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Crystallization of the Complex Between Cyclophilin A and Cyclosporin Derivatives: the Use of Cross-Seeding

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Abstract

With ammonium sulfate as the precipitant, different crystal forms of the complex between cyclosporin A (CsA) and cyclophilin A (CypA) have been crystallized [Zurini, Kallen, Mikol, Pflügl, Jansonius & Walkinshaw (1990). *FEBS Lett.* **276**, 63–66]. All have large unit cells and contain a pentamer or a decamer in the asymmetric unit. Using a more water soluble CsA analog, orthorhombic crystals containing only one molecule per asymmetric unit could be grown. They diffract to significantly higher resolution (2.1 Å). In this crystal form, CsA has no packing interactions with neighbouring molecules and these crystals could be used to cross-seed other CypA/CsA analog complexes. Nine different CypA/CsA analog complexes could be crystallized using this technique, most of them yielding highly diffracting crystals, quickly solvable by Fourier difference methods.

Abbreviations

CypA, cyclophilin A; CsA, cyclosporin A; MeBmt, (4*R*)-4-[(*E*)-2-butenyl]-4,*N*-dimethyl-L-threonine; $MeBm_2t$, 4-[(*E*)-2-butenyl]-4,4,*N*-trimethyl-L-threonine; Abu, L-α-aminobutyric acid; Sar, sarcosine; MeLeu, *N*-methylleucine; MeVal, *N*-methylvaline; PEG, polyethylene glycol; DMSO, dimethyl sulfoxide; DH, dihydro.

Introduction

Cyclosporin A (Fig. 1), a cyclic hydrophobic undecapeptide, is the major drug used to prevent graft rejection after transplant surgery (Borel, 1989). CsA impedes the activation and cell-cycle progression of quiescent T-cells in response to foreign antigens. The drug penetrates T-cells and blocks the calcium-dependent signal-transduction pathway leading to the transcription of T-and-B-cell growth factors such as interleukin-2 (Emmel *et al.*, 1989). It binds cyclophilin A, a cytosolic peptidyl-prolyl *cis–trans* isomerase (165 residues) with a high affinity. The cyclosporin/cyclophilin complex binds and inhibits calcineurin, a calcium- and calmodulin-dependent Ser/Thr phosphatase (Liu *et al.*, 1991) that was shown to modulate the phosphorylation state of the cytoplasmic subunit of the transcription factor of activated T-cells, which is involved in turning on genes encoding various cytokines (McCaffrey, Perrino, Soderling & Rao, 1993).

Using ammonium sulfate as the precipitant, different crystal forms of the complex between CsA and CypA could be grown: a monoclinic form, a hexagonal form and a tetragonal form (Zurini *et al.*, 1990). All have large unit cells (volume greater than 2×10^6 Å^3) and contain a pentamer or a decamer of complex in the asymmetric unit. The tetragonal form was used to solve the first X-ray structure of the complex which was refined at 2.8 Å resolution (Pflügl, Kallen, Schirmer, Jansonius & Walkinshaw, 1993). In this latter structure, the crystal contains a pentamer of the complex in the asymmetric unit. The cyclophilin pentamers pack in pairs to form decamer discs which sandwich the CsA molecules. In the 'decameric sandwich,' about 80% of the CsA surface is inaccessible to the solvent. These multimeric forms are not suitable for the routine crystallographic study of series of CsA derivatives complexed with CypA. Attempts to crystallize CypA/CsA under different conditions failed to give a crystal form with a smaller asymmetric unit content, but using a CsA analog (denoted CsA*) crystals containing one complex per asymmetric unit could be grown. We report in this paper the growth and use of these crystals to cross-seed other CypA/CsA analog crystallization experiments.

Materials and methods

(*a*) *Solutions*

Recombinant human cyclophilin A was overexpressed in *E. coli* and purified as described elsewhere (Zurini *et al.*, 1990) and concentrated to 18 mg ml^{-1} in 0.02% NaN_3. CsA analogs were synthesized according to the methods previously reported (Wenger, 1989). CsA and its analogs were dissolved in DMSO at a concentration of 10 mg ml^{-1} (*ca* 8.3 m*M*). For the preparation of the complex, an

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equimolar solution of CypA and of the CsA analog was made and the solution was incubated at 310 K for 30 min and then centrifuged at 14000 rev min^{-1} in a table centrifuge. PEG 8000 purchased from Sigma, was recrystallized in acetone and washed with ethyl ether solution to remove aldehydes and peroxides. The powder was subsequently dissolved in water at 277 K to give a 25%(*w*/*w*) solution. It was then mixed with a deionizing resin (Biorad, AG 501-X8D) to remove ions, filtered and then sodium azide was added to give a 0.05%(*w*/*v*) solution. All subsequent solutions containing PEG were made by diluting this stock solution of PEG by volume. For example an '18% PEG solution' was made by mixing 720 μl of the stock solution of PEG with 280 μl of water. Sodium azide was present in all other solutions at a concentration of 0.02%(*w*/*v*).

(*b*) *Preparation of the parent crystals*

Crystals of the CypA/CsA analog complex were grown by vapor diffusion at 295 K by the hanging-drop method (Ducruix & Giegé, 1992). The precipitating solution in the 1 ml well consisted of 100 m*M* Tris–HCl pH 8.0, 19% PEG 8000, 5%(*v*/*v*) DMSO, 0.04%(*w*/*v*) NaN_3. The initial 6 μl drop consisted of 50 m*M* Tris–HCl pH 8.0, 9.5% PEG 8000, 3.0%(*v*/*v*) DMSO, 0.02%(*w*/*v*) NaN_3, 0.5 m*M* cyclophilin, 0.5 m*M* cyclosporin analog.

(*c*) *Cross-seeding*

The cross-seeding experiments were performed basically as described by Stura & Wilson (1992). A single crystal of the CypA/CsA* complex was placed into a drop (15 μl) of reservoir solution and washed two times with reservoir solution. It was then finely crushed and individual seeds (about 10 μm) were then transfered into drops of a solution containing the CypA/CsA analog to be crystallized. This solution had been equilibrated for 4 d. The compositions of the solutions used for the growing of the CypA/CsA analog crystals were identical to those used for

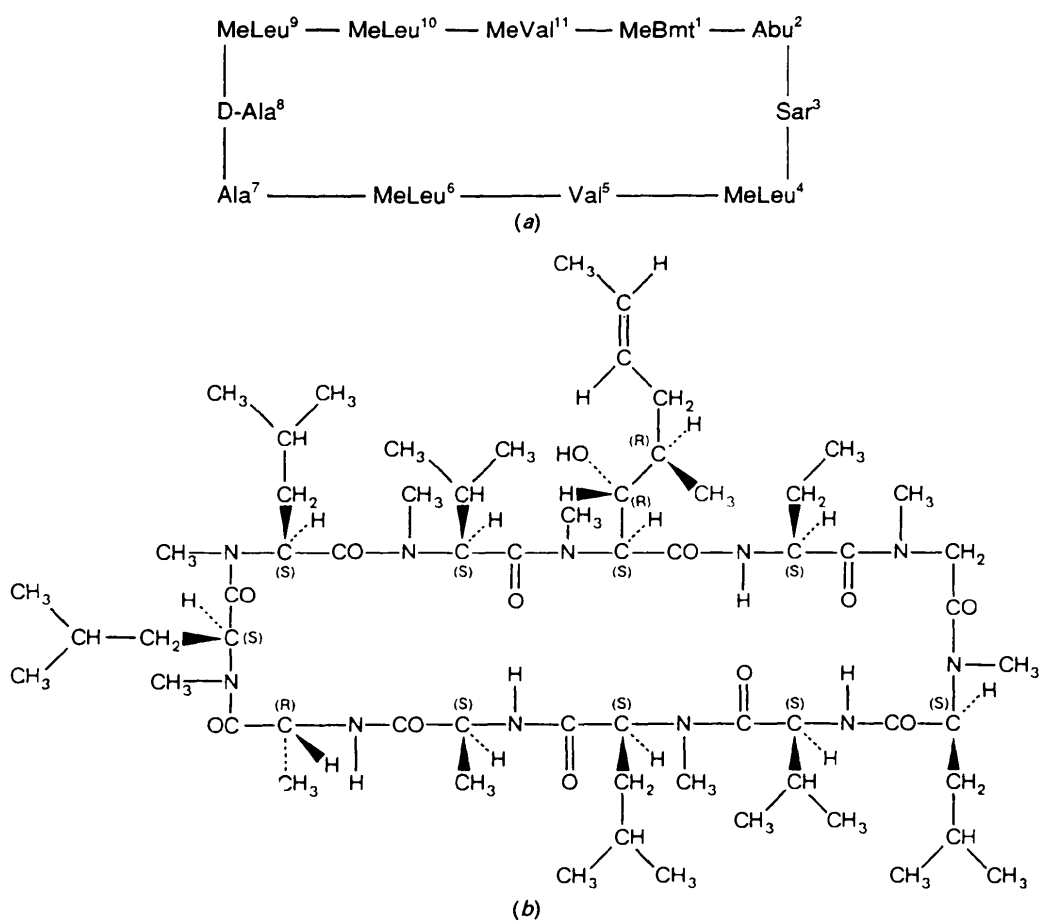


Fig. 1. The cyclosporin molecule. (*a*) CsA with the standard numeration of the amino-acid residues. Abbreviations: MeBmt, (4*R*)-4-[(*E*)-2-butenyl]-4,*N*-dimethyl-L-threonine; Abu, L-α-aminobutyric acid; Sar, sarcosine; MeLeu, *N*-methylleucine; MeVal, *N*-methylvaline. (*b*) Chemical structure of CsA.

Table 1. *Crystallographic data for CypA/CsA derivative complexes grown in PEG solution*

The first four derivatives for which space-group information is present, were grown by spontaneous nucleation. The others were crystallized by cross-seeding and are accordingly isomorphous to the CypA/CsA* crystal. The question mark indicates that the corresponding parameter was not determined. $MeBm_2t$ is 4-[(*E*)-2-butenyl]-4,4,*N*-trimethyl-L-threonine.

CSA analog	Space group	Cell dimensions *a*, *b*, *c* (Å)	V_M (Å^3 Da^{-1})	Molecules per asymmetric unit	Resolution (Å)
[D-MeSer^3]-CsA	$P2_12_12$	62.8, 65.3, 40.9	2.3	1	2.4
[*O*-(Cholinylester)-D-Ser^8]-CsA	$P2_12_12_1$	36.4, 61.7, 72.3	2.1	1	2.5
[Thr^2,Leu^5,D-Hiv^8,Leu^{10}]-CsA	$P2_12_12_1$	79.1, 125.0, 150.0	?	?	2.8
CsA*	$P2_12_12_1$	36.3, 60.0, 72.3	2.1	1	2.1
CsA					2.1
[$MeBm_2t^1$]-CsA					2.2
[*O*-Acetyl-Thr^2]-CsA					Small crystals
[Me-Thr^2]-CsA					Small crystals
[D-(αSMe^3)Val^2]-CsA					1.9
[D-MeAla^{3}Val^2]-CsA					1.9
DH-[Val^2(αSMe^3)]-CsA					1.9
[*N*-Me-γ-OH-Leu^4]-CsA					1.8
[Me-Ile^4]-CsA					1.9

the CypA/CsA* complex except for the concentration of PEG 8000 in the reservoir, which was varied from 15 to 18%, and the pH, which was between 7.8 and 8.4. Crystals obtained from cross-seeding were used to seed one or two subsequent experiments diluting out the effect of the heterogeneous seeds.

(*d*) *X-ray diffraction measurements*

Crystals were mounted and sealed in Debye–Scherrer capillary tubes with a plug of well solution. The X-ray intensity data were collected on a FAST television area-detector diffractometer. The X-ray source was a rotating-anode generator (FR571) operating at 40 kV 80 mA. The evaluation of the measured intensities and the determination of the cell dimensions were performed by the program *MADNES* (Messerschmidt & Pflugrath, 1987) and the space group was found by inspection of *hk*0, *h*0*l* and 0*kl* precession pictures using the *HKLPLOT* program from the *CCP*4 package (SERC Daresbury Laboratory, 1979).

Results and discussion

(*a*) *Spontaneous nucleation*

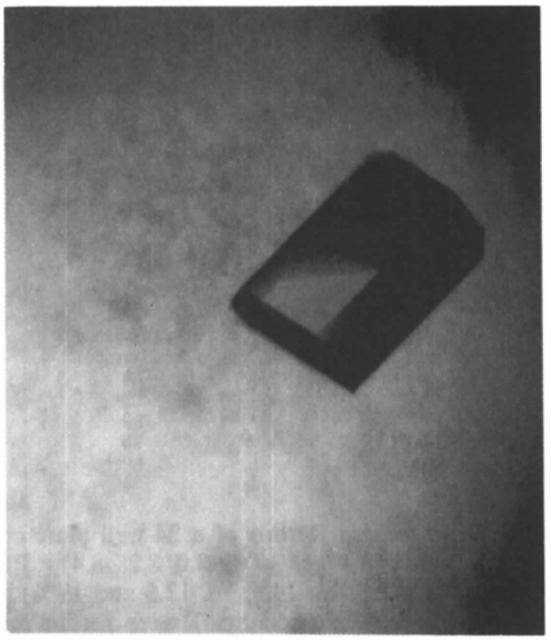
Using the conditions given in *Materials and methods* (*b*) for the parent crystals, four different CsA analogs could be crystallized with CypA (Table 1). Crystals grow to their final sizes within one to three weeks. Crystals of the CypA/[*O*-(cholinylester)-D-Ser^8]-CsA complex were of sufficient size (0.4 × 0.2 × 0.1 mm) to be analysed by X-ray diffraction (Fig. 2*a*). They diffracted to 2.5 Å and were found to contain only one molecule per asymmetric unit in space group $P2_12_12_1$.

The only crystal of the CypA/CsA* complex looked like a crystalline agglomerate (Fig. 2*b*) and was used for seeding. Drops of a 24-well plate containing a matrix of pH (7.8, 8.0 8.2, 8.4) × PEG concentration (11.5, 13, 14.5, 16, 17.5 and 19%) (all other conditions being identical) were seeded with microparticles from the crushed crystalline agglomerate. This procedure yielded large single crystals within 2–7 d in all drops equilibrated against a reservoir having a concentration of PEG higher than 13% (Fig. 2*e*). These crystals were found to be isomorphous to the CypA/[*O*-(cholinylester)-D-Ser^8]-CsA crystal complex and to diffract to 2.1 Å]. The structure indicated that the CsA analog had no packing contacts with any neighbouring molecules in contrast to the previously determined crystal forms (Mikol, Kallen, Pflügl & Walkinshaw, 1994). Furthermore, because of the high success rate of the seeding experiment, this derivative was chosen to perform cross-seeding experiments with other CsA derivative/CypA complexes.

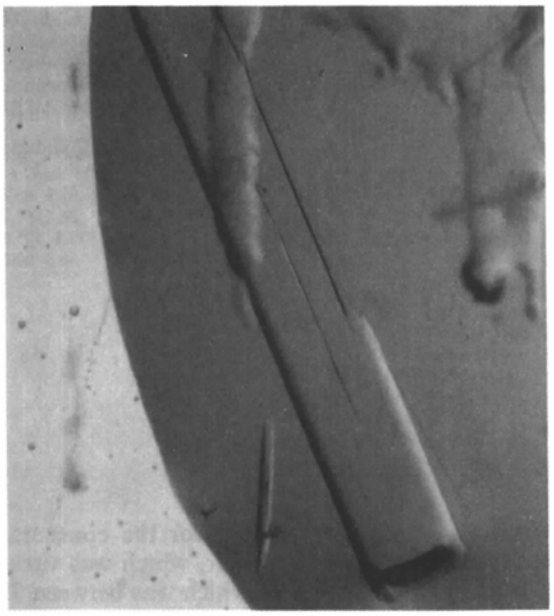
Crystals of the CypA/[D-MeSer^3]-CsA displayed a different morphology (Fig. 2*c*) and crystal size was also increased by seeding which proved not to be as successful as for the CypA/CsA* crystals. They belong to a different space group $P2_12_12$ and diffract to 2.5 Å. In this packing, CsA does not contact any crystallographically related molecule. Crystals of the CypA/[Thr^2,Leu^5,D-Hiv^8,Leu^{10}]-CsA complex were shown to diffract to medium resolution (2.8 Å) and to have multiple protomers in the asymmetric unit (Fig. 2*d*).

(*b*) *Cross-seeding*

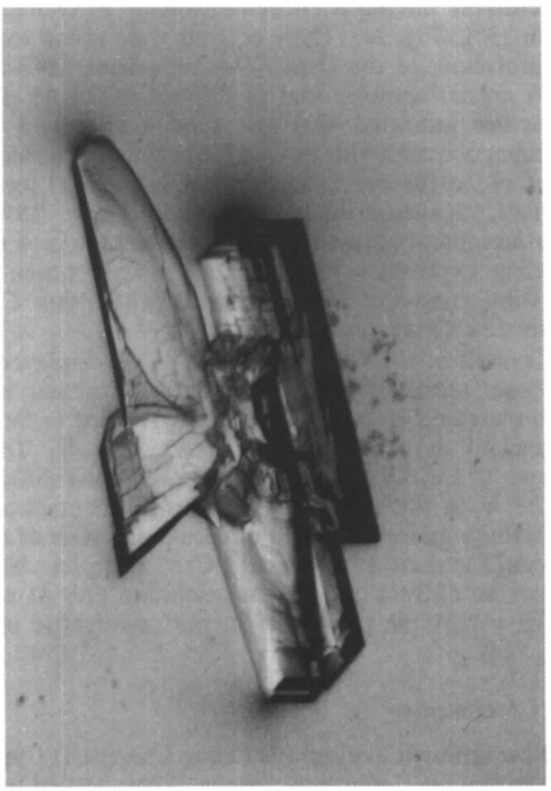
Nine different analogs (including CsA itself)/CypA crystallization solutions were cross-seeded as described in *Materials and methods* (*c*) and all of them yielded crystals (Table 1). Two of the CypA/CsA analog complex crystals were too small to be analysed and the remaining seven CypA/CsA analog complexes gave isomorphous crystals to their parent



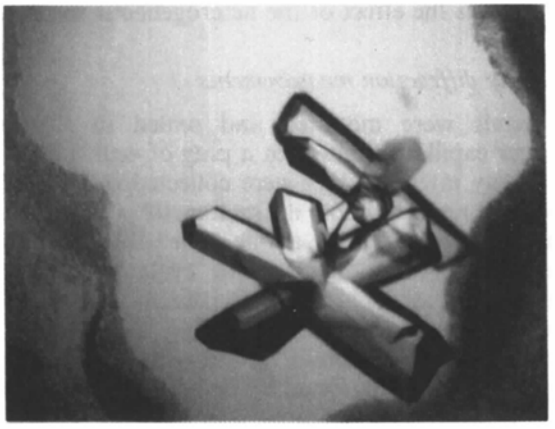
(*a*)



(*c*)



(*b*)



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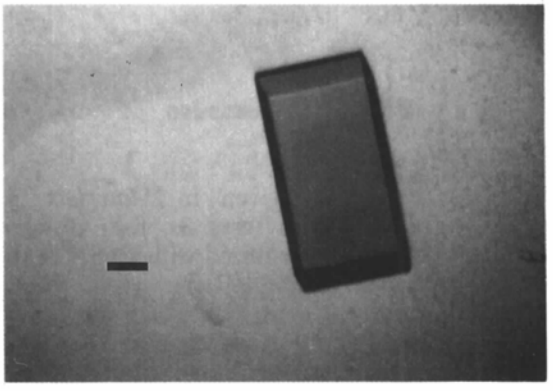

(*e*)

Fig. 2. Photomicrographs of crystals of CypA/CsA analog complexes grown by spontaneous nucleation of CypA/[*O*-(cholinylester)-D-Ser^{8}]-CsA (*a*), CypA/CsA* (*b*), CypA/[D-MeSer^{3}]-CsA (*c*), and CypA/[Thr^{2},Leu^{5},D-Hiv^{8},Leu^{10}]-CsA (*d*). (*e*) Habit of the crystal of CypA/CsA* after homogeneous seeding with microseeds resulting from the crushed crystalline agglomerate seen in (*b*). The bar in (*e*) represents 100 μm.

Table 2. *Quality of the X-ray data of a crystal grown by cross-seeding: the case of the* [D-*MeAla*^{3}*Val*^{2}]-*CsA/CypA complex*

Data reduction was performed with the programs *ROTAVATA* and *AGROVATA* from the *CCP*4 package (SERC Daresbury Laboratory, 1979). $R_{sym} = (\sum|I - \langle I \rangle)/\sum I$ and includes all reflections with $I > 0$ within a certain resolution shell given by D_{min}. R_{cum} is the R_{sym} up to this range.

D_{min} (Å)	R_{sym} (%)	R_{cum} (%)	No. of measurements	No. of independent *hkl*'s
8.47	5.2	5.2	243	81
6.00	6.2	6.0	810	232
4.90	5.6	5.7	1130	317
4.25	6.1	5.9	1166	326
3.80	6.6	6.2	1534	418
3.47	6.9	6.4	1769	473
3.21	6.6	6.4	1882	503
3.00	7.3	6.5	2178	563
2.83	7.9	6.6	2178	573
2.69	8.5	6.8	2328	627
2.56	9.1	6.9	2294	627
2.45	9.5	7.0	2235	634
2.36	9.4	7.1	2231	671
2.27	10.7	7.2	2230	683
2.19	10.4	7.3	2247	676
2.12	11.6	7.5	2226	694
2.06	14.9	7.6	2208	700
2.00	12.7	7.7	1672	625
1.95	13.4	7.7	701	309
1.90	12.4	7.8	168	81

crystals and diffracted between 1.8 and 2.2 Å (Fig. 3). The quality of the data sets of the crystals grown by cross seeding was excellent, yielding low R_{sym} values even in the high-resolution shells (Table 2). The difference in diffraction quality seems to be essentially correlated with the size of the crystals. The concentration of PEG 8000 required for optimal growth had to be reduced in the reservoir from 19% for parent crystals to 15–18% according to the CsA analog. With higher pH (*e.g.* 8.4) the growth is more rapid (2–3 d) and it often results in more crystals, agglomeration and clustering of crystals. This can be readily explained by the fact that CypA has a isoelectric point of 9.0 and is, therefore, less soluble at more basic pH. Crystals with CsA derivatives having a reduced affinity for CypA such as [MeBm$_2$t]^1-CsA (about 1% that of CsA) grow much slower (two to three weeks) than that containing CsA for example. CsA analogs which are more soluble in water than CsA such as [*N*-Me-γ-OH-Leu^4]-CsA yielded larger crystals (1.0 × 0.5 × 0.4 mm).

(*c*) *Additive*

The exact concentration of isopropanol in the droplet was reported to be crucial for the growth of the different crystal forms of the CypA/CsA complex from ammonium sulfate solutions (Zurini *et al.*, 1990). In the cross-seeding and spontaneous nucleation experiments in PEG solution, the growth of the crystals seemed rather insensitive to the presence of 1–5%(*v*/*v*) isopropanol, 0.2–0.5%(*v*/*v*) β-octylglucoside or 1–2%(*v*/*v*) dioxane (the concentration of the additive mentioned is the initial concentration in the drop).

Concluding remarks

CsA is a very hydrophobic cyclic peptide which is scarcely soluble in water. When crystallized from ammonium sulfate, CsA minimizes its contacts with the solvent (20%) and establishes hydrophobic interactions with neighbouring molecules forming a decamer (Pflügl, Kallen, Schirmer, Jansonius & Walkinshaw, 1993). Using more water soluble CsA analogs, several crystal forms containing a monomer of the complex between CypA and CsA analog in the asymmetric unit could be grown in which 56% of the cyclosporin molecule is exposed to the solvent. Structure analysis of CypA/CsA crystals grown by cross-seeding has revealed that CsA adopts the same backbone conformation to that found in the decameric complex (Mikol, Kallen, Pflügl & Walkinshaw, 1994). The use of cross-seeding in the crystallization of CypA/CsA analog complexes has proved to be very successful and is likely to become more popular. The combination of cross-seeding and molecular replacement will allow one to determine quickly the structure of a great number of X-ray structures of receptor/drug complexes often required in the process of structure-based drug design.

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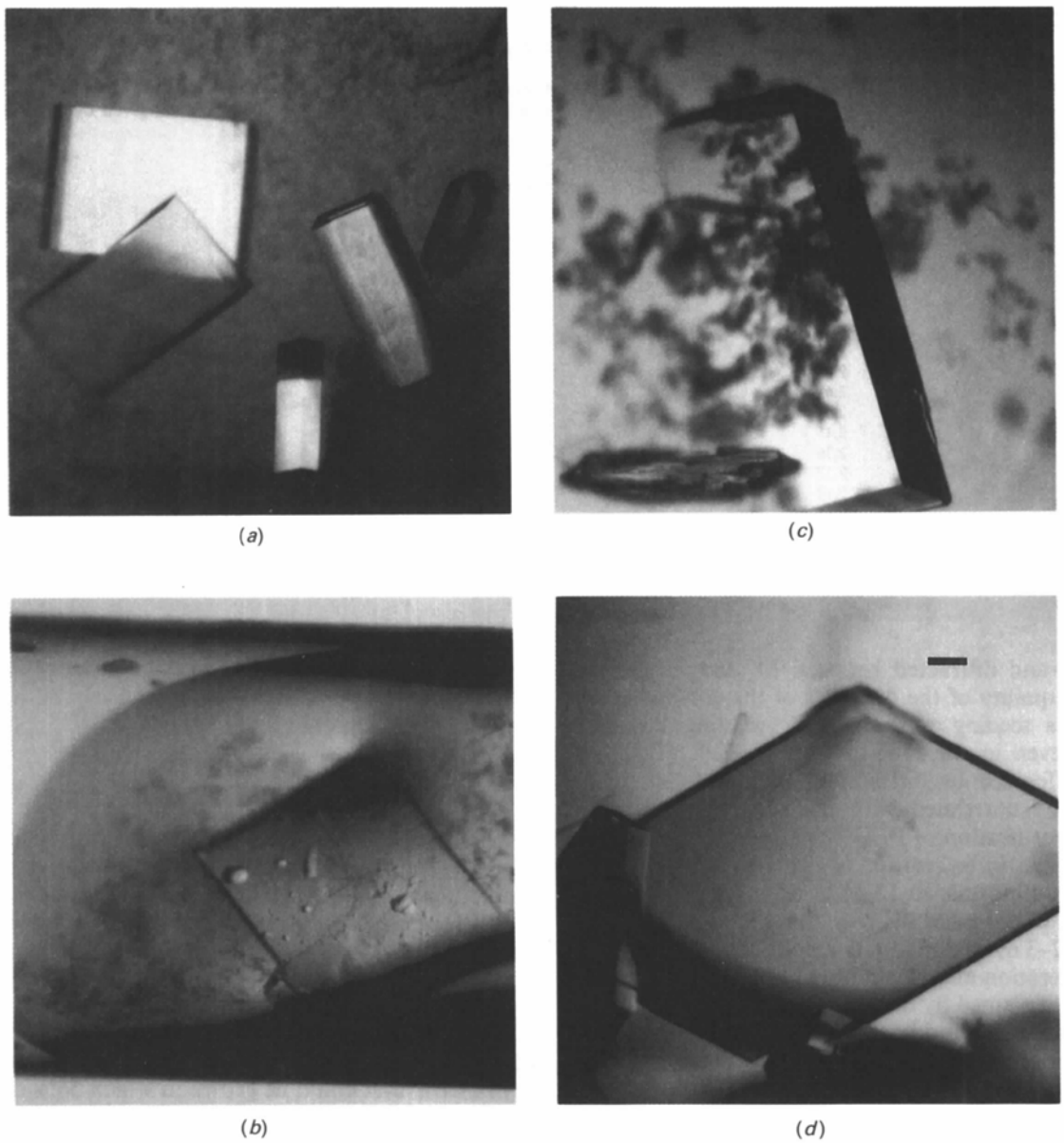

Fig. 3. Photomicrographs of the habit of representative crystals of CypA/CsA analog complexes grown by cross-seeding. CsA/CypA (*a*), [*N*-Me-γ-OH-Leu^4]-CsA/CypA (*b*), [MeBm$_2$t^1]-CsA/CypA (*c*), DH-[Val^2(αSMe^3)]-CsA/CypA (*d*). The bar in (*d*) represents 100 μm.

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