CYCLOSPORINS — NEW ANALOGUES BY PRECURSOR DIRECTED BIOSYNTHESIS†

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Cyclosporin A (ciclosporin), a potent and clinically important immunosuppressive drug (Sandimmun), represents the main component of a group of over 25 closely related, cyclic undecapeptides produced by the fungus *Tolypocladium inflatum*. By feeding experiments using DL-α-allylglycine as precursor, specific incorporation in position 2 was attained leading to [Allylgly^2]cyclosporin A. Exogenously supplied L-β-cyclohexylalanine results in the almost exclusive production of [MeCyclohexylala^1]cyclosporin A (replacement of methylbutenyl-methylthreonine-1). D-Alanine in position 8 can be successfully substituted by D-serine. The new [D-Ser^8] analogues of the cyclosporins A, C, D and G as well as [Allylgly^2]cyclosporin A exhibit high immunosuppressive effects.

Cyclosporins represent a group of biologically active secondary metabolites produced by members of the genus *Tolypocladium* in submerged culture[1,2]. The main component in normal fermentation broths, cyclosporin A (INN; ciclosporin)[3], has proven to be a powerful immunosuppressant (Sandimmun) and is used clinically in organ and bone marrow transplantations[4]. Besides cyclosporin A, a plethora of over 25 closely related congeners (cyclosporins B to Z) have been isolated so far from large scale fermentations[5~8]. All natural cyclosporins are neutral cyclic oligopeptides composed of 11 amino acids of which several are *N*-methylated and one or two belong to the D-series. A characteristic feature consists in the occurrence of the unique building element (2*S*,3*R*,4*R*,6*E*)-2-methylamino-3-hydroxy-4-methyl-6-octenoic acid (*N*-methyl-(4*R*)-4-((*E*)-2-butenyl)-4-methyl-L-threonine, abbreviation MeBmt). Variations in the amino acid sequence have been observed in all positions except 3 and 8; some cyclosporins differ from their congeners only by *N*-demethylation of one amino acid residue[5~8].

The large number of minor components as well as the unusual structural units, *e.g.* MeBmt, L-α-aminobutyric acid and D-alanine, indicate a nonribosomal biosynthetic pathway for cyclosporins[9,10]. Feeding experiments using carbon-13 labeled acetate and methionine revealed that the constituent amino acid MeBmt is built up by head-to-tail coupling of 4 acetate units, whereas the *C*-methyl in the carbon chain and the 7 *N*-methyl groups in cyclosporin A originate from the *S*-methyl of methionine[11]. Recently, the *N*-demethylated amino acid Bmt was obtained from a cyclosporin-blocked mutant of *Tolypocladium inflatum*[12].

The course of cyclosporin biosynthesis can be strongly influenced by an exogenous supply of amino acid precursors to the fermentation medium. Thus, the specific amino acids L-α-aminobutyric acid (Abu), L-alanine, L-threonine, L-valine and L-norvaline (Nva), at position 2 in the natural cyclosporins, are preferably well incorporated giving rise to enhanced yields of the corresponding cyclosporins A, B, C, D and G, respectively[10].

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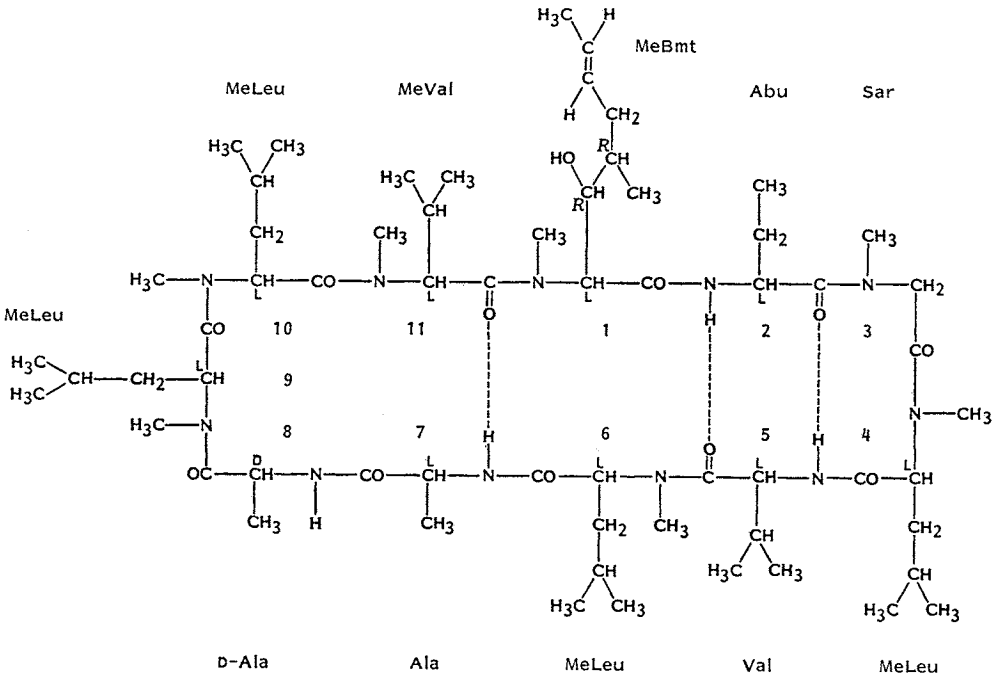

Cyclosporin A (INN; ciclosporin)

In this paper we report on the successful incorporation of 'foreign' amino acids into various positions of the cyclosporin molecule leading to the synthesis of new analogues.

Materials and Methods

Strain

The strain used in the experiments of this report is a high producing mutant (~500 mg cyclosporin A per liter) obtained by UV treatment from the original strain of *T. inflatum* NRRL 8044.

Cultivation

The spore- and mycelium-suspension used for inoculation was produced from a culture of *T. inflatum* cultivated for 21 days at 27°C on an agar medium containing malt extract 20 g, yeast extract 4 g and agar 20 g per liter of demineralized water. The spores of this preculture were taken up in physiological saline to give a final concentration of 5×10^6 conidia/ml. Five ml of this suspension were used for inoculation of 500 ml of a nutrient solution containing maltose 50 g, the precursor (*e.g.* L-β-cyclohexylalanine) 5 g, KH_2PO_4 0.75 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $CaCl_2 \cdot 6H_2O$ 0.1 g and casein-peptone 8 g per liter. The inoculated production-medium was transferred in 100 ml portions to 50 500-ml Erlenmeyer flasks which then were incubated for 14 days at 27°C on a rotary shaker at 180 rpm.

For the preparation of [D-Ser^8] compounds, *e.g.* [D-Ser^8]cyclosporin G ([Nva^2, D-Ser^8]cyclosporin A), the fermentation medium was supplemented with DL-norvaline 5 g and D-serine 8 g per liter.

Analytical Methods

The culture medium was thoroughly mixed with an equal amount of methanol in order to extract the cyclosporins from the mycelial mass. After filtration an aliquot was analyzed on a HPLC Shandon Hypersil column (5 μm, 250×4.6 mm) at 75°C, mobile phase: acetonitrile - water - *ortho*-phosphoric acid (630 : 370 : 0.1), flow: 1.5 ml/minute, detection: UV-absorption at 210 nm.

Table 1. Mobilities on TLC and HPLC.

Cyclosporin	TLC (silica gel, Rf values)		HPLC (α values)
	Ethyl acetate[a]	Acetone - hexane[b] (1 : 1)	
Cyclosporin A	0.40	0.37	10.00
[MeCyclohexylala[1]]cyclosporin A	0.53	0.49	19.79
[Allylgly[2]]cyclosporin A	0.48	0.40	10.14
[D-Ser[8]]cyclosporin A	0.28	0.27	5.45
[D-Ser[8]]cyclosporin C	0.14	0.14	3.64
[D-Ser[8]]cyclosporin D	0.43	0.34	7.20
[D-Ser[8]]cyclosporin G	0.37	0.31	6.64

[a] Distance: 3×10 cm. [b] Distance: 1×10 cm.

Chromatographic purification was monitored by the same HPLC system and by TLC (silica gel plates, solvent systems: ethyl acetate or acetone - hexane (1 : 1), detection: iodine vapor, see also ref 8).

Isolation and Purification Procedure

The fermentation broth (5 liters) was separated into the mycelial cake and the culture filtrate. The former was homogenized three times with methanol - water (9 : 1) and the combined filtrates were concentrated *in vacuo* to remove the methanol. The aqueous solution was extracted three times with ethyl acetate. The organic layer were combined and evaporated to dryness. The crude residue was subjected to gel filtration on Sephadex LH-20 with methanol and then to repeated column chromatography on silica gel (Merck, 0.063~0.2 mm) using ethyl acetate saturated with water as eluent. Final purification, when necessary, was achieved by reversed-phase medium pressure column chromatography on LiChroprep RP18 (Merck) with methanol - water (4 : 1) as eluent.

Characterization of the New Cyclosporin Analogues

The chromatographic and physico-chemical properties are compiled in Tables 1 and 2, respectively. The spectroscopic data (UV, IR, ^{1}H and ^{13}C NMR) of all new cyclosporins reported are in agreement with the assigned structures.

Biological Assays

The immunosuppressive activity of the cyclosporins was determined in various *in vitro* and *in vivo* assays such as inhibition of proliferation of lymphocytes (MLR mixed lymphocyte reaction), inhibition of direct plaque-forming cells in mice, hemagglutination test, skin hypersensitivity reaction to oxazolone in mice[4].

Results and Discussion

In a series of precursor studies published some years ago[10], we observed a strong influence on the biosynthesis of cyclosporins exerted by exogenously supplied constituent amino acids at position 2 (Abu, Ala, Thr, Val, Nva), resulting in enhanced yields of the desired natural cyclosporin (A, B, C, D or G, respectively).

In the present study we have investigated the feasibility of incorporating foreign amino acids into the cyclosporin molecule. Regarding position 1, several naturally occurring variations of MeBmt have already been detected; deoxy analogues (cyclosporins F and K), *N*-methyl-L-2-aminooctanoic acid (cyclosporin Z) and *N*-methyl-L-leucine (cyclosporin O)[8]. In feeding experiments using L-β-cyclohexylalanine as a mimetic of MeBmt, this precursor was incorporated in position 1 leading to the almost exclusive formation of [MeCyclohexylala[1]]cyclosporin A. The biosynthesis of the 'normal'

Table 2. Physico-chemical properties of the new cyclosporin analogues.

Cyclosporin	MP (°C)	$[\alpha]_D^{20}$		Molecular formula	Elemental analysis		FAB-MS (m/z)
		Chloroform	Methanol		Found	Calcd	
[MeCyclohexylala[1]]-cyclosporin A	150~153	−292° (c 0.94)	−227° (c 0.87)	$C_{62}H_{111}N_{11}O_{11}$	C 62.5, H 9.5, N 13.1, O 15.0	C 62.8, H 9.4, N 13.0, O 14.8	1,186 $(M+H)^+$
[Allylgly[2]]cyclosporin A	147~151	−228° (c 0.75)	−175° (c 0.78)	$C_{63}H_{111}N_{11}O_{12}$	C 62.0, H 9.4, N 12.4, O 15.9	C 62.3, H 9.2, N 12.7, O 15.8	1,214 $(M+H)^+$
[D-Ser[8]]cyclosporin A	158~160	−268° (c 0.43)	−187° (c 0.53)	$C_{62}H_{111}N_{11}O_{13}$	C 60.6, H 9.3, N 12.4, O 17.6	C 61.1, H 9.2, N 12.6, O 17.1	1,218 $(M+H)^+$
[D-Ser[8]]cyclosporin C	147~152	−258° (c 0.39)	−178° (c 0.40)	$C_{62}H_{111}N_{11}O_{14}$	C 60.0, H 9.2, N 12.3, O 18.6	C 60.3, H 9.1, N 12.5, O 18.1	1,234 $(M+H)^+$
[D-Ser[8]]cyclosporin D	152~158	−257° (c 0.35)	−210° (c 0.37)	$C_{63}H_{113}N_{11}O_{13}$	C 61.2, H 9.4, N 12.4, O 16.8	C 61.4, H 9.2, N 12.5, O 16.9	1,232 $(M+H)^+$
[D-Ser[8]]cyclosporin G	142~146	−260° (c 0.48)	−191° (c 0.59)	$C_{63}H_{113}N_{11}O_{13}$	C 61.3, H 9.4, N 12.4, O 17.0	C 61.4, H 9.2, N 12.5, O 16.9	1,232 $(M+H)^+$

cyclosporins A, B and C was completely inhibited. Surprisingly, DL-*threo*-β-cyclohexylserine, a closer analogue to MeBmt, was not employed by the organism; only the usual pattern of cyclosporins A, B and C was found.

In systematic trials to vary the amino acid at position 2, DL-α-allylglycine, as an example of an unsaturated amino acid, was provided in the fermentation medium. As expected, [Allylgly^2]cyclosporin A was produced in addition to a large amount of cyclosporin G containing L-norvaline in position 2. The formation of the latter compound can be explained by enzymatic reduction of the olefinic double bond.

Additional efforts to substitute for D-alanine at place 8 by other amino acids with D-configuration, *e.g.* D-serine proved successful, yielding [D-Ser^8]cyclosporin A. Its potent immunosuppressive activity (see below) prompted investigations to prepare the D-serine-8 analogues of the natural, highly active cyclosporins C, D and G. By combined addition of the specific amino acid of position 2 (threonine, valine or norvaline, respectively) and D-serine, the desired new metabolites were obtained in good yield (20~100 mg/liter). Separation from the natural cyclosporins, formed by competitive incorporation of D-alanine in position 8, was easily achieved because of the distinctive more polar character of the D-serine-8 congeners.

Incorporation of foreign amino acids occurs only with a relatively limited number of specific amino acids; many substrates used were not incorporated into cyclosporin. For example, whereas an external supply of DL-2-aminobutyric acid enabled the organism to produce cyclosporin A in high quantity, addition of DL-3-aminobutyric acid suppressed cyclosporin biosynthesis. On the other hand, DL-4-aminobutyric acid exerted no effect on the course of the fermentation process.

Regarding structure-activity relationships, biological studies with the novel analogue [MeCyclohexylala^1]cyclosporin A confirmed earlier findings (see ref 1) that modification of the essential structural unit MeBmt, in general, results in a more or less dramatic decrease of immunosuppressive efficacy. Replacement of this amino acid residue by mimetics such as *N*-methyl-L-2-aminooctanoic acid (cyclosporin Z), *N*-methyl-L-β-cyclohexylalanine or by the common amino acid *N*-methyl-L-leucine

Table 3. Immunosuppressive activity of cyclosporins.

Cyclosporin	Trivial name	Source[a]	Activity[b]
Cyclosporin A	CyA	Nat	+++
[(3′-Deoxy)MeBmt^1]CyA	CyF	Nat	−
[(3′-Deoxy)MeBmt^1, Val^2]CyA	CyK	Nat	−
[*N*-Me-2-Aminooctanoic acid^1]CyA	CyZ	Nat	−
[MeLeu^1, Nva^2] CyA	CyO	Nat	−
[MeCyclohexylala^1]CyA		Dbs	−
[Ala^2]CyA	CyB	Nat	+
[Thr^2]CyA	CyC	Nat	++
[Val^2]CyA	CyD	Nat	+
[Nva^2]CyA	CyG	Nat	+++
[Allylgly^2]CyA		Dbs	++
[D-Ser^8]CyA		Dbs	+++
[D-Ser^8]CyC		Dbs	++
[D-Ser^8]CyD		Dbs	+++
[D-Ser^8]CyG		Dbs	+++

[a] Nat: Natural, Dbs: by directed biosynthesis.

[b] +++: Strong immunosuppressive activity, ++: moderate activity, +: weak activity, −: no significant activity.

(as in cyclosporin O), indeed led to only marginally potent or inactive compounds.

On the contrary, structural variations at position 2 in the side chain of the amino acid are rather well tolerated, as proven by the high potency of cyclosporin A (α-aminobutyric acid), cyclosporin G (norvaline) and [Allylgly^2]cyclosporin A. Shortened or branched alkyl residues as found in cyclosporin B (alanine) or cyclosporin D (valine) cause a slight decrease of activity. Interestingly, cyclosporin C which contains a polar threonine unit in position 2 is also a strong immunosuppressant.

Substitution of the D-alanine in position 8 by D-serine as illustrated by the series of [D-Ser^8]-cyclosporins A, C, D and G had little influence on the biological activity. The new derivatives display equipotent or, as in the case of [D-Ser^8]cyclosporin D, even higher immunosuppressive effects, compared to the native compounds (see Table 3).

The study presented in this communication has provided further evidence that precursor directed biosynthesis is a useful and efficient way to prepare cyclosporin analogues not encountered in nature. The successful incorporation of constituent and foreign amino acids demonstrates convincingly the low specificity in the biosynthesis of cyclosporins which is characteristic for a non-ribosomal biosynthetic pathway directed by multienzyme thiotemplates. Similar results have been reported for other secondary metabolites from prokaryotes and eukaryotes, *e.g.* in the actinomycin-family[13], the enniatins[14] and ergot alkaloids[15,16].

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