# CRYSTAL STRUCTURES OF TWO NEW CYCLOSPORIN CLATHRATES

Michal HUŠÁK[a1,d], Bohumil KRATOCHVÍL[a2], Ivana CÍSAŘOVÁ[b] and Alexandr JEGOROV[c,*]

[a] *Department of Solid State Chemistry, Institute of Chemical Technology, Prague, Technická 5, 166 28 Prague 6, Czech Republic; e-mail:* [1] *michal.husak@vscht.cz,* [2] *bohumil.kratochvil@vscht.cz*

[b] *Department of Inorganic Chemistry, Charles University, Hlavova 2030, 128 43 Prague 2, Czech Republic; e-mail: cisarova@prfdec.natur.cuni.cz*

[c] *Galena a.s., Research Unit, Branišovská 31, 370 05 České Budějovice, Czech Republic; e-mail: alexandr_jegorov@galena.cz*

[d] *Laboratory of Biomembranes, South Bohemian University, Branišovská 31, 370 05 České Budějovice, Czech Republic*

Received November 27, 2000
Accepted December 28, 2000

Two isomorphous clathrates formed by dihydrocyclosporin A or cyclosporin V with *tert*-butyl methyl ether are reported and compared with the structures of related $P2_1$-symmetry cyclosporin clathrates. The cyclosporin molecules in both structures are associated *via* van der Waals interactions forming cavities occupied by solvent molecules (cyclosporin : *tert*-butyl methyl ether is 1 : 2).

**Key words**: Cyclosporins; Cyclic peptides; Clathrates; X-Ray diffraction; Crystal structure; Immunosuppressants.

Cyclosporins are cyclic undecapeptides produced as fungal metabolites. Immunosuppressive 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]], where MeBmt = (4*R*)-4-[(*E*)-but-2-enyl]-4,*N*-dimethyl-L-threonine, is therapeutically used in transplantation surgery (*e.g.*, Consupren®, Galena). Cyclosporin molecules are associated *via* van der Waals forces in crystals forming cavities occupied mostly by solvent molecules. These assemblies are called cyclosporin clathrates[1] and described as isomorphous $P2_1$-symmetry structural types of cyclosporins.

The present study deals with two new crystal structures of cyclosporins crystallized in the form of $P2_1$ clathrates.

## EXPERIMENTAL

### Preparation of Crystals

Dihydrocyclosporin A bis(*tert*-butyl methyl ether) clathrate (**1**): *cyclo*-[**Dihydro-MeBmt**[1]-Abu[2]-Sar[3]-MeLeu[4]-Val[5]-MeLeu[6]-Ala[7]-D-Ala[8]-MeLeu[9]-MeLeu[10]-MeVal[11]] (Dihydro-CsA, 10 mg, Galena a.s, Czech Republic) was dissolved in *tert*-butyl methyl ether (200 μl) under heating (50–80 °C, 3 min), heptane (1 ml) was added in one portion with stirring, and the solution was allowed to evaporate under laboratory temperature. Crystals of **1** were formed within 24 h. A cyclosporin sample isolated from *Mycelium sterilae*[2] and submitted for identification (2 mg) was treated in the same way providing thus single crystals of cyclosporin V bis(*tert*-butyl methyl ether) clathrate (**2**). Since clathrates are prone to desolvation, they were directly transferred to a holder and immediately cooled to 150 K. Crystal structure determination of **2** revealed that the sample was *cyclo*-[MeBmt[1]-Abu[2]-Sar[3]-MeLeu[4]-Val[5]-MeLeu[6]-**Abu**[7]-D-Ala[8]-MeLeu[9]-MeLeu[10]-MeVal[11]] (CsV), which was later confirmed also by MS and NMR (Fig. 1).

### X-Ray Structure Analysis

The starting coordinates of non-H atoms were taken from the almost isostructural model of cyclosporin A dimethyl isosorbide clathrate[3]. The positions of the solvent atoms were found from subsequent Fourier series. All non-H atoms of the cyclosporin molecule were refined anisotropically by full-matrix least-squares based on *F* values. Two terminal C-atoms of the

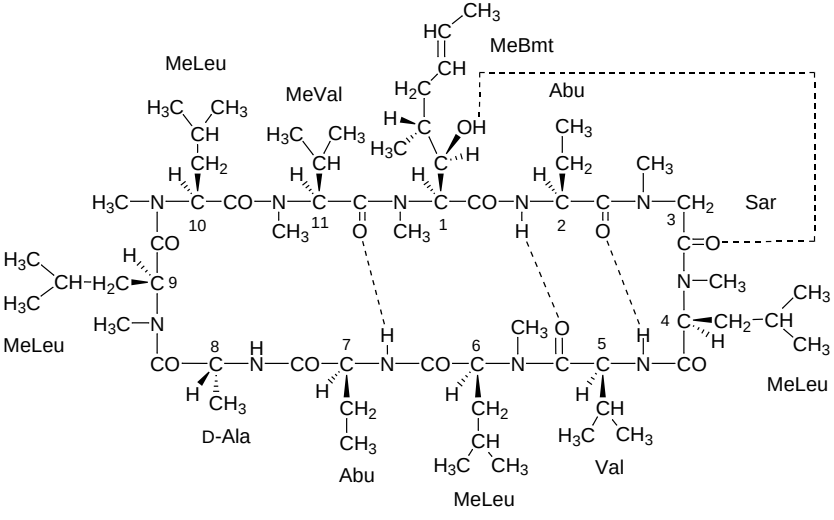


FIG. 1
Schematic representation of structure and hydrogen bonds in cyclosporin V crystallizing with *tert*-butyl methyl ether. Dashed lines indicate hydrogen bonds. System of intramolecular H-bonds is the same as found in dihydro-CsA or CsA dimethyl isosorbide clathrate[3]

MeBmt[1] side chain were found disordered in **2**, therefore the both were placed in two positions with occupancy factors fixed at 0.5 with restrained bond distances. This disorder is caused by the free rotation around the C7–C8 single bond. Solvent atoms were refined anisotropically for **1** and only isotropically for **2**. C- and N-linked hydrogens were found from the expected geometry and constrained during refinement, OH-hydrogen was fixed as localised at the difference Fourier map. The same numbering scheme as for cyclosporin A dimethyl isosorbide clathrate[3] was used both for **1** and **2** (the additional C-atom of Abu[7] was numbered as C63). The crystal data and common measurement and refinement details are summarised in Table I. Crystallographic data for the structures reported in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication numbers: **1** CCDC-153171 and **2** CCDC-153170. Copies of the data can be obtained free of charge on application to CCDC, e-mail: deposit@ccdc.cam.ac.uk.

## RESULTS AND DISCUSSION

When we recently evaluated the contributions of various instrumental methods to the identification of cyclosporins we stated[4] that MS and NMR techniques are much faster and that due to several disadvantages of diffraction techniques (amount of sample, lack of crystals, time for measurements), these techniques can only contribute in the case of confirmation of the absolute chirality. Since current X-ray instrumentation makes it possible to determine structure of such size within few hours, we must correct a little bit this conclusion and the only remaining problem seems to be the preparation of the single crystals. We reported recently that the use of some ethers directs the crystallization of cyclosporins into the form of clathrates having $P2_1$-symmetry[1]. Since these clathrates crystallise easily and furthermore, crystals have a shape suitable for X-ray diffraction, it seems that this approach can be used as a new method of choice also for the identification of yet unidentified cyclosporin samples.

The conformation of cyclosporins in clathrates differs from the conformation of cyclosporin A in monohydrate[5] or dihydrate[6] practically only by the orientation of the MeBmt side chain. Anyway, this difference is rather subtle in comparison with conformation changes caused, *e.g.*, by the interaction of cyclosporin with cyclophilin or antibodies. The conformation of the cyclosporin backbone is identical within an experimental error in CsV, dihydro-CsA and CsA crystallised with dimethyl isosorbide[3]. The presence of either Abu[7] or dihydro-MeBmt[1], respectively, does not influence the overall molecular shape. The ORTEP view of **2** with the atom-numbering scheme is given as a typical representative of the clathrate series (Fig. 2). With regard to the Cambridge database, the X-ray structure of dihydro-MeBmt, 3-hydroxy-4-methyl-2-(methylamino)octanoic acid, is reported for

TABLE I
Crystal data and collection and refinement parameters

| Parameter | **1** | **2** |
|---|---|---|
| Formula | $C_{62}H_{113}N_{11}O_{12}\cdot 2C_5H_{12}O$ | $C_{63}H_{113}N_{11}O_{12}\cdot 2C_5H_{12}O$ |
| $M_w$ | 1 380.96 | 1 392.97 |
| Space group | $P2_1$ (No. 4) | $P2_1$ (No. 4) |
| $a$, Å | 15.2910(2) | 15.3452(4) |
| $b$, Å | 21.3764(2) | 21.2590(7) |
| $c$, Å | 12.7657(2) | 12.8041(4) |
| β, °; $Z$; $V$, Å$^3$; $\rho_{calc}$, g cm$^{-3}$ | 96.7276(8); 2; 4 144.0; 1.11 | 97.020(2); 2; 4 145.8; 1.12 |
| μ(MoKα), mm$^{-1}$ | 0.08 | 0.08 |
| $F$(000) | 1 516.33 | 1 528.34 |
| Crystal dimensions, mm | 0.5 × 0.4 × 0.35 | 0.4 × 0.3 × 0.3 |
| Diffractometer and radiation used | Nonius Kappa CCD area detector, λ(MoKα) = 0.71073 Å | |
| Scan technique | ψ and ω scans to fill the Ewald sphere | |
| Temperature, K | 150(2) | |
| No. and θ range of reflections for lattice parameter refinement | 39 720, 1.02–26.02° | 14 393, 1.02–25.03° |
| Range of $h$, $k$ and $l$ | –18→18, –26→26, –15→15 | –18→18, –24→25, –15→14 |
| Total number of reflections measured; 2θ range | 15 505; 0–26° | 13 733; 0–25° |
| No. of observed unique reflections | 8 300 | 7 394 |
| Criterion for observed reflections | $I \geq 1.96\sigma(I)$ | |
| Function minimised | $\sum w(F_o - F_c)^2$ | |
| Weighting scheme | Chebychev polynomial with 3 parameters | |
| Parameter refined | 873 | 768 |
| Value of $R$, $wR$ and $S$ | 0.058, 0.064, 1.04 | 0.11, 0.12, 0.94 |
| Ratio of the maximum least-squares shift to e.s.d. in the last cycle | 0.094 | 0.030 |
| Minimum and maximum heights in final Δρ map, e Å$^{-3}$ | –0.43, 0.37 | –0.65, 1.58 |
| Software used | CRYSTALS (ref.[9]) | |

the first time ($\phi_1$ = –91°, $\psi_1$ = 157°, $\omega_1$ = 167°, $\chi_1^{11}$ = –45°, $\chi_1^{12}$ = 81°, $\chi_1^{21}$ = –174°, $\chi_1^{22}$ = –51°, $\chi_1^{3}$ = –168°, $\chi_1^{4}$ = 178°, $\chi_1^{5}$ = 175°).

There are several ways how to estimate, which largest molecule could be potentially incorporated into the cavity without changing the cell parameters of the structure. The assumption that the average packing coefficient for non-hydrogen atoms is 19.4 in such type of organic compound[7] is a possible approach. Using this rough estimation, it can be calculated that a solvent molecule having more than 20 non-hydrogen solvent atoms (in addition to 85 or 86 ones in dihydrocyclosporin A or cyclosporin V, respectively) might be incorporated into the asymmetric unit of the $P2_1$ form. Obviously, this value makes the limit of non-linear function for the infinite volume of cavity, and the size of molecules that could be incorporated, depends also on their number, structure and ability to accommodate the shape of the cavity. In fact, molecules that are actually incorporated, have approximately one half of non-hydrogen atoms than the calculated number.

Alternatively, the programme PLATON (ref.[8]) was used to calculate the shape and the volume of the cavity, which is occupied by a guest molecule in clathrates (Fig. 3). This volume is defined as the space in which could be placed atoms having a larger distance from the van der Waals surface of the nearest atom than 1.2 Å. Note that with regard to this definition, the total

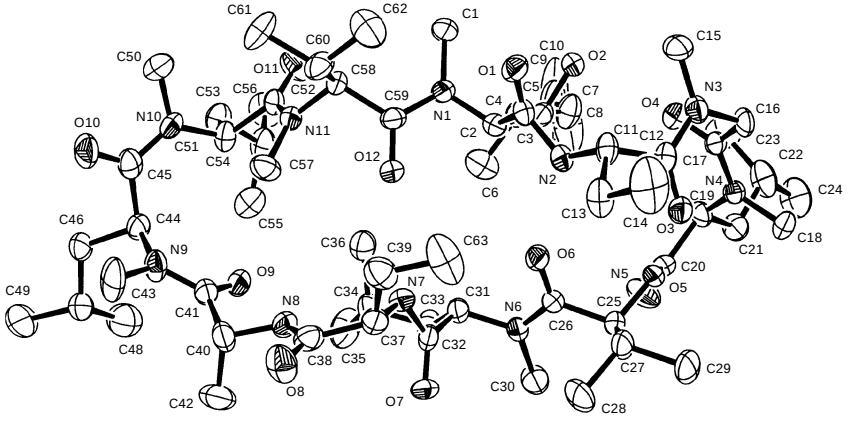


FIG. 2

ORTEP drawing of cyclosporin V bis(*tert*-butyl methyl ether) clathrate with the atom-numbering scheme. Thermal ellipsoids are drawn at the 50% probability level. Hydrogens, solvent molecules, and the second position of two disordered terminal MeBmt[1] atoms were omitted for clarity

accessible area in the unit cell is not equal to the total volume of cavity, but refers only to a part of total volume, which the solvent can utilise. The results of these calculations are summarised in Table II. In the case of cyclosporin clathrates, the vectors of the free space volume indicate that the shape of the cavity is similar in all structures. The computed maximal continuous solvent accessible volume in the unit cell was found in the range from 391 to 562 Å$^3$.

Evaluation of the residual volume after subtraction of the solvent volume provides more realistic information on the additionally available free space in the particular cavity. As follows from the Table II, there is relatively large free volume in some structures, but, *e.g.*, dimethyl isosorbide seems to be the largest molecule, which can be incorporated into the CsA clathrate. Evaluation of the continuous solvent-accessible area offers the possibility to propose some additional substituents in the solvent molecules, and/or to design chiral molecules, which could be separated by preferential crystallization. However, it is necessary to keep in mind that the side chains of amino acids in cyclosporins are flexible and that the presence of a particular solvent can slightly affect modification of the shape of the cavity. On the contrary, slight changes in amino acids in cyclosporins can also cause unexpected differences in the packing of solvent molecules in otherwise

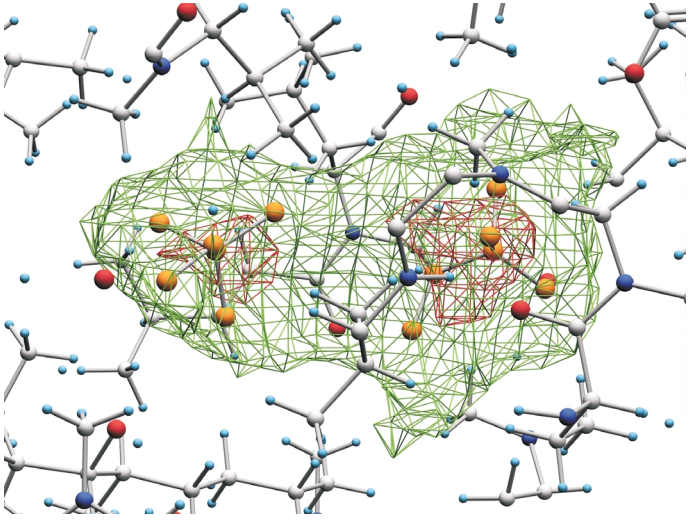

FIG. 3
Visualisation of the cavity in dihydro-CsA structure calculated by void function of CRYSTALS (ref.[9]). The green and red wire surfaces define space in distance of 1 and 2 Å from VdW sphere of the nearest surrounding atoms. The orange balls represent the solvent atoms

TABLE II
Comparison of different $P2_1$ cyclosporin crystal forms

| Cyclosporin[a] Solvent (reference) | *a*, Å | *b*, Å | *c*, Å | β, ° | Total cell volume Å$^3$ | Accessible area[b,c] Å$^3$ (%) | Continuous area[b,d] Å$^3$ | Residual area[b,e] Å$^3$ | Continuous residual area[b,f] Å$^3$ |
|---|---|---|---|---|---|---|---|---|---|
| CsA[g] DMI (ref.[3]) | 15.521(2) | 20.833(3) | 12.949(3) | 100.21(1) | 4 121 | 875 (21.23) | 438 | 71 | 16 |
| CsA[g] THF (ref.[1]) | 15.551(2) | 21.216(7) | 12.862(2) | 98.23(1) | 4 200 | 974 (23.19) | 487 | 666 | 326 |
| CsA[g] DBE (ref.[1]) | 15.47(1) | 21.115(5) | 12.843(7) | 98.96(4) | 4 144 | 926 (22.35) | 463 | [h] | [h] |
| CsA[i] DBE (ref.[1]) | 15.37(1) | 20.910(4) | 12.496(6) | 99.44(4) | 3 962 | 781 (19.71) | 391 | 67 | 34 |
| CsE[g] acetone, water (ref.[10]) | 15.698(2) | 21.333(3) | 13.224(2) | 103.74(1) | 4 302 | 1 142 (26.55) | 562 | 546 | 188 |
| CsE[i] bis(2-butanol) (ref.[8]) | 15.575(6) | 20.584(9) | 13.280(5) | 105.95(3) | 4 093 | 949 (23.19) | 453 | 179 | 56 |
| Dihydro-CsA[i] bis(*t*-BME)[j] | 15.2910(2) | 21.3764(2) | 12.7657(2) | 96.7276(8) | 4 144 | 924 (22.30) | 462 | 57 | 17 |
| CsV[i] bis(*t*-BME)[j] | 15.3452(4) | 21.2590(7) | 12.8041(4) | 97.020(2) | 4 146 | 910 (21.95) | 455 | 215 | 107 |

[a] Cs, cyclosporin; DMI, dimethyl isosorbide; THF, tetrahydrofurane; DBU, dibutyl ether; *t*-BME, *tert*-butyl methyl ether. [b] The programme PLATON (ref.[8]) was used to calculate the slope and volume of the cavity. [c] Total solvent accessible area in the unit cell, solvent atoms not included in the calculation. [d] Maximum continuous solvent accessible area in the unit cell, which can be potentially utilised by one solvent. [e] Total additional solvent accessible area in the unit cell, solvent atom included in the calculation. [f] Maximal continuous additional solvent accessible area, solvent atoms included in calculation; this area is suitable for additional solvent molecules. [g] Ambient temperature. [h] Impossible to calculate, solvent atoms were not localised. [i] 150 K. [j] This work.

virtually identical cavities. These phenomena can be best exemplified just on the example of different arrangement of one of two *tert*-butyl methyl ether molecules in CsV and dihydro-CsA clathrates (Fig. 4). The difference in packing of two otherwise identical solvent molecules results in the substantially different potentially free residual area (Table II). Hence, whereas we can predict the size of molecule, which might be incorporated into the structure, its exact orientation in the cavity remains the only fact to be found experimentally.

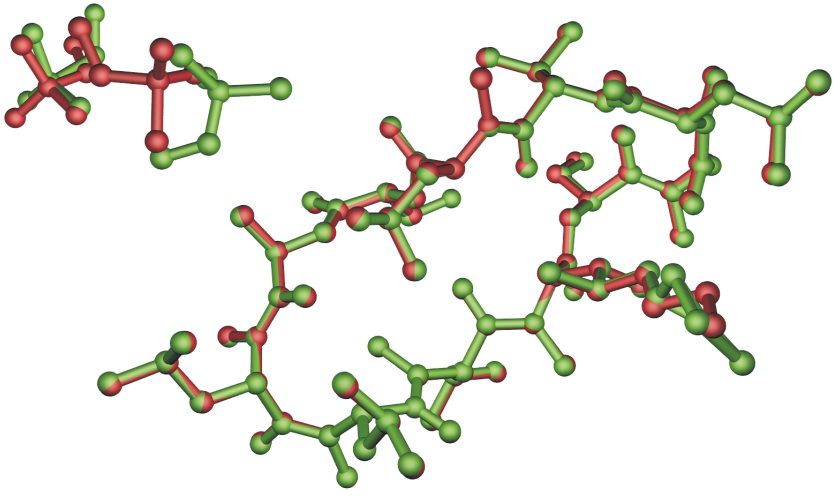

FIG. 4
Comparison of solvent position in dihydro-CsA (red) and CsV (green)

*Authors thank to M. Buchta (Galena, a.s.) for the sample of cyclosporin V. This work was supported by the Grant Agency of the Czech Republic (grant No. 203/99/1190) and by the Ministry of Education, Youth and Sports of the Czech Republic (research project No. CEZ: MSM 223100002).*

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