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Characterization of the *N*-Methyltransferase Activities of the Multifunctional Polypeptide Cyclosporin Synthetase

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SUMMARY

This study demonstrates a critical role for *N*-methylation in cyclosporin biosynthesis and maintenance of the biologically active cyclosporin conformation. The structural requirements for the AdoMet binding to CySyn were defined. *N*-methylation of specific amide positions in the cyclosporin backbone is critical for the complete assembly and cyclization of the cyclosporin peptide. A maximum of two desmethyl positions is tolerated before peptide assembly stalls. Subinhibitory concentrations of AdoMet analogs directed peptide assembly towards cyclosporins with less than seven *N*-methylated amide bonds. Molecular modeling and nuclear magnetic resonance analyses indicate that *N*-methylation of specific amide bond positions in the cyclosporin backbone is mandatory for the formation of a product-like conformation and recognition by the acceptor site of the downstream peptide bond forming C-domain.

INTRODUCTION

Many peptide secondary metabolites of microbial origin possess clinically useful pharmacological activities due to their expanded structural spectrum of nonproteinogenic amino- and hydroxy-acid monomeric units, together with unique structural modifications (Grünewald and Marahiel, 2006; Konz and Marahiel, 1999; Velkov and Lawen, 2003b; Walsh, 2004; Walsh et al., 2001). The enormous structural and functional diversity of these low-molecular weight peptides is attributable to their mode of biosynthesis. Peptide secondary metabolites are assembled nonribosomally by multifunctional modular enzymes, termed nonribosomal peptide synthetases (NRPS) (Grunewald and Marahiel, 2006; Konz and Marahiel, 1999; Velkov and Lawen, 2003b; Walsh, 2004; Walsh et al., 2001). In addition to their ability to utilize a broad spectrum of proteinogenic and non-proteinogenic monomeric units, NRPS introduce further structural diversity into their products via auxiliary modifying functions that reside in either externally associated or integral enzyme activities (Grünewald and Marahiel, 2006; Konz and Marahiel, 1999; Velkov and Lawen, 2003b; Walsh, 2004; Walsh et al., 2001). Integral tailoring domains, embedded within the modular assembly line, act in *cis* to modify the peptide chain during elongation (Konz and Marahiel, 1999; Velkov and Lawen, 2003b; Walsh et al., 2001). External enzyme species act in *trans* via protein-protein interactions with the synthetase while intermediates are still covalently tethered, or postassembly, after release (Konz and Marahiel, 1999; Walsh et al., 2001). Auxiliary enzymes that catalyze modification reactions such as methylations, glycosylations, halogenations, epimerizations, and heterocyclizations, have been documented (Grünewald and Marahiel, 2006; Konz and Marahiel, 1999; Vaillancourt et al., 2005; Velkov and Lawen, 2003b; Walsh, 2004; Walsh et al., 2001). The understanding of these tailoring functions is of great relevance because the structural modifications they introduce are essential for the biological activities of the natural products. Moreover, the exploitation of these tailoring enzymes is of considerable utility in the synthesis of structural variants of therapeutic natural products (Grünewald and Marahiel, 2006; Kohli et al., 2001, 2002; Lin and Walsh, 2004; Mahlert et al., 2005; Oberthür et al., 2005; Trauger et al., 2000; Vaillancourt et al., 2005; Velkov and Lawen, 2003b).

A characteristic property of many peptides of non-ribosomal origin is the methylation of their backbone, including their amide nitrogens (Mahlert et al., 2005; Miller et al., 2001, 2002; Patel and Walsh, 2001; Velkov and Lawen, 2003b; Welker and von Döhren, 2006). An example of an *N*-methylated non-ribosomal peptide is the cyclic undecapeptide cyclosporin A (CsA) (see Figure S1A available online). CsA exhibits potent immunosuppressive bioactivity and is in clinical use worldwide for the treatment of allograft rejection (Borel et al., 1996). In CsA, 7 of the 11 amino acid amide nitrogens are *N*-methylated (positions 1, 3, 4, 6, 9, 10, and 11). The undecapeptide cyclosporin backbone is assembled in an assembly line fashion on the multifunctional protein thiotemplate cyclosporin synthetase (CySyn) (Dittmann et al., 1994; Velkov and Lawen, 2003b). The presence of functional *N*-methyltransferase (*N*-MTase) activity in the CySyn polypeptide was first demonstrated by photoaffinity labeling with *S*-adenosyl-L-[*methyl*-^{14}C]methionine and by the ability of the purified enzyme to transfer the sulfonium methyl group from *S*-adenosyl-L-methionine (AdoMet) to CsA (Lawen and Zocher, 1990; Velkov and Lawen, 2003a).

CySyn is one of the most complex NRPS systems known, consisting of a single 1.7 MDa polypeptide capable of catalyzing a total of 40 partial reaction steps in the synthesis of CsA. The CySyn polypeptide can be divided into eleven semi-autonomous modular units, which are composed of homologous NRPS functional domains responsible for the activation, modification, and polymerization of the constituent amino acids of CsA (Velkov and Lawen, 2003b; Weber et al., 1994). The modular architecture of CySyn conforms to an iterative organization such that the amino acid sequence of the peptide product is colinear with the order of the protein modules. The fundamental modular unit consists of catalytic domains responsible for substrate amino acid activation (A-domain), a peptidyl carrier protein (PCP) domain that acts to translocate the 4′-phosphopantetheine (4′-Ppant) covalently tethered peptidyl chain intermediate between modular active sites, and a condensation (C-domain) responsible for amide bond formation. CySyn contains four (C)-(A)-(PCP) modules (modules 1, 6, 9, 11) and seven modules that display an additional 430 amino acid *N*-MTase domain insert between the A- and PCP-domains (modules 2, 3, 4, 5, 7, 8, 10), presenting a modular domain order of (C)-(A)-(*N*-MTase)-(PCP) (Figure S1B). *N*-methylation takes place before peptide bond formation where these integral *N*-MTase domains catalyze the transfer of the *S*-methyl group of AdoMet to the amide nitrogen of the thioiesterfied amino acid, releasing *S*-adenosyl-L-homocysteine as a reaction product (Dittmann et al., 1990; Lawen and Zocher, 1990; Velkov and Lawen, 2003a).

Presently very little is known about the selectivity of the AdoMet binding sites and the steric course of *N*-methyltransfer in NRPS systems. This work provides an insight into the chemical selectivity of the AdoMet binding site(s) and the effects of inhibitory cofactor analogs on cyclosporin biosynthesis.

RESULTS AND DISCUSSION

Structural Requirements for Cofactor Binding to the *N*-MTase Centers of CySyn

The cofactor selectivity of the AdoMet binding pockets of CySyn was assessed by screening a number of natural and synthetic AdoMet analogs for inhibition of *N*-MTase activity and photoaffinity labeling with [^{14}C-*methyl*]AdoMet (Figure 1A; Table S1). It should be noted that the radiolabel *N*-MTase activity assay is a measure of *N*-methylation of the final enzymatic product, CsA. Therefore, in addition to the contribution of the *N*-MTase domains, the assay is also dependent on the perpetual contribution from the A-, PCP-, and C-domains from each module. *S*-adenosyl-L-homocysteine (AdoHcy), sinefungin, and *S*-adenosyl-L-ethionine (AdoEth) were the only compounds found to act as potent inhibitors. IC_{50} measurements indicated an inhibitory efficacy in decreasing order of AdoHcy > sinefungin > AdoEth. The *N*-MTase binding centers appear to be stereo-selective for the L-isomer of AdoHcy as the D-isomer was not inhibitory. The AdoMet precursor methionine and the amino acid components of each of the inhibitory analogs produced no overt effects on *N*-MTase activity. The effect of the purine component of these compounds was also tested, which resulted in no inhibition.

Several salient features are evident from a pharmacophore structure-activity-relationship analysis of the inhibitory AdoMet analogs (Figure 1B). Common structural features across the *N*-MTase inhibitors include a closed backbone configuration such that the hydroxyl groups of the purine ring are hydrogen-bonded to the carboxyl oxygen of the amino acid component and a zwitterionic tail that appears to be required for binding. Amino acid 5′-modifications of these compounds appear to be tolerated. The three carbon distance between the sulfonium center and the asymmetric amino acid carbon also appears to be important for binding, as a reduced length (even by a single C-linkage) seems to preclude binding, as is the case for *S*-adenosyl-L-cysteine.

Docking solutions with a homology model of the *N*-MTase domain of module 8 suggest the adenosyl moiety and amino acid 5′-side chain of each analog binds to a similar set of residues within the AdoMet binding pocket (Figure 1C). The length of the central S^{δ} (AdoMet, AdoHcy, and AdoEth) or C^{δ} (sinefungin) substituent appears to be the critical structural feature for differentiating selectivity and binding affinity, as an increase in substituent length results in steric hindrances with the side chains of Asn^{10701} and Met^{10734} (Figure 1C). The side chains of Lys^{10604} and Met^{10607} are also proximal to the central S^{δ} substituent and may also be involved conferring selectivity. These residues are largely conserved across all seven *N*-MTase domains of CySyn (Velkov and Lawen, 2003a, 2003b). The inhibition of photoaffinity labeling observed in the presence of various sulfhydryl blocking agents (after loading of the enzyme with amino acids, in an attempt to avoid preferential alkylation of the 4′-PPant cofactor thiols) also suggests the involvement of cysteine side-chains in AdoMet binding (Table S2). It follows that the highest inhibitory activity of AdoHcy results from the lack of substitution on the central S^{δ} atom. The intermediate inhibitory activity of sinefungin results from its amino substituted C^{δ} center, which is similar in size to the S^{δ} methyl of AdoMet. AdoEth displays a bulky ethyl S^{δ} substituent, and as such, binding is poorly accommodated.

The Effect of *N*-MTase Inhibitors on In Vitro Cyclosporin Biosynthesis

The effects of increasing concentrations of inhibitory AdoMet analogs on the enzymatic biosynthesis of CsA were examined (Figure 2A). The complete inhibition of the *N*-MTase reactions of CySyn results in the inhibition of CsA biosynthesis. Densitometric analysis (not shown) of high performance thin layer chromatography (HPTLC) autoradiographs indicated that AdoHcy was the most potent inhibitor of CsA biosynthesis, followed by sinefungin and AdoEth, respectively. When the inhibitors were introduced at a subinhibitory concentration (10 μM), cyclosporin biosynthesis was preferentially shifted from CsA to cyclosporins that exhibit five to six *N*-methylated amides, with the following abundance: CsU ≥ CsL > CsE ≥ CsQ > CsA > CsR > > CsT (Figure S1C). The cyclosporin product profiles observed at 10 μM of each *N*-MTase inhibitor were comparable; the data obtained for the sinefungin reaction are given as an example (Figures 2B–2D). The identity of the cyclosporin products was confirmed by HPTLC, high performance liquid chromatography (HPLC), and matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry, with reference to authentic standards calibrated in each analytical method (Figures 2B–2D). In the control CsA biosynthetic reaction, the main reaction

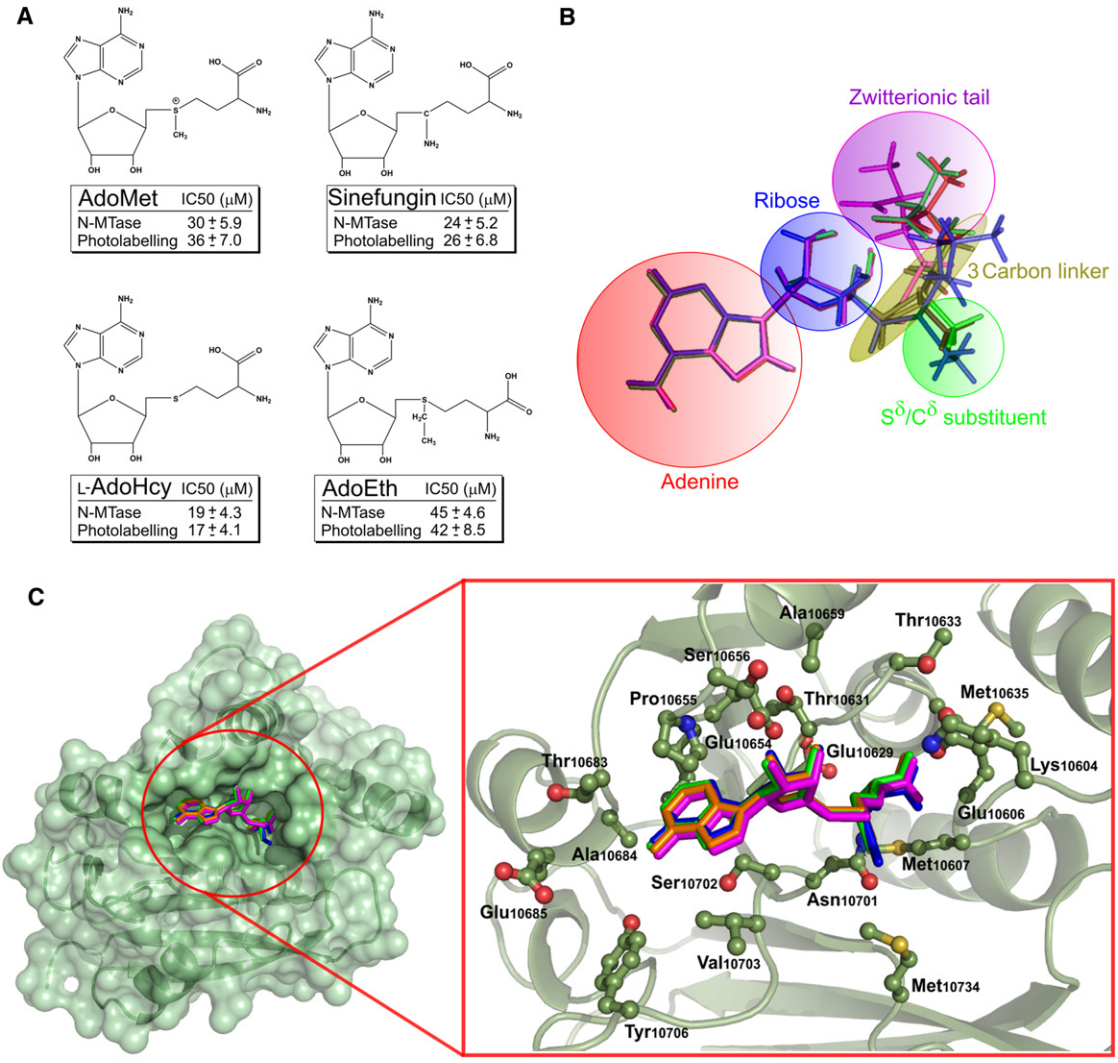


Figure 1. Structural Requirements for Cofactor Binding to the *N*-MTase Centers of CySyn

(A) Chemical structures and IC_{50} values for *S*-adenosyl-L-methionine (AdoMet), sinefungin, *S*-adenosyl-L-homocysteine (AdoHcy) and *S*-adenosyl-L-ethionine (AdoEth). The IC_{50} value for AdoMet reflects its ability to compete with [*methyl*-^{14}C]AdoMet and is not indicative of inhibitory activity.

(B) Superimposed energy minimized models of the 3D structures of AdoMet (green), sinefungin (orange), AdoHcy (magenta), AdoEth (blue). Structural moieties important for binding affinity are highlighted by colored spheres.

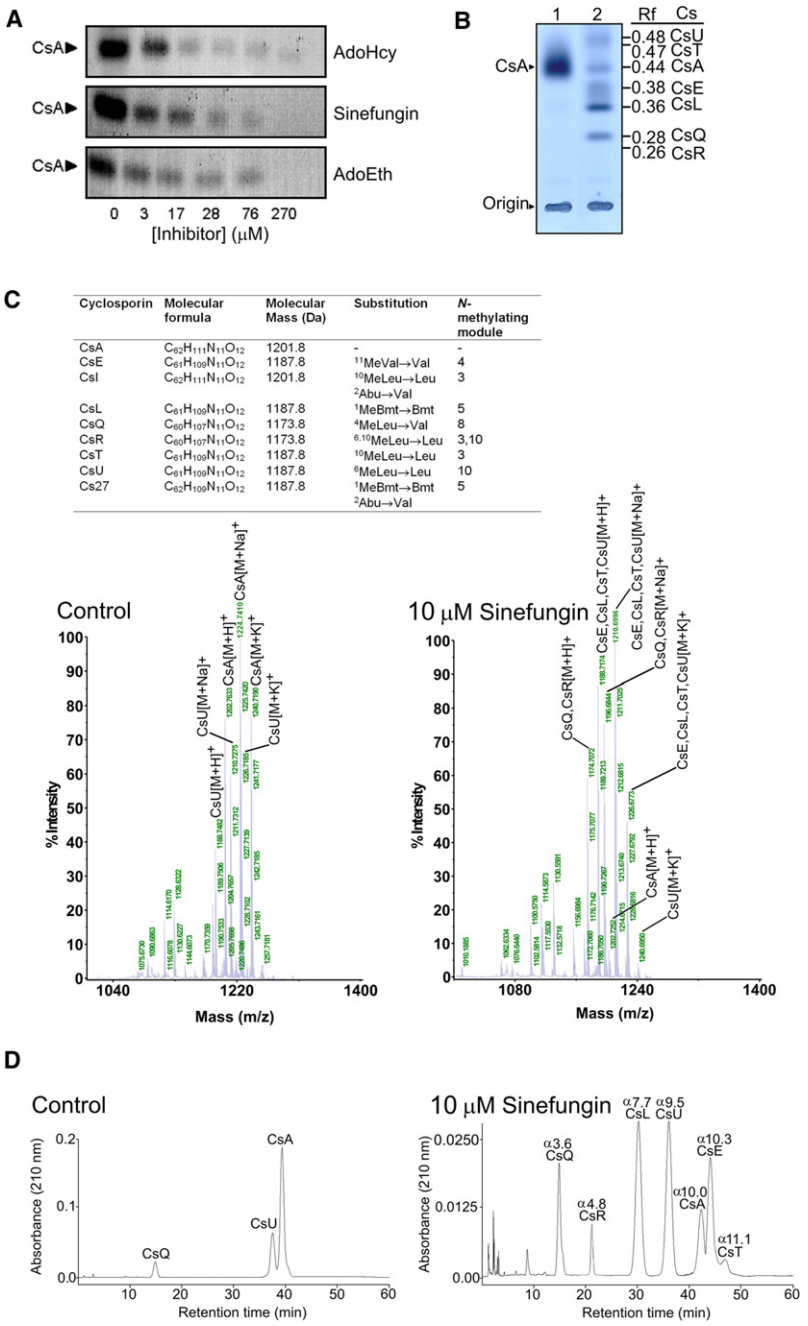
(C) Homology model of the *N*-MTase domain of CySyn module 8 with docking solutions for AdoMet and the aforementioned *N*-MTase inhibitors.

See also Tables S1 and S2.

product is CsA. As by-products, the desmethyl cyclosporins CsU and CsQ are also observed, with CsA > > CsU > CsQ in abundance, in agreement with earlier published data (Lawen et al., 1989). Furthermore, integration of the HPLC traces of the cyclosporin reaction products synthesized in the presence of 10 μM *N*-MTase inhibitor indicate an overall lower yield of cyclosporin products, suggesting biosynthesis of cyclosporins with less than seven *N*-methyl amides is an unfavored process. This observation is in line with the frequency and abundance (compared to CsA) of desmethylated cyclosporins observed across the 32 cyclosporins isolated from *Tolypocladium inflatum* nutrient broths (Lawen, 1996). The desmethyl cyclosporin profile observed in the presence of subinhibitory concentrations of *N*-MTase inhibitors, indicates the enzyme has the ability to "skip" amide *N*-methylations at certain backbone positions. It appears that *N*-methylations are most conserved at positions 3, 9, and 10, whereas unmethylated peptide bonds are most frequent at positions 1, 4, 6, and 11. However, no more than two desmethyl positions are tolerated before peptide assembly stalls, and these will never occur in direct succession.

The Effect of *N*-MTase Inhibitors on the Partial Reactions of Peptide Assembly

To examine the possibility that the inhibition of CsA biosynthesis by the presence of *N*-MTase inhibitors is due to effects on adjacent catalytic domains rather than the direct inhibition of *N*-MTase, the effect of the *N*-MTase inhibitors on some of the individual catalytic activities of A-, PCP-, and C-domains were



Cyclosporin	Molecular formula	Molecular Mass (Da)	Substitution	*N*-methylating module
CsA	$C_{62}H_{111}N_{11}O_{12}$	1201.8	-	-
CsE	$C_{61}H_{109}N_{11}O_{12}$	1187.8	^{11}MeVal→Val	4
CsI	$C_{62}H_{111}N_{11}O_{12}$	1201.8	^{10}MeLeu→Leu ^{2}Abu→Val	3
CsL	$C_{61}H_{109}N_{11}O_{12}$	1187.8	^{1}MeBmt→Bmt	5
CsQ	$C_{60}H_{107}N_{11}O_{12}$	1173.8	^{4}MeLeu→Val	8
CsR	$C_{60}H_{107}N_{11}O_{12}$	1173.8	^{6,10}MeLeu→Leu	3,10
CsT	$C_{61}H_{109}N_{11}O_{12}$	1187.8	^{10}MeLeu→Leu	3
CsU	$C_{61}H_{109}N_{11}O_{12}$	1187.8	^{6}MeLeu→Leu	10
Cs27	$C_{62}H_{109}N_{11}O_{12}$	1187.8	^{1}MeBmt→Bmt ^{2}Abu→Val	5

Figure 2. The Effect of *N*-MTase Inhibitors on In Vitro Cyclosporin Biosynthesis

(A) Inhibition of CsA biosynthesis by AdoMet analogs. CsA biosynthesis was monitored in the presence of the indicated concentrations of *N*-MTase inhibitors. The reaction products were separated by HPTLC in solvent I and visualized by autoradiography.

(B) Autoradiograph of the HPTLC separation of reaction products. Lane 1: Control CsA biosynthetic reaction. Lane 2: Cyclosporins (Cs) produced in the presence of 10 μM sinefungin. R_f values for Cs reference compounds are indicated on the ordinate. The running span was 1 × 10 cm in solvent I.

(C) MALDI-TOF analysis of solvent extracted Cs products synthesized in the absence and presence of 10 μM sinefungin. The insert table lists the characteristics and calculated mass for each desmethyl Cs.

(D) Reversed-phase HPLC traces of Cs products synthesized in the absence and presence of 10 μM sinefungin. The retention α-factor for each Cs is indicated.

See also Figure S1.

examined. There were no discernible effects on the degree of amino adenylation of the substrate amino acid L-leucine, which is activated by A-domains associated with at least four of the seven modules harboring *N*-MTase domains (Figure 3A). Comparably, there were no effects on the activation of the substrate amino acid D-alanine, which is activated by the A-domain of the non-*N*-MTase initiator module of CySyn (Figure 3A).

The loading of amino acid precursors onto the PCP-domain as thioesters was not inhibited in the presence of *N*-MTase inhibitors (Figure 3B; Table S3). The proportional quantities of amino acid released in the presence and absence of *N*-MTase inhibitors indicates that the covalent attachment of amino acid substrates L-leucine and L-2-aminobutyric acid to their respective *N*-MTase and non-*N*-MTase modules remains unaffected in the presence of *N*-MTase inhibitors (Figure 3B). For glycine and L-alanine similar data were obtained (Table S3). Furthermore, to demonstrate that the recognition and ATP-dependent carboxyl activation of each amino acid is mediated by the A-domain of each respective module, control incubations for each amino acid were performed in the absence of ATP (Figure 3B, lanes 6 and 12).

The biosynthesis of the diketopiperazine *cyclo*-(D-alanyl-L-leucyl) (DKP) partial reaction product was monitored in the presence of *N*-MTase inhibitors. If L-leucine and D-alanine are the only available amino acid substrates in an AdoMet-free reaction mixture, the main reaction product is *cyclo*-(D-alanyl-L-leucyl), representing positions 8 and 9 of the CsA molecule. AdoHcy, sinefungin and AdoEth did not produce any discernible effects on desmethyl-DKP formation (Figure 3C).

The *N*-MTase domain physically separates the PCP from the A-domain (Figure S1B). Despite its intervening physical positioning, our data indicate that the function of the *N*-MTase domain is not critical for the functionality of adjacent A and PCP domains. Thus, the stalling of CsA biosynthesis with the inhibition of *N*-MTase function appears to evolve from a mechanism that operates independently of the fundamental amino acyl-adenylation and thioester formation reactions.

The Effects of *N*-MTase Inhibitors on Peptidyl-*S*-Intermediate Elongation

To determine the importance of amide *N*-methylation for the progression of peptide assembly and the mechanism whereby *N*-MTase inhibitors stall CsA biosynthesis, we characterized

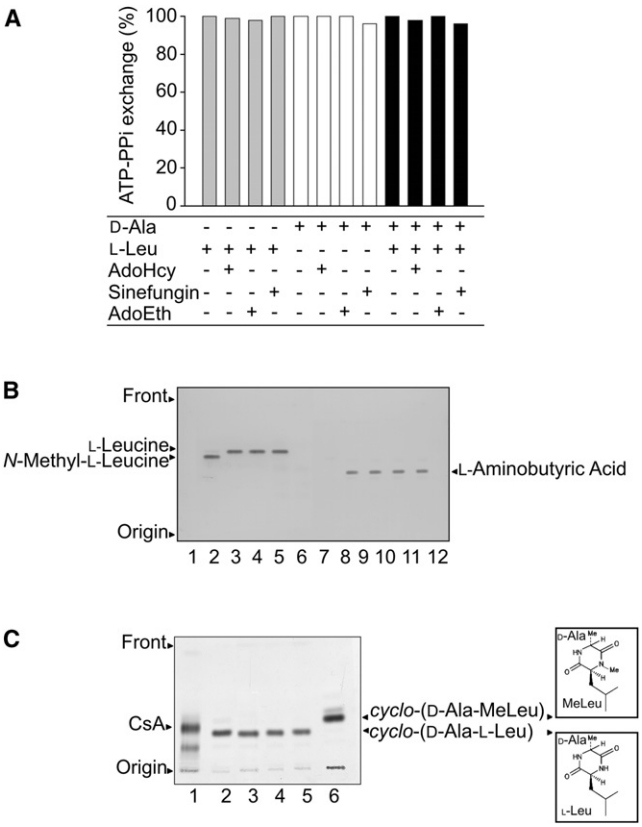


Figure 3. The Effect of *N*-MTase Inhibitors on the Partial Reactions of Peptide Assembly

(A) The effect of *N*-MTase inhibitors on the activity of A-domains. The ATP-PP_i exchange rate in the absence of *N*-MTase inhibitors was set as 100%; measurements from individual experiments are expressed as relative percentages of this value.

(B) The effect of *N*-MTase inhibitors on the covalent loading of substrate amino acids as thioesters onto the PCP-domain. Lane 1: [^{14}C]-L-leucine control reaction, performic acid treatment omitted. Lane 2: Products released after performic acid treatment of the [^{14}C]-L-leucine control reaction. Lanes 3–5: As for lane 2 but in the presence of 270 μM L-AdoHcy, sinefungin and L-AdoEth, respectively. Lane 6: As for lane 2, but excluding ATP. Lane 7: [^{14}C]-L-aminobutyric acid control reaction, performic acid treatment omitted. Lane 8: Products released after performic acid treatment of the [^{14}C]-L-aminobutyric acid control reaction Lanes 9–11: As for lane 8 but in the presence of 270 μM L-AdoHcy, sinefungin and AdoEth, respectively. Lane 12: As for lane 8, but excluding ATP.

(C) Lane 1: The effect of *N*-MTase inhibitors on the C-domain peptide bond condensation reaction. Lane 2: Control CsA biosynthetic reaction. Lane 3: Control *cyclo*-(D-alanyl-[^{14}C]-L-leucyl, *desmethyl*-diketopiperazine biosynthetic reaction. Lane 4: *Cyclo*-(D-alanyl-[^{14}C]-L-leucyl biosynthetic reaction in the presence of 270 mM L-AdoHcy. Lane 5: In the presence of 270 mM sinefungin. Lane 6: In the presence of 270 mM AdoEth. Control *cyclo*-(D-alanyl-*methyl*-[^{14}C]-L-leucyl), *methyl*-diketopiperazine biosynthetic reaction in the presence of nonlabeled AdoMet.

See also Table S3.

the peptidyl-*S*-intermediates from *N*-MTase inhibited reactions. CsA is assembled via a single linear precursor peptide that is covalently bound to the modular 4′-Ppant-PCP, with chain elongation occurring by the stepwise *trans*-peptidation of the growing peptide chain to the next module for incorporation of the subsequent activated amino acid in the CsA sequence (Figure 4A) (Dittmann et al., 1994). The formation of enzyme-bound linear CsA peptide intermediates was detected by incubating CySyn with all of the components for CsA biosynthesis and radiolabeled L-leucine. After thioester breakage, the 2–10 residue intermediate peptides of CsA were identified by LC-ESI-mass spectrometry and high performance 2D thin layer chromatographic (2D-HPTLC) separation of the products (Figure 4B; Table S4). CsA chain growth normally occurs to the undecapeptidyl stage before cyclization and product release. The final undecapeptide stage in the biosynthesis of CsA was not detected by 2D-HPTLC, suggesting the linear undecapeptide is liberated from the enzyme simultaneously with cyclization and/or the cyclization reaction is very fast. Omission of AdoMet or the inclusion of saturating levels of an inhibitory AdoMet analog from the otherwise complete reaction mixture stops chain elongation at the D-Ala-L-Leu didpeptidyl stage, observed as the cyclo-diketopiperazine (Figures 4C and 4D; Table S4). Thus, *N*-methylation of amide bonds appears to be critical for linear chain elongation beyond the two residue stage (cf. Figure 5B).

N-Methylation of Backbone Amides May Preserve Recognition of the Amino Acyl-*S*-PCP Acceptor Nucleophile by Upstream C-Domains

Condensation domains act as the gate-keepers of peptide bond formation and display rigid substrate selectivity for the incoming acceptor amino acid, thereby preserving the directionality of elongation and preventing misinitiation at internal modules (Belshaw et al., 1999; Doekel and Marahiel, 2000; Ehmann et al., 2000; Keating et al., 2002; Linne and Marahiel, 2000; Mootz et al., 2000; Samel et al., 2007; Tanovic et al., 2008). A widely established mechanism for why nonribosomal peptide assembly stalls when a tailoring domain is deleted, mutated, or exchanged into another module is that the upstream C-domain does not recognize the non-native amino acyl-*S*-PCP acceptor nucleophile (Clugston et al., 2003; Linne et al., 2001; Luo et al., 2001, 2002; Luo and Walsh, 2001; Schauwecker et al., 2000; Stachelhaus et al., 1998; Stachelhaus and Walsh, 2000; Stein et al., 2005, 2006). Crystallographic studies have revealed NRPS C-domains have a V-shaped architecture with a central canyon-like groove into which the upstream and downstream PCP-bound condensation substrates can be positioned from the opposing donor and acceptor sites, respectively (Keating et al., 2002; Samel et al., 2007; Tanovic et al., 2008) (Figure 5A). In the PCP-C di-domain structure of TycC5-C6, the A/H conformation (Koglin et al., 2006; Weber et al., 2000) of the PCP domain interacts with the C-domain donor site in a catalytically un-productive orientation for peptide bond formation (Samel et al., 2007). Whereas, in the structure of the termination module of SrfA (Tanovic et al., 2008), the A/H conformation of the PCP is stalled in the C-domain acceptor site with its 4′-PPant binding site situated 16 Å away from the HHxxxDG core catalytic motif (Bergendahl et al., 2002; Stachelhaus et al., 1998), indicative of a catalytically productive orientation. We have modeled the C-domain of module 2 of CySyn (where CsA elongation stalls) with the upstream (donor) and downstream (acceptor) PCP domains (in the A/H state), and docked them into their respective C-domain donor and acceptor sites according to the productive PCP-C-domain contacts defined by mutagenesis studies (Finking et al., 2004; Lai et al., 2006a, 2006b), and seen in

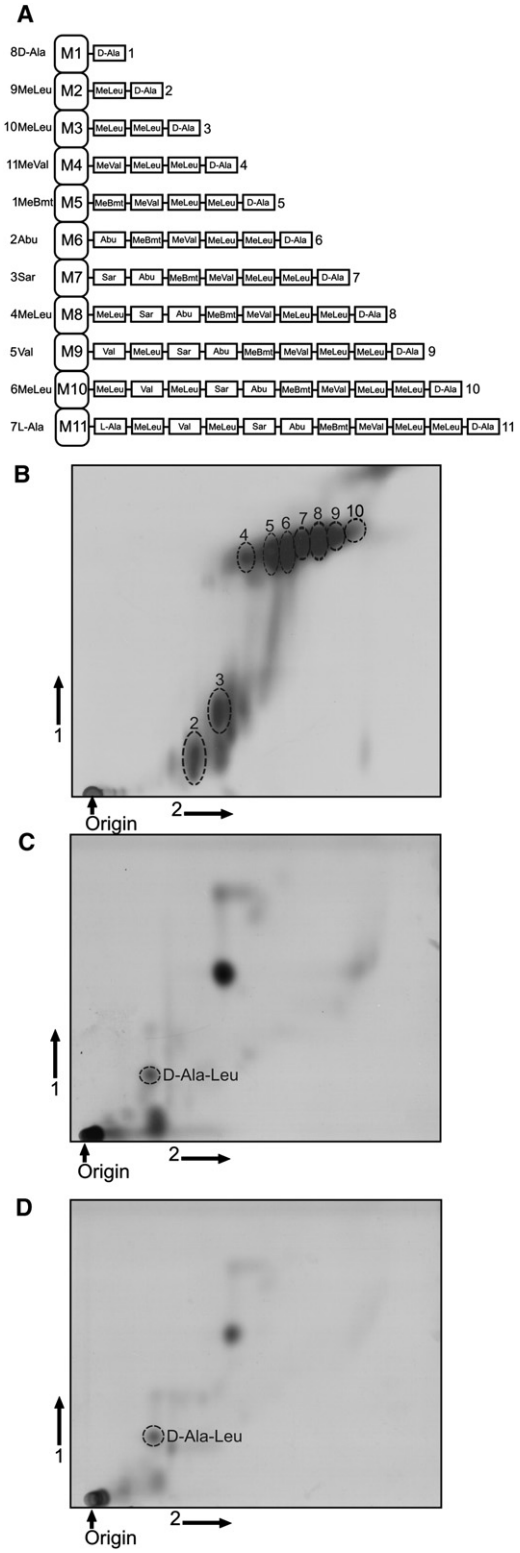


Figure 4. The Effects of *N*-MTase Inhibitors on Peptidyl-*S*-Intermediate Elongation

(A) Linear peptidyl-*S*-enzyme intermediate stages in CsA biosynthesis. M1 to M11 represent the CySyn modular units responsible for binding and incorporation of individual amino acid components of CsA (cognate amino acid substrates are indicated to the left of each module).

(B–D) Autoradiographs of 2D-HPTLC chromatograms of CsA peptide intermediates after performic acid liberation. (B) Peptides released from the uninhibited control CsA biosynthetic reaction, identified by numbering as per Figure 4A, (C) in the absence of AdoMet, (D) in the presence of AdoMet and 1 mM sinefungin.

See also Table S4.

the SrfA termination module structure (Tanovic et al., 2008). The docking solutions with the 4′-PPant donor and acceptor substrates suggest one of the mechanisms that stall CsA assembly at the dipeptidyl stage on *N*-MTase inhibition or absence of AdoMet is the inability of the acceptor site of the C-domain of module 2 to recognize the unnatural desmethyl Leu^3-*S*-PCP acceptor (Figure 5). The C-domain acceptor channel is formed by secondary elements αC1 and αC10. The side chains of Leu^{1124}, Trp^{1136}, Tyr^{1137}, Ile^{1244}, Phe^{1399} form a hydrophobic pocket surrounding the catalytic HHxxxDG core that cradles the methylated amide of the $MeLeu^3$-*S*-PCP acceptor (Figure 5A). These residues are largely conserved across all of the C-domains of CySyn (Velkov and Lawen, 2003b), with the exception of Phe^{1399}, which is substituted by Cys or Pro only in the non-*N*-MTase modules 1, 6, 9, and 11. In the methyl-substrate acceptor site model, the side chain of Phe^{1399} makes hydrophobic contacts with the methyl amide of the $MeLeu^3$-*S*-PCP acceptor. Whereas, in the corresponding desmethyl-substrate acceptor site model, the polar amide of Leu^3-*S*-PCP is poorly accommodated by the Phe^{1399} side chain and orientated away from the catalytic HHxxxDG. The docking solution for the donor site model suggests the D-Ala^1-$MeLeu^2$ dipeptidyl-*S*-PCP approaches the HHxxxDG core from the opposing face of the channel formed by αC5, αC6, and αC9. In light of the nondiscriminatory nature of the donor site, it is not surprising that the residues forming the donor channel are conserved across all C-domains of CySyn (Velkov and Lawen, 2003b).

Desmethylation Affects Cyclosporin Backbone Conformation and Intramolecular Hydrogen Bonding

CsA and the partially unmethylated congeners CsE, CsQ, CsU, and CsT were studied by 1D and 2D ^{1}H-nuclear magnetic resonance (NMR) spectroscopy with the focus on the effect of desmethylation on backbone conformation and intrachain hydrogen bonding (Figure 6A; Figures S2 and S3). The amide region of the ^{1}H-NMR spectra of each cyclosporin in $CDCl_3$ is shown in Figure 6A. An examination of the amide signals shows CsA and CsE exhibit one major conformer in $CDCl_3$, indicated by a dominant set of signals for each amide proton, whereas CsU, CsQ, and CsT all display >10 major peaks indicating the presence of at least two slowly interconverting conformations (Figure 6A).

The ^{1}H assignment of CsA, CsE, and CsU in $CDCl_3$ at 298.15 K was determined by means of standard homonuclear 2D experiments (DQF-COSY, ROESY, and TOCSY) (Figure S3 and Table S5). The spectra for CsQ and CsT could not be assigned under these conditions due to the existence of multiple interchanging conformers. A comparison of the C_α proton shifts of the desmethyl cyclosporins to CsA indicates significant conformational differences in the loop region over residues 6 and 4 and the antiparallel β-pleated sheet over residues 4–10, suggesting the preferred conformation of the desmethyl congeners differ from

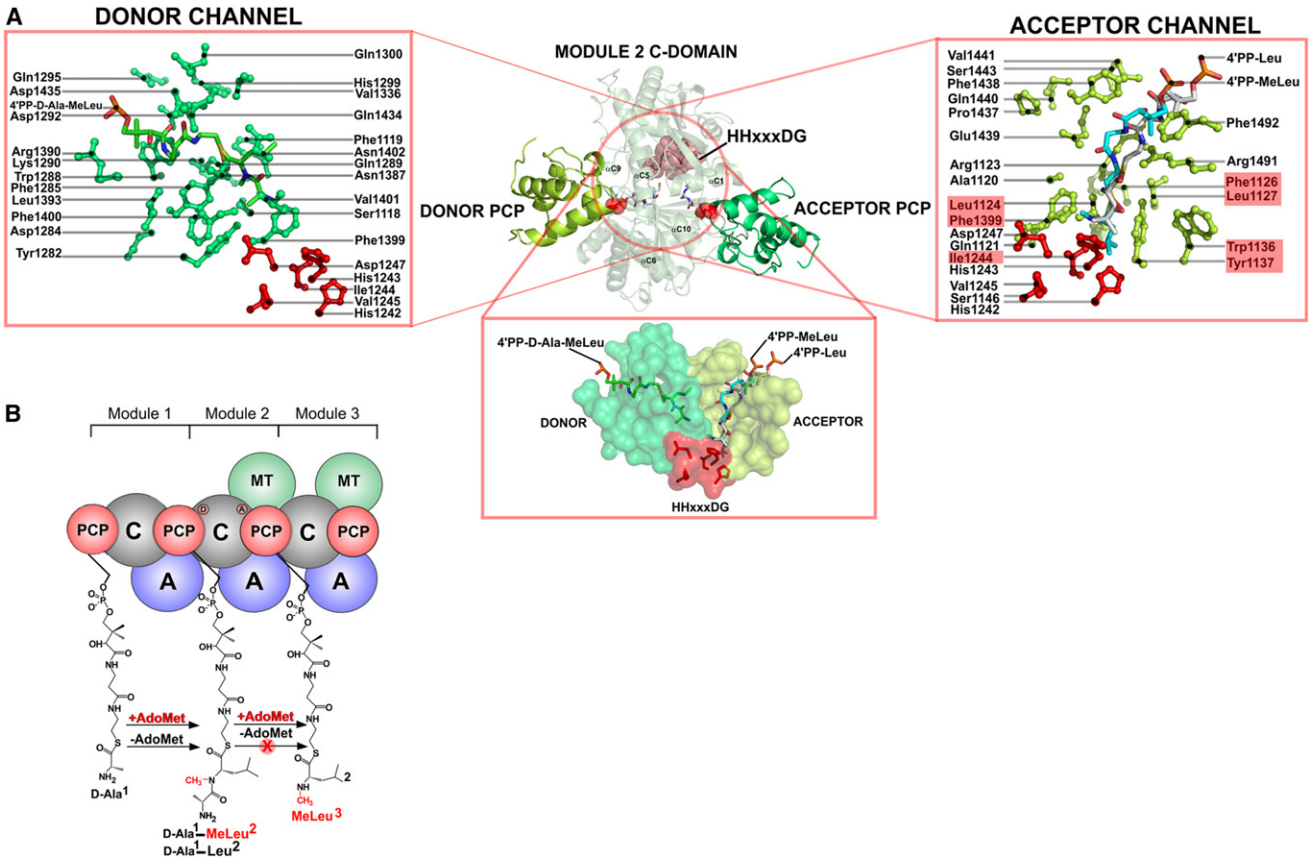


Figure 5. Modeling the Recognition of Donor and Acceptor Intermediates by the C-Domain of Module 2

(A) Tentative C-domain model of CySyn module 2 with the upstream (donor) and downstream (acceptor) PCP domain substrates docked into their respective C-domain donor and acceptor sites.

(B) Schematic diagram of the catalytic events depicted in the C-domain model.

that of CsA (Table S5). The largest chemical shift differences are for the C_α-protons of MeBmt^1 and Val^{11} in CsE, and the C_α-protons of MeLeu^6 in CsE, and Leu^6 in CsU.

To verify the involvement of the free amide protons in hydrogen bonding, we examined the temperature dependence of their chemical shifts in the 25–45°C range (Figure 6B). A temperature coefficient of less than −4.5 ppb/K, indicates the involvement in hydrogen bonding (Stevens et al., 1980). However, it must be noted that in the case of cyclic peptides, small temperature dependencies can also be observed if the NH group is shielded from the solvent and remains shielded over the temperature range of the ^{1}H-NMR experiment (Stevens et al., 1980). The plot of δ_{NH} versus T (K) for CsA indicates the four exchangeable protons display a linear dependence on temperature, the rates of shift for the NH groups agree with their participation in hydrogen bonds: −3.6 ppb/K (Abu^2); −3.7 ppb/K (Ala^7); −1.8 ppb/K (Val^5); −1.1 ppb/K (D-Ala^8). The rates of shift for the NH groups for CsE also agreed with their participation in stable hydrogen bonds: −2.9 ppb/K (Abu^2); −1.6 ppb/K (Ala^7); −1.7 ppb/K (Val^5); −2.7 ppb/K (D-Ala^8); −2.5 ppb/K (Val^{11}). The small temperature gradient for the additional free NH at the Val^{11} desmethyl position of CsE suggests it is involved in stable hydrogen bonding. The occurrence of one additional intrachain hydrogen bond was also observed in the crystal structure of CsE (Hušák et al., 1998). The temperature dependence data for CsU indicate that the NH groups of Ala^7 (−2.8 ppb/K), Val^5 (−1.4 ppb/K), and D-Ala^8 (−2.0 ppb/K) are involved in stable hydrogen bonds. The faster rate of shift for Abu^2 (−4.1 ppb/K) and the additional desmethyl position Leu^6 (−4.0 ppb/K) suggest that the hydrogen bonds of these groups are markedly weaker. A large temperature dependence is known to occur when a NH, is initially shielded through intramolecular hydrogen bonding and becomes transferred into a solvated environment with increasing temperature (Stevens et al., 1980). We interpret the large temperature dependence of the Abu^2 and Leu^6 NH amides in CsU as indicating the presence of intramolecularly hydrogen bonded NH amides that are thermally disrupted over the temperature range of the ^{1}H-NMR experiment.

Collectively the NMR data indicate that desmethylation at even a single amide position significantly affects the hydrogen bonding and backbone conformation of cyclosporins. In CsA the backbone is constrained by *N*-methylation of specific amide bonds that limits the hydrogen bonding potential. Removal of an *N*-methyl from position eleven as in CsE (MeVal^{11} → Val^{11}) results in changes over the loop region formed by residues 6–11 with the formation of a hydrogen bond Ala^7-CO—HN-Val^{11} (Figure 6C).

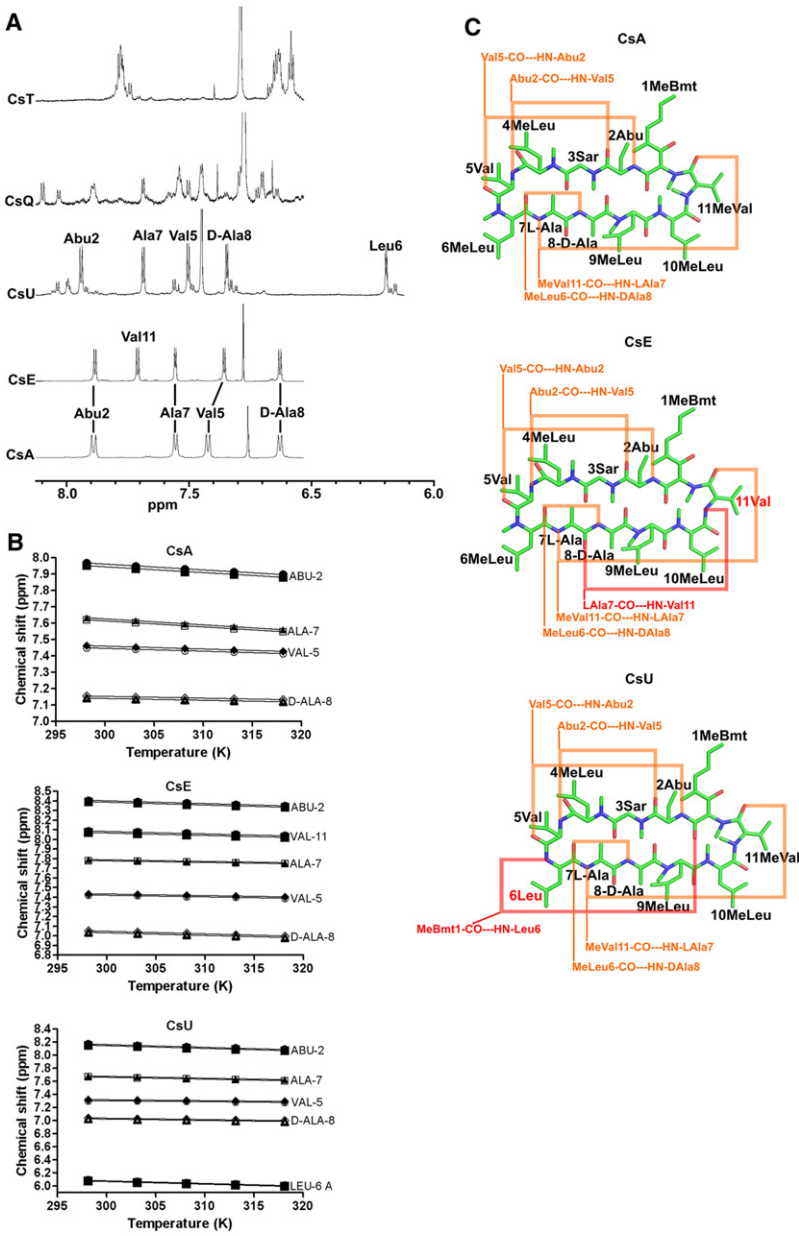


Figure 6. NMR Characterization of the Effect of Desmethylation on Cyclosporin Backbone Conformation and Intramolecular Hydrogen Bonding

(A) Amide proton region of the ^{1}H NMR spectra in (Figure S2).

(B) Plots of the chemical shift variation as a function of temperature for amide protons, the temperature coefficients were obtained by linear regression fitting of the data points (solid line).

(C) Inferred backbone hydrogen bonding pattern of CsA and the desmethyl congeners, CsE and CsU.

See also Figures S2 and S3, and Table S5.

Similarly, in CsU the loss of the position 6 amide *N*-methyl group (MeLeu $^{6}\rightarrow$Leu^{6}) allows for the formation of an additional hydrogen bond MeBmt^{1}-CO—HN-Leu^{6} (Figure 6C).

N-Methylation of Backbone Amides May Facilitate Preorganization of the Peptide for Cyclization

The total chemical synthesis of CsA and its precursors is complex (Wenger, 1984), and limited by the lack of commercial availability and the difficulty to synthesize the amino acid Bmt (Savignac et al., 1994). To overcome these limitations, we used molecular modeling to examine the effects of *N*-methylation patterns on backbone conformer preferences of linear CsA peptidyl-*S*-enzyme intermediates. Molecular models of the 3–11 amino acid stages of fully *N*-methylated and desmethyl CsA peptidyl-*S*-enzyme intermediates were constructed (Figure S4). The models suggest that the specific set of amide *N*-methylations present in CsA is critical at the five residue peptidyl-*S*-enzyme intermediate stage. The simulation predicts that the desmethyl backbone folds in on itself into a tight cyclic structure with the termini almost in contact. In comparison, the fully *N*-methylated form is more open and elongated because the four consecutive amide *N*-methylations over the loop region formed by the first four residues, act to prevent the structure from closing up on itself before elongation is complete. Over the 6–11 residue stages, each desmethyl peptide is predicted to cyclize back onto itself toward the carboxyl termini anchor point on the enzyme PCP-4′-Ppant, with both termini too close together, which would possibly interfere with elongation. This results from extensive intramolecular hydrogen bonding. In comparison, over the 6–11 residue stages of the fully *N*-methylated backbone, the intermediates adopt a product-like conformation via the formation of the ordered succession of hydrogen bonds seen in the CsA structure (Figure S1A and Figure S4). These include hydrogen bonds between, Abu^{2}-CO—HN-Val^{5} at the nine residue intermediate stage, then at the 10 residue intermediate stage between the Val^{5}-CO—HN-Abu^{2}, and finally at the mature length 11 residue stage between MeVal^{11}-CO—HN-Ala^{7}, and MeLeu^{6}-CO—HN- D-Ala^{8} (Figure 6C; Figure S1A). The latter hydrogen-bond serves to hold the backbone in a folded L-shape. The fully *N*-methylated peptidyl-*S*-enzyme intermediates fold back toward the protein anchor point but with the free amino terminus end folding from above the anchor point, so as not to interfere with the assembly process.

Overall the modeling data suggest that the amide *N*-methylation pattern of CsA only allows for specific intrachain hydrogen bonds that may operate to stabilize the open undecapeptide chain into a product-like conformation, thereby bringing the amino and carboxyl termini of position 7 and 8 together to assist cyclization. Preorganization of the growing peptidyl-*S*-intermediate into a product-like conformation is an emerging consensus mechanism across NRPS systems (Kohli et al., 2001, 2002; Trauger et al., 2000, 2001; Tseng et al., 2002). It appears

structural modifications to the peptide backbone introduced during assembly such as amide *N*-methylation help maintain the growing peptide in a product-like conformation that ensures that *trans*-peptidation and cyclization reactions proceed un-hindered by futile intramolecular bonding events. In the assembly of the peptide tyrocidine A, the peptide substrate is preorganized for cyclization via intramolecular backbone hydrogen bonds similar to those in the product to allow for the proper presentation of the termini to the thioesterase domain for ring closure (Kohli et al., 2001, 2002). The overall rate of product cyclization/release was shown to be dependent on the rate of substrate pre-organization (Kohli et al., 2001, 2002). Structural modifications such as *N*-methylation that inhibit backbone hydrogen bonding are, therefore, likely to influence the pre-organization of the peptide backbone for cyclization. Thus, the slower rate of biosynthesis of cyclosporins with five to six backbone *N*-methylations may be attributable to a less facile preorganization rate. Coincidentally, *N*-methylation of a specific amide bond position was shown to be mandatory for a high substrate turnover in the biosynthesis of streptogramin B antibiotics (Mahlert et al., 2005). In the biosynthesis of tyrocidine A, key residues near the N- and C-termini are involved in the formation of intramolecular hydrogen bonds to allow for preorganization of the linear peptide backbone, such that the N- and C-termini are presented in the correct orientation for macrocyclization (Trauger et al., 2001). In the CsA structure, the hydrogen bond between the amide nitrogen of D-Ala^8 and the carbonyl oxygen of L-Ala^7 helps to bring the ends of the molecule together for cyclization. The small side chains of the N- and C-terminal alanine residues of CsA also facilitate cyclization by allowing the ends to come close together into a suitable orientation for condensation. The D-configuration of the C-terminal alanine facilitates ring formation over side reactions as has been shown with other examples in the literature (Brady et al., 1979). This is coincident with the strict selectivity of the A-domain of the last module incorporating for an L-amino acid and the strict D-chiral selectivity of the first A-domain module incorporating D-Ala^8 (Lawen and Traber, 1993). Similarly, N- and C-terminal amino acid side-chain stereochemistry and size constraints appear to be in place for other non-ribosomal peptide cyclization reactions (Tseng et al., 2002). Wenger (1984) described the first total synthesis of the CsA molecule using a fragment-condensation technique. Chemical cyclization of the synthetic undecapeptide between positions 7 and 8, analogous to the enzymatic cyclization reaction, was the most efficient route. Moreover, L-Ala^7 and D-Ala^8 are the only consecutive pair of *N*-desmethyl amino acids in the CsA molecule. This further facilitates ring closure, as chemical amide bond formation between *N*-methylated amino acid residues is more difficult (Gund and Veber, 1979; Nitecki et al., 1968). Additional structural features of the CsA molecule that facilitate the reorganizational folding process include the presence of the invariant *N*-methyl glycine (sarcosine) in the middle of the open peptide that serves as a β-turn forming element. A β sheet appears to be a critical substructure requisite for proper preorganization for cyclization and release of nonribosomal peptides (Trauger et al., 2000, 2001). Accordingly, backbone *N*-methylation is crucial for conservation of the proper hydrogen bonding pattern that preserves this β sheet structure of CsA.

SIGNIFICANCE

The *N*-methylation of enzyme-bound intermediates is of general importance in the synthesis of many peptide and depsipeptide antibiotics. In future, it is hoped that *N*-methyltransferase tailoring functions can be routinely exploited in the genetic engineering of hybrid synthetases for the rational design of peptide antibiotics. These new biosynthetic approaches require detailed studies into the enzymology of the reactions involved and the effect of these modifications have on active conformation of the cyclic peptide. This study indicates *N*-methylation of specific amide positions in the backbone of CsA is critical for the efficient progression of peptide assembly to the mature undecapeptidyl stage. The desmethylation of even a single amide appears to destabilize the main conformation of CsA. Given the mechanistic similarities across NRPS, these findings provide a biochemical model for *N*-methylation processes of NRPS in general.

EXPERIMENTAL PROCEDURES

Materials

S-adenosyl-L-[*methyl*-^{14}C]methionine ([^{14}C-*methyl*]AdoMet, 58 Ci/mol), [^{14}C]-iodoacetamide (21 mCi/mmol), and [^{14}C]-L-leucine ([^{14}C]-L-Leu, 30 mCi/mmol) were purchased from New England Nuclear (Boston, MA). (4*R*)-4-[(*E*)-2-*butyl*]-4-*methyl*-L-threonine (Bmt) and the cyclosporin standards were kindly donated by Dr. R. Traber (Novartis Ltd., Basel, Switzerland) and Dr. A. Jegerov (Galena Co. Ltd., Czech Republic). AdoMet and analogs were purchased from Sigma (Castle Hill, NSW, Australia). Sinefungin was a generous gift from Eli Lilly Research Laboratories (Indianapolis, IN).

Growth of *Tolypocladium inflatum* and Purification of CySyn

These methods were performed as previously described (Velkov et al., 2006).

Assay of *N*-MTase activity

N-MTase activity was assayed by monitoring the incorporation of [^{14}C]-methyl groups from *S*-adenosyl-L-[*methyl*-^{14}C]methionine into the amide nitrogens of CsA, according to Lawen et al. (1989) and Lawen and Zocher (1990). Where indicated in the results, the assay mixture was supplemented with an AdoMet analog dissolved in buffer A (0.1 M Tris, pH 7.8; 4 mM EDTA; 4 mM dithiothreitol; 15% (v/v) glycerol).

Photoaffinity Labeling of CySyn with [^{14}C-*methyl*]AdoMet

These methods were performed as previously described (Lawen and Zocher, 1990; Velkov and Lawen, 2003a).

Liberation of Covalently Bound Amino Acids and Linear Peptide Intermediates

The release and development of covalently-bound peptide intermediates from CySyn was performed as previously described (Dittmann et al., 1994). Two-dimensional HPTLC analysis of isolated peptidyl-intermediates was performed as previously described (Dittmann et al., 1994). Peptide spots were visualized by superimposition of autoradiographs from 2D-HPTLC analysis of [^{14}C]-L-leucine radiolabeled biosynthetic reactions. Peptides were identified by liquid chromatography electrospray ionization mass spectrometry (LC ESI-MS). The measurement of thioester formation was as previously described (Dittmann et al., 1990, 1994).

Assay of Amino Acid Adenylation (Substrate Amino Acid-Dependent ATP-^{32}PP$_i$ Exchange Activity)

The quantification of the aminoacyl adenylation of the amino acid substrates was performed by the ATP-PP$_i$ exchange method (Linne and Marahiel, 2004).

Enzymatic and Chemical Synthesis of Cyclosporins and Diketopiperazines

The enzymatic biosynthesis of cyclosporins and *cyclo*-(D-Ala-L-Leu) diketopiperazine was monitored by the incorporation of [^{14}C]-L-leucine as previously described (Dittmann et al., 1990).

HPTLC

HPTLC of cyclosporins and *cyclo*-(D-Ala-L-Leu) diketopiperazines was performed on silica gel sheets (Merck, Darmstadt, FGR) developed with water-saturated ethyl acetate (EtOAc) (solvent I). Radiolabeled material was visualized by autoradiography and superimposed onto iodine vapor stained HPTLC plates and correlated with bona fide reference compounds. Radioactive amino and *N*-methyl amino acids released from the enzyme after performic acid hydrolysis were separated on silica plates developed in butanol/acetic acid/H_2O (4:1:1) (solvent II).

HPLC Analysis of In Vitro Cyclosporin Products

Cyclosporin products were concentrated by SpeedVac centrifuge and resuspended in 20 μl acetonitrile (ACN)/H_2O (60:40). Reverse phase HPLC of cyclosporins was performed on a Zorbax 300 Å column (C_{18}, 3.5 μm, 4.6 × 250 mm) (Agilent Technologies, Melbourne Victoria, Australia). Separation was performed isocratically at 75°C with ACN/H_2O/H_3PO_4 (630:370:0.1) as the mobile phase, at a flow rate of 1 ml/min, cyclosporins were detected at 210 nm. The identity of enzymatic cyclosporin products was ascertained by reference to the retention α-factor of bona fide reference compounds and MALDI-TOF MS analysis of individual peak fractions. The α-factor is defined as the relative retention time $[(t_{R,1} - t_o)/(t_{R,2} - t_o)] \times 10$ where $t_{R,1}$ and $t_{R,2}$ are the corresponding retention times and t_o is the dead retention time of the column. CsA is taken as the reference compound and assigned as $\alpha = 10$.

MALDI-TOF mass spectrometry

The positive ion MALDI-TOF mass spectra were recorded on a MALDI VOYAGER DE STR time of flight mass spectrometer (PerSeptive Biosystems, Framingham MA). The ion acceleration voltage was set at 20 kV. Samples were irradiated with a pulsed nitrogen laser at 337 nm. When delayed extraction was used, a potential difference between the probe and extraction lens was applied at a time delay of 300 ns subsequent to each laser pulse. For the MALDI matrix, a solution of saturated α-cyano-hydroxy-cinnamic acid in 50% ACN (v/v)/0.1% trifluoroacetic acid (TFA) (v/v) in H_2O was employed. Samples were applied onto the MALDI probe by the dried-drop method: 1 μl matrix solution was spotted directly onto the plate and allowed to air dry at room temperature, followed by 1 μl of sample, and dried at room temperature.

NMR Methods

Cyclosporin ^{1}H-NMR spectra were assigned by standard sequential assignment methods (Wüthrich, 1986). NMR spectra were collected on a Varian INOVA 600 MHz spectrometer equipped with a cryogenically cooled triple-resonance probe at 25°C in deuterated $CDCl_3$. The chemical shifts were referenced relative to the solvent peak and tetramethylsilane set to 0.0 ppm. Data were processed with NMRPipe software and analyzed in SPARKY.

Molecular Modeling

Molecular modeling operations for linear CsA intermediates were performed using the Accelrys Discovery Studio V2.1 using previously described methods (O'Donohue et al., 1995). Initial CsA coordinates were extracted from the Cambridge Crystallographic databank (access code: DEKSAN). A structural model of the AdoMet binding site of the *N*-MTase domain of module 8 of CySyn was constructed using the crystallographic structure of catechol-*O*-methyltransferase (RCSB PBD: 1VID) as the modeling template (Velkov and Lawen, 2003a). A homology model of the C-domain of module 2 and PCP domains of modules 2 and 3 were constructed using the 1.8 Å crystallographic structure of the C-domain of tyrocidine synthetase (RCSB PBD: 2JGP) (Samel et al., 2007) and termination module of SrfA-C (RCSB PBD: 2VSQ) (Tanovic et al., 2008) as the templates. All modeling and docking operations were performed with Accelrys Discovery Studio V2.1.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and five tables and can be found with this article online at doi:10.1016/j.chembiol.2011.01.017.

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