



Halogenation-Guided Chemical Screening Provides Insight into Tjipanazole Biosynthesis by the Cyanobacterium *Fischerella ambigua*

Tomasz Chilczuk,^[a] Till F. Schäberle,^[b, f] Sahel Vahdati,^[c] Ute Mettal,^[b, f] Mustafa El Omari,^[d] Heike Enke,^[e] Michael Wiese,^[c] Gabriele M. König,^[d] and Timo H. J. Niedermeyer^{*,[a]}

Halogenated natural products (HNPs) show a wide range of interesting biological activities. Chemistry-guided screening with a software tool dedicated to identifying halogenated compounds in HPLC-MS data indicated the presence of several uncharacterised HNPs in an extract of the cyanobacterium *Fischerella ambigua* (Näg.) Gomont 108b. Three new natural products, tjipanazoles K, L, and M, were isolated from this strain together with the known tjipanazoles D and I. Taking into account the structures of all tjipanazole derivatives detected in this strain, reanalysis of the tjipanazole biosynthetic gene

cluster allowed us to propose a biosynthetic pathway for the tjipanazoles. As the isolated tjipanazoles show structural similarity to arcyriaflavin A, an inhibitor of the clinically relevant multidrug-transporter ABCG2 overexpressed by different cancer cell lines, the isolated compounds were tested for ABCG2 inhibitory activity. Only tjipanazole K showed appreciable transporter inhibition, whereas the compounds lacking the pyrrolo[3,4-c] ring or featuring additional chloro substituents were found to be much less active.

Introduction

More than 5000 halogenated natural products (HNPs) are known today, and the number is constantly growing.^[1] HNPs constitute a diverse group of metabolites with a wide range of bioactivities, including antibacterial, antifungal, antiparasitic,

antiviral, anti-inflammatory and antitumor activities.^[2] Some of these HNPs are used as antibiotics (e.g., vancomycin,^[3] chloramphenicol^[4]) or are studied as potential antitumor agents (e.g., rebeccamycin^[5], cryptophycins^[6]). The presence of halogen substituents often enhances the bioactivity of natural products,^[7] as exemplified by vancomycin, where mono- and didechlorovancomycin show two to four times lower activity in binding to aliphatic peptides than vancomycin.^[8] Halogenation is also a widely used strategy to modify the activity of small synthetic drug molecules.^[9]

The majority of known HNPs are biosynthesised by marine bacteria,^[10,11] marine algae^[10,12] and marine invertebrates.^[13] However, their production has been also reported in terrestrial plants,^[14] fungi,^[15] soil bacteria,^[3,16] insects,^[17] and higher animals.^[18] The search for HNPs from cyanobacteria is particularly interesting because cyanobacteria are considered to be a rich source of bioactive and chemically diverse compounds,^[19] and are well known to synthesise HNPs, such as aeruginosins,^[20] cyanopeptolins,^[21] cryptophycins,^[22] microginins,^[23] lyngbyabellins,^[24] and the indole alkaloids ambiguines,^[25] hapalindoles,^[26] welwitindolinones,^[27] and fischerindoles.^[28] Other cyanobacterial HNPs from various chemical classes are the ambigols,^[29] bartolosides,^[30] carbamidocyclophanes,^[31] malyngamides,^[32] and tjipanazoles.^[33]

In an effort to identify novel HNPs from cyanobacteria, a chemistry-guided screening was conducted with the software tool HaloSeeker.^[34] In full-scan HPLC-HRMS data of an extract of *Fischerella ambigua* (Näg.) Gomont 108b, we could detect far more polyhalogenated compounds than described from this strain to date. As currently only 23 HNPs from the genus *Fischerella* are listed in the Dictionary of Natural Products (18 indole alkaloids, 3 ambigols, 2,4-Dichlorobenzoic acid, and tjipanazole D),^[29,35,36] this suggested the strain to be a promising

[a] T. Chilczuk, Prof. Dr. T. H. J. Niedermeyer
Department of Pharmaceutical Biology/Pharmacognosy
Institute of Pharmacy
Martin-Luther-University Halle-Wittenberg
Hoher Weg 8, 06120 Halle (Saale) (Germany)
E-mail: timo.niedermeyer@pharmazie.uni-halle.de


[b] Prof. Dr. T. F. Schäberle, Dr. U. Mettal
Institute for Insect Biotechnology
Justus-Liebig-University Gießen
Heinrich-Buff-Ring 26–32, 35392 Gießen (Germany)


[c] S. Vahdati, Prof. Dr. M. Wiese
Department of Pharmaceutical and Cell Biological Chemistry
University of Bonn
An der Immenburg 4, 53121 Bonn (Germany)


[d] Dr. M. El Omari, Prof. Dr. G. M. König
Institute of Pharmaceutical Biology, University of Bonn
Nussallee 6, 53115 Bonn (Germany)

[e] Dr. H. Enke
Cyano Biotech GmbH
Magnusstraße 11, 12489 Berlin (Germany)

[f] Prof. Dr. T. F. Schäberle, Dr. U. Mettal
Department of Bioresources
Fraunhofer Institute for Molecular Biology and Applied Ecology
Winchesterstrasse 2, 35394 Gießen (Germany)

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source for new HNPs. Subsequent isolation of some of the HNPs detected in this strain led to the identification of the new natural products tjipanazoles K (3), L (4) and M (5), along with the known tjipanazoles D (1) and I (2). Their structures were elucidated using 1D and 2D NMR and HRMS.

Tjipanazole D has been proposed to be synthesised from two L-tryptophans by five enzymes, an L-tryptophan halogenase, an L-tryptophan oxidase, a chromopyrrolic acid synthase-like protein, a CYP 450 enzyme, and a FAD-binding monooxygenase. The first two enzymes are supposed to catalyse the conversion of L-tryptophan to 5-chloro-indole-3-pyruvic acid (chlorination and oxidative desamination), whereas the latter three enzymes use this intermediate in subsequent reactions (dimerisation, aryl-aryl-coupling, side-chain reduction, aromatisation) for the assembly of tjipanazole D.^[37] The structures of the three new tjipanazole derivatives and the detectable halogenated intermediates of the tjipanazole biosynthesis, as well as a more detailed analysis of the genome of this *F. ambigua* strain, allowed us to revisit and substantiate the formerly described proposed putative tjipanazole biosynthesis pathway.

All three new tjipanazoles share an indolocarbazole core structure and show structural similarities to the ABCG2 inhibitors K252c (7) and arcylriaflavin A (8).^[38] ABCG2, also known as breast cancer resistance protein, is an ATP-binding cassette transporter that protects many tissues against xenobiotics by transporting them across extracellular and intracellular membranes. It is known to contribute to multidrug resistance in cancer by affecting the pharmacokinetics of anticancer drugs.^[39] Due to the clinical relevance of ABCG2-mediated multidrug chemotherapy resistance, extensive efforts have been devoted to the development of ABCG2 inhibitors that could be used in combination with anticancer drugs to reduce their secretion from cancer cells.^[40] Based on the studies of Rubey et al.,^[38] ABCG2 is regarded as a promising new target for indolocarbazoles. Thus, all isolated compounds were tested for their capability to inhibit ABCG2.

Results and Discussion

Isolation and structure elucidation

In a chemistry-guided screening for HNPs in cyanobacteria extracts using HaloSeeker, a post-acquisition processing tool designed to annotate signals from halogenated ions within HRMS data sets, an extract of *F. ambigua* (Näg.) Gomont 108b was found to be rich in HNPs. Evaluation of the HRMS data resulted in the detection of 105 analyte species with isotope patterns typical for halogenated substances (Figure S1 in the Supporting Information). Fractionation of the biomass extract of this strain using flash chromatography resulted in 24 fractions. The fractions containing the major HNPs were combined.

Several compounds were isolated from the combined fraction using semi-preparative HPLC, among them compounds 1–5. The UV spectra of the compounds suggested that they are structurally related (Figure S2). The molecular formulas were deduced from HRMS data, the structures were elucidated based on 1D and 2D NMR (Figure 1). The NMR and HRMS data of 1 and 2 matched the published data of tjipanazole D and I, respectively (Figures S3–S5).^[33] Tjipanazole D (1) has been isolated from the same strain before,^[29,36,41] whereas tjipanazole I (2) has already been reported as a natural product from the cyanobacterium *Tolypothrix tjipanasensis*.^[33]

Compound 3 was isolated as yellow crystals. Its molecular formula was established as C₂₀H₁₁Cl₂N₃O by the [M–H][–] ion at *m/z* 378.0207 (calcd. 378.0206, Δ 0.01 ppm). Compared to 1, the sum formula of 3 indicated the presence of an additional nitrogen as well as two carbon atoms, suggesting it to be more closely related to tjipanazoles containing an additional pyrrolo substructure. Indeed, the mass difference between 3 and the known tjipanazole J (6) was 16 Da, suggesting 3 being a tjipanazole J derivative missing one oxygen. The ¹H NMR data of 3 showed close similarities to those of 6 (Figures S6–S8).^[33] However, H-5 of 3 (δ_H 4.98 ppm) is stronger shielded than the respective proton of 6 (δ_H 6.42 ppm), suggesting the lack of the hydroxy group at C-5. The position of the methylene group at the pyrrolo[3,4-c] ring was further confirmed comparing the NMR data of 3 with those of the staurosporine aglycone K252c (7). Compound 7 is an indolo[2,3-a]pyrrolo[3,4-c]carbazole with-

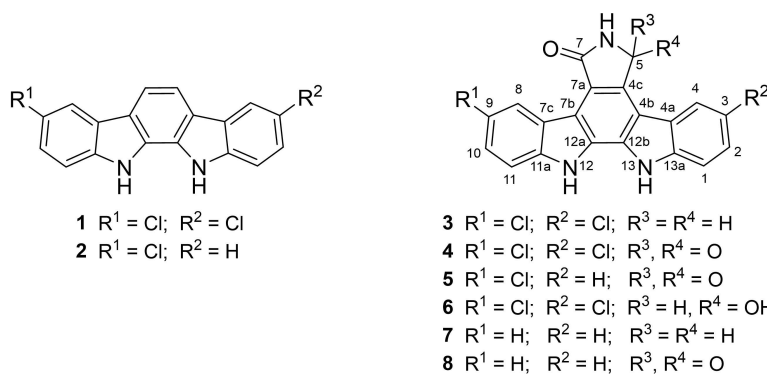


Figure 1. Chemical structures of tjipanazoles 1–5 isolated from *F. ambigua*, tjipanazole J (6), K252c (7), and arcylriaflavin A (8).

out chlorine substituents. The ^1H and ^{13}C chemical shifts of the pyrrolo substructures of **3** and **7** were in good agreement, confirming the methylene group at position C-5.^[42] Thus, **3** was identified as the new natural product tjipanazole K. Compound **3** has already been reported as synthetic substance, and has been tested for protein kinase C (PKC) inhibition and treatment of AIDS.^[43]

Compound **4** was isolated as yellow crystals. Its molecular formula was established as $\text{C}_{20}\text{H}_9\text{Cl}_2\text{N}_3\text{O}_2$ by the $[M-H]^-$ ion at m/z 391.9997 (calcd. 391.9999, Δ 0.49 ppm), two protons less than **6**. The ^1H spectrum of **4** showed just three signals for aromatic protons (δ_{H} 8.96, 7.81, 7.56 ppm) in addition to two NH protons (δ_{H} 12.98, 11.11 ppm), thus indicating a symmetrical molecule. Due to the obvious symmetry of **4**, both protons can only be missing at the pyrrolo[3,4-*c*] ring, resulting in an indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole skeleton with two carbonyl groups for **4**. This was confirmed by 2D NMR experiments (HSQC-DEPT, HMBC; Figures S9–S12). Compound **4** has already been described as a synthetic substance.^[44,45] NMR data of **4** were in good agreement with the published data, thus **4** was identified as the new natural product tjipanazole L.

Compound **5** was isolated as yellow crystals. Its molecular formula was established as $\text{C}_{20}\text{H}_{10}\text{ClN}_3\text{O}_2$ by the $[M-H]^-$ ion at m/z 358.0390 (calcd. 358.0389, Δ 0.46 ppm), one chlorine atom less than **4**. Indeed, the ^1H spectrum of **5** showed seven aromatic protons, whereas in **3** and **4**, only six aromatic protons can be observed. The splitting patterns in the ^1H spectrum showed the presence of one 1,2,4-trisubstituted and one 1,2-disubstituted benzene ring (Figure S13–S15), confirming the structure of the new natural product tjipanazole M (**5**). Compound **5** has already been described as a substance resulting from a combinatorial biosynthesis study being produced by a recombinant strain of *Streptomyces albus*.^[46] The ^1H chemical shifts from this publication are in good agreement with our ^1H NMR data. However, taking into consideration our 2D NMR data of **3**, **4**, and **5**, as well as the published NMR data of other indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazoles, we reassign the ^{13}C chemical shifts for **5** as given below.

Tjipanazole biosynthesis

Over the years, hundreds of bisindole alkaloids have been isolated from natural sources. The structures of the respective biosynthetic precursors, two L-tryptophans, are usually easily recognisable in the final structure. Taking into account previous work on tjipanazole biosynthesis,^[37] the reported biosynthetic pathways for the structurally related natural products rebeccamycin and staurosporine,^[46,47] based on a bioinformatic analysis of the *F. ambigua* (Näg.) Gomont 108b genome sequence and the tjipanazoles detected in the strain, we propose the biosynthesis pathway of the tjipanazoles as follows (Figure 2B):

In analogy to the biosynthesis of rebeccamycin, halogenation of tryptophan is most likely the first step in the tjipanazole biosynthesis. This reaction is catalysed by the putative FADH_2 -dependent tryptophan halogenase Tjp10. Subsequently, the monoamine oxidase Tjp1 converts 5-chloro-tryptophan to 5-

chloro-indole-3-pyruvate imine. Then, Tjp2, showing homology to previously described chromopyrrolic acid synthases, installs a carbon-carbon bond between the β -carbon atoms of the two indole-pyruvate imines, thereby generating a dimer. As we found that both mono- and dichlorinated tjipanazoles are produced by the strain, the presence of a chlorine atom at C-3 does not seem to be essential for substrate recognition.

Tjp9 is a cytochrome P450 enzyme showing homology to RebP and StaP. This oxygenase can be expected to react with the chromopyrrolic acid-like intermediate. The catalytic action of the cyp450 enzyme enables an aryl-aryl coupling between the two C-2 carbons of the indole systems. A mechanism via the generation of two indole cation radicals is supported by the crystal structure of the homologues StaP.^[48] Consecutively, the FAD-binding monooxygenases Tjp7 and/or Tjp8 could catalyse the modification of the pyrrole substructure, comparable to the reaction catalysed by the homologous proteins RebC and StaC (Figure 2A, Table S1). Interestingly, Tjp7, which is only 251 amino acids in size, shows homology with the N-terminal half of RebC/StaC proteins (~550 amino acids), whereas the 314 amino acids of Tjp8 show homology to the C-terminal part of RebC/StaC. We assume that Tjp7 and Tjp8 together form a multifunctional enzyme that is able to modify the pyrrole substructure in various degrees, resulting in multiple products, tjipanazole K (**3**), J (**6**), L (**4**) and M (**5**).

In contrast to staurosporine and rebeccamycin, some tjipanazoles, that is, tjipanazole D (**1**) and I (**2**), lack the pyrrolo[3,4-*c*] ring, which is cleaved during biosynthesis. One possibility for this cleavage could be that the decarboxylation and hydrolysis reactions take place prior to the formation of the pyrrole ring (Figure S16). The second C–C bond between the indole rings might then be formed in the dimeric intermediate to finally yield an aromatic ring connecting the tryptophan units. The accessory atoms might finally be cleaved off by decarboxylation, hydrolysis and oxidation reactions to give the tjipanazoles D (**1**) and I (**2**). Alternatively, the oxidative degradation of tjipanazoles might start from the hemiaminal tjipanazole J (Figure 2B). We think that the latter (removal of the pyrrolo[3,4-*c*] ring) is likely the pathway used for the biosynthesis of tjipanazoles D (**1**) and I (**2**), as we could detect almost all intermediates of the latter proposed pathway as $[M-H]^-$ ions in the extract of *F. ambigua* (Figures 2B, S17 and S18), while no intermediates of the other potential pathway were detected.

The genes encoding for the enzymes Tjp3–6 have no counterparts in the rebeccamycin and/or staurosporine biosynthetic gene clusters (BGCs, Figure 2A, Table S1). From the producer organisms harbouring these gene clusters, no alkaloids without the pyrrolo ring are reported. Therefore, involvement of Tjp3–6 in the cleavage of this pyrrolo substructure can be expected. The detailed investigation of these enzymatic steps during tjipanazole biosynthesis will be subject to further studies.

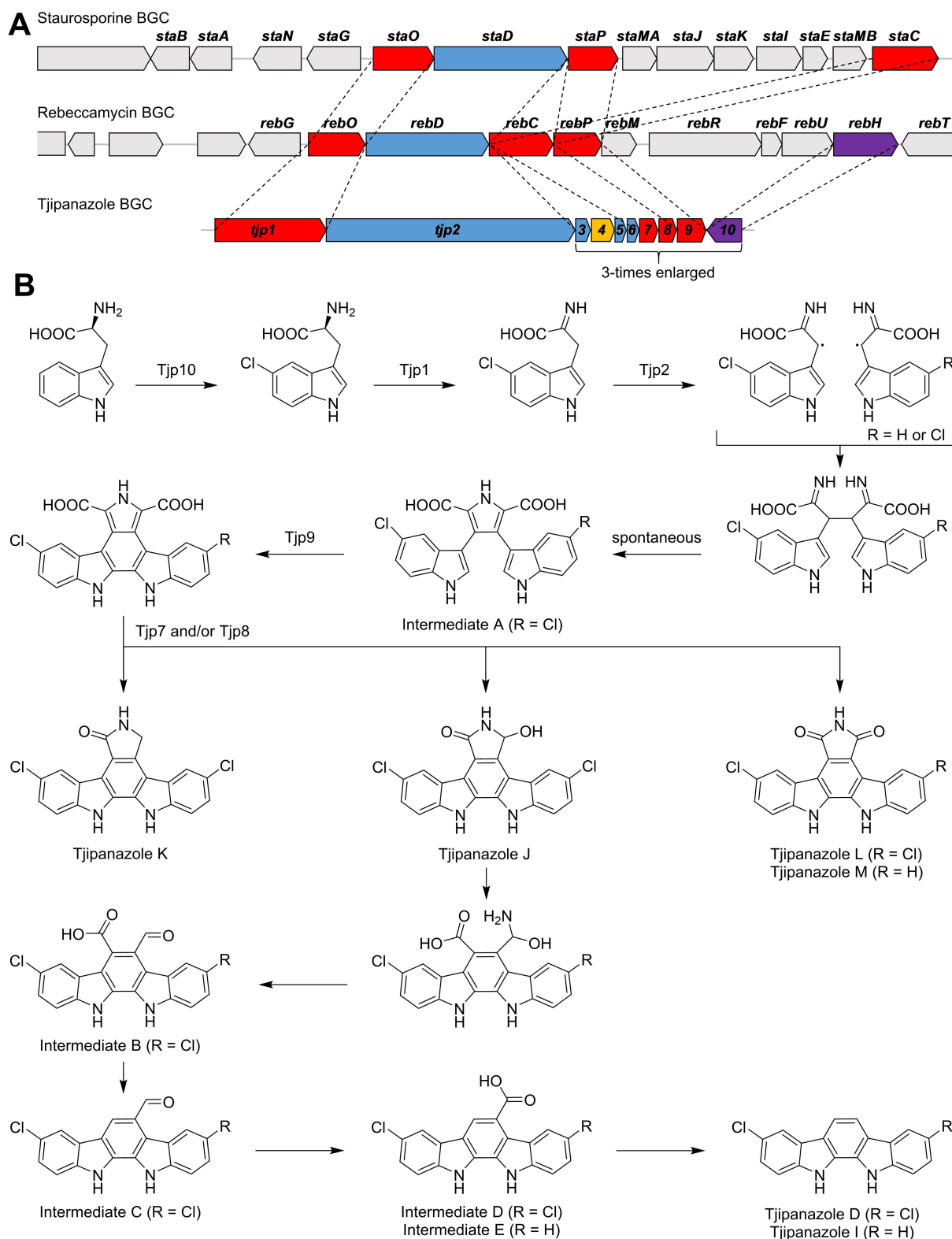


Figure 2. A) Comparison of the putative biosynthetic gene cluster (BGC) of tjipanazole biosynthesis in *F. ambigua* (Näg.) Gomont 108b with the BGC of staurosporine in *Streptomyces* sp. TP-A0274 and the BGC of rebecamycin in *Lechevalieria aerocolonigenes* ATCC 39243. Oxidase-like genes are coloured red, the methyltransferase-like gene in yellow, halogenase-like genes in violet, and the remaining in blue. Genes are drawn to scale, tjp3–10 are enlarged. Dashed lines show homology of the genes between the BGCs (for details concerning the homologous genes, see Table S1). B) Putative biosynthetic pathway of the tjipanazoles in *F. ambigua* (Näg.) Gomont 108b. All named metabolites were detected by HPLC-HRMS as $[M - H]^-$ ions in the extract of the strain (R = Cl or H). For the intermediates, the position of the functional groups has not been experimentally proven.

Bioactivity of tjipanazoles

Tjipanazoles A1 and A2 showed *in vitro* antifungal activity against rice blast and leaf rust wheat infections, as well as weak, non-selective cytotoxicity against leukaemia and solid tumour cell lines. Protein kinase C was not inhibited.^[33] Tjipanazole D exhibited moderate antibacterial activity against Gram-positive bacteria, whereas no antialgal, antifungal, antiparasitic, molluscicidal, or cytotoxic activity was found.^[36,49]

ABCG2, an ATP-binding cassette transporter with importance in drug resistance, was identified as target of indolocarbazoles such as K252c (7) and arcyriaflavin A (8). Both 7 and 8 showed inhibitory activity against the ABCG2-mediated efflux of pheophorbide A.^[38,50] Due to structural similarities of the tjipanazoles to 7 and 8, compounds 1–5 and 8 were tested for inhibitory activity against ABCG2 (Figure 3).

Tjipanazole M (5) and arcyriaflavin A (8) showed the highest inhibitory activity against ABCG2 from all tested compounds with an IC₅₀ of 11 and 9 μM, respectively. Weaker activity was found for tjipanazoles I (2, 35 μM), D (1, 43 μM), and K (3, 75 μM). Tjipanazole L (4, estimated 100 μM) was the least active compound. Based on these results, the following structure-activity relationships could be deduced: I) the loss of the pyrrolo[3,4-c] ring as well as II) the presence of two chlorine substituents at position C-3 and C-9 decreases inhibitory activity against ABCG2, whereas III) the exchange of a carbonyl group with a methylene group at position C-5 increases it.

The importance of the pyrrolo[3,4-c] ring for inhibitory activities against PKC or cyclin-dependent kinases (CDK) has been shown for indolocarbazoles by co-crystallisation studies.^[51] Indolo[2,3-a]pyrrolo[3,4-c]carbazoles, such as staurosporine or arcyriaflavin A and derivatives, are able to bind to the ATP binding site of various kinases by creating hydrogen bonds between amino acids residues and the pyrrolo[3,4-c] ring, whereby the binding of ATP to the kinase is prevented. Interestingly, 7 and 8 did not appreciably affect the ATPase activity of ABCG2.^[38] The modification of the pyrrolo[3,4-c] ring of 8 showed different results regarding the PKC and D1-CDK4

inhibitory activity. The exchange of a carbonyl group with a methylene or hydroxy group decreased the inhibitory activity against the D1-CDK4 enzyme complex.^[52] However, a methylene or hydroxy group increased the inhibitory activity against PKC and enhanced the antiproliferative activities against murine b16 melanoma cells.^[53] The modification of the C-3 and/or C-9 position by halogenation has been shown to reduce various activities of 8. Extensive structure-activity relationship studies revealed that a bromine substituent at C-3 position of 8 shows lower CDK4 inhibition,^[54] whereas the addition of chlorine at positions C-3 and C-9 decreases the inhibitory activity against PKC.^[44] Although the addition of two chlorine substituents reduced the PKC inhibitory activity, the activity against the human cytomegalovirus was maintained.^[44] Our results are thus in agreement with the literature regarding the importance of the pyrrolo[3,4-c] ring as well as the absence of two chlorine substituents for inhibitory activity against PKC and CDK, and strengthen the key role of these structural features for the inhibition of the ABCG2 transporter.

Conclusion

In conclusion, three tjipanazoles new to nature (3–5), were isolated along with two previously described tjipanazoles (1, 2) from the cyanobacterium *F. ambigua* after a chemical screening using the software tool HaloSeeker. The putative BGC of the tjipanazoles has been refined. We substantiated the putative tjipanazole biosynthesis pathway based on new insights into the BGC as well as the structural diversity of the tjipanazoles produced by this strain. Tjipanazole M (5) showed similar inhibitory activity against ABCG2 as arcyriaflavin A. Structure-activity relationships point to the importance of the pyrrolo[3,4-c] ring as well as the chlorination as key features for the inhibition of ABCG2.

Experimental Section

General experimental procedures: HRMS data were acquired using a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a heated ESI interface coupled to an UltiMate 3000 HPLC system (Thermo Fisher Scientific, Bremen, Germany). HaloSeeker 1.0 was applied to HRMS data of extracts and fractions to detect HNPs.^[34] Flash chromatography was performed on a GX-271 Liquid Handler system equipped with a 322 series pump and a 171 series diode array detector (Gilson, Middleton, USA). Semi-preparative HPLC was conducted on an UltiMate 3000 HPLC system (Thermo Fisher Scientific, Bremen, Germany). NMR spectra were either recorded at 600 MHz/150 MHz (¹H/¹³C) on a Varian/Agilent V NMR spectrometer, or at 400 MHz (¹H frequency) on an Agilent DD2 spectrometer. NMR spectra were analysed with ACD/Structure Elucidator Suite (2018.2, Toronto, Canada). Arcyriaflavin A (purity ≥ 95%) was purchased from Cayman Chemical Company. Ko143 (purity ≥ 99%) was purchased from Tocris Bioscience (Bristol, United Kingdom). All other chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany). Stock solutions of the compounds at a concentration of 10 mM were prepared in DMSO and stored at –18 °C for further usage. The prepared Krebs-HEPES buffer (KHB) contained 118.6 mM NaCl,

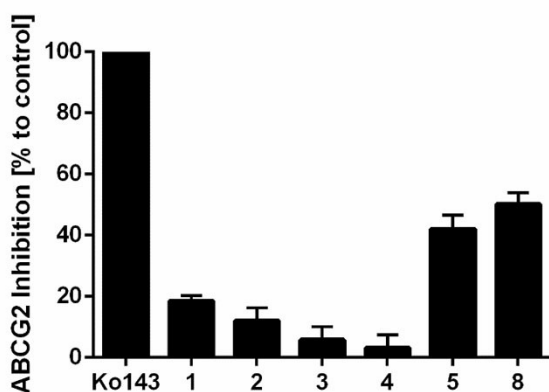


Figure 3. Inhibitory activity of tjipanazoles 1–5 and arcyriaflavin A (8) against ABCG2 at 10 μM, *n* = 4. Ko143 was used as positive control, indicating complete inhibition.

4.7 mM KCl, 1.2 mM MgSO₄, 2.5 mM CaCl₂, 11.7 mM D-glucose monohydrate, and 10.0 mM HEPES in doubly distilled water. The pH of the buffer has been adjusted to 7.41 at 37 °C using NaOH solution and then sterilised using a 0.2 µm pore membrane filter (Whatman, Maidstone, UK).

Cyanobacteria material: *Fischerella ambigua* (Näg.) Gomont 108b was classified as *Fischerella* based on its morphology and is deposited in the culture collection of the Cyano Biotech GmbH, Germany, under the accession number CBT 45. The strain was cultivated in BG-11 medium^[55] at 28 °C, illuminated continuously by Sylvania GROLUX fluorescent lamps (50–200 µmol photons m⁻² s⁻¹), and aerated with 0.5–5% CO₂ in sterile filtrated air in 20 L polycarbonate carboys. To minimise cell death and lysis, the cultures were harvested weekly and diluted with fresh medium (semi-continuous cultivation to avoid entering into stationary phase). Sufficient amounts of cyanobacteria biomass for further processing were obtained after a cultivation duration of about 10 weeks. After separation of the biomass from the medium by centrifugation, the biomass was lyophilised.

Extraction and isolation of compounds 1–5: 38 g of lyophilised dry biomass were suspended in 500 mL of 50% methanol in water (v/v), treated with an ultrasonication rod (Bandelin, Berlin, Germany), and extracted on a shaker for 30 min at room temperature. After centrifugation (20 min, 10800), the biomass was extracted using 500 mL 80% methanol (v/v). The solutions were combined and dried *in vacuo*, yielding 3.2 g of biomass extract. For preparative isolation of the active compounds, 1.6 g of the extract was dissolved in MeOH and fractionated by flash chromatography using a C₁₈ flash cartridge (43 g sorbent, RS 40 C₁₈ ec, Chromabond Flash, MACHEREY-NAGEL, Düren, Germany) and a binary gradient from 5–100% MeOH in water (0.1% formic acid each) as the mobile phase at 20 mL/min in 35 min, and a final elution step with 100% MeOH for 10 min to afford 24 fractions. Fractions 18–20 (t_R = 32–38 min) were combined, since most HNPs could be found in these fractions. The combined fraction (790 mg) was further purified by semi-preparative HPLC using a C₁₈ column (250 x 100 mm, 5 µm, 100 Å, Luna, Phenomenex, Aschaffenburg, Germany) and a step gradient of ACN in water (0.1% formic acid each) at 4.7 mL/min, starting with 57% ACN for 16 min, stepping to 70% for 6 min, increasing to 100% within 5 min and a final step with 100% ACN for 10 min. Several rounds of separation afforded tjipanazole K (3; t_R = 10.8 min, 0.9 mg), tjipanazole M (5; t_R = 13.3 min, 1.0 mg), tjipanazole L (4; t_R = 19.9 min, 8.0 mg), tjipanazole I (2; t_R = 25.9 min, 0.8 mg) and tjipanazole D (1; t_R = 28.6 min, 14.8 mg).

Tjipanazole D (1): brown, amorphous powder; NMR spectra: see Figure S3; HRMS (ESI): *m/z* calcd for C₁₈H₁₁Cl₂N₂-H⁻: 323.0148 [M-H]⁻; found: 323.0146.

Tjipanazole I (2): brown, amorphous powder; NMR spectra: see Figures S4 and S5; HRMS (ESI): *m/z* calcd for C₁₈H₁₁ClN₂-H⁻: 289.0538 [M-H]⁻; found: 289.0538.

Tjipanazole K (3): yellow crystals; NMR (¹H 600 MHz, ¹³C 150 MHz, [D₆]DMSO): δ_H 4.98 (s, 1H; H-5), 7.40 (dd, *J* = 8.4, 2.2 Hz, 1H; H-10), 7.46 (dd, *J* = 8.5, 1.9 Hz, 1H; H-2), 7.63 (d, *J* = 8.4 Hz, 1H; H-11), 7.68 (d, *J* = 8.6 Hz, 1H; H-1), 8.05 (d, *J* = 1.8 Hz, 1H; H-4), 9.23 (d, *J* = 2.0 Hz, 1H; H-8), 13.95 (br s, 1NH), 14.22 (br s, 1NH); δ_C 45.8 (C-5), 113.3 (C-11), 113.9 (C-1), 120.9 (C-4), 124.5 (C-8), 125.1 (C-10), 125.3 ppm (C-2); NMR spectra: see Figures S6–S8; HRMS (ESI): *m/z* calcd for C₂₀H₁₁Cl₂N₃O-H⁻: 378.0206 [M-H]⁻; found: 378.0207.

Tjipanazole L (4): yellow crystals; NMR (¹H 400 MHz, ¹³C 100 MHz, [D₆]DMSO): δ_H 7.57 (dd, *J* = 8.8, 2.2 Hz, 2H; H-2, H-10), 7.81 (d, *J* = 8.6 Hz, 2H; H-1, H-11), 8.96 (d, *J* = 2.2 Hz, 2H; H-4, H-8), 11.1 (br s, 1NH), 12.98 ppm (br s, 2NH); δ_C 113.7 (C-1, C-11), 114.6 (C-4b, C-7b), 120.2 (C-4c, C-7a), 122.5 (C-4a, C-7c), 123.1 (C-4, C-8), 124.3 (C-3, C-

9), 126.5 (C-2, C-10), 129.6 (C-12a, C-12b), 138.7 (C-11a, 13-a), 171.1 ppm (C-5, C-7); NMR spectra: see Figures S9–S12; HRMS (ESI): *m/z* calcd for C₂₀H₉Cl₂N₃O₂-H⁻: 391.9999 [M-H]⁻; found: 391.9997.

Tjipanazole M (5): yellow crystals; NMR (¹H 600 MHz, ¹³C 150 MHz, [D₆]DMSO): δ_H 7.32 (t, *J* = 7.5 Hz, 1H; H-3), 7.53 (m, 2H; H-2, H-10), 7.69 (m, 2H; H-1, H-11), 8.97 (m, 2H, H-4, H-8), 10.96 (s, 1NH), 14.48 (br s, 1NH), 14.74 (br s, 1NH); δ_C 111.7 (C-1), 113.1 (C-11), 119.6 (C-3), 122.9 (C-8), 123.9 (C-4), 126.0 (C-10), 126.1 ppm (C-2); NMR spectra: see Figures S13–S15; HRMS (ESI): *m/z* calcd for C₂₀H₁₀ClN₃O₂-H⁻: 358.0389 [M-H]⁻; found: 358.0390.

DNA isolation and sequencing: Axenic *F. ambigua* cells were inoculated in 100 mL BG-11 medium and grown at 25 °C with shaking at 120 rpm under constant illumination. Cells were harvested after 2–3 weeks of growth, washed three times with sterile water and resuspended in 20 mL SET buffer (75 mM NaCl, 25 mM EDTA, 10 mM Tris-HCl). The filaments were mechanically broken to separate the cells using a homogeniser. SDS (0.5%), proteinase K (500 µg/mL) and lysozyme (2.5 mg/mL) were added and the suspension was incubated at 55 °C for 2 h. Subsequently, one volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added for extraction. This step was repeated until no precipitated proteins could be seen between the aqueous and the organic phase. The resulting supernatant was purified using genomic tips, which were supplied with the Blood & Cell Culture DNA Mini Kit (Qiagen, Hilden, Germany) based on the manufacturer's instruction manual. The resulting DNA was submitted to 454 sequencing applying the Roche GS FLX Titanium sequencer (GATC, Konstanz, Germany). The sequence of the putative tjipanazole BGC has been deposited in GenBank (MT078730).

Quantification by evaporative light scattering detection: The concentrations of test compound solutions for the ABCG2 assay were quantified using HPLC coupled with an evaporative light scattering detector (ELSD; Sedex 85, Sedere).^[56] Tjipanazole L (4) was used as standard substance to establish the calibration curve (injection of 0.5 to 6 µL of a 150 ng/µL solution in DMSO). Solutions were injected in triplicate on a Kinetex C₁₈ column (100 x 3.0 mm, 2.6 µm, 100 Å, Phenomenex) and eluted with a step gradient of ACN in water (0.1% formic acid each) at 0.85 mL/min, starting with 40% ACN for 1 min and stepping to 60% ACN for 6 min. ELSD response areas were averaged and log(ELSD response area) was plotted against log(amount) to generate a linear calibration curve. Solutions of the compounds in DMSO were injected in triplicate under identical conditions. ELSD response areas were averaged, and the corresponding compound concentration was calculated using the tjipanazole L (4) calibration curve.

Cell culture: MDCK II BCRP cell line was a kind gift from Dr. A. Schinkel (The Netherlands Cancer Institute, Amsterdam, Netherlands). Cell culture was performed in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum (FCS), 2 mM L-glutamine, 50 µg/mL streptomycin and 50 U/mL penicillin G. MDCK II BCRP cells were incubated at 37 °C under humidified atmosphere containing 5% CO₂. At a confluence of 80–90%, sub-culturing was performed using 0.05% trypsin and 0.02% EDTA for detaching the cells from the inner surface of the culture flask. Then cells were resuspended with the fresh medium into a 50 mL falcon. A cell pellet was obtained by centrifugation (4 °C, 4 min, 266 x g) and the supernatant was removed by aspiration of the liquid. Then cells were again resuspended in fresh medium. The amount of cells was determined using a CASY1 model TT cell counter equipped with a 150 µm capillary (Schaerfe System GmbH, Reutlingen, Germany). Before using the cells in Hoechst 33342 accumulation assay, they were washed three times with KHB to remove residual medium.

Hoechst 33342 accumulation assay: The inhibitory effect of the compounds was investigated in the Hoechst 33342 accumulation assay using ABCG2 transfected MDCK II BCRP cells as described in the literature with minor modifications.^[57] Bisbenzimidazole derivative, Hoechst 33342 is a fluorescent compound and a substrate of ABCG2 which has a stronger fluorescence after binding into adenine- and thymine-rich minor grooves of double-stranded DNA or being embedded in a lipophilic environment like the cell membrane. For this aim, a stock solution of the compounds was prepared at a final concentration of 10 mM in DMSO. From this solution, 11 different concentrations were prepared using sterile KHB and a small amount of methanol. The final concentration of the DMSO and methanol in the presence of cells was always below 0.1% and 5%, respectively. Assays were performed on BMG POLARstar microplate reader (BMG Labtech, Offenburg, Germany) at 37 °C (excitation: 355 nm/emission: 460 nm) using 96-well black plates (Greiner, Frickenhausen, Germany). Black plates were favourable as they provided much lower background fluorescence than transparent plates when irradiated in the UV. The cells were prepared as described above. 160 µL cell suspension in KHB at a cell density of approximately 30 000 cells per well was added to each well together with 20 µL of the different concentrations of the test compounds. The plate was then incubated for 30 min at 37 °C and 5% CO₂ followed by quick addition of 20 µL of a 10 µM Hoechst 33342 solution (protected from light) to each well. Fluorescence intensity was measured immediately for the next 120 min at constant time intervals of 1 min. The average fluorescence between 100 and 109 min in the steady state was calculated and plotted against the logarithm of the compound concentration. Dose-response curves were generated using non-linear regression analysis, the four-parameter logistic equation with variable slope compared to the three-parameter logistic equation. The statistically preferred model was selected for calculating IC₅₀ values (GraphPad Prism, version 6.0, San Diego, USA). For compounds reaching the maximal effect of Ko143 up to 10 µM, IC₅₀ values were calculated by constraining the maximal response to that of Ko143. Percentage of the response of the compounds in comparison to the reference inhibitor (100% inhibition), has been calculated by comparing the fluorescence intensity of the compounds at their highest concentration in comparison to Ko143.

Conflict of Interest

H.E. is CSO and co-owner of Cyano Biotech GmbH; the company does not have any financial interest in the research presented here. The authors declare no conflict of interest. The funding sponsors had no role in the design, writing or publishing strategy of the study, or in the collection, analysis or interpretation of the data.

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Keywords: biosynthesis · cancer resistance protein · *Fischerella* · natural products · tjipanazoles

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