Journal of Industrial Microbiology (1996) 17, 397–401

Occurrence of cyclosporins and cyclosporin-like peptolides in fungi

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Apart from 17 previously listed fungal taxa producing cyclosporin A and its natural congeners B to Z, several additional and taxonomically diverse strains producing the single novel component [Thr^2,Leu^5,Leu^{10}]cyclosporin or cyclosporin-like peptolides (eg SDZ 214-103 = [Thr^2,Leu^5,D-Hiv^8,Leu^{10}]cyclosporin) have recently been described. We report here the isolation of two further and novel cyclosporins, [Thr^2,Leu^5,Ala^{10}]cyclosporin (2) and [Thr^2,Ile^5]cyclosporin (3), from strains classified as *Acremonium luzulae* (Fuckel) W Gams and *Leptostroma* anamorph of *Hypoderma eucalyptii* Cooke & Harkn, respectively. In both new strains the usual pattern of cyclosporins A to Z is not found. The structure elucidations of 2 and 3 are based on NMR spectroscopy, and biological data (immunosuppressive activity, cyclophilin-binding affinity and antifungal effects) are presented.

Keywords: cyclosporins; cyclosporin-like peptolides; fungal taxa; *Acremonium luzulae*; *Leptostroma* anamorph of *Hypoderma eucalyptii*

Introduction

The cyclic peptide cyclosporin A (1) (Table 1), which is in clinical use worldwide as an immunosuppressive drug (Sandimmun®/Neoral®) [1], has been reported from 17 fungal taxa [6]. Although only one case has been investigated in detail, we presume that natural, non-mutated cyclosporin A-producing fungi potentially co-produce the natural analogues B through Z [28] by merit of a common multienzyme characterized in detail from *Tolypocladium inflatum* W Gams NRRL 8044 [14].

In our microbiological research, cyclosporin A-producing fungal strains initially originated exclusively from cooler or temperate climates [5]. Recently, however, we have also found several cyclosporin A-producing *Chaunopycnis alba* W Gams strains from Brazilian rainforest soils and from leaf litter samples collected at approximately 2000 meters altitude on Mt Kinabalu, Borneo [19,20].

Apart from strains producing the usual cyclosporin A to Z spectrum, different patterns of cyclosporin biosynthesis in fungi are now beginning to emerge. The strain S 22651/F of *Cylindrotrichum* Bonorden which was isolated from leaf litter collected in the Swiss Jura produces the peptolide SDZ 214-103 = [Thr^2,Leu^5,D-Hiv^8,Leu^{10}]cyclosporin [4] but none of the other known natural cyclosporins. More recently, a strain of *Stachybotrys chartarum* No. 19392 was reported to produce [Thr^2,Leu^5,Leu^{10}]cyclosporin, and, as in the former case, no other natural member of this metabolite group was observed as a by-product [23].

We report here two further examples, namely the production of the novel [Thr^2,Leu^5,Ala^{10}]cyclosporin (2) by strain F/88-3089/11 of *Acremonium luzulae* (Fuckel) W Gams and of [Thr^2,Ile^5]cyclosporin (3) by strain F/93-4641/04 of *Leptostroma* anamorph of *Hypoderma eucalyptii* Cooke & Harkn.

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Received 28 May 1996; accepted 30 August 1996

Materials and methods

Isolation of the producer strains

The *Acremonium luzulae* strain F/88-3089/11 was isolated from a leaf litter sample collected by RA Foote in an Oak-Pine-Rhododendron forest at an altitude of 2200 meters in Nepal, and the *Leptostroma* anamorph of *Hypoderma eucalyptii* strain F/93-4641/04 was isolated from a *Eucalyptus* leaf litter sample collected by K Hyde in Koam Cairns, Australia.

For fungal isolations leaf litter samples were homogenized in sterile water and 0.2-ml aliquots of serial dilutions streaked on petri dishes containing 2% malt extract agar supplemented with 50 ppm terramycin and 1 ppm cyclosporin A. Plates were incubated at 21°C and fungal colonies isolated onto agar slants containing 2% malt extract agar.

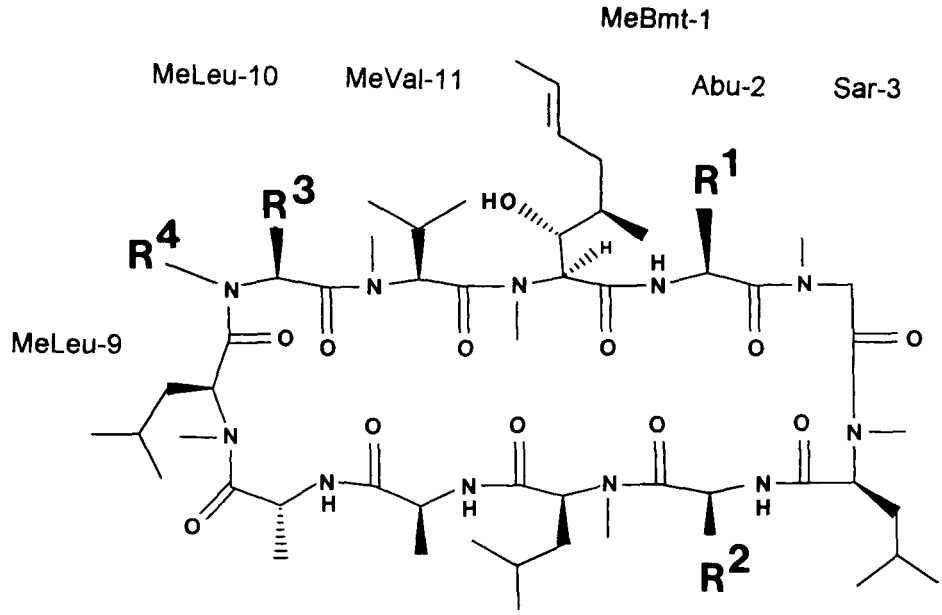
Extracts for screening

Cultures were grown in Erlenmeyer flasks at 21°C for 7 days on a rotary shaker (200 rpm) containing a medium composed of 3% maltose-monohydrate and 0.5% yeast extract in deionized water. Broths were lyophilized and extracted with equivalent amounts of methanol.

Biological screening assays

Methanol extracts were tested in agar diffusion assays against *Neurospora crassa* strain S 261 and *Aspergillus niger* strain S 7281 to detect antifungal activities. The immunosuppressive activity was determined in various *in vitro* assays such as inhibition of proliferation of lymphocytes (mixed lymphocyte reaction) and inhibition of proliferation of tumor cells [26]. The cyclophilin-binding assay was performed as described by Quesniaux *et al* [22].

Table 1 Chemical structures



Compound	R^1	R^2	R^3	R^4
Cyclosporin A (1)	CH_2CH_3	$CH(CH_3)_2$	$CH_2CH(CH_3)_2$	CH_3
[Thr^2,Leu^5,Ala^{10}]Cy (2)	CH(OH)-CH_3	$CH_2CH(CH_3)_2$	CH_3	H
[Thr^2,Ile^5]Cyclosporin (3)	CH(OH)-CH_3	$CH(CH_3)$-CH_2CH_3	$CH_2CH(CH_3)_2$	CH_3

Fermentation and isolation procedure

Isolation of (2) and (3) were performed from 50 L of culture broth produced in Erlenmeyer flasks using the same conditions as described above. Activity against *Neurospora crassa* strain S 261 was used for monitoring the optimal time for harvest, which was reached at day 7. The culture broth was filtered using Hyflo Super-Cel and the mycelium cake was extracted three times with *n*-butanol (2 L each). The organic solutions were evaporated *in vacuo* to yield 178 g crude extract which was distributed between hexane and *n*-butanol (4 : 1) (3 × 2 L each) to remove fatty materials. The combined butanol phases yielded after evaporation 10.08 g of an oily residue which was separated by repeated medium pressure column chromatography using silica gel 60 H (Merck, Darmstadt, Germany) and CH_2Cl_2-MeOH-H_2O (88 : 11 : 1) as eluent. After final purification by gel filtration on Sephadex LH-20 with methanol, 557 mg 2 was obtained as a white amorphous powder.

Compound 3 was isolated from a 50-L fermentation of the strain F/93-4641/04 following analogous procedures to yield 169 mg.

Analytical methods

TLC was done on silical gel 60 F_{254} plates (Merck); system I: ethyl acetate saturated with water; system II: methylene chloride-methanol-water (88 : 11 : 1). The running span was 2 × 10 cm (I) or 10 cm (II).

HPLC was performed on a Shandon Hypersil column (5 μm, 250 × 4.6 mm) at 75°C; mobile phase: acetonitrile-water-ortho phosphoric acid (630 : 370 : 0.1, by vol); flow rate 1.2 ml min^{-1}; detection: UV absorption at 210 nm.

Instrumental analyses

IR spectra were measured on a Perkin-Elmer spectrophotometer model 21 (Norwalk, CT, USA). ^{1}H NMR spectra were recorded on a Bruker AM-360 instrument (Karlsruhe, Germany). FAB mass spectra were obtained on a VG70-SE mass spectrometer (Vacuum Generator, Manchester, UK) at 8 keV using thioglycerol as liquid matrix.

Amino acid analysis

1 mg of 2 was hydrolyzed with 6 N HCl at 115°C for 24 h. The hydrolysate was analyzed with an automatic amino acid analyzer HP1090. Column: Hypersil ODS (4.6 × 250 mm); phase A: acetonitrile-methanol-buffer solution (5 : 40 : 60, by vol; buffer solution: 1 ml acetic acid + 3 ml triethylamine filled up to 1 L, pH 4.2); phase B: acetonitrile-buffer solution (60 : 40, by vol); eluent: phase A-phase B (45 : 55, by vol); gradient: 55% B to 100% B in 25 min; flow: 0.8 ml min^{-1}; detection by fluorescence: excitation at 266 nm, emission at 310 nm (FMOC derivatization).

Results

Taxonomy and characteristics of the producer strains

Isolates F/88-3089/11 and F/93-4641/04 were selected based on their pronounced antifungal activity and their profiles in a mixed lymphocyte reaction bioassay. Microscopic examination revealed that they did not belong to any of the genera so far known to produce cyclosporins.

Isolate F/88-3089/11 was identified as an *Acremonium* species using the identification keys of von Arx [30] and to species level using the identification keys of Gams [8] and assigned to *Acremonium luzulae* (Fuckel) W Gams. This strain has a growth optimum between 18°C and 24°C. At an incubation temperature of 21°C on 2% malt extract agar, colonies attain a diameter of 10–12 mm in 14 days and up to 20 mm in 21 days. Between 27°C and 33°C strain F/88-3089/11 reaches its upper growth limit. Colonies incubated at 18–24°C are initially fluffy white and gradually develop dark olive-green due to the production of conidia from the center towards the colony margin. The reverse side of colonies is light orange brown to dark brown, maroon in age, sometimes producing a reddish-brown diffusable pigment. The conidiogenesis is phialidic. Phialides are cylindrical, tapering towards the apex, hyaline, covered with warts, especially dense at the apex and measure 23–34 μm in length, 2.5–3 μm at the base, and 1–2 μm in width at the apex. They are formed singly but are numerous on hyphae or hyphal strands and are occasionally branched at the base, especially in older cultures. Conidia are formed in long dry chains, are barrel shaped, ellipsoidal with truncate ends, light olive brown with a dark equatorial area and dark ends, and measure 5–8 × 2–3 μm.

Isolate F/93-4641/04 was identified to genus level as *Leptostroma* Fr ex Fr using identification keys in Sutton [27]. Based on its host (*Eucalyptus*) the strain can best be assigned to *Leptostroma* anamorph of *Hypoderma eucalyptii* Cooke & Harkn [7]. The growth optimum of *H. eucalyptii* strain F/93-4641/04 is between 24°C and 27°C. At an incubation temperature of 24°C on 2% malt extract agar, colonies attain a diameter of 14–16 mm in 14 days. Between 33°C and 37°C *H. eucalyptii* strain F/93-4641/04 reaches its upper growth limit. Colonies incubated at 24°C are initially cream to light gray, gradually becoming darker gray to grayish-brown, mycelium velutinous appressed in small irregular leathery tufts. Conidiomata are black measuring 170–400 μm in diameter, singly and scattered over the colony, superficial to semi-immersed, stromatic, multilocular, nonostiolate; basal part *textura angularis*, cells 3–4 × 2–3 μm, light brown, covering layer loose *textura intricata*, hyphal diameter 2–3 μm. Conidiogenesis is blastic sympodial. Conidiogenous cells are hyaline, cylindrical, tapering towards the apex, measuring 6–10 × 1.5–2 μm. Conidia are hyaline, allantoid to cylindrical with obtuse ends, measuring 4–5 × 1–1.5 μm, and are extruded from conidiomata in whitish slimy masses.

Structure elucidations

[Thr²,Leu⁵,Ala¹⁰]Cyclosporin (2) The physico-chemical properties are compiled in Table 2. The IR spectrum clearly indicated the peptide character of 2 and the membership to the group of cyclosporins. The molecular formula $C_{59}H_{105}N_{11}O_{13}$ was deduced from FAB-MS (m/z) 1176 MH^+, ^{13}C NMR spectra (59 C-atoms) and elemental analysis. The first indication of the presence of the amino acid Bmt, characteristic for most natural cyclosporins, came from mass spectral data (m/z 1064 = $MH^+ - 112$) showing a fragment of $C_7H_{12}O$, split off from the Bmt side chain. Acidic hydrolysis of 2 revealed the amino acids Ala (3×), Thr (1×), Leu (1×) and the *N*-methylated amino acids Sar (1×), MeVal (1×), MeLeu (3×) and the cyclic artifact of MeBmt [32]. In comparison to CyA, neither α-aminobutyric acid nor valine was obtained, giving evidence for the absence of these two structural units in 2. Assuming the replacement of Abu-2 of CyA by threonine in 2, still a deficiency in the molecular formula by three CH_2 units remains between 2 and CyA; one of the four MeLeu units of CyA is *N*-demethylated.

Table 2 Physico-chemical properties of [Thr²,Leu⁵,Ala¹⁰]-(2) and [Thr²,Ile⁵]Cyclosporin (3)

	[Thr²,Leu⁵,Ala¹⁰]-(2)	[Thr²,Ile⁵]Cyclosporin (3)
Melting point	165°	144–147°
$[\alpha]^{20}_D$		
($CHCl_3$)	−234° (*c* 0.74)	−244° (*c* 0.77)
(MeOH)	−161° (*c* 0.67)	−175° (*c* 0.61)
FAB-MS (m/z)	1176 $(M+H)^+$	1232 $(M+H)^+$
Molecular formula	$C_{59}H_{105}N_{11}O_{13}$	$C_{63}H_{113}N_{11}O_{13}$
TLC R_f		
system I	0.23	0.23 (CyA: 0.43)
system II	0.51	0.52 (CyA: 0.58)
HPLC α-value	4.96	8.42 (CyA: 10.00)

The ^1H NMR spectrum of 2 in $CDCl_3$ showed five NH-amide protons as doublets between 7.0 and 9.3 ppm and six singlets in the region of *N*-methyl signals, corresponding to the five primary amino acids and the six *N*-methylated amino acids, respectively, as detected in the amino acid analysis. In contrast to most natural cyclosporins, a mixture of at least two main conformers of 2 was present in the chloroform solution making an unambiguous interpretation in the important region of the α-protons not possible. Upon addition of H_2O the resolution of signals was improved and two-dimensional techniques, ^1H-^1H COSY, TOCSY and ROESY NMR spectra, enabled the assignments of all relevant proton signals. The confirmation of the proposed amino acid sequence emerged from the ^1H NMR spectrum recorded in d_6-benzene (Figure 1); in this solvent the α-protons of Thr-2, Leu-5 and Ala-10 appeared as separated signals.

[Thr²,Ile⁵]Cyclosporin (3) The FAB-MS data (1232 MH^+/$C_{63}H_{113}N_{11}O_{12}$) indicated for 3 one methylene group and one oxygen atom in addition to cyclosporin A. The structure elucidation was based on detailed NMR studies. ROESY spectra (Figure 2) revealed a threonine unit in position 2 instead of the α-aminobutyric acid in CyA and, for position 5, the replacement of the usual valine unit by isoleucine in 3.

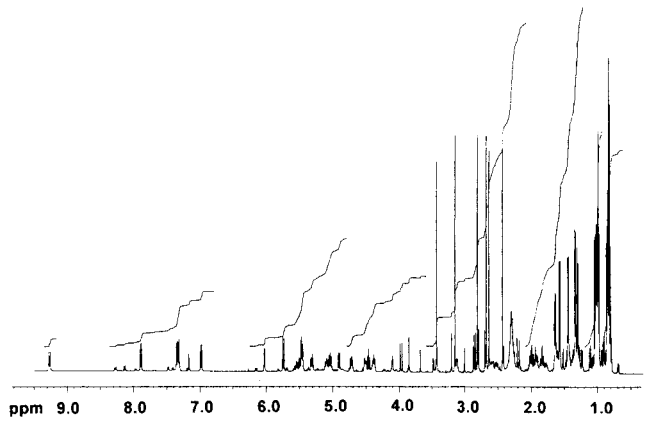


Figure 1 ^{1}H NMR spectrum of 2 in d_6-benzene.

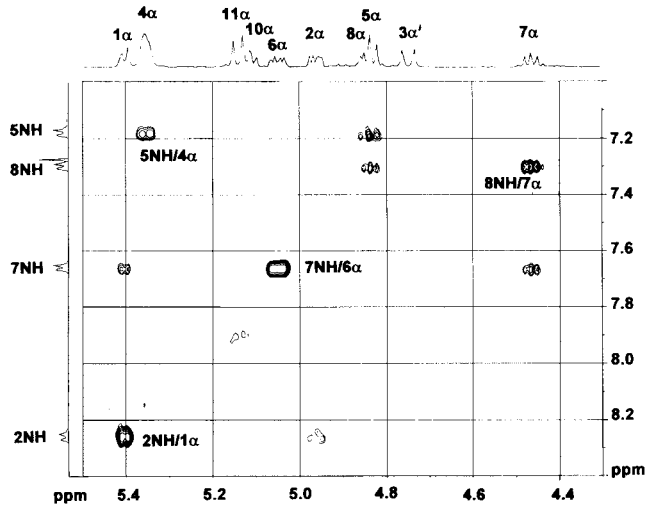


Figure 2 ROESY NMR spectrum of 3 in $CDCl_3$.

Biological properties

[Thr^2,Leu^5,Ala^{10}]-(2) and [Thr^2,Ile^5]cyclosporin (3) inhibited filamentous fungi, eg *Neurospora crassa*, as observed for most natural cyclosporins [2]. The immunosuppressive activity *in vitro* of 2 is approximately 100 times less compared to CyA while the binding affinity to the cellular CyA-binding protein cyclophilin [11] is retained. For [Thr^2,Ile^5]cyclosporin in contrast, the immunosuppressive potency is decreased only by a factor of 2–3, and, as in the case of 2, the binding capacity to cyclophilin lies in the same range as for cyclosporin A.

Discussion

Both [Thr^2,Leu^5,Ala^{10}]-(2) and [Thr^2,Ile^5]cyclosporin (3) represent unique new members in the series of natural cyclosporins. In 2 the *N*-methylleucine unit in position 10 is exchanged for alanine. In all other natural cyclosporins known so far, position 10 is occupied by *N*-methylleucine or its *N*-demethylated form as eg in cyclosporin T = [Leu^{10}]CyA. Whereas threonine in position 2 is a well-known modification encountered in cyclosporin C [32], leucine substituting valine in position 5 represents a structural variation hitherto only found in the recently disclosed [Thr^2,Leu^5,Leu^{10}]cyclosporin [23] and the natural congener cyclosporin 26 = [Nva^2,Leu^5]CyA (Traber *et al*, unpublished results).

In [Thr^2,Ile^5]cyclosporin produced by a *H. eucalyptii* F/93-4641/04 of *Leptostroma* anamorph of *Hypoderma eucalyptii*, for the first time an isoleucine unit occurs in position 5 of the peptide ring.

Regarding conformational studies, in CyA a strong NOE is observed in the ^{1}H NMR spectrum between the 9-α and the 10-α proton, originating from the *cis* peptide bond between the two MeLeu units 9 and 10 [18]. This NOE is absent in 2, therefore, a *trans* configuration of the peptide bond MeLeu-9 → Ala-10 is indicated as a consequence of the *N*-demethylation in position 10. The strong decrease in the immunological potency of 2 is explained by the conformational change of the peptide backbone. On the other hand, in [Thr^2,Ile^5]cyclosporin (3), according to NMR spectra, the backbone conformation is similar to that of CyA: this is reflected by the nearly unchanged biological properties of 3.

Interestingly, addition of D-serine to the fermentation medium did not result in incorporation of this 'foreign' amino acid into the cyclosporin molecule, whereas, in the case of the fungus *Tolypocladium inflatum* NRRL 8044, D-serine^8 analogues were obtained in high yield [29].

Noteworthy, the *Cylindrotrichum* strain S 22651/F produces a structurally related cyclosporin-analogue, the cyclic peptolide SDZ 214-103 [4], containing threonine in position 2 and leucine in positions 5 and 10 ([Thr^2,Leu^5,D-Hiv^8,Leu^{10}]Cyclosporin). It is biosynthesized by the multienzyme polypeptide SDZ 214-103 synthetase [15], which is similar in size and properties to cyclosporin synthetase [25], but shows a much higher substrate specificity [16]. From large-scale fermentations only a few minor metabolites of the peptolide SDZ 214-103 have been isolated [4].

Our current knowledge about the occurrence of cyclosporins and cyclosporin-like peptolides renders it worthwhile to discuss the taxonomic distribution of the producing fungi. A large group of fungal taxa produces cyclosporins A–D as major metabolites and cyclosporins E–Z as minor ones, probably by merit of a common multienzyme [14]. Seventeen taxa have been listed belonging to this group [3,6] and further taxa may be found in the future. Within this group *Nectria* [10] and *Neocosmospora* strains [3,21] (both *Hypocreaceae*), *Trichoderma viride* [9] and several strains of *Fusarium* [3,12,24] (possibly anamorphs of *Hypocreaceae*) have been reported, besides a cluster of rather slow growing, whitish hyphomycetes with small conidia, eg *Tolypocladium, Acremonium, Paecilomyces, Isaria, Verticillium, Beauveria, Chaunopycnis* [3,6]. With the exception of *Chaunopycnis*, representatives of the other genera of this latter cluster are well known to be parasitic on higher fungi, nematodes, rotifers and insects and can be potentially expected to be anamorphs of *Clavicipitaceae* [3,8,31].

More recently, single strains producing novel cyclosporins or cyclosporin-like peptolides, but lacking the usual pattern of cyclosporins A–D and the known minor congeners, are being discovered: *Cylindrotrichum* species [4], *Stachybotrys chartarum* [23], *Acremonium luzulae* (F/88-

3089/11), and *Leptostroma* (F/93-4641/04), the latter being the first true coelomycete shown to produce a cyclosporin.

The occurrence of cyclosporins and cyclosporin-like peptolides in such a broad range of fungal taxa, and concurrently the conservation of the genes coding for the corresponding multienzyme synthetase, suggest that there must be some significance for the producing fungi. *Tolypocladium inflatum* must possess a self-protecting system in order to survive in the presence of cyclosporin, which binds to its own cyclophilin [17,33]. This is confirmed by our observations that all producers of cyclosporin and cyclosporin-like peptolides are resistant towards these metabolites, whereas non-producers generally are sensitive. In *Cryphonectria parasitica* cyclosporin can stimulate gene transcription, indicating that cyclophilin is the cellular receptor that mediates this activity [13]. Beyond these published findings the relevance of cyclosporins for fungi in general and their producers in particular remains unknown.

Acknowledgements

We thank Dr Chr Möller (Sandoz Pharma Ltd, Basel, Switzerland), Dr W Gams (CBS, Netherlands) and Dr L Petrini (Comano, Switzerland) for strain identifications and descriptions, and Dr L Pfenning (Campinas, Brazil) for two Brazilian strains of *Chaunopycnis*. We further thank Ch Beerli for amino acid analysis, and L Oberer and M Ponelle for interpretation of NMR spectra, and we acknowledge the biological assays performed in the laboratories of Dr MH Schreier (immunological testing) and Dr V Quesniaux (cyclophilin binding) (all Sandoz Pharma Ltd).

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