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Cyclosporin A and C

New Metabolites from *Trichoderma polysporum* (Link ex Pers.) Rifai

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Summary. Cyclosporin A and C are new antifungal antibiotics from *Trichoderma polysporum* (Link ex Pers.) Rifai. The metabolites are produced in submerged culture and are extracted therefrom by organic solvents. Cyclosporin A is a nonpolar cyclic peptide with a molecular weight of 1202.6. The cyclosporins exhibit a narrow spectrum of antifungal activity and in addition have immunosuppressive properties.

INTRODUCTION

In the course of a screening program for new metabolites from microorganisms the antifungal antibiotics cyclosporin A and C[1] were isolated from the fermentation broth of a fungus identified as *Trichoderma polysporum* (Link ex Pers.) Rifai. This report includes the taxonomy of the producing strain, the fermentation procedure, isolation and characterization of the metabolites as well as their antimicrobial activity. The structure of cyclosporin A, a neutral cyclopeptide containing 11 amino acids, is presented. Chemical investigations, X-ray analysis and the immunosuppressive properties of the metabolite are reported in separate publications (Rüegger et al., 1976; Petcher & Weber, 1976; Borel et al., 1976).

MATERIALS AND METHODS

Materials. Chemicals and solvents, obtained from Merck, Darmstadt, W.Germany, were of analytical grade. Polyoxin A was isolated from a commercial sample of polyoxin obtained from Kaken Chemical Ltd., Tokyo, Japan. Materials for chromatography were obtained as indicated in the text. Ingredients for media were

[1] Cyclosporin B is produced by another fungus and will be reported elsewhere.

commercial products. Antifoam used was Silicon M 30 supplied by Dow Corning.

Media. The following culture- and protection media for deep-freezing and liquid nitrogen conservation were used:
Medium 1: Malt extract 20 g, yeast extract 4 g, agar 20 g, dist. water to 1 l.
Medium 2: Malt extract 70 g, glucose 50 g, dist. water to 1 l.
Medium 3: Bacto brain heart infusion 37 g, glycerol 100 g, dist. water to 1 l.
Medium 4: Glucose 40 g, caseinpeptone 5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, KH_2PO_4 2 g, $NaNO_3$ 3 g, KCl 0.5 g, $FeSO_4 \cdot 7H_2O$ 10 mg, dist. water to 1 l. pH: 5.2 after sterilization.
Medium 5: Medium 4 with additional 5 g of caseinpeptone.
Medium 6: Medium 3 without glycerol.
All media were sterilized at 120°C for 20 min.

Incubation Temperature. If not stated otherwise incubations were carried out at 27°C in the dark.

Preparation of Inoculum. A single conidium isolate was used for all subsequent procedures involving cyclosporin production. Cultures were grown for at least 10 days on medium 1, flushed with medium 2 or 3 to obtain suspensions of conidia which were deep-frozen in small portions at -40°C or in liquid nitrogen and used as stock suspension for preparing fresh inocula. Inoculating medium 1 with this suspension followed by incubation for 10 to 12 days yielded cultures containing 5×10^8 conidia/cm^2 of agar surface which were used as fresh inoculum.

Shake Cultures. Generally 0.5 l Erlenmeyer flasks containing 100 ml of liquid medium plugged with cotton were incubated on rotary shakers (2 inch excursion, 200 rpm).

Fermenters. Stainless steel fermenters (with 4 baffles, ring-shaped air-inlet and a stirrer with two 8-bladed wings) equipped with automatic foam, temperature and pH control were used in three sizes, holding 50 l, 500 l and 3000 l of medium.

Analytical Methods. A strain of *Aspergillus niger,* grown on medium 1 was used as a sensitive test organism for detection of the antifungal activity in culture broths and crude extracts.

Agar diffusion tests were made using paper disks of a diameter of 6 mm (Schleicher and Schüll, Feldbach, Switzerland). For quantitative determination and for the separate identification of the two metabolites a thin layer chromatographic method on silicagel plates (G Merck) with the following solvent systems was developped:

Solvent system (v/v)	Rf cyclosporin A	Rf cyclosporin C
diethyl ether-methanol 90:10	0.57	0.36
ethyl acetate	0.28	0.09
ethyl acetate-isopropanol 95:5	0.34	0.20
benzene-isopropanol 90:10	0.35	0.21

Polygram Sil G plates were used as well for TLC. Detection was possible after staining with iodine vapor. By comparing spot sizes the amounts of cyclosporins in crude extracts were estimated. More precise quantitative results were obtained by measuring the remitted light in the range 205 - 240 nm, using a Zeiss-TLC-Spectrophotometer.

Antibiotic Activity. Minimal inhibitory concentrations were obtained by manual serial dilution using Brain Heart Infusion Broth (medium 6) for growing bacteria and malt extract broth (medium 1 without agar) for yeasts and fungi.

Incubation temperatures were 37° or 27°C and the end points were read after 48 h.

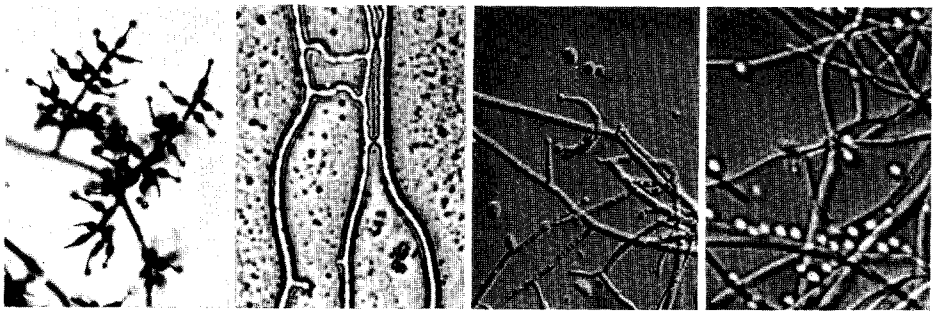
Strains used were from the collection of the Microbiology Department of Sandoz Ltd. if not otherwise indicated.

RESULTS AND DISCUSSION

Taxonomy

The fungus strain (NRRL 8044) producing cyclosporin A and C was isolated by conventional soil dilution technique from soil collected at Hardanger, Norway, and was identified as *Trichoderma polysporum* (Link ex Pers.) Rifai (von Arx, 1970; Rifai, 1969). The strain grows on medium 1 within the temperature range of 6° to 33°C. The optimal temperature for growth is about 24°C. The colonies attain a diameter of 24 - 30 mm in ten days. They appear whitish and floccose at the surface and yellowish on the reverse side. Sporulation is distributed evenly throughout the colony but tends to form in tufts at the edge of older colonies.

The main conidiophore axis measures 2.0 - 3.8 µ in width and usually terminates into a sterile, flexuous thread. The phialide-bearing lateral branches, (which can be unbranched or branched) bear clusters or verticills of phialides and usually terminate into a singel phialide (Fig.1a). The phialides are swollen at the base and elongate into a long, thin neck, where the hyaline, subglobose to ellipsoidal, 2.0 - 3.1 × 1.5 - 2.0 µ measuring conidia accumulate in small heads. Some of the strain's characteristics do not agree completely with the species description of *T.polysporum* given by Rifai (1969), e.g. the relatively slow growth, the width of the main conidiophore axis, the thin and long-necked phialides and the relatively



a b c d

Fig.1 a - d
Morphological characteristics of *Trichoderma polysporum*. (a) side branches of a conidiophore with phialides. (b) hyphal anastomoses. (c) young submers mycelium with phialides. (d) submers culture after 8 days with hyphae and conidia. Magnification 500 ×

small conidia. Grown in a moist chamber on a cover slide with a thin layer of medium 1 plenty of anastomoses between hyphae were observed as shown in Figure 1b.

Fermentation

A. Course of Growth and Cyclosporin Formation in Shake Cultures

Preculture. An Erlenemyer flask with medium 4 was seeded with fresh inoculum of about 10^8 conidia and incubated on a rotary shaker. Germination became evident after 16 h, loose flaky particles of conglomerated young hyphae were predominant after 32 h and after 72 h the mycelial dry weight was 7 g/l. At this time the pH of the broth was 4.5 and cyclosporins were present in small amounts.

Intermediate Culture. 10 ml of the 72 h preculture were inoculated to the same medium in Erlenmeyer flasks, and during 48 h incubation small loose pellets of mycelia and masses of conidia developed, giving a total dry weight of 8 g/l. The pH then had dropped to 4.2 and the cyclosporins were found to be present in small quantities.

Production Culture. Erlenmeyer flasks containing medium 5 were inoculated with 10 ml of the 48 h intermediate culture. Two stages of development are illustrated in Figure 1c and d. The course of growth and cyclosporin production during the 12-day incubation period is presented in Figure 2a.

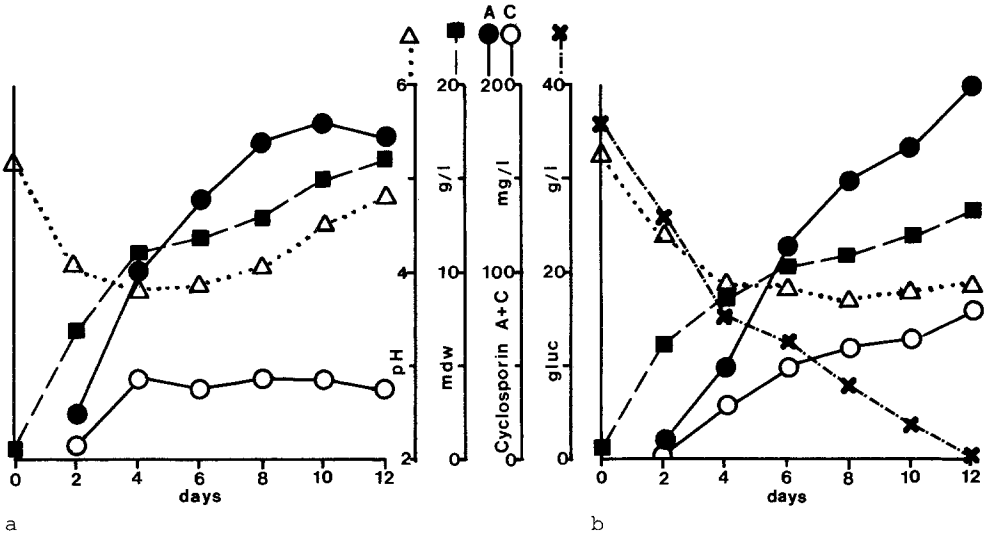


Fig.2 a and b
Mycelial dry weight (mdw), pH, glucose consumption and formation of cyclosporins during growth of *Trichoderma polysporum*. (a) in shake cultures. (b) in steel fermenters

B. Cyclosporin Production in Fermentation Tanks

All fermentations were aerated with 1 l air/min/l medium at a pressure of 0.5 bar.

Preculture. A 75-l fermenter containing 50 l of medium 4 was seeded with 5×10^9 conidia from a fresh inoculum. The tank was stirred at 200 rpm for 72 h and the pH dropped from 5.2 to 4.3.

Intermediate Culture. The preculture was inoculated to a 750 l fermenter containing 500 l of medium 5 and stirred at 150 rpm for six days. From the fourth day on the culture broths became yellowish and contained large amounts of variously shaped conidia.

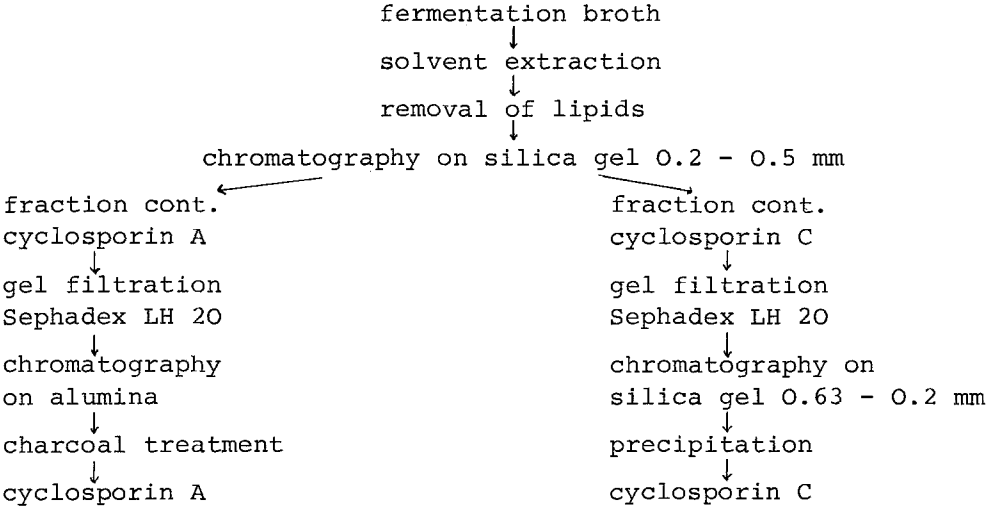
Production Culture. 300 l of the intermediate culture were inoculated to 3000 l of medium 5 in a 4500 l fermenter. The culture was stirred at 100 rpm for 12 days, after seven days the mycelium began to disintegrate, leaving a large amount of conidia in the yellowish to brown, somewhat slimy culture broth. In Figure 2 b the course of growth and production is presented.

Isolation and Characterization of Cyclosporin A and C

The fermentation broth was extracted with an equal volume of butyl acetate and after separation the organic layer was evaporated under reduced pressure. From 3000 l of broth extracted in this way approximately 3300 g of crude material were obtained. Details of the extraction and purification method were described by Rüegger et al. (1976).

Yields were in the range of 150 - 200 mg/l for cyclosporin A and 50 - 100 mg/l for cyclosporin C.

The isolation procedure is outlined in the following flow chart:



The amorphous cyclosporin A and C obtained in this way could be crystallized from the 2- to 3-fold amount of acetone after cooling to -15°C to give white prismatic needles with the following data:

	cyclosporin A	cyclosporin C
melting point	148-151°	152-155°
α_D^{20} C 0.5, $CHCl_3$	-244°	-255°
α_D^{20} C 0.5, CH_3OH	-189°	-182°
formula	$C_{62}H_{111}N_{11}O_{12}$	$C_{62}H_{111}N_{11}O_{13}$
molecular weight	1202.6	1218.6

The structure of cyclosporin A was investigated by chemical, spectroscopical and crystallographic methods. The metabolite is characterized as a neutral cyclic peptide containing 11 amino acids, seven of which are N-methylated, therefore explaining the highly nonpolar character of the compound. The complete structure is given in Figure 3 (Petcher & Weber, 1976).

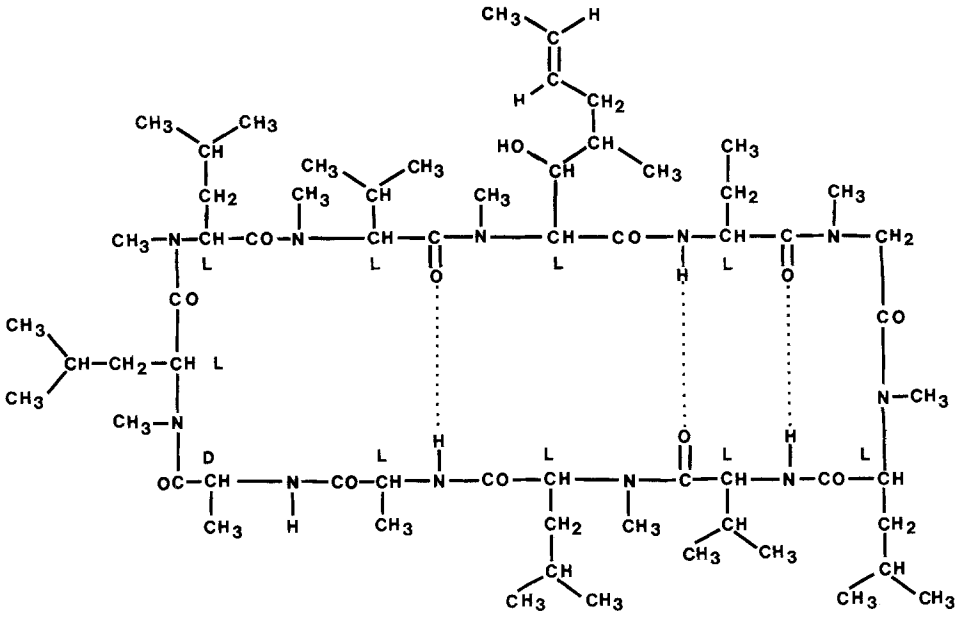


Fig.3 Structure of cyclosporin A

Biological Activity

a) Spectrum of Activity

The cyclosporins exhibit a rather narrow spectrum of activity. Among the yeasts only a few species are sensitive and no inhibition of bacteria has been observed. Strains of some mucorales, ascomycetes and fungi imperfecti show differing sensitivity against cyclosporins and inhibition frequently becomes evident as deformation and branching of the growing hyphal tips or simple growth rate reduction in sensitive yeasts when grown in solid media and in contact with the agent. Cyclosporins do not affect the aerial mycelium, nor do they prevent germination of conidia and spores of sensitive fungi. In Table 1 the minimal inhibitory concentrations for the growth of some yeasts and fungi are shown.

Table 1
Minimal inhibitory concentrations for cyclosporin A and C in µg/ml

	Cyclosporin A	Cyclosporin C
Rhodotorula rubra	100	100
Oospora lactis	31.6	100
Aspergillus niger	3	1
Curvularia lunata	1	1
Neurospora crassa	10	10
Anixiopsis stercoraria	100	100
Trichophyton quinckeanum	10	31.6

The cyclosporins were found not to inhibit growth of the following organisms up to a concentration of 100 µg/ml: *Staphylococcus aureus, Streptococcus faecalis, Bacillus subtilis, Escherichia coli* K 12, *Salmonella typhimurium, Pseudomonas aeruginosa, Saccharomyces cerevisiae, Kloeckera apiculata, Hansenula anomala, Candida albicans* and *Pythium debaryanum.*

b) Mode of Antifungal Action

If the spectrum of activity of the cyclosporins is compared with the taxonomic position of the sensitive organisms an inhibition of cell wall synthesis, especially chitin synthesis, seems likely (Bartnicki-Garcia, 1968). Blocking of chitin synthesis is so far reported only for the polyoxins, a class of antibiotics with a similar narrow spectrum of activity (Bartnicki-Garcia & Lippmann, 1972).

From the therapeutic point of view an antifungal antibiotic which inhibits cell wall synthesis would be of great value. In analogy to the β-lactam antibiotics a low toxicity could be expected from such an antibiotic due to its highly selective action. To prove the hypothesis of blocking chitin synthesis in sensitive organisms by the cyclosporins the activities of these metabolites were compared with polyoxin A using the following tests:

I. Comparison of the sensitivity of *Neurospora crassa* and the cell wall defective "slime" mutant of *Neurospora crassa* (Bigger et al., 1972).

II. Comparison of the sensitivity of yeasts which contain chitin as a major component of their cell wall (*Rhodotorula* and *Sporobolomyces*).

The results are summarized in Table 2. The slime mutant of

Table 2. Inhibition zones in mm on medium 1

	Organisms	Polyoxin A	Cyclosporin A	Cyclosporin C
I	*Neurospora crassa*	25	20	20
	Neurospora crassa "slime"	0	0	0
II	*Rhodotorula rubra* ETHZ H 1079	40	20	17
	Sporobolomyces roseus LBH 1269 ETHZ	23	0	0
	Sporobolomyces antarcticus CBS 5955	18	0	0

For *N.crassa* "slime" sorbose was added as osmotic stabilizer. Tested concentration of the compounds 1 mg/ml.

Neurospora crassa is insensitive to both cyclosporins and to polyoxin A, but the difference in activity towards the tested *Sporobolomyces* strains indicates another mode of action of the cyclosporins than inhibition of chitin synthesis.

c) Other Activity

The narrow spectrum of the cyclosporins restricts their use as antifungal antibiotics. A careful pharmacological investigation of these compounds however showed that they might be useful as a new type of immunosuppressive agent. Their activities in several pharmacological models indicating immunosuppressive and antiphlogistic action is reported in greater detail by Borel et al., 1976.

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