Pergamon 0031-9422(94)00711-X

Phytochemistry, Vol. 38, No. 2, pp. 403–407, 1995

0031-9422/95 $9.50 + 0.00

CYCLOSPORINS FROM *TOLYPOCLADIUM TERRICOLA*

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(*Received* 6 *June* 1994)

Key Word Index—*Tolypocladium terricola*; fungi imperfecti; cyclosporins.

Abstract—New natural cyclosporins were isolated from the mycelium of surface cultivated fungus *Tolypocladium terricola*. The chemical structures of [Leu^4] CS and [MeLeu^1] CS = cyclosporin-J, were deduced from the NMR and mass spectral data. Biological activity of new cyclosporins is reported based on the proliferative mitogen stimulation test.

INTRODUCTION

Cyclosporins are cyclic undecapeptides produced by a number of imperfect fungi [1–4]. A representative, cyclosporin A (CS) (**3**) cyclo-(-MeBmt^1-Abu^2-Sar^3-MeLeu^4-Val^5-MeLeu^6-Ala^7-D-Ala^8-MeLeu^9-MeLeu^{10}-MeVal^{11}-), MeBmt = (2*S*, 3*R*, 4*R*, 6*E*)-3-hydroxy-4-methyl-2-methylamino)-6-octenoic acid, is a well known drug used particularly to prevent graft rejection in organ transplantations [5]. The majority of other cyclosporins are derived from **3** by substitution at the position of the second amino acid [6, 7].

In this paper we report the structures of new natural cyclosporins isolated from the mycelium of the surface cultivated fungus *Tolypocladium terricola* [8, 9].

RESULTS AND DISCUSSION

New cyclosporins **1** and **2** were isolated from the methanolic extract of the mycelium of the fungus *T. terricola*. The crude extract was separated by column chromatography on a silica gel with a mixture of methylene chloride–methanol as eluent. [Leu^4]CS (**1**) was obtained by preparative HPLC on a reversed phase column (C-18) using a mixture of methanol–water as eluent. [MeLeu^1]CS (**2**) was purified by preparative HPLC on a reversed phase column (C-18) with a mixture of methanol–water as eluent and finally separated from cyclosporin-D by a preparative HPLC on a cyano-bonded column in the system isopropanol–*n*-heptane.

Molecular weight information was obtained by positive-ion FAB mass spectrometry. Compound **1** exhibited the protonated molecule $[M + H]^+$ at *m/z* 1188.8, i.e. at *m/z* of 14 amu lower than **3**. The fragment $[M + H - C_7H_{13}O]^{+\cdot}$, *m/z* 1075.4, indicated the presence of MeBmt in the molecule [6, 7]. Eleven carbon signals out of a total of 61 in the NMR spectrum belonged to carbonyls. According to the ^{1}H NMR spectrum, they represented five CONH and six CONMe groups. Individual amino acids found either in acid hydrolysate of **1** or by a COSY experiment (L-alanine, D-alanine, L-α-*n*-aminobutyric acid, L-valine, L-leucine, three *N*-methyl-L-leucines, MeBmt, sarcosine and *N*-methyl-L-valine) correspond to that of **3** with one *N*-methyl-leucine demethylated. However, the comparison of the ^{13}C NMR spectrum with that of known natural *N*-demethyl derivatives of **3** ([Leu^{10}] CS = cyclosporin T and [Leu^6] CS = cyclosporin U) revealed that our compound was not identical to any of them and also exhibited different chromatographic properties [6, 7]. Sequence determination by ROESY (based on cross-peaks between N-H or N-Me and H_α) confirmed the same structure as **3**, but missing the *N*-methyl at the fourth residue. The cross-peak between the new 4-NH (Leu) and the downfield sarcosine doublet was diagnostic. Thus, **1** is cyclo-(-MeBmt^1-Abu^2-Sar^3-Leu^4-Val^5-MeLeu^6-Ala^7-D-Ala^8-MeLeu^9-MeLeu^{10}-MeVal^{11}-). The ^{13}C NMR spectra of **1** and **3** (Table 1) are very similar except for C-4$_\alpha$ and its neighbours. The ^{1}H NMR parameters are also very close. The absence of an *N*-methyl causes a characteristic upfield shift of the corresponding H_α and some conformational changes. Affected are also the sarcosine protons.

Compound **2** exhibited the protonated molecule $[M + H]^+$ at *m/z* 1146.9. However, the fragment $[M + H - C_7H_{13}O]^{+\cdot}$ was not observed. The deduced molecular formula $C_{59}H_{107}N_{11}O_{11}$ was smaller than all cyclosporins known so far. The only abundant ion in the high mass range appeared at *m/z* 1089.8 $[M + H - C_4H_9]^{+\cdot}$. The absence of $[M + H - H_2O]^+$ and $[M + H - C_7H_{13}O]^{+\cdot}$ in the mass spectrum suggested a lack of MeBmt moiety. COSY experiments and amino acid

Table 1. NMR data of [Leu^4]-cyclosporin A (**3**)

Residue	Amino acid	Group	δ_C	δ_H	Mult.	J [Hz]
1	MeBmt	MeN	33.5	3.47	*s*	
		1α	58.5	5.43	*d*	7.1
		1β	74.2	3.85	*dd*	7.1, 9.8
		OH	—	3.25	*bs*	
		1γ	33.5	1.59	*m*	
		Me(γ)	16.8	0.79	*d*	6.8
		1δ	34.9	2.27	*m*	
				1.77	*m*	
		1ε	129.4	5.35	*m*	
		1ν	126.5	5.36	*m*	
		Me(ω)	17.8	1.63	*dd*	4.5, 1.0
2	Abu	NH	—	8.27	*d*	9.7
		2α	49.2	4.98	*dt*	9.7, 7.4
		2β	24.8	1.68	*m*	
				1.68	*m*	
		Me(γ)	9.8	0.85	*t*	7.3
3	Sar	MeN	39.1	3.36	*s*	
		3α	54.8	4.26	*d*	13.4
				3.33	*d*	13.4
4	Leu	N-H	—	6.09	*d*	9.8
		4α	52.0	4.49	*ddd*	9.8, 7.2, 3.7
		4β	34.9	1.98	*m*	
				1.48	*m*	
		4γ	25.0	1.65	*m*	
		Me(δ)	23.2	0.92	*d*	6.4
		Me(δ′)	21.1	0.91	*d*	6.5
5	Val	N–H	—	7.70	*d*	8.5
		5α	55.5	4.57	*dd*	9.4, 8.5
		5β	31.2	2.35	*dqq*	9.4, 6.5, 6.5
		Me(γ)	19.6	1.05	*d*	6.5
		Me(γ′)	18.3	0.88	*d*	6.6
6	MeLeu	MeN	31.4	3.25	*s*	
		6α	55.0	5.11	*dd*	9.1, 6.1
		6β	37.1	2.08	*m*	
				1.18	*m*	
		6γ	25.1	1.31	*m*	
		Me(δ)	23.8	0.94	*d*	6.4
		Me(δ′)	21.9	0.87	*d*	6.3
7	Ala	N–H	—	7.59	*d*	7.3
		7α	48.7	4.47	*dq*	7.3, 7.3
		Me(β)	15.8	1.35	*d*	7.3
8	Ala	N–H	—	7.18	*d*	7.7
		8α	45.0	4.83	*dq*	7.7, 6.9
		Me(β)	18.0	1.26	*d*	6.9
9	MeLeu	MeN	29.7	3.15	*s*	
		9α	48.3	5.67	*dd*	10.8, 4.3
		9β	40.6	2.08	*m*	
				1.23	*m*	
		9γ	24.7	1.47	*m*	
		Me(δ)	23.7	1.01	*d*	6.7
		Me(δ′)	22.0	1.01	*d*	6.7
10	MeLeu	MeN	29.8	2.69	*s*	
		10α	57.5	5.09	*m*	
		10β	40.8	1.99	*m*	
				1.37	*m*	
		10γ	24.6	1.70	*m*	
		Me(δ)	23.3	0.86	*d*	7.1
		Me(δ′)	23.7	0.82	*d*	7.0
11	MeVal	MeN	29.8	2.66	*s*	
		11α	58.2	5.10	*d*	11.1
		11β	28.2	2.14	*m*	
		Me(γ)	20.0	0.95	*d*	6.4
		Me(γ′)	18.8	0.84	*d*	6.5

Carbonyls: 173.7, 173.5, 173.4, 173.2, 171.6, 171.4, 171.2, 170.4, 170.2, 170.1, 169.0.

Table 2. NMR data of [MeLeu[1]]-cyclosporin A (3)

Residue	Amino acid	Group	δ_C	δ_H	Mult.	J [Hz]
1	MeLeu	MeN	31.7	3.35	*s*	
		1α	55.3	5.13	*dd*	9.0, 6.5
		1β	33.9	1.99	*m*	
				1.18	*m*	
		1γ	24.4	1.41	*m*	
		Me(δ)	23.8	1.03	*d*	6.5
		Me(δ')	23.8	1.01	*d*	6.5
2	Abu	NH	—	8.44	*d*	9.9
		2α	48.7	4.94	*ddd*	9.9, 8.5, 6.0
		2β	24.8	1.69	*m*	
				1.59	*m*	
		Me(γ)	9.9	0.87	*t*	7.3
3	Sar	MeN	39.3	3.40	*s*	
		3α	49.9	4.17	*d*	13.7
				3.17	*d*	13.7
4	MeLeu	MeN	31.2	3.09	*s*	
		4α	55.1	5.33	*dd*	11.6, 3.9
		4β	36.2	1.96	*m*	
				1.58	*m*	
		4γ	24.9	1.45	*m*	
		Me(δ)	23.8	0.94	*d*	6.7
		Me(δ')	21.9	0.90	*d*	6.4
5	Val	N–H	—	7.51	*d*	9.0
		5α	55.0	4.71	*dd*	9.5, 9.0
		5β	31.4	2.42	*dqq*	9.5, 6.9, 6.6
		Me(γ)	19.6	1.04	*d*	6.6
		Me(γ')	18.5	0.84	*d*	6.9
6	MeLeu	MeN	31.2	3.27	*s*	
		6α	54.1	5.19	*dd*	10.8, 5.0
		6β	37.5	2.11	*m*	
				1.19	*m*	
		6γ	24.5	1.76	*m*	
		Me(δ)	23.4	0.90	*d*	6.6
		Me(δ')	21.1	0.69	*d*	6.5
7	Ala	N–H	—	8.07	*d*	6.9
		7α	48.3	4.47	*dq*	7.2, 6.9
		Me(β)	15.1	1.34	*d*	7.2
8	Ala	N–H	—	7.49	*d*	8.0
		8α	44.7	4.85	*dq*	8.0, 7.0
		Me(β)	17.7	1.26	*d*	7.0
9	MeLeu	MeN	29.7	3.19	*s*	
		9α	47.9	5.69	*dd*	11.2, 4.1
		9β	39.3	2.14	*m*	
				1.18	*m*	
		9γ	24.7	1.31	*m*	
		Me(δ)	23.8	0.95	*d*	6.6
		Me(δ')	21.3	0.86	*d*	6.5
10	MeLeu	MeN	30.0	2.68	*s*	
		10α	57.2	5.10	*dd*	7.0, 6.9
		10β	40.7	2.02	*m*	
				1.35	*m*	
		10γ	24.5	1.53	*m*	
		Me(δ)	23.6	0.86	*d*	6.5
		Me(δ')	22.2	0.73	*d*	6.5
11	MeVal	MeN	29.8	2.68	*s*	
		11α	58.2	5.11	*d*	10.9
		11β	29.7	2.15	*m*	
		Me(γ)	20.3	0.82	*d*	6.5
		Me(γ')	18.3	0.88	*d*	6.5

Carbonyls: 173.8, 173.5, 173.2, 172.8, 171.7, 171.6, 171.2, 170.9, 170.7, 170.6, 170.1.

analysis of **2** hydrolysate revealed L-alanine, D-alanine, L-α-*n*-aminobutyric acid, L-valine, five *N*-methyl-L-leucines, sarcosine, *N*-methyl-L-valine, and the absence of MeBmt. The sequence of **2** was determined (Table 2) in the same manner as above, and led to the structure cyclo(-MeLeu[1]-Abu[2]-Sar[3]-MeLeu[4]-Val[5]-MeLeu[6]-Ala[7]-D-Ala[8]-MeLeu[9]-MeLeu[10]-MeVal[11]-).

To obtain a comparison of biological activity of new cyclosporins with cyclosporin A (**3**), the proliferative response of lymphocytes to mitogen stimulation was tested (Table 3). Compound **1** showed *ca* 30% and **2** showed 10% of the activity of **3**. Both **1** and **2** significantly ($P < 0.05$) increased cell proliferation at low concentrations. Cyclosporin A is metabolized *in vivo* with retention of its cyclic structure. Metabolites originate usually by hydroxylation of alkyl chains of amino acids at the position 1, 4, 6 and 9, or by *N*-demethylation at the position 4 = **1** [10, 11]. Among these metabolites, only **1** seems to contribute significantly to the nephrotoxicity accompanying the cyclosporin-A therapy [12]. Its concentration in kidney is comparable with that of **3** [11]. Whereas the majority of cyclosporin metabolites can only be obtained from urine or bile of living subjects, the natural production of **1** thus makes it easily available as an analytical standard, as well as for additional biological testing.

EXPERIMENTAL

Instruments and methods. Mps were determined between cover plates on air and are uncorr. IR spectra were recorded with a Nicolet 205 FT-IR spectrometer; UV spectra were measured with a Varian DMS 300 spectrometer. NMR spectra were measured on a Varian VXR-400 spectrometer (400 MHz observing frequency for ^{1}H and 100 MHz for ^{13}C). The chemical shifts are reported in δ-scale, tetramethylsilane was used as an int. standard. ^{1}H and ^{13}C 1D NMR, APT, DEPTGL, COSY, delay-COSY, HOM2DJ, NOESY, ROESY, two-step RELAY and HETCOR experiments were performed with the standard pulse sequences and programming as supplied by Varian. FAB-MS were recorded on a Finnigan MAT 90 double-focusing instrument (Finnigan MAT, Bremen, F.R.G.) of BE geometry (magnetic sector preceding the electrostatic one). For more MS experimental details see ref. [13].

Amino acid analysis. Each cyclosporin (0.5 mg) was hydrolysed in 1 ml 6 M HCl at 115° for 24 hr. The hydrolysate was divided into 3 parts for subsequent analyses. In the first portion, the released components were identified as their *tert*-butyldimethylsilyl derivatives by GC-MS, using a method described elsewhere [14]. The absolute configuration of the primary amino acids found in the hydrolysates was determined in the second portion by reversed-phase HPLC of the corresponding diastereomeric isoindolyl derivatives, formed by pre-column derivatization with *o*-phthaldialdehyde and 1-thio-β-D-glucose [15]. Chirality of MeVal and MeLeu was obtained by gas chromatography on a chiral 50 m × 0.25 mm (i.d.) XE-60-*S*-valine-*S*-α-phenyl-ethylamide WCOT capillary column (Chrompack, Middelburg, The Netherlands) after treatment of the third portion with phosgen at pH = 10 and subsequent CH_2Cl_2 extraction [16].

Isolation of cyclosporins. Stationary cultivation of the fungus *T. terricola* has been described elsewhere [9]. A crude extract of cyclosporins was obtained by the extraction of sepd mycelia (*ca* 100 kg) with MeOH. The resulting extract was roughly fractionated by CC on silica-gel using a stepwise MeOH–CH_2Cl_2 gradient (up to 10% vol. of MeOH in CH_2Cl_2). The resulting CC frs were pooled according to the content of principal cyclosporins in the following order: cyclosporin-D, cyclosporin-A, cyclosporin-B and cyclosporin-C. The fr. of cyclosporin-C was purified by prep. HPLC (column 250 × 25 mm, i.d., SGX-C18 7 μm from Tessek, Prague, Czech Republic), isocratic elution with 80% vol. of aq. MeOH, flow rate: 6 ml min^{-1}, 50°, det. 245 nm) to give pure **1** (100 mg). The crude cyclosporin-D fr. was purified on the same C-18 column using 85% vol. of aq. MeOH to separate accompanying cyclosporins A, G, F. Chromatography afforded a mixt. of **2** with cyclosporin-D which was not sepd by any of the tested RP systems. Final purification of **2** was carried out on a cyano-modified column 250

Table 3. Effect of cyclosporins on the proliferative responsivness on BALB/c spleen lymphocytes, activated by concanavalin A (1 μg ml^{-1})

Agent	Concentration (ng ml^{-1})	Absorbance mean (± s.d.)	Inhib. effect* (%)
Cyclosporin-A (CS) (**3**)	100	0.164 (0.024)	76.9
	50	0.277 (0.031)	61.0
	10	0.534 (0.042)	24.9
[Leu^4]CS (**1**)	100	0.511 (0.007)	28.1
	50	0.686 (0.045)	3.5
	10	0.786 (0.037)	− 10.5
[MeLeu^1]CS (**2**)	100	0.638 (0.042)	10.3
	50	0.807 (0.037)	− 13.5
	10	0.768 (0.036)	− 8.0
None (control)	—	0.711 (0.075)	0

*Inhibition effect is expressed in relative % with respect to control.

× 8 nm, i.d., SGX-CN 7 μm from Tessek (Prague, Czech Republic), isocratic elution with the *i*-PrOH–*n*-heptane mixt. (1:9), 50°, 240 nm, yielding pure **2** (50 mg).

[Leu^4]*CS* (**1**). Amorphous powder, mp 142°, $[\alpha]_D^{25}$ − 220°. $CHCl_3$; *c* 9.1 mg ml^{-1}. (Found: C 61.6%, H 9.5% $C_{61}H_{109}N_{11}O_{12}$ requires C 61.64%, H 9.24%). UV (MeOH) end absorption 200 nm: IR ν_{max}^{KBr} cm^{-1}: 1663 vs (CO), 1097 m, 2961 m: MS (FAB) protonated molecule $[M + H]^+$ *m/z* 1188.8, ions belonging to dominating 2–3 cleavage [13] (obtained from daughter ion scan): 1104.5, 920.2, 808.1, 679.9, 552.8, 482.7, 411.9, 283.9, 186.4. NMR data :Table 1.

[MeLeu^1]*CS* = *cyclosporin-J* (**2**). Amorphous powder mp 136°. $[\alpha]_D^{25}$ − 283.5°. $CHCl_3$; *c* 8.8 mg ml^{-1}. (Found: C 62.0% H 9.6%, $C_{61}H_{109}N_{11}O_{12}$ requires C 61.80%, H 9.41%). UV (MeOH) end absorption 200 nm: IR ν_{max}^{KBr} cm^{-1}: 1627 vs (CO), 1097 m, 2963 m; MS (FAB) protonated molecule $[M + H]^+$ *m/z* 1146.9, ions originated from the 2–3 cleavage (daughter ion scan): 1062.1, 934.8, 821,5, 694.2, 567.1, 495.9, 424.9, 297.7, 198.2: NMR data: Table 2.

Proliferative response of lymphocytes to mitogen stimulation. Stock solns of individual cyclosporins (1 mg ml^{-1} in EtOH) were diluted to a final concn with serum-free RPMI 1640 medium. Mononuclear spleen cells (2.5 × 10^5) from female BALB/c mice were placed into a 96-well microplate and incubated for 72 hr with concanavalin A (1 μg ml^{-1}, Sigma, U.S.A.) and an appropriate concn of tested compounds in a humid atm. with 5% CO_2 at 37°. Cell proliferation was assessed by a colorimetric assay using MTT (3-[4,5-dimethylthiazoly-2-yl]2,5-diphenyl tetrazolium bromide, Serva, F.R.G.) as described earlier [17]. Each variant was tested at least × 3.

Acknowledgement—This research was in part supported by the EC grant 27ERB40450PL 93-2014 (Commission of the European Communities).

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