CELL-FREE BIOSYNTHESIS OF NEW CYCLOSPORINS

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An enzyme preparation, isolated from extracts of the fungus *Beauveria nivea* (previously designated *Tolypocladium inflatum*), is able to synthesize cyclosporins (Cy's) *in vitro*. At suboptimal temperature it was possible to yield about 50 μg of CyA per ml. The enzyme also produces several of the naturally occuring congeners of CyA, such as the Cy's B, C, D, G, M, O, Q, U and V and some of the analogues known to be produced by the fungus *via* precursor directed biosynthesis, like dihydro-CyA, [*N*-methyl-L-β-cyclohexylalanine[1]]CyA, [L-allylglycine[2]]CyA and [D-serine[8]]CyA.

Furthermore, Cy's not obtainable by the fungus could be prepared by the enzyme system in the presence of the appropriate precursor amino acids; the synthesis of [*N*-methyl-(+)-2-amino-3-hydroxy-4,4-dimethyloctanoic acid[1]]CyA, [L-norvaline[2,5], *N*-methyl-L-norvaline[11]]CyA, [L-norvaline[5], *N*-methyl-L-norvaline[11]]CyA, [L-*allo*-isoleucine[5], *N*-methyl-L-*allo*-isoleucine[11]]CyA, [L-*allo*-isoleucine[5,11]]CyA, [D-2-aminobutyric acid[8]]CyA and [β-chloro-D-alanine[8]]CyA could be established.

The immunosuppressive effects of the new derivatives are discussed.

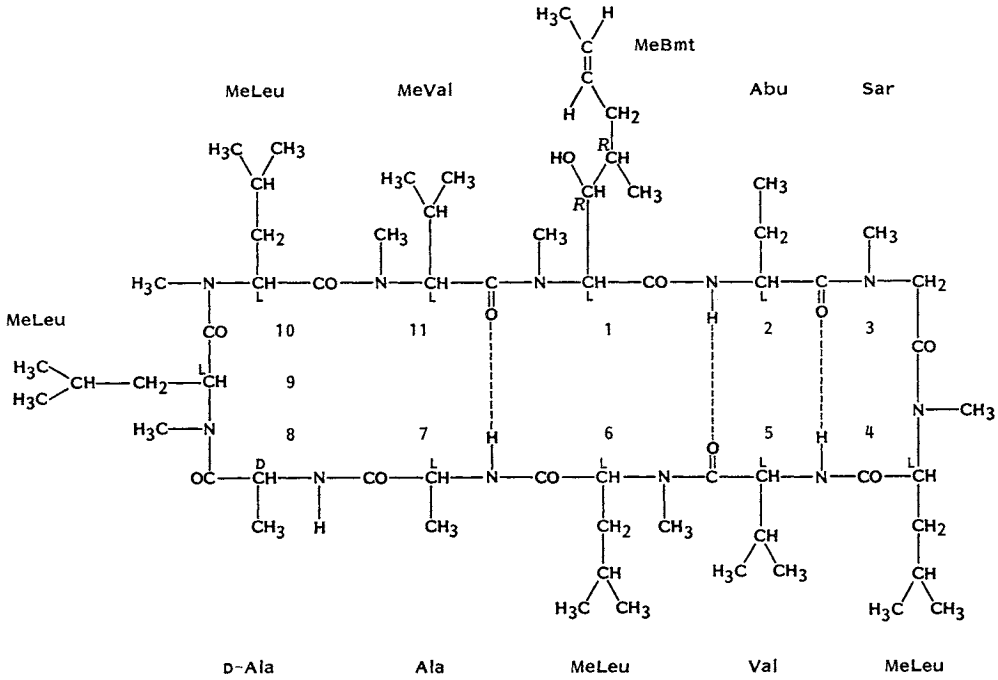
The cyclosporins (Cy's) represent a class of cyclic undecapeptides, which possess the structure shown in Fig. 1 for CyA (Sandimmun), but differ in their amino acid composition. CyA exerts antifungal and antiparasitic, antiinflammatory and immunosuppressive activities[1] and is used in transplantation surgery and the treatment of autoimmune diseases[2,3].

So far twenty-four naturally occuring Cy's beside CyA have been described[4,5], which are modified in nine of the eleven amino acid positions. The only non-variable building units are sarcosine in position 3 and D-alanine (D-Ala) in position 8 of the Cy ring. In positions 6, 9 and 10, the only modification compared to CyA consists in the lack of the *N*-methyl (Me) groups in these positions. The greatest variability in the Cy molecule is observed in position 2, which can be occupied with L-2-aminobutyric acid (Abu), L-alanine (Ala), L-threonine (Thr), L-valine (Val) or L-norvaline (Nva).

In addition to these natural Cy's several Cy's were obtained by precursor directed biosynthesis, like [*N*-methyl-L-β-cyclohexylalanine[1]]CyA, [L-allylglycine[2]]CyA and [D-serine[8]]CyA[6].

In a previous paper[7] the isolation of an enzyme fraction from *Beauveria nivea* (previously designated *Tolypocladium inflatum*[8]), strain 7939/F, actively synthesizing CyA and some homologues, which differ in positions 2 and 8 from CyA, has been reported. The present communication describes an enzyme fraction of another strain of *B. nivea* (7939/45), able to synthesize beside these homologues of CyA some further Cy's which naturally occur or can be attained by precursor directed biosynthesis *in vivo*. New Cy analogues have become available with this enzyme fraction which were not produced *in vivo*.

Fig. 1. The structure of CyA.



Materials and Methods

Growth of Organism

B. nivea, strain 7939/45, was maintained on agar slants (malt extract 2%, yeast extract 0.4%). Preculture fermentation was carried out as described earlier[7]. After 48 hours the preculture was used as inoculum for 10 flasks with 100 ml of the same medium, but containing EHC pepton (Amber, Milwaukee, U.S.A.) instead of casein pepton Tryptone (Oxoid, Wesel, FRG). After 7 days of cultivation cells were harvested by suction filtration and lyophilized.

Enzyme Preparation

Enzyme preparation was performed as described[7], but buffer A contained Tris-HCl 0.2 M (pH 7.8), KCl 0.3 M, EDTA 4 mM, dithioerythritol 10 mM, glycerol 40% (w/v) and buffer B contained Tris-HCl 0.1 M (pH 7.8), EDTA 4 mM, dithioerythritol 4 mM and glycerol 15% (w/v). The ammonium sulfate precipitation from 30 to 50% of saturation in buffer B was used. Active fractions of Ultrogel AcA 34 gel filtration were detected by measuring directly the synthesis of CyA.

Enzymatic Synthesis of Cy's

For synthesis of CyA, 100 μl of the enzyme preparation were incubated together with a mixture of 0.69 mM of each of L-leucine (Leu), D-Ala, Ala, Val, Abu, and glycine (Gly); (4*R*)-4-[(*E*)-2-butenyl]-4-methyl-L-threonine (Bmt)[9] 0.17 mM, ATP 3.5 mM, $MgCl_2$ 8.3 mM and *S*-adenosyl-L-[*methyl*-^{14}C]-methionine ([^{14}C]AdoMet, 58 Ci/mol) 0.25 μCi in a total volume of 120 μl for 2 hours at 25°C. Stopping of the reaction and extraction of Cy's were done as described[7]: For the production of higher yields of CyA, concentrations were increased to ATP 8 mM, $MgCl_2$ 16 mM, [^{14}C]AdoMet (0.5 μCi) 0.83 mM, Bmt 0.3 mM and 1.3 mM of each of the other constituent amino acids of CyA. Usually 1 ml of enzyme preparation was incubated in a total volume of 1.25 ml for 1 week at 6°C. For the production of analogues of CyA the amino acid composition of the incubation mixture was changed according to the amino acid content of the analogue.

Chromatographic Procedures (*cf.* Table 1)

Cy's were routinely separated on Silica gel HPTLC plates (Merck, Darmstadt, FRG). Solvents were water - saturated EtOAc (I), EtOAc - MeOH - H_2O (100 : 5 : 5) (II) or diisopropyl ether - $CHCl_3$ - MeOH (6 : 3 : 1) (III). HPLC separation was performed on a Shandon Hypersil column (5 μm, 250×4.6 mm) at 75°C with MeCN - H_2O - H_3PO_4 (630 : 370 : 0.1) as mobile phase. The flow rate was 2 ml per minute, Cy's were detected at 210 nm. Isolation of Cy's: Two incubations were performed; a smaller volume with [^{14}C]AdoMet and a larger one without labeling. The crude products from the enzymatic reactions were separated by preparative HPLC. Those fractions corresponding to the ^{14}C-label in the analytical run were combined, evaporated and extracted with ethyl acetate.

Radioactivity

Radioactivity (^{14}C) was measured either with a β-counter MR 300 (Kontron Analytic, Zürich, Switzerland) using Riatron (Kontron) scintillation solution or with a β-counter Tri-Carb (Canberra-Packard, Frankfurt, FRG) using Quickszint 501 scintillation solution (Zinsser Analytic, Frankfurt, FRG). For quantitative analysis of TLC plates, an automatic TLC-linear analyzer (Tracemaster 20, Berthold, Wildbad, FRG) was used.

Mass Spectra

Fast atom bombardment mass spectra (FAB-MS) were recorded on a VG 70-SE mass spectrometer (Vaccum Generator, Manchester, UK) at 8 keV using nitrobenzylalcohol as liquid matrix.

Biological Assays

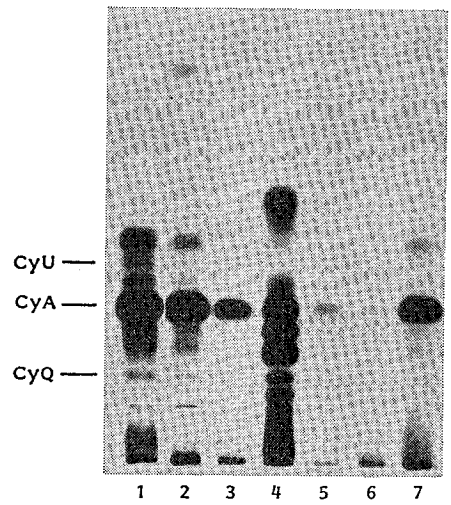
The immunosuppressive activity of Cy's purified on HPLC was determined in various *in vitro* assays such as inhibition of proliferation of lymphocytes (mixed lymphocyte reaction: MLR) and inhibition of proliferation of tumor cells[10].

Results

It was previously shown that CyA can be synthesized *in vitro* by an enzyme fraction of a crude extract of the fungus *B. nivea*, strain 7939/F[7]. However the specific activity of this enzyme preparation was rather low. We therefore prepared an enzyme from extracts of a high producer strain (7939/45). Fig. 2 demonstrates, that this enzyme is highly active. The main EtOAc-extractable reaction product comigrates with authentic CyA in TLC and HPLC. This compound can be labeled with [^{14}C]AdoMet, D-[^{14}C]-Ala, L-[^{14}C]-Ala, L-[^{14}C]-Val, [^{14}C]Gly, DL-[^{14}C]-Abu or L-[^{14}C]Leu, respectively (Fig. 2) and shows strong immunosuppressive activity; all these data confirm the identity of CyA.

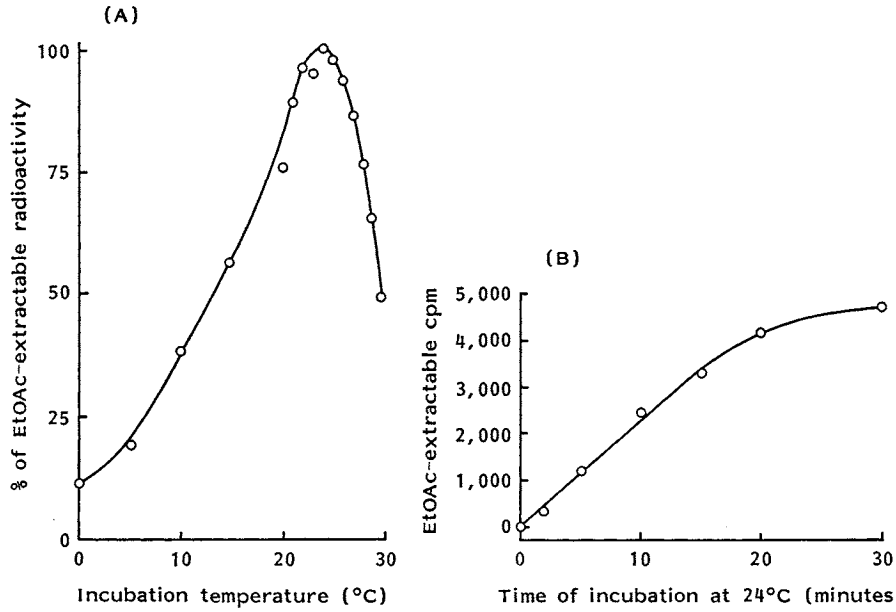
In vitro synthesis of CyA has an optimal temperature of 24°C (Fig. 3). Since the enzyme has been proven to be much more stable at

Fig. 2. Identification of *in vitro* synthesized [^{14}C]-CyA.



CyA was synthesized as described in Materials and Methods, but in each incubation vial another constituent amino acid was omitted and replaced by the ^{14}C-labeled one. Lane 1 represents the standard assay with [^{14}C]AdoMet (0.25 μCi), the radiolabel in the other lanes were 0.5 μCi D-[^{14}C]-Ala (40 Ci/mol) (2), 0.5 μCi L-[^{14}C]Ala (171 Ci/mol) (3), 0.5 μCi L-[^{14}C]Val (250 Ci/mol) (4), 0.5 μCi [^{14}C]Gly (114 Ci/mol) (5), 1 μCi DL-[^{14}C]Abu (26.8 Ci/mol) (6) and 0.5 μCi L-[^{14}C]Leu (348 Ci/mol) (7). The TLC plate was developed in solvent III and autoradiographed. The positions of CyA, CyQ and CyU are indicated.

Fig. 3. Temperature dependence of *in vitro* synthesis of CyA.



The reaction mixtures were incubated at the indicated temperatures (A). The reaction proceeded linear for about 15 minutes at 24°C (B). After 10 minutes of reaction, Cy's were extracted with 2 ml of ethyl acetate. The content of radioactivity in a 100-μl aliquot of this extract was measured in a scintillation counter. 100% value was 3,591 cpm; identity of CyA was checked by TLC analysis.

lower temperature, the yields of CyA at optimal (24°C) and suboptimal (6°C) temperature were compared in incubations of long duration: The reaction stops at a CyA concentration of about 30 μg/ml when the incubation was carried out at 24°C, whereas the incubation at 6°C yields more than 50 μg/ml of CyA. Therefore all incubations for preparative HPLC separations were performed at 6°C.

Among the natural Cy's some variations in position 1 have been detected: The deoxy-analogue in CyF and CyK, the non-*N*-methylated Bmt in CyL and CyP, MeLeu in CyO and *N*-methyl-L-2-aminooctanoic acid in CyZ. With the enzyme system described here, the incorporation of MeLeu in position 1 (CyO) was also possible. L-β-Cyclohexylalanine (CyclohexylAla), a mimetic of Bmt, which has been successfully incorporated in position 1 in feeding experiments[6], was also accepted by the enzyme, leading to formation of [MeCyclohexylAla^1]CyA as the main EtOAc-extractable reaction product. Two other Cy's with modifications in position 1, the second of which was accessible until now only by chemical synthesis, were formed also in good yields: Dihydro-CyA† and [*N*-methyl-(+)-2-amino-3-hydroxy-4,4-dimethyloctanoic acid^1]CyA ([MeAhdo^1]CyA), both cochromatographing exactly with authentic references in TLC and HPLC.

The greatest variability in the Cy ring occurs at position 2. As shown previously[7], the enzyme complex is able to introduce into this position the amino acids Abu, Ala, Thr, Val and Nva (Cy'sA, B, C, D and G). These Cy's were available with the enzyme preparation described here, too. Furthermore the [L-allylglycine^2]CyA ([AllylGly^2]CyA), which was obtained by precursor feeding to the fungus[6] could also be synthesized *in vitro* and was identified by cochromatography with an authentic

† Dihydro-CyA was isolated from fermentations using dihydro-Bmt as precursor. (P. BOLLINGER and J. J. BOELSTERLI (Sandoz Ltd.); unpublished results).

Table 1. Chromatographic data of enzymatically formed Cy's.

Cy	TLC (Rf value)			HPLC α value
	Solvent I	Solvent II	Solvent III	
CyA	0.37	0.44	0.42	10.00
CyO=[MeLeu^1, Nva^2]CyA	0.65	0.59	0.68	13.95
Dihydro-CyA	0.40	0.47	0.49	12.18
[MeAhdo^1]CyA	0.43	0.48	0.55	13.74
[MeCyclohexylAla^1]CyA	0.48	0.54	0.57	19.79
[AllylGly^2]CyA	0.53	0.55	0.48	10.14
CyQ=[Val^4]CyA	0.19	0.28	0.20	4.84
CyM=[Nva^{2,5}]CyA	0.53	0.57	0.58	13.61
[Nva^{2,5}, MeNva^{11}]CyA	0.56	0.61	0.58	14.34
[Nva^5, MeNva^{11}]CyA	0.38	0.46	0.45	10.20
[aIle^5, aMeIle^{11}]CyA	0.55	0.57	0.53	15.77
[aIle^{5,11}]CyA[a]	0.30	0.42	0.41	10.67
CyU=[Leu^6]CyA	0.45	0.48	0.52	8.92
CyV=[Abu^7]CyA	0.48	0.53	0.49	11.97
[D-Abu^8]CyA	0.43	0.47	0.51	13.09
[β-Chloro-D-Ala^8]CyA	0.49	0.54	0.47	11.73

The running span was 2×10 cm (I) or 10 cm (II and III).

The α-factor is defined as relative retention time $[(t_{R,1}-t_0)/(t_{R,2}-t_0)]\times 10$ whereat $t_{R,1}$ and $t_{R,2}$ mean the corresponding retention times and t_0 is the dead retention time. As reference compound, CyA is taken (α=10.00).

[a] The Rf values of this compound were determined after HPLC fractionation.

sample. When Nva, in place of Abu, was added in excess, the production of [Nva^{2,5}]CyA (CyM) was achieved.

On the other hand, substitution of Val by Nva led, as expected, to the formation of the new analogue [Nva^5,MeNva^{11}]CyA. When both precursors Abu and Val were substituted by Nva, [Nva^{2,5}, MeNva^{11}]CyA was obtained as main product. Exchange of Val in positions 5 and 11 by L-*allo*-isoleucine (aIle) successfully yielded the homologues compound [aIle^5,aMeIle^{11}]CyA and, in low quantity, the corresponding 11-demethylated derivative [aIle^{5,11}]CyA. The structural assignments for the above new Cy's were based on chromatographic parameters (TLC, HPLC; see Table 1) and mass spectrometry. The $(M+H)^+$-values were determined as 1,216.7 for [Nva^{2,5},MeNva^{11}]CyA, 1,202.7 for [Nva^5, MeNva^{11}]CyA and 1,230.9 for [aIle^5,aMeIle^{11}]CyA.

Two naturally occuring Cy's with variations in positions 4 and 6 of the Cy ring could be identified by TLC and HPLC among the minor reaction products of CyA synthesis reactions, namely CyQ and CyU, representing [Val^4]CyA and [Leu^6]CyA, respectively (Fig. 2).

So far the Ala residue in position 7 can only be replaced by Abu *in vivo*, yielding CyV. The latter Cy was formed *in vitro* as the main reaction product, when Ala was omitted in the reaction mixture.

Recently, several Cy's containing D-serine (D-Ser) instead of D-Ala in position 8 have been described[6]. Nevertheless, the variability of position 8 by feeding other D-amino acids to the fungus was found to be low, *e.g.*, supplying D-Abu results in an increase of CyA formation *via* epimerization of D-Abu to L-Abu in the fungus (P. BOLLINGER (Sardoz Ltd.); personal communication).

Therefore the *in vitro* synthesis seems to be a better way to yield Cy's with various D-amino acids in position 8. The *in vitro* synthesis of [D-Ser^8]CyA has previously been demonstrated[7]. When in our *in vitro* system D-Ala is exchanged either by β-chloro-D-Ala or D-Abu, the main reaction com-

ponents are [β-chloro-D-Ala^8]CyA or [D-Abu^8]-CyA, respectively. The identity of the first homologue was established by cochromatography in HPLC and TLC with a reference compound synthesized chemically. The [D-Abu^8]-CyA structure was suggested by the assay conditions (ATP- and D-Abu-dependent formation) and by the CyV-like behavior on HPLC and TLC. Analysis in a FAB-MS gave the correct molecular ion peak (1,216 $(M+H)^+$) for [D-Abu^8]CyA.

Table 2. Immunosuppressive activity of new Cy's.

Cy	Biosynthesis	Activity
CyA (Sandimmun)	Natural	+++
CyA	Enzymatic	+++
[Nva^{2,5}, MeNva^{11}]CyA	Enzymatic	++
[Nva^5, MeNva^{11}]CyA	Enzymatic	++(+)
[aIle^5, aMeIle^{11}]CyA	Enzymatic	++
[aIle^{5,11}]CyA	Enzymatic	+
[β-Chloro-D-Ala^8]CyA	Enzymatic	++
[D-Abu^8]CyA	Enzymatic	++

+++: Strong immunosuppressive activity, ++: moderate activity, +: weak activity.

Structure-activity Relationships

The structure-activity relationships of several of the Cy's described here have been discussed elsewhere[4~6]. Therefore only some remarks about the new Cy's listed in Table 2 have to be made in this context. All new compounds isolated from *in vitro* reactions show immunosuppressive effects indicating strongly that they represent new Cy's.

[Nva^5,MeNva^{11}]CyA, containing Nva in positions 5 and 11 instead of Val, retains fairly strong immunosuppressive activity but does not reach the activity level of CyA. Exchange of Val by aIle as in [aIle^5,aMeIle^{11}]CyA considerably reduces the immunosuppressive potency. The corresponding *N*-demethylated compound [aIle^{5,11}]CyA is even less active, a phenomenon which is also observed in the case of CyE, the 11-*N*-demethylated CyA.

Replacement of both Abu in position 2 and Val in positions 5 and 11 by Nva leads to an unfavorable modification; [Nva^{2,5},MeNva^{11}]CyA displays only intermediate activity.

Regarding position 8, substitution of D-Ala by β-chloro-D-Ala or D-Abu causes some decrease of immunosuppressive efficacy.

Discussion

In a previous paper[7] BILLICH and ZOCHER have reported on an enzyme fraction from *B. nivea* extracts, capable of synthesizing CyA and some homologues *de novo*. Using a strain of *B. nivea* which produces higher Cy concentrations in submerged cultures, we were able to yield highly active fractions which allowed a systematic search for biosynthesis of a wide spectrum of homologues of CyA. Cy's were obtained in sufficient quantities to allow examination of their immunosuppressive activities.

In each case where we could adjust the proper amino acid compositions for Cy's obtained either naturally or by precursor feeding to the fungus, the expected Cy's were also produced in the *in vitro* system.

Moreover the enzyme system makes Cy's available, which could not be synthesized by providing the specific precursors to the fungus, as in the case of [D-Abu^8]CyA and [aIle^5,aMeIle^{11}]CyA. In the fungus, these amino acids seem to be metabolized, as indicated by the fact that supplementing of D-Abu increases the CyA production.

In summary, the methodology of enzymatic *in vitro*-synthesis enabled directed modifications, so far, in positions 1, 2, 5, 7, 8 and 11 of the CyA peptide ring.

Acknowledgments

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