SYNTHESIS AND CRYSTAL STRUCTURE DETERMINATION OF CYCLOSPORIN H

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Dedicated to Professor Josef Loub on the occasion of his 70th birthday.

Acid-catalyzed degradation of cyclosporin A was studied in various solvents and products of reaction were monitored by HPLC. Identification of amino acids and their chirality were determined after hydrolysis and derivatization by GC-MS. Cyclosporin H was isolated as the principal product and its structure was determined by X-ray diffraction: Cyclosporin H–diethyl ether–water (1 : 0.5 : 1) crystallizes in the monoclinic space group *I*2 with a = 12.338(2) Å, b = 18.963(2) Å, c = 34.074(3) Å, β = 96.47(2)°, Z = 4, and V = 7 921.4(17) Å^3.
Key words: Cyclosporins; Conformation analysis; Crystal structure determination; X-Ray diffraction; Hydrolysis; Amino acids; Peptides; Racemization; Immunosuppressants.

Racemization of amino acids is one of the most important pathways leading in most cases to the loss of biological activity in peptides and proteins[1]. Whereas racemization was studied in detail for amino acids, small peptides and some industrially important peptides, the information on racemization of amino acids in cyclic peptides is limited.

Cyclosporins are natural undecapeptides derived from cyclosporin A, cyclo(-MeBmt[1]-Abu[2]-Sar[3]-MeLeu[4]-Val[5]-MeLeu[6]-Ala[7]-D-Ala[8]-MeLeu[9]-MeLeu[10]-

MeVal[11]-) by formal substitution of one or two amino acids. They differ from most peptides by the presence of several *N*-methylated amino acids and an unusual amino acid MeBmt = (2*S*,3*R*,4*R*,6*E*)-3-hydroxy-4-methyl-2-(methylamino)oct-6-enoic acid), Fig. 1. Recently, cyclosporin A has widely been used as immunosuppressant for organ transplantations and for treatment of autoimmune diseases (*e.g.*, Consupren®, Galena, Neoral®, Novartis). Due to its cyclic structure and lipophilic character, cyclosporin A is considerably stable in organic solvents in a wide range of experimental conditions; however, its stability is not unlimited. The following reactions have been described: N→O peptidyl shift providing iso-cyclosporins[2–10], acid- and Lewis-acid-catalyzed opening of the cycle[9–12], intramolecular alkoxy addition to double bond of MeBmt[2,3,6], singlet oxygen oxidation[13], dehydration and the loss of [MeBmt[1]] side chain[14]. In this paper we report a convenient synthesis of cyclosporin H (*i.e.*, [D-MeVal[11]]cyclosporin) and its crystal structure determination.

EXPERIMENTAL

Amino Acid Analysis

Cyclosporin samples (typically about 2 mg) were hydrolyzed by 6 M HCl (1 ml) at 105 °C for 48 h; aliquots of the acid hydrolyzate (100 μl) were evaporated to dryness in a stream of dried nitrogen and derivatized prior to gas chromatographic analysis. Cyclosporin amino acids were identified by gas chromatography-mass spectrometry as *tert*-butyldimethylsilyl derivatives on a 30 m × 0.25 mm i.d. DB-1 capillary column[15]. Chiral analysis was performed on a 30 m × 0.25 mm i.d. DB-210 capillary column after conversion of amino acids to *N*-((1*R*)-menthyloxycarbonyl)methyl esters or (*S*)-2-methylbutyl ester derivatives[16,17] (Fig. 2). Standards of individual D- and L-amino acids were from Sigma except of MeBmt, derivatives of which were identified by GC-MS. GC-MS was performed on a Hewlett–Packard 5890 gas chromatograph directly coupled to a Kratos Profile mass spectrometer (Mass Spectrometry Int., Manchester, U.K.) operated in EI mode.

Preparation of Cyclosporin H

Preliminary experiments were carried out with cyclosporin A (1 g, 99% HPLC purity, Galena Co., Czech Republic) dissolved in an organic solvent (20 ml; methanol, ethanol, or dioxane) and methanesulfonic acid (99.5%, 0.1–0.3 ml) at various temperatures (60–100 °C). Reaction products were extracted with dichloromethane and evaporated to dryness. HPLC method with sodium dodecyl sulfate-containing mobile phase[18] was used for the analysis of reaction mixtures, chromatographic fractions and purity of isolated cyclosporin H.

For laboratory scale synthesis, cyclosporin A (100 g) was dissolved in dioxane (2 000 ml) and methanesulfonic acid (99.5%, 15 ml) was added. The mixture was heated under stirring for 1 h at 100 °C. After cooling, pH was adjusted to 9.0 with 10% aqueous sodium hydroxide. Dichloromethane (800 ml) and water (800 ml) were added and the organic layer was separated. The aqueous layer was extracted with dichloromethane (800 ml) and pooled ex-

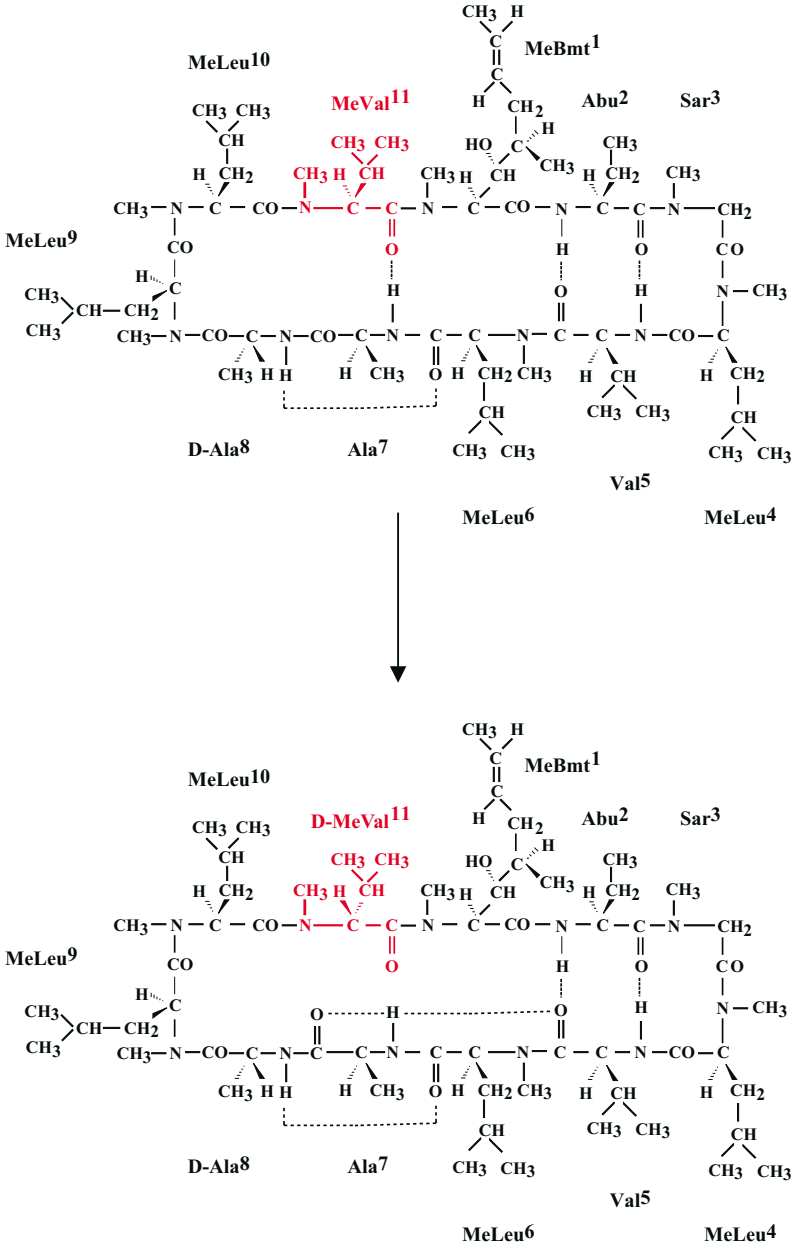

FIG. 1

Schematic representation of structure and hydrogen bonds in cyclosporin A dihydrate (upper) and cyclosporin H–diethyl ether–water (1 : 0.5 : 1)

tracts were evaporated to dryness. The crude mixture of cyclosporins H and A (approximately 1 : 2 w/w) was subjected to column chromatography on silica gel 60 (Merck) using stepwise gradient of methanol (0–5% v/v) in dichloromethane. Fractions containing cyclosporin H were pooled, evaporated, and cyclosporin H was finally purified by repeated crystallization from diethyl ether. The yield was roughly 15 g of pure cyclosporin H (98%, HPLC).

Molecular Weight and Sequence Analysis

The static fast atom bombardment (FAB) ionization in combination with collision-induced dissociation (CID) of protonated molecules were used for the molecular and sequence determination of [D-MeVal[11]]cyclosporin. The CID mass spectrum of $[M + H]^+$ ions was recorded on a Finnigan MAT 95 instrument of BE geometry using B/E linked scan. Helium was used as collision gas and 3-nitrobenzyl alcohol (Aldrich, Steinheim, Germany) as FAB matrix. The positive-ion FAB mass spectrum revealed the protonated molecule of cyclosporin H at *m/z* 1 202.8, in good agreement with the published FD and EI mass spectra[19]. The CID mass spectrum of $[M + H]^+$ ion is completely interpretable in terms of primary ring cleavages between amino acid residues 2–3, 1–11 and 5–6 (refs[5,9]). Dominating fragmentation mechanism is the 2–3 splitting producing the corresponding linear undecapeptide[5]. Its further fragmentation leads to the b_i^{2-3} daughter ion series, which is used for sequence determination (for the ion nomenclature see refs[5,20]). The corresponding CID spectrum is nearly iden-

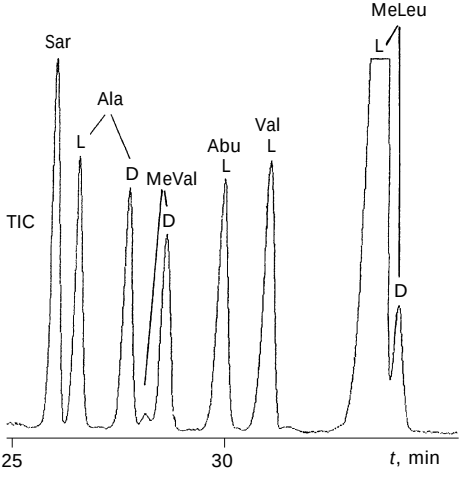

FIG. 2
Chiral GC-MS analysis of amino acid as their *N*-((1*R*)menthyloxycarbonyl)-(*S*)-2-methylbutyl esters in cyclosporin H acid hydrolyzate. Column: 30 m DB-210, temperature program: 120 °C, 5 °C/min, 225 °C, then hold for 10 min. Injection: split 30 : 1, injector temperature: 250 °C, tranfer line temperature: 225 °C. Detector: mass spectrometer; EI mode; TIC, total ion current

tical to that of cyclosporin A (mass spectrometry does not provide any information on chirality) and exhibited the following b_i^{2-3} ions: 934.8 (b_9), 821.7 (b_8), 694.6 (b_7), 567.5 (b_6), 496.4 (b_5), 425.3 (b_4), 298.2 (b_3), 199.2 (b_2).

^{1}H and ^{13}C NMR spectra were measured on a Varian VXR-400 spectrometer (399.95 MHz for ^{1}H, 100.58 MHz for ^{13}C) in $CDCl_3$ at 25 °C (δ, ppm; *J*, Hz). The residual solvent signal was employed as an internal reference (δ_H 7.265, δ_C 77.0). Carbon signal multiplicity was determined by APT (Attached Proton Test). 2D NMR experiments, HOM2DJ, COSY, delay-COSY, and ROESY were performed using the manufacturer's software. NMR spectra confirmed the presence of diethyl ether in the solvate. However, its ^{1}H NMR spectrum was very complicated, indicating the equilibrium of several forms with one prevailing [methyl on the double bond at 1.656 (*J* = 5.0), two alanine methyls at 1.264 (*J* = 7.1) and 1.153 (*J* = 6.9), seven *N*-methyl singlets (3.316, 3.292, 3.200, 3.106, 2.931, 2.895, 2.812), four N-H doublets at 8.618 (*J* = 9.4), 6.814 (*J* = 9.0), 6.723 (*J* = 8.0) and 5.730 (*J* = 9.4)]. The overall picture resembled the published spectrum[19]. There were 11 carbonyls in the ^{13}C NMR spectrum, which was also complex. The number of components was determined as seven on the basis of seven pairs of double-bond carbon resonances. No exchange between conformers was detected by ROESY (mixing times 0.125, 0.2, 0.5 s), which means that this process is slow on the NMR time scale. Whereas all cyclosporins show one prevailing conformation in non-polar solvents (contrary to multiple conformations in polar solvents[21–23]), cyclosporin H already exhibits such behaviour in chloroform, similarly to iso-cyclosporins[22].

Crystal Structure Determination of Cyclosporin H

A small vial with cyclosporin H (440 mg, 98%) dissolved in acetone (3 ml) was placed in a capped flask containing diethyl ether. Single crystals of cyclosporin H were formed within two days. Since the crystals were prone to desolvation, a suitable crystal was mounted in a capillary with residual amount of mother liquor. For data collection and refinement parameters see Table I.

The structure was solved by direct methods and anisotropically refined by full-matrix least squares. The presence of solvate molecules was revealed from a difference map. Diethyl ether molecule had a half occupancy factor and was restrained using the geometry obtained from low-temperature structure analysis of diethyl ether[24]. Hydrogen atoms were located from a Fourier map and from the expected geometry. Only hydrogen atoms from NH and OH groups were refined isotropically with distance restrains. Hydrogen atoms of ether molecule were not found because of large thermal parameters of its non-hydrogen partners. The absolute structure was assigned based on that of cyclosporin A. Data collection and structure refinement parameters are listed in Table I, Fig. 3 shows an ORTEP drawing of the molecule. Crystallographic data for the structure reported in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication number CCDC-139242. Copies of the data can be obtained free of charge on application to CCDC, e-mail: deposit@ccdc.cam.ac.uk.

RESULTS AND DISCUSSION

The novel GC-MS method was used for chiral analysis of various amino acids present in hydrolyzates of cyclosporins. Some partial racemization of *N*-methyl amino acids representing few per cent was observed in hydrolysis

of cyclosporins (Fig. 2), whereas free and *N*-methyl amino acids were virtually stable under the conditions used. The extent of this racemization was used as the background correction for evaluation of synthetic experiments. Preliminary experiments indicated that, besides the well known conversion of cyclosporin A to iso-cyclosporin A, some new peaks appeared in HPLC chromatograms and simultaneously the amount of D-MeVal increased at

TABLE I
Data collection and structure refinement parameters for cyclosporin H–diethyl ether–water (1 : 0.5 : 1), $C_{64}H_{118}N_{11}O_{13.5}$, M_r = 1 257.7

Crystal system, space group	Monoclinic, *I2* (No. 5), a = 12.338(2) Å, b = 18.963(2) Å, c = 34.074(3) Å, β = 96.47(2)°, V = 7 921.4(17) Å^3, Z = 4, D_{calc} = 1.055 g cm^{-3}
Crystal size	0.25 × 0.38 × 0.50 mm
Temperature	293 K
Difractometer and radiation used	Enraf–Nonius CAD4, λ(MoKα) = 0.70930 Å
Absorption coefficient	0.74 mm^{-1}
Scan technique	ω/2θ
θ range	1.23–24.91°
No. of unique observed reflections	7 229
Criterion for observed reflections	$I \geq 1.96\sigma(I)$
Function minimized	$\Sigma w(F_o^2 - F_c^2)^2$
Weighting scheme	$w = 1/[\sigma^2(F_o^2) + (0.0996P)^2]$, where $P = (F_o^2 + 2F_c^2)/3$
Parameters refined	874
Final *R* indices	R = 0.056, $wR(F^2)$ = 0.137
Value of *S*	1.101
Extinction coefficient	0.0024(3)
Ratio of max. least-squares shift to e.s.d.	0.001
Largest difference peak and hole	0.30, –0.19 eÅ^{-3}
Source of atomic scattering factors	SHELXL97 (ref.[44])
Programs used	SDP (ref.[45]), SHELXS86 (ref.[46]), SHELXL97 (ref.[44])

the lower pH and higher temperature used in the synthesis. The use of HPLC method with sodium dodecyl sufate in the mobile phase[18] made it possible to distinguish both cyclosporins A and H, and their respective iso-cyclosporins as the main products. Upon raising the pH, iso-cyclosporins quickly transformed into cyclosporins A and H, which were separated by column chromatography on silica gel. The reaction was optimized for the laboratory scale preparation of cyclosporin H.

The migration of acyl groups in acyl derivatives of substances having a primary amino group and a hydroxyl group on adjacent carbon atoms was first observed in 1921 (ref.[25]) and for cyclosporin A was described in 1976 (ref.[2]). There are two general distinct mechanisms proposed for N→O-acyl migration leading to the different chirality of the carbon atom directly bonded to the hydroxyl moiety[6]. Since the crystal structure determination proved[26] that the chirality of MeBmt is retained in iso-cyclosporin A, the reaction proceeds *via* hydroxyoxazolidine-type intermediate[6]. Similarly as with other threonine-containing peptides[27–29], the creation of an ester bond contributes to the further degradation in acidic media providing linear 1,11-seco-cyclosporins[10]. Racemization of *N*-methylvaline as a side reaction in the formation of iso-cyclosporins was not found in the preceding works[12]. Apparently, it is due to the pH range used for the reaction and possibly also because cyclosporins A and H are difficult to resolve on some

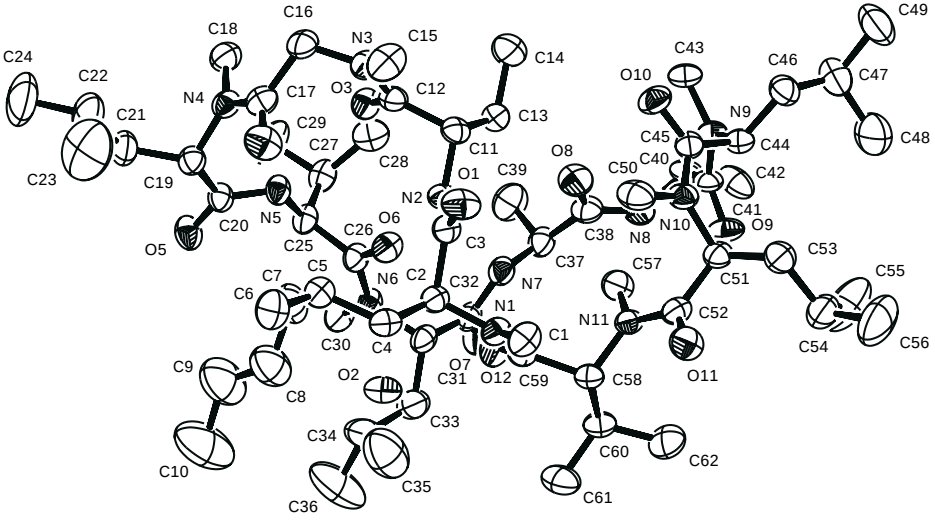

FIG. 3

Ortep drawing of cyclosporin H–diethyl ether–water (1 : 0.5 : 1). Hydrogen atoms are omitted for clarity

chromatographic columns. However, this reaction becomes very important with regard to the fact that iso-cyclosporins were proposed as potential prodrugs[7,30,31]. Whereas cyclosporin A possesses an immunosuppresive activity utilized to prevent graft rejection during, *e.g.*, kidney transplantations, cyclosporin H lacks the immunomodulating activity[32,33]. However, cyclosporin H itself is not a useless substance, exhibiting a number of interesting activities[32–36]. It can serve as a model for the study of mechanism of action and side effects of cyclosporins[32].

X-Ray crystal structure determination confirmed the expected absolute configuration of cyclosporin H and revealed the presence of water and diethyl ether in the crystal structure. The intramolecular hydrogen bond network is nearly the same as in other cyclosporins[37] (Fig. 1). Hence, it might seem, also on the basis of inspection of torsion angles (Table II), that besides obvious changes in the vicinity of D-MeVal (ϕ_{11}, ψ_{11}) and some minor differences (ψ_5, ψ_6, ψ_7, ϕ_7), conformation of the cyclosporin backbone is not much affected. In fact, conformation of the MeBmt^1-Abu^2-Sar^3-MeLeu^4-Val^5-MeLeu^6 amino acid chains in both cyclosporins H and A is very similar (Fig. 4, almost identical bottom parts of the drawings). However, just the above-mentioned changes in the torsion angles result in the opposite folding of the D-Ala^8-MeLeu^9-MeLeu^{10}-MeVal^{11} loop in cyclosporin H (Fig. 4, the upper parts of the drawings). A detailed investigation of the structure also revealed some differences in the orientation of side chains of amino acids MeBmt^1, Val^5, and MeLeu^6.

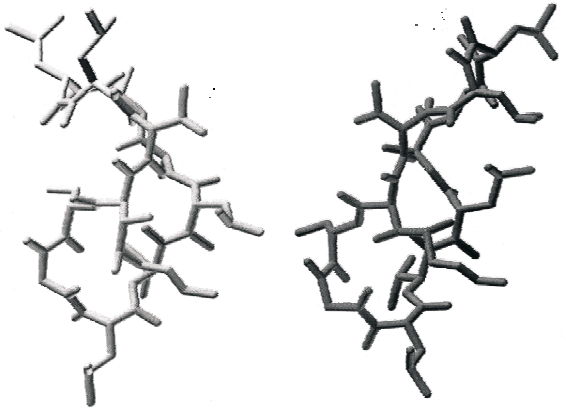

FIG. 4
Comparison of cyclosporin H (left) and cyclosporin A (right) conformation

TABLE II
Comparison of cyclosporin conformations in the solid state

Cyclosporin	ϕ_1	ψ_1	ω_1	ϕ_2	ψ_2	ω_2	ϕ_3	ψ_3	ω_3	ϕ_4	ψ_4	ω_4	ϕ_5	ψ_5	ω_5	ϕ_6	ψ_6
CsH[a]	–103	105	–177	–105	107	–173	61	–132	176	–105	23	–176	–121	155	174	–99	85
CsA·2H_2O[b]	–84	122	–175	–120	90	–177	71	–128	173	–99	22	–180	–113	125	167	–90	100
CsA·H_2O[b]	–100	103	–169	–108	105	–175	66	–136	173	–105	33	177	–109	120	167	–87	105
CsA·DMI[c]	–93	156	167	–118	99	–167	68	–133	172	–98	9	–177	–85	134	176	–79	128
CsA·CypA[d]	–109	171	–178	–116	93	–179	128	–72	173	–108	103	–177	–76	122	–176	–96	168

Cyclosporin	ω_6	ϕ_7	ψ_7	ω_7	ϕ_8	ψ_8	ω_8	ϕ_9	ψ_9	ω_9	ϕ_{10}	ψ_{10}	ω_{10}	ϕ_{11}	ψ_{11}	ω_{11}
CsH[a]	–173	–157	142	164	67	–128	–175	–102	120	–7	–146	70	178	127	–86	170
CsA·2H_2O[b]	–165	–83	52	178	88	–125	–167	–119	100	–6	–139	65	–167	–103	125	173
CsA·H_2O[b]	–173	–86	52	–180	82	–127	–169	–120	100	3	–146	66	–176	–98	122	178
CsA·DMI[c]	–174	–98	–5	180	150	–133	–173	–120	99	–1	–139	64	–178	–95	142	166
CsA·CypA[d]	179	–63	156	173	100	–141	178	–123	72	–178	–108	170	174	–123	83	179

Data from: [a] This work. [b] Cambridge Structural Database (CsA·2H_2O, DEKSAN; CsA·H_2O, KEPNAU). [c] Ref.[22]. [d] Ref.[38] (monomeric CsA·CypA)[47]; Cs, cyclosporin; Cyp, cyclophilin.

Regardless cyclosporins represent an important class of biologically active substances, only several X-ray structures have been reported. Three different polymorphs have been described for cyclosporin A: dihydrate (tetragonal, $P4_1$)[22], monohydrate (orthorhombic, $P2_12_12_1$)[38], and dimethylisosorbide solvate (1 : 1) (monoclinic, $P2_1$)[37]. It was found only recently that various cyclosporins crystallizing in symmetry $P2_1$ represent a series of clathrates with various guest molecules[39,40]. Whereas iso-cyclosporin A possesses quite different conformation (orthorhombic $C222_1$)[26], some cyclosporin derivatives crystallize either in the same symmetry, *e.g.*, acetylcyclosporin A (orthorhombic, $P2_12_12_1$)[41] and thiocyclosporin A (monoclinic, $P2_1$)[42], or have almost identical conformation as described already for cyclosporin A, *e.g.*, [3,*O*-didehydro-MeBmt[1],Val[2]]cyclosporin ($P3_221$)[43]. Hence, cyclosporin H described in this work represents a new cyclosporin conformation.

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