Rational Design of Cyclosporin A Derivatives

for Selective Enzyme Inhibition

Dissertation zur Erlangung des akademischen Grades

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Dedicate to my

parents and yun

Content

Abbreviation

Abz	Aminobenzoic acid
Ac-	Acetyl
Boc-	*tert*-Butoxycarbonyl
CaM	Calmodulin
CaN	Calcineurin
CD	Circular dichroism
CE	Capillary electrophoresis
CNA	Calcineurin subunit A
CNB	Calcineurin subunit B
CsA	Cyclosporin A
CsH	Cyclosporin H
CTD	C-terminal domain
Cyp	Cyclophilin
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCM	Dichloromethane
DIPEA	Diisopropylethylamine
DMAP	4-(Dimethylamino)pyridine
DMS	Dimethyl sulfide
DMSO	Dimethyl sulfoxide
EDT	1,2-Ethanedithiol
FKBP	FK506 binding protein
Fmoc-	9-Fluorenylmethoxycarbonyl
Hepes	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
IC_{50}	50% inhibition constant
IL	Interleukin
LDA	Lithium diisopropylamide
MeBmt	(4R)-4-[(E)-2-Butenyl]-4-methyl-L-threonine
MHC	Major histocompatibility complex
MMF	Mycophenolate mofetil
n-ButLi	*n*-Butyl lithium
NES	Nuclear export signal
NFAT	Nuclear factor of activated T cells
NH-Np	4-Nitroanilide
NLS	Nuclear localisation signal
NMR	Nuclear magnetic resonance
PP	Protein phosphatase
PPIase	Peptidyl-prolyl *cis/trans* isomerase.
r.t.	Room temperature
Sar	Sarcosine
Suc-	Succinyl
TBTU	2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
TCR	T cell receptor
TFA	Trifluoroacetic acid
TFE	Trifluoroethanol
TFMSA	Trifluoromethanesulfonic acid
TGF	Transforming growth factor
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TMS-	Trimethyl silyl
TMSOTf	Trimethylsilyl trifluoromethanesulfonate

	A list of important synthetic CsA derivatives throughout this work
Cs3	[Phosphoryl-D-serine][8] CsA
Cs4	[H-phosphoryl-D-serine][8] CsA
Cs5	[Dimethyl phosphoryl-D-serine][8] CsA
Cs6	[O-Carboxymethyl D-serine][8] CsA
Cs7	[O-4-bromo-n-butyl D-serine][8] CsA
Cs8	[O-2-aminoethoxy-ethyl D-serine][8] CsA
Cs9	[O-($NH_2(CH_2)_5NHC(O)CH_2$-) D-serine][8] CsA
Cs10	[O-($BocNH(CH_2)_5NHC(O)CH_2$-) D-serine][8] CsA
Cs11	[(R)α-methyl sarcosine][3] CsA (R)
Cs12	[(S)α-methyl sarcosine][3] CsA (S)
Cs13	[(R)α-methylthio sarcosine][3] CsA (R)
Cs14	[(S)α-methylthio sarcosine][3] CsA (S)
Cs15	[(R)α-methyl sarcosine][3] CsH (R)
Cs16	[(S)α-methyl sarcosine][3] CsH (S)
Cs18	[O-Phosphoryl MeBmt][1] CsA
Cs19	[O-Dimethyl phosphoryl MeBmt][1] CsA
Cs20	[O-Diallyl phosphoryl MeBmt][1] CsA
Cs21	[O-monoallyl phosphoryl MeBmt][1] CsA
Cs22	[Phosphoryl MeBmt][1] [Phosphoryl-D-serine][8] CsA
Cs23	[Phosphoryl MeBmt][1] [O-($BiotinNH(CH_2)_5NHC(O)CH_2$-) D-serine][8] CsA
Cs24	[O-($BiotinNH(CH_2)5NHC(O)CH_2$-) D-serine][8] CsA
Cs25	[1-amino-2,6-octadienoic acid][1] CsA
Cs26	[O-sulfonyl MeBmt][1] CsA
Cs27	[β-Oxo MeBmt][1] CsA
Cs28	[Acetyl MeBmt][1] CsA
Cs29	[β-Oxo MeBmt][1] [(R)α-methyl sarcosine][3] CsA
Cs30	[Acetyl MeBmt][1] [(R)α-methyl sarcosine][3] CsA
Cs31	[Acetyl MeBmt][1] CsH
Cs33	[Me-Asp(O-Bu-t)][3] CsA (R&S)
Cs35	[Me-Asp][3] CsA (R&S)
Cs40	[α-Ethyl sarcosine][3] CsA, (R)
Cs41	[α-Ethyl sarcosine][3] CsA, (S)
Cs42	[O-($AcNH(CH_2)_5NHC(O)CH_2$-) D-serine][8] CsA
Cs44	[O-($HOOC(CH_2)_5NHC(O)CH_2$-) D-serine][8] CsA
Cs54	[$^4\psi^5$ CSNH] [$^7\psi^8$ CSNH] CsA
Cs55	[$^4\psi^5$ CSNH] CsA
Cs56	[$^7\psi^8$ CSNH] CsA
Cs57	[O-Aectyl MeBmt][1] [$^4\psi^5$ CSNH] [$^7\psi^8$ CSNH] CsA
Cs58	[O-Aectyl MeBmt][1] [$^4\psi^5$ CSNH] CsA
Cs59	[O-Aectyl MeBmt][1] [$^7\psi^8$ CSNH] CsA
Cs61	[O-Phosphoryl MeBmt][1] CsH

Chapter 1. Introduction

Cyclosporin A (CsA) is an immunosuppressive drug, which has been established as a standard drug to prevent allograft rejection after transplantation of organs and bone marrow (Borel, 1989). The immunosuppressive mechanism of CsA requires the interactions of two sets of residues of CsA with two distinct proteins (Fischer, et al., 1989; Liu, et al., 1991, Schreiber & Crabtree, 1992), respectively. The bi-functioned double-headed drug molecule has been investigated intensively during the past decades. Studying the structure-function relationship of these drug/protein complexes provides a molecular basis for understanding signal transduction pathways in stimulated T cells, as well as the preciseness and beauty of nature.

1.1 Peptidyl-Prolyl *cis/trans* Isomerases.

Rates of protein folding reactions vary considerably (Debe, et al., 1999). Some denatured proteins regain the native conformation within milliseconds or seconds at room temperature, whereas others refold very slowly in the time range of minutes or hours. The slow processes frequently involved are *cis/trans* conformational inter-conversions of certain peptide backbone, in particular the peptide bonds preceding proline (Fischer, 1994 & 1998). The partial double bond character of the carbon-nitrogen bond leads to planarity of the peptide bond unites creating a high barrier of rotation about this bond, and it allows a peptide bond to occur in just two conformations, *cis* (ω about 0°) or *trans* (ω about 180°). The distinction between the *cis* and *trans* isomer of a molecule originates from a geometry-based classification of structure. The vicinity of the C_α atoms of neighboring amino acids, in close contact to each other in the *cis* conformation, may be important for the low percentage of secondary *cis* peptide bonds because the steric strain is released in the *trans* conformer (Fischer, 2000; Scherer, 1998). In a recent analysis, 59 non-proline *cis* peptide bonds were found in a database of 747 native proteins (Jabs, et al., 1999; Reimer, et al., 1998). In contrast to amide bonds, the two lowest energy arrangements of the prolyl peptide bond (Scheme **1.1**) are of comparable thermodynamic stability.
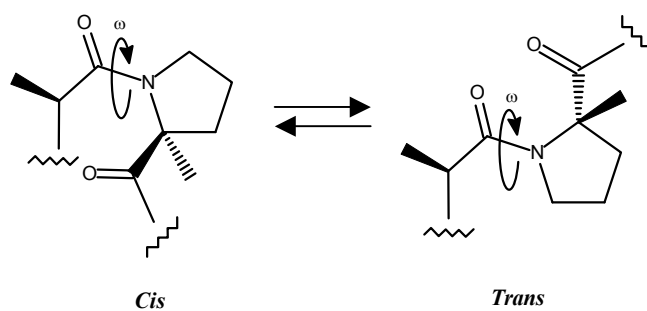
Scheme 1.1 *Cis/trans* isomerization of peptidyl-prolyl bond. The peptide bond between proline and its preceding residue has two stable conformations: *trans* (ω about 180°) and *cis* (ω about 0°).

Peptidyl prolyl *cis/trans* isomerases (PPIases) are ubiquitous and abundant enzymes conserved from procaryotes to eucaryotes (Fischer, 1994). PPIases accelerate the slow peptidyl prolyl *cis/trans* isomerization in oligopeptides and polypeptides of different folding steps. Three different families of PPIases have been described (Fischer, et al., 1998): cyclophilins, FK506 binding proteins (FKBPs, with the subfamily of the ribosome-bound trigger factors), and the parvulins. Cyclophilins and FKBPs are natural receptors for immunosuppressants cyclosporin A and FK506 respectively and are also imprecisely called immunophilins (Schreiber & Crabtree, 1992). In contrast to the enormous number of cyclophilins and FKBPs, only a small number of parvulins or homologues are now known. The human parvulin Pin1 is a mitotic regulator essential for G2M transition of eukaryotic cell cycle. A direct requirement of PPIase for *in vivo* protein folding has already been demonstrated. Deletion of the nonessential gene encoding trigger factor results in a doubling of the fraction of nascent polypeptides interacting with DnaK, the major *E. coli* Hsp70 that chaperones *de novo* protein folding (Teter, et al., 1999). Some knockout mutants of single or multiple PPIases were viable without significant phenotype under normal conditions (Dolinski K. et al., 1997; Fujimori, et al., 1999). Recent work gave lethal deletion of a single Cyp in *D. melanogaster* (Reissmann, Ph.D. thesis, Halle). Although the natural cellular functions of all but a few remain obscure, some new findings demonstrated the great variety of PPIases functions in the living cells and place them at the intersection of protein folding, signal transduction, trafficking, assembly and cell cycle regulation.

1.2 Immunosuppressive drug cyclosporin A (CsA).

Cyclosporin A (Sandimmun®) is a cyclic undecapeptide (Figure **1.1**) that is first isolated from natural sources (Rüegger, et al., 1976) and prepared later through total synthesis (Wenger, 1983 & 1984). CsA contains several unusual amino acids like L-2-aminobutyric acid, D-Ala and (4R)-4-[(E)-2-butenyl]-4-methyl-L-threonine (MeBmt). In addition, the cyclosporin molecule possesses seven N-methylated peptide bonds. It exhibits many biological activities, including anti-inflammatory, anti-fungal, anti-parasitic as well as immunosuppressive activities. It is commonly used in transplantation surgery and in the treatment of autoimmune diseases.

Before discussing CsA and its mode of action in more detail, let me briefly review a currently well-accepted immunosuppressive mechanism of CsA. The identification of calcineurin as the common cellular target of immunosuppressive drug CsA and FK506 (Flanagan, et al., 1991; Schreiber, et al., 1992, Liu, et al., 1991, O'Keefe, et al., 1992, Clipstone, et al., 1992) has revealed an important transcriptional regulatory mechanism in the immune system: CsA and FK506 bind to two distinct immunophilins, cyclophilin (Cyp) and FKBP, respectively; and inhibit their PPIase activities (Fischer, et al., 1989; Tropschug, et al., 1990; Standaert, et al., 1990). However, such inhibition alone is not sufficient for immunosuppression. Cyp/CsA and FKBP/FK506 complexes share a common cellular target phosphatase calcineurin (CaN). In stimulated T cells, CaN dephosphorylates

NFAT (nuclear factor of activated T cells) proteins, promotes their nuclear translocation and transcriptional activation of gene for T-cell growth factors including IL-2 and IL-4. Blocking of this pathway is thought to contribute to the immunosuppressive activity of both drugs.
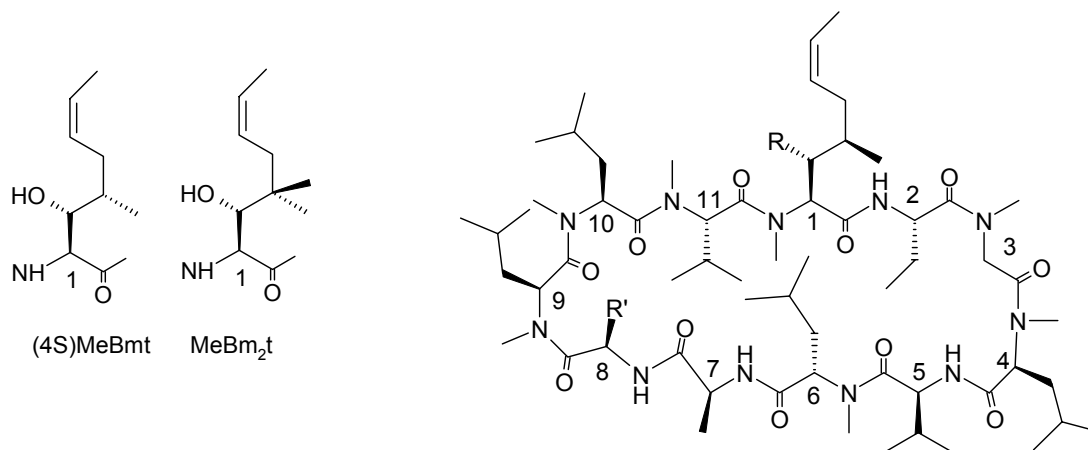
CsA		Immunosuppressive, strong CaN inhibition, strong Cyp inhibition
CsH	MeVal[11] → D-MeVal[11]	Nonimmunosuppressive, no CaN inhibition, no Cyp inhibition. (Traber, 1987)
[MeBm$_2$t]^{1}Cs	MeBmt^1→ MeBm$_2$t^1	Immunosuppressive, strong CaN inhibition, low Cyp inhibition (Sigal et al., 1991, Nelson, 1993).
[(4S)MeBm$_2$t]^{1}Cs	MeBmt^1→(4S)MeBm$_2$t^1	The epimer is only 2-4% as active as CsA in immunosuppression assay (Rich et al., 1982)
[MeAla]^{6}Cs	MeLeu^6 → MeAla^6	Nonimmunosuppressive, no CaN inhibition, strong Cyp inhibition (Sigal et al., 1991).
[MeIle]^{4}Cs	MeLeu^4 → MeIle^4	Nonimmunosuppressive, no CaN inhibition, strong Cyp inhibition (Rosenwirth, et al., 1994)

Figure 1.1 Cyclosporin A and some of its derivatives. The function-structure relationship of these compounds, as well as genetic and chemical biology studies were used to unravel the immunosuppressive mechanism of CsA and FK506 to reach our present level of knowledge.

1.2.1 The inhibition of cyclophilins by CsA

S. L. Schreiber et al. (1990) introduced the term 'immunophilins' for the families of PPIases in order to point out the role of PPIases as receptor proteins for immunosuppressive drugs (CsA, FK506, and rapamycin) in the mammalian immune system. However, there are much more

immunophilic proteins than just those with proven PPIase activity (reviewed by Fischer, 1994). On the other hand, amongst the PPIases of cyclophilin and FKBP families are proteins with relatively little affinity for CsA and FK506 and which therefore can not be classified as immunophilins (Table **1.1**, reviewed by Fischer, 1994). Furthermore, the third family of PPIases, the parvulin, has not been shown being immunophilic.

Table 1.1 The inhibition of CsA to various cyclophilins.

Cyclophilins	PPIase activities $K_{cat}/k_m[\mu M^{-1}s^{-1}]$ [a]	Inhibition by CsA (nM)	References
human18cy	22	1.6 (K_i) 3.7 (IC_{50}) [b] 7 (K_d) [c]	Bergsma, et al., 1991; Levy, et al., 1991; Holzman, et al., 1991
(porcine) ovine18cy	13.4	2.6 (K_i); 20 (IC_{50})	Fischer, et al., 1989; Kofron, et al., 1991; Kieffer, et al., 1992
rat18cy	+	+	Thalhammer, et al., 1992
*L. esc.*18cy	+	+	Gasser, et al., 1990
*P. vulgaris*18cy	+	+	Luan, et al., 1993
*C. albicans*18cy	+	+	Koser, et al., 1990
*S. cerevis*17cy	19	40 (IC_{50}); 4.1 (K_i)	Zydowsky, et al., 1992; Hasumi, et al., 1993
*N. crassa*24mito	+	200 (K_d)	Tropschug, et al., 1988
*N. melanog*26mem	[d]	[e]	Shieh, et al., 1989
human41cy	+	300 (IC_{50})	Kieffer, et al., 1993
human24sec	+	+	Spik, et al., 1991
human23sce	+	84 (IC_{50})	Price, et al., 1991
mouse22sec	+	400 (K_d)	Hasel, et al., 1991
human22mito	8.0	8 (K_i)	Bergsma, et al., 1991
mouse23sec	+	+	Liu, et al., 1991
*S. cerevis*20mito	9.2	9.3 (K_i)	McLaughlin, et al., 1992
*S. cerevis*23sec	+	101 (IC_{50})	Zydowsky, et al., 1992; Tanida, et al., 1991
*S. chryso*18	+	25 (IC_{50})	Pahl, et al., 1992
*E. coli*18cy	67.4	[f]	Compton, et al., 1992
*E. coli*21peri	57.1	[f]	Compton, et al., 1992

K_d: dissociation constant; K_i: inhibition constant

[a] Suc-Ala-Ala-Pro-Phe-NH-Np were used as substrate for PPIase activity measurements. [b] (Zhang, et al., unpublished results) [c] (Fanghänel, et al., unpublished results) [d] not determined [e] probably has activity [f] > 10μM

1.2.2 Cyclosporin synthetase.

CsA is synthesized by cyclosporin synthetase, a multi-enzyme polypeptide. Its molecule mass has been estimated to be about 1,400 kDa. Sequencing of the open reading frame of the cyclosporin synthetase gene resulted in a molecule mass of 1,689,243. The multifunctional polypeptide catalyzes CsA formation in at least 40 reaction steps with an assembly belt-like mechanism. It activates all constituent amino acids of CsA to thioesters via amino acyladenylates and carries out

specific N-methylation reactions. During elongation, the activated amino acids are linked by peptide bonds leading to enzyme-bound nascent peptide chains. D-Alanine at position 8 of the cyclosporin A molecule was found to be a starting amino acid in the biosynthetic process (Dittmann, et al., 1994).

Exchange of one or more of the amino acids gives a picture of the substrate specificity of the enzyme *in vitro* (Lawen, et al., 1993). The MeBmt in position 1 can be exchanged by an unexpected large spectrum of different amino acids, showing a great flexibility of this site. Position 3 has a very high degree of specificity, whereas position 8 shows only low substrate specificity *in vitro*.

1.2.3 Cyclosporin derivatives.

A plethora of modified cyclosporins have been isolated from natural sources or were obtained through synthetic efforts (Traber, et al., 1982 & 1987). All cyclosporins are cyclic undecapeptides differing from each other by minor variations in the amino sequence. Interestingly, the specificity of cyclosporin syntheses *in vivo* shows difference from the preferences of cyclosporin synthetase *in vitro* (Section 1.2.1 and Lawen, et al., 1993). Position 3 and 8 are highly conserved, whereas position 2 have the greatest flexibility. Most of these derivatives are not as active as CsA (Traber, 1987). This indicates that almost each residue in CsA molecule plays a role in the interactions to its cellular targets, and/or restricts the conformation through intramolecular non-covalent bond. In the past two decades, these information concerning structure-activity relationship as well as genetic and cell biological efforts revealed us the immunosuppressive mechanism of CsA and an immune response pathway in activated T cells. For instance, [MeAla][6]CsA is a nonimmunosuppressive analogue, but bound well to cyclophilin and was active as a PPIase inhibitor. Another analogue, [MeBm$_2$t][1] CsA, which was immunosuppressive *in vitro*, but possessed low activity as a PPIase inhibitor (Sigal et al., 1991). These finding promoted scientist to find new targets for cyclosporin.

1.2.4 Medical side-effects of CsA treatment.

The clinical trails show that treatment with CsA is accompanied by significant side effects such as hypertension and nephrotoxicity. The mechanism of these side effect is now considered to be vasoconstriction of the afferent arterioles (Ishikawa, 1999). Many factors, such as nitric oxide (NO), cytoplasmic calcium and endothelin (ET) are nominated as the mediator of these side effects. Among them, ET is thought to play a pivotal role as a key substance of CsA-induced nephrotoxicity.

Recent results indicate that growth factors are critically important in both chronic rejection and chronic CsA toxicity, suggesting that these two entities share a common pathophysiological pathway, leading to progressive allograft failure (Pascual, et al. 1998). In human T cells, CsA enhanced the production of TGF-β1 (transforming grow factor β1) as well as the expression of its receptor (Li, et al. 1991). Elevated TGF-β expression is correlated with increasing production of ET

in endothelial cells and muscle cells (Markewitz, et al, 2001, Gonzalez, et al. 2001,). However, many puzzles still remain unsolved, for example, what is the role of CsA in the activation of TGF-β; are Cyp or CaN inhibition involved in these pathways?

Malignancy is also a common and dreaded complication following CsA treatment in organ transplantation. The high incidence of neoplasm and its aggressive progression, which are associated with immunosuppressive therapy, are thought to be due to the resulting impairment of the organ recipient's immune-surveillance system. However, some new finding suggested that these side effects maybe independent of the effect on the host's immune cells. CsA induced TGF-β production by tumor cells and promoted cell invasiveness by a cell-autonomous mechanism (Hojo, 1999).

1.3 Calcineurin.

Calcineurin (also known as PP-2B) is a calcium and calmodulin-dependent protein serine/threonine phosphatase with narrow substrate specificity. It plays pivotal roles in a number of physiological processes including T lymphocyte activation, muscle cell differentiation, learning and memory (Hemenway, et al., 1999; Rusnak, et al., 2000).

1.3.1 Calcineurin structure.

Purified calcineurin is a heterodimer consisting of a catalytic subunit, calcineurin A (CNA), and a "regulatory" subunit, calcineurin B (CNB) (Scheme **1.2**). CNA exhibits some, although minimal, phosphatase activity even in the absence of other subunits and, thus, has been recognized as the catalytic subunit. CNA genes are highly conserved across species and encode for a polypeptide consisting of a catalytic domain homologous to other serine/threonine protein phosphatases (including protein phosphatases 1 (PP1), PP2A and PP2C), and three regulatory domains at the C terminus that distinguish CaN from other family members. These domains have been identified as the CNB binding domain, the calmodulin (CaM)-binding domain, and the amino-terminal autoinhibitory domain, which binds in the active site cleft in the absence of Ca^{2+}/calmodulin and inhibits the enzyme activity. The CNB subunit is also highly conserved throughout evolution and shares much structural homology with CaM. The gene for mammalian CNB encodes a protein of 170 amino acids containing four Ca^{2+} binding EF-hand motifs.

CaN phosphatase activity is highly dependent on the interaction of CNA, CNB, calmodulin and Ca^{2+} (reviewed by Hemenway, et al., 1999; Rusnak, et al., 2000). Reconstitution experiments, limited proteolysis and kinetic data implied that CNB likely alters the substrate binding pocket of the catalytic subunit and the activation is Ca^{2+} dependent. Calmodulin binding may displace the autoinhibitory domain from its position adjacent the catalytic site and render it more accessible to substrate entry.
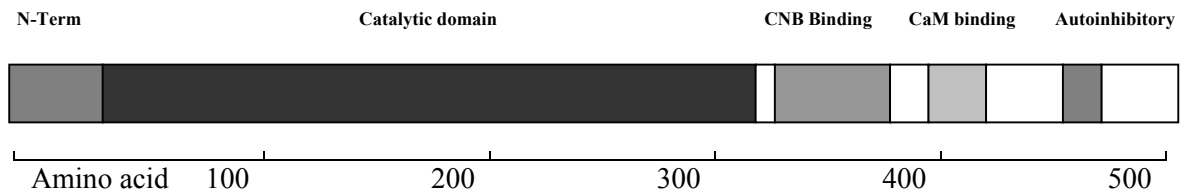
Scheme 1.2 Calcineurin A domain structure.

1.3.2 Phosphatase activity of calcineurin.

In addition to CaN (PP2B), the serine/threonine protein phosphatase family members, including protein phosphatases 1 (PP1), 2A (PP2A), and 2C (PP2C), are essential for a number of signal transduction pathways in eukaryotic cells. The original classification of this family was proposed by Ingebritsen and Cohen (1983), separating almost all the serine/threonine phosphatase activity in mammalian tissue extracts into two classes (Cohen, 1989). Type 1 protein phosphatases (PP1) were found to dephosphorylate the β-subunit of phosphorylase kinase, whereas type 2 protein phosphatases (PP2) dephosphorylate the α-subunit of phosphorylase kinase. A difference in divalent metal ion dependence led to the resolution of the PP2 enzymes into PP2A, PP2B (CaN), and PP2C. PP2A was originally described as having no requirement for divalent metal ion, CaN is regulated by Ca^{2+}/CaM, and PP2C is Mg^{2+} dependent. Differences among the various phosphatases are also found with their specific inhibitors. PP2A and PP1 are inhibited by okadaic acid, whereas CaN is specifically inhibited by CsA and FK506, in the presence of cyclophilin and FKBP, respectively.

The narrow substrate specificity is another unique characteristics of CaN. Dephosphorylation of phosphopeptides by CaN showed that the specificity is determined by a variety of primary and higher-order structural features, which confer to it an overall selectivity that is different from those of any other known protein phosphatases (Donella-Deana, et al., 1994). For instance, while PP2A and PP2C were also very active on short phosphopeptides, an extended N-terminal stretch appears to be a necessary, albeit not sufficient, requirement for an optimal dephosphorylation by CaN. Peptides that are appreciably dephosphorylated by CaN contain basic residues on the N-terminal side. Conversely, acidic residues adjacent to the C-terminal are powerful negative determinants, preventing the dephosphorylation of otherwise suitable peptide substrates. Furthermore, CaN dephosphorylates the phospho-Ser/Thr-Pro motif in peptides where this motif prevents dephosphorylation by other classes of Ser/Thr-specific protein phosphatases.

1.3.3 CaN/NF-AT pathway in stimulated T cells.

The nuclear factors of activated T cells (NF-ATs) constitute a family of transcription factors that transduce Ca^{2+} signals in the immune, cardiac, muscular and nervous systems (Rao, et al., 1997). It

directs transcription of several cytokine genes, including that encoding IL-2. In resting cells, NF-AT is cytoplasmic, hyperphosphorylated and inactive. Costimulation of the TCR and CD28 on cell surfaces results in tyrosine phosphorylation of the TCR by membrane-associated protein kinases. By virtue of its SH2 domains, phospholipase C-γ (PLCγ) is then recruited to these phosphorylated sites on the TCR, therefore positioning PLCγ in the vicinity of the plasma membrane. Hydrolysis of phospholipids by PLC gives rise to secondary signal molecules (IP3) that trigger the release of Ca^{2+} from intracellular storage sites.
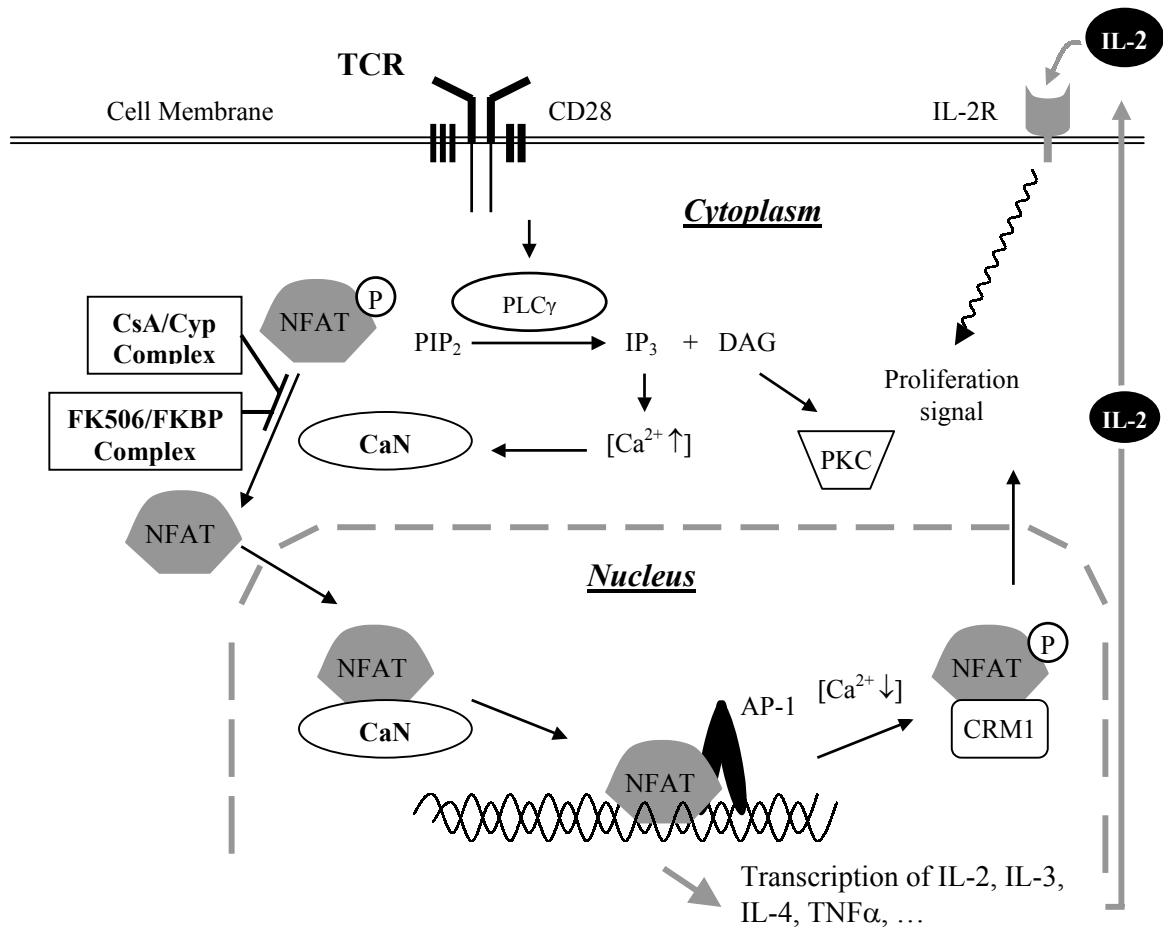
Figure 1.2 A simplified view of some signaling pathways in stimulated T cells. Activated CaN binds and dephosphorylate NFAT. CaN is cotranslocated with NFAT to the nucleus. The CsA/Cyp or FK506/FKBP complex inhibit CaN and block this pathway.

The calcium-activated CaN then binds to NF-AT during calcium signaling and dephosphorylates the NLS (nuclear localization signal) masking domain of NF-AT (Zhu, et al., 1999 & 2000), resulting in NLS exposure and nuclear import of the nuclear factors by importins. Calcineurin is cotransported with NF-AT into the nucleus, where it continues to bind to NF-AT via sites containing NESs (nuclear export signals). The exportin, CRM1, cannot bind or export NF-AT until calcium signaling ends and calcineurin dissociates from NF-AT. In the absence of calcineurin

activity, NF-AT kinases rephosphorylate the NLS mask sequence on NF-AT, further assuring the cytoplasmic disposition of this molecule. The immunosuppressive activity of CsA and FK506 is suggested to block this pathway by binding and inhibiting CaN phosphatase activity together with their own immunophilins (Figure **1.2**).

1.3.4 Calcineurin inhibitors.

The most potent, specific, and well-known inhibitors of CaN are immunophilin/immunosuppressant complexes, Cyp/CsA and FKBP/FK506. A number of other natural products have demonstrated inhibitory activity against CaN and other serine/threonine protein phosphatases. Okadaic acid (Scheme **1.3**), often used as a potent and specific inhibitor of PP2A, can also inhibit PP1 and CaN at higher concentrations (Bialojan, et al. 1998). The cyclic peptide microcycstin LR is a potent inhibitor of PP1 and PP2A, with a K_i value <1nM. The inhibition of CaN by microcystin LR (Scheme **1.3**) occurs at over 1,000-fold higher concentrations (Mackintosh, et al. 1990). Dibefurin (Scheme **1.3**), a novel fungal metabolite, also has modest inhibitory activity against CaN (Brill, et al. 1996). Due to the unspecificity of these phosphatase inhibitors to CaN, they cannot be applied for unraveling the role of CaN in CsA or FK506 mediated immunosuppression and the side effects.

Endothal Derivatives

Metal-ligating phosphonates

X = SH, COOH, Me
n = 1 - 3

Dibefurin

Okadaic Acid

Microcystin LR
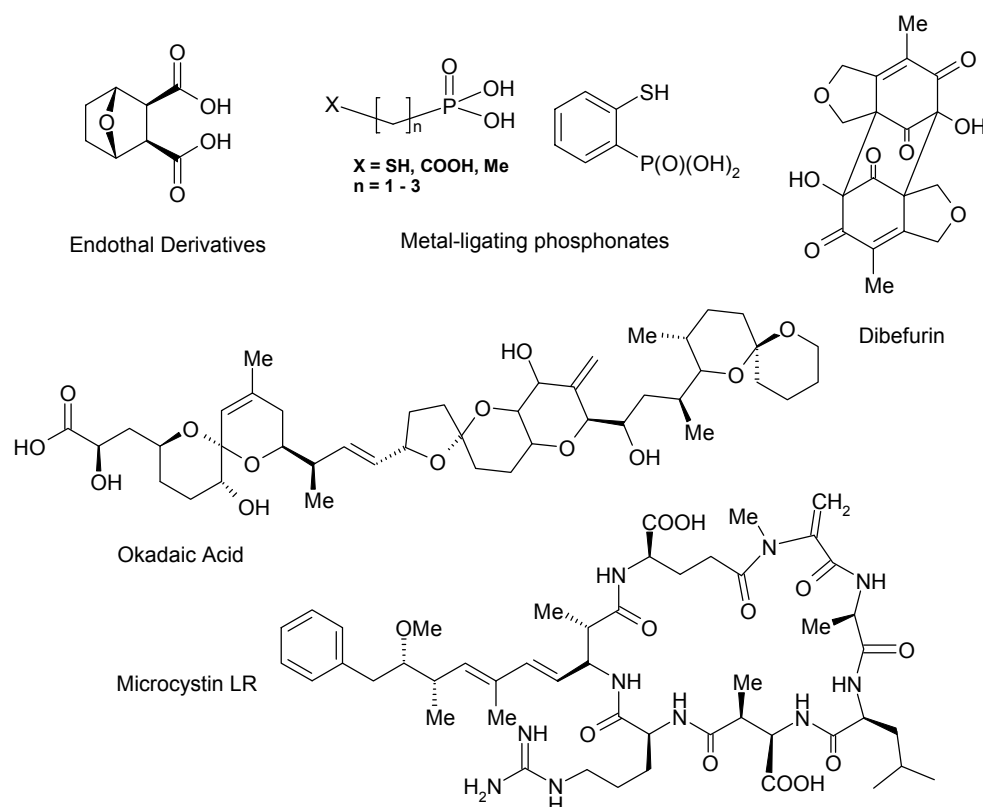
Scheme 1.3 Some other phosphatases inhibitors.

Several new synthetic compounds have been found to be reasonable inhibitors of CaN and other phosphatases. A variety of alkylphosphonic acid derivatives containing an additional thiol or carboxylate group were explored as inhibitors of alkaline phosphatase and purple acid phosphatase (Scheme **1.3**) (Myers, 1997). Tatlock et al. (1997) utilized computational docking experiments and synthetic derivatives of the *exo*, *exo*-7- oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid ring system (Scheme **1.3**) of endothall to search for enhanced ligand binding to CaN. Among these compounds, the most potent inhibitor gave an apparent K_i of 0.5 μM.

Peptide inhibitors of CaN has been also been introduced. The 25-residue peptide based on the sequence of the auto-inhibitory domain of the CNA subunit from residue 457-481 is also an inhibitor of CaN phosphatase activity (Hashimoto, et al. 1990).

Recently, a high-affinity CaN-binding peptide (VIVIT peptide) was selected using a combinatorial peptide library based on the CaN docking motif of NF-AT. (Aramburu, et al. 1998, 1999). The peptide inhibited NF-AT activation and expression of NF-AT-dependent cytokine genes in T cells, but did not inhibit CaN phosphatase activity toward phospho-RII peptide (DLDVPIPGRFDRRVSVAAE, a partial sequence of the subunit of the bovine c-AMP-dependent protein kinase (PKA)), and thus did not affect the expression of other cytokines that require CaN but not NF-AT. The latter point is significant because compounds such as this peptide that selectively interfere with CaN NF-AT interaction without disrupting CaN phosphatase activity may prove to be less toxic immunosuppressants compared with CsA and FK506.

Most of these compounds are not specific inhibitors for CaN. VIVIT peptide did not inhibit the phosphatase activity of CaN and its immunosuppressive activity is still under investigation (Aramburu, et al. 1999). To distinguish the PPIase and CaN inhibition by CsA and FK506 in their diverse pharmaceutical effects (as discussed in the following section), an ideal inhibitor which exhibits potent CaN inhibitory activity in the absence of immunophilins and still remains most structural properties of the parent drugs for high and specific interaction will be greatly appreciated.

1.4 Puzzles to cyclosporin immunosuppressive mechanism still remain unknown.

IL-2 is thought to play a key role in the immune response of mammalian cells. Complexes of immunophilins with immunosuppressive drugs bind and inhibit calcineurin, and in turn block NF-AT nuclear translocation and transcription of IL-2 and other T cell growth factors. The blockage of this pathway by CsA and FK506 has revealed an important transcriptional regulatory mechanism in immune system. However, the explanation is not complete. Calcineurin Aα deficient mice and mice lacking IL-2, as well as NFAT1 knockout mice show the effects which do not match the current model of CsA mediated immunosuppression (Table **1.2**).

Treatment with potent inhibitor or transgenic experiments should not be interpreted as the *sine qua non* for defining the participation of a protein in signaling pathways. Some other action mechanisms of CsA and FK506 for the blockade of T-cell activation were suggested. Recent studies indicate that

CsA also blocks the activation of JNK and p38 signaling pathways triggered by antigen recognition, making CsA a highly specific inhibitor of T cell activation (Su, et al., 1994, Matsuda, et al., 1998 & 2000).

Table 1.2 Knockout experiments of genes in CsA immunosuppressive mechanism.

	Phenotypes of knockout animals
CNA$\alpha^{-/-}$ mice (Zhang, 1996)	The composition and distribution of T and B cells appeared to be normal. CNA$\alpha^{-/-}$ T cells responded normally to mitogenic stimulation. CNA$\alpha^{-/-}$ mice generated defective antigen-specific T cell responses *in vivo*, indicating that CNAα is required for T cell function. CNA$\alpha^{-/-}$ T cells remained sensitive to both CsA and FK506. An explanation could be that CNAβ or another CNA-like molecule can mediate the action of these immunosuppressive drugs.
NFTA1$^{-/-}$ mice (Xanthoudakis, 1996)	Unexpectedly, cells from NFAT1$^{-/-}$ mice showed increased primary response under stimulation and mounted increased secondary responses *in vitro* and *in vivo*. CsA completely inhibited the response of both NFAT1$^{-/-}$ and wild-type cells to stimulation. In an *in vivo* model of allergic inflammation, eosinophils accumulate and the levels of serum IgE were increased. These results suggest that NFAT1 exerts a negative regulatory influence on the immune response.
IL-2$^{-/-}$ mice (Sadlack, 1993) (Kündig, 1993) (Schorle, 1991)	In IL-2-deficient mice, the normal *in vivo* T cell and B cell responses are not in agreement with the importance of IL-2 as defined by *in vitro* assay. Moreover, older IL-2$^{-/-}$ mice had a high number of activated T and B cells, elevated immunoglobin secretion, anti-colon antibodies and aberrant expression of class II major histocompatibility complex molecules.
Cyp18$^{-/-}$ mice (Colgan, 2000a) (Colgan, 2000b)	Cyclophilin 18 is not essential for survival neither in yeast Saccharomyces cerevisiae nor in mammalian cells. Cyclophilin 18 (Cyp18)-null animal is resistant to the effects of CsA, though sensitive to FK506, indicating that of all the family members, Cyp18 is the major CsA target. However, intracellular CsA must decrease in response to Cyp18 deletion considerably, rendering dose dependent curve unpredictable. T-cells from Cyp18 null mice are hypersensitive to TCR-mediated stimulation and express elevated levels of IL-2 and IL-4. These animals exhibit splenomegaly, tissue infiltrates with eosinophils and increased IgE. These results suggest that Cyp18 play a role not only in presenting CsA to its cellular target CaN, but also an indispensable member in immune system.

To answer these questions, a preferable approach is to distinguish the cyclophilin and calcineurin inhibitory activities of CsA by designing and synthesizing CsA derivatives that can inhibit calcineurin but does not inhibit cyclophilin and vice versa.

1.5 The parvulin type PPIase Pin1.

There are abundant cyclophilins in cells and the Cyp18 is probably the major receptor protein for CsA (Colgan, 2000a, 2000b). However, the resistance of Cyp18$^{-/-}$ animal to CsA treatment is a

matter of various factors. Another approach could be to investigate the effects of CsA metabolites on immunosuppression. CsA could be modified in cells. It has already been shown that several metabolites contribute to the immunosuppressive activity and/or some side effects of CsA. Phosphorylation, sulfoylation, acetylation and oxidation are among the most ubiquitous cellular events. We studies the Cyp18 inhibition, CaN inhibition and immunosuppressive activity of modified CsA derivatives. Besides the interesting results from phosphorylated CsA about its immunosuppressive behavior, it was surprising for us to find that phosphorylated CsA is a potent inhibitor to a member of the parvulin family of PPIase – the human Pin1.

Phosphorylation of critical substrates by the Ser/Thr kinase Cdc2/cyclinB complex is believed to underlie many of the structural rearrangements which occur as cells enter mitosis (Nurse, 1990). An additional Ser/Thr kinase, NIMA, is also required for mitotic entry in the filamentous fungus, *A. nidulans* (Osmani et al., 1987; Osmani et al., 1988). Cloning strategies have been unsuccessful in identifying functional NIMA homologs in higher eukaryotes. In contrast, Pin1, which was uncovered by using a yeast two hybrid screen as a protein that interacts with NIMA and suppresses its mitosis-promoting activity, has been identified in all eukaryotic organisms where examined (Hanes et al. 1989; Lu et al. 1996; Maleszka et al. 1996). Pin1 can perform both negative regulation of entry into mitosis and progression through mitosis in human cells and yeast.

Phosphorylation-specific prolyl isomerization catalyzed by Pin1 provides a novel mechanism essential for regulating dephosphorylation of certain phosphoSer/Thr-Pro motifs by conformation specific phosphatases (Zhou, et al., 1999). The major Pro-directed phosphatase PP2A is conformation-specific and effectively dephosphorylates only the *trans* phorphoSer/Thr-Pro isomer. Pin1 catalyzes prolyl isomerization of specific phosphoSer/Thr-Pro motifs both in Cdc25C and tau to facilitate their dephosphorylation by PP2A. Furthermore, Pin1 and PP2A show reciprocal genetic interactions, and prolyl isomerase activity of Pin1 is essential for cell division *in vivo*. Moreover, it was recently shown that Pin1 catalyzes a conformational change on Cdc25 (Stukenberg, et al., 2001) and in turn influences the enzymatic activity of the phosphatase. These results suggest that prolyl isomerization may play an important regulatory role in the cell cycle. Winkler et al. reported recently that Pin1 appears to be required for the DNA replication checkpoint in *Xenopus laevis* (Winkler et al. 2000). In contrast, Pin1 is not critical for any readily observable function in *Drosophila melanogaster* or mice (Maleszka et al., 1996; Fujimori et al., 1999). This may can be explained by the new finding that Cyp18 and Pin1/ESS1 can act on common targets required for mitosis and the PPIase activity is linked to their essential function (Wu, et al., 2000, Arevalo-Rodriguez, et al., 2000). Interestingly, it was reported that Pin1 binds to mitosis-specific phosphorylated tau protein and Alzheimer's disease tau to only one phospho-Thr-Pro motif, but not to any tau in extracts from age-matched normal brain cells (Lu et al. 1999b).

Is there any relationship between Pin1 inhibitory activity by phosphorylated CsA and the immunosuppressive effects its parent drug? We tried to design and synthesize potent Pin1 inhibitors

basing on the minimal sequence requirement for Pin1 catalysis (Füssel, Susanne, Ph.D. thesis). Peptide libraries containing nonnatural amino acids were also constructed for scaning Pin1 inhibitors.

1.6 Other immunosuppressants.

Immunosuppressive drugs can be classified according to their mechanism of action: inhibitors of cytokines (e.g. glucocorticoids, cyclosporins, FK506, rapamycin) and inhibitors of DNA synthesis (e.g. azathioprine, mycophenolate). Among all the new immunosuppressive drugs being investigated either preclinically or clinically, three stand out: FK506, rapamycin, and mycophenolate mofetil. Each drug has distinct mechanisms of immunosuppression, and in the past years significant advances have been made in our understanding of the actions of these drugs at the molecular levels. Searching for new cellular targets of CsA, several recently discovered immunosuppressive compounds attracted our attention due to their intricate structures and/or novel biological effects (Sanglifehrin, FR901483 and Stevastelins).

FK506 (tacrolimus). CsA and FK506 inhibit CaN after binding to an endogenous receptor (section 1.3.3). Although its mechanism of action is similar to that of CsA, FK506 has a completely different structure. The risk for acute graft-versus-host disease, nephrotoxocity, infections or leukemia relapse of CsA and FK506 were compared in a lot of different studies. However, it is difficult to draw firm conclusions.

Besides immunosuppressive activity, both CsA and FK506 demonstrate neuroprotective actions, but only FK506 and its derivatives have been clearly shown to exhibit significant neuroregenerative activity (Gold, 2000). The neuroregenerative property of FK506 does not involve CaN inhibition. A major breakthrough for the development of this class of compounds for the treatment of human neurological disorders was the ability to separate the neuroregenerative property of FK506 from its immunosuppressant action via the development of non-immunosuppressive (non-calcineurin inhibiting) derivatives.

Rapamycin (Sirolimus). Rapymycin shares structural similarity with FK506 and binds to the same family of FK506 receptor, the FK506 binding proteins (FKBPs). However, the action of FK506 and rapamycin is different. Neither rapamycin nor rapamycin/FKBP complex binds and inhibits CaN phosphatase activity. The FKBP12/rapamycin complex interacts with a recently defined target protein termed the mammalian target of rapamycin (mTOR) (Brown, et al., 1994, Sabatini, et al., 1994). Accumulating data suggest that mTOR functions in a previously unrecognized signal transduction pathway required for the progression of IL-2-stimulated T cells from G(I) into the S phase of the cell cycle (Schmelzle, et al., 2000).

MMF. Mycophenolate mofetil (MMF) is an inactive prodrug and releases the active form *in vivo*, mycophenolic acid (MPA). T- and B- lymphocytes are more dependent than other cells on new synthesis of guanosine and deoxyguanosine nucleotides. Inosine monophosphate dehydrogenase

FK506

Rapamycin

Sanglifehrin

FR901483

Stevastelin A: R = SO$_3$H
Stevastelin B: R = H
Stevastelin P: R = PO$_3$H$_2$

Mycophenolate mofetil (Prodrug)

Mycophenolic acid (immunosuppressant)
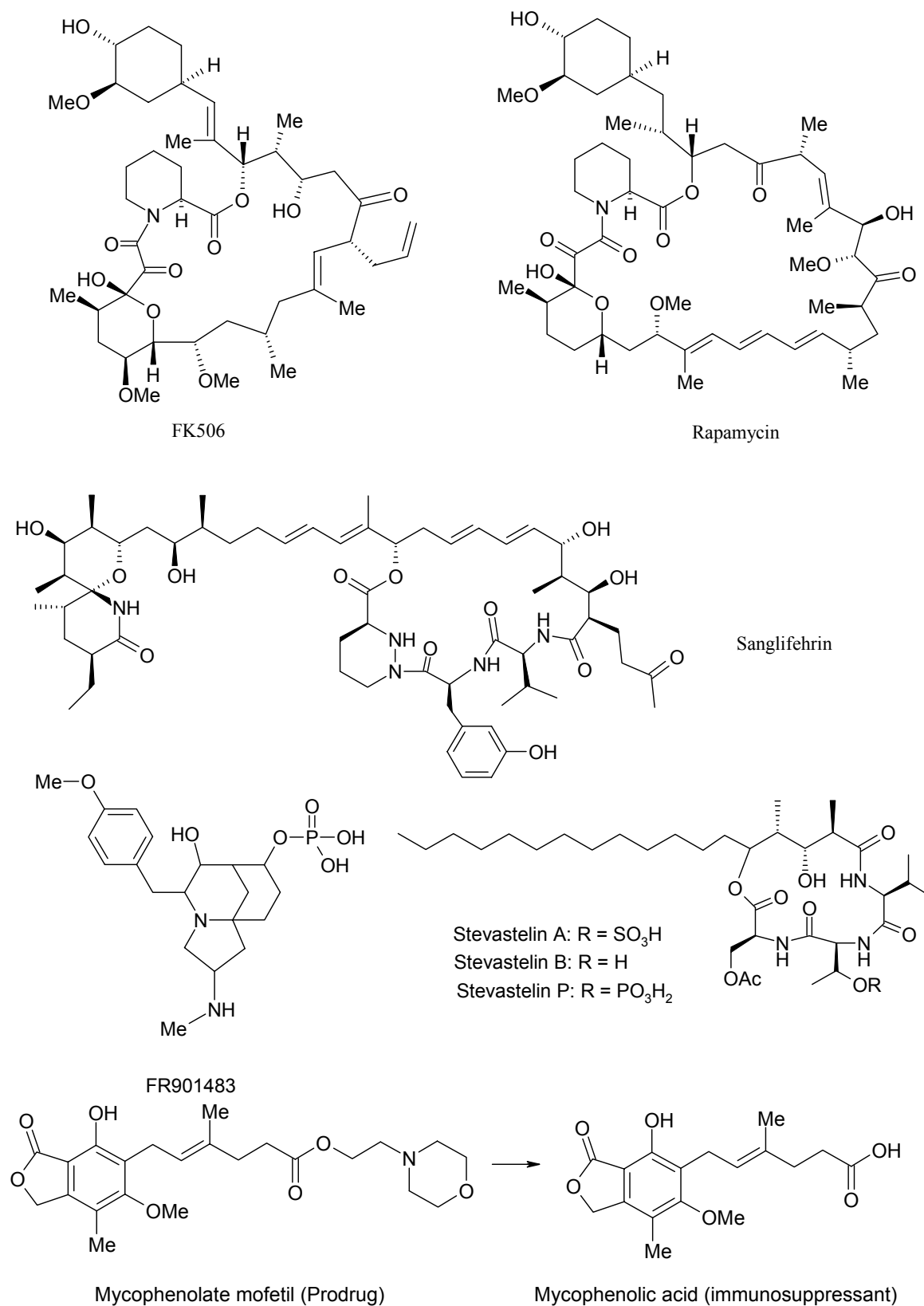
Scheme 1.4 Some other immunosuppressants

(IMPDH) is the rate-limiting enzyme for the *de novo* purine biosynthetic pathway and MPA inhibits IMPDH. MMF is used in transplantation in combination with other agents such as CsA and corticosteroids (Allison, et al., 1996).

Sanglifehrin. Recently, a new immunosuppressive compound, sanglifehrin A (Sanglier, et al., 1999; Fehr, et al., 1999), was discovered in screening for compounds that would interfere with signaling molecules other than calcineurin.

Sanglifehrin A represents the fourth class of immunophilin-binding immunosuppressants and the mode of its action is different from any known immunosuppressive drugs (Zhang, et al., 2001; Zenke, et al., 2001). It exhibits 20-fold higher affinity for Cyp18 than CsA and the immunosuppressive activity are independent of Cyp binding. Distinct from CsA, sanglifehrin A does not affect calcium-dependent IL-2 production. It blocks T cell proliferation induced by IL-2 in G(1) with no appreciable effect on IL-2 receptor expression in a manner similar to that of rapamycin. However, sanglifehrin A has no effect on the enzymatic activity of p70(s6k) kinase, distinguishing it from rapamycin in their mode of action. The activity of sanglifehrin A is also different from that of other known late-acting immunosuppressants, e.g., mycophenolate mofetil or brequinar, as it does not affect *de novo* purine and pyrimidine biosynthesis. Among the events involved in controlling G(1) progression, it was found that sanglifehrin A inhibited the hyper-phosphorylation of Rb by cyclin E-Cdk2 in an indirect manner (Zhang, et al., 2001).

FR901483. A novel immunosuppressant, FR901483 (Sakamoto, et al., 1996), has been recently isolated from the fermentation broth of *Cladobotryum*. FR901483 is not an inhibitor of IL-2 production, whereas CsA and FK506 specifically inhibit endogenous production of IL-2. FR901483 suppressed both types of lymphocyte proliferation, namely those that are independent and dependent on endogenous IL-2 production. Primary experimental results suggested that FR901483 is an anti-metabolite, which is likely to interfere with purine nucleotide biosynthesis by inhibiting the enzyme(s) adenylosuccinate synthetase and/or adenylosuccinase. Interestingly, this compound is an intriguing tricyclic structure possessing a phosphate ester in its molecule (Scheme **1.4**). This ester residue may play an important role in exerting immunosuppressive activity because the dephosphorylated derivative is nonimmunosuppressive.

Stevastelins. These are a novel group of immunosuppressants, which were originally discovered as inhibitors of the IL-2 and IL-6 dependent gene expression. Stevastelins with a free hydroxyl group in their threonine residues showed immunosuppressive activities (stevastelin B). In contrast, derivatives that have O-sulfoyled or phosphorylated threonine residues are inactive (stevastelin A & P). However, the phosphorylated and sulphonyled derivatives were more potential as phosphatase inhibitors. The authors suggested that stevastelin B with a free hydroxy group, which could permeate the cell membrane easily, is phosphorylated or sulphonylated by cellular enzyme(s) and have an inhibitory effect on cellular protein phosphatases. The acidic functionality of stevastelin A

& P result in its low membrane permeability and poor immunosuppressive activity (Hamaguchi, et al., 1997).

<div align="center">*   *   *</div>

To answer the questions involving CsA and its diverse biological effects, the main aim of my work is to design and synthesize CsA derivatives, as well as to investigate their PPIase and CaN inhibitory activities:

* To distinguish the Cyp and CaN inhibitory activities of CsA, we designed and synthesized direct CaN inhibitors basing on CsA structure. The first CsA derivative, which could inhibit CaN in the absence of Cyp was reported (in chapter 2).

* To get nonimmunosuppressive Cyp inhibitors, we describe a facile synthesis of CsA derivatives with potent Cyp18 inhibitory activity but minor CaN inhibition (in chapter 3).

* Thioxylated cyclosporins were synthesized and they exhibited interesting inhibitory activities to Cyp18 and CaN. Furthermore, the conformations of these compounds could undergo photoisomerization as characterized by biophysical measurements and enzymatic assays (in chapter 4).

* The chemical synthesis of phosphorylated CsA was described. The inhibitory activities to Cyp18, CaN, and Pin1 by phosphorylated CsA as well as a series of CsA residue 1 derivatives were investigated (in chapter 5).

* Basing on the minimal requirements for Pin1 catalysis, potent reversible Pin1 inhibitors were designed and synthesized (in chapter 6).

Chapter 2. Direct CaN inhibition by CsA derivatives.

2.1 CsA structure.

Due to the unique signal-function mode of action of CsA and FK506, much effort has been devoted to obtain structural information. NMR structure, computer modeling, chemical modification (so called chemical genetics), protein surface residues mutations as well as X-ray crystal structures have provided us different perspectives to their free ligands, binary complexes and ternary complexes structures.

2.1.1 Free ligand structure of CsA, FK506 and rapamycin.

The crystal structures of CsA, FK506 and rapamycin have been reported (Loosli et al., 1985; Tanaka et al., 1987; Swindells et al., 1978). CsA has four intramolecular hydrogen bonds and one *cis* amide bond, which is placed between MeLeu^9 and MeLeu^{10}. A β-II turn involving residue 2, 3, 4 and 5 was observed (Figure **2.1**). The NMR structure in CDCl$_3$ is very similar to the X-ray structure. Although the MeBmt^1 side chain is folded over the backbone, the formation of an H-bridge involving MeBmt^{1}OH and MeBmt^{1}CO was proposed by NMR spectra (Kessler, et al., 1990). A second minor conformation has been suggested by NMR structure, which contains two *cis* peptide bonds, and the additional *cis* bond is between residue 3 and 4. In solvents of higher polarity, changes in the backbone conformation are directly visible in the NMR spectra and many conformations are in equilibrium and inter-converting slowly (Kessler, et al., 1990).

Either FK506 or rapamycin has only one amide bond, containing the N7 atom of the pipecolinyl ring and C8 and O3 atoms of the dicarbonyl. Interestingly, the amide bond of FK506 is in *cis* conformation whereas the rapamycin amide bond is in *trans* conformation.

2.1.2 The CsA/Cyp18 binary complex structure.

X-ray structure of Cyp18/CsA complex (Mikol, et al., 1993) shows that CsA adopts a dramatically different conformation when bound to Cyp18. Namely, all peptide bonds are in *trans* conformation and all four hydrogen bonds disappear. X-ray structure of FKBP12/FK506 and FKBP12/rapamycin complexes have shown that in the bound immunosuppressants the amide bonds are also in the *trans* conformation (Van Duyne et al., 1991 & 1993). Cyp18 seems to be a rather rigid protein that does not undergo significant structural changes upon ligand binding. The binding pocket is a hydrophobic crevice defined by 13 residues that are within 4Å of the bound CsA. Five direct hydrogen bonds between Cyp18 and CsA as well as a network of water-mediated contacts stabilize the interactions between the protein and ligand.

2.1.3 The Cyp18/CsA/CaN ternary complex structure.

According to the Cyp and CaN inhibitory activities of plenty of CsA derivatives, the eleven residues in the cyclic peptide can be divided into two domains. Only residues 9, 10, 11, 1 and 2 (Cyp binding
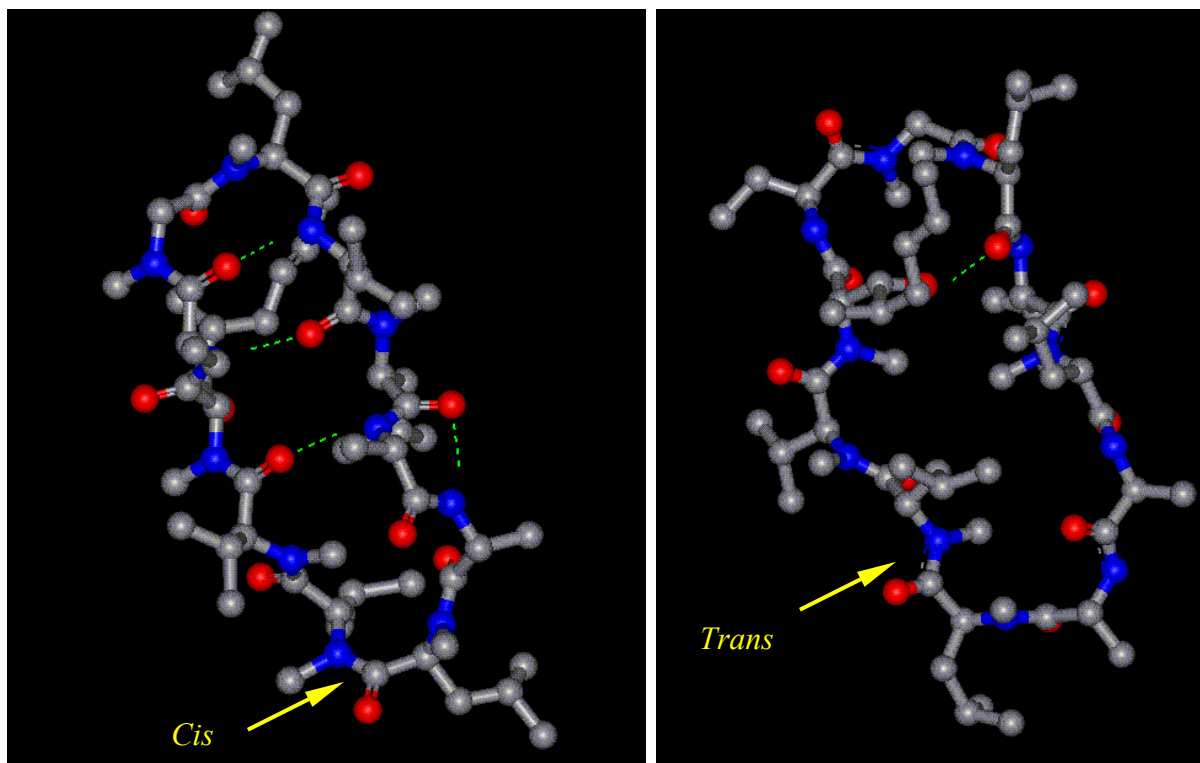
Figure 2.1 Comparison of CsA structures in the free form (left) and in the ternary complex (right) (the proteins are hidden, and the nitrogen atoms are in black)
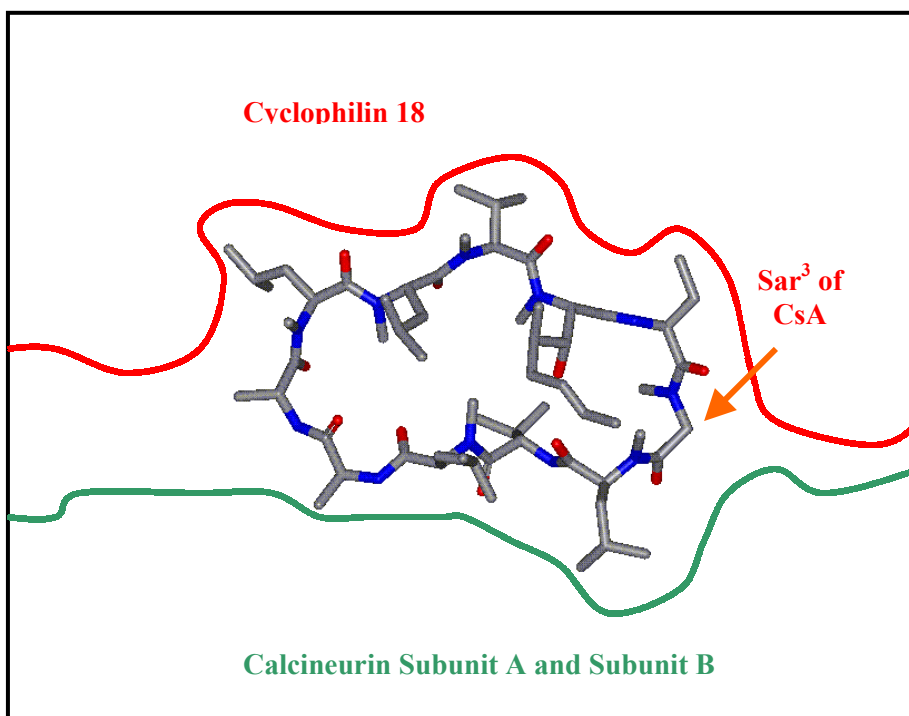

Figure 2.2 The interface among CsA, Cyp18 and CaN in the ternary complex. Sarcosine 3 of CsA is on the concourse of the ternary structure. (Cyp18 in red, CNA and CNB in green, CsA in stick).

domain) are in contact with Cyp18. The remaining residues (CaN binding domain) are involved in the specific interactions with CaN. The crystal structure of Cyp18/CsA/CaN complex was reported recently (Zhao, et al, 2000). These structural informations, together with the FKBP/FK506/CaN complex structure, provided us the molecular basis of how CsA and FK506 (Kissinger, et al., 1995) can induce the same biological effects through the same enzymatic inhibition with their differences in the active complexes.

The conformations of Cyp18 and CsA in the Cyp18/CsA/CaN (Zhao, et al., 2000) (Figure **2.1**) complex are similar to those in the Cyp18/CsA binary complex, respectively. The CaN structures in the free form, in the FKBP/FK506/CaN ternary complex as well as in the Cyp18/CsA/CaN complex are also similar. CsA alone has no any detectable affinity to CaN. Cardenas, et al. (1994) reported that both Cyp18 and FKBP12 could bind calcineurin A in the absence of exogenous immunosuppressive ligands, CsA and FK506 respectively. However, we were not able to detect any inhibitiory activities of either FKBP12 or Cyp18 to CaN (Baumgrass, et al., unpublished results). CsA adopts a 'wrong' conformation to CaN binding pocket in the uncomplexed form. CaN prefers the new conformation of CsA when it is bound to Cyp18. This binding brings Cyp18 and CaN together and the interactions between the interfaces of both proteins enhance the affinity. Mutations of Cyp18 surface residues (Etzkorn, et al., 1994) as well as X-ray structure of Cyp18/CsA/CaN complex (Zhao, et al., 2000) revealed a network of contacts between Cyp18 and CaN. Another example of ligand-protein interaction with similar recognition mechanism is the specific recognition of foreign peptides bound to MHC molecules by TCR (Garcia, 1999), which is the central event in the cellular immune response to invading.

2.2 Design a conformation for direct calcineurin inhibition.

The structural information have provided us a static picture of the intermolecular and intermolecular interactions of CsA in either free form or in the Cyp18/CsA complex. However, the puzzle about the interplay between the two sets of residues (Cyp binding domain & CaN binding domain) still remains. The principle, by which nature designs such a conformation-switchable molecule, hides itself behind these non-covalent bond forming and breaking events. This principle should have two aspects: First, what are the restrictions in the free CsA molecule which prevent it from CaN binding. Second, what intramolecular and intermolecular interactions are necessary for constructing a new 'active' conformation? Furthermore, is it possible to design and synthesize a cyclosporin derivative which loses the intramolecule restrictions in the uncomplexed CsA structure, and mimics the 'active' conformation of the ligand in CsA/Cyp18 complex which could fit the CaN binding pocket?

Noncompetitive inhibition of CaN by immunosuppressant/immunophilin complex. The Cyp/CsA complex neither blocks nor induces an observable conformational change in the active site of CaN. It inhibits CaN in a classical noncompetitive fashion (Etzkorn, 1994). This has been shown

to be the case of FKBP12/FK506 inhibition as well (Kissinger, 1995). The Cyp/CsA complex masks the active site of CaN and inhibits its activity to large phosphorylated substrates. Interestingly, the CaN phosphatase activity toward nitrophenylphosphate was stimulated about 3-fold in the presence of Cyp/CsA (Liu, et al., 1991). In the absence of an immunophilin above the CaN binding pocket (Figure **2.3**), can a small molecule ligand like CsA or FK506 alone block the phosphopeptides or phosphoproteins to access the active site? The immunophilins are much larger than CsA. It maybe also function as a huge block near the active site, as well as a conformation-switch device for CsA or FK506.

Studies of the biosyntheses (Lawen, et al., 1993; Dittmann, et al., 1994) of cyclosporins *in vivo* and *in vitro* by cyclosporin synthetase revealed that the sarcosine residue at position 3 is highly conserved. The side chains of residues 3-6 of CsA make hydrophobic contacts with CNA subunit. However, sarcosine is not a hydrophobic amino acid and there is no evidence to show that Sar[3] is as critical as MeLeu[4] and MeLeu[6] in the multiple hydrophobic interactions. The exact role of this highly conserved residue remains unknown (Figure **2.2**).

Sarcosine and glycine are often found in β-turn conformations. Free CsA adopts a βII - turn in CDCl₃ (Kessler, et al., 1990) and X-ray crystal structure. All the hydrogen bonds disappear in the CsA/Cyp18 complex. Cyp18 binding disrupts the β-turn structure. We proposed that the fungus produces cyclosporins with a conserved sarcosine residue at position 3 to facilitate the β-turn formation. We hypothesize that losing the turn structure will probably create a compound that has calcineurin inhibitory activity in the absence of Cyp. For unknown reasons, such a product must be prevented in the fungus' cyclosporins biosyntheses. To test our proposal, we synthesized some CsA derivatives by Sar[3] residue chemical modifications.

2.3 Syntheses of [α-substituted Sar][3] cyclosporins.

There's no need to perform a total synthesis to get these derivatives because an amazing chemical reaction has been reported by Seebach and coworkers (Seebach, et al., 1993). They synthesized a series of CsA position 3 derivatives by generation of an enolate at the sarcosine residue and reaction with various electrophiles. Using the same synthetic strategy, we have synthesized a series of CsA sarcosine[3] derivatives. After a short discussion about the reaction and its mechanism, we will come to the biochemical results of these compounds.

Strong base (lithium diisopropylamide (LDA) or n-BuLi) converts CsA to a hexalithio derivative containing a lithium alkoxide, four lithium azaenolate and one lithium enolate units (Scheme **2.1**). The lithium-alkoxide group on MeBmt[1] can be alkylated to from an ether. The four lithium azaenolates could be N-alkylated. Epimerization and/or C-alkylation with formation of an α,α-disubstituents could be side reactions. However, this reaction showed high regio-selectivity. Reaction with electrophiles (alkyl halides, ClCOOR, RSSR) at low temperature give main products containing new side chains in sarcosine 3 of the cyclic undecapeptide (Scheme **2.1**) in moderate to
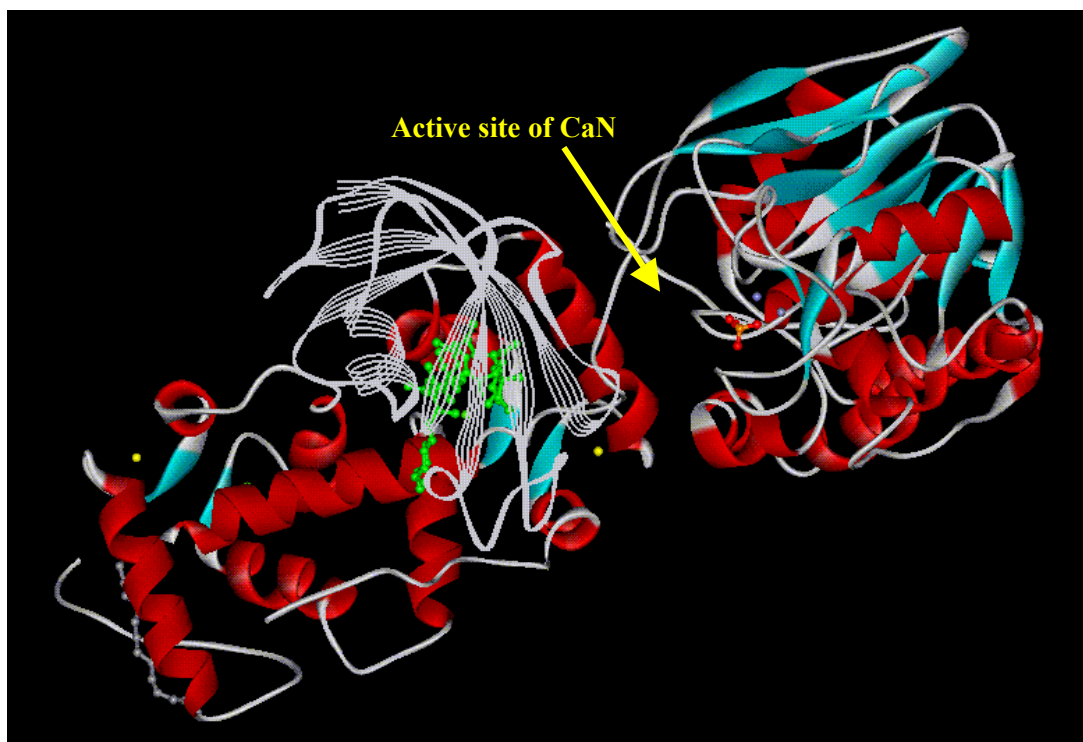
Figure 2.3 Noncompetitive CaN inhibition by FKBP12/FK506 complex: Is the size of CsA or FK506 large enough to block voluminous substrate from entering the active site in the absence of Immunophilin (Cyp and FKBP, respectively)? FKBP12 is in Gray, CaN was colored according to the secondary structure, and FK506 is in green.

Scheme 2.1 Synthesis of [α-substitited Sarcosine]³ CsA

Cs**11**: R = Me (R)

Cs**12**: R = Me (S)

Cs**13**: R = -SMe (R)

Cs**14**: R = -SMe (S)

Cs**33**: R = CH₂COOBu-t (mixture of R and S)

Cs**35**: R = CH₂COOH (R and S)

Cs40: R = Et (R)

Cs**41**: R = Et (S)
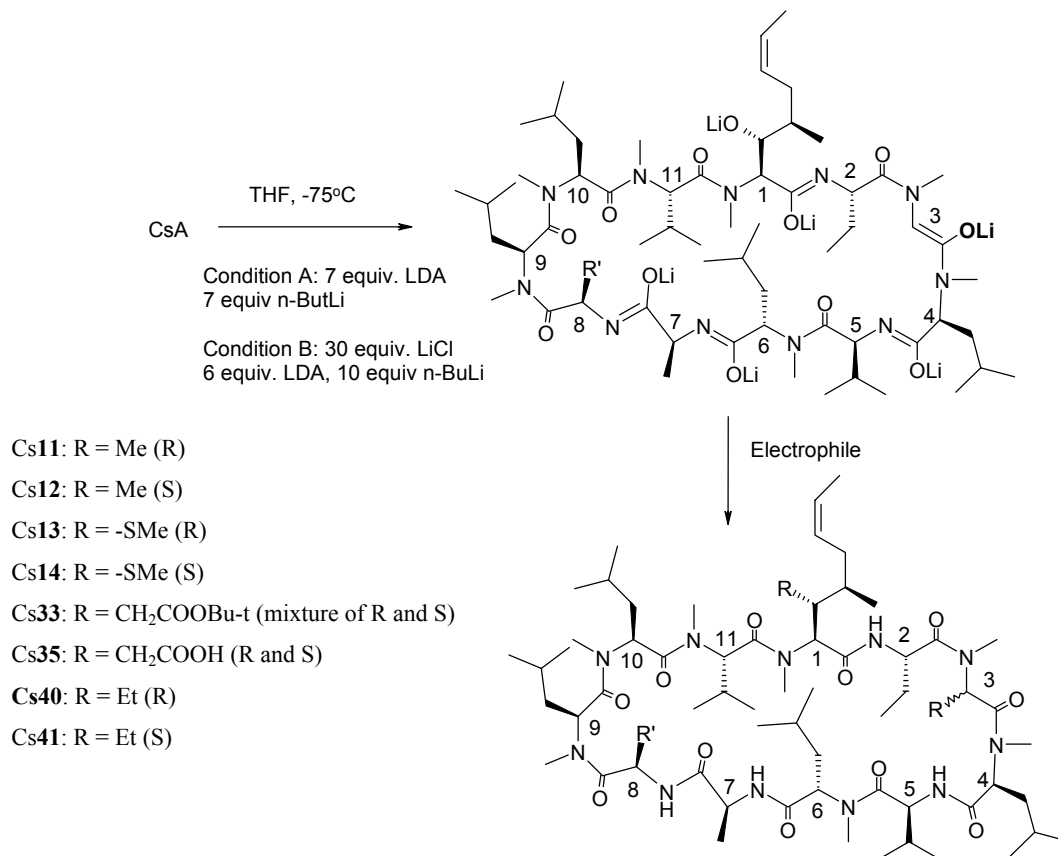
high yields. N-alkylation turns out to be a competing process only at elevated temperature (Seebach, et al., 1993).

Interestingly, this reaction also showed reverse stereochemical courses in salt-free and LiCl-containing solutions (Seebach, et al., 1993). LiCl can be used to solubilize the CsA hexalithio derivatives. It turns out that CsA/LiCl mixtures are soluble in THF at dry ice/aceton temperature (-78°C) with up to 30 equiv. of LiCl. No precipitates were observed when the CsA/LiCl solutions were combined with LDA and n-BuLi. In salt-free THF solution, the reaction gave the major diastereoisomer of *R* configuration. The high selectivity for producing the *S* configuration was observed in the presence of LiCl. The structure of the enolate formed with LDA alone is such that the *R* face of the trigonal center on the double bond is more readily available for electrophilic attack. The enolate generated in the presence of LiCl reacts preferentially from the *S*-face. Room temperature NMR spectroscopy showed structural disruption of CsA molecule in 30 equivalent LiCl/(D8)THF solution (Seebach, et al., 1993): no intramolecular hydrogen bonds between NH and carbonyl oxygen atom and all peptide bonds in *trans* conformation. It seems that the presence of LiCl changes the CsA conformation dramatically. This difference might provide a speculative interpretation of the reverse stereochemical course of the reactions in two different solvent systems.

2.4 hr Cyp18 and CaN enzymatic activity assays.

PPIase activity assay. The methods of measuring PPIase activity are often based on the production of pure *cis* or *trans* isomers or at least an isomeric distribution different from that expected based on the isomerization reaction condition (for example, in another solvent system, or buffer of different pH value). This allows investigation of the kinetics of the reestablishment of equilibrium and that is identical to the rate of the *cis/trans* isomerization. The rate constant of *cis* to *trans* isomerization is generally assessed by an assay that is based on isomer-specific proteolysis using tetrapeptide derivatives (Suc-Ala-Xaa-Pro-Yaa-(4-)nitroanilides with Xaa, Yaa for any natural non-proline amino acid) as standard substrates (Fischer, et al., 1984). Many proteases are $>10^4$ more specific for the all-*trans* conformer of a substrate. In high protease concentration, all *trans* conformers are cleaved immediately and pure *cis* isomer is left over. The rate of *cis* to *trans* isomerization is corresponding to the subsequent production of 4-nitroaniline because only the newly produced *trans* isomer can be cleaved immediately by protease. 4-Nitroaniline has a characteristic absorbance at 390nm. This makes it possible to monitor the reaction with an UV/VIS spectrometer. The major part of the reported kinetic constants of PPIase catalysis has been evaluated by this method (Some novel methods for PPIase assay will be discussed in chapter 6). Figure **2.4** shows the reaction course for the cleavage of Suc-Ala-Phe-Pro-Phe-4-nitroanilide by isomer-specific protease α-chymotrypsin in the absence (black line) and presence (dot) of Cyp18, or Cyp18 plus CsA (gray line).

The IC_{50} value of the inhibitors can be calculated according to the formula:
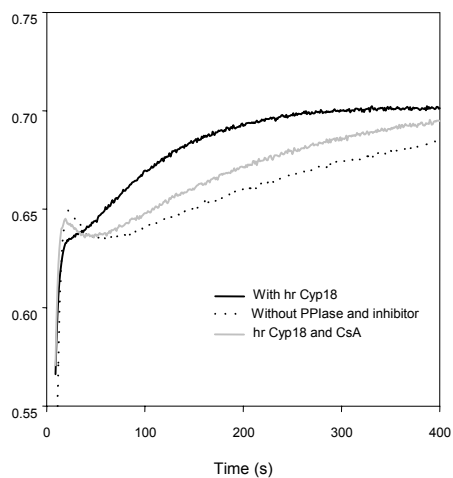
Figure 2.4 *Cis*/*trans* isomerization of peptide substrate Suc-Ala-Phe-Pro-Phe-NH-Np in Hepes pH 7.8 at 7°C was measured with the protease coupled assay. The time course is monitored with UV absorbency at 390nm, in the absence of PPIase (dots); in the presence of 2.5nM hr Cyp18 (black line), and in the presence of 2.5nM hr Cyp18 and 10nM CsA (gray line).
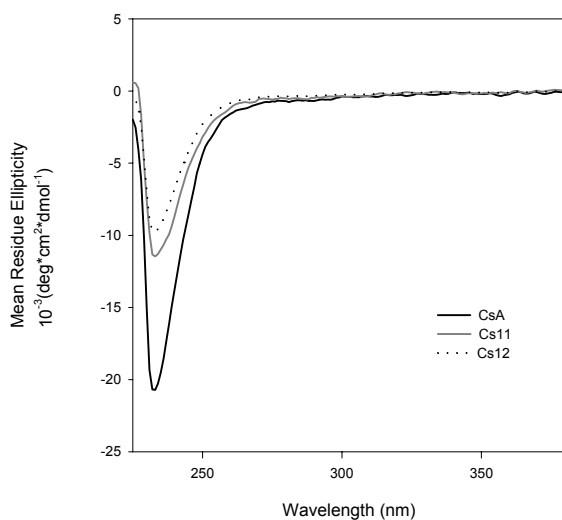
Figure 2.5 CD spectra of CsA, Cs**11**, and Cs**12**. The CD spectra of CsA, Cs**11**, and Cs**12** were measured in DMSO/water (10/90) at 10°C. A significant difference of the band around 332nm could be observed. (The noise from DMSO is from 190-220nm, and its intensity is below 1.0).
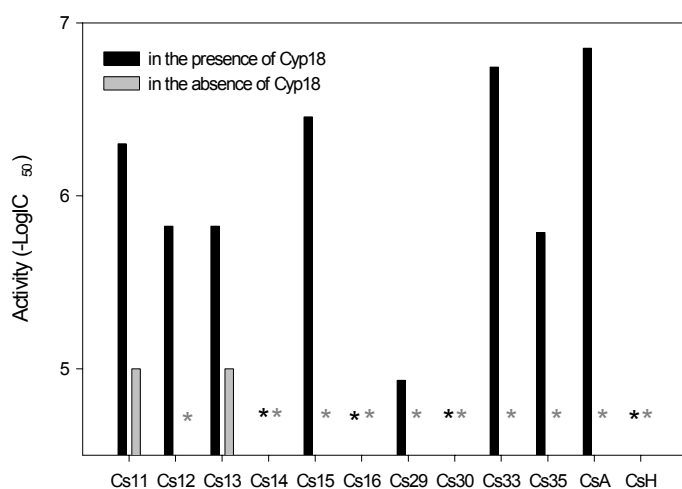
Figure 2.6 CaN inhibitory activities of [α-substituted Sar][3] cyclosporins. CaN phosphatase activities were measured, using biotinylated RII peptide as substrate with Scintillation assay at 30°C. For measuring the direct CaN inhibitory activities (gray), inhibitors of desired concentrations were added in the absence of Cyp18. For measuring the indirect CaN inhibitory activities (black), the concentration of inhibitors were kept in constant and Cyp18 of desired concentrations were added. (See the list on page ii for compounds and their corresponding codes)
* Undetectable.

$$K_i = k_{obs}/k_u; \qquad K_{max} = k_{enz}/K_u$$

$$(K_i - K_{min}) = (K_{max} - K_u)/(1 + C_i/IC_{50})$$

k_{obs} is the rate of *cis/trans* isomerization being measured. k_u is the reaction rate in the absence of inhibitor and enzyme. k_{enz} is the maximal rate in the presence of PPIase without addition of inhibitor. C_i is the concentration of inhibitor. The IC_{50} of CaN inhibition was also calculated with this formula, but CaN activities were used and the dephosphorylation were measured under initial rate conditions.

Calcineurin phosphatase activity assay. The scintillation proximity concept has been applied for measuring CaN phosphatase activity using ScintiStrip surfaces coated with streptavidin (Sullivan et al., 1997; Nakayama et al., 1998). Biotinylated [γ-^{33}P] RII peptide was used as a standard substrate for CaN. After dephosphorylation by CaN in the presence or absence of CaN inhibitor, the reaction mixture was transferred to the streptavidin coated well. The biotinylated peptide is then immobilized on the streptavidin surface and RII associated [γ-^{33}P] was measured.

2.5 Biophysical properties of [α-methyl Sarcosine]^3 CsA.

2.5.1 CD spectra of CsA and [α-methyl Sarcosine]^3 CsA.

CD (circular dichroism) spectra is one of the most popular physical techniques for monitoring overall structural changes of biomolecules. It can directly interpret the changes of protein secondary structure, even though the method is empirical. Due to the low solubility of CsA in water and its multiple conformations in solvents of high polarity, the NMR structure of CsA in water is not available. However, using CD spectra, a strong signal of the turn can be observed in water/ethanol (50/50) (Hasumi, et al., 1994) and water/DMSO (90/10). The solubility of [α-methyl Sarcosine]^3 CsA is as poor as its parent molecule. By CD spectra, we were able to demonstrate that sarcosine α-methylation of CsA impairs the turn structure significantly. As shown in figure **2.5**, with the same concentration (0.1mM in water/DMSO (90/10)), the band corresponding to the turn at around 232 nm decrease about 50% for the derivative.

2.5.2 Time courses of fluorescence during the binding of CsA, Cs11 and Cs13 to Cyp18.

CsA is a slow-binding inhibitor of Cyp18. Both the initial inhibitory activity and the subsequent time-dependent inhibition are sensitive to the solvent system (ethanol/water, DMSO, THF, LiCl-THF, and non-ionic surfactant cremophor EL (CEL)) in which CsA is dissolved prior to the assay. The kinetics of the conformational change during the binding to Cyp18 was investigated indirectly using solvent jump assays (Kofron, et al., 1992; Janowski, et al., 1997). Only minor inhibition of Cyp18 could be detected if CsA was added from THF or CEL. On the contrary, significant inhibition of PPIase activity occured during mixing time when CsA was added from LiCl/THF or polar solvents (DMSO, ethanol/water). This fast phase could not be resolved with manual mixing

procedures, but its amplitude can be calculated by extrapolation (Janowski, et al., 1997). The second kinetic phase of inhibition proceeded at a time scale sufficiently slow to be evaluated quantitatively. A *cis* to *trans* isomerization of the *cis* peptide bond between MeLeu[9] and MeLeu[10] and a resulffling of an initially formed Cyp18/[*trans*-MeLeu[9,10]] CsA complex, both occurring monomolecularly, was suggested to be involved in this second phase. When CsA was added from DMSO or ethanol/water, a third phase of decrease of PPIase activity could be observed. The amplitude of this third phase is relatively small. A direct observation of the binding course of CsA to Cyp18 is of great interest for understanding the dynamics of the conformational changes.

Cyp18 contains one tryptophan residue at position 121 (Trp121) (Gastmans, et al., 1999). The fluorescence intensity of this single tryptophan residue increases upon binding to CsA. Trp121 is in close contact to the bound CsA and is conserved in almost all immunophilins. Using time-resolved fluorescence measurement, the authors could demonstrate that the strong enhancement is mainly due to a selection of a major microconformation of Trp. The amino acids in the immediate environment, especially Glu120, that move upon binding, have only minor influences. We measured the fluorescence time course of Cyp18 Trp121 after the addition of CsA from DMSO.

The *cis* peptide bond between MeLeu[9] and MeLeu[10] as well as the β-turn conformation maintained by four intramolecular hydrogen bonds in the free form of CsA disappear in the Cyp18/CsA complex. Replacing the Sar[3] that is important for the β-turn formation by an α-substituted amino acid, we presumed that Cs**11** and Cs**13** could adopt conformations similar to the active CsA in the complex. Although the inhibitory activities of Cs**11** and Cs**13** to Cyp18 are very similar to their parent drug (in the next section), the dynamics of backbone could be different due to losing the intramolecular restrictions. Using time-dependent Cyp18 PPIase activity inhibition assays as well as fluorescence time course of Cyp18 during ligands binding, we were able to show that the binding of Cs**11** and Cs**13** to Cyp18, different from CsA, is a fast process.

As shown in Figure **2.7**, three phases could be observed in the binding course of CsA to Cyp18. The first phase is very fast and so could not be resolved with manual mixing procedures. The amplitude of this phase is similar to the second phase. The k_{obs} of second slow phase is $17.57 \pm 1.48 \times 10^{-2}$/s. The third phase is extremely slow and could not be evaluated exactly. These results are in agreement with the former studies (Kofron, et al., 1992; Janowski, et al., 1997) and provided a real time observation of the binding of CsA to this receptor protein. The fluorescence time course of Cyp18 upon binding to Cs**11** or Cs**13** was different from that of Cyp18/CsA binding. Only two kinetic phases could be observed in each case: the first fast phase and the last extremely slow phase. The second slow phase, which involves the *cis* to *trans* isomerization of the *cis* MeLeu[9, 10] peptide bond is absent. This indicates that a slow *cis*/*trans* isomerization does not occur in the binding process. Losing the β-turn conformation dominating the inactive CsA structure in the free form, Cs**11** or Cs**13** could adopt conformations similar to that of active CsA in the Cyp18/CsA complex,

or the structures of both compounds are flexible and in quick equilbrium among various conformations. These resulted were also confirmed by time dependent Cyp18 PPIase inhibition assays (data not shown). Due to the poor solubility of Cs**11** and Cs**13** in aqueous solution, we could not get direct evidence of the structural changes upon Sarcosine[3] α-carbon modification of CsA. However, using the CD and fluorescence spectra, we were able to demonstrate that Cs**11** and Cs**13** lose some structural property, which restrict CsA's conformation in its inactive from.
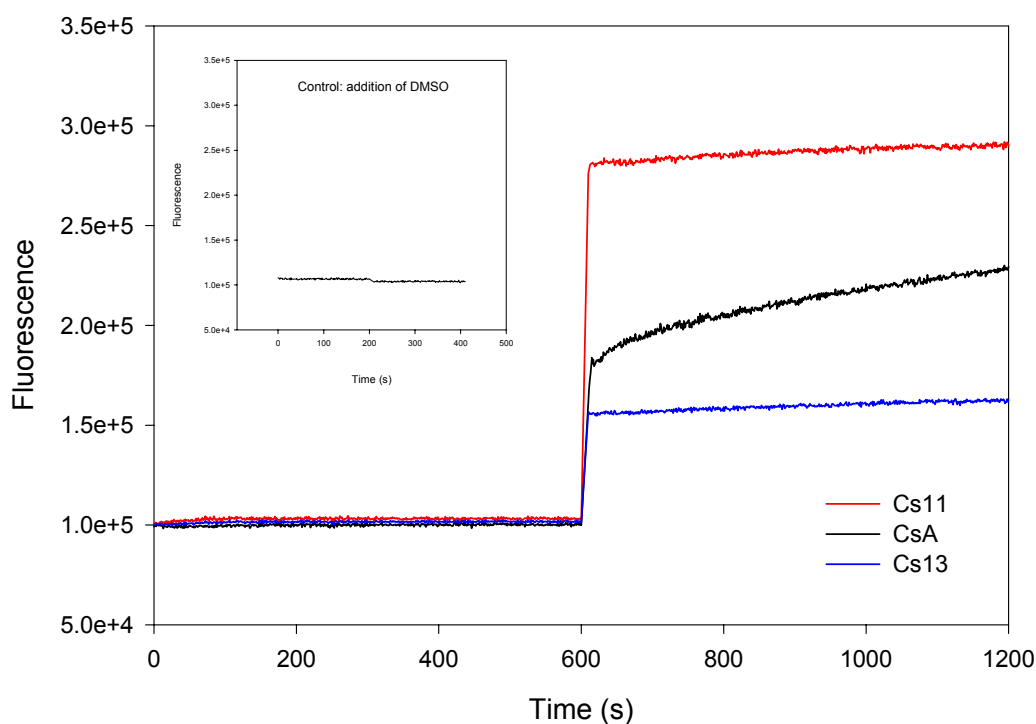
Figure 2.7 Time courses of fluorescence at 339 nm of Cyp18 after addition of various cyclosporin derivatives The measurement was performed at 5°C in 35mM Hepes buffer pH 7.8. The concentration of Cyp18 is 8mM. The cyclosporin derivatives were added to a final concentration of 8.8 mM. Excitation wavelength was 280 nm with a spectral bandwidth of 3nm. Emission was detected at 339 nm with a spectral bandwidth of 3 nm.

2.6 Inhibitory activity of [α-substituted Sar]^3 CsA

To test our proposal of direct CaN inhibition by [α-substituted Sar]^3 cyclosporins, we measure their PPIase inhibitory activities and CaN inhibition in the presence or absence of rh Cyp18.

2.6.1 Cyp18 PPIase activity inhibition.

Sar^3 derivatives (Table **2.1**) showed different Cyp18 inhibitory activities. Losing the turn-like structure partially, [(R)α-Methyl sarcosine]^3 CsA (Cs**11**) still exhibit high Cyp18 inhibitory activity. This indicates that the turn, which is stabilized by the highly conserved sarcosine residue on

position 3, is not essential for the recognition of the ligand by its receptor protein. [(R)α-methylthio sarcosine][3] CsA (Cs**13**), [Me-Asp(t-Bu)][3] CsA (Cs**33**) also gave similar Cyp18 PPIase inhibitory activity. The diastereoisomers with a new substituent in *S* position (type *S*) showed higher IC$_{50}$ value to Cyp18 than the diastereoisomers with a new substituent in *R* position (type *R*). Cs**12**, Cs**14** display 2 – 20 fold lower inhibitory activity than their *R* type diastereoisomers. This can be partially explained by the NMR spectra of the two series of diastereoisomers, which indicated that type *S* derivatives causes more dramatic structural disruptions than type *R* derivatives (Seebach, et al., 1993).

Table 2.1 Inhibition of hr Cyp18 PPIase activity by some of [α-substituted Sar][3] cyclosporins. The PPIase activities were measured in Hepes buffer, pH 7.8 at 7°C, Suc-Ala-Phe-Pro-Phe-NH-Np as substrate with the protease coupled assay.

Label	Name of the derivatives	IC$_{50}$ value of hr Cyp18 inhibition[*]
CsA		3.7 nM
CsH	[D-MeVal][11] CsA	11.0 μM
Cs**11**	[(R)α-methyl sarcosine][3] CsA	4.0 nM
Cs**12**	[(S)α-methyl sarcosine][3] CsA	8.0 nM
Cs**13**	[(R)α-methylthio sarcosine][3] CsA	3.8 nM
Cs**14**	[(S)α-methylthio sarcosine][3] CsA	80 nM
Cs**15**	[(R)α-methyl sarcosine][3] CsH	600.0 nM
Cs**16**	[(S)α-methyl sarcosine][3] CsH	No inhibition at 1μM
Cs**29**	[β-Oxo MeBmt][1] [(R)α-methyl sarcosine][3] CsA	No inhibition at 1μM
Cs**30**	[Acetyl MeBmt][1] [(R)α-methyl sarcosine][3] CsA	No inhibition at 1μM

* The errors of these IC$_{50}$ value are less the +/- 20%.

** Alternative naming of α-Methyl sarcosine is N-methyl alanine. We used this name in this work for emphasizing the α-substitution.

*** The syntheses of Cs**11**, Cs**12**, Cs**13**, and Cs**14** have been reported by Seebach, et al.(1993).

2.6.2 Indirect CaN phosphatase activity inhibition.

Cs**11**, Cs**12**, Cs**13,** Cs**14** and Cs**33** inhibit CaN less efficient than CsA in the presence of Cyp18 (Figure **2.6**). The inhibitory activity of Cs**12** is much less than its diastereoisomer Cs**11**. Cs**13** gave an IC$_{50}$ value of 1.5μM. Its diastereoisomer Cs**14** does not inhibit CaN up to 10 μM concentration. These results are not unexpected because the residue 3 is involved in the interaction to CaN in the crystal structure of Cyp/CsA/CaN complex (Zhao, et al., 2000). On the other hand, the changes in the overall conformation of the molecule might also contribute to the loss of activity.

2.6.3 Direct CaN phosphatase activity inhibition

The CaN phosphatase inhibitory activity of both Cs**11** and Cs**13** demonstrated our hypothesis about the structural basic of a conformation-switchable molecule as CsA. The CaN binding domain of CsA is locked in a conformation inactive to CaN due to multiple intramolecular hydrogen bonds to the Cyp binding domain (Figure **2.1**). The sarcosine 3 is important for forming this turn-like structure. Upon Cyp binding, the Cyp binding domain are involved in contacts with the receptor protein. The turn disappears and the CaN binding domain is released from the intramolecular restrictions. The new conformation in this set of residue then can fit well to the CaN binding pocket. Using chemical modification on residue 3 to impair the turn, a similar effect could be achieved. The inhibitory activity of the ligand alone is much lower than the immunosuppressant/immunophilin complex because the contacts between the interfaces of both proteins can enhance the affinity.

Neither their diastereoisomers Cs**12**, Cs**14**, nor other derivatives with larger side chains like **R** and **S** type of [α-ethyl sarcosine]³ CsA (Cs**40** and Cs**41**), the mixture of two diastereoisomers of [Me-Asp]³ CsA (Cs**35**) and [Me-Asp(t-Bu)]³ CsA (Cs**33**) can inhibit CaN without forming a ligand/Cyp complex.

2.7 Design direct CaN inhibitor with low Cyp affinity.

Unfortunately, we can not use these compounds in T cell experiments for distinguishing the Cyp and CaN inhibitory activities of CsA. The abundance of Cyp is very high in the T cells. These direct CaN inhibitors, like its parent drug, would bind and inhibit Cyp in the cells. A CsA derivative which can inhibit CaN directly with low Cyp binding affinity will be greatly appreciated. We tried to achieve this by changing the Cyp binding domain of Cs**11**. Residue 1 and 11 are essential for Cyp binding. We proposed that these changes had low influence on the active conformation of Cs**11**, but could decrease the inhibitory activity to Cyp18 (Scheme **2.2**)

2.7.1 Residue 1 modifications.

The hydroxy group of MeBmt¹ is necessary for the high affinity between CsA and Cyp18. Acetylation or oxidation of the OH group results in almost total loss of Cyp18 inhibitory activity. The hydroxy group of Cs**11** was acetylated by Ac₂O in pyridine at room temperature. However, [acetyl MeBmt]¹ [(R)α-methyl sarcosine]³ CsA (Cs**30**) is neither a Cyp18 inhibitor nor a direct or indirect CaN inhibitor. Oxidation of the hydroxy group by Jones oxidation produced [β-oxo MeBmt]¹ [(R)α-methyl sarcosine]³ CsA (Cs**29**) which gave similar results.

2.7.2 Residue 11 modifications.

The crystal structure of the Cyp18/CsA complex shows residue 11 directly contacting Cyp18, binding in a deep hydrophobic pocket in the active site of Cyp18. Changes at this position were expected to reduce the binding of cyclosporin to Cyp significantly. CsH is a natural cyclosporin with a D-MeVal at position 11 and is more than 1000-fold less active than CsA, presumable through
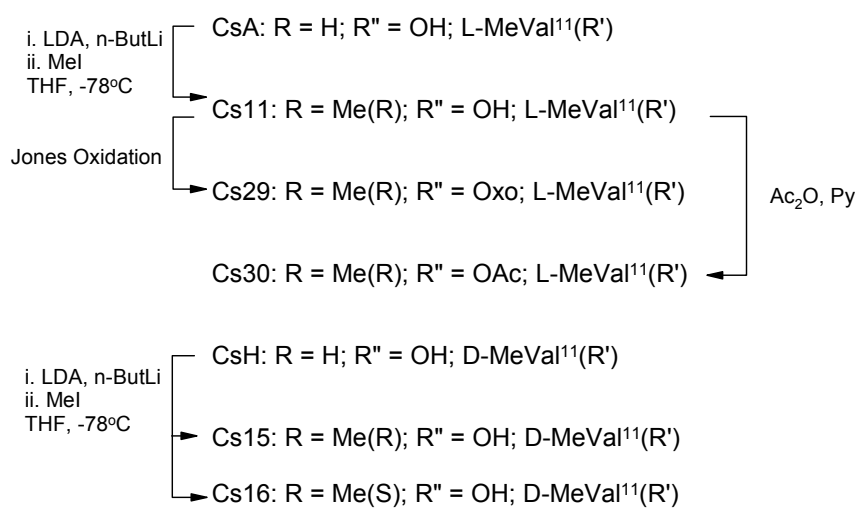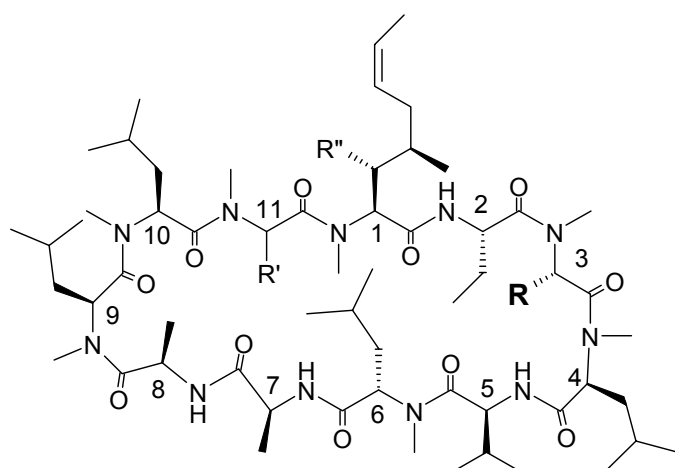
Scheme 2.2 Design and synthesis of CaN inhibitor with deceased the Cyp18 affinity basing on the structure of direct CaN inhibitor Cs**11**.

steric interaction between the side chain and the receptor. Unfortunately, [α-methyl Sarcosine][3] CsH (Cs**15**) does not act as a direct inhibitor to CaN. Losing intramolecular restrictions and turn-like structure by Sar[3] α-carbon substitution, Cs**11** and Cs**13** could adopt a more flexible structure. Modifications on other amino acids in the direct CaN inhibiting molecule result in loss of its affinity to CaN binding pocket.

It was reported that a rationally modified CsA, the [α-cyclopentylsarcosine][11] cyclosporin, does not bind to wild type Cyp18. Compensatory mutations in the CsA-binding pocket of Cyp18 (F113G, C115M, S99T) can recover the ligand binding ability of the protein (Belshaw, et al., 1997). In our case, it came out as a surprise that one diastereoisomers of [α-Methyl sarcosine][3] CsH (Cs**15**) gives an IC_{50} value of 600 nM. Structural disruption by sarcosine substitution may cause some changes in the side chain orientation, and in turn recovers the interaction between the protein and the ligand.

A comparison of the ratios of $IC_{50Cyp18}/IC_{50CaN}$ for CsA and its derivatives could reflect the diverse binding affinities between the immunophilin/immunosuppressant binary complexes and CaN. The CaN inhibitory activities for Cs**11**, Cs**12**, Cs**13**, Cs**14**, Cs**16**, Cs**33** can be correlated to their Cyp18 binding affinities approximately. However, [α-Methyl sarcosine][3] CsH (Cs**15**) is an exception. The IC_{50CaN} value of Cs**15** is only 3.5 folds lower than CsA and its $IC_{50Cyp18}/IC_{50CaN}$ ratio is much higher than CsA and other analogues (Figure **2.6**). Another CsA derivative [MeBm$_2$t][1] CsA has been reported to have alike property. Although [MeBm$_2$t][1] CsA has weaker affinity for Cyp18 (K_i 540nM) than CsA, [MeBm$_2$t][1] CsA/Cyp18 complex inhibits CaN phosphatase activity with a K_i of 67nM (Nelson, et al., 1993). One explanation for these observations could be that the binding of CaN to Cs**15**/Cyp18 complex could stabilize the interaction between Cs**15** and Cyp18. CsH is not a Cyp18 inhibitor, and in turn cannot inhibit CaN in the presence of Cyp18. The turn-like structure as well as the intramolecular restrictions in CsH could be impaired by replacing the Sar[3] by an α-substituted amino acid. Changes of the dynamics and conformations of the backbone in the cyclic peptide may cause the reorientation of side chains. Without changing the side chains responsible for Cyp18 or CaN interaction, Sar[3] modification in CsH can enhance its PPIase inhibition and the binary ligand/Cyp18 complex is more active than CsA/Cyp18.

Calcineurin phosphatase activity. From the crystal structure of the Cyp/CsA/CaN ternary complex, it seems that, in the absence of Cyp, the size of CsA molecule is not large enough to mask the active site and to prevent large phosphorylated substrates from accessing. Binding to the distal site could induce a conformational switch in the active site. However, in both Cyp/CsA/CaN and FKBP/FK506/CaN complexes, no conformational change has been found. Furthermore, the phosphatase activity of CaN to small substrate like nitrophenylphosphate can be stimulated upon FKBP/FK506 or Cyp/CsA binding. The binding of Cs**11** to CaN may effect the phosphatase activity in a more subtle manner. The narrow substrate specificity is one of the unique characteristics of CaN (Donella-Deana, et al., 1994). While Ser/Thr phosphatases of other families are also very

active to short peptide substrates, an extended N-terminal stretch appears to be required for an optimal dephosphorylation by CaN. Basic residues on the N-terminal of the substrate is a positive determinant for high activity. Besides the active site, a second binding site of CaN to peptide is required for optimal activity. CsA could block the second bind site, and in turn impair the phosphatase activity.

2.8 Conclusion.

In solvents of high polarity, changes in the CsA backbone conformation are directly visible in the NMR spectra, and many conformations are in equilibrium, interconverting slowly on the NMR time scale (Kessler, 1990) (e.g. in DMSO, at least seven conformations can be observed). This was also demonstrated by kinetic analysis of reversed-phase liquid chromatography (HPLC) of CsA. None of these conformations can fit the CaN binding pocket which has high affinity to CsA/Cyp18 complex. Cyp18 alone also has no detectable inhibitory activity to CaN (R. Baumgrass, et al., unpublished results). We presumed that the turn-like structure of CsA found in the crystal structure and in CDCl$_3$ solution also presents a common feature of the equilibrium conformations in an aqueous environment, as observed in the CD spectra in water/DMSO (90/10) solution. Turn disruption by chemical modifications can mimic a crucial event in the active conformation formation during the Cyp18/CsA binding process, generating a new structure sharing similarity with the CsA in the binary complex. Now, the CaN binding domain of Cs**11** and Cs**13** displays affinity to the binding pocket of CaN and exhibits moderate inhibitory activity to CaN in the absence of Cyp18.

However, losing β-turn is not sufficient for forming an active conformation. ***S*** position substitution as Cs**12** and Cs**14** undergoes much more dramatic structural disruption (Seebach, et al., 1993) and results in reduced Cyp affinity. These compounds are not direct CaN inhibitors. A larger substituent in either ***R*** or ***S*** (Cs**33**, Cs**35**, Cs**40**, and Cs**41**) position also does not result in a direct CaN inhibitor. Changes on other residues in Cs**11** impair the activity to CaN. We failed to generate a direct CaN inhibitor with reduced Cyp affinity through changing the residues in Cyp binding domain on Cs**11**. However, in the case of Cs**15**, that is a weak Cyp18 inhibitor and is nonimmunosuppressive, sarcosine[3] modification can switch the ligand's affinity to Cyp as well as the CaN inhibitory activity of the ligand-Cyp complex remarkably.

Chapter 3. Design of novel nonimmunosuppressive and Cyp18 inhibiting CsA derivatives.

3.1 Two concourses of Cyp18/CsA/CaN ternary complex.

To alter the ligand/protein or protein/protein affinity, the residues involved in the contacts are frequently the targets for chemical modifications and/or genetic mutations (Schreiber, 1998). This strategy was also applied in the study of CsA (Traber, et al., 1987, Etzkorn, et al., 1994, Gothel, et al., 1996). To impair its Cyp18 affinity, residue 9, 10, 11, 1 and 2 (so called Cyp binding domain) were reasonable candidates for substitutions. Changing residue 4 or 6 (in the so called CaN binding domain or effector domain comprising residue 3-7) resulted in nonimmunosuppressive Cyp18 inhibitors with low CaN inhibitory activity. The Cyp18/CsA/CaN ternary complex X-ray crystal structure (Zhao, et al., 2000) confirms this structure-activity relationship. Due to the special feature of the protein/ligand/protein ternary complex, two additional amino acids in the cyclic peptide stand out and distinguish themselves from other residues, the Sar[3] and D-Ala[8] (Figure **3.1**). They are located in the interface of the two proteins and in the concourses of the ternary structure. But they are not considered being as critical as some other residues (e.g. MeBmt[1] in the Cyp18 binding domain or MeLeu[6] in the CaN binding domain) for ligand-protein interactions.

In the former chapter, we have showed that Sar[3] is responsible for forming a β-turn, which is decisive for CsA to adopt an inactive conformation for CaN binding. Disruption of the β-turn by either Cyp18 binding or chemical modifications confers the immunosuppressive drug CaN inhibitory activity. In this chapter, to achieve a facile synthesis of potent Cyp18 inhibitors with low CaN inhibitory activity, we investigated the possibility to impair the ternary complex formation by introducing a side chain on the second concourse – the residue 8. It also provides a novel approach to study the interaction between the interfaces of two proteins.

3.2 Biological effects of mono-functional CsA derivatives.

Host factors are required for the life cycle and replication of all retroviruses, including human immunodeficiency virus type 1 (HIV-1). During the reproductive cycle of HIV-1 in the host cell, the retrovirus incorporates Cyp18 into its virion (Luban, et al., 1993 & 1996; Franke, et al., 1994). Cyp18 specifically interacts with a single exposed loop of the Gag polyprotein capsid domain via a network of nine hydrogen bonds, which mainly implicates a 7-mer fragment of the loop. In subsequent steps of the viral maturation, the polyprotein is cleaved into three distinct proteins: matrix protein (MA), capsid protein (CA), and nucleo-capsid protein (NC), which remain stacked together. About 250 Cyp18 molecules are packaged into each virion in a ratio of 1 Cyp18 to 10 CA. Though Cyp18 is incorporated into HIV-1 virions it is not required for virion assembly per se: virions rendered Cyp18-deficient by gag mutation, or by production in the presence of CsA, are produced at normal levels and are otherwise indistinguishable from wild-type virions by standard

biochemical criteria (Braaten et al., 1996b; Franke et al., 1994 ; Thali et al., 1994). Although the exact function of Cyp18 in the HIV-1 viral cycle is still controversial, nonetheless, disruption of Cyp18 incorporation causes a quantitative reduction in virion infectivity, with the block occuring early in the virus life cycle, after membrane fusion, but prior to the initiation of reverse transcription. Target cell Cyp18 is not required for these early events and cannot rescue Cyp18-deficient virions (Luban, 1996).
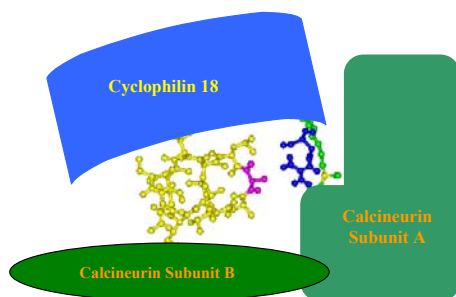
Figure 3.1 A concourse in the Cyp18/CsA/CaN ternary complex. CsA is in yellow and the residue 8 is in pink. Two subunits of CaN are in green. Cyp18 is in blue. The Arg148 of Cyp18 and Arg122 of CNA is shown as ball and stick.

Numerous anti-HIV drugs have been designed to target viral enzymes. However error-prone reverse transcription and high rates of retroviral recombination as well as the decreasing adhesion of many patients to long-term treatments have led to the emergence of viruses resistant to combined therapies(Hecht, et al., 1998; Hirsch, et al., 1998). Consequently, the definition of novel pharmacological targets is critical. Host proteins implicated in the viral replication cycle, such as hCyp18, are not prone to genetic instability and offer new potential therapeutic applications.

Due to the high cellular concentration of Cyp, for inhibiting Cyp by CsA, the amount of agent must be much higher than that used in immunosuppressive treatment. However, the high dosage will result in toxicity because of complete CaN inhibition. Nonimmunosuppressive CsA derivatives such as [N-Methyl Ile][4] CsA (SDZ NIM 811) are interesting inhibitors for Cyp18 incorporation (Mlynar, et al., 1997; Dorfman, et al., 1996, Billich, et al., 1995; Steinkasserer, et al., 1995). However, total synthesis of series of cyclic undecapetides is generally expensive and time-consuming. Although some CsA derivative has been synthesized by solid phase synthesis, the solid phase synthesis of CsA has not been achieved (Li, et al., 2000; Angell, et al., 1994). It will be ideal to get such drugs by facile synthetic approach from abundant resource. [D-serine][8] CsA is a cyclosporin derivative, which can be obtained in high amount by fermentation. However, this position does not play an essential role in either Cyp or CaN binding (Zhao, et al., 2000). A new strategy should be considered to switch it to an important component in the ternary complex. In this study, we investigated the influence of different functionalities as well as various long side chains at this position on its biological activities.

3.3 Acidic, basic and hydrophobic functional group on residue 8 – syntheses and activities.

Because the D-serine[8] is more reactive than the hydroxy group in residue 1(Eberle, et al., 1995), all the chemical reactions were performed without protecting the OH on MeBmt[1]. The chemical modifications are summerized in figure **3.2**.

3.3.1 Acidic functional groups on residue 8.

First we investigated the influence of acidic function groups. We phosphorylated the primary hydroxy group of D-serine using global phosphorylation (phosphorylation methodology will be discussed in more detail in chapter 5) (Perich, 1991) approach with diallyl N,N-diisopropylphosphoramidite. Allyl group was deemed most desirable since diallyl phosphate esters or diallyl phosphite esters can be removed under very mild condition with palladium catalysis. Oxidation of phosphite ester intermediate in the presence of aqueous *tert*-butyl hydroperoxide yielded diallyl phosphate ester. After deprotection with *tetrakis*(triphenylphosphine)palladium(0) in the presence of formic acid and amine, [O-phosphoryl-D-serine][8] CsA (Cs**3**) was obtained in high yield. Deprotection of the phosphite ester intermediate with palladium catalysis led to [H-phosphoryl-D-serine][8] CsA (Cs**4**). The phosphite triester is much less stable than its deprotected form (Cs**4**). It can be oxidized readily in air. On the contrary, Cs**4** is stable to air exposure (Hoffmann, et al., 1996). After phosphorylation with dimethyl N, N-diisopropylphosphoramidite and oxidation with the same method, [O-dimethyl-phosphoryl-D-serine][8] CsA (Cs**5**) was prepared in high yield. Phosphorylation by one equiv. di-benzyl N,N-diisopropylphosphoramidite, oxidation with *tert*-butyl hydroperoxide and deprotection in TFA also produced Cs**3** in moderate yield (50%). Due to the steric hindrance, di-*tert*-butyl N,N-diisopropylphosphoramidite does not react with the hydroxyl group. [O-(carboxymethyl)-D-serine][8] CsA (Cs**6**) was prepared according to the reported method (Eberle, et al., 1995).

CsA and its four derivatives (Cs**3**, Cs**4**, Cs**5** and Cs**6**) (Figure **3.3**) bind to Cyp18 with a similar affinity (increase or decrease of IC_{50} value less than 4-fold). Negative charge at this position does not influence the inhibition of PPIase activity significantly. It is not unexpected because Cs**6** was reported to be as immunosuppressive as CsA (Eberle, et al., 1995). Then we measured their inhibitory activities to CaN (Figure **3.4**). Phosphite Cs**4**, dimethyl-phosphate Cs**5**, carboxylic acid Cs**6** and CsA show relatively similar inhibitory activity. However, the phosphate Cs**3** inhibits CaN phosphatase activity much less than CsA (50-fold decrease). Phosphorylation and dephosphorylation of proteins play special roles in signal transduction, the cell cycle, transcriptional control and carbonogenesis. Phosphorylation can induce local comformational changes, as well as influence protein-protein or protein/ligand interactions. In our case, among these three derivatives with acidic functional groups, carboxylic acid, phosphate and phosphite, only phosphate Cs**3** shows a dramatic impairment of CaN phosphatase inhibitory activity. Compared with H-phosphopeptides,

Cs8: R = NH$_2$CH$_2$CH$_2$-O-CH$_2$CH$_2$-O-

Cs7: R = Br(CH$_2$)$_4$O-

Cs5: R = OP(O)(Me)$_2$

ZnCl$_2$/Et$_2$-O

Br(CH$_2$)$_4$Br
30% NaOH

a. 1-*H* Tetrazole
iPr$_2$NP(OMe)$_2$
b. t-BuOOH, -5°C

R = BocNHCH$_2$CH$_2$-O-CH$_2$CH$_2$-O-

BocNHCH$_2$CH$_2$-O-CH$_2$CH$_2$Br
30%, NaOH, PTC

PTC

R = OCH$_2$COOBu-t

BrCH$_2$COOBu-t
30% NaOH

KOH, MeOH

Cs60:
R = OMe

Cs6: R = OCH$_2$COOH

[D-Serine]^8 CsA, R = OH

PyBOP, DIPEA
BocNH(CH$_2$)$_5$NH$_2$

Cs10: R = OCH$_2$CONH(CH$_2$)$_5$NHBoc

1-*H* Tatrazole
iPr$_2$NP(OAll)$_2$

ZnCl$_2$/Et$_2$-O

Pd[PPh$_3$]$_4$
n-Bu-NH$_2$, HCOOH

Cs9: R = OCH$_2$CONH(CH$_2$)$_5$NH$_2$

R = O-P(All)$_2$ ⟶ **Cs4**: R = OP(OH)$_2$

NH$_2$(CH$_2$)$_5$COOMe
PyBOP, DIPEA

AC$_2$O, Py

t-BuOOH, -5°C

C42: R = OCH$_2$CONH(CH$_2$)$_5$NHAc

R = OP(O)(All)$_2$

Pd[PPh$_3$]$_4$
n-Bu-NH$_2$, HCOOH

R = OCH$_2$CONH(CH$_2$)$_5$COOMe

Cs3: R = OP(O)(OH)$_2$

1M KOH

Cs44: R = OCH$_2$CONH(CH$_2$)$_5$COOH

Cs24: R = OCH$_2$CONH(CH$_2$)$_5$NHBiotin

Figure 3.2 Derivation of [D-Ser]^8 CsA. The reaction conditions will be described in details in the experimental chapter. Position 8 modification was also used in other studies. The syntheses are described elsewhere. For the parent drug CsA, R = H.
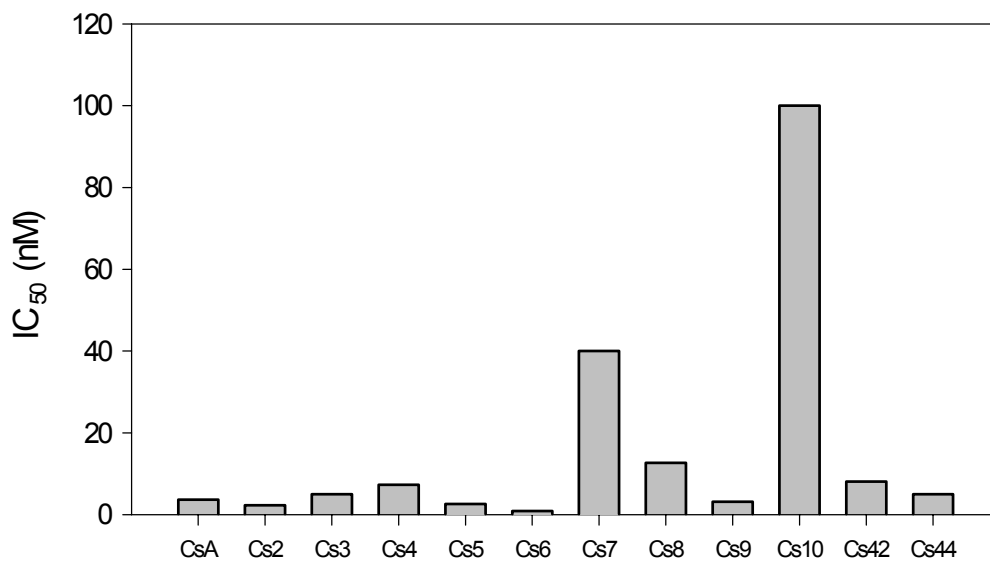
Figure 3.3 Inhibition of rh Cyp18 PPIase activities by various [O-substituted D-Ser][8] cyclosporins. PPIase activities of Cyp18 were measured with protease coupled assay and Suc-Ala-Phe-Pro-Phe-NH-Np as substrate in Hepes buffer, pH7.8, at 7°C, in the presence of 2.5nM Cyp18 and inhibitors of desired concentrations.
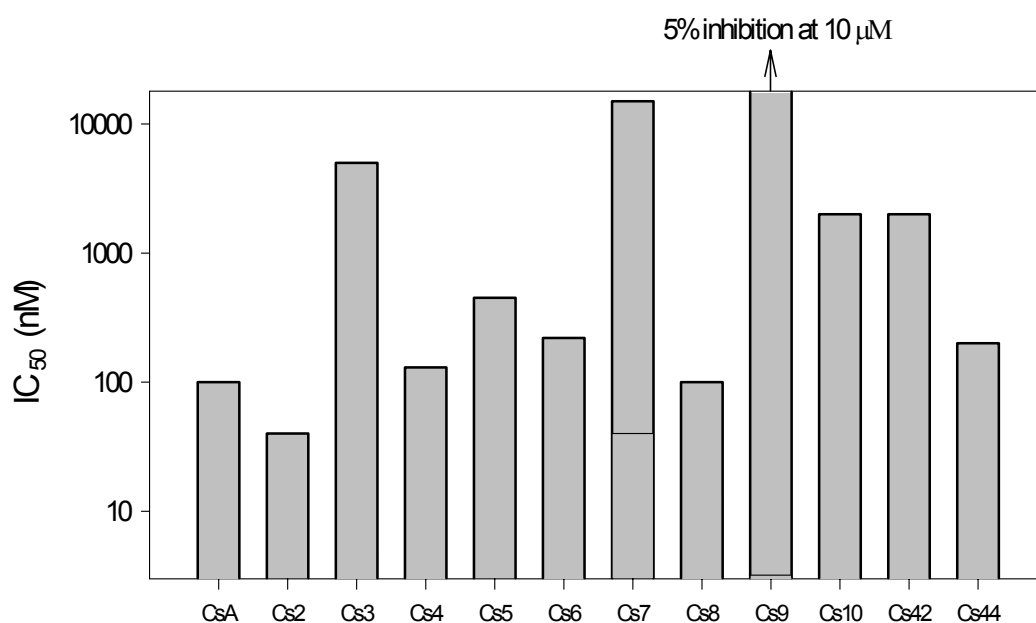
Figure 3.4 Inhibition of calcineurin phosphatase activities by various Cyp18/[O-substituted D-Ser][8] cyclosporin complexes. The calcineurin phosphatase activities were measured using scintillation assay and biotinylated RII peptide as substrate at 30°C, in the presence of 1.32nM calcineurin, 10μM inhibitor and desired Cyp18 concentration.
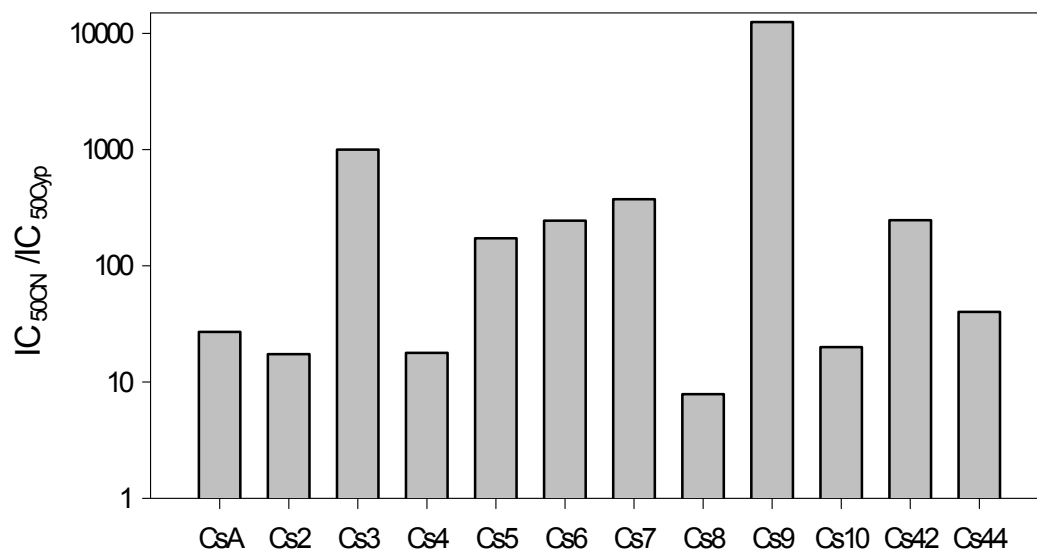
Figure 3.5 Ratios of CN inhibitions by various Cyp18/[O-substituted D-Ser]^8 cyclosporin complexes to Cyp18 inhibition by /[O-substituted D-Ser]^8 cyclosporin.

Table 3.1 IC$_{50}$ of Cyp18 inhibition by various [O-substituted D-Ser]^8 cyclosporins and CaN inhibition by various Cyp18//[O-substituted D-Ser]^8 cyclosporin complexes.

	Inhibitors	IC$_{50cyp}$ [a]	IC$_{50CaN}$ [b]
	Cyclosporin A	3.7	100
	[D-Serine]^8 CsA	2.3	40
Cs3	[Phosphoryl-D-serine]^8 CsA	5.0	5000
Cs4	[H-phosphoryl-D-serine]^8 CsA	7.3	130
Cs5	[Dimethyl phosphoryl-D-serine]^8 CsA	2.6	450
Cs6	[O-Carboxymethyl D-serine]^8 CsA	0.9	220
Cs7	[O-4-bromo-n-butyl D-serine]^8 CsA	40	15000
Cs8	[O-2-aminoethoxy-ethyl D-serine]^8 CsA	12.7	100
Cs9	[O-(NH$_2$(CH$_2$)$_5$NHC(O)CH$_2$-) D-serine]^8 CsA	3.2	Low inhibition [c]
Cs10	[O-(BocNH(CH$_2$)$_5$NHC(O)CH$_2$-) D-serine]^8 CsA	100.0	2000
Cs42	[O-(AcNH(CH$_2$)$_5$NHC(O)CH$_2$-) D-serine]^8 CsA	8.1	2000
Cs44	[O-(HOOC(CH$_2$)$_5$NHC(O)CH$_2$-) D-serine]^8 CsA	5.0	200

a. The IC$_{50}$ (nM) of Cyp18 inhibition by various [O-substituted D-Ser]^8 cyclosporins;

b. The IC$_{50}$ (nM) of CaN inhibition by Cyp18//[O-substituted D-Ser]^8 cyclosporin complexes.

c. 5% inhibition at 10μM Cs**9**.

phosphopeptides are more hydrophilic and with more negative charge. A little difference of the ionic state between phosphite Cs**4** and phosphate Cs**3** leads to a nearly 40-fold decrease of the binding affinity to CaN. Although this modification does not likely occur naturally, it provides a novel example of the subtle effect attributed to phosphorylation.

3.3.2 Basic function groups on residue 8.

Then, we investigated the influence of basic groups. Hydroxyl of D-Ser was etherified by BocNHCH$_2$CH$_2$OCH$_2$CH$_2$Br under phase transfer-catalyzed (PTC) reaction condition (Eberle, et al., 1995). After deprotection by Lewis acid ZnCl$_2$-Et$_2$O in ether (Frank, et al., 1996; Wildemann, et al., 1999), [O-(aminoethoxy-ethyl)-D-serine]^8 CsA (Cs**8**) was obtained in 50% yield. Using PyBop activation, [O-(BocNH(CH$_2$)$_5$-NHC(O)-CH$_2$)-D-serine]^8 CsA (Cs**10**) was synthesized in nearly quantitative yield. After deprotection by ZnCl$_2$-Et$_2$O complex in ether, [O-(NH$_2$(CH$_2$)$_5$NHC(O)CH$_2$)-D-serine]^8 CsA (Cs**9**) was obtained in 70% yield. CsA and the two derivatives with free amino group Cs**8** and Cs**9** give very similar PPIase inhibitory activity (Figure **3.3**). Like those acidic functional groups, amino group at residue 8 has little influence on their Cyp18 inhibition. Then we measured their CaN inhibitory activity (Figure **3.4**). Cs**8** inhibits CaN as well as CsA. Surprisingly, Cs**9** shows less than 5% CaN inhibitory activity at a drug concentration as high as 10 µM.
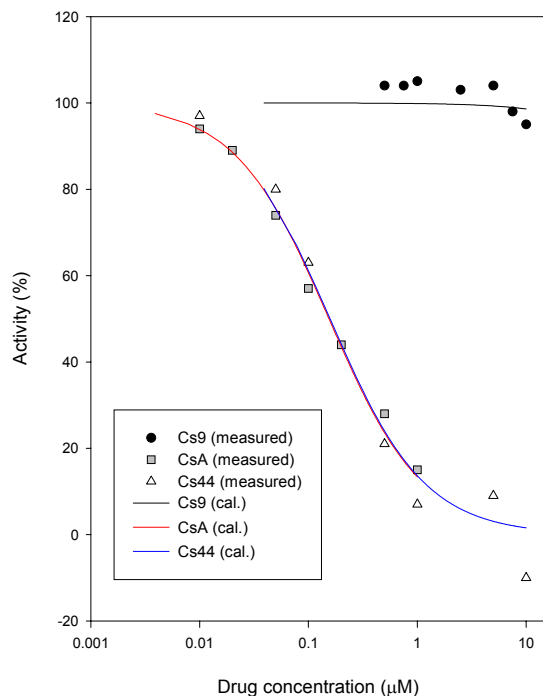
Figure 3.6 Inhibition of CN phosphatase activity by CsA, Cs**9** and Cs**44** in the presence of Cyp18. The calcineurin phosphatase activities were measured using scintillation assay and biotinylated RII peptide as substrate at 30°C, in the presence of 1.32nM calcineurin, 10µM inhibitor and desired Cyp18 concentration.

It was reported that mutation of three cationic surface residues of human cyclophilin A, R69, K125 and R148, to either anionic or neutral residues, does not change its PPIase activity, but alter its CaN inhibitory activity (Etzkorn, et al., 1994). R69E was 13-fold less effective than wild-type Cyp18,

while R148E was 17-fold more effective, and K125Q impaired the inhibitory ability by 6.3-fold. These cationic surface residues are important for the interaction between cyclophilin and CaN. Whereas R69 and K125 could stabilize the complex, the positive charge of R148 impairs the affinity. There should be a cationic region on the surface of CaN which repulse R148 preventing optimal protein-protein interaction. The dramatic effect of Cs**9** on CaN inhibition gives further evidence for this hypothesis. The difference between the fully extended length of the side chain of Cs**8** (8.39Å) and Cs**9** (12.29Å) is 3.9 Å. Observing the crystal structure of CsA/Cyp18 complex (Figure **3.7**), we found that the side chain of Cs**9**, but not that of Cs**8**, can approach R148 guanidino of Cyp18 in a van der Waals distance. Due to the normal PPIase inhibitory activity of Cs**8**, Cs**9**, it's reasonable to propose that a cationic side chain does not change the molecular conformation. CsA is a hydrophobic cyclopeptide, a hydrophilic side chain prefers to extending outwards into aqueous environment. The amino group at the tip of a 12.29Å side chain inserts into the region and strengthen the repulsion between R148 and CaN, and causes a dramatic decrease of affinity between Cyp18 and CaN.

Our hypothesis was proved by the recently resolved Cyp18/CsA/CaN ternary crystal structure (Zhao, et al., 2000). The short distance between Arg148 of Cyp18 and Arg122 of CaN indicates a contact between the two cationic surfaces of both proteins. To decrease the energetic expense of this contact, the side chains of both residues reorientate and form a van der Waals interaction. The enhanced energy is compensated by other interactions in the ternary complex. R148E mutation resulted in a more stable complex. Inserting a third cationic residue into this region by D-Ser[8] modification enhance the repulsion force.
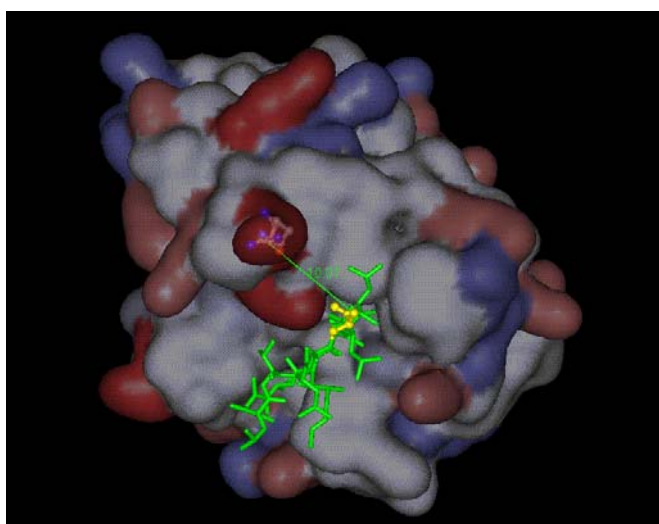
Figure 3.7 An unfavorite contact between Cyp18 and CaN interface. In the Cyp18/CsA complex, CsA is in green, its residue 8 is in yellow. The β-carbon of the D-Ala[8] is 10.07Å from the terminal carbon of Arg148 which is responsible for a basic surface of Cyp18. It contacts with a basic surface on CNA (Arg122, not shown) through hydrophobic interaction.

To exclude the possibility that other functionality of Cs**9** causes the remarkable impairment of CaN inhibition, we tested Cyp18 inhibitory activity and CaN inhibitory activity in the presence of Cyp18 of Cs**42** and Cs**44** (Figure **3.6**). Using PyBop activation, [O-(MeOOC(CH$_2$)$_5$NHC(O)-CH$_2$)-D-

serine]^8 CsA was synthesized in nearly quantitative yield. Saponification with 1M NaOH produced [O-(HOOC(CH$_2$)$_5$-NHC(O)-CH$_2$)-D-serine]^8 CsA (Cs**44**) in high yield. Cs**9** is acetylated with 1 equiv. Ac$_2$O in pyridine. [AcNH(CH$_2$)$_5$-NHC(O)-CH$_2$)-D-serine]^8 CsA was synthesize in nearly quantitative yield. Both Cs**42** and Cs**44** displayed normal Cyp18 inhibitory activities and CaN inhibition in the presence of Cyp18. Neither neural nor acidic functional groups affect the Cyp18-CaN interaction.

SDZ IMM125, (Baumann, et al., 1992; Donatsch, et al., 1992) with an addition of an ethanol unit onto the Ser^8 hydroxyl group, possesses a similar immunosuppressive activity as CsA. Two related analogs, [D-Dap]^8 CsA and [D-Dab]^8 CsA (Nelson, et al., 1993), also retained significant activity, albeit with a 10-20 fold decrease. A photo-affinity probe of CsA (PL-CS) (Ryffel, et al., 1992) prepared by attachment of a photo-affinity probe to the hydroxy group of serine 8, is active as evidenced by its photo-dependent irreversible inhibition of T cell activation. Together with most of our CsA position 8 derivatives, these results indicated that this position is not as important as other residues in the ligand-protein interactions. In the Cyp18/CsA/CaN crystal structure, authors gave structural evidence that among the different positions on CsA, the side chain of D-Ala^8 is most tolerable to modifications without significant loss of immunosuppressive activity. However, the interaction between the surface residues in both protein in the crystal structure also prove our hypothesis how Cs**9** inhibits Cyp18 without exhibiting CaN inhibitory effects in the complex form.

3.3.3 Hydrophobic function groups on residue 8.

When the amino group of Cs**9** is protected by Boc, compared with Cs**9**, the IC$_{50}$ value of Cyp18 inhibition by Cs**10** decreases significantly (27 folds) (Figure **3.3**). CsA is a neutral cyclic undecapeptide containing only lipophilic amino acids, seven of which are N-methylated. It is a very hydrophobic molecule. In aqueous environment, the bulky hydrophobic *tert*-butyl group of Boc may fold back to the Cyp18 binding domain of the cyclopeptide and prevents its binding to Cyp18. To confirm this assumption, we measured [O-(4-bromobutyl)-D-serine]^8-cyclosporin Cs**7** which was synthesized under PTC reaction condition. It gave more than 10-fold lower PPIase inhibitory activity. Then we measured their CaN inhibitory activity (Figure **3.4**). The 20 fold decrease of IC$_{50}$ values to CaN of Cs**10** can be correlated to its 25 fold decreased Cyp18 inhibition. The side chain has not much influence on the CaN binding affinity. On the contrary, the decrease of CaN inhibition by Cs**7** is much more significant than that of Cyp18 inhibition. It seems that the side chain can affect both the Cyp18 binding domain and the CaN binding domain.

The correlation of Cyp18 and CaN inhibitory activities of these position 8 is shown in figure **3.5**. They could partially reflect the capability of CaN inhibition by the ligand/protein binary complex. For obtaining a potent Cyp inhibitor with minor CaN inhibitory activity, Cs**9** and Cs**3** are good candidate for anti-HIV-1 infection study.

Cyp/CsA/CaN and FKBP/FK506/CaN complexes are not the unique models of protein/ligand/protein ternary structures. For example, TCRs (T cell receptors) recognize processed antigens, such as peptides, but only when bound to an MHC molecule. The formation of a complex between a TCR and a peptide-MHC ligand (pMHC) represents the molecular solution to the recognition of an antigen in the cellular immune response (Hennecke, et al., 2001; Garcia, et al., 1999). A number of general conclusions about TCR structure and its recognition of antigen can already be derived from the relatively few TCR structures that have been determined. However, a lot of puzzles still remain. Our novel strategy, by inserting a side chain from the peptide into the protein/protein interface, would provide additional information for understanding the molecular basis of such ternary complex structure.

3.4 Conclusion.

We investigated the effect of cationic, anionic and neutral hydrophobic side chains at residue 8 of CsA (synthesized from [D-serine]^8 cyclosporin) on their Cyp18 inhibitory activities and CaN inhibition in their complexes with Cyp18. Besides those proofs from genetic Cyp18 variants (Etzkorn, et al., 1994), we gave another mean to investigate the protein-protein interaction. Insertion of an amino group into a defined region of the protein-protein interface can decrease the affinity between Cyp18 and CaN dramatically. Cyclophilin is not able to distinguish a phosphate or a phosphite moiety at position 8, however, the small differences between phosphate Cs**3** and phosphite Cs**4** leads to a nearly 40-fold change in CaN inhibition by the Cyp/ligand complex. Furthermore, a hydrophobic side chain could fold back to the hydrophobic cyclopeptide in water and impair its Cyp18 inhibitory activity. Cs**3** and Cs**9** are mono-functional non-immunosuppressive Cyp inhibitors. Their inhibitory activities to Cyp18 are similar to their parent drug CsA. The syntheses of both compounds are facile and with high yields. They could be potential anti-HIV drug candidates for preventing Cyp from incorperation into the virions and reducing the infectivity.

Chapter 4. Reversible Switch of Cyclosporin Conformation.

4.1 Physical and Chemical Properties of Thioxo amide.

Peptide backbone modifications have received much attention in recent years. The thioxo amide replacement Ψ[CSNH] (Table **1**), in which the amide O-atom has been substituted by an S-atom, constitutes one example of seemingly minimal and subtle backbone modification. Their novel physical and chemical properties may lead to surprising conformational change for polypeptides and provide us a useful tool in studying peptide and protein structure-function relationship (Kessler, et al., 1992, Seebach, et al., 1991). On the other hand, backbone modifications may afford analogues possessing an enhanced stability to enzymatic hydrolysis, (Bartlett, et al., 1982; Campbell, et al., 1982; Maziak, et al., 1986; Beattie, et al., 1987; Schutkowski, et al., 1994) as well as a greater affinity and specificity towards biological receptors (Lajoie, et al., 1984; Claussen, et al., 1984; Salvadori, et al., 1984; Majer, et al., 1988; Kruszynski, et al., 1985). Some other backbone modifications has also been investigated, for instance, Oligopeptides consisting exclusively of β-amino acids can adopt a large variety of secondary structures. The main structural elements of proteins (helices, parallel and anti-parallel pleated sheets, turns and tubular stacks) have been identified in the realm of β-peptides (Sifferlen, et al., 1999 and references cited therein).

X = O amide bond
X = S thioamide bond
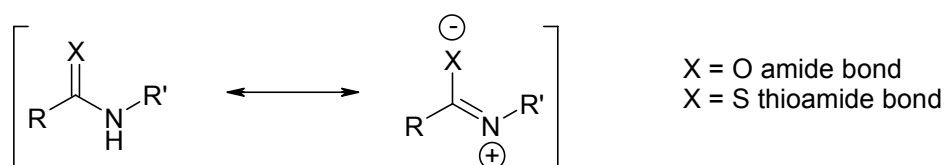
Table 1. Some physical and chemical properties of amide and thioxo amide.

Amide	Thioxo amide
C=O bond length approx. 1.24 Å	C=S bond length approx. 1.65 – 1.68 Å
C=O bond energy approx. 170kcal/mol	C=S bond energy approx. 130kcal/mol
Covalent radius of the O approx. 0.73 Å	Covalent radius of the S approx. 1.02 Å
Van der Waals redius: O < S approx. 0.45 Å	
Electronegativity O > S	
pK$_a$ of NH ca. 17	pK$_a$ of NH ca. 11-13
170-195nm ($\pi \rightarrow \pi^*$ transition) 210-220nm (n $\rightarrow \pi^*$ transition)	210nm (n$_\sigma \rightarrow \pi^*$ transition) 250-280nm ($\pi \rightarrow \pi^*$ transition) 340-400nm (n$_s \rightarrow \pi^*$ transition)
Rotation barrier: amide < thioxo amide by 2-3kcal/mol	

In comparison to the oxygen in an amide, the S-atom is a weaker hydrogen bond acceptor (Abboud et al., 1988 & 1993; Laurence, et al., 1995). On the other hand, the donor ability of the adjacent NH is enhanced due to the increased polarity and acidity (Dudeck, et al., 1967). The length of a

hydrogen bond involving an S-atom shows an increase of about 0.5Å when compared to a hydrogen bond with an O-atom as acceptor. It was shown that a thioxo amide unit in a peptide destabilizes a β-turn, if the S-atom acts as acceptor in an intramolecular hydrogen bond, whereas the donor property of the NH is enhanced (Sherman, 1990). Thioxo amide bonds also showed different population of the *cis* isomer compared to an amide bond (Walter, et al., 1968; Sandström et al., 1967). The rotation barrier around the thioxo amide bond is higher than that around a normal amide bond. This may increase the conformational stability of peptide that is thioxylated in the key position of the backbone. Recently, 'freezing' the dynamics of peptide backbone has been achieved by peptide bond thioxylation (Schutkowski, et al, 1994, 1995, & 1997).

4.2 Photoisomerization of amide & thioxo amide bonds.

The classical view of the amide resonance first discussed by Linus Pauling in 1948 is based on the ability of the nitrogen atom to delocalize its electron lone pair over the whole moiety. The resulting partial double bond character of the C-N bond restricts the amide moiety to only two lowest energy arrangements – *cis* and *trans* conformations. *Cis-trans* isomer ratio of a prolyl peptide bond can be effected by its environments, e.g. different pH values or salt concentrations, solvent composition. However, these conditions are not suitable for many biological assays. Incorporation of some nonnatural amino acids also can influence the ratio significantly. The rotational barriers for *cis/trans* isomerization of different proline analogues have been investigated by dynamic NMR spectroscopy (Kern, et al., 1997). NMR studies of a series of pseudo-proline containing peptides reveal a pronounced effect of the 2-C substituents upon the *cis* to *trans* ratio of the adjacent amide bond in solution. 2-C unsubstituted systems show a preference similar to that of the proline residue for the *trans* form, whereas 2,2-dimethylated derivatives adopt the *cis* amide conformation in high content (Dumy, et al., 1997). L-5,5-dimethylproline (dmP) was used as a substitute to the prolyl to restrict the bond in a *cis* conformation in peptides and proteins (An, et al., 1999).

Photoisomerization of peptide bonds. Excitation into the amide π to π^* transitions on N-methyllactamide and small peptides such as di- and tri-glycine (Song, et al., 1991) results in the photochemical conversion of a fraction of ground state *trans*-amide into ground state *cis*-amide with a high quantum yield. UV/Vis spectroscopy of thioxo-peptides have shown the characteristic absorption bands for the $^1\pi$ to $^1\pi^*$ and n_s to π^* transitions of the thioxo amide bond (Figure **4.1**). Using model small molecule substrate such as N-methylthioacetamide or short thioxo peptides, the *cis/trans* photoisomerization of thioxo amide bond has been studied (Harada, et al., 1980). Recently, a thioxylated oligopeptide containing Ψ[CSN]-Pro moiety without aromatic side chains was demonstrated to undergo reversible *cis/trans* photoisomerization by laser irradiation, without influence on adjacent C(O)NH moieties (Frank, et al., 2000). No photo-decomposition was observed after several cycles of excitation/re-equilibration.

Isomerization of Nonproline peptide bond. To investigate the biological behavior of peptides with non-proline *cis* peptide bonds are of great interest for understanding the molecule basic of biomolecule recognition events as well as in the study of peptide and protein folding. However, comparing with prolyl peptide bonds, we lack general methods to generate a *cis* conformer in a normal secondary amide bond. Due to the one atom variation of the constitution and the reversible photoisomerization feature (Frank, et al., 2000), secondary thioxo peptide bond is thought to be an ideal candidate. Early works concerning the photoisomerization of small model substrates with secondary thioxo peptide bond have been reported (Harada, et al., 1980; Kato, et al., 1984; Ataka, et al., 1984)). To apply this method to a biologically important molecule, such as CsA, are of great interest.
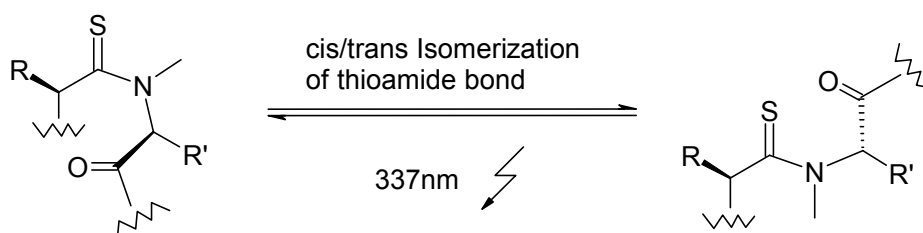
Figure 4.1 Cis/trans isomerization of thioxo amide bond by laser irradiation. The thioxo amide bond undergoes *cis/trans* photoisomerization by 337 nm N_2 laser ($n_s \rightarrow \pi^*$) illumination.

4.3 Multiple conformations in CsA structure.

For biological assays, we hope that the peptide in its different conformations can be relatively stable under room temperature during a time of hours or days. This is the case with the thioxo amide substitution that increases the rotational barrier of *cis/trans* isomerization considerably. At room temperature, the re-equilibration time for a Ψ[CSNH-Pro bond is in the range of hours. In a secondary amide or thioxo amide bond, the *trans* conformation is greatly stablized by releasing the steric strain in the *cis* conformation. If the new-generated *cis* conformer can be stablized by intramolecular forces, the rate of the reverse reaction will be decreased. In solvents of high polarity, many conformations of CsA are in equilibrium to each other (Kessler, et al., 1990; also discussed in chapter 2). These conformations must be of comparable energy. In NMR and crystal structure, the peptide bond between residue 9 and 10 adopt a *cis* conformation. In $CDCl_3$, a second minor conformation has been identified which contains two *cis* peptide bonds: between residue 3-4 and 9-10. These results hinted that a high-energy local conformation of CsA could be stabilized by the drug molecule itself. We wonder if *cis/trans* isomerization is involved in these interconversion processes. The intricate structural properties of CsA in its free form or in its binary and tertiary complex, as well as its biological activities, provide an ideal model in the study of photoisomerization of a secondary thioxo peptide bond.

S-substitution of CsA backbone on single or multiple residues would limit the *cis/trans* isomerization of the thioxo amide moiety, in turn influence the thermodynamic behavior of the whole molecule. On the other hand, the hydrogen bond property of thioxo amide moiety could influence the formation of the binary and tertiary complex. It was reported that thioxylation of a cyclic peptide resulted in a conformational change of the peptide backbone (Kessler, et al., 1992). Furthermore, a thioxo amide bond could undergo *cis/trans* photoisomerization. We expected that a new conformer generated by laser irradiation would be stabilized by intramolecular noncovalent bonds, and biological activities, by means of cyclophilin (Cyp) and calcineurin (CaN) inhibition, can then be measured.

4.4 Thioxo CsA conformations.

The preparation, structure and immunosuppressive activity of thioxo CsA had been reported by Seebach and coworkers (Seebach et al., 1991). There are seven N-methyl residues in CsA molecule. Thioxylation of CsA (Eberle, et al., 1994; Seebach, et al., 1991) with thioxylation reagent (e.g. Lawesson reagent) showed high regio-selectivity to secondary amide bonds. None of the nine thioxo CsAs was found to be as immunosuppressive as their parent drug (Seebach et al., 1991). Interestingly, NMR and MD (molecular dynamics) simulation of [MeBmt^1-ψ-CSNH-Aba^2] CsA ([$^1\psi^2$ CSNH] CsA*) which is most active among all these thioxo derivatives, showed a significant increase of population of the minor conformation in CsA that contains two *cis* bonds. The crystal structure of another derivative, the [$^4\psi^5$ CSNH] [$^7\psi^8$ CSNH] CsA (Cs**54**), is similar to that found in the X-ray structure of CsA.

The blockage of CaN-NFAT pathway by immunosuppressant/immunophilin complexes (CsA/Cyp and FK506/FKBP) in stimulated T cells (Chapter 1) is believed to be the immunosuppressive mechanism of CsA and FK506. We wondered if the lack of immunosuppressive activities of thioxo CsAs could be correlated to their inhibitory activities to the cellular receptor (Cyp) and cellular target (CaN). Various CsA structures as well as the structure-activity relationship studies of many CsA analogues provided us a relatively complete picture about how each residue of the cyclic peptide contacts with its cellular and the pharmaceutical target. Besides the gross information from chemical genetic and genetic studies, an elegant backbone reversible switch strategy would provide us a dynamic insight into the structure.

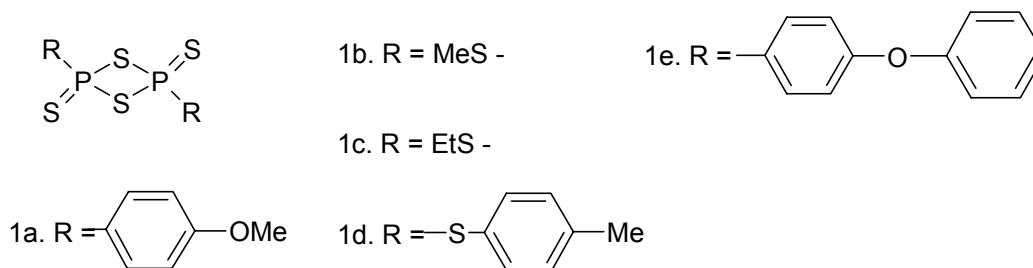* We will use such abbreviation throughout this chapter.

Figure 4.2 Some important thioxylation reagents

1a: Lawesson's reagent; b: Davy-reagents methyl;

1c. Davy-reagents ethyl; d: Davy-reagents p-tolyl   1e: Lajoie reagent

4.5 Syntheses of thioxo CsA.

4.5.1. Thioxylation reagents.

We tried to thioxylate CsA with different thioxylation reagents and under various reaction conditions. Since the first successful synthesis of thioxopeptides with P_2S_5 in 1926, important developments in the field of thioxylation have been achieved. The introduction of the Lawesson's reagents, a convenient racemization-free thioxylation is a major and notable breakthrough of the synthetic methodology. Lawesson's reagent (Pederson, et al., 1978; Scheibye, et al., 1978), along with some of its analogues is listed in figure **4.2**. The size of peptide substrates for the Lawesson reaction, however, is usually limited to two amino-acid residues, owning to the problems of regio-selectivity. Therefore, large thioxopeptides are usually prepared via fragment coupling from separately prepared thioxo-dipeptides (Thorsen, et al., 1983; Clausen, et al., 1984; Jensen, et al., 1985). Ideally, a synthetic strategy generating 11 thioxo-cyclosporins with a thioxo amide on each position will be mostly appreciated. The solid phase synthesis of CsA has not been achieved because of the difficult coupling and cyclization reaction of sequence containing seven N-methyl amino acids. The total synthesis of thioxylated CsAs should be much more difficult. We synthesized several thioxo CsAs by random thioxylation reaction with various reagents in reasonable yield.

4.5.2. The chromatographic behavior of thioxo CsA and its purification.

Typically, CsA as well as some of its derivatives give a broad peak on reverse phase HPLC. This chromatographic behavior can be accounted for the interconversion among several conformations of the molecule (Nishikawa, et al., 1994). The organic syntheses performed in former and following chapters normally gave one or two major products and C8 reverse phase HPLC provided

satisfactory separations in most cases. However, it took long time for us to realize the optimal separation condition for the mixture of randomly thioxylated cyclosporins. C4 and C8 reverse phase HPLC chromatography and capillary electrophoresis (CE) always showed multiple peaks, which could not be separated. Because of the small scale of reaction, products separation by silica gel column alone as described in the literature (Eberle, et al., 1994; Seebach, et al., 1991) also did not give favorable results. For long time thin layer chromatography (TLC) was the method of choice. Currently, we are using silica gel column followed by TLC to purify thioxo CsAs and get optimal separation.

Cs57 R = Ac; X = S; Y = S

Cs58 R = Ac; X = S; Y = O

Cs59 R = Ac; X = O, Y = S

Cs54 R = H; X = S; Y = S

Cs55 R = H; X = S; Y = O

Cs56 R = H; X = O, Y = S
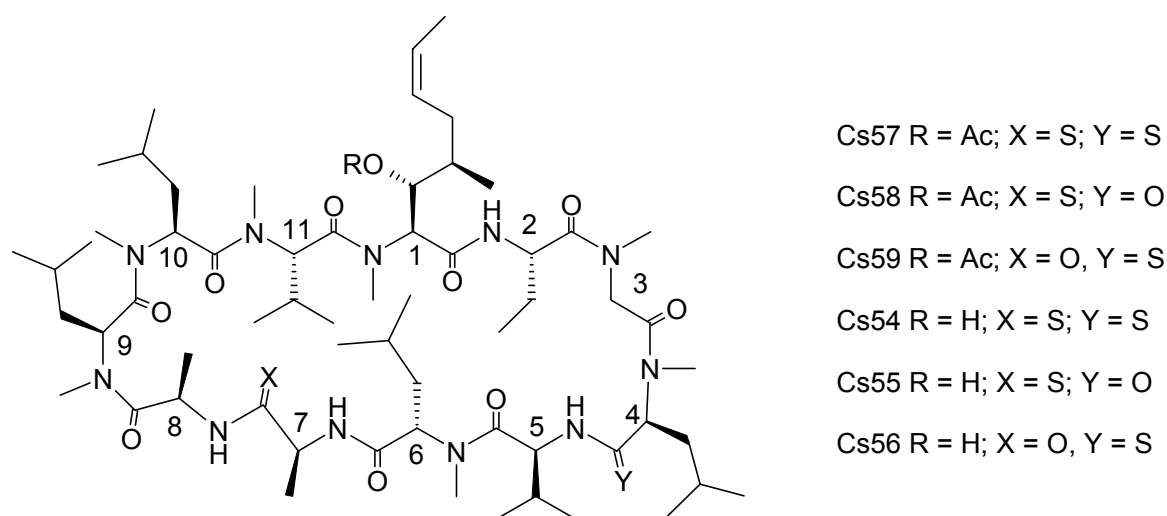
Figure 4.3 Thioxylated CsA derivatives. [O-Acetyl MeBmt][1] CsA was thioxylated by Lawesson reagent and deprotected by NaOMe in MeOH. Three main products were separated and used in the following studies.

4.5.3. Thioxylation of CsA.

CsA thioxylation with Lawesson reagent had been reported (Seebach, et al., 1991) for investigating the immunosuppressive activities of these thioxo derivatives. Using their protocol on a small synthetic scale, we failed to get pure thioxylated products. Thioxylation of [O-acetyl MeBmt][1] CsA showed high regioselectivity and yield (Eberle, et al., 1994). The hydroxyl group of CsA was protected by acetylation with Ac_2O in pyridine at reflux temperature. [O-acetyl MeBmt][1] CsA was thioxylated by Lawesson's reagent in toluene under 130°C for two hours. Two mono-thioxylated compounds, [O-acetyl MeBmt][1] [$^4\psi^5$ CSNH] CsA (Cs**58**), [O-acetyl MeBmt][1] [$^7\psi^8$ CSNH] CsA (Cs**59**) and the di-thioxylated derivative, [O-acetyl MeBmt][1] [$^4\psi^5$ CSNH] [$^7\psi^8$ CSNH] CsA (Cs**57**) were separated by silica gel chromatography. Using Davy reagent-methyl (Figure **4.2**), several new thioxylated CsAs were obtained (these compounds are still under investigation). The acetyl protection of Cs**56**, Cs**57**, and Cs**58** were then deprotected by NaOMe in methanol for 2 hours at

room temperature. Using TLC, [$^4\psi^5$ CSNH] CsA (Cs**55**), [$^7\psi^8$ CSNH] CsA (Cs**56**), and [$^4\psi^5$ CSNH] [$^7\psi^8$ CSNH] CsA (Cs**54**) were separated with moderate yields (Figure **4.3**). The positions of thioxylation were decided by NMR spectra according to the ^{1}H chemical shifts reported by Seebach, et al. (1991). These results have also been confirmed by mass fragmentation experiments (data not shown) and CD spectra, and are in agreement with the literature.

4.5.4 Different conditions for CsA thioxylation.

Trying to get other thioxo CsA derivatives, we investigated direct thioxylation with various reagents under different condition.

Using strong thioxylation reagent. It seems that Lawesson reagent is not reactive enough for CsA thioxylation. Compound **1e** (Figure **4.2**) is a strong thioxylation reagent. CsA and **1e** in DMPU (3,4,5,6-tetrahydro-1,3-dimethylpyrimidin-2(1H)-one) were stirred at room temperature overnight. HPLC with a C8 column gave more than 20 peaks. Each peak is corresponding to one or several thioxo cyclosporins with 1, 2, 3 or 4 thioxo amide moieties (determined by mass spectra). Several of the multiply thioxylated compounds are very hydrophobic. They were eluted out at about 100% acetonitrile from the column. Using a C4 HPLC column did not enhance the resolution. Running the crude sample on a TLC gave a continuous band. We performed thioxylation reaction at lower temperature (5°C), but no improvement was observed. To facilitate the purification, the regioselectivity of the thioxylation reaction should be enhanced.

Thioxylation using basic conditions. Thioxylation with Lawesson reagent in pure pyridine as scavengers for the phosphoric acids gave low reaction yield. It was reported recently that lowering the amount of pyridine can gradually increase the thioxylation yield. Using 1 equiv. of pyridine and 4 equiv. of Lawesson reagent in anh. THF, the authors could thioxylate a class of sugar-modified nucleosides regioselectively (Wojczewski, et al. 2000).

With 1 equiv. of Lawesson reagent and 1 equiv. of pyridine in toluene, the reaction showed higher selectivity. Under reflux temperature, the starting material disappeared in one hour and the HPLC gave only 3 major peaks. These compounds can be separated by silica gel chromatography, but proved to be unstable during preparative HPLC purification. Although it is well known that thioxo amide bond is very sensitive to acidic condition, the decomposition in 0.5% TFA under HPLC conditions is unusual. Analyzing with MALDI and ESI mass spectroscopy, besides the [M+H]$^+$ peak, a signal of [M-1] have often been observed. The intensity of these peaks is not reproducible in different MS measurements. We are not able to explain this phenomena until now. Using other thioxylation reagents as Davy reagent-tolyl and Davy reagent-methyl in the presence of 1 equiv. of pyridine gave similar results.

4.6. Biological activities of thioxo CsAs.

IL-2 and IL-8 reporter gene assays showed that Cs**54**, Cs**55** and Cs**56** were less active than CsA (Seebach, et al., 1991). We measured the Cyp18 and CaN inhibitory activities of these analogues.
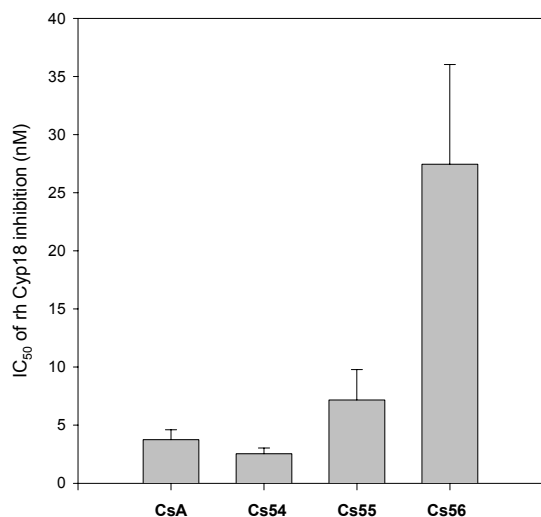
Figure 4.4a Cyp18 inhibition by CsA and thioxylated CsA derivatives Cs**54**, Cs**55**, and Cs**56**. PPIase activities of Cyp18 were measured with protease coupled assay and Suc-Ala-Phe-Pro-Phe-NH-Np as substrate in Hepes buffer, pH7.8, at 7°C, in the presence of 2.5nM Cyp18 and inhibitors of desired concentrations.
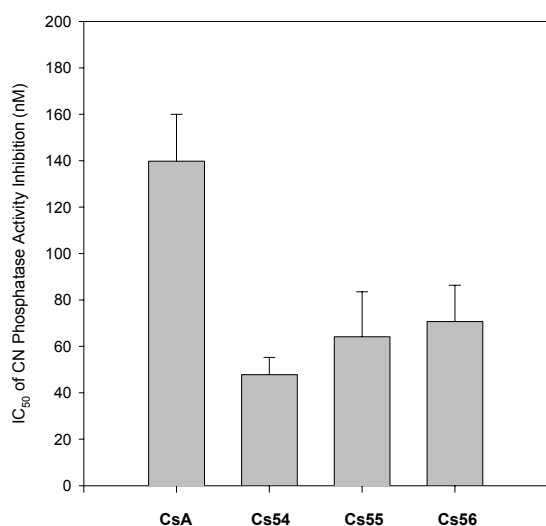
Figure 4.4b Calcineurin inhibition by CsA and Thioxylated CsA derivatives Cs**54**, Cs**55**, and Cs**56**. The calcineurin phosphatase activities were measured using scintillation assay and biotinylated RII peptide as substrate at 30°C, in the presence of 1.32nM calcineurin, 10μM inhibitor and Cyp18 of desired concentrations.

4.6.1. Cyp18 PPIase activity inhibition.

Thioxo CsA derivatives displayed different inhibitory activities to rh Cyp18 (Figure **4.4a**). In the Cyp18/CsA complex, there are five direct hydrogen bonds beween CsA and Cyp18 (Kallen, et al., 1998). Figure **4.5** represents the interactions of CsA with Cyp18 and CaN. Residues 1, 2, 3, 9, and

10 of CsA are involved, while residues 4, 5, 7, and 8 are not in the Cyp binding domain of CsA. The latter contact CaN in the Cyp18/CsA/CaN tertiary structure. The thioxylation on the CaN binding domain of CsA may influence the PPIase binding affinity via stabilizing or destabilizing the ligand structure in its free form because all four secondary amide moieties participate in intramolecular hydrogen bonds. As shown in figure **4.4a**, two monothioxo CsA analogues Cs**55** and Cs**56** show an increase of IC_{50} value of 2-fold and 7-fold, respectively. On the contrary, Cs**54** displays higher inhibitory activity than its parent drug CsA.
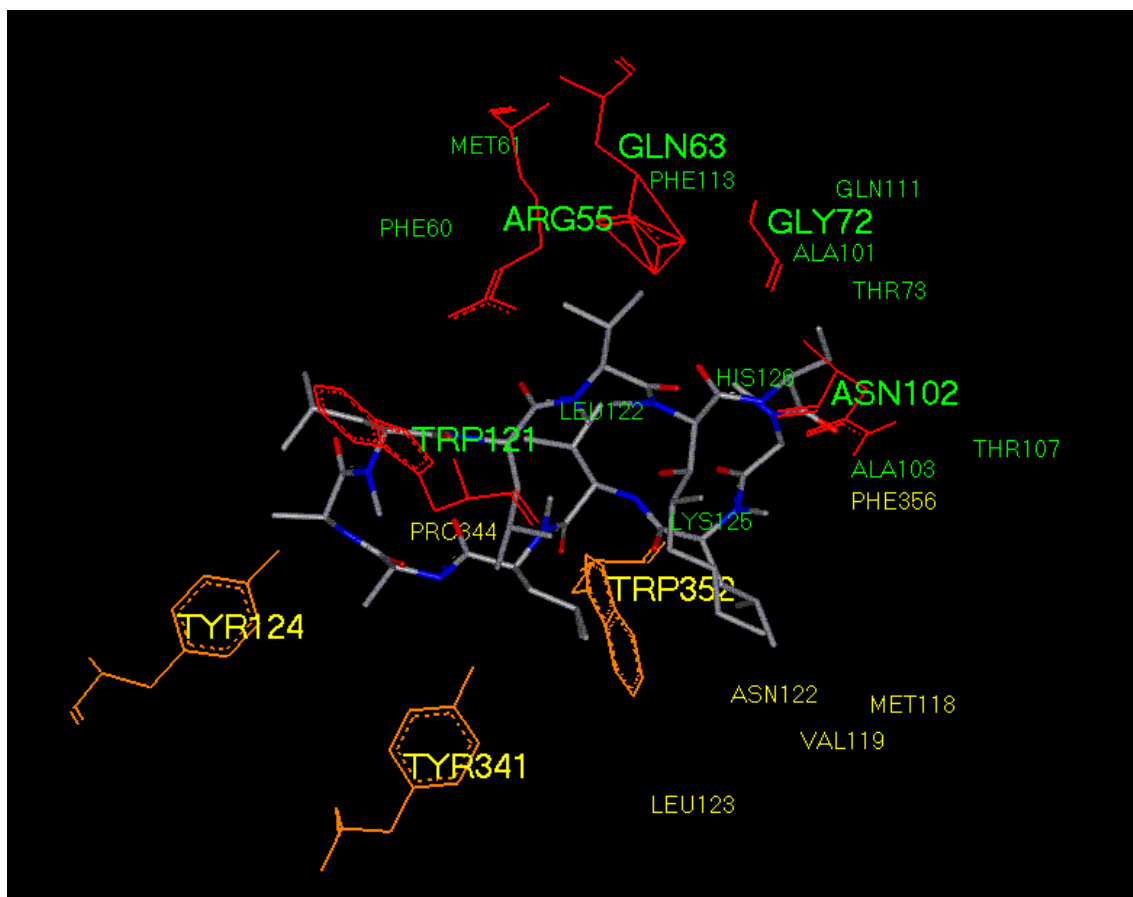
Figure 4.5 The interactions of CsA with Cyp18 and CaN. The residues from Cyp18 involved in contacts with CsA was in green. Among them, the amino acids which form Hydrogen bond with CsA was displayed as stick in red. The residues from CaN involved in contacts with CsA was in yellow. Among them, the amino acids, which form H-bond with CsA was displayed as stick in orange.

4.6.2. CaN phosphatase activity inhibition.

Surprisingly, all three Cyp18/thioxylated CsA analogue complexes show higher CaN inhibitory activity than CsA (figure **4.4b**). Compared with C(O)NH, secondary thioxo amide bonds are stronger hydrogen bond donors and weaker acceptors (Hollosi, et al., 1990). In the Cyp/CsA/CaN

complex structure (Zhao, et al., 2000) (Figure **4.5**), the W352 of CaN forms two hydrogen bonds with the O-atom of MeLeu[4] and the NH of Val[5], respectively. The enhanced hydrogen bond between W352 and the NH of [4ψ5 CSNH] moiety contributes more to the interaction with CaN. An increase of inhibitory activity of Cs**56** was observed. There is no hydrogen bond between the oxygen atom of Ala[7] and CaN. On the contrary, a hydrogen bond between the NH of D-Ala[8] and the hydroxyl group of Y124 of CaN was observed. S-substitution could enhance this interaction. Thioxylation of this amide bond (Cs**55**) also causes an increase of CaN inhibition by the immunophilin/ligand complex. Dithioxylation of CsA (Cs**54**) gave similar results.

However, in an immunosuppressive assay, all these thioxo cyclosporins were much less active than CsA (Seebach, et al., 1991). The immunosuppressive mechanism of CsA and FK506 through blocking the CaN-NFAT pathway is not necessarily complete and has been challenged by many genetic and cell biological studies (in Chapter 1). All these puzzles will prompt new finding and deep understanding of immunosuppression.

4.7 CD spectroscopy of cyclosporins.

Circular dichroism (CD) has special advantages for exploration of the conformational environment of a thioxo amide group, which may be considered as a chromophoric derivative of an amide group. The thioxo amide $n\pi^*$(C=S) band is by 130 - 180 nm red-shifted relative to the amide $n\pi^*$ (C=O) band, and the $\pi\pi^*$(C=S) band appears in the spectral range of 250 - 280 nm and is, therefore, well-separated from that of amides. All CD bands are influenced by the stereochemistry around the chromophore and the sign pattern of the $n\pi^*$ and $\pi\pi^*$ bands mainly reflect the local conformation of the residue following the thioxo amide group in the chain (Milewska, et al., 1997; Khan, et al, 1996). We studied the CD spectra of thioxylated CsA analogues (Cs**54**, Cs**55**, Cs**56**) and their acetylated forms (Cs**57**, Cs**58**, Cs**59**).

4.7.1 CD spectra of thioxo CsA and thioxylated [O-acetyl MeBmt][1] CsA.

The CD spectra of CsA in ethanol/H$_2$O (50/50) indicated turn structure around 226 nm (Hasumi, et al., 1994). In DMSO, this peak red-shifts to 232 nm. Thioxo CsAs as well as the acetylated thioxo CsAs have similar spectra around this wavelength (Figure **4.**6 and Figure **4.7**). The difference in the intensity among these bands indicates some changes in their turn structure. For instance, in the CsA X-ray structure, besides a βII′-turn involving residues 2 – 5, a γ-turn involving D-Ala[8]NH and MeLeu[6]CO were observed. Inverse γ-turn have a negative $n\pi^*$ band at around 230 nm. The CD spectrum is the composite of their chiral contribution. In the X-ray crystal structure of the di-thioxylated CsA (Cs**54**) (Seebach, et al., 1991), the γ-turn hydrogen bond was not found between residues 7 and 8 due to the bulk of the S-atom. The decrease of the band around 230 nm might reflect the loss of the γ-turn structure.
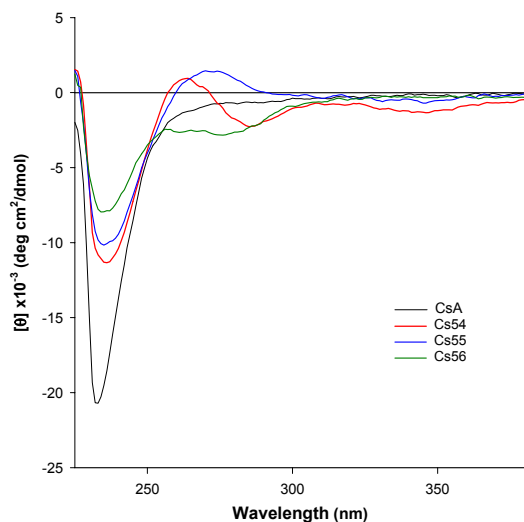
Figure 4.6 CD spectra of CsA and thioxo CsA (Cs**54**, Cs**55** & Cs**56**)
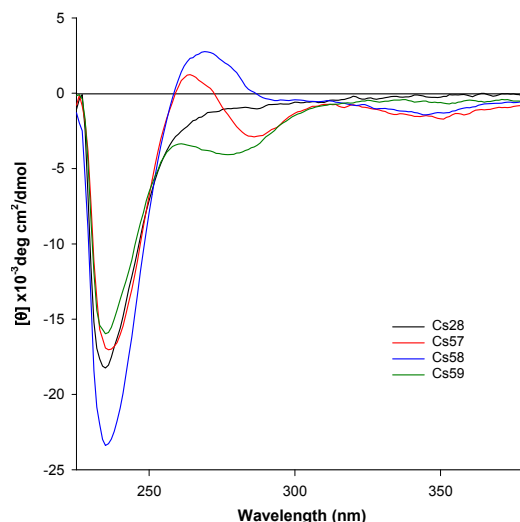
Figure 4.7 CD spectra of CsA-Ac (Cs**28**) and thioxo CsA-Ac (Cs**57**, Cs**58** & Cs**59**)
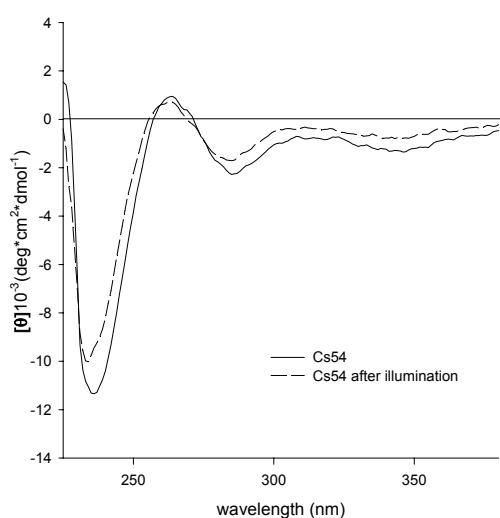
Figure 4.8 Photo-induced switch of CD spectra of dithioxylated CsA (Cs**54**).
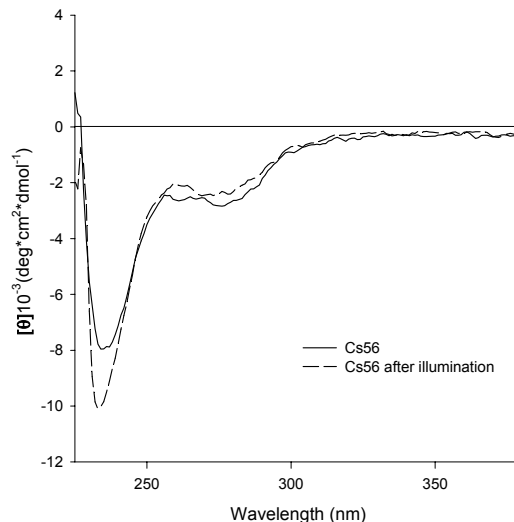
Figure 4.9 Photo-induced switch of CD spectra of an acetylated monothioxylated CsA (Cs**56**)
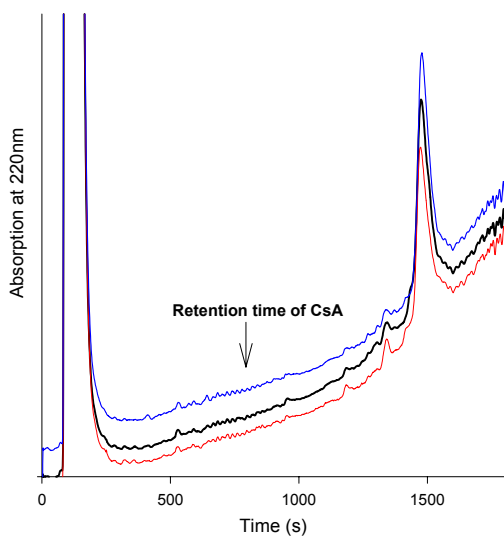
Figure 4.10a Stability of thioxylated CsA. 1mM Cs**54** (black) in DMSO was irradiated with N_2 laser (337 nm) for five minutes (red) or 35 minutes (blue). The samplers were analyzed by RP C8-HPLC.

The spectra at long wavelengths reflect the chemical environment around the thioxo amide moiety. A negative band at 260 – 300 nm is typical of thioxo peptides ($\pi \rightarrow \pi^*$ transition). The band above 340 nm corresponds to the $n_s \rightarrow \pi^*$ transition of thioxo amide bond. All three bands can be observed in the CD spectra of Cs**56** and Cs**59**, which contain thioxo amide bonds between L-MeLeu[4] and L-Val[5]. In the spectra of Cs**55** and Cs**58**, in which thioxo amide bonds are located between L-Ala[7] and D-Ala[8], the positive bands of $\pi \rightarrow \pi^*$ transition reflect the stereochemistry of the residue following the thioxo amide group. Interestingly, the CD spectrum of the dithioxo CsA (Cs**54**) is likely a combination of the spectra of the two monothioxo Cs**55** and Cs**56**. It contains a band around 232 nm indicating a turn structure, a positive and a negative band between 260-300 nm that correspond to the two thioxo amide moieties with different stereochemical environments, as well as the $n_s \rightarrow \pi^*$ transition band at long wavelength. The dithioxo CsA-Ac (Cs**57**) has very similar CD spectrum.

4.7.2 Photoisomerization and stability under laser illumination of thioxylated cyclosporins.

The thioxo-CsAs and [O-acetyl MeBmt][1] thioxo CsAs underwent light-induced *cis/trans* isomerization (by excitation of the $^1n \rightarrow {}^3\pi^*$ transition with a N_2 laser at 337 nm, 10 pulse per second, 90μJ, at room temperature). The intensity of the negative band at about 232 nm decreased (for Cs**54**, Cs**57**, Cs**58,** and Cs**59**) or increased (in the case of Cs**55**, Cs**56**) after laser flashing reflecting changes in the turn structure. The spectra in the range of 260-300 nm are corresponding to the $\pi \rightarrow \pi^*$ transition of the thioxo amide bond. In all these six samples investigated, the intensity of both the positive and negative bands decreased (Figure **4.8** and **4.9**). Photoisomerization induces changes in the local chemical environment around the thioxo amide bonds. Similar light-induced effects were also observed in the CD spectra of linear thioxo peptides (Rascher-Bang, et al., unpublished results). HPLC measurements after illumination of the samples (1mM in DMSO) at room temperature demonstrated that no photodecomposition occurs (Figure **4.10a**).

4.7.3 Reversible photo-switch of UV spectra of thioxylated CsAs.

The UV spectra of thioxylated cyclosporins have a characteristic band around 270nm corresponding to the $\pi \rightarrow \pi^*$ transition of thioxo amide bond (Figure **4.10b** D). After 30min of laser illumination at room temperature, the absorbency around 270nm of Cs**54**, Cs**55**, and Cs**56** exhibited minor but detectable enhancements. As in the CD spectra of thioxylated cyclosporins, this $\pi \rightarrow \pi^*$ transition band is sensitive to the *cis/trans* conformation of the thioxo peptide bond. Interestingly, the spectral changes of Cs**54** and Cs**56**, but not Cs**55**, are reversible (Figure **4.10** A, B, C). At room temperature, the k_{obs} of the reverse processes, as determined by time course of UV absorbency at 270nm, are $8.1 \times 10^{-4}s^{-1}$ and $10.9 \times 10^{-4}s^{-1}$ for Cs**54** and Cs**56,** respectively (Figure **4.10b** A and B). Obviously, due to the high rotation barrier around thioxo amide bonds, the inter-conversions among different conformations of thioxylated CsAs are much slower than that of CsA (as discussed in
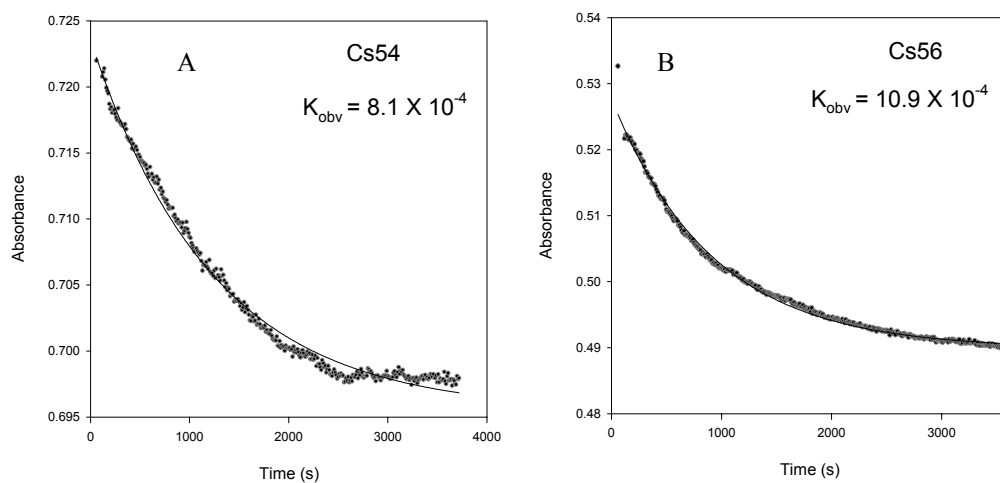
Figure 4.10b. Reversible photo-switch of UV spectra of thioxo cyclosporins Cs**54**, Cs**55**, and Cs**56**. The UV spectra of the samples were measured. Then the samples were irradiated (in DMSO) with N_2 laser at 337 nm for 30 min and the UV spectra were measured again. The time course of UV absorbance at 270 nm (A, B, C) during re-equilibration were monitored. After one hour, the UV spectra were measured again. The UV spectra of Cs**56** (D) before (black), after (red) laser flashing, and after one hour re-equilibration (blue) were shown. All UV spectra were measured in H2O/ethanol/DMSO (5/4/1)
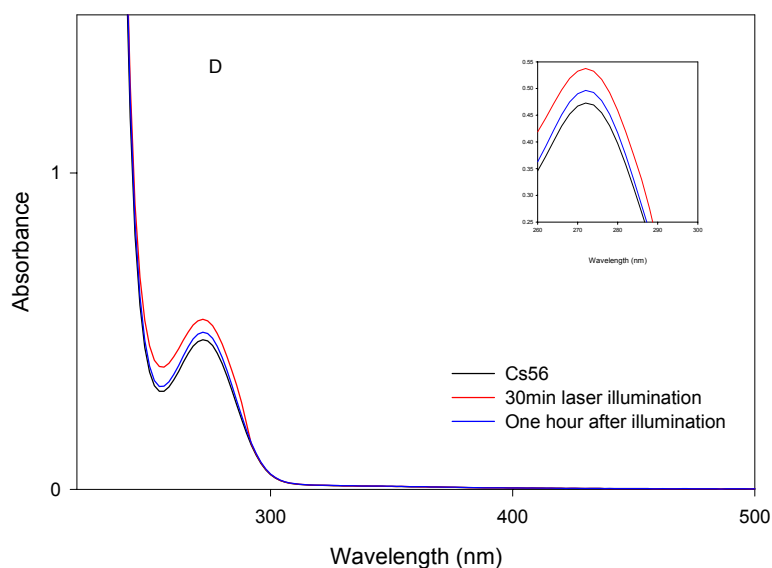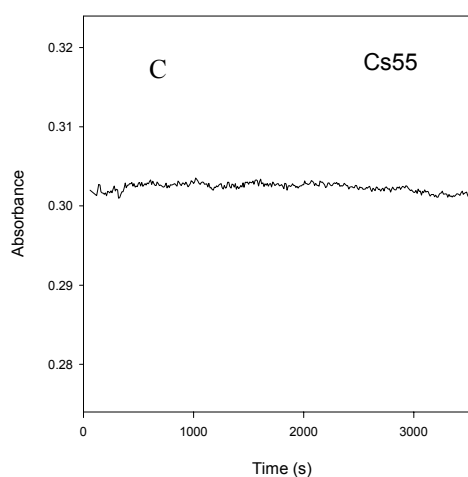
chapter 2). Furthermore, the amplitudes of both kinetic curves are approximately equal to the enhancement of UV absorbance upon laser illumination. These results also confirmed that Cs**54** and Cs**56** are stable under illumination condition. Although the photo-switch of Cs**55** cannot be reversed after staying at room temperature over night, there is no evidence that Cs**55** is chemically less stable than the other two analogues. Long time exposure to air or laser illumination did not result in decomposition of Cs**55**, as determined by HPLC.

4.8 Photoswitch of thioxo CsAs' biological activity.

The photoisomerization of thioxo amide bonds has not yet been shown for cyclic peptides. Due to the behavior of CsA and its thioxo derivatives on HPLC, it is almost impossible to separate individual conformers and study their kinetics independently to each other like the normal thioxo peptides comtaining Ψ[CSN]-Pro moiety (Frank, et al., 2000). However, the Cyp18 and CaN enzyme inhibitory activity of CsA and its analogues could provide us a direct evaluation of the structural changes after photoisomerization of the thioxo amide bonds in thioxo CsAs.

4.8.1 Photoswitch of Cyp18 inhibition.

Solutions of Cs**54**, Cs**55,** and Cs**56** were irradiated by N_2 laser at 337 nm (^{1}n $\rightarrow$ $^3\pi^*$ transition) for 30 min at room temperature. Half of each solution was then diluted ten times with cold Hepes buffer and kept on ice bath for measurement. The remaining solutions were kept in darkness overnight and the Cyp and CaN inhibition was measured again. Due to its special absorption at 337 nm, only the thioxo amide bond could undergo *trans*/*cis* isomerization upon laser flashing. A part of the molecules could adopt a switched conformation (thioxo CsA*) and contribute to the changes in spectra and biological activities. The uncomplexed structure of CsA is maintained by multiple intramolecular noncovalent bonds. Although both thioxylated amide bonds are not involved in the Cyp18/ligand interaction, changes in a local conformation by photoisomerization result in structural alterations in the entire molecule. As shown in figure **11**A, **11**C, and **11**E, laser illumination impair the Cyp18 enzyme inhibitory activity of Cs**54**. The impairments are reversible. On the contrary, laser irradiation has minor effects on Cs**55** and Cs**56**.

Some of the functional biomolecules change their activities according to expressed locations and involved cellular processes, so it is often important to inactivate them in a spatiotemporally controlled manner. Chromophore-assisted laser inactivation (CALI) is an excellent method for achieving this purpose (Jay, 1988 & 1990). In CALI, chromophore-labeled antibody molecules are introduced into cells, which are then subjected to laser irradiation. Upon absorbing the laser energy, the chromophores mediate generation of radical species, and in turn inactivates their target proteins. Recently the implementation of a small molecule-based CALI was reported (Inoue, et al., 2001). However, all these inactivation reactions are irreversible. Thioxo cyclosporins Cs**54** exhibit distinct
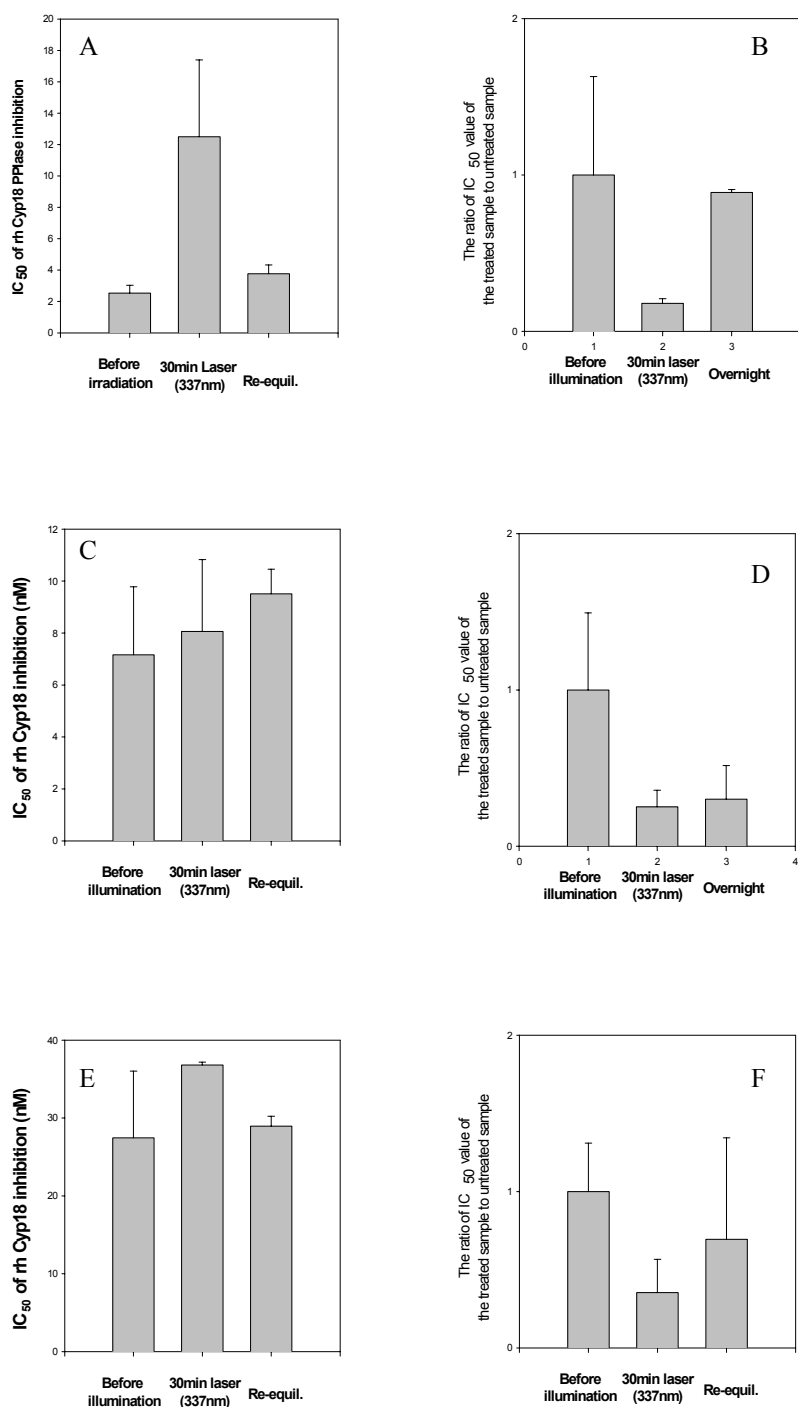
Figure 4.11 Photoswitching and reequilibration of Cyp18 inhibition by thioxo-CsAs Cs**54** (A), Cs**55** (C), and Cs**56** (E) and CaN inhibition by Cs**54** (B), Cs**55** (D), and Cs**56** (F) in complexes with Cyp18. Cyp18 activities were measured with protease coupled assay and Suc-Ala-Phe-Pro-Phe-NH-Np as substrate in Hepes buffer, pH7.8, at 7°C, in the presence of 2.5nM Cyp18 and inhibitors of desired concentrations. CaN phosphatase activities were measured using biotinylated RII peptide as substrate with Scintillation assay at 30°C, in the presence of 1.32nM CaN, 10 μM inhibitor, and Cyp18 of desired concentration. Solutions of Cs**54**, Cs**55,** and Cs**56** in DMSO were irradiated by N_2 laser at 337 nm for 30 min at r.t.. Half of each solution was then diluted ten times with cold Hepes buffer and kept on ice bath for measurements. The remaining solutions were kept in darkness overnight at room temperature and the Cyp and CaN inhibition was measured again.

Cyp18 inhibitory activities in a light-dependent manner. PPIase activity of the Cyp18 could be partially recovered upon laser light irradiation. This process is reversible.

4.8.2 Photoswitch of CaN inhibition.

We measured CaN inhibitory activity of Cs**54**, Cs**55**, and Cs**56** in complex with Cyp18. Surprisingly, all three thioxylated cyclosporins in the presence of Cyp18 showed enhanced CaN inhibitory activities after laser illumination (Figure **11**B, **11**D, **11**F). This is not due to the direct inhibitory activities of the analogues to CaN because they cannot inhibit CaN in the absence of Cyp18. Two thioxo amide bonds are located in the CaN binding domain of CsA ($[^4\psi^5$ CSNH] and $[^7\psi^8$ CSNH]). There are two possibilities of the photo-induced effect on forming the tertiary complex.

1. Interacting with CsA/Cyp binary complex, CaN prefers a *trans* peptide bond rather than *cis* peptide bond in CsA molecule.
2. The switched structure of the whole molecule results in enhanced affinity.

Interestingly, the photo-induced increase of inhibitory activity of Cs**55**/Cyp18 complex towards CaN is an irreversible process. Laser irradiation might switch the structure of Cs**55** to a state of relatively high stability. Because of the high rotation barrier of thioxo amide bond, the ligand is 'frozen' in its new conformation. This gives another proof that the new-generated structure of Cs**55** is relatively thermodynamic stable, which was shown in the UV experiments.

4.9 Conclusion.

Although all thioxylated CsA are less immunosuppressive than their parent drug in biological assays, three thioxo derivatives investigated in our study displayed enhanced CaN inhibitory activity, along with either unaffected or impaired Cyp binding affinity. Photoisomerization had significant influences on their CD spectra, Cyp and CaN inhibitory activities. Our results proved that changing the *cis/trans* conformation of a peptide bond could affect the ligand structure and the recognition by its receptor and target proteins.

Chapter 5. CsA Phosphorylation

5.1 Metabolites of cyclosporin A.

Cyclosporin A (CsA) is extensively metabolized in the body. More than ten metabolites have been isolated from human liver (Vine, et al., 1987; Wang, et al., 1989). All these compounds identified so far have their cyclic structure intact. Most of them were hydroxylated by oxidation. Another compound, [O-Sulfonyl MeBmt][1] CsA (Cs**26**), has also been isolated from human bile and plasma (Henricsson, 1990, Johansson et al., 1990). Several metabolites contribute in blocking IL-2 production (Johansson et al., 1990, Freed, et al., 1987).

Protein phosphorylation touches on most aspects of cell physiology (Hunter, 1995 & 2000). Almost every known signaling pathway eventually impinges on protein kinases, or in some instances, protein phosphatases. Especially, Ser, Thr, and Tyr phosphorylation and dephosphorylation can modulate cellular events and exert diverse effects. Furthermore, each cell is programmed to express a specific combination of kinases and phosphatases, and different cells can respond differently to these signal molecules. The function of some small molecules can be regulated in the same way. For example, the study of G proteins revealed the principle that hydrolysis of protein-bound GTP could act as a signaling switch (Gilman, 1987). Is it possible that phosphorylation and dephosphorylation can modulate a drug in the same manner, to switch on/off its activity, to cause different distribution among various cell types and tissues, and to change its subcellular localization? A lot of evidences (reviewed in chapter 1) have suggested that some other cellular components involved in immune responses are targeted by CsA (Su, et al., 1994, Matsuda, et al., 1998, Matsuda, et al., 2000). On the other hand, the side effects of CsA and FK506 can neither been explained totally by calcineurin (CaN) phosphatase activity inhibition by Cyp/CsA or FKBP/FK506 complex (Hojo, 1999). Besides searching for other cellular targets of CsA, investigating the effects of CsA metabolites on the immune system or some other cellular events could be another approach.

Looking at CsA molecule, four structural features of this potent immunosuppressive drug are noticeable: **I.** Most side chains of the cyclopeptide are hydrophobic. **II.** The uncommon MeBmt[1] residue contains a hydroxyl group and a long unsaturate side chain. **III.** Seven of the eleven amino acids are N-methylated. **IV.** Most side chains are chemically inert.

In free CsA, the MeBmt[1] side chain is folded over the backbone (Kessler, 1990). It was proposed that the single hydroxyl group was involved in a H-bridge with the MeBmt[1] carbonyl. Upon Cyp18 binding, the ligand undergoes a dramatic conformational change. None of the intramolecular hydrogen bonds found in the free structure were present. The single intramolecular hydrogen bond exists between the hydroxyl group and the carbonyl oxygen of MeLeu[4] (Pflugl et al., 1993, 1994, Mikol, et al., 1993, Kallen, et al., 1998). In the Cyp18/CsA/CaN tertiary complex structure, the long side chain is responsible for CaN binding (Zhao, 2000).

Removal of the hydroxyl group resulted in almost total loss of activity. Replacing MeBmt[1] with N-methylthreonine or with MeLeu(3-OH) reduced the compound's immunosuppressive activity drastically (Miller, 1989). Modifications at this position can abolish the primary activity of CsA as a Cyp and CaN inhibitor and, probably, gains new biological functions. Phosphate moieties are observed in some other immunosuppressive drugs (Chapter 1). A novel immunosuppressant FR901483 is active only in its phosphorylated form (Sakamoto, 1996). It was suggested that stevastelins could be phosphorylated by cellular enzyme(s) after permeating cell membrane, and in turn exhibits immunosuppressive activity because the phosphorylated form is a more potent phosphatase inhibitor than its parent drug (Hamaguchi, et al., 1997). Phosphorylated rapamycin was obtained by microbial conversion. Phosphorylation of rapamycin in the FKBP binding domain did not change its affinity to FKBP and immunosuppressive effect dramatically (Kuhnt, et al., 1997).

We investigated the MeBmt[1] phosphorylation of CsA chemically and biochemically. Some other CsA position 1 derivatives (Figure **5.1**) were also synthesized chemically. We measured the rh Cyp18 and CaN inhibitory activities of these analogues. The immunosuppressive activities to cell line and human lymphocytes were also evaluated. Cell membrane permeability and other cell biological effects of [O-phosphoryl MeBmt][1] CsA (Cs**18**) were investigated. For comparison, [O-Phosphoryl-D-Serine][8] CsA and [O-Phosphoryl-D-Serine][8] [O-Phosphoryl MeBmt][1] CsA were also synthesized from [D-Ser][8] CsA and the inhibitory activities were investigated.

5.2 Chemical phosphorylation of CsA.

Study of the chemical phosphorylation of amino acid derivatives can be documented back to the 1950s. Several groups investigated serine, threonine and tyrosine phosphorylation on the premise that these phosphorylated residues were the possible source of protein-bound phosphate in phosphoproteins. Although early chemical studies employed harsh phosphorylation reagents such as phosphoryl trichloride/pyridine and orthophosphoric acid/phosphorus pentoxide for the phosphorylation of amino acids and their simple peptide derivatives, the subsequent introduction of protected monofunctional diaralkyl phosphorochloridate and phosphoramidite reagents provided new avenues for the mild preparation of phosphorylated biomolecules with improved chemical efficiency (Perich, 1991). There are two general strategies for phospho-oligopeptide synthesis: i) using protected phospho-amino acids as building blocks in solid phase synthesis and ii) the global or post-assembly phosphorylation approach. In our case of CsA chemical modification, the post-assembly phosphorylation approach using different dialkyl N, N-diisopropylphosphoramidites was investigated.

The phosphoramidites are activated by weak acids such as 5-methyltetrazole (Beaucage, et al., 1981) or 1*H*-tetralzole (Perich, et al., 1987). The reagents react with alcohols, forming phosphites, which can be transferred to phosphates by oxidation. The phosphate moiety of serine and threonine derivatives can be protected in various ways, including methyl, allyl, *tert*-butyl and benzyl groups.

Figure 5.1 CsA position 1 derivatives.
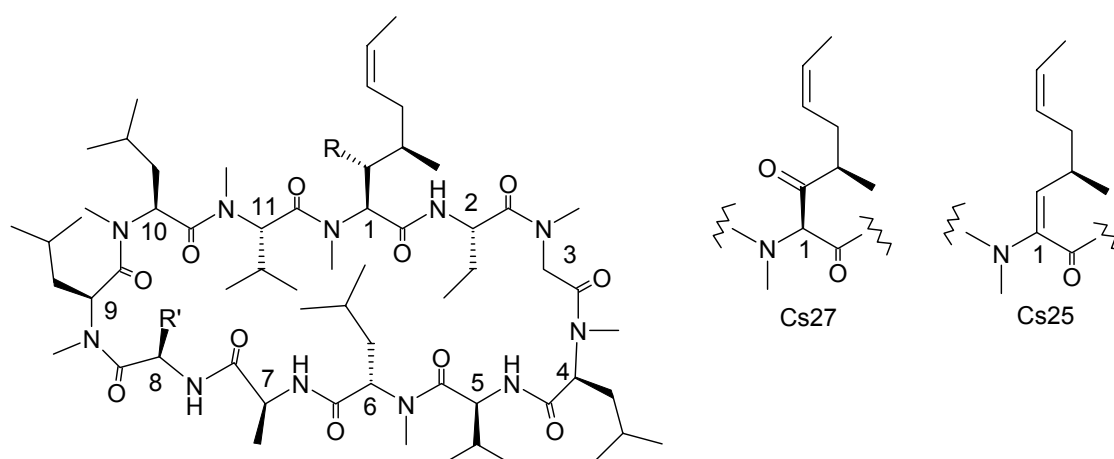
Label	
CsA	R = OH; R' = H
Cs18	R = -O-P(O)(OH)$_2$; R' = H
Cs19	R = -O-P(O)(OMe)$_2$, R' = H
Cs20	R = -O-P(O)(OAll)$_2$; R' = H
Cs21	R = -O-P(O)(OAll)(OH); ; R': H
Cs22	R, R' = -O-P(O)(OH)$_2$
Cs23	R = -O-P(O)(OH)$_2$, R' = -OCH$_2$C(O)NH(CH$_2$)$_5$NHBiotin
Cs25	[1-Amino-2, 6-octadienoic acid][1] CsA
Cs26	R = -OSO$_3$H, R' = H
Cs27	[β-Oxo MeBmt][1] CsA
Cs28	R = OC(O)CH$_3$, R' = H
Cs31	R = OC(O)CH$_3$, R' = H; Residue 11 = D-N-methyl valine
Cs61	R = -O-P(O)(OH)$_2$; R' = H; Residue 11 = D-N-methyl valine
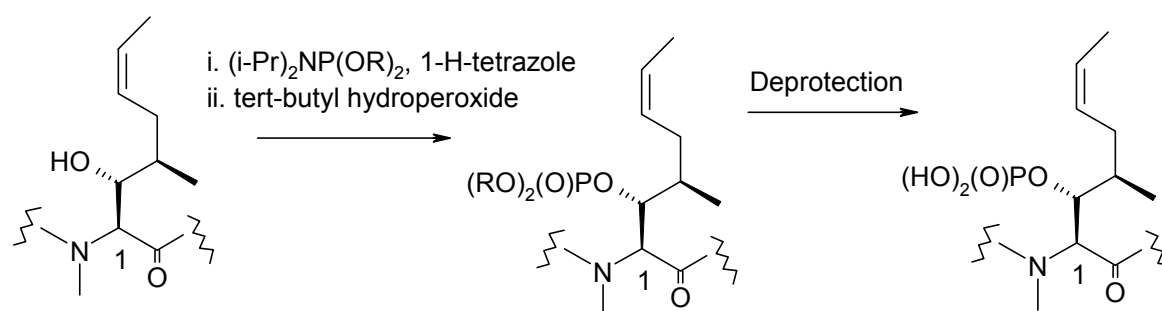
R = Methyl, Allyl, *tert*-Butyl, Benzyl

Figure 5.2 Phosphorylation of CsA by diisopropylphosphoramidites.

Each protection group has its advantage and disadvantage in phosphorylation or deprotection reactions (Figure **5.2**).

5.2.1 Phosphorylation with dimethyl N, N-diisopropylphosphoramidite.

CsA phosphorylation was carried out with 4-equivalent dimethyl N, N-diisopropylphosphoramidite and 8-equivalent 1-*H* tetrazole in THF at room temperature. Oxidation of the phosphite was achieved *in situ* by addition of *tert*-butyl hydroperoxide to give [O-dimethylphosphoryl MeBmt][1] cyclosporin Cs**19** in high yield.

Harsh acid treatment for complete removal of the methyl protection group of phospho-peptides results in side reactions. Various deprotection methodologies have been developed. Trimethylsilyl bromide (TMSBr), a strong cleavage reagent, was normally used to cleave the methyl or ethyl groups from the phosphate (Kitas, 1989). An efficient deprotection was achieved with a mixture of trifluoromethanesulfonic acid (TFMSA), TFA, dimethyl sulfide (DMS) and *m*-cresol (Lee et al, 1994). An optimized two-step deprotection strategy was suggested (Otaka et al., 1995) which consists of a combination of the first-step reagent (trimethylsilyl trifluoromethanesulfonate (TMSOTf)-thioanisole in TFA, *m*-cresol, 1,2-ethanedithiol (EDT)) and the second-step reagent (first step reagent plus DMS-TMSOTf). Unfortunately, all these conditions are too harsh for the deprotection of Cs**19** and resulted in unidentified mixtures.

Phosphoric acid diester could mimic a phosphate moiety. Base treatment is commonly used for mono-deprotection of phosphoric acid esters (Figure **5.3**). However, mono-deprotection of dialkyl phosphoserine and phosphothreonine was problematic due to β-elimination of the phosphate esters. On treatment with 1M NaOH aqueous solution, Cs**19** undergoes β-elimination and gave [1-amino-2, 6-octadienoic acid][1] CsA (Cs**25)** as main product. The problems for deprotection and monodeprotection involved in [O-Dimethylphosphoryl MeBmt][1] CsA were overcome by using allyl as protection group (section 5.2.3).
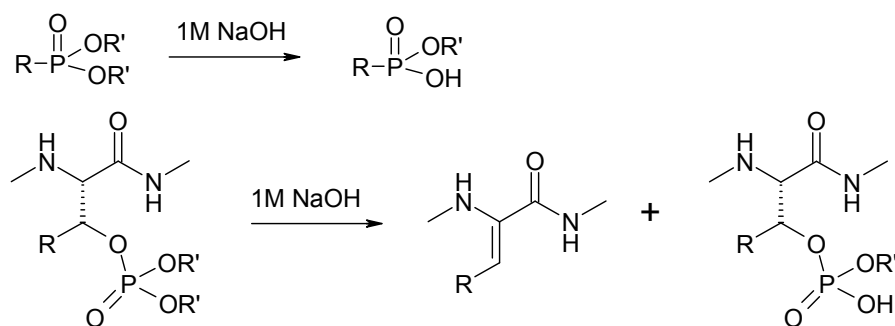

Figure 5.3 β-elimination of Cs**18**.

5.2.2 Acidic labile protection groups – phosphorylation with dibenzyl & di-*tert*-butyl N, N-diisopropylphosphoramidites and bis(dimethylamino)-phosphoryl chloride.

Benzyl and *tert*-butyl groups can be easily removed by TFA treatment. Because of the steric hindrance, we failed to phosphorylate both hydroxyl groups in [D-Ser][8] CsA in the presence of 1 equivalent of di-*tert*-butyl N, N-diisopropylphosphoramidite (in chapter 3). Treating CsA with 4-equivalent of di-*tert*-butyl N, N-diisopropylphosphoramidite over night, no reaction occurred. The steric hindrance of the secondary hydroxyl of MeBmt[1] is more remarkable than the primary hydroxyl of D-Ser in [D-Ser][8] CsA. With one equiv. of dibenzyl N, N-diisopropylphosphoramidite, the D-Ser[8] residue of [D-Ser][8] CsA was phosphorylated selectively (in chapter 3). This reagent phosphorylated the hydroxyl group of MeBmt[1] residue of CsA with very low yield (10%).

Phosphorus-nitrogen bond in a phosphoramidate group is stable under alkaline conditions but unstable in acids, hydrolyzing readily to the corresponding substituted phosphate compounds. O-[Bis(dimethylamino)-phosphono]-tyrosine underwent hydrolysis in 2N HCl/dioxane solution (Chao, et al., 1995). However, because of the steric hindrance, bis(dimethylamino)-phosphoryl chloride did not react with CsA in the presence of DMAP and DBU in CH_2Cl_2.

5.2.3 Phosphorylation of CsA and its analogues.

Allyl phosphate esters can be removed under very mild conditions with palladium catalysis in the presence of a nucleophile. CsA phosphorylation was carried out in 4-equivalent of diallyl N, N-diisopropylphosphoramidite and 8-equivalent of 1-*H* tetrazole. Oxidation of the phosphite was achieved *in situ* by addition of *tert*-butyl hydroperoxide to give [O-diallylphosphoryl MeBmt][1] CsA (Cs**20**) in high yield. After deprotection with *tetrakis*(triphenylphosphine)palladium(0) in the presence of formic acid and amine (Sander et al., 1999), [O-phosphoryl MeBmt][1] CsA (Cs**18**) was obtained in high yield (Figure **5.4**). Interesting, [O-monoallyl phosphoryl MeBmt][1] CsA was also separated as a monodeprotected side product (Cs**21**). With the same method, both hydroxyl groups of [D-Ser][8]CsA could be phosphorylated and the diphosphorylated CsA (Cs**22**) was obtained in high yield. CsH was also phosphorylated (Cs**61**) in this way with high yield. A biotinlated phosphorylated CsA (Cs**23**) was also synthesized (Figure **5.5**) for affinity absorption on streptavidin bead in order t extract potential cellular receptor of Cs**18**.

5.3 CsA position 1 modifications and their inhibitory activities.

Besides phosphorylation, other chemical modifications such as oxidation, acetylation, sulfate conjugation were performed on the MeBmt[1] residue of CsA to investigate the particular role of phosphate moiety. The rh Cyp18 and CaN inhibitory activities of these compounds are measured.

5.3.1 [O-Phosphoryl MeBmt][1] CsA.

As expected (Table **5.1**), the interaction between CsA and Cyp18 is impaired dramatically by phosphorylation on MeBmt[1]. The compound inhibited half of the PPIase activity at a concentration as high as 10μM. It did not exhibit any CaN inhibition up to 10μM. Interestingly, the double

F101306'1 (4.465) Ba(73.2%)Sm(DF;4x8): (MS), ES+
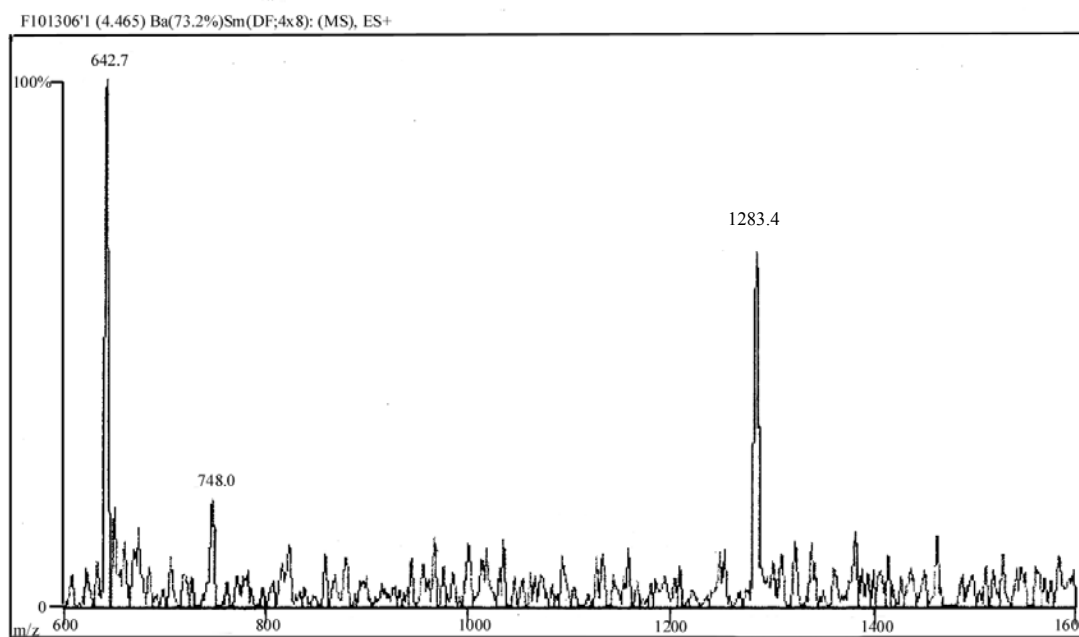
Figure 5.4 ESI Mass spectra of [O-phosphoryl MeBmt][1] CsA. The calculated mass is 1282.2.

R = OH, R' = OH

BrCH$_2$COOBu-t, 30% NaOH, r.t.

R = OH, R' = OCH$_2$COOBu-t

KOH/MeOH

R = OH, R' = OCH$_2$COOH

NH$_2$(CH$_2$)$_5$NHBoc, PyBoP, EDIPA

R = OH,
R' = OCH$_2$CONH(CH$_2$)$_5$NHBoc
i. 1-H Tetrazole, (i-Pr$_2$N)P(OAll)$_2$
ii. t-but-OOH

R = O-P(O)(OAll)$_2$,
R' = OCH$_2$CONH(CH$_2$)$_5$NHBoc

Pd(PPh$_3$)$_4$, butyl amine

R = O-P(O)(OH)$_2$,
R' = OCH$_2$CONH(CH$_2$)$_5$NHBoc

ZnCl$_2$/ether

R = O-P(O)(OH)$_2$,
R' = OCH$_2$CONH(CH$_2$)$_5$NH$_2$

Biotin

R = O-P(O)(OH)$_2$,
R' = OCH$_2$CONH(CH$_2$)$_5$NHBiotin

TBTU, Biotin, EDIPA

Figure 5.5. Synthesis of Biotinlated phosphorylated CsA

phosphorylated derivative Cs**22**, showed better Cyp18 inhibitory activity with an IC_{50} of 2μM. It also did not display any detectable CaN inhibition.

5.3.2 Oxidation.

We tried to oxidize the hydroxyl group of MeBmt[1] with Swern oxidation and Jones reagent. CsA was treated with oxalyl chloride and dimethyl sulfoxide. However, this reaction resulted in a mixture of unidentified products. [β-Oxo MeBmt][1] CsA (Cs**27**) was obtained in moderate yield by treating CsA with Jones reagent. As expected, this compound did not show Cyp18 inhibition at drug concentration of 1μM (Table **5.1**).

Table 5.1 Cyp18 and CaN inhibitory activity of CsA and its derivatives

	Cyclosporin Derivatives	IC_{50} of Cyp18 inhibition	IC_{50} of CaN Inhibition
CsA	Cyclosporin A	3.7nM	100nM
Cs**3**	[Phosphoryl-D-serine][8] CsA	5.0 nM	5μM
Cs**18**	[O-Phosphoryl MeBmt][1] Cs	10μM	NI[b]
Cs**19**	[O-Dimethylphosphoryl MeBmt][1] Cs	2.8μM	NI[b]
Cs**20**	[O-Diallylphosphoryl MeBmt][1] Cs	2.3μM	NI[b]
Cs**21**	[O-Monoallylphosphoryl MeBmt][1] Cs	0.46μM	NI[b]
Cs**22**	[O-Phosphoryl-D-Serine][8] [O-Phosphoryl MeBmt][1] Cs	2.9μM	NI[b]
Cs**25**	[1-Amino-2,6-octadienoic acid][1] Cs	251nM	1.1μM
Cs**26**	[O-Sulfonyl MeBmt][1] Cs	10μM	*
Cs**27**	[β-Oxo MeBmt][1] Cs	NI[a]	-
Cs**28**	[O-Acetyl MeBmt][1] Cs	NI[a]	NI[b]
Cs**31**	[O-Acetyl MeBmt][1] [N-methyl-D-Val][11] Cs	NI[a, c]	NI[b]
Cs**61**	[O-Phosphoryl MeBmt][1] [N-methyl-D-Val][11] Cs	10μM	-

* CaN inhibition activity by CsA sulfate conjugate is due to the trace amount of CsA from degradation.

NI: No Inhibition ([a] at 1μM; [b] at 10μM; [c] 20% inhibition at 10μM)

** The error in these measurement are less than 20%.

5.3.3 [O-Sulfonyl MeBmt][1] CsA

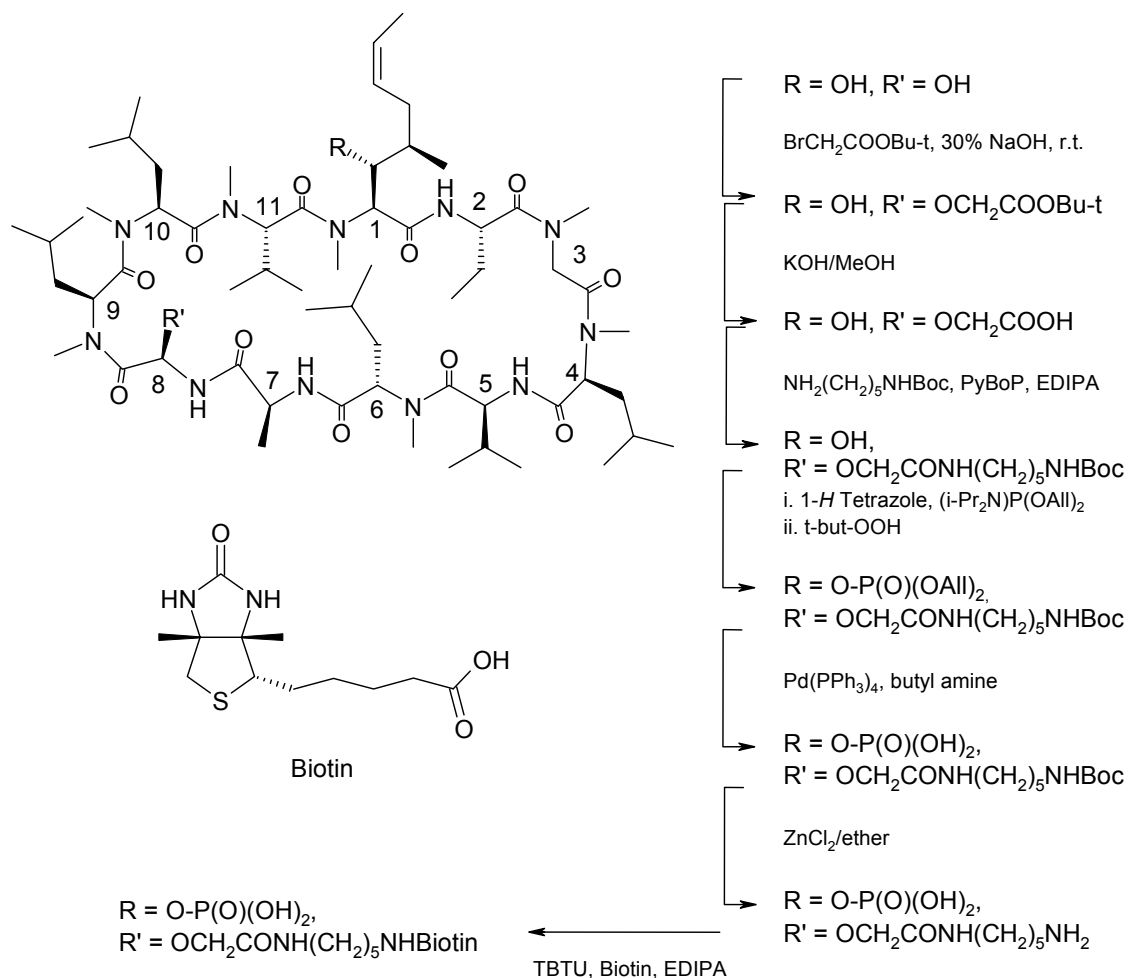Another identified metabolite of CsA with modification on residue 1 is a sulfate conjugate. The concentration of the [O-Sulfonyl MeBmt][1] Cs in plasma was estimated to exceed that of the parent compound by a factor of 50 (Johansson, 1990). The concentrations of [O-Sulfonyl MeBmt][1] Cs needed for immunosuppression *in vitro* exceed those of CsA about 1000 times, whereas the *in vivo*
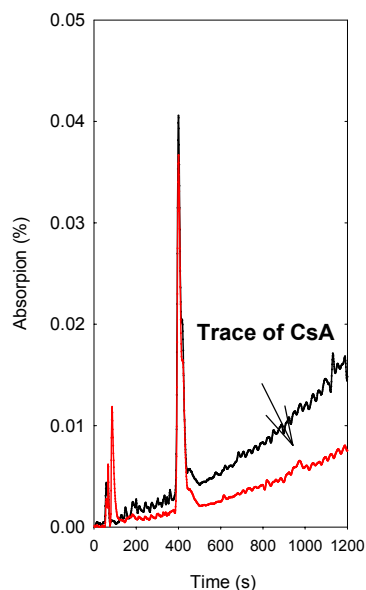
Figure 5.6 Cs**26** is not stable in DMSO at room temperature, as analyzed by HPLC after preparation (black) and after three days at room temperature (gray).
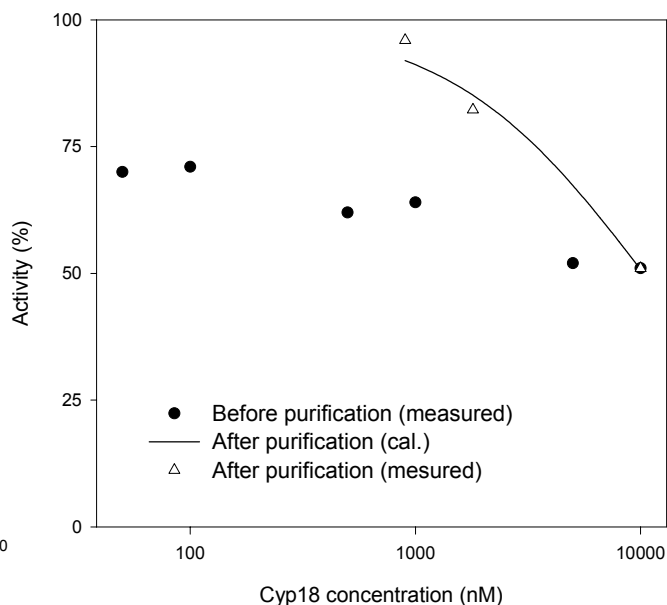
Figure 5.7 Purification of Cs**26** with Cyp18 affinity column. The sample before and after affinity column purification were analyzed using their inhibitory activities to Cyp18.

Inhibition of Pin1 PPIase activity by
phosphorylated cyclosporins and [O-Sulfonyl MeBmt][1] Cs
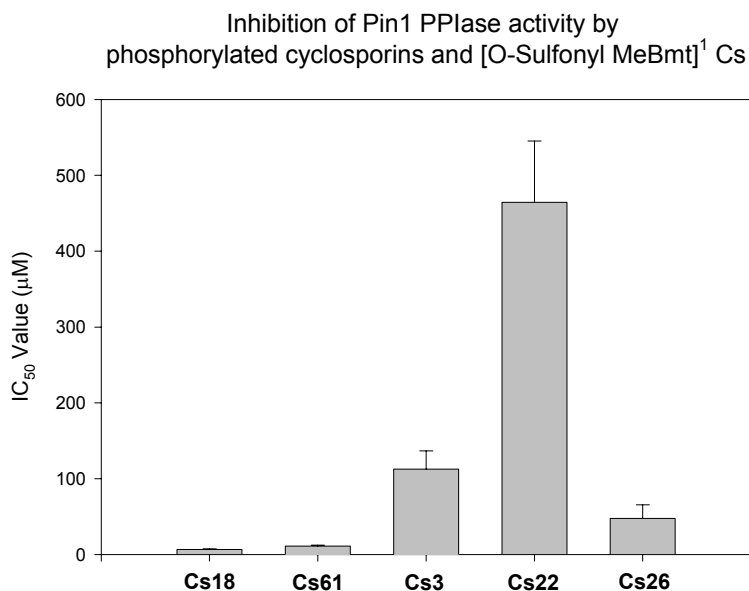
Figure 5.8 Pin1 inhibition by phosphorylated CsAs and . The Pin1 PPIase activity was measured with protease coupled assay and Ac-Ala-Ala-Ser(PO_3H_2)-Pro-Arg-NH-Np as substrate, in Hepes buffer pH 7.8, at 7^oC, in the presence of 2.7 nM Pin1 and inhibitor of desired concentration.

concentration of the metabolite are approximately 50 times higher than those of the parent drug (Johansson, 1990). However, due to the minor tendency of such a more polar compound to cross lipoprotein membrane, these results did not reflect the behavior of the metabolite that is produced in the cells. To identify if the role of this sulfate conjugate is distinct from CsA, it's necessary to study its Cyp and CaN inhibitory activity.

[O-Sulfonyl MeBmt][1] Cs was synthesized by refluxing CsA with three equivalent of SO_3-DMF complex in dry pyridine for 3 hours. The product was more hydrophilic than CsA and could be separated easily by reverse phase HPLC (Cs**26**). Then we measured its rh Cyp18 inhibitory activity. The sulfate conjugation also impairs the Cyp18 affinity significantly like phosphorylation and gave an IC_{50} value of 4.1 μM. However, the inhibitory activity of this compound showed distributed concentration dependence. At a concentration as low as 100nM it showed 30% inhibition of the PPIase activity (Figure **5.7**). One explanation could be that a minor portion of CsA is still remained after separation. However, further purification did not give different result. Then we checked the stability of this compound. The freshly prepared sample was analyzed by HPLC. After three days at room temperature in DMSO, a small peak with the same migration time as CsA was observed (Figure **5.6**). The sample was incubated with a rh Cyp18 affinity column and its Cyp18 inhibition was measured again (Figure **5.7**). Decreased inhibitory activity as well as the normal concentration dependence demonstrated that the sulfate conjugate is not as stable as other CsA residue 1 derivatives in DMSO.

5.3.4 Acetylation.

CsA was O-acetylated by Ac_2O in pyridine (Cs**28**). Acetylation of CsA (Elrouby, et al., 1992; Tullbergreinert, et al., 1991) impaired its Cyp18 binding affinity remarkably (Table **5.1**). Nonimmunosuppressive CsA analogues with or without Cyp18 inhibitory activity are used as negative controls in the study of Cyp inhibition, CaN inhibition, immunosuppressive activity as well as searching for new members of Cyp family protein or new cellular targets for the immunosuppressant. However, most of these compounds such as CsH showed weak Cyp18 inhibitory activity at high drug concentration. Further purification did not decrease the inhibitory activity. The IC_{50} value of CsH is around 2 to 3 μM from different preparations. There are two possibilities: i) less than 0.1% active component can not be identified and separated by HPLC or silica gel chromatography. ii) The compound itself exhibits activity at high concentration. To overcome this problem for CsH and obtain an excellent negative control, we acetylated CsH by Ac_2O in pyridine. If the IC_{50} observed for CsH is due to the weak affinity of CsH to Cyp18, changes in two critical amino acids (residue 1 and 11) in Cyp18 binding domain of CsA after CsH acetylation should abolish the affinity between the cyclosporin and its receptor. On the other hand, the trace amount of the active component (CsA) could also undergo the chemical modification and lose its activity (Scheme **5.1**). Indeed, [acetyl MeBmt][1] CsH showed only 5% Cyp18 inhibition at a

concentration as high as 10μM. Using this 'double inactivation strategy', we obtained an excellent negative control of CsA.

Scheme 5.1. A novel strategy for abolishing minor Cyp18 inhibitory activity of CsA derivative.

CsH $\xrightarrow{\text{Acetylation}}$ [O-acetyl MeBmt][1] CsH

+   +

CsA (Trace amount)   [O-acetyl MeBmt][1] CsA (Trace)
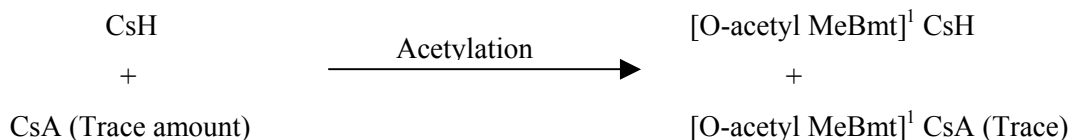
5.4 Pin1 inhibition.

Whereas cyclophilins and FKBPs isomerize unmodified Xaa-Pro bonds specifically, Pin1 recognizes sequence motifs containing a phosphorylated serine or threonine proceeding proline (Yaffe, et al., 1997; Schutkowski, et al., 1998). CsA has no any detectable inhibitory activity to Pin1. However, MeBmt[1] of CsA is a threonine analogue with a long unsaturated branch from its γ carbon. The N-methylated Val[11] on position –1 to MeBmt[1] mimics a proline residue. Therefore, CsA binds to Cyp18 in a reversed-chain manner. [Phosphoryl MeBmt][1] CsA is a potent Pin1 inhibitor with an IC_{50} value of 6.78μM. Surprisingly, the IC_{50} value of [Phosphoryl MeBmt][1] CsH to Pin1 is only a little higher. On the contrary, the [O-phosphoryl D-Ser][8] cyclosporin (Cs**3** in chapter 3), which contains a phosphorylated residue preceding the proline mimic N-methylated Leucine[9], inhibited Pin1 much less efficiently. The IC_{50} value of diphosphorylated [D-Ser][8] CsA (Cs**22**) is about 100-fold higher than [Phosphoryl MeBmt][1] CsA (Figure **5.8**). The sulfate group is a phosphate mimic in some respects. Cs**26** inhibited Pin1 with a moderate IC_{50} value of 47.7μM (Figure **5.8**).

A phosphorylated Ser/Thr-Pro motif is necessary but not sufficient for high Pin1 catalysis or inhibition (in following chapter). The IC_{50} value of [O-phosphoryl D-Ser][8] CsA is normal for phosphorylated peptides (Verdecia, et al., 2000). The stereochemistry of the residue following MeBmt[1] in a putative reverse orientation has not much influence on the binding because the [phosphoryl MeBmt][1] CsH has similar inhibitory activity to Pin1.

For a linear peptide, multiple phosphorylation could enhance its affinity to Pin1. In the case of CTD peptide YSPTSPS, the double phosphorylated form YpSPTpSPS displayed lower dissociation equilibrium constant to Pin1, Pin1 WW domain and Pin1 PPIase domain than its mono phosphorylated forms, YpSPTSPS or YSPTpSPS (Verdecia, 2000). Did the low affinity of double phosphorylated CsA (Cs**22**) to Pin1 indicate that multiple phosphorylation influences the interaction between Pin1 and a cyclopeptide in a manner different from its binding to a linear sequence?

We designed and synthesized phosphorylated cyclo-peptides to elucidate this question. Furthermore, cyclopeptides have attracted more and more interest in recent years because they are good candidates as inhibitors or drugs due to their proteolysis resistance.
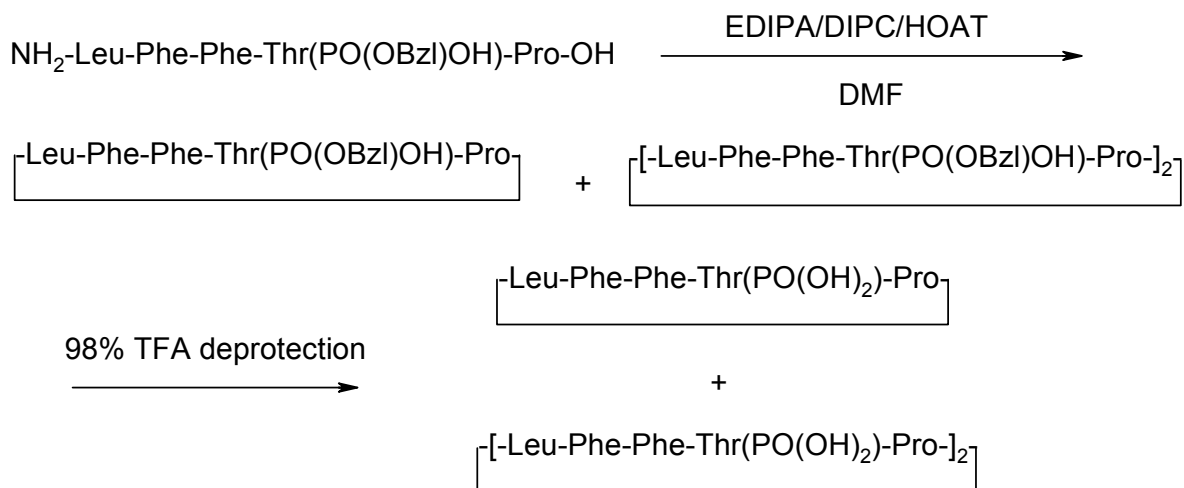
Cyclodecapeptide syntheses and their Pin1 inhibitory activities. Cyclization of penta or tetra peptides are normally less efficient due to the production of cyclic dimers. However, it makes that possible to synthesize a cyclodecapeptide or cyclooctapeptide from a penta peptide or tetra peptide directly. The cyclization of peptide NH_2-Leu-Phe-Phe-Thr(PO(OBzl)OH)-Pro-OH was performed with 4 equiv. EDIPA/DIPC/HOAT in DMF. Even in highly diluted solution (500μM) we got both the monomer cyc[Phe-Phe-Thr(PO(OBzl)OH)-Pro-Leu] and dimer cyc[Phe-Phe-Thr(PO(OBzl)OH)-Pro-Leu]$_2$. After deprotection in TFA and purification with HPLC, both peptides were obtained in moderate yields.

We measured the Pin1 inhibition by the diphosphorylated cyclodecapeptide. Phe-Phe-Thr(PO$_3$H$_2$)-Pro-Leu is a typical sequence for Pin1 catalysis (in following chapter). Cyclo[Phe-Phe-Thr(PO$_3$H$_2$)-Pro-Leu]$_2$ is a potent Pin1 inhibitor with an IC$_{50}$ values of 4.4 μM. The IC$_{50}$ of the cyclo-monomer was 18.2μM. Not like [O-Phosphoryl-D-Serine][8] [O-Phosphoryl MeBmt][1] Cs (Cs**22**), introducing two phosphate groups into the cyclopeptide did not result in low affinity. There are two possible explanations for the 100-fold decreased inhibitory activity of the double phosphorylated cyclosporin Cs**22**:

1. Pin 1 interacts with [phosphoryl MeBmt][1] CsA in a manner different from the CsA-Cyp18 complex and residue 8 is involved in the contacts. Phosphorylation on residue 8 impaired their binding affinity significantly.

2. Like in the CsA/Cyp18 complex, residue 8 of [Phosphoryl MeBmt][1] CsA is not involved in the protein/ligand interactions. However, double phosphorylation caused re-orientation of both side chain (residue 1 and 8) and/or a whole conformational change in the molecule.

In many cases, multiple phosphorylation enhances the interaction between a phosphoprotein or phosphopeptide and its target. However, the effects of phosphorylation are much subtler in some cases. For instance, the signaling from the phosphorylation of TCR does not behave as a simple on/off switch. Different phosphorylation statues could provoke any one of several different cellular responses, depending on the stage of differentiation of the T cell and on its prior antigen recognition history (reviewed by Alberola-Ila, et al., 1997). Phosphorylation of CsA on distinct residues also showed diverse influences on its Pin1 PPIase inhibitory activity. Monophosphorylation, especially on MeBmt[1], generated a potent Pin1 inhibitor. Double phosphorylation switched this effect off. This consequence is unique to cyclosporin. Although this does not likely happen naturally, it gives a novel example of intricate functional modulation by phosphorylation.

There are few drugs inhibiting their target proteins by a mechanism like CsA and FK506: both immunosuppressants inhibit CaN dependent on immunophilin binding. Interestingly, in the case of

NH$_2$-Leu-Phe-Phe-Thr(PO(OBzl)OH)-Pro-OH $\xrightarrow[\text{DMF}]{\text{EDIPA/DIPC/HOAT}}$

[Leu-Phe-Phe-Thr(PO(OBzl)OH)-Pro] + [-Leu-Phe-Phe-Thr(PO(OBzl)OH)-Pro-]$_2$

$\xrightarrow{\text{98\% TFA deprotection}}$

[Leu-Phe-Phe-Thr(PO(OH)$_2$)-Pro]

+

[-Leu-Phe-Phe-Thr(PO(OH)$_2$)-Pro-]$_2$

Scheme 5.2 Synthesis of phosphorylated cyclic peptide.
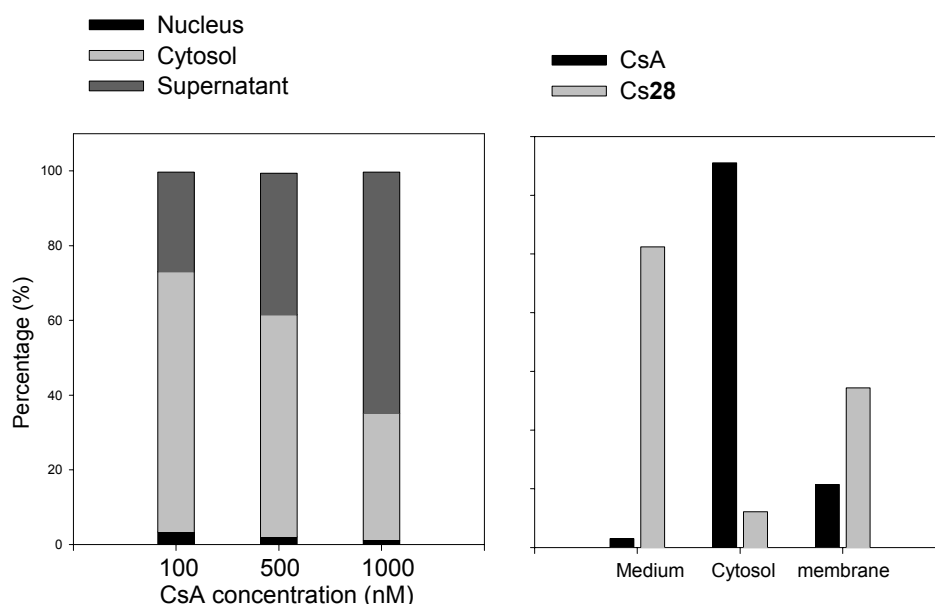
Figure 5.15 Concentration-dependent uptake of CsA by Jurkat T cell (left) and uptake of CsA and [phosphoryl MeBmt][1] CsA by human T cells. Rapid separation of particulate and soluble fractions of ^{3}H-CsA and [O-^{32}phosphoryl MeBmt][1] CsA-treated cells without redistribution was accomplished by a digitonin fractionation method. Cells were resuspended in Hank`s balanced salt solution at 1 x 10^6 cells/ml and ^{3}H-CsA (200,000 cpm) or [O-^{32}phosphoryl MeBmt][1] CsA (25,000 cpm) was added for up to 2 hour at 37°C. The cells were sedimented by centrifugation and the medium was analyzed for labeled CsA. Resuspension of cells was done in a fractionation medium containing either 20 µM digitonin (cytolsol release) or 0.1 % Triton X-100 (rupture of all membranes). Fractionated cell samples were layered on a density gradient and centrifuged. The top aqueous layers were counted and calculated as percentage of whole radioactivity.

[Phosphoryl MeBmt][1] CsA, it exhibits potent inhibition to another family of PPIase without fulfilling the general requirements for Pin1 binding. Homologs of human Pin1 (Lu et al., 1996; Maleszka et al., 1996) are present in *Drosophila* (Dodo) and the yeast Ess1/Ptf1 (Hanes et al., 1989; Hani et al., 1995, 1999). It was reported recently that Cyp18 becomes essential in yeast when Ess1 function is compromised. Overexpression of cyclophilin A suppresses *ess1* conditional and null mutations, and that Cyp18 enzyme activity is required for suppression. These results indicate that Cyp18 and Ess1 function in parallel pathways and act on common targets by a mechanism that requires prolyl isomerization (Wu, et al., 2000). The phenotype of the Cyp18 knockout mice suggested that the PPIase could play an important role in inmmune system (Colgan, et al. 2000a&b). Pin1 and Cyp belong to two distinct PPIase families. In parallel with blocking the CaN-NFAT pathway by CsA/Cyp complex, inhibition of Pin1 by a phosphorylated form of CsA suggests a new mechanism of the immunosuppression. The design and synthesis of Pin1 inhibitor will be discussed in the following chapter.

5.5 Stability of [Phosphoryl MeBmt][1] CsA (Cs18) and its analogues.

5.5.1 Membrane permeability of [Phosphoryl MeBmt][1] CsA.

The cell membrane permeability is another critical parameter for evaluating the immunosuppressive effect of [O-phosphoryl MeBmt][1] CsA because the acidic functional group may interfere the ligand molecule from cell entrance. Uptake and distribution of ^{3}H-CsA and [O-^{32}phosphoryl MeBmt][1] CsA were investigated.

The ^{3}H-CsA uptake was very rapid, and reached a steady state level within 30 min (data not shown). Jurkat T cells were incubated with different concentrations of ^{3}H-CsA for two hours. Rapid separation of cells without redistribution was accomplished by a digitonin fractionation method. The radioactivity of each fraction was then counted. Increasing the ^{3}H-CsA concentration, the percentage of CsA uptake by Jurkat T cells decreases. Cyp18 is the main cellular receptor of CsA. When cellular Cyp is saturated by the drug molecule, the percentage of CsA uptake by T cells reduces significantly. As shown in figure **5.9**, CsA locates mainly in the cytosol. At 100nM drug concentration, about 70% of the drug was found in cytosol. Only 35% of ^{3}H-CsA was recovered from cytosol at a drug concentration of 1000nM.

[O-^{32}phosphoryl MeBmt][1] CsA was generated by protein kinase phosphorylation (data not shown). Due to its acidic functional group and low Cyp binding affinity, the uptake and cellular distribution of [O-^{32}phosphoryl MeBmt][1] CsA (Cs**18**) is different from its parent drug. (Figure **5.9**). The cell membrane permeability of Cs**18** is lower than CsA. Whereas most ^{3}H-CsA is found in cytosol fraction, Cs**18** was recovered mainly from the cell membrane. Further work must be done to clarify if the membrane association of Cs**18** is due to the amphipathic property of the ligand molecule or because of the existence of receptors on cell membrane.
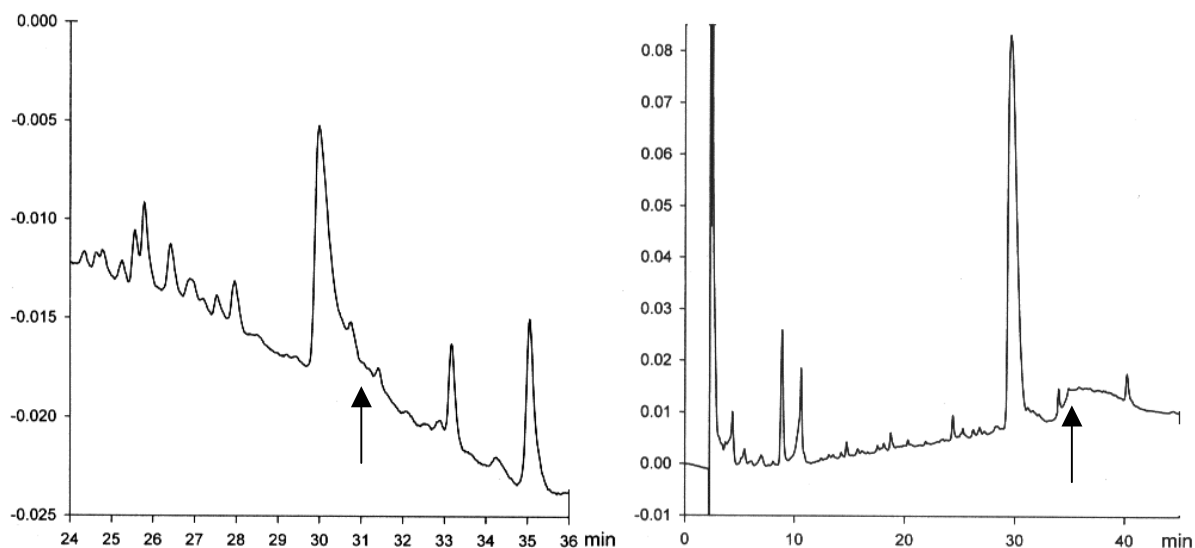
Figure 5.10 HPLC analysis of phosphorylated CsA in Jurkat T cells cytosol (A) and the supernatant (B). A. Jurkat T-Cells were incubated for five hours with Cs**18**. The T cells were stimulated by addition of phorbol ester. After three hours, acetonitrile/ethanol precipitation and lyophilization were performed. B. Jurkat T-Cells were incubated for five hours with Cs**18**. The T cells were stimulated by addition of phorbol ester. After two hours, acetonitrile/ethanol precipitation, lyophilization, resuspendation in 200mM NaAc (pH = 4) and ethyl acetate extraction were performed. These results were confirmed by rh Cyp18 inhibitory activity assay of different HPLC fractions. (The arrows indicate the retention time of CsA)
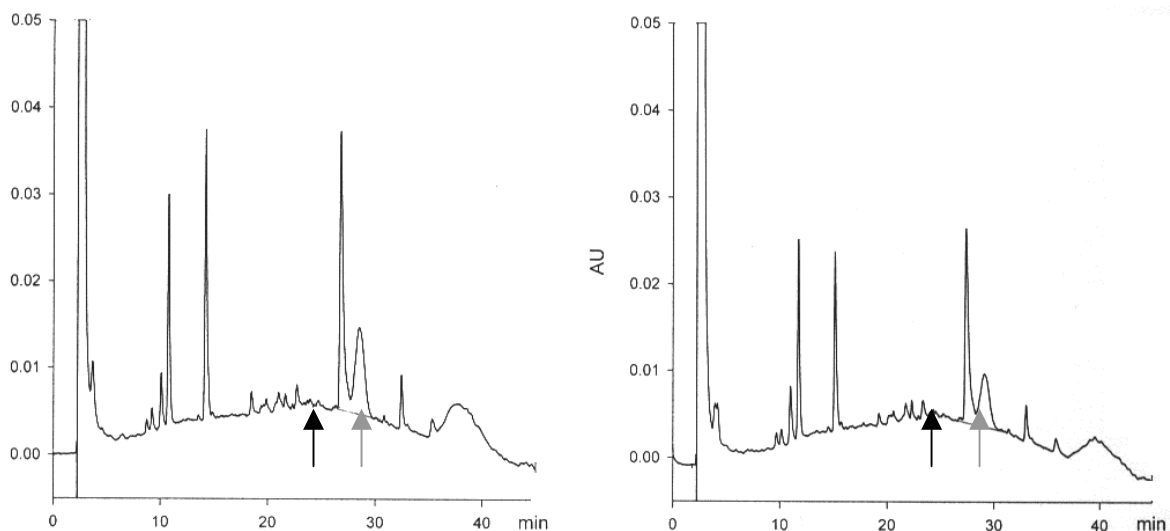
Figure 5.11. HPLC analysis of [O-sulfonyl MeBmt][1] Cs (Cs**26**) in Jurkat T cell cytosol. Phorbol ester stimulated cells were incubated with Cs**26** for different time, followed by acetonitrile/ethanol precipitation, ethyl acetate extraction and lyophilization. A. One hour after stimulation. B. Three hours after stimulation. (The black arrows indicate the retention time of CsA sulfate conjugate, and the gray arrows indicate the retention of CsA).

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5.5.2 Stability of Cs18 and Cs26.

To exclude the possibility that any suspected activity of phosphorylated CsA (Cs**18**) is caused by dephosphorylation by cellular phosphatases, the stability of the CsA analogues were examined. Jurkat T cells were incubated with CsA or its analogues ([O-phosphoryl MeBmt][1] Cs (Cs**18**), [O-dimethylphosphoryl MeBmt][1] Cs (Cs**19**), [O-Sulfonyl MeBmt][1] Cs (Cs**26**), [O-Acetyl MeBmt][1] Cs (Cs**28**)). With or without phorbol ester stimulation, HPLC analyses of supernatant and cytosol fraction were performed. No dephosphorylation of Cs**18** was observed in these experiments (Figure **5.10**). These results were confirmed by measuring the Cyp18 inhibitory activities of different HPLC fractions (data not shown). In human lymphocytes, the [O-phosphoryl MeBmt][1] CsA (Cs**18**), displays similar stability (data not shown). [O-Dimethylphosphoryl MeBmt][1] CsA and [O-acetyl MeBmt][1] CsA are also stable in T cells. Like in DMSO solution at room temperature, the CsA sulfate conjugate (Cs**26**) is less stable than other CsA derivatives in cells (Figure **5.11**). In stimulated (phorbol ester) as well as unstimulated Jurkat T cells, Cs**26** undergoes degradation. CsA was identified from the Cs**26** treated cells, as determined by HPLC and MS spectroscopy. Considering the high concentration of Cs**26** in body (Johansson, 1990), the sulfate conjugate (Cs**26**) production in cells could be a deposition pathway of CsA metabolite. However, its low stability also indicates that it is in a partially reversible equilibrium with its parent drug.

5.6 Conclusion

CsA was phosphorylated chemically at position 1. It is stable in stimulated T cells and exhibits low cell membrane permeability. As expected, the modification results in a dramatic decrease of either Cyp18 or CaN inhibitory activity. Furthermore, the phosphorylated CsA (Cs**18**) displays potent Pin1 inhibitory activity. Further work will be done to elucidate the biological effects of this metabolite in lymphocytes.

Chapter 6. Design of linear peptidic Pin1 Inhibitors

6.1 Inhibitors for Cyp, FKBP, and parvulins.

The isomerase activity of human parvulin Pin1 is essential for normal cell growth and this function seems to be a target for anticancer drug development because depletion of Pin1 induces mitotic arrest followed by apoptosis (Zhou, et al., 1999, Lu, et al., 1996).

6.1.1 A short review of inhibitors for Cyp and FKBP.

Members of both the cyclophilin and the FKBP-type PPIase family had proved to be valuable drug targets. A variety of natural products were found to bind and reversibly inhibits such PPIases with K_i values down to the low nanomolar range. Examples are cyclosporin A (Fischer et al., 1989; Takahashi et al., 1989), Sanglifehrin (Sanglier et al., 1999; Fehr et al., 1999) for cyclophilin and FK506 (Siekierka et al. 1989; Harding et al., 1989), ascomycin (Petros et al., 1991), rapamycin (Bierer et al., 1990a & b), meridamycin (Salituro et al., 1995), and cycloheximide (Christner et al., 1999) for FKBP, respectively. CsA, FK506 and rapamycin are of outstanding importance as immunosuppressive drugs in clinical use.

Additionally, various designed inhibitors of PPIase activity are known. Nonmacrocyclic compounds containing the minimal FKBP12-binding elements of FK506 were prepared and are capable of binding to FKBP12 with low nanomolar affinity (Teague, et al., 1993; Teague, et al. 1994; Armistead, et al., 1995; Tatlock., et al., 1995). For N-(glyoxy) pipecolyl esters (Holt, et al., 1993; Holt, et al. 1994) and N-(glyoxy) prolyl esters, low nanomolar to picomolar affinities to FKBP were reported. Replacement of the diketo portion of FK506 with other functionalities, such as sulfonamide (Holt, et. al., 1994), urea (Dragovich, et al., 1996) or *(E)*-alkene (Andres et al., 1993) resulted in compounds with inhibition constants in the low micromolar range. In general, for FKBPs peptidic inhibitors are less potent than N-(glyoxy)pipecolyl derivatives (Albers, 1990, Hauske, et al., 1992). In the case of cyclophilins several peptide bond surrogates were used to generate peptide derived inhibitors like fluoroolefins (Boros et al., 1994), *(Z)*-alkenes (Hart and Etzkorn, 1999), and thioxo peptides (Schutkowski et al., 1995). However, the estimated K_i values were more than 1000-fold higher than that of CsA. A more general principle of inhibition was discovered mapping the stereospecificity of PPIases (Schiene et al., 1998). It was demonstrated that a substrate derived diastereomer containing a D-amino acid residue preceding proline represents a compettitive inhibitor for various PPIases with substrate like affinities.

6.1.2 Pin1 inhibitors.

In contrast to the various inhibitors of cyclophilins and FKBPs, for a long time no inhibitor was known for the parvulins. Recently it was discovered that juglone, 5-hydroxy-1, 4-naphthoquinone, can irreversibly inhibit the enzymatic activity of several parvulins such as the E. coli parvulin 10,
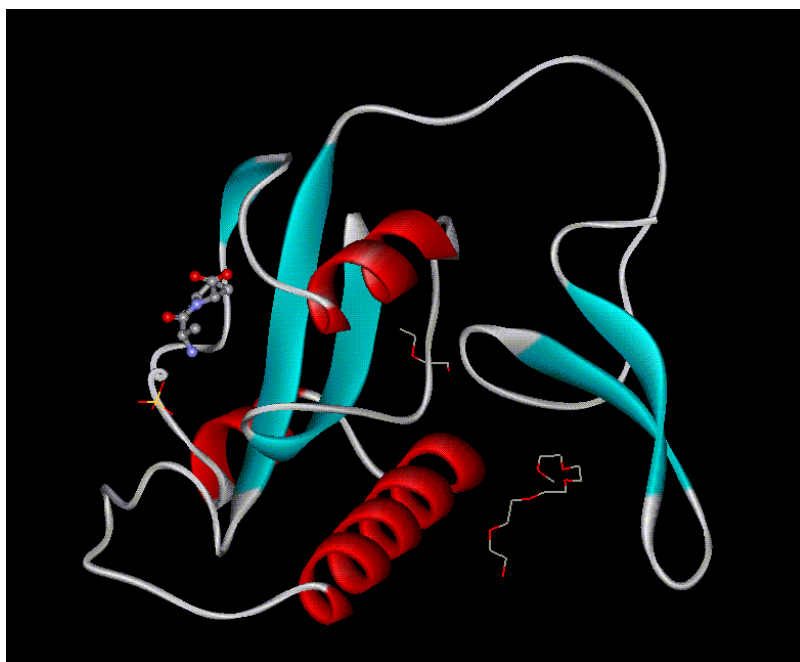
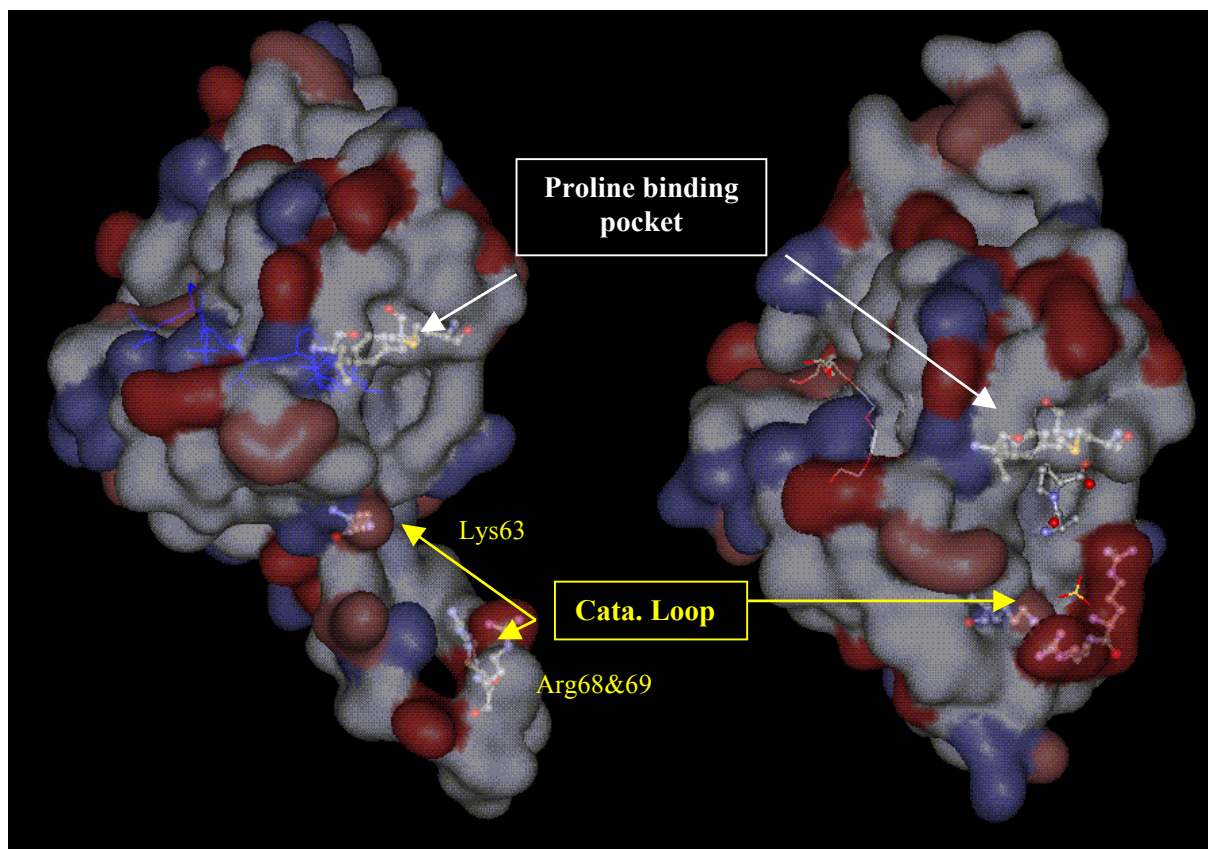**Figure 6.1** The crystal structure of Pin1 bound to Ala-Pro.

Figure 6.2 The different conformation in the catalytic loop of the Pin1-dipeptide (right) and Pin1-CTD peptide (left) structures.

the yeast ESS1/ptf1, and human Pin1 by binding two juglone molecules to the side chains of Cys41 and Cys69 covalently. However, partial unfolding of the active site was assumed to be the reason for the deterioration of PPIase activity (Hennig et al., 1998, Rippmann et al., 2000).

In chapter 5, the finding that [O-phosphoryl MeBmt][1] CsA as a Pin1 inhibitor suggested a possible relationship between Pin1 inhibition and the phosphorylation state of the cyclic peptide. Interestingly, phosphorylation on the MeBmt[1], but not on the D-Ser[8], conferred the ligand potent Pin1 binding affinity. The structure-activity relationship of Pin1 and its substrates as well as the biological and pharmaceutical significance of Pin1 inhibitors prompted us to study the minimal requirements for Pin1 catalysis and design reversible Pin1 inhibitors.

6.2 Bimolecular character of Pin1

6.2.1 WW domains.

Pin1 contains an amino-terminal WW domain, residues 1-39, and a carboxy-terminal PPIase domain, residues 45-163. The WW domain is a 38–40 amino acid structural motif that functions as an interaction module in a diverse set of signaling proteins. There are five distinct groups of WW domains, classified basing upon current understanding of their binding specificity. Group I WW domains, like those found in dystrophin and YAP65 (Yes kinase-associated protein), recognize 'PPxY' motifs (Chen, et al., 1997), where x is any amino acid. Group II WW domains, like that of FE65, bind the 'PPLP' motif (Ermekova, et al., 1997). Group III WW domains, such as those found in a subset of FBPs, interact with 'PGM' motifs (Bedford, 1998). A fourth group, which includes the WW domain of Pin1, specifically interacts with phospho-Ser/Thr-Pro motifs (Lu, et al., 1999b). The group V WW domains, including the WW domain of FBP30, recognize Pro-Arg motifs (Komuro, et al., 1999, Beford, et al., 2000). Recently, a combinatorial library of group I WW domain was constructed by SPOT synthesis on a cellulose membrane to investigate the structure-function relationship of this domain (Toepert, et al., 2001).

The Pin1 WW domain functions (Zhou et al., 1999) as a phosphoserine- or phosphothreonine binding module and mediates Pin1 interaction with most substrates (Lu, et al., 1999b). Of particular interest is the C-terminal domain (CTD) of RNA Pol II, which in humans consists of 23 tandem repeats of the sequence YSPTSPS. Pol II's behaviors during different stages of transcription are controlled by CTD phosphorylation and dephosphorylation. WW domain of Pin1 interacts specifically with the hyperphosphorylated CTD of Pol II (Morris et al, 1999). Pin1 was shown to be active in restoration of the microtubule-binding activity of Tau, a protein of neurofibrillar tangles found in the brains of Alzheimer's patients. The WW domain mediates Pin1 binding to the pT231 sequence of tau protein (Lu, et al., 1999a).

6.2.2 PPIase domain.

Like the WW domain, PPIase domain of Pin1 also prefers an acidic residue preceding proline. Pin1 has been shown to be a phosphorylation-dependent PPIase that specifically recognises the phosphoserine/threonine-proline bonds presenting in mitotic phosphoproteins (Yaffe, et al., 1997, Schutkowski, et al., 1998). Phosphorylation affected the rate of the *cis/trans* isomerization of the Ser/Thr-Pro peptide bonds. As determined by a protease-coupled assay, the isomerization rate of phosphorylated Thr-Pro bond was found to be 8-fold slower than that of the nonphosphorylated analogue. These effects are not simply due to the negatively charged side chain since neither phosphotyrosine nor the widely used phosphorylated Ser/Thr mimic glutamic acid is able to influence the isomerization in the same manner. Pin1 preferentially isomerized the phosphorylated Thr/Ser-Pro bond with up to 1300-fold higher selectivity compared to unphosphorylated peptide substrates.

Two X-ray crystal structures of Pin1, one with a dipeptide presented in the PPIase domain (Ranganathan, et al., 1997) (Figure **6.1**) and one with a CTD peptide associated on the WW domain (Verdecia, et al., 2000), provided us information about how this two-domain architecture assembles a platform on which peptide specificities are built.

6.2.3 Structure of PPIase domain and WW domain.

The structural basis for Pin1 substrate specificity has been investigated by molecular model-building basing on the Pin1-dipeptide crystal structure (Yaffe, et al., 1997) as determined by Ranganathan et al., (1997). Using the optimal WFYpSPR peptide substrate, the model showed that R68 and R69 of Pin1 coordinate the phospho-serine phosphate, a hydrophobic groove accepts the preceding aromatic tripeptide, and the side chain of C113 and H59 coordinate the isomerizing phospho-Ser-Pro peptide bond with an angle of 90°, stabilizing the transition state between the *cis* and *trans* conformation. Substitution of both R68 and R69 by Ala resulted in reduced the k_{cat}/K_m value for the phosphorylated substrate by a factor of about 500. Recent study suggests that the side chain of C113 is required for the catalytic reaction per se, whereas the two basic residues R68 and R69 coordinate the binding of the phosphorylated substrate, with R69 being more important than R68 (Rippmann, et al., 2000). In *E. coli* parvulin, R68 and R69 of Pin1 are replaced by Glu; this enzyme failed to catalyze the isomerization of the phosphorylated Pin1 substrate, although it can catalyze Pro isomerization in unphosphorylated peptides. H59 appears to play an important role in catalyzing Pro isomerization or in binding the substrate Pro residue. Replacement of the critical H59 residue of Pin1 with Ala markedly reduced PPIase activity toward both phosphorylated and unphosphorylated peptides, however, the preference for phosphorylated over unphosphorylated substrates was unchanged.

Comparing the Pin1-dipeptide structure, the crystal structure of Pin1 bound to Tyr-pSer-Pro-Thr-pSer-Pro-Ser (CTD peptide) by its WW domain exhibits two significant differences (Figure **6.2**):

I. The extended CTD peptide contacts to the concave of WW domain by multiple hydrogen bonds and hydrophobic interactions. An exaggerated twist in the triple-stranded antiparallel β-sheet of WW domain was observed due to the binding.

II. In the PPIase catalytic site of Pin1, two sets of residues determine the substrate specificity. In both crystal structure, no significant difference was observed in the main hydrophobic binding pocket for proline pyrrolidine, which is defined by Leu122, Met130, and Phe134. The phosphate binding site, which is defined by Lys63, Arg68, and Arg69 and thought to be the catalytic loop, is occupied by a sulfate moiety in the Ala-Pro/Pin1 structure and the amide bond of the dipeptide adopts a *cis* conformation. However, this pocket disappear in the Pin1-CTD complex structure. The distance among these residues, either the α carbons or the side chains, increase remarkably (Figure **6.2**). Furthermore, the distance between this loop and the proline binding pocket also increases. The catalytic loop is in an open conformation in the Pin1-CTD complex. Does this reflect the conformation of the catalytic loop in the absence of a substrate binding to the active site, or it is switched upon ligand binding to the WW domain?

From the crystal structure of human Pin1, a mechanism including acid-base catalysis and catalysis *via* a covalent intermediate was suggested (Ranganathan et al., 1997). However, are Ala-Pro or phosphorylated Thr/Ser-Pro the minimal substrates for Pin1 PPIase activity? Because the normal PPIase assays can not be applied for examining short peptides, we wonder if the closed loop conformation in Ala-Pro/Pin1 complex and the *cis* peptide bond represents the structure basis for understanding the binding events of Pin1 to its phosphorylated substrates.

6.2.4 Comparison of proline binding pockets

Structural analysis of the active site of different PPIases does not reveal conserved residues or motifs (Fischer, 1994; Ranganathan, et al., 1997). However, a similarity of the environments around the peptidyl-prolyl bonds could be found in the Cyp18/Ala-Pro, FKBP12/FK506, and Pin/Ala-Pro structures. Rotation around the imide bond is the central event being concerned. We introduced a vertical plane across the imide bond and divide this motif into two regions. In the ring binding regions, hydrophobic residues (Figure **6.4** the right figures) form a pocket to hold the ring (Phe112 and Leu121 in Cyp18; Phe46, and Trp59 in FKBP12, and Phe134 and Leu122 in Pin1) (Figure **6.4** the left figures). In the carbonyl regions, the environments are relatively hydrophilic (Figure **6.4** the right figures) and the amino acids that are thought to be good candidate for catalysis are found around the plane or in the carbonyl region. For instance, the His125 in Cyp18, the Phe36 and Asp37 in FKBP (reviewed by Fischer, 1994), the Cyc113 and His59 in Pin1 (Ranganathan, et al., 1997) point toward this section (Figure **6.4** the right figures). These observations do not provide a chemical mechanism for the catalytic activity of these enzymes. However, it shows the scaffold of a local environment which enzyme catalyses base on.
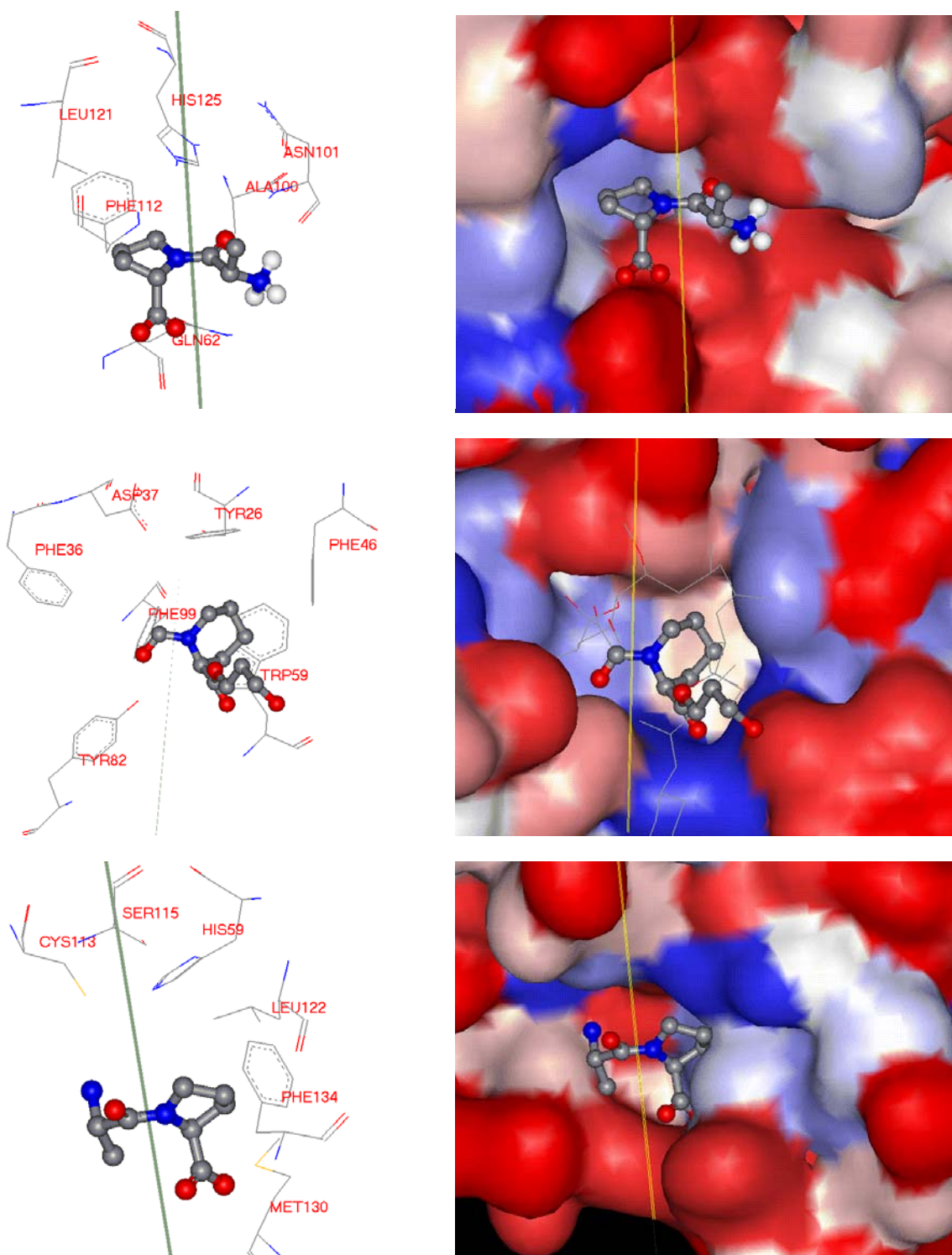
Figure 6.4 The similarity of the proline-binding pockets in the Cyp18/Ala-Pro (up), FKBP12/FK506 (middle), and Pin1/Ala-Pro structures (down). A vertical plane was introduced to across the imide bond and separates the binding pocket into two regions, the ring binding region and the carbonyl region. Hydrophobic residues form the ring binding regions, and the environments around the carbonyl regions are more hydrophilic. Residues, which are thought to be good candidate for catalysis could be found in the carbonyl region or around the plane (left up, left middle, left down). Only residues that are close to the imide bonds were displayed. Ligands are shown as balls and sticks. For FK506, only the atoms around the pipecolinyl ring were shown in balls and sticks. The surface of proteins are colored according to their hydrophobicity (red: hydrophilic, blue: hydrophobic)

We used a sensitive assay (S. Füssel, Ph.D. thesis) to investigate if short peptides like Ala-Pro or phospho-Ser-Pro are substrates for Pin1. As shown in this chapter, phosphoSer-Pro as well as Ala-Pro are not a substrates for Pin1, but are weak inhibitors. Together with the information from those structural studies, these results would provide us a new insight into the catalysis mechanism of Pin1 as well as its substrate specificity.

6.3 The minimal requirement for Pin1 catalysis.

Generally, long peptides are not good candidates as drugs or inhibitors because they themselves are immunogens and can be digested by various proteases. To circumvent these disadvantages, small molecular ligands are discovered from nature resources, by screening combinatorial libraries as well as by rational design. However, the interaction between the protein and its cellular targets provides us the primary clue for drug discovery. A strategy for searching potent inhibitors of Pin1is summarized in Scheme **6.1**.

Scheme 6.1
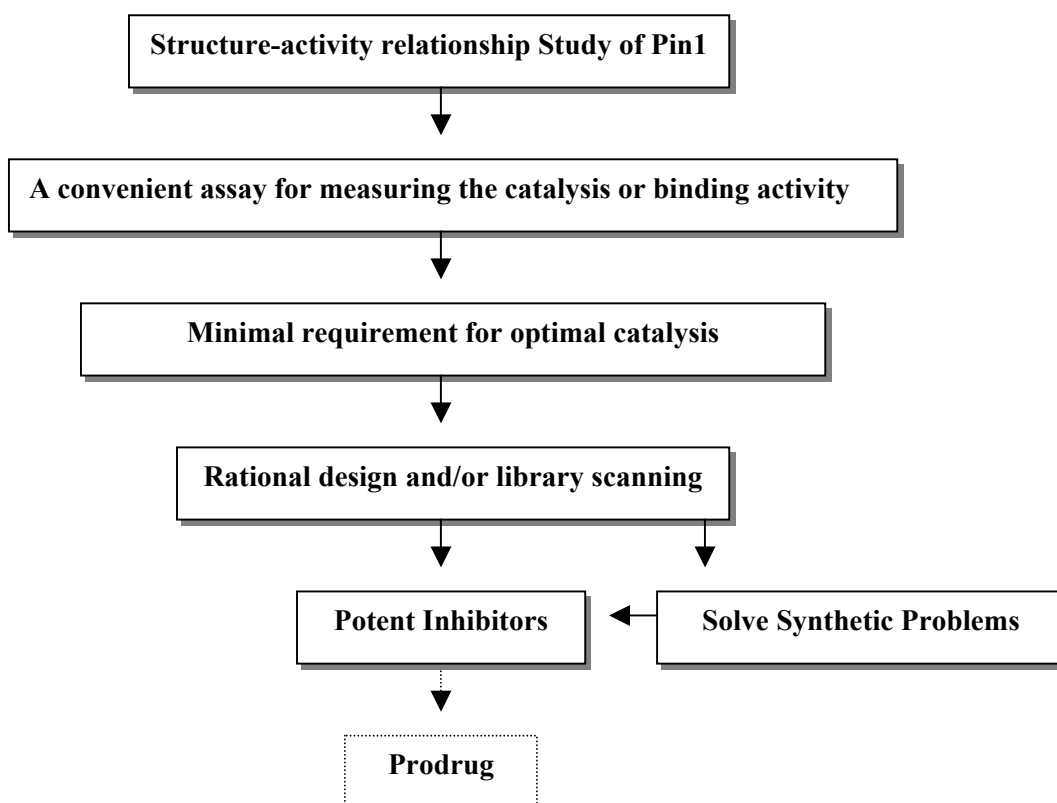
6.3.1. The assay for measuring *cis/trans* isomerization of short peptide.

The methods of measuring PPIase activity are often based on the production of pure *cis* or *trans* isomers or at least an isomeric distribution different from that expected based on the isomerization reaction condition (for example, in another solvent system, or buffer of different pH value). This

allows investigation of the kinetics of the reestablishment of equilibrium and the rate of the *cis-trans* isomerization. The standard protease-coupled assay as well as the newly developed protease-free spectrophotometric and fluorometric assays are restricted to substrates equipped with artificial moieties (Kofron et al., 1991; Garcia-Echeverria et al., 1993; Janowski et al., 1997). This prevents the *in vitro* characterisation of short peptide and naturally occurring PPIase substrates.

A panel of dipeptides was used (Füssel, Susanne Ph.D. thesis) to analyze the minimal structural requirements for efficient catalysis by the active site of Pin1. Based on the different absorption coefficients for the *cis* and *trans* conformers of Xaa-Pro peptide bonds, peptidyl prolyl bond isomerization could be followed directly at 210 or 215 or 220 nm dependent on the largest absorbance change during the interconversion (data does not shown). This novel sensitive assay allowed the determination of specificity constants ($k_{cat}/K_m > 5$ mM^{-1} s^{-1}) for the Pin1 mediated catalysis of the solvent or pH induced re-equilibration of the *cis/trans* isomers (Füssel, Susanne Ph.D. thesis).

6.3.2 The minimal requirement for optimal Pin1 catalysis.

Using this assay we were able to demonstrate that dipeptides like Ala-Pro, Ser-Pro, and even Ser(PO$_3$H$_2$)-Pro are poor reversible inhibitors for Pin1. In contrast, the addition of one amino acid residue to Ser(PO$_3$H$_2$)-Pro switches the inhibitor to a substrate with k_{cat}/K_m values of 100 mM^{-1}s^{-1} for Ala-Ser(PO$_3$H$_2$)-Pro and 38 mM^{-1}s^{-1} for Ser(PO$_3$H$_2$)-Pro-Arg. The combination of these sequences in Ala-Ser(PO$_3$H$_2$)-Pro-Arg further increases the specificity constant for Pin1 (270 mM^{-1}s^{-1}). With a k_{cat}/K_m value of 9300 mM^{-1}s^{-1} Ac-Ala-Ala-Ser(PO$_3$H$_2$)-Pro-Arg-NH-Np indicates an extended subsite specificity ranging at least from P3- to P3`-position. Moreover, we were able to demonstrate a very similar requirement for secondary binding sites in the case of the yeast parvulin Ptf1.

One reason for this effect could be the shift of the terminal charges of the isomerizing prolyl bond. On the other hand the fixation of the extended peptide backbone by the formation of hydrogen bonds to the enzyme molecule could be discussed. The importance of extended binding sites of other enzymes recognizing peptide backbones is illustrated by proline-specific peptidases (Yaron, 1970; Walter, 1978; Walter, 1980; Yoshimito, 1980; Yoshimito, 1982; Yaron, 1993). In the case of prolyl oligopeptidase an amide bond between P2 and P3 is necessary for switching a competitive inhibitor to a substrate. This finding was underlined by the recognition of appropriate hydrogen bonds between the substrate peptide bonds and prolyl oligopeptidase in the crystal structure (Fulop et al., 1998). Additionally, other phosphopeptide binding enzymes use ligand backbone interactions resulting in highly favorable binding (Kimber et al., 2000; Yaffe et al., 1997)

While the catalytic efficiency increases in the same extent as the affinity to the active site by prolongation of the dipeptide to the tripeptide, additional elongation seems also affect the turnover

number. Therefore the conclusion can be drawn that the full catalytic power of the Pin1 is performed only when three preconditions are fulfilled:

First: The pyrrolidine ring of proline has to be present for interactions with the hydrophobic binding pocket defined by Leu122, Met130 and Phe134.

Second: The phosphorylated Ser/Thr residue preceding proline is necessary to generate a main portion of the appropriate affinity to the active site by interaction with the triad of basic side chains consisting of Lys63, Arg68 and Arg69.

Third: A minimum of three-residue peptide backbone is needed to enable productive interactions with Pin1 active site.

6.4 Synthesis of substrates and inhibitors.

The phosphopeptides Ser(PO_3H_2)-Pro, Ala-Ser(PO_3H_2)-Pro, Ala-Ser(PO_3H_2)-Pro-Arg were synthesized by solid phase peptide synthesis using chlorotritylchloride-resin, TBTU and HOBt as coupling reagents, Fmoc-Ser(PO(OBzl)-OH)-OH as phosphoamino acid building block and DMF as reaction solvent. D-Ser(PO_3H_2)-Pro and Ac-Ala-Ala-D-Ser(PO_3H_2)-Pro-Arg-NH-Np were synthesized using Fmoc-D-Ser(P(O)(OBzl)$_2$ as phosphoamino acid building block and PyBOP as coupling reagent. After detachment of the peptides from the resin and side-chain deprotection with TFA, these phosphopeptide derivatives were purified by HPLC. Ac-Ala-Ala-D-Ser-Pro-Leu-NH-Np was phosphorylated by (i-Pr$_2$N)P(OAll)$_2$/tetrazole in THF and oxidized by *tert*-butyl hydroperoxide at room temperature. Deprotection of allyl ester was performed in THF with tetrakis(triphenylphosphine)palladium/butyl amine/formic acid at 50°C.

Allyl, *tert*-butyl and benzyl groups are commonly used to protect the phosphate moiety in the chemical phosphorylation. Allyl group can be deprotected by palladium catalyst. Benzyl and *tert*-butyl groups can be removed under acidolytic deprotection condition in TFA. Thioxo amide bond is very sensitive to acidic condition. In the presence of trace amount of TFA in solution, thioxo peptides showed low stability at room temperature. Beside dethioxylation, thioxylated products often undergo a side reaction similar to the Edman degradation. The sulfur atom could cause toxicity to palladium catalyst. These disadvantages prevent the usage of these well-established phosphorylation methodologies. Lewis acids as SnCl$_4$ and ZnCl$_2$ in organic solvents act as extremely mild reagents for Boc deprotection even in the presence of the acid-labile thioxo amide moiety with excellent yield (Wildemann et al., 1999; Frank, et al., 1996). Mg(ClO)$_4$ deprotects Bpoc/Ddz during solid phase peptide synthesis of thioxo peptides.

We used Lewis acid as deprotection reagent in the synthesis of phosphorylated thioxo peptides (Scheme **6.2**). Fmoc-Phe-Ser-Ψ[CS-N]-Pro-Phe-NH-Np was phosphorylated using (i-Pr$_2$N)P(OBzl)$_2$/tetrazole in THF at room temperature. To prevent oxidation of thioxo amide bond, phosphite was treated with *tert*-butyl hydroperoxide under –15°C for 20 min. HPLC and mass spectroscopic analyses indicated that the oxidation of phosphite was complete and no dethioxylation

Scheme 6.2 Synthesis of Thioxo phosphorylated Pin1 inhibitor.

Fmoc-Ser(t-Bu)-OH $\xrightarrow{\text{1. NMM, i-BuOCOCl}}$ Fmoc-Ser(Bu-t)-NH... $\xrightarrow{P_4S_{10}, \text{THF}}$

H-Pro-Phe-NH-Np $\longrightarrow$ Fmoc-Ser(t-Bu)-Ψ[CS-N]-Pro-Phe-NH-Np $\xrightarrow{\text{Morpholine}}$

$\xrightarrow[\text{Fmoc-Phe-OH}]{\text{TBTU, DIPEA}}$ Fmoc-Phe-Ser(t-Bu)-Ψ[CS-N]-Pro-Phe-NH-Np $\xrightarrow{\text{ZnCl}_2/\text{ether complex}}$

Fmoc-Phe-Ser-Ψ[CS-N]-Pro-Phe-NH-Np $\xrightarrow{\text{1-}H \text{ tatrazole, i-Pr}_2\text{NP(O)(OBzl)}_2}$ $\xrightarrow{\text{t-Bu-OOH, -15°C}}$

Fmoc-Phe-Ser(P(O)(OBzl)$_2$)-Ψ[CS-N]-Pro-Phe-NH-Np $\xrightarrow{\text{ZnCl}_2}$ $\xrightarrow{\text{Morpholine}}$

H-Phe-Ser(P(O)(OH)$_2$)-Ψ[CS-N]-Pro-Phe-NH-Np
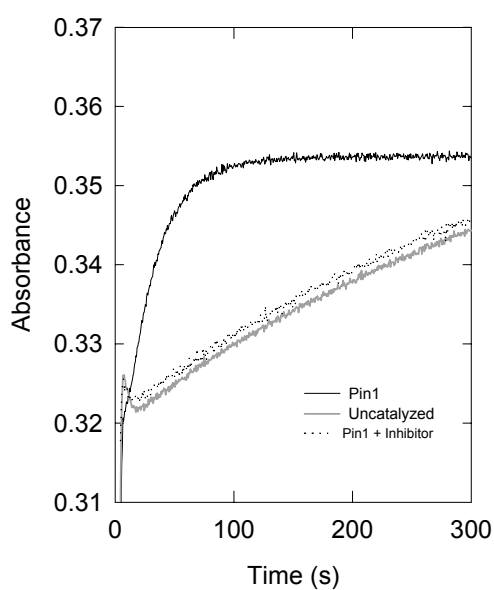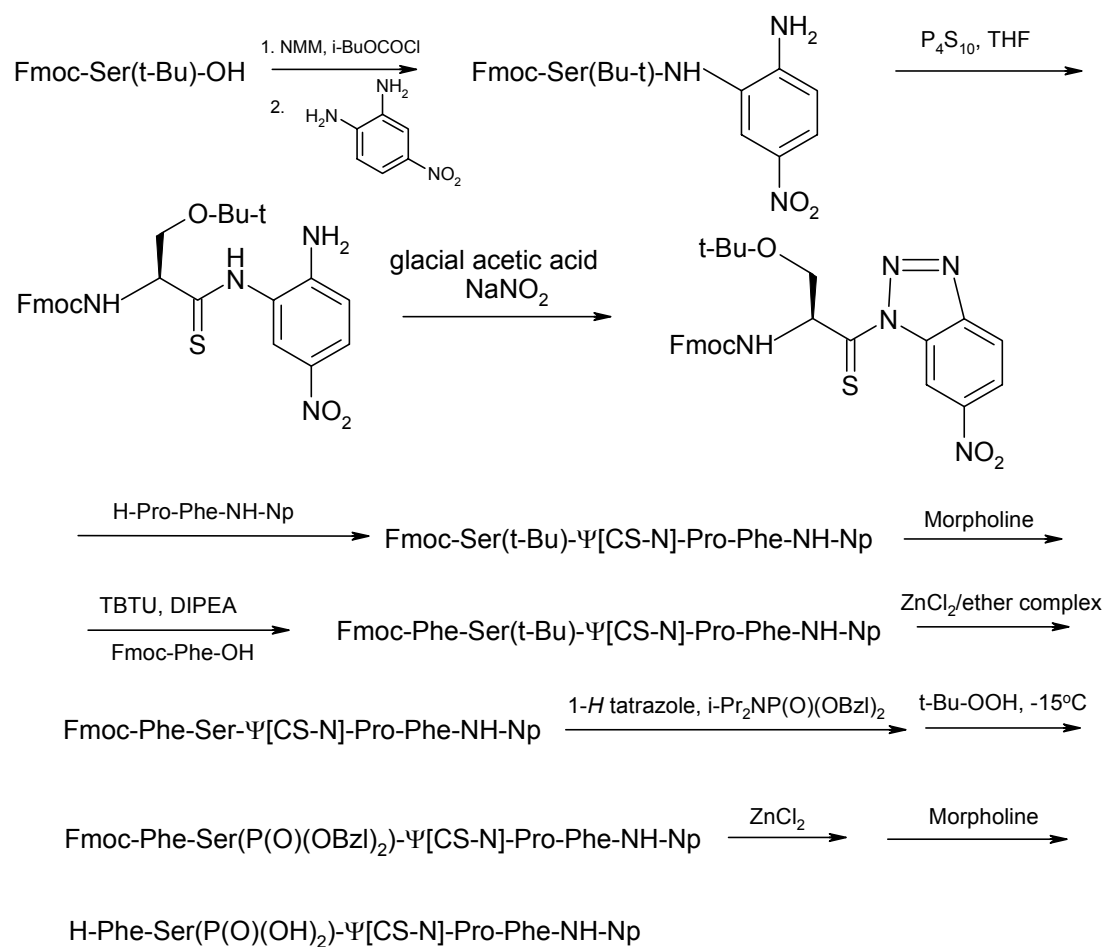
Figure 6.3 Inhibition of the PPIase activity of Pin1 by Ac-Ala-Ala-D-Ser(PO$_3$H$_2$)-Pro-Leu-NH-Np. The *Cis/trans* isomerization of Ac-Ala-Ala-Ser(PO$_3$H$_2$)-Pro-Leu-NH-Np was monitored under 7°C at 390nm, with or without Pin (4nM). The concentration of inhibitor is 15μM.

of thioxo amide occurred. After deprotection with $ZnCl_2$/Et_2O complex under argon followed by 20% piperidin in DMF, it was purified by RP8-HPLC and gave moderate yield. In conclusion, we have demonstrated that this novel deprotection strategy is an extraordinarily mild method for synthesis of phosphorylated compounds containing acid sensitive functionalities.

6.5 Pin1 inhibition by phospho peptides.

Both PPIase domain and WW domain could recognize the phospho-Ser/Thr-Pro motifs selectively. It's not clear if the open catalytic loop conformation in the Pin1-CTD peptide structure is induced upon ligand binding to the WW domain. To exclude these confusing points in our investigations, we applied trypsin as protease in our assay. Trypsin can digest a proline peptide in *trans* conformation. On the other hand, trypsin is also able to cleave the WW domain from Pin1 and remains the PPIase activity of the enzyme intact (data does not shown). Basing on the extended subsite specificity of Pin1 we designed and synthesized potential Pin1 inhibitors and measured their PPIase inhibitory activities using standard protease coupled assay.

Table 6.1 Pin1 inhibition. The Pin1 PPIase activity was measured with protease coupled assay and Ac-Ala-Ala-Ser(PO_3H_2)-Pro-Arg-NH-Np as substrate, in Hepes buffer pH 7.8, at 7°C, in the presence of 2.7 nM Pin1 and inhibitor of desired concentration.

	Peptides	IC$_{50}$
1.	Ser-Pro	>> 1mM[*]
2.	Ser-Ψ[CS-N]-Pro	>> 1mM[*]
3.	Ser(PO_3H_2)-Pro	1.0 ± 0.2 mM
4.	D-Ser(PO_3H_2)-Pro	20% inhibition at 1mM
5.	Ac-Ala-Ala-D-Ser-Pro -Leu-NH-Np	85 ± 9 μM
6.	Phe-Ser-Ψ[CS-N]-Pro -Phe- NH-Np	97 ± 11 μM
7.	Phe-Ser(PO_3H_2)-Ψ[CS-N]-Pro -Phe- NH-Np	4.0 ± 0.5 μM
8.	Ac-Ala-Ala-D-Ser(PO_3H_2)-Pro -Leu-NH-Np	1.0 ± 0.1 μM

* less that 5% inhibition at 1mM.

A very successful tool in the field of drug design is the utilization of peptide analogues that differ from naturally occurring peptides at certain amino-acid residue(s). These analogues are expected to have higher potency and enhanced stability against various proteolytic enzymes. Generally, there are two nearly general methods available for the generation of substrate peptide derived PPIase inhibitors: incorporation of D-amino acids (Schiene et al., 1998) and replacement of backbone

linkages by thioxo peptide bonds (Chapter 4; Schutkowski et al., 1994, 1995, 1997; Frank et al., 2000, Mario Jacob Ph.D. thesis).

6.5.1 Pin1 inhibition by peptidic inhibitors.

According to Pin1 substrate specificity (X-X-phospho Ser/Thr-Pro-X), we were able to demonstrate that the incorporation of a phosphoserine residue in the D-configuration preceding proline resulting in Ac-Ala-Ala-D-Ser(PO_3H_2)-Pro-Leu-NH-Np yielded a peptide derivative with an IC_{50} value of 1.1 μM (Table 6.1) (Figure **6.3**). Using trypsin as an isomer-specific protease in this assay, the C-terminal leucine residue was designed for resistance to the protease. The binding seems to be phosphorylation-dependent because the appropriate control peptide derivative Ac-Ala-Ala-D-Ser-Pro-Leu-NH-Np is approximately 100-fold less active (Table 6.1). However, the high affinity is also coupled to the peptide chain length because dipeptides D-Ser(PO_3H_2)-Pro and D-Ser-Pro showed only poor inhibition to Pin1 (Table 6.1) A very similar situation was obtained by the incorporation of a thioxylated peptidyl-prolyl bond into a phosphopeptide (Table 6.1). This one-atom-substitution represents a minimal deviation of the structure but again gave a potent inhibitor for Pin1 with an IC_{50} value of 4.0 ± 0.5 μM.

To investigate if these unnatural peptides are inhibitors or potent substrates which compete with Pin1 substrate in this assay, we measured the *cis/trans* isomerization of Ac-Ala-Ala-D-Ser(PO_3H_2)-Pro-Arg-NH-Np catalyzed by Pin1. Under 40 nM Pin1 concentration, no acceleration of isomerization could be observed. This single change in stereochemistry indeed switches a substrate to an inhibitor of Pin1.

6.5.2 Stability of Pin1 inhibitor Ac-Ala-Ala-D-Ser(PO_3H_2)-Pro-Leu-NH-Np

Replacing a L-Ser by a D-Ser in peptides, substrate specificity studies have shown that the non-natural peptides cannot be phosphorylated as efficiently as their parent sequences by kinases (Toomik, et al., 1994; Eller, et al., 1993). We investigated the stability of a phospho-D-Serine containing Pin1 inhibitor against cellular proteases and phosphatases. The Pin1 inhibitor Ac-Ala-Ala-D-Ser(PO_3H_2)-Pro-Leu-NH-Np was incubated with cell lysate (whole cell lysate of Hela cellls) for 15min, 1h, 2h, and 3h and analyzed with capillary electrophoresis, using Ac-Ala-Ala-D-Ser-Pro-Leu-NH-Np as control. No dephosphorylation of the Pin1 inhibitor was found. The production of a degraded component during incubation time could be observed (3.5% after 1 h of incubation at room temperature, 8.5% after 2 h and 13% after 3 h). The retention time as well as the UV spectrum of this peak indicates that it is a truncated product and both the Np and phosphate moieties remain intact.

6.5.3 Selective and reversible inhibition of Pin1 by Ac-Ala-Ala- D-Ser(PO_3H_2)-Pro-Leu-NH-Np

The inhibition of Pin1 by Ac-Ala-Ala-D-Ser(PO_3H_2)-Pro-Leu-NH-Np is reversible. 227 nM Pin1 was incubated with 10μM inhibitor for 1 min for full inhibition. The solution was diluted to a final

concentration containing 1.77nM Pin1 and 78nM inhibitor. 42.8% and 67.7% of Pin1 PPIase activities could be recovered after 2 and 5 min of dilution, respectively. Longer incubation is not possible due to the absorption of Pin1 to the vessel wall.

Ac-Ala-Ala-D-Ser(PO_3H_2)-Pro-Leu-NH-Np is highly specific to Pin1. No inhibitory activity to Cyp18 and FKBP12 could be detected at a concentration as high as 20μM.

6.5.4 The inhibitory activity of peptidic inhibitors to entire Pin1 and the catalytic domain.

Both the PPIase domain and the WW domain of Pin1 interact with phospho-Ser/Thr-Pro motifs specifically. WW domain has been shown to have higher binding affinity to phosphorylated peptides than PPIase domain (Verdecia, et al., 2000). The presence of the WW domain could have three possible functional influences on the PPIase activity of the enzyme.

I. It could act as a helper to direct the enzyme to the phospho-Ser/Thr-Pro motifs.

II. It competes with the catalytic domain for substrates or ligands binding.

III. The binding of a phospho-Ser/Thr-Pro motif to the WW domain could suppress the catalytic activity either by inducing a conformational change in the active site, or by enhancing steric hindrance. The dramatic conformational change (Figure **6.2**) observed in the active site of Pin1 upon phosphorlyted CTD peptide binding (Section **6.2.3**) (Verdecia, et al., 2000) may reflect such inhibitory effect.

Studies of the relationship between the two domains in Pin1 could help us to understand the molecular basis of the bimolecular character of the enzyme.

To prevent the cleavage of Pin1 by trypsin, the PPIase activity of Pin1 and the inhibitory activities of some inhibitors were measured with a protease free assay (Garcia-Echeverria, et al., 1992; Schiene, et al., 1998). The measurements were performed with Abz-Ala-Glu-Pro-Phe-NH-Np as substrate. The substrate was dissolved in anhydrous trifluoroethanol/0.5M LiCl. The time course of fluorescence signal at 416nm subsequent to a solvent jump was followed in the presence of 22 nM Pin1. As we see in table **6.2**, the inhibitory activity of Ac-Ala-Ala-D-Ser(PO_3H_2)-Pro-Leu-NH-Np to entire Pin1 is about 8 fold lower than that to Pin1 PPIase domain, which was measured by protease coupled assay. To exclude the difference between two assays, we measured the inhibitory activity of the inhibitor to Pin PPIase domain by the protease free assay. The results are in agreements with the protease coupled assay. Using the protease free assay, we also measured the inhibition of Ac-Ala-Ala-D-Ser(PO_3H_2)-Pro-Arg-NH-Np to entire Pin1 and the PPIase domain. A 4-fold difference was observed.

The binding of the phosphorylated ligands to the WW domain does neither induce an inactive conformation in the catalytic site nor facilitate to present the ligands to the active site, as shown by the enhanced enzymatic activities in the identical inhibitor concentration. It seems that the WW domain could compete with the PPIase domain for ligands binding.

Table 6.2 IC$_{50}$ of Pin1 inhibitors from different assays. PPIase activity of Pin1 was also measured with a protease free assay, Abz-Ala-Glu-Pro-Phe-NH-Np as substrate, in the presence of 22 nM Pin1, at 7°C, in Hepes buffer, pH7.8.

Inhibitors	IC$_{50}$[a]	IC$_{50}$[b]	IC$_{50}$[c]
Ac-Ala-Ala-D-Ser(PO$_3$H$_2$)-Pro-Leu-NH-Np	1.0μM	0.96μM	7.6 μM
Ac-Ala-Ala-D-Ser(PO$_3$H$_2$)-Pro-Arg-NH-Np		1.0μM	3.6 μM

a. Protease coupled assay

b. Protease free assay to PPIase domain of Pin1

c. Protease free assay to entire Pin1

6.6 Conclusion.

For peptides, a phosphorylated Ser/Thr residue preceding proline is required but not enough for both effective Pin1 binding and catalysis. Like Ser-Pro and Ser(PO$_3$H$_2$)-Pro, the dipeptide derivatives Ser-Ψ[CS-N]-Pro and D-pSer-Pro were also weak inhibitors to Pin1. Oligopeptides Phe-Ser-Ψ[CS-N]-Pro-Phe-NH-Np and Ac-Ala-Ala-D-Ser-Pro-Leu-NH-Np showed higher affinity than the appropriate dipeptide derivatives. Obviously, extended binding sites were beneficial for the affinity. As expected, phosphorylation of these peptide derivatives increased their inhibitory activity dramatically (Table 6.1). According to these minimal requirements for Pin1 inhibition, the current work in our lab is a systematic approach to the construction of intelligent phosphopeptide derivative libraries and the screening for potent and reversible Pin1 inhibitors.

Chapter 7. Experiments

7.1 General procedures.

Chromatography. Reverse phase high performance liquid chromatography (RP-HPLC) was applied for monitor, analysis and separation in chemical reactions. Analytic and preparative HPLC from Sykam and Abimed were used. Lichrosorb Hibar RP-select B(7µm) C8 250-25 column, Nucleosil 300-7 C8 250-10 column; Nucleosil 300-7 C4 250-4 column; Lichrosorb 100 RP8 125-4 column, Lichrosorb 100 RP18 125-4 column, were applied in different studies. Merck silica gel 60 (0.063-0.200mm) and Merck silica gel 60 (0.040-0.063mm) were used for silica gel chromatography. Merck Kieselgel 60 F_{254} was used in thin layer chromatography (TLC) for either analysis or preparation.

Software. Sigma Plot 5.0 is used for data analysis and calculation. Weblab Viewer 3.5 is applied for protein structure analysis.

NMR: DRX NMR spectrometer 500 MHz with active shirmed Z-gradient (Bruker). The spectra of Cs**18** and Cs**25** were measured at 295K. The spectra of Cs**9**, Cs**11**, Cs**13**, Cs**54**, Cs**55**, and Cs**56** were measured at 278K. The assignments of most of the signals are tentative and based on the chemical shifts observed for the corresponding signals of CsA.

MS: MALDI-TOF-mass spectrometer REFLEX (Bruker-Daltonik); ESI-Iontrap-mass spectrometer ESQUIRE-LC with HP1100 HPLC and Nanospray source (Bruker-Daltonik)

Laser: N2-Laser MSG 800 with pigment laser und doubler (Lasertechnik Berlin)

CD: CD-Spectrometer J-710 (Jasco).

Capillary electrophoresis: Applied Biosystems 270A-HT capillary Electrophoresis system; Beckman P/ACE^{TM} system MDQ capillary electrophoresis.

The synthesized compounds were measured with MS and/or NMR and purity was confirmed with RP C8 HPLC and thin layer chromatography.

7.1.1 rh Cyp18 and Pin1 PPIase activity and inhibition assays.

Materials. The Pin1-vector was from K.P. Lu (Ranganathan et al., 1997). The purified PPIases Pin1 and rh Cyp18 was kindly provided by Dr. J.-U. Rahfeld. The proteases α-chymotrypsin and trypsin were products from Merck (Darmstadt, Germany).

Assay. The PPIase activity was measured at 10°C with a protease coupled assay on a Hewlett-Packard 8452a diode array spectrophotometer according to (Fischer et al., 1984). To 1220 µl Hepes buffer pH 7.8, PPIase and inhibitor of desired concentration are added. After 10 min of incubation, protease and substrate were added. The final substrate concentratioin is 40 µM in 1280 µl. The *cis/trans* isomerization was monitor at 390 nm with 510 nm as a reference. The rate is calculated according to first order reaction. rh Cyp18 PPIase activity was measured with the peptide Suc-Ala-Phe-Pro-Phe-NH-Np as substrate. The substrate was dissolved in DMSO (10 mg/ml). α-Chymotrypsin was used as auxillary protease. Pin1 PPIase activity was measured with the phosphorylated peptide Ac-Ala-Ala-Ser(PO$_3$H$_2$)-Pro-Arg-NH-Np as stubstrate. Trypsin was used as auxillary protease. The

substrate peptides for Pin1 were dissolved in 20 mM TRIS pH 7.8 because DMSO hass moderate inhibitory activity to Pin1 at high concentration. The residual enzymatic activity was plotted versus the inhibitor concentration and used for calculating the inhibitor constants K_i as described by Schutkowski et al., (1995).

7.1.2 Calcineurin phosphatase activity and inhibition assays.

Materials. ScintiStrip plates coated with streptavidin were purchased from Wallac (Turku, Finland). The biotinylated and non-biotinylated RII peptide (biotin-DLDVPIPGRFDRRVSVAAE-OH), a partial sequence of the subunit of the bovine c-AMP-dependent protein kinase (PKA), were synthesized by Fmoc based solid phase peptide synthesis (SPPS). The catalytic subunit of bovine heart PKA and cAMP were purchased from Boehringer Mannheim (Mannhiem, Germany). Calmodulin, buffer and salts used were purchased from Sigma (Steinheim, Germany). Expression and purification of the recombinant human calcineurin α from the *Escherichia coli* strain BL21-(pLysS)/pETCNα/pBB131 (kindly provided by J. O. Liu) was performed exactly as described (Mondragon, et al., 1997).

Calcineurin activity assay using RII peptide substrate. The biotinylated and non-biotinylated RII peptides were phosphorylated by PKA. The reaction mixture containing 700μM peptide, 0.2 μM cAMP, 100μCi[γ-^{32}P]ATP and 10mU PKA in a final volume of 100 μl in the buffer (containing 20mM MES, pH6.5, 0.4mM EDTA, 0.2mM EGTA, 50μM CaCl$_2$ and 2mM MgCl$_2$) was incubated for one hour at 30°C. The phosphorylated peptides were separated from unincorporated labeled ATP by 1ml RP-C2 clean up extraction column (Amchto, Sulzbach, Germany). The eluted peptide (with 70% acetonitrile) was lyophilized and reconstituted with de-ionized water.

Scintillation Assay. The scintillation proximity concept has been applied for measuring calcineurin activity using ScintiStrip surfaces coated with streptavidin. Pre-incubation of calmodulin (50nM), calcineirin (1.32nM) and inhibitors of desired concentrations in the assay buffer (40nM Tris/HCl, pH7.5; 100mM NaCl, 6mM MgCl$_2$, 0.5mM DTT, 1mM CaCl$_2$, 0.1mg/ml BSA) was carried out for 30 min at 30°C in a 96-well microtiter plate (Costar, Bodenhtim, Germany). Biotinylated [γ-^{32}P] RII peptide was added to a final concentration of 100nM and the total assay volume is 100μl. After an incubation at 30°C for 30 min, 90 μl of the reaction mixture was transferred to the ScintiStrip well coated with streptavidin. The streptavidin immobilizes the biotinylated RII peptide on the wells. After 20 min at 22°C the well was washed once with water and the RII associated [γ-^{32}P] was measured in the top-counter MicroBeta (Wallac, Turku, Finland).

7.2 Experiments in chapter 2.

The syntheses of CsA residue 3 derivative were performed exactly as described (Seebach, et al., 1993). Solvents and other chemical reagents were bought from Fluka (Germany) and of the best available quality. THF was eventually dried by refluxing with Na. LiCl was dried under vacuum overnight.

Synthesis of [α-methyl sarcosine]³ CsA (R) (Cs11). A solution of 0.07 ml (i-Pr)$_2$NH (0.5mmol) in 5ml THF under N$_2$ was cooled to −78°C (with dry ice/i-PrOH) and then treated with 0.19 ml n-BuLi (2.7M) in hexane and stirred for half hour. To the result LDA solution, 80mg CsA (0.067mmol) in 1ml THF was added. After one hour, 0.02ml MeI (0.33mmol) was added. Within 10 hours, the mixture was warmed to room temperature and 4ml water was added. The solvent was evaporated. 40ml ethyl ether was added and the organic layer was washed with half saturated NaCl aqueous solution twice, dried with Na$_2$SO$_4$, evaporated, and purified by C-8 RP-HPLC (31%). ^{1}H NMR, δ:7.96(d), 7.68(d), 7.49(d), 7.14(d), 5.67(m), 5.48(d), 5.20-5.40(m),   5.07(d), 4.98-5.05(m), 4.92(d), 4.82-4.90(m), 4.78(t), 4.59(t), 4.52(t), 3.66(m), 3.48(s), 3.23(d), 3.06(s), 2.67(d), 2.41(m), 2.05-2.15(m), 1.90-2.05(m), 1.62-1.90(m), 1.60(m), 1.38(d), 1.32(d), 1.23(d), 1.06(d), 0.92-1.02(m), 0.83-0.92(m), 0.73-0.83(m), 0.63(m) m/z cal. 1215.9, found 1216.9 [M+H]$^+$.

Synthesis of [α-methyl sarcosine]³ CsA (S) (Cs12). 60mg CsA (0.05mmol) and 64mg eventually dried LiCl (Fluka) in 2ml THF was prepared under N$_2$ and was cooled to −78°C. Separately, to the solution of 0.047ml (i-Pr)$_2$NH (0.33mmol) in 5ml THF under N$_2$, 0.122ml n-BuLi (2.7M) in hexane was added at 0°C. After 30min of stirring at 0°C, the solution was cooled to −78°C. The LDA solution was then added to the CsA/LiCl solution. After 3h, additional 100µl n-BuLi and 1mmol MeI were added. The mixture was stirred at −20°C for 4 hours, then warmed up to room temperature. NH$_4$Cl aqueous solution was added. The aqueous phase was extracted 3 times with ether and the combined organic layer was washed with a half-saturated NaCl twice, dried with Na$_2$SO$_4$, evaporated and purified by C-8 RP-HPLC (33%). m/z cal. 1215.9, found 1217.2 [M+H]$^+$.

Synthesis of [α-methylthio sarcosine]³ CsA (R) (Cs13). To the solution of 0.106 ml (i-Pr)$_2$NH (0.75mmol) in 5ml THF under N$_2$, 0.277ml n-BuLi (2.7M) in hexane was added at 0°C. After 30min of stirring at 0°C, the solution was cooled to −78°C. 60mg CsA (0.05mmol) in 1ml THF was added. After 3h, additional 100µl n-BuLi and 1mmol MeSSMe were added. The temperature was allowed to rise to 0°C overnight. 10ml 1N HCl was added and the mixture was worked up with ether. The organic phase was washed two times with saturated NaHCO$_3$ and two times with saturated NaCl solution, dried with Na$_2$SO$_4$. After evaporating the solvent, the reaction mixture was purified by C8 RP-HPLC (34%). ^{1}H NMR, δ: 7.96(m), 7.78(m), 7.70(d), 7.36(d), 7.16(d), 6.26(s), 5.74(s), 5.64-5.70(m), 5.59-5.64(m), 5.47(d), 5.45-5.32(m), 5.31(m), 5.18-5.25(m), 5.10-5.16(m), 5.08(s), 4.96-5.07(m), 4.84-4.95(m), 4.75-4.83(m), 4.62(t), 4.52(t), 4.23(m), 3.96-4.10(m), 3.96(t), 3.91(s), 3.09(q), 2.70-3.02(m), 2.68(d), 1.50-1.80(m), 1.20-1.42(m), 1.06(d), 0.70-1.00(m), m/z cal. 1248.0, found 1247.9.

Synthesis of [α-methyl sarcosine]³ CsA (S) (Cs14). 60mg CsA (0.05mmol) and 64mg eventually dried LiCl in 2ml THF was prepared under N$_2$ and was cooled to −78°C. Separately, to the solution of 0.047ml (i-Pr)$_2$NH (0.33mmol) in 5ml THF under N$_2$, 0.122ml n-BuLi (2.7M) in hexane was added at 0°C. After 30min of stirring at 0°C, the solution was cooled to −78°C. The LDA solution was then added to the CsA/LiCl solution. After 3h, additional 100µl n-BuLi and 1mmol MeSSMe were added. The temperature was allowed to rise to 0°C overnight. 4ml water was added. The solvent was

evaporated. 40ml ethyl ether was added and the organic layer was washed with half saturated NaCl twice, dried with Na_2SO_4, evaporated, and purified by C-8 RP-HPLC (31%). m/z cal. 1215.9, found 1247.9.

Synthesis of [α-methyl sarcosine][3] CsH (R&S) (Cs15 & Cs16). CsH was methylated according to the method used for CsA (without LiCl). The main as well as a minor product could be separated by C-8 RP-HPLC. According to the stereo-selectivity of this condition, the major product (33%) was considered as R conformation whereas the minor product (7%) was considered as S conformation. m/z cal. 1215.9, found 1216.7 $[M+H]^+$ and 1216.8 $[M+H]^+$ respectively.

Synthesis of [α-ethyl sarcosine][3] CsA (R&S) (Cs40 & Cs41). CsA was ethylated according to the method used for CsA methylation (without LiCl). The main as well as a minor product could be separated by C-8 RP-HPLC. According to the stereo-selectivity of this condition, the major product (24%) was considered as R conformation, whereas the minor product (9%) was considered as S conformation. For both compounds, m/z cal. 1229.9, found 1231.1 $[M+H]^+$.

Synthesis of [Me-Asp(Bu-t)][3] CsA (R&S) (Cs33) and [Me-Asp][3] CsA (R&S) (Cs35). A solution of 0.07 ml $(i-Pr)_2NH$ (0.5mmol) in 5ml THF under N_2 was cooled to $-78^{\circ}C$ (with dry ice/i-PrOH) and then treated with 0.19 ml n-BuLi (2.7M) in hexane and stirred for half hour. To the result LDA solution, 80mg CsA (0.067mmol) in 1ml THF was added. After one hour, 0.05ml tert-butyl bromoacetate (0.33mmol) was added. Within 15 hours, the mixture was warmed to room temperature and 4ml water was added. The solvent was evaporated. 40ml ethyl ether was added and the organic layer was washed with saturated NaCl twice, dried with Na_2SO_4, and evaporated. Two isomers were separated by analytic C-8 RP-HPLC. The yields of both products are about 20% (as estimated by HPLC). For both compounds, m/z cal. 1316.0, found 1317.0 $[M+H]^+$. Half of the crude product was purified by C8 RP-HPLC and two isomers were obtained as mixture for calcineurin inhibitory activity assay.

To the another half of the crude product, 1ml THF was added at ice-bath temperature. The residue was taken up in 5ml 2N NaOH and MeOH was added until the solution becoming clear. At the ice cooling, 2N HCl was added to reach pH = 2. The mixture was then extracted 3 times with 20ml ether and the combined organic layers was washed with 20ml saturated NaCl twice. The organic phase was dried with Na_2SO_4, evaporated and purified by C8 RP-HPLC (85%). 1260.0, found 126.9 $[M+H]^+$.

Synthesis of [β-oxo MeBmt][1] [(R)α-methyl sarcosine][3] CsA (Cs29). 6mg CrO_3, 10mg pyridine were added to 10mg Cs**11** (0.008mmol) in 1 ml CH_2Cl_2. The reaction was stirred at room temperature for 3 hours. 20 ml CH_2Cl_2 was added and the organic phase was washed with aqueous $NaHCO_3$ and brine solution. The solvent was evaporated and the mixture was separated by C8 RP-HPLC. The reaction yield is 20%, as determined by HPLC (the main component in the mixture was starting material Cs**11**). m/z cal. 1213.9, found 1214.4.

Synthesis of [O-acetyl MeBmt][1] [(R)α-methyl sarcosine][3] CsA (Cs30). 1 equiv. Ac_2O was added to 10mg Cs**11** (0.008mmol) in 1 ml pyridine. The reaction was stirred at room temperature overnight.

30ml ethyl acetate was added and the organic phase was washed with aqueous $NaHSO_4$, aqueous $NaHCO_3$, and brine solution. The solvent was evaporated and the reaction mixture was separated by C8 RP-HPLC (87%). m/z cal. 1258.0, found 1258.9 $[M+H]^+$.

7.3 Experiments in chapter 3.

Synthesis of [phosphoryl D-Ser][8] CsA (Cs3). Tetrazole was eventually dried in vacuum overnight. 8mg tetrazole and 4Å molecule sieve were added to 60mg [D-Ser][8] CsA in 2ml CH_2Cl_2. After half hour at room temperature, 1 equiv. of (i-Pr$_2$N)P(OAll)$_2$ was added. The reaction was stirred at room temperature overnight. 20μl t-BuOOH in 1 ml THF was added at -10^oC. After half hour, 10ml 10% $Na_2S_2O_3$ and 50 ml ethyl acetate were added. The organic layer was separated, washed with 5% $NaHSO_4$, 5% $NaHCO_3$ and saturated NaCl aqueous solution, and dried with Na_2SO_4. The solvent was evaporated and the crude product was separated with HPLC (95%). 20mg [Diallyl phosphoryl D-Ser][8] CsA, 6mg Pd[PPh$_3$]$_4$, 10μl HCOOH, 10μl triethyl amine in 5ml THF was stirred at 50^oC for five hours. 5 ml acetonitrile was added and the reaction mixture was separated by HPLC (80%). m/z cal. 1297.9, found 1298.5 $[MH]^+$.

Synthesis of [H-phosphoryl D-Ser][8] CsA (Cs4). 8mg tetrazole and 4Å molecule sieve were added to 60mg [D-Ser][8] CsA in 2ml CH_2Cl_2. After half hour at room temperature, 1 equiv. of (i-Pr$_2$N)P(OAll)$_2$ was added. The reaction was stirred at room temperature overnight. The organic layer was separated, washed with 5% $NaHSO_4$, 5% $NaHCO_3$ and saturated NaCl aqueous solution, and dried with Na_2SO_4. Without separation, 20mg crude product, 6mg Pd[PPh$_3$]$_4$, 10μl HCOOH, 10μl triethyl amine in 5ml THF was stirred at 50^oC for five hours. 5 ml acetonitrile was added and the reaction mixture was separated by HPLC (80%). m/z cal. 1282.0, found 1282.9 $[MH]^+$.

Synthesis of [dimethyl phosphoryl D-Ser][8] CsA (Cs5). Synthesis of [dimethyl phosphoryl D-Ser][8] CsA is similar to the synthesis of [diallyl phosphoryl D-Ser][8] CsA. The crude was separated with HPLC (89%). m/z cal. 1326.0, found 1327.0 $[MH]^+$.

Synthesis of [O-carboxymethyl D-Ser][8] CsA (Cs6). A solution of 60mg [D-Ser][8] CsA, *tert*-butyl bromoacetate (20mg) and 5mg benzyltriethylammonium chloride in 1ml CH_2Cl_2 was stirred vigorously for two hours at room temperature with 2ml 30% NaOH aqueous solution. The reaction was then diluted with 10ml water and extracted with ether twice. The organic layer was dried with Na_2SO_4. Without separation, the product was added to 60mM KOH methanol solution and stirred at room temperature for 3 hours. Acetic acid was added. Most of the acetic acid and methanol were evaporated. Ethyl acetate was added and the mixture was washed with water. The organic layer is separated, dried with Na_2SO_4, evaporated and separated with RP HPLC (94%). m/z cal. 1275.7, found 1276.8 $[MH]^+$.

Synthesis of [bromo-n-butyl D-Ser][8] CsA (Cs7). A solution of 50mg [D-Ser][8] CsA, 20mg tetrabutylammonium chloride and 22mg 1,4-dibromobutane in 1ml CH_2Cl_2 was stirred vigorously for two hours at room temperature with 2ml 30% NaOH aqueous solution. Ether was added and the

organic layer was washed with water. The solvent was evaporated and the reaction mixture was separated with C8 HPLC (63%). m/z cal. 1352.9, found 1354.2 [MH]$^+$.

Synthesis of [2-aminoethoxyethyl D-Ser]^8 CsA (Cs8). A solution of 100mg [D-Ser]^8 CsA, 40mg tetrabutylammonium chloride and 50mg BocNHCH$_2$CH$_2$OCH$_2$CH$_2$Br in 2ml CH$_2$Cl$_2$ was stirred vigorously overnight at room temperature with 4ml 30% NaOH aqueous solution. Ether was added and the organic layer was washed with water. The solvent was evaporated and the reaction mixture was separated with C8 HPLC. The product was treated with 5ml ZnCl$_2$/ether in ether under N$_2$ for 3 hours for Boc deprotection. 0.1ml water and 10ml acetonitrile was added. The precipitated salt was filtered. The organic solvent was evaporated, and the reaction mixture was separated by C8 HPLC (two steps, 37%). m/z cal. 1305.1, found 1306.2 [MH]$^+$.

Synthesis of [O-(BocNH(CH$_2$)$_5$NHC(O)CH$_2$-) D-Ser]^8 CsA (Cs10) and [O-(NH$_2$(CH$_2$)$_5$NHC(O)-CH$_2$-) D-Ser]^8 CsA (Cs9). 100mg Cs6, 3equiv. of NH$_2$(CH$_2$)$_5$NHBoc, 4 equiv. of PyBop, 8 equiv. or DIPEA in 5 ml CH$_2$Cl$_2$ was stirred at room temperature overnight. 40ml ethyl acetate was added and the organic layer was washed with 5% NaHSO$_4$, 5% NaHCO$_3$ and saturated NaCl aqueous solution, and dried with Na$_2$SO$_4$. The solvent was evaporated and the product (Cs10) was separated by C8 HPLC (96%). m/z cal. 1460.2, found 1461.3 [MH]$^+$. Cs10 was treated with 5ml ZnCl$_2$/ether in ether under N$_2$ for 3 hours for Boc deprotection. 0.1ml water and 15ml acetonitrile was added. The precipitated salt was filtered. The organic solvent was evaporated, and the reaction mixture was separated by C8 HPLC (86%). ^{1}H NMR, δ: 9.50(m), 8.42(m), 8.08(m), 7.56(m), 6.84(m), 5.62(s), 5.52(s), 5.40(m), 5.28(m), 5.19(s), 4.60(m), 4.10(m), 3.58-3.40(m), 3.14(m), 3.03(m), 2.84(m), 2.75(m), 2.70(s), 1.81(m), 1.72(m), 1.59(m), 1.18-1.40(m), 0.70-1.15(m). m/z cal. 1360.1, found 1361.3 [MH]$^+$.

Synthesis of [O-(BiotinNH(CH$_2$)$_5$NHC(O)CH$_2$-) D-Ser]^8 CsA (Cs24). 20mg Cs9, 2 equiv. of biotin, 3 equiv. of PyBop, 5 equiv. of DIPEA in CH$_2$Cl$_2$ was stirred at room temperature for two days. 20ml ethyl acetate was added and the organic layer was washed with 5% NaHSO$_4$, 5% NaHCO$_3$ and saturated NaCl aqueous solution, and dried with Na$_2$SO$_4$. The solvent was evaporated and the product was separated by C8 HPLC (63%). m/z cal. 1586.5, found 1587.3

Synthesis of [O-(AcNH(CH$_2$)$_5$NHC(O)CH$_2$-) D-Ser]^8 CsA (Cs42). 1 equiv. Ac$_2$O was added to 10mg Cs9 (0.008mmol) in 1 ml pyridine. The reaction was stirred at room temperature overnight. 30ml ethyl acetate was added and the organic phase was washed with aqueous NaHSO$_4$, aqueous NaHCO$_3$, and brine solution. The solvent was evaporated and the reaction mixture was separated by C8 RP-HPLC (89%). m/z cal. 1402.1, found 1403.2 [M+H]$^+$.

Synthesis of [O-(HOOC(CH$_2$)$_5$NHC(O)CH$_2$-) D-Ser]^8 CsA (Cs44). 10mg Cs6, 3equiv. of NH$_2$(CH$_2$)$_5$COOMe, 4 equiv. of PyBop, 8 equiv. or DIPEA in 5 ml CH$_2$Cl$_2$ was stirred at room temperature overnight. 20ml ethyl acetate was added and the organic layer was washed with 5% NaHSO$_4$, 5% NaHCO$_3$ and saturated NaCl aqueous solution, and dried with Na$_2$SO$_4$. The solvent was evaporated and the crude product was treat with 1N NaOH aqueous solution. Acetic acid was added

after one hour. After removing the solvent, 40ml ethyl acetate was added. The organic lay was washed with saturated NaCl aqueous solution twice, and dried with Na_2SO_4. The solvent was evaporated and the product was separated by C8 HPLC (89%). m/z cal. 1389.0, found 1389.4.

Synthesis of [O-methyl D-Ser][8] CsA (Cs60). A solution of 60mg [D-Ser][8] CsA, 20mg tetrabutylammonium chloride and 20mg MeI in CH_2Cl_2 was stirred overnight at room temperature in 30% NaOH aqueous solution. More solvent was added and the mixture was washed with water. The organic layer was separated, dried with Na_2SO_4 and evaporated. The compound was separated with C8 HPLC (65%). m/z cal. 1231.9, found 1233.0 $[MH]^+$.

7.4 Experiments in chapter 4.

The Lawesson reagent, Davy-methyl reagent, Davy-Tolyl reagent, 3,4,5,6-tetrahydro-1,3-dimethylpyrimidin-2(1*H*)-one (DMPU) were purchased from Fluka.

Thioxylation of CsA with Lawesson reagent. 60mg CsA and 60mg Lawesson reagent in 2ml DMPU was stirred at room temperature for one week as described (Seebach, et al., 1991). The mixture was diluted with 12ml ether and washed with water three times and saturated NaCl aqueous solution twice. The aqueous phase was combined and extracted with ether twice. The combined organic phases were dried with Na_2SO_4 and evaporated. The crude product was analyzed with analytic HPLC and thin layer chromatography (TLC, ethyl acetate/petroleum(9:1) or water saturated ether). The main component in the mixture is CsA, as determined by mass spectroscopy, and the multiple minor peaks were identified as mixture of mono-, di-, or tri- thioxylated CsA derivatives (m/z cal. 1218 (monothioxo); 1234 (dithioxo) 1250 (trithioxo), found around 1218, 1234 or 1250 in various HPLC fractions.

The same reactions using Davy-methyl reagent or Davy-tolyl reagent and/or with HMPT (hexamethylphosphoric triamide) as solvent were performed and similar results were obtained.

Thioxylation of CsA with reagent 1e. 60mg CsA, 1equiv. of **1e** in 2ml DMPU was stirred at room temperature for one day. 40ml ether was added and washed with saturated NaCl aqueous solution twice. The organic phase was dried with Na_2SO_4 and evaporated. The crude product was analyzed with analytic HPLC and mass spectroscopy. The multiple peaks on HPLC were identified as mono-, di-, or tri- thioxylated CsA derivatives (m/z cal. 1218 (monothioxo); 1234 (dithioxo) 1250 (trithioxo), found around 1218, 1234 or 1250 in various HPLC fractions.

The same reaction was performed at 5°C for two days and similar result was obtained.

Thioxylation of CsA with Lawesson reagent in the presence of base. 60mg CsA, 1 equiv. Lawesson reagent, 1 equiv. pyridine in 5ml toluene was stirred at 100°C for 1 – 2 hours. Ether was added and washed with water. The organic layer was dried with Na_2SO_4, and evaporated. Three main peaks were observed on HPLC. (m/z cal. 1218 (monothioxo); 1234 (dithioxo) 1250 (trithioxo), found around 1216, 1218, 1232, 1234, 1248 or 1250 in various HPLC fractions.

Higher reaction temperature resulted in undesired side reactions. Lower reaction temperature resulted in lower yield. Using Davy-methyl reagent or Davy-tolyl reagent gave similar results.

Thixoxoylation of [O-acetyl MeBmt][1] CsA with Lawesson reagent. 200mg [O-acetyl MeBmt][1] CsA, 1equiv. Lawesson in 5ml toluene was stirred at 120°C for one hour. The reaction mixture was cooled to room temperature and 50ml ether was added. The organic phase was washed with water, dried with Na_2SO_4 and evaporated under reduced pressure. The crude product was purified by silica gel chromatography with a gradient ether → 1%MeOH/ether → 3%MeOH/ether → 5%MeOH/ether (ether was saturated with water). Three compounds were separated. Further purification was performed with TLC. The purity of each compound was further confirmed by HPLC. [O-acetyl MeBmt][1] [$^4\psi^5$ CSNH] CsA (23%) (Cs**58**) cal. m/z 1259.9, found 1260.1; [O-acetyl MeBmt][1] [$^7\psi^8$ CSNH] CsA (21%) (Cs**59**) cal. m/z 1259.9, found 1260.3; and a di-thioxylated derivative [O-acetyl MeBmt][1] [$^4\psi^5$ CSNH] [$^7\psi^8$ CSNH] CsA (33%) (Cs**57**) cal. m/z 1275.7, found 1276.1.

Each [O-acetyl MeBmt][1] thioxo CsA was dissolved in 5ml 1M NaOMe/MeOH. The mixture was stirred at room temperature for one hour. Acetic acid was added to adjust to pH 7. 20ml Ether was added and washed with water and NaCl saturated aqueous solution twice, dried with Na_2SO_4 and evaporated. The crude produet was purified with TCL.

[$^4\psi^5$ CSNH] CsA (Cs**55**) (89%) ^{1}H NMR, δ: 8.83(d), 7.64(d), 7.53(d), 7.45(m),5.69(q), 5.43(d), 5.31(m), 5.15(d), 5.06(t), 5.02(q), 4.92(m), 4.73(m), 4.70(m), 4.61(t), 4.26(d), 3.50(s), 3.36(s), 3.22(s), 3.09(d), 2.67(d), 2.40(m), 2.19(m), 2.10(m), 1.98(m), 1.58(d), 1.97(s), 1.32(d), 1.22(s), 1.05(d), 0.98(m), 0.95(d), 0.90(d), 0.85(m), 0.66(d) cal. m/z 1217.8, found 1218.3;

[$^7\psi^8$ CSNH] CsA (91%) (Cs**56**) ^{1}H NMR, δ: 8.72(d), 7.91(d), 7.58(d), 7.11(d), 5.67(q), 5.48(d), 5.45(d), 5.43(d), 5.31(m), 5.15(m), 5.12(s), 5.09(s), 5.01(m), 4.93(m), 4.92(m), 4.80(m), 4.18(d), 3.65(m), 3.50(s), 3.20(d), 3.05(d), 3.68(d), 2.08(m), 2.00(m), 1.66(m), 1.60(d), 1.45(m), 1.33(d), 1.12(m), 1.09(d), 1.00(m), 0.93(m), 0.86(m), 0.83(m), 0.61(d)cal. m/z 1217.8, found 1218.4;

The di-thioxylated derivative [$^4\psi^5$ CSNH] [$^7\psi^8$ CSNH] CsA (62%) (Cs**54**) ^{1}H NMR, δ: 8.81(d), 8.73(d), 7.68(d), 7.74(d), 5.69(q), 5.46(d), 5,43(d), 5.26-5.40(m), 5.15(s), 5.13(s), 5.11(d), 5.08(s), 4.98-5.06(m), 4.92(t), 4.72-4.80(m), 4.71(s), 4.46(d), 3.63(m), 3.51(s), 3.36(s), 3.32(s), 3.09(s), 3.05(s), 2.68(s), 1.90-2.20(m), 1.60(d), 1.35(s), 1.23(m), 1.08(d), 1.01(m), 1.97(m), 1.93(m), 0.85(m), 0.61(m). cal. m/z 1233.8, found 1234.1.

The positions of thioxylation for these CsA analogues also can be determined using mass spectroscopy fragmentation, CD spectra and are in agreement with the reported results (Eberle, et al., 1994).

Assays of Thioxo CsA analogues. 300μl 1mM stock solutions in DMSO of these thioxo CsA analogues (Cs**54**, Cs**55**, Cs**56**, Cs**57**, Cs**58**, and Cs**59**) were prepared. 100μl of each stock solution was taken for PPIase inhibitory activitiy assays and/or CD spectra measurements. The rest 200μl of each stock solution was irradiated with laser (337nm, 90μJ and 10Hz) for half hour. 100μl of each was taken for PPIase inhibitory activitiy assays and/or CD spectra measurements. The rest 100μl of each irradiated sample was kept in darkness overnight, and the PPIase inhibitory activity was measured.

For measuring the calcineurin inhibitory activity, 10mM stock solutions in DMSO of Cs**54**, Cs**55** and Cs**56** were prepared. The samples were treated as described above, and the calcineurin inhibition was measured.

7.5 Experiment in chapter 5.

Synthesis of [O-diallyl-phosphoryl MeBmt][1] CsA (Cs20), [O-monoallyl-phosphoryl MeBmt][1] CsA (Cs21) and [O-phosphoryl MeBmt][1] CsA (Cs18). Tetrazole was eventually dried in vacuum overnight. 32mg tetrazole and 4Å molecule sieve were added to 60mg CsA in 2ml CH_2Cl_2. After half hour at room temperature, 4 equiv. of $(i\text{-}Pr_2N)P(OAll)_2$ was added. The reaction was stirred at room temperature overnight. 20μl t-BuOOH in 1 ml THF was added at $-10^{o}C$. After half hour, 10ml 10% $Na_2S_2O_3$ and 50 ml ethyl acetate were added. The organic layer was separated, washed with 5% $NaHSO_4$, 5% $NaHCO_3$ and saturated NaCl aqueous solution, and dried with Na_2SO_4. The solvent was evaporated and the crude product was separated with HPLC (95%). m/z cal. 1360.8, found 1361.8 $[MH]^+$. 20mg [O-Diallyl phosphoryl MeBmt][1] CsA, 6mg $Pd[PPh_3]_4$, 10μl HCOOH, 10μl triethyl amine in 5ml THF was stirred at $50^{o}C$ for five hours. 5 ml acetonitrile was added and the reaction mixture was separated by HPLC. Both Cs**18** (70%)and Cs**21** (10%) were identified in this reaction. For Cs**18**, ^{1}H NMR, δ: 7.93(d), 7.64(d), 7.44(d), 7.13(d), 5.66(d), 5.44(d), 5.3(s), 5.10(d), 5.04(m), 5.00(m), 4.96(m), 4.80(m), 4.70(d), 4.63(m), 4.49(m), 3.79(m), 3.48(s), 3.36(s), 3.23(s), 3.17(d), 3.09(d), 2.67(d), 2.38(m), 1.96-2.12(m), 1.72(m), 1.67(s), 1.60(s), 1.38-1.46(m), 1.33(d), 1.23(d), 1.04(d), 0.79-1.01(m), 0.69(d). m/z cal. 1282.8, found 1283.4$[MH]^+$, and for Cs**21**, m/z cal. 1321.8, found 1323.5$[MH]^+$.

Synthesis of [O-dimethyl phosphoryl MeBmt][1] CsA (Cs19). 30mg tetrazole and 4Å molecule sieve were added to 61mg CsA in 2ml CH_2Cl_2. After half hour at room temperature, 4 equiv. of $(i\text{-}Pr_2N)P(OMe)_2$ was added. The reaction was stirred at room temperature overnight. 20μl t-BuOOH in 1 ml THF was added at $-10^{o}C$. After half hour, 10ml 10% $Na_2S_2O_3$ and 50 ml ethyl acetate were added. The organic layer was separated, washed with 5% $NaHSO_4$, 5% $NaHCO_3$ and saturated NaCl aqueous solution, and dried with Na_2SO_4. The solvent was evaporated and the crude product was separated with HPLC (95%). m/z cal. 1310.8, found 1311.3 $[MH]^+$.

Synthesis of [O-phosphoryl D-Ser][8] [O- phosphoryl MeBmt][1] CsA (Cs22). 40mg tetrazole and 4Å molecule sieve were added to 60mg [D-Ser][8] CsA in 2ml CH_2Cl_2. After half hour at room temperature, 5 equiv. of $(i\text{-}Pr_2N)P(OAll)_2$ was added. The reaction was stirred at room temperature overnight. 40μl t-BuOOH in 1 ml THF was added at $-10^{o}C$. After half hour, 10ml 10% $Na_2S_2O_3$ and 50 ml ethyl acetate were added. The organic layer was separated, washed with 5% $NaHSO_4$, 5% $NaHCO_3$ and saturated NaCl aqueous solution, and dried with Na_2SO_4. The solvent was evaporated and the crude product was separated with HPLC (80%). m/z cal. 1534.9, found 770.1 $[M+2H]^{2+}$. The product, 9mg $Pd[PPh_3]_4$, 15μl HCOOH, 15μl triethyl amine in 5ml THF was stirred at $50^{o}C$ for five hours. 5 ml acetonitrile was added and the reaction mixture was separated by HPLC (89%). m/z cal. 1378.1, found 1378.8 $[MH]^+$.

Synthesis of [O-(BiotinNH(CH$_2$)$_5$NHC(O)CH$_2$-) D-Ser]^8 [O- phosphoryl MeBmt]^1 CsA (Cs23). 40mg tetrazole and 4Å molecule sieve were added to 100mg Cs**10** in 3ml CH$_2$Cl$_2$. After half hour at room temperature, 4 equiv. of (i-Pr$_2$N)P(OAll)$_2$ was added. The reaction was stirred at room temperature overnight. 20µl t-BuOOH in 1 ml THF was added at −10°C. After half hour, 10ml 10% Na$_2$S$_2$O$_3$ and 50 ml ethyl acetate were added. The organic layer was separated, washed with 5% NaHSO$_4$, 5% NaHCO$_3$ and saturated NaCl aqueous solution, and dried with Na$_2$SO$_4$. Without separation, the crude product, 6mg Pd[PPh$_3$]$_4$, 10µl HCOOH, and 10µl triethyl amine in 5ml THF was stirred at 50°C for five hours. 5 ml acetonitrile was added and the reaction mixture was separated by HPLC (3 steps, 51%). m/z cal. 1440.0, found, 1441.1 [MH]$^+$. (The allyl deprotection must be performed before biotin coupling reaction for preventing the sulfur atom of biotin moiety to be toxic to palladium catalyst).

Biotin was coupled to [O-(NH$_2$(CH$_2$)$_5$NHC(O)CH$_2$-) D-Ser]^8 [O- phosphoryl MeBmt]^1 CsA using 3 equiv. PyBop and 3.5 equiv. DIPEA. The reaction was stirred at room temperature for two days. 20ml ethyl acetate was added and the organic layer was washed with 5% NaHSO$_4$, 5% NaHCO$_3$ and saturated NaCl aqueous solution, and dried with Na$_2$SO$_4$. The solvent was evaporated and the product was separated by C8 HPLC (73%). m/z cal. 1666.6, found 834.1 [M+2H]$^{2+}$.

Synthesis of [1-amino-2,6-octadienoic acid]^1 CsA (Cs25). 20mg Cs**21** in 5 ml 1N NaOH aqueous solution was stirred at room temperature for one hour. Acetic acid was added and the solvent was evaporated. The crude was separated by C8 HPLC (62%). ^{1}H NMR, δ: 8.41(d), 7.85(d), 7.30(d), 7.03(d), 6.96(d), 6.87(m), 6.72(m), 6.06(d), 5.68(m), 5.41(m), 5.34(m), 5.27(m), 5.14(m), 5.08(m), 4.65-5.00(m), 4.42(s), 3.73(m), 3.62(m), 3.48(s), 3.30(m), 3.26(s), 3.20(s), 2.80-3.17(m), 2.69-2.78(m), 1.85-2.33(m), 1.73(m), 1.60(m), 1.20-1.40(m), 0.59-1.13(m) m/z cal. 1184.1, found 1185.7 [MH]$^+$.

Synthesis of [O-sulfonyl MeBmt]^1 CsA (Cs26). 60mg CsA and 1.1 equiv. of SO$_3$/DMF complex in 2 ml pyridine were refluxed for 2 h. Ethyl acetate was added and the organic layer was washed with saturated NaCl aqueous solution, dried with Na$_2$SO$_4$, evaporated and separated with C8 HPLC (71%). m/z cal. 1282.1, found 1282.7 [MH]$^+$.

Synthesis of [β-oxo MeBmt]^1 CsA (Cs27). 6mg CrO$_3$, 10mg pyridine were added to 10mg CsA (0.008mmol) in 1 ml CH$_2$Cl$_2$. The reaction was stirred at room temperature for 3 hours. 20 ml CH$_2$Cl$_2$ was added and the organic phase was washed with aqueous NaHCO$_3$ and saturated NaCl aqueous solution. The solvent was evaporated and the mixture was separated by C8 RP-HPLC (70%). m/z cal. 1199.9, found 1200.5.

Synthesis of [O-acetyl MeBmt]^1 CsA (Cs28). 1 equiv. Ac$_2$O was added to 10mg CsA (0.008mmol) in 1 ml pyridine. The reaction was stirred at room temperature overnight. 30ml ethyl acetate was added and the organic phase was washed with aqueous NaHSO$_4$, aqueous NaHCO$_3$, and brine solution. The solvent was evaporated and the reaction mixture was separated by C8 RP-HPLC (87%). m/z cal. 1244.0, found 1244.9 [M+H]$^+$.

Synthesis of [O-acetyl MeBmt][1] CsH. The synthesis of this inactive control compound is similar to the synthesis of Cs**28.**

Synthesis of cyc[Leu-Phe-Phe-Thr(PO₃H₂)-Pro]₂ and cyc[Leu-Phe-Phe-Thr(PO₃H₂)-Pro]. Leu-Phe-Phe-Thr(PO₃H₂)-Pro was synthesized using Fmoc based SPPS. Cyclization reaction of Leu-Phe-Phe-Thr(PO(OBzl)OH)-Pro was performed with 4 equiv. DIPEA/DIPC/HOAT in highly diluted DMF solution. Treated with 98%TFA, the peptides were separated by RP8-HPLC and lyophilized.

Synthesis of [O-phosphoryl MeBmt][1] CsH (Cs61). The synthesis of Cs**61** is similar to the synthesis of Cs**18**.

7.6 Experiments in chapter 6.

The peptide derivatives Suc-Ala-Ala-Pro-Phe-NH-Np, Suc-Ala-Pro-Phe-NH-Np, Suc-Ala-Glu-Pro-Phe-NH-Np, Ala-Pro, Ser-Pro, Ala-Pro-Ala, Ala-Ala-Pro and Ala-Ala-Pro-Ala were purchased from Bachem (Heidelberg, Germany).

The synthesis of the phosphorylated peptides Ac-Ala-Ala-Ser(PO₃H₂)-Pro-Arg-NH-Np and Ser(PO₃H₂)-Pro-Arg-NH-Np was carried out according to (Yaffe et al., 1997b; Schutkowskiet al., 1998). Ac-Ala-Ala-Pro-Ala-Lys-NH₂ was synthesized as described in (Reimer et al., 1998). The peptide Ser(PO₃H₂)-Pro-Arg was obtained by tryptic digestion of Ser(PO₃H₂)-Pro-Arg-NH-Np. The phosphopeptides Ser(PO₃H₂)-Pro, Ala-Ser(PO₃H₂)-Pro, Ala-Ser(PO₃H₂)-Pro-Arg and Ac-Ala-Ala-D-Ser(PO₃H₂)-Pro-Arg-pNA were synthesized by solid phase peptide synthesis using chlorotritylchloride-resin, TBTU, HOBt (Alexis Biochemicals, Germany) as coupling reagent, 20% peperidin in DMF as Fmoc deprotection reagent, and 98% TFA as the final cleavage reagent. Fmoc-Ser(PO(OBzl)-OH)-OH (Calbiochem-Novabiochem-AG, Switzerland) as phosphoamino acid building blocks and dimethylformamide as reaction solvent. D-Ser(PO₃H₂)-Pro and Ac-Ala-Ala-D-Ser(PO₃H₂)-Pro-Arg-pNA were synthesized using Fmoc-D-Ser(P(O)(OBzl)₂ as phosphoamino acid building blocks and PyBOP as coupling reagent. After detachment of the peptides from the resin and side-chain deprotection with TFA these phosphopeptides were purified by RP C8 HPLC and lyophilized.

Synthesis of H-Phe-Ser(PO₃H₂)-Ψ[CS-N]-Pro-Phe-NH-Np. General procedure for peptide phosphorylation has been described in details in chapter 5. Fmoc-Phe-Ser-Ψ[CS-N]-Pro-Phe-NH-Np was phosphorylated by (i-Pr₂N)P(OBzl)₂/tetrazole in THF at room temperature and oxidated by *tert*-Butyl hydroperoxide under −15°C. After deprotection with ZnCl₂/Et₂O complex under argon in CH₂Cl₂ for 2 hours and then with 20% piperidin in DMF, it was purified by RP C8-HPLC and lyophilized.

Synthesis of Ac-Ala-Ala-D-Ser(PO₃H₂)-Pro-Leu-pNA. Ac-Ala-Ala-D-Ser-Pro-Leu-NH-Npwas phosphorylated by (i-Pr₂N)P(OAll)₂/tetrazole in THF and oxidated by *tert*-Butyl hydroperoxide at room temperature. Deprotection of allyl ester was performed in THF with tetrakis(triphenylphosphine)palladium/butyl amine/formic acid under 50°C for four hours. After

separation by RP8-HPLC, the peptide was lyophilized. The purity of all peptides was checked by CE and/or RP18-HPLC.

Stability of Pin1 inhibitor Ac-Ala-Ala-D-Ser(PO$_3$H$_2$)-Pro-Leu-NH-Np

Pin1 inhibitor Ac-Ala-Ala-D-Ser(PO$_3$H$_2$)-Pro-Leu-NH-Np was incubated with cell lysate (whole cell lysate of Hela cells, 8.8×10^5/ml) for 15min, 1h, 2h, and 3h and analysized with CE (fused silica gel, 50/60cm, 50µm inner diameter, 50mM phosphate buffer, pH8, separation voltage 30kV, 10s injection with 0.5psi), using Ac-Ala-Ala-D-Ser-Pro-Leu-NH-Np as control.

Summary

Cyclosporin A (CsA) is a potent immunosuppressive drug. To distinguish the Cyp inhibition by CsA and CaN inhibition by Cyp/CsA complex, a main aim of this work is to design CsA derivatives for selective enzyme inhibition. The structure-activity relationship of CsA backbone as well as the biological effects of CsA metabolites were also investigated.

Two [α-substituted sarcosine][3] CsA derivatives (Cs**11** and Cs**13)** can inhibit CaN phosphatase activities in the absence of Cyp18　with IC_{50} value of 10μM. This is the first report that CsA derivatives can inhibit CaN directly. Compared to CsH, [α-methyl sarcosine][3] CsH showed a dramatic enhancement of Cyp18 inhibition and CaN inhibitory activity of the Cyp18/ligand complex. CsA is a slow-binding inhibitor of Cyp18, as measured by the time course of intrinsic fluorescence of Cyp18. On the contrary, the binding of Cs**11** and Cs**13** to Cyp18 is fast.

We investigated the effect of cationic, anionic and neutral hydrophobic side chains at residue 8 of CsA on their Cyp18 inhibition and CaN inhibitory activity of the Cyp18/ligand complexes. Two mono-functional CsA derivatives (Cs**3** and Cs**9)** were obtained with potent Cyp18 inhibitory activities and weak CaN inhibition by Cyp18/ligand complexes.

We investigated various conditions for CsA thioxylation and synthesized several thioxylated CsA derivatives. We studied the influences of thioxylation on conformation by CD spectra. Three thioxo CsA derivatives investigated in our study displayed enhanced CaN inhibition by the Cyp18/ligand complexes, along with either unaffected or impaired Cyp18 binding affinities. These compounds are stable under laser illumination. Two of the three thioxo CsA undergo reversible photoisomerization, as studied by UV spectra. The re-equilibration courses are very slow, reflecting the high rotation barrier around thioxo amide bond. Laser illumination of these thioxylated CsA derivatives resulted in significant changes in their CD spectra, Cyp18 inhibitions, and CaN inhibitory activities of the Cyp18/thioxo-CsA complexes.

CsA was phosphorylated chemically at MeBmt[1] (Cs**18**). Some other CsA residue 1 derivatives were also synthesized. A biotinylated [O-phosphoryl MeBmt][1] CsA was synthesized for affinity absorption on streptavidin bead in order to extract potential cellular receptors of [O-phosphoryl MeBmt][1] CsA. Cs**18** is stable in stimulated T cells and exhibits low cell membrane permeability. The modification results in a dramatic decrease of both Cyp18 inhibition and CaN inhibitory activity in the presence of Cyp18 . [O-Sulfonyl MeBmt][1] CsA is more active than [O-phosphoryl MeBmt][1] CsA. However, this activity is partially due to its low stability. [O-phosphoryl MeBmt][1] CsA displays potent Pin1 inhibitory activity.

Phosphorylated Ser/Thr residue preceding proline is required but not enough for both effective Pin1 binding and catalysis. Like Ser-Pro and Ser(PO$_3$H$_2$)-Pro, the dipeptide derivatives Ser-Ψ[CS-N]-Pro and D-phospho-Ser-Pro are weak inhibitors to Pin1. Oligopeptides Phe-Ser-Ψ[CS-N]-Pro-Phe-NH-Np and Ac-Ala-Ala-D-Ser-Pro-Leu-NH-Np showed higher affinity than the appropriate dipeptide derivatives. Phosphorylation of these peptide derivatives increased their inhibitory activity dramatically. Ac-Ala-Ala-D-phospho-Ser-Pro-Leu-NH-Np is stable against cellular phosphatases and proteases. It is a reversible and selective inhibitor to Pin1. The differences between the inhibitory activities of the inhibitors to PPIase domain and entire Pin1 indicated that the WW domain of Pin1 compete with the PPIase domain for ligand binding.

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