

Cyclosporin C Is the Main Antifungal Compound Produced by *Acremonium luzulae*

MUSTAPHA MOUSSAÏF,^{1*} PHILIPPE JACQUES,¹ PETER SCHAARWÄCHTER,²
HERBERT BUDZIKIEWICZ,² AND PHILIPPE THONART^{1*}

Centre Wallon de Biologie Industrielle, University of Liege, Liege 4000, Belgium,¹ and Institut für Organisch Chemie, University of Cologne, 50939 Cologne, Germany²

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A strain of *Acremonium luzulae* (Fuckel) W. Gams was selected in screening new microorganisms for biological control of fruit postharvest diseases, especially gray and blue mold diseases on apples and strawberries. This strain manifests a very strong activity against a large number of phytopathogenic fungi. In this work, the product responsible for this antifungal activity was isolated from modified Sabouraud dextrose broth cultures of *A. luzulae*. It was purified to homogeneity by reverse-phase column chromatography. On the basis of UV, infrared, and ¹H and ¹³C nuclear magnetic resonance spectra, mass spectral analysis, and the amino acid composition of the acid hydrolysates, the antibiotic was determined to be cyclosporin C. Cyclosporin C showed a broad-spectrum activity against filamentous phytopathogenic fungi but no activity against bacteria or yeasts. Its antifungal activity is only fungistatic. In contrast to *Tolypocladium inflatum*, another cyclosporin-producing strain, *A. luzulae*, did not produce additional cyclosporins. This was confirmed by in vivo-directed biosynthesis.

Cyclosporins are a family of neutral, highly lipophilic, cyclic undecapeptides containing some unusual amino acids. They can be produced in a fungal fermentation process by aerobic filamentous fungi which were originally classified as *Trichoderma polysporum* (Link ex Pers.) Rifai (13) and more recently as *Tolypocladium inflatum* W. Gams (15) or *Beauveria nivea* Von Arx (39).

Cyclosporin A (cyclosporine) (Fig. 1), the main representative of the series, is a potent antifungal and immunosuppressive compound (8, 17), and is thus widely used in patient management after transplantation surgery and in autoimmune-disease treatment (2). The mode of action of cyclosporin A has been studied. Apparently, it binds to intracellular proteins, the cyclophilins (10), which are abundant, ubiquitous, highly conserved, and found in multiple forms in different intracellular compartments. These cyclophilins catalyze protein folding by *cis-trans* isomerization of peptidyl-prolyl bonds (14, 34).

The fermentation of cyclosporins follows the general characteristic of "directed biosynthesis" in peptide production, in which the supplemented amino acids modify the endogenous amino acid pool of the producer fungus and direct the biosynthesis as precursors (6, 21). Cyclosporin biosynthesis is catalyzed by a single multienzyme polypeptide (22) called cyclosporin synthetase, which has a molecular mass of about 1,500 kDa (33). It proceeds via the so-called nonribosomal thiotemplate mechanism, which leads to a number of very similar cyclosporins (36). The thiotemplate mechanism is a less specific pathway than the ribosomal mechanism with respect to the amino acids used as substrates. This phenomenon has also been described for other peptide synthetases (20). Cyclosporin synthetase has relatively low substrate specificity, and thus it is able to catalyze the synthesis of more than two dozen cyclosporins in vitro and in vivo depending on the available precursors (23, 35). According to the thiotemplate mechanism, each amino acid is first activated as adenylate, then bound as thio-

ester, and finally attached to the following amino acid by a peptide bond. During the formation of cyclosporins, seven of the amino acids bound as thioesters are N methylated before they are linked to the preceding amino acid. This methylation function is an integral part of the action of the enzyme (22). Including cyclization, cyclosporin synthetase performs at least 40 chemical reactions.

In this work, we studied the antifungal activity of a strain of *Acremonium luzulae* (Fuckel) W. Gams (27) isolated from strawberry fruits. This strain, also reported as *Gliomastix luzulae* (Fuckel) Mason ex Hughes (11), shows a very strong antagonistic activity against a large number of plant-pathogenic fungi (data not shown).

According to our knowledge, *A. luzulae* produces ergosterol metabolites, ergosterol peroxide, and some diterpenes such as virescenosides (β -D-altropyranosides of virescenols) (3, 5, 28). A further terpenoid metabolite, named ascochlorin, was also isolated from a spontaneous albino mutant of *A. luzulae* (4). The antifungal action of *A. luzulae* was supposed to be related to the production of the terpenoid compounds.

In this work, we show for the first time that the antifungal activity of *A. luzulae* toward a range of pathogenic filamentous fungi is due mainly to cyclosporin C and that in contrast to *T. inflatum*, *A. luzulae* does not produce additional cyclosporins, suggesting that the specificity of the enzymatic cyclosporin C biosynthesis system in *A. luzulae* is high.

MATERIALS AND METHODS

Maintenance of stock cultures of *A. luzulae*. *A. luzulae*, originally isolated by the Centre Wallon de Biologie Industrielle, was deposited as strain 34683 in the Mycothèque de l'Université Catholique de Louvain in Belgium. For current use, it can be maintained on potato dextrose agar (PDA; Merck) slants or plates or both. Spores were kept for a long time by freeze-drying with 5% maltose as a cryoprotector.

Isolation and screening for antifungal activity of *A. luzulae* in vitro. The antifungal activity of *A. luzulae* was determined in petri dishes containing PDA (Merck) or malt agar medium (3% malt extract, 0.3% Bacto-Peptone, 2% agar). Two mycelial plugs (diameter, 1 cm), one from the pathogenic strain culture and the other from the antagonistic strain culture, were placed at opposite ends of the plate and grown for 5 days at 25°C (this test was carried out with controls).

* Corresponding author. Phone: 00-32-4-366-2861. Fax: 00-32-4-366-2862. E-mail: CWBIULG@Vm1.ulg.ac.be.

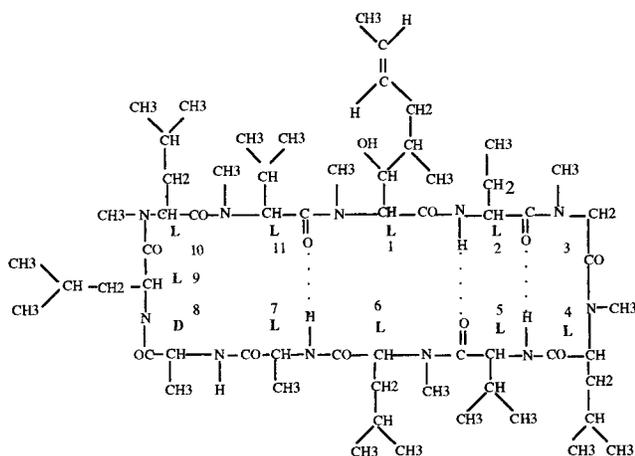


FIG. 1. Structure of cyclosporin A (13).

Inhibition was determined by measuring the width of the zone between the edge of the *A. luzulae* and pathogenic strain colonies.

Culture conditions for production and directed biosynthesis of antibiotic. PDA plates containing 1% NaCl, incubated at 27°C and under light, were used to produce dense sporulation. Spore suspensions were used to inoculate 500 ml of sterile (121°C and 1.2×10^5 Pa for 20 min) modified Sabouraud dextrose broth medium in a 1-liter Erlenmeyer flask (2% dextrose, 0.5% casein peptone, 0.5% meat peptone, 0.05% chloramphenicol [pH 6.5 to 6.9]) for antibiotic production and to inoculate 200 ml of sterile minimal medium as suggested by Kobel and Traber (21) for directed metabolite biosynthesis experiments, where we used separately 6 g of threonine, valine, and alanine per liter as the sole nitrogen sources for induced biosynthesis. Samples were inoculated and incubated at 25 to 27°C on a gyratory shaker at 120 rpm for 10 to 12 or 10 to 24 days.

Antimicrobial assays. Routinely, the antimicrobial activities in the culture broth, as well as that of fractions isolated during purification, were determined by the agar diffusion bioassay method against *Botrytis cinerea* isolated from strawberries grown in the field. The MICs of cyclosporin C for several species of bacteria, yeasts, and molds were determined by the paper disk agar diffusion method (16).

Isolation and purification of the antibiotic from shaken culture. After 10, 12, or 24 days of growth, the fermentation broth was freed from the mycelial cake by centrifugation. The liquid phase was either subjected to ultrafiltration through membranes with a cutoff of 5,000 and 1,000 Da, or treated with a 55% saturated $(\text{NH}_4)_2\text{SO}_4$ solution, and the low-density phase was recovered by centrifugation and dissolved in 20% methanol. The solutions obtained by either method were extracted with Mega Bond ODS (octadecyl silanol type Varian size 20 cc) as previously described (29). The eluted solutions were evaporated to dryness, and the combined extracts were subjected to repeated high-pressure liquid chromatography (HPLC) with two different columns (Pharmacia Pep RPC 10/10 C_2/C_{18} or semipreparative Chromspher C_{18} column [5 μm ; 250 by 10 mm; Chrompack]) with H_2O -trifluoroacetic acid (100:0.1, vol/vol) with increasing concentrations of either acetonitrile-water (70:30, vol/vol) or methanol-water (85:15, vol/vol). Fractions containing the antibiotic were pooled, concentrated under vacuum, and freeze-dried; this resulted in an amorphous colorless powder.

Homogeneity test. The homogeneity of the antibiotic was determined by silica gel thin-layer chromatography (precoated glass plate; silica gel Si G60 [Merck]) with several solvent systems (chloroform-methanol-water, 65:25:4; ethyl acetate; benzene-isopropanol, 90:10; ethyl acetate-isopropanol, 95:5; diethyl ether-methanol, 90:10) and detected by UV and by spraying with water, ninhydrin, 4,4'-tetramethyldiaminodiphenylmethane reagent (38) and Liberman reagent (H_2SO_4 -ethanol [11:9, vol/vol]), as well as by analysis by reverse-phase HPLC (Chromspher RP, 5 μm , C_{18}) with 80% methanol-water (the column effluent was monitored at 214 nm with a constant flow rate of 1 ml/min).

Structural analysis. Structural determination of the antibiotic was carried out by spectroscopy (UV and visible spectra, infrared spectra, and ^1H and ^{13}C nuclear magnetic resonance [NMR] spectroscopy measurements), mass spectrometry, and amino acid analysis as previously described (18). NMR measurement in d_4 -methanol was not successful. Cyclosporins build up to six different solution structures in methanol-water (19). One- and two-dimensional ^1H and ^{13}C NMR measurements in d -chloroform and benzene- d_6 were successful.

Biomass determination. A 20-ml sample of culture broth was centrifuged at $12,100 \times g$ for 15 min, and the fungal biomass was washed twice with distilled water. The supernatant was then filtered through a preweighed filter (0.45 μm ; HA Millipore) and dried at 110°C for 48 h.

Analytical cyclosporin determination. A 20-ml sample of the culture broth was centrifuged at $12,100 \times g$ for 15 min. The supernatant was filtered through a

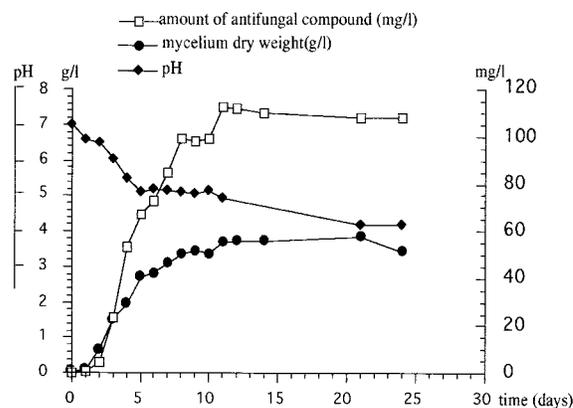


FIG. 2. Evolution of biomass (mycelium dry weight), pH, and antibiotic production during a batch culture of *A. luzulae* in modified Sabouraud dextrose broth. The culture was carried out in a flask and was incubated at 30°C for 24 days on a gyratory shaker (120 rpm).

0.45- μm filter paper, and 2 ml was extracted by analytical octadecyl silanol bonded phase (bond elut: size 3 cc/500 mg [Varian]). The 100% methanolic solutions were evaporated to dryness, and the precipitate was redissolved in a defined volume of methanol. The extracted samples were analyzed by HPLC with a 7-cm reverse-phase column (internal diameter, 4 mm; packed with 5- μm C_{18} and maintained at 60°C on a 4×10^5 Pa mobile phase of methanol-water [80:20, vol/vol] at a flow rate of 1 ml/min). The HPLC pump (KONTRON system 325), the UV detector (HPLC KONTRON detector 432), and the KONTRON integration pack were used. Samples were injected via an autosampler (Hewlett-Packard series 1050).

RESULTS

Production of the antibiotic. A typical fermentation diagram of *A. luzulae* in modified Sabouraud dextrose broth batch culture in a flask is shown in Fig. 2. Production of the antibiotic was observed after 3 days and reached its maximal yield of 114 mg/liter between 10 and 12 days. Thereafter, the antibiotic content of the medium remained constant along with the pH of the medium and the packed mycelium volume. The kinetic production of antibiotic was parallel to the *A. luzulae* growth.

Purification and characterization of the antibiotic. The original purification scheme, summarized in Materials and Methods, yielded an amorphous colorless powder with high homogeneity, as checked by thin-layer chromatography and HPLC analyses. By compilation of all data (data not shown) given by mass spectroscopic analyses ($m/z = 1,217.836$), amino acid analysis, and NMR measurements, the active compound was identified as cyclosporin C in which threonine at position 2 replaces α -aminobutyric acid in cyclosporin A (13, 37). All the physicochemical properties tested (solubility, infrared spectrum, reaction with ninhydrin or 4,4'-tetramethyldiaminodiphenylmethane) can be correlated with those of cyclosporin C.

Antibiotic activity. The MICs of the antibiotic for selected microorganisms are presented in Table 1. It is not active against either gram-positive or gram-negative bacteria. Only one strain of yeast is weakly sensitive to the antibiotic at high concentrations (between 100 and 150 $\mu\text{g}/\text{ml}$). In contrast, it shows excellent activity against several members of the Fungi Imperfecti including the postharvest fruit pathogens *Botrytis cinerea* Pers and *Penicillium expansum* Link and also fungi involved in soil-borne plant diseases. Its antifungal activity is not fungicidal but fungistatic, since spore germination is possible even if the antibiotic is present (data not shown).

Feeding experiments. To check whether other cyclosporins in addition to cyclosporin C were produced by *A. luzulae*, we

TABLE 1. MICs for selected microorganisms of cyclosporin C produced by *A. luzulae* in comparison to previous studies

Microorganism	MIC ($\mu\text{g/ml}$)	
	Data found	Literature data (reference)
<i>Alternaria kikuchiana</i> Tanaka		1 (26)
<i>Alternaria mali</i> Roberts		>100 (32)
<i>Alternaria</i> sp.	0.1–0.5	
<i>Aspergillus niger</i> van Tieghem	1–2	1 (13)
<i>Bacillus subtilis</i>	>150	
<i>Botrytis cinerea</i> Pers ^a	0.1–0.5	
<i>Botrytis cinerea</i> Pers ^b	>150	
<i>Botrytis cinerea</i> Pers ^c	0.1–0.5	
<i>Botrytis cinerea</i> Pers ^d	0.1–0.5	
<i>Botrytis cinerea</i> Pers ^e	0.1–0.5	
<i>Curvularia lunata</i> (Wakker) Boedijn		1 ^f (13)
<i>Cryptococcus albidus</i> (Saito) Skinner	100–150	
<i>Escherichia coli</i>	>150	
<i>Fusarium oxysporum</i> Schlechtendahl	5–10	
<i>Gaeumannomyces graminis</i> (Sacc) Von Arx & Olivier	>40	
<i>Glomerella cingulata</i> (Stoneman) Spaulding & Von Schrenk		>100 ^f (32)
<i>Helminthosporium oryzae</i> Van Breda de Hann		30 ^f (32)
<i>Mucor miehei</i> ATCC 16457 Cooney & Emerson	1–2	
<i>Mucor javanicus</i> ATCC 8770 Yamasaki	2–5	
<i>Mucor</i> sp.	2–5	
<i>Neurospora crassa</i> Shear & Dodge		10 ^f (13)
<i>Penicillium roqueforti</i> Thom	1–2	
<i>Penicillium expansum</i> Link	1–2	
<i>Penicillium</i> sp.	2–5	
<i>Pseudomonas fluorescens</i>	>150	
<i>Rhizopus arrhizus</i> Fischer	0.5–1	
<i>Rhizopus</i> sp.	2–5	
<i>Rhodotorula rubra</i> (Demme) Lodder		100 ^f (13)
<i>Saccharomyces cerevisiae</i> Meyen ex Hansen	>150	
<i>Sarcina lutea</i>	>150	
<i>Sterium purpureum</i> (Persoon: Fries) Fries		90 ^f (32)
<i>Tolypocladium inflatum</i> W. Gams	>40	
<i>Trichoderma hamatum</i> (Bonorden) Bainier	1–2	
<i>Trichoderma harzianum</i> Rifai	>60	
<i>Trichoderma reesei</i> D369 Simmons	2–5	
<i>Trichoderma reesei</i> Gx 1N 169 Simmons	1–2	

^a Isolated from apples.

^b Isolated from pears.

^c Isolated from wheat.

^d Isolated from strawberries grown in a field.

^e Isolated from strawberries grown in a greenhouse.

^f MICs in references 13, 26, and 32.

followed the procedure developed by Kobel and Traber (21), in which the proportion of a single cyclosporin in a cyclosporin mixture can be increased by adding its characteristic amino acid. The amino acids of position 2 were added in excess (alanine, valine, and threonine, 6 g/liter in each case) to influence the composition of the amino acid pool of the cell in the desired direction. The results are presented in Fig. 3. In all cases, only cyclosporin C was produced.

DISCUSSION

We demonstrated in this study that cyclosporin C is the main antifungal compound produced by *A. luzulae*. The antifungal activity of *A. luzulae* was previously demonstrated to be related to the production of diterpenic aglycones including virescensides (3, 28) and of a terpenoid metabolite called ascochlorin

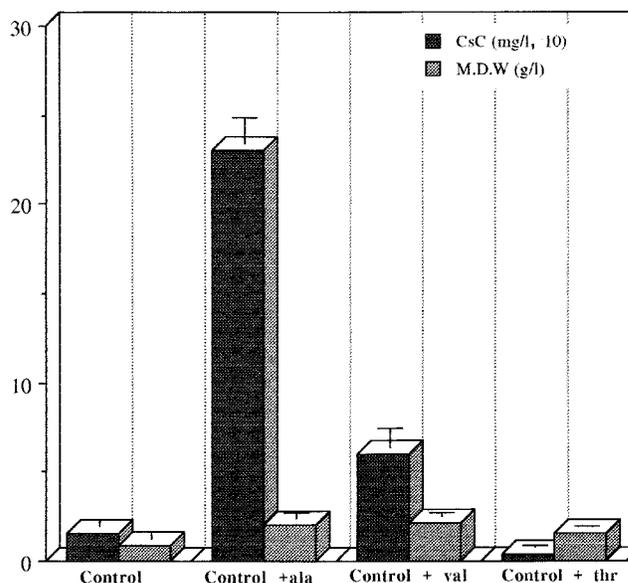


FIG. 3. Induction of *A. luzulae* cyclosporin C production by adding amino acids to the basal Borel medium (21). CsC, amount of cyclosporin C produced; M.D.W, mycelium dry weight.

(4). Ascochlorin was isolated from an albino mutant of *A. luzulae*. In our study, a small amount of a compound corresponding to one of these terpenoid metabolites was detected by mass spectrometric analysis when the cyclosporin C extraction method was changed (data not shown). No explanation of the absence of the other terpenoid metabolites could be given on the basis of our work. Nevertheless, this could be related to their expression level in different strains and could reflect the heterogeneity of this fungal species, since the isolate is not the same as that used in the previous studies (3, 4, 28).

A total of 25 cyclosporins (cyclosporins A to I and cyclosporins K to Z) have been isolated so far from the fermentation broth of *T. inflatum* (40). However, the majority of natural cyclosporins other than cyclosporin A were isolated as minor metabolites of the same structural type. Here, the antifungal activity produced by *A. luzulae* is at least due to cyclosporin C, and even trace amounts of other cyclosporins were not detected at all in the fermentation broth. Various fungal species have been reported to produce more than one cyclosporin. Among them are the ascomycete *Neocosmospora vasinfecta* E. F. Smith var. *africana* Von Arx (26) and different moniliaceous hyphomycetes including *T. inflatum* (13, 15), other *Tolypocladium* species (12), *Cylindrocarpum lucidum* Booth (9), *Fusarium solani* (Martius) Saccardo (32), and *Beauveria bassiana* (Balsamo) Vuillemin (1). In this paper, we report for the first time that the moniliaceous hyphomycete species *A. luzulae* is a unique cyclosporin C producer.

Cyclosporin C was reported to show a narrow spectrum of antibiotic activity (13, 32). The members of the Fungi Imperfecti showed different sensitivities: while *Alternaria kikuchiana* Tanaka (26), *Aspergillus niger* Van Tieghem, and *Curvularia lunata* (Wakker) Boedijn were very sensitive, *Alternaria mali* Roberts and *Glomerella cingulata* (Stoneman) Spaulding & Von Schenk were not affected by cyclosporin C at 100 $\mu\text{g/ml}$. This phenomenon was also observed in our assays, but in addition we showed that most of the plant-pathogenic filamentous fungi tested were very sensitive.

Cyclosporin A is widely used to prevent graft rejection and

was recently shown to have promising therapeutic effects in a number of autoimmune diseases (2, 7); however, its therapeutic use is limited by its nephrotoxicity (24, 25). In addition, the recent comparative studies on the immunosuppressive potency *in vitro* and *in vivo* of cyclosporin A and cyclosporin C showed roughly the same effect (31). In contrast, the *in vitro* nephrotoxicity of cyclosporin C was shown to be lower than that of cyclosporin A (30). Since cyclosporin C is the sole cyclosporin metabolite produced by *A. luzulae*, the method used for its purification is much easier than the one used to purify the mixture of cyclosporins produced by *T. inflatum*. As its low nephrotoxicity has been proved, it would be interesting to study ways to increase the productivity of cyclosporin C by mutagenesis and to study optimized growth and production conditions for *A. luzulae* as a step in direction toward the use of cyclosporin C instead of cyclosporin A in clinical domains.

On the other hand, in contrast to *T. inflatum*, which produces a mixture of cyclosporins, we showed that *A. luzulae* produces only cyclosporin C. Several feeding experiments involving directed biosynthesis to natural cyclosporins other than cyclosporin C were performed. By adding key exogenous amino acids to the culture medium as described by Kobel and Traber (21), the amount of cyclosporin C was modulated; nevertheless, no other cyclosporin metabolites were detected by HPLC analysis. To explain this phenomenon, two hypotheses could be suggested: either the *trans*-membrane amino acid transport is insufficient or the biosynthesis mechanism in *A. luzulae* is more specific than the one in *T. inflatum*. Since growth is similar in all cases, and considering that the amino acids used in these feeding experiments were the only nitrogen sources for vegetative growth, the first hypothesis could be dismissed.

The unique fungus *A. luzulae* is a good tool to study the enzymatic biosynthesis specificity of cyclic peptides such as cyclosporins. It would be interesting to isolate the genes encoding the cyclosporin synthetase of *A. luzulae* and compare their sequences with those of the equivalent *T. inflatum* genes, since the entire sequences of the latter are already known (41). This could provide more information about the correlation between sequences and the capabilities of the various domains of different enzymes to activate and incorporate a specific spectrum of amino acids.

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