

Photocontrol and Structural Analysis of Amyloid Fibril
Formation Using Azobenzene and Novel Spiropyran
Photoswitches

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Dedicated to S., who has always believed that I will find my way.

“The supreme question about a work of art is out of how deep a life does it spring. Paintings of Moreau are paintings of ideas. The deepest poetry of Shelley, the words of Hamlet bring our mind into contact with the eternal wisdom; Plato’s world of ideas. All the rest is the speculation of schoolboys for schoolboys.”

“A man of genius makes no mistakes. His errors are volitional and are the portals of discovery.”

James Joyce - Ulysses

Eigenständigkeitserklärung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Arbeit selbstständig und ohne fremde Hilfe verfasst habe. Andere als die angegebenen Quellen und Hilfsmittel wurden nicht benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen wurden als solche kenntlich gemacht. Außerdem erkläre ich, die vorliegende Dissertation an keiner anderen wissenschaftlichen Einrichtung zur Erlangung eines akademischen Grades eingereicht zu haben.

Halle (Saale), den 12.05.2025

André Paschold

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List of abbreviations

A β	amyloid- β
AFM	atomic force microscopy
Ac	acetyl
3,4'-AMPB	3-((4-(aminomethyl)phenyl)diazenyl)benzoic acid
AMPP	[3-(3-aminomethyl)phenylazo]phenylacetic acid
ATR	attenuated total reflectance
Boc	<i>tert</i> -butoxycarbonyl
CD	circular dichroism
CyHex	cyclohexane
DCM	dichloromethane
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
EE	ethyl acetate
EM	electron microscopy
eq.	equivalent
Et	ethyl
ESI	electrospray ionization
FA	formic acid
FASC	5'-amino-1',3',3'-trimethylspiro[chromene-2,2'-indoline]-6-carboxylic acid
Fmoc	fluorenylmethoxycarbonyl
IR	infrared
HPLC	high-performance liquid chromatography
HR-MS	high-resolution mass spectrometry
MALDI	matrix-assisted laser desorption/ionization
MC	merocyanine
MD	molecular dynamics
Me	methyl
MS	mass spectrometry
NMR	nuclear magnetic resonance
PSS	photostationary state
PrP	prion protein
PTH	parathyroid hormone
QS	quorum sensing

SAFC	6-amino-1',3',3'-trimethylspiro[chromene-2,2'-indoline]-5'-carboxylic acid
SP	spiropyran
SPPS	solid-phase peptide synthesis
ssNMR	solid-state nucleus magnetic resonance
Su	succinimide
TEA	triethylamine
TEM	transmission electron microscopy
THF	tetrahydrofuran
TMFA	thioflavin T-monitored fluorescence assay
ToF	time-of-flight
UV	ultraviolet radiation
UV/Vis	ultraviolet visible
WAXS	wide-angle X-ray scattering

Abstract

Amyloidogenic peptides are involved in many biological processes. Their self-organization, termed as amyloid formation, comprises of complex inter- and intramolecular interactions and is an efficient process to transform peptides from a soluble into an insoluble state. They play a crucial role in diseases such as Alzheimer disease or Parkinson disease or in physiological functionalities, like the storage of peptide hormones. Thus, the control of this process is a powerful tool to gain insight into the kinetics of fibrillization and the structural nature of the supramolecular assemblies. Furthermore, it promises the potential to develop novel medical treatments. Peptide hormones in particular, such as parathyroid hormone (PTH), are suitable candidates for the establishment of a model system due to their reversible fibril formation under physiological conditions.

In this work, various photoswitches were used to develop a model system based on PTH-derived peptides that is able to switch between a fibrillar and a non-fibrillar state by light. For this purpose, the respective building blocks were synthesised and incorporated into the fibril-forming sequence of the parathyroid hormone (PTH₂₅₋₃₇) at different positions

Therefore, the azobenzene photoswitch 3-((4-(aminomethyl)phenyl)diazanyl)benzoic acid (3,4'-AMPB) was chosen and the novel spiropyran building blocks 5'-amino-1',3',3'-trimethylspiro[chromene-2,2'-indoline]-6-carboxylic acid (FASC) and 6-amino-1',3',3'-trimethylspiro[chromene-2,2'-indoline]-5'-carboxylic acid (SAFC) were designed, which can be incorporated into the peptide backbone. More than 20 peptides were produced using solid-phase peptide synthesis (SPPS) and their isomerisation behaviour, fibrillization behaviour and the morphology of the fibrils were studied.

The novel spiropyran building blocks are the first report of spiropyran introduced in the backbone of peptides and thus, expand the toolbox of available switchable entities for modulating peptide behaviour. Solid-state nuclear magnetic resonance (ssNMR) and molecular dynamics (MD) studies of the unmodified PTH₂₅₋₃₇ and the azobenzene-containing derivatives allowed conclusions to be drawn about the structural details of the fibrils produced. While the unmodified peptide mainly forms fibrils consisting of parallel β -sheets, the fibrils of the 3,4'-AMPB containing peptides adopts an antiparallel arrangement. Furthermore, it was demonstrated that the isomeric conformations of the photoswitch modulated the fibrillization in a contrary way, which unlocked the control of the fibrillization. Thus, this is the first reversible (de)fibrillating peptide model and insights hereof, can be used as basis for further developments.

Zusammenfassung

Amyloidogene Peptide sind an vielen biologischen Prozessen beteiligt. Ihre Selbstorganisation, die als Amyloidbildung bezeichnet wird, besteht aus komplexen inter- und intramolekularen Wechselwirkungen und ist ein effizienter Prozess, um Peptide von einem löslichen in einen unlöslichen Zustand zu überführen. Sie spielen eine entscheidende Rolle bei Krankheiten wie der Alzheimer- oder Parkinson-Krankheit oder bei physiologischen Funktionen wie der Speicherung von Peptidhormonen. Die Kontrolle über diesen Prozesses bietet daher eine einzigartige Möglichkeit, um Einblicke in die Kinetik der Fibrillierung und die strukturelle Beschaffenheit der supramolekularen Aggregate zu gewinnen. Darüber hinaus bietet sie das Potential neue medizinische Behandlungen zu entwickeln. Insbesondere Peptidhormone, wie das Parathormon, sind aufgrund ihrer reversiblen Fibrillenbildung unter physiologischen Bedingungen geeignete Kandidaten für die Etablierung eines Modellsystems.

In diesem Werk wurden verschiedene Photoschalter genutzt um ein Modellsystem auf Basis von PTH abgeleiteten Peptiden zu entwickeln, dass durch Licht in der Lage ist zwischen einem fibrillierenden und einem nicht fibrillierenden Zustand zu wechseln. Dazu wurden die jeweiligen Bausteine synthetisiert und in die fibrillenbildende Sequenz des Parathormons (PTH₂₅₋₃₇) an verschiedenen Positionen eingebaut.

Die Grundlage dafür bildeten der Azobenzen-Schalter 3,4'-AMPB, sowie die neuartigen hierfür konzipierten Spiropyran-Bausteine FASC and SAFC, die in das Peptidrückgrat eingebaut werden können. Es wurden mehr als 20 Peptide mittels Festphasenpeptid-Synthese hergestellt und deren Isomerisierungsverhalten, Fibrillierungsverhalten, sowie die Morphologie der Fibrillen studiert.

Die hier entwickelten Spiropyran-Bausteine sind die erste Möglichkeit, Spiropyrane in das Rückgrat von Peptiden einzubauen und erweitern somit die Palette an schaltbaren Rückgratsmodifikationen zur Modulation des Peptidverhaltens. Festkörper-NMR und MD-Studien des unmodifizierten PTH₂₅₋₃₇ und der azobenzenhaltigen Derivate ließen Rückschlüsse auf strukturelle Details der erzeugten Fibrillen zu. Während das unmodifizierte Peptid hauptsächlich Fibrillen bildet, die aus parallelen β -Faltblättern bestehen, konnte gezeigt werden, dass eines der 3,4'-AMPB-haltigen Peptide eine antiparallele Anordnung annimmt. Darüber wiesen die isomeren Formen des Photoschalters gegensätzliche Effekte auf die Fibrillierung auf und es konnte demonstriert werden, dass es möglich ist zwischen einer fibrillierenden und einer nicht fibrillierenden Form reversibel zu wechseln. Dies ist somit der erste Bericht über ein reversibel (de)fibrillierendes Peptidsystem und bietet damit eine Basis für weitere Entwicklungen.

Introduction

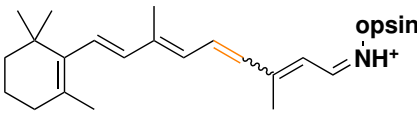
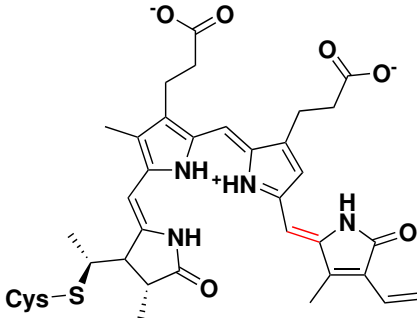
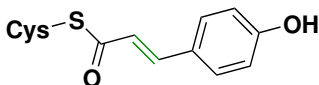
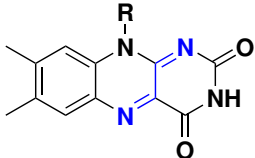
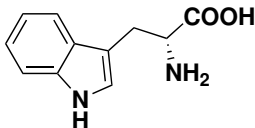
The first process that takes place in the human body as we read these lines, is the a million times occurring phototriggered *cis* $\rightarrow$ *trans*-isomerization of a 11-*cis*-retinal chromophore. The chromophore is attached to proteins called opsins, which are present in the rod and cone cells of the retina.^{1,2} It is one of the most astonishing systems that we can find in nature concerning switching processes on a molecular level reacting to an external trigger. In general, molecular switches are molecules or entities of molecules, which can be activated through an external trigger in a directed manner between bistable or multistable states.^{3–5} A plethora of stimulus exists to trigger the process: *e.g.* light,^{6,7} temperature,^{8,9} electric fields,^{10,11} tunneling electrons,^{12,13} changes in polarity and pH,^{14,15} and other chemical stimuli,^{16,17} enable the manipulation of electronic properties, dipole moments, and molecular conformations. They can differ in their size from a small chemical entity inside a molecule^{6,18} up to large macromolecules.^{19,20}

The generic definition of molecular switches already indicates that the research in this field covers a broad scope and even more that it is a highly interdisciplinary field. The chemical and the physical aspects refer mostly to the structural motives of molecular switches and describe the isomerization process itself. In biological context switches are responsible for signal transduction^{21–23} in living tissue and are a powerful tool in synthetic biology to control cellular processes.²⁴ In material sciences molecular switches are explored as molecular machines,^{25,26} in displays,^{27,28} and in smart materials.²⁹ For computer technology they are potential candidates to gain access to new materials like sensors and logical gates in molecular computers,^{30,31} which could undergo the physical limits of silicon-based computer chips.^{32,33}

As life on the earth is mainly fueled by the light energy of the sun, beneath some archaeobacteria living under extreme conditions, there exist several behaviours of cells in reaction of changing light conditions. Beneath our ability of vision, described *vide supra*, the main purposes are: *e.g.* establishment of the circadian rhythm, sensing different wavelengths of light, phototaxis,³⁴ and ATP synthesis.³⁵ Most of these processes are triggered by photochromic molecules, whereby photochromism describes the ability to undergo photoinduced isomerization reactions between stable and (meta)stable states.

Usually the chromophore is bound to a protein, *e.g.* G-protein coupled receptor,¹ ion channels,^{38,39} kinases,⁴⁰ proton/ion pumps,³⁵ or gene expression regulator.⁴⁷ Absorption of a photon induces an isomerization process that leads to conformational change in the protein and starts a signal cascade. The chromophores can be separated after their modes of action: retinal-based chromophores, bilin-based chromophores and cinnamic acid undergo a *E/Z*-isomerization, flavenoid-based chromophores and tryptophan change their electronic state. The deactivation of the protein, respectively the recovery of the photoswitch, can occur thermodynamically³⁹ or accelerated *via* enzymes.⁴³ In Table 1.1 a short

Table 1.1: Natural photoswitches and where they can be found. The changing bonds are highlighted in colour.

Chromophor	Protein (organisms)	Function (light color)
 retinal-based	melanopsin ³⁶ (animals)	circadian ryrthm (blue)
	rhodopsins ¹ (animals)	vision (visible)
	neuropsin ³⁷ (animals)	circadian rythm (UV)
	channelrhodopsin ³⁸ (algae)	phototaxis (green)
	bacterial rhodopsins ^{35,39} (bacteria, archae)	light sensing ATP synthesis (green)
 bilin-based	phytochrome ^{40,41} (plants, bacteria, fungi)	phototaxis cell regulation (blue)
	cyanobacteriochromes ⁴² (cyanobacteria)	phototaxis cell regulations (blue and green)
 cinnamic acid	xanthopsin ⁴³ (bacteria)	unkwown (blue)
 flavenoid-based	cryptochrome ^{44,45} (animals)	circadian rythm (blue)
	phototropin ⁴⁶ (algae, plants)	chloroplast movement (UV and blue)
 tryptophan	UVR8 ⁴⁷ (plants)	phototaxis (UV-B)

overview is given about known chromophores and examples of photoswitchable proteins, which occur in nature.

Occasionally, the first emergence of a photochromic behaviour is reported for Alexander III of Macedon, better known as Alexander the Great.^{48,49} During his reign from 336–323 BC it was told, that his warriors used wristbands, changing their colour through the presence or absence of sunlight, to synchronize their military actions. It is not known if these should be taken seriously as it might be based on a play of words from an article in an American news magazine in 1961. It refers to the wristband as "Alexanders Rag Time Band", a homophonous word play to the song "Alexander's Ragtime Band" from Irvine Berlin.⁵⁰ However, the first "real" discovery of the photochromic effect was attributed to a German chemist in the second half of the nineteenth century. J. Fritsche reported the decolourization of an orange tetracene solution in the daylight and their recolourisation during the night.⁵¹ In the following decades several similar phenomena were described^{52,53} and the nowadays used term photochromism was introduced 1950 by Y. Hirshberg.⁵⁴ Light is a nearly perfect stimulus as it encodes information in wavelength and intensity. It exhibits a high resolution in space and time and does not permanently contaminate a treated sample. Furthermore, it does not cause undesired interactions as the photoisomerization is only triggered by light of specific wavelengths. This offers possibilities, like using two photoswitchable entities in a molecule, where each can be activated separately,⁵⁵ or dual wavelength activation of initiators for polymerization reactions, which offer high spatial control.⁵⁶

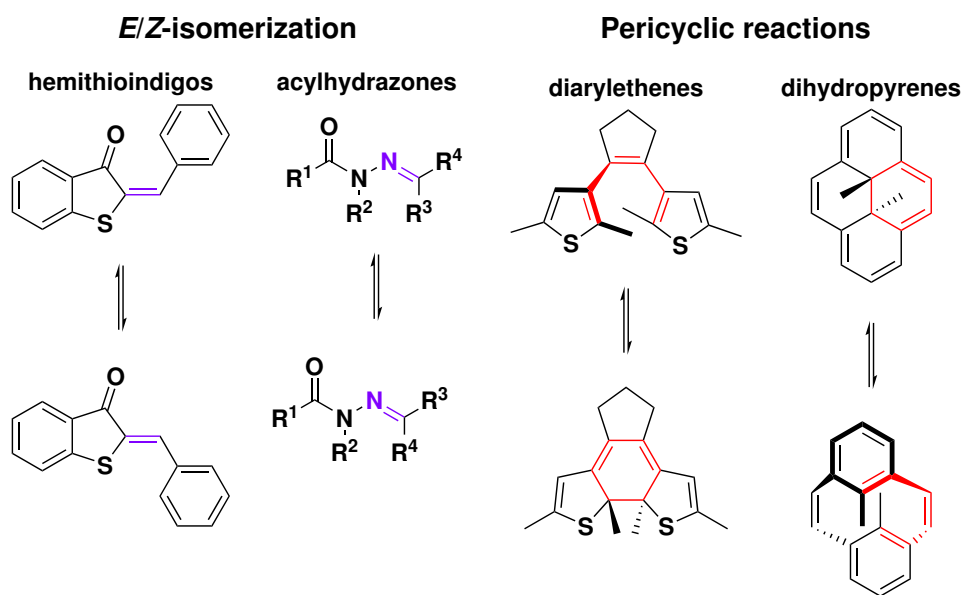


Figure 1.1: Selection of different types of synthetic photoswitches: (left) Photoswitches undergoing *E/Z*-isomerization. (right) Photoswitches undergoing pericyclic reactions.

Since Hirshberg introduced the term photochromism, several molecular structures were synthesised that possess this ability. However, for application in materials, they have to fulfill some requirements: large extinction coefficients, high quantum yields, a elevated ratio of the isomers in the respective photostationary state (PSS), low photobleaching, and facile synthesis and chemical functionalization.

Further requirements can occur in the different fields, *e.g.* in *in vivo* systems the switching wavelengths should be in the visible or far-red range to avoid ultraviolet radiation (UV) based cell damages.⁵⁷ Common photoswitchable entities can be divided into two groups (Figure 1.1):⁵⁷⁻⁵⁹ The first group involves molecular entities, where the absorption of a photon causes *E/Z*-isomerization of a N=N, C=C or N=C double bond, *e. g.* stilbenes, hemithioindigos, overcrowded alkenes, acylhydrazones, and azobenzenes. While the second group undergoes pericyclic reactions (primary cyclization and ring opening), like diarylethenes, fulginimides, dihydropyrenes, and spiropyrans. In the following two sections we will gain further insights in the groups of azobenzenes, which exhibit *E/Z*-isomerization, and in the spiropyrans, which undergo cyclization/ring opening reactions.

1.1 Azobenzenes

Azobenzenes are a class of molecules that represents a diazene ($\text{HN}=\text{NH}$) derivative, where both hydrogen atoms were replaced by arenes. The eponymous functionality is the azo-bridge (highlighted in Figure 1.2 in purple), two nitrogen atoms connected through a double bond. Similarly to stilbenes,⁶⁰ the analogs with a carbon-carbon double bond, azobenzenes can exist in two isomeric forms. According to the arrangement of the arenes in relation to the double bond, we have either a *trans*-isomer, also called *E*-, or a *cis*-isomer, also called *Z*-. (Figure 1.2). In the further oeuvre exclusively the *trans*-/ *cis*-nomenclature will be used for the azobenzenes.

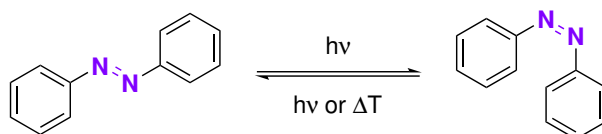


Figure 1.2: The simplest representative of the azobenzenes in its isomeric forms: *trans*-azobenzene and *cis*-azobenzene.

Both forms can undergo isomerization to generate the other isomer. The isomerization process in both direction can be accessed *via* irradiation with light of different wavelengths. Further possible external triggers are mechanical stress⁶¹ or electrostatic stimulation.^{62,63} In general, the *trans*-isomer is the thermodynamically favoured state and therefore the metastable *cis*-form isomerizes spontaneously in the dark. Their beneficial switching properties and the facile synthetic access made them one of the most used and best studied photoswitches.^{64–67}

1.1.1 History

The discovery of azobenzene occurred in 1834 by E. Mitscherlich (Figure 1.3).⁶⁸ He described a red compound, which was obtained by heating nitrobenzene in the presence of aqueous KOH-solution. After more than twenty years A. Nobel was the first who reported a method that could produce azobenzenes on an industrial scale,⁶⁹ which marks the beginning of the success story of azobenzenes. He adapted the Bechamp modification of the Zinin's procedure, an actual procedure to reduce nitrobenzol to aniline with iron and acetic acid.⁷⁰ Two years afterwards P. Griess reported the diazotation reaction.⁷¹ It is the nowadays singularly most used reaction in the synthetic dyes industry,⁷² as azo dyes represent almost 70% of dyes used in industry.⁷³ The textile industry, growing through the industrial revolution,⁷⁴ had an increasing demand of dyes. The first commercial azobenzene dye Aniline Yellow was reported by C. Mene in 1861.⁷⁶ Closely afterwards, in 1863 Bismarck Brown Y was introduced by C. A. von Martius.⁷² It is the first bis azo dye and was synthesised through allowing nitrous acid to act on 1,3-phenyldiamine, an diazotation reaction followed by an azo coupling. Even though azobenzene had been known for more than thirty years, A. Kekulé reported the correct molecular formula $\text{C}_{12}\text{H}_{10}\text{N}_2$ for the first time in 1866.⁷⁷ A further application for azobenzenes was reported 1935 by G. Domagk,⁷⁸ who showed that sulfamidochrysoidine, a prodrug for sulfanilamide and better known under its trade name "Prontosil", possesses an antibacterial activity against various bacteria. He was awarded for his achievement 1939 with the Nobel prize in physiology and medicine. The next significant step was

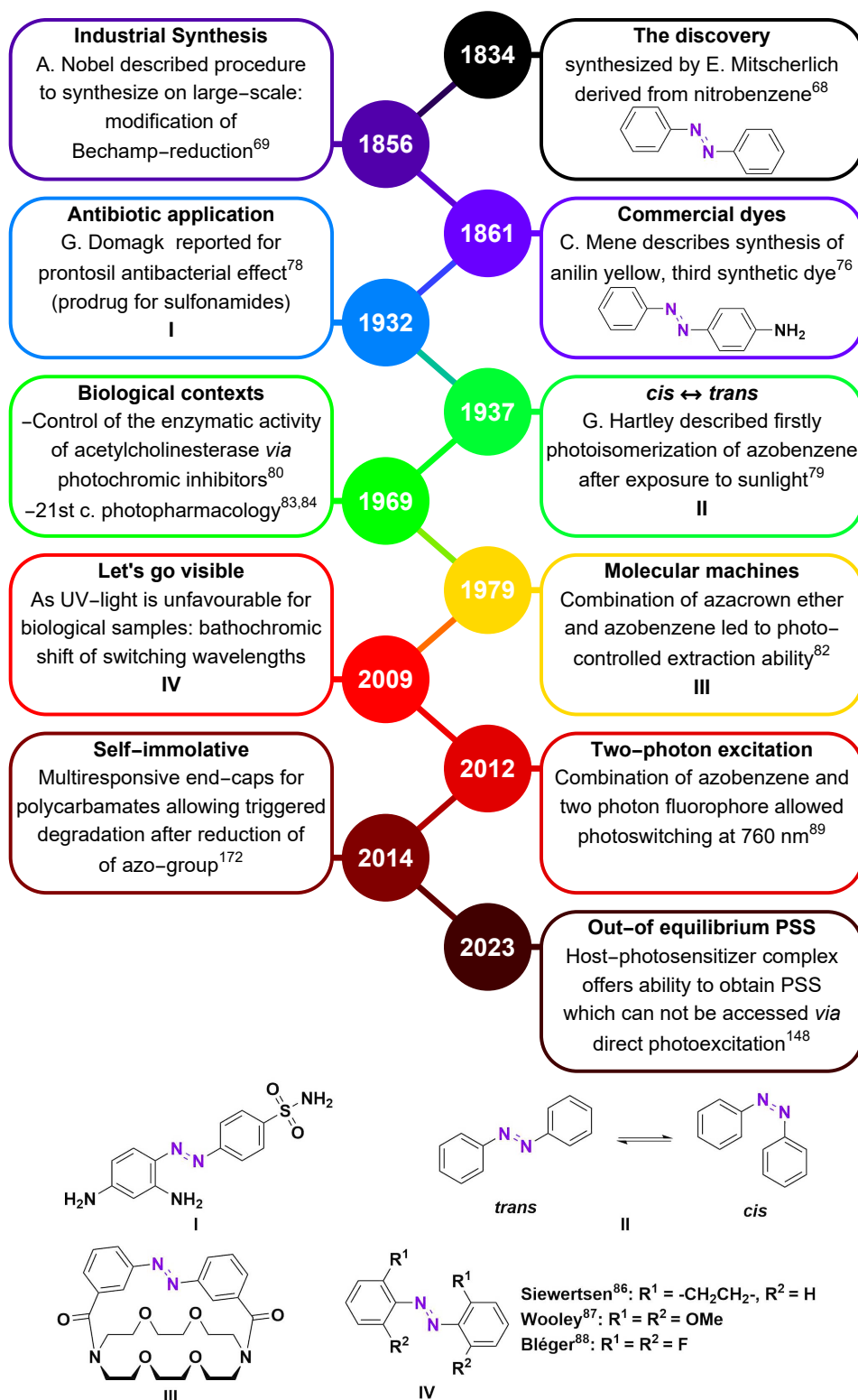


Figure 1.3: Milestones in the development of azobenzenes.

made by G. S. Hartley in 1937, when he observed a lack of reproducibility in solubility experiments upon exposure to light. His conclusions led him to the discovery of the photochemical *cis-trans*-isomerization.⁷⁹ This result offered another possible application for azobenzenes. Beneath the industrial use as a dye, azobenzenes could also be used as photoswitchable entities to control properties of materials. Major achievements are the first use in a biological example, which was in 1969,⁸⁰ the induced photochemically alignment of a liquid crystal phase in 1971,⁸¹ or the first report of a molecular machine based on an azobenzene.⁸² In the last two decades the field of photopharmacology emerged, where pioneering research was contributed by the groups of Trauner, Krahmer, and Feringa based on azobenzene photoswitches.^{83,84} Even nowadays azobenzenes are the most used photoswitches in this field,⁸⁵ as they possess the most suitable properties especially for *in vivo* use. This correlates with another trend, which started at the end of the first decade of this century: the red-shifting of the azobenzene-switching.^{86–89}

1.1.2 Synthesis

In the almost 200 years that have passed since the discovery of azobenzenes, a plethora of synthetic methods have been described to obtain them.^{90,91} Five main approaches have emerged (Figure 1.4):

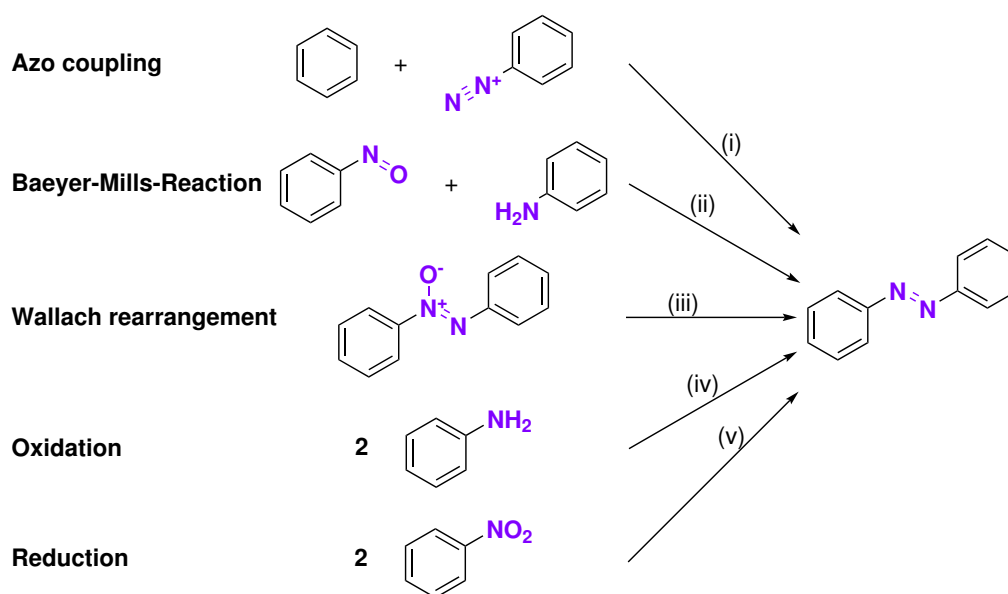


Figure 1.4: Synthetic methods towards azobenzenes.

(i) The azo coupling reaction, discovered by the victorian brewer P. Griess, is still the most used chemical reaction to synthesize azobenzene derivates. It is based on the initial formation of the diazonium-salt, from an aromatic amine, which then reacts with an electron-rich aromatic compound, *e.g.* aromatic amines or phenols.⁹²

(ii) The Bayer-Mills reaction is based on the condensation reaction between a nitroso compound and an aniline derivate. It was first published by A. Baeyer in 1874⁹³ and further investigated by C. Mills.⁹⁴

(iii) A chemoselective deoxygenation reaction of *N*-oxides, named after the German chemist O. Wallach, who discovered the reaction in 1890.⁹⁵ The Wallach-rearrangement describes the transformation of azoxybenzenes into hydroxyazobenzene. The reaction is promoted by strong acids and dependent of the substituents in position 4 of the phenyl rings. It can lead either to 2-hydroxy or 4-hydroxy products.^{96,97}

(iv) The latest discovered of these methods is the oxidation of aromatic amines to obtain azobenzenes. The first chemical oxidation was reported in 1953⁹⁸ and the first electrolytical oxidation in 1972.⁹⁹ There exist various metallic and non-metallic oxidation reagents, which lead to symmetrical azobenzenes.

(v) The reduction of nitro compounds was the first method discovered to synthesize azobenzenes (see 1.1.1).⁶⁸ It was the first method to obtain azobenzenes on an industrial scale and, like the oxidation of anilines, it leads to symmetrical azobenzenes.

1.1.3 Transition process in detail

Azobenzenes belong to the group of photoswitches, which undergoes *trans/cis*-isomerization of a N=N double bond. The *trans*-isomer is the thermodynamically favoured one and adopts a planar conformation with C_{2h} symmetry,¹⁰⁰ while the *cis*-isomer is distorted in the planarity and possesses C_2 symmetry.¹⁰¹ The energy difference ΔG between two isomers is ~ 56 kJ/mol¹⁰² and the thermal barrier between both isomers ΔG^* is approximately 100 kJ/mol in heptane¹⁰³ and benzene.¹⁰⁴ The *trans* $\rightarrow$ *cis*-isomerization is achieved by the irradiation of UV light, while the opposing *cis* $\rightarrow$ *trans*-isomerization proceeds thermally or is triggered by the irradiation of visible light. Beneath altering the shape, the isomerization process has two main impacts: Change in the dipole moment¹⁰⁵ and in the end-to-end distance of the carbon atoms at the *para*-position of the rings (Figure 1.5A).¹⁰⁶ While the *trans*-configuration has a dipole moment near zero and an end-to-end distance of ~ 9 Å, the dipole moment increases in the *cis*-form to ~ 3 D and the end-to-end distance is decreased to ~ 5.5 Å.

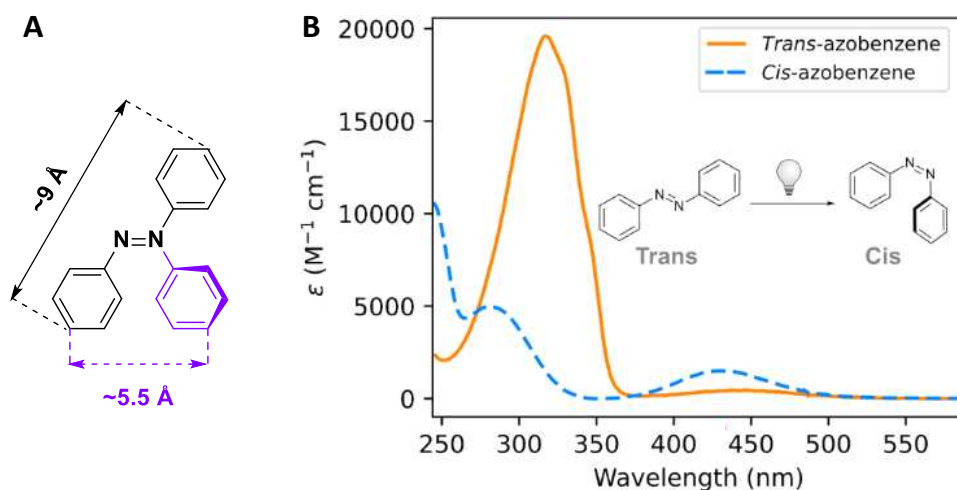


Figure 1.5: (A) Distance of the carbon atoms in the *para*-positions of the unsubstituted azobenzene. Black - structure of the *trans*-isomer. Purple - corresponds to the phenylring in *cis*-configuration. (B) Ultraviolet visible (UV/Vis)-absorption spectra of the isomers of unsubstituted azobenzene. Reprinted with permission from the Beilstein-Institute, copyright ©2024 (open access).¹⁰⁷

In Figure 1.5B the extinction coefficients of the unsubstituted *trans*- and *cis*-azobenzene are shown. In both spectra we can observe two well separated bands.⁶⁶ In the UV region we observe for the *trans*-azobenzene a band at 320 nm and for the *cis*-isomer a band at 270 nm, which both correspond to the symmetry allowed $\pi \rightarrow \pi^*$ transition. The weaker band in the visible region, for both isomers the maximum is ~ 440 nm, corresponds to the symmetry forbidden $n \rightarrow \pi^*$ transition.¹⁰⁸ As the *cis*-isomer is slightly distorted in the planarity, the $n \rightarrow \pi^*$ transition absorbs more strongly than the one for the *trans*-isomer. While the $\pi \rightarrow \pi^*$ transition excites the molecule to the $S_2(\pi\pi^*)$ state, the $n \rightarrow \pi$ transition excites the molecule to the $S_1(n\pi^*)$ state. In stilbene *cis/trans*-photoisomerization occurs only *via* a rotational pathway,¹⁰⁹ whereby the sum of the quantum yields of photoinduced *cis* $\rightarrow$ *trans*- and *trans* $\rightarrow$ *cis*-isomerization equals one. However, in azobenzene the sum of the quantum yields does not,¹¹⁰ which suggests, that the photoisomerization can occur *via* different mechanisms (Figure 1.6): in the rotation mechanism (upper mechanism), a cleavage of the π bond between the two nitrogens allows a free out-of-plane rotation of the dihedral angle of C-N-N-C, whereby the angle of N-N-C remains fixed.¹⁰⁰ In the inversion mechanism (lower mechanism) an inversion occurs in the plane. It corresponds to a transition state with linear geometry, where one of the nitrogen is *sp* hybridized.¹¹¹ One of the N=N-C angles is increased to 180° , while the C-N=N-C dihedral angle remains unchanged. Further possible mechanisms are the concerted inversion of both phenyl rings or the inversion supported rotation.⁶⁶

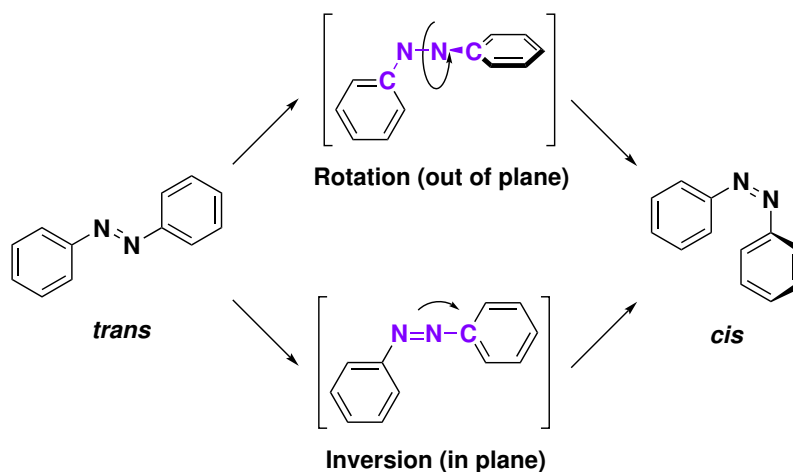


Figure 1.6: Changes in the configuration of unsubstituted azobenzene from the thermostable *trans*-isomer to the metastable *cis*-isomer through irradiation with UV light. Shown here are, as examples: The rotation, an out of plane torsion of the C-N-N-C dihedral angle, and the inversion, which corresponds to an in plane inversion of the N=N-C angle between the azo moiety and the adjacent carbon atom of the phenyl ring.

For the *trans* $\rightarrow$ *cis*-isomerization it has been proposed that excitation with UV (upper mechanism), which leads to the high-energy $S_2(\pi\pi^*)$ state, causes a cleavage of the π bond between the two nitrogens. This allows a free out-of plane rotation of the C-N-N-C dihedral angle, whereby the N-N-C angle remains fixed.¹¹² The excitation with visible light (lower mechanism), which excites in the $S_1(n\pi^*)$ state, should therefore proceed *via* an in-plane inversion. However, a few years later investigations of the isomerization process after excitation in the $S_2(\pi\pi^*)$ state revealed that the S_2 state quantitatively relaxes in the S_1 state and isomerization occurs uniformly from this state.¹¹³ Up to today it is not completely

clear by which pathway *trans* $\rightarrow$ *cis* isomerization occurs, independent of the excitation wavelength. While computational analysis indicates a more rotational mechanism, ultra-fast time-resolved techniques tend to show a relaxation mechanism dominated by inversion with a rotational part.⁶⁶ For the reversed *cis* $\rightarrow$ *trans*-isomerization we have a thermal and a photomediated process. Both are less investigated than the photostimulated *trans*-process. For the photoisomerization computational analysis, *e.g.* dynamic simulations¹¹⁴ and *ab initio* calculations,¹¹⁵ rather indicate a mechanism dominated by rotation. The half-life time of *cis*-isomer is $\sim$ two days.¹⁰⁴ As mechanism in solution rotation and inversion are possible mechanisms,^{116,117} while the inversion pathway seems to be the dominant one in liquid crystals¹¹⁸ and under high pressure.¹¹⁹ While the rate of thermal isomerization is independent of solvent polarity^{120,121} and solvent viscosity, it is dependent on temperature and accelerated by acids.¹²² As well for the photoisomerization processes solvent viscosity does not have an effect. For solvent polarity, the situation is different: Although it does not show an effect on the $\pi \rightarrow \pi^*$ excitation, the quantum yield of the *trans* $\rightarrow$ *cis*-photoisomerization following the $n \rightarrow \pi^*$ is increased and for the reversed photoisomerization it is decreased with increasing solvent polarity.⁶⁶ It was assumed that, only one excited state, the $n\pi^*$ state itself, or a state more easily reachable from the $n\pi^*$ than from the $\pi\pi^*$ state, is responsible for the phototriggered isomerization process.¹¹⁰

As the *trans*-isomer is thermally more stable, the equilibrium is strongly shifted towards it. Under ambient temperature and in the dark more than 99.9% of the *trans*-isomer are possible.⁶⁵ The photoisomerization process, however, is incomplete. At the optimal wavelength of 313 nm, 90% of the *cis*-isomer can be obtained, while in the reversed isomerization process 80% of the *trans*-form can be generated using light with a wavelength of 436 nm.¹²³ The reason is observable in the UV/Vis spectra (Figure 1.5B): Both, the UV band and the band in the visible region, overlap for the isomers.

1.1.4 Structure-activity relationship

The photophysical properties of azobenzenes are highly dependent on the substitution pattern. Especially, the electron withdrawing/donating propensity and the position of the substituent have a great influence on the switching behaviour. Derivates bearing substituents, *e.g.* alkyl,¹³⁰ halides,¹³¹ carboxylic acids,¹³² amides,¹³³ esters,¹³⁴ carbonyl, nitrile, and nitro¹³⁵ in the 3/3'/4/4'-positions as well as 3/3'-alkoxy¹³⁶ and -amino¹³⁷ have in common, that the UV/Vis spectra resemble mostly the spectra of unsubstituted azobenzene, whereby the $\pi\pi^*$ transition band is slightly red-shifted, but both transition bands are well separated (Table 1.2). They possess a shorter half-life of the *cis*-isomer as the substituents decrease the energy barrier for thermal isomerization. This effect correlates with the electron-withdrawing propensity of the substituents,¹³⁸ *e.g.* carboxylic acid and nitro-containing azobenzenes exhibit a rather fast thermal isomerization.

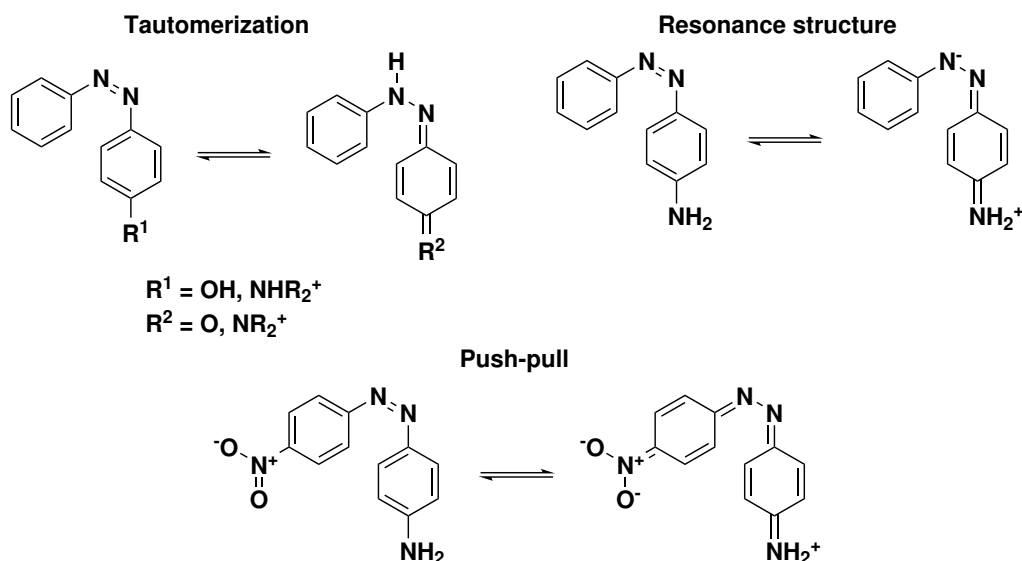
Electron-donating substituents with a +M effect (amino, *N*-amides, hydroxy, and alkoxy) in the 4/4'-position have a stronger effect on the absorption spectra. The $\pi\pi^*$ transition is red-shifted and overlaps with the $n\pi^*$ transition. This effect is minor for the weaker donors of electron density, *N*-amides¹²⁵ and alkoxy substituents,¹³⁶ and the two transition bands are still observable. For stronger electron-donating substituents, *e.g.* hydroxy or amino groups, both transition bands are overlapping partially or completely.^{125,139} While in the latter case both transition bands lie in the range of visible

Table 1.2: Substituent effect on $\pi\pi^*$ transition in the *trans*-isomer and on the half-life time $\tau_{1/2}$ of *cis*-isomer in 4,4' modified azobenzenes.

#	R^1	R^2	$\tau_{1/2,cis}$ (solvent)	$\lambda_{\pi\pi^*,trans}$ [nm]	Ref.
-	H	H	2880 min	320	[107]
1	Cl	Cl	430 min (CyHex)	331	[124]
2	Me	Me	401 min (CyHex)	330	[124]
3	H	OMe	221 min (CyHex)	357	[124]
4	OMe	OMe	221 min (CyHex)	353	[124]
5	NC(O)CH ₃	NC(O)CH ₃	159 min (-)	~370	[125]
6	H	NO ₂	94 min (CyHex)	330	[124]
7	H	NEt ₂	69 min (CyHex)	407	[124]
8	NH ₂	NH ₂	2 min (-)	420	[125]
9	H	OH	31 min (toluene) 205 ms (ethanol)	~350	[126]
push-pull azobenzenes					
10	NO ₂	OH	4.6 ms (EtOH)	-	[127]
11	N(Me) ₂	NO ₂	717 s (pentane)	408	[128, 129]
			5 ms (DMSO)	450	

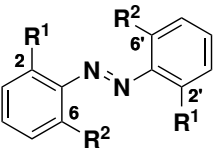
light, the overlapping of the transition states hampers the correct assignment of them and lowers the photoisomerization yield (Table 1.2). A different tendency can be observed in thermal *cis* $\rightarrow$ *trans*-isomerization. *N*-amides and alkoxy substituents reduce the half-life time moderately, as they increase the electron density in the π^* orbital and thus lower the energy barrier of isomerization. However, the introduction of tautomerisable amino and hydroxy groups cause a significant decrease. Despite increasing the electron density in the π^* orbital the even greater influence arises from the resonance structures formed or from the tautomerization in the case of hydroxy groups (Figure 1.7).^{125,139} These effects favour a hydrazone like-distribution with a N-N bond, which enables a rotation pathway to easily isomerize to the *trans*-isomer. The thermal isomerization is significantly affected by solvent polarity: in case of 4,4'-dihydroxyazobenzene the half-life time increases by four orders of magnitude in toluene compared to ethanol.^{126,140} Furthermore, in the case of amino groups we can observe a dependence of the pH value. Below the pK_a value a protonated form of the azobenzene exists, where the $n\pi^*$ transition is red-shifted to ~680 nm and therefore, both transition bands are separated again. Unfortunately, the hydrazone-tautomer is in this case the preferred one, which disables the photoisomerization.¹⁴¹

If both electron-donating and electron-withdrawing substituents are present in the 4,4'-positions, it's called a push-pull-system (Figure 1.7). In general, the $\pi\pi^*$ transition is red-shifted and can as well overlap with the $n\pi^*$ transition band.¹³⁹ The *cis*-isomer is less stable and therefore the half-life time is significantly reduced and can be in the range of milliseconds or microseconds (Table 1.2).¹²⁷ A reason

Figure 1.7: Transition states increasing the thermal rate for *cis*→*trans*-isomerization.

hereby is again the formation of resonance structures, which enables the rotation around the azo-bond. The half-life time is furthermore dependent on the solvent polarity, whereby a increase of the rate constant about 10^5 -fold is possible for polar solvents over non-polar, because the isomerization occurs *via* a highly polar charge-separated transition state.¹²⁹

Table 1.3: Substituent effect on $n\pi^*$ transition in both isomers and on the half-life time $\tau_{1/2}$ of *cis*-isomer in *ortho*-modified azobenzenes.

						
#	R ¹	R ²	$\tau_{1/2,cis}$ (solvent)	$\lambda_{n\pi^*,trans}$ [nm]	$\lambda_{n\pi^*,cis}$ [nm]	Ref.
-	H	H	2880 min	440	440	[107]
1	H	Me	10 h (hexane)	460	439	[142, 143]
2	Me	Me	-	462	454	[142]
3	Cl	Cl	20.7 h	455	443	[144]
4	H	F	11 h (MeCN, 60°C)	448	420	[145]
5	F	F	~700 d (MeCN)	458	416	[88]
6	H	OMe	1.9 h (DMSO)	474 (calc.)	472 (calc.)	[87]
7	OMe	OMe	14 d (DMSO)	480	444	[87]

While the *para*-substitution has an effect on the electronic properties of the azobenzene, spatial and steric effects play a role if substitution occurs in *ortho*-position. Alkyl substituents do not alter the electronic properties significantly. While *para*-alkyl substituents cause for the *trans*-isomer a slight red-shift of the $\pi\pi^*$ and a slight blue-shift of the $n\pi^*$ transition, *ortho*-alkyl substituents cause an opposite effect, a slight blue-shift of $\pi\pi^*$ and red-shifting of $n\pi^*$ (Table 1.3).¹⁴² This effect is further

intensified with the number and size of the substituents and is steric in nature, as it causes a twisting of the phenyl rings. It results from the repulsion of the *ortho*-substituent and the distant lone-pair of the azo-nitrogen. Therefore, the effect is stronger for tetra-alkylated compared to dialkylated compounds, as in the second case the azobenzene can adopt a conformation, where the substituent does not interfere with the lone-pair. The same effect occurs for the *cis*-isomer. Interestingly, the dialkylated compound has a larger separation of the $n\pi^*$ transition between both isomers than the tetraalkylated one, as the transition is not shifted for the *cis*-isomer. Compared to unsubstituted azobenzene the half-life time of the *cis*-isomer is reduced.¹⁴³ Electronic effects seem to have a minor impact compared to sterical concerns, *e.g.* replacing the alkyl-substituents by chlorine-atoms have the same effects on the $n\pi^*$ transition.¹⁴⁴ The separation of the $n\pi^*$ transition allows to use exclusively visible light for photoswitching. For example the tetrachloro-derivate can be switched to a PSS of 60% *cis*-isomer with 530 nm or 660 nm and *vice versa* to 88% *trans*-isomer with 410 nm.

To achieve higher PSS ratios a further separation of the $n\pi^*$ transition is necessary, which can be achieved with either *ortho*-methoxy or *ortho*-fluoro substituents. While a disubstitution with *ortho*-methoxy groups only cause a significant red-shift, but not a separation of the transition bands, the tetramethoxy substituted derivate has a large separation of 36 nm.⁸⁷ This allows a PSS of 80% *cis*-isomer in dimethyl sulfoxide (DMSO) with 530–560 nm and 85% *trans*-isomer with 450–460 nm. Additionally, a strong increase of the half-life time was observed (2 d for the unsubstituted *vs.* 14 d for tetramethoxy substituted). Again this effect results from a large sterical repulsion, which brings the *trans*-isomer in a highly twisted conformation, compared to nearly planar unsubstituted or 4/4'-substituted azobenzenes. This brings the HOMO, which is located at the lone-pair of the nitrogen close to the electron-rich oxygen and therefore raises the relative energy of the HOMO. Photoisomerization in the *cis*-form relieves this interaction. Another approach, which nearly maintains the planarity, is the introduction of fluorine-atoms in the *ortho*-position.⁸⁸ Instead of the repulsion effect of the lone-pairs of nitrogen and oxygen, the fluorine, as a σ -electron withdrawing group, reduces the *n*-electron density in the azo-bond and therefore lowering the *n*-orbital energy. As the planarity is not distorted, the $\pi\pi^*$ -transition is only slightly blue-shifted. Furthermore, it's causing a separation of the $n\pi^*$ transition bands by 42 nm. Light of wavelengths above 450 nm leads to a PSS of 91% *cis*-isomer and light of 410 nm leads to 86% *trans*-isomer. The most exceptional property, however, is the thermal stability of the *cis*-isomer with a half-life time of more than two years. Further research with the fluorine-substituents showed, that if only the 2/2'-positions is substituted the effect on the switching behavior is diminished and the half-life time is reduced.¹⁴⁵

Despite, the shown effects of substitution patterns, there exist a plethora of different azobenzene compounds. Some with more exotic behavior can be seen in Figure 1.8: *e.g.* introduction of an ethylen bridge in the *ortho*-positions leads to an highly twisted *trans*-isomer, which is thermodynamically disfavoured compared to the *cis*-conformer, with an half-life time of 4.5 h in hexane.⁸⁶ Furthermore the distortion of the planarity causes a separation of the $n\pi^*$ bands of 95 nm, which enables a isomerization yield of >90% *trans*-isomer with light between 370–400 nm and 100% *cis*-isomer with green light (480–550 nm).

An *ortho*-hydroxy group¹³⁹ leads to a similar behavior like a *para*-hydroxy group. It favours an hydrazone like structure and therefore possesses a half-life time below a second. In contrary to the

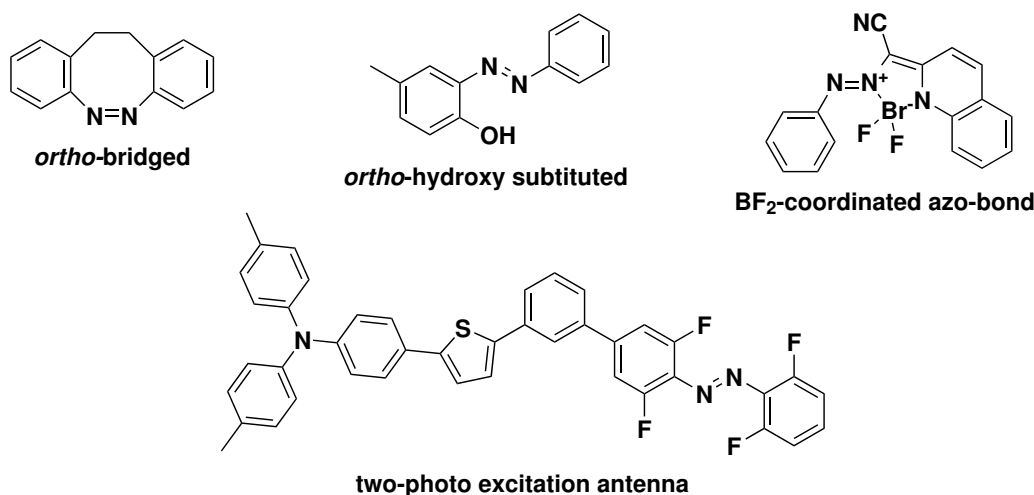


Figure 1.8: Further examples of rather exotic azobenzenes with interesting properties

para-position the *ortho*-position enables this behavior independently of the solvent.

The complexation of a BF_2 -unit with the nitrogen lone-pair, coupled with an extended π -electron system, alternates the energy of the $n\pi^*$ and $\pi\pi^*$ transition and leads to well separated transitions bands for both isomers of the latter in the visible range.¹⁴⁶

The last example shows an *ortho*- F_4 -azobenzene with a conjugated "antenna". The antenna is able to harvest low energy light in the near-infrared (IR) region (750 nm), which is used for *cis* $\rightarrow$ *trans*-isomerization triggered by two-photon excitation. In contrast, the reverse isomerization occurs with green light (510 nm).¹⁴⁷ Recent research also showed that two-photon isomerization with near-IR excitation is possible *via* intermolecular Förster resonance energy transfer.⁸⁹

Another approach is the use of photosensitizers and cage-like structures. While the photosensitizer enables the use of different wavelengths, the cage has a significantly higher binding ability towards the *trans*-isomer. This enables a PSS out-of-equilibrium of nearly 100%, which is not achievable under normal photoisomerization conditions.¹⁴⁸

1.1.5 Photoswitching of materials

From the myriad of photoswitches, azobenzenes offer currently the largest range of switching wavelengths and stabilities of photoactivated states. Therefore, they are widely distributed in material science. The Web of Science™-database counts more than 18.000 entries for the keyword "azobenzene" (effective 11.05.2025). There exists a plethora of reviews covering the different applications, *i.e.* movable materials,¹⁴⁹ optical materials,^{150,151} energy storage,¹⁵² polymers for biological use,¹⁵³ or in biological systems, respectively, in biomimetical systems.^{85,154,155}

The scientific discipline of investigating ways to influence the biologic ability of synthetic compounds is termed "photopharmacology".⁸⁵ To obtain photocontrol of proteins or peptides in particular, the photoresponsive entity has to stabilize or destabilize their interacting conformation. Thus, several approaches exist for photoswitches to achieve this for the protein of interest (Figure 1.9):

(i) Incorporation into ligand or inhibitor

(ii) Attachment in the side-chain or at the N-terminal/C-terminal end

(iii) Incorporation in the backbone

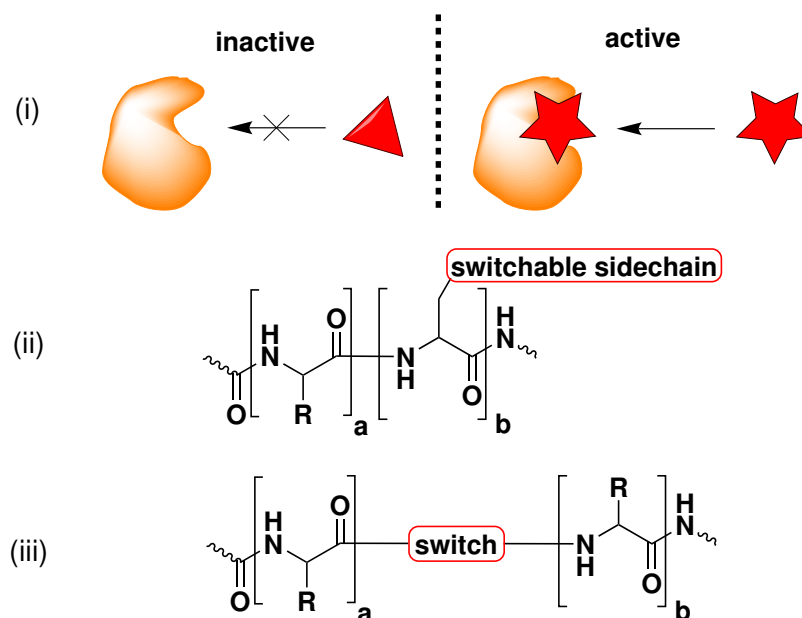


Figure 1.9: Approaches to achieve photocontrol of protein of interest. (i) Photochromic ligand switching between active (triangle) and inactive form. (ii) Photoswitch is attached to side chain. (iii) Photoswitch is introduced as a backbone modification.

In the first approach (i) the azobenzene is incorporated into the structure of a ligand or an inhibitor of a protein. These are usually smaller molecules and the azobenzene is introduced during the synthesis process. One of the azobenzene isomers forms the active molecule, while the other isomer forms an inactive form. The first use of azobenzenes in proteins is an example therefore (Table 1.4 #1).⁸⁰ A reversible inhibitor of acetylcholin esterase was synthesised bearing an azobenzene moiety. As both azobenzene isomers possess different effectivity, it was possible to modulate the activity of the enzyme over several switching cycles *in vitro* with UV light and sunlight.

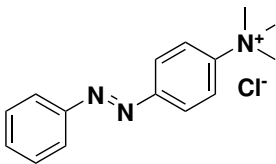
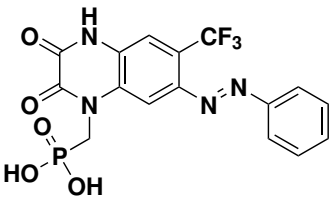
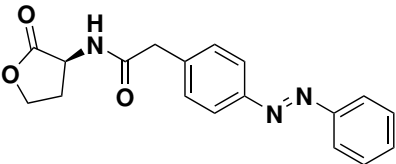
The molecule ShuBQX-3 (Table 1.4 #2) is a derivate of an antagonist of the AMPA receptor.¹⁵⁶ AMPA receptors are transmembrane receptors for glutamate and play a crucial role in excitatory neurotransmission. It could be shown that ShuBQX-3 was able to reduce selectively the activity of AMPA receptors *in vivo* with the *trans*-form as the active one. Interestingly, interaction of the photoswitchable antagonist and the receptor caused a red-shift of the $\pi\pi^*$ band and therefore enabled isomerization to the inactive *cis*-form with blue light.

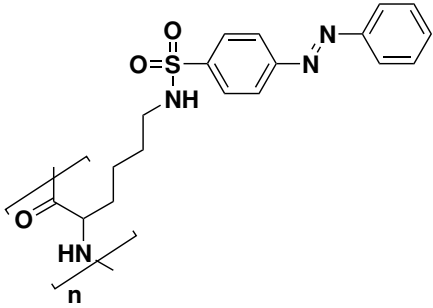
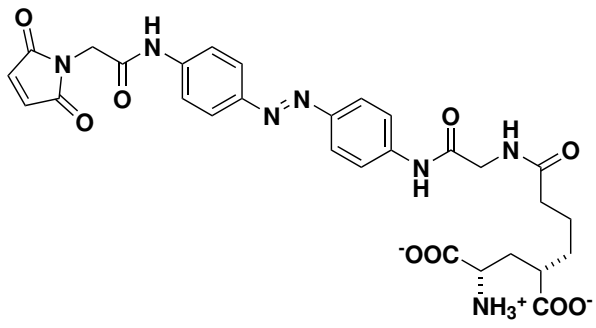
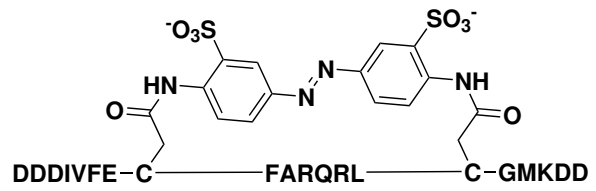
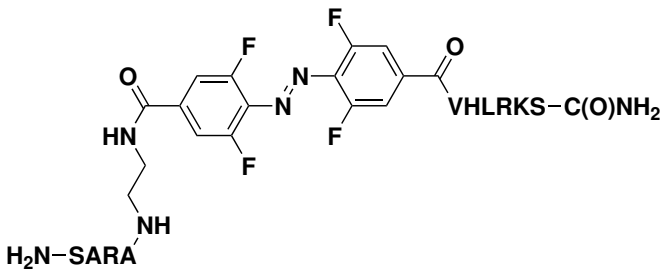
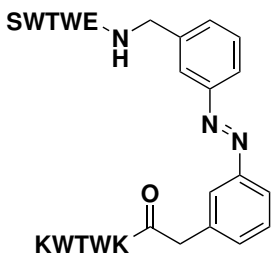
The third example (Table 1.4 #3) acts in the regulation of quorum sensing (QS) in bacteria. QS describes a process to detect cell density and synchronize gene expression *via* extracellular signal molecules. The combination of such an azobenzene and a signal molecule, an *N*-acyl homoserine lactone, enabled control over this process and therefore QS-related gene expression.¹⁵⁷ The *trans*-form was inactive, while the *cis*-isomer, generated with light of 365 nm wavelength, induces the production of luminescent molecules in an *E. coli* strain.

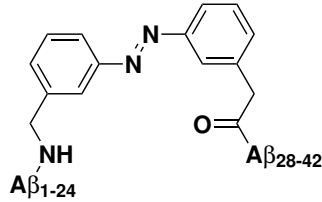
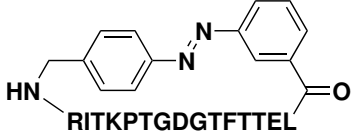
For the second approach (ii) the azobenzene is attached to the side-chains or at one of the terminal ends. Therefore, the protein can change spatial distances in the target molecule or between the target molecule and an attached structure. In this case the azobenzene is usually introduced after the protein was synthesised. Therefore, it has to bear moieties which can be linked to the protein. An example for that is azo-modified poly(L-lysine) (Table 1.4 #4).¹⁵⁸ The azobenzene was attached at the amino groups of the lysine side chain *via* a sulfonamide group. It was demonstrated that in mixtures of organic solvents, the conformation of photoresponsive polypeptides could be switched between a rather random coil and higher α -helical content with light of 340 nm and 417 nm wavelength.

Ionotropic glutamate receptors (iGluR) are ligand-gated ion channels responding to glutamate. A engineered iGluR was modified with the reaction of the thiol residue of cysteine with a maleimide bearing an azobenzene and a tethered glutamate residue (Table 1.4 #5).¹⁵⁹ The *trans*-isomer is the inactive form as it separates the glutamate entity from the binding site of the receptor, while the *cis*-isomer brings the glutamate close to the binding site. In *ex vivo* experiments the receptor was activated after irradiation with light of 380 nm wavelength and turned off with light of 500 nm wavelength.

Table 1.4: Selected examples of azobenzene modified materials

#	Structure	Target/construct	Ref.
1	 The structure shows an azobenzene core (two benzene rings connected by an -N=N- group). One benzene ring is substituted with a quaternary ammonium group, represented as -N ⁺ (CH ₃) ₃ Cl ⁻ .	acetylcholin esterase <i>in vitro</i>	[80]
2	 The structure features a central benzene ring. It has a trifluoromethyl group (-CF ₃) at the 2-position and an azobenzene group (-N=N-C ₆ H ₅) at the 4-position. At the 1-position, there is a maleimide ring system, which is further substituted with a phosphate group (-CH ₂ -P(=O)(OH) ₂).	AMPA receptor <i>ex vivo</i>	[156]
3	 The structure shows a maleimide ring system (a five-membered ring with two carbonyl groups and one nitrogen). This maleimide is linked via its nitrogen atom to a glutamate residue, represented as -CH ₂ -CH ₂ -COO ⁻ . The glutamate side chain is further substituted with an azobenzene group (-N=N-C ₆ H ₅).	quorum sensing <i>in vivo</i>	[157]

#	structure	target/construct	Ref.
4		poly(L-lysine) <i>in vitro</i>	[158]
5		iGluR <i>ex vivo</i>	[159]
6		β -adaplin <i>ex vivo</i>	[160]
7		WDR5 <i>in vitro</i>	[161]
8		Trp-zipper <i>in vitro</i>	[162]

#	structure	target/construct	Ref.
9		A β <i>in vitro</i>	[163]
10		neuronal NO synthase <i>in vitro</i>	[164]

Another linker strategy involving the thiol residue is based on the nucleophilic substitution of alkyl halides. In Table 1.4 #6 an endocytosis process was turned on and off *via* protein-protein interaction with β -adaptin.¹⁶⁰ The azobenzene bearing two *para*-chloroacetamido groups was attached by reaction with two cysteine residues in the designed peptides. Furthermore, the azobenzene possesses two *meta*-sulfonate groups to increase solubility. The resulting peptides were cell-permeable and taken up by various cell lines. The *cis*-isomer induced an α -helical conformation, generated by light of 380 nm, while the *trans*-isomer favors a random coil conformation, generated by light of 500 nm. The α -helical conformation binds the β -adaptin and inhibits the endocytosis process in *ex vivo* experiments.

The last approach (iii) comprises azobenzenes, which are incorporated into the protein backbone. Therefore, the azobenzene has to bear an amino and a carboxy moiety. In general standard SPPS technics are sufficient to integrate the switch directly in the peptide. However, if groups are not stable under standard SPPS conditions, *i.e.* *ortho*-fluoro groups can undergo nucleophilic substitution reactions,¹⁶¹ chemical ligation of peptides acts as an alternative synthetic route.¹⁶⁵ For the azobenzene in Table 1.4 #7 the chemical ligation was used to introduce the photoswitch into a peptidomimetic, which interacts with WDR5.¹⁶¹ WDR5 is essential for the histone-methyltransferase activity of MLL1. The novel peptidomimetics showed affinity to WDR5, but the configuration of the azo double bond showed only a minor impact. This was the first attempt of incorporating visible light switchable *ortho*-fluoro azobenzenes into peptides.

The AMPP ([3-(3-aminomethyl)phenylazo]phenylacetic acid) photoswitch is designed to link two peptide chains.¹⁶⁶ Therefore it bears the necessary groups in the *meta*-position linked through a methylene group. The β -hairpin is the smallest known structural motif consisting of short β -strands. However, they are only marginally stable and are more difficult to characterize. In Table 1.4 #8 the AMPP was incorporated into the known β -hairpin forming Trp-zipper motif.¹⁶² The *meta*-, *meta*-substitution has proven to be suitable in the *cis*-form to stabilize the β -hairpin. In contrary, the *trans*-form preferred a disordered structure.

Based on this the AMPP switch was introduced in amyloid- β (A β , Table 1.4# 9)¹⁶³ to test the hypothesis whether turn nucleation affects self-assembly of A β . They replaced three amino acids in the turn region by the AMPP switch and were able to show that the *trans*-isomer self-assembles into

aggregates, while the *cis*-isomer only forms amorphous structures.

Another used azobenzene is the 3,4'-AMPB switch, which bears the carboxy group in the *meta*-position and the amino group with an methylene linker in the *para*-position.¹⁶⁷ For example in Table 1.4 # 10, the 3,4'-AMPB entity was included into the internal recognition motif of neuronal NO synthase.¹⁶⁴ The peptidomimic is a cyclic peptide that adopts in the *cis*-form a β -finger, an internal β -hairpin, which activates the enzyme. The *trans*-isomer, however, showed almost no binding.

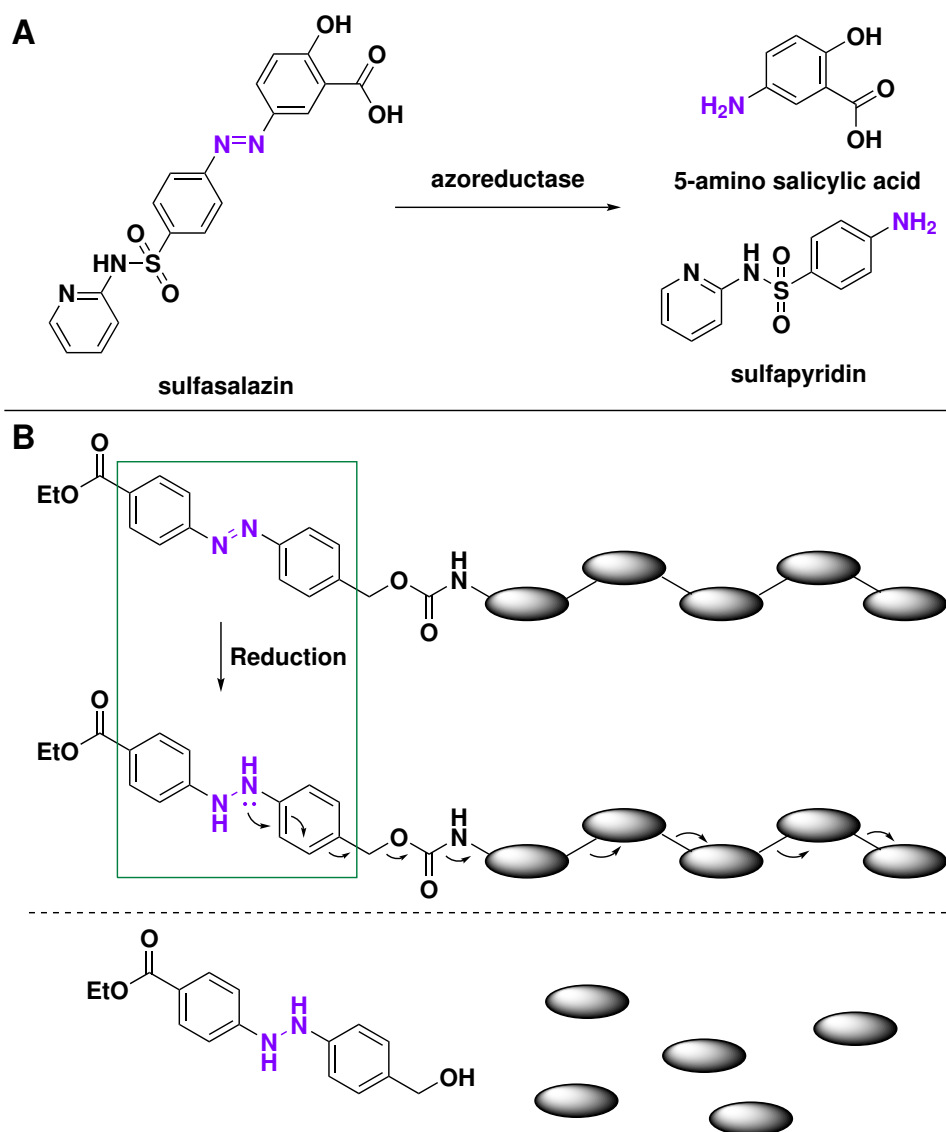


Figure 1.10: Applications of azobenzene degradation. (A) The sulfasalazin prodrug is metabolized through bacterial azoreductases to active compounds 5-amino salicylic acid and sulfapyridin. (B) Self-immolative polymers: reduction of azobenzene group causes depolymerization. The ellipsoids represent the monomeric units. Reprinted and adapted with permission from the American Chemical Society, copyright ©2014.¹⁷²

It has to be taken into consideration, that in *in vivo* and *ex vivo* experiments reduction reactions and reductive environments in cells can limit the availability of the respective molecule. The main

degradation pathways are the NADPH dependent reduction *via* azoreductases to anilines¹⁶⁸ and the glutathion mediated reduction to hydrazines.¹⁶⁹ In other contexts, however, this can be a desired property, *i.e.* prodrugs,¹⁷⁰ mycel based drug release,¹⁷¹ or in self-immolative polymers.^{172,173} In medicine for example the 1935 discovered Prontosil, serves as a prodrug that is metabolized to sulfanilamide. Sulfasalazin (Figure 1.10A) is metabolized in the colon through bacterial azoreductases and the metabolites sulfapyridin and 5-amino salicylic acid serve as anti-rheumatic agents against inflammatory diseases.¹⁷⁰ The sensitivity to reducing agents can also be used in polymer science as a trigger for self-immolative polymers. In Figure 1.10B the schematic structure of a self-immolative polycarbamate with a single azobenzene end-cap is shown.¹⁷² Upon reduction, including hydrazine and dithiothreitol to hydrazobenzene, an 1,6-elimination reaction occurs followed by depolymerization of the polymer.

1.2 Spiropyrans

Another class of widely used photoswitches are the spiropyrans. These photoswitches belong in contrast to the azobenzene to the second group of photoswitches, which undergo isomerization through pericyclic reactions. In the case of spiropyrans, irradiation with UV light generates the coloured merocyanine (MC)-isomer *via* ring-opening and visible light regenerates the colourless spiropyran (SP)-form through cyclization (Figure 1.11). The MC-form is a hybrid of its two resonance structures, the quinoidal and the zwitterionic MC. As a chemist the term spiropyran is somewhat misleading: from nomenclature aspects spiropyran would refer to all pyran-isomers, where the protons at one position are replaced by a spiro functionality, a second ring system linked to the carbon atom. However, nowadays it is associated with indolino-benzospiropyrans, a 2H-pyran-isomers bearing an indole as the second ring (Figure 1.11),^{174–176} and we will focus on this type in the following section.

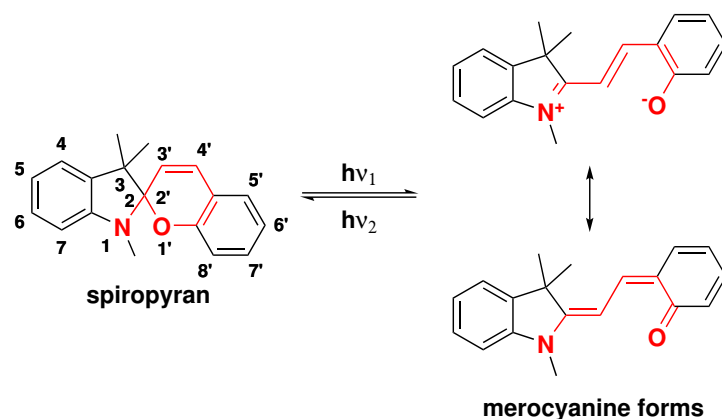


Figure 1.11: Isomerization process of spiropyrans: (left) spiropyran-form (SP), (right) resonance structures of the merocyanine-form (MC). The ring system participating in isomerization is highlighted in red.

What makes spiropyrans special compared to most other photoswitches are the strongly different properties of the SP- and the MC-form. Furthermore, beneath the photoisomerization there exist several further triggers: *e.g.* solvent polarity,¹⁷⁷ metal ions,¹⁷⁸ acidity/basicity,¹⁷⁹ temperature,¹⁸⁰ redox potential,¹⁸¹ and mechanical force.¹⁸² This enables, dependent of the environmental properties both positive, the colourization under UV light, and negative photochromism, the decolourization under visible light.

1.2.1 History

The story of spiropyrans begun more than 70 years after the discovery of azobenzenes in the year 1908. The first description is attributed to H. Decker and H. Felser, who synthesised dipheno-spiropyran (IUPAC: 2,2'-spirobi[chromene], Figure 1.12) starting from dicumarketon, the aldol condensation product of one equivalent (eq.) acetone and two eq. salicylaldehyde.¹⁸³ Spiropyrans first attracted attention when their thermochromism was reported. Three articles appeared in 1926 describing, that dinaphtho-spiropyran, colourless in solution and as solid, turn to a violet-blue colour simply by heating.^{184–186} Furthermore, they assumed the formation of a zwitter ionic species. During the following

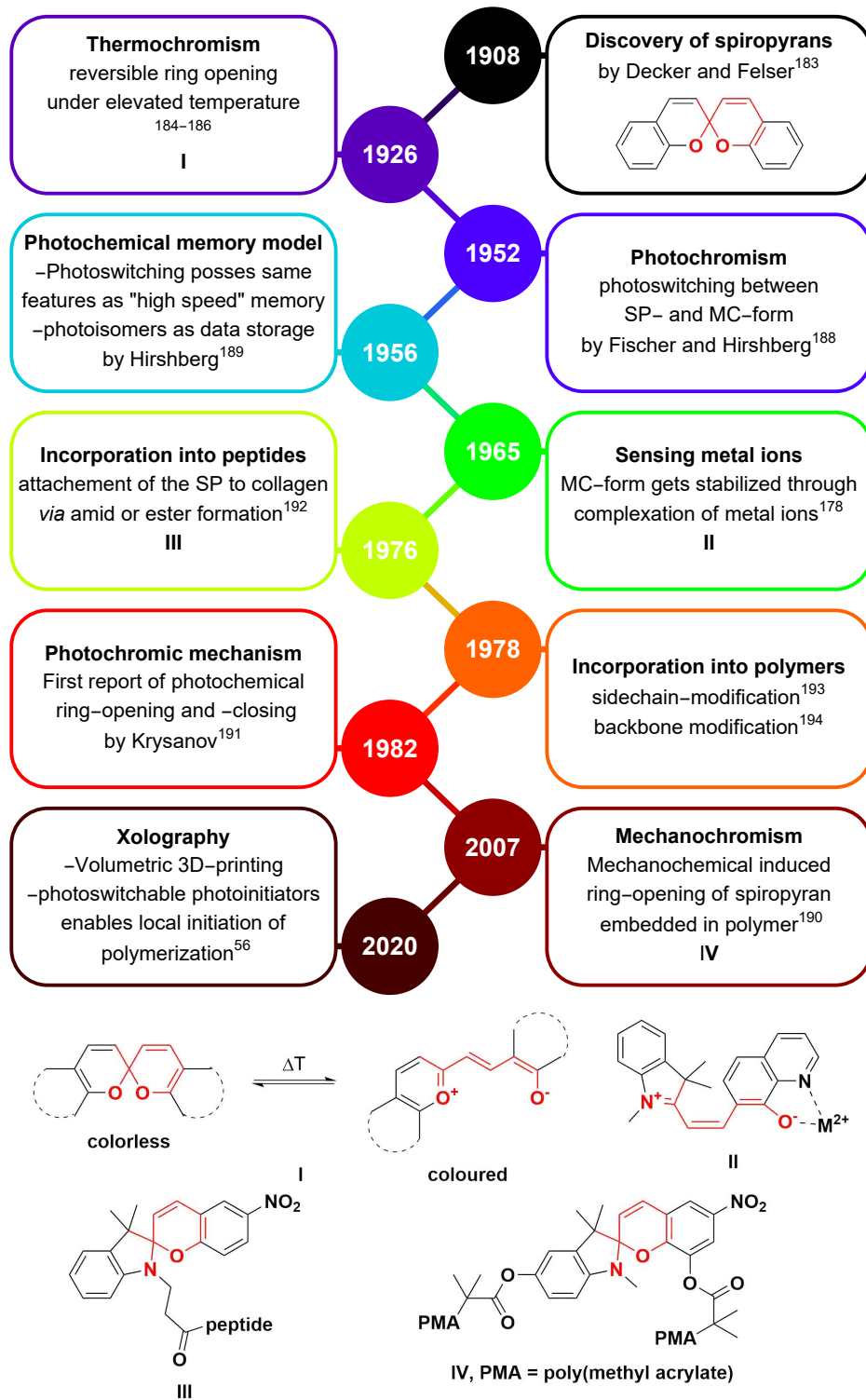


Figure 1.12: Milestones in the development of spiropyrans.

decades, the non-photochromic spiropyrans were further investigated and new properties were reported, *e.g.* their acidchromic behavior.¹⁸⁷ However, it was not until 1952, that E. Fischer and Y. Hirshberg reported the photochromic behavior of the nowadays with the term spiropyran associated structure (Figure 1.11), the condensation product of salicylaldehydes with a Fischer base.¹⁸⁸ Based on these insights Y. Hirshberg reported 1956 a reversible photochemical process, which possess the same features as computer memory, the first photochemical memory model.¹⁸⁹ In the following years, further findings were obtained about the switching behavior and the photoswitching process in detail. The metal ion sensitivity was reported 1965,¹⁷⁸ while the mechanochromic properties were not known until 2007.¹⁹⁰ The mechanism of the phototriggered isomerization process, however, was elucidated three decades after the discovery in 1982.¹⁹¹ To use the property of photochromic molecules in materials, the photoswitch has to be brought into spatial proximity of the respective material. The first application on proteins was reported 1976, when a spiropyran was attached to the sidechains of a collagen membrane and enabled the control of the activity of urease.¹⁹² The first introduction in the side-chain of synthetic polymers was two years later.¹⁹³ A spiropyran modified methacrylate was copolymerized with methylmethacrylate allowing the photoreversible change of the polymer viscosity. The introduction in the backbone of macromolecules did not take place until 2013, when spiropyran backbone modified polymers were synthesised *via* Suzuki-polycondensation.¹⁹⁴

1.2.2 Synthesis

Compared to the variety of synthetic methods towards azobenzenes, the synthetic approaches towards spiropyrans can be reduced to one standard method: The condensation of *ortho*-hydroxy aromatic aldehydes, with methylene bases, in our case indole derivatives, or their precursors (Figure 1.13). The proton, which interacts in the reaction is derived from the solvent molecules, most often alcohols.¹⁹⁵

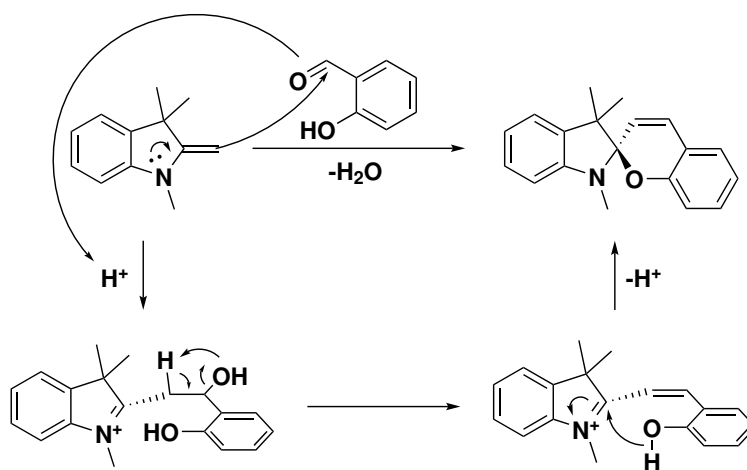


Figure 1.13: General reaction mechanism for condensation of indolene species with *ortho*-hydroxy aromatic aldehydes towards spiropyrans.

Despite the single synthetic method, a variety of different structural features are possible due to simple access to the precursors. However, especially alterations in the pyran ring require a slightly different reaction management (Figure 1.14).¹⁹⁵ Two example are, on the one hand, the introduction of a

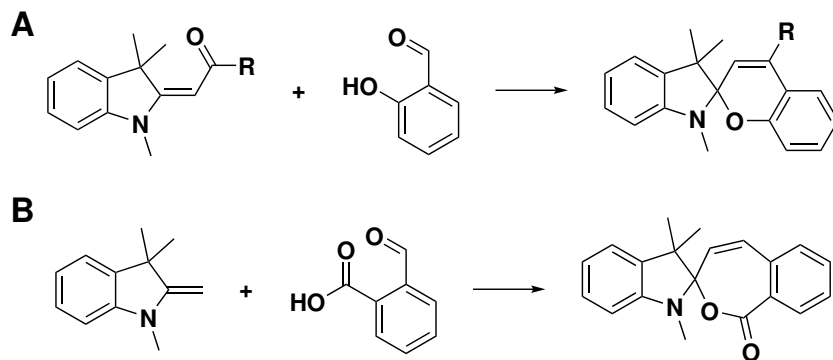


Figure 1.14: Modification of the reaction procedure to obtain pyran-ring modified spiropyrans. (A) Reaction procedure towards 4'-substitution of the pyran ring. (B) Reaction procedure towards spiropyrans containing instead of the pyran ring a oxepin-2(3H)-one ring.

substituent in the 4'-position, which is obtained by replacing the indole(-precursor) with an acyl derivate thereof (Figure 1.14A) and adding phosphoroxychloride as an agent. On the other hand, replacing the *ortho*-hydroxy group by a carboxy group of the aromatic aldehyde led to spirooxyepinones with a seven-membered ring instead of the six-membered pyran ring (Figure 1.14B).

Spiropyran isomers differ vastly in their physicochemical properties and show significant differences in their structures. The SP-form, with the tetrahedral C_{spiro} , occupies less volume than the planar structure of the MC-isomer.¹⁹⁶ The alteration in the electronic structure results further in a large charge separation of the MC-form and therefore, gives a large rise in the electric dipole moment, ~ 14 D, compared to the SP-form, ~ 6 D.^{197,198} Additionally, the changes in the electronic structures strongly alter the spectroscopic properties. The colourless SP-isomer, with the closed pyran-ring, possesses two transition bands. The $\pi\pi^*$ transition of the indole moiety is located around ~ 285 nm and the $\pi\pi^*$ transition of the chromene part is red-shifted towards ~ 340 nm.¹⁹⁹ The MC-isomer, however, appears with a deep blue colour and exhibits a new absorption maxima in the range of 550–600 nm.¹⁷⁴ In addition, they differ not only in their absorption behaviour, but also in their emission behaviour. While the SP-form does not show any strong emission, the extended π -conjugated system of the MC-form exhibits a strong emission of red light centered ~ 650 nm.²⁰⁰ The MC-form possesses further special properties compared to the SP-form: It has a significantly increased basicity. The protonation leads to the protonated MCH^+ -isomer, which exhibits a characteristic band in absorption spectrum ~ 420 nm.²⁰¹ The MC-form also tends to aggregation, mainly driven by dipol-dipol interaction and promoted by $\pi\pi$ stacking, whereby the aggregation takes place readily in non-polar solvents.^{202,203} To understand the isomerization process, we must be aware that the MC-form adopts, additionally to its two resonance structures, eight isomers (Figures 1.15A). These isomers, firstly reported 1953,²⁰⁴ result through rotation around three bonds (indicated through α , β , γ), and are energetically distinct. At any given temperature a weighted average of all states is obtained. The nomenclature consisting of three letters follows an *cis/trans*-orientation over the three adjoining bonds.¹⁷⁵ The isomers can be divided into two forms dependent on the configuration of the double bond (indicated with β , Figure 1.15A): The cisoid-MCs (*cis*-configuration) and the transoid-MCs (*trans*-configuration).

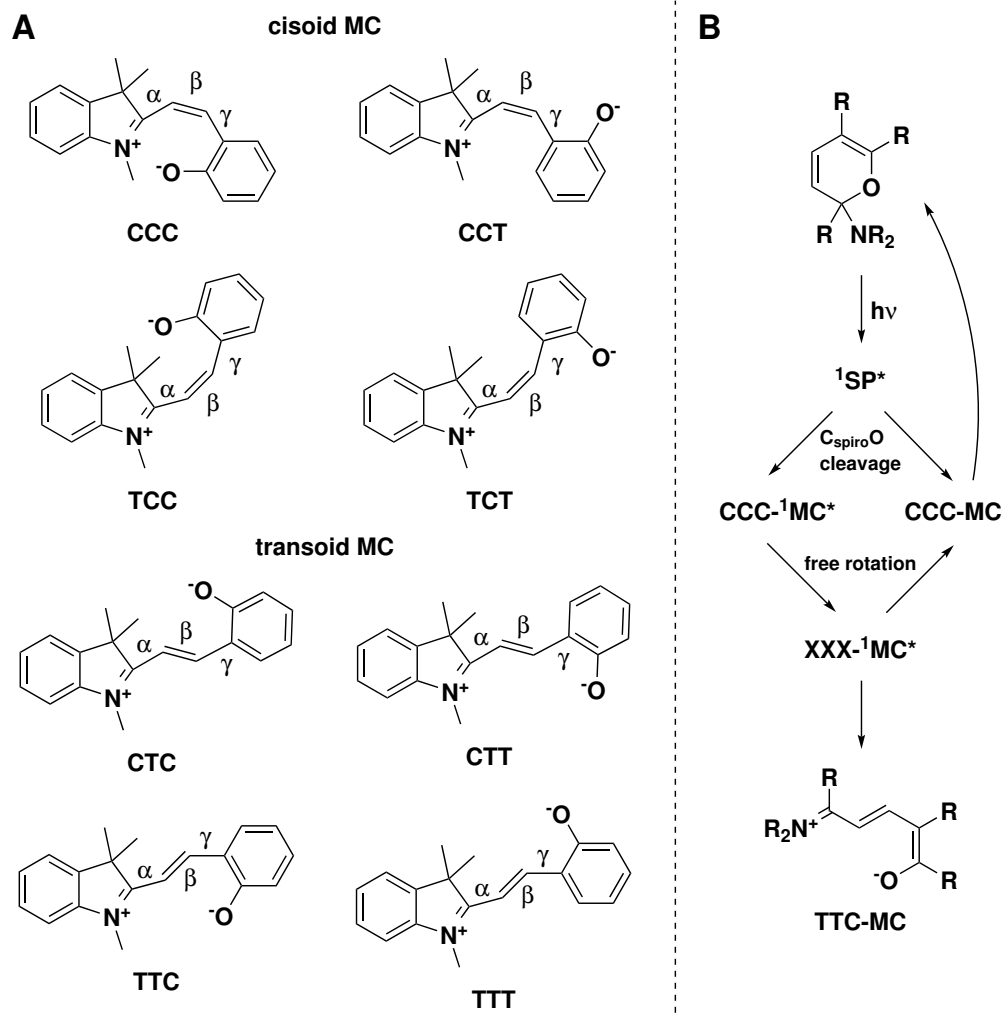


Figure 1.15: (A) The distinct isomers of the MC-form. The nomenclature refers to the spatial orientation (C - Z, T - E) of the three bonds (indicated as α , β , and γ) between the C_{spiro} and the phenolate ring. The isomers are divided into two groups, cisoid and transoid, depending on the configuration of the double bond (β). (B) The general mechanism of SP $\rightarrow$ MC-photoisomerization. XXX- $^1MC^*$ refers to species with unknown geometry, X can be either C or T. Reprinted and adapted with permission from the Royal Society of Chemistry, copyright ©2019 (open access).¹⁷⁵

The first step in the UV triggered SP $\rightarrow$ MC-isomerization is electronic excitation of the SP-isomer, which undergoes subsequently cleavage of the $C_{spiro}O$ -bond, thus, resulting in the CCC-MC* (Figure 1.15B).¹⁹¹ The exact mechanism depends on the substitution pattern of the spiropyran. Spiropyrans without additional substituents access a singlet state $^1SP^*$, where it loses its double bond character through a $\pi\pi^*$ transition. As electronic processes are faster than nuclear motions, the cleavage of the $C_{spiro}O$ bond reduces the energy before rotation processes take place. Through rotation processes of the three bonds (α , β , γ) a metastable species XXX- $^1MC^*$ is obtained, whereby X can be either C or T. This species can either undergo pericyclic rearrangement towards the SP-form or relaxate into the ground state. This ground state leads to thermally unstable cisoid MCs or thermally stable transoid MCs.¹⁷⁵ In contrast, 6'-nitro substitution facilitates the access to triplet state *via* intersystem crossing from the

singlet $^1\text{SP}^*$ to $^3\text{SP}^*$ state,²⁰⁵ which is similar to the $\text{S}_1\text{-T}_1$ intersystem crossing of nitro substituted stilbenes.¹⁷⁵ Furthermore, the $\text{SP} \rightarrow \text{MC}$ -isomerization is also accessible through two-photon excitation in the near IR range.²⁰⁶ The inverse photoreaction of the $\text{MC} \rightarrow \text{SP}$ is more complex, as the MC-form not only exist in an equilibrium of the zwitterionic and the quinodial form, but also in a mixture of the most stable MC-isomer TTC and a minor amount of TTT.²⁰⁷ A possible mechanism starts with the excitation of the MC ground state in a excited S_1 state with a destabilized double bond (β), which lowers the activation energy to adopt a twisted form. The twisted or *cis*-intermediate undergoes either ring closure towards the SP-form or re-forms the excited *trans*-MC S_1 , which relaxes in the ground state MC S_0 .²⁰⁸

Despite the irradiation with light, the equilibrium of the MC-form and the SP-form is dependent of a variety of other environmental factors. The influence known for the longest time is the temperature, as mentioned in the history section (1.2.1).^{184–186} The thermochromism of spiropyrans was even in the early 1960s one of best studied.²⁰⁹ Interestingly, the reversible colour change was not reported for the first spiropyran, the dibenzospiropyran. The insight, that the polarization of the spiro-centre is accessible through introduction of a indolino group,²¹⁰ was a breakthrough. While in non-polar solvents SP is the dominant form, elevated temperatures lead to a reversible shift in the equilibrium towards the MC-form. While the photoisomerization mechanism ends with the formation of the zwitterionic resonance structure, the thermal isomerization leads through an electrocyclic reaction path to the quinodial resonance structure (Figure 1.16).¹⁷⁴

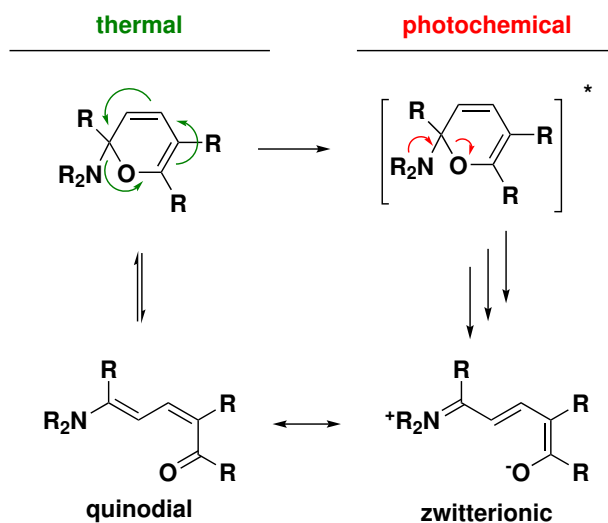


Figure 1.16: Simplified mechanism of the thermal and the photochemical isomerization of spiropyrans.

Another factor to alter the equilibrium is the solvent polarity. The solvatochromism of spiropyrans is known since the end of the first half of the twentieth century.²¹¹ In non-polar solvents the SP is the dominant form and therefore positive photochromism occurs. Interactions with polar solvents, however, stabilizes and lowers the energy level of the MC-isomer,²¹² which is why the MC is the dominant form and negative photochromism occurs. A phenomenon we could already observe in the solvatochromism of the spiropyrans is the charge-induced ionization, which leads to the coloured MC-form. This effect is further enhanced by the presence of protons, referred to as acidochromism, or the presence of metal

ions, referred to as metalochromism.¹⁷⁵ The formation of coloured species through addition of acids is a general phenomenon of spiropyrans and was already known before the photochromism.²¹⁰ Important for the protonation driven ring-opening is the strength of the acid. In 1929 it was demonstrated, that acetic acid is not able to induce acidochromism, whereas the replacement of the hydrogen atoms with chlorines in trichloroacetic acid enabled the acidochromic behavior of dinaphthospiropyrans.¹⁸⁷ For indolino spiropyrans this effect was reported nearly seven decades later in 1995.²¹³ Interestingly, the acidochromism enables an access to the actually non-available cisoid-MCs. Both, the transoid and cisoid forms, differ in their pK_a .¹⁷⁵ The use of acids, like hydrochloric acid or trifluoroacetic acid promotes the formation of transoid-MCs. Use of stronger acids, *e.g.* promotes the formation of a relative stable *cis*-MC form undergoing slow thermal isomerization towards the *trans*-form. This enables a further photochromic behavior, as the *cis*-MCH⁺ is generated from the *trans*-MCH⁺ by irradiation of light of 455 nm and *vice versa* with irradiation of 365 nm.²¹⁴

The equilibrium of the MC-form and the SP-form can be adjusted by using different environment conditions. Similarly, applying mechanical force can cause a change of the molecular shape and therefore induce the ring-opening of the spiropyran.^{190,215} It is necessary that the stress acts on both sides, the indolene and the chromene unit, of the spiro moiety (Figure 1.17). The pulling apart causes a breakage of the C_{spiro} -O bond and therefore a formation of the MC-form. Furthermore, grinding or ultrasound can induce strain that cause also a mechanochromic response.^{190,216}

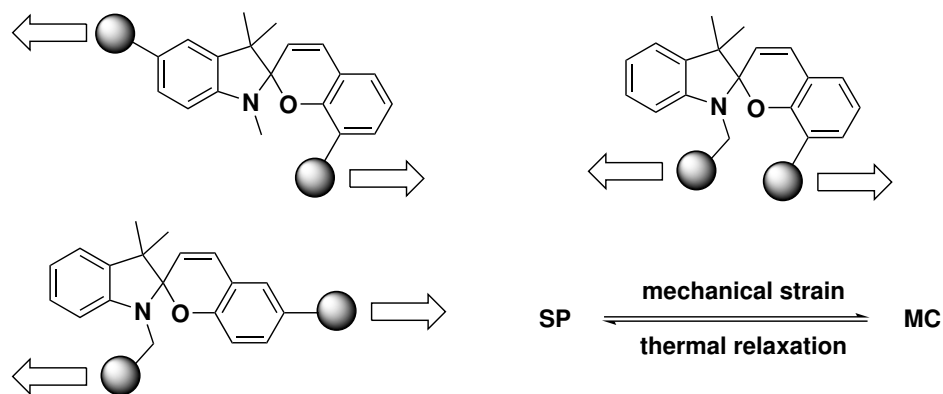


Figure 1.17: Types of mechano-activated spiropyrans. The types are based on the position of the attachments (direction of the force is indicated by the arrows). Reprinted and adapted with permission from the Royal Society of Chemistry, copyright ©2019 (open access).¹⁷⁵

1.2.3 Structure-activity relationship

As we could already observe, the thermochromism of spiropyrans is dependent on the structural modification. Dibenzospiropyran is not able to undergo reversible thermochromism, but dinaphtho- or indolinospirans, which can be easier polarized, exhibit this property.²⁰⁹ Similarly, changes in the substitution pattern of indolinospirans offer an access to modify the equilibrium between the SP- and the MC-form, as well as between the zwitterionic and the quinoidal MC. An important factor are substituents on the pyran ring, which can stabilize or destabilize the phenolate formed upon the ring-opening. Electron withdrawing substituents, *e.g.* a nitro group, in the 6'- and 8'-position stabilize

the phenolate. Therefore, they enable instead of the singlet a triplet pathway for the photoinduced SP $\rightarrow$ MC-isomerization and shift the thermal equilibrium in polar solvents to the zwitterionic MC-form, as the ring-opening reaction is promoted.^{175,205} However, the electron withdrawing substituents reinforce as well photobleaching processes. In contrast, electron donating substituents, which lower stability of the phenolate, reduce the formation of the MC-form, respective its thermal stability.²¹⁷ This observation is supported by comparison of the pK_a value of the corresponding MCH^+ . The less stable phenolate anion of MCs with electron donating substituents possesses a higher pK_a value than the phenolate anion of MCs with electron withdrawing substituents. While the *vide supra* described substituent effects on the 6'- and 8'-position favour the zwitterionic resonance structure, electron donating substituents, e.g. in the 5'- or 7'-position enhance the amount of the quinoidal resonance structure of the MC-form without charge separation.^{175,218}

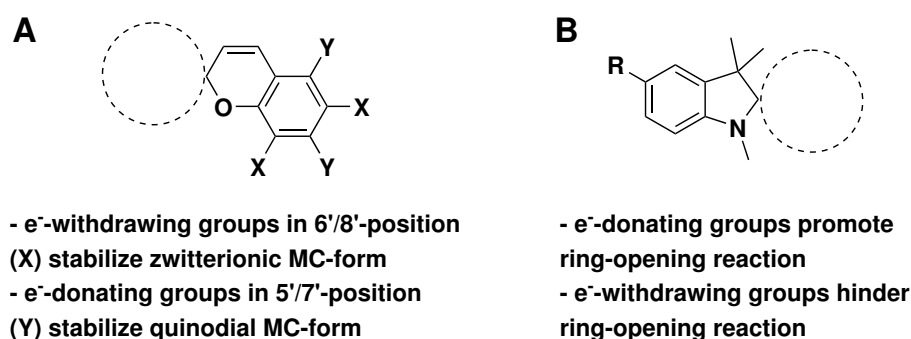


Figure 1.18: Influence of the substituents on the SP-MC-equilibrium. (A) Substituent influence of the chromene unit. (B) Substituent influence on the indolene unit. Reprinted and adapted with permission from the Royal Society of Chemistry, copyright ©2019 (open access).¹⁷⁵

Substituents on the chromene unit mainly (de)stabilize the phenolate anion, whereas substituents at the indolene unit affect the stability of the N^+ -cation of the indolene ring. It was shown, that the introduction of electron donating substituents can shift the equilibrium also to the MC-form and *vice versa*. Electron withdrawing groups at the 5-position of the indolene destabilize the cation and therefore, decrease the rate of ring opening.^{217,219}

1.2.4 Photoswitching of materials

Spiropyrans are not as widely distributed as azobenzenes. Nevertheless, the Web of ScienceTM-database counts still more than 3.000 entries for the keyword "spiropyran" (effective 26.02.2025) and they are used in material science, due to their multiple possibilities to trigger isomerization and their facile synthesis.^{174,175,220,221} For polymers, methods are known to incorporate them in the side chain¹⁹³ and the backbone.¹⁹⁴ However for peptides, only methods are known to introduce spiropyrans in the side chain of amino acids and not use them as backbone modifications.¹⁹²

In general, the spiropyran structure offers four easy accessible positions, where linker units/functional groups can be introduced to attach the spiropyran to materials (Figure 1.19, Table 1.5). These are at the indolene subunit the 5-position and on the chromene side the 6'- and the 8' position, whereby the attachment occurs *via* amide, esters, or alkyl linkers. A special case is the 1-position at the

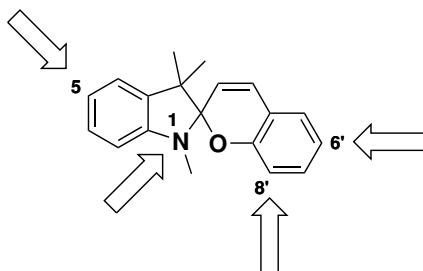


Figure 1.19: Positions used to incorporate/attach spiropyran moieties to polymers and peptides.

nitrogen-atom, where only alkyl linkers are available.

The first report of a spiropyran attached to a polymer was in 1978 (Table 1.5, #1).¹⁹³ Two spiropyran units containing an alkylester linker to polyethylacrylate at the 8'-position were connected *via* a xylylene linker at each 1-position. The aim of the study was to investigate the photocontractile behavior of the obtained photochromic rubbers and they could show, that the SP-MC equilibrium is able to mediate shrinking/recovery behaviour of the surrounding media *via* photoisomerization and thermal relaxation.

Table 1.5: Selected examples of spiropyran modified materials

#	Structure	Target/construct	Ref.
1		polyethylacrylate (PEA)	[193]
2		self-assembled micelles	[222]

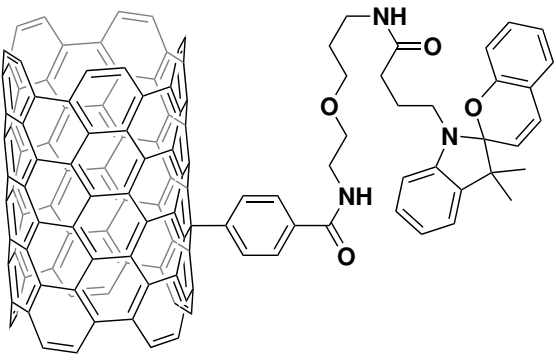
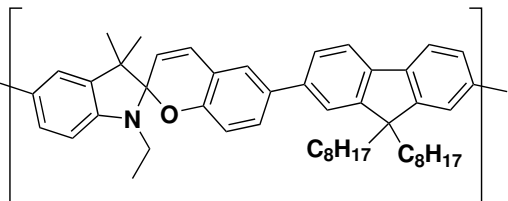
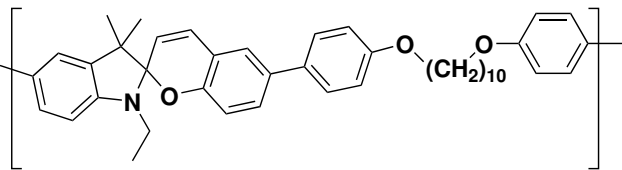
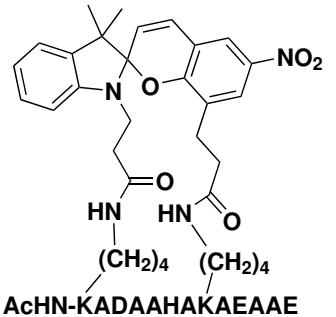
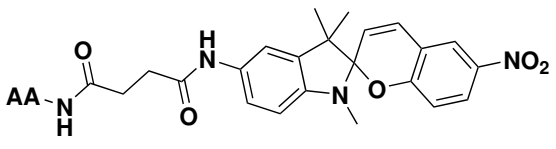
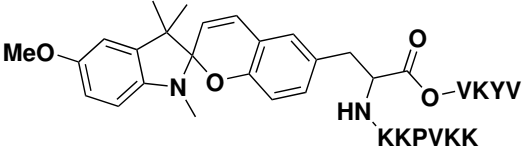
#	structure	target/construct	Ref.
3		single-walled nanotubes	[223]
4		alternating copolymer	[194]
5		alternating copolymer	[182]
6	 AcHN-KADAHAKEAEAE	helix forming peptide	[224]
7		peptide hydrogel	[225]
8	 MeO-CH2-CH2-CO-NH- KKPVKK	membrane active peptide	[226]

Table 1.5 #2 shows a spiropyran, bearing at the 1-position an 2-hydroxy ethyl group, that was used to initiate ring-opening multibranching polymerization of glycidol.²²² The so formed spiropyran-

hyperbranched glycerol-conjugates comprise of an hydrophobic head, the spiropyran moiety, and an hydrophilic tail, the hyperbranched glycol. This enables reversible self-assembly of polymeric micelles in the SP-form, while photoswitching transform the hydrophobic head to the hydrophilic MC-form and therefore the micelles disassemble. This assembly/disassembly process was especially interesting as it shows potential for smart drug delivery systems of hydrophobic therapeutics. The next example (Table 1.5, #3) shows a spiropyran bearing a propionic acid group at the 1-position. This was anchored to a single-walled carbon nanotube scaffold, modified by Tour reaction,²²⁷ *via* a bifunctional amide linker.²²³ It was demonstrated that the spiropyran was able to reversibly complexate Zn^{2+} ions in the MC-form and release them upon photoisomerization to the SP-form. Furthermore, the effect could be extended to potential drug delivery system, as the MC- Zn^{2+} -complex can bind an additional molecule, *e.g.* salicylic acid, which is released as well upon photoswitching.²²⁸ Completely different approaches are listed in Table 1.5 #4 and #5, where the spiropyran is part of the polymer backbone. #4 shows the first reported example for alternating copolymers with a spiropyran as one monomer.¹⁹⁴ The synthesis followed a Suzuki polycondensation, with a spiropyran bearing two bromine substituents in the 5- and 6'-position and the fluorene monomer bearing two pinacolborane substituents. It was furthermore demonstrated, that the SP→MC-isomerization can be accessed *via* mechanical strain induced through ultrasonication. In #5 polymers were obtained in a similar manner, except that another monomer was used.¹⁸² The C_{10} -alkyl chain made the polymer more flexible and enabled to create nanofibers through electrospinning from dimethylformamide (DMF)/ tetrahydrofuran (THF). The obtained fibers were further embedded in an polydimethylsiloxane matrix and the composites showed reversible mechanoresponsiveness upon stretching. Remarkably, the use of fibers caused an anisotropic behaviour of the mechanochromism, depending if the material is stretched perpendicular or parallel to the fiber direction. The mechanochemical properties of such spiropyran mainchain polymers can be further tuned through variation in the substitution pattern of the spiropyran.²²⁹

In peptides, the use of spiropyrans is limited to modifications of the side chain and of the N-/C-termini. To date, no use as a modification of the peptide backbone is known. A disadvantageous factor might be, that spiropyrans in the MC-form suffer hydrolysis in aqueous media at neutral pH²³⁰ and therefore, their use is often limited to non-aqueous media, aqueous media with acidic pH, or targets with locally modified pH. Table 1.5 #6 shows a peptide cross linking agent composed of a spiropyran with two propionic acid linkers in the 1- and the 8'-position.²²⁴ The carboxy groups were attached to the lysine side-chains *via* amide formation to a short peptide sequence known to form helical structures. With circular dichroism (CD) spectroscopy it was demonstrated that the spiropyran form, obtained with indoor lighting, stabilizes the helical structure, while the MC-form, obtained in the dark through thermal equilibration, destabilizes the helical structure. The hydrogel forming dipeptide conjugate in Table 1.5 #7 is composed of two D-alanine aminoacids linked at the N-terminal end to a spiropyran bearing an amino group at the 6'-position with a succinic acid.²²⁵ The MC-form, which is known to form aggregates through $\pi\pi$ -stacking interactions of the conjugate,²³¹ tends to form self-assembled hydrogels at low pH in aqueous solution. Photoisomerization to the SP-form, which prevents the $\pi\pi$ -stacking, causes a disassembly of the hydrogel. Furthermore, the use of the two D-alanine moieties enable specific ligand-interaction with vancomycin.²³² This adds a second response mechanism MC-hydrogel, as the addition of vancomycin causes as well a disassembly of the peptide hydrogel. While usually the

photoswitchable moiety is attached *via* a linker unit to the respective peptide, the approach in Table 1.5 #8 shows a spiropyran-derived amino acid. In the 6'-position of the chromene ring an L-alanyl is directly affixed.²²⁶ The amino acid was introduced with SPPS to generate analogues of BP100, a membrane active peptide. BP100 is known to form amphiphilic helices. In the analogues the polar MC-form is destroying this amphiphicity, while the less polar SP-form maintains it. Furthermore, it was demonstrated, that the SP-form interacts with membranes in a similar manner than the original peptide, whereby the MC-form with destroyed amphiphicity does not exhibit an interaction.

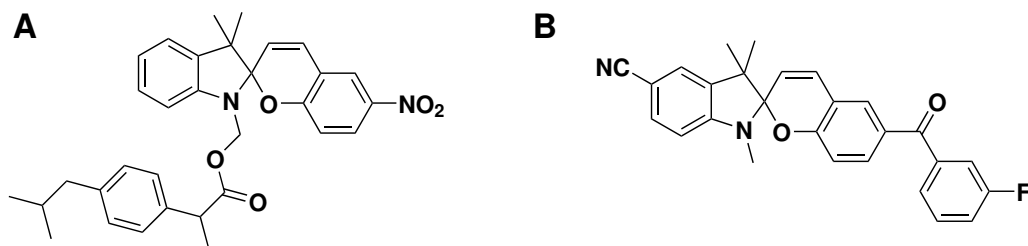


Figure 1.20: Other modes of actions of spiropyrans. (A) Ibuprofene prodrug for micellular release.²³³ (B) Dual colour photoinitiator for xolography.⁵⁶

Another approach to use spiropyrans with a potential medical application is shown in Figure 1.20A. The smart prodrug is a conjugate of a spiropyran and ibuprofene, which are connected *via* a hemiaminal.²³³ The study uses different hydrophilic/hydrophobic behaviours of the SP- and the MC-form in a photo-activated drug delivery system. The nonpolar SP-form of the conjugate can be encapsulated in micelles with hydrophobic cores. Photoisomerization generates the polar MC-form, which is subsequently released from the micelles into the surrounding environment. The released pro-drug can be activated through esterases, which releases the ibuprofen moiety. A last example of spiropyrans in material science is the use in a volumetric 3D-printing method termed as xolography (Figure 1.20B).⁵⁶ The shown spiropyran contains a benzophenone type II photoinitiator. A first light sheet in this technique excites the dormant photoinitiator molecules, SP-form, in a latent state, which is regenerated through thermal isomerization after a specific lifetime. A projector orthogonal to this light sheet uses a second wavelength and focus sectional images of a 3D-model in the light sheet, whereby only initiators in the latent form absorb the light of the projector and therefore cause polymerization in the current layer.

1.3 Amyloidogenic proteins and peptides

Self-organization of molecules without guidance from the outside is referred to as molecular self-assembly. It is one of the key concepts in supramolecular chemistry²³⁴ and provides an alternative way to creating (functional) nano structures using a bottom-up approach.²³⁵ Furthermore, it is one of the fundamental principles of life: lipids and biopolymers (*e.g.* polysaccharides, polypeptides and polynucleotides) are able to arrange into nano- and microscale architectures.²³⁷ These architecture are able to perform a variety of functions, *e.g.* separation of cells and cell compartments,²³⁸ acting as recognition elements,²³⁹ store and transmit informations,²⁴⁰ record and react to changing external influences.²⁴¹ In particular, proteins and peptides are of great interest^{237,242} due to their compositional complexity, their simple synthesis,²⁴³ and as well their inspiration from natural systems.²⁴⁴ The first discovery of self-assembling proteins was in 1990.²⁴⁵ In the following the term proteins is used representatively as well for peptides. A class of self-assembling proteins are the so called amyloidogenic proteins. The term "amyloid" arises from the protein aggregates (Figure 1.21A), formed by a representative of these proteins, in human brain tissue and underlies a mistaken identification of Rudolf Virchow in 1854.²⁴⁶ He implied from a positive iodine staining test, that the substance has to be starch (*amylum* in Latin). Despite their differences in size, primary structure and natural functionality, all amyloidogenic proteins have one ability in common: They form so called amyloid fibrils, the protein aggregates mentioned before, that are unbranched, have a diameter usually between 70 and 120 Å, and if they are stained with Congo Red they exhibit a birefringence in polarized light (Figure 1.21B).²⁴⁷

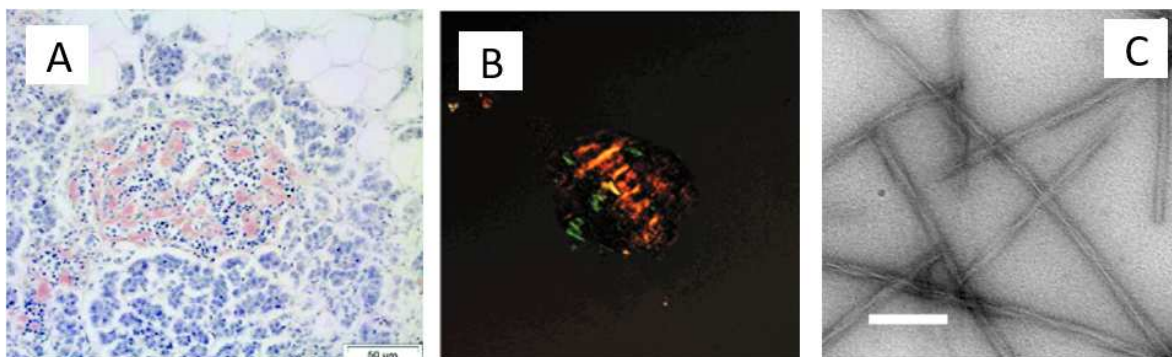


Figure 1.21: (A) Human pancreatic islet with extracellular amyloid deposits (red). Stained with Congo red. Scale bar = 50 μm. Reprinted and adapted with permission from American Physiological Society, copyright ©2011(open access).²⁴⁸ (B) *In vitro* Aβ40 amyloid fibrils stained with Congo Red using polarized filter. Reprinted and adapted with permission from the American Chemical Society, copyright ©2000 (open access).²⁴⁹ (C) Negatively stained transmission electron microscopy (TEM) image of recombinant PrP fibrils *in vitro*. Scale bar = 200 nm. Reprinted and adapted with permission from the American Chemical Society, copyright ©2013.²⁵⁰

1.3.1 Amyloids in nature

The first report of amyloid plaques, or at least tissue with a similar macroscopic appearance to that described by Virchow, occurred four centuries ago in 1639 as lardaceous or waxy liver and spongy and "white stone" containing spleens.²⁵¹ Just five years after Virchow's misidentification as a polysaccharid, Friedrich and Kekulé were able to correct the error. They demonstrated the presence of proteins in the

mass and the absence of carbohydrates by identification of a high nitrogen content.²⁵¹ While it was initially assumed that the amyloids are structurally unordered, improvements in histological dyes and microscopy revealed further structural information. In 1927 the finding of the birefringence mentioned *vide supra* suggested that amyloids consist of highly ordered organized proteins.²⁵² This achievement motivated in 1959 Cohen and Calkins to undertake electron microscopy (EM) studies, which revealed a general fibrillar ultrastructure of amyloids from patients and animals with amyloidosis.²⁵³ For a long time these amyloid fibrils were only associated with diseases. Today we know more than fifty disease related amyloidogenic peptides, among them serious diseases (Table 1.6), *e.g.* Alzheimer disease, which was linked to amyloid fibrils by Alois Alzheimer at the beginning of the nineteenth century,²⁵⁴ Parkinson disease,²⁵⁵ Creutzfeld-Jacob disease,²⁵⁶ or diabetes mellitus.²⁴⁸

Table 1.6: Selected examples of disease with appearance of amyloidogenic peptides

Clinical appearance	Affected organs (causation)	Protein	Structure (number of residues)
Alzheimer disease	CNS (s, h) ^a	Amyloid- β peptide (A β)	disordered (40 or 42) ^b
Parkinson disease	CNS (s, h) ^a	α -Synuclein	disordered (140)
Creutzfeld-Jakob disease	CNS (s, h, ia, in)	Prion protein (PrP)	disordered (1-102), α -helical (103-208)
Huntington disease	CNS (h)	Huntington exon I	disordered (03-187) ^b
Familial Danish dementia	CNS (h)	ADan peptide	disordered (34)
Type II diabetes	pankreas (s)	Islet amyloid polypeptide	disordered (37)
Light-chain amyloidosis	systemic (s, h) ^a	Ig light chain or fragments	all- β , Ig-like (~100) ^b
Heavy-chain amyloidosis	systemic (s, h) ^a	Ig heavy chain or fragments	all- β , Ig-like (~190) ^b
Injection localized amyloidosis	skin (ia)	Insulin	all- α , insulin-like (30+21)
Aortic medial amyloidosis	cardiovascular (s)	Medin	disordered (50)
C-Cell thyroid tumors	thyroid glands (s)	Calcitonin	disordered (32)
Familial amyloidosis, Finnish-type	PNS, cornea (h)	Gelsolin	disordered (53 or 71) ^b
Dialysis-related amyloidosis	HMS (ia)	β_2 microglobulin (h β_2 m)	all- β , Ig-like (99) ^b

CNS - central nervous system, HMS - human musculoskeletal system, Ig - immunoglobuline, PNS - peripheral nervous system, s - sporadic, h - hereditary, ia - iatrogenic, in - infectious. ^a Predominantly sporadic, although hereditary forms are documented. ^b Fragments of various length were reported. Data were taken from Ref.[257–259]

In all cases, aggregation occurs on the basis of misfolding of the respective proteins.²⁶⁰ What initiates the fibril formation remains unclear, however, many of the diseases are related with ageing and involve the spontaneous aggregation of wild-type protein (Table 1.6), which suggests that the phenomenon of protein aggregation originates from loss in regulatory control with ageing.²⁶¹ Inherited mutations in the precursor proteins can cause diseases to have an earlier onset.^{262,263} Further factors can be medical treatment²⁶⁴ or, like forms of the PrP, are even transmissible through contact with infected tissue.²⁶⁵

For more than a century amyloids were solely associated with these diseases. It was therefore surprising that several amyloid structures with different physiological functions were discovered at the turn of the millennium (Table 1.7). The first reports were about functional amyloids in bacteria, *e.g.* *E. coli*²⁶⁶ or *Streptomyces*,²⁶⁷ fungi^{268,269} and even higher organisms like insects²⁷⁰ or spiders.²⁷¹ The first functional amyloid in mammals was discovered in 2006²⁷² and to date more than 35 functional amyloid-forming proteins or protein families are known, suggesting that functional amyloids may rather be the rule than the exception.^{260,273,274} In their functionality they can be divided into several classes. Especially in microorganisms they have six main purposes: Biofilm formation & stabilization, cell-to-cell adhesion & flocculation, surface tension modulation, cell cycle & gametogenesis, antimicrobials & toxins, symbiosis & host-pathogen interaction.²⁷⁴ In higher organisms we have additional functions, *e.g.* controlled cell death, templating of melanin biosynthesis and the storage form of various peptide hormones.²⁶⁰ Especially, the amyloid formation of peptide hormones is of interest. Several endocrine hormones, *e.g.* amylin, calcitonin, or atrial natriuretic factor, may be stored as functional amyloids in secretory granules in amyloid state and are also present as amyloids in amyloid diseases.²⁷⁵

Table 1.7: Selected examples of functional amyloid forming peptides

Protein (number of residues)	Organism (year) ^a	Function Soluble form	Function Amyloid form
Bacteriae & Archaea			
Curli protein CsgA (~112) ^b	Enterobacteriaceae (2002) ²⁶⁶	None known	Biofilm formation
Microcin E492 (84)	<i>K. pneumoniae</i> (2005) ²⁷⁶	Bacteriocin, membrane pore-formation	inactive/storage
Chaplins (63-225) ^b	<i>S. coelicolor</i> (2003) ²⁶⁷	None known	Aerial hyphae forma- tion, modulates surface tension
FapC (225)	<i>P. fluorescence</i> (2010) ²⁷⁷	None known	Biofilm stabiliza- tion, cell-to-surface adhesion, host invasion
Fungi			
Het-s (289)	<i>P. anserina</i> (1997) ²⁶⁸		Prion form is active: in- volved in heterokaryon incompatibility
Sup35 (685)	<i>S. cerevisiae</i> (2000) ²⁷⁸	Translation regulator	Prion form is inactive
Rim4 (718)	<i>S. cerevisiae</i> (2015) ²⁷⁹	Sporulation-specific RNA-binding protein	Translation suppressor, regulates meiosis
Hydrophobins (~100) ^b	Filamentous fungi (2000) ²⁸⁰	None known	Surface attache- ment, aerial hyphae formation

Protein (number of residues)	Organism (year) ^a	Function Soluble form	Function Amyloid form
Plants			
Rubber elongation factor (137)	<i>H. brasiliensis</i> (2012) ²⁸¹	Latex biosynthesis ^c	
Vicilin (394)	<i>P. sativum</i> (2019) ²⁸²	Mediate protein storage in seeds ^c	
Animals			
Chorion (~100) ^b	<i>B. mori</i> (2000) ²⁷⁰	None known	Protects moth oocyte and embryo
Pmel17 (661)	<i>H. sapiens</i> (2006) ²⁷²	None known	Templates melanin synthesis in melanosomes
RIPK1 (671), RIPK3 (518)	<i>H. sapiens</i> (2012) ²⁸³	None known	Signaling and cell death of necroptosis
Bombesin (14)	<i>B.bombina</i> (2009) ²⁷⁵	Involved in hormone signaling	Sorting, storage, and release of hormone

^a Year of first report of functional amyloids. ^b Proteins of various length were reported. ^c Functionality could not be clearly assigned to the soluble protein or the amyloid form. Data were taken from Ref.[260, 273, 274, 284]

1.3.2 Structure, mechanism of aggregation and how to analyse

Despite, that amyloid fibrils are composed of proteins with little or no sequence homology, they share a common underlying architecture: a translational symmetry element that lies parallel to the fibril axis. In the repeating substructure, the cross- β -sheet motif,^{285,286} the protein chains are organized in β -sheets with the β -strands perpendicular to the fibril axis (Figure 1.22A).²⁴⁷ X-ray diffraction studies^{287,288} revealed characteristic patterns with a meridional reflection at ~ 4.8 Å corresponding to the interstrand spacing and an equatorial more diffuse reflection at ~ 10 Å arising from the distance between adjacent β -sheets (Figure 1.22B).²⁸⁹

The self-assembling process of the amyloid formation consists of multiple stages (Figure 1.23). In general the amyloid formation possesses a typical sigmoidal reaction time course. Herein, a lag phase is initially observed, followed by a rapid growth/elongation phase. If the total quantity of protein is limited, it reaches a stationary or plateau phase.²⁹⁰ This behaviour is a feature of nucleated polymerization.²⁹¹ The triggering event is usually the complete or partial unfolding of monomeric precursor proteins, which generates aggregation-prone intermediates.²⁹² Alternatively, the assembly starts from intrinsically disordered proteins,²⁹³ and in rare cases the aggregation is initiated by the folded protein itself.²⁹⁴ In the nucleation phase the first step is the formation of oligomeric species by the precursor proteins. These oligomers can further associate to higher-ordered species, which either serve as critical nucleus for the fibril formation or are dead-end assemblies, which are not able to undergo fibrillization.²⁹⁵ The probability of the formation of the critical nuclei is partially decisive for the length of lag phase as the rapid assembly/polymerization into amyloid fibrils takes place starting from them.²⁵⁸ At a later point in the self-assembly a second structural transformation causes the assembling peptides to adopt the typical cross- β -sheet structure, irrespective of their initial fold.²⁹⁶

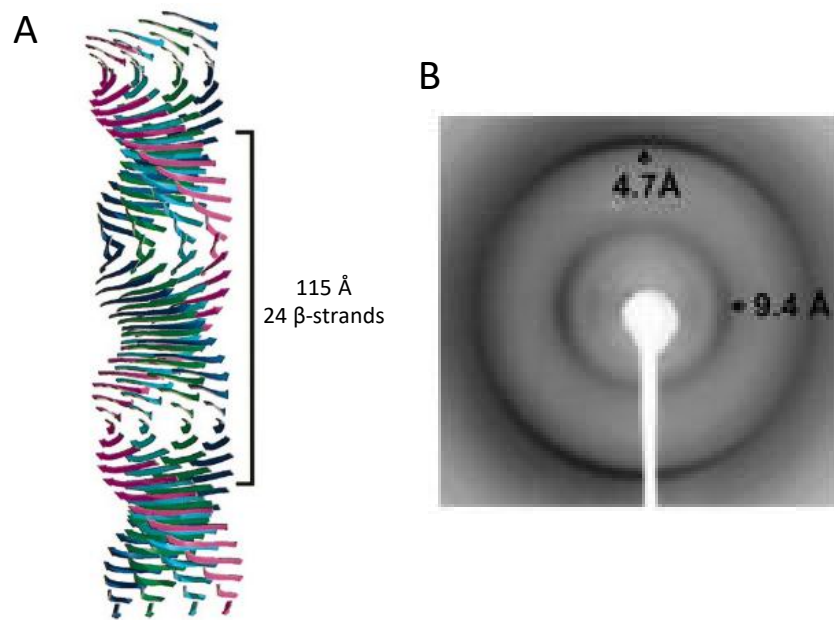


Figure 1.22: (A) Molecular model of the common core protofilament structure in amyloid fibrils. Exemplary four β -sheets make up the protofilament structure. The sheets run parallel to the axis of the protofilament, with their component β strands perpendicular to the fibril axis. Twisting of β -sheets gives helical repeat of 115.5 Å containing 24 β -strands (depicted by the box). Reprinted with permission from Elsevier, copyright ©1997.²⁴⁷ (B) X-ray diffraction of partially aligned RIP1/RIP3 fibrils. Arrows indicating the meridional (4.7 Å) and the equatorial reflection (9.4 Å). Reprinted with permission from Elsevier, copyright ©2012.²⁸³

While the just described can be grouped under the term primary mechanisms, secondary mechanisms describe processes occurring starting from fibrils with the cross- β form. These include fragmentation, which forms new fibril ends for further assembly, reduces the length of the lag-time, and result in exponential growth.²⁹⁷ Another one is the secondary nucleation on the surface of preformed fibrils. The surface thereby, catalyses oligomer formation, which also enhances the rate of fibril formation.²⁹⁸

The ability of the amyloid fibrils to seed its own assembly is a characteristic of prion diseases, *e.g.* transmissible spongiform encephalopathies in mammals,²⁵⁶ as it allows to spread between cells and in some cases between organisms.²⁵⁸ However, in yeast prion-like spreading of functional amyloids is beneficial. It stimulates metabolic responses of daughter cells under certain selective pressures.²⁹⁹

To analyse the amyloidogenic proteins, with regard of fibril formation and structure, a variety of methodologies is available. The main tool used to monitor the fibrillization kinetic *in vivo* is the thioflavin T-monitored fluorescence assay (TMFA).³⁰⁰ It is based on the histological used dye Thioflavin T (Figure 1.24A), which shows an increased fluorescence quantum yield and a bathochromic shift of its fluorescence excitation upon binding to the cross- β -sheet motif of the amyloid fibrils.^{301,302} Other *in situ* techniques that can be used are nuclear magnetic resonance (NMR),²⁹⁶ CD,³⁰³ or IR spectroscopy,³⁰⁴ whereby these offer additional information about the secondary structure.³⁰⁵ Microscopic approaches, *e.g.* thermophoretic trapping combined with fluorescence microscopy³⁰⁶ or two-colour super resolution microscopy,³⁰⁷ allow to investigate the fibrillization of single fibrils. Congo Red (Figure 1.24A) has a long history of identifying amyloid fibrils³⁰⁸ and the appearance of birefringence is one of the three criteria for the presence of amyloids.³⁰⁹ The other two criteria are a fibrillar morphology, which can

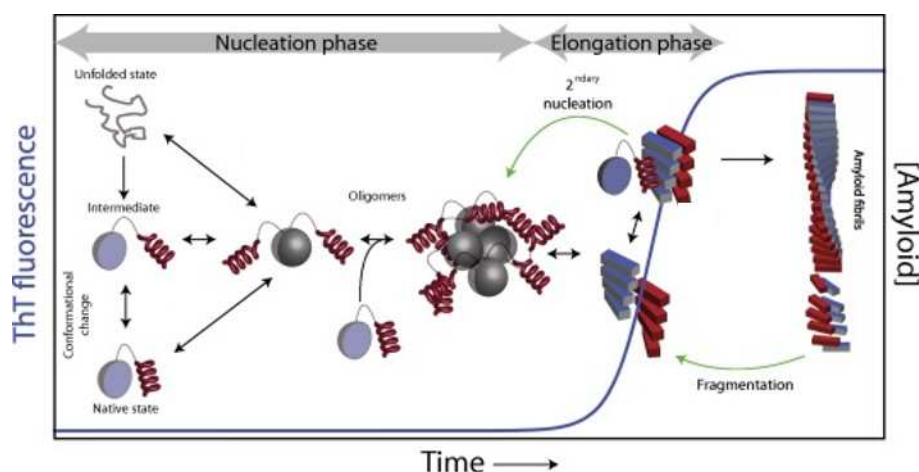


Figure 1.23: Mechanisms of amyloid self-assembly. The scheme shows primary (black arrows) and secondary (green arrows) pathways in the process of amyloid self-assembly, which starts from the unfolded state. Reprinted and adapted with permission from Elsevier, copyright ©2015 (open access).²⁹⁶

be proven by EM (Figure 1.24B)²⁵¹ and atomic force microscopy (AFM, Figure 1.24C),³¹⁰ and a cross- β -structure, which is usually analyzed by X-ray fibre diffraction.²⁸⁶ During the last decades, other methods have become particularly important to gain insights at a near atomic level. Especially breakthroughs in cryo-EM^{311,312} and ssNMR^{313,314} brought first impressions how individual protein subunits are able to form cross- β -sheets (Figure 1.24D).²⁵⁸

1.3.3 Parathyroid hormone

Functional amyloids are widely distributed in nature. Among other things, they serve as the storage form of several peptide or protein hormones. In the cell they are synthesised as monomers and subsequently stored in secretory granules in the amyloid form.²⁷⁵ When they are released into the blood, the fibrils dissociate from the end to release the active monomers.³¹⁸ In several studies it was proven that functional amyloids could release their constituents by being exposed to a different pH or by simply diluting.^{319–322} The reason for this is that the fibrils have a much lower thermodynamic stability than, for example, amyloids of disease-related proteins. The Gibbs free energy ΔG^0 for the entire fibrillization process is for A β , from Alzheimer disease, and for h β_2 g, from dialysis-related amyloidosis, $-36.0 \text{ kJ mol}^{-1}$ ³²³ and $-33.0 \text{ kJ mol}^{-1}$,³²⁴ respective, while for parathyroid hormone (PTH) ΔG^0 it is reported to be only $-23.8 \text{ kJ mol}^{-1}$.³²⁵

PTH is ubiquitously distributed in vertebrates.^{326–328} In mammals and birds it is the major secretory product of the chief cells of the parathyroid glands and was isolated 1959,^{329,330} even though its physiological role was already investigated since 1925, when it was possible to produce an active extract of the glands.³³¹ As a hormone it plays a central role in maintaining the Ca^{2+} and phosphate homeostasis of extracellular fluid.³³⁰ Diseases associated to PTH can generally be attributed to an insufficient or an excess availability of PTH.³³¹ An excess of PTH (hyperparathyroidism) arises from hormone overproduction, *e.g.* in case of tumors. In this case the typical symptoms are hypercalcaemia, bone disease,

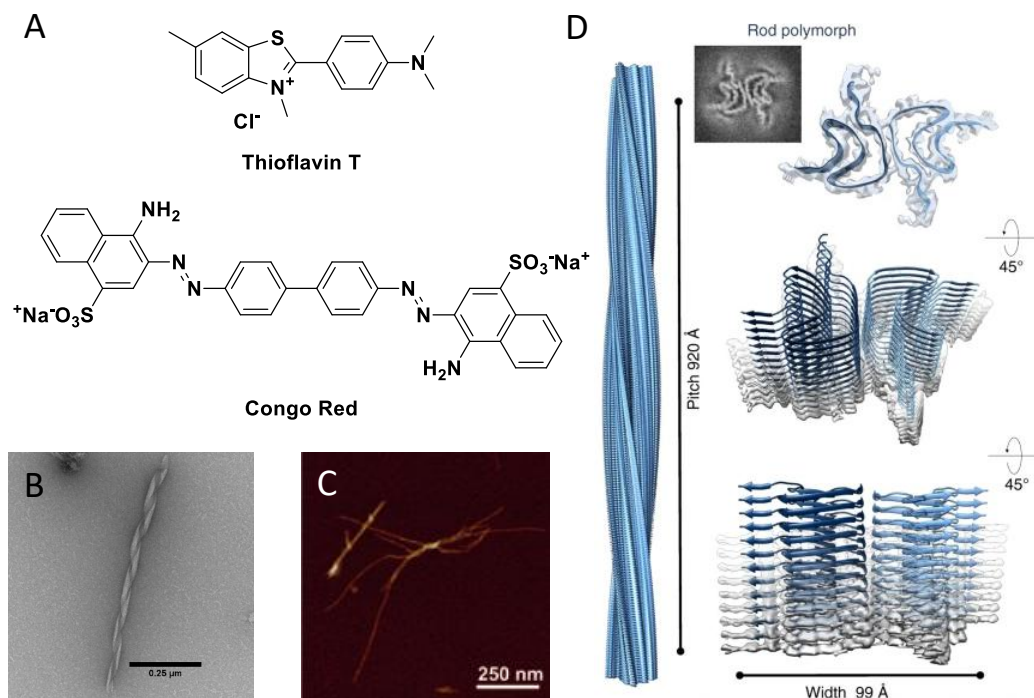


Figure 1.24: Methods to analyse amyloidogenic proteins. (A) Structure formulas of histological dyes thioflavin T and Congo Red used in assays to identify amyloid fibrils and analyse the fibrillization kinetic. (B) TEM image of fibril from azobenzene modified PTH-fragment. Reprinted and adapted with permission from American Chemical Society, copyright ©2024(open access).³¹⁵ (C) AFM image of fibrils from endcapped heptapeptide $\text{CH}_3\text{CONH-}\beta\text{A}\beta\text{AKLVFF-CONH}_2$ after 10 h at 25°C. Reprinted and adapted with permission from Wiley, copyright ©2011.³¹⁶ (D) Cryo-EM structures and atomic model of α -Synuclein rod (left), as density slice (top inlet), viewed from two different angles (lower panels). Reprinted and adapted with Permission from Springer Nature, copyright ©2018 (open access).³¹⁷

and kidney stones.³³¹ The contrary, an insufficient parathyroid action, arises from either deficient hormone production (hypoparathyroidism) or hormone resistance (pseudohypoparathyroidism).³³² Typical symptoms are hypocalcemia, hypophosphatemia (low blood level of Ca^{2+} , respective phosphate), and tetany.³³¹ In the case of hypoparathyroidism recombinant PTH (Natpara™) or the N-terminal PTH fragment PTH_{1-34} (Forteo™) are approved drugs.³³³

The human PTH is 84 amino acids in length (Figure 1.25A). The cellular formation occurs *via* two precursor peptides. Initially, *prepro*-PTH (115 amino acids) is the product of translation of the respective mRNA (Figure 1.25B).³³⁴ The *pre*-region is a signal sequence, that comprises the first 25 amino acids and directs the protein into the cell secretory pathway (Figure 1.25B).³³⁵ In the endoplasmatic reticulum the proteolytical cleavage of the signal sequence takes place.³³⁶ The formed *pro*-PTH (90 amino acids) is transferred to the Golgi apparatus, where the *pro*-region is removed proteolytically. The function of this region is not completely understood, however, it seems to prevent premature fibrillization of *pro*-PTH.³²² The mature PTH is packed and stored as functional amyloid fibrils in secretory granules.³²¹ After the release to the blood, PTH activates G-protein coupled receptors presented on the plasma membrane of kidney and bone cells.³³⁷ It is known that the N-terminal first 34 amino acids are responsible for the activation of the receptor (Figure 1.25A).³³⁷ In addition, the

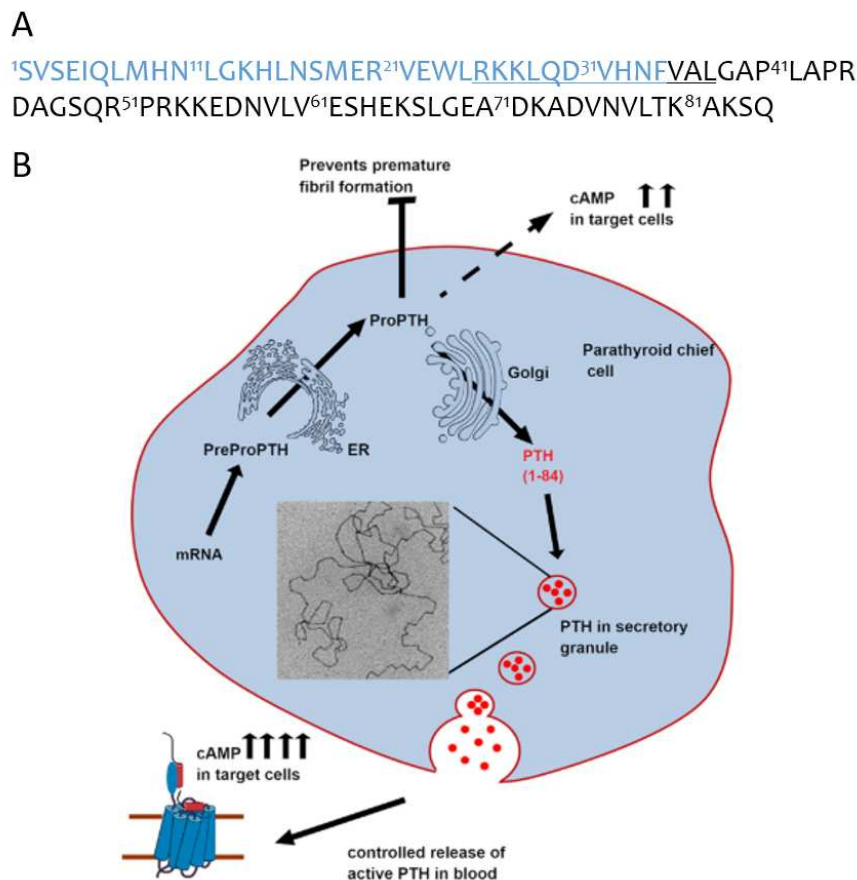


Figure 1.25: A PTH overview. (A) Primary structure of full-length PTH₁₋₈₄. PTH fragments are highlighted: N-terminal PTH₁₋₃₄ is highlighted in blue & fibril forming core PTH₂₅₋₃₇ is underlined. (B) Overview of the cell maturation and release of PTH. Reprinted and adapted with Permission from John Wiley & Sons Inc., copyright ©2023 (open access).³²²

amino acid sequence R25–L37, which is responsible for fibril formation, was identified by *in silico* predictions and proteolysis with chymotrypsin of preformed fibrils.³²¹ However, fibril formation and in particular its reversibility have hardly been investigated and are therefore the subject of current research.^{321,322,325,338,339,346}

Amyloid fibrils play key roles in biological systems, both in connection with diseases and to maintain normal physiological functionalities.^{259,260} In case of the first one, *e.g.* Alzheimer disease, diabetes type II, or AL amyloidosis, studies support, that cell death and resulting diseases are linked directly to the aggregation of peptides and formation of oligomeric peptide structures.^{340–342} In this context it is of major interest to understand and control amyloid aggregation, as it can be crucial for the development of potential therapies. However, due to that amyloid formation comprises complex processes, *e.g.* extensive conformational changes at the level of secondary structure and inter- and intrapeptide interactions, it is a significant challenge to obtain control. Light seems to be therefore a powerful external trigger, as it is generally noninvasive and offers a high spatiotemporal resolution. While for

proteins in general a large number of examples are already known where the functionality is influenced by photoswitches,^{85,155,343} there are only a few reports about photoswitching of amyloidogenic peptides, mainly focusing on synthetic sequences^{344,345} or investigating the presence of β -turns (see Table 1.4 #9).¹⁶³ The low thermodynamic stability of PTH therefore makes it a potential candidate to study light-switchable fibrillation. To the best of our knowledge, to date no reversibly switchable system of a natural amyloidogenic protein is known.

Aim of the thesis

The aim of this thesis is to establish a model system to control the fibrillization of amyloidogenic peptides with irradiation of light. It comprises the design and synthesis of peptides, derived from a peptide hormone and including a photoswitchable moiety incorporated into the peptide backbone (Figure 2.1, left), which are able to form amyloid fibrils *in vitro* under physiological conditions. Furthermore, the modified peptides shall be switchable between two photostationary states, whereby one is able to form fibrils and the other one not, to enable photocontrol over the fibrillization process (Figure 2.1, right).

Thus, this thesis covers the following scientific issues represented in Figure 2.1: What is the most beneficial class of photoswitches to enable sufficient stability in relation to the fibrillization time? What is the influence of the position of the photoswitch? Does one isomer fibrillize and the other one not? And finally, is the system able to undergo multiple fibrillization/disaggregation cycles?

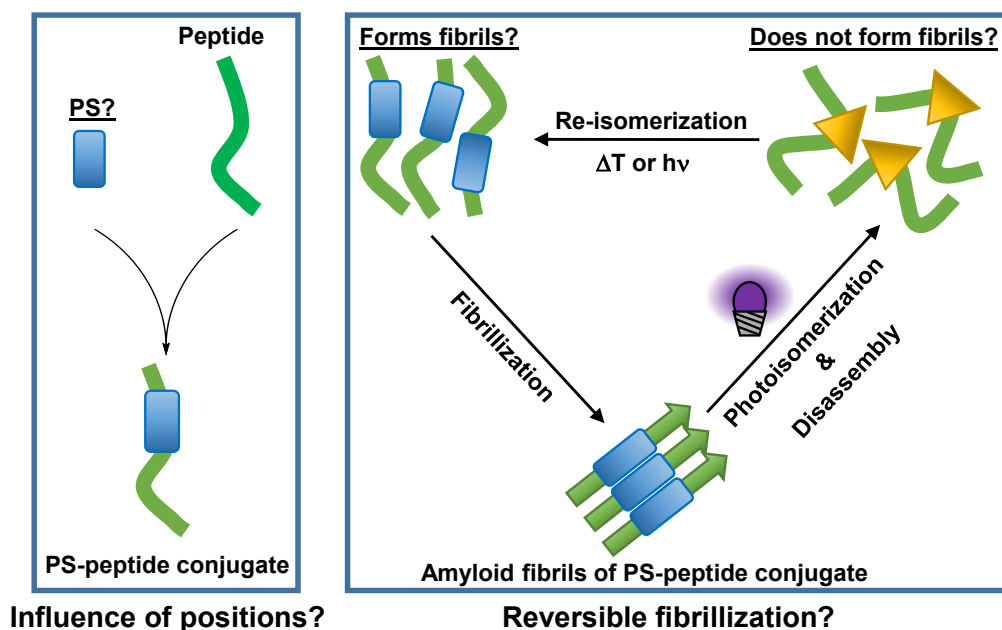


Figure 2.1: (left) Schematic interpretation of the incorporation of a photoswitchable moiety (PS) into an amyloidogenic peptide. (right) Concept for a light driven (de)fibrillization process of a photoswitch (PS) peptide conjugate.

Scientific concept

The first step involved identifying suitable model peptides and photoswitches. Among other peptide hormones,²⁷⁵ the fibril forming core sequence of PTH, PTH₂₅₋₃₇ (Figure 1.25), is a potential candidate. Based on the current state of research it is assumed that the thermodynamic stability of fibrils formed by PTH is low enough to allow disaggregation under physiological conditions in contrast to disease-related amyloids, *e.g.* A β . Furthermore, as the sequence in PTH participating in the fibril formation is only 13 amino acids in length,³²¹ the synthesis is less complex.

As potential candidates for the photoswitches the focus was on two classes: azobenzenes and spiropyrans. Both classes offer a facile synthesis and functionalization. While azobenzenes induce upon *cis/trans*-isomerization a huge spatial strain in the peptide (their end-to-end distance changes by ~ 3.5 Å, see section 1.1.3), spiropyrans undergo a major change in their dipole-moment, thus, changing local polarity.

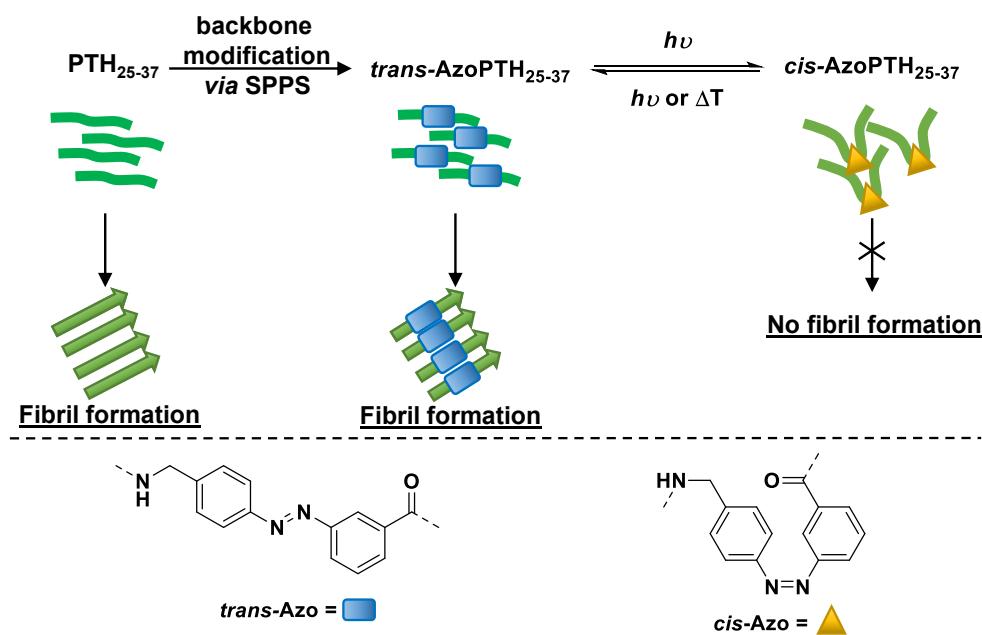


Figure 3.1: Schematic representation of the proof-of-principle. Azo-modified PTH₂₅₋₃₇, bearing a 3,4'-AMPB moiety in the backbone, is able to undergo fibrillization in the *trans*-configuration of the photoswitch, while the *cis*-configuration is not able to.

In case of PTH previous research on the fibrillization mainly focused on the influence of the *pro*-sequence³²² and on the impact of environmental parameters on the fibril formation.^{339,346} However, there is only minor knowledge of the structural parameters of fibrils and the fibrillization of PTH₂₅₋₃₇ has not been studied at all. Thus in the initial study, the proof-of-principle shows that both isomers

of the switch alter the propensity to form fibrils in opposite manners (Figure 3.1). An azobenzene photoswitch is introduced in the backbone of PTH_{25–37} at various positions, replacing amino acids or being inserted between two adjacent amino acids. SPPS with fluorenylmethyloxycarbonyl (Fmoc) chemistry is the method of choice, as it has milder deprotection conditions than *tert*-butyloxycarbonyl (Boc) chemistry. The chosen 3,4'-AMPB building block possesses an elaborated synthesis and a sufficient thermodynamical stability.¹⁶⁷ The photoswitchable peptides will be investigated regarding their photophysical properties, such as half-life time of the *cis*-isomer and PSSs after photoisomerization, using UV/Vis spectroscopy and high-performance liquid chromatography (HPLC). Subsequently, the fibrillization process of both isomers is analyzed with TMFA and TEM.

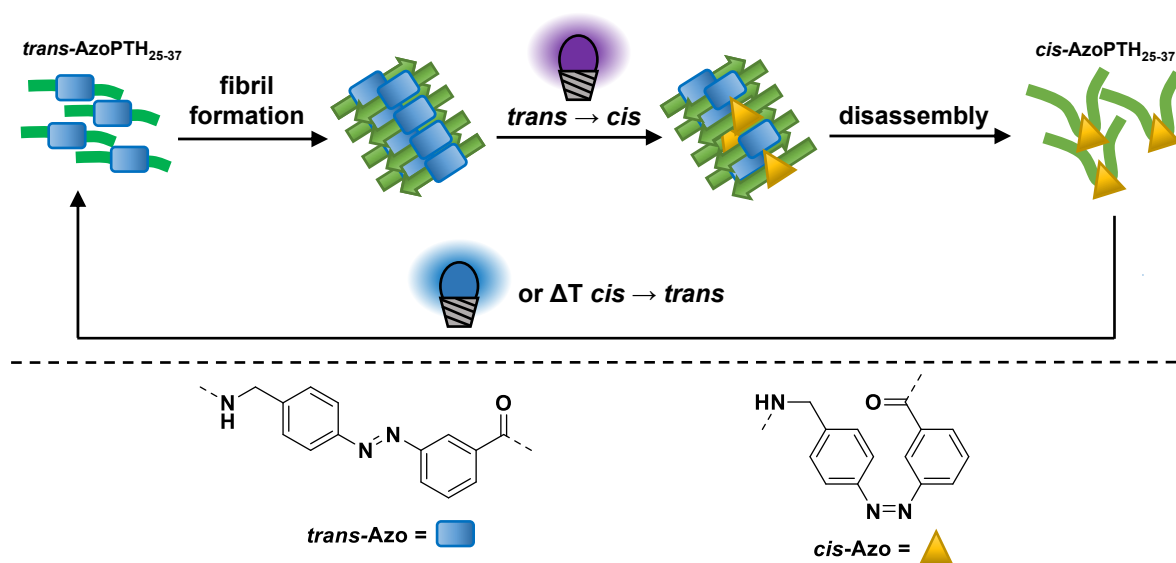


Figure 3.2: Concept of the light-controlled (de)-assembly of Azo-modified PTH_{25–37}. Reprinted and adapted with permission from the American Chemical Society, ©2024 (open access).³¹⁵.

The second concept comprises the determination of structural information of the fibrils from PTH_{25–37} and modified peptides with wide-angle X-ray scattering (WAXS), ssNMR and MD simulations and determination of fibrillization parameters of the modified peptides with TMFA. The aim thereof is to gain information about the influence of the position regarding the control of the fibrillization process. The most promising candidate, with the largest difference in fibrillization for the *trans*- and the *cis*-isomer, is then used to test whether amyloid formation can be toggled on and off *via* light (Figure 5.4). The fibril formation is in each cycle tracked by TMFA, while the fibrils/degraded fibrils are investigated by CD spectroscopy and transmission measurements.

The last part covers the impact of spiropyran moieties in PTH_{25–37}. As already described in section 1.2.4, there is still no reported spiropyran suitable for the incorporation into peptide backbone (Figure 3.3A). Thus, the first challenge is to design spiropyran building blocks suitable for SPPS. Two target structures (Figure 3.3B) were selected, the FASC switch bearing an amino group at the 5-position of the indolene unit and a carboxy group at the 6'-position and the SAFC switch bearing the amino group at the 6'-position and the carboxy group at the 5-position. The respective Fmoc-protected building

blocks are used in SPPS to incorporate the switches in the primary structure of PTH₂₅₋₃₇ at similar positions as the 3,4'-AMPB switch. Subsequently, the photophysical properties are characterized with UV/Vis-spectroscopy and the fibrillization behaviour will be investigated with TMFA and TEM.

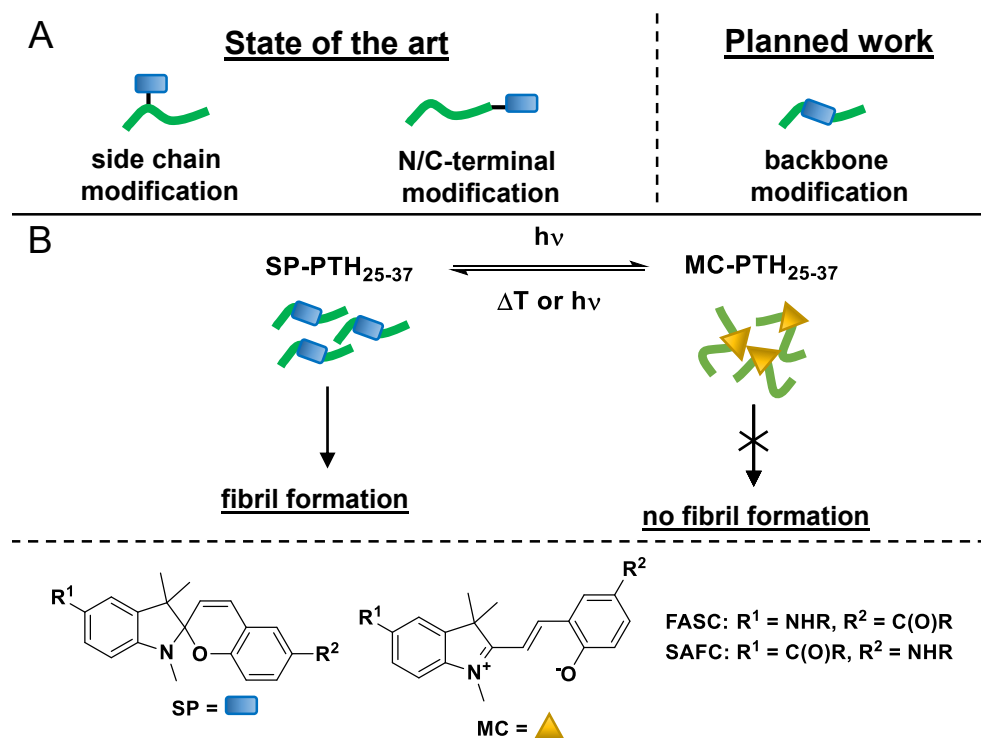


Figure 3.3: (A) State-of-the-art strategies to introduce spiropyran in peptides comprise side-chain modifications and N-/C-terminal modifications. In this thesis it is planned to incorporate the spiropyran in the peptide backbone *via* SPPS. (B) Spiropyran-modified PTH₂₅₋₃₇ is able to undergo fibrillization in the SP-form, while the MC-form is not able to fibrillize.

List of publications

The results of this thesis are published in the following four publications. The three first-author publications [P1] – [P3] are reprinted as part of this cumulative thesis with the permissions from MDPI³⁴⁸ and the American Chemical Society.^{315,347}

[P1] Modulating the Fibrillization of Parathyroid-Hormone (PTH) Peptides: Azo-Switches as Reversible and Catalytic Entities.

André Paschold, Bruno Voigt, Gerd Hause, Tim Kohlmann, Sven Rothmund, Wolfgang H. Binder*
Biomedicines **2022**, 10 (7), 1512, DOI: <https://doi.org/10.3390/biomedicines10071512>.

The conceptualization and writing of this publication were performed by myself. Furthermore, I was responsible for the syntheses as well as chemical and structural characterization of all synthetic products. Together with B. Voigt I investigated the fibrillization behaviour and together with T. Kohlmann I conducted the photophysical analysis. G. Hause conducted the TEM recordings and evaluated the data. S. Rothmund was responsible for the peptide synthesis and characterization thereof. All steps of this project were supervised by W. H. Binder. All authors were involved in the reviewing process of this publication.

[P2] Photocontrolled Reversible Amyloid Fibril Formation of Parathyroid Hormone-Derived Peptides.

André Paschold, Moritz Schäffler, Xincheng Miao, Luis Gardon, Stephanie Krüger, Henrike Heise, Merle I. S. Röhr, Maria Ott, Birgit Strodel*, Wolfgang H. Binder*
Bioconjugate Chemistry **2024**, 35 (7), 981–995, DOI: <https://doi.org/10.1021/acs.bioconjchem.4c00188>.

The conceptualization and writing of this publication were performed by myself. Furthermore, I was responsible for the syntheses as well as chemical and structural characterization of all products, the analysis of the fibrillization behaviour and together with S. Krüger I conducted the TEM recordings and evaluated this data. M. Schäffler conducted together with X. Miao, M. I. S. Röhr, and B. Strodel the computational analysis. L. Gardon and H. Heise were responsible for the NMR-experiments and evaluated the data. M. Ott conducted the WAXS measurements and evaluated the data. All steps of this project were supervised by W. H. Binder. All authors were involved in the reviewing process of this publication.

[P3] Spiropyran as building block in peptide synthesis and modulation of photochromic properties.

André Paschold, Niclas Starke, Sven Rothmund, Wolfgang H. Binder*
Organic Letters **2024**, 26 (49), 10542–10547, DOI: <https://doi.org/10.1021/acs.orglett.4c03929>.

The conceptualization and writing of this publication were performed by myself. Furthermore, I investigated the photophysical behaviour of the peptides and together with N. Starke, I was responsible for the syntheses as well as chemical and structural characterization of all synthetic products. S. Rothmund conducted the peptide synthesis and analysis. All steps of this project were supervised by W. H. Binder. All authors were involved in the reviewing process of this publication.

Article

Modulating the Fibrillization of Parathyroid-Hormone (PTH) Peptides: Azo-Switches as Reversible and Catalytic Entities

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Abstract: We here report a novel strategy to control the bioavailability of the fibrillizing parathyroid hormone (PTH)-derived peptides, where the concentration of the bioactive form is controlled by an reversible, photoswitchable peptide. PTH_{1–84}, a human hormone secreted by the parathyroid glands, is important for the maintenance of extracellular fluid calcium and phosphorus homeostasis. Controlling fibrillization of PTH_{1–84} represents an important approach for in vivo applications, in view of the pharmaceutical applications for this protein. We embed the azobenzene derivate 3-[(4-aminomethyl)phenyl]diazetylbenzoic acid (3,4'-AMPB) into the PTH-derived peptide PTH_{25–37} to generate the artificial peptide AzoPTH_{25–37} via solid-phase synthesis. AzoPTH_{25–37} shows excellent photostability (more than 20 h in the dark) and can be reversibly photoswitched between its *cis*/*trans* forms. As investigated by ThT-monitored fibrillization assays, the *trans*-form of AzoPTH_{25–37} fibrillizes similar to PTH_{25–37}, while the *cis*-form of AzoPTH_{25–37} generates only amorphous aggregates. Additionally, *cis*-AzoPTH_{25–37} catalytically inhibits the fibrillization of PTH_{25–37} in ratios of up to one-fifth. The approach reported here is designed to control the concentration of PTH-peptides, where the bioactive form can be catalytically controlled by an added photoswitchable peptide.

Keywords: azobenzene; photoswitchable peptides; fibrillization; parathyroid hormone; aggregation



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1. Introduction

Fibrillization of proteins and peptides is a supramolecular process [1,2] that leads to the formation of peptide aggregates, containing a cross- β -sheet motif [3]. It involves multiple steps [4] and is associated with many diseases such as Alzheimer's disease, Parkinson's disease or diabetes type II [5–7]. However, in the past decades, it has also been associated with amyloids with distinct physiological functions, so-called functional amyloids, which are found in lower organisms [8–11]. Subsequently, functional amyloids were also discovered in humans, whereby the amyloid can be the active physiological form [12,13] or the storage form of peptide hormones [14].

The parathyroid hormone, abbreviated PTH, is a human hormone secreted by the parathyroid glands [15], with PTH-like peptides also known from other animals [16,17]. It is expressed as a 115 residue pre-pro-protein, whereby the first 25 amino acids at the N-terminus (referred to PTH_{1–25}) serve as a signaling peptide for the transport to the endoplasmic reticulum and are removed by a signal peptidase [18]. The formed pro-peptide is subsequently transferred to the Golgi apparatus and the N-terminal six amino acids (referred to PTH_{1–6}) are proteolytically removed [19]. Before mature PTH_{1–84} is released into the blood, it is stored in secretory granules as amyloid fibrils [20]. The physiological role

is well studied [21,22], being important in the maintenance of extracellular fluid calcium and phosphorus homeostasis. The receptor is mainly activated through the first 34 *N*-terminal amino acids [23], wherefore recombinant PTH_{1–84} and recombinant PTH_{1–34} are approved drugs against osteoporosis, Natpara® and Forteo®, respectively. However, its fibrillization has barely been investigated. Thus far, it is known that the amyloid fibrils of PTH_{1–84} are formed by the amino acid residues R25–L37, and the thermodynamic stability of the fibrils is sufficiently low to dissociate after dilution [20]. Thus, control over the fibrillization of amyloids and PTH specifically represents an important approach for controlling its factual concentration for in vivo applications, placing modulators of fibrillization and thus reversible fibrillization into the focus of pharmaceutically applicable proteins [24–27].

In the past decades, the photoinduced switching of protein functionalities has emerged as an important concept to modulate protein function, often by modulations in binding specificity between proteins and ligands. Thus, not only enzymes have been equipped with photosensitive switches, but also larger protein complexes, involved in many physiological or neurological functions [28]. To this end, artificial photoswitches are embedded into either the main chain or side chains of polypeptides, in order to change their secondary structures by photoinduced conformational changes of the photoswitches. Thereby, a plethora of different photoswitches, such as those based on *cis-trans*-isomerization of azo-dyes [29,30] stilbenes [31] and hemithioindigos [32,33], have been developed. Important for the proper use of a specific photoswitch inside a polypeptide chain is not only the quest to retain the initial (functional) secondary structure of the protein, but also to achieve a reasonably stable conformation after photoswitching, so as to allow for sufficient time to exert the desired effect. Many examples of such sufficiently stable and also reversible photoswitches have been reported, allowing one to modulate several aspects of protein function [34–39]. Here, we report on an approach to modulate the fibrillization of PTH, equipped with a photoswitch at a specific position in the peptide sequence, in order to reversibly trigger its aggregation/disaggregation (see Figure 1).

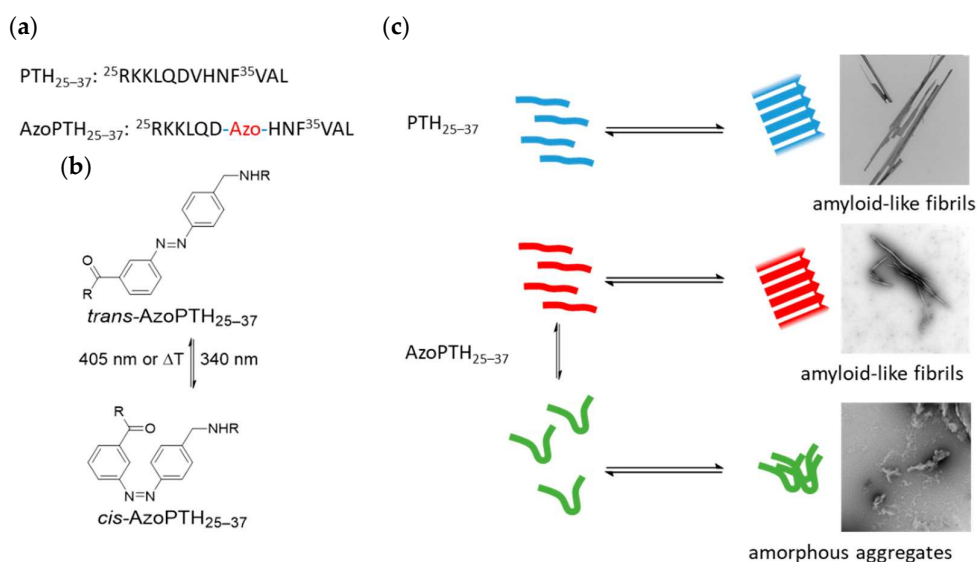


Figure 1. (a) Primary sequence of PTH_{25–37} and the azobenzene-modified PTH_{25–37} (AzoPTH_{25–37}, azobenzene-moiety highlighted in red). (b) *Cis-trans*-isomerization of the incorporated 3,4'-AMPB switch. (c) Equilibrium of the monomeric peptides PTH_{25–37} and AzoPTH_{25–37} in both forms and their aggregates.

In view of the functional design of the modified PTH_{25–37}, we sought to embed the photoswitch into a region of the protein where aggregation is still possible, but only in a specific (untriggered) conformation of the photoswitch, whereby fibrillization should be inhibited after the conformational change. As a model system, we chose peptides derived

from the PTH fibril core structure, including the amino acids 25R-37L (Figure 1a) [20], which is able to form fibrils itself. In addition, we investigated the influence of both conformations on the fibrillization of the unmodified peptide. As the photoswitch we chose a structural motif from the class of azobenzenes, as they are well known for enabling reversible control of peptide conformation [29,34,39–41]. Specifically we chose the azobenzene derivate 3-[[[(4-aminomethyl)phenyl]diazenyl]benzoic acid (3,4'-AMPB; Figure 1b) [42], which is known to introduce a significant geometric change. 3,4'-AMPB displays both: a high photoisomerization yield and a sufficient thermodynamically stability of the *cis*-isomer [41]. If desired, the photoswitch can be reversed via irradiation at 405 nm, or thermally, with a half-life time of more than 20 h in the dark. We hypothesized that the incorporation of the azobenzene into the backbone would allow us to switch between the *cis*- and the *trans*-conformation, whereby one of them is able to fibrillize and the other one is not. Furthermore, azobenzenes in their *cis*-conformation are known to mimic β -hairpins, which allowed us to investigate the hypothesis if the PTH fibrils possess a turn region like amyloid fibrils from other peptides [43–45].

2. Materials and Methods

2.1. General

All technical solvents were distilled prior to use. Air- and moisture-sensitive reactions were carried out in flame-dried glassware under atmospheric pressure of nitrogen. 2-(6-Chloro-1-H-benzotriazole-1-yl)-1,1,3,3-tetramethylammonium hexafluorophosphate (HCTU), *N*-methyl-morpholine (NMM), *N,N*-dicyclohexylcarbodiimide (DIC), *N*-Hydroxybenzotriazole (HOBT), trifluoroacetic acid, 4-aminobenzylamine, and oxone[®] were purchased from Sigma Aldrich (Taufkirchen, Germany). 9-Fluorenylmethyl-*N*-succinimidylcarbonat (Fmoc-OSu) was received from Fluorochem. 3-Aminobenzoic acid was purchased from Merck (Darmstadt, Germany). All these chemicals were used without further purification.

NMR spectra were recorded on a Varian Gemini 400 or 500 spectrometer (400 MHz or 500 MHz; Agilent Technologies, Waldbronn, Germany) at 27 °C in DMSO-*d*₆ (99.8 Atom%D; Chemotrade, Düsseldorf, Germany) or D₂O (99.8 Atom%D; Sigma-Aldrich, Taufkirchen, Germany). Chemical shifts are given in ppm and referred to the solvent residual signal (DMSO-*d*₆: δ = 2.50 ppm and δ = 39.5 ppm; D₂O: δ = 4.79 ppm). The following abbreviations were used for ¹H- and ¹³C-NMR peaks' assignment: s = singlet, d = doublet, t = triplet, td = triplet of doublet, and m = multiplet. MestReNova (version 6.0.2–5475, Mestrelab Research S.L., Santiago de Compostela, Spain) was used for data interpretation.

ESI-ToF mass spectrometry was performed on a Bruker Daltonics microTOF (Bruker Corporation, Billerica, MA, USA). Samples were dissolved in HPLC-grade solvents (MeOH, THF, or mixtures; Sigma Aldrich, Taufkirchen, Germany) at concentrations of 0.1 mg/mL and measured via direct injection with a flow rate of 180 μ L/h using the positive mode with a capillary voltage of 4.5 kV. The spectra were analyzed with otofControl (version 3.4, Bruker Daltonik, Bremen, Germany).

2.2. Organic Synthesis

Fmoc-protected 3,4'-AMPB was synthesized in two steps according to literature procedures [42,46].

2.3. Peptide Synthesis and Purification

Solid-phase peptide synthesis was utilized on an automated peptide synthesizer MultiPep RS (Intavis AG, Koeln, Germany) using standard Fmoc-chemistry and preloaded resins. Standard coupling of all protected natural amino acids was performed as single couplings in dimethylformamid (DMF) using 5 equivalents of amino acids, HCTU as coupling reagents, and 10 equivalents of NMM as base for 1 h at room temperature. Special building groups, such as Fmoc-3,4'-AMPB, were coupled with 3 equivalents using DIC and HOBT in DMF/*N*-methyl-2-pyrrolidone (NMP) at room temperature and with gentle shaking in the dark overnight.

The *N*-terminal Fmoc-protecting group was removed by washing the resin with 20% piperidine for 20 min. The final side chain deprotection and cleavage from the resin employed a mixture of trifluoroacetic acid and water (90:10 Vol%) with gentle agitation for 2 h at room temperature.

The crude peptides were purified to >95% purity using preparative RP-HPLC (Gilson, Limburg, Germany). For both analytical and preparative use, the mobile phase was a mixture of water (eluent A) and acetonitrile (eluent B), respectively, each containing 0.1% trifluoroacetic acid. Samples were eluted with a linear gradient from 5% B to 95% B in 15 min for analytical runs and in 90 min for preparative runs on a semipreparative PLRP-S column (300 × 25 mm, 8 µm; Agilent Technologies, Waldbronn, Germany). Finally, all peptides were characterized by analytical HPLC Dionex Ultimate 3000 (Thermo Fisher Scientific, Dreieich, Germany) using a PLRP-S column (150 × 4.6 mm, 3 µm; Agilent Technologies, Waldbronn, Germany) and MALDI-MS (Bruker Microflex LT, Bremen, Germany), which gave the expected $[M+H]^+$ mass peaks.

2.4. Azobenzene Peptide Photoisomerization

Trans → *cis* isomerization was performed by irradiating the dissolved peptide in a 1 cm quartz cuvette for 30 min with light of 340 nm wavelength using a 50 W mercury lamp (VEB) and a 340 nm band pass filter (FB340-10, Thorlabs, Bergkirchen, Germany) under stirring. For *cis* → *trans* isomerization, the dissolved peptide was irradiated with light of 405 nm wavelength using a 1.4 W LED (M405L4, Thorlabs, Bergkirchen, Germany) for 30 min under stirring.

2.5. Aggregation Kinetics

ThT-monitored fibrillization assays of artificial peptides and mixtures with PTH_{25–37} were investigated by fluorescence intensity measurements using thioflavin T (ThT) as fluorescent dye. Lyophilized peptides were dissolved in 50 mM Na₂HPO₄ buffer solution with a pH value of 7.4 in a concentration of 2 mg/mL and kept on ice for the next steps. The samples were centrifuged at 13,000 × *g* rpm for 10 s and the concentrations were determined with a JASCO V-660 absorbance spectrometer (JASCO, Pfungstadt, Germany; PTH_{25–37} by absorbance at 205 nm and the molar extinction coefficient of 49,310 cm^{−1}M^{−1}; *trans*-AzoPTH_{25–37} by absorbance at 327 nm and the molar extinction coefficient of 13,000 cm^{−1}M^{−1}). *Cis*-AzoPTH_{25–37} was produced as described before. The solutions were centrifuged at 10,000 rpm for 1 h at 4 °C, the supernatant was transferred to another tube. The protein solutions were mixed in the desired ration and diluted with 50 mM Na₂HPO₄ buffer (pH 7.4) to obtain final concentrations of 0/100 µM PTH_{25–37}, 50 µM ThT, and 0/10/20/50/100 µM AzoPTH_{25–37}. For each sample, a total volume of 480 µL was prepared and 3 × 150 µL were transferred to a medium binding 96-well plate (Greiner Bio-One, Kremsmünster, Austria). The plate was sealed with a microplate cover. The fluorescence intensity was monitored at 37 °C using a BMG FLUOStar Omega multi-mode plate reader (BMG LABTECH, Ortenberg, Germany) using fluorescence excitation and emission wavelengths at 460 nm and 485 nm, respectively. One measurement cycle of 5 min consisted of double-orbital shaking for 150 s and incubating for 150 s.

2.6. Transmission Electron Microscopy (TEM)

TEM images were taken with an electron microscope (EM 900; Zeiss, Oberkochen, Germany) at 80 kV acceleration voltage. For preparation, 5 µL of the peptide solution were added on Formvar/Cu grids (mesh 200). After 3 min of incubation, the grids were gently cleaned with water for 0.1 min and then negatively stained using uranyl acetate (1%, *w/v*) for 1 min.

2.7. Seeding Assay

The seeding assay follows the same procedure as the ThT-monitored fibrillization assay for the determination of the aggregation kinetics. In addition, the final samples

contained 20 μM of seeds from *trans*-AzoPTH_{25–37} fibrils. The seeds were prepared via ultrasonication of a 100 μM mature *trans*-AzoPTH_{25–37} fibrils solution (Sonifier W-250 D, Branson Ultrascall, Dietzenbach, Germany; 15 times, 1 s 10% amplitude, 1 s pause).

3. Results & Discussion

3.1. Chemistry

To investigate the fibrillization behavior of PTH_{25–37}, the azobenzene switch was incorporated directly into the peptide backbone. We selected the 3,4'-azobenzene motif (Figure 1b) [42]. As it possesses suitable photochemical properties, e.g., an excellent half-life time with a stability larger than 20 h and switching wavelengths >300 nm. These are easily addressable by our photophysical equipment and also avoid eventual photodegradation. The synthesis was conducted in two steps (Figure 2a): in the first step, we conducted the Fmoc-protection of **2** [46], which in the second step reacts in a Mills reaction with an in situ-generated nitroso compound **3** to obtain the Fmoc-protected 3,4'-AMPB **5** in an overall yield of 68%.

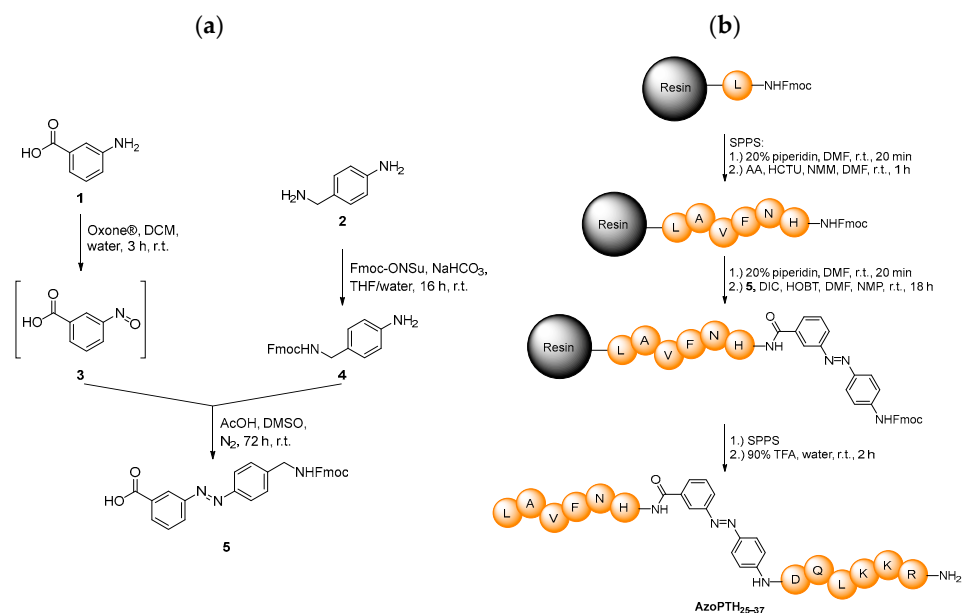


Figure 2. (a) Synthesis of Fmoc-protected *trans*-3,4'-AMPB **5**. (b) Solid-phase peptide synthesis strategy towards the peptide AzoPTH_{25–37}.

The modified azobenzene switch **5**, bearing the proper functionalities for Fmoc-chemistry, was incorporated into the peptide backbone of PTH_{25–37} via solid-phase peptide synthesis (Figure 2b). It replaces V31 in the artificial peptide AzoPTH_{25–37}, due to its central position along the peptide, expecting the largest impact on fibrillization after photoswitching. Furthermore, we probed the replacement of D30 or the insertion between D30 and V31, which led to a greater loss of solubility in the fibrillization buffer (240 μM vs. 25 μM vs. 60 μM ; Table S1). Thus, several of the generated peptides displayed strongly reduced solubility—an effect that is important for the subsequent investigations. All peptides were obtained in yields of 10–19%, and high purities as proven by both HPLC and MALDI-ToF measurements, in addition to 500 MHz NMR spectroscopy (Figures S1–S5 and S13–S15).

3.2. Photophysical Properties

We first studied the photophysical properties of the *cis-trans*-isomerization of AzoPTH_{25–37} (Figure 1b) by UV/Vis spectroscopy and HPLC analysis in pure water in order to minimize effects of a potential self-assembly and to quantify the generated amounts of the respective *cis*/*trans*-modified peptides before and after photoswitching. The UV/Vis spectra for the

pure isomers (Figure S6) were separated from the spectra of *trans*-enriched AzoPTH_{25–37} in the thermodynamically stable state after synthesis and in the *cis*-enriched photostationary state (PSS, Figure 3) with Wolfram Mathematica 12.2. The *trans*-isomer displays an absorption maximum at 327 nm ($\epsilon = 13,000 \text{ cm}^{-1}\text{M}^{-1}$) and a second maximum at 427 nm, while the *cis*-isomer possesses maxima at 288 nm and 433 nm. Both isomers display two isobestic points at 278 nm and 388 nm. They represent in the thermodynamically stable state a *cis-trans* ratio of 3:97. Under irradiation with UV light (340 nm), the *cis*-content could be increased of up to 82% in the *cis*-enriched PSS. Visible light (405 nm) yields 76% of the *trans*-isomer in the *trans*-enriched PSS via the back reaction. The difference of the *trans*-content between the *trans*-enriched PSS at 405 nm and the thermodynamically stable state arises from the overlapping of the $n \rightarrow \pi^*$ transitions of both isomers at this wavelength [47]. The rate of thermal *cis*-to-*trans* isomerization of AzoPTH_{25–37} follows first-order kinetics, and was determined by monitoring the increase of the $\pi \rightarrow \pi^*$ absorption band at 327 nm (Figure S7) via time-dependent UV measurements. In the absence of light at 37 °C, *cis*-AzoPTH_{25–37} isomerizes thermally with a rate constant of $3.53 \times 10^{-6} \text{ s}^{-1}$, corresponding to a half-life time of 79 h.

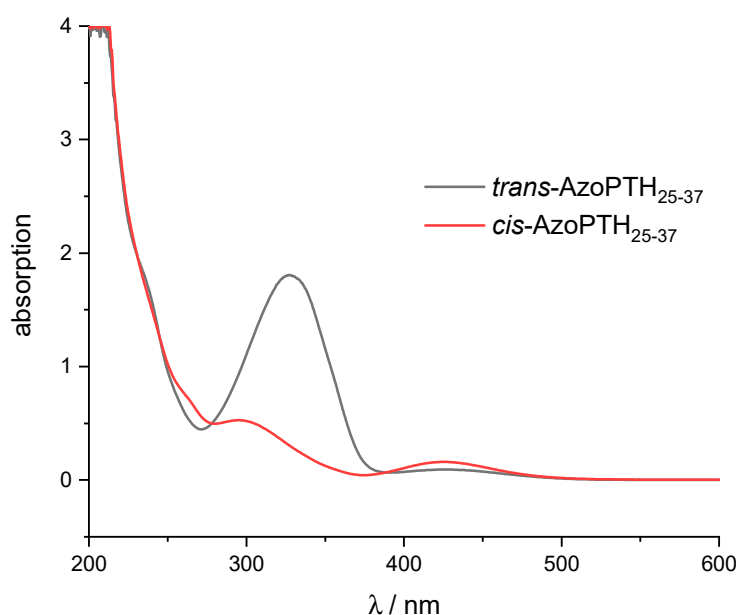


Figure 3. UV/Vis absorption spectra for *trans*-AzoPTH_{25–37} after synthesis and for the *cis*-enriched photo-stationary state after irradiation at 340 nm, which almost corresponds to *cis*-AzoPTH_{25–37}.

3.3. Aggregation Kinetics and TEM-Recordings

In order to determine the kinetics of fibril formation of both modified AzoPTH_{25–37} isomers a thioflavin T (ThT)-monitored fibrillization assay was conducted and compared to PTH_{25–37}. ThT is a benzothiazole compound that binds to the cross- β -sheet structure of amyloid fibrils [48]. Causing a large red shift of fluorescence excitation of ThT, which in turn enables the selective excitation of amyloid fibril-bound ThT and therefore the in situ observation of fibril formation.

In a first attempt, the fibrillization kinetics for pure *trans*-AzoPTH_{25–37}, *cis*-AzoPTH_{25–37}, and the PTH-derived peptide PTH_{25–37} were measured at 37 °C and the results are shown in Figure 4. Two characteristic times were used to characterize the fibrillization (Figure 4, Table 1): the lag time t_{lag} corresponds to the time before an increase in the fluorescence signal occurs; the characteristic time t_{char} indicates at which time 50% of the maximum fluorescence was reached.

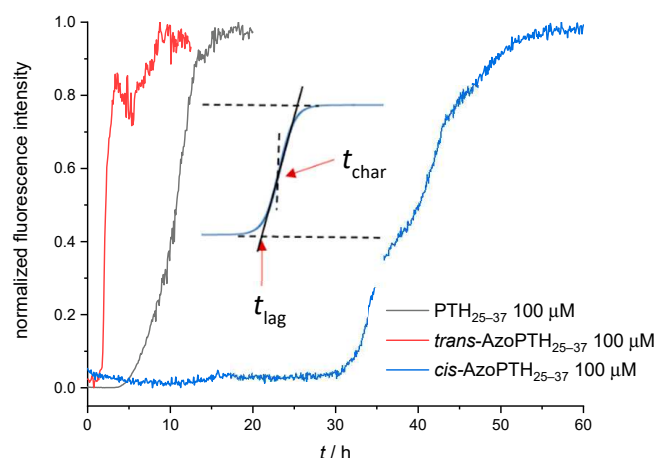


Figure 4. ThT-monitored fibrillization assay of PTH₂₅₋₃₇, *cis*-AzoPTH₂₅₋₃₇, and *trans*-AzoPTH₂₅₋₃₇ (average of triplets; $T = 37^\circ\text{C}$, buffer = 50 mM Na₂HPO₄, pH = 7.4): (black) PTH₂₅₋₃₇ (100 μM), (red) *trans*-AzoPTH₂₅₋₃₇ (100 μM), and (blue) *cis*-AzoPTH₂₅₋₃₇ (100 μM).

Table 1. Fibrillization parameters (t_{lag} , t_{char}) of PTH₂₅₋₃₇, *cis*-AzoPTH₂₅₋₃₇, *trans*-AzoPTH₂₅₋₃₇, and mixtures thereof ($T = 37^\circ\text{C}$, buffer = 50 mM Na₂HPO₄, pH = 7.4).

Sample	t_{lag} [h]	t_{char} [h]
PTH ₂₅₋₃₇ (100 μM)	7.2	10.9
<i>cis</i> -AzoPTH ₂₅₋₃₇ (100 μM)	34.4	42.4
<i>cis</i> -AzoPTH ₂₅₋₃₇ :PTH ₂₅₋₃₇ (100 μM :100 μM)	27.9	35.7
<i>cis</i> -AzoPTH ₂₅₋₃₇ :PTH ₂₅₋₃₇ (50 μM :100 μM)	16.3	21.2
<i>cis</i> -AzoPTH ₂₅₋₃₇ :PTH ₂₅₋₃₇ (20 μM :100 μM)	10.1	14.5
<i>cis</i> -AzoPTH ₂₅₋₃₇ :PTH ₂₅₋₃₇ (10 μM :100 μM)	6.9	7.9
<i>trans</i> -AzoPTH ₂₅₋₃₇ (100 μM)	1.6	2.1
<i>trans</i> -AzoPTH ₂₅₋₃₇ :PTH ₂₅₋₃₇ (100 μM :100 μM)	3.0	4.8
<i>trans</i> -AzoPTH ₂₅₋₃₇ :PTH ₂₅₋₃₇ (50 μM :100 μM)	8.0	8.6
<i>trans</i> -AzoPTH ₂₅₋₃₇ :PTH ₂₅₋₃₇ (20 μM :100 μM)	8.7	9.7
<i>trans</i> -AzoPTH ₂₅₋₃₇ :PTH ₂₅₋₃₇ (10 μM :100 μM)	7.5	8.9

The self-assembly of the *trans*-AzoPTH₂₅₋₃₇ was accelerated compared to PTH₂₅₋₃₇, while *cis*-AzoPTH₂₅₋₃₇ exhibited the opposite effect (Figure 4). The first increase of ThT fluorescence was observable after >30 h. Furthermore, *cis*-AzoPTH₂₅₋₃₇ shows a biphasic fibrillization behavior, while *trans*-AzoPTH₂₅₋₃₇ and PTH₂₅₋₃₇ show monophasic fibrillization. Compared to PTH₂₅₋₃₇, the magnitude of the ThT fluorescence of both AzoPTH₂₅₋₃₇ isomers was significant lower (Figure S9). This effect might arise from fluorescence quenching via the azobenzene moiety. To test this hypothesis, the fluorescence lifetime of ThT was measured either alone, in the presence of PTH₂₅₋₃₇ fibrils, or in the presence of *trans*-AzoPTH₂₅₋₃₇ fibrils (Figure S8). As expected the lifetime is increased in the presence of PTH₂₅₋₃₇ fibrils compared to the control experiment, while it is decreased significantly in the presence of *trans*-AzoPTH₂₅₋₃₇, which further supports our concept. In addition, this effect could be enhanced from a reduced binding affinity of ThT through a different peptide conformation of the fibril.

The observations of the ThT-monitored fibrillization assay were supported by negative stain transmission electron microscopy (TEM) after different time points (Figure 5). After 20 h, amyloid fibrils were only observable for PTH₂₅₋₃₇ and *trans*-AzoPTH₂₅₋₃₇ (Figure 5a,b), while *cis*-AzoPTH₂₅₋₃₇ formed amorphous aggregates (Figure 5e). Both peptides produced straight fibrils, whereby the single fibrils of PTH₂₅₋₃₇ were larger (>6 μm vs. <1.5 μm) and tend to aggregate further. Interestingly, we found fibrils after 60 h for *cis*-AzoPTH₂₅₋₃₇ (Figure 5g), which matched in the morphology those of *trans*-AzoPTH₂₅₋₃₇ even if they were

significantly shorter (<300 nm). This may result from the thermal *cis-trans*-isomerization, as the *cis*-content decreases and is reduced to 48% after 60 h.

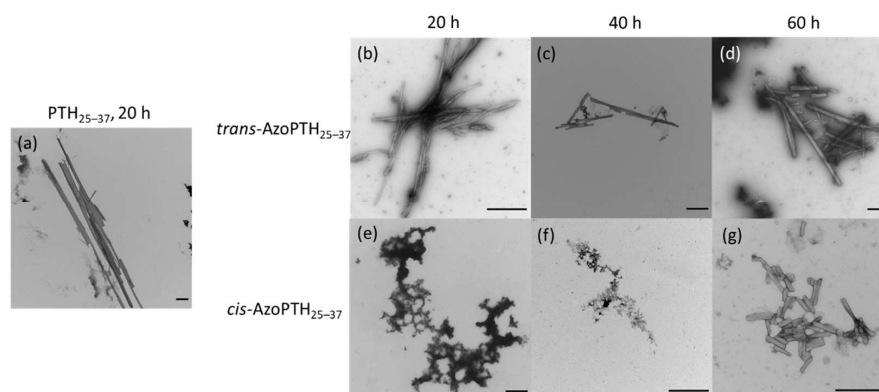


Figure 5. TEM recordings of fibrils obtained from PTH_{25–37}, *cis*-AzoPTH_{25–37}, and *trans*-AzoPTH_{25–37} at ($T = 37\text{ }^{\circ}\text{C}$, buffer = 50 mM Na₂HPO₄, pH = 7.4) after different time points (all scale bars corresponds to 500 nm). (a). PTH_{25–37} after 20 h, (b) *trans*-AzoPTH_{25–37} after 20 h (100 μM), (c) *trans*-AzoPTH_{25–37} after 40 h (100 μM), (d) *trans*-AzoPTH_{25–37} after 60 h (100 μM), (e) *cis*-AzoPTH_{25–37} after 20 h (100 μM), (f) *cis*-AzoPTH_{25–37} after 40 h (100 μM), and (g) *cis*-AzoPTH_{25–37} after 60 h (100 μM).

In further experiments, we investigated the (catalytic) influence of the AzoPTH_{25–37} isomers on the fibrillization of PTH_{25–37} (Figure 6). We previously observed such catalytic effects of β -turn modified amyloids (A β) on the fibrillization of the Alzheimer peptide A β _{1–40} [49]. Thus 100 μM of PTH_{25–37} were fibrillized in the presence of various concentrations of the respective AzoPTH_{25–37} isomer (10/20/50/100 μM). Kinetic measurements revealed that the fibrillization behavior of PTH_{25–37} was affected in the same way as the pure AzoPTH_{25–37} isomers.

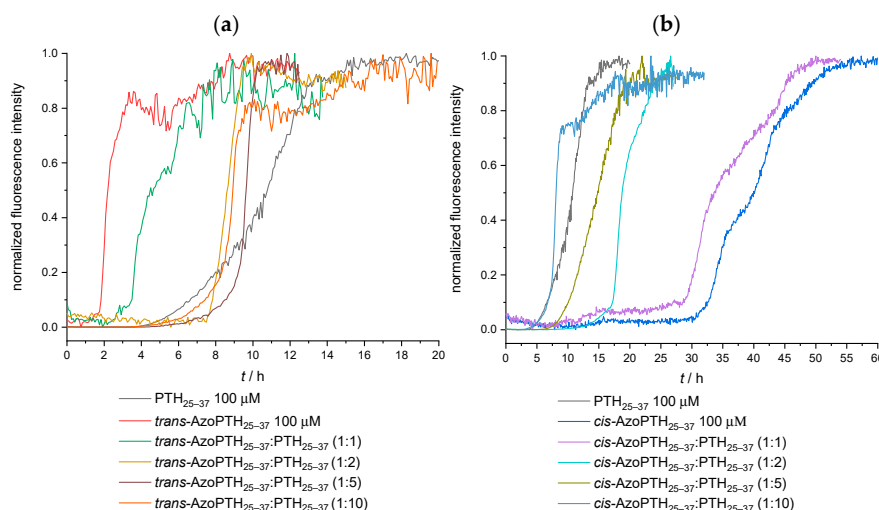


Figure 6. (a) ThT-monitored fibrillization assay of PTH_{25–37} and mixtures with *trans*-AzoPTH_{25–37} (average of triplets; $T = 37\text{ }^{\circ}\text{C}$, buffer = 50 mM Na₂HPO₄, pH = 7.4): (black) PTH_{25–37} (100 μM), (red) *trans*-AzoPTH_{25–37} (100 μM), (green) *trans*-AzoPTH_{25–37}:PTH_{25–37} (100 μM :100 μM), (dark yellow) *trans*-AzoPTH_{25–37}:PTH_{25–37} (50 μM :100 μM), (brown) *trans*-AzoPTH_{25–37}:PTH_{25–37} (20 μM :100 μM), and (orange) *trans*-AzoPTH_{25–37}:PTH_{25–37} (10 μM :100 μM) (b) ThT-monitored fibrillization assay of PTH_{25–37} and mixtures with *cis*-AzoPTH_{25–37} at 37 $^{\circ}\text{C}$: (black) PTH_{25–37} (100 μM), (blue) *cis*-AzoPTH_{25–37} (100 μM), (purple) *cis*-AzoPTH_{25–37}:PTH_{25–37} (100 μM :100 μM), (cyan) *cis*-AzoPTH_{25–37}:PTH_{25–37} (50 μM :100 μM), (olive) *cis*-AzoPTH_{25–37}:PTH_{25–37} (20 μM :100 μM), and (light blue) *cis*-AzoPTH_{25–37}:PTH_{25–37} (10 μM :100 μM).

While *trans*-AzoPTH_{25–37} accelerated the fibrillization and therefore reduced t_{lag} and t_{char} of the mixtures (Figure 6a), *cis*-AzoPTH_{25–37} inhibited the fibrillization and extended t_{lag} and t_{char} (Figure 6b). Interestingly, the biphasic fibrillization behavior of *cis*-AzoPTH_{25–37} was also observable for the *cis*-AzoPTH_{25–37}:PTH_{25–37} (100 μ M:100 μ M) mixture. These effects are reduced with decreasing concentration of the respective AzoPTH_{25–37} isomer. While the mixtures with *trans*-AzoPTH_{25–37} exhibited a concentration below 50 μ M, *trans*-AzoPTH_{25–37} had a higher t_{lag} than pure PTH_{25–37}. However, t_{char} was still shorter, and the stationary phase of the fibrillization was reached earlier.

TEM images were recorded for the peptide mixtures after 20 h (Figure 7). In contrast to the pure peptides, we could observe fibrils for all investigated ratios. Interestingly, the fibrils formed by the mixtures exhibit a similar twisted morphology regardless of the used AzoPTH_{25–37} isomer. Furthermore, the formation of larger aggregates like for the pure PTH_{25–37} (Figure 5) were only observed for a ratio of 1:10, indicating that the AzoPTH_{25–37} inhibits the formation of larger fibril aggregates.

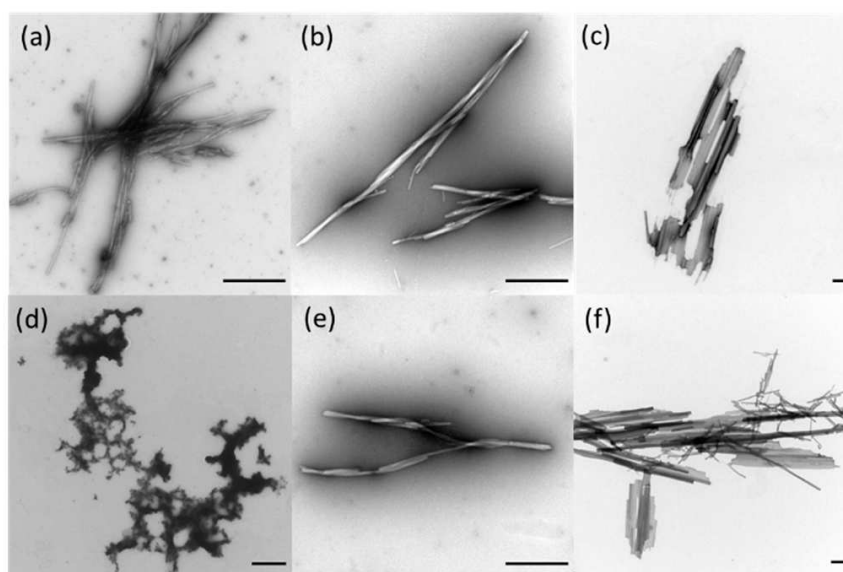


Figure 7. TEM recordings of fibrils obtained from PTH_{25–37}, *cis*-AzoPTH_{25–37}, and *trans*-AzoPTH_{25–37} at 37 °C after 20 h (scale bar = 500 nm): (a) *trans*-AzoPTH_{25–37} (100 μ M), (b) *trans*-AzoPTH_{25–37}:PTH_{25–37} (100 μ M:100 μ M), (c) *trans*-AzoPTH_{25–37}:PTH_{25–37} (10 μ M:100 μ M), (d) *cis*-AzoPTH_{25–37} (100 μ M), (e) *cis*-AzoPTH_{25–37}:PTH_{25–37} (100 μ M:100 μ M), and (f) *cis*-AzoPTH_{25–37}:PTH_{25–37} (10 μ M:100 μ M).

3.4. Seeding Experiments

To determine whether both isomers of AzoPTH_{25–37} are able to form fibrils or only the *trans*-isomer, we investigated, if *trans*-AzoPTH_{25–37} fibrils were able to induce seeding [50]. A 100 μ M solution of each isomer was treated with 20 μ M of mature *trans*-AzoPTH_{25–37} fibrils, and the kinetics of the fibril formation were investigated via a ThT-monitored fibrillization assay (Figure 8). While the fibrillization of the *trans*-isomer was accelerated compared to the unseeded monomer, we were not able to observe fibrillization for the *cis*-isomer. This indicates that the *cis*-isomer is unable to nucleate amyloid formation as well as elongate preformed fibrils. The observed fibrils after 60 h for the *cis*-isomer are presumably formed by the thermally isomerized *trans*-isomer.

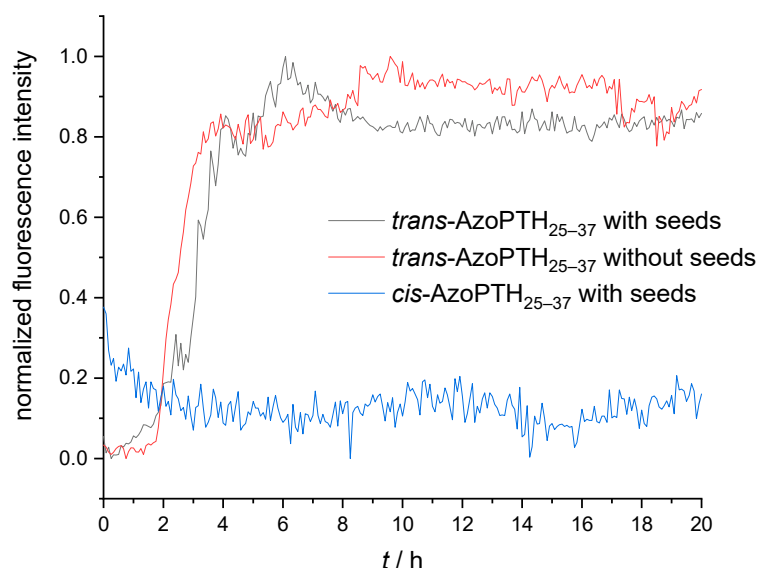


Figure 8. ThT-monitored fibrillization assay of cross-seeding studies with *cis*-AzoPTH₂₅₋₃₇ and *trans*-AzoPTH₂₅₋₃₇ monomeric peptides and mature *trans*-AzoPTH₂₅₋₃₇ fibrils as seeds (average of triplets; $c_{\text{monomer}} = 100 \mu\text{M}$, $c_{\text{seed}} = 20 \mu\text{M}$, $T = 37^\circ\text{C}$, buffer 50 mM Na₂HPO₄, pH = 7.4): (black) *trans*-AzoPTH₂₅₋₃₇ with seeds, (red) *trans*-AzoPTH₂₅₋₃₇ without seeds, and (blue) *cis*-AzoPTH₂₅₋₃₇ with seeds.

4. Conclusions

We here report for the first time a photoswitchable fibrillizing PTH-derived peptide, which is able to modulate its fibrillization by embedding an azobenzene photoswitch in the middle of PTH₂₅₋₃₇. PTH₁₋₈₄ is a peptide hormone, which is stored as functional amyloids in secretory granules. Its physiological role is well studied, but it still lacks detailed information about its exact fibril structure. We used the 3,4'-AMPB photoswitch to investigate the fibril formation of the fibril core fragment of PTH₁₋₈₄ by incorporating the azobenzene into the peptide backbone, yielding the modified PTH-derived peptide AzoPTH₂₅₋₃₇. We could show that the *trans*-isomer is able to form fibrils, while the *cis*-isomer induces a conformational change that inhibits fibril formation. Hypothetically, we can also conclude that there might not be a β -turn in the fibril structure of PTH₁₋₈₄, as the *cis*-conformer would be reminiscent of such a structure, whereas the *trans*-conformer would not. Most importantly, we were able to show that the modified peptides can catalytically inhibit fibrillization of the PTH₂₅₋₃₇, underscoring the importance of seeding during this fibrillization process, which in the future allows for a reversible triggering of the fibrillization by light as an external stimulus. Studies are in progress to investigate if the photocontrol is also possible with the photoswitch at other positions of the backbone and if we can also control the fibrillization of full-length PTH₁₋₈₄ with ours or other modified peptides. This represents a novel strategy to control bioavailability of proteins, specifically of PTH peptides and other fibrillating peptides, where not only the concentration of the bioactive form can be controlled by an added photoswitchable peptide, but also the fibrillization as such, important to guide nerve cell regeneration and other directed growth processes in euryotic cells. For a potential clinical perspective, we want to investigate the cytotoxicity of our peptides as well as the ability to influence the fibrillization of larger PTH-derived peptides (e.g., PTH₁₋₃₄ and PTH₁₋₈₄) in vitro and in vivo. As known from other azobenzene containing drugs/prodrugs (e.g., Prontosil), the azobenzene moiety is metabolized in liver tissue via azoreductases, yielding two aniline moieties or through intestinal microbes [51,52]. This is potentially important for the photoswitching inside cells by light, allowing them to tune the reversible fibrillization of other amyloidogenic peptides, which important for regeneration of nerve cells, as reported earlier. Thus, peptide fibrils

can seed potential harmful amyloidogenic peptides, which is known from recent work quite prominently [53]. This is a strategy to trigger fiber-formation from the outside via photochemical triggering—thus avoiding the toxic effects of the fibers outside the cells but enabling triggered fibrillization inside the cell to exert the desired effects, allowing them to promote the recovery of spinal cord injuries.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/biomedicines10071512/s1>, Figure S1: (A) HPLC-trace of AzoPTH_{25–37} (*cis*-isomer at 5.353, *trans*-isomer at 5.530). (B) MALDI-spectrum of AzoPTH_{25–37}; Figure S2: (A) HPLC-trace of SP1 (*cis*-isomer at 5.557, *trans*-isomer at 5.790). (B) MALDI-spectrum of SP1; Figure S3: (A) HPLC-trace of SP2 (*cis*-isomer at 5.223, *trans*-isomer at 5.460). (B) MALDI-spectrum of SP2; Figure S4: (A) HPLC-trace of SP3 (*cis*-isomer at 5.560, *trans*-isomer at 5.807). (B) MALDI-spectrum of SP3; Figure S5: (A) HPLC-trace of SP4 (*cis*-isomer at 5.363, *trans*-isomer at 5.547). (B) MALDI-spectrum of SP4; Figure S6: Separated UV/Vis-spectra of the pure isomers of AzoPTH_{25–37}; spectra were separated with Wolfram Mathematica 12.2; Figure S7: (A) UV/Vis-spectra of *trans*-isomer, *cis*-enriched PSS, and *cis*-enriched PSS sample after distinct time points in the dark. (B) logarithmic application of the absorption change over time to determine rate constant k and half-life time $t_{1/2}$; Figure S8: Time-resolved fluorescence measurement (excitation wavelength = 460 nm, emission wavelength = 480 nm) of unbound ThT (black), ThT bound to PTH_{25–37} fibrils (dark green), ThT bound to *trans*-AzoPTH_{25–37} fibrils (light green); Figure S9: ThT monitored fibrillation assays ($c = 100 \mu\text{M}$, 37°C , $50 \text{ mM Na}_2\text{HPO}_4$, $\text{pH } 7.4$). (A) PTH_{25–37}, (B) *trans*-AzoPTH_{25–37}, (C) *cis*-AzoPTH_{25–37}; Figure S10: ThT monitored fibrillation assays of mixtures of PTH_{25–37}, *trans*-AzoPTH_{25–37}, and *cis*-AzoPTH_{25–37} (37°C , $50 \text{ mM Na}_2\text{HPO}_4$, $\text{pH } 7.4$). (A) *trans*-AzoPTH_{25–37}:PTH_{25–37} ($100 \mu\text{M}:100 \mu\text{M}$), (B) *cis*-AzoPTH_{25–37}:PTH_{25–37} ($100 \mu\text{M}:100 \mu\text{M}$), (C) *trans*-AzoPTH_{25–37}:PTH_{25–37} ($50 \mu\text{M}:100 \mu\text{M}$), (D) *cis*-AzoPTH_{25–37}:PTH_{25–37} ($50 \mu\text{M}:100 \mu\text{M}$), (E) *trans*-AzoPTH_{25–37}:PTH_{25–37} ($20 \mu\text{M}:100 \mu\text{M}$), (F) *cis*-AzoPTH_{25–37}:PTH_{25–37} ($20 \mu\text{M}:100 \mu\text{M}$), (G) *trans*-AzoPTH_{25–37}:PTH_{25–37} ($10 \mu\text{M}:100 \mu\text{M}$), (H) *cis*-AzoPTH_{25–37}:PTH_{25–37} ($10 \mu\text{M}:100 \mu\text{M}$); Figure S11: ^1H -NMR spectrum (top; 400 MHz , $\text{DMSO-}d_6$) and ^{13}C -NMR spectrum (bottom; 100 MHz , $\text{DMSO-}d_6$) of (9H-Fluoren-9-yl)methyl (4-aminobenzyl)carbamate; Figure S12: ^1H -NMR spectrum (top; 400 MHz , $\text{DMSO-}d_6$) and ^{13}C -NMR spectrum (bottom; 100 MHz , $\text{DMSO-}d_6$) of Fmoc-3,4'-AMPB (mixture of isomers); Figure S13: ^1H -NMR spectra (500 MHz , D_2O) of AzoPTH_{25–37} (top, *trans*-isomer) and SP1 (bottom, *trans*-isomer); Figure S14: ^1H -NMR spectra (500 MHz , D_2O) of SP2 (top, *trans*-isomer) and SP3 (bottom, *trans*-isomer); Figure S15: ^1H -NMR spectrum (500 MHz , D_2O) of SP4 (*trans*-isomer); Scheme S1: Synthesis of Fmoc-protected 3,4'-AMPB 7. (a) Fmoc-ONSu, triethylamin, DMF/MeCN, 16 h, room temperature. (b) Oxone®, DCM, water, 3 h, room temperature. (c) AcOH, DMSO, N_2 , 72 h, room temperature; Table S1: Primary sequence and solubility in $50 \text{ mM Na}_2\text{HPO}_4$ buffer ($\text{pH } 7.4$) of peptides AzoPTH_{25–37} and SP1–SP4.

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Photocontrolled Reversible Amyloid Fibril Formation of Parathyroid Hormone-Derived Peptides

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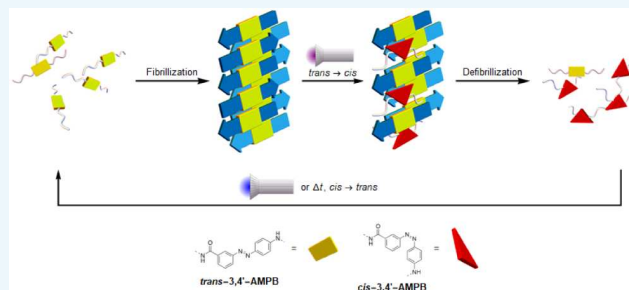


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ABSTRACT: Peptide fibrillization is crucial in biological processes such as amyloid-related diseases and hormone storage, involving complex transitions between folded, unfolded, and aggregated states. We here employ light to induce reversible transitions between aggregated and nonaggregated states of a peptide, linked to the parathyroid hormone (PTH). The artificial light-switch 3-[[[(4-aminomethyl)phenyl]diazanyl]benzoic acid (AMPB) is embedded into a segment of PTH, the peptide PTH_{25–37}, to control aggregation, revealing position-dependent effects. Through *in silico* design, synthesis, and experimental validation of 11 novel PTH_{25–37}-derived peptides, we predict and confirm the amyloid-forming capabilities of the AMPB-containing peptides. Quantum-chemical studies shed light on the photoswitching mechanism. Solid-state NMR studies suggest that β -strands are aligned parallel in fibrils of PTH_{25–37}, while in one of the AMPB-containing peptides, β -strands are antiparallel. Simulations further highlight the significance of π – π interactions in the latter. This multifaceted approach enabled the identification of a peptide that can undergo repeated phototriggered transitions between fibrillated and defibrillated states, as demonstrated by different spectroscopic techniques. With this strategy, we unlock the potential to manipulate PTH to reversibly switch between active and inactive aggregated states, representing the first observation of a photostimulus-responsive hormone.



INTRODUCTION

Modulating a protein's secondary structure stands as a pivotal strategy to define and harness its functionality.¹ While numerous protein structures are identified, predicted, and engineered, the concept of inducing conformational changes by external triggers to alter their biological activities remains rare. Temperature,² pH,³ polarity,⁴ or light offer avenues for such a dynamic control, in particular when applied to sensitive functional groups inserted inside the protein. Among these, light emerges as a particularly advantageous stimulus, providing precise temporal control across vast time scales, noninvasiveness, and compatibility with intricate matrices like living tissues.⁵ Leveraging light-induced conformational changes has demonstrated success in various proteins, from transporter-proteins like rhodopsins to enzymes, showcasing potential applications in photopharmacology and diverse enzymatic processes.^{5,6} Moreover, external photoswitches have successfully enabled photomodulation in a diverse array of enzymes,⁷ modifying binding affinities,⁸ facilitating peptide purification through photoaffinity,⁹ and controlling secondary structure alterations.¹⁰ Beyond the structure of individual peptides or proteins, there is a strong interplay between secondary structure and aggregation of peptides. This process is particularly pronounced in the context of amyloid

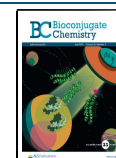
aggregation, where proteins form β -sheet-rich structures, leading to the formation of highly ordered fibrillar aggregates.¹¹ Such fibrils are a hallmark of several neurodegenerative diseases, including Alzheimer's and Parkinson's. Understanding and controlling amyloid aggregation can thus be crucial for developing therapies for diseases associated with these pathological protein assemblies. However, controlling peptide aggregation proves to be a significant challenge, given the complex processes involved. This challenge arises from intricate intrapeptide and interpeptide interactions, coupled with extensive conformational changes on a large scale, particularly when considering the regulation of primary and secondary nucleation preceding fibrillization. Therefore, the utilization of light to control amyloid assembly processes represents a groundbreaking advancement, enabling control over the bioavailability of hormones, such as the parathyroid hormone (PTH). PTH is reversibly stored in functional

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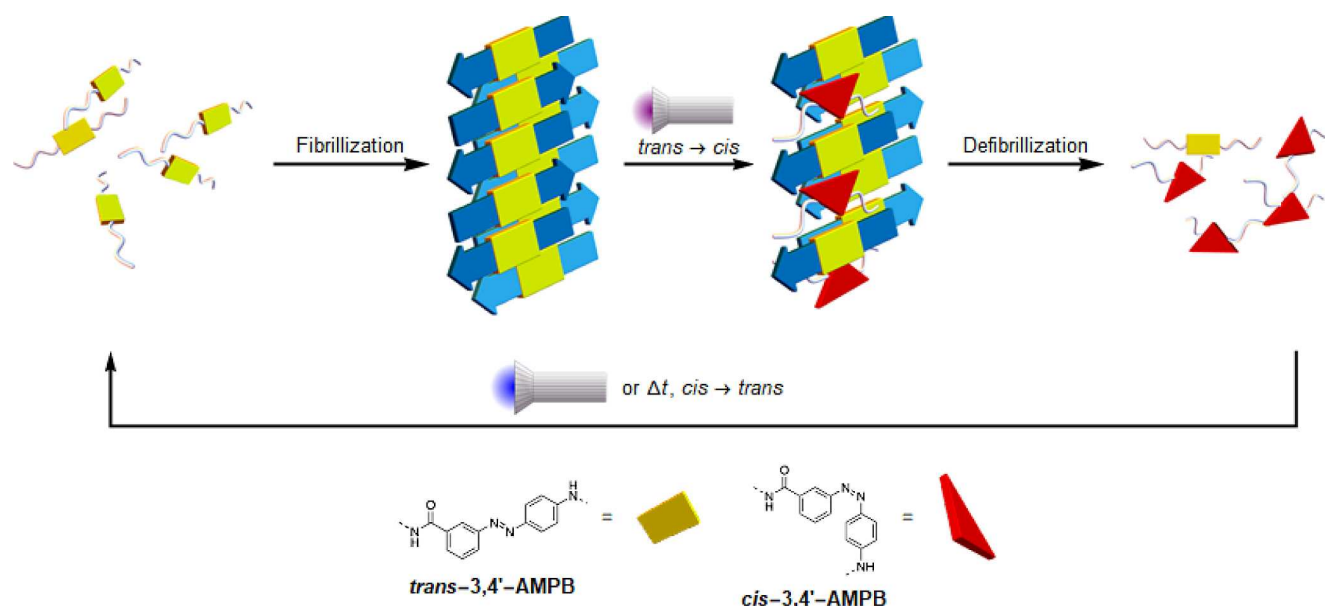


Figure 1. Concept for a light-driven (de-)aggregation of the parathyroid-hormone (PTH). The artificial light-switch, 3-[(4-aminomethyl)-phenyl]diazanylbenzoic acid (*cis/trans*-3,4'-AMPB) is embedded at various positions of a peptide-fragment of PTH, switching between the *cis*/*trans*-form, in this mode regulating the reversible assembly of the peptide into fibrils.

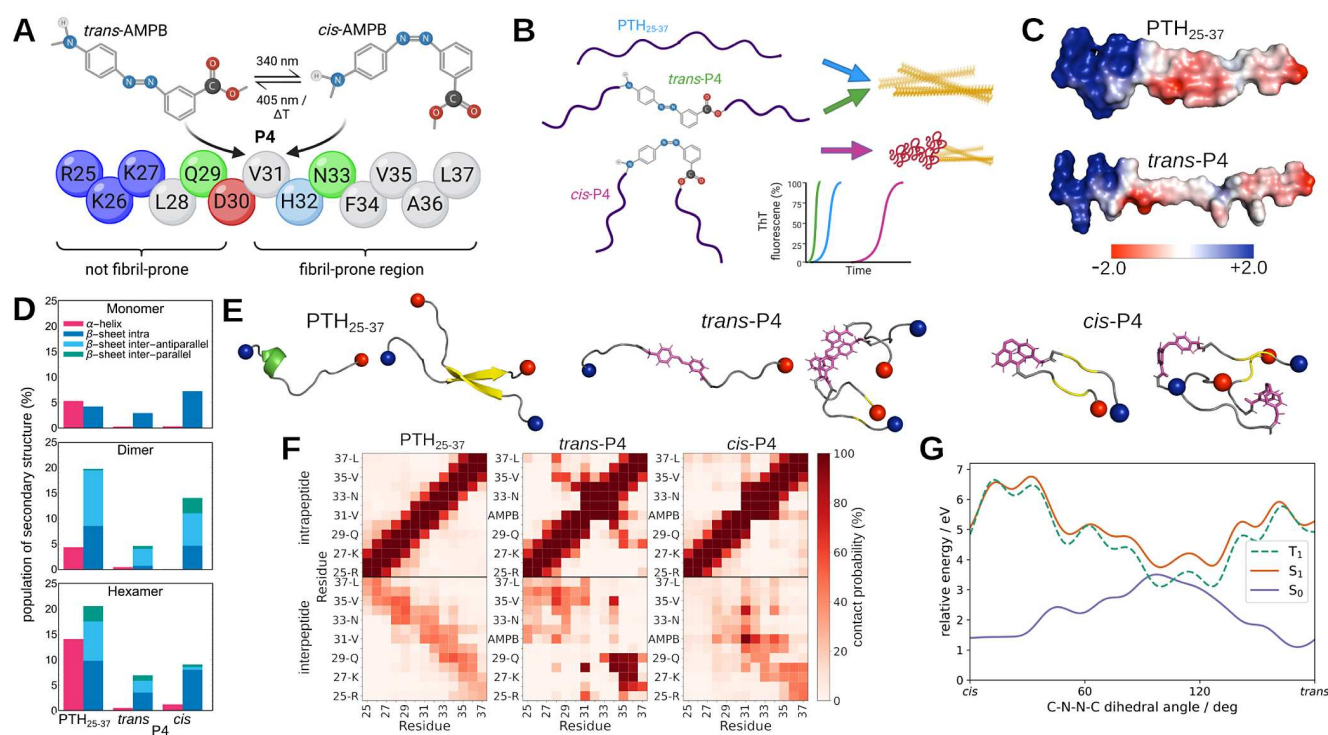


Figure 2. (A) Sequence of PTH_{25–37}, where the fibril-forming region identified by bioinformatic analysis is labeled, and the chemical structure of AMPB, which replaces V31 in P4, is shown. The side chains of the amino acids, shown in blue and red, are positively and negatively charged at pH 7.4, respectively, with H32 shown in light blue as a borderline case, while green and gray indicate polar and hydrophobic amino acids, respectively. (B) PTH_{25–37} and *trans*-P4 form fibrils, as shown in the ThT fluorescence cartoon below (blue and green line), while *cis*-P4 initially forms amorphous aggregates, which then transform into fibrils (red line in ThT cartoon). (C) Electrostatic potentials surface (values in kTe^{−1} according to the color scale at the bottom) of PTH_{25–37} and *trans*-P4. (D) Average simulated secondary structure population, divided into α -helical, intrapeptide β -sheets, interpeptide parallel, and antiparallel β -sheets as indicated by the color code. Results are shown for simulations of monomers, dimers, and hexamers of PTH_{25–37}, *trans*-P4, and *cis*-P4. (E) Representative snapshots of the monomer and dimer simulations, with the α -helix shown in green, the β -sheets in yellow, the random coil in gray, the N- and C-termini as blue and red spheres, respectively, and AMPB in purple. (F) Contact matrix of dimer simulations for PTH_{25–37}, *trans*-P4, and *cis*-P4. (G) Quantum chemical potential energy scan of the *trans* $\rightarrow$ *cis* isomerization of the AMPB photoswitch in P4.

amyloid fibrils, as these fibrils, unlike, e.g., amyloid-A β fibrils, can disintegrate again after aggregation. In this study, we pioneer the utilization of light to achieve a reversible transition between the aggregated and nonaggregated states of PTH (Figure 1), allowing the regeneration of PTH fibrils through precise light control, presenting a transformative advancement in the field.¹²

PTH, a reversibly fibrillating 84-amino acid hormone, is ubiquitously distributed in animals.¹³ Responsible for regulating calcium and phosphate homeostasis,¹⁴ mature PTH_{1–84} is stored in functional amyloids before secretion,¹⁵ wherein its N-terminal pro-sequence potentially prevents premature aggregation.¹⁶ PTH-related diseases prompt the use of approved drugs, Natpara (PTH_{1–84}) and Forteo (PTH_{1–34}), addressing hormone imbalances.¹⁷ While the physiological role of the N-terminal 34 amino acids of PTH_{1–84} is well investigated, being crucial for the activation of G-protein coupled receptors of bone and kidney cells¹⁸ and in the nervous system for calcium and phosphate homeostasis,¹⁹ there remains a knowledge gap regarding the fibrillization process and the resulting fibrils. Current knowledge indicates that under physiological conditions, the thermodynamic stability of PTH_{1–84} fibrils is low enough to allow dissociation upon dilution, with the fibril-forming sequence encompassing amino acids R25–L37.¹⁵ Previous research explored the impact of the pro-sequence¹⁶ and environmental factors on the fibrillization process^{20,21} of PTH, however, with an only limited insight into the precise structural parameters controlling assembly and disassembly of the fibrils.²² The aim of the current work is to gain deeper insights into the fibrillization of PTH_{25–37}, coupled with the ability to reversibly control this process and understand the structural principles of the assembly process. We employ a synthetic approach for synthesizing PTH_{25–37} peptides bearing the azobenzene photoswitch, 3-[(4-aminomethyl)phenyl]-diazanyl}benzoic acid (AMPB), guided by bioinformatics to strategically place AMPB for optimal photocontrol, with biophysical techniques such as thioflavin T (ThT) fluorescence, CD spectroscopy, and transmission electron microscopy (TEM) to assess the peptide aggregation dynamics and aggregate morphology. This is further combined with molecular dynamics (MD) simulations to elucidate the impact of AMPB on the structure and dynamics of the designed peptides and their small oligomers (amounting to a total of 285 μ s simulation time), while wide-angle X-ray scattering (WAXS) and solid-state nuclear magnetic resonance (ssNMR) spectroscopy in conjunction with MD are employed to provide structure models for selected amyloid fibrils. Finally, quantum-chemical potential energy scans of both the ground (S_0) and excited states (S_1 and T_1) reveal insights into the photo-switching mechanism of the AMPB group and its electronic interaction when integrated into PTH_{25–37}, considering both the monomeric and dimeric peptide state. Providing a thorough understanding of the aggregation behavior of PTH_{25–37} and its derivatives, we have engineered a peptide analogue with the unique capability of reversible light-induced switching of its aggregation state.

RESULTS AND DISCUSSION

Aggregation Characteristics of Unmodified PTH_{25–37} and Engineered Peptide. To understand the aggregation of PTH_{25–37} and make informed design decisions, we began our analysis with a detailed examination of the original peptide PTH_{25–37} and one engineered peptide (P4),²² bearing the

photoswitch in the central part of the peptide (Figure 2A). Unmodified PTH_{25–37} and *trans*-P4 form amyloid fibrils within 15 and 10 h, respectively, whereas *cis*-P4 initially forms amorphous aggregates and only forms amyloid fibrils after about 50 h (Figure 2B).²² The increased rate of aggregation of *trans*-P4 compared to PTH_{25–37} is assigned to the physicochemical properties of the amino acids and the phototrigger, AMPB, that make up the peptide (Figure 2A). PTH_{25–37} has three positive charges (RKK) at the N-terminus, followed by a mixture of hydrophobic, polar and one negatively charged residue (D30) in the middle, while the C-terminal residues are mainly hydrophobic. This uneven distribution of physicochemical properties across the sequence is also reflected in the electrostatic potential surface, which shows a strongly positively charged N-terminus and a more hydrophobic C-terminal half with some negative charge accumulation beyond the first three residues (Figure 2C). The assumption therefore is that amyloid aggregation of PTH_{25–37} is driven by the residues after the initial RKK sequence. To test this assumption, we used four aggregation predictors: PASTA,²³ AGGRESCAN,²⁴ AmyloGRAM,²⁵ and FoldAmyloid,²⁶ which show that the sequence ³²HNFVA³⁷L is an aggregation hotspot and that the first five amino acids ²⁵RKKL²⁹Q should not contribute to fibrillization. For ³⁰DV, a low tendency to aggregation was observed. In P4, one of the residues of the later sequence, V31, is replaced by AMPB. This increases the overall hydrophobicity of the peptide, while the azo group itself adds some positive charge to the electrostatic potential, which compensates for the predominant negative charge in the C-terminal part of the peptide. These two effects, therefore, explain the faster aggregation kinetics of *trans*-P4 compared to PTH_{25–37}.

To understand the aggregation mechanisms and elucidate the structures that form during aggregation, namely first oligomers and finally fibrils, we performed MD simulations and ssNMR spectroscopy of both PTH_{25–37} and P4, the latter simulated in the *trans*- and *cis*-states (see Table S1 for an overview of simulations performed). In this way, we can also develop a structural understanding of why the fibrillization of *cis*-P4 is slowed down and generally reduced. The monomers of either PTH_{25–37}, *trans*-P4, and *cis*-P4 are mainly in a random coil state (Figure 2D). Nevertheless, small differences between the monomeric peptides can be observed. One of them is that PTH_{25–37} shows a certain tendency to form an α -helix in the N-terminal residues up to D30, which is lost when V31 is replaced by AMPB (Figure 2E). When the azo group is in the *cis*-state, this allows more intrapeptide contacts, including the formation of a β -hairpin, whereas in the *trans*-state, P4 is mostly in a fully elongated state. The dimer simulations showed an increase in β -sheets for all three peptides, but most for PTH_{25–37}. This is a result of peptide aggregation, as there is a particular increase in β -sheets between the peptides (Figure 2D), which are mainly arranged antiparallel, as shown by the contact matrix (Figure 2F). In an antiparallel arrangement, the three positively charged N-terminal residues RKK can interact with the negative charge at the C-terminus of the neighboring peptide. Interestingly, the *trans*-P4 peptide, which we expected to aggregate the fastest based on the fibrillization data, forms fewer β -sheets at the oligomer level. Instead, the interpeptide interactions are dominated by contacts between the two AMPB groups, while the peptides are aligned antiparallel to each other. In contrast, although the *cis*-P4 peptide adopts β -sheet structures to some extent due to intrapeptide hairpins, it

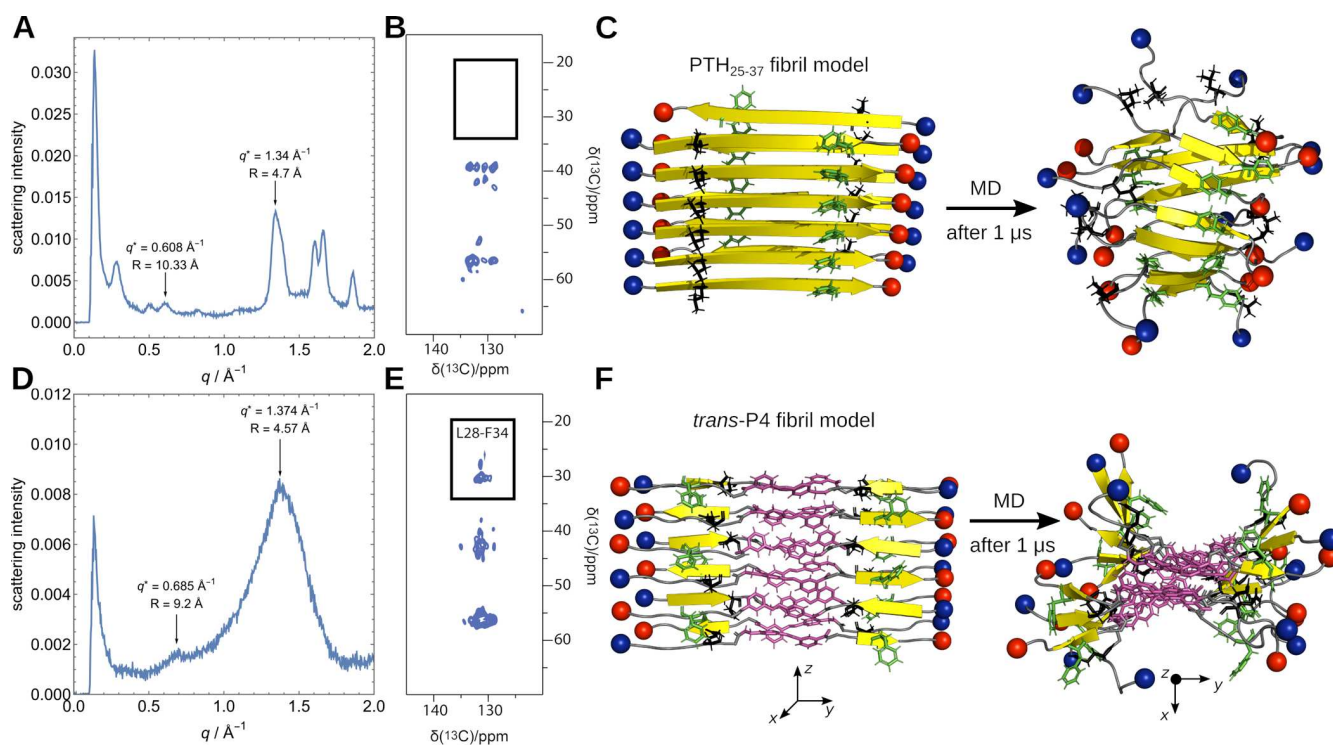


Figure 3. (A, D) WAXS results for PTH_{25–37} and the AMPB-modified PTH_{25–37} peptide *trans*-P4. (B, E) Solid-state NMR (ssNMR) 2D ¹³C–¹³C proton driven spin diffusion spectra close to rotational resonance (mixing time 1 s) of fibrils of the PTH_{25–37} and *trans*-P4 peptide with uniformly ¹³C-labeled L28 and F34. (C, F) Fibril models of PTH_{25–37} and *trans*-P4 constructed based on the ssNMR (left) and at the end of MD simulations (right), where β -sheets are shown in yellow, random coil in gray, the N- and C-termini as blue and red spheres, respectively, AMPB in purple, and the side chains of L28 and F34 are highlighted as black and green sticks, respectively. Note that in panel F, the structure on the right-hand side is rotated by 90° to better represent the fibrillar arrangement.

aggregates mainly randomly, as confirmed by the many interpeptide contacts, which is consistent with the amorphous aggregates observed *in vitro*. The distinct differences between peptide aggregation involving *cis*- and *trans*-AMPB allow fibril formation to be controlled at the molecular level by isomerization, providing a solid basis for photocontrol of amyloid formation.

In an effort to identify the nucleus of fibril formation, we also simulated the hexamer formation of PTH_{25–37}, *trans*-P4, and *cis*-P4. However, this system size is still too small (or the simulation time too short) to observe the emergence of fibrillar structures. On the contrary, the hexamers of these peptides are less ordered than the dimers. This confirms the experimental observation that the propensity of PTH_{25–37} to form amyloid is much lower than that of other peptides, such as that of A β _{16–22}. For the latter, we observe the formation of ordered hexamers when simulated under the same conditions as here,²⁷ while ThT experiments for this peptide show that fibrils are already present at the beginning of the measurements.²⁸ For both PTH_{25–37} and P4, we even see a decrease in interpeptide β -sheets when we increase the system size to the hexamer, which can be explained by the increase in dimensionality of the conformational space, which allows for more interpeptide interactions and makes it less likely to see ordered aggregates on short time scales. Another interesting observation is that the amount of helix formed increased in the hexamer system of the PTH_{25–37} compared to its monomer and dimer. This again reflects the helical propensity of this peptide, which can be stabilized by interpeptide interactions, a common observation

in intrinsically disordered peptides that can fold after binding to interaction partners.²⁹

We further investigated the electronic interactions and the photoswitching mechanism of AMPB integrated into PTH_{25–37}, both in the monomer and dimer states. To this end, we generated 34 switching trajectories using MD simulations to model the *trans* $\rightarrow$ *cis* isomerization of the AMPB photoswitch along the CNNC dihedral angle. The resulting ensemble of pathways was individually analyzed using ONIOM-based QM/QM2³⁰ (NEVPT2³¹/xTB³²) calculations, yielding potential energy scans for the ground and first excited triplet and singlet states (Figures 2G and S1). While the calculations clearly indicate that photoswitching is feasible, we also found that several scans exhibit structural barriers, that may impact the fluorescence wavelength and lifetime compared to the pure photoswitch (Figure S2).³³ Further elucidation through principal component analysis of the distance matrices between peptide residues (Figures S3 and S4) revealed critical structural motifs potentially responsible for the observed S₁ barrier in *trans* $\rightarrow$ *cis* isomerization paths. These motifs particularly involve configurations of R25 interacting with A36/L37 and H32/Q33, in both monomeric and dimeric forms (Figure S5).

Structures of PTH_{25–37} and P4 Fibrils. The final state of aggregation, the amyloid fibrils, was characterized by wide-angle X-ray scattering (WAXS) and ssNMR, and the resulting data were used to generate structural models whose stability was evaluated in MD simulations. The WAXS measurement was performed with preformed fibrils of either PTH_{25–37} or *trans*-P4. Due to the isotropic orientations of the fibrils and the

Table 1. Designed PTH_{25–37} Peptides Containing the AMPB Photoswitch (Azo) Inserted between Two Amino Acids (i) or an Amino Acid Exchanged by AMPB (e)^a

	peptide	primary sequence	modification	<i>t</i> _{1/2} (cis) [h]
	PTH _{25–37}	²⁵ RKKLQ ³⁰ DVHNF ³⁵ VAL	-	-
central	P1 (i)	²⁵ RKKLQ ³⁰ D-Azo-VHNF ³⁵ VAL	D30-Azo-V31	90
	P2 (i)	²⁵ RKKLQ ³⁰ DV-Azo-HNF ³⁵ VAL	V31-Azo-H32	90
	P3 (e)	²⁵ RKKLQ-Azo-VHNF ³⁵ VAL	D30 → Azo	-
	P4 (e)	²⁵ RKKLQ ³⁰ D-Azo-HNF ³⁵ VAL	V31 → Azo	97
	P5 (e)	²⁵ RKKLQ-Azo-HNF ³⁵ VAL	D30,V31 → Azo	-
	P6 (i)	²⁵ RKKLQ-Azo- ³⁰ DVHNF ³⁵ VAL	Q29-Azo-D30	-
N-terminal	P7 (i)	²⁵ RKKL-Azo-Q ³⁰ DVHNF ³⁵ VAL	L28-Azo-Q29	72
	P8 (i)	²⁵ RK-Azo-KLQ ³⁰ DVHNF ³⁵ VAL	K26-Azo-K27	89
	P9 (e)	²⁵ R-Azo-KLQ ³⁰ DVHNF ³⁵ VAL	K26 → Azo	86
C-terminal	P10 (i)	²⁵ RKKLQ ³⁰ DVHN-Azo-F ³⁵ VAL	N33-Azo-F34	-
	P11 (i)	²⁵ RKKLQ ³⁰ DVHNF ³⁵ V-Azo-AL	V35-Azo-A36	-
	P12 (e)	²⁵ RKKLQ ³⁰ DVHNF-Azo-AL	V35 → Azo	63

^aThe corresponding peptides were prepared by on-resin synthesis (Merrifield-synthesis) using Fmoc-based building blocks. The synthesis of the AMPB photoswitch follows previously published methods.²² The half-life time *t*_{1/2} (hours) of the cis-form of the peptides was investigated via UV/vis spectroscopy (see the experimental section for further experimental details).

resulting isotropic scattering pattern (see Figure S6), the scattering intensities were angular averaged and are displayed in Figure 3 A, D. For PTH_{25–37}, we observed a diffraction pattern typical for β -sheet-containing amyloid fibrils.³⁴ The reflection at 4.7 Å indicates a structural repeat corresponding to the distance between two β -strands within a sheet, whereas the reflection at 10.3 Å corresponds to the distance between two β -sheets in a fibril. A reflection at 9.4 Å, which would correspond to the repeating unit of two antiparallel β -strands within a β -sheet (i.e., 2×4.7 Å), is not observed, suggesting a parallel alignment of the strands within the β -sheets, which allows the hydrophobic, aggregation-prone residues on the C-terminal side of the peptide to lie adjacent to each other. To test this conclusion, we performed ssNMR measurements of synthesized PTH_{25–37} with uniformly ¹³C-labeled L28 and F34. In the 2D ¹³C–¹³C spin diffusion measurements with longitudinal mixing times of 500 ms to 1 s, we did not observe any cross-peaks between these residues (Figures 3B, S7). This finding is indicative of a distance of >6 Å between these residues³⁵ and thus supports parallel β -sheets in PTH_{25–37} fibrils. To answer the question of how two sheets of parallel stacked peptides could be arranged in the fibril,³⁶ we constructed four possible fibril models (with 6 peptides per sheet) consistent with the NMR distance data and tested their stability in MD simulations. Only one of these arrangements proved to be stable, even after 1 μ s MD. In this model, the β -sheets, which consist of parallel and in-register strands, are oriented antiparallel and their R25 side chains point inward (Figure 3C). This also agrees with our findings from the oligomer simulations, which revealed a preferred antiparallel arrangement between the PTH_{25–37} peptides, as this allows the positive charges at the N-terminus to interact with the negatively charged C-terminus. In the fibril, this is realized via intersheet interactions, while within the sheets, the hydrophobic residues are adjacent to each other for optimal β -sheet stability. The fibril model at the end of the simulation confirms that the β -sheets are stable. The β -conformation is partially lost only at the terminal residues, which is due to the electrostatic repulsion between the three positive charges on the RKK residues, which also cause twisting of the fibril. The simulation-averaged distance between β -strands is 4.8 Å, and

between two sheets, it is 10.3 Å, in agreement with the WAXS data.

The WAXS signals for *trans*-P4 (Figure 3D) are much broader than for PTH_{25–37}, indicating less structured fibrils for P4. Nevertheless, the peak for strand spacing at 4.6 Å dominates the signal, clearly indicating β -sheet formation. The next peak is at 9.2 Å, which corresponds to twice the interstrand distance. This indicates an antiparallel arrangement of the *trans*-P4 peptides within a sheet, as here the antiparallel unit consisting of two peptides is another repeat unit leading to a scattering signal. A signal for the intersheet distance is not visible, but could be hidden under the 9.2 Å signal, considering that the signal at 10.3 Å for the PTH_{25–37} fibrils is also not strong. The conclusion that the *trans*-P4 peptides are antiparallel in the fibrils is supported by the ssNMR measurements showing an L28-F34 cross-peak in 2D ¹³C–¹³C spin diffusion spectra, which is indicative of an inter-residual distance <6 Å (Figures 3E and S8). Based on this information and in addition to the results of our dimer and hexamer simulations of *trans*-P4, which revealed a strong interaction between the AMPB groups, we created several possible fibril structures and tested their stability in MD simulations. The structure that met all our experimental specifications and was also stable during the 1 μ s simulation is the one shown in Figure 3F. The structure at the end of the simulation shows a larger deviation from the idealized fibril model and with shorter β -sheets than observed for PTH_{25–37}, which explains the broader WAXS signal. Stabilizing aspects are that the oppositely charged N- and C-terminal ends are directly adjacent within and between the β -sheets and that the central AMPB groups are aligned parallel to each other for π – π stacking interactions. The antiparallel β -sheets themselves are less stable, however, as they are formed between the hydrophobic C-terminal half and the nonamyloid-prone N-terminal half on both sides of the AMPB group. Moreover, the AMPB group in the center of the peptide breaks the β -sheet structure, which explains that the P4 fibrils are shorter than those of PTH_{25–37}. In addition, the π – π stacking and β -sheet stacking have opposite spacing requirements with ≈ 3.8 Å and ≈ 10 Å, respectively, which can be clearly seen in the MD

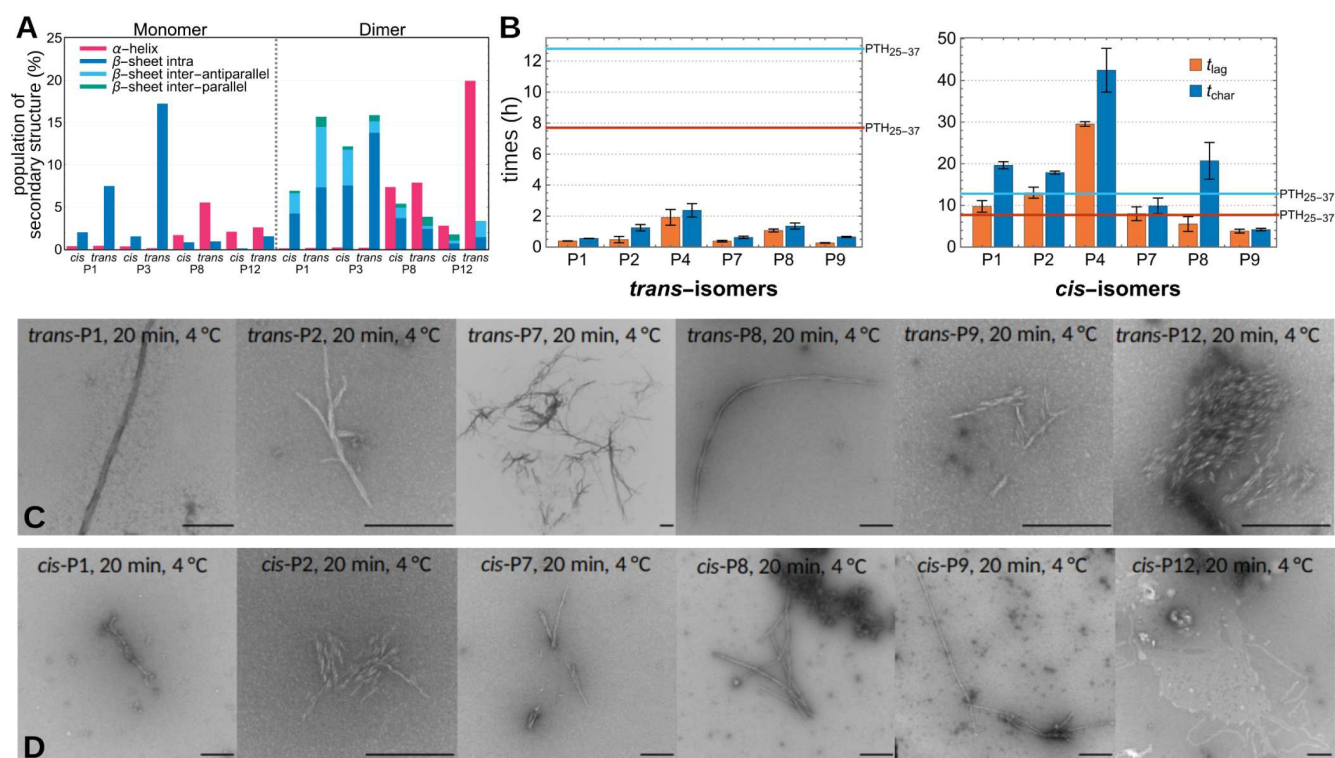


Figure 4. (A) Simulated average secondary structure population, divided into α -helical, intrapeptide β -sheets, and interpeptide parallel and antiparallel β -sheets as indicated by the color code. Results are shown for simulations of monomers and dimers of the *trans*- and *cis*-forms of P1, P3, P8, and P12. (B) Lag time t_{lag} (orange) and characteristic time t_{char} (blue) of the *trans*-forms (left) and *cis*-forms (right) of the peptides P1, P2, P4, P7, P8, and P9. The corresponding values of PTH_{25-37} are indicated as orange (t_{lag}) and blue line (t_{char}). (C) TEM images of the *trans*-isomers of P1, P2, P7, P8, P9, and P12 after 20 min at 4 °C. Scale bar = 250 nm. (D) TEM images of the *cis*-isomers of P1, P2, P7, P8, P9, and P12 after 20 min at 4 °C. Scale bar = 250 nm. TEM data for the photoswitching of P4 are presented in Figure S23.

snapshot shown in Figure 3F and further explains the broadness of the corresponding WAXS signal.

Peptide Design. Based on all simulations and structural investigations made for PTH_{25-37} and the P4 variant, we designed 11 novel PTH_{25-37} -derived peptides, wherein the amyloid-forming capabilities of the AMPB group in view of fibrillization were probed by placing the photoswitch at positions in the center of the peptide (P1–P6), at the N-terminal (P7–P9) or at the C-terminal part (P10–P12), with an amino acid either being exchanged by AMPB or AMPB being inserted between two amino acids (Table 1).

Our design strategy is based on the assumption that moving the AMPB group to the N-terminal side will enhance fibrillization by introducing the hydrophobic AMPB into the polar and charged N-terminal region, whereas moving it to the C-terminal side should impair amyloid formation by disrupting the amyloid-prone peptide region. In addition, we expect that the difference between the *cis*- and *trans*-forms of AMPB will become less important as it is shifted toward the termini, since hairpinning should no longer be possible in the *cis*-form. To refine these predictions, we performed MD simulations of the P1, P3, P8, and P12 variants, considering both the *cis*- and *trans*-configurations of each peptide as monomer and dimer (Figure 4A; P1 was also simulated as hexamer, see Figure S9). The simulations of P1 revealed a similar tendency for β -sheet formation as seen for P4 (Figure 2D), which makes sense given that AMPB is at the same position in both peptides, but instead of replacing V31 as in P4 it is added between D30 and V31, thereby extending the hydrophobic stretch on the C-terminal side of the peptide (Figure S10), which might lead to

a faster aggregation kinetics in the experiments. In P3, the AMPB group is shifted by one position toward the N-terminus compared to P4, replacing D30. This increases the hydrophobicity of the peptide in support of aggregation, while the removal of the negative charge increases the overall positive peptide charge, which could discourage aggregation. The simulations revealed that the increase in hydrophobicity prevails, as random dimerization with mainly intrapeptide instead of interpeptide β -sheets dominated in the simulations of *trans*-P3. P8 was simulated as a representative peptide in which AMPB is significantly shifted toward the N-terminus and inserted between K26 and K27. The electrostatic potential surface (Figure S10) shows that the insertion of the hydrophobic AMPB into the positively charged N-terminal region indeed significantly increases its hydrophobicity, promoting fibrillization. However, the secondary structure preferences are somewhat shifted away from β -sheet toward the formation of α -helices in both *cis*- and *trans*-configurations and both as monomers and dimers, which was not seen for the P3 and P4 variants and may counteract amyloid fibrillization. Finally, we examined the P12 mutant in which the AMPB photoswitch is introduced into the aggregation-prone C-terminal region in the form of an exchange of V35. As predicted, the simulations confirm a drastic decrease in β -sheet formation compared to PTH_{25-37} and the other simulated peptide designs. As with P8, where AMPB is placed at the N-terminal side, its C-terminal position in P12 also leads to the formation of α -helices, which could also counteract amyloid aggregation.

Next, we investigated the influence of the AMPB site experimentally. The designed peptides displayed varying solubility in a buffered aqueous solution (50 mM Na₂HPO₄, pH 7.4), ranging from 370 μ M for P4 to 7 μ M for P10 (Table S2), thus limiting the assay-conditions to those wherein sufficient critical concentrations could be reached. The critical concentration (c_{cr}) of monomers in a fibril-forming system is the minimum concentration required to form fibrils in the dynamic equilibrium between the fibrils and the monomeric peptide form, which can be converted to a standard free energy of the fibril elongation reaction (ΔG^0). Since the c_{cr} of PTH_{25–37} is 42 μ M at the chosen conditions, we decided to exclude the peptides with lower solubility, also motivated by the observation that no fibrillization was observed in P3 and P5, due to their low solubility. The peptides with small c_{cr} are the ones in which the hydrophobicity in the otherwise rather polar and positively charged peptide region was increased (P3, P5, P6), or in which the already hydrophobic C-terminal region was made more hydrophobic by placing the AMPB there (P10, P11). The reduced solubility of P3 is consistent with our MD simulation prediction of random aggregation. For the remaining peptides (P1, P2, P7, P8, P9, and P12, in addition to P4), we determined the photophysical characteristics of the isomerization reaction, such as the half-life time $t_{1/2}$ of the thermodynamically less favorable *cis*-isomer, which is influenced by the position of the azobenzene switch (Table 1). To ensure probing of the fibrillization kinetics, it is important that the stability of the *cis*-isomer is significantly larger than the lag time of the fibrillization process. As both process are competing we strived to minimize the rate of *cis* $\rightarrow$ *trans* isomerization to primarily investigate the behavior of the *cis*-isomer in the relevant time range. These half-life times range from 63 to 97 h, with the longest time observed for P4, where the central V31 was replaced by AMPB, and the shortest time, when the azo switch was shifted toward the C-terminus, replacing V35. The increased half-life time can be explained by the energy barrier for the *cis* $\rightarrow$ *trans* isomerization in the ground state due to interaction between the residues at either side of the AMPB group (Figure 2G), which is also reflected in the *cis:trans* ratio. After synthesis, this ratio is between 3:97 and 6:94 for the freshly prepared peptides, subsequently increasing during the photo-induced *trans* $\rightarrow$ *cis* isomerization to the photostationary state (PSS), reaching values of 90:10 to 82:18. Isomerization back under dark conditions reduces this ratio to 19:81 and 24:76 in the PSS of *cis* $\rightarrow$ *trans* isomerization (Table S3). To exclude a photobleaching effect of the AMPB photoswitch, we repetitively conducted alternating *trans* $\rightarrow$ *cis* and *cis* $\rightarrow$ *trans* isomerizations over 5 cycles (Figure S11). When monitoring the absorption at two wavelengths (absorption maxima of *trans*- and *cis*-isomer, respectively) we did not observe a decline in the overall absorption, except for the first cycle, which can be explained by the fact that the initial peptides directly after synthesis possess an increased *trans*-content compared to the subsequent photostationary state. To assess the toxicity of the peptides with the incorporated photoswitch, we conducted cytotoxicity assays with the N-terminally modified P8, the centrally modified P4, and the C-terminally modified P12 (Figure S12), and we did not observe any toxicity toward 3T3 and NHDF cells.

We then examined the fibrillization process of the peptides using the ThT fluorescence assay after an established protocol for PTH_{1–84}²⁰ (Figures S13–S20) and followed the formation of fibrils with TEM images after 20 min at 4 °C and after 1, 3,

24, and 96 h at 37 °C (Figures S21–27, original data for the photoswitching (TEM) for P4 are presented in Figure S23). The ThT fluorescence curves were fitted using eq. 1 and yielded the lag time t_{lag} , which corresponds to the onset of the fibril growth phase, and the characteristic time t_{char} , the point at which the fluorescence intensity reaches 50% of its maximum (Figures 4B, S13–20). Compared to PTH_{25–37}, the fluorescence intensity was significantly lower, resulting in a poorer signal-to-noise ratio. We attribute this to fluorescence quenching by the azobenzene moiety, which is an already known property of this molecular building block.^{10, g.22} Nevertheless, a sigmoidal fibrillization curve is clearly visible for the three replicate measurements of each peptide. Peptides P1, P2, P4, P7, P8 and P9 show typical fibrillization behavior with lag phase, growth phase, and stationary phase (Table S4). This observation is consistent with our prediction that the placement of AMPB in the middle or at the N-terminus of PTH_{25–37} should not prevent the amyloid aggregation ability of the peptide. All *trans*-peptides exhibited, irrespective of the positioning of the photoswitch, a faster fibrillization process than PTH_{25–37} with significantly shortened lag phases (t_{lag} of 8 h for PTH_{25–37} vs 0–2 h for the peptide designs) and reduced t_{char} (Figure 4B). We assume that the increased hydrophobicity and the organizing effect of the AMPB group are the driving forces for the increased tendency of amyloid aggregation. TEM images of the fibrils were obtained for all peptides after only 20 min at 4 °C (Figure 4C). The *cis*-forms of peptides P1, P2, and P4, in which AMPB was inserted at the central position next to the aggregation hot-spot sequence H32-L37, showed slower fibrillization compared to PTH_{25–37}, whereby for *cis*-P1 and *cis*-P2 the fibrillization occurred in a similar time range, while the fibrillization of *cis*-P4 was strongly delayed. In agreement with our predictions, the incorporation of the photoswitch in its *cis*-form at the N-terminal part had almost no influence on the fibrillization kinetics: *cis*-P7 and *cis*-P8 aggregated at a similar rate as PTH_{25–37}, while the fibrillization of *cis*-P9 was even faster (due to the increased hydrophobicity of the peptide). The TEM images (Figures 4D, S21–27) confirm these observations. For the *cis*-isomers of P1 and P2, we mainly observed amorphous aggregates in the early images (after 20 min); the first objects, which could be clearly assigned to fibrillar aggregates, as with the *trans*-forms, were only detected after 24 h. For P4 (Figure S20) the difference was even more significant: in the *cis*-form, the first fibrillar aggregates were visible in the image only after 60 h. For the N-terminally modified peptides *cis*-P7, *cis*-P8, and *cis*-P9, however, fibrils were already visible after 20 min.

For the fibrillization behavior of the C-terminally modified P12, where V35 was replaced by AMPB, we found that it is entirely different. Unlike other peptides, ThT fluorescence curves for both *trans*- and *cis*-P12 show a linear increase after a lag phase of approximately 20 h, with no saturation observed even after 140 h, indicating reduced fibrillization. This also holds true for fibril morphologies, as both P12 isomers exhibit similar shorter fibrils in the TEM images, with *trans*-P12 forming fibril-like structures after 1 h and *cis*-P12 after 3 h.

In summary, our study revealed that the impact of the photoswitch varies depending on its placement: when positioned within the fibril-forming segment, such as in P12, fibril formation is significantly hindered, whereas outside this crucial region, the *trans*-isomers demonstrate faster fibril formation compared to PTH_{25–37}. Notably, *cis*-isomers display a strongly hindered fibril formation or the formation of

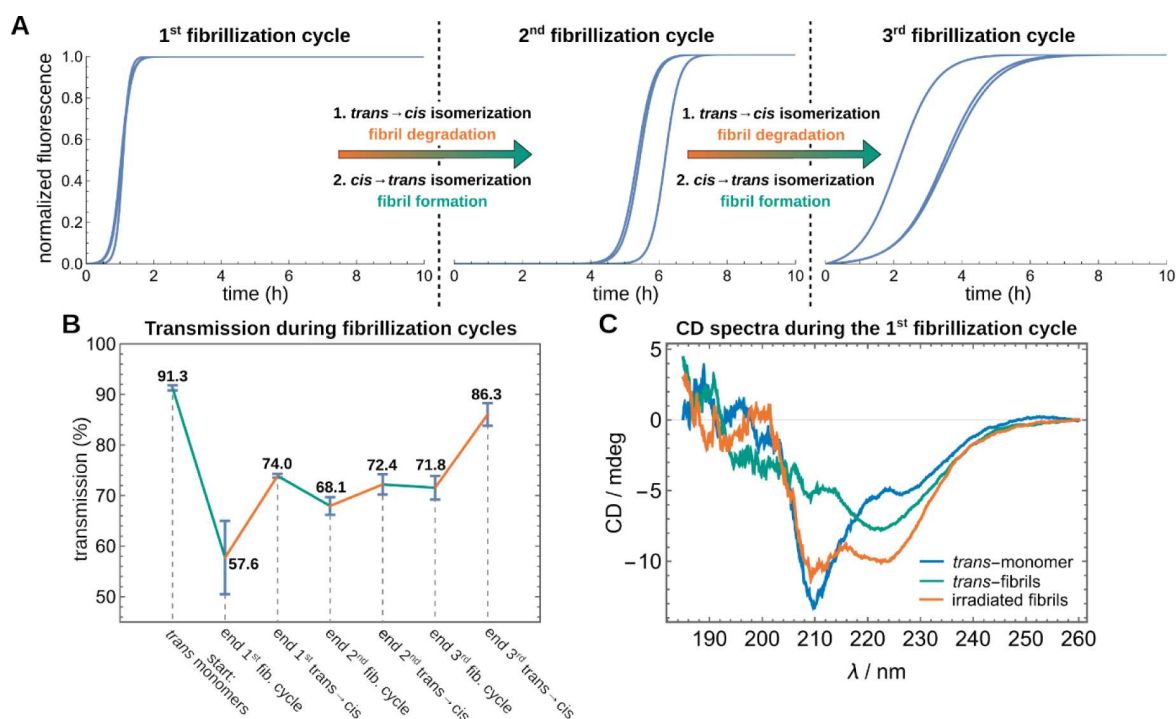


Figure 5. Multiple fibrillization and degradation cycles of *trans*-P4 fibrils through photoisomerization. (A) Fitted ThT signal monitored by the fluorescence assay of three fibrillization cycles of *trans*-P4 comprising degradation of fibrils by photoinduced *trans* → *cis* isomerization and subsequent photoinduced *cis* → *trans* isomerization leading to renewed fibrillization. (B) Change in the transmission of a P4 sample over several switching cycles (cyan for fibrillization periods and orange during *trans* → *cis* isomerization). (C) CD spectra of monomeric *trans*-P4 (blue), *trans*-P4 fibrils (green), and the fibrils after *trans* → *cis* isomerization via irradiation with 340 nm for 5 h (orange).

amorphous aggregates, with the inhibition decreasing as the distance from the fibril-forming C-terminal region increases.

Photocontrolled Multiple Switching of Fibril Formation. By computationally analyzing the primary structure, modeling the peptide structures in their monomeric and aggregated forms, and studying the fibrillation process of PTH_{25–37} and the designed peptides, we aimed to determine the most suitable position for the incorporation of the photoswitch into the peptide sequence to control fibril formation. Based on the results obtained, we applied several criteria to select the most promising candidate from the designed peptides (Table 1) to test whether amyloid formation can be reversibly switched on and off by light. After excluding peptides with insufficient solubility (P10 and P11) as well as the poorly aggregating P12, the focus was placed on the peptides P1, P2, and P4, which show accelerated fibrillization due to the central placement of the photoswitch in the *trans*-conformation, while the *cis*-form slows down fibrillization. Of these peptides, P4 exhibited both the best solubility and the largest difference in fibrillization for *trans*- and *cis*-P4. Therefore, we selected this peptide P4 to further test whether we could achieve reversible fibril formation (Figures 5, S19, S28). It should be noted that during the following sequence of experiments, the cuvettes were not changed; moreover, also no new peptides were added to the solution. Starting from the *trans*-isomer of P4, fibrils were formed in the first cycle at 37 °C in phosphate buffered aqueous solution and the aggregation kinetics followed via ThT fluorescence (Figure 5A, left). After the fibrillization passed over to the stationary phase, the solution was shaken another 20 h to affirm that the equilibrium has been established. Subsequently, the suspension was irradiated at a wavelength of 340 nm for 5 h under stirring,

after which UV/vis spectroscopy was used to verify that the *cis*-photostationary state of P4 was reached. Now, the solution was irradiated for 20 min with light of 405 nm wavelength to switch the *cis*-PSS to the *trans*-PSS to produce monomeric *trans*-peptides, which underwent another fibrillization cycle (Figure 5A, middle). The newly formed fibrils were again exposed to the photoisomerization treatment involving *trans* → *cis* isomerization to degradate the fibrils. The ThT-monitored fibrillization assay was repeated with a portion of the solution, and fibril formation was observed for a third time (Figure 5A, right). For the second and the third fibrillization cycle, a decreased starting concentration of the *trans*-isomer was observed (UV/vis spectra, Figure S29). As we could already exclude photobleaching, we suppose that aggregates adhere to the wall of the 96-well plates and are, therefore, not available for further fibrillization cycles. The fibrils formed during the fibrillization process can precipitate and thus disturb the measurement via scattering effects, causing noise in the signal after the first stationary phase. Because the *trans* → *cis* isomerization of the AMPB-switch is not reaching 100% due to the photostationary state, there certainly are fibrillar aggregates present in the solution, which, however, may interfere in the subsequent fibrillization processes and thus change the following fibrillation kinetics. Nonetheless, for each new fibrillization process monitored here, a lag phase, growth phase, and stationary phase were observed.

While monomers remain soluble, fibrils tend to precipitate, causing the solution to become turbid during the fibrillization assay and leading to a decrease in transmission. Observing the transmission across three switching cycles, it was evident that transmission decreases during the fibrillization process (Figure 5B, cyan lines). Degradation of the fibrils via *trans* → *cis*

isomerization, however, led to an increase in transmission, as released monomers dissolve back into the solution (Figure 5 B, orange lines). The incomplete recovery of the transmission during degradation results from the aforementioned observation that the *trans* $\rightarrow$ *cis* isomerization only leads to a maximum *trans:cis* ratio of 18:82. Therefore, the degradation process of the fibrils is an only partial one, presumably as a full isomerization cannot be reached. The changes in secondary structure during fibrillization were probed by CD spectroscopy (Figures 5C, S28). In its monomeric form, *trans*-P4 displays a shoulder around 220–230 nm and a minimum around 210 nm. In the CD spectrum of the *trans*-P4 fibrils, a minimum is observable between 220 and 230 nm, while the minimum around 210 nm is nearly completely absent. Degradation of the fibrils by the *trans* $\rightarrow$ *cis* isomerization leads to a CD spectrum where the minimum around 210 nm reappeared and the other minimum between 220 and 230 nm still exists. The minimum at 210 nm arises from the monomeric peptides, while the minimum between 220 and 230 nm is indicative for the fibril form. The observed CD spectrum is thus a superposition of both forms, the monomeric and the fibrillated form. From the CD spectra, we can, therefore, conclude that fibrillization from the *trans*-form and incomplete fibril degradation during *trans* $\rightarrow$ *cis* isomerization can be observed.

Fibrillization of proteins are complex, often irreversible processes, which are characterized by a strong thermodynamic negative free energy, as e.g., in A β fibrillization, and thus conventionally termed as “irreversible.”⁵⁸ This is often connected with a high kinetic barrier, usually preceding the fibrillization process, wherein nucleation is central to initiate that nucleation process. The PTH peptides studied here are “more reversibly” fibrillating peptides, wherein the thermodynamic stabilization of the amyloid state is less and, therefore, also the kinetic barriers.^{15,59} Nonetheless, until now, it has not been possible to control the reversibility of this process, as accomplished here via the introduction of a photoswitch. However, in contrast to other reversibly (photo)-switchable systems, such as reported for small molecule assemblies,⁶⁰ adhesives,⁶¹ or photoswitchable enzymes,⁶² a full reversion of the aggregation of the current system is not reached, as expected, as the fibrillization and defibrillization processes depend on many factors, such as the kinetic barriers in either direction, the kind and number of nuclei present during the different switching cycles, or also the precipitation of fibrils.

CONCLUSION

In this study, we have demonstrated precise control over reversible peptide fibrillization by strategically positioning a photoswitch within the central region of a fibril-forming peptide. Utilizing the artificial light switch 3-[[[(4-aminomethyl)phenyl]diazanyl]benzoic acid (AMPB) embedded in a peptide containing residues 25–37 of the parathyroid hormone (PTH), we investigated the impact of position on peptide aggregation. Through a comprehensive approach involving computational modeling, peptide synthesis, aggregation assays, and structural analyses, we elucidated key features governing the fibrillization of both unmodified and modified PTH_{25–37} peptides. Notably, the *trans*-peptides with the modification positioned adjacent to the fibril-forming region in the center of the peptide (P1, P2, and P4) displayed enhanced fibrillization compared to unmodified PTH_{25–37}, while the aggregation is slowed down for their *cis*-isomers. Peptides featuring the photoswitch in the nonamyloidogenic

N-terminal region behaved similarly to unmodified PTH_{25–37} (P7, P8, and P9), whereas peptide P12, where the azobenzene unit replaced V35 in the amyloid-prone region, showed decreased fibrillization, largely unaffected by the photoswitch's isomer state. Our bioinformatics and simulation analysis uncovered that modification with AMPB typically boosts the peptides' hydrophobicity, thereby augmenting their tendency to aggregate. Furthermore, AMPB facilitates self-interaction among peptides through π – π interactions, further enhancing their aggregation propensity. Structural investigations of P4 employing WAXS and ssNMR suggest that β -strands in amyloid fibrils of P4 are—in contrast to fibrils of unmodified PTH_{25–37}—aligned antiparallel. Simulations suggest that AMPB might not fully adhere to the amyloid fold, owing to the distinct demands for interpeptide distances in π – π interactions and fibril formation. This elucidates the diminished fibrillization observed when the photoswitch is positioned within the amyloid-forming segment of the peptide, as seen in P12. Importantly, our results showcase the potential of strategically placing the azo photoswitch, particularly exemplified by peptide P4, to control reversible amyloid aggregation. The phototriggered degradation of fibrils formed in the *trans*-state of AMPB enables repeated fibril formation, which in fact may allow to reversibly modulate the fibrillization of the PTH hormone, with the monomeric peptides released upon fibril degradation serving as the active form and the fibrillar structures acting as an inert peptide reservoir. Given the peptides' nontoxic nature (Figure S12), this light-triggered approach, therefore, presents a promising method for controlled drug delivery and release of such reversibly fibrillating peptides. We regard our system here as a switchable fibrillization system, where a phototrigger from the outside is able to induce fibrillization, as e.g., needed in many modern neuro-cellbiology systems.⁶³

EXPERIMENTAL SECTION

Materials. All technical solvents were distilled prior use; air- and moisture-sensitive reactions were carried out in flame-dried glassware under atmospheric pressure of nitrogen. 2-(6-Chloro-1-H-benzotriazole-1-yl)-1,1,3,3-tetramethylammonium hexafluorophosphate (HCTU), *N*-methyl-morpholine (NMM), *N,N*-dicyclohexylcarbodiimide (DIC), *N*-Hydroxy-benzotriazole (HOBT), trifluoroacetic acid, 4-amino-benzylamine, and oxone were purchased from Sigma-Aldrich. 9-Fluorenylmethyl-*N*-succinimidylcarbonat (Fmoc-OSu) was received from Fluorochem. 3-Aminobenzoic acid was purchased from Merck and was used without further purification.

UV/Vis, CD, and Transmission Spectroscopy Measurements. UV/vis-absorbance spectroscopy was measured on a JASCO V-660 absorbance spectrometer in a 1 cm quartz glass cuvette. For PTH_{25–37}, the absorbance was measured at 205 nm with a molar extinction coefficient of 49.310 cm^{−1} M^{−1}; *trans*-azobenzene containing peptides were measured at 327 nm with a molar extinction coefficient of 13.000 cm^{−1} M^{−1}. CD spectroscopy was measured with a JASCO J-1500 CD Spectrometer in either a 1 mm. As buffered solution, a 50 mM aqueous Na₂HPO₄ buffer solution was used with a pH value adjusted to 7.4. Transmission was measured with a Litesizer DLS 500 from Anton Paar using a 3 mm $\times$ 3 mm quartz glass cuvette. The irradiation wavelength was 658 nm. The equilibration time was 1 min, and the measurement time was 10 s. The temperature was maintained at 25 °C.

Peptide Synthesis. The 3,4'-AMPB photoswitch was synthesized in two steps according to our published procedure.²² Solid-phase peptide synthesis was utilized on an automated peptide synthesizer MultiPep RS (Intavis AG, Koeln, Germany) using standard Fmoc-chemistry and preloaded resins. Standard coupling of all protected natural amino acids was performed as single couplings in dimethylformamid (DMF) using 5 equiv of amino acids, HCTU as coupling reagents, and 10 equiv of NMM as base for 1 h at room temperature. Special building groups, such as Fmoc-3,4'-AMPB, were coupled with 3 equiv using DIC and HOBT in DMF/ *N*-methyl-2-pyrrolidone (NMP) at room temperature and with gentle shaking in the dark overnight. The N-terminal Fmoc protecting group was removed by washing the resin with 20% piperidine for 20 min. The final side chain deprotection and cleavage from the resin employed a mixture of trifluoroacetic acid and water (90:10 Vol%) with gentle agitation for 2 h at room temperature. The crude peptides were purified to >95% purity using preparative RP-HPLC (Gilson, Limburg, Germany). For both analytical and preparative use, the mobile phase was a mixture of water (eluent A) and acetonitrile (eluent B), respectively, each containing 0.1% trifluoroacetic acid. Samples were eluted with a linear gradient from 5% B to 95% B in 15 min for analytical runs and in 90 min for preparative runs on a semipreparative PLRP-S column (Agilent Technologies, 300 × 25 mm, 8 μm). Finally, all peptides were characterized by analytical HPLC Dionex Ultimate 3000 (Thermo Scientific, Germany) using a PLRP-S column (Agilent Technologies, 150 × 4.6 mm, 3 μm) and MALDI-MS (Bruker Microflex LT, Bremen, Germany), which gave the expected [M + H]⁺ mass peaks. The full molecular characterizations are shown for all peptides in Figures S30–S47.

Aggregation Kinetics. The fibrillization process was investigated using a thioflavin T (ThT) monitored fluorescence assay following the established process for PTH_{1–84}.²⁰ Therefore, the fluorescence intensity of ThT was measured. Lyophilized peptides were dissolved in buffered solution and kept on ice for the next steps. The sample solutions were centrifuged at 13 000 rpm for 10 s, and the concentration of the respective peptide was determined using UV/vis-absorbance spectroscopy. If required, the *cis*-isomer of the azobenzene containing peptides was produced as described below. The sample solutions were centrifuged at 10 000 rpm for 1 h at 4 °C, and the supernatant was transferred to another tube. The solutions were diluted with buffer, and ThT was added as a 1 mM stock solution to obtain a final concentration of 100 μM for the peptides, with a solubility above 100 μM, and 50 μM for ThT. For the other peptides, the solutions were solely diluted with the ThT stock solution, to achieve the highest possible concentration. For each sample, a total volume of 480 μL was prepared, and three aliquots with 150 μL were transferred to a medium binding 96-well plate (GREINER Bio-One 675 076). The plate was sealed with a microplate cover, and the fluorescence intensity was monitored at 37 °C using a BMG FLUOStar Omega multimode plate reader using fluorescence excitation and emission wavelengths at 460 and 485 nm, respectively. One measurement cycle lasted 5 min, consisting of double-orbital shaking for 150 s and incubating for 150 s. To describe the fibrillization process, the source data were fitted. As the description of fibrillar growth in terms of the molecular rate, kinetic can be considered as two main fibrillation processes.³⁷ Their contributions to the increase in

fluorescence $\Delta F(t)$ can be analyzed by a function, which was derived by Dear et al.³⁸ and has been used to characterize the fibrillation kinetics of full-length PTH.²⁰

$$\Delta F(t) = \left(1 - \frac{1}{\frac{\kappa^3}{\lambda^3}(e^{\kappa t} - 1) + 1} \right) \Delta F_{\text{Pl}} \quad (1)$$

ΔF_{Pl} corresponds to the plateau value of the fluorescence in the stationary phase, while λ and κ are the rate constants of the primary and secondary nucleation processes.

Determination of the Solubility Parameters. To determine the maximal solubility of a peptide, small amounts (0.5 mg) of the respective peptide were added to 1 mL of buffered solution and shaken for 10 s until an insoluble precipitate was visible. The suspensions were centrifuged at 10 000 rpm for 1 h at 4 °C, and the supernatant was transferred to another tube. The remaining monomer concentration was determined using UV/vis-absorbance spectroscopy. To determine the critical concentration c_{cr} , two samples were prepared for each peptide according to the procedure for the ThT monitored fibrillization assay, one with ThT as a reference sample and the other one without ThT. Twenty hours after the reference sample reached the stationary phase, the fibril containing solutions from the sample without ThT were transferred to a tube and centrifuged at 10 000 rpm for 1 h at room temperature. The supernatant was transferred to another tube, and the concentration of the remaining monomers was determined using UV/vis-absorbance spectroscopy at 278 nm with a molar extinction coefficient of 3750 cm^{−1} M^{−1} for the azobenzene containing peptides and at 205 nm with a molar extinction coefficient of 49 310 cm^{−1} M^{−1} for PTH_{25–37}.

Photoisomerization. The photoisomerization of the *trans*-azobenzene moiety in the peptides was performed by irradiating the dissolved peptides in a 1 cm quartz cuvette for 30 min with light of 340 nm wavelength using a 69.2 mW LED (Thorlabs, M340L5) equipped with a controller (Thorlabs, LEDD1B). The photoisomerization of the *cis*-azobenzene moiety in the peptides was performed by irradiating the dissolved peptides in a 1 cm quartz cuvette for 30 min with light of 405 nm wavelength using a 1.4 W LED (Thorlabs, M405L4) equipped with a controller (Thorlabs, LEDD1B).

Photobleaching. To test whether photobleaching occurs during the photoisomerization, a solution of 50 μM P4 was irradiated alternately for 30 min with light of 340 nm wavelength (*trans* → *cis* isomerization) and light of 405 nm wavelength (*cis* → *trans* isomerization). The absorption was measured after each isomerization step at two wavelengths: 295 nm (absorption maximum of the *cis*-isomer) and 327 nm (absorption maximum of the *trans*-isomer).

Transmission Electron Microscopy. TEM images were taken with an electron microscope (EM 900; Zeiss) at 80 kV acceleration voltage. For preparation, 5 μL of the peptide solution were added on Formvar/Cu grids (mesh 200). After 3 min of incubation, the grids were gently rinsed two times with water and then negatively stained using uranyl acetate (1%, w/v) for one minute.

Fibril Degradation. To investigate the fibril degradation through photoisomerization, 3 mL of a 100 μM *trans*-P4 in buffer was prepared according to the procedure for the ThT monitored fibrillization assay, except that ThT was excluded,

and aliquots of 150 μL were transferred to a medium binding 96-well plate. As a reference sample, 480 μL of a 100 μM *trans*-P4 in buffer was prepared with ThT, and both approaches were incubated as described for the ThT monitored fibrillization assay. Twenty hours after the reference sample reached the stationary, the fibril containing solutions of the sample without ThT were collected in one tube. UV/vis-absorbance and CD were measured, and the fibril solution was transferred in a 1 cm quartz glass cuvette and treated according to the procedure for the *trans* $\rightarrow$ *cis* isomerization, except that the irradiation time was prolonged to 5 h. Again UV/vis-absorbance and CD were measured. Subsequently, the *cis*-isomer was photoisomerized back to the *trans*-form. 456 μL of the solution was mixed with 24 μL of a 1 mM ThT stock solution and transferred as 150 μM aliquots to a medium binding 96-well plate for a ThT monitored fibrillization assay. The remaining solution was transferred as well in 150 μL aliquots to the 96-well plate and incubated under the same conditions. This procedure was repeated for every degradation cycle.

Solid-State NMR Spectroscopy. For the solid-state NMR spectroscopy, 6 mL of a 100 μM solution of the respective peptides (PTH_{25–37} and P4 with uniformly $^{15}\text{N}/^{13}\text{C}$ labeled residues L28 and F34) was prepared according to the procedure for the ThT monitored fibrillization assay and transferred in 150 μL aliquots to a medium binding 96-well plate. Twenty hours after the stationary phase was reached, the fibril containing solutions were collected in a tube and centrifuged at 60 000 rpm for 15 min at 10 $^{\circ}\text{C}$. The supernatant was discarded, the remaining fibril pellet was frozen with liquid nitrogen, and the residual water was removed using lyophilization. The lyophilized peptides were packed into Bruker MAS rotors with outer diameters of 3.2 mm (PTH_{25–37}) and 1.9 mm (P4), respectively. Solid-state NMR experiments were conducted on an 18.8 T (800 MHz ^1H frequency) Bruker Avance III spectrometer equipped with a triple resonance HCN 3.2 mm MAS Efree probe and a 1.9 mm triple resonance probe. For determination of intermolecular long-range contacts between L28 and F34 resonances, proton-driven spin diffusion (PDSF) experiments with an MAS frequency of 20 kHz close to the rotational resonance condition between aromatic ^{13}C resonances of F34 and aliphatic $\text{C}_\gamma/\text{C}_\delta$ resonances of L28 were recorded with mixing times ranging from 50 ms to 1 s. For PTH_{25–37}, additional PDSF and DQSQ spectra were recorded on a 14.1 T (600 MHz ^1H frequency) Bruker Avance wide bore spectrometer equipped with a 3.2 mm MAS triple resonance ^1H , ^{13}C , ^{15}N probe. Typical radiofrequency field strengths were 91–100 kHz for ^1H , and 55.6 kHz for ^{13}C Spinal64 ^1H decoupling³⁹ (rf field of 85 kHz) was applied during ^{13}C evolution and acquisition. The VT gas temperature was set to 263 K (thermocouple reported temperature); the sample temperature was estimated to be around 10–20 K higher due to frictional heating under MAS.

WAXS Measurements. The WAXS measurements were performed in transmission mode with a SAXSLAB laboratory setup (Retro-F) equipped with an AXO microfocus X-ray source. As a monochromator, the AXO multilayer X-ray optic (AXO Dresden GmbH, Dresden, Germany) was used for Cu-K α ($\lambda = 0.154$ nm). The two-dimensional scattering patterns were recorded with a two-dimensional detector (PILATUS3 R 300 K, DECTRIS, Baden, Switzerland). The preformed fibrils were prepared as described for the measurement of the aggregation kinetics in a total volume of 1 mL. Twenty hours

after the fibrillization reached the stationary phase, the fibril suspension was ultracentrifuged at 60.000 g for 15 min, and the obtained pellet was transferred into a glass capillary and dried overnight. The scattering measurements were performed at room temperature in vacuum and corrected for background.

Cytotoxicity Tests. Cell viability was determined for *trans*-P4, *trans*-P8, and *trans*-P12 on NHDF as well as 3T3 fibroblasts with a resazurin reduction assay. Briefly, cells were seeded at desired cell densities in corresponding culture media supplemented with 10% FCS and penicillin/streptomycin in 96-well plates on day 0 and incubated overnight under standard cell culture conditions. On day 1, serial dilutions of the peptides in cell culture media were prepared. Then, medium (background and negative control–100% viability), TritonX in medium f.c. 0.025% (positive control–0% viability), and the peptide dilutions were added with one treatment per column ($n = 8$). Incubation for 24 h or 96 h under standard cell culture conditions followed. On day of measurement, resazurin stock solution was added to a final concentration of 44 μM into each well. After 2 h incubation, the resorufin fluorescence was measured with a Cytation 5 plate reader system. Means and standard deviation of each column were calculated. Experiments were repeated independently 3 times, and average values were evaluated (see Figure S12).

MD Simulations. For all MD simulations of the PTH_{25–37} peptide, as well as the mutants P1, P3, P4, P8, and P12, we used the GROMACS simulation package.⁴⁰ Since the PTH_{25–37} sequence classifies as an intrinsically disordered protein (IDP), we have used the CHARMM36m force field⁴¹ to model protein interaction, which has previously been shown to be a suitable choice for IDPs.⁴² For the azobenzene photoswitch (denoted AZO in our force field implementation), we parametrized the interactions following the cgenff standard protocol⁴³ and refined the parametrization using data from QM/MM simulations,⁴⁴ by fitting our parameters to reproduce the bond, angle, and dihedral angle distributions of the *cis*- and *trans*-state as obtained from QM/MM. The resulting force field parameters are available at https://github.com/strodel-group/Charmm36m_Azobenzene-FF. During the MD simulations of the modified PTH_{25–37} peptides, the $\angle\text{CNNC}$ dihedral angle was restricted to either the *cis*- or *trans*-states; thus, transitions between the two states of the AMPB photoswitch were not modeled. The MD simulations of all systems were prepared following the same protocol: first, the peptide(s) were placed in the simulation box, where in case of the dimer and hexamer simulations, the box size was always chosen to achieve a peptide concentration of 10 mM. Then, the box was filled with TIP3P water molecules,⁴⁵ as well as Na⁺ and Cl[−] ions to neutralize the system and achieve a physiological salt concentration of 150 mM. After equilibration of the systems, a production run of 10 μs per system (1 μs for the fibril models) was carried out under NpT conditions at constant number of particles N , pressure $p = 1$, bar and temperature $T = 300$ K. The pressure and temperature were regulated using the Parrinello–Rahman pressure coupling scheme⁴⁶ and Nosé–Hoover thermostat,⁴⁷ respectively. To exclude edge effects, periodic boundary conditions in all directions were applied, and the particle-mesh Ewald method⁴⁸ was used to calculate electrostatic interactions. For the calculation of van der Waals and Coulomb interaction in real space, a cutoff of 12 Å was applied. An overview over all simulations performed is given in Table S1, yielding 285 μs of total simulation time across all systems. All MD simulations

were run on the high-performance cluster JURECA-DC.⁴⁹ Analysis of the MD data was performed with python using the MDAnalysis⁵⁰ package for reading of the MD trajectory and the calculation of distances between groups of atoms, while the MDTraj⁵¹ package was used for secondary structure analysis. For visualization of the MD structures, the PyMOL⁵² software was used. Additionally, PyMOL in combination with the APBS⁵³ plugin was used for the calculation and visualization of electrostatic potential surfaces.

QM Calculations. The photoswitching mechanism of AMPB integrated into PTH_{25–37} was monitored in the ground and excited states along the $\angle$ CNNC dihedral angle for the *cis* $\leftrightarrow$ *trans*-transition of the azobenzene photoswitch. As a starting point for the QM calculations, we constructed pathways from *cis* to *trans* and *vice versa*, using MD simulations. To consider the structural ensemble along the pathway, we simulated 40 *cis* $\leftrightarrow$ *trans* switching trajectories. We started the switching from equilibrated *cis*- and *trans*-P4 structures obtained from equidistant time steps of 250 ns from the 10 μ s MD simulations. The switching in the MD simulations was achieved by imposing a restraining potential on the $\angle$ CNNC dihedral angle and changing it every 2 ns in increments of 10° between 0° and 180°. All other MD simulation parameters were the same as described above. The resulting trajectories were used as input for the subsequent QM calculations. The QM calculations were conducted using the ONIOM-based QM/QM2 method,³⁰ with NEVPT2(2,2)/def2-TZVP^{31,54} for QM and xTB2³² for QM2, as implemented in the ORCA program package.⁵⁵ The QM region was focused on the azobenzene core, including the peptide in π -conjugation with it. The solvent was implicitly modeled using the ALPB method.⁵⁶ Due to convergence issues, analysis was limited to 34 of the original 40 trajectories. For these paths, the energies of electronic states S₀, S₁, and T₁ were interpolated to obtain potential energy curves using Gaussian process regression from the scikit-learn package.⁵⁷ Some of the paths that have an energy barrier at dihedral angles around 170° were analyzed at the structural level. To this end, the inter-residue distances based on the centers of mass of the residues were calculated for all structures exhibiting a dihedral angle near 170° and then analyzed through dimensionality reduction via principal component analysis (PCA). The first three principal components were transferred back into the original distance matrix format and illustrated with three representative distance matrices from the corresponding cluster of structures. Representative structures for the distance matrices were also extracted and visualized.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.bioconjchem.4c00188>.

Supplementary figures S1, QM potential energy curves for 34 *cis* $\leftrightarrow$ *trans* isomerization paths; S2, fluorescence lifetime measurements; S3, PCA analysis of selected P4 structures; S4, principal components projected onto inter-residue distance space; S5, selected P4 snapshots; S6, WAXS data of P4 and PTH_{25–37}; S7 ssNMR data for PTH_{25–37} fibrils; S8, ssNMR data for *trans*-P4 fibrils; S9, summary of all MD simulation results; S10, electrostatic potential surface of PTH_{25–37} and selected peptides; S11, absorption data to exclude photobleaching; S12,

cell viability assay of *trans*-P4, *trans*-P8, and *trans*-P12; S13–S20, ThT fluorescence data; S21–S27, TEM images of fibrils; S28, CD spectra during reversible fibrillization of P4; S29, UV–vis spectra of *trans*-P4 before each fibrillization cycle; S30–S47, ¹H NMR, HPLC traces, and MALDI data of the peptides P1–P12. **Supplementary tables** S1, list of all MD simulations performed; S2, sequence of PTH_{25–37} and P1–P12 and their respective solubility; S3, photophysical properties of peptides; S4: fibrillization parameters of peptides (PDF)

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Notes

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■ ABBREVIATIONS

AMPB, 3-[(4-aminomethyl)phenyl]diazanyl]benzoic acid; CD, circular dichroism; MD, molecular dynamics; NMR, nuclear magnetic resonance; PSS, photostationary state; PTH, parathyroid hormone; ssNMR, solid state nuclear magnetic resonance; TEM, transmission electron microscopy; ThT, thioflavin T; UV/vis, ultraviolet/visible; WAXS, wide-angle X-ray scattering

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Spiropyran as Building Block in Peptide Synthesis and Modulation of Photochromic Properties

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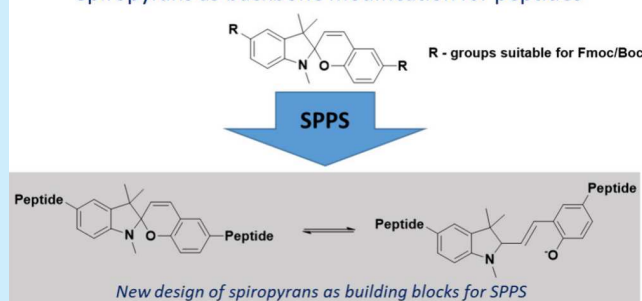
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Supporting Information

ABSTRACT: Light-controlled triggering of materials requires efficient embedding of molecular photoswitches into larger molecules. We herein present the synthesis of two new building blocks for the synthesis of photoswitchable peptides, embedding spiropyranes as a central unit into peptide-backbones via a novel, yet unreported approach. The synthesis presented here allows us to embed spiropyranes directly into solid-phase peptide synthesis (SPPS), further describing the resulting photophysical properties of the as-prepared photoswitchable peptides.

Spiropyranes as backbone modification for peptides



The incorporation of photoswitchable moieties into functional materials¹ allows to address molecular conformation² and dynamics³ by light as external trigger.⁴ Besides azobenzenes,⁵ diarylethenes,⁶ and thioindigos,⁷ spiropyranes are important photoswitches.⁸ Spiropyranes exist in two forms (Figure 1A): a colorless, closed form of the cyclic spiropyran (SP) and the colored merocyanin (MC) form, the latter displaying an extended molecular shape. Irradiation by UV-light causes cleavage of the C–O bond at the spiro-carbon and its isomerization to the MC-form, reversing isomerization by visible light.⁹ There is a strong change in the dipole-moment upon isomerization:¹⁰ while the dipole moment of the SP-form is ~4–6 D, it is increased in the MC-form to ~14–18 D. Spiropyranes are not only sensitive to light¹¹ but can also be modulated by temperature,¹² pH,¹³ the redox potential,¹⁴ solvent polarity,¹⁵ ions,¹⁶ and even by mechanical force.¹⁷ Thus, they are of great interest in the generation of smart materials, where multiple stimuli can be transferred to the shape, strength, and dynamic properties of a material they are embedded into.^{10,18} Their incorporation into biological and synthetic macromolecules was first reported in 1976 for peptides¹⁹ and in 1978 for synthetic polymers²⁰ but only in the side-chain of the respective monomer unit. Thus, for decades, side-chain or end-group modification of peptides and polymers²¹ (Figure 1B) remained the only method to incorporate spiropyranes into (biological) macromolecules to transfer their adaptive and stimuli-responsive properties therein. In 2013 the spiropyran unit was embedded directly into a polymer-backbone, revealing significant property changes upon their photoswitching.²² While spiropyranes play an important role in the control of peptide properties,^{8b,23} a general methodology to embed spiropyranes into the main

backbone of a peptide is still missing. We herein report new spiropyran building blocks (Figure 1C) and their incorporation into peptides via SPPS (solid-phase peptide synthesis): two blocks via a Fmoc-synthesis strategy and two blocks via a Boc-strategy. Based on suitable indole and salicylaldehyde precursors, equipped with either a protected amine- or a carboxy functionality, the desired spiropyran can be formed subsequently be embedded into peptides via Fmoc-SPPS at different positions of a fibrillating peptide (see Table 1), and further investigated for their photophysical properties.

As non-natural amino acid building blocks for SPPS, we synthesized the four spiropyranes **1a**, **1b**, **2a**, and **2b** (Scheme 1). Spiropyranes **1a** and **1b** are designed for introducing the FASC building block containing the N-terminal protected amino group at the indolinium entity and the C-terminal carboxy function at the chromene unit. The synthetic strategy was reversed for spiropyranes **2a** and **2b**, introducing the SAFC building block.

Synthesis of the Photoswitch

We designed the building blocks **1a** and **2a** bearing a Fmoc-protection group, and **1b** and **2b** with a Boc-protection group.²⁴ The key step in both strategies was a condensation reaction of an indoline compound with an *o*-hydroxy-aromatic aldehyde. The synthesis of the spiropyranes **1a** and **1b** (Scheme

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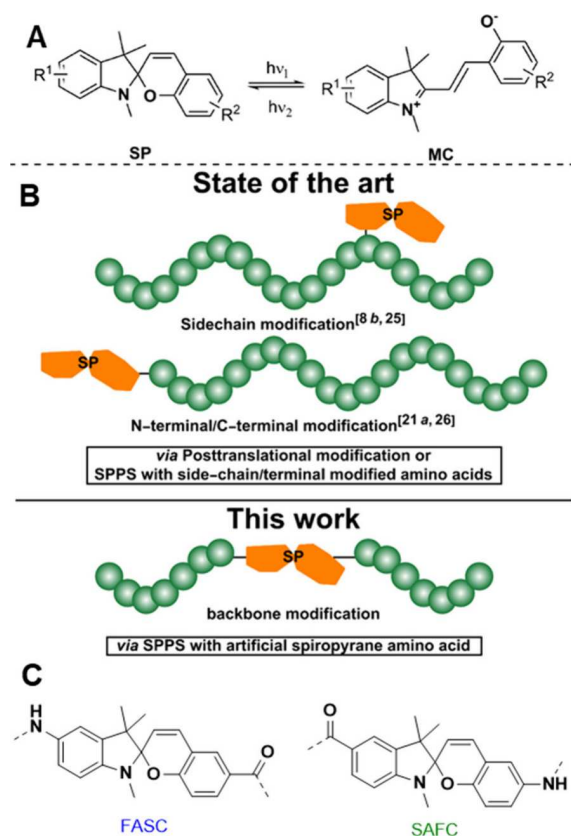


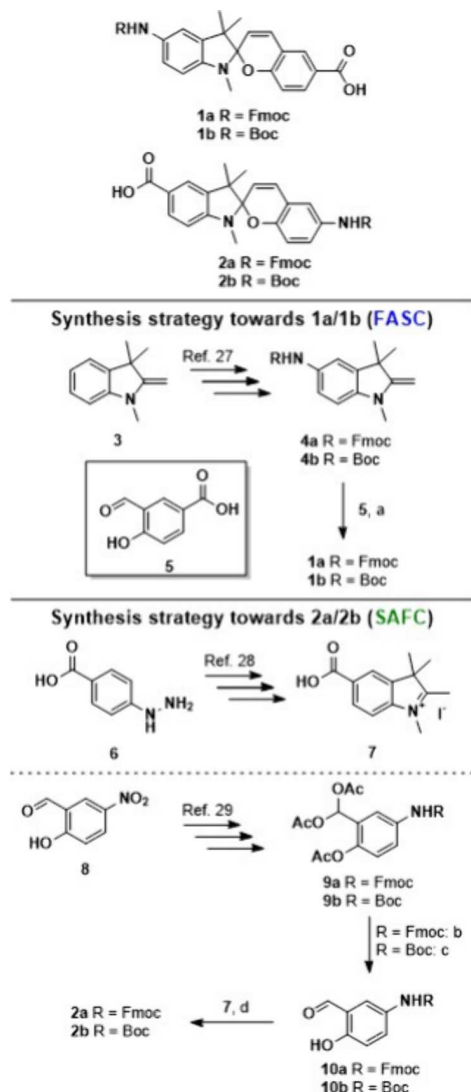
Figure 1. (A) Isomerization of spiropyrans between their closed form (SP) and their ring-opened merocyanine form (MC). (B) State-of-the-art strategies to modify peptides with spiropyrans consists of post-translational modifications of the synthesized peptide at the side chain^{8b,25} and at the N-/C-terminal end of the peptide chain (above).^{21a,26} This work: solid-phase peptide synthesis (SPPS) to incorporate a spiropyran building block into the peptide backbone (below). (C) Structures of the spiropyran two different building blocks, FASC and SAFC.

Table 1. Peptides Containing the Photoswitchable Spiropyran Moiety (SAFC, FASC) Synthesized *via* Fmoc Solid-Phase Peptide Synthesis

peptide	primary sequence	embedded spiropyran
P1	²⁵ RKKLQ ³⁰ D-FASC-VHNF ³⁵ VAL	1a
P2	²⁵ RK-FASC-KLQ ³⁰ DVHNF ³⁵ VAL	1a
P3	GSGSGS-FASC-GSGSGS	1a
P4	²⁵ RKKLQ ³⁰ D-SAFC-HNF ³⁵ VAL	2a

1) started with commercially available **5**, where a nitro group was introduced at position 5.²⁷ Subsequently, the nitro group was reduced to the amine²⁷ and protected with a Boc- or Fmoc-group. Finally a condensation reaction with commercially available carboxylic acid **5** leads to the desired product **1a** with an overall yield of 5% over 4 steps and the spiropyran **1b** in a yield of 18%. The convergent synthesis toward spiropyran **2a** and **2b** (Scheme 1) started with the indole precursor. 4-Hydrazinobenzoic acid **6** was converted in a Fischer indole like reaction²⁸ followed by a methylation to yield the indolinium iodide **7**.²⁸ In contrast to the spiropyran **1a** and **1b** we additionally prepared *o*-hydroxy benzaldehydes **10a** and **10b**. First step was to protect the hydroxy- and the aldehyde-functionality of nitro salicyl aldehyde **8** with acetoxy groups,²⁹ followed by reduction of the nitro group and attaching the

Scheme 1. Synthesis of the Spiropyran **1a/1b and **2a/2b****



group (Boc or Fmoc).²⁹ To remove the acetoxy protecting groups, we probed de-esterification under different conditions. The Boc-protected **9a** was treated under basic conditions, while the Fmoc-protected **9b** was treated under acidic conditions. To obtain the final compounds, the indolinium iodide and the respective protected *o*-hydroxybenzaldehyde were converted in a condensation reaction. Spiropyran **2a** was obtained in an overall yield of 4% and spiropyran **2b** in 3%, both in 6 steps.

Peptide Synthesis and Photophysical Properties

To test if the Fmoc-protected spiropyran **1a** and **2a** are compatible with standard SPPS conditions and to investigate the photophysical properties we synthesized four different peptides (Table 1). We chose the fibril core sequence of the parathyroid hormone (PTH_{25–37}) as a model peptide³⁰ to probe a modulation of the fibrillization of the peptide, in addition to the GlySer-sequence at the N-terminus and the C-terminus. The peptides were synthesized using standard SPPS conditions and characterized with ESI-ToF, MALDI-ToF, and ¹H NMR (see the Supporting Information). The isomerization between the MC and the SP-form was probed via UV/vis-spectroscopy and HPLC. As spiropyran display an acid-

Scheme 2. Photophysical Isomerization Process of the FASC (top) and SAFC (bottom) Building Blocks

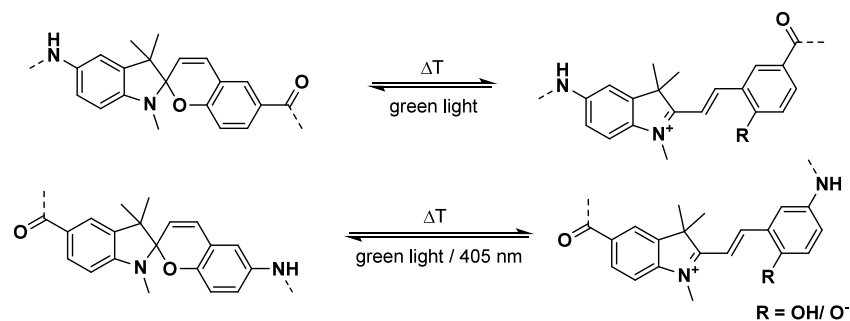


Table 2. Photochromic Properties of Peptides P1–P4 in Aqueous Media

peptide	$\lambda_{\text{max, SP}}$ [nm]	$\lambda_{\text{max, MC}}$ [nm]	λ_{iso} [nm]	ϵ^a [mol ⁻¹ cm ⁻¹]	$\tau_{1/2}$ at 37 °C (4°) [min]
P1 ^b	244, 511	244, 376, 518	292	— ^f	3.74 ± 0.06 (171.43 ± 0.28)
^c	250	257, 321, 428	314	6201 ± 386 (314 nm)	10.74 ± 0.02 (1155.2 ± 0.4)
P2 ^b	242	286, 376, 518	282	— ^f	2.92 ± 0.04 (321.44 ± 0.30)
^c	n.d.	257, 321, 427	314	5910 ± 553 (314 nm)	11.31 ± 0.02 (1151.1 ± 0.2)
P3	240	244, 375, 515	283	— ^f	5.95 ± 0.10 (358.88 ± 0.18)
^c	n.d.	257, 323, 426	313	5644 ± 66 (313 nm)	15.78 ± 0.03
P4 ^c	247, 305	253, 379, 442	265, 326	— ^f	1.65 ± 0.04
^d	305, 357, 450	253, 379, 442	329	6153 ± 90 (329 nm)	10.04 ± 0.21 (384.88 ± 0.86)

^aAt the isosbestic point. ^b50 μM NaH_2PO_4 , 0.01% NaN_3 , pH 7.4. ^c100 μM citric acid/sodium citrate, pH 2.5. ^d1 M HCl, pH 1. ^fNot determined as MC-form is hydrolyzed. n.d.: not determined.

ochromic behavior, we investigated the behavior at physiological pH (FASC; buffered aqueous solution, pH 7.4), in a citrate buffered solution (FASC and SAFC; pH 2.5), and in 0.1 M HCl (SAFC; pH 1). For both spiropyran-building blocks (peptides P1–P4, Figures S1–S4) the colored MC-form showed absorption in the UV and visible range, while the colorless SP-isomer usually showed absorption only in the UV range. Furthermore, the MC-isomer was the thermodynamically favored form in aqueous solution (Scheme 2, Figures S5–S14), which is well-known for this class of spiropyranes in the presence of water.³¹ Irradiation with green light (525 nm) of P1–P4 led to the SP-isomer, determined by UV/vis-spectroscopy. The thermal (dark) relaxation toward the MC-form follows a first-order kinetic^{15b,32} and occurred fast at 37 °C. At all tested conditions, a half-life time of less than 20 min was observed which increased to up to 1155 min if the temperature was decreased to 4 °C (Table 2, Figures S5–S16). A competitive reaction is the degradation process of the MC-form via hydrolysis of the bridging double bond, which was first described by Stafforst et al.³⁵ Decomposition led to a peptide with the Fischer's base moiety and a peptide bearing a salicyl aldehyde moiety, both detected via MALDI-ToF measurements (Figure S17–S19). The hydrolysis rate compared to the thermal isomerization of the MC-FASC unit is slower, whereby the rate constant of the hydrolysis is 13 (0.116 min⁻¹ vs 0.00921 min⁻¹ for P3) to 150-times lower (0.237 min⁻¹ vs 0.00162 min⁻¹ for P2, Table S1). In contrast, the SAFC building block could not be handled at a physiological pH value, as the hydrolysis occurs within several minutes.

If FASC is kept in the SP-form through continuous irradiation with green light, it remains stable until the light source is switched off, and the isomerization and degradation kinetics can be followed via UV/vis-spectroscopy (irradiation time of 5 h). Spiropyranes are known to act as photoacids; therefore, we further investigated the behavior at pH values,

where the MC-form should mainly exist in its protonated form. As the pK_a value of the MC-form was determined to be ~ 7.2 for several spiropyranes,³⁴ strong acidic conditions (pH 2.5 or pH 1) were used. The FASC-containing peptides are stable ($\tau_{1/2}$ of hydrolysis >15,000 min) at a pH value of 2.5. The SP-to-MC isomerization reaches thermal equilibrium usually after 2 h, while the SP-PSS (photostationary state) was obtained through irradiation with green light after 10 min. Analysis with HPLC revealed that the thermal equilibrium consists of nearly 100% of the MC-form, while the photoconversion was almost quantitative (Table S2 and Figures S23 and 24). The SAFC-switch was not stable at pH 2.5, but the decomposition was strongly inhibited (the rate constant was ~ 1300 fold smaller than the thermal isomerization, Table S1), so that photo-physical parameters could be determined (Table 2). Irradiation at 405 nm leads to a conversion of at least $\sim 75\%$ to the SP-form, whereby the thermal back-isomerization at 37 °C exhibits a half-life time of 1.65 min, completely stable at pH 1. The photoisomerization at pH 1 could be achieved with irradiation of light of two different wavelengths, where 405 nm led to an SP content of 51% at the PSS, while green light generated 62% (Figure S21).

The thermal back-isomerization exhibits a half-life time of 10 min at 37 °C and 385 min at 4 °C, respectively (Figures S17–19).

We observed that the stability of the spiropyranes in our peptides against hydrolysis was strongly pH-dependent. Thus, the peptide P2 at pH 7.4 and 37 °C is almost six times more stable than the peptide P1. Furthermore, the substituents at the spiropyran rings also have a significant impact, as the FASC building block could at least be investigated at pH 7.4, while the SAFC building block was hydrolyzed already within a few minutes. Increasing the stability of the spiropyranes against hydrolysis at higher pH values by different substituents is currently a subject of intensive research strategies. Thus, substituents can influence the SP-MC ratio in thermal

equilibrium,³⁵ wherein the SP-form does not undergo hydrolysis but the hydrolytic rate of the MC-form remains almost unchanged. Another approach is to modulate the stability of the bridging double bond against hydrolysis.³⁶ Introducing an electron-donating methoxy group in the *para*-position conjugated with the double bond improved stability. Recently a spiropyran was reported bearing a naphthalimide moiety, which was stable at pH 7.³⁷

Conclusion

Herein, we for the first time presented an approach to use Boc- and Fmoc-protected spiropyran photoswitches directly as a backbone modification, applicable via solid phase peptide synthesis (SPPS). We have developed an easy synthetic route and demonstrated the incorporation with standard Fmoc-chemistry as well as the photophysical behavior of the generated, fibrillating peptides under acidic conditions. Our future efforts will focus on the design of new photoswitches to increase the stability at higher pH values and to further modulate aggregation of peptides and proteins.

■ ASSOCIATED CONTENT

Data Availability Statement

Some data underlying this study are not publicly available due to patent issues. Most data of this study however are available within this article and its [Supporting Information](#). The raw data that support the findings of this study are stored electronically according to the requirements of the DFG and are available from the corresponding author upon reasonable request by email (please contact the corresponding author, WHB, via Email (Wolfgang-binder@chemie.uni-halle.de)).

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.orglett.4c03929>.

General information, experimental procedures, characterization data, and copies of ¹H, ¹³C NMR spectra; HPLC data; spectroscopic investigations; Supplementary Tables: (S1) Kinetic parameters of thermal isomerization and decomposition of peptides P1–P4; (S2) isomeric ratio in the thermal equilibrium and at the respective PSS of P1–P4; Supplementary Figures: (S1–S4) UV/vis-spectra of P1–P4 at different pH values; (S5–S19) UV/vis kinetic measurements of thermal isomerization and decomposition of P1–P4; (S20–S22) MALDI-ToF measurements of P1, P2, and P4 at different pH values; (S23–S24) UV/vis spectra, measured (thermal equilibrium and PSS) and calculated (pure isomers) of P1–P4; (S25–S42) NMR data of compounds and peptides; (S43–S46) HPLC and MS data of peptides (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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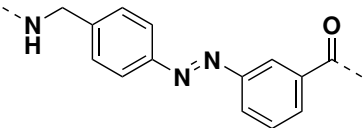
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Conclusion

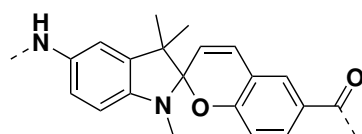
According to the scope of this thesis, the three photoswitchable building blocks 3,4'-AMPB, FASC and FASC were successfully synthesised as their Fmoc-protected forms. The respective photoswitches were incorporated into the backbone of PTH₂₅₋₃₇ at various positions using SPPS with Fmoc-chemistry (see Table 5.1). In total 22 variants of the modified sequence were generated, whereby twelve peptides contained the 3,4'-AMPB moiety, nine containing the FASC moiety and one peptide bears the SAFC moiety. The photophysical properties of the peptides were characterized with UV/Vis spectroscopy, in particular the optimal switching wavelengths, the composition in the PSS, and the half-life time of the metastable state (for azobenzenes the *cis*-form and for spiropyrans the SP-form). The investigation of the fibrillization behaviour was mainly conducted with the azobenzene containing peptides (see section 5.1 and 5.2). The spiropyran containing peptides were not able to fibrillize under physiological conditions and therefore their switching behaviour was investigated at different pH values, which is described in section 5.3. Additionally, two peptides with uniformly ¹⁵N/¹³C labeled L28 and F34 were used in the ssNMR studies (see section 5.2).

Table 5.1: Primary sequences of the PTH-fragments, and the PTH₂₅₋₃₇ derived azobenzene and spiropyran containing peptides. Position of the azobenzene moiety is highlighted in violet. Position of the spiropyran moieties is highlighted in red.

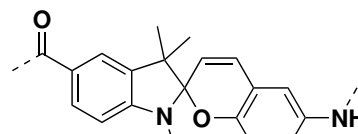
peptide	primary sequence	modification
PTH fragments		
PTH ₁₋₈₄	¹ SVSEIQLMHN ¹¹ LGKHLNSMER ²¹ VEWLRKKLQD ³¹ VHNFVALGAP ⁴¹ LAPRDAGSQR ⁵¹ PRKKEDNVLV- ⁶¹ ESHEKSLGEA ⁷¹ DKADVNVLT ⁸¹ AKSQ	
PTH ₂₅₋₃₇	²⁵ RKKLQ ³⁰ DVHNF ³⁵ VAL	-
Azobenzene containing peptides		
		
3,4'-AMPB		
A1	²⁵ RKKLQ ³⁰ D- 3,4'-AMPB -VHNF ³⁵ VAL	insertion D30-V31
A2	²⁵ RKKLQ ³⁰ DV- 3,4'-AMPB -HNF ³⁵ VAL	insertion V31-H32
A3	²⁵ RKKLQ ³⁰ - 3,4'-AMPB -VHNF ³⁵ VAL	exchange D30
A4	²⁵ RKKLQ ³⁰ D- 3,4'-AMPB -HNF ³⁵ VAL	exchange V31
A5	²⁵ RKKLQ ³⁰ - 3,4'-AMPB -HNF ³⁵ VAL	exchange D30-V31

peptide	primary sequence	modification
A6	²⁵ RKKLQ-3,4'-AMPB- ³⁰ DVHNF ³⁵ VAL	insertion Q29-D30
A7	²⁵ RKKL-3,4'-AMPB-Q ³⁰ DVHNF ³⁵ VAL	insertion L28-Q29
A8	²⁵ RK-3,4'-AMPB-KLQ ³⁰ DVHNF ³⁵ VAL	insertion K26-K27
A9	²⁵ R-3,4'-AMPB-KLQ ³⁰ DVHNF ³⁵ VAL	exchange K26
A10	²⁵ RKKLQ ³⁰ DVHN-3,4'-AMPB-F ³⁵ VAL	insertion N33-F34
A11	²⁵ RKKLQ ³⁰ DVHNF ³⁵ V-3,4'-AMPB-AL	insertion V35-A36
A12	²⁵ RKKLQ ³⁰ DVHNF-3,4'-AMPB-AL	exchange V35

Spiropyran containing peptides



FASC

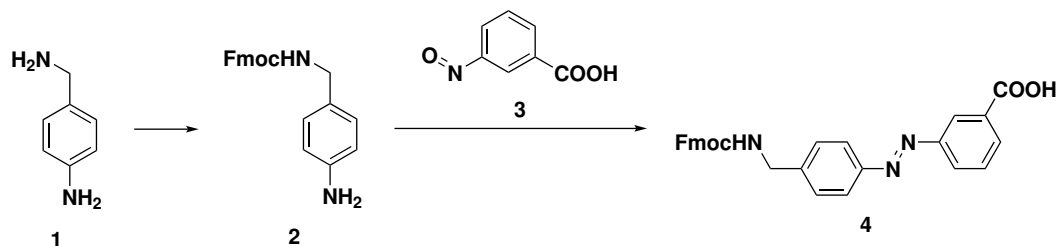


SAFC

S1	²⁵ RKKLQ ³⁰ D-FASC-VHNF ³⁵ VAL	insertion D30-V31
S2	²⁵ RKKLQ ³⁰ DV-FASC-HNF ³⁵ VAL	insertion V31-H32
S3	²⁵ RKKLQ ³⁰ -FASC-VHNF ³⁵ VAL	exchange D30
S4	²⁵ RKKLQ ³⁰ D-FASC-HNF ³⁵ VAL	exchange V31
S5	²⁵ RKKLQ ³⁰ -FASC-HNF ³⁵ VAL	exchange D30-V31
S7	²⁵ RKKL-FASC-Q ³⁰ DVHNF ³⁵ VAL	insertion L28-Q29
S8	²⁵ RK-FASC-KLQ ³⁰ DVHNF ³⁵ VAL	insertion K26-K27
S9	²⁵ R-FASC-KLQ ³⁰ DVHNF ³⁵ VAL	exchange K26
S10	²⁵ RKKLQ ³⁰ D-SAFC-HNF ³⁵ VAL	exchange V31

5.1 Photocontrol of azo-modified PTH₂₅₋₃₇-derived peptides

The aim of the initial study was to test the hypothesis, whether it is possible to control the fibrillization behaviour of the model peptide PTH₂₅₋₃₇ with the 3,4'-AMPB switch, *i.e.* one isomer is able to form amyloid fibrils and the other is not. Therefore, the Fmoc-protected 3,4'-AMPB building block **4** was synthesized according to literature (Figure 5.1)¹⁶⁷ and introduced into the PTH₂₅₋₃₇ backbone *via* SPPS. Peptide A4 (primary structure in Table 5.1) had the highest solubility and was therefore chosen

Figure 5.1: Synthesis scheme of Fmoc-protected 3,4'-AMPB building block.¹⁶⁷

to be investigated in accordance to the fibrillization behaviour of both isomers (Figure 5.2). While the fibrillization kinetic was characterized by the lag time t_{lag} , which corresponds to the onset of the process, and the characteristic time t_{char} , displaying the point fluorescence reaches 50% of the stationary phase, the presence of fibrils was verified with TEM-recordings taken after distinct time points. In Figure 5.2A it is visible that fibrillization of *trans*-A4 is accelerated compared to the unmodified PTH_{25–37}, while the fibrillization of the *cis*-isomer is inhibited. Similar conclusions can be drawn from the TEM images (Figure 5.2B), where fibrils are observed in all recordings with the exception of *cis*-A4, where fibrils are firstly present in the recording after 60 h.

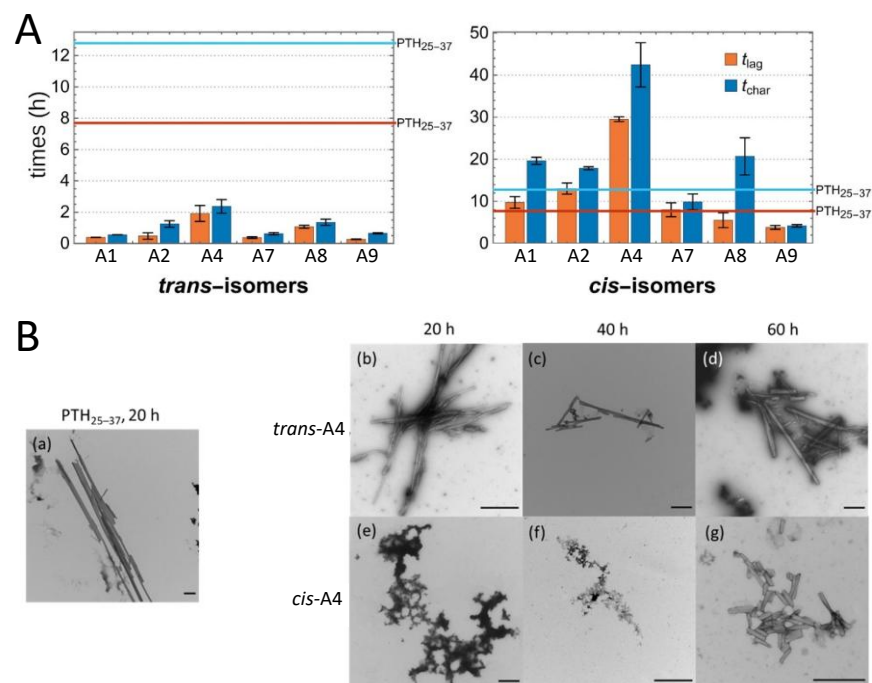


Figure 5.2: (A) Kinetic parameters t_{lag} (orange) and t_{char} (blue) of unmodified and modified PTH_{25–37}-derived peptides. Reprinted and adapted with permission from American Chemical Society, copyright ©2024 (open access).³¹⁵ (B) TEM-recordings of unmodified PTH_{25–37}, *trans*-A4, and *cis*-A4. (a), (b), and (e) were taken after 20 h. (c) and (f) after 40 h. (d) and (g) after 60 h. Scale bar = 500 nm. Reprinted and adapted with permission from MDPI, copyright ©2022 (open access).³⁴⁸

Based on these findings, the structural nature of fibrils formed by *trans*-A4 and PTH_{25–37} was investigated using WAXS, ssNMR with ¹³C-labeled peptides, and, combined with these data, MD simulations. While WAXS measurements could verify the fibrillar structure (Figure 5.3A,D) and allows an assumption towards the arrangement of the β -sheets, ssNMR measurements could prove that PTH_{25–37} β -strands are arranged in a parallel manner (Figure 5.3B), while β -strands of *trans*-A4 adopt an antiparallel order (Figure 5.3E). According to the MD simulations, it is anticipated that in PTH_{25–37} mainly the C-terminal amino acids form the β -sheet (Figure 5.3C). In the MD simulations of *trans*-A4, however, the photoswitch breaks partially the β -sheet, which is formed by the C- and N-terminal regions (Figure 5.3F). The antiparallel arrangement is herein favoured, as electrostatic interactions, between positively charged N-termini and negatively charged C-termini, and the π - π -interactions of the azobenzene switch are stabilizing.

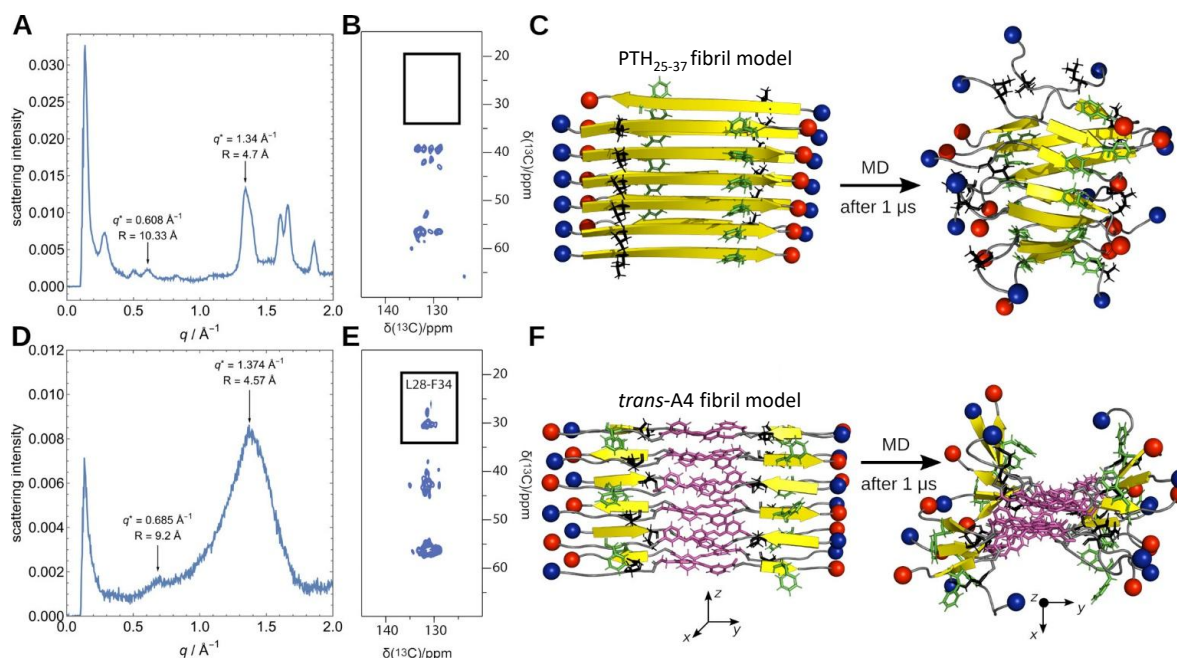


Figure 5.3: Structural investigations of fibrils of PTH₂₅₋₃₇ and *trans*-A4. (A, D) WAXS results. (B, E) ssNMR 2D ¹³C–¹³C proton driven spin diffusion spectra close to rotational resonance (mixing time 1s) of fibrils of the PTH₂₅₋₃₇ and *trans*-A4 peptide with uniformly ¹³C-labeled L28 and F34. (C, F) Fibril models of PTH₂₅₋₃₇ and *trans*-A4 constructed based on the ssNMR (left) and at the end of MD simulations (right), where β -sheets are shown in yellow, random coil in gray, the N- and C-termini as blue and red spheres, respectively, 3,4'-AMPB in purple, and the side chains of L28 and F34 are highlighted as black and green sticks, respectively. Note that in panel F, the structure on the right-hand side is rotated by 90° to better represent the fibrillar arrangement. Reprinted with permission from American Chemical Society, copyright ©2024 (open access).³¹⁵

In total twelve peptides were synthesised containing the 3,4'-AMPB switch at various positions, three bearing the switch at the N-terminal end, six in the central region and three at the C-terminus. The determination of the fibrillization parameters t_{lag} and t_{char} of both isomers (Figure 5.2) of all peptides was in accordance with the prediction drawn from the MD simulations: the *trans*-configuration does not prevent the ability to form fibrils, if placed in the N-terminal and central region, moreover, the fibrillization was significantly faster compared to the unmodified peptide due to the increased hydrophobicity and the organizing effect of the 3,4'-AMPB. The *cis*-configuration caused a delay of fibrillization, whereby the effect decreases with increasing distance to the aggregation-prone region at the C-terminus. However, if the switch is positioned in the fibril forming region, *e.g.* A12, the fibrillization is hindered for both isomers.

The reversible fibrillization was conducted with A4, as it had the highest solubility and largest difference in fibrillization between the two isomers. In Figure 5.4 the TMFAs are shown, subsequently followed by a degradation process. The presence of a new fibrillization process, comprising of lag phase, growth phase and stationary phase for each cycle indicates that monomers in a sufficient amount were released during each fibril degradation by *trans* $\rightarrow$ *cis*-photoisomerization.

Thus, the herein described system is the first report of an amyloidogenic peptide model, which is able to undergo reversibly fibril formation/fibril degradation cycles *via* light. Furthermore, the results showcased the importance of the strategical placement of the photoswitchable entity in the peptide.

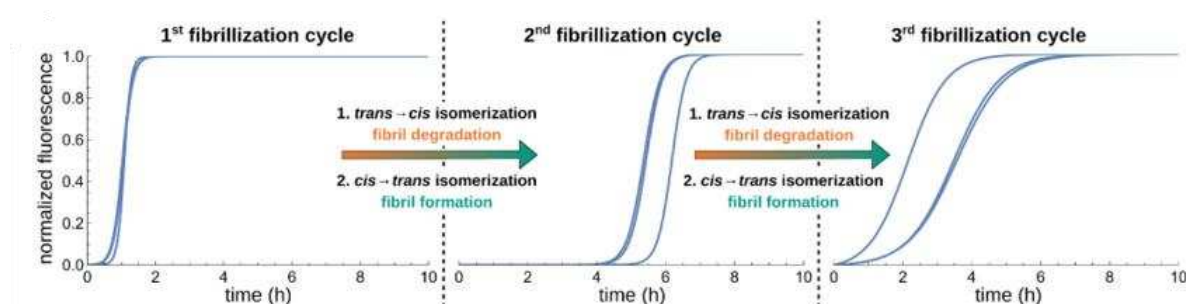


Figure 5.4: Three (de)-aggregation cycles via photoisomerization of A4. Fitted signals of TMFAs of each fibrillization of *trans*-A4 followed by fibril degradation through *trans* → *cis*-isomerization and reisomerization towards the *trans*-form. Reprinted with permission from American Chemical Society, copyright ©2024 (open access).³¹⁵

5.2 Co-fibrillization of azo-modified PTH_{25–37} and PTH(-derived peptides)

From previous studies it is known that heterologous sequences are known to undergo cross-amyloid aggregation³⁴⁹ and that as well synthetic β -turn modified A β -derivatives are able to catalytically effect the fibrillization of the Alzheimer peptide A β _{1–40}.³⁵⁰ This raises the question, whether it is possible to modulate fibrillization of the full-length PTH with the azobenzene modified peptides as well. As the fibrils of PTH serve as an inactive peptide storage, the monomers released upon degradation represents the active form. Thus, controlled (de)-fibrillization would be a promising method for regulating the bioavailability of the hormone or triggering drug release.

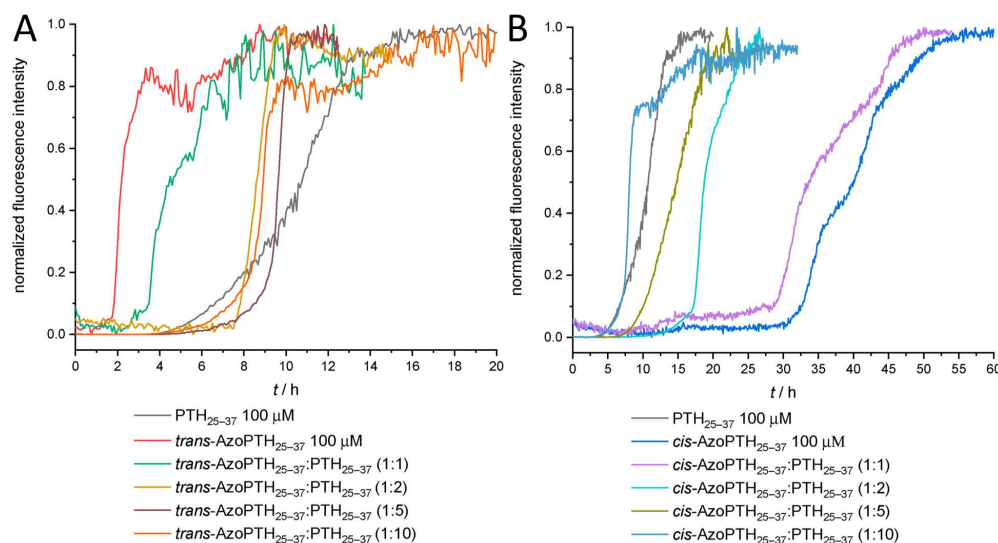


Figure 5.5: TMFA of mixtures of A4 and unmodified PTH_{25–37}. (A) *trans*-A4. (B) *cis*-A4. Reprinted and adapted with permission from MDPI, copyright ©2022 (open access).³⁴⁸

A4 has proven to be suitable to catalytically modulate the fibrillization process of PTH and PTH-derived peptides. In case of PTH_{25–37} mixtures (Figure 5.5) the fibrillization process was affected in the same

manner as for the modified peptide: *trans*-A4 accelerated the fibrillization, while *cis*-A4 hindered the process. Even the full-length peptide is affected by the A4-isomers (Figure 5.6). Unfortunately, t_{lag} of PTH₁₋₈₄ is significantly larger than the half-life time of *cis*-A4. Nevertheless, it is observable that the *cis*-isomer hinders the fibrillization compared to the *trans*-isomer, however, both isomers still accelerate the aggregation compared to the native peptide. Interestingly, the morphology of the fibrils is altered in presence of A4. While the native peptide produces curly fibrils, only straight fibrils were observable in the case of the mixture.

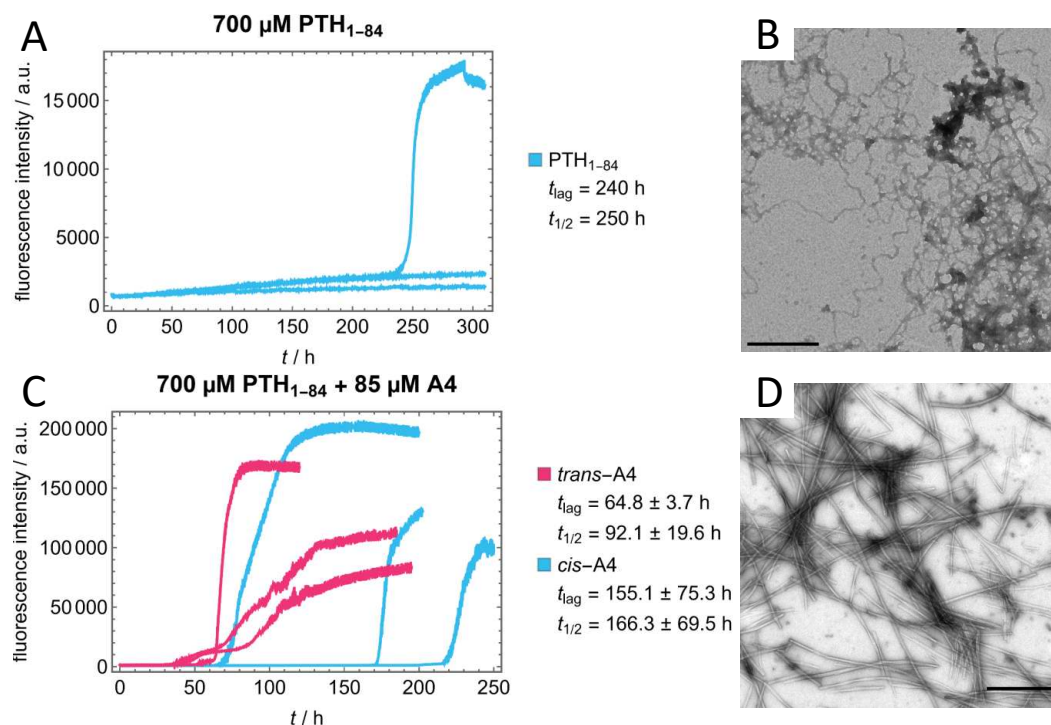


Figure 5.6: (A) TMFA and kinetic parameters of PTH₁₋₈₄. (B) TEM recording of PTH₁₋₈₄ fibrils. (C) TMFA and kinetic parameters of PTH₁₋₈₄-A4-mixture. (D) TEM recording of heterologous fibrils from PTH₁₋₈₄ and *trans*-A4. Scale bar = 500 nm.

Even if the half-life time of *cis*-A4 was too short, this light-triggered approach is a promising method regarding the controlled drug delivery of PTH. It is clearly visible that the A4 isomers had a different influence on the fibrillization of native PTH. In further studies the use of another azobenzene moiety with a higher stability of the *cis*-form, *e.g.* *ortho*-fluoro azobenzenes⁸⁸ or *ortho*-methoxy azobenzenes,⁸⁷ could enable full control of the fibrillization of PTH.

5.3 Novel spiropyran building blocks for SPPS

While for the azobenzene class several suitable building blocks for SPPS exist,^{161,167,351} there is no known example of a spiropyran backbone modification suitable for SPPS using either Boc-chemistry or Fmoc-chemistry. Thus, during this thesis spiropyran backbone modifications, FASC and the SAFC (Figure 5.7), were designed. FASC contains the N-terminus at the indolene unit and the C-terminus

at the chromene, while SAFC bears the N-terminus at the chromene unit and the C-terminus at the indolene unit. A facile synthetic route was elaborated to obtain both switches as ready-to-use building blocks for SPPS, either Boc- (**9**, **18**) or Fmoc-protected (**10**, **19**). To investigate their behaviour in the peptide backbone, the photoswitches were introduced using SPPS with Fmoc chemistry at similar positions as the 3,4'-AMPB photoswitch in 5.1 and 5.2 (see Table 5.1, S1-10). Unfortunately, it was not possible to investigate the fibrillization behaviour of the SP- and MC- isomer of the modified peptides, as the spiropyrans are unstable at neutral pH. Nevertheless, it was proven that the FASC and SAFC switch are stable at acidic pH and the photophysical properties were characterized for different pH values. The herein presented photoswitchable entities are the first reported spiropyran building blocks for SPPS and extend the possible modification options in peptide chemistry by a powerful tool even if future research has taken improvements in pH stability in account.

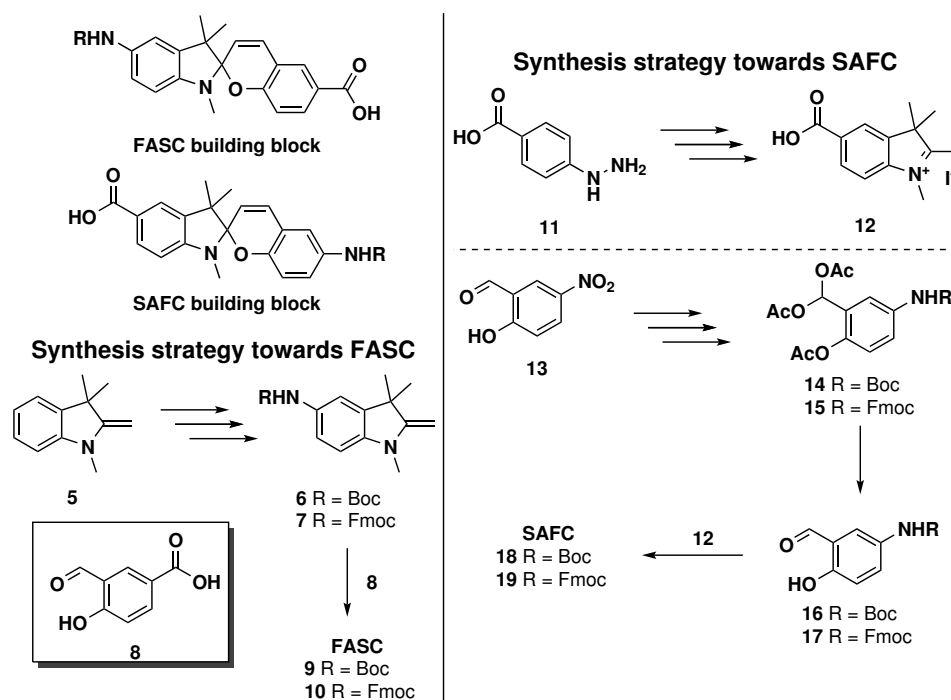
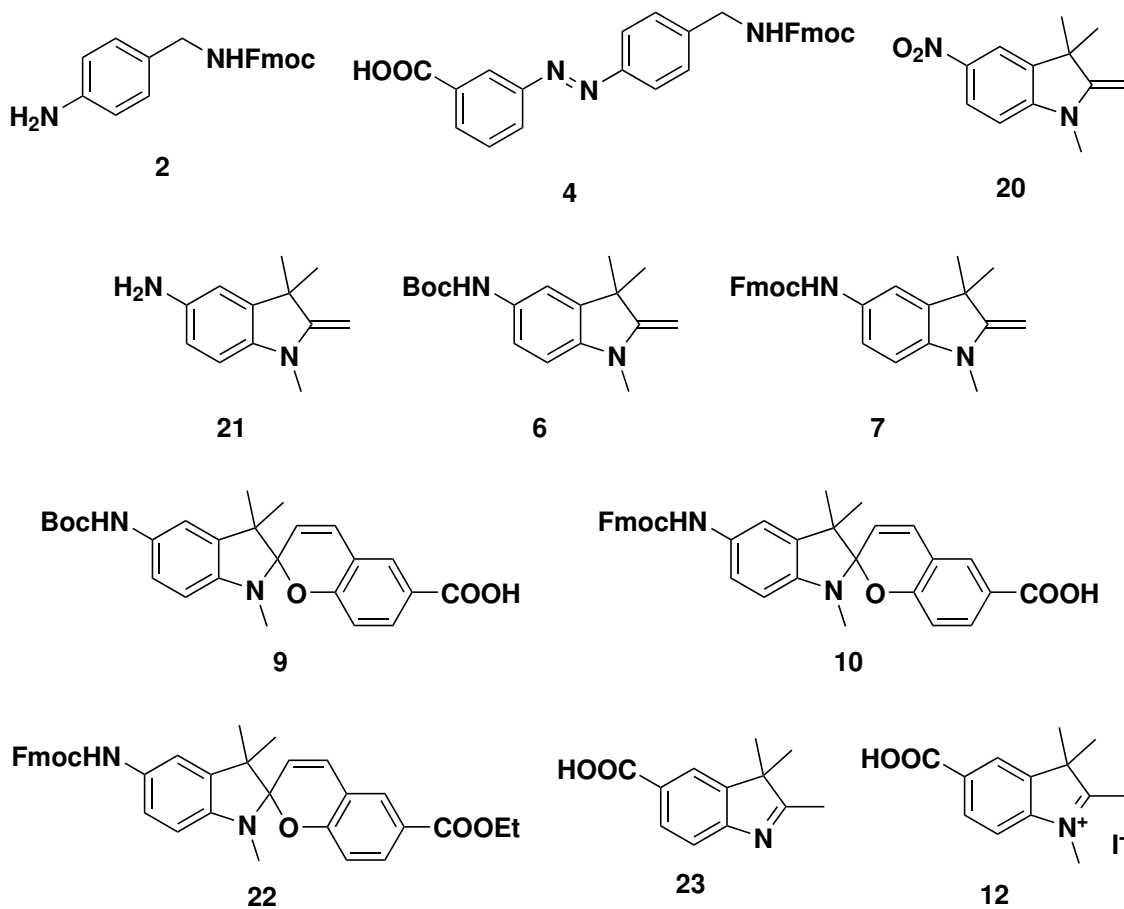


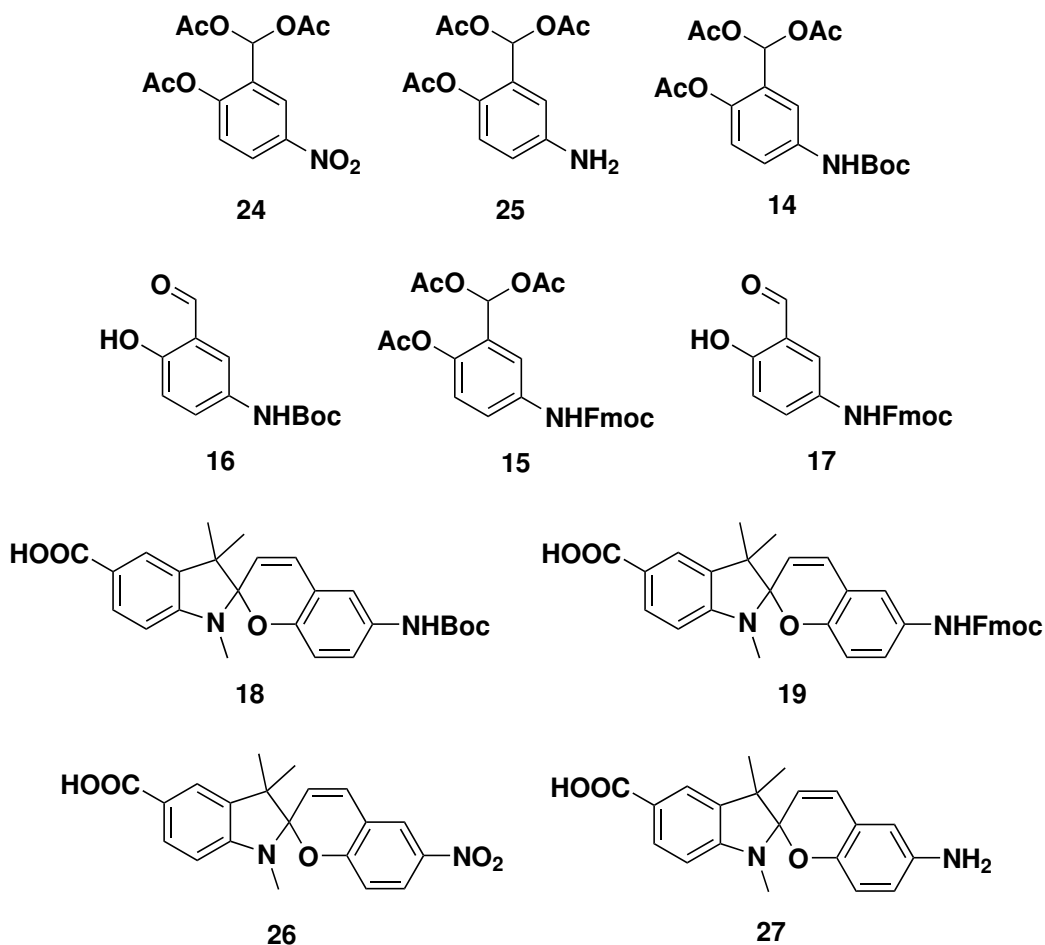
Figure 5.7: Synthesis schemes of Boc- and Fmoc-protected FASC and SAFC building blocks.

In this thesis photoswitchable PTH-derived peptides were successfully generated. It was demonstrated that the azobenzene moiety is able to influence the fibrillization process in different ways, depending on the configuration of the azo bond. This made it possible to control the fibrillization in a reversible and switchable manner. In addition, the structural understanding of the fibrillization of PTH₂₅₋₃₇ was improved and it was shown that azobenzene-modified peptides have a catalytic effect on the fibrillization of PTH₂₅₋₃₇ and PTH. It could therefore be a potential method for modulating the bioavailability of hormones.

Syntheses

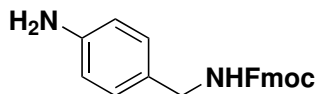
The following compounds were synthesized and characterized together with Niclas Starke during his master thesis.





6.1 Azobenzenes

(9*H*-Fluoren-9-yl)methyl (4-aminobenzyl)carbamate (**2**)



4-Aminobenzylamine **1** (2.5 g, 20.5 mmol, 1 eq.) was dissolved in a mixture of 24 mL MeCN and 2 mL DMF and triethylamine (TEA) (3 mL, 22.5 mmol, 1.1 eq.) was added. Fmoc-OSu (7.6 g, 22.5 mmol, 1.1 eq.) was dissolved in 50 mL MeCN and slowly added over a duration of 90 min to the reaction solution at ambient temperature. The formed precipitate was filtrated and washed with EtO₂ and water. Compound **2** was obtained as a white powder (6.6 g, 19.2 mmol, 84%).

Analytical data match reported literature values.³⁵²

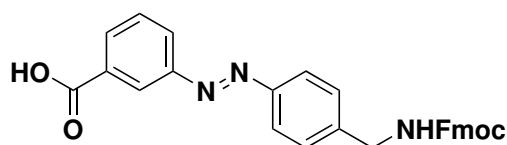
$R_f = 0.2$ (hexane:EE / 3:2).

¹H-NMR (500 MHz, CDCl₃): δ = 7.76 (dt, J = 7.6, 1.0 Hz, 2H), 7.59 (d, J = 7.5 Hz, 2H), 7.40 (tt, J = 7.5, 0.9 Hz, 2H), 7.31 (td, J = 7.5, 1.2 Hz, 2H), 7.07 (d, J = 7.9 Hz, 2H), 6.64 (d, J = 8.4 Hz, 2H), 4.98 (s, 1H), 4.44 (d, J = 6.9 Hz, 2H), 4.31–4.16 (m, 3H), 3.65 (s, 2H).

¹³C-NMR (125 MHz, CDCl₃): δ = 156.3, 145.9, 144.0, 141.3, 129.0, 128.2, 127.6, 127.0, 125.1, 120.0, 115.2, 66.6, 47.3, 44.8.

MS (ESI+, MeCN/MeOH): m/z = 383.11 [M+K]⁺.

(trans)-3-((4-((((9H-Fluoren-9-yl)methoxy)carbonyl)amino)-methyl)phenyl)diazenyl)benzoic acid (4)



3-Aminobenzoic acid (2 g, 14.6 mmol) were suspended in 36 mL dichloromethane (DCM) and Oxone (17.9 g, 29 mmol) dissolved in 160 mL water was added dropwise. The reaction mixture is stirred at ambient temperature for 3.5 h. The formed precipitate is separated through filtration, washed with water and dried *in vacuo*. 3-Nitrosobenzoic acid **3** (1.8 g, 13.2 mmol) were obtained as a greenish solid and used without further purification. Compound **3** (1.8 g, 13.2 mmol, 2 eq.) was dissolved in 150 mL DMSO and 150 mL acetic acid were added. The solution was degassed for 30 min with nitrogen and compound **2** (2.28 g, 6.6 mmol, 1 eq.) was added. The reaction solution was stirred for 3 d at ambient temperature and 300 mL water were added. The solution was extracted three times with ethyl acetate (EE). The combined organic phases were dried over Na₂SO₄ and the solvent was removed *in vacuo*. The residue was purified by flash chromatography on normal phase silica gel (solvent A: DCM + 0.1% FA, solvent B: MeOH + 0.1% FA, 9% A) and compound **4** was obtained as an orange solid (1.5 g, 3.2 mmol, 47%).

Analytical data match reported literature values.¹⁶⁷

R_f = 0.7 (hexane:EE + 0.1% FA / 1:4).

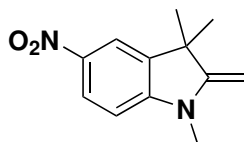
¹H-NMR (500 MHz, DMSO-*d*₆): δ = 8.38 (t, J = 1.8 Hz, 1H), 8.13 (ddt, J = 10.5, 7.7, 1.4 Hz, 2H), 7.90 (dd, J = 7.8, 5.1 Hz, 4H), 7.76–7.68 (m, 3H), 7.47–7.39 (m, 4H), 7.34 (td, J = 7.4, 1.1 Hz, 2H), 4.40 (d, J = 6.7 Hz, 2H), 4.30 (d, J = 6.1 Hz, 2H), 4.25 (t, J = 6.8 Hz, 1H).

¹³C-NMR (125 MHz, DMSO-*d*₆): δ = 167.1, 156.9, 152.3, 151.2, 144.5, 144.3, 141.2, 132.6, 132.2, 130.4, 128.4, 128.1, 127.8, 127.5, 125.6, 123.3, 122.6, 120.6, 65.8, 47.3, 43.9.

MS (ESI+, MeCN/MeOH): m/z = 516.13 [M+K]⁺.

6.2 Spiropyrans

1,3,3-Trimethyl-2-methylene-5-nitroindoline (20)



1,3,3-Trimethyl-2-methylenindolin **5** (10 g, 57.7 mmol, 1 eq.) was slowly added to 60 mL concentrated H_2SO_4 and cooled down to 0°C . NaNO_3 (7.9 g, 63.5 mmol, 1.1 eq.) was added in small portions and the reaction mixture is stirred for 1 h. The solution was poured onto 100 g ice and neutralized with solid Na_2CO_3 . The formed precipitate was filtrated and recrystallized in a mixture of acetone/water (1:1). Compound **20** was obtained as a red solid. (7.3 g, 33.2 mmol, 58%).

Analytical data match reported literature values.³⁵³

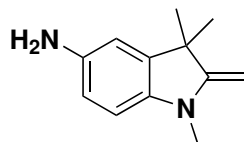
$R_f = 0.7$ (hexane:EE / 3:2).

$^1\text{H-NMR}$ (500 MHz, CDCl_3): $\delta = 8.12$ (dd, $J = 8.8, 2.3$ Hz, 1H), 7.92 (d, $J = 2.3$ Hz, 1H), 6.51 (d, $J = 8.8$ Hz, 1H), $4.16\text{--}4.06$ (m, 2H), 3.12 (s, 3H), 1.36 (s, 6H).

$^{13}\text{C-NMR}$ (125 MHz, CDCl_3): $\delta = 161.2, 151.5, 140.0, 138.2, 126.2, 118.1, 103.9, 78.9, 43.4, 29.7, 29.1$.

MS (ESI+, MeCN/MeOH): $m/z = 225.122$ $[\text{M}+\text{Li}]^+$.

1,3,3-Trimethyl-2-methyleneindolin-5-amine (21)



Compound **20** (2 g, 9.1 mmol, 1 eq.) and dried tin(II) chloride (10.4 g, 55 mmol, 6 eq.) were dissolved in 60 mL concentrated aqueous hydrogen chloride solution and refluxed for 2 h. The reaction solution was alkalized and extracted three times with 60 mL Et_2O . The organic phases were combined and the solvent removed *in vacuo*. The residue was recrystallized in hexane and compound **21** was obtained as a green solid (1 g, 5.3 mmol, 58%).

Analytical data match reported literature values.³⁵⁴

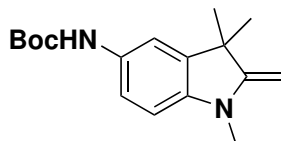
$R_f = 0.1$ (DCM:MeOH + 0.1% FA / 9:1).

¹H-NMR (500 MHz, CDCl₃): δ = 6.56 (d, J = 2.3 Hz, 1H), 6.51 (dd, J = 8.1, 2.3 Hz, 1H), 6.35 (d, J = 8.0 Hz, 1H), 3.79–3.72 (m, 2H), 3.35 (s, 2H), 2.99 (s, 3H), 1.33 (s, 6H).

¹³C-NMR (125 MHz, CDCl₃): δ = 163.4, 139.7, 138.9, 138.5, 114.1, 111.3, 105.1, 71.5, 44.4, 29.9, 29.0.

MS (ESI+, MeCN/MeOH): m/z = 189.14 [M+H]⁺.

***tert*-Butyl (1,3,3-trimethyl-2-methyleneindolin-5-yl)carbamate (6)**



Compound **21** (585 mg, 3.1 mmol, 1 eq.) was dissolved in 40 mL THF. Boc₂O (680 mg, 3.1 mmol, 1 eq.) and TEA (440 μ L, 3.1 mmol, 1 eq.) were added under a nitrogen atmosphere and the reaction mixture was stirred at ambient temperature for 18 h. The solvent was removed *in vacuo* and the residue was dissolved in 50 mL DCM. The solution was extracted three times with 50 mL of an aqueous solution with 5% acetic acid. The combined aqueous phases were washed two times with 50 mL Et₂O and the solution was made alkaline (pH 9–10) with NaHCO₃. The aqueous solution was extracted three times with 50 mL DCM. The combined organic phases were dried over Na₂SO₄ and the solvent was removed *in vacuo*. The residue of compound **6** (yellowish oil, 760 mg) was used without further purification.

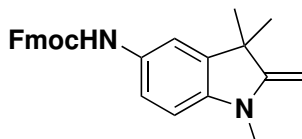
R_f = 0.4 (DCM:MeOH / 19:1).

¹H-NMR (400 MHz, CDCl₃) δ = 7.17 (s, 1H), 7.02–6.90 (m, 1H), 6.41 (d, J = 8.3 Hz, 1H), 6.26 (s, 1H), 3.79 (q, J = 2.0 Hz, 2H), 2.99 (s, 3H), 1.49 (s, 9H), 1.31 (s, 6H).

¹³C-NMR (100 MHz, CDCl₃) δ = 163.0, 155.0, 142.7, 138.2, 129.7, 118.7, 114.7, 104.5, 79.6, 72.7, 44.3, 29.9, 28.9, 28.4.

MS (ESI+, MeCN/MeOH): m/z = 289.19 [M+H]⁺.

(9H-Fluoren-9-yl)methyl (1,3,3-trimethyl-2-methyleneindolin-5-yl)carbamate (7)



To a mixture of compound **21** (0.5 g, 2.7 mmol, 1 eq.) and Fmoc-Cl (0.82 g, 3.2 mmol, 1.2 eq.) were added 4 mL water and the suspension was stirred at 60°C for 2 h. The reaction suspension was neutralized with saturated aqueous NaHCO₃ solution and extracted three times with 60 mL DCM. The

organic phases were combined and the solvent removed *in vacuo*. The residue was purified by flash chromatography on normal phase silica gel (solvent A: DCM + 0.1% FA, solvent B: MeOH + 0.1% FA, 95% A) and compound **7** was obtained as a lightpurple solid. (0.38 g, 0.93 mmol, 35%).

$R_f = 0.2$ (DCM:MeOH + 0.1% FA / 9:1).

$^1\text{H-NMR}$ (500 MHz, CDCl_3): $\delta = 7.78$ (d, $J = 7.5$ Hz, 2H), 7.69–7.52 (m, 2H), 7.41 (t, $J = 7.4$ Hz, 2H), 7.37–7.29 (m, 2H), 7.24–7.15 (m, 1H), 7.05 (s, 1H), 6.52 (s, 1H), 6.45 (d, $J = 8.3$ Hz, 1H), 4.50 (d, $J = 6.8$ Hz, 2H), 4.28 (t, $J = 6.9$ Hz, 1H), 3.84 (q, $J = 2.0$ Hz, 2H), 3.02 (s, 3H), 1.34 (s, 6H).

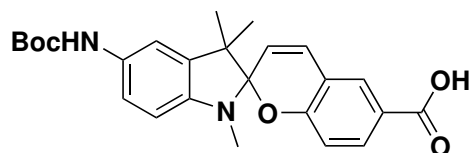
$^{13}\text{C-NMR}$ (125 MHz, CDCl_3): $\delta = 162.8, 154.1, 143.9, 143.2, 141.3, 138.3, 129.2, 127.7, 127.1, 125.0, 120.0, 119.2, 114.9, 104.6, 73.1, 66.7, 47.2, 44.3, 29.9, 28.9$.

IR (ATR): $\nu = 3303\text{w}, 2959\text{w}, 1697\text{s}, 1499\text{s}, 1208\text{s cm}^{-1}$.

UV/Vis ($c = 0.06 \mu\text{mol mL}^{-1}$ in MeOH): $\lambda_{\text{max},1} = 289.2, \lambda_{\text{max},2} = 277.3, \lambda_{\text{max},3} = 265.3 \text{ nm}$.

HR-MS (ESI+, MeCN/MeOH): calcd. for $[\text{C}_{27}\text{H}_{26}\text{N}_2\text{O}_2 + \text{H}]^+ = 411.210$ found: 411.210.

5'-((*tert*-Butoxycarbonyl)amino)-1',3',3'-trimethylspiro[chromene-2,2'-indoline]-6-carboxylic acid (9**)**



The unpurified residue of compound **6** (700 mg, ~2.4 mmol, 1 eq.) and 3-formyl-4-hydroxybenzoic acid **8** (400 mg, 2.43 mmol, 1 eq.) were dissolved in 50 mL MeCN and the solution was refluxed (heated with an oil bath) for 4.5 h under a nitrogen atmosphere. The solvent was removed *in vacuo* and the residue was purified by flash chromatography in normal phase silica (solvent A: hexane + 0.1% FA, solvent B: EE + 0.1% FA, 60% A). Compound **9** was obtained as a redish solid (0.680 mg, 1.56 mmol, 64%).

$R_f = 0.4$ (hexane:EE + 0.1% FA / 3:2).

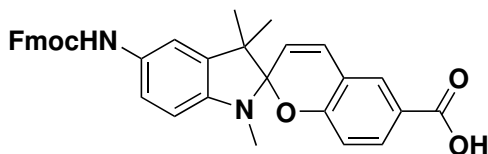
$^1\text{H-NMR}$ (500 MHz, $\text{DMSO}-d_6$): $\delta = 12.65$ (s, 1H), 8.97 (s, 1H), 7.80 (d, $J = 2.3$ Hz, 1H), 7.68 (dd, $J = 8.5, 2.3$ Hz, 1H), 7.25 (s, 1H), 7.18–6.99 (m, 2H), 6.74 (d, $J = 8.6$ Hz, 1H), 6.48 (d, $J = 8.3$ Hz, 1H), 5.83 (d, $J = 10.3$ Hz, 1H), 2.61 (s, 3H), 1.46 (s, 9H), 1.17 (s, 3H), 1.10 (s, 3H).

$^{13}\text{C-NMR}$ (125 MHz, $\text{DMSO}-d_6$): $\delta = 166.7, 157.7, 153.1, 143.2, 136.3, 131.3, 129.0, 128.5, 122.7, 122.0, 119.8, 118.4, 117.5, 114.4, 113.6, 106.7, 105.3, 78.4, 51.6, 28.7, 28.2, 25.6, 19.7$.

IR (ATR): $\nu = 3319\text{w}, 3069\text{w}, 2973\text{br}, 1683\text{s}, 1158\text{s cm}^{-1}$.

HR-MS (ESI+, MeCN/MeOH): calcd. for $[\text{C}_{25}\text{H}_{28}\text{N}_2\text{O}_5\text{Na}]^+ = 459.1890$ found 459.1909.

5'-((((9*H*-Fluoren-9-yl)methoxy)carbonyl)amino)-1',3',3'-tri-methylspiro[chromene-2,2'-indoline]-6-carboxylic acid (10**)**



Compound **7** (0.3 g, 0.73 mmol, 1 eq.) and 3-formyl-4-hydroxybenzoic acid **8** (0.16 g, 0.95 mmol, 1.3 eq.) were dissolved in 25 mL MeCN for 6 h and refluxed under a nitrogen atmosphere. The solvent was removed *in vacuo* and the residue was purified by flash chromatography on normal phase silica gel (solvent A: hexane + 0.1% FA, solvent B: EE + 0.1% FA, 60% A). compound **10** was obtained as a redish solid (0.17 g, 0.3 mmol, 42%).

$R_f = 0.5$ (hexane:EE + 0.1% FA / 3:2).

$^1\text{H-NMR}$ (500 MHz, DMSO- d_6): $\delta = 12.60$ (s, 1H), 9.40 (s, 1H), 7.89 (d, $J = 7.0$ Hz, 2H), 7.79 (d, $J = 2.2$ Hz, 1H), 7.77–7.69 (m, 2H), 7.67 (dd, $J = 8.5, 2.2$ Hz, 1H), 7.41 (t, $J = 7.4$ Hz, 2H), 7.34 (t, $J = 7.4$ Hz, 2H), 7.23 (s, 1H), 7.15 (s, 1H), 7.10 (d, $J = 10.5$ Hz, 1H), 6.73 (d, $J = 8.5$ Hz, 1H), 6.49 (d, $J = 8.3$ Hz, 1H), 5.82 (d, $J = 10.2$ Hz, 1H), 4.41 (d, $J = 6.9$ Hz, 2H), 4.28 (t, $J = 6.9$ Hz, 1H), 2.60 (s, 3H), 1.17–1.07 (m, 6H).

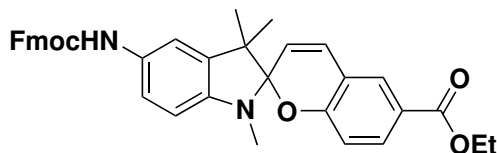
$^{13}\text{C-NMR}$ (125 MHz, DMSO- d_6): $\delta = 167.2, 158.1, 154.2, 144.3, 141.2, 137.3, 136.9, 131.8, 131.2, 129.5, 129.0, 128.1, 127.6, 125.6, 123.2, 120.6, 120.2, 118.9, 118.0, 114.9, 107.3, 105.7, 65.9, 52.1, 47.2, 40.6, 40.4, 40.3, 40.1, 39.9, 39.8, 39.6, 29.2, 26.1, 20.1$.

IR (ATR): $\nu = 3338\text{w}, 2968\text{br}, 1693\text{s}, 1234\text{m}, 1208\text{s cm}^{-1}$.

UV/Vis ($c = 0.46 \mu\text{mol mL}^{-1}$ in MeOH): $\lambda_{\text{max},1} = 443.7 \text{ nm}$.

HR-MS (ESI+, MeCN/MeOH): calcd. for $[\text{C}_{35}\text{H}_{30}\text{N}_2\text{O}_5]^+ = 558.220$ found 558.214.

Ethyl 5'-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-1',3',3'-trimethylspiro[chromene-2,2'-indoline]-6-carboxylate (22**)**



Compound **10** (0.15 g, 0.27 mmol, 1 eq.) was dissolved in a mixture of 30 mL ethanol and 3 mL concentrated H_2SO_4 and the solution was refluxed for 2 h. Subsequently, the reaction solution was neutralized with saturated aqueous NaHCO_3 solution and extracted 3 times with 50 mL EE. The combined organic phases were dried over Na_2SO_4 and the solvent was removed *in vacuo*. The residue

was purified by flash chromatography on normal phase silica gel (solvent A: hexane + 0.1% FA, solvent B: EE + 0.1% FA, 90% A) and compound **22** was obtained as a purple solid (0.09 g, 0.15 mmol, 57%).

$R_f = 0.5$ (hexane:EE + 0.1% FA / 4:1).

$^1\text{H-NMR}$ (500 MHz, CDCl_3): $\delta = 9.42$ (s, 1H), 7.91 (d, $J = 7.5$ Hz, 2H), 7.83 (d, $J = 2.2$ Hz, 1H), 7.74 (s, 2H), 7.71 (dd, $J = 8.6, 2.2$ Hz, 1H), 7.43 (t, $J = 7.4$ Hz, 2H), 7.35 (t, $J = 7.4$ Hz, 2H), 7.24 (s, 1H), 7.20–7.09 (m, 2H), 6.78 (d, $J = 8.6$ Hz, 1H), 6.51 (d, $J = 8.3$ Hz, 1H), 5.85 (d, $J = 10.3$ Hz, 1H), 4.43 (d, $J = 6.8$ Hz, 1H), 4.33–4.21 (m, 3H), 1.29 (t, $J = 7.1$ Hz, 3H), 1.17 (s, 3H), 1.10 (s, 1H).

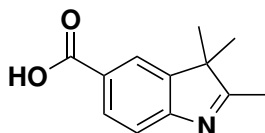
$^{13}\text{C-NMR}$ (125 MHz, CDCl_3): $\delta = 165.7, 158.4, 154.2, 144.3, 141.2, 136.9, 131.6, 129.4, 128.8, 128.1, 127.6, 125.6, 122.3, 120.6, 120.4, 119.1, 118.9, 115.1, 115.2, 107.3, 105.8, 65.9, 60.8, 52.1, 47.2, 29.1, 26.1, 20.1, 14.7$.

IR (ATR): $\nu = 3320\text{br}, 2959\text{w}, 1711\text{s}, 1486\text{m}, 1203\text{s cm}^{-1}$.

UV/Vis ($c = 0.13 \mu\text{mol mL}^{-1}$ in MeOH): $\lambda_{\text{max},1} = 288.4, \lambda_{\text{max},2} = 277.6 \text{ nm}$.

HR-MS (ESI+, MeCN/MeOH): calcd. for $[\text{C}_{35}\text{H}_{34}\text{N}_2\text{O}_5 + \text{Li}]^+ = 593.261$ found: 593.262.

2,3,3-Trimethyl-3H-indole-5-carboxylic acid (**23**)



4-Hydrazinobenzoic acid **11** (5 g, 33 mmol, 1 eq.) and 3-methyl-2-butanone (5 mL, 59 mmol, 1.8 eq.) were added to 50 mL acetic acid and refluxed for 16 h. The solvent was removed *in vacuo*, the residue redissolved in 50 mL DCM and neutralized with saturated aqueous NaHCO_3 -solution. The combined organic phases were dried over Na_2SO_4 and the solvent was removed *in vacuo* to obtain compound **23** as an orange solid (1.3 g, 6.4 mmol, 19%).

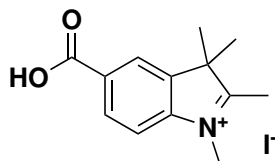
Analytical data match reported literature values.³⁵⁵

$R_f = 0.7$ (DCM:MeOH + 0.1% FA / 9:1).

$^1\text{H-NMR}$ (500 MHz, CDCl_3): $\delta = 12.76$ (s, 1H), 7.97 (d, $J = 1.6$ Hz, 1H), 7.90 (dd, $J = 8.1, 1.5$ Hz, 1H), 7.49 (dd, $J = 8.0, 1.2$ Hz, 1H), 2.23 (s, 3H), 1.25 (s, 6H).

$^{13}\text{C-NMR}$ (125 MHz, CDCl_3): $\delta = 192.1, 167.9, 157.9, 146.6, 130.0, 127.7, 123.2, 119.6, 53.9, 22.7, 15.8$.

MS (ESI+, MeCN/MeOH): $m/z = 204.10$ $[\text{M} + \text{H}]^+$.

5-Carboxy-1,2,3,3-tetramethyl-3*H*-indol-1-ium iodide (12)

Compound **23** (5 g, 25 mmol, 1 eq.) was dissolved in 70 mL toluene and 35 mL MeCN under nitrogen atmosphere. Iodomethane (1.8 mL, 30 mmol, 1.2 eq.) was added and the reaction mixture was refluxed for 16 h. The formed precipitate was separated, washed with hexane and cold ethanol and dried. Compound **12** was obtained as an orange-pinkish solid (2.9 g, 8.4 mmol, 34%).

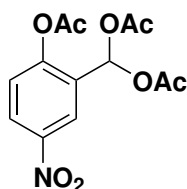
Analytical data match reported literature values.³⁵⁵

$R_f = 0.2$ (DCM:MeOH + 0.1% FA / 9:1).

¹H-NMR (400 MHz, DMSO-*d*₆): δ = 8.35 (s, 1H), 8.17 (d, *J* = 8.4 Hz, 1H), 8.02 (d, *J* = 8.4 Hz, 1H), 3.98 (s, 3H), 2.80 (s, 3H), 1.55 (s, 6H).

¹³C-NMR (100 MHz, DMSO-*d*₆): δ = 199.5, 166.9, 145.7, 142.4, 132.0, 130.8, 124.7, 115.8, 54.7, 35.5, 21.9, 15.0.

MS (ESI+, MeCN/MeOH): *m/z* = 218.12 [M]⁺.

(2-Acetoxy-5-nitrophenyl)methylene diacetate (24)

2-Hydroxy-5-nitrobenzaldehyd **13** (2.5 g, 15 mmol, 1 eq.) was dissolved in 50 mL acetic anhydride. Three drops of concentrated H₂SO₄ were added and the solution was stirred for 1 h under nitrogen atmosphere at ambient temperature. The reaction mixture was poured in 500 mL water and stirred for 30 min. The formed precipitate was separated, washed with water and dried. Compound **24** was obtained as white crystals (3.57 g, 11.5 mmol, 76%).

Analytical data match reported literature values.³⁵⁶

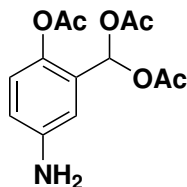
$R_f = 0.2$ (hexane:EE/ 8:2).

¹H-NMR (400 MHz, CDCl₃): δ = 8.53 (d, *J* = 2.8 Hz, 1H), 8.29 (dd, *J* = 8.9, 2.8 Hz, 1H), 7.93 (s, 1H), 7.35 (d, *J* = 8.9 Hz, 1H), 2.38 (s, 3H), 2.13 (s, 6H).

$^{13}\text{C-NMR}$ (100 MHz, CDCl_3): δ = 168.3, 168.2, 152.7, 145.6, 129.6, 125.8, 124.4, 123.8, 84.1, 20.8, 20.6.

MS (ESI+, MeCN/MeOH): m/z = 318.08 $[\text{M}+\text{Li}]^+$.

(2-Acetoxy-5-aminophenyl)methylene diacetate (25)



Compound **24** (1.5 g, 4.8 mmol, 1 eq.) was dissolved in 40 mL ethanol and PtO_2 (109 mg, 480 μM , 0.1 eq.) was added. The reaction suspension was stirred for 12 h under a hydrogen atmosphere (5 bar) at ambient temperature. The suspension was filtrated over Celite® and the solvent of the filtrate was removed *in vacuo*. The residue was purified by flash chromatography on normal phase silica gel (solvent A: hexane, solvent B: EE, 60% A) and compound **25** was obtained as a yellowish solid (0.95 g, 3.4 mmol, 70%).

Analytical data match reported literature values.³⁵⁶

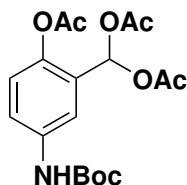
R_f = 0.4 (hexane:EE / 1:1).

$^1\text{H-NMR}$ (500 MHz, CDCl_3): δ = 7.79 (s, 1H), 6.95–6.83 (m, 2H), 6.69 (dd, J = 8.7, 2.8 Hz, 1H), 3.72 (s, 2H), 2.30 (s, 3H), 2.10 (s, 6H).

$^{13}\text{C-NMR}$ (125 MHz, CDCl_3): δ = 170.1, 168.4, 144.6, 140.1, 128.1, 123.8, 117.0, 113.5, 85.4, 20.8, 20.7.

MS (ESI+, MeCN/MeOH): m/z = 288.11 $[\text{M}+\text{Li}]^+$.

(2-Acetoxy-5-((tert-butoxycarbonyl)amino)phenyl)methylene diacetate (14)



Compound **25** (1 g, 3.6 mmol, 1 eq.) and Boc_2O (1.55 g, 7 mmol, 2 eq.) were dissolved in 10 mL DCM and stirred at ambient temperature for 12 h under a nitrogen atmosphere. The solvent was removed *in vacuo* and the residue was purified by flash chromatography on normal phase silica gel (solvent A: hexane, solvent B: EE, 80% A) to obtain compound **14** as a transparent gel (0.57 g, 1.5 mmol, 42%).

Analytical data match reported literature values.³⁵⁶

$R_f = 0.6$ (hexane:EE / 3:2).

$^1\text{H-NMR}$ (400 MHz, CDCl_3): $\delta = 7.84$ (s, 1H), 7.64 (d, $J = 2.5$ Hz, 1H), 7.41 (d, $J = 8.8, 2.7$ Hz, 1H), 7.03 (d, $J = 8.8$ Hz, 1H), 6.52 (s, 1H), 2.31 (s, 3H), 2.09 (s, 6H), 1.52 (s, 9H).

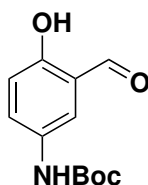
$^{13}\text{C-NMR}$ (100 MHz, CDCl_3): $\delta = 169.6, 168.4, 152.5, 143.3, 136.5, 128.2, 123.7, 120.7, 117.6, 85.1, 80.9, 28.3, 20.8, 20.7$.

IR (ATR): $\nu = 3316\text{m}, 2985\text{w}, 1764\text{s}, 1689\text{s}, 1151\text{s cm}^{-1}$.

UV/Vis ($c = 0.07 \mu\text{mol mL}^{-1}$ /MeOH): $\lambda_{\text{max},1} = 287 \text{ nm}$, $\lambda_{\text{max},2} = 242 \text{ nm}$.

MS (ESI+, MeCN/MeOH): $m/z = 388.157$ $[\text{M}+\text{Li}]^+$.

***tert*-Butyl (3-formyl-4-hydroxyphenyl)carbamate (16)**



Compound **14** (0.5 g, 1.3 mmol, 1 eq.) was dissolved in 10 mL methanol and 5 g NaOH dissolved in 5 mL water were added. The solution was refluxed for 3 h and neutralized with an aqueous 1 M HCl-solution. The solution was extracted three times with 20 mL EE and the combined organic phases were dried over Na_2SO_4 . The solvent was removed *in vacuo* and the residue was purified by flash chromatography on normal phase silica gel (solvent A: hexane, solvent B: EE, 90% A) to obtain compound **16** as a yellow solid (0.21 g, 890 μmol , 45%).

Analytical data match reported literature values.³⁵⁶

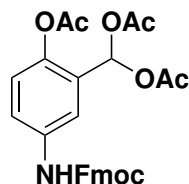
$R_f = 0.5$ (hexane:EE / 4:1).

$^1\text{H-NMR}$ (400 MHz, CDCl_3): $\delta = 10.76$ (s, 1H), 9.86 (d, $J = 0.5$ Hz, 1H), 7.83 (s, 1H), 7.29 (dd, $J = 8.9, 2.7$ Hz, 1H), 6.92 (d, $J = 8.9$ Hz, 1H), 6.45 (s, 1H), 1.52 (s, 6H).

$^{13}\text{C-NMR}$ (100 MHz, CDCl_3): $\delta = 196.5, 156.7, 153.0, 130.8, 128.4, 123.3, 120.2, 118.0, 80.9, 28.3$.

MS (ESI+, MeCN/MeOH): $m/z = 244.11$ $[\text{M}+\text{Li}]^+$.

(5-(((9H-Fluoren-9-yl)methoxy)carbonyl)amino)-2-acetoxy-phenyl)methylene diacetate (15)



Compound **25** (2 g, 7.1 mmol, 1 eq.) and Fmoc-Cl (2.2 g, 8.5 mmol, 1.2 eq.) were suspended in 10 mL water and stirred for 2 h at 60°C. The reaction mixture was extracted three times with 60 mL EE and the combined organic phases were dried over Na₂SO₄. The solvent was removed *in vacuo* and the residue was purified by flash chromatography on normal phase silica gel (solvent A: hexane, solvent B: EE, 80% A) to obtain compound **15** as transparent crystals (1.45 g, 2.9 mmol, 41%).

$R_f = 0.1$ (hexane:EE / 4:1).

¹H-NMR (500 MHz, CDCl₃): δ = 7.85 (s, 1H), 7.79 (d, J = 7.6 Hz, 2H), 7.68 (s, 1H), 7.61 (d, J = 7.5 Hz, 2H), 7.44–7.40 (m, 3H), 7.33 (t, J = 7.4 Hz, 2H), 7.05 (d, J = 8.8 Hz, 1H), 6.74 (s, 1H), 4.56 (d, J = 6.6 Hz, 2H), 4.28 (t, J = 6.5 Hz, 1H), 2.32 (s, 3H), 2.09 (s, 6H).

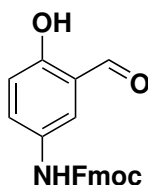
¹³C-NMR (125 MHz, CDCl₃): δ = 169.5, 168.4, 153.2, 143.8, 143.6, 141.4, 135.8, 128.4, 127.8, 127.2, 124.9, 123.8, 120.8, 120.1, 117.8, 85.0, 67.0, 47.1, 20.8, 20.7.

IR (ATR): ν = 3399m, 1749s, 1727s, 1543m, 1195s cm⁻¹.

UV/Vis (c = 0.05 μ mol mL⁻¹ /MeOH): $\lambda_{max,1}$ = 300, $\lambda_{max,2}$ = 289, $\lambda_{max,1}$ = 264, $\lambda_{max,1}$ = 244 nm.

HR-MS (ESI+, MeCN/MeOH): calcd. for [C₂₈H₂₅NO₈+Li]⁺ = 510.172 found: 510.173.

(9H-Fluoren-9-yl)methyl (3-formyl-4-hydroxyphenyl)carbamate (17)



Compound **15** (0.1 g, 0.2 mmol, 1 eq.) was dissolved in 20 mL ethanol and 1 mL of concentrated H₂SO₄ was added. The solution was refluxed for 2 h and neutralized with a saturated aqueous NaHCO₃-solution. The solution was extracted three times with 50 mL Et₂O and the combined organic phases were dried over Na₂SO₄. The solvent is removed *in vacuo* and compound **17** was obtained as beige crystals (52 mg, 0.14 mmol, 73%).

$R_f = 0.3$ (hexane:EE / 4:1).

¹H-NMR (400 MHz, THF-*d*₈): δ = 10.58 (s, 1H), 9.91 (s, 1H), 8.82 (s, 1H), 7.88 (s, 1H), 7.79 (d, *J* = 7.5 Hz, 2H), 7.67 (d, *J* = 7.3 Hz, 2H), 7.51 (s, 1H), 7.36 (t, *J* = 7.5 Hz, 2H), 7.28 (t, *J* = 7.4 Hz, 2H), 6.85 (d, *J* = 8.9 Hz, 1H), 4.55 (d, *J* = 6.3 Hz, 2H), 4.26 (t, *J* = 6.2 Hz, 1H).

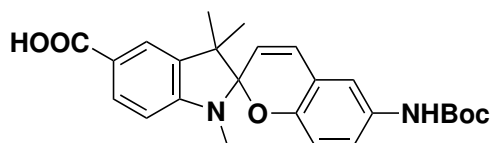
¹³C-NMR (100 MHz, THF-*d*₈): δ = 196.8, 157.8, 154.3, 145.0, 142.2, 132.8, 128.2, 128.0, 127.6, 125.5, 122.6, 121.5, 120.5, 118.0, 66.6, 48.1.

IR (ATR): ν = 3294m, 1693s, 1658s, 1243s, 732m cm⁻¹.

UV/Vis (c = 0.07 μ mol mL⁻¹ /MeOH): $\lambda_{max,1}$ = 356, $\lambda_{max,2}$ = 256, $\lambda_{max,3}$ = 235 nm.

HR-MS (ESI+, MeCN/MeOH): calcd. for [C₂₂H₁₇NO₄+H]⁺ = 360.123 found: 360.123.

6-((*tert*-Butoxycarbonyl)amino)-1',3',3'-trimethylspiro-[chromene-2,2'-indoline]-5'-carboxylic acid (18**)**



Compound **16** (0.5 g, 2.1 mmol, 0.5 eq.) and compound **12** (1.45 g, 4.2 mmol, 2 eq.) were dissolved in 100 mL ethanol under a nitrogen atmosphere and TEA (1.2 mL, 8.4 mmol, 4 eq.) was added slowly. The solution was refluxed for 3 h and neutralized with an aqueous 1M HCl-solution. The solution was extracted three times with 60 mL EE and the combined organic phases were dried over Na₂SO₄. The solvent was removed *in vacuo* and the residue was purified by flash chromatography on normal phase silica gel (solvent A: hexane, solvent B: EE, 20% A) to obtain compound **18** as a pink solid (0.3 g, 0.7 mmol, 33%).

R_f = 0.4 (DCM:MeOH / 19:1).

¹H-NMR (400 MHz, DMSO-*d*₆): δ = 12.25 (s, 1H), 9.10 (s, 1H), 7.76 (dd, *J* = 8.2, 1.7 Hz, 1H), 7.61 (d, *J* = 1.6 Hz, 1H), 7.34 (s, 1H), 7.04 (dd, *J* = 8.8, 2.4 Hz, 1H), 6.98 (d, *J* = 10.2 Hz, 1H), 6.59 (dd, *J* = 8.4, 6.2 Hz, 2H), 5.75 (d, *J* = 10.2 Hz, 1H), 2.70 (s, 3H), 1.44 (s, 3H), 1.21 (s, 3H), 1.08 (s, 3H).

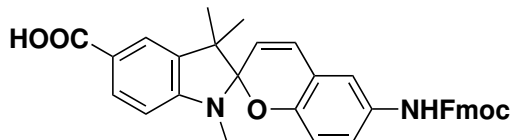
¹³C-NMR (100 MHz, DMSO-*d*₆): δ = 167.9, 153.4, 153.0, 149.3, 136.8, 132.9, 131.1, 130.2, 123.3, 121.4, 120.9, 119.5, 118.6, 117.4, 114.8, 106.3, 104.0, 79.2, 51.3, 28.9, 28.6, 26.0, 20.2.

IR (ATR): ν = 3448w, 2980m, 1724s, 1667s, 1155s cm⁻¹.

UV/Vis (c = 0.03 μ mol mL⁻¹ /MeOH): $\lambda_{max,1}$ = 301, $\lambda_{max,2}$ = 233 nm.

HR-MS (ESI+, MeCN/MeOH): calcd. for [C₂₅H₂₈N₂O₅+Li]⁺ = 443.214 found: 443.212.

6-(((9*H*-Fluoren-9-yl)methoxy)carbonyl)amino)-1',3',3'-tri-methylspiro[chromene-2,2'-indoline]-5'-carboxylic acid (19**)**



Compound **17** (0.3 g, 0.83 mmol, 1 eq.) and compound **12** (0.58 g, 1.7 mmol, 2 eq.) were dissolved in 100 mL ethanol under a nitrogen atmosphere and TEA (0.47 mL, 3.3 mmol, 4 eq.) was added slowly. The solution was refluxed for 3 h and neutralized with an aqueous 1M HCl-solution. The solution was extracted three times with 60 mL EE and the combined organic phases were dried over Na₂SO₄. The solvent was removed *in vacuo* and the residue was purified by flash chromatography on normal phase silica gel (solvent A: DCM, solvent B: MeOH, 98% A) to obtain compound **19** as a red solid (0.12 g, 0.21 mmol, 26%).

$R_f = 0.4$ (DCM:MeOH / 19:1).

¹H-NMR (400 MHz, THF-*d*₈): δ = 12.83 (s, 1H), 8.64 (s, 1H), 7.87 (dd, J = 8.2, 1.7 Hz, 1H), 7.80 (d, J = 7.6 Hz, 2H), 7.72 (d, J = 1.7 Hz, 1H), 7.68 (d, J = 7.4 Hz, 2H), 7.43–7.34 (m, 3H), 7.29 (td, J = 7.4, 1.2 Hz, 2H), 7.10 (d, J = 8.7 Hz, 1H), 6.93 (d, J = 10.2 Hz, 1H), 6.58 (d, J = 8.7 Hz, 1H), 6.53 (d, J = 8.2 Hz, 1H), 5.76 (d, J = 10.2 Hz, 1H), 4.51 (d, J = 6.5 Hz, 2H), 4.27 (t, J = 6.4 Hz, 1H), 2.77 (s, 3H), 1.33 (s, 3H), 1.17 (s, 3H).

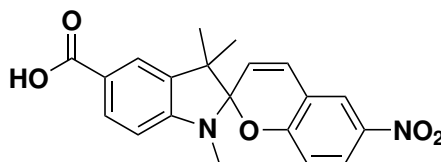
¹³C-NMR (100 MHz, THF-*d*₈): δ = 167.6, 154.2, 152.6, 150.5, 145.1, 142.2, 137.3, 133.3, 131.6, 130.6, 128.1, 127.6, 125.6, 123.8, 122.3, 120.5, 119.8, 119.4, 117.5, 115.3, 106.2, 104.8, 66.5, 51.8, 48.2, 28.7, 25.9, 20.3.

IR (ATR): ν = 3303br, 3064br, 1671s, 1583s, 1199s cm⁻¹.

UV/Vis (c = 0.09 μ mol mL⁻¹ /MeOH): $\lambda_{max,1}$ = 474, $\lambda_{max,2}$ = 378, $\lambda_{max,3}$ = 299, $\lambda_{max,4}$ = 264 nm.

HR-MS (ESI+, MeCN/MeOH): calcd. for [C₃₅H₃₀N₂O₅+H]⁺ = 559.222 found: 559.220.

1',3',3'-Trimethyl-6-nitrospiro[chromene-2,2'-indoline]-5'-carboxylic acid (26**)**



Compound **12** (1 g, 2.9 mmol, 1 eq.), 2-hydroxy-5-nitrobenzaldehyde **13** (0.54 g, 3.2 mmol, 1 eq.), TEA (1.1 mL, 7.6 mmol, 1 eq.) were dissolved in 100 mL ethanol under a nitrogen atmosphere. The solution was refluxed for 3 h and quenched with 100 mL aqueous 0.1 M HCl-solution. The formed precipitate is

separated and solved in aqueous 10% KOH-solution. The solution is washed with DCM and the pH value is adjusted to 4. The formed precipitate is recrystallized in ethanol and compound **26** is obtained as a brownish solid (0.05 g, 0.14 mmol, 5%).

Analytical data match reported literature values.³⁵⁷

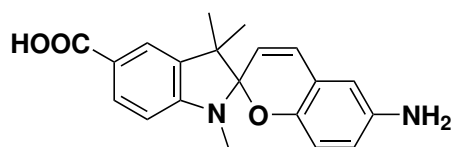
$R_f = 0.3$ (hexane:EE + 0.1% FA / 4:1).

¹H-NMR (500 MHz, CDCl₃): $\delta = 12.35$ (s, 1H), 8.24 (d, $J = 2.8$ Hz, 1H), 8.02 (dd, $J = 9.0, 2.8$ Hz, 1H), 7.82 (dd, $J = 8.2, 1.7$ Hz, 1H), 7.68 (d, $J = 1.7$ Hz, 1H), 7.26 (d, $J = 10.4$ Hz, 1H), 6.92 (d, $J = 9.0$ Hz, 1H), 6.70 (d, $J = 8.3$ Hz, 1H), 6.02 (d, $J = 10.4$ Hz, 1H), 2.76 (s, 3H,), 1.25 (s, 3H), 1.14 (s, 3H).

¹³C-NMR (125 MHz, CDCl₃): $\delta = 167.8, 159.4, 151.7, 141.2, 136.3, 131.3, 128.9, 126.3, 123.4, 123.3, 122.0, 121.3, 119.2, 115.9, 106.8, 106.4, 52.0, 28.8, 25.9, 20.0$.

MS (ESI+, MeCN/MeOH): $m/z = 373.136$ [M+Li]⁺

6-Amino-1',3',3'-trimethylspiro[chromene-2,2'-indoline]-5'-carboxylic acid (**27**)



Compound **18** (0.2 g, 0.46 mmol, 1 eq.) was dissolved in 10 mL of a 1:1 mixture of trifluoroacetic acid and DCM and stirred ambient temperature for an hour. The solvent is removed *in vacuo* and the residue is purified by flash chromatography on normal phase silica gel (solvent A: DCM, solvent B: MeOH, 96% A) to obtain compound **27** as a red solid (0.14 g, 0.42 mmol, 91%).

Analytical data match reported literature values.³⁵⁸

$R_f = 0.3$ (DCM:MeOH / 19:1).

¹H-NMR (500 MHz, CDCl₃): $\delta = 10.09$ (s, 1H), 7.78 (dd, $J = 8.2, 1.7$ Hz, 1H), 7.62 (d, $J = 1.7$ Hz, 1H), 7.10–7.04 (m, 2H), 6.97 (dd, $J = 8.6, 2.6$ Hz, 1H), 6.74 (d, $J = 8.6$ Hz, 1H), 6.62 (d, $J = 8.2$ Hz, 1H), 5.85 (d, $J = 10.3$ Hz, 1H), 2.71 (s, 3H), 1.21 (s, 3H), 1.09 (s, 3H).

¹³C-NMR (125 MHz, CDCl₃): $\delta = 167.9, 152.2, 151.9, 136.6, 131.2, 129.4, 123.5, 123.3, 121.6, 120.6, 120.4, 119.6, 115.9, 106.5, 104.6, 51.6, 28.9, 25.9, 20.2$.

IR (ATR): $\nu = 2889\text{m}, 2641\text{br}, 1658\text{s}, 1605\text{s}, 1182\text{s cm}^{-1}$.

UV/Vis ($c = 0.15 \mu\text{mol mL}^{-1}$ /MeOH): $\lambda_{\text{max},1} = 407, \lambda_{\text{max},2} = 300 \text{ nm}$.

HR-MS (ESI+, MeCN/MeOH): calcd. for [C₂₀H₂₁N₂O₃+H]⁺ = 337.154 found: 337.153.

6.3 Solid-phase peptide synthesis

The Fmoc-protected photoswitches 3,4'-AMPB **4**, FASC **10**, and SAFC **19** were incorporated into the PTH₂₅₋₃₇ sequence *via* SPPS. Therefore, an automated peptide synthesizer (MultiPep RS; Invatis AG, Cologne, Germany) was used together with standard Fmoc-chemistry and preloaded resins (210 µmol/g resin). Coupling of protected natural amino acids was performed as single couplings in DMF using 5 eq. of the respective amino acid, 5 eq. of HCTU as coupling reagent, and 10 eq. of *N*-methylmorpholine as base for 1 h at ambient temperature.

For the coupling of the special Fmoc-building blocks **4**, **10**, and **19**, 3 eq. of the respective building block, 3 eq. *N,N'*-diisopropylcarbodiimide, and 3 eq. hydroxybenzotriazole were used in a mixture of DMF and *N*-methyl-2-pyrrolidone (50:50 vol.-%) at ambient temperature gently shaken in the absence of light overnight.

The N-terminal Fmoc-protecting group was removed by washing the resin with 20% piperidine in DMF. The final side chain deprotection and cleavage from the resin occurred in a mixture of trifluoroacetic acid and water (90:10 vol.-%) with gentle agitation for 2 h at ambient temperature.

The crude peptides were further processed to a purity of >95% using preparative RP-HPLC (Gilson, Limburg, Germany). For both, analytical and preparative purification, the mobile phase was a mixture of water (eluent A) and acetonitrile (eluent B), each containing 0.1% trifluoroacetic acid. The crude peptides were purified using a linear gradient from 95% A to 15% A in 15 min for analytical separation and 90 min for preparative runs. The column was a semi.preparative PLRP-S column (Agilent Technologies, 300x25 mm, 8 µm).

All peptides were characterized by analytical HPLC (Dionex Ultimate 3000; Thermo Scientific, Germany) using a PLRP-S column (Agilent Technologies, 150x4.6mm, 3µm), MALDI-Tof-MS (Bruker Microflex LT; Bruker, Bremen, Germany), and ¹H-NMR.

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Rupali, J. B.; Thomas, S.; Sen, N.; Paschold, A.; Binder, W. H.; Kumas, S., Bioinspired synthetic polymers-based inhibitors of Alzheimer's amyloid- β peptide aggregation, *Polym. Chem.* **2023**, *23*, 392–411 *Polym Chem*, *14* (2023), pp. 392-411, DOI: <https://doi.org/10.1039/D2PY01217K>

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Paschold, A.; Starke, N.; Rothmund, S.; Binder, W. H., Spiropyran as building block in peptide synthesis and modulation of photochromic properties *Organic Letters* **2024**, *26* (49), 10542–10547, DOI: <https://doi.org/10.1021/acs.orglett.4c03929>.

Konferenzbeiträge

Paschold, A.; Voigt, B.; Rothmund, S.; Kohlmann, T.; Balbach, J.; Binder, W. H., Switchable PTH_{25–37} fibrillation via photoisomerization of an azobenzene motif, *Bordeaux Polymer Conference 2022*, Poster.

Appendix

Supporting Information to: Modulating the Fibrillization of Parathyroid-Hormone (PTH) Peptides: Azo-Switches as Reversible and Catalytic Entities, *Biomedecines* **2022**, 10 (7), 1512,
DOI: <https://doi.org/10.3390/biomedicines10071512>.

Supporting Information to: Photocontrolled Reversible Amyloid Fibril Formation of Parathyroid Hormone-Derived Peptides, *Bioconjugate Chemistry* **2024**, 35 (7), 981-995,
DOI: <https://doi.org/10.1021/acs.bioconjchem.4c00188>.

Supporting Information to: Spiropyran as building block in peptide synthesis and modulation of photochromic properties, *Organic Letters* **2024**, 26 (49), 10542-10547,
DOI: <https://doi.org/10.1021/acs.orglett.4c03929>.

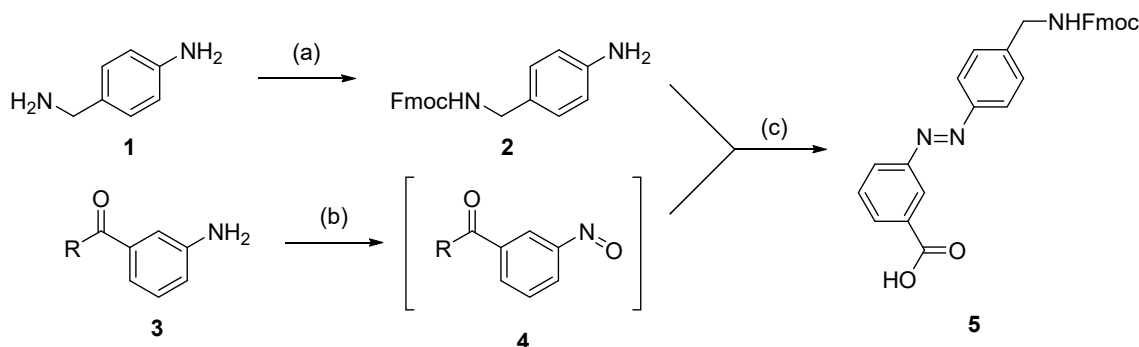
Modulating the fibrillization of parathyroid-hormone (PTH) peptides: azo-switches as reversible and catalytic entities.

Authors: André Paschold, Bruno Voigt, Gerd Hause, Tim Kohlmann, Sven Rothemund, and Wolfgang H. Binder

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1. Synthesis of Fmoc-3,4'-AMPB



Scheme S1: Synthesis of Fmoc-protected 3,4'-AMPB **7**. (a) Fmoc-ONSu, triethylamin, DMF/MeCN, 16 h, room temperature. (b) Oxone[®], DCM, water, 3 h, room temperature. (c) AcOH, DMSO, N₂, 72 h, room temperature.

1.1. Synthesis of (9H-Fluoren-9-yl)methyl (4-aminobenzyl)carbamate **2**

4-Aminobenzylamine (1.85 mL, 16.4 mmol, 1 equiv.) was dissolved in a mixture of 12 mL THF and 1.5 mL aqueous 5% NaHCO₃ solution. A suspension of Fmoc-*N*-hydroxysuccinimide (Fmos-OSu) (5.52 g, 16.4 mmol, 1 equiv.) in 20 mL THF was added slowly and the reaction mixture was stirred for 18 h at ambient temperature. 500 mL of water were added and the suspension was filtrated. The precipitate was dissolved in THF and dried over Na₂SO₄. The solvent was removed *in vacuo* and **2** was obtained as a white solid (5.45 g, 15.3 mmol, 96% yield).

¹H-NMR (400 MHz, DMSO-*d*₆) δ = 7.86 (d, J = 7.6 Hz, 2H, C_{ar}H), 7.82 (d, J = 7.6 Hz, 2H, C_{ar}H), 7.40 (td, J = 7.5 Hz, 1.2 Hz, 1H, C_{ar}H), 7.32 (td, J = 7.5 Hz, 1.2 Hz, 1H, C_{ar}H), 6.97 (t, J = 6.3 Hz, 1H, NH), 6.88 (d, J = 8.2 Hz, 2H, C_{ar}H), 6.48 (d, J = 8.2 Hz, 2H, C_{ar}H), 6.26 (s, 2H, CH₂), 4.88 (s, 2H, NH₂), 3.93 (d, J = 5.9 Hz, 1H, CH₂).

¹³C-NMR (100 MHz, DMSO-*d*₆) δ = 157.8 (C(O)N), 147.8 (C_{ar}N), 143.0 (C_{ar}), 139.8 (C_{ar}), 137.8 (C_{ar}), 141.2 (C_{ar}), 129.4 (C_{ar}H), 128.4 (C_{ar}H), 127.7 (C_{ar}), 121.1 (C_{ar}H), 120.5 (C_{ar}H), 114.1 (C_{ar}H), 110.1 (CH₂), 43.9 (CH₂).

MS (ESI⁺): m/z = 383.11 [M+K]⁺.

Analytical data match reported literature values.^[1]

1.2. Synthesis of Fmoc-3,4'-AMPB **5**

To a suspenison of 3-aminobenzoic acid (2.00 g, 14.6 mmol, 2 equiv.) in 36 mL DCM is a solution of Oxone[®] (8.80 g, 29.17 mmol, 4 equiv.) added dropwise. The reaction mixture is stirred at ambient temperature for 4 h. DCM is removed *in vacuo* and the formed precipitate is filtrated and washed with 100 mL water. The residue is dissolved in 175 mL DMSO and 175 mL of acetic acid are added. The solution is degassed for 30 min with N₂. (9H-Fluoren-9-yl)methyl (4-aminobenzyl)carbamate **2** (2.51 g, 7.29 mmol, 1 equiv.) is added and the mixture is stirred for 72 h at ambient temperature. 100 mL water

and 400 mL EtOAc were added and the phases were separated. The aqueous phase is washed two times with 400 mL EtOAc and the combined organic phases were washed two times with 200 mL of a saturated aqueous NaCl solution. The organic phase was dried over Na₂SO₄ and the solvent was removed *in vacuo*. The residue was purified by flash chromatography on normal phase silica gel (solvent: EtOAc + 0.1% FA) to obtain **5** as a mixture of the *E*- and the *Z*-isomer (1.51 g, 3.17 mmol, 43.5% yield).

¹H-NMR (400 MHz, DMSO-*d*₆) *E*:*Z*-ratio = 29:11, *E*-isomer: δ = 13.27 (s, 1H, C(O)OH), 8.37 (t, J = 1.9 Hz, 1H, C_{ar}H), 8.15 – 8.08 (m, 2H, C_{ar}H), 7.93 (t, J = 6.2 Hz, 1H, C_{ar}H), 7.90 – 7.81 (m, 4H, C_{ar}H), 7.76 – 7.66 (m, 3H, C_{ar}H, NH₂), 7.46 – 7.48 (m, 6H, C_{ar}H), 4.38 (d, J = 6.7 Hz, 2H, CH₂), 4.28 (d, J = 6.0 Hz, 2H, CH₂), 4.24 (t, J = 6.8 Hz, 1H, CH), *Z*-isomer: 7.86 (d, J = 7.6 Hz, 2H, C_{ar}H), 7.79 – 7.69 (m, 2H, C_{ar}H), 7.66 (d, J = 7.5 Hz, 2H, C_{ar}H), 7.46 – 7.35 (m, 4H, C_{ar}H, C(O)NH), 7.28 (t, J = 7.5 Hz, 2H, C_{ar}H), 7.08 (d, J = 8.0 Hz, 2H, C_{ar}H), 6.99 (d, J = 7.8 Hz, 1H, C_{ar}H), 6.81 (d, J = 8.2 Hz, 2H, C_{ar}H), 4.36 (d, J = 6.6 Hz, 2H, CH₂), 4.20 (t, J = 6.7 Hz, 1H, CH), 4.09 (d, J = 6.2 Hz, 2H, CH₂).

¹³C-NMR (100 MHz, DMSO-*d*₆) *E*-isomer: δ = 167.2 (C(O)OH), 156.9 (C(O)N), 152.4 (C_{ar}N), 151.3 (C_{ar}N), 144.5 (C_{ar}), 144.3 (C_{ar}), 141.2 (C_{ar}), 132.7 (C_{ar}H), 132.2 (C_{ar}H), 130.4 (C_{ar}), 128.4 (C_{ar}H), 128.0 (C_{ar}H), 127.8 (C_{ar}H), 127.5 (C_{ar}H), 125.6 (C_{ar}H), 123.7 (C_{ar}H), 122.6 (C_{ar}H), 120.5 (C_{ar}H), 65.8 (CH₂), 47.3 (CH), 44.0 (CH₂). *Z*-isomer: δ = 167.1 (C(O)OH), 156.8 (C(O)N), 154.3 (C_{ar}N), 152.3 (C_{ar}N), 144.3 (C_{ar}), 143.8 (C_{ar}), 141.2 (C_{ar}), 133.3 (C_{ar}), 132.2 (C_{ar}H), 130.4 (C_{ar}H), 128.2 (C_{ar}H), 128.0 (C_{ar}H), 127.7 (C_{ar}H), 127.5 (C_{ar}H), 125.6 (C_{ar}H), 123.1 (C_{ar}H), 122.8 (C_{ar}H), 120.5 (C_{ar}H), 65.6 (CH₂), 47.3 (CH), 43.5 (CH₂).

UV-Vis *E*-isomer: λ_{max} = 291 nm, 301 nm, 330 nm, 441 nm; *Z*-isomer: λ_{max} = 290 nm, 301 nm, 434 nm; isobestic point (*E*:*Z*): λ_{iso} = 386 nm.

MS (ESI⁺): m/z = 516.13 [M+K]⁺.

Analytical data match reported literature values.^[2]

2. Peptide Characterisation

2.1. Peptide sequences

Table S1: Primary sequence and solubility in 50 mM Na₂PO₄ buffer (pH 7.4) of peptides AzoPTH₂₅₋₃₇ and SP1-SP4.

peptide	primary sequence ^a	solubility [μM] ^b
PTH ₂₅₋₃₇	H ₂ N- ²⁵ RKKLQ ³⁰ DVHNF ³⁵ VAL-OH	> 1000
AzoPTH ₂₅₋₃₇	H ₂ N- ²⁵ RKKLQ ³⁰ D-Azo-HNF ³⁵ VAL-OH	250
SP1	H ₂ N- ²⁵ RKKLQ ³⁰ D-Azo-VHNF ³⁵ VAL-OH	60
SP2	H ₂ N- ²⁵ RKKLQ ³⁰ DV-Azo-HNF ³⁵ VAL-OH	55
SP3	H ₂ N- ²⁵ RKKLQ ³⁰ -Azo-VHNF ³⁵ VAL-OH	35
SP4	H ₂ N- ²⁵ RKKLQ ³⁰ -Azo-HNF ³⁵ VAL-OH	25

^a Azo = 3,4'-AMPB; ^b maximum solubility was determined *via* UV-Vis absorption spectroscopy ($\epsilon = 49310 \text{ M}^{-1}\text{cm}^{-1}$ at 205 nm for PTH₂₅₋₃₇, $\epsilon = 13000 \text{ M}^{-1}\text{cm}^{-1}$ at 327 nm for 3,4'-AMPB containing peptides).

2.2. HPLC- and MALDI-ToF-data

AzoPTH₂₅₋₃₇: MS (MALDI+): m/z calc. 1705.990 found 1706.254.

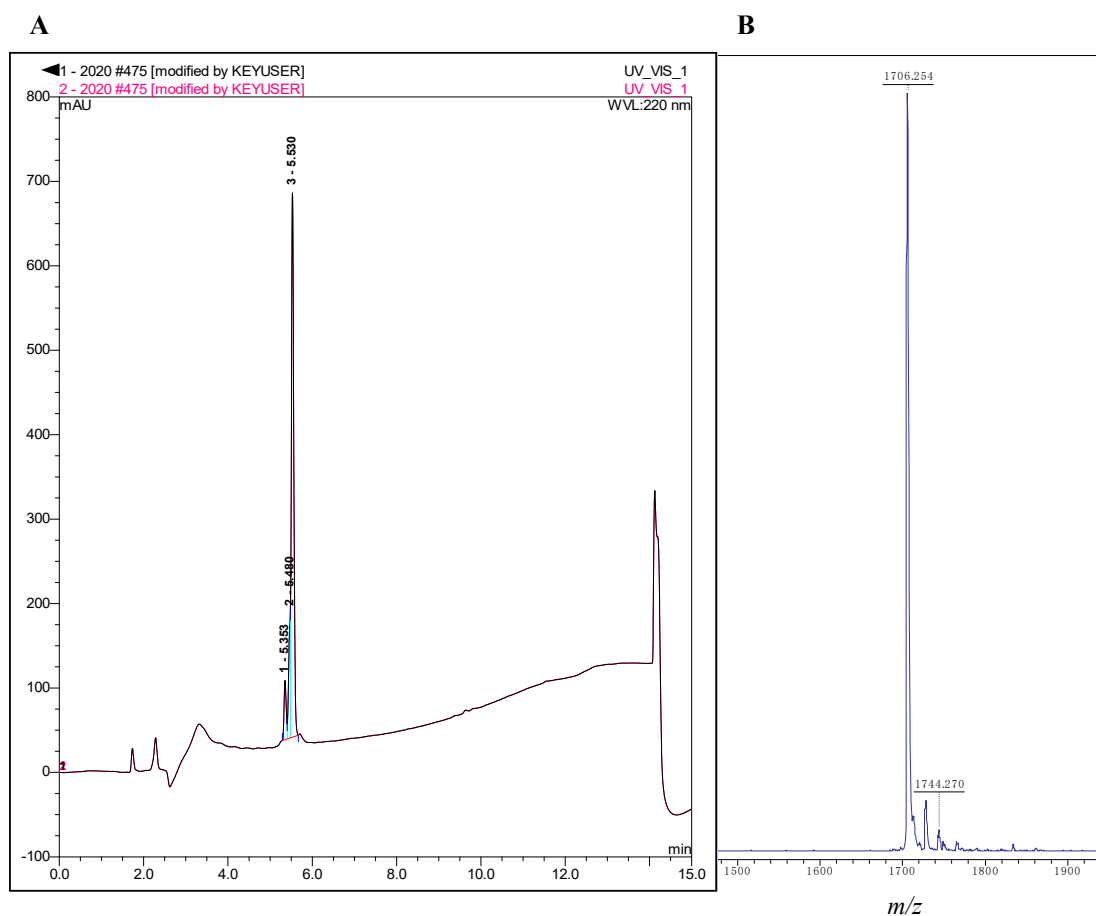


Figure S1: (A) HPLC-trace of AzoPTH₂₅₋₃₇ (*cis*-isomer at 5.353, *trans*-isomer at 5.530). (B) MALDI-spectrum of AzoPTH₂₅₋₃₇.

SP1: MS (MALDI+): m/z calc. 1805.123 found 1805.938.

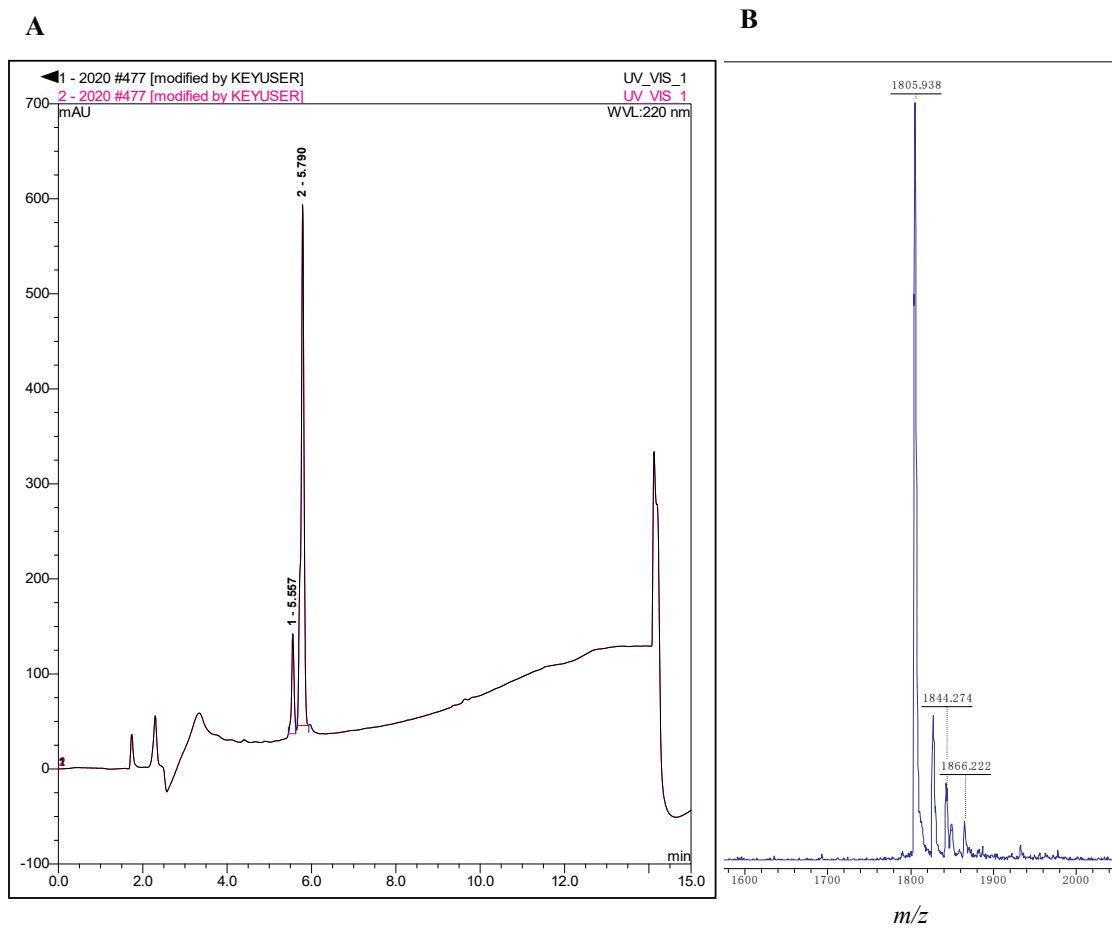


Figure S2: (A) HPLC-trace of SP1 (*cis*-isomer at 5.557, *trans*-isomer at 5.790). (B) MALDI-spectrum of SP1.

SP2: MS (MALDI+): m/z calc. 1805.123 found 1806.162.

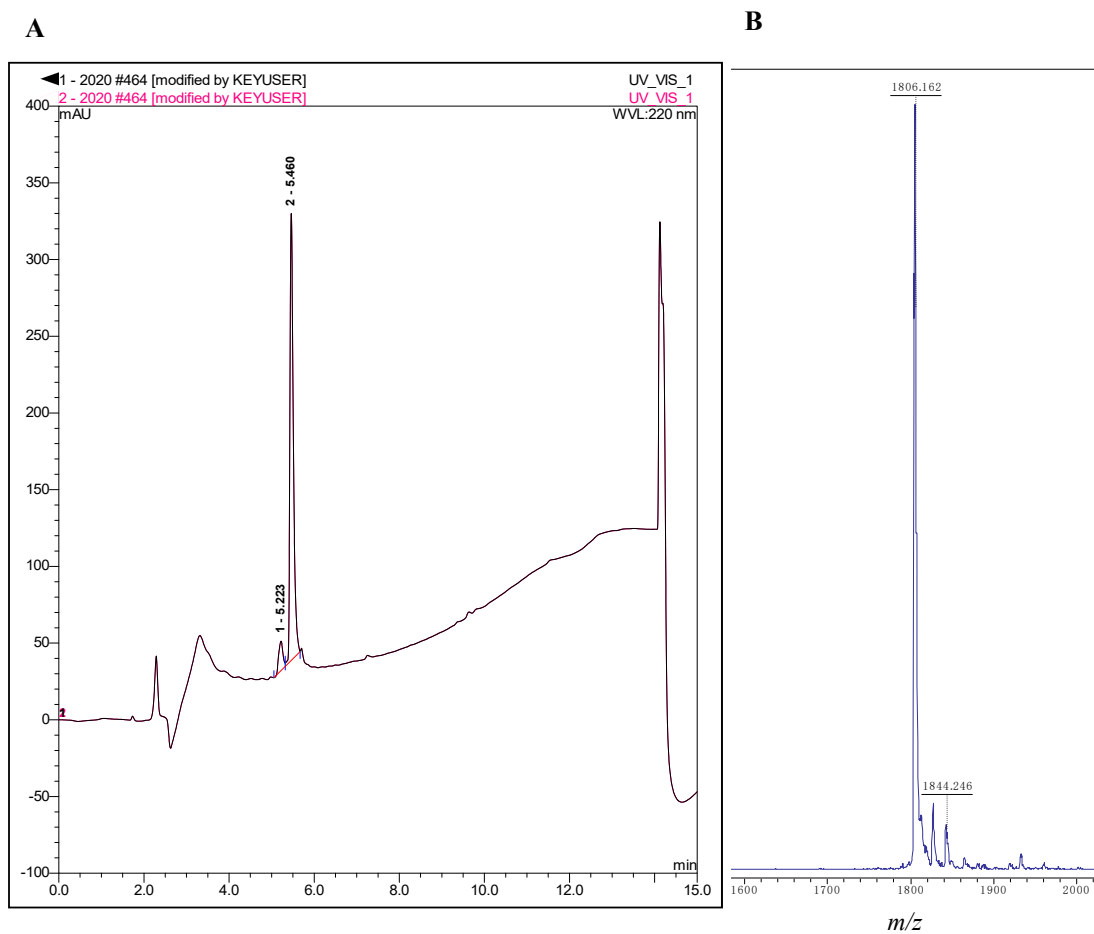


Figure S3: (A) HPLC-trace of SP2 (*cis*-isomer at 5.223, *trans*-isomer at 5.460). (B) MALDI-spectrum of SP2.

SP3: MS (MALDI+): m/z calc. 1690.035 found 1688.964.

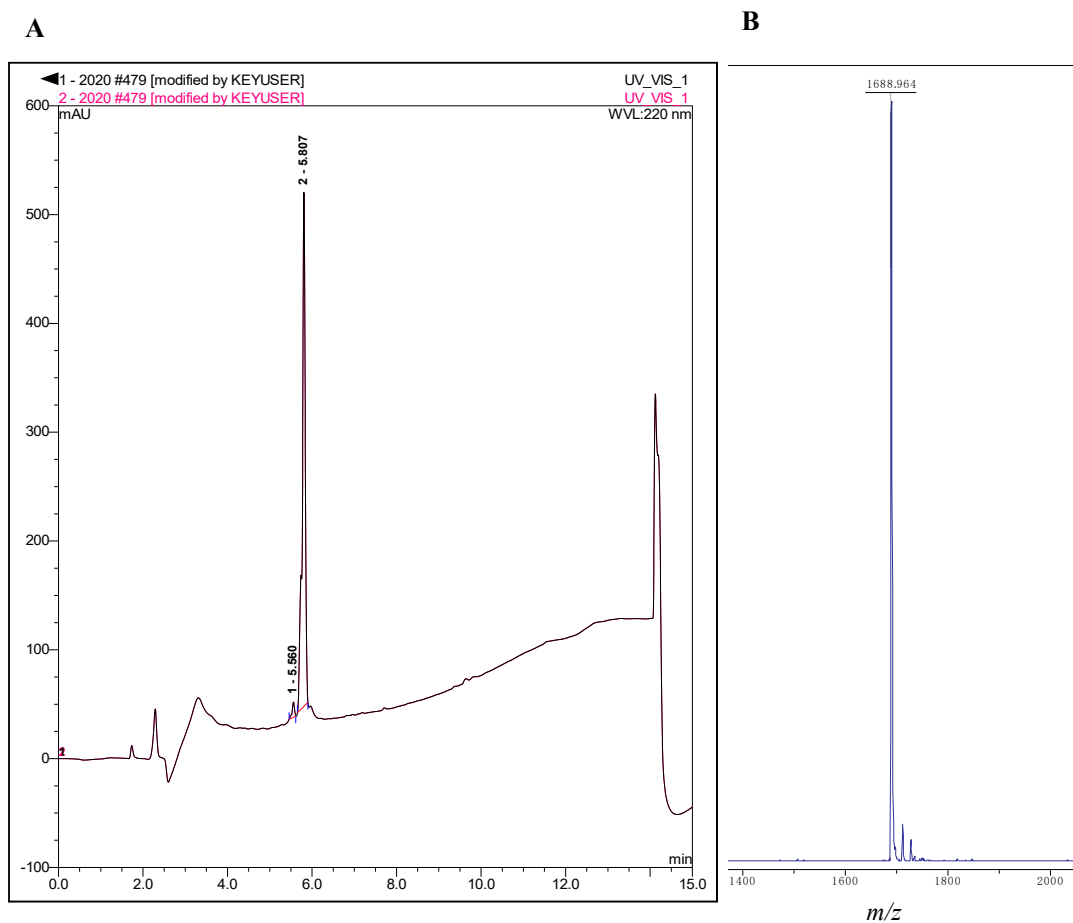


Figure S4: (A) HPLC-trace of SP3 (*cis*-isomer at 5.560, *trans*-isomer at 5.807). (B) MALDI-spectrum of SP3.

SP4: MS (MALDI+): m/z calc. 1590.902 found 1591.069).

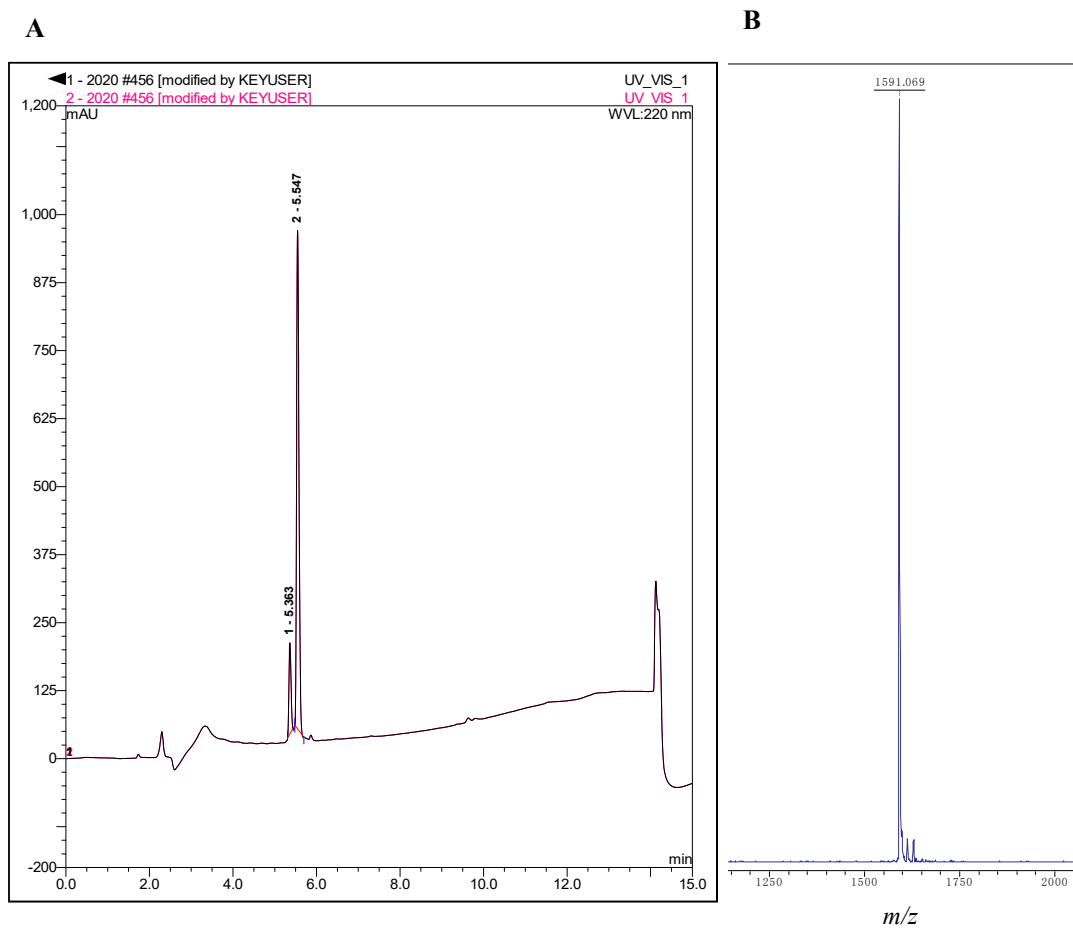


Figure S5: (A) HPLC-trace of SP4 (*cis*-isomer at 5.363, *trans*-isomer at 5.547). (B) MALDI-spectrum of SP4.

2.3. Photophysical properties of AzoPTH₂₅₋₃₇

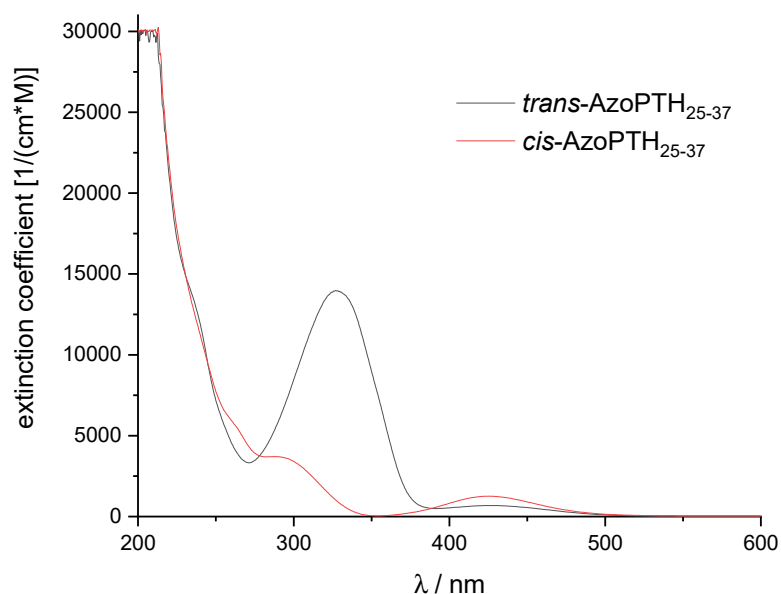


Figure S6: Separated UV/Vis-spectra of the pure isomers of AzoPTH₂₅₋₃₇; spectra were separated with Wolfram Mathematica 12.2.

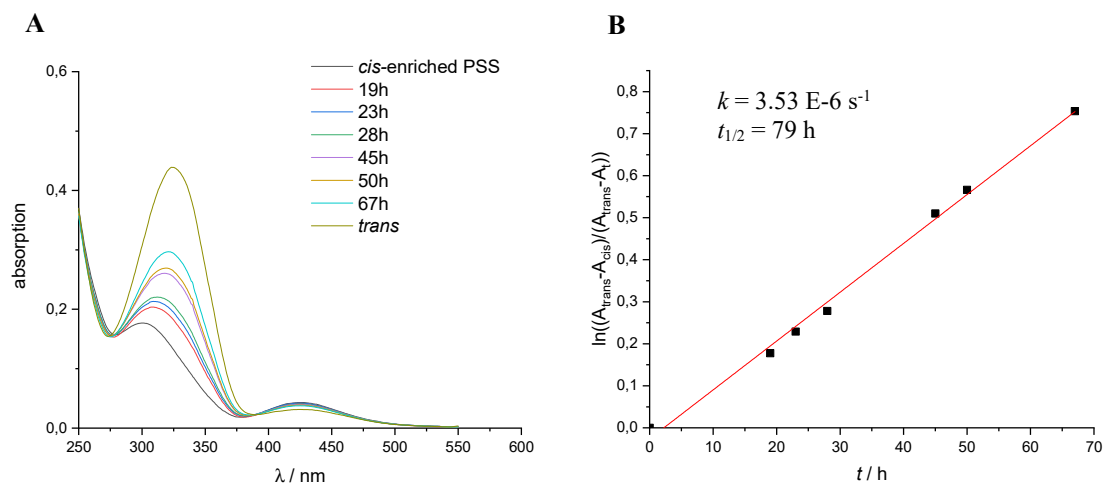


Figure S7: (A) UV/Vis-spectra of *trans*-isomer, *cis*-enriched PSS, and *cis*-enriched PSS sample after distinct time points in the dark. (B) logarithmic application of the absorption change over time to determine rate constant k and half-life time $t_{1/2}$.²

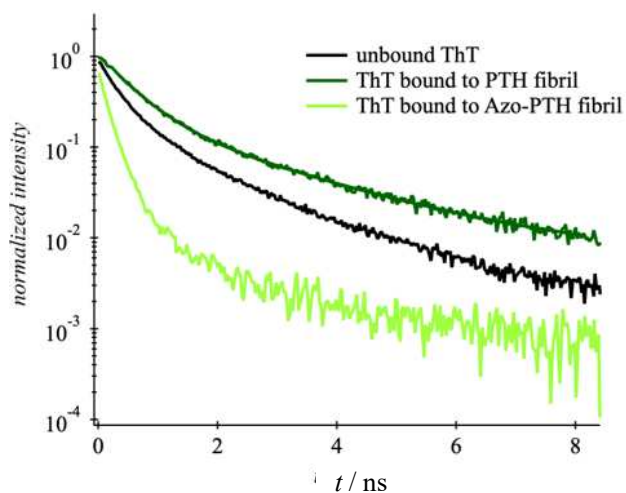


Figure S8: Time- resolved fluorescence measurement (excitation wavelength = 460 nm, emission wavelength = 480 nm) of unbound ThT (black), ThT bound to PTH₂₅₋₃₇ fibrils (dark green), ThT bound to *trans*-AzoPTH₂₅₋₃₇ fibrils (light green).

2.4. Aggregation Kinetics of PTH₂₅₋₃₇, *trans*-AzoPTH₂₅₋₃₇, *cis*-AzoPTH₂₅₋₃₇, and mixtures

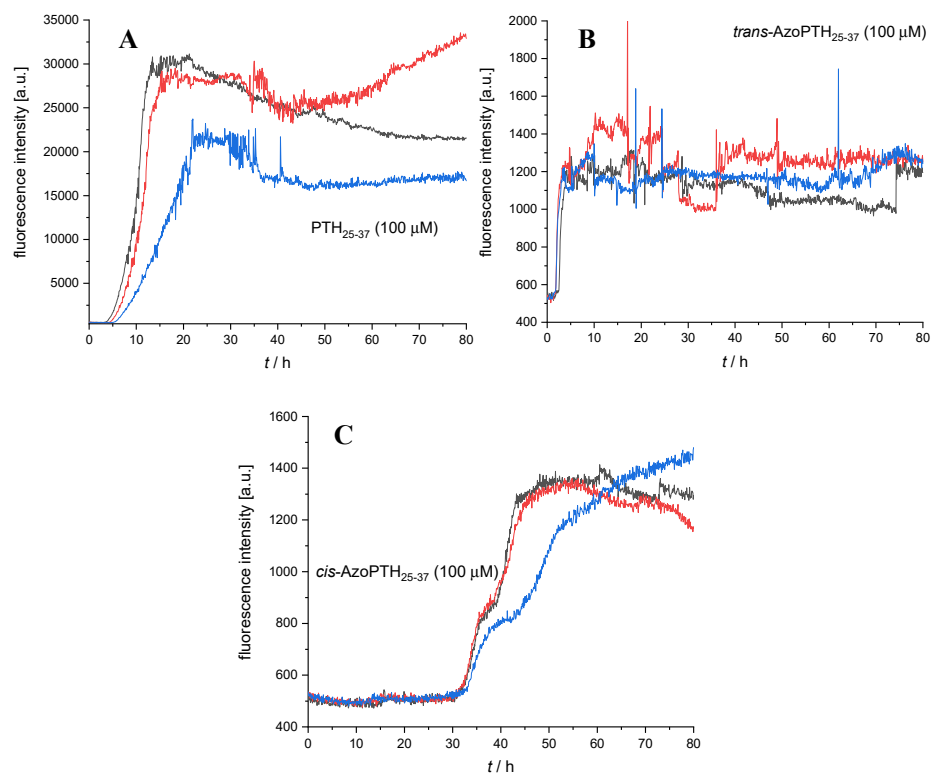


Figure S9: ThT monitored fibrillation assays ($c = 100 \mu\text{M}$, 37°C , $50 \text{ mM Na}_2\text{HPO}_4$, $\text{pH}7.4$). (A) PTH₂₅₋₃₇, (B) *trans*-AzoPTH₂₅₋₃₇, (C) *cis*-AzoPTH₂₅₋₃₇.

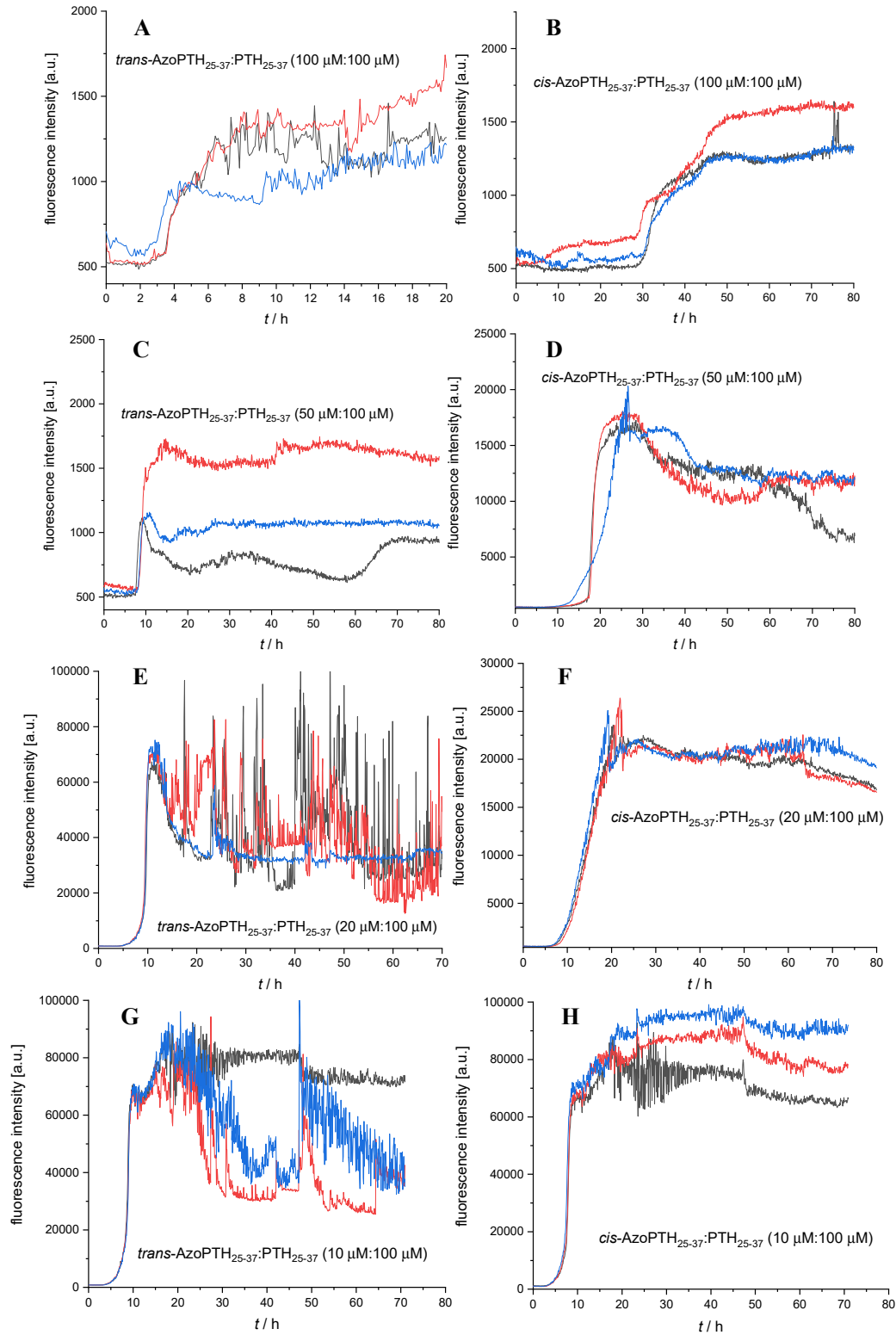


Figure S10: ThT monitored fibrillization assays of mixtures of PTH₂₅₋₃₇, *trans*-AzoPTH₂₅₋₃₇, and *cis*-AzoPTH₂₅₋₃₇ (37 °C, 50 mM Na₂HPO₄, pH7.4). (A) *trans*-AzoPTH₂₅₋₃₇:PTH₂₅₋₃₇ (100 μM:100 μM), (B) *cis*-AzoPTH₂₅₋₃₇:PTH₂₅₋₃₇ (100 μM:100 μM), (C) *trans*-AzoPTH₂₅₋₃₇:PTH₂₅₋₃₇ (50 μM:100 μM), (D) *cis*-AzoPTH₂₅₋₃₇:PTH₂₅₋₃₇ (50 μM:100 μM), (E) *trans*-AzoPTH₂₅₋₃₇:PTH₂₅₋₃₇ (20 μM:100 μM), (F) *cis*-AzoPTH₂₅₋₃₇:PTH₂₅₋₃₇ (20 μM:100 μM), (G) *trans*-AzoPTH₂₅₋₃₇:PTH₂₅₋₃₇ (10 μM:100 μM), (H) *cis*-AzoPTH₂₅₋₃₇:PTH₂₅₋₃₇ (10 μM:100 μM).

3. Analytical NMR-Spectra

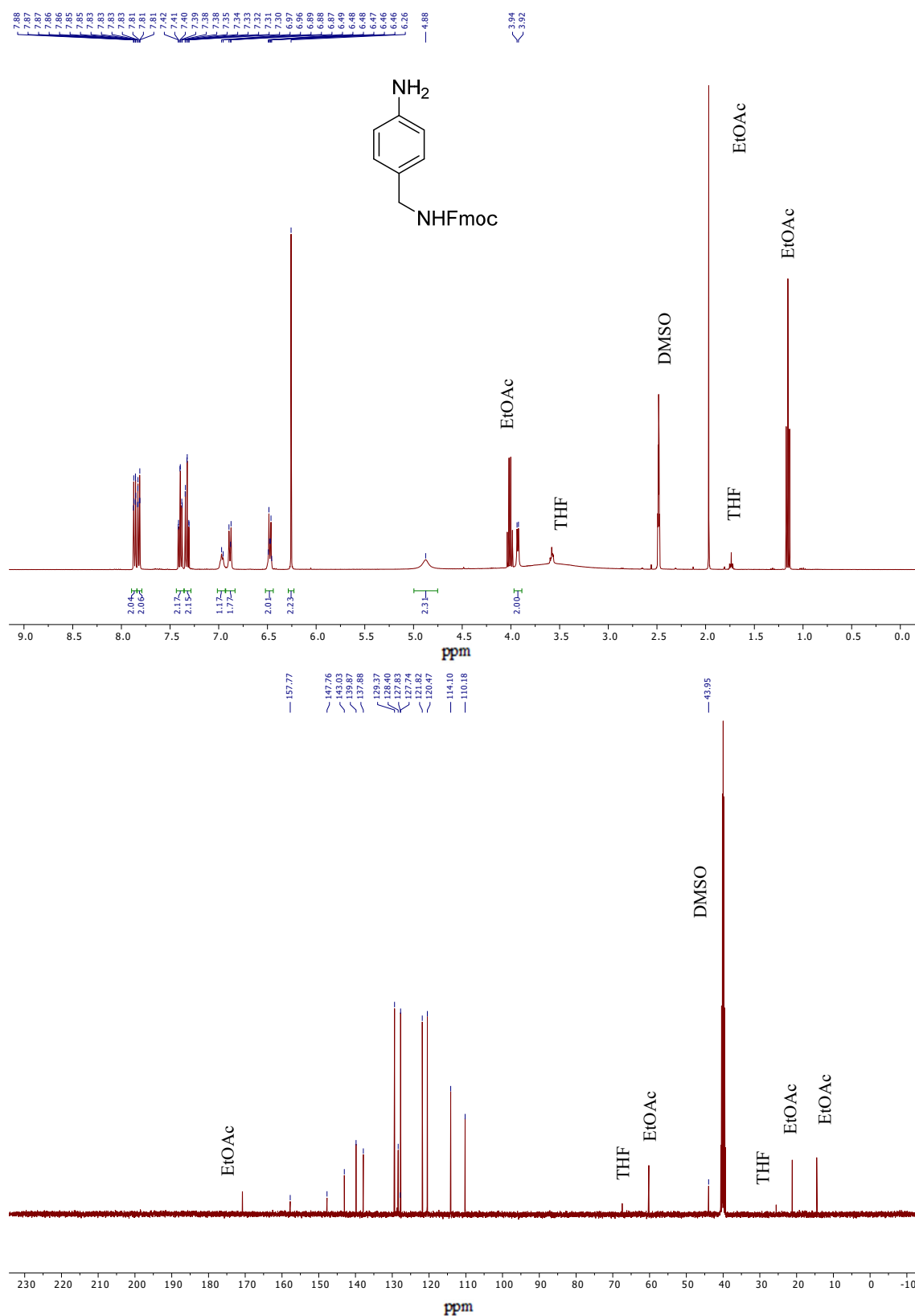


Figure S11: ¹H-NMR spectrum (top; 400 MHz, DMSO-*d*₆) and ¹³C-NMR spectrum (bottom; 100 MHz, DMSO-*d*₆) of (9H-Fluoren-9-yl)methyl (4-aminobenzyl)carbamate.

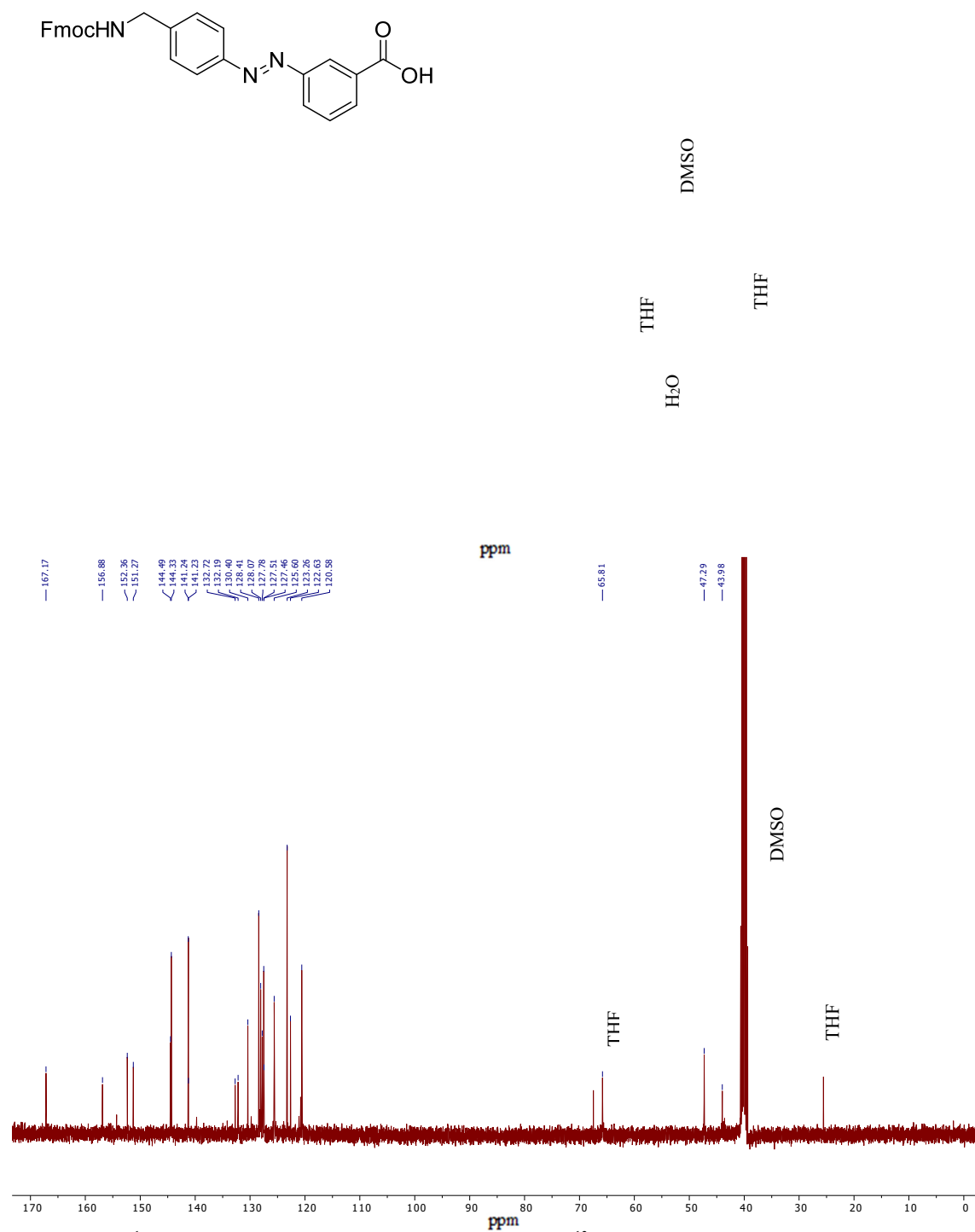


Figure S12: ¹H-NMR spectrum (top; 400 MHz, DMSO-*d*₆) and ¹³C-NMR spectrum (bottom; 100 MHz, DMSO-*d*₆) of Fmoc-3,4'-AMPB (mixture of isomers).

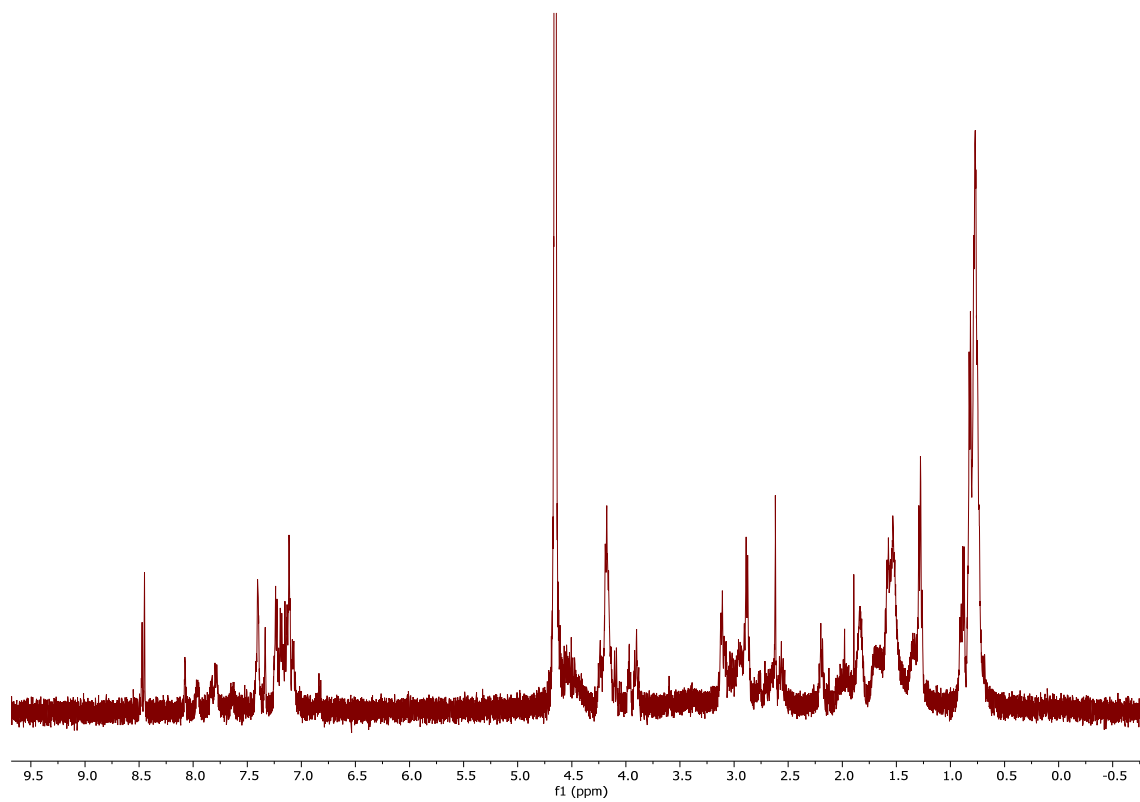
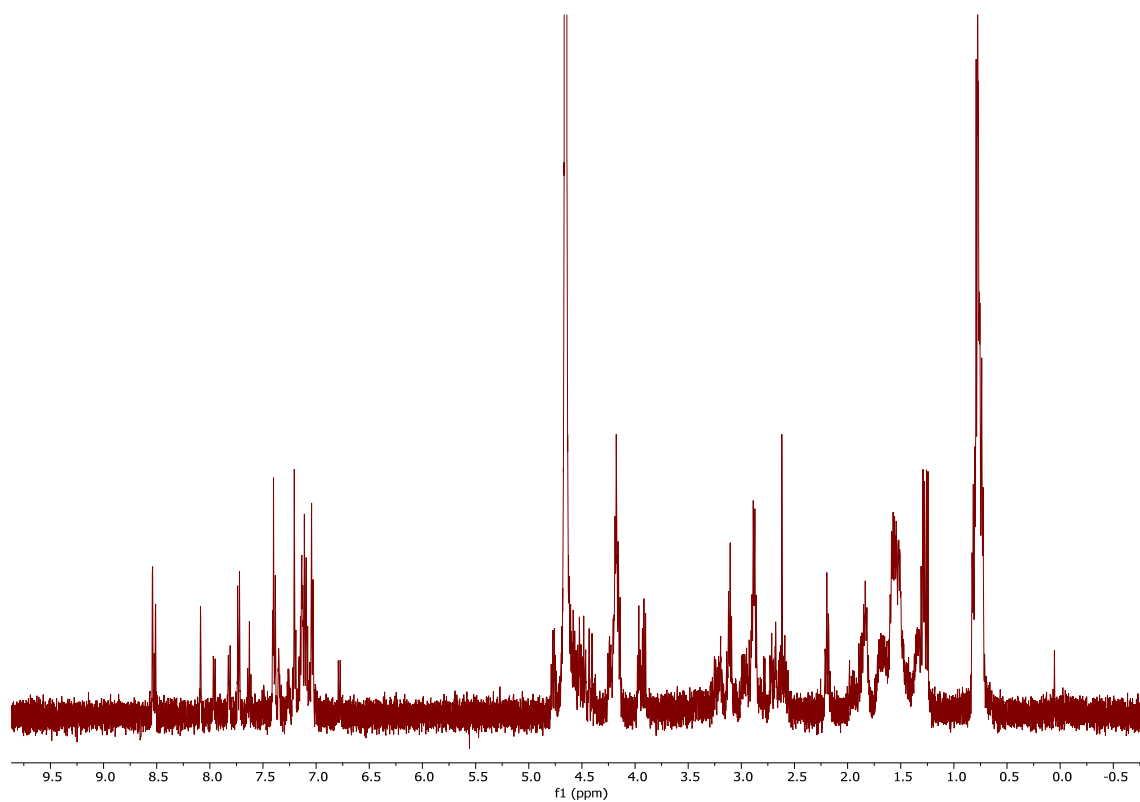


Figure S13: ¹H-NMR spectra (500 MHz, D₂O) of AzopTH₂₅₋₃₇ (top, *trans*-isomer) and SP1 (bottom, *trans*-isomer).

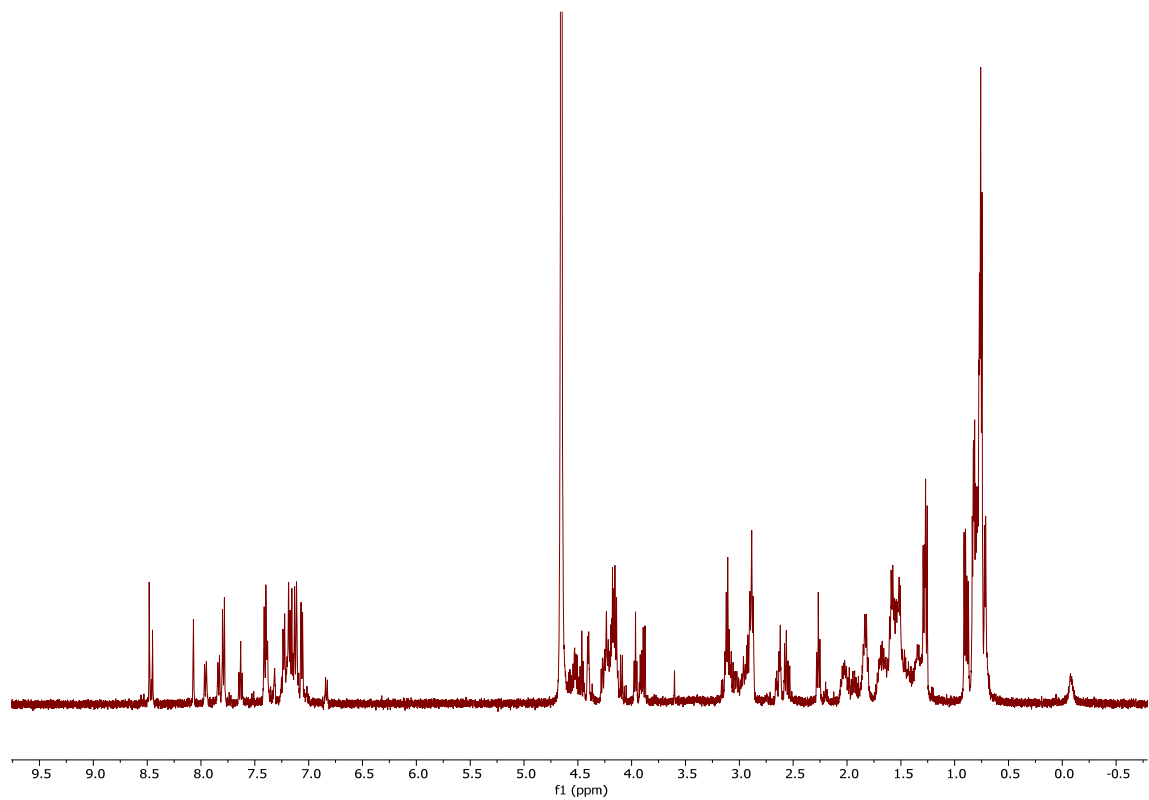
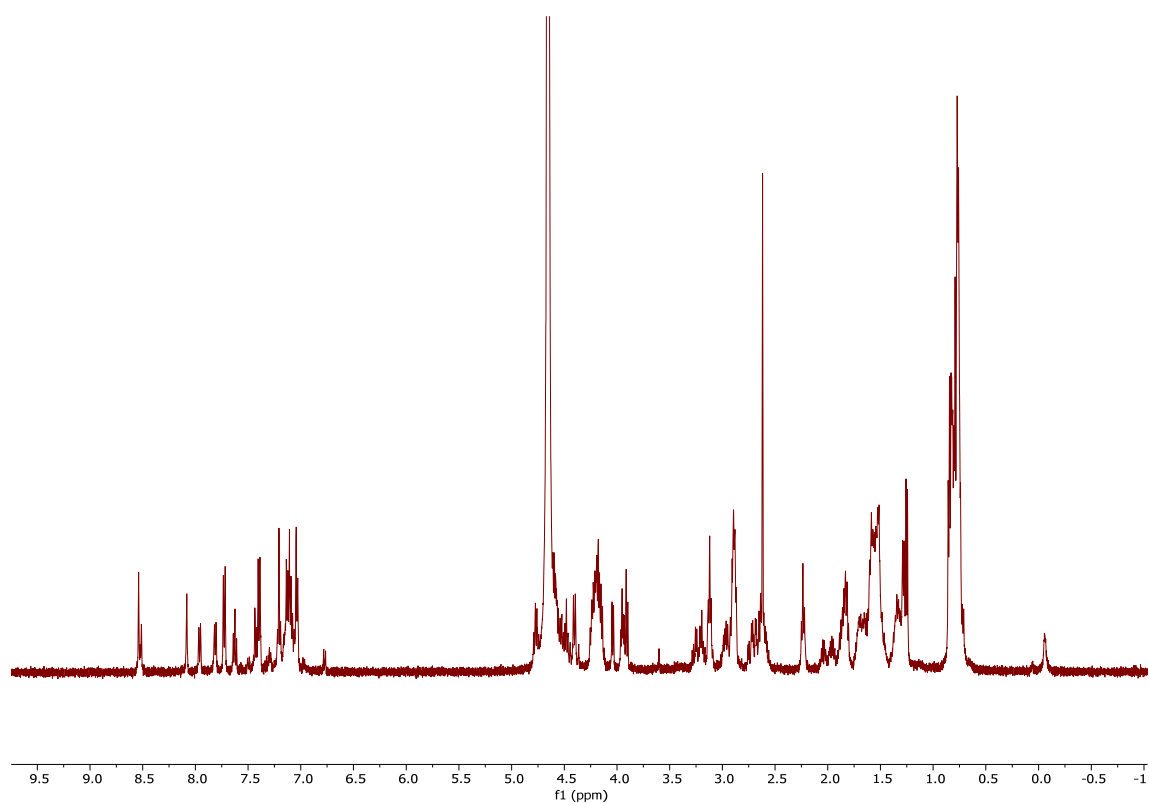


Figure S14: ¹H-NMR spectra (500 MHz, D₂O) of SP2 (top, *trans*-isomer) and SP3 (bottom, *trans*-isomer).

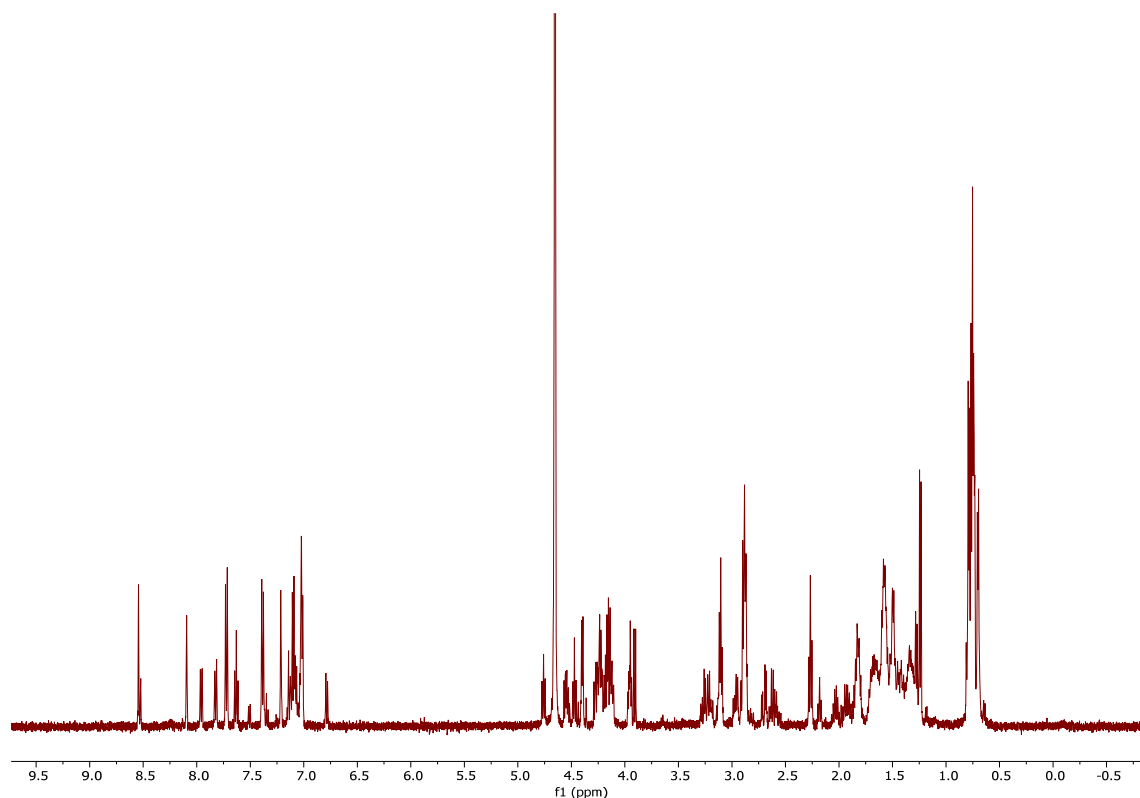


Figure S15: ^1H -NMR spectrum (500 MHz, D_2O) of SP4 (*trans*-isomer).

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Supporting Information

Photocontrolled reversible amyloid fibril formation of parathyroid hormone-derived peptides

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1. Supplementary figures

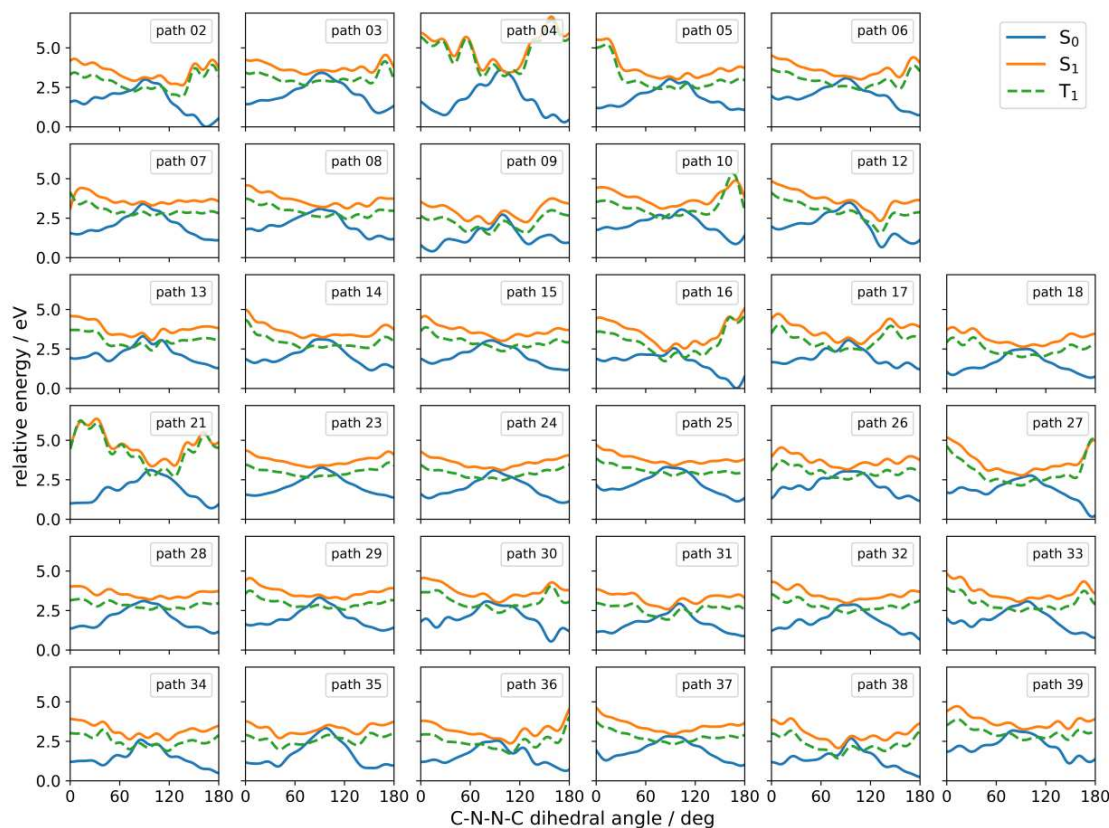


Figure S1. The smoothed potential energy curves from 34 converged *cis* ↔ *trans* isomerization paths. Single-point calculations are performed using NEVPT2(2,2)/xTB with implicit water through ALPB. The smoothing is carried out using Gaussian process regression. A subset of the paths has an energy barrier in the *trans*-*cis*-isomerization, notably paths 04, 10 and 21, with excitation energies before the barrier range from 2.5 to 3.0 eV. This correlates with the experimental fluorescence peak at 485 nm (**Figure S2**), albeit with an expected ipsochromic effect caused by the minimum active space considered in the calculations. We attribute the extended fluorescence lifetime, compared to azobenzene, to structures hindered by this barrier, preventing radiationless relaxation. These barriers predominantly arise from structures characterized by dihedral angles around 170°, which exhibit a heightened S0-S1 excitation energy in the scan.

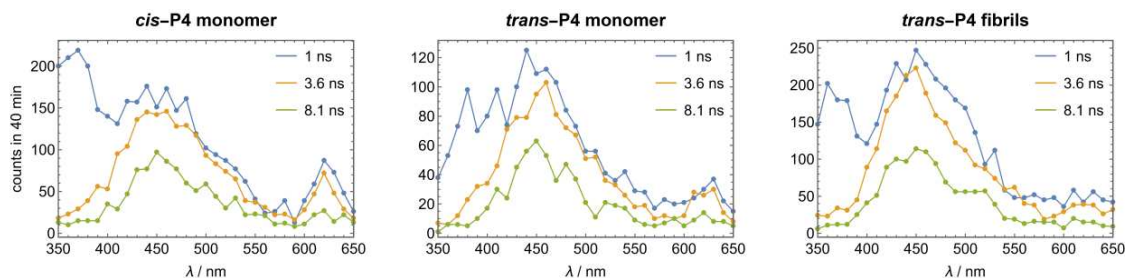


Figure S2. Fluorescence life-time measurements. Fluorescence spectra measured at various points in time after the excitation pulse.

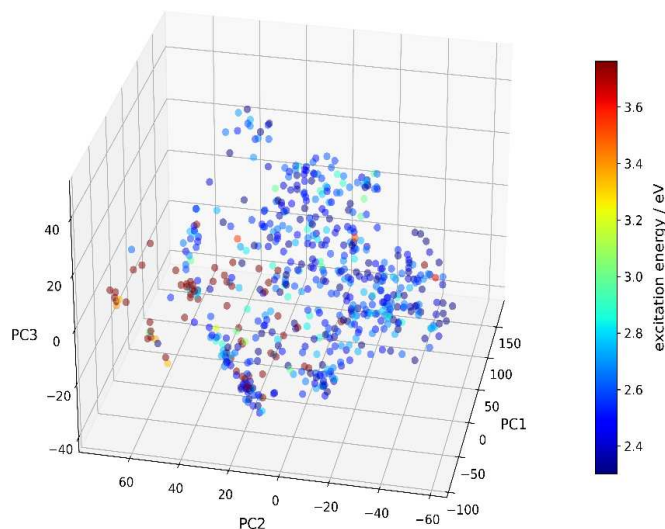


Figure S3. All geometries with dihedral angles near 170° projected on to the three-dimensional PCA space. The colors encode the S0–S1 excitation energies. The reduced three-dimensional space accounts for 86% of the total variance. Several clusters can be detected, notably one predominantly comprising structures with high excitation energies (visually represented in dark red in the lower left corner of the projection). This particular cluster is positioned at the negative extremity of the PC1-axis, advances towards the positive end of the PC2-axis and is situated approximately mid-way along the PC3-axis.

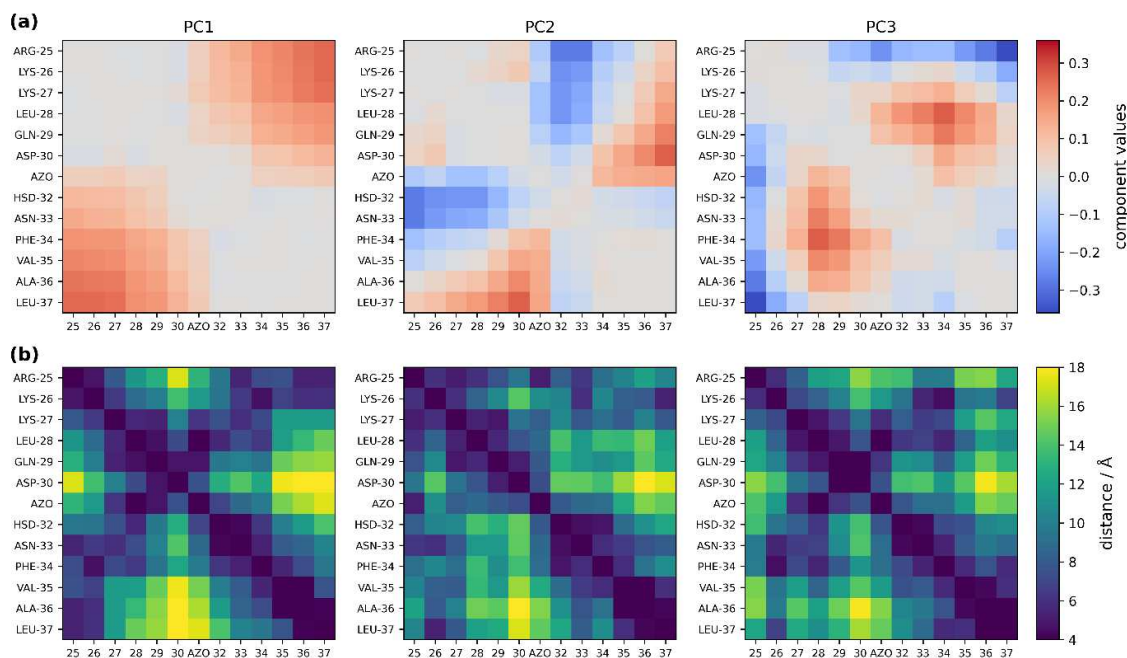


Figure S4. (a) The first three principal components represented in the distance matrix space. The first principal component signals a considerable separation between the residues R25 and A36/L37. Given this cluster's positioning at the PC1-axis's negative end, it implies that within these geometries, the distances between these residues are notably reduced. The second principal component reveals negative values for the separation between R25 and H32/N33, alongside positive distances between D30 and L37. The positive projection of this cluster on the PC2 suggests these distance matrix characteristics directly, without reversing the sign. With almost negligible projection on PC3, this component scarcely influences the distance matrices for structures within this cluster. (b) Distance matrices for clusters with high excitation energies. Despite originating from different paths, these matrices exhibit consistent patterns.

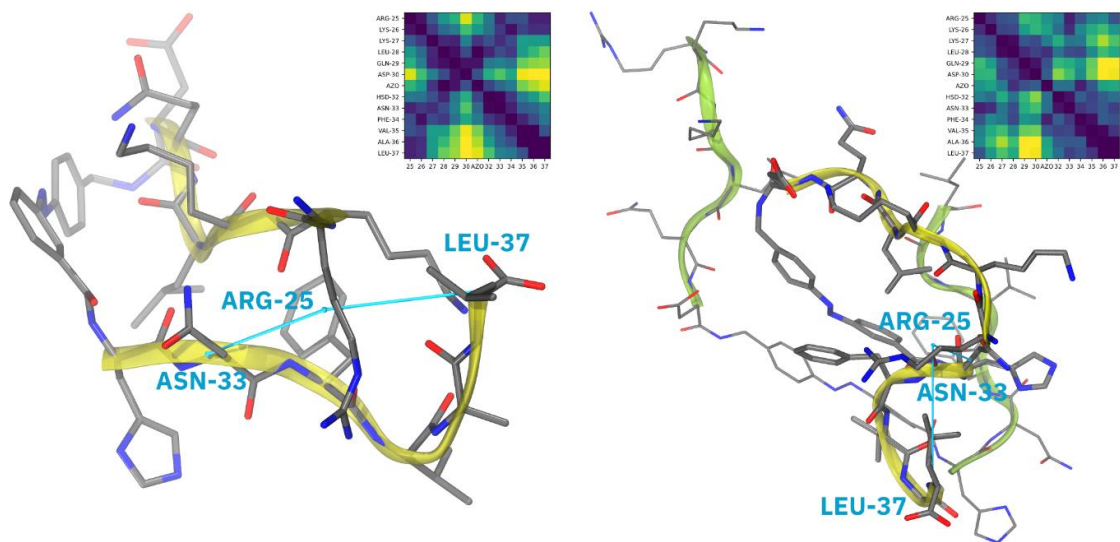


Figure S5. Selected monomer structure (left) with distance matrix shown in Figure S4b and dimer structure (right) with a similar distance matrix. The corresponding distance matrices are shown in the upper right corners. It can be seen that the simultaneous interactions of R25 with A36/L37 and H32/N33 potentially contribute to the S1-barrier during the *trans*→*cis* isomerization path, leading to longer fluorescence lifetimes. This structural motif is also found in the dimer, while their configurations stabilized by intermolecular interactions could also lead to an increased barrier.

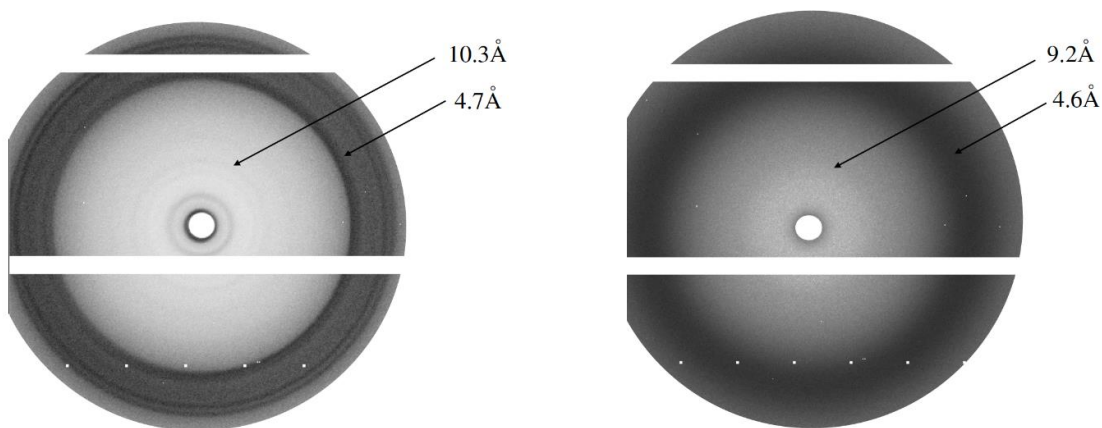


Figure S6: Wide-angle x-ray diffraction patterns from dried fibrillar samples of PTH₂₅₋₃₇ (left) and P4 (right).

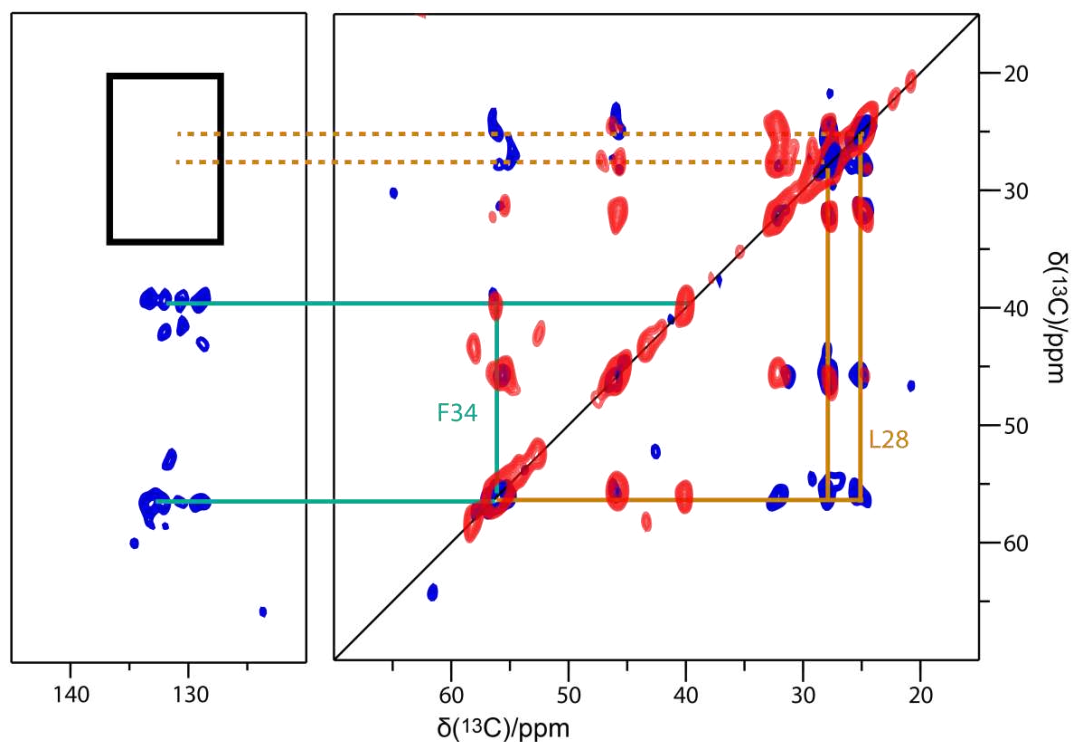


Figure S7. 2D ^{13}C , ^{13}C spin diffusion spectra of fibrils from PTH₂₅₋₃₇, uniformly ^{13}C labeled for L28 and F34, recorded under different conditions. Red: magnetic field strength 14.1 T (corresponding to 600 MHz ^1H resonance frequency), spinning speed 11 kHz, mixing time 10 ms. Blue: magnetic field strength of 18.8 T (corresponding to 800 MHz ^1H resonance frequency), spinning frequency of 20 kHz, mixing time 1 s. Figure S6 shows the 2D ^{13}C , ^{13}C spin diffusion spectra of fibrils from PTH₂₅₋₃₇, uniformly ^{13}C labeled for L28 and F34, recorded under different conditions. In the red spectrum, intraresidual cross-peaks between neighboring ^{13}C sites of L28 and F34, respectively, are visible. At a longer mixing time of 1 s (blue), long-range correlations between all ^{13}C spins within one residue are obtained. Spin systems of the labeled amino acids F34 (cyan) and L28 (brown) are marked by solid lines. Dashed lines show a possible contact between L28 $\text{C}_\gamma/\text{C}_\delta$ and an aromatic F34 carbon. The spinning speed of 20 kHz corresponds to the first order rotational resonance condition for resonances with a chemical shift difference of 100 ppm, leading to a recoupling of dipolar couplings between aromatic ring carbon atoms of F34 and aliphatic C_γ and C_δ signals of L28, facilitating magnetization transfer between those residues if the distance between these residues would not exceed 6 Å.²⁰ The fact that no inter-residual cross-peaks between L28 and F34 can be observed is thus a strong indication against an antiparallel arrangement of β -strands within the β -sheet.

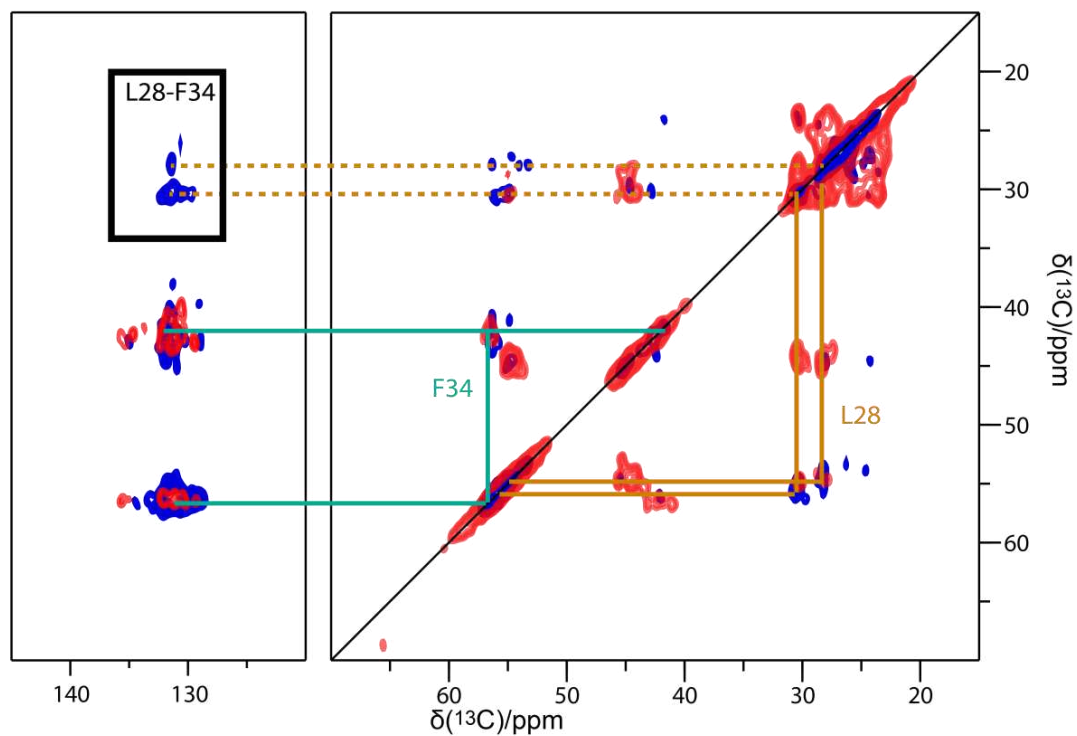


Figure S8. 2D ^{13}C , ^{13}C spin diffusion spectra of fibrils from P4, uniformly ^{13}C labeled for L28 and F34, recorded at a magnetic field strength of 18.8 T (corresponding to 800 MHz ^1H resonance frequency) at a spinning speed of 20 kHz, corresponding to the first order rotational resonance condition for signals with a chemical shift difference of 100 ppm. Red: Mixing time of 50 ms. Blue: Mixing time of 1 s. See for more information in Fig. S6. Here, inter-residual cross-peaks between the aromatic ring signals of F and L $\text{C}\delta$ resonances are clearly visible for a mixing time of 1 s (blue spectrum).

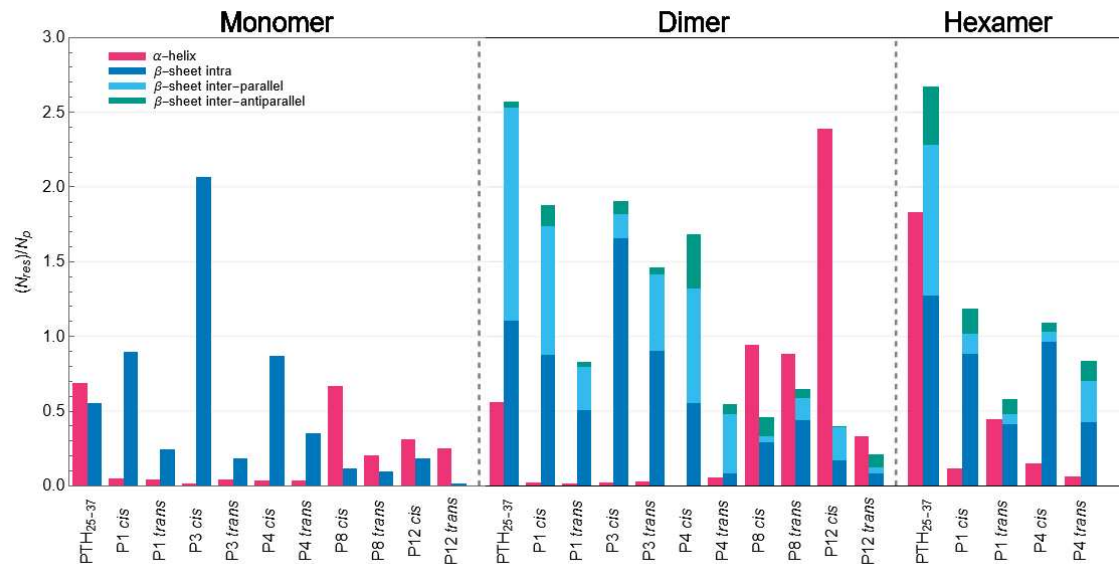
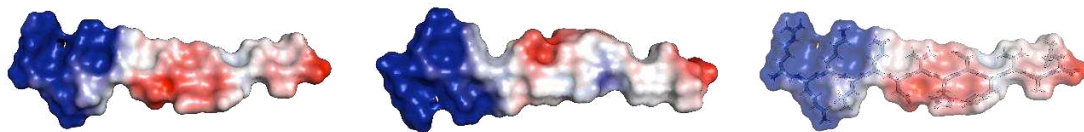


Figure S9. Summary of all MD simulation results. The average numbers of residues $\langle N_{\text{res}} \rangle$ forming secondary structure elements, divided into α -helical (magenta), intrapeptide β -sheets (blue), interpeptide parallel β -sheets (cyan) and interpeptide antiparallel β -sheets (green) are given. The average was taken over the number of frames of the simulation and normalized by the number of peptides N_p present in the corresponding simulation.

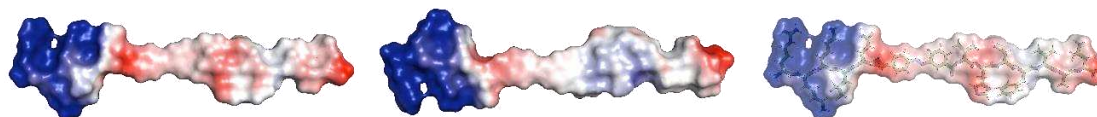
PTH₂₅₋₃₇



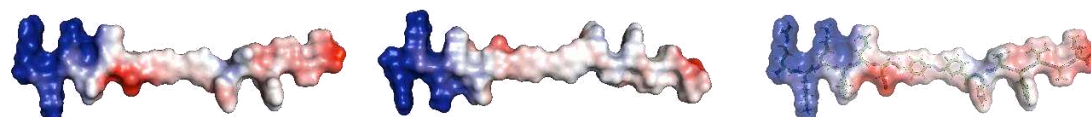
P1



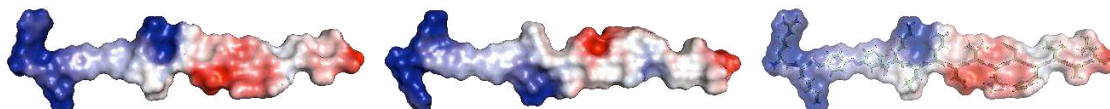
P3



P4



P8



P12

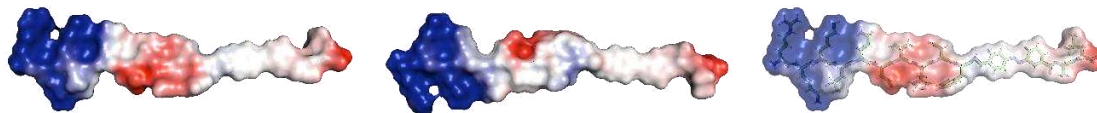


Figure S10. Electrostatic potential surface of PTH₂₅₋₃₇ and the peptides P1, P3, P4, P8, and P12, with values according to the color map at the bottom, ranging from -2 (red) to +2 kTe⁻¹ (blue). The electrostatic potential mapped to the molecular surfaces was calculated using the Adaptive Poisson-Boltzmann Solver (APBS¹) plugin for the pymol² software package. For each peptide, the two views that are rotated by 180° around the backbone axis are shown, as well as a transparent front view.

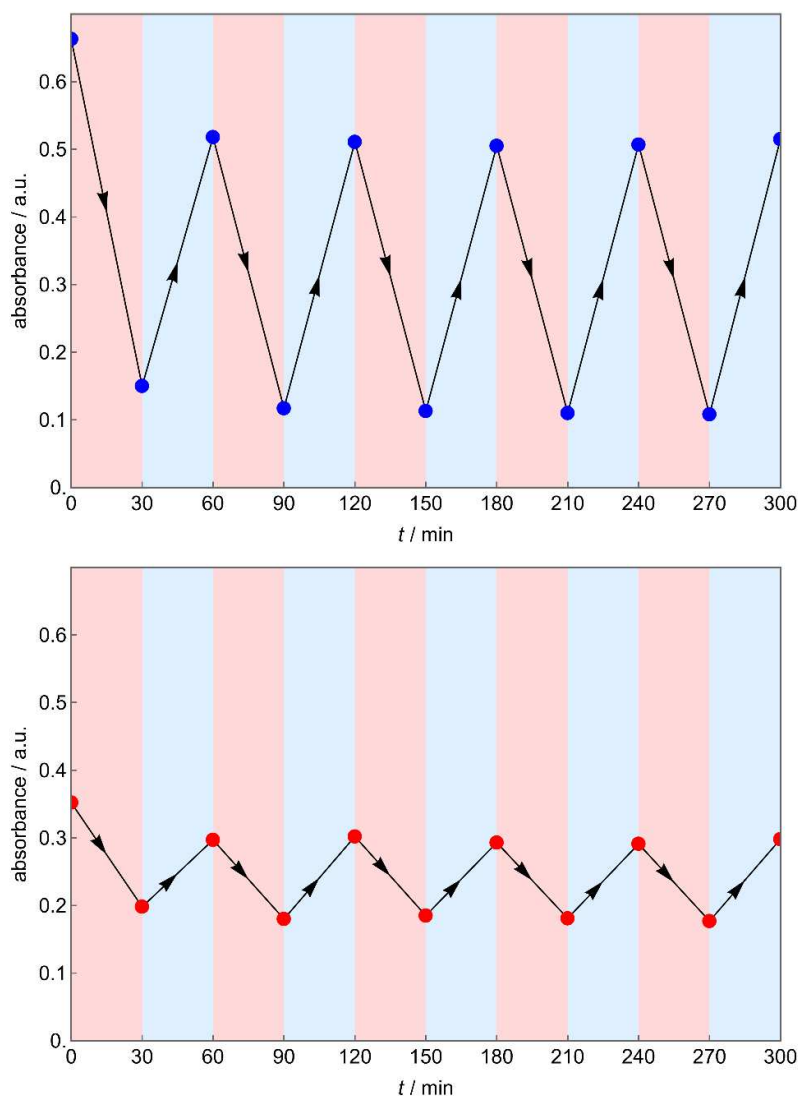


Figure S11: Control experiment to investigate photobleaching of the azobenzene unit during the photoisomerization. Absorption of P4 was measured after each isomerization step at 327 nm (top, blue dots) and 295 nm (bottom, red dots). Light red area corresponds to irradiation with light of 340 nm wavelength for 30 min to achieve *trans*→*cis* isomerization. Light blue area corresponds to irradiation with light of 405 nm wavelength for 30 min to achieve *cis*→*trans* isomerization.

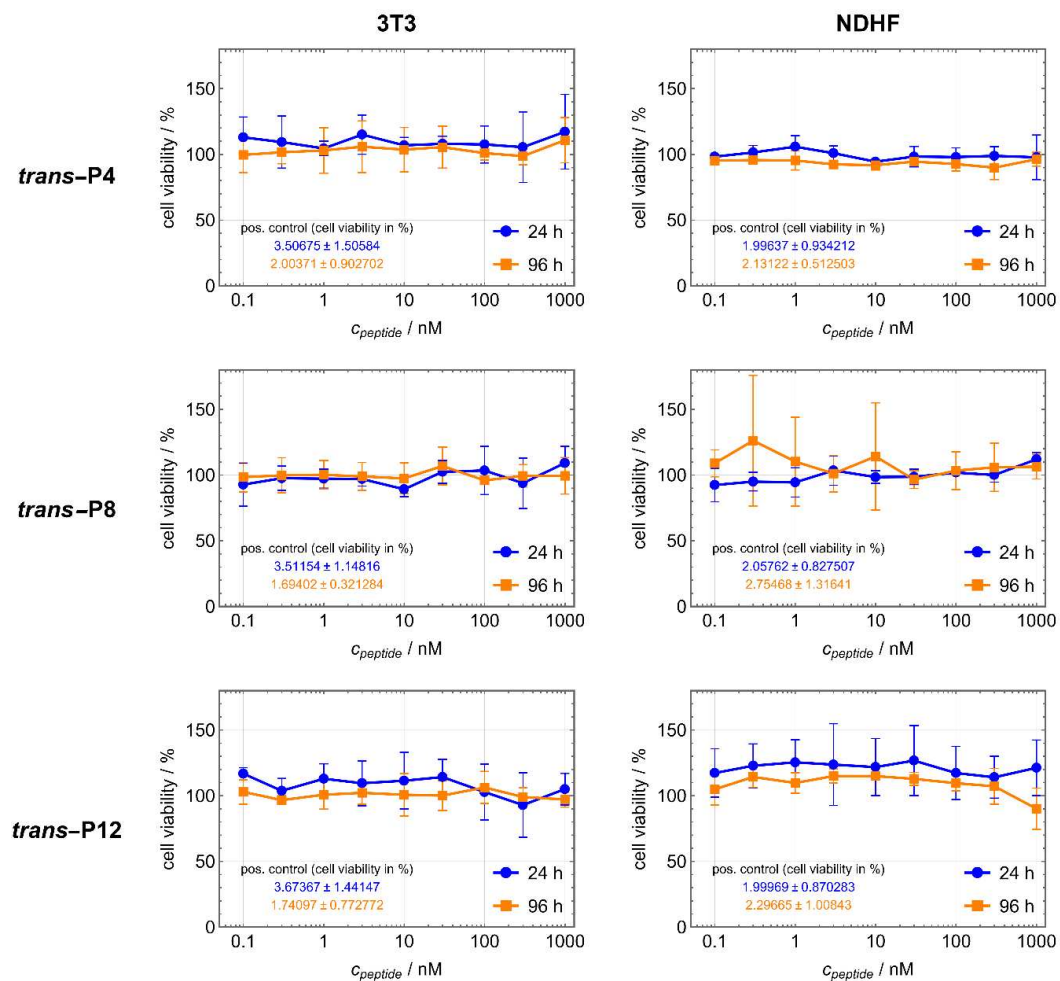


Figure S12: Cell viability assay of *trans*-P4, *trans*-P8, and *trans*-P12 on NDHF (human, adult, fibroblasts) and 3T3 (murine, embryonal, fibroblasts) cells after 24 h (blue) and 96 h (orange). 100% cell viability corresponds to the value of the negative, untreated control.

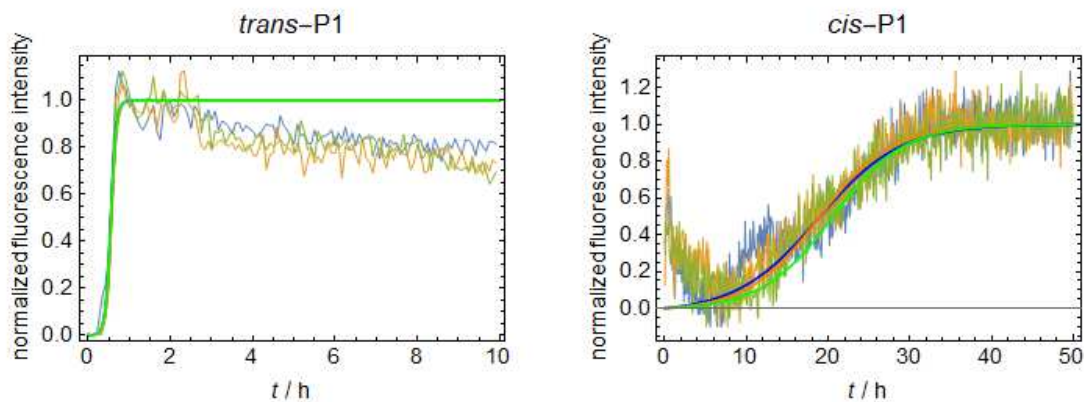


Figure S13. Fibrillization kinetics of 85 μM P1 as *trans*- (left) and *cis*-isomer (right). Measured in a ThT-monitored fluorescence assay at 37 $^{\circ}\text{C}$ in 50 μM Na_2HPO_4 buffered aqueous solution (pH 7.4).

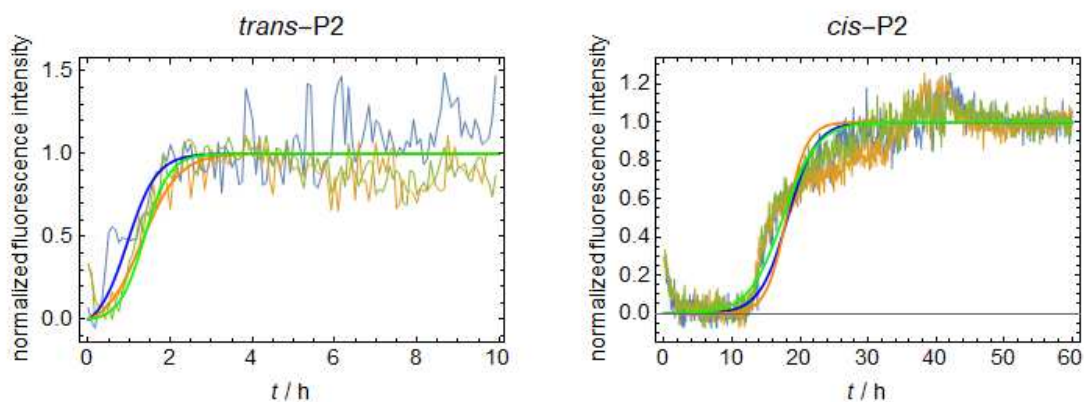


Figure S14. Fibrillization kinetics of 55 μM P2 as *trans*- (left) and *cis*-isomer (right). Measured in a ThT-monitored fluorescence assay at 37 $^{\circ}\text{C}$ in 50 μM Na_2HPO_4 buffered aqueous solution (pH 7.4).

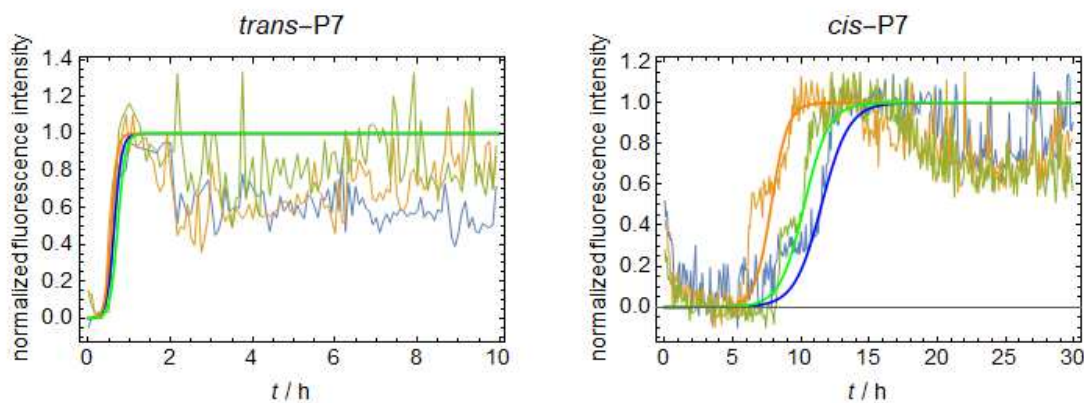


Figure S15. Fibrillization kinetics of 100 μM P7 as *trans*- (left) and *cis*-isomer (right). Measured in a ThT-monitored fluorescence assay at 37 $^{\circ}\text{C}$ in 50 μM Na_2HPO_4 buffered aqueous solution (pH 7.4).

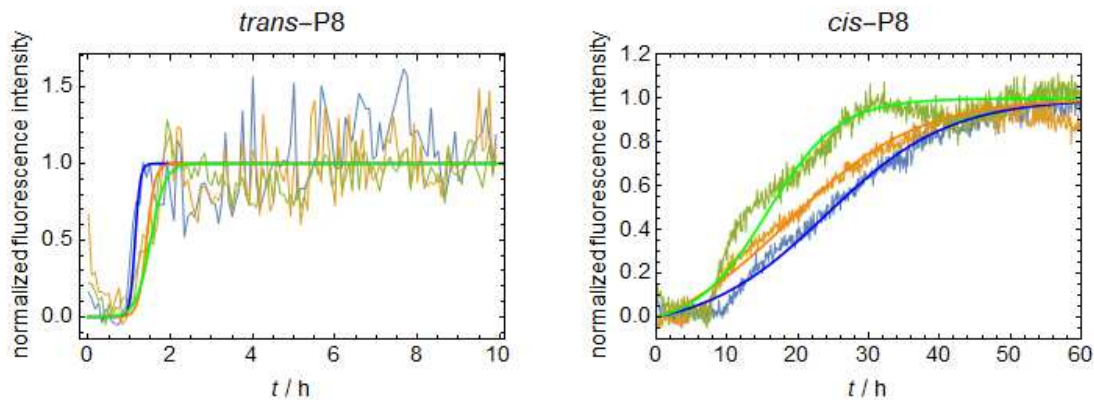


Figure S16. Fibrillization kinetic of 100 μM P8 as *trans*- (left) and *cis*-isomer (right). Measured in a ThT-monitored fluorescence assay at 37 $^{\circ}\text{C}$ in 50 μM Na_2HPO_4 buffered aqueous solution (pH 7.4).

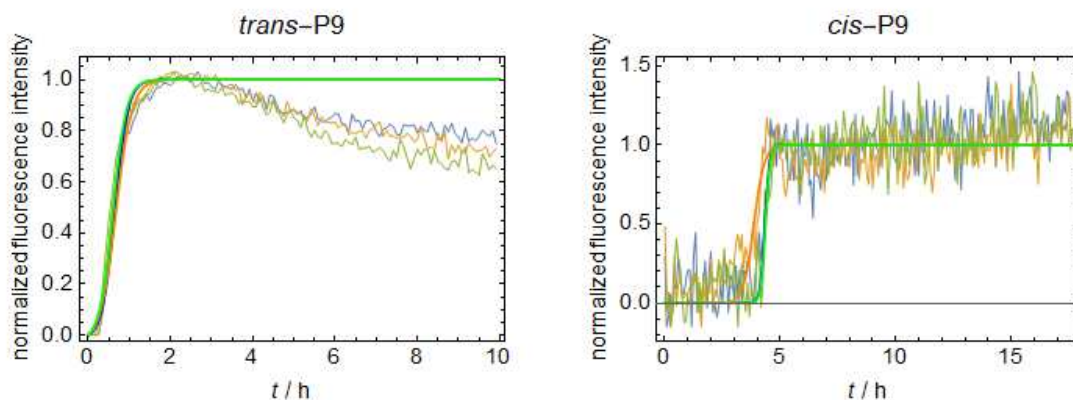


Figure S17. Fibrillization kinetics of 100 μM P9 as *trans*- (left) and *cis*-isomer (right). Measured in a ThT-monitored fluorescence assay at 37 $^{\circ}\text{C}$ in 50 μM Na_2HPO_4 buffered aqueous solution (pH 7.4).

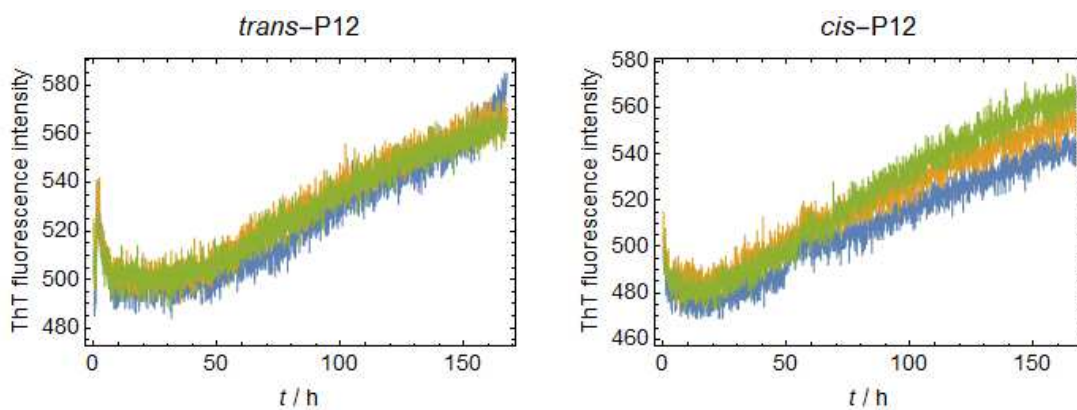


Figure S18. Fibrillization kinetics of 100 μM P12 as *trans*- (left) and *cis*-isomer (right). Measured in a ThT-monitored fluorescence assay at 37 $^{\circ}\text{C}$ in 50 μM Na_2HPO_4 buffered aqueous solution (pH 7.4).

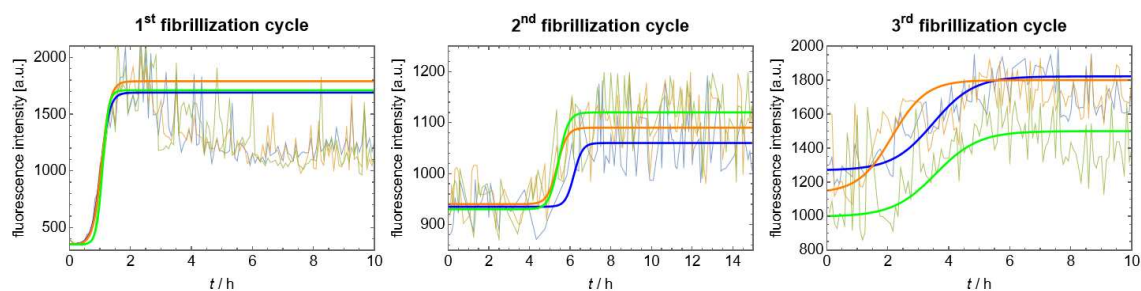


Figure S19. Fibrillization kinetics of P4 over three cycles of alternating fibrillization and fibril degradation through *trans*→*cis* isomerization measured in a ThT-monitored fibrillization assay at 37 °C in 50 μM Na_2HPO_4 buffered aqueous solution (pH 7.4).

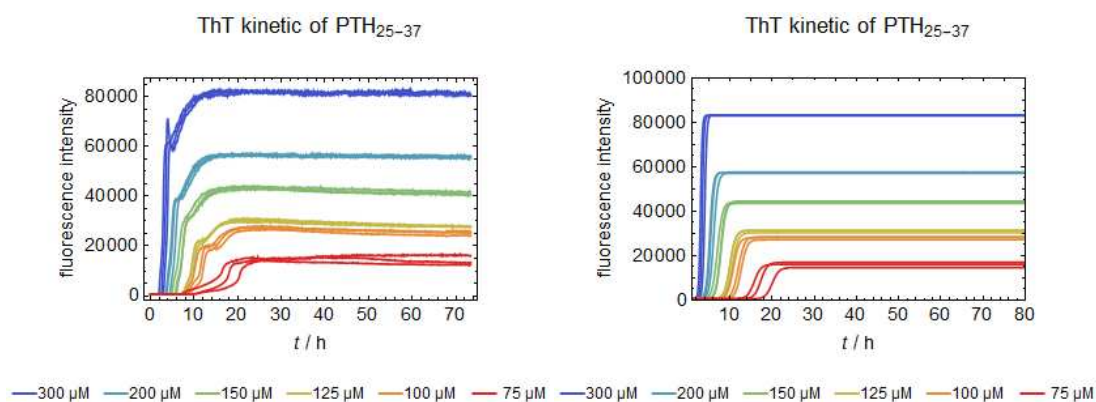


Figure S20. Fibrillization kinetics of PTH₂₅₋₃₇ in different concentrations measured in a ThT-monitored fluorescence assay at 37 °C in 50 μM Na_2HPO_4 buffered aqueous solution (pH 7.4); (left) raw data, (right) fitted data using Eq. (1).

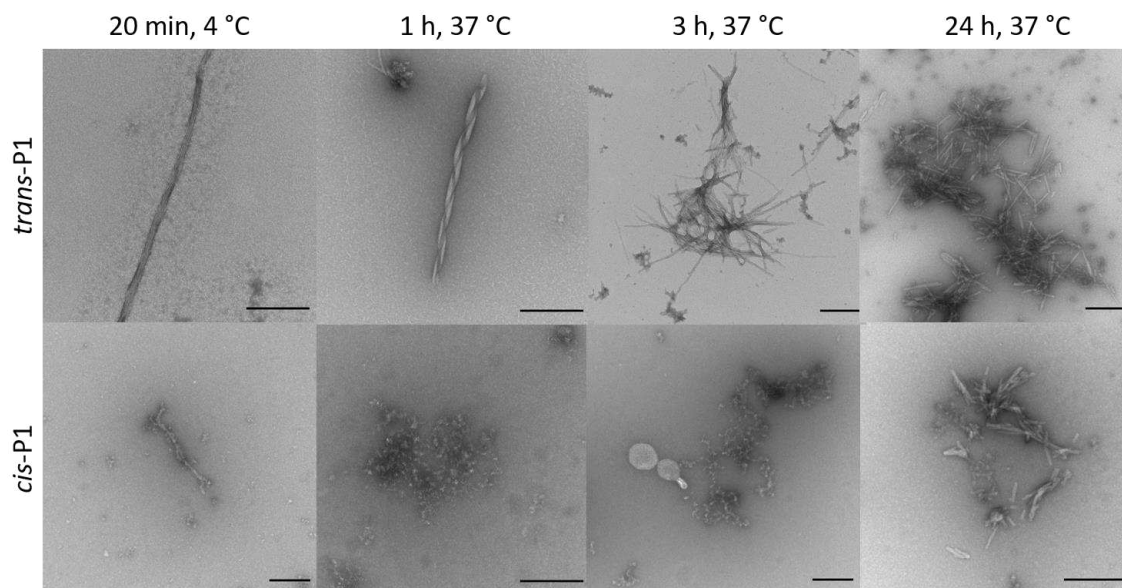


Figure S21. TEM images of P1 at different times and temperatures; scale bar = 250 nm.

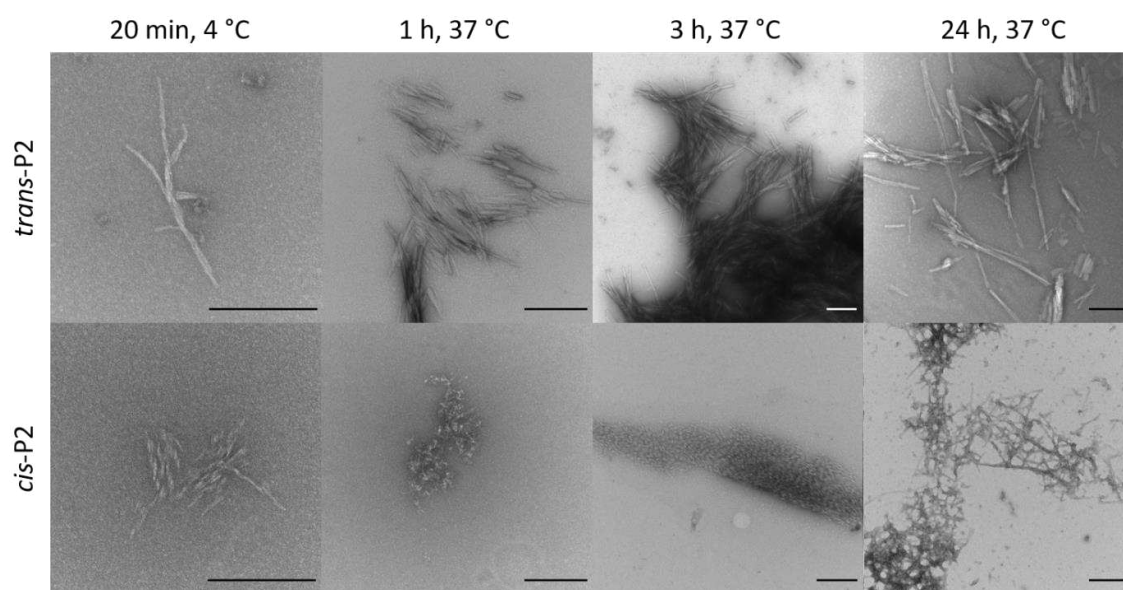


Figure S22. TEM images of P2 at different times and temperatures; scale bar = 250 nm.

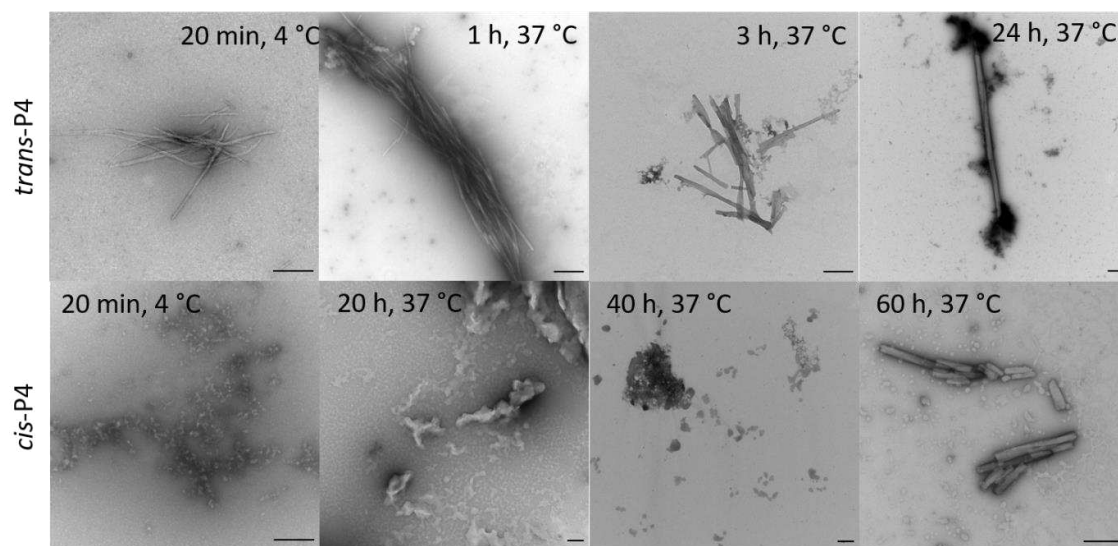


Figure S23. TEM images of P4 at different times and temperatures; scale bar = 250 nm.

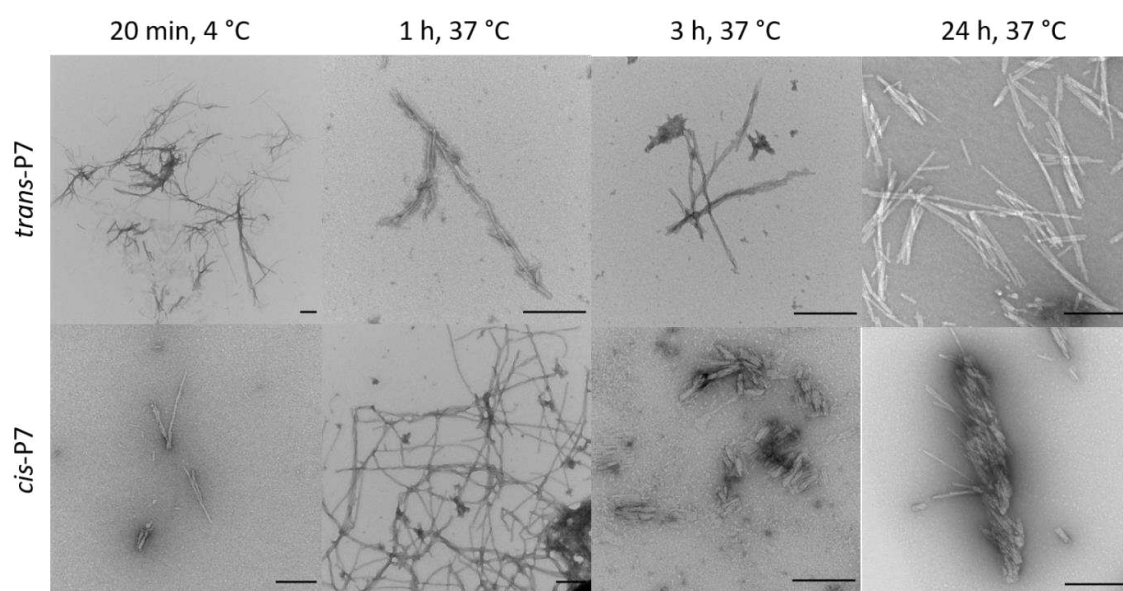


Figure S24. TEM images of P7 at different times and temperatures; scale bar = 250 nm.

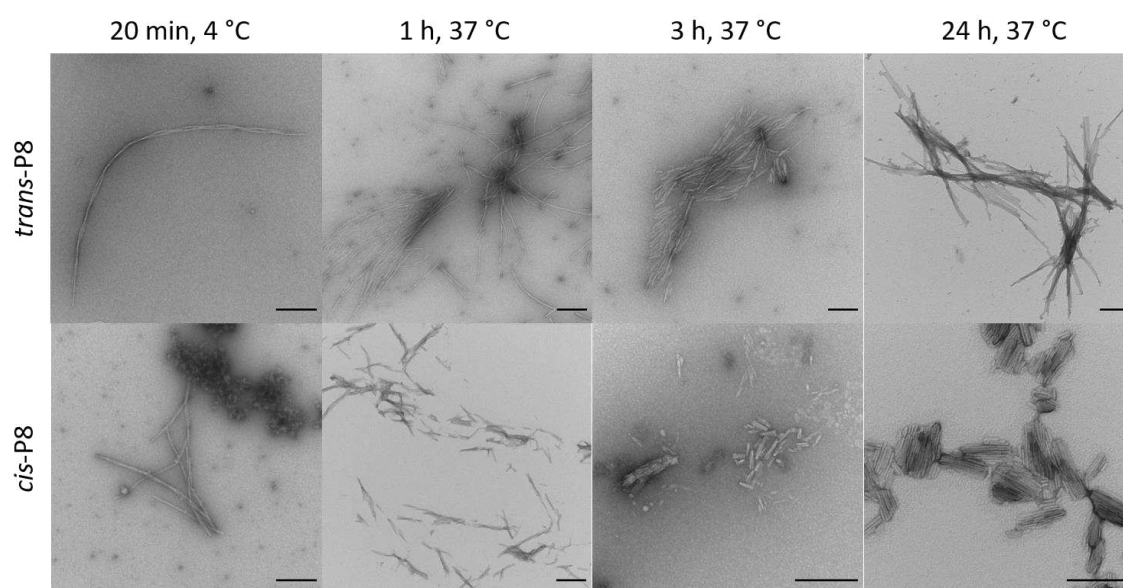


Figure S25. TEM images of P8 at different times and temperatures; scale bar = 250 nm.

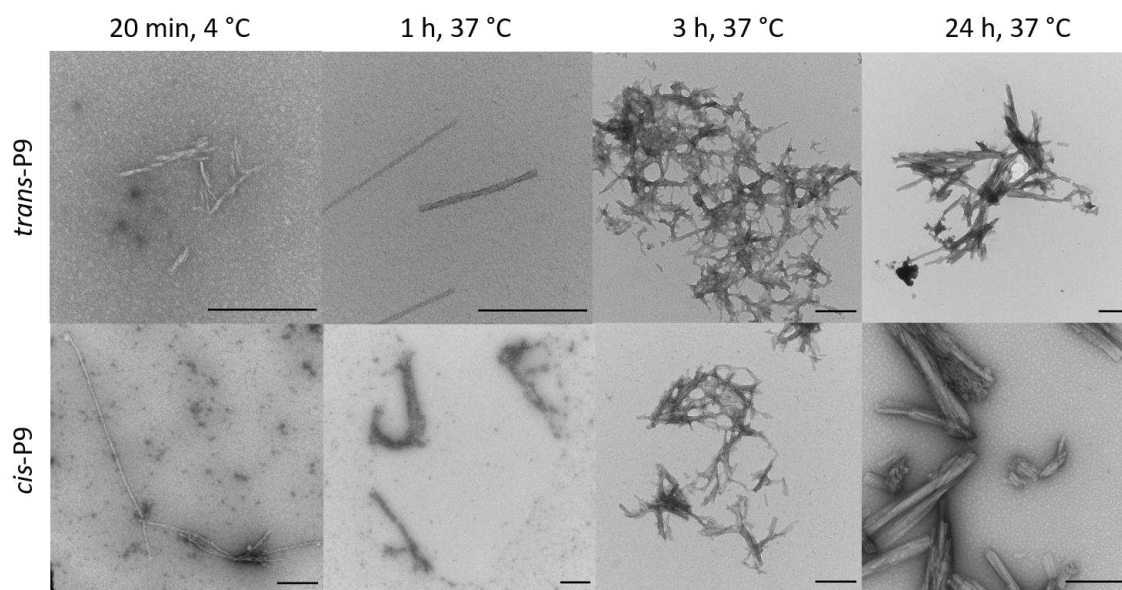


Figure S26. TEM images of P9 at different times and temperatures; scale bar = 250 nm.

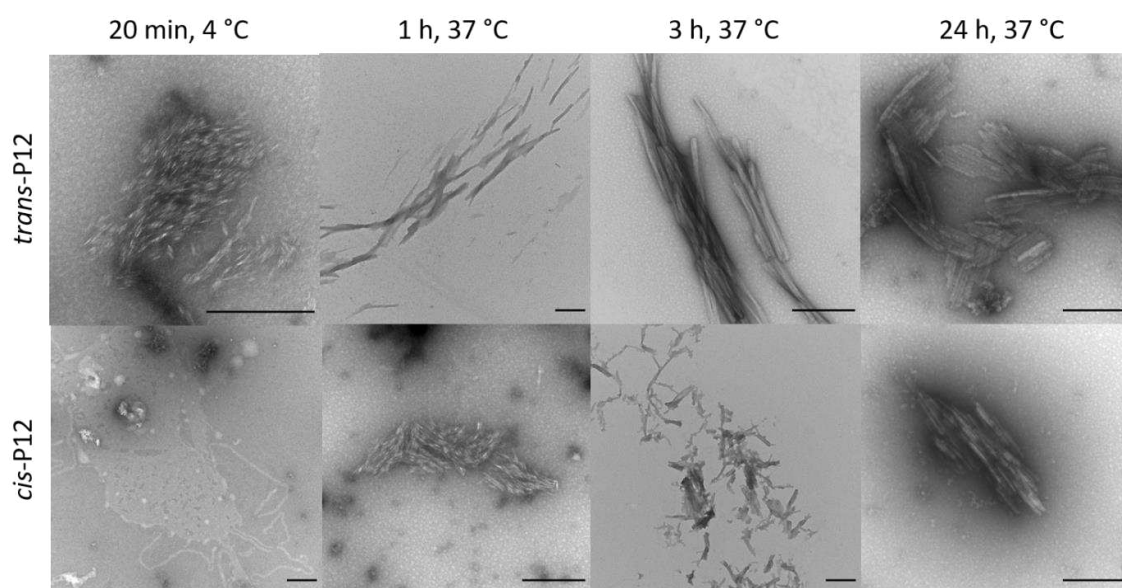


Figure S27. TEM images of P12 at different times and temperatures; scale bar = 250 nm.

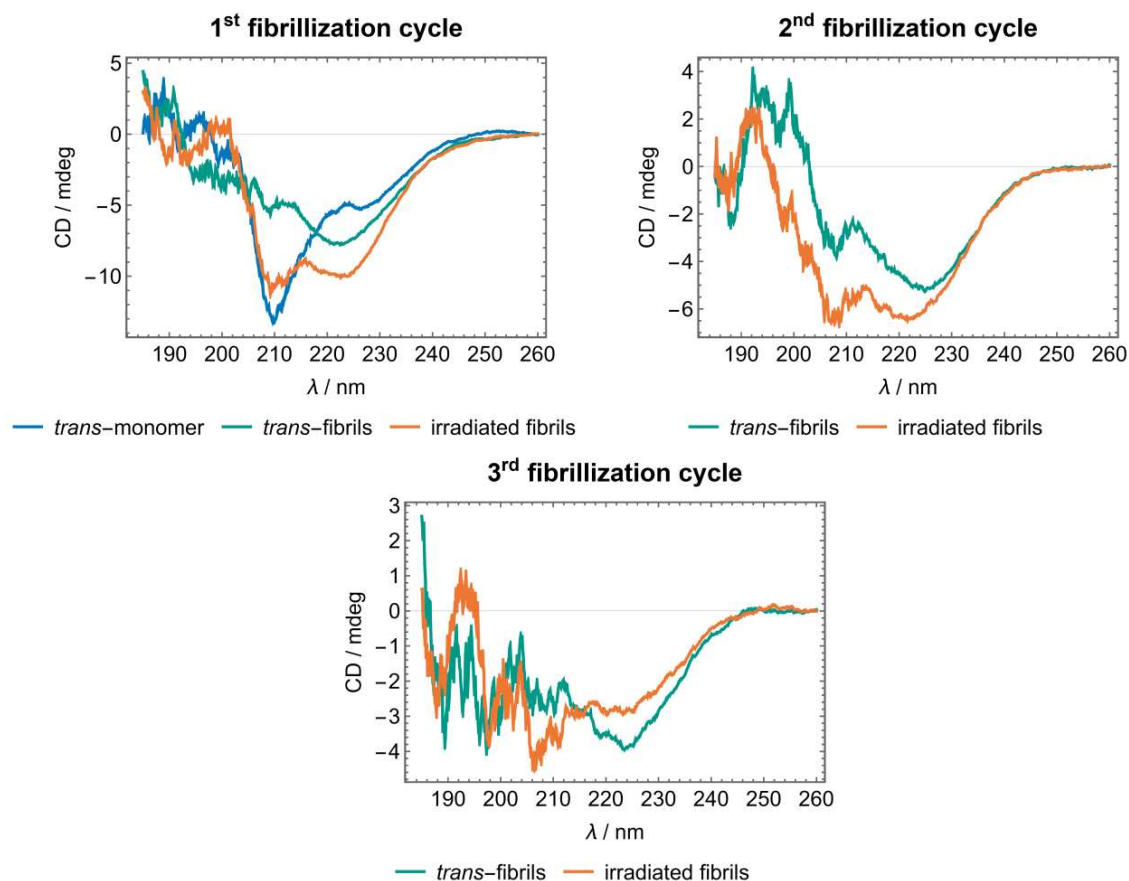


Figure S28: CD-spectra of the reversible fibrillization of P4 over three cycles (1st, 2nd, and 3rd). *trans*-Isomer (blue) was measured directly after dissolving the peptide, *trans*-fibrils (green) were measured 20 h after reference sample reached the stationary phase, and irradiated fibrils (orange) were measured directly after irradiating the fibrils with 340 nm for 5 h.

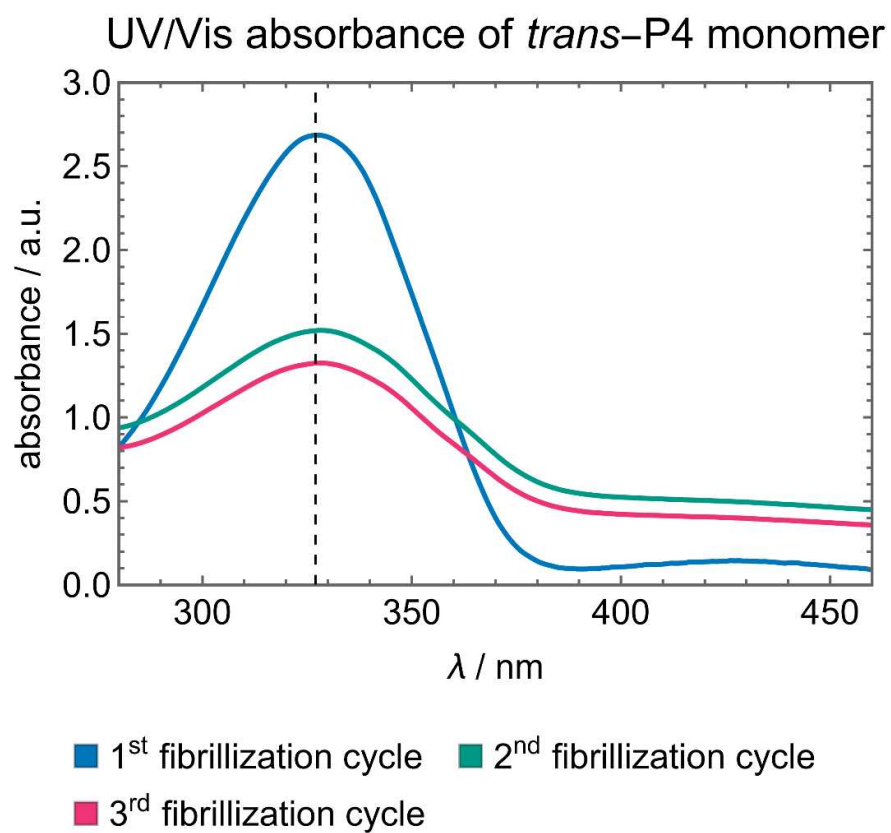


Figure S29: UV/Vis-spectra of *trans*-P4 monomer before each fibrillization cycle. Dashed line corresponds to the absorption maxima of the *trans*-isomer at 327 nm.

2. Supplementary tables

Table S1. All Simulations performed, with their respective simulation time

Peptide	Simulation	Simulation Time [μ s]
PTH ₂₅₋₃₇	Monomer/Dimer/Hexamer	3 systems x 10
P1	Monomer/Dimer/Hexamer - <i>cis/trans</i>	6 systems x 10
P3	Monomer/Dimer - <i>cis/trans</i>	4 systems x 10
P4	Monomer/Dimer/Hexamer - <i>cis/trans</i>	6 systems x 10
P8	Monomer/Dimer - <i>cis/trans</i>	4 systems x 10
P12	Monomer/Dimer - <i>cis/trans</i>	4 systems x 10
PTH ₂₅₋₃₇	fibril	4 models x 1
P4	fibril	11 models x 1

Table S2. Sequence of PTH₂₅₋₃₇ and P1 – P12 and their respective solubility in buffered solution (50 mM aqueous Na₂HPO₄) with pH 7.4, the critical fibrillization concentration (c_{cr}), and standard free energy of the fibrillization reaction ΔG^0 .

Peptide	Primary sequence	Solubility [μ M]	c_{cr} [μ M]	ΔG^0 [kJ/mol]
PTH ₂₅₋₃₇	²⁵ RKKLQ ³⁰ DVHNF ³⁵ VAL	>500	42	26
P1	²⁵ RKKLQ ³⁰ D- Azo -VHNF ³⁵ VAL	90	28	27
P2	²⁵ RKKLQ ³⁰ DV- Azo -HNF ³⁵ VAL	60	27	27
P3	²⁵ RKKLQ- Azo -VHNF ³⁵ VAL	35	n.d.	n.d.
P4	²⁵ RKKLQ ³⁰ D- Azo -HNF ³⁵ VAL	370	23	28
P5	²⁵ RKKLQ- Azo -HNF ³⁵ VAL	25	n.d.	n.d.
P6	²⁵ RKKLQ- Azo - ³⁰ DVHNF ³⁵ VAL	20	n.d.	n.d.
P7	²⁵ RKKL- Azo -Q ³⁰ DVHNF ³⁵ VAL	140	32	27
P8	²⁵ RK- Azo -KLQ ³⁰ DVHNF ³⁵ VAL	200	6	31
P9	²⁵ R- Azo -KLQ ³⁰ DVHNF ³⁵ VAL	130	26	27
P10	²⁵ RKKLQ ³⁰ DVHN- Azo -F ³⁵ VAL	7	n.d.	n.d.
P11	²⁵ RKKLQ ³⁰ DVHNF ³⁵ V- Azo -AL	20	n.d.	n.d.
P12	²⁵ RKKLQ ³⁰ DVHNF- Azo -AL	135	-	-

n.d. – not determined

Table S3. Photophysical properties of P1, P2, P4, P7, P8, P9, P12 in buffered solution. *cis*-PSS – *cis*-photostationary state at 340 nm after 30 min. *trans*-PSS – *trans*-photostationary state at 405 nm after 20 min.

Peptide	$t_{1/2}$ (<i>cis</i> , 37 °C) [h]	after synthesis	<i>cis</i> -PSS	<i>trans</i> -PSS
<i>cis:trans</i> ratio				
P1	90	5:95	86:14	19:81
P2	90	6:94	87:13	19:81
P4	97	3:97	82:18	24:76
P7	72	8:92	94:6	23:77
P8	89	6:94	85:15	23:77
P9	86	9:91	91:9	16:84
P12	63	4:96	90:10	19:81

Table S4. Fibrillization parameters of PTH₂₅₋₃₇, P1, P2, P4, P7, P8, and P9 in buffered solution. t_{lag} - lag time. t_{char} - characteristic time.

Peptide	t_{lag} (<i>trans</i>) [h]	t_{char} (<i>trans</i>) [h]	t_{lag} (<i>cis</i>) [h]	t_{char} (<i>cis</i>) [h]
PTH ₂₅₋₃₇	7.770 ± 1.111	12.797 ± 3.341	-	-
P1	0.392 ± 0.013	0.556 ± 0.008	9.759 ± 1.383	19.640 ± 0.842
P2	0.482 ± 0.207	1.247 ± 0.208	13.052 ± 1.336	17.867 ± 0.360
P4	1.917 ± 0.507	2.371 ± 0.433	29.525 ± 0.558	42.444 ± 5.251
P7	0.381 ± 0.056	0.627 ± 0.078	8.014 ± 1.643	9.875 ± 1.881
P8	1.069 ± 0.096	1.356 ± 0.197	5.524 ± 1.781	20.701 ± 4.415
P9	0.267 ± 0.019	0.653 ± 0.040	3.833 ± 0.458	4.188 ± 0.290

3. Peptide characterization after synthesis

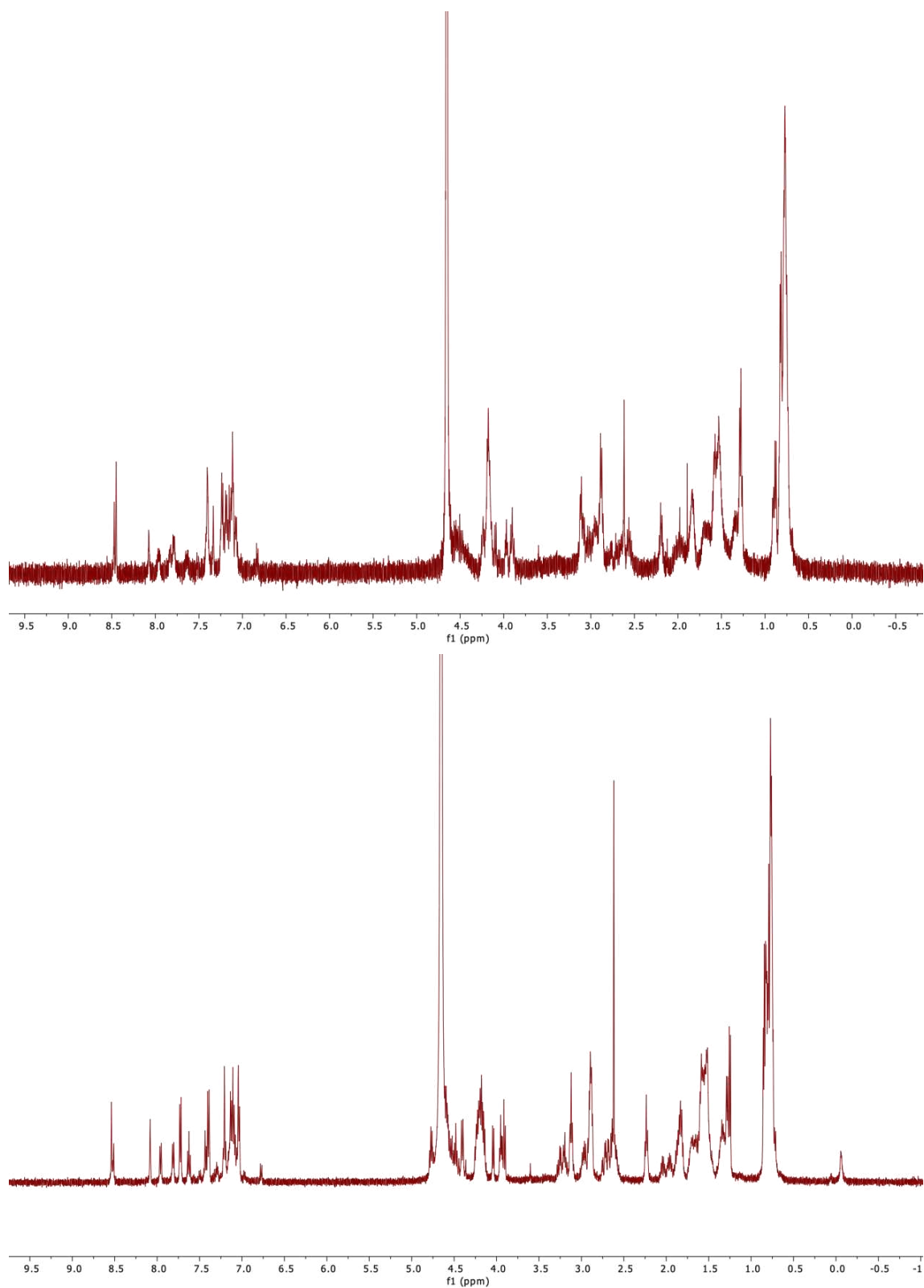


Figure S30: ¹H-NMR spectra (500 MHz, D₂O) of P1 (top, *trans*-isomer) and P2 (bottom, *trans*-isomer).³

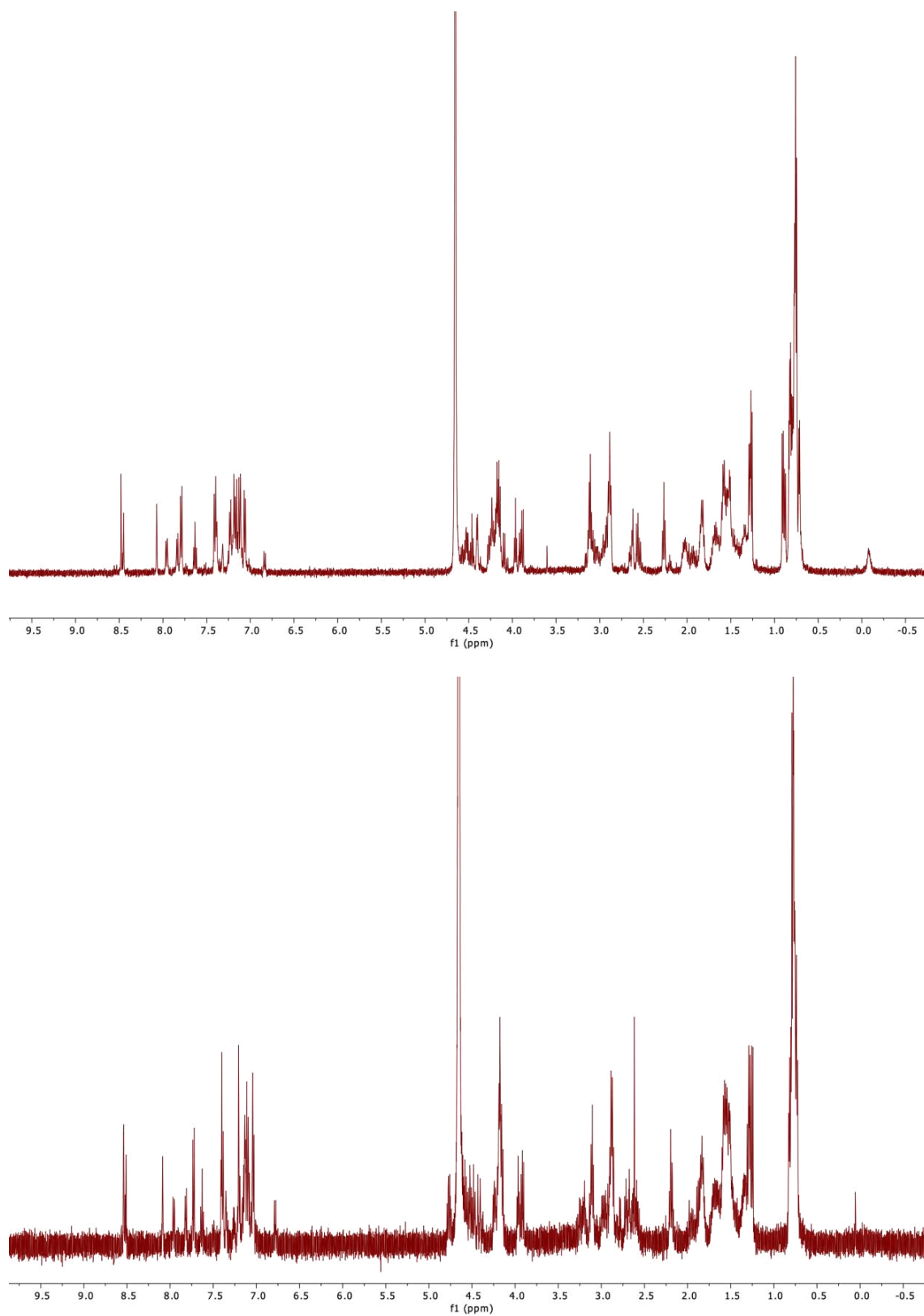


Figure S31: ^1H -NMR spectra (500 MHz, D_2O) of P3 (top, *trans*-isomer) and P4 (bottom, *trans*-isomer).³

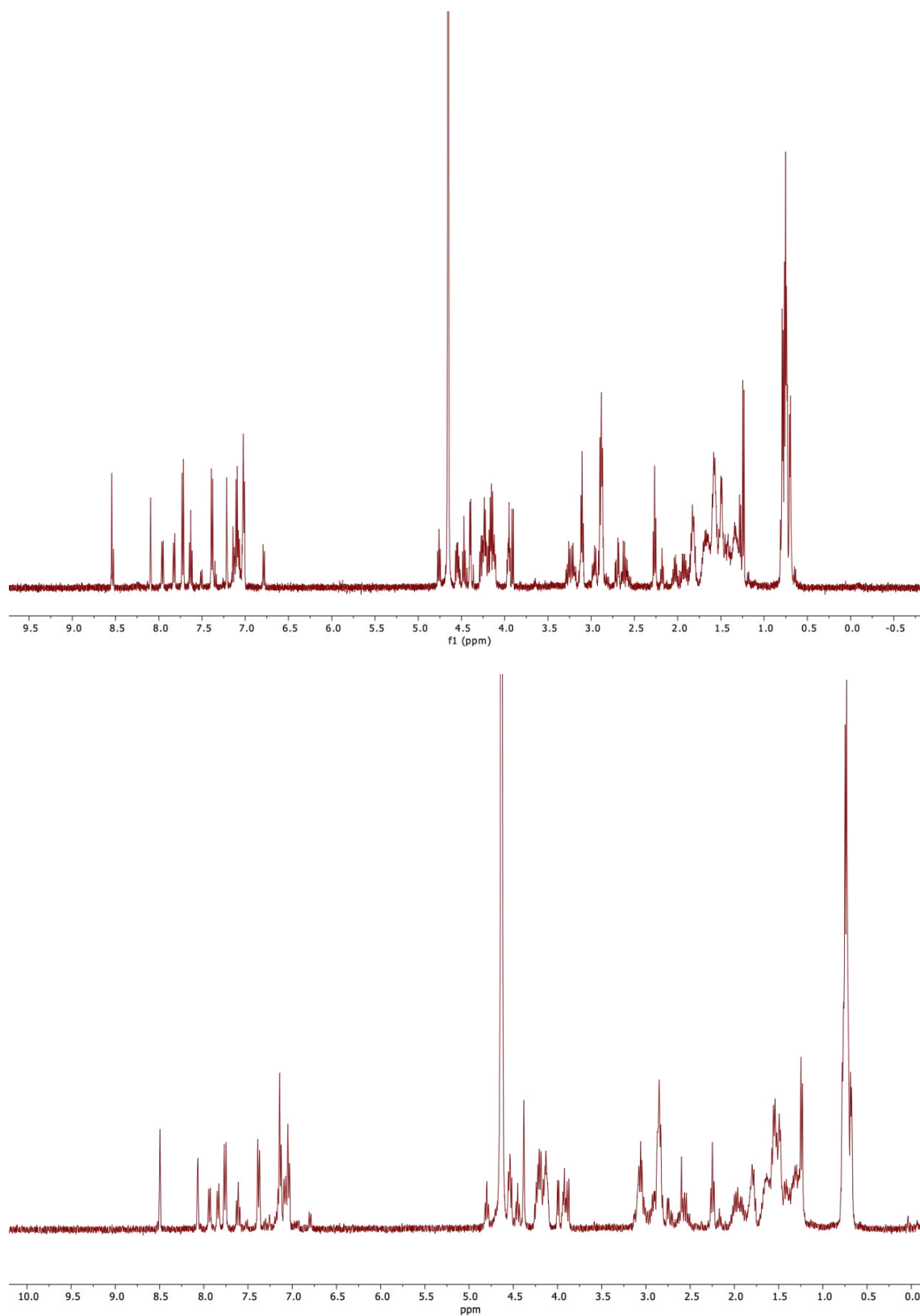


Figure S32: ¹H-NMR spectra (500 MHz, D₂O) of P5 (top, *trans*-isomer)³ and P6 (bottom, *trans*-isomer).

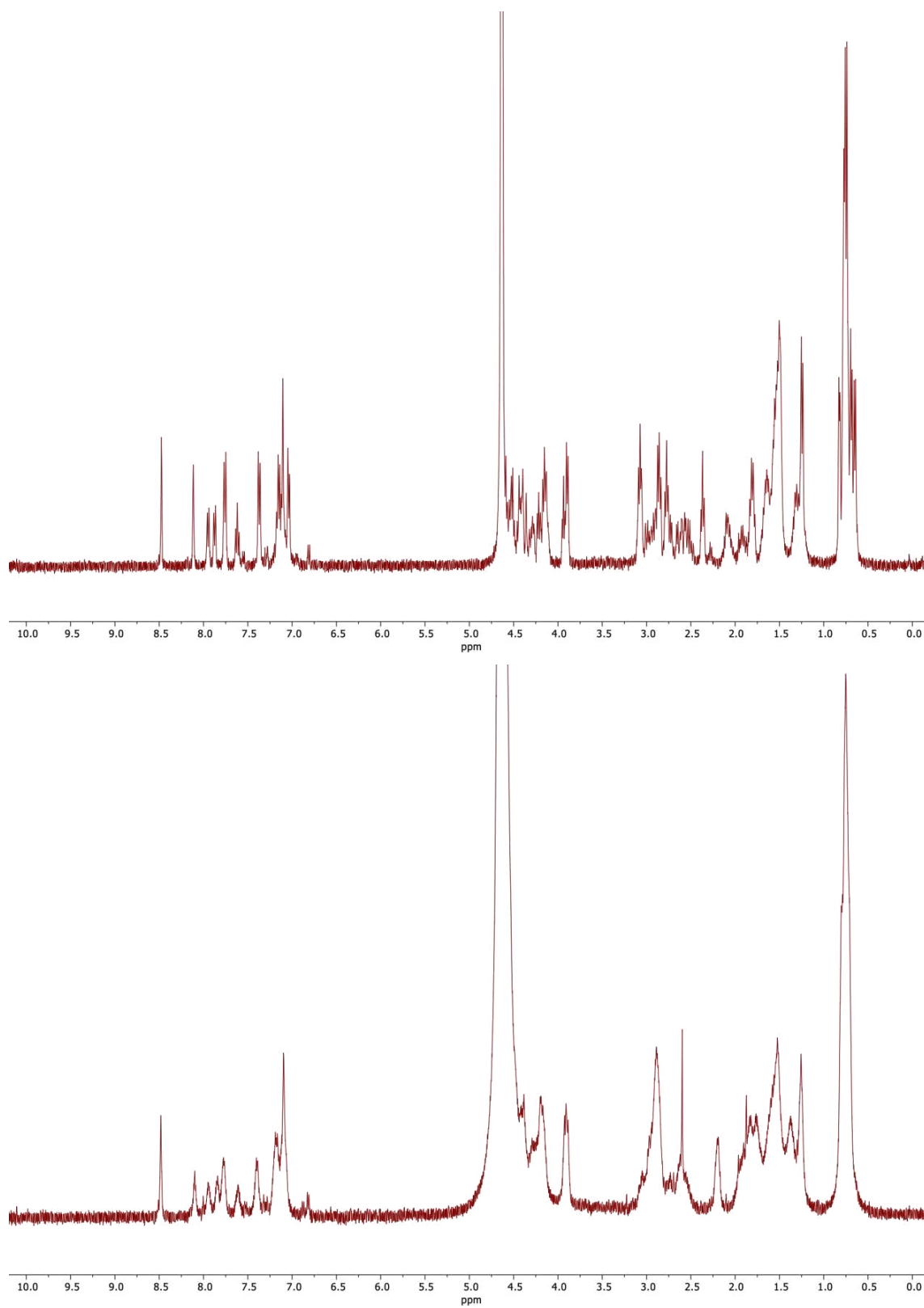


Figure S33: ^1H -NMR spectra (500 MHz, D_2O) of P7 (top, *trans*-isomer) and P8 (bottom, *trans*-isomer).

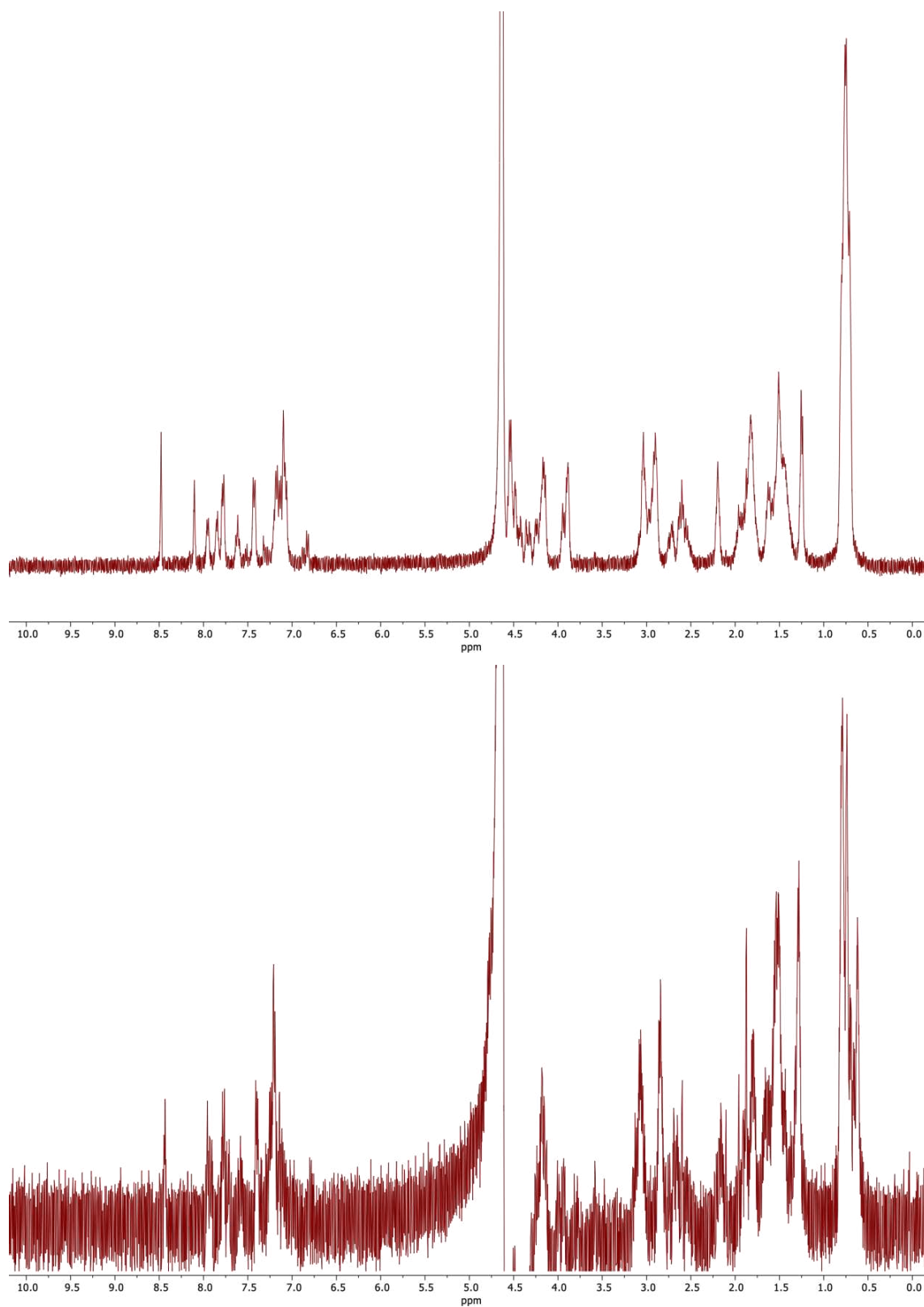


Figure S34: ^1H -NMR spectra (500 MHz, D_2O) of P9 (top, *trans*-isomer) and P10 (bottom, *trans*-isomer).

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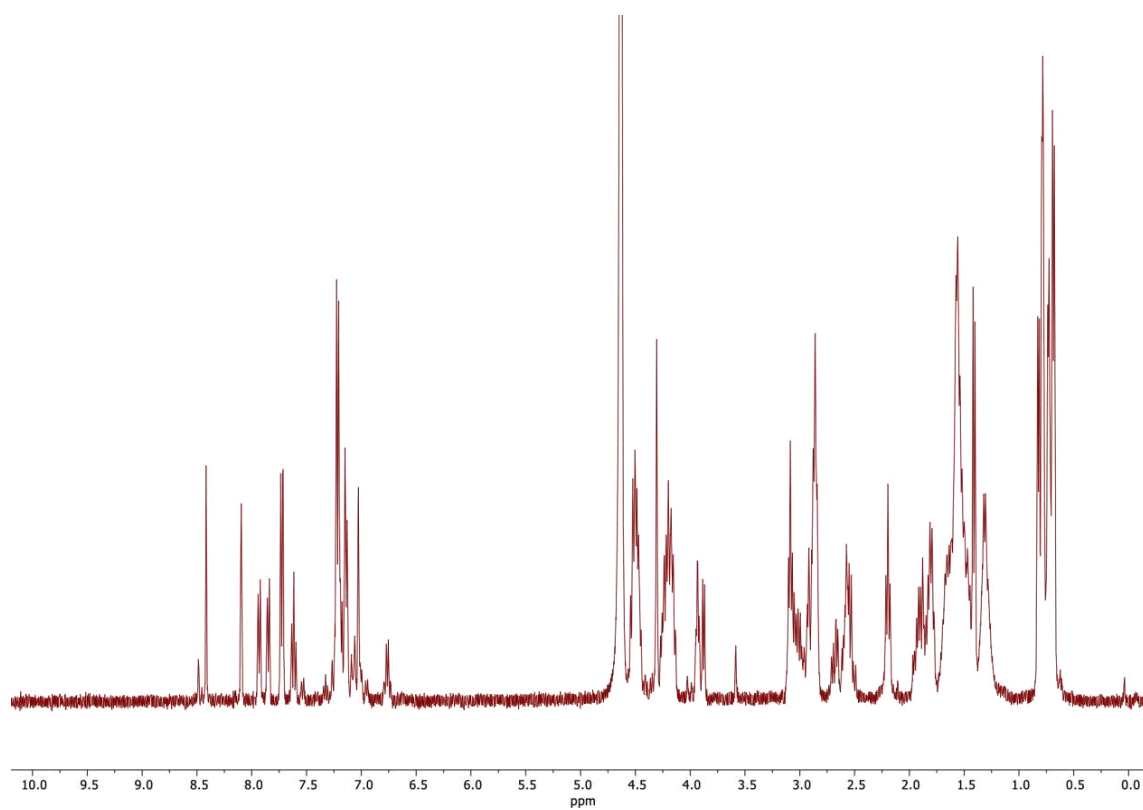
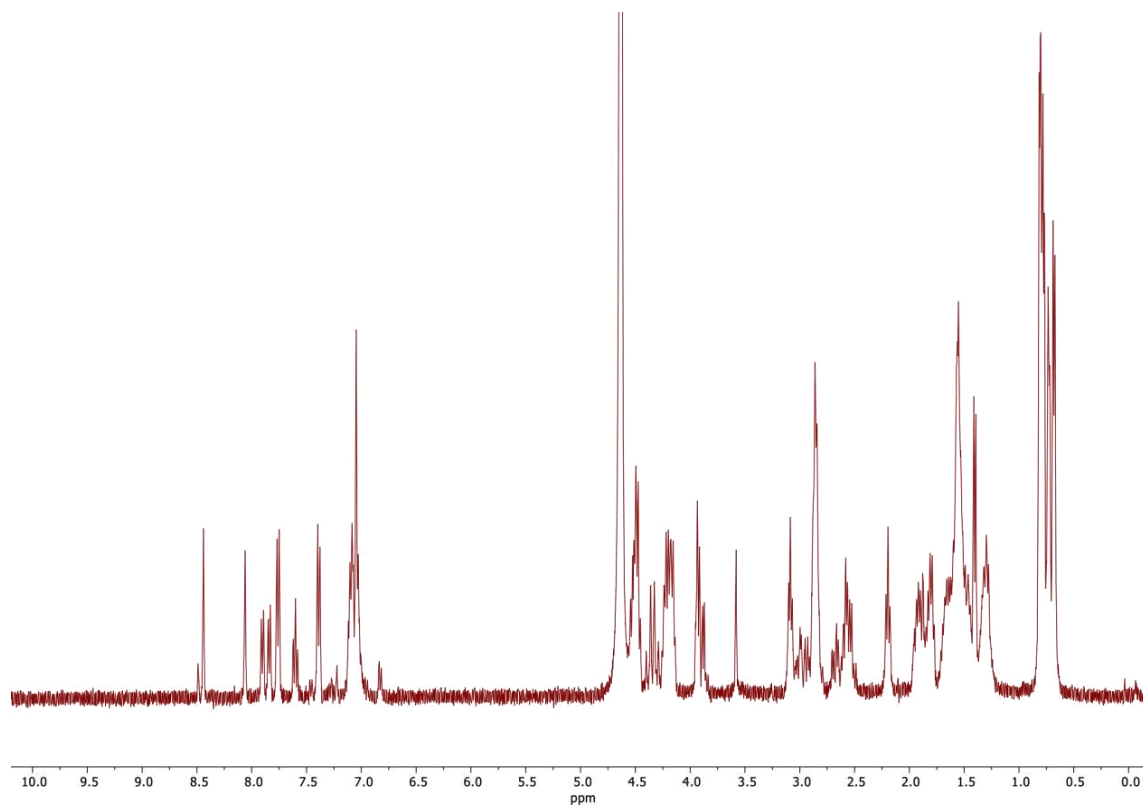


Figure S35: ¹H-NMR spectra (500 MHz, D₂O) of P11 (top, *trans*-isomer) and P12 (bottom, *trans*-isomer).

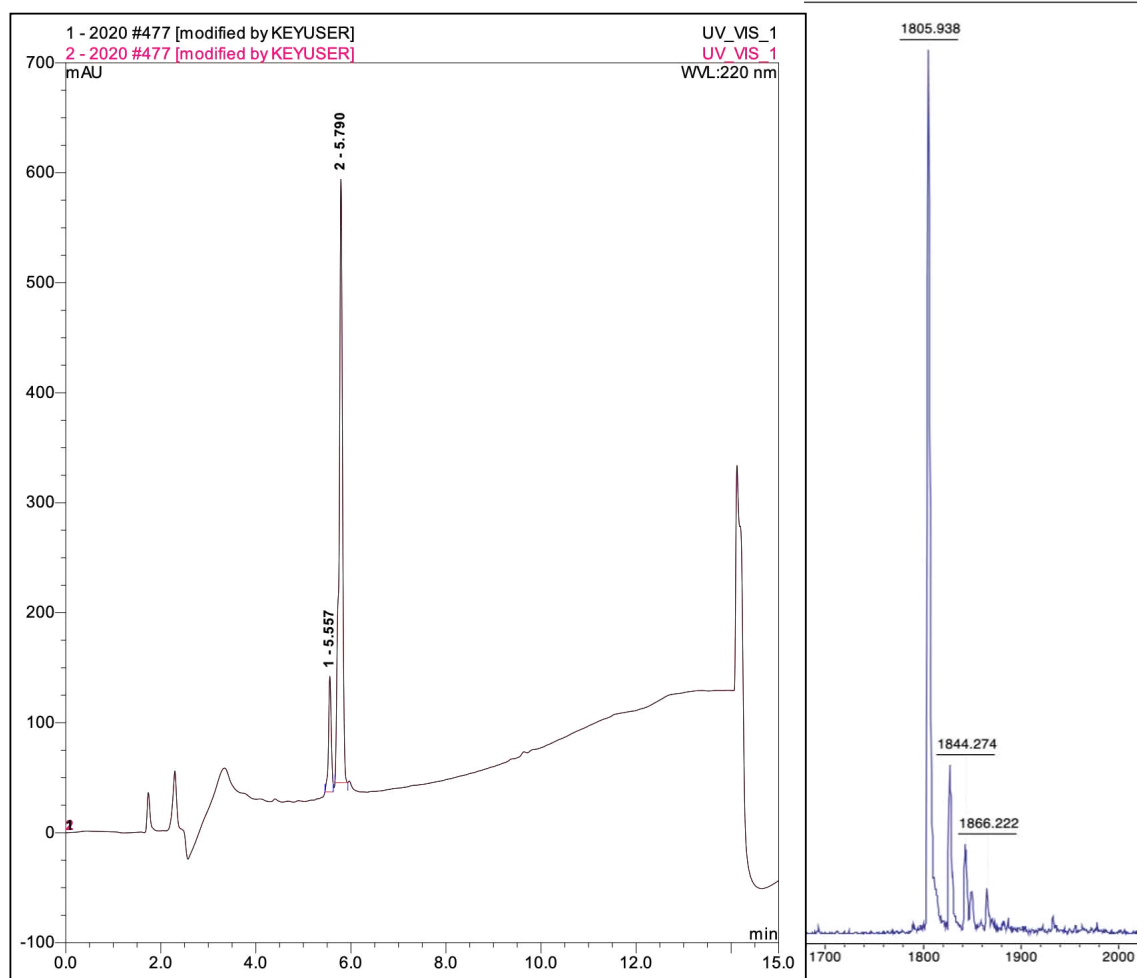


Figure S36: (left) HPLC-trace of P1 (*cis*- and *trans*-isomer between 5 and 6 min). (right) MALDI-spectrum of P1 (m/z calculated: 1805.00 found: 1805.938).³

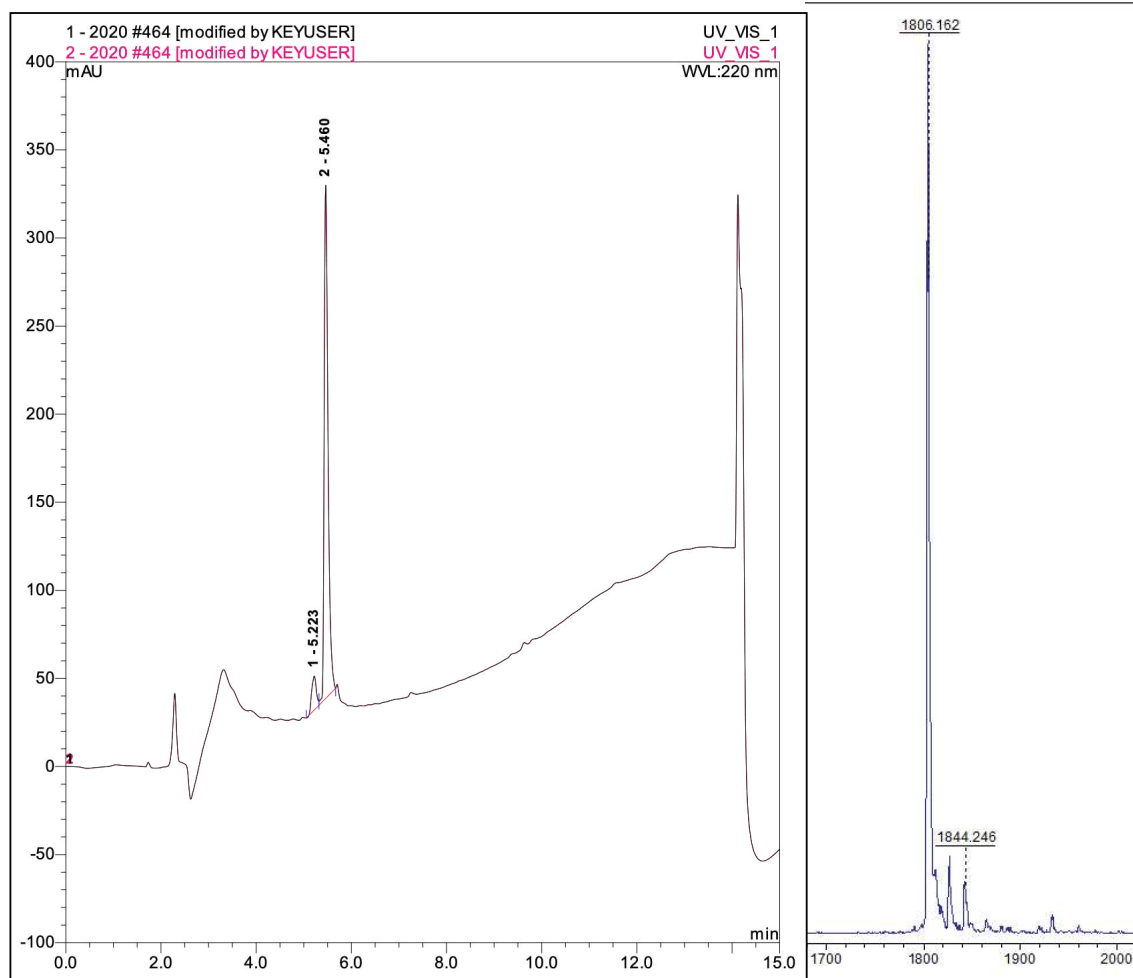


Figure S37: (left) HPLC-trace of P2 (*cis*- and *trans*-isomer between 5 and 6 min). (right) MALDI-spectrum of P2 (m/z calculated: 1805.00 found: 1806.162).³

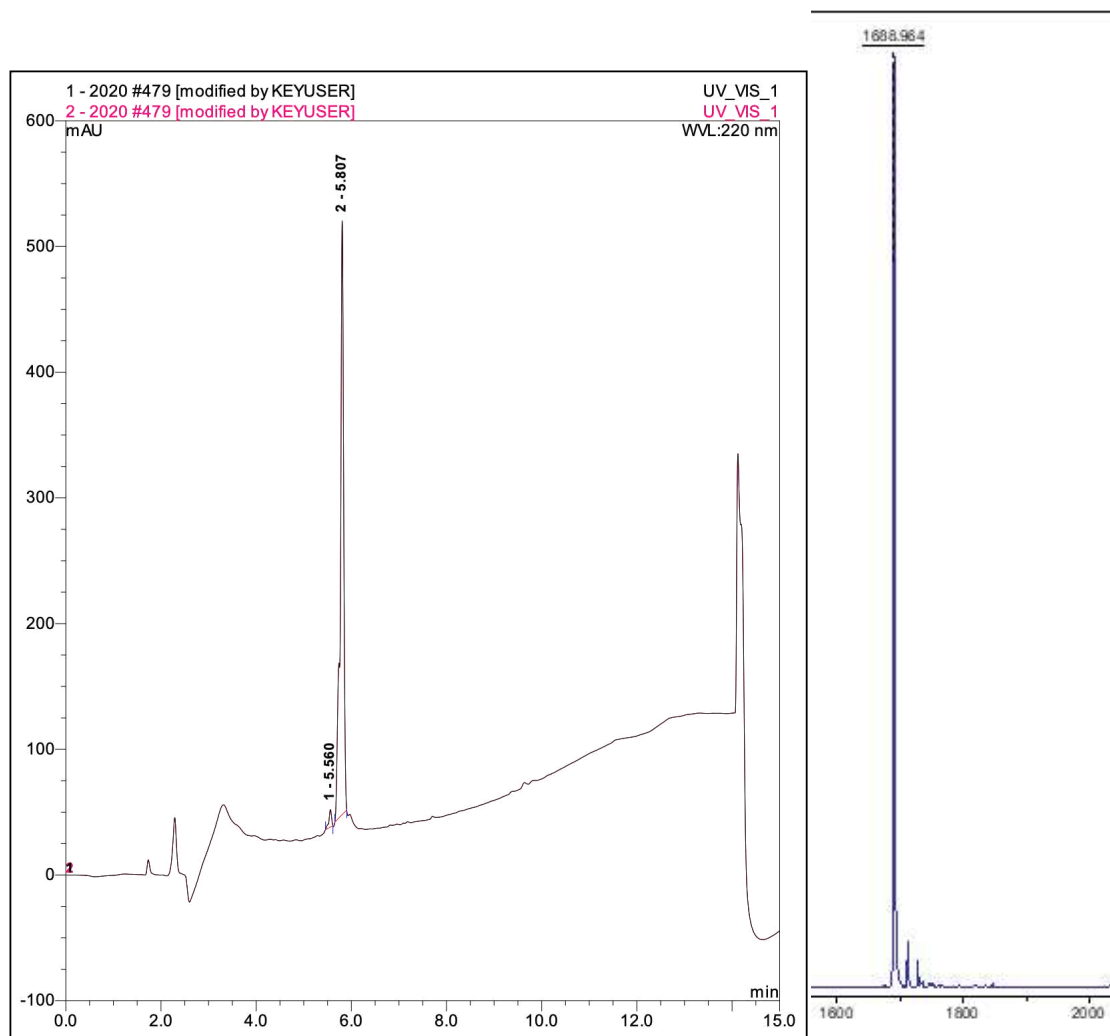


Figure S38: (left) HPLC-trace of P3 (*cis*- and *trans*-isomer between 5 and 6 min). (right) MALDI-spectrum of P3 (m/z calculated: 1689.97 found: 1688.964).³

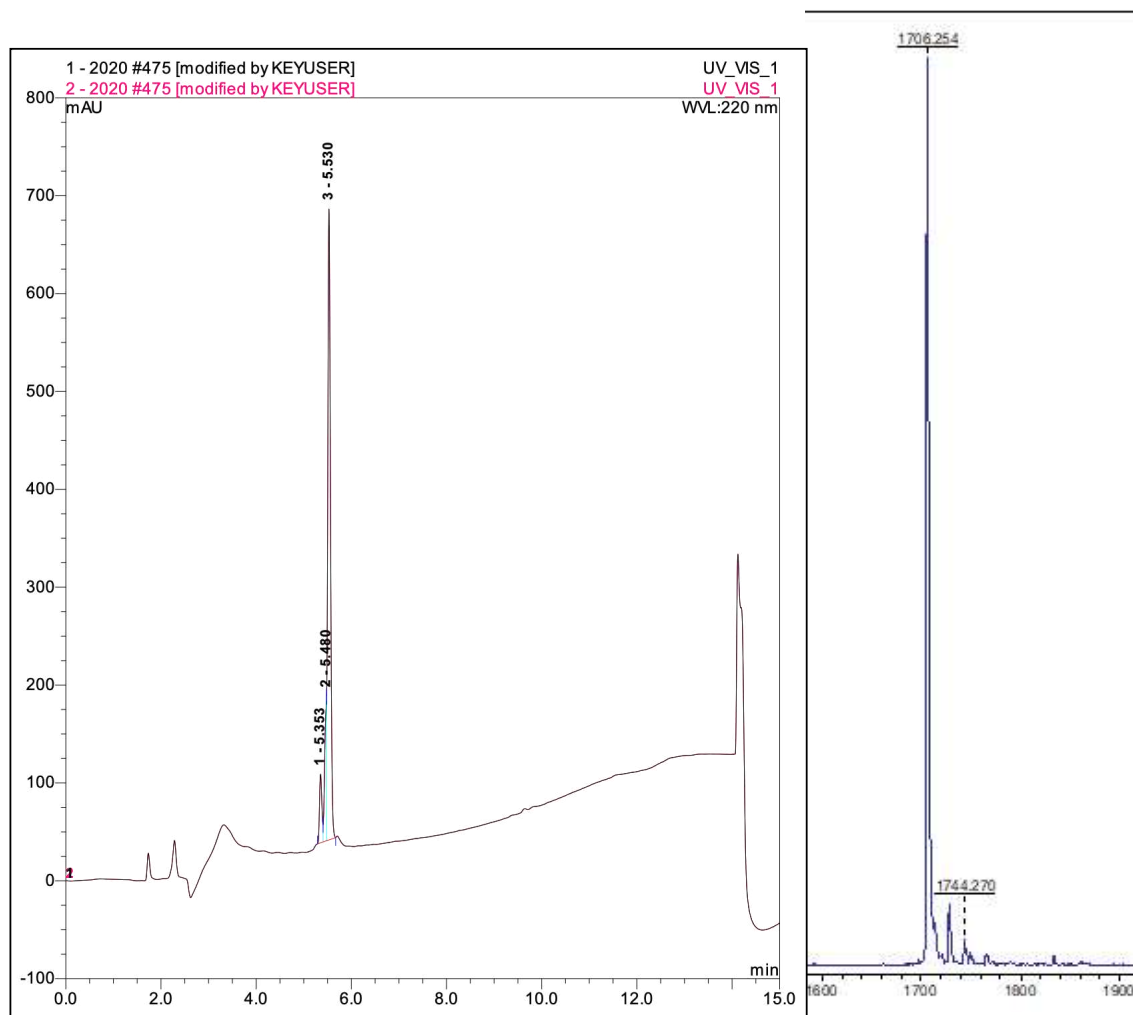


Figure S39: (left) HPLC-trace of P4 (*cis*- and *trans*-isomer between 5 and 6 min) (right) MALDI-spectrum of P4 (m/z calculated: 1705.93 found: 1706.254).³

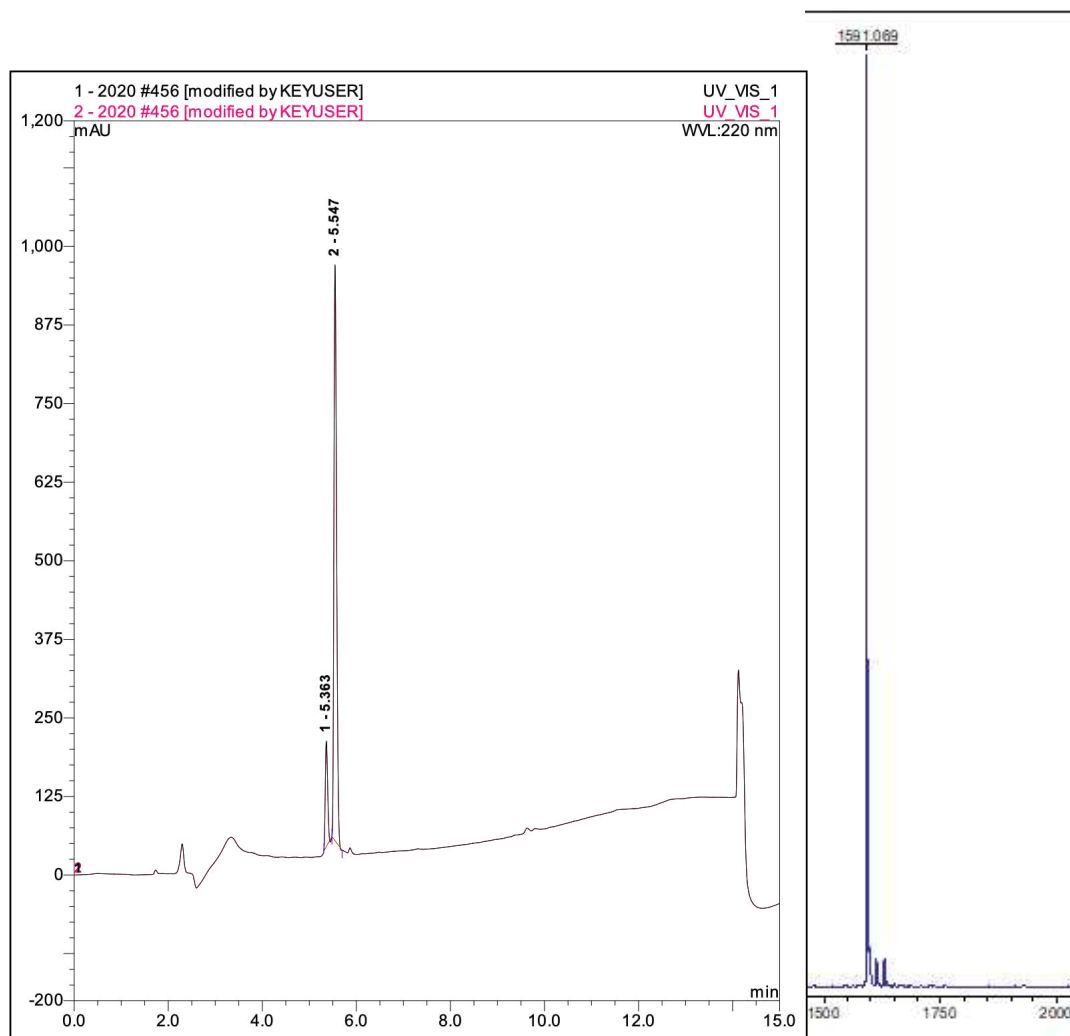


Figure S40: (left) HPLC-trace of P5 (*cis*- and *trans*-isomer between 5 and 6 min). (right) MALDI-spectrum of P5 (m/z calculated: 1590.90 found: 1591.069).³

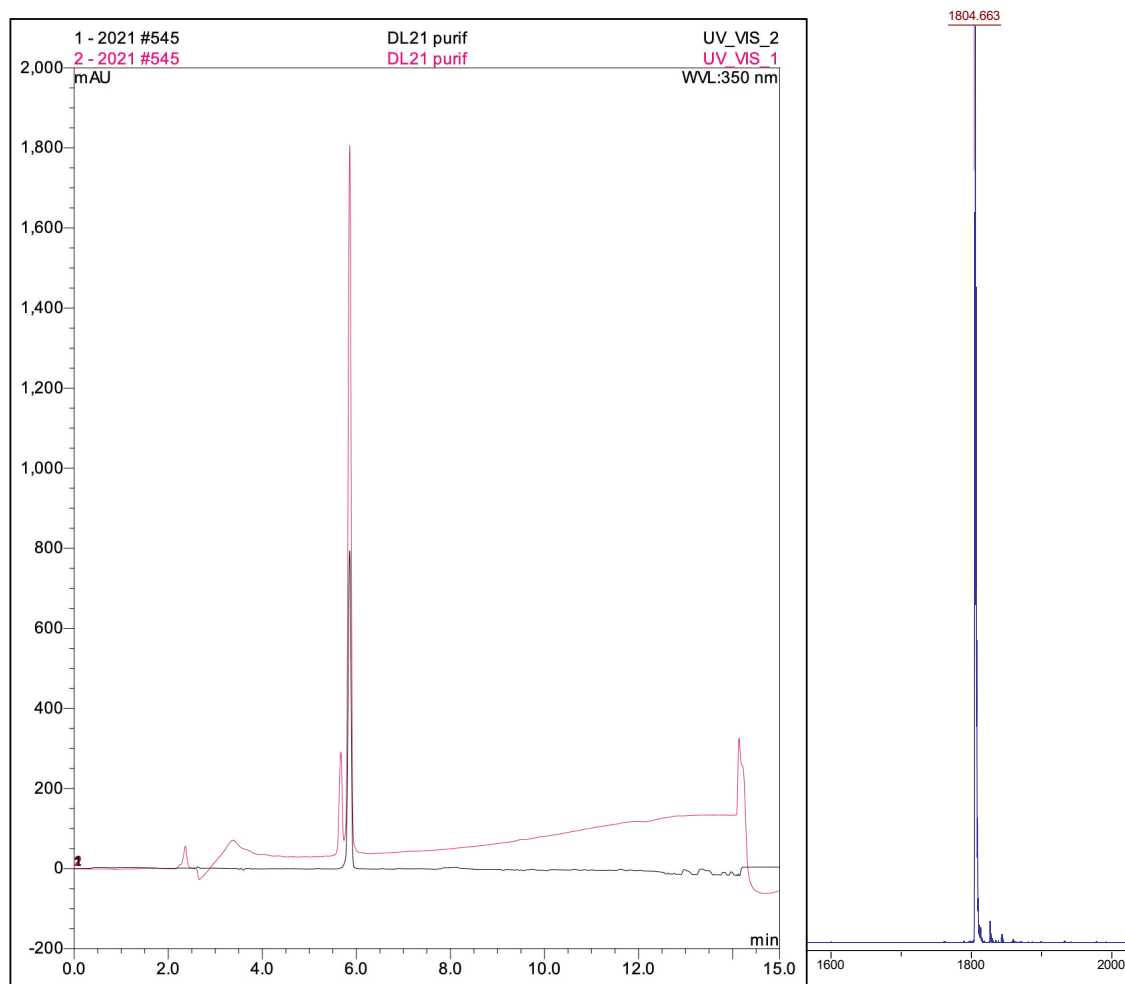


Figure S41: (left) HPLC-trace of P6 (*cis*- and *trans*-isomer between 5 and 6 min). (right) MALDI-spectrum of P6 (m/z calculated: 1805.00 found: 1804.663).

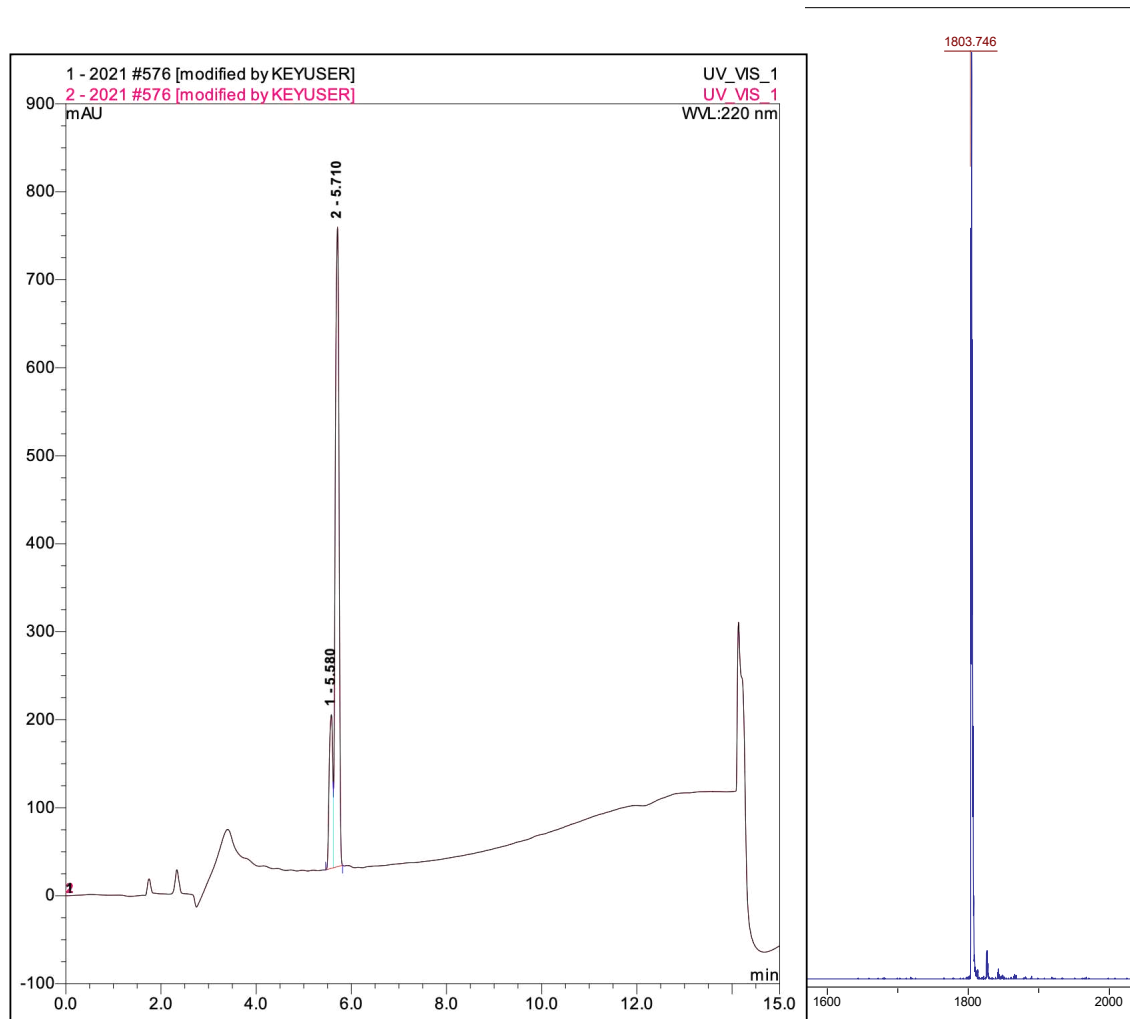


Figure S42: (left) HPLC-trace of P7 (*cis*- and *trans*-isomer between 5 and 6 min). (right) MALDI-spectrum of P7 (m/z calculated: 1805.00 found: 1803.746).

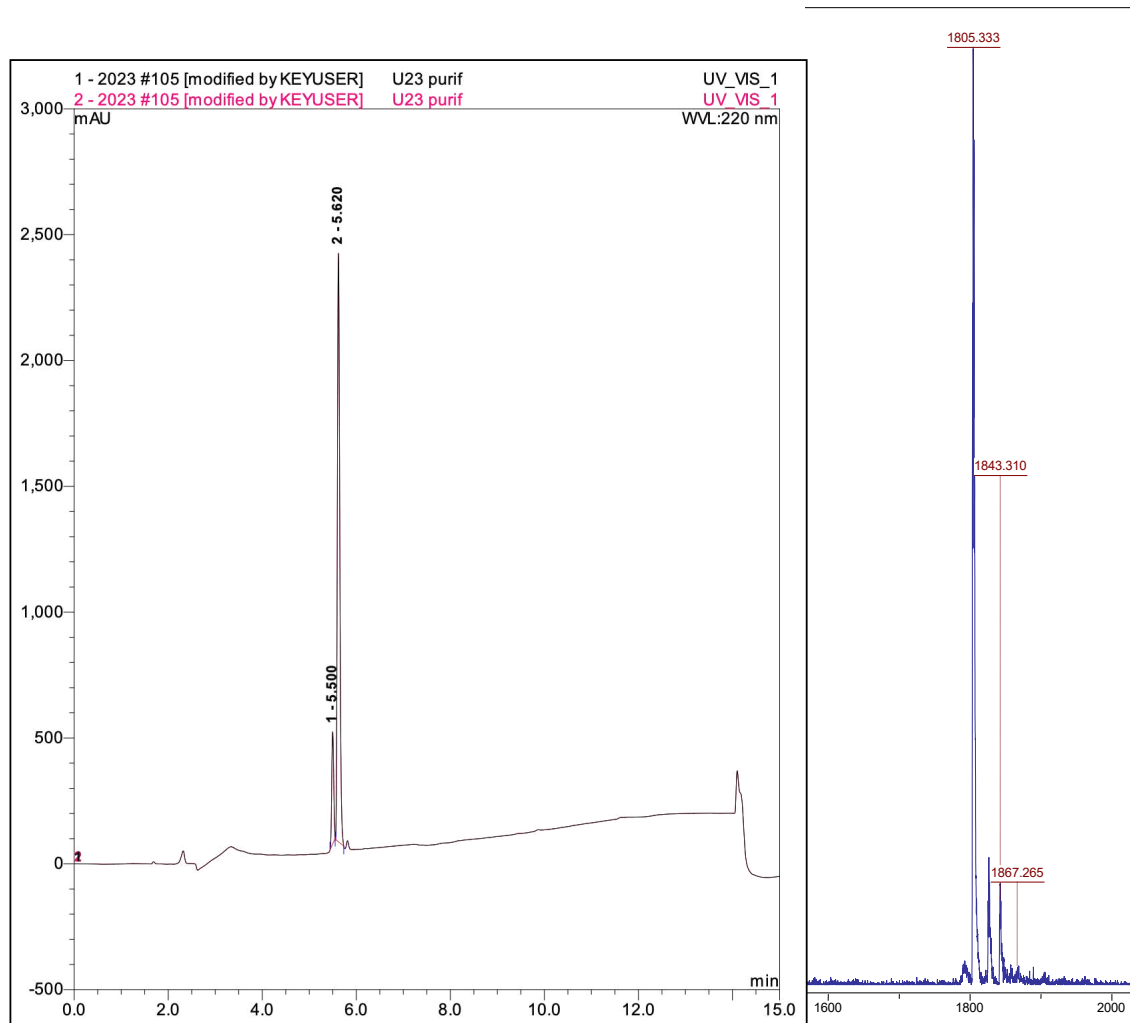


Figure S43: (left) HPLC-trace of P8 (*cis*- and *trans*-isomer between 5 and 6 min). (right) MALDI-spectrum of P8 (*m/z* calculated: 1805.00 found: 1805.333).

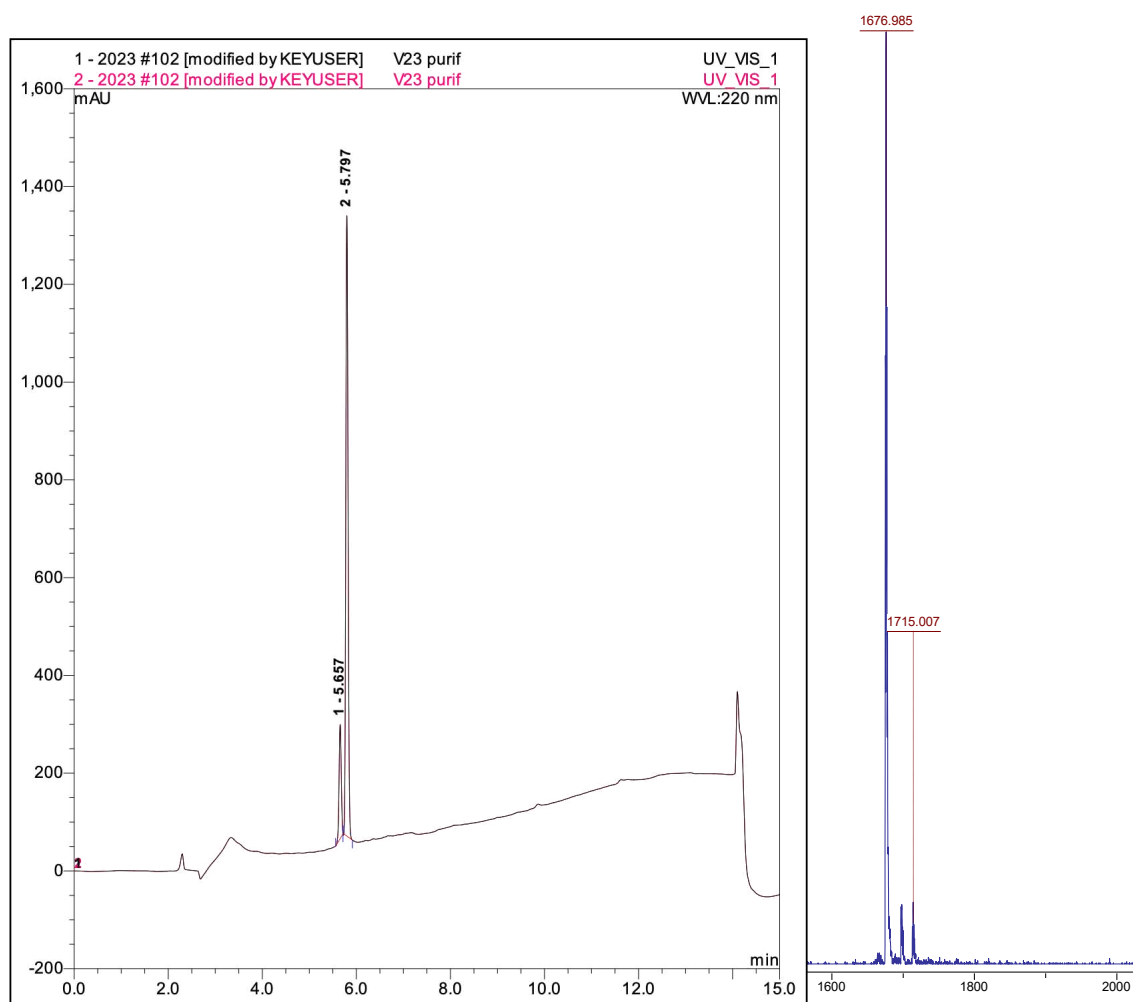


Figure S44: (left) HPLC-trace of P9 (*cis*- and *trans*-isomer between 5 and 6 min). (right) MALDI-spectrum of P9 (m/z calculated: 1676.90 found: 1676.985).

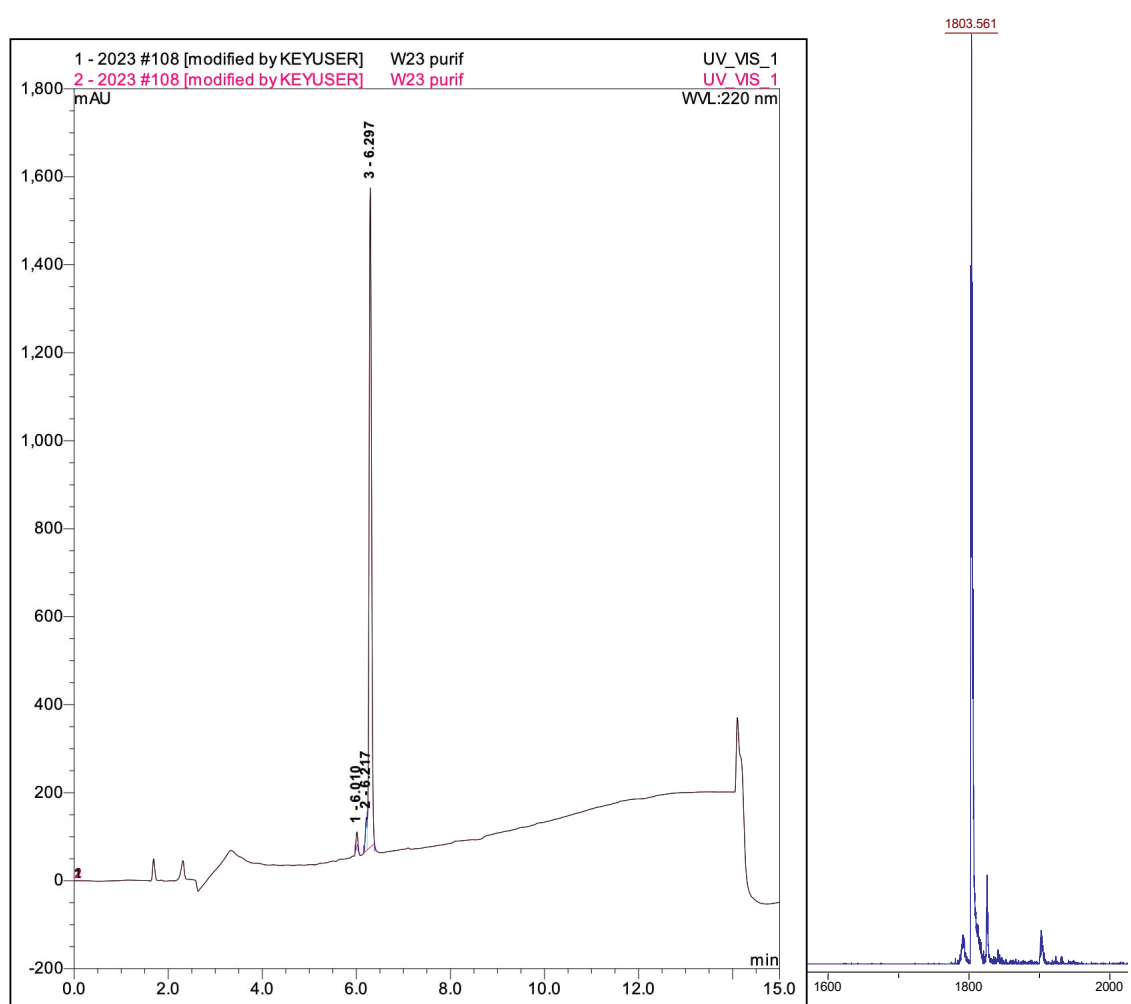


Figure S45: (left) HPLC-trace of P10 (*cis*- and *trans*-isomer between 6 and 7 min). (right) MALDI-spectrum of P10 (m/z calculated: 1805.00 found: 1803.561).

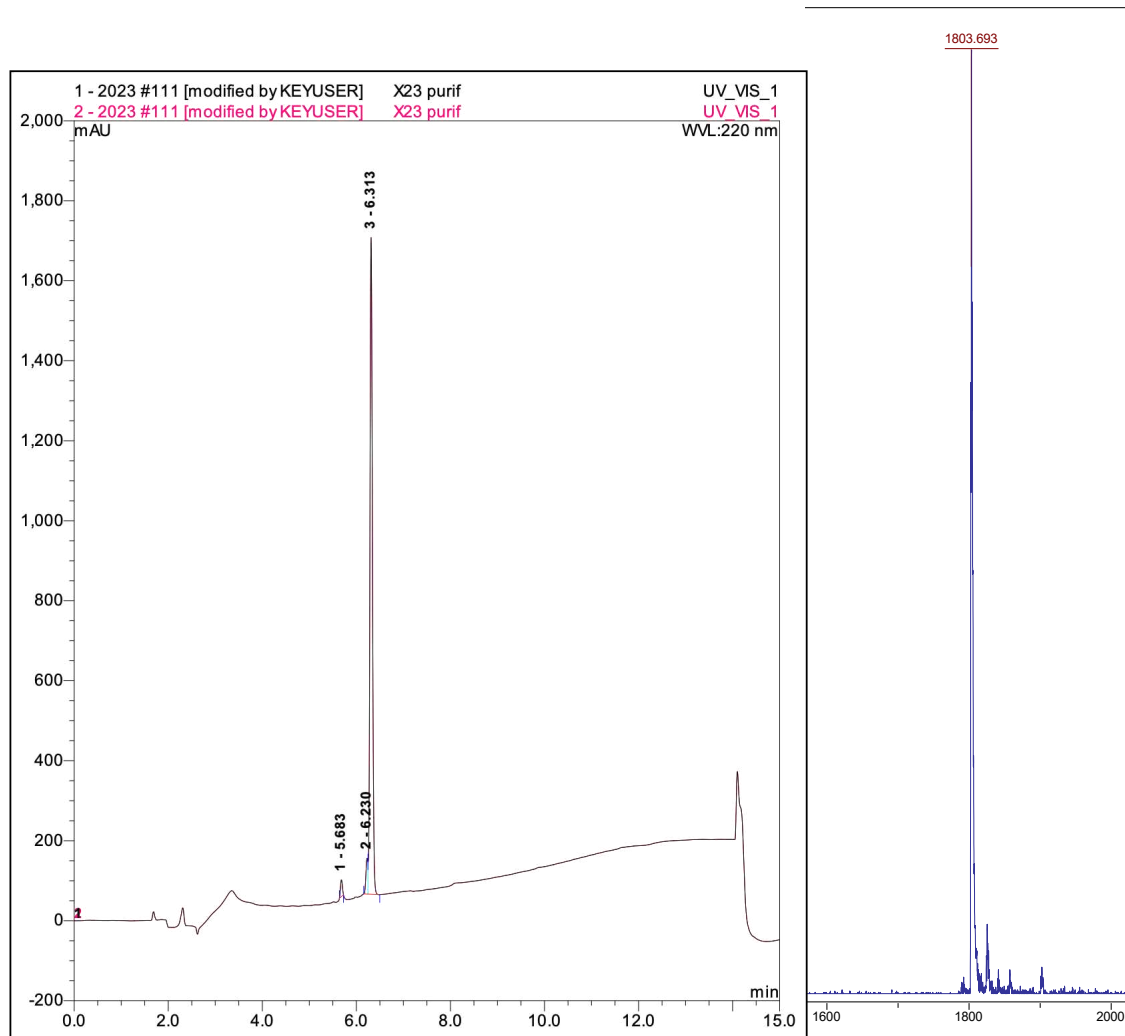


Figure S46: (left) HPLC-trace of P11 (*cis*- and *trans*-isomer between 6 and 7 min). (right) MALDI-spectrum of P11 (m/z calculated: 1805.00 found: 1803.693).

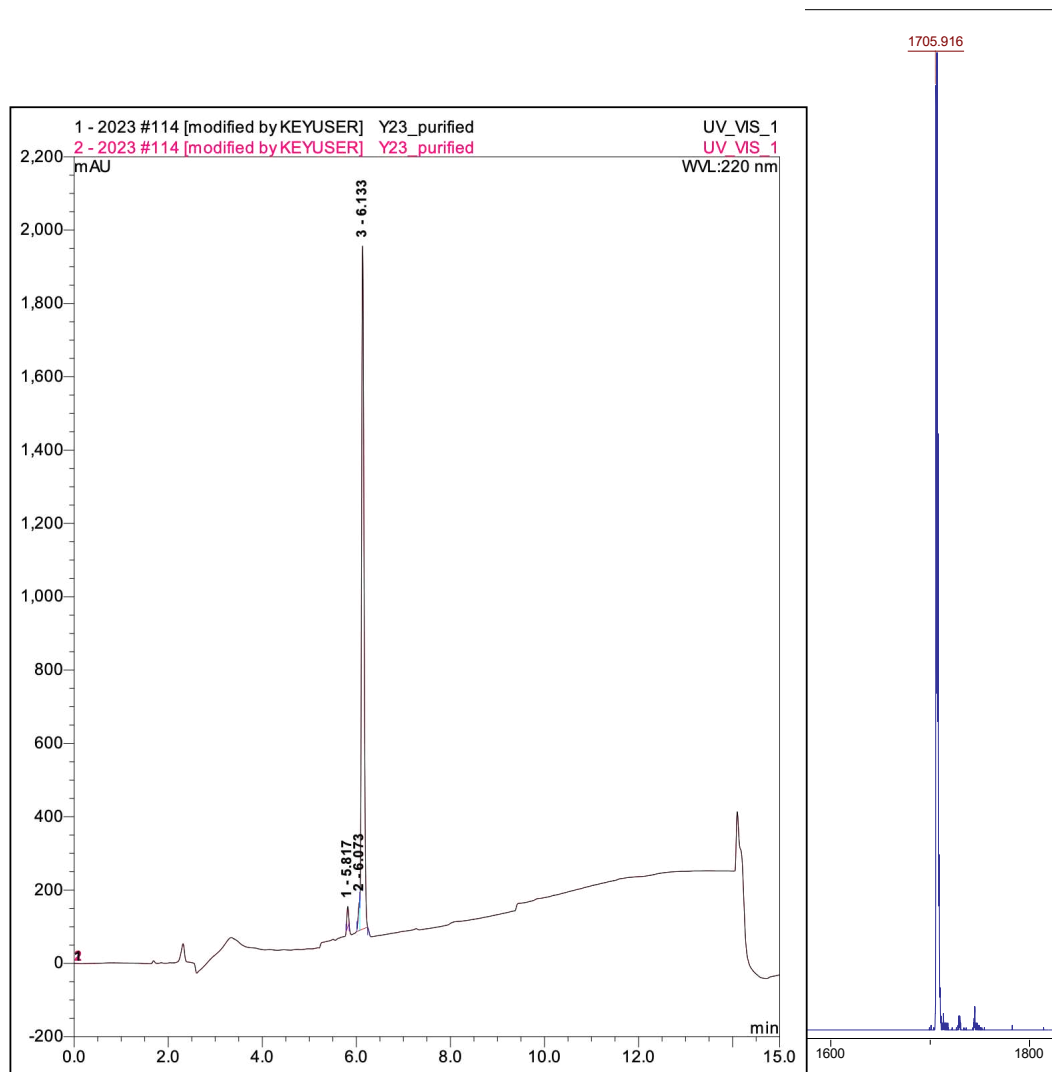


Figure S47: (left) HPLC-trace of P12 (*cis*- and *trans*-isomer between 5.5 and 6.5 min) (right) MALDI-spectrum of P12 (m/z calculated: 1705.93 found: 1705.916).

References

- (1) Jurrus, E.; Engel, D.; Star, K.; Monson, K.; Brandi, J.; Felberg, L. E.; Brookes, D. H.; Wilson, L.; Chen, J.; Liles, K. Improvements to the APBS biomolecular solvation software suite. *Protein Sci.* **2018**, 27 (1), 112-128. DOI: 10.1002/pro.3280.
- (2) Schrödinger, L. The PyMOL Molecular Graphics System, Version 1.8. In *(No Title)*, 2015.
- (3) Paschold, A.; Voigt, B.; Hause, G.; Kohlmann, T.; Rothmund, S.; Binder, W. H. Modulating the Fibrillization of Parathyroid-Hormone (PTH) Peptides: Azo-Switches as Reversible and Catalytic Entities. *Biomedicines* **2022**, 10 (7), 1512. DOI: 10.3390/biomedicines10071512.

Supporting Information

Spiropyran as building block in peptide synthesis and modulation of photochromic properties

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SUPPORTING INFORMATION

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1. Experimental procedures

1.1. Chemicals

All technical solvents were distilled prior use. Air- and moisture- sensitive reactions were carried out in flame-dried glassware under atmospheric pressure of nitrogen. 1,3,3-Trimethyl-2-methylenindolin and 4-hydrazino benzoic acid were purchased from TCI Deutschland GmbH. NaOH and NaNO₃ were purchased from Gruessing GmbH. H₂SO₄ Boc₂O, iodomethane, citric acid, sodium citrate, NaH₂PO₄, 2-hydroxy-5-nitrobenzaldehyd, and PtO₂ were purchased from Sigma Aldrich. SnCl₂ and 3-methyl-2-butanone were purchased from Merck GmbH, triethylamin (TEA) and Na₂HPO₄ from Alfa Aesar, Fmoc-Cl from Fluorochem, 3-fomyl-4-hydroxybezoic acid from abcr GmbH, Ac₂O from VWR, and Celite® from Carl Roth GmbH.

The phosphate buffer for pH 7.4 was made as a mixture of an aqueous 50 mM Na₂HPO₄ and 50 mM NaH₂PO₄-solution. The citrate buffer for pH 2.5 was made as a mixture of an aqueous 100 mM citric acid and a 100 mM sodium citrate solution. Each buffer solution was adjusted with a NaOH- and HCl-solution to the correct pH measured with a Titroline 7500 KF from SI Analytics.

1.2. Instrumentation and Analysis

ESI-ToF-MS measurements were performed on a Bruker Daltonics microTOF via direct injection at a flow rate of 180 µL h⁻¹ in positive mode with an acceleration voltage of 4.5 kV. Samples were prepared by dissolving in either LC-MS grade THF, LC-MS grade acetonitrile or LC-MS grade methanol or a mixture thereof. The instrument was calibrated using the ESI-L low concentration tuning mix from Agilent Technologies (product no. G1969-85000). The software Data Analysis (version 4.0) was used for data evaluation.

MALDI-ToF-MS measurements were performed on a Bruker Autoflex III system (Bruker Daltonics) using a nitrogen laser operating at a wavelength of $\lambda = 337$ nm in reflection mode. The used matrix:analyte ratio was 1:1 and 1 µL of the solution was spotted on the MALDI target. The peptide samples were either dissolved or suspended in MeCN + 0.1% trifluoroacetic acid (TFA) with a concentration of 20 mg/mL. Data evaluation was carried out via flexAnalysis software (3.4) and simulation of the isotopic pattern was performed by Data Analysis software (version 4.0).

NMR spectra were measured on an Agilent Technologies 400 MHz VNMRs and 500 MHz DD2 at 27°C. Chemical shifts (δ) are reported in ppm and referred to the solvent residual signal (CDCl₃ 7.26 ppm for ¹H and 77.0 ppm for ¹³C, DMSO-*d*₆ 2.50 ppm for ¹H and 39.52 ppm for ¹³C, THF-*d*₈ 1.72 and 3.58 ppm for ¹H and 67.2 and 25.3 ppm for ¹³C, and D₂O 4.66 ppm for ¹H). The following abbreviations were used for ¹H- and ¹³C-NMR peaks assignment: s = singlet, d = doublet, t = triplet, td = triplet of doublet, m = multiplet.

ATR-IR spectra were measured on a Bruker Tensor Vertex 70 equipped with a Golden Gate Heated Diamond ATR Top-plate.

UV/VIS-absorption measurements were performed on a Perkin Elmer LAMBDA 365 UV/Vis Spectrophotometer using Helma analytics quartz glass cuvettes (d = 10 mm). Temperature control was achieved using the Perkin Elmer Peltier System L365. To describe the kinetic processes, the kinetic measurements were fitted according to a first-order reaction. In the measurements at 4 °C condensation of water occurred at the cuvette walls during the measurements. The respective parts during the kinetic measurements were excluded for the fit.

MC-to-SP-isomerization was achieved by irradiation of the respective peptide dissolved in a buffered aqueous solution in a quartz glass cuvette (d = 10 mm) under stirring with light with a distinct wavelength, whereby the light source was placed 1 cm away from the cuvette. Green light (peak at 525 nm) was generated using a LED (2 W, UHP-T-520-DI, Prizmatix) together with a driver (Ultra-High-Power LED Controller, Prizmatix). For light with a wavelength of 405 nm a mounted LED (1.3 W, M405L4, Thorlabs) was used together with a LED-driver (LEDD1B, Thorlabs).

High-performance liquid chromatography (HPLC) was performed on a Hitachi Chromaster (VWR) with the modules 5430 Detector, 5310 Column Oven, 5260 Autosampler, 5160 Pump. The mobile phases were water (eluent A) and acetonitrile (eluent B), respectively, each containing 0.1% formic acid. Samples were eluted for 60 min with a linear gradient, which had to be adjusted for each peptide individually. As column a Atlantis T3 5 µm (Waters Corporation) was used. The oven temperature was set to 20 °C and the autosampler temperature to 4 °C.

Solid-phase peptide synthesis was utilized on an automated peptide synthesizer MultiPep RS (Intavis AG, Koeln, Germany) using standard Fmoc-chemistry and preloaded resin. Standard coupling of all protected natural amino acids were performed as single couplings in dimethylformamid (DMF) using 5 equivalents of amino acids and 2-(6-chloro-1-H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU) as coupling reagents and 10 equivalents of N-methyl-morpholine (NMM) as base for 1 h at room temperature. Special building groups, such as FASC-Fmoc **1a** or SAFC-Fmoc **2a**, were coupled with 3 equivalents using N,N'-diisopropylcarbodiimide (DIC) and N-Hydroxybenzotriazole (HOBT) in DMF/NMP at room temperature and with gentle shaking in the dark overnight. The N-terminal Fmoc protecting group was removed by washing the resin with 20% piperidine for 20 min. The final side chain deprotection and cleavage from the resin employed a mixture of trifluoroacetic acid and water (90:10 Vol%) with gentle agitation for 2h at room temperature.

The crude peptides were purified to >95% purity using preparative RP-HPLC (Gilson, Limburg, Germany). For both analytical and preparative use, the mobile phases were water (eluent A) and acetonitrile (eluent B), respectively, each containing 0.1% TFA. Samples were eluted with a linear gradient from 5% B to 95% B in 15 min for analytical runs and in 90 min for preparative runs on a

semipreparative PLRP-S column (Agilent Technologies, 300x25mm, 8 μ m). Finally, all peptides were characterized by analytical HPLC Dionex Ultimate 3000 (Thermo Scientific, Germany) using a PLRP-S column (Agilent Technologies, 150x4.6mm, 3 μ m) and MALDI-MS (Bruker Microflex LT, Bremen, Germany), which gave the expected $[M+H]^+$ mass peaks.

2. Supplementary tables

Table S1. Peptide primary sequences, the half-life time $\tau_{1/2}$ and rate constant k of the thermal back-isomerization from SP-to-MC and, the half-life time $\tau_{1/2}$ and rate constant k of the hydrolysis of the MC-form at different temperatures and pH values.

peptide	pH	T [°C]	thermal isomerization		decomposition	
			$\tau_{1/2}$ [min]	k [min ⁻¹]	$\tau_{1/2}$ [min]	k [min ⁻¹]
P1	7.4	37	3.7 ± 0.1	0.185	68.7 ± 0.1	0.101
		4	171.4 ± 0.3	0.0040	4418 ± 11	0.000156
	2.5	37	10.7 ± 0.0	0.0645	15620 ± 21	4.44 * 10 ⁻⁵
		4	1155 ± 0	0.000600	-	-
P2	7.4	37	2.9 ± 0.0	0.237	427.8 ± 2.1	0.00162
		4	321.4 ± 0.3	0.00216	n.d.	n.d.
	2.5	37	11.3 ± 0.0	0.0613	18733 ± 10	3.70 * 10 ⁻⁵
		4	1151 ± 2	0.000602	n.d.	n.d.
P3	7.4	37	6.0 ± 0.1	0.116	75.3 ± 0.2	0.00921
		4	358.9 ± 0.2	0.00193	n.d.	n.d.
	2.5	37	15.8 ± 0.0	0.440	n.d.	n.d.
		4	2070 ± 1	0.000335	n.d.	n.d.
P4	2.5	37	1.7 ± 0.0	0.429	2072 ± 1	0.000334
	1	37	10.04 ± 0.2	0.0666	-	-
		4	384.9 ± 0.9	0.00180	-	-

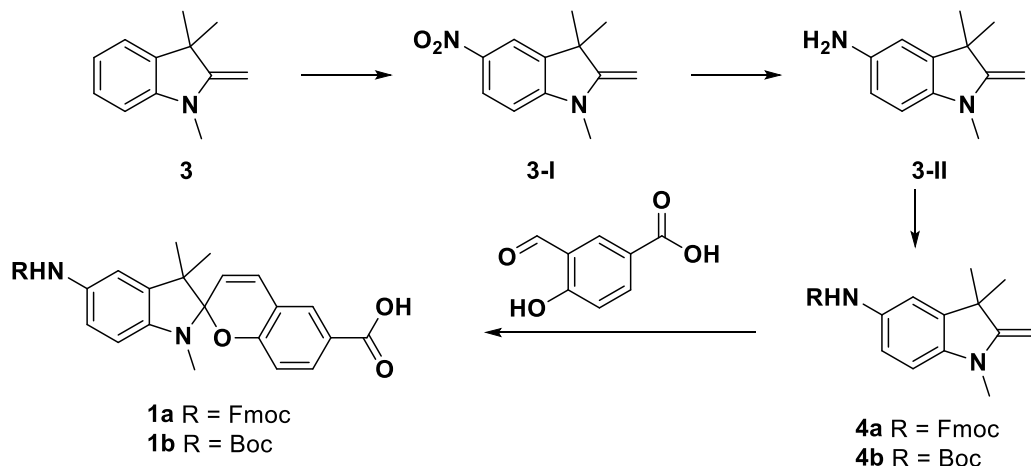
n.d. – not determined.

Table S2. Isomeric ratio in the thermal equilibrium and the SP-PSS at pH 2.5 (P1 – P3) and pH 1 (P4).

Peptide	ratio thermal equilibrium [MC:SP]	ratio PSS [MC:SP] (wavelength)
P1	98:2	4:96 (520 nm)
P2	98:2	3:97 (520 nm)
P3	98:2	5:95 (520 nm)
P4	98:2	49:51 (405 nm) 38:62 (520 nm)

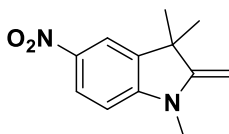
3. Synthetis procedures & analytical data

Synthesis strategy 1



Scheme S1. Synthesis pathway for spiropyrans **1a** and **1b**.

The overall yield over 4 steps was for **1a** 4% and for **1b** 18%, respectively. Especially **3-II**, **4a**, and **4b** are not completely stable at air and should therefore be processed faster.



Synthesis of 3-I

1,3,3-Trimethyl-2-methylenindolin (10 g, 57.7 mmol, 1 eq.) was slowly added to 60 mL concentrated H_2SO_4 and cooled down to 0 °C. Sodiumnitrate (7.9 g, 63.5 mmol, 1.1 eq.) was added in small portions and the reaction mixture is stirred for 1 h. The solution was poured onto 100 g ice and neutralized with solid Na_2CO_3 . The formed precipitate was filtrated and recrystallized (heated with an oil bath) in a mixture of acetone/water(1:1). **3-I** was obtained as a darkred solid. (7.3 g, 33.2 mmol, 58%).

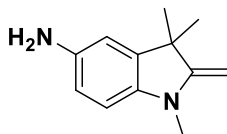
Analytical data match reported literature values.¹

$R_f = 0.74$ (hexane:EE / 3:2).

$^1\text{H-NMR}$ (500 MHz, CDCl_3): $\delta = 8.12$ (dd, $J = 8.8, 2.3$ Hz, 1H), 7.92 (d, $J = 2.3$ Hz, 1H), 6.51 (d, $J = 8.8$ Hz, 1H), 4.16–4.06 (m, 2H), 3.12 (s, 3H), 1.36 (s, 6H).

$^{13}\text{C-NMR}$ (125 MHz, CDCl_3): $\delta = 161.2, 151.5, 140.0, 138.2, 126.2, 118.1, 103.9, 78.9, 43.4, 29.7, 29.0$.

MS (ESI) m/z : $[\text{M}+\text{Li}]^+$ Calcd. for $[\text{C}_{12}\text{H}_{14}\text{N}_2\text{O}_2\text{Li}]^+$ 225.12; Found 225.12.



Synthesis of **3-II**

3-I (2 g, 9.1 mmol, 1 eq.) and dry tin(II) chloride (10.4 g, 55 mmol, 6 eq.) were dissolved in 60 mL concentrated aqueous hydrogen chloride solution and refluxed (heated with an oil bath) for 2 h. The reaction solution was alkalized and extracted three times with 60 mL Et₂O. The organic phases were combined and the solvent removed *in vacuo*. The residue was recrystallized in hexane (heated with an oil bath) and **3-II** was obtained as a green solid (1 g, 5.3 mmol, 58%).

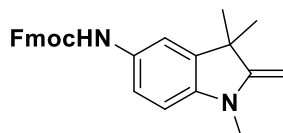
Analytical data match reported literature values.²

R_f = 0.1 (DCM:MeOH + 0.1% FA / 9:1).

¹H-NMR (500 MHz, CDCl₃): δ = 6.56 (d, *J* = 2.3 Hz, 1H), 6.51 (dd, *J* = 8.1, 2.3 Hz, 1H), 6.35 (d, *J* = 8.0 Hz, 1H), 3.79–3.72 (m, 2H), 3.35 (s, 2H), 2.99 (s, 3H), 1.33 (s, 6H).

¹³C-NMR (125 MHz, CDCl₃): δ = 163.4, 139.7, 138.9, 138.5, 114.1, 111.3, 105.1, 71.5, 44.4, 29.9, 29.0.

MS (ESI) *m/z*: [M+H]⁺ Calcd. for [C₁₂H₁₇N₂]⁺ 189.14; Found 189.14.



Synthesis of **4a**

To a mixture of **3-II** (0.5 g, 2.7 mmol, 1 eq.) and Fmoc-Cl (0.82 g, 3.2 mmol, 1.2 eq.) were added 4 mL water and the suspension was stirred at 60 °C (heated with an oil bath) for 2 h. The reaction suspension was neutralized with saturated aqueous NaHCO₃ solution and extracted three times with 60 mL DCM. The organic phases were combined and the solvent removed *in vacuo*. The residue was purified by flash chromatography on normal phase silica gel (solvent A: DCM + 0.1% FA, solvent B: MeOH + 0.1% FA, 95% A) and **4a** was obtained as a lightpurple solid. (0.38 g, 0.93 mmol, 35%).

R_f = 0.16 (DCM:MeOH + 0.1% FA / 9:1).

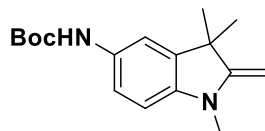
¹H-NMR (500 MHz, CDCl₃): δ = 7.78 (d, *J* = 7.5 Hz, 2H), 7.69–7.52 (m, 2H), 7.41 (t, *J* = 7.4 Hz, 2H), 7.37–7.29 (m, 2H), 7.24–7.15 (m, 1H), 7.05 (s, 1H), 6.52 (s, 1H), 6.45 (d, *J* = 8.3 Hz, 1H), 4.50 (d, *J* = 6.8 Hz, 2H), 4.28 (t, *J* = 6.9 Hz, 1H), 3.84 (q, *J* = 2.0 Hz, 2H), 3.02 (s, 3H), 1.34 (s, 6H).

¹³C-NMR (125 MHz, CDCl₃): δ = 162.8, 154.1, 143.9, 143.2, 141.3, 138.3, 129.2, 127.7, 127.1, 125.0, 120.0, 119.2, 114.9, 104.6, 73.1, 66.7, 47.2, 44.3, 29.9, 28.9.

IR (ATR): $\nu = 3303_{\text{w}}, 2959_{\text{w}}, 1697_{\text{s}}, 1499_{\text{s}}, 1208_{\text{s}} \text{ cm}^{-1}$.

UV/VIS ($c = 0.06 \mu\text{mol mL}^{-1}$ in MeOH): $\lambda_{\text{max},1} = 289.2, \lambda_{\text{max},2} = 277.3, \lambda_{\text{max},3} = 265.3 \text{ nm}$.

HR-MS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calcd. for $[\text{C}_{27}\text{H}_{27}\text{N}_2\text{O}_2]^+$ 411.2106; Found: 411.2119.



Synthesis of 4b

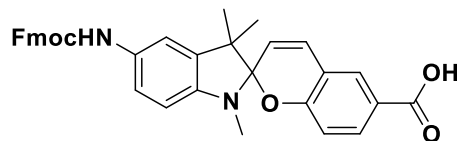
3-II (585 mg, 3.1, mmol, 1 eq.) were dissolved in 40 mL THF. Boc_2O (680 mg, 3.1 mmol, 1 eq.) and TEA (440 μL , 3.1 mmol, 1 eq.) were added under a nitrogen atmosphere and the reaction mixture was stirred at ambient temperature for 18 h. The solvent was removed *in vacuo* and the residue was dissolved in 50 mL DCM. The solution was extracted three times with 50 mL of an aqueous solution with 5% acetic acid. The combined aqueous phases were washed two times with 50 mL Et_2O and the solution was made alkaline (pH 9 – 10) with NaHCO_3 . The aqueous solution was extracted three times with 50 mL DCM. The combined organic phases were dried over Na_2SO_4 and the solvent was removed *in vacuo*. The residue of **4b** (yellowish oil, 760 mg) was used without further purification.

$R_f = 0.39$ (DCM:MeOH / 19:1).

$^1\text{H-NMR}$ (400 MHz, CDCl_3) $\delta = 7.17$ (s, 1H), 7.02 – 6.90 (m, 1H), 6.41 (d, $J = 8.3 \text{ Hz}$, 1H), 6.26 (s, 1H), 3.79 (q, $J = 2.0 \text{ Hz}$, 2H), 2.99 (s, 3H), 1.49 (s, 9H), 1.31 (s, 6H).

$^{13}\text{C-NMR}$ (100 MHz, CDCl_3) $\delta = 163.0, 155.0, 142.7, 138.2, 129.7, 118.7, 114.7, 104.5, 79.6, 72.7, 44.3, 29.9, 28.9, 28.4$.

MS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calcd. for $[\text{C}_{17}\text{H}_{25}\text{N}_2\text{O}_2]^+$ 289.19; Found 289.19.



Synthesis of 1a

4a (0.3 g, 0.73 mmol, 1 eq.) and 3-formyl-4-hydroxybenzoic acid (0.16 g, 0.95 mmol, 1.3 eq.) were dissolved in 25 mL MeCN for 6 h and refluxed (heated with an oil bath) under a nitrogen atmosphere. The solvent was removed *in vacuo* and the residue was purified by flash chromatography on normal phase silica gel (solvent A: hexane + 0.1% FA, solvent B: EE + 0.1% FA, 60% A). **1a** was obtained as a redish solid (0.17 g, 0.3 mmol, 42%).

$R_f = 0.45$ (hexane:EE + 0.1% FA / 3:2).

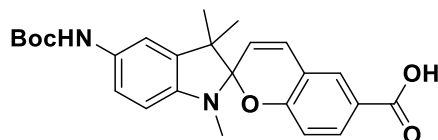
^1H -NMR (500 MHz, $\text{DMSO}-d_6$): δ = 12.60 (s, 1H), 9.40 (s, 1H), 7.89 (d, J = 7.0 Hz, 2H), 7.79 (d, J = 2.2 Hz, 1H), 7.77 – 7.69 (m, 2H), 7.67 (dd, J = 8.5, 2.2 Hz, 1H), 7.41 (t, J = 7.4 Hz, 2H), 7.34 (t, J = 7.4 Hz, 2H), 7.23 (s, 1H), 7.15 (s, 1H), 7.10 (d, J = 10.5 Hz, 1H), 6.73 (d, J = 8.5 Hz, 1H), 6.49 (d, J = 8.3 Hz, 1H), 5.82 (d, J = 10.2 Hz, 1H), 4.41 (d, J = 6.9 Hz, 2H), 4.28 (t, J = 6.9 Hz, 1H), 2.60 (s, 3H), 1.17 – 1.07 (m, 6H).

^{13}C -NMR (125 MHz, $\text{DMSO}-d_6$): δ = 167.2, 158.1, 154.2, 144.3, 141.2, 137.3, 136.9, 131.8, 131.2, 129.5, 129.0, 128.1, 127.6, 125.6, 123.2, 120.6, 120.2, 118.9, 118.0, 114.9, 107.3, 105.7, 65.9, 52.1, 47.2, 40.6, 40.4, 40.3, 40.1, 39.9, 39.8, 39.6, 29.2, 26.1, 20.1.

IR (ATR): ν = 3338w, 2968br, 1693s, 1234m, 1208s cm^{-1} .

UV/VIS (c = 0.46 $\mu\text{mol mL}^{-1}$ in MeOH): $\lambda_{\text{max},1}$ = 443.7 nm.

HR-MS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calcd. for $[\text{C}_{35}\text{H}_{31}\text{N}_2\text{O}_5]^+$ 559.2227; Found 559.2258.



Synthesis of **1b**

The unpurified residue of **7** (700 mg, ~2.43 mmol, 1 eq.) and 3-formyl-4-hydroxybenzoic acid (400 mg, 2.43 mmol, 1 eq.) were dissolved in 50 mL MeCN and the solution was refluxed (heated with an oil bath) for 4.5 h under a nitrogen atmosphere. The solvent was removed *in vacuo* and the residue was purified by flash chromatography in normal phase silica (solvent A: hexane + 0.1% FA, solvent B: EE + 0.1% FA, 60% A). **1b** was obtained as a redish solid (0.680 mg, 1.56 mmol, 64%).

R_f = 0.41 (hexane:EE + 0.1% FA / 3:2).

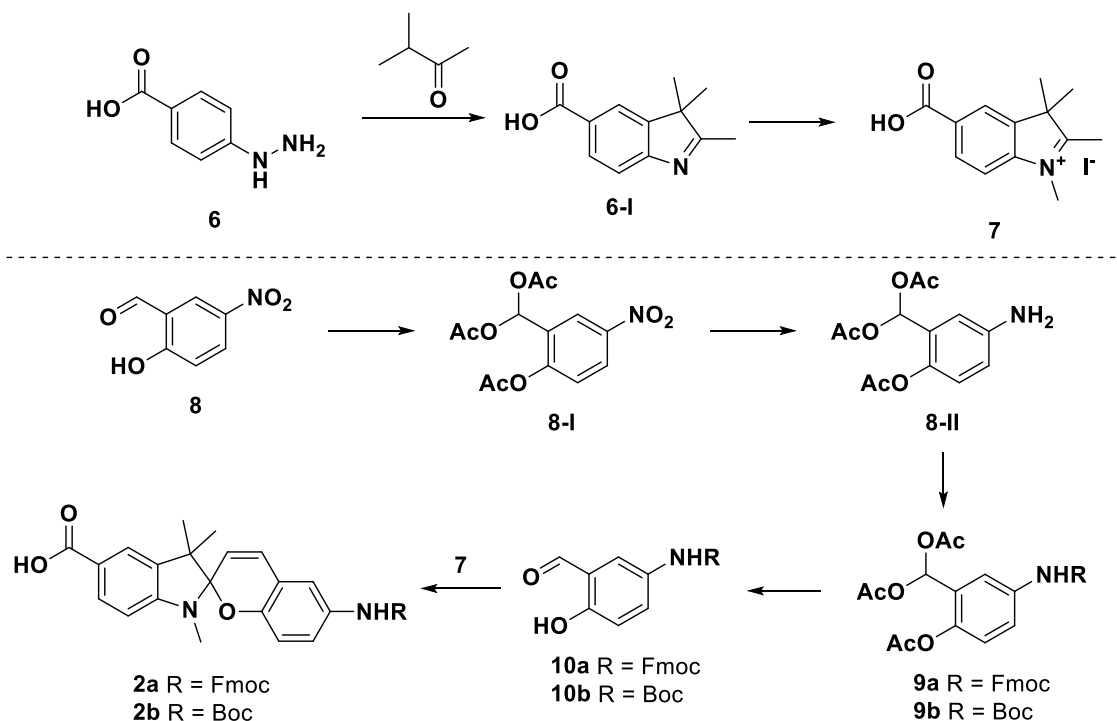
^1H -NMR (500 MHz, $\text{DMSO}-d_6$): δ = 12.65 (s, 1H), 8.97 (s, 1H), 7.80 (d, J = 2.3 Hz, 1H), 7.68 (dd, J = 8.5, 2.3 Hz, 1H), 7.25 (s, 1H), 7.18 – 6.99 (m, 2H), 6.74 (d, J = 8.6 Hz, 1H), 6.48 (d, J = 8.3 Hz, 1H), 5.83 (d, J = 10.3 Hz, 1H), 2.61 (s, 3H), 1.46 (s, 9H), 1.17 (s, 3H), 1.10 (s, 3H).

^{13}C -NMR (125 MHz, $\text{DMSO}-d_6$): δ = 166.7, 157.7, 153.1, 143.2, 136.3, 131.3, 129.0, 128.5, 122.7, 122.0, 119.8, 118.4, 117.5, 114.4, 113.6, 106.7, 105.3, 78.4, 51.6, 28.7, 28.2, 25.6, 19.7.

IR (ATR): ν = 3319w, 3069w, 2973br, 1683s, 1158s cm^{-1} .

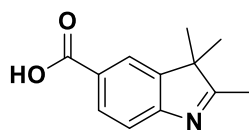
HR-MS (ESI) m/z : $[\text{M}+\text{Na}]^+$ Calcd. for $[\text{C}_{25}\text{H}_{28}\text{N}_2\text{O}_5\text{Na}]^+$ 459.1890; Found 459.1909.

Synthesis strategy 2



Scheme S2. Synthesis pathway for spiropyrans **2a** and **2b**.

The overall yield over 6 consecutive steps was for **2a** 4% and for **2b** 3%, respectively. Especially **8-II**, was not completely stable at air and should therefore be processed faster. Furthermore, the actual indole precursor was generated in the last step from **7** *in situ* through deprotonation. A more convenient method could be to produce and isolate the respective indole precursor in an additional step.



Synthesis of 6-I

4-Hydrazinobenzoic acid **6** (5 g, 33 mmol, 1 eq.) and 3-methyl-2-butanone (5 ml, 59 mmol, 1.8 eq.) were added to 50 mL acetic acid and refluxed (heated with an oil bath) for 16 h. The solvent was removed *in vacuo*, the residue redissolved in 50 mL DCM and neutralized with saturated aqueous NaHCO₃-solution. The combined organic phases were dried over Na₂SO₄ and the solvent was removed *in vacuo* to obtain **6-I** as an orange solid (1.3 g, 6.4 mmol, 19%).

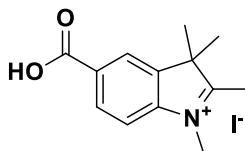
Analytical data match reported literature values.³

R_f = 0.74 (DCM:MeOH + 0.1% FA / 9:1).

¹H-NMR (500 MHz, CDCl₃): δ = 12.76 (s, 1H), 7.97 (d, *J* = 1.6 Hz, 1H), 7.90 (dd, *J* = 8.1, 1.5 Hz, 1H), 7.49 (dd, *J* = 8.0, 1.2 Hz, 1H), 2.23 (s, 3H), 1.25 (s, 6H).

¹³C-NMR (125 MHz, CDCl₃): δ = 192.1, 167.9, 157.9, 146.6, 130.0, 127.7, 123.2, 119.6, 53.9, 22.7, 15.8.

MS (ESI) *m/z*: [M+H]⁺ Calcd. for [C₁₂H₁₄NO₂]⁺ 204.10; Found 204.10.



Synthesis of **7**

6-I (5 g, 25 mmol, 1 eq.) was dissolved in 70 mL toluene and 35 mL MeCN under nitrogen atmosphere. Iodomethane (1.8 ml, 30 mmol, 1.2 eq.) was added and the reaction mixture was refluxed (heated with an oil bath) for 16 h. The formed precipitate was separated, washed with hexane and cold ethanol and dried. **7** was obtained as an orange-pinkish solid (2.9 g, 8.4 mmol, 34%).

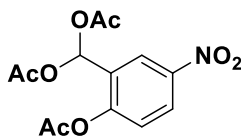
Analytical data match reported literature values.³

R_f = 0.21 (DCM:MeOH + 0.1% FA / 9:1).

¹H-NMR (400 MHz, DMSO-*d*₆): δ = 8.35 (s, 1H), 8.17 (d, *J* = 8.4 Hz, 1H), 8.02 (d, *J* = 8.4 Hz, 1H), 3.98 (s, 3H), 2.80 (s, 3H), 1.55 (s, 6H).

¹³C-NMR (100 MHz, DMSO-*d*₆): δ = 199.5, 166.9, 145.7, 142.4, 132.0, 130.8, 124.7, 115.8, 54.7, 35.5, 21.9, 15.0.

MS (ESI) *m/z*: [M-I]⁺ Calcd. for [C₁₃H₁₆NO₂]⁺ 218.12; Found 218.12.



Synthesis of **8-I**

2-Hydroxy-5-nitrobenzaldehyd **8** (2.5 g, 15 mmol, 1 eq.) was dissolved in 50 mL acetic anhydride. Three drops of concentrated H₂SO₄ were added and the solution was stirred for 1 h under nitrogen atmosphere at ambient temperature. The reaction mixture was poured in 500 mL water and stirred for 30 min. The formed precipitate was separated, washed with water and dried. **8-I** was obtained as white crystals (3.57 g, 11.5 mmol, 76%).

Analytical data match reported literature values.⁴

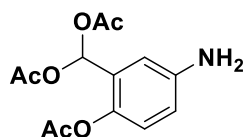
$R_f = 0.23$ (hexane:EE/ 8:2).

$^1\text{H-NMR}$ (400 MHz, CDCl_3): $\delta = 8.53$ (d, $J = 2.8$ Hz, 1H), 8.29 (dd, $J = 8.9, 2.8$ Hz, 1H), 7.93 (s, 1H), 7.35 (d, $J = 8.9$ Hz, 1H), 2.38 (s, 3H), 2.13 (s, 6H).

$^{13}\text{C-NMR}$ (100 MHz, CDCl_3): $\delta = 168.3, 168.2, 152.7, 145.6, 129.6, 125.8, 124.4, 123.8, 84.1, 20.8, 20.6$.

MS (ESI+, MeCN/MeOH): $m/z = 318.081$ $[\text{M}+\text{Li}]^+$. 318.0801

MS (ESI) m/z : $[\text{M}+\text{Li}]^+$ Calcd. for $[\text{C}_{13}\text{H}_{13}\text{NO}_8\text{Li}]^+$ 318.08; Found 318.08.



Synthesis of 8-II

8-I (1.5 g, 4.8 mmol, 1 eq.) was dissolved in 40 mL ethanol and PtO_2 (109 mg, 480 μM , 0.1 eq.) was added. The reaction suspension was stirred for 12 h under a hydrogen atmosphere (5 bar) at ambient temperature. The suspension was filtrated over Celite® and the solvent of the filtrate was removed *in vacuo*. The residue was purified by flash chromatography on normal phase silica gel (solvent A: hexane, solvent B: EE, 60% A) and **8-II** was obtained as a yellowish solid (0.95 g, 3.4 mmol, 70%).

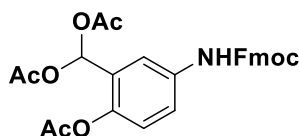
Analytical data match reported literature values.⁴

$R_f = 0.4$ (hexane:EE / 1:1).

$^1\text{H-NMR}$ (500 MHz, CDCl_3): $\delta = 7.79$ (s, 1H), 6.95–6.83 (m, 2H), 6.69 (dd, $J = 8.7, 2.8$ Hz, 1H), 3.72 (s, 2H), 2.30 (s, 3H), 2.10 (s, 6H).

$^{13}\text{C-NMR}$ (125 MHz, CDCl_3): $\delta = 170.1, 168.4, 144.6, 140.1, 128.1, 123.8, 117.0, 113.5, 85.4, 20.8, 20.7$.

MS (ESI) m/z : $[\text{M}+\text{Li}]^+$ Calcd. for $[\text{C}_{13}\text{H}_{15}\text{NO}_6\text{Li}]^+$ 288.11; Found 288.11.



Synthesis of 9a

8-II (2 g, 7.1 mmol, 1 eq.) and Fmoc-Cl (2.2 g, 8.5 mmol, 1.2 eq.) were suspended in 10 mL water and stirred for 2h at 60 °C (heated with an oil bath). The reaction mixture was extracted three times with 60 mL EE and the combined organic phases were dried over Na_2SO_4 . The solvent was removed *in vacuo*

and the residue was purified by flash chromatography on normal phase silica gel (solvent A: hexane, solvent B: EE, 80% A) to obtain **9a** as transparent crystals (1.45 g, 2.9 mmol, 41%).

$R_f = 0.12$ (hexane:EE / 4:1).

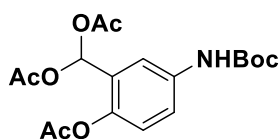
$^1\text{H-NMR}$ (500 MHz, CDCl_3): $\delta = 7.85$ (s, 1H), 7.79 (d, $J = 7.6$ Hz, 2H), 7.68 (s, 1H), 7.61 (d, $J = 7.5$ Hz, 2H), 7.44–7.40 (m, 3H), 7.33 (t, $J = 7.4$ Hz, 2H), 7.05 (d, $J = 8.8$ Hz, 1H), 6.74 (s, 1H), 4.56 (d, $J = 6.6$ Hz, 2H), 4.28 (t, $J = 6.5$ Hz, 1H), 2.32 (s, 3H), 2.09 (s, 6H).

$^{13}\text{C-NMR}$ (125 MHz, CDCl_3): $\delta = 169.5, 168.4, 153.2, 143.8, 143.6, 141.4, 135.8, 128.4, 127.8, 127.2, 124.9, 123.8, 120.8, 120.1, 117.8, 85.0, 67.0, 47.1, 20.8, 20.7$.

IR (ATR): $\nu = 3399\text{m}, 1749\text{s}, 1727\text{s}, 1543\text{m}, 1195\text{s cm}^{-1}$.

UV/VIS ($c = 0.05 \mu\text{mol mL}^{-1}$ /MeOH): $\lambda_{\text{max},1} = 300, \lambda_{\text{max},2} = 289, \lambda_{\text{max},1} = 264, \lambda_{\text{max},1} = 244 \text{ nm}$.

HR-MS (ESI) m/z : $[\text{M}+\text{Li}]^+$ Calcd. for $[\text{C}_{28}\text{H}_{25}\text{NO}_8\text{Li}]^+$ 510.1740; Found 510.1724.



Synthesis of **9b**

8-II (1 g, 3.6 mmol, 1 eq.) and Boc_2O (1.55 g, 7 mmol, 2 eq.) were dissolved in 10 mL DCM and stirred at ambient temperature for 12 h under a nitrogen atmosphere. The solvent was removed *in vacuo* and the residue was purified by flash chromatography on normal phase silica gel (solvent A: hexane, solvent B: EE, 80% A) to obtain **9b** as a transparent gel (0.57 g, 1.5 mmol, 42%).

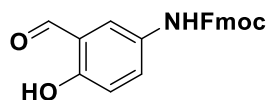
Analytical data match reported literature value.⁴

$R_f = 0.63$ (hexane:EE / 3:2).

$^1\text{H-NMR}$ (400 MHz, CDCl_3): $\delta = 7.84$ (s, 1H), 7.64 (d, $J = 2.5$ Hz, 1H), 7.41 (d, $J = 8.8, 2.7$ Hz, 1H), 7.03 (d, $J = 8.8$ Hz, 1H), 6.52 (s, 1H), 2.31 (s, 3H), 2.09 (s, 6H), 1.52 (s, 9H).

$^{13}\text{C-NMR}$ (100 MHz, CDCl_3): $\delta = 169.6, 168.4, 152.5, 143.3, 136.5, 128.2, 123.7, 120.7, 117.6, 85.1, 80.9, 28.3, 20.8, 20.7$.

MS (ESI) m/z : $[\text{M}+\text{Li}]^+$ Calcd. for $[\text{C}_{18}\text{H}_{23}\text{NO}_8\text{Li}]^+$ 388.16; Found 388.16.



Synthesis of **10a**

9a (0.1 g, 0.2 mmol, 1 eq.) was dissolved in 20 mL ethanol and 1 mL of concentrated H₂SO₄ was added. The solution was refluxed (heated with an oil bath) for 2 h and neutralized with a saturated aqueous NaHCO₃-solution. The solution was extracted three times with 50 mL Et₂O and the combined organic phases were dried over Na₂SO₄. The solvent is removed *in vacuo* and **10a** was obtained as beige crystals (52 mg, 0.14 mmol, 73%).

R_f = 0.28 (hexane:EE / 4:1).

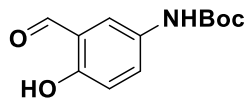
¹H-NMR (400 MHz, THF-*d*₈): δ = 10.58 (s, 1H), 9.91 (s, 1H), 8.82 (s, 1H), 7.88 (s, 1H), 7.79 (d, *J* = 7.5 Hz, 2H), 7.67 (d, *J* = 7.3 Hz, 2H), 7.51 (s, 1H), 7.36 (t, *J* = 7.5 Hz, 2H), 7.28 (t, *J* = 7.4 Hz, 2H), 6.85 (d, *J* = 8.9 Hz, 1H), 4.55 (d, *J* = 6.3 Hz, 2H), 4.26 (t, *J* = 6.2 Hz, 1H).

¹³C-NMR (100 MHz, THF-*d*₈): δ = 196.8, 157.8, 154.3, 145.0, 142.2, 132.8, 128.2, 128.0, 127.6, 125.5, 122.6, 121.5, 120.5, 118.0, 66.6, 48.1.

IR (ATR): ν = 3294m, 1693s, 1658s, 1243s, 732m cm⁻¹.

UV/VIS (c = 0.07 μmol mL⁻¹ /MeOH): λ_{max,1} = 356, λ_{max,2} = 256, λ_{max,3} = 235 nm.

HR-MS (ESI) *m/z*: [M+Na]⁺ Calcd. for [C₂₂H₁₈NO₄Na]⁺ = 382.1055; Found 382.1052.



Synthesis of **10b**

9b (0.5 g, 1.3 mmol, 1 eq.) was dissolved in 10 mL methanol and 5 g NaOH dissolved in 5 mL water were added. The solution was refluxed (heated with an oil bath) for 3 h and neutralized with an aqueous 1 M HCl-solution. The solution was extracted three times with 20 mL EE and the combined organic phases were dried over Na₂SO₄. The solvent was removed *in vacuo* and the residue was purified by flash chromatography on normal phase silica gel (solvent A: hexane, solvent B: EE, 90% A) to obtain **10b** as a yellow solid (0.21 g, 890 μmol, 45%).

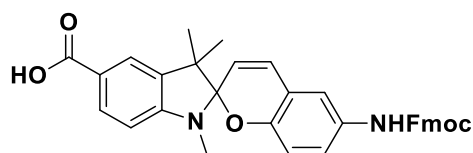
Analytical data match reported literature values.⁴

R_f = 0.45 (hexane:EE / 4:1).

¹H-NMR (400 MHz, CDCl₃): δ = 10.76 (s, 1H), 9.86 (d, *J* = 0.5 Hz, 1H), 7.83 (s, 1H), 7.29 (dd, *J* = 8.9, 2.7 Hz, 1H), 6.92 (d, *J* = 8.9 Hz, 1H), 6.45 (s, 1H), 1.52 (s, 6H).

^{13}C -NMR (100 MHz, CDCl_3): δ = 196.5, 157.6, 153.0, 130.8, 128.4, 123.3, 120.2, 118.0, 80.9, 28.3.

MS (ESI) m/z : $[\text{M}+\text{Li}]^+$ Calcd. for $[\text{C}_{12}\text{H}_{15}\text{NO}_4\text{Li}]^+$ 244.12; Found 244.11.



Synthesis of **2a**

10a (0.3 g, 0.83 mmol, 1 eq.) and **7** (0.58 g, 1.7 mmol, 2 eq.) were dissolved in 100 mL ethanol under a nitrogen atmosphere and TEA (0.47 mL, 3.3 mmol, 4 eq.) was added slowly. The solution was refluxed (heated with an oil bath) for 3 h and neutralized with an aqueous 1 M HCl-solution. The solution was extracted three times with 60 mL EE and the combined organic phases were dried over Na_2SO_4 . The solvent was removed *in vacuo* and the residue was purified by flash chromatography on normal phase silica gel (solvent A: DCM, solvent B: MeOH, 98% A) to obtain **2a** as a red solid (0.12 g, 0.21 mmol, 26%).

R_f = 0.41 (DCM:MeOH / 19:1).

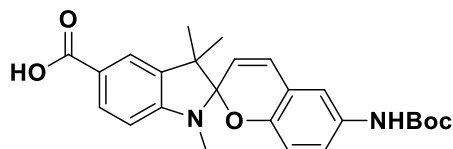
^1H -NMR (400 MHz, $\text{THF}-d_8$): δ = 12.83 (s, 1H), 8.64 (s, 1H), 7.87 (dd, J = 8.2, 1.7 Hz, 1H), 7.80 (d, J = 7.6 Hz, 2H), 7.72 (d, J = 1.7 Hz, 1H), 7.68 (d, J = 7.4 Hz, 2H), 7.43 – 7.34 (m, 3H), 7.29 (td, J = 7.4, 1.2 Hz, 2H), 7.10 (d, J = 8.7 Hz, 1H), 6.93 (d, J = 10.2 Hz, 1H), 6.58 (d, J = 8.7 Hz, 1H), 6.53 (d, J = 8.2 Hz, 1H), 5.76 (d, J = 10.2 Hz, 1H), 4.51 (d, J = 6.5 Hz, 2H), 4.27 (t, J = 6.4 Hz, 1H), 2.77 (s, 3H), 1.33 (s, 3H), 1.17 (s, 3H).

^{13}C -NMR (100 MHz, $\text{THF}-d_8$): δ = 167.6, 154.2, 152.6, 150.5, 145.1, 142.2, 137.3, 133.3, 131.6, 130.6, 128.1, 127.6, 125.6, 123.8, 122.3, 120.5, 119.8, 119.4, 117.5, 115.3, 106.2, 104.8, 66.5, 51.8, 48.2, 28.7, 25.9, 20.3.

IR (ATR): ν = 3303br, 3064br, 1671s, 1583s, 1199s cm^{-1} .

UV/VIS (c = 0.09 $\mu\text{mol mL}^{-1}$ /MeOH): $\lambda_{\text{max},1}$ = 474, $\lambda_{\text{max},2}$ = 378, $\lambda_{\text{max},3}$ = 299, $\lambda_{\text{max},4}$ = 264 nm.

HR-MS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calcd. for $[\text{C}_{35}\text{H}_{31}\text{N}_2\text{O}_5]^+$ 559.2227; Found: 559.2207.



Synthesis of **2b**

10b (0.5 g, 2.1 mmol, 0.5 eq.) and **7** (1.45 g, 4.2 mmol, 2 eq.) were dissolved in 100 mL ethanol under a nitrogen atmosphere and TEA (1.2 mL, 8.4 mmol, 4 eq.) was added slowly. The solution was refluxed (heated with an oil bath) for 3 h and neutralized with an aqueous 1 M HCl-solution. The solution was

extracted three times with 60 mL EE and the combined organic phases were dried over Na₂SO₄. The solvent was removed *in vacuo* and the residue was purified by flash chromatography on normal phase silica gel (solvent A: hexane, solvent B: EE, 20% A) to obtain **2b** as a pink solid (0.3 g, 0.7 mmol, 33%).

R_f = 0.35 (DCM:MeOH / 19:1).

¹H-NMR (400 MHz, DMSO-*d*₆): δ = 12.25 (s, 1H), 9.10 (s, 1H), 7.76 (dd, *J* = 8.2, 1.7 Hz, 1H), 7.61 (d, *J* = 1.6 Hz, 1H), 7.34 (s, 1H), 7.04 (dd, *J* = 8.8, 2.4 Hz, 1H), 6.98 (d, *J* = 10.2 Hz, 1H), 6.59 (dd, *J* = 8.4, 6.2 Hz, 2H), 5.75 (d, *J* = 10.2 Hz, 1H), 2.70 (s, 3H), 1.44 (s, 3H), 1.21 (s, 3H), 1.08 (s, 3H).

¹³C-NMR (100 MHz, DMSO-*d*₆): δ = 167.9, 153.4, 153.0, 149.3, 136.8, 132.9, 131.1, 130.2, 123.3, 121.4, 120.9, 119.5, 118.6, 117.4, 114.8, 106.3, 104.0, 79.2, 51.3, 28.9, 28.6, 26.0, 20.2.

IR (ATR): ν = 3448w, 2980m, 1724s, 1667s, 1155s cm⁻¹.

UV/VIS (c = 0.03 μmol mL⁻¹ /MeOH): λ_{max,1} = 301, λ_{max,2} = 233 nm.

HR-MS (ESI) *m/z*: [M+Li]⁺ Calcd. [C₂₅H₂₈N₂O₅Li]⁺ 443.2158; Found: 443.2121.

4. Supplementary figures

UV/Vis-spectra

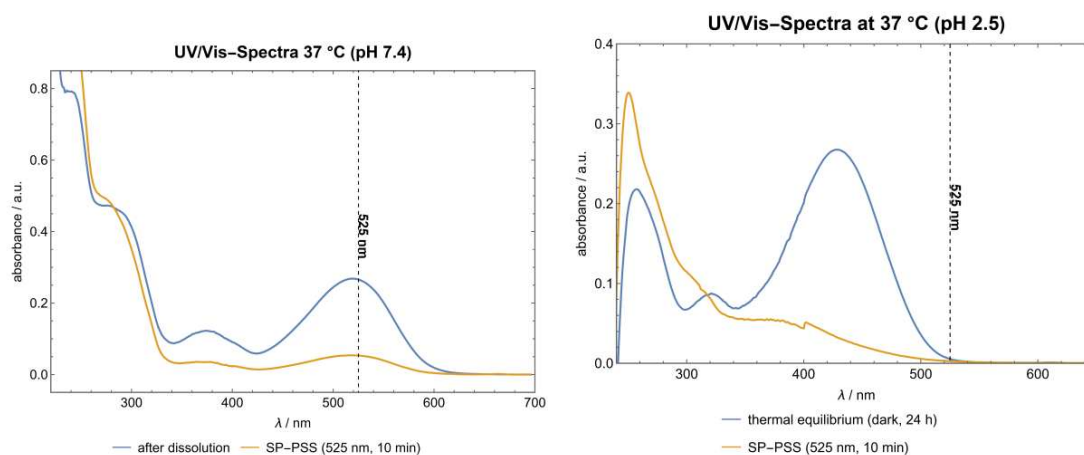


Figure S1. UV/Vis-spectra of **P1** at 37 °C and different pH values: pH 7.4 (left, 55 μ M), pH 2.5 (right, 13 μ M).

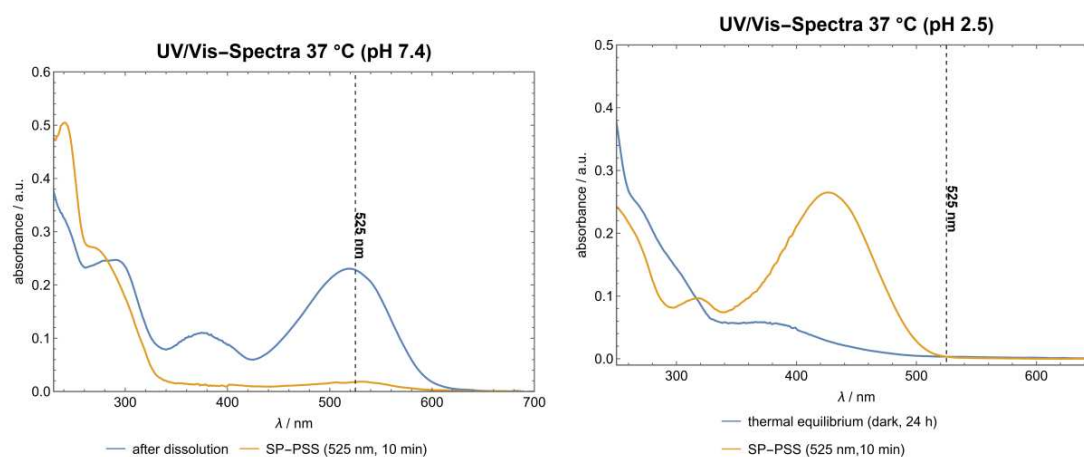


Figure S2. UV/Vis-spectra of **P2** at 37 °C and different pH values: pH 7.4 (left, 30 μ M), pH 2.5 (right, 16 μ M).

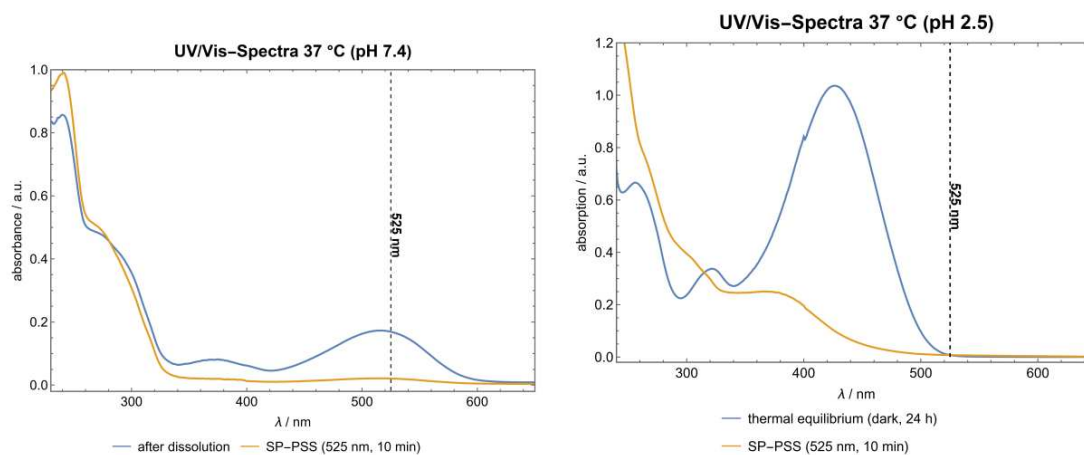


Figure S3. UV/Vis-spectra of **P3** at 37 °C and different pH values: pH 7.4 (left, 50 μ M), pH 2.5 (right, c = 55 μ M).

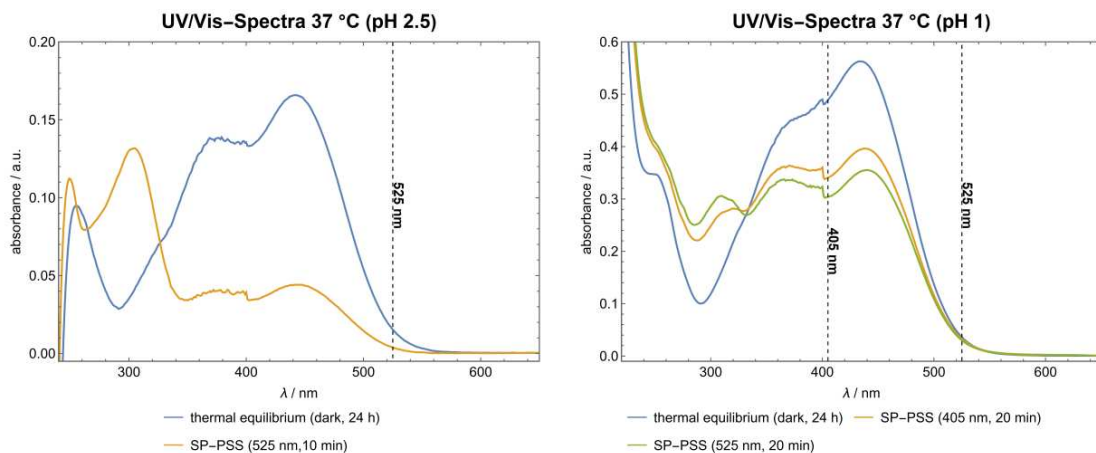


Figure S4. UV/Vis-spectra of **P4** at 37 °C and different pH values: pH 2.5 (left, 15 μ M), pH 1 (right, 44 μ M).

Kinetics

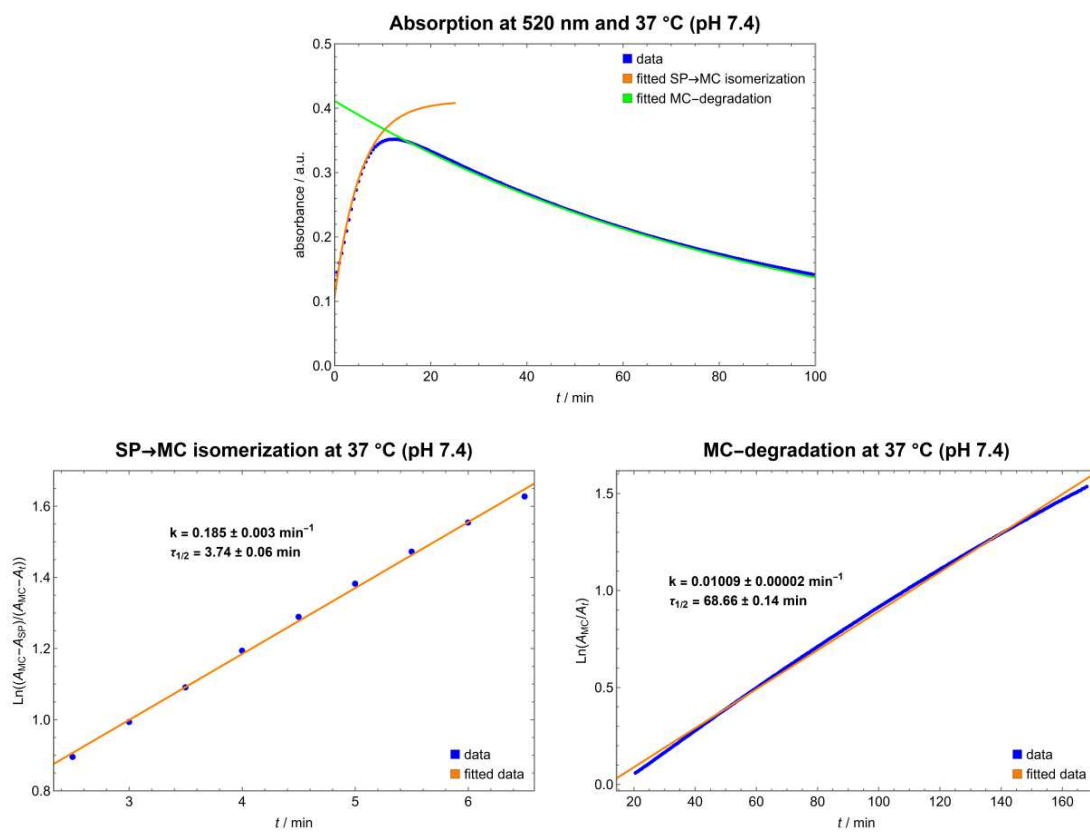


Figure S5. Kinetic measured at 520 nm (top) of SP-to-MC isomerization (rate constant k , lower left) and hydrolysis (rate constant k , lower right) of **P1** (55 μ M) at pH 7.4 and 37 °C.

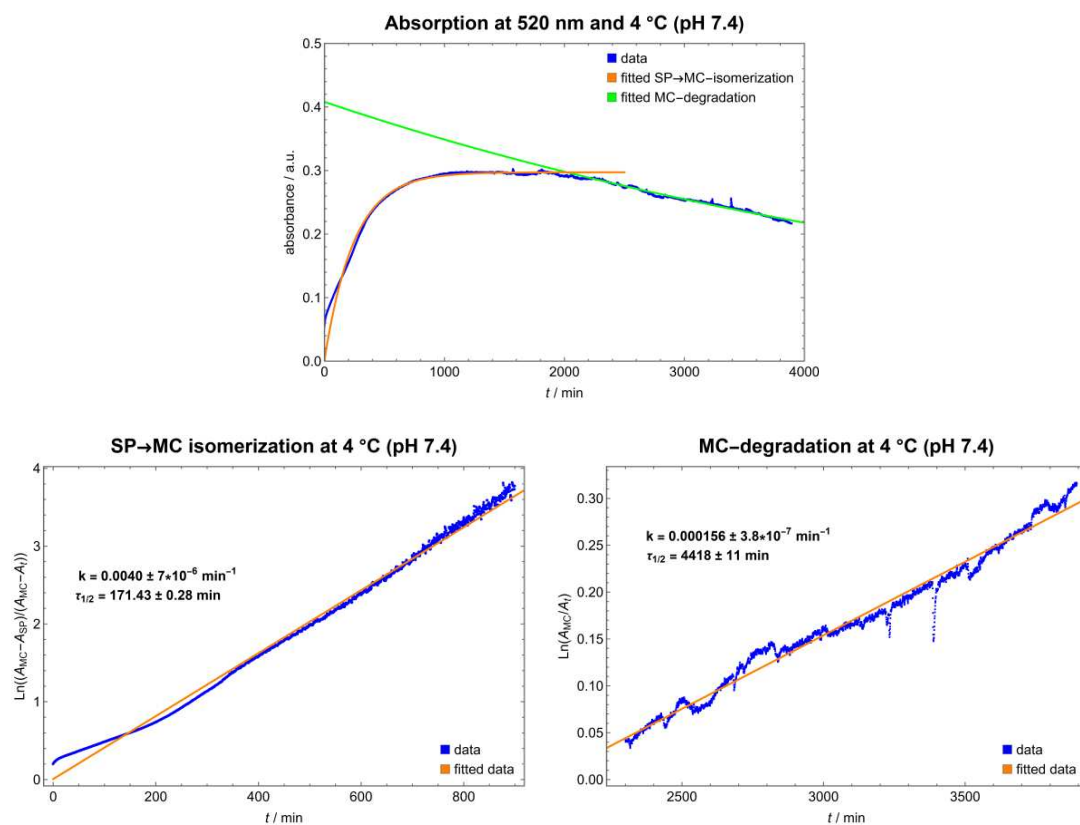


Figure S6. Kinetic measured at 520 nm (top) of SP-to-MC isomerization (rate constant k , lower left) and hydrolysis (rate constant k , lower right) of **P1** (40 μM) at pH 7.4 and 4 °C.

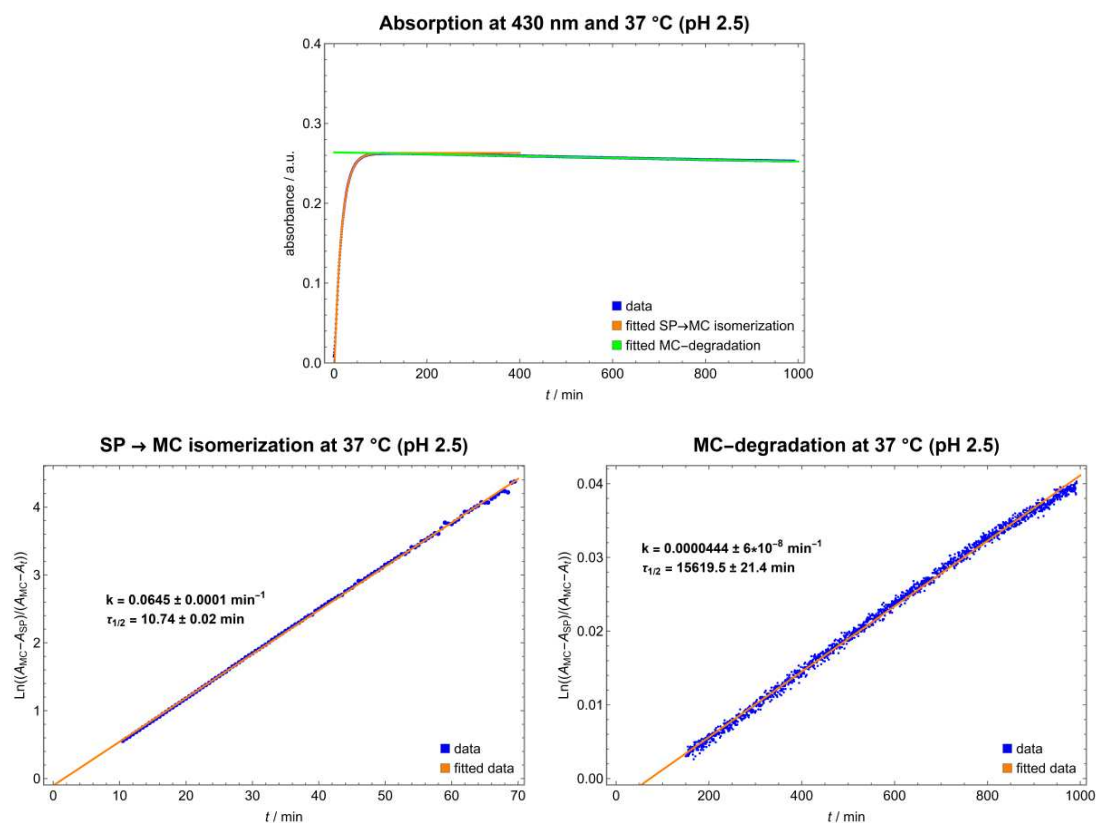


Figure S7. Kinetic measured at 430 nm (top) of SP-to-MC isomerization (rate constant k , lower left) and hydrolysis (rate constant k , lower right) of **P1** (13 μM) at pH 2.5 and 37 °C.

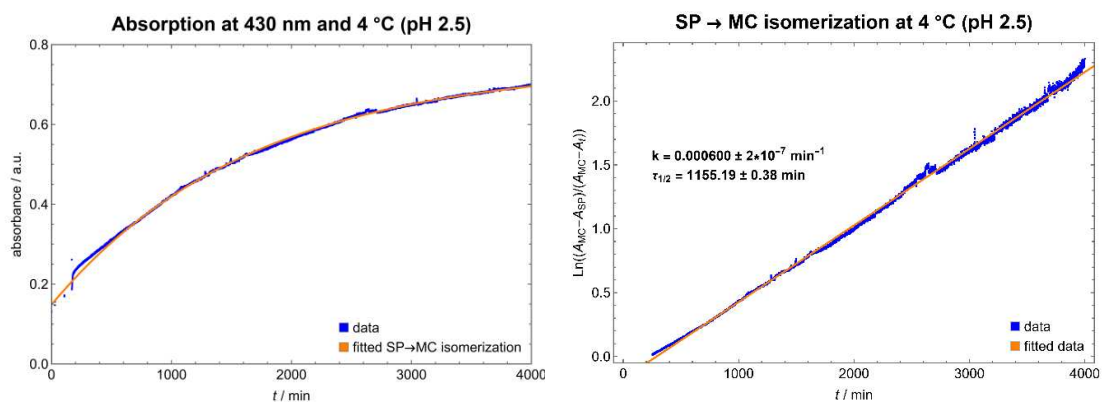


Figure S8. Kinetic measured at 430 nm (left) of SP-to-MC isomerization (rate constant k , right) of **P1** (13 μM) at pH 2.5 and 4 °C.

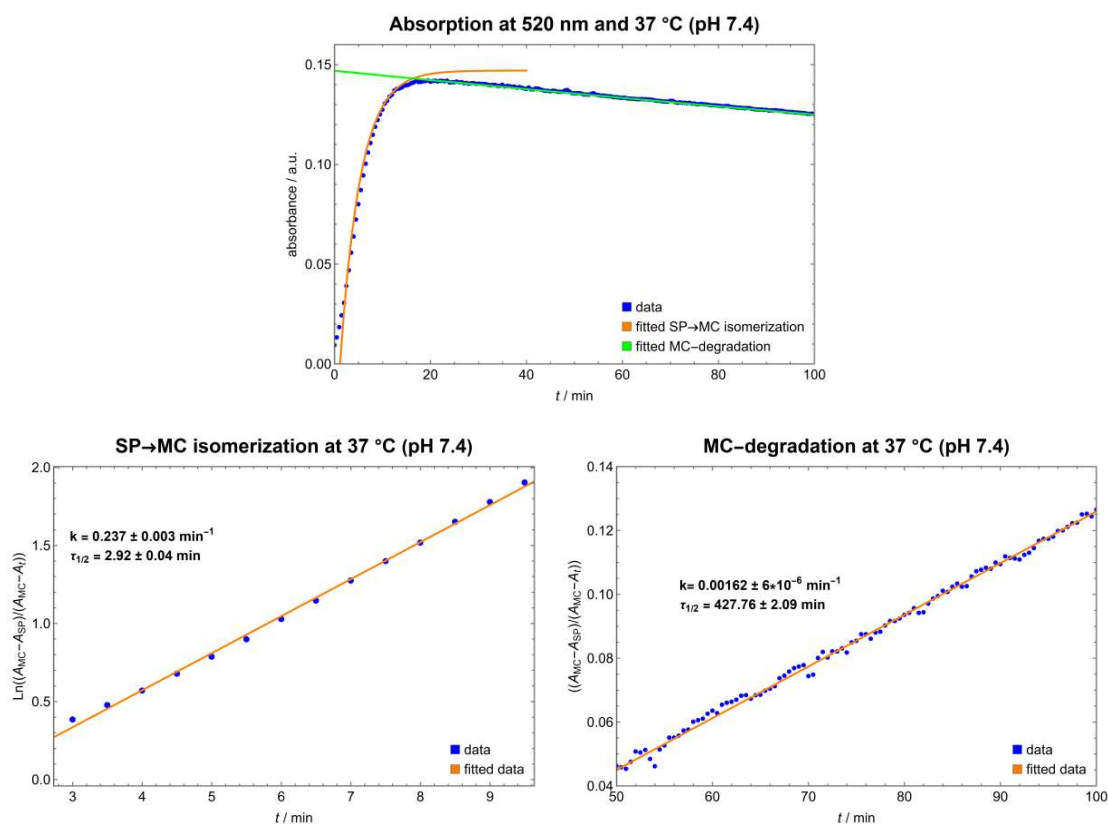


Figure S9. Kinetic measured at 520 nm (top) of SP-to-MC isomerization (rate constant k , lower left) and hydrolysis (rate constant k , lower right) of **P2** (20 μM) at pH 7.4 and 37 $^{\circ}\text{C}$.

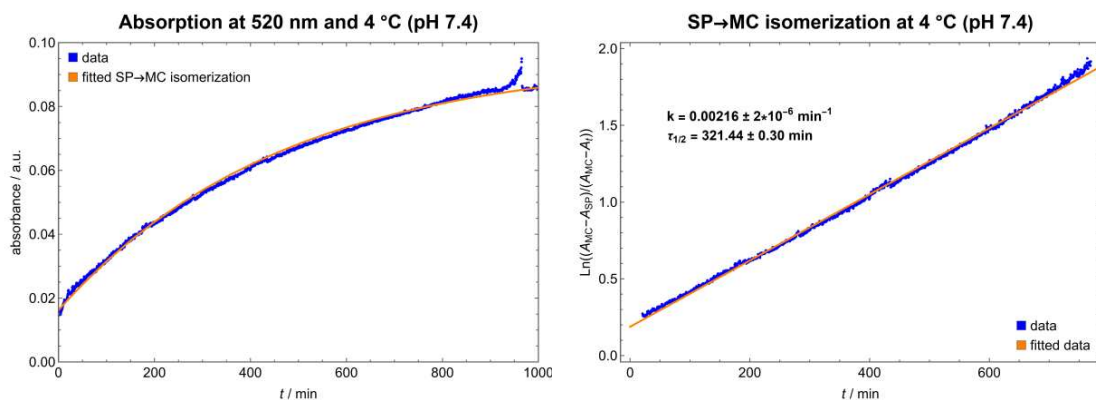


Figure S10. Kinetic measured at 520 nm (left) of SP-to-MC isomerization (rate constant k , right) of **P2** (12 μM) at pH 7.4 and 4 $^{\circ}\text{C}$.

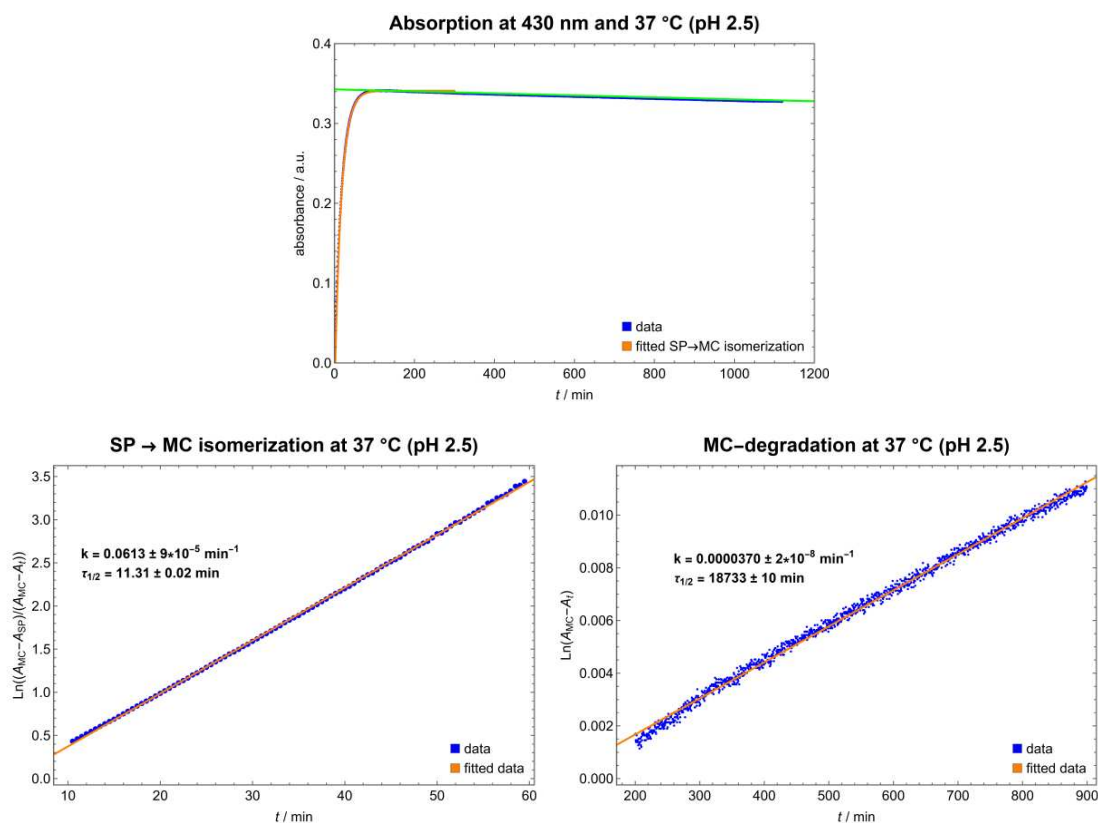


Figure S11. Kinetic measured at 430 nm (top) of SP-to-MC isomerization (rate constant k , lower left) and hydrolysis (rate constant k , lower right) of **P2** (16 μ M) at pH 2.5 and 37 $^{\circ}$ C.

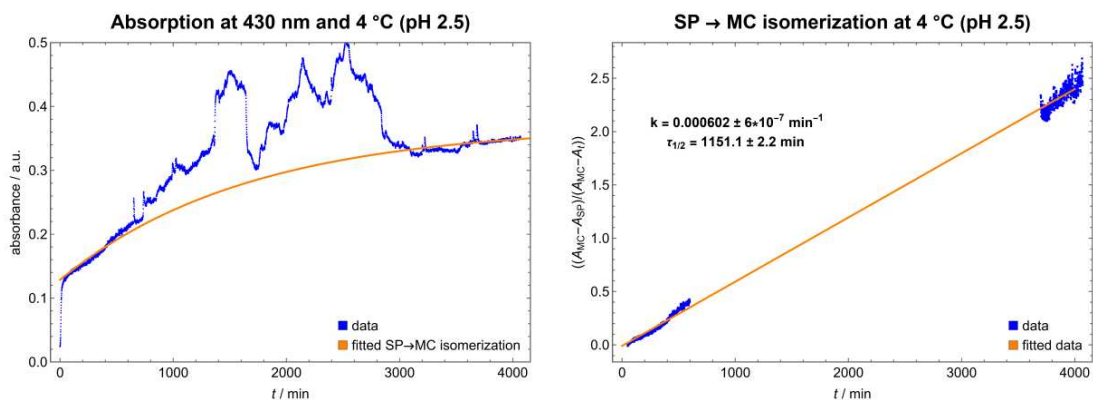


Figure S12. Kinetic measured at 430 nm (left) of SP-to-MC isomerization (rate constant k , right) of **P2** (16 μ M) at pH 2.5 and 4 $^{\circ}$ C.

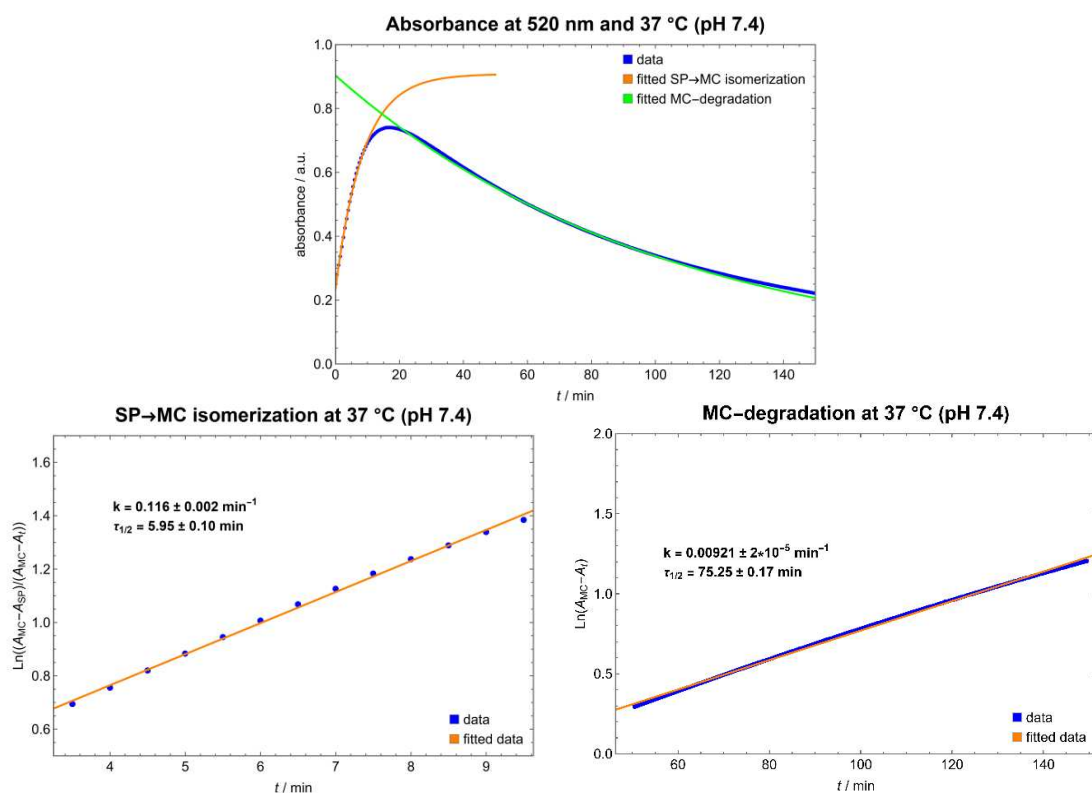


Figure S13. Kinetic measured at 520 nm (top) of SP-to-MC isomerization (rate constant k , lower left) and hydrolysis (rate constant k , lower right) of **P3** (120 μM) at pH 7.4 and 37 $^{\circ}\text{C}$.

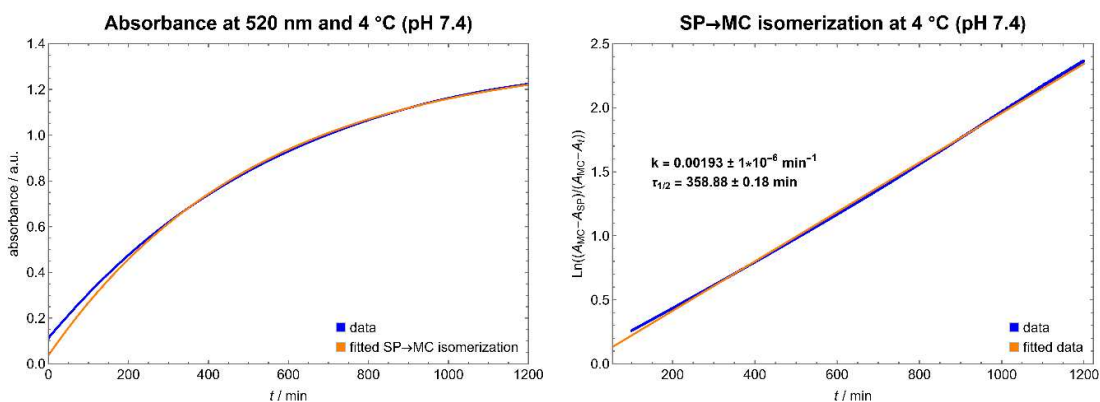


Figure S14. Kinetic measured at 520 nm (left) of SP-to-MC isomerization (rate constant k , right) of **P3** (160 μM) at pH 7.4 and 4 $^{\circ}\text{C}$.

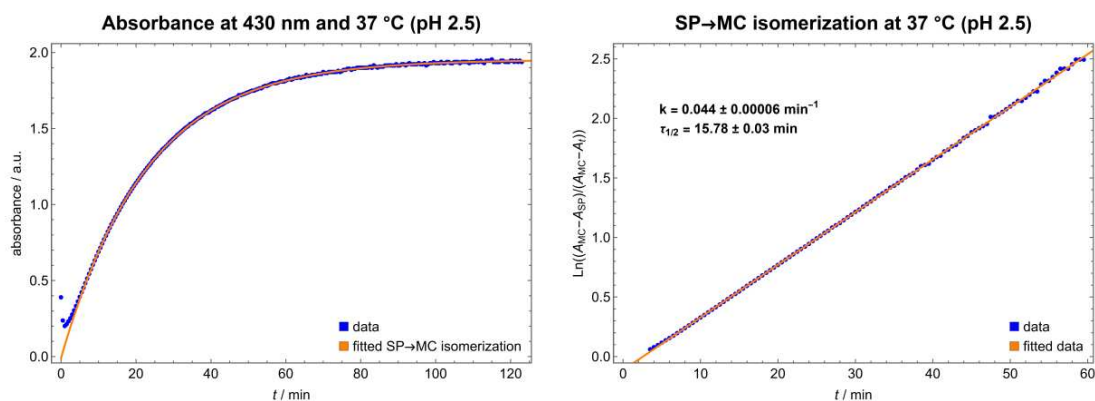


Figure S15. Kinetic measured at 430 nm (left) of SP-to-MC isomerization (rate constant k , right) of **P3** (100 μM) at pH 2.5 and 37 °C.

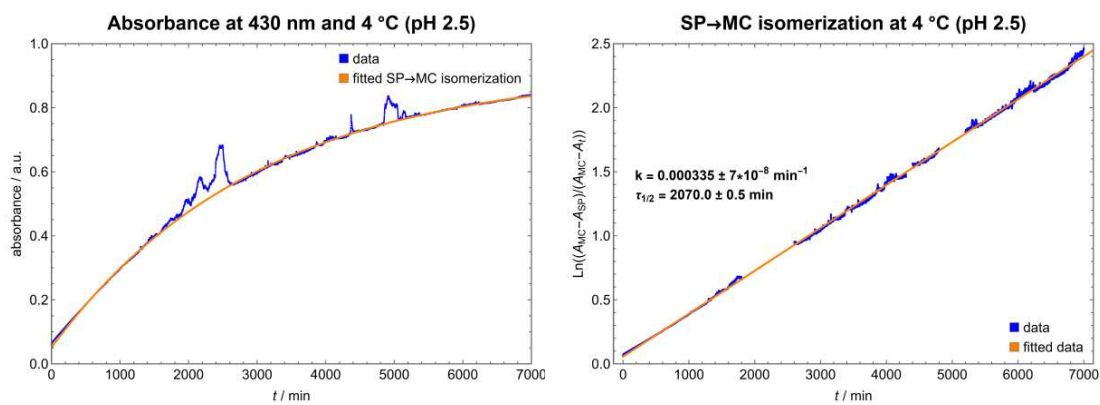


Figure S16. Kinetic measured at 430 nm (left) of SP-to-MC isomerization (rate constant k , right) of **P3** (50 μM) at pH 2.5 and 4 °C.

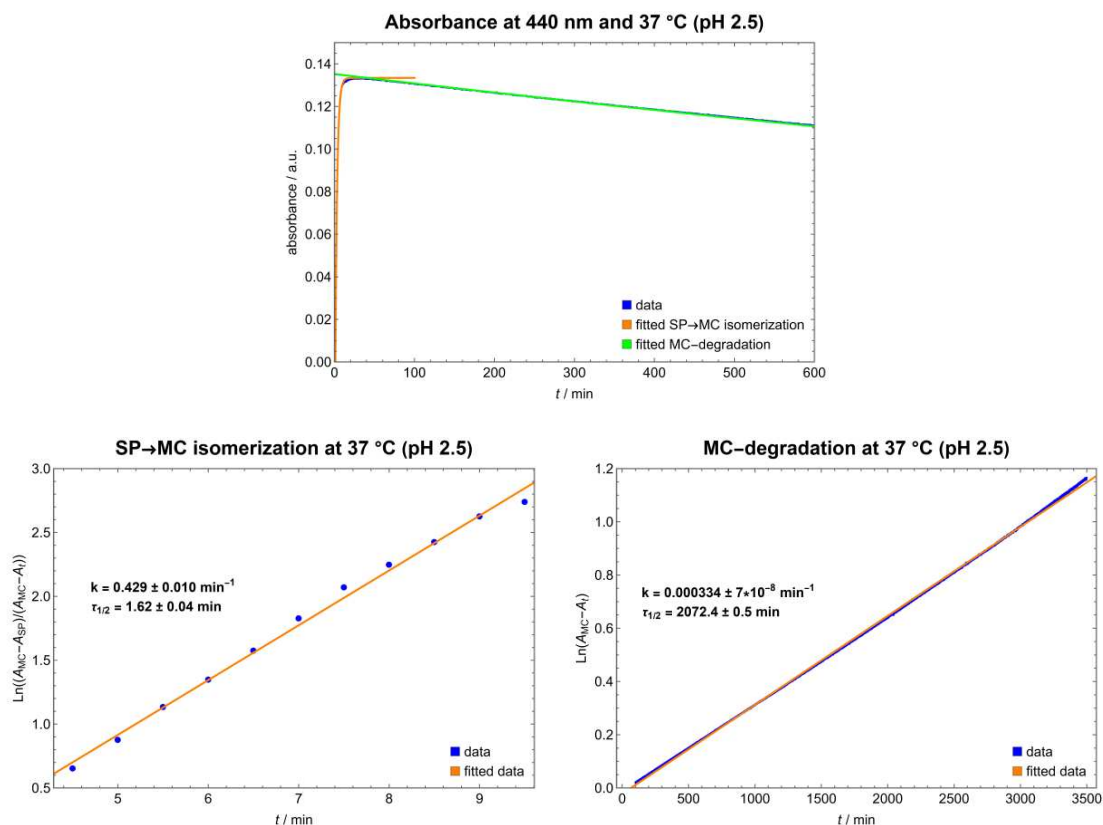


Figure S17. Kinetic measured at 440 nm (top) of SP-to-MC isomerization (rate constant k , lower left) and hydrolysis (rate constant k , lower right) of **P4** (15 μ M) at pH 2.5 and 37 $^{\circ}$ C.

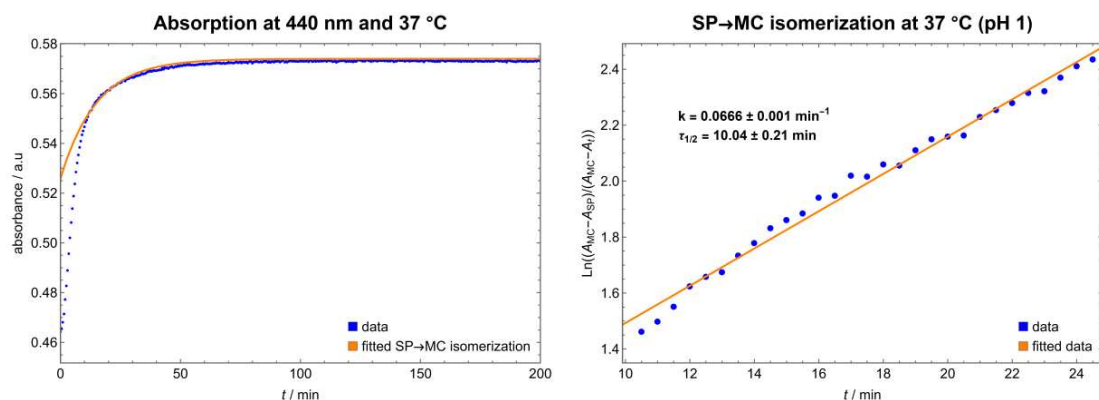


Figure S18. Kinetic measured at 440 nm (left) of SP-to-MC isomerization (rate constant k , right) of **P4** (44 μ M) at pH 1 and 37 $^{\circ}$ C.

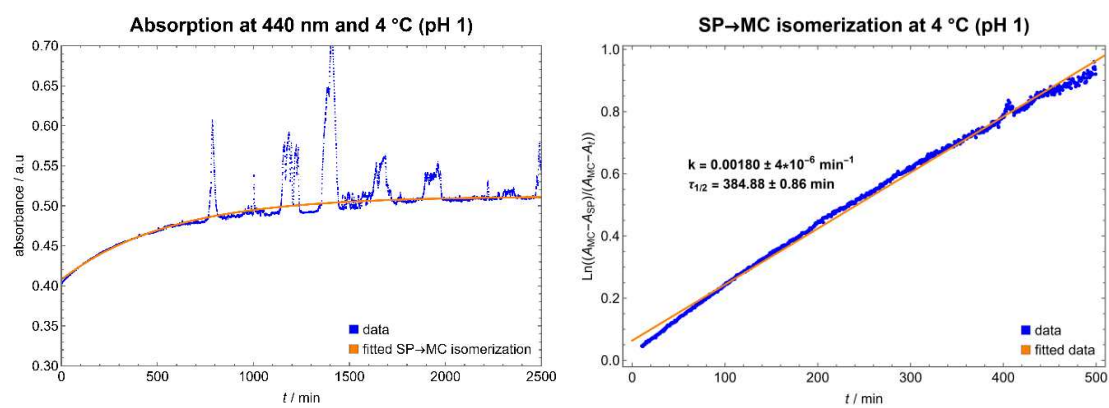


Figure S19. Kinetic measured at 440 nm (left) of SP-to-MC isomerization (rate constant k , right) of **P4** (39 μM) at pH 1 and 4 °C.

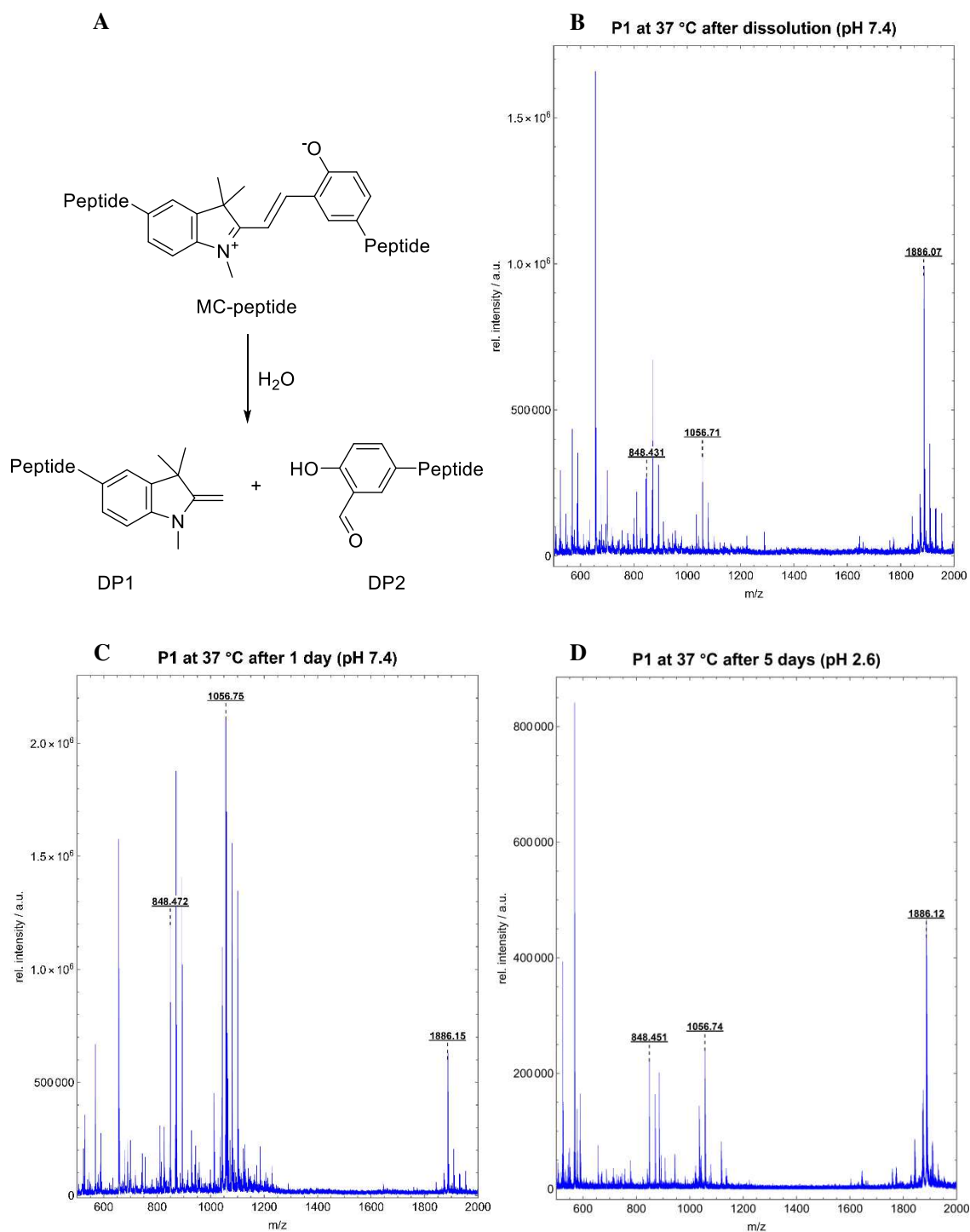


Figure S20. (A) Products of the hydrolysis reaction. (C – D) MALDI-measurements of **P1** and hydrolysis products at 37 °C after dissolution at pH 7.4 (B), after 1 day at pH 7.4 (C), and after 5 days at pH 2.6 (D). Calculated mass of **P1**+H⁺: 1886.0435, calculated mass for DP1+H⁺: 1056.6600, calculated mass for DP2+H⁺: 848.3997.

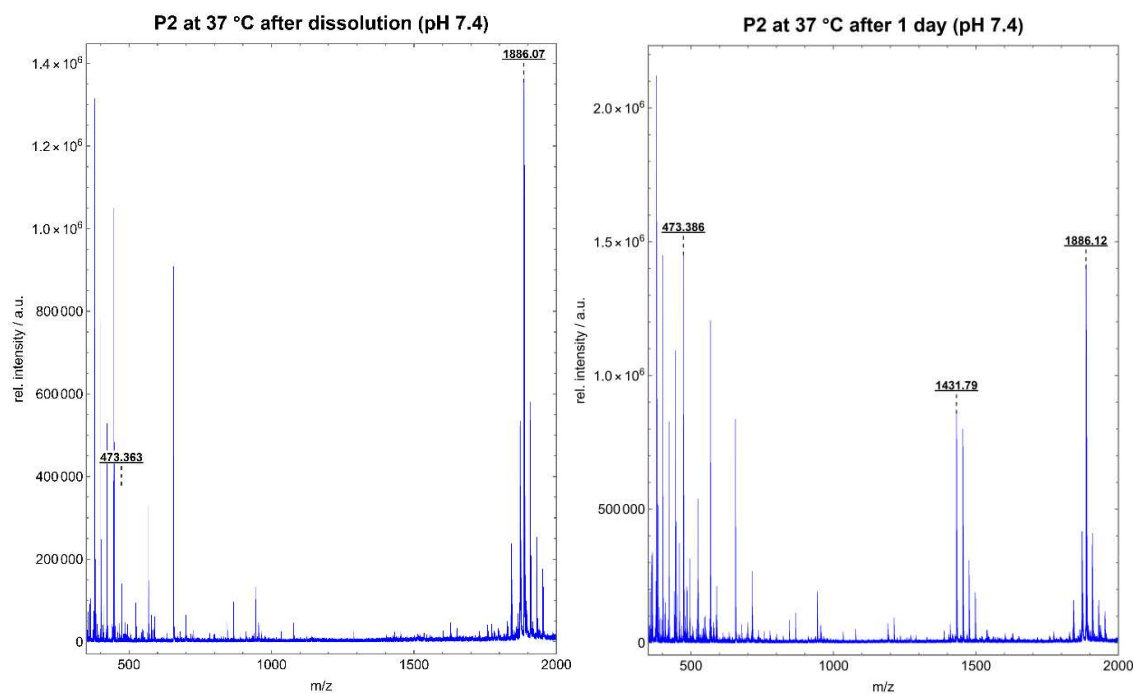


Figure S21. MALDI-measurements of **P2** and hydrolysis products at 37 °C after dissolution at pH 7.4 (left) and after 1 day at pH 7.4 (right). Calculated mass of **P2**+H⁺: 1886.0435, calculated mass for DP1+H⁺: 473.3347, calculated mass for DP2+H⁺: 1431.7267.

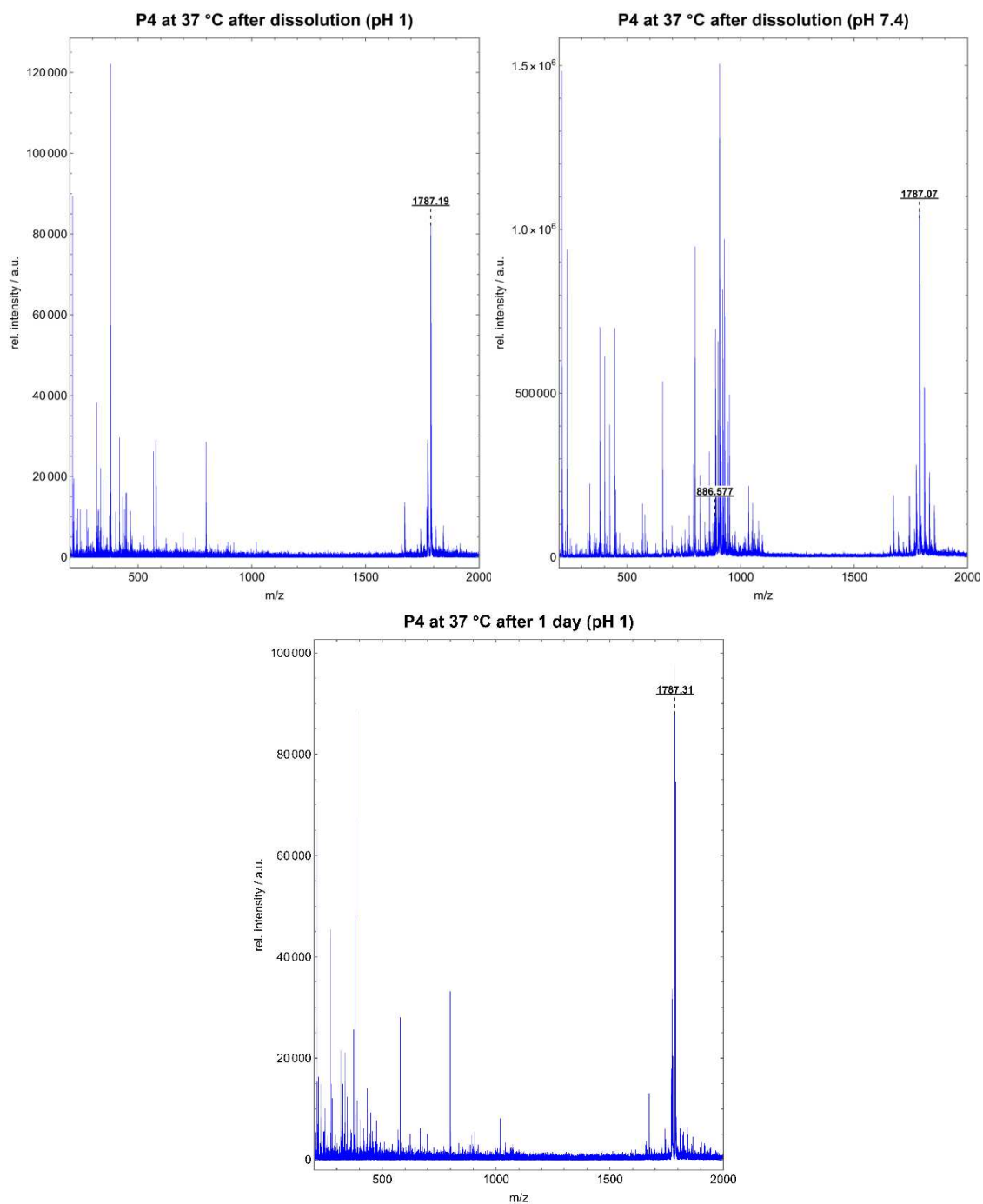


Figure S22. MALDI-measurements of **P4** and hydrolysis products at 37 °C after dissolution at pH 7.4 (top, left), after dissolution at pH 1 (top, right), and after 1 day at pH 1 (bottom). Calculated mass of **P4**+H⁺: 1786.9571, calculated mass for DP1+H⁺: 886.4934, calculated mass for DP2+H⁺: 919.4996.

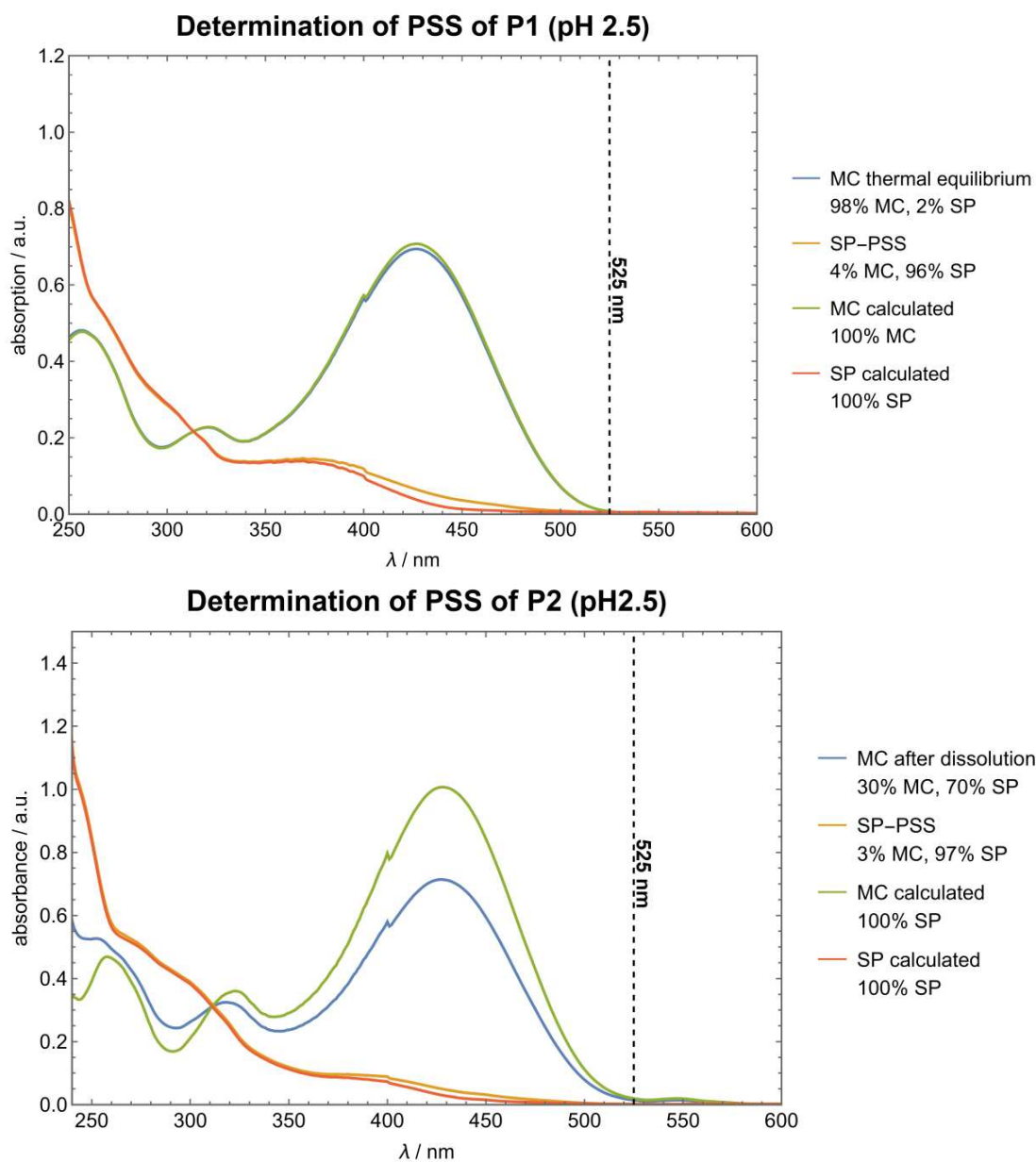


Figure S23. UV/Vis-spectra of **P1** (top, 35 μ M) and **P2** (bottom, 56 μ M) at pH 2.5. Spectra were measured at thermal equilibrium (P1), after dissolution (P2) and in the SP-PSS (after irradiation with green light for 10 min). SP:MC ratio was analyzed with HPLC and the spectra of the pure MC- and SP-form were calculated using Mathematic 13.3.1.0.

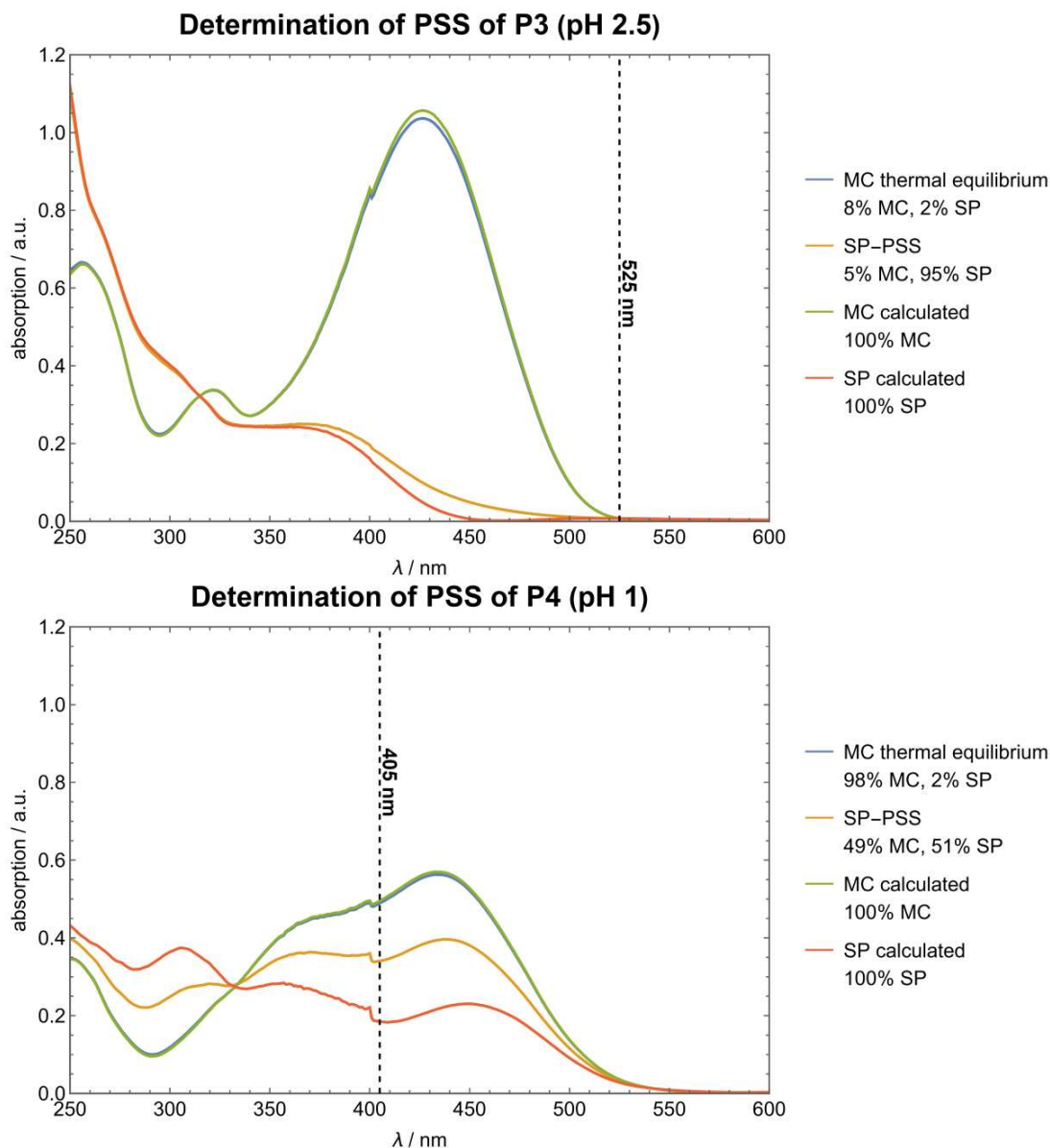


Figure S24. UV/Vis-spectra of **P3** at pH 2.5 (top, 55 μ M) and **P4** at pH 1 (bottom, 44 μ M). Spectra were measured at thermal equilibrium and in the SP-PSS (for **P3** after irradiation with green light for 10 min, for **P4** after irradiation with 405 nm for 10 min). SP:MC ratio was analyzed with HPLC and the spectra of the pure MC- and SP-form were calculated using Mathematic 13.3.1.0.

5. NMR data of compounds and peptides and LC-MS of peptides

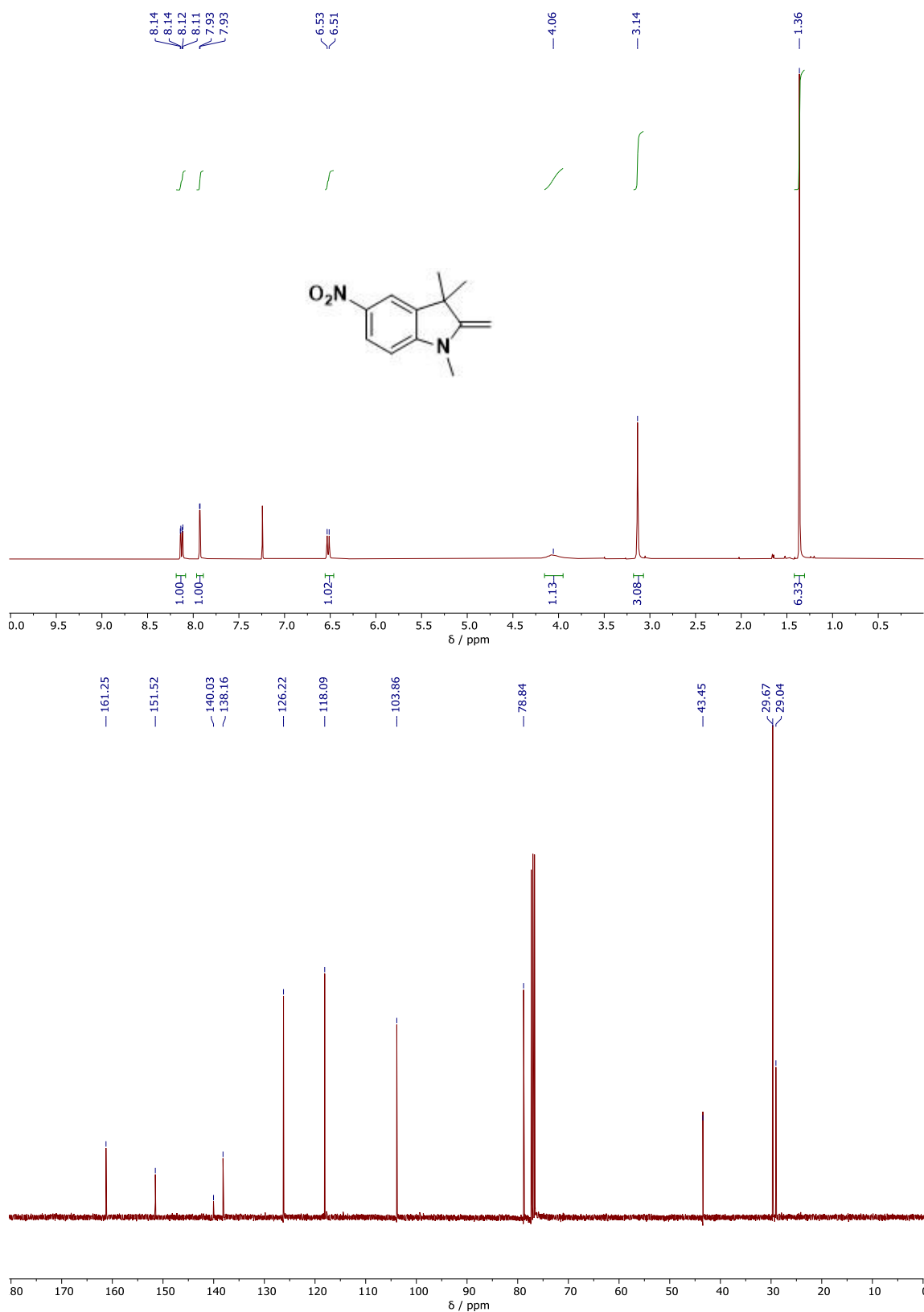


Figure S25. ¹H-NMR (top, 500 MHz) and ¹³C-NMR (bottom, 125 MHz) in CDCl₃ of **3-I**.

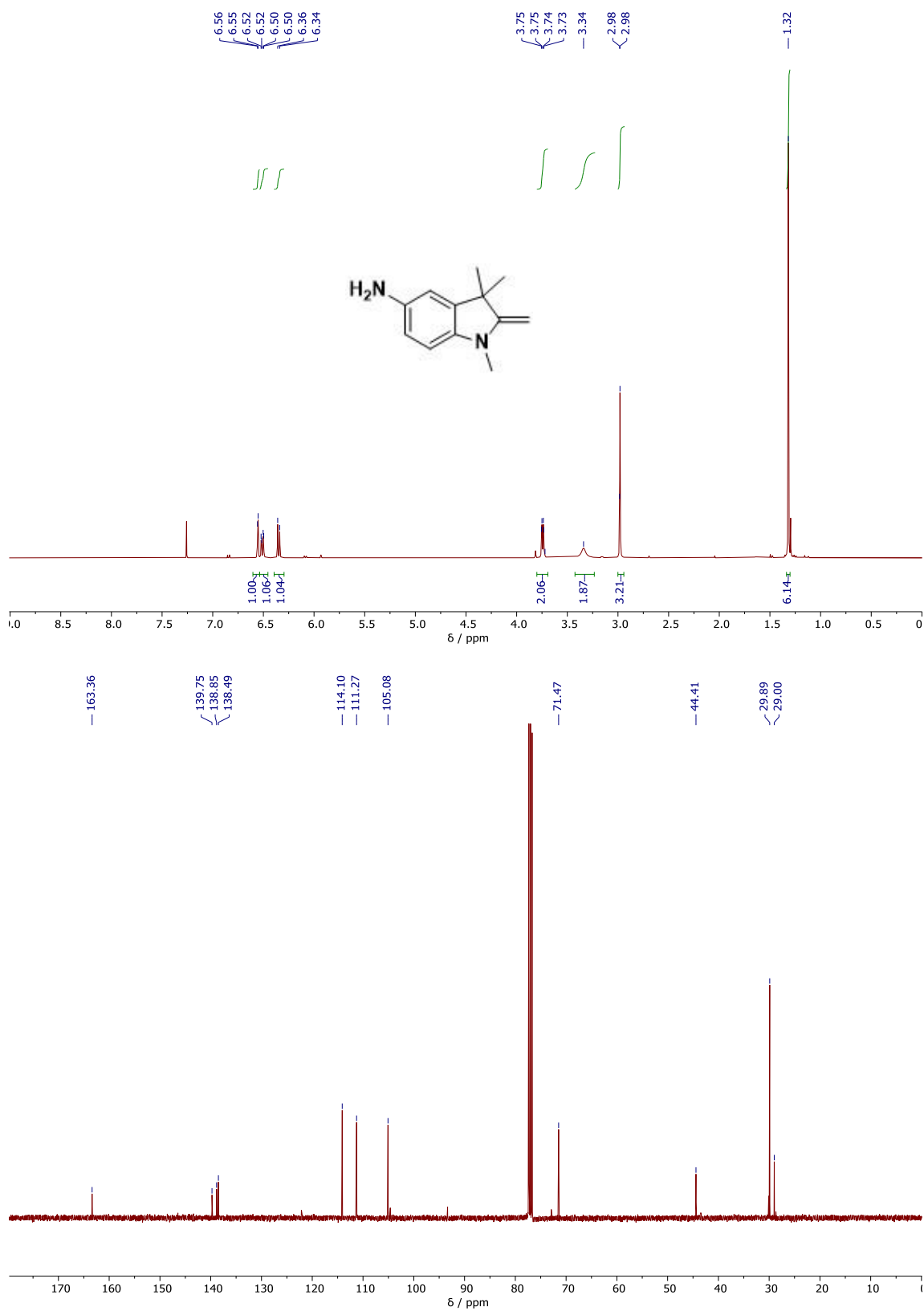


Figure S26. ¹H-NMR (top, 500 MHz) and ¹³C-NMR (bottom, 125 MHz) in CDCl₃ of **3-II**.

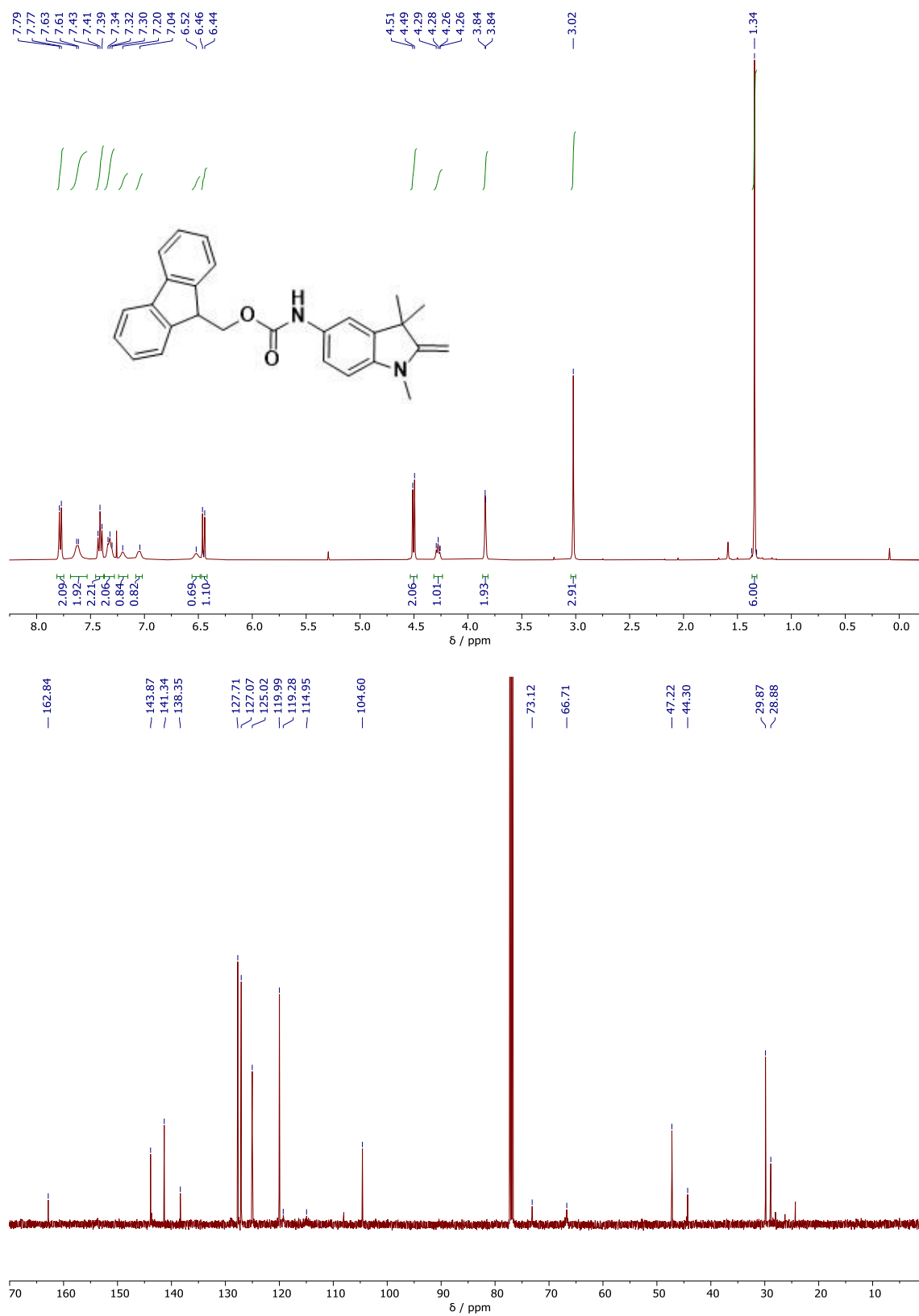


Figure S27. ¹H-NMR (top, 500 MHz) and ¹³C-NMR (bottom, 125 MHz) in CDCl₃ of **4a**.

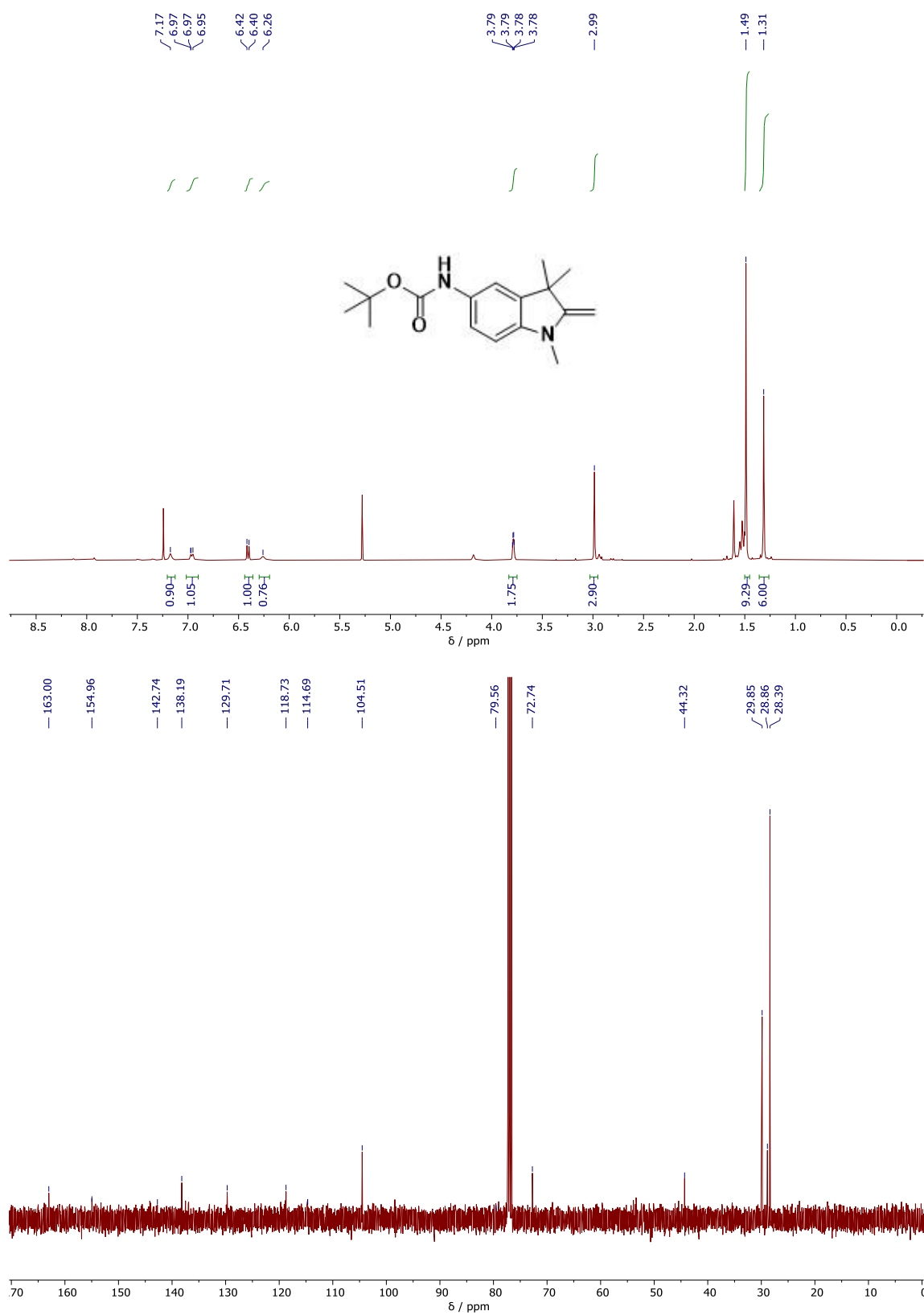


Figure S28. ¹H-NMR (top, 400 MHz) and ¹³C-NMR (bottom, 100 MHz) in CDCl₃ of **4b**.

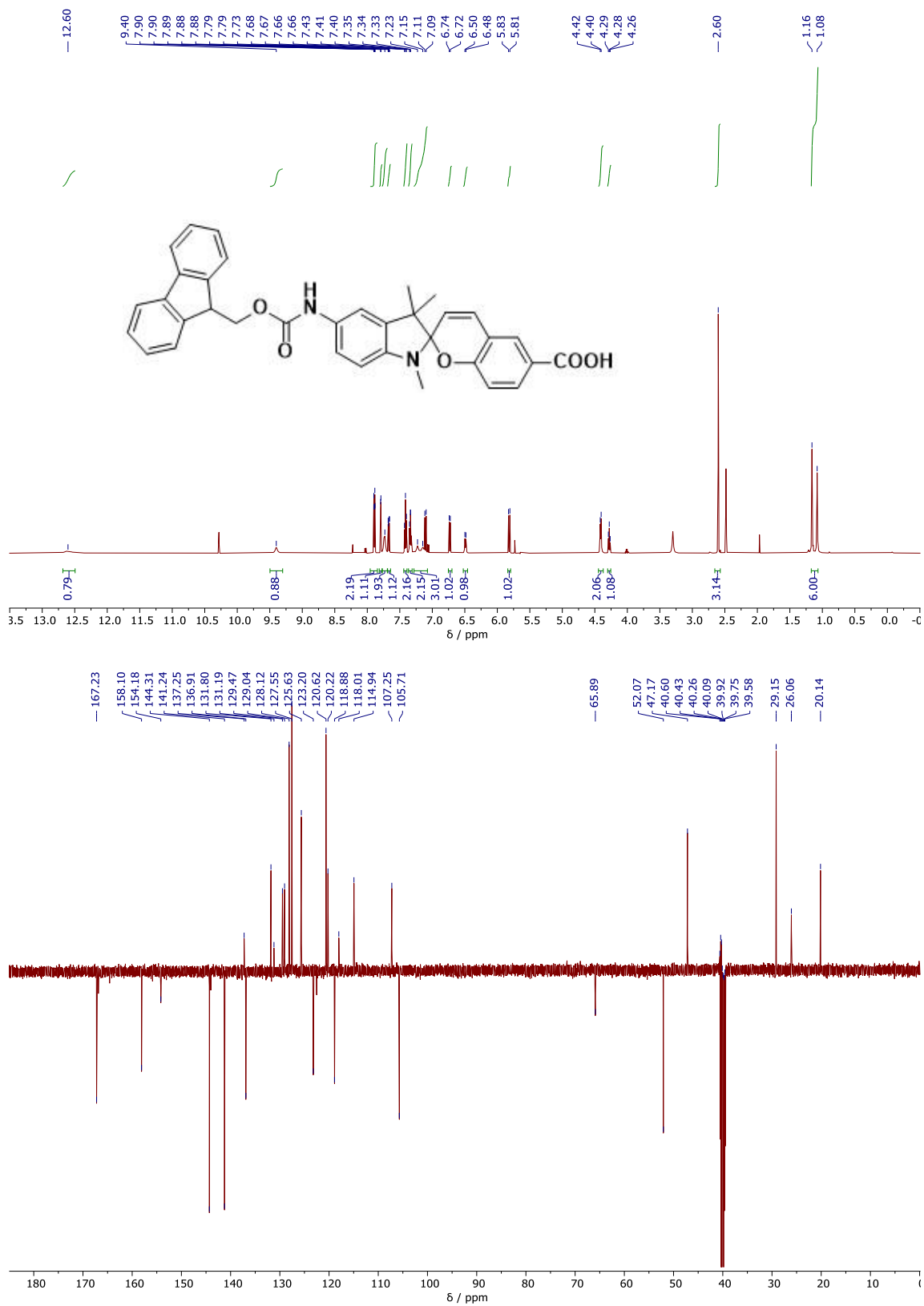


Figure S29. ¹H-NMR (top, 500 MHz) and ¹³C-NMR (bottom, 125 MHz) in DMSO-*d*₆ of **1a**.

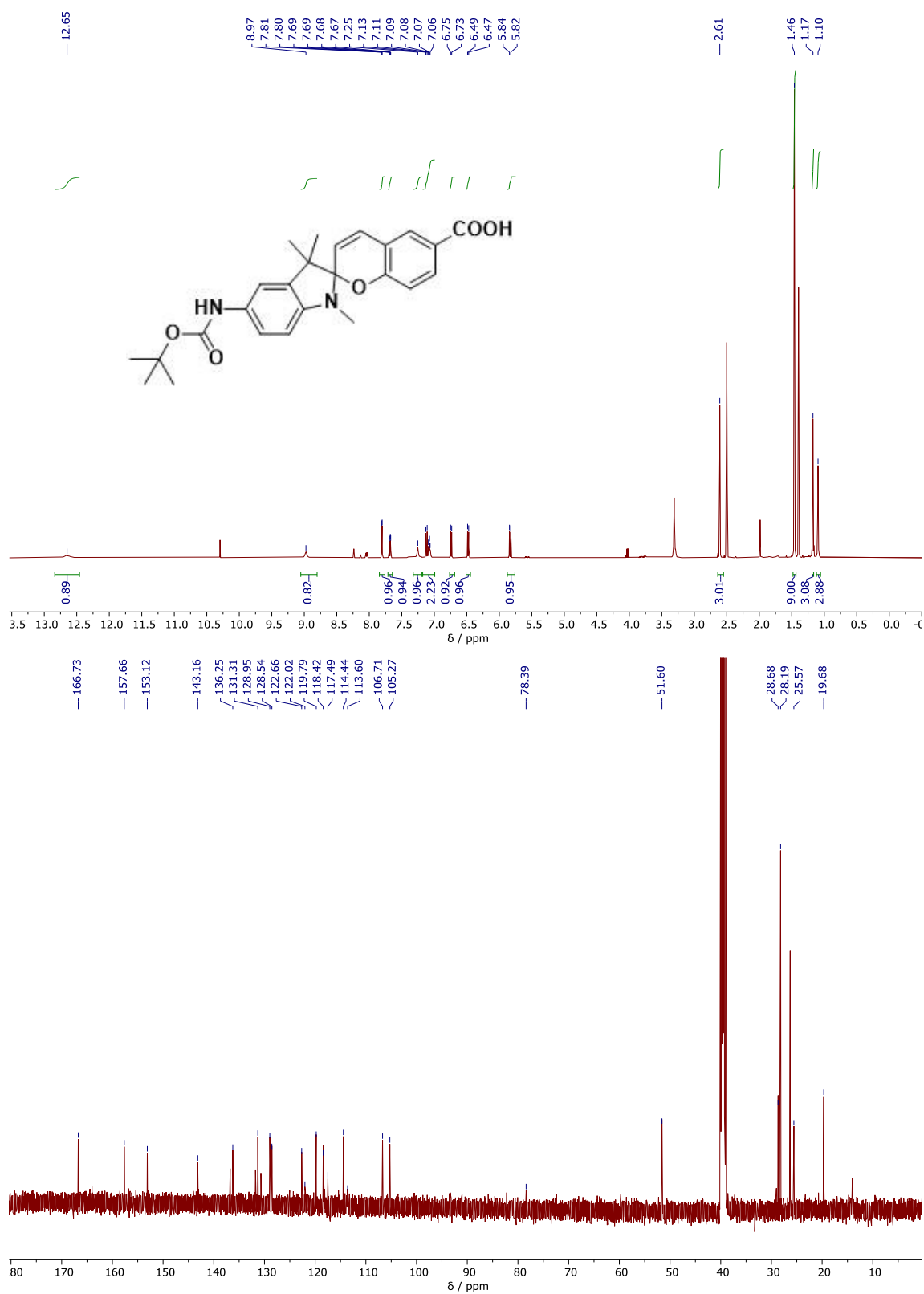


Figure S30. ^1H -NMR (top, 500 MHz) and ^{13}C -NMR (bottom, 125 MHz) in $\text{DMSO}-d_6$ of **1b**.

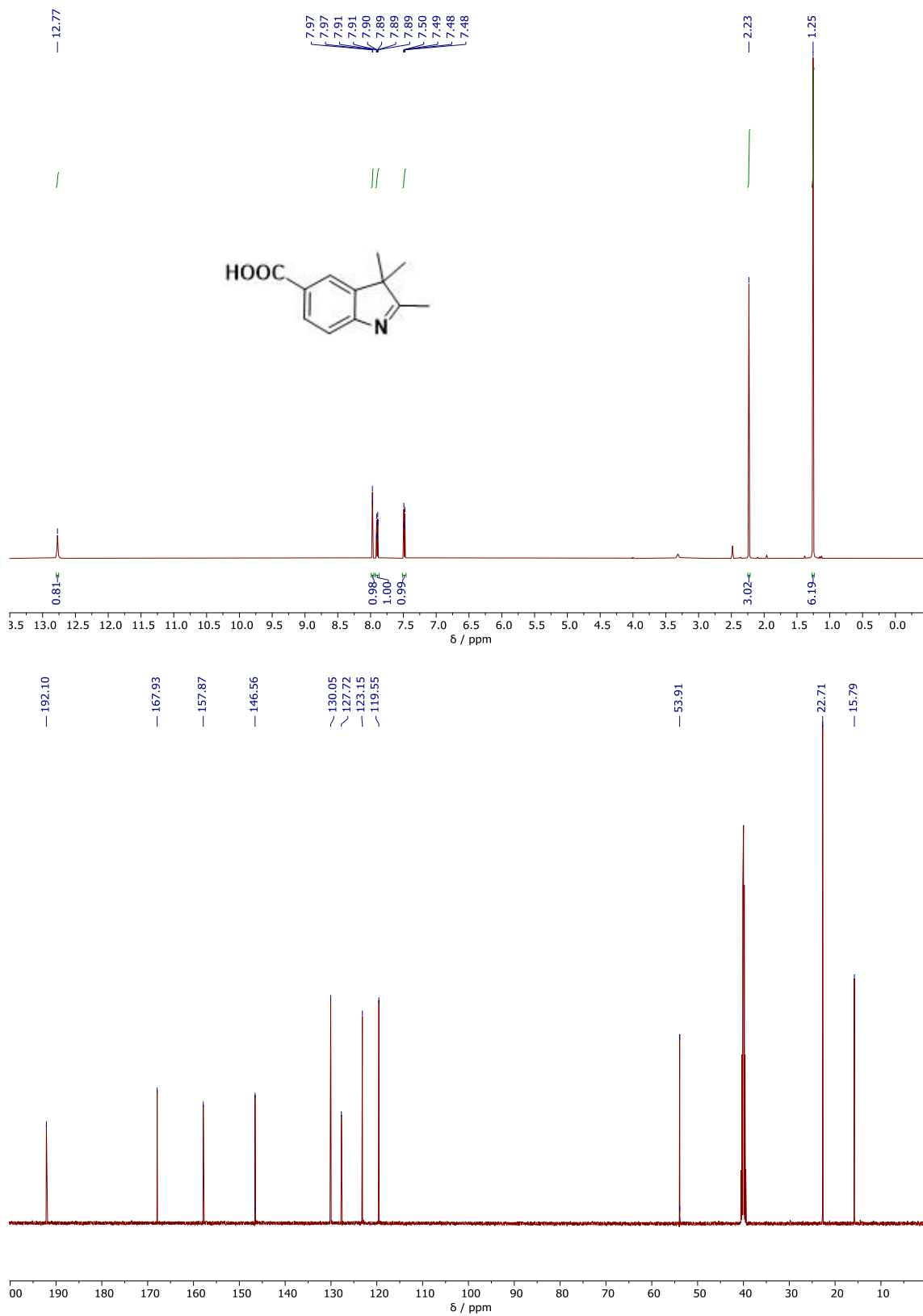


Figure S31. ¹H-NMR (top, 500 MHz) and ¹³C-NMR (bottom, 125 MHz) in CDCl₃ of **6-I**.

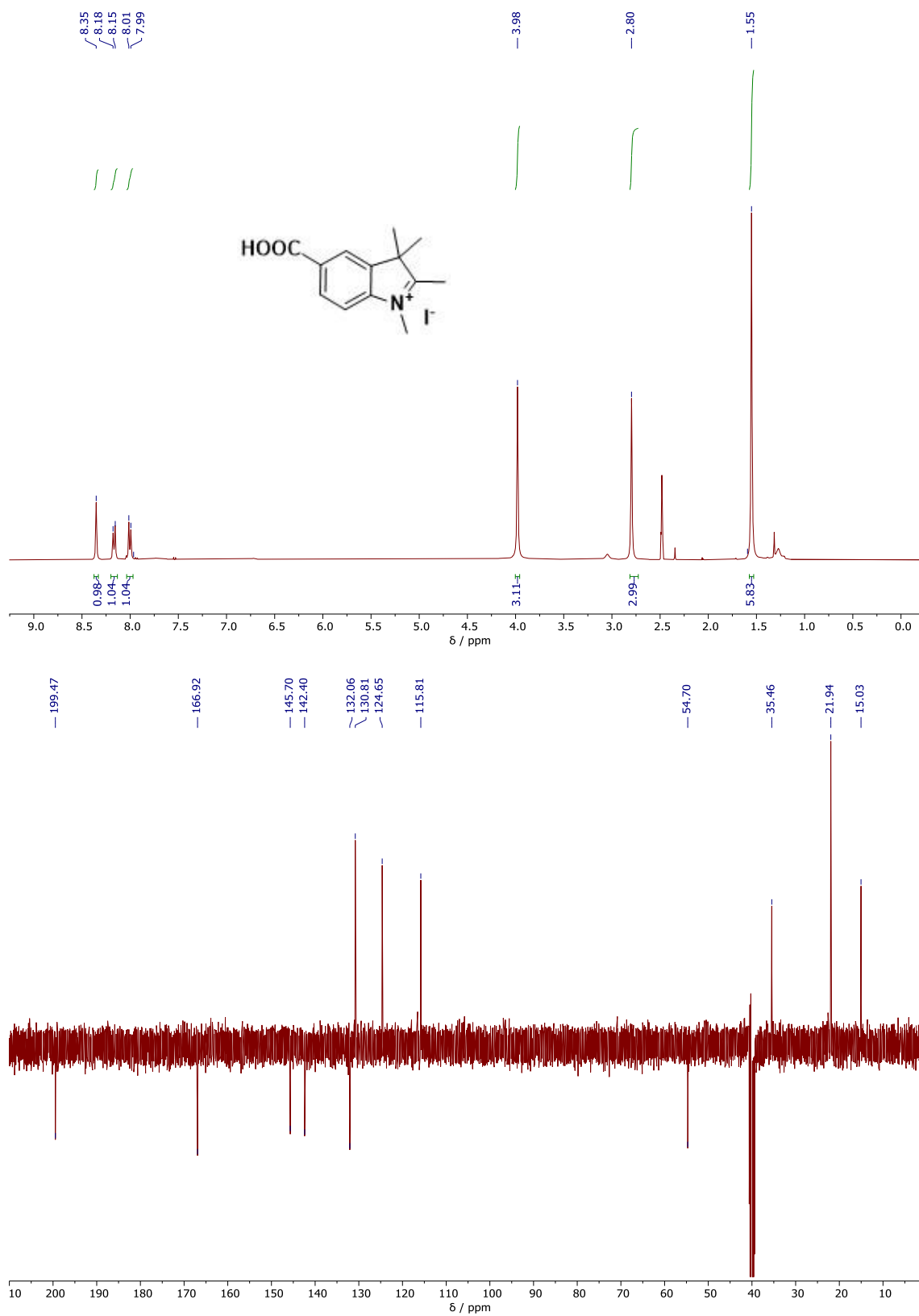


Figure S32. ¹H-NMR (top, 400 MHz) and ¹³C-NMR (bottom, 100 MHz) in DMSO-*d*₆ of **7**.

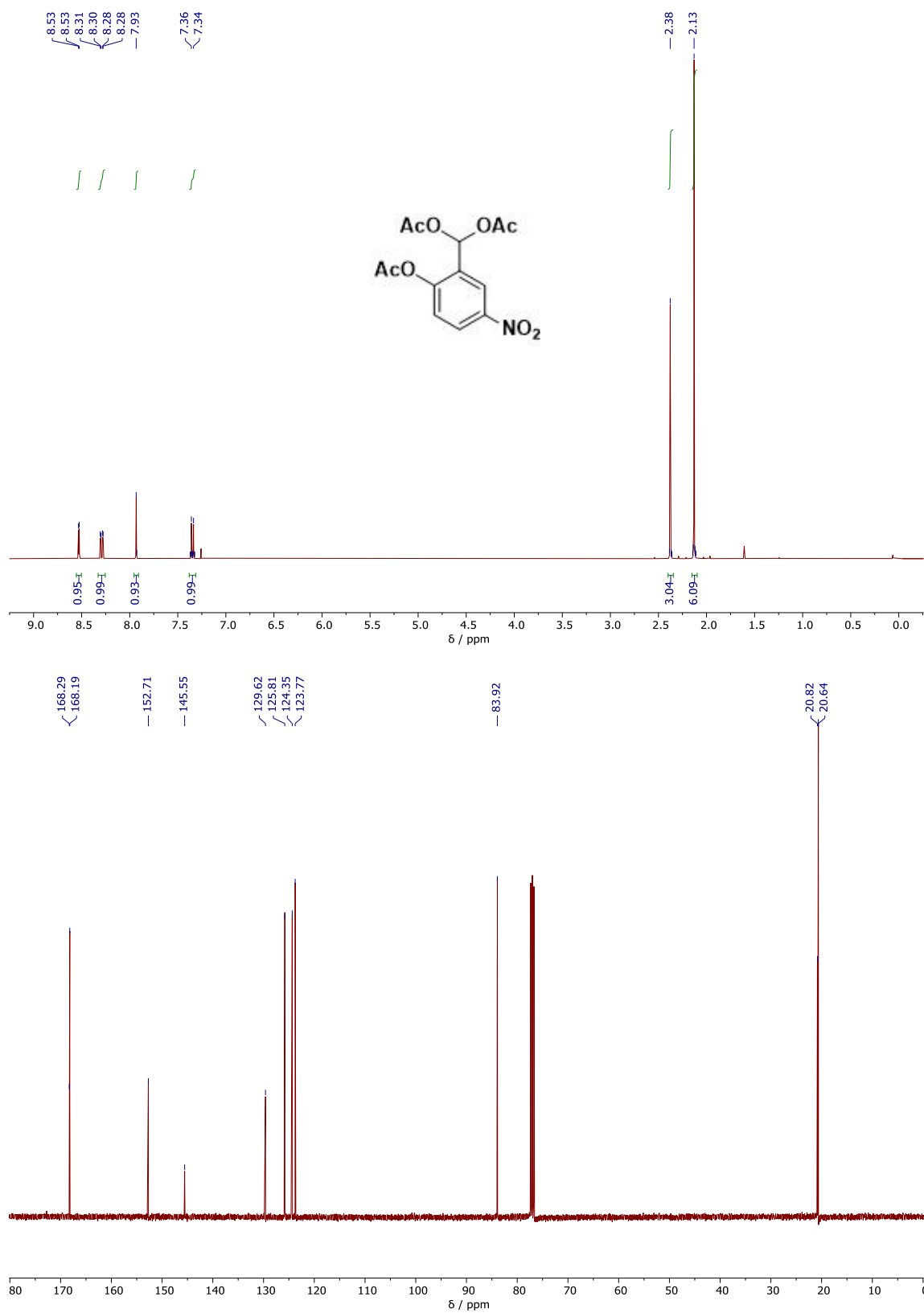


Figure S33. ¹H-NMR (top, 400 MHz) and ¹³C-NMR (bottom, 100 MHz) in CDCl₃ of **8-I**.

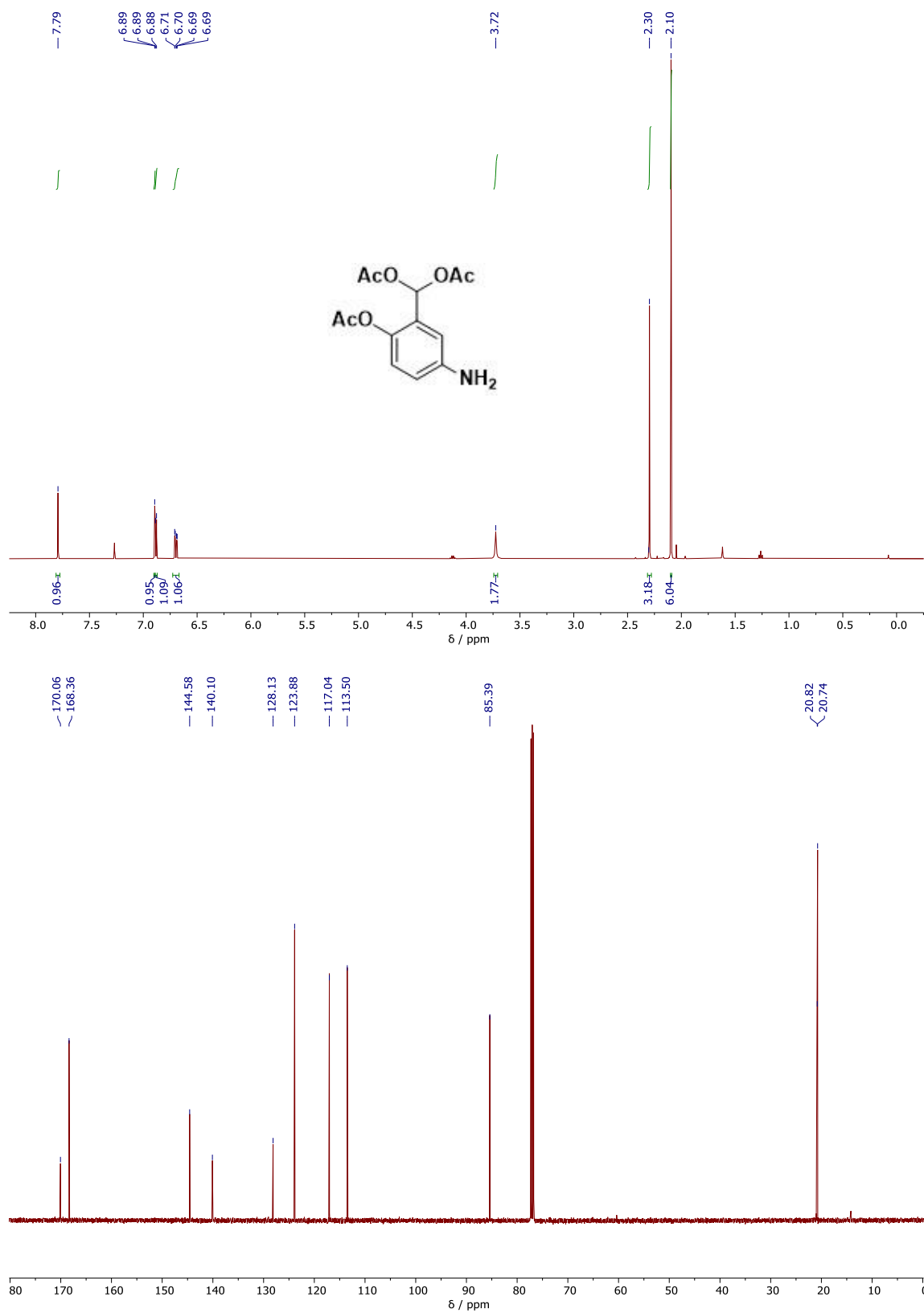


Figure S34. ¹H-NMR (top, 500 MHz) and ¹³C-NMR (bottom, 125 MHz) in CDCl₃ of **8-II**.

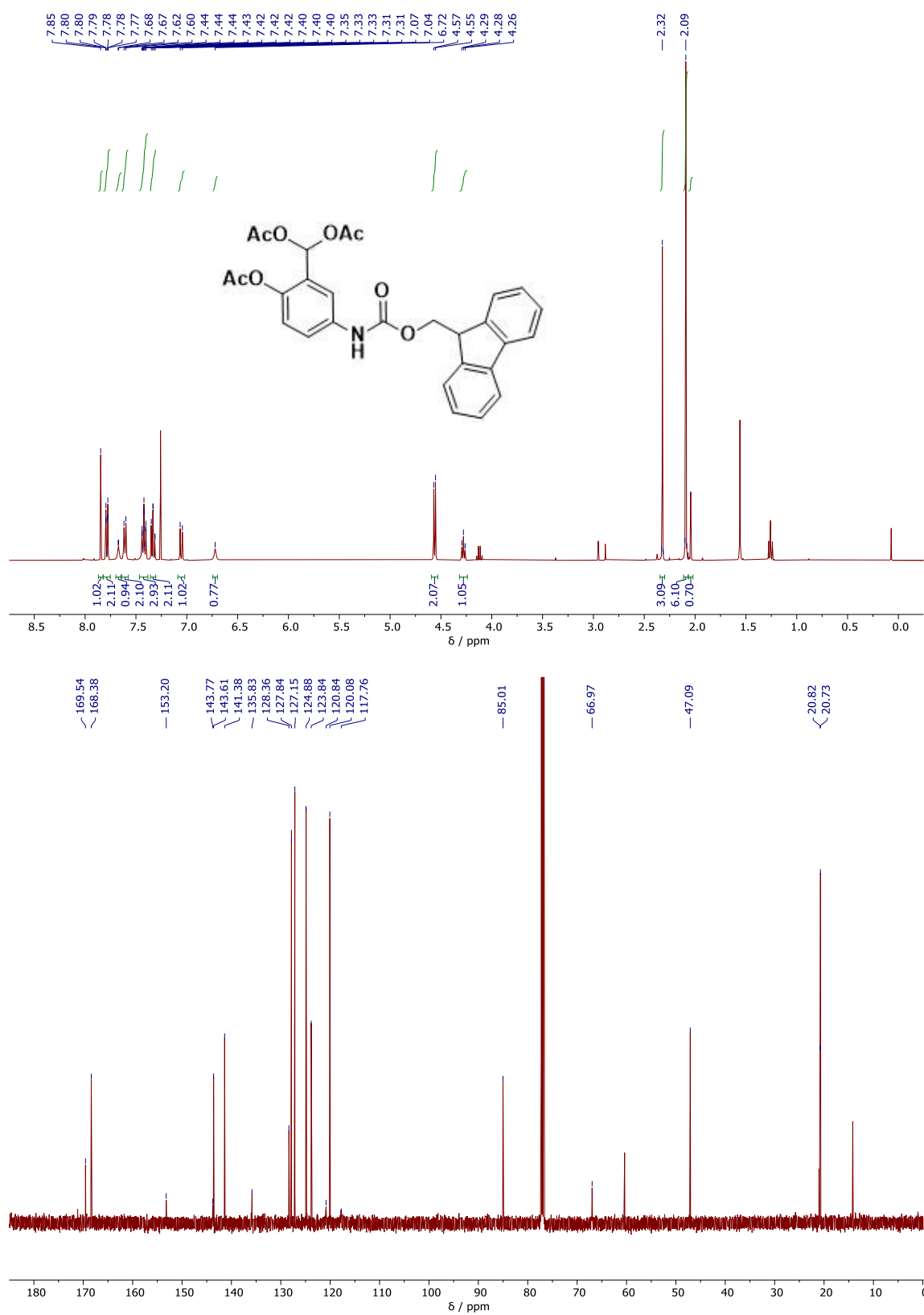


Figure S35. ¹H-NMR (top, 500 MHz) and ¹³C-NMR (bottom, 125 MHz) in CDCl₃ of **9a**.

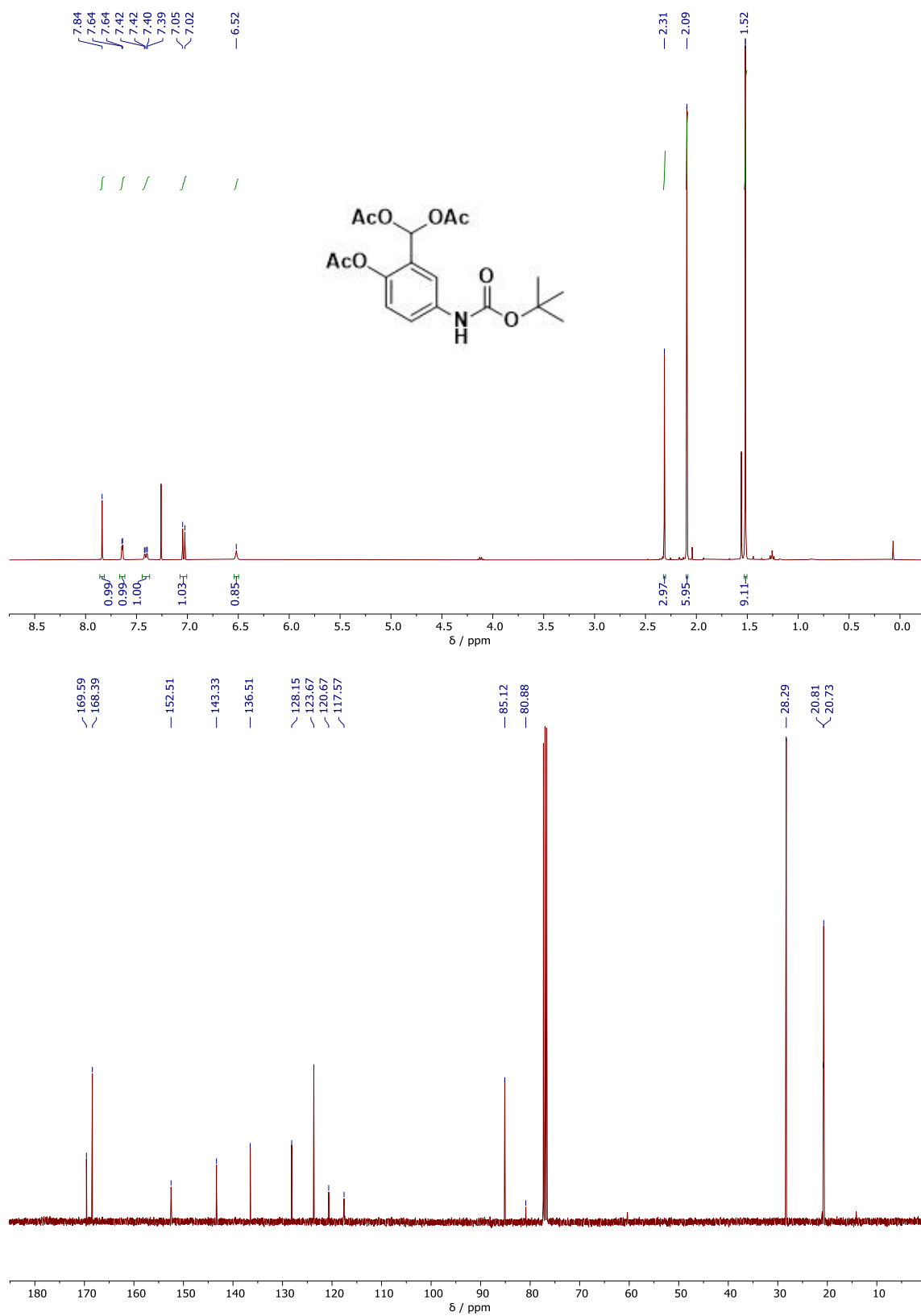


Figure S36. ¹H-NMR (top, 400 MHz) and ¹³C-NMR (bottom, 100 MHz) in CDCl₃ of **9b**.

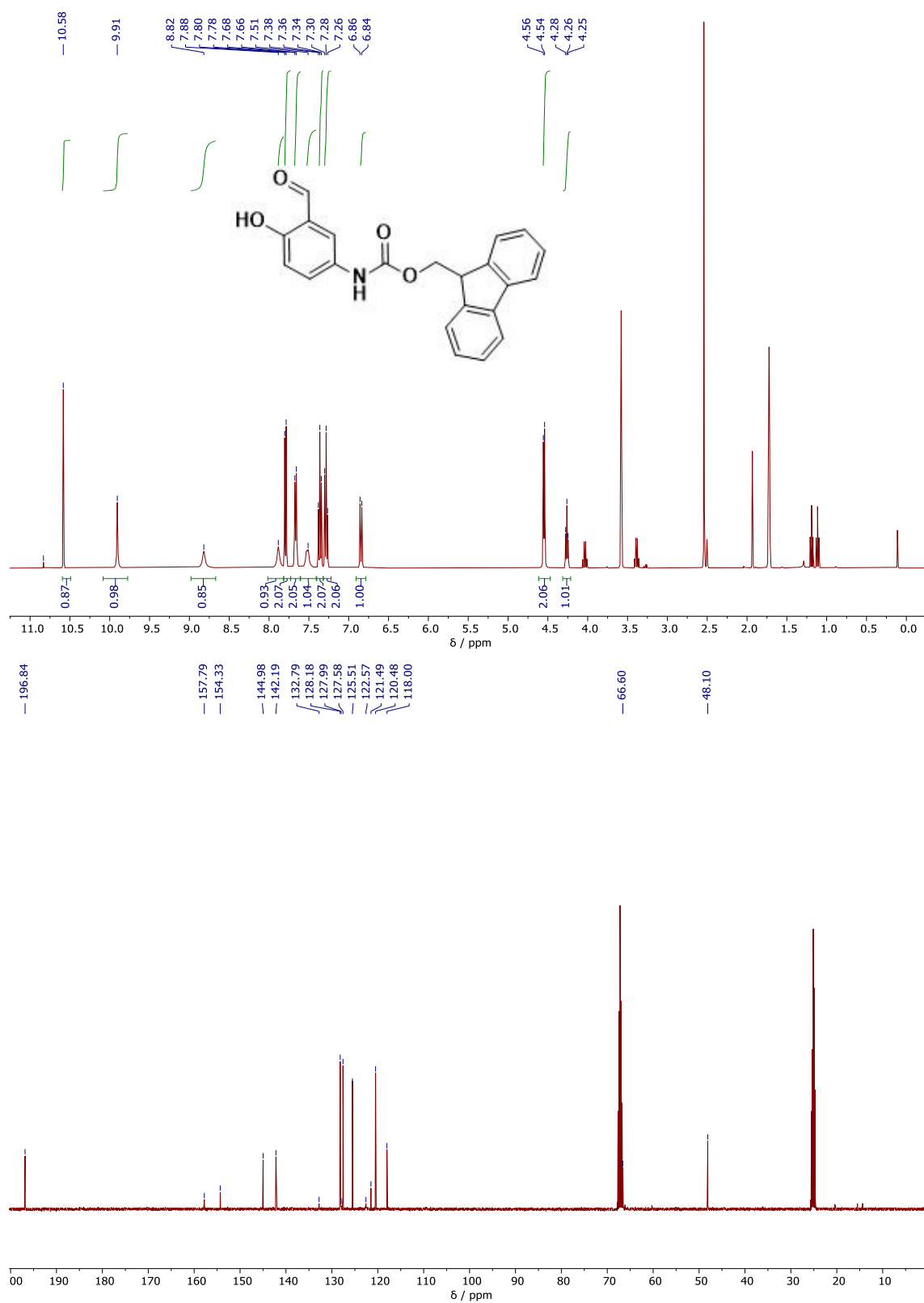


Figure S37. ¹H-NMR (top, 400 MHz) and ¹³C-NMR (bottom, 100 MHz) in THF-*d*₈ of **10a**.

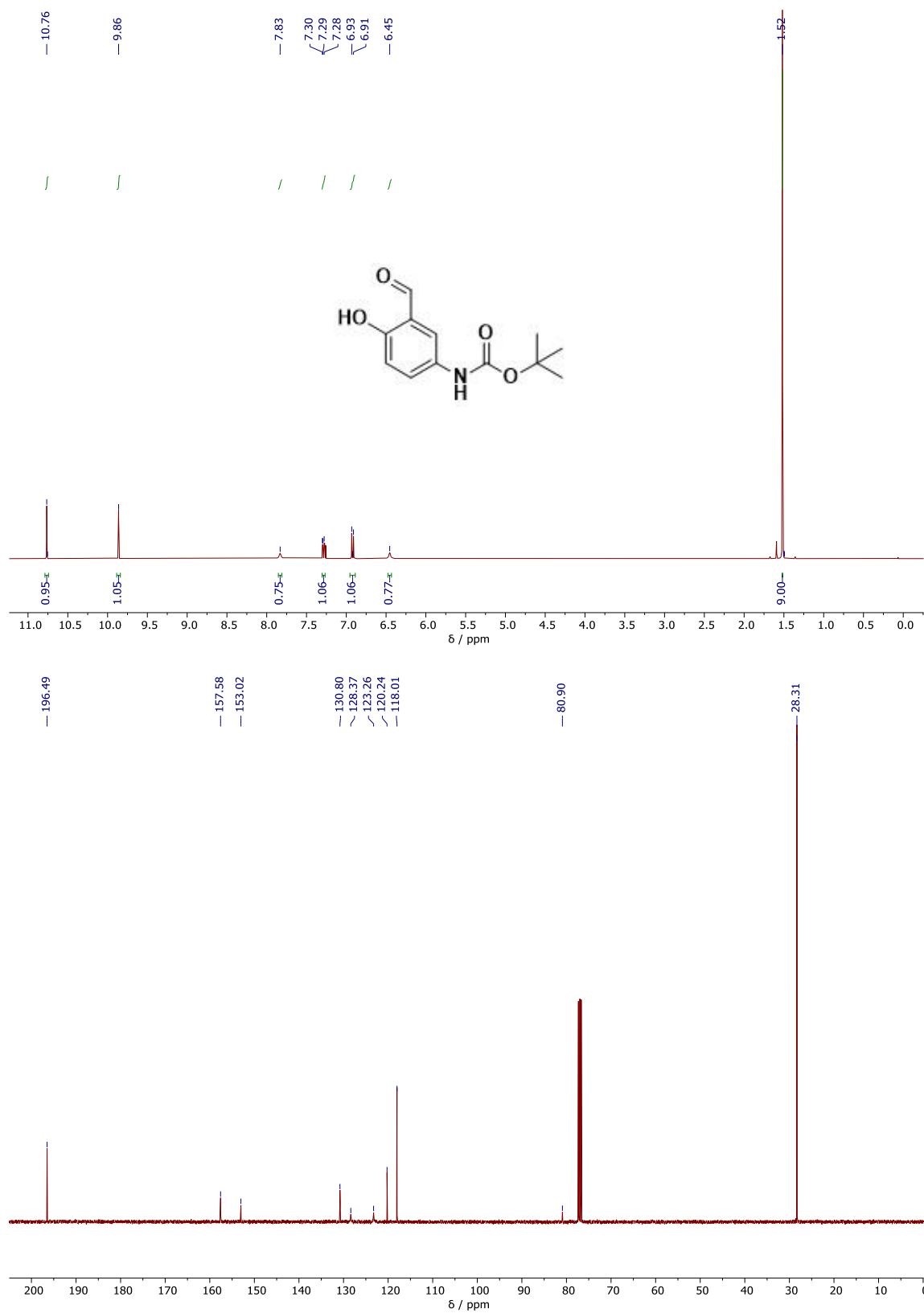


Figure S38. ¹H-NMR (top, 400 MHz) and ¹³C-NMR (bottom, 100 MHz) in CDCl₃ of **10b**.

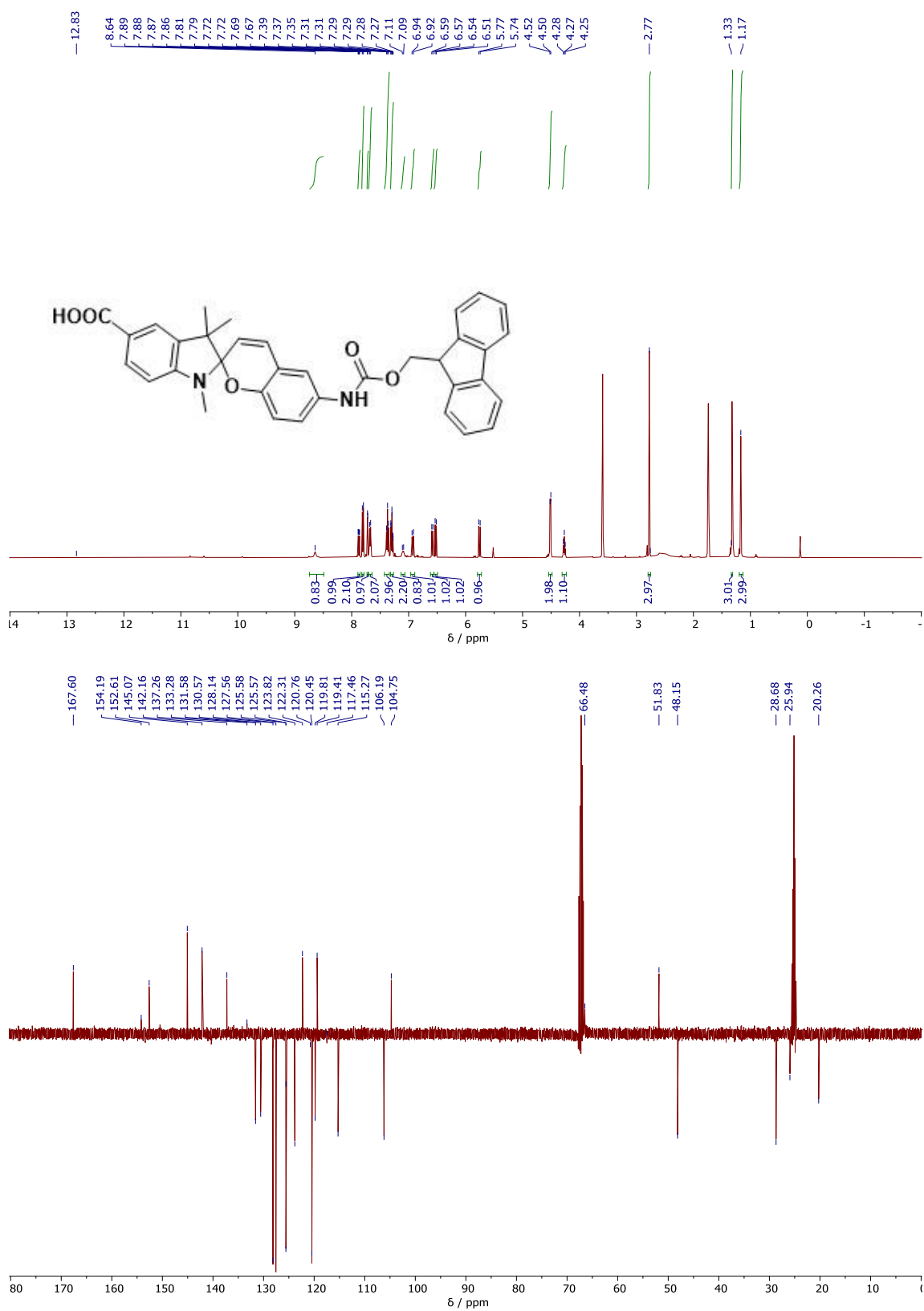


Figure S39. ¹H-NMR (top, 400 MHz) and ¹³C-NMR (bottom, 100 MHz) in THF-*d*₈ of **2a**.

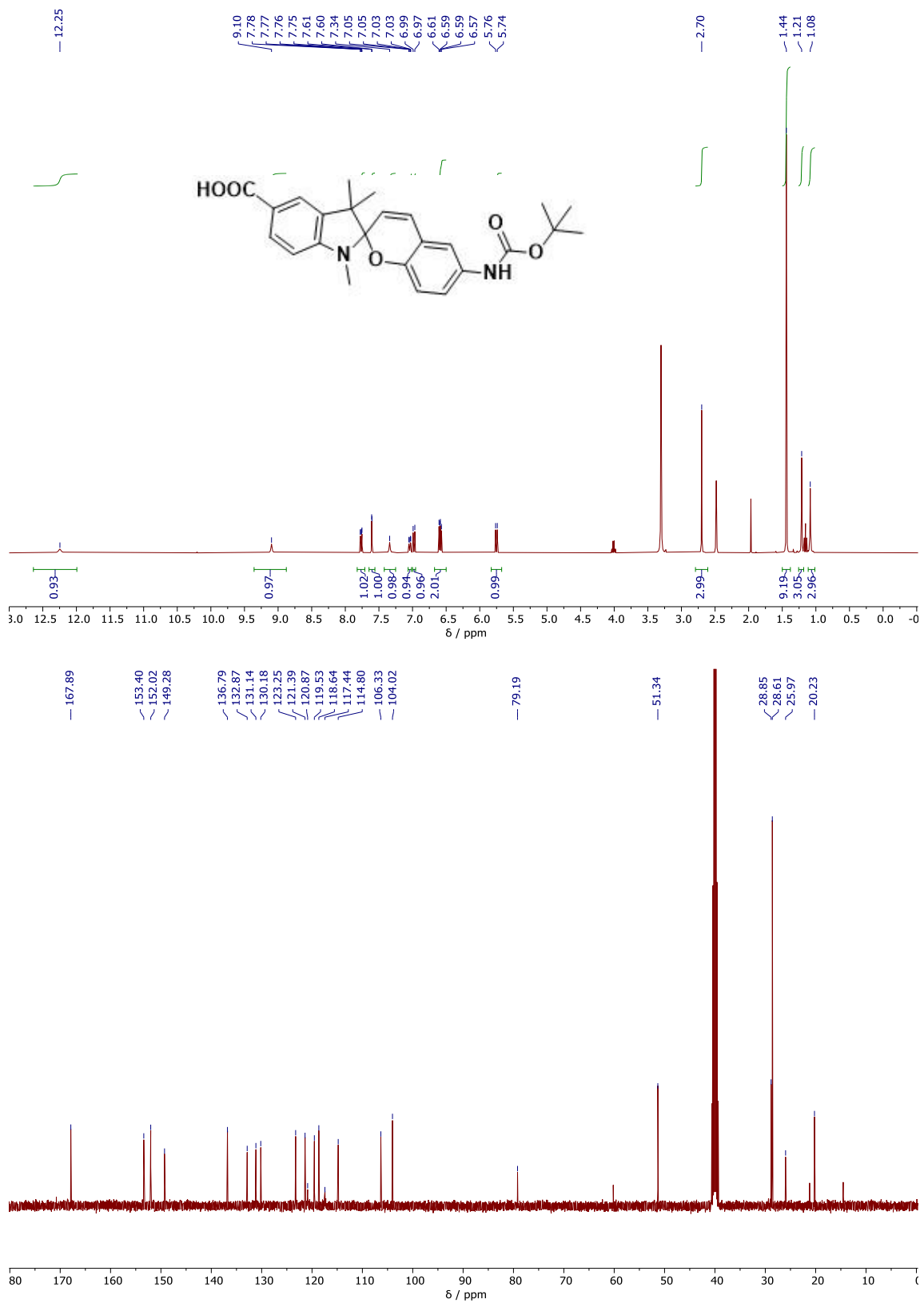


Figure S40. ¹H-NMR (top, 400 MHz) and ¹³C-NMR (bottom, 100 MHz) in DMSO-*d*₆ of **2b**.

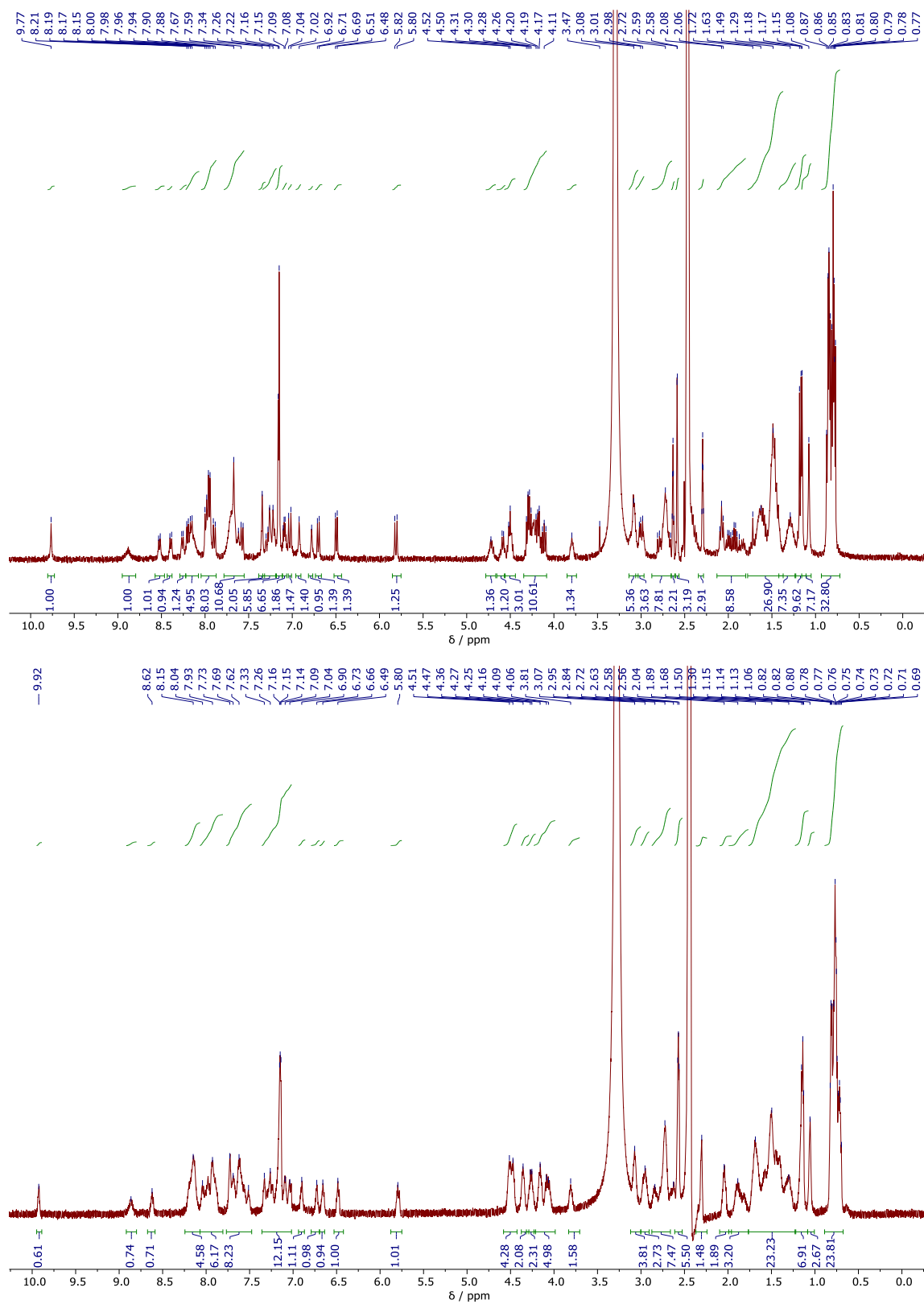


Figure S41: ^1H -NMR spectra (400 MHz, $\text{DMSO}-d_6$) of P1 (top, mixture of isomers) and P2 (bottom, mixture of isomers).

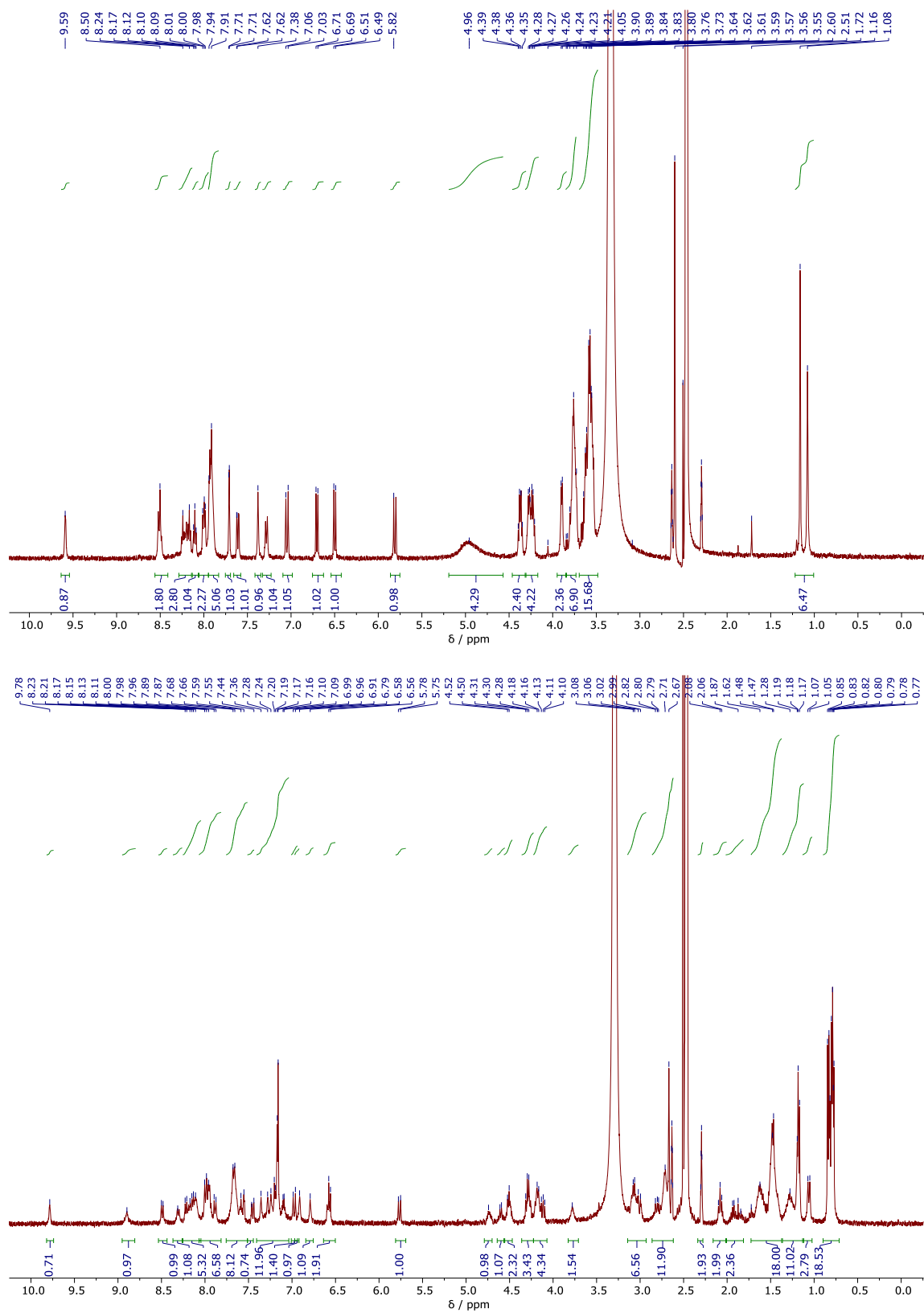


Figure S42: ^1H -NMR spectra (400 MHz, $\text{DMSO-}d_6$) of **P3** (top, mixture of isomers) and **P4** (bottom, mixture of isomers).

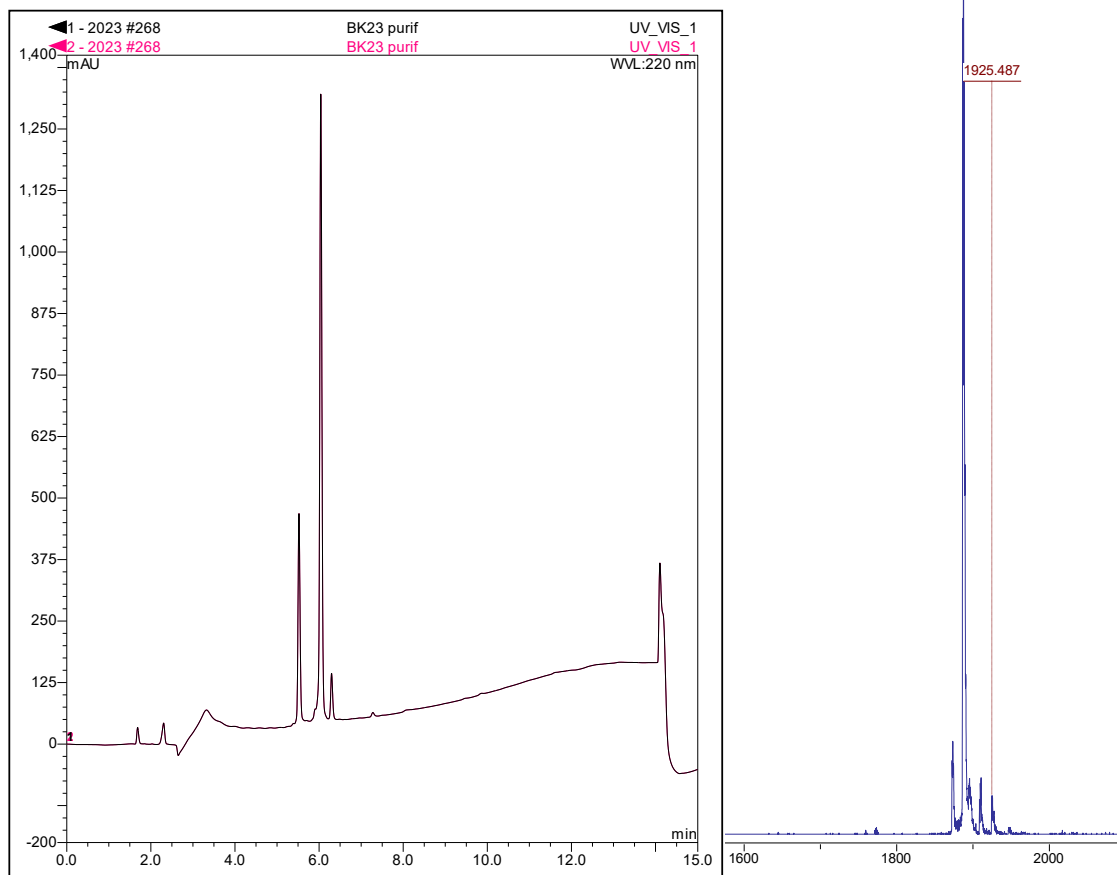


Figure S43: (left) HPLC-trace of P1 (MC- and SP-isomer between 5 and 7 min). (right) MALDI-spectrum of **P1** (m/z calculated: 1886.0435 found: 1887.496).

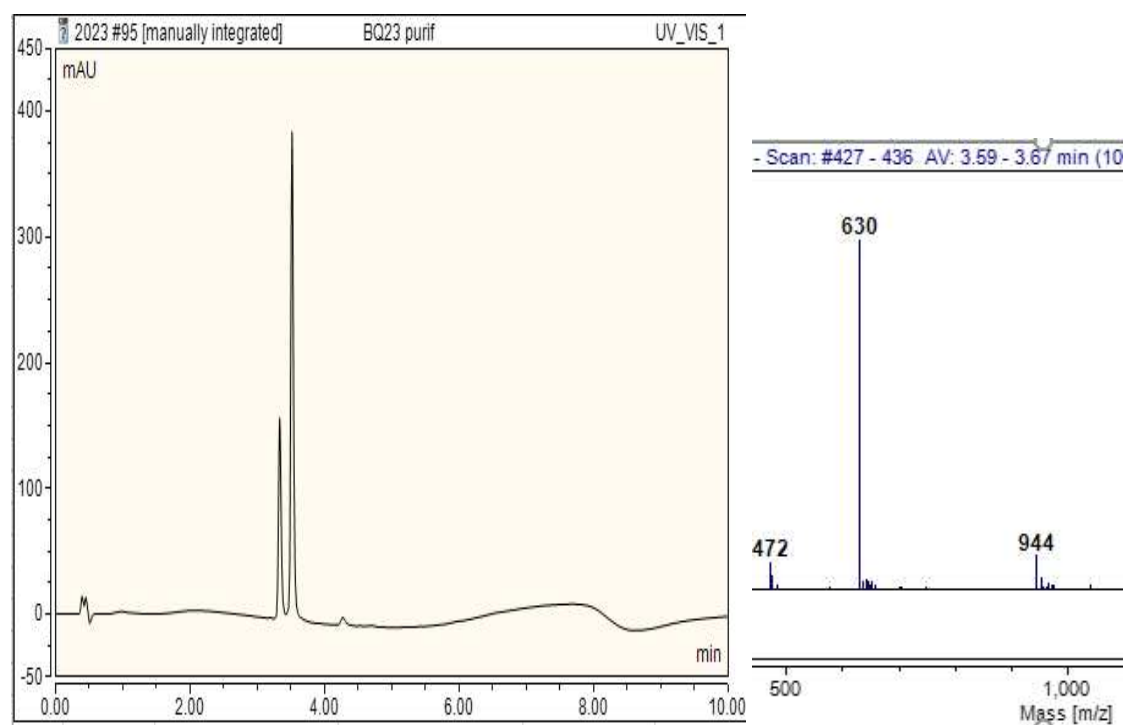


Figure S44: (left) HPLC-trace of P2 (MC- and SP-isomer between 3 and 4min). (right) LC-MS spectrum of **P2** (m/z calculated $[M+2H]^{2+}$: 945 found 945; calculated $[M+3H]^{3+}$: 630 found 630).

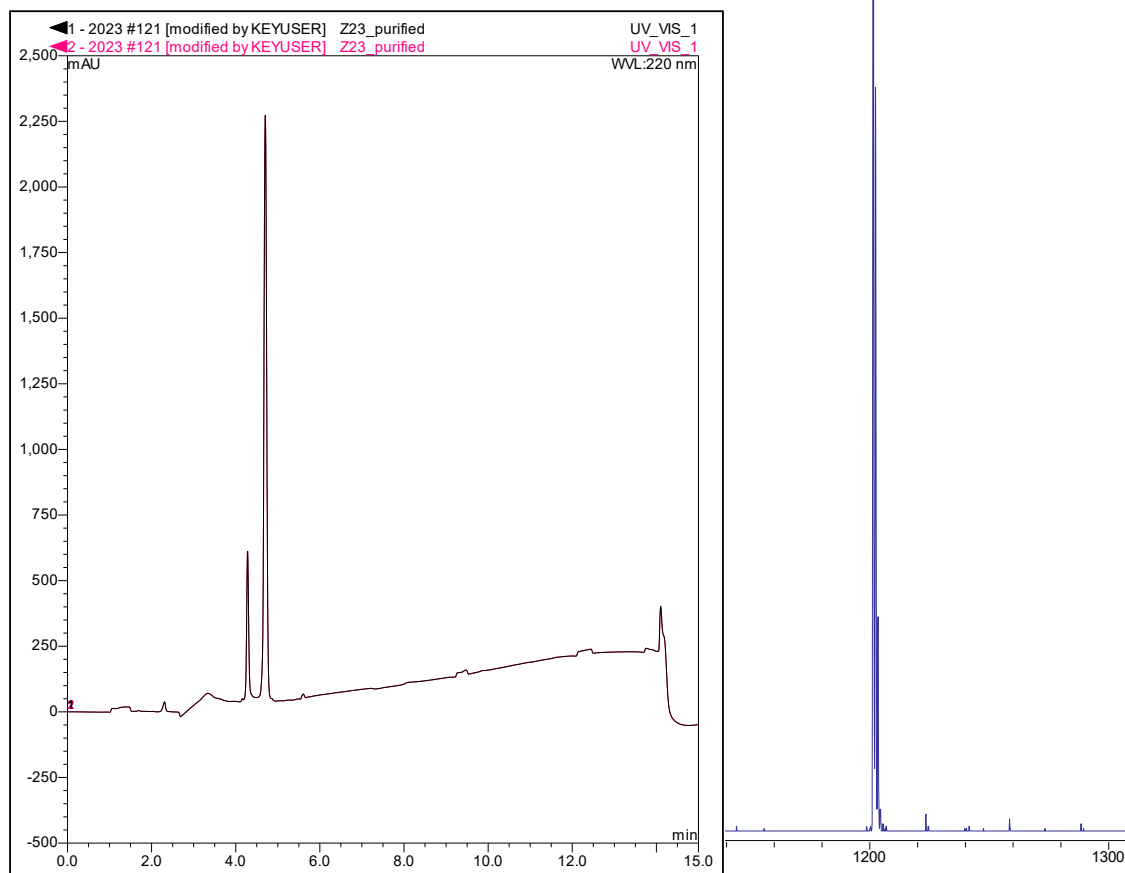


Figure S45: (left) HPLC-trace of P3 (MC- and SP-isomer between 4 and 5 min). (right) MALDI-spectrum of **P3** (m/z calculated: 1201.4756 found: 1201.373).

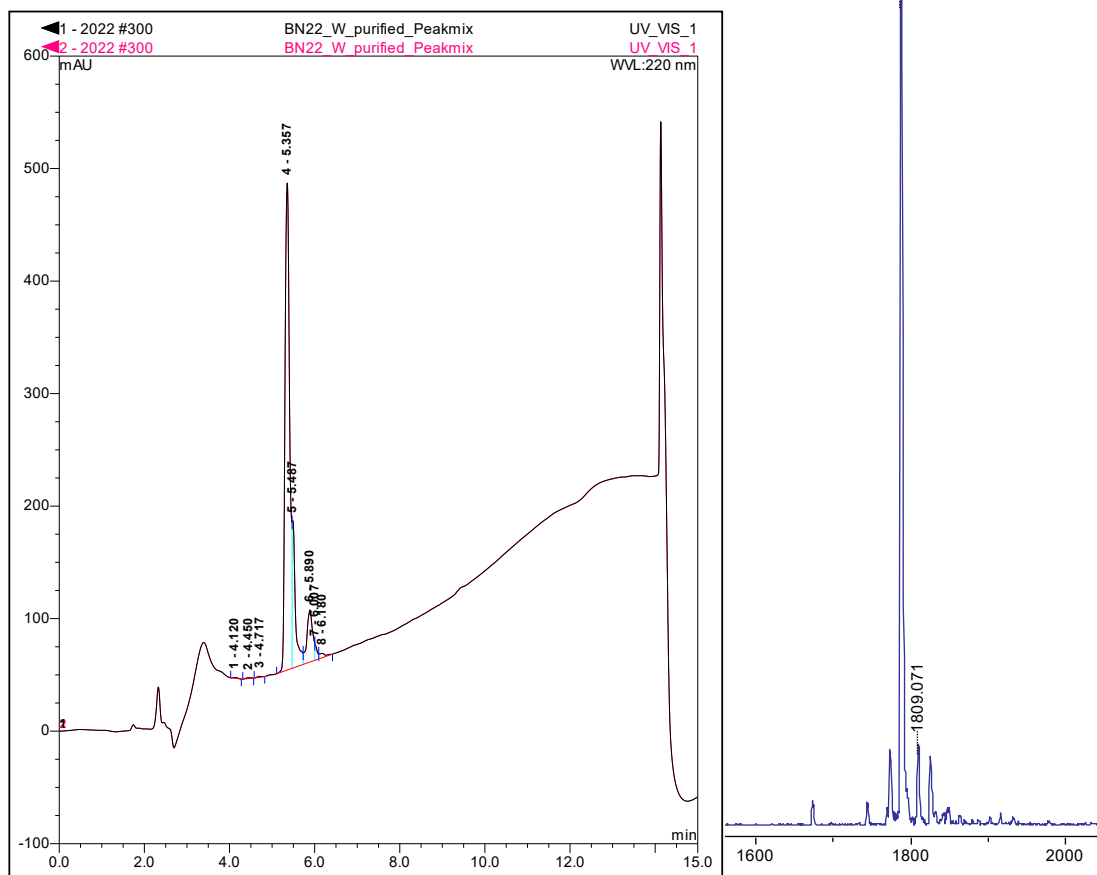


Figure S46: (left) HPLC-trace of P4 (MC- and SP-isomer between 6 and 7 min). (right) LC-MS spectrum of **P4** (m/z calculated $[M+H]^+$: 1787 found 1787).

- (1) Sepehr, Z.; Nasr-Isfahani, H.; Mahdavian, A. R.; Amin, A. H. Synthesis, characterization, and UV–visible study of some new photochromic formyl-containing 1', 3', 3'-trimethylspiro [chromene-2, 2'-indoline] derivatives. *J. Iran. Chem. Soc.* **2021**, *18* (11), 3061-3067. DOI: 10.1007/s13738-021-02253-5.
- (2) GALE, D. J.; WILSHIRE, J. F. Fibre–reactive Basic Dyes I–Polymethine Dyes Containing the N–Chloroacetyl Group. *J. Soc. Dye. Colour.* **1974**, *90* (3), 97-100. DOI: 10.1111/j.1478-4408.1974.tb03188.x.
- (3) Tomasulo, M.; Kaanumal, S. L.; Sortino, S.; Raymo, F. M. Synthesis and properties of benzophenone– spiropyran and naphthalene– spiropyran conjugates. *J. Org. Chem.* **2007**, *72* (2), 595-605. DOI: 10.1021/jo062004d.
- (4) Di Bella, S.; Consiglio, G.; Leonardi, N.; Failla, S.; Finocchiaro, P.; Fragalà, I. Film polymerization– a new route to the synthesis of insoluble polyimides containing functional nickel (II) schiff base units in the main chain. *Eur. J. Inorg. Chem.* **2004**, *2004* (13), 2701-2705. DOI: <https://doi.org/10.1002/ejic.200300959>.