Pergamon

PII: S0031-9422(98)00160-5

Phytochemistry, Vol. 48, No. 7, pp. 1195–1198, 1998

Printed in Great Britain
0031-9422/98 $19.00+0.00

A CYCLOSPORIN FROM *MYCELIUM STERILAE*

MARTIN BUCHTA,* ALEXANDR JEGOROV,†,¶ LADISLAV CVAK,* VLADIMÍR HAVLÍČEK,‡ MILOŠ BUDĚŠÍNSKÝ§ and PETR SEDMERA‡

*Galena Co., R. & D., 747 70 Opava-Komárov, Czech Republic; †Galena Co., Research Unit, Branišovská 31, 370 05 České Budějovice, Czech Republic; ‡Institute of Microbiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague 4, Czech Republic; §Institute of Organic Chemistry and Biochemistry, Flemingovo nám. 2, 166 10 Prague 6, Czech Republic

(*Received in revised form* 9 *February* 1998)

Key Word Index—*Mycelium sterilae*; fungi imperfecti; cyclosporins; [Ala^2,Val^{11}]cyclosporin.

Abstract—A new natural cyclosporin, [Ala^2,Val^{11}]cyclosporin, has been isolated from the submerged culture of *Mycelium sterilae* MS 2929. Its structure was deduced from the NMR and mass spectral data. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Cyclosporins are cyclic undecapeptides produced extraribosomally by multifunctional enzymes of several fungi imperfecti. The most important representative of this group, cyclosporin A, (**1**), is a potent immunosuppressant widely used in human medicine to prevent graft rejection or in the treatment of various autoimmune diseases [1] (Galena, Consupren®). Some related cyclosporins, derived from cyclosporin A by substitution of one or two amino acids, are also pharmacologically active with distinct potential for the treatment of multidrug resistance (MDR) [2–5], and acquired immunodeficiency syndrome (AIDS) [6–8]. We report here a new member of cyclosporin series [Ala^2, Val^{11}]cyclosporin (**2**) which is the third example of demethylation at the 11th-amino acid residue of the cyclosporin skeleton.

RESULTS AND DISCUSSION

Submerged cultivation of *Mycelium sterilae* MS 2929 [9], followed by extraction of the mycelium with methanol and chromatographic separation provided a series of already known cyclosporins, predominantly A, B, C, D, G, and F. Among other less abundant cyclosporins, a new member of this family of cyclic undecapeptides was detected and isolated by a combination of LC on silica gel and HPLC on C18 reversed-phase column.

The Matrix Assisted Laser Desorption Ionization (MALDI) mass spectrum of the new compound (**2**) exhibited the sodiated $[M+Na]^+$ and potassiated $[M+K]^+$ ions at *m/z* 1196.8 and 1212.7, respectively. The sixty carbons observed in the ^{13}C NMR spectrum represented 11 carbonyls, 20 methines, 6 methylenes, and 23 methyls. A total of 101 hydrogens were attached to the carbons, five of them were part of amidic N-H groups, one formed a secondary alcoholic group. The number of carbonyls (11), N-methyls (6) and N-H's (5) indicated that the compound was an undecapeptide. There was an indirect evidence for 11 nitrogens and 12 oxygens (carbonyls + CHOH) present in the molecule. Therefore, the deduced molecular formula was $C_{60}H_{107}N_{11}O_{12}$. According to COSY, LR COSY, and RELAY experiments, MeBmt, Sar, three Ala, two Val, and four MeLeu were the building blocks. The closest cyclosporin analogue with respect to amino acid composition, cyclosporin B, has a molecular weight 14 daltons higher. The sequence information was obtained from NOE's between H-α and N-H (H-1α *vs.* 2-NH, H-8α *vs.* 7-NH, H-10α *vs.* 11-NH) or H-α and NMe (H-2α *vs.* 3-NMe, H-3α *vs.* 4-NMe, H-5α *vs.* 6-NMe, H-8α *vs.* 9-NMe, H-11α *vs.* 1-NMe) plus H-9α *vs.* H-10α. This information was independently provided by Post Source Decay (PSD) mass spectra of $[M+Na]^+$ ions. Dominating sodiated fragment ions originated from the primary splitting between the 2nd and 3rd and between the 1st and 11th amino acid. This ring opening mechanism is similar to that described for the fragmentation of $[M+H]^+$

¶Author to whom correspondence should be addressed: Fax: 420-38-7312728, e-mail: husakm@marvin.jcu.cz

ions of cyclosporins [10–12]. A comparison of the PSD fragmentation of our compound and CsB, revealed b_5-b_8 ions (1–11 bond splitting) and b_3–b_8 ions (2–3 bond splitting) at the same masses indicating identical amino acid composition at positions 1 to 10. On the contrary, a difference was observed with the b_9 ion mass (2–3 bond splitting). The shift from *m/z* 956.1 (observed with CsB) to *m/z* 943.0 (with [Ala^2, Val^{11}]cyclosporin) revealed an amino acid change at the eleventh position of the cyclosporin molecule. Therefore, the new compound **(2)** was *cyclo*(-MeBmt^1-Ala^2 - Sar^3 - MeLeu^4 - Val^5 - MeLeu^6 - Ala^7 - D - Ala^8 - MeLeu^9-MeLeu^{10}-Val^{11}-), assuming the same chirality of all centers as in cyclosporin A.

The new compound is formally 11-demethyl cyclosporin B; analogous derivatives of CsA and CsC are known (cyclosporin E, cyclosporin W) [13]. Demethylation is one of the diversity principles of this group of natural compounds. It has been described at the positions 1, 4, 6, 9, 10, and 11 [10, 11, 13] and, besides oxidation, it is also an ongoing process during cyclosporin metabolism [14, 15].

EXPERIMENTAL

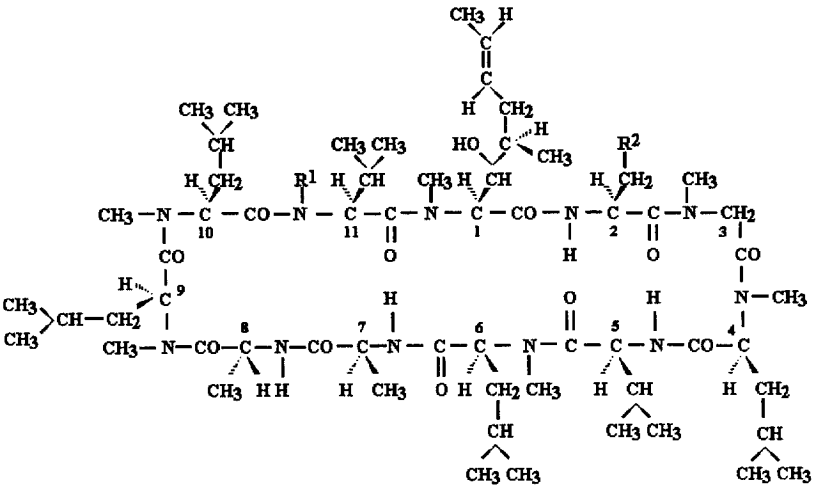
Instruments and methods

NMR spectra were measured on a Varian VXR-400 spectrometer (400 MHz observing frequency for ^{1}H and 100 MHz for ^{13}C) and Varian Unity-500 (500 MHz for ^{1}H, 125 MHz for ^{13}C) in $CDCl_3$ at 25°C. The HMQC experiment was performed on a Varian Unity 500 device. The reported assignment (Table 1) is based on APT, DEPT, LR COSY, COSY, RELAY, HOM2DJ, ROESY, and HMQC experiments. The chemical shifts are reported in ppm (δ) units downfield from tetramethylsilane used as internal standard. Positive-ion MALDI (Matrix-Assisted Laser Desorption Ionization) mass spectra were measured on a Bruker BIFLEX reflectron time-of-flight mass spectrometer (Bruker–Franzen, Bremen, Germany) equipped with a multiprobe inlet and a gridless delayed extraction ion source. Ion acceleration voltage was 19 kV and the reflectron (ion mirror) voltage was set at 20 kV. For delayed extraction, a 5 kV potential difference between the probe and the extraction lens was applied with a time delay in the range of 150–200 ns after each laser pulse. Samples were irradiated at a frequency of 5 Hz by 337-nm photons from a pulsed Laser Science (Cambridge, MA, U.S.A.) nitrogen laser. Typically 20–50 shots were summed into a single mass spectrum. The spectra were calibrated externally using the monoisotopic $[M+H]^+$ ion of a peptide standard (bombesin, Aldrich, Germany). Post source decay (PSD) spectra were typically recorded in 10–18 segments, each successive segment representing a 20% reduction in reflector voltage. About 200 shots were averaged per segment. The segments were pasted, calibrated and smoothed under computer control by Bruker XTOF 3.0 software.

Table 1. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) data of [Ala^2,Val^{11}]cyclosporin ($CDCl_3$, 25°C)

Atom	δ_C	δ_H	mult.	*J* [Hz]
N-CH_3	35.36	3.498	*s*	
1α	58.91	5.753	*d*	4.8
1β	76.42	3.615	*ddd*	9.6, 7.3, 4.8
1γ	37.67	1.442	*m*	
1γ-CH_3	15.96	0.578	*d*	5.9
1δ	36.19	1.448	*m*	
		2.620	*m*	
1γ-OH	—	4.932	*d*	7.3
1ϵ	129.78	5.385	*dd*	15.3, 4.1
1η	126.27	5.358	*m*	
1ω	17.96	1.642	*dm*	5.6
2-NH	—	8.208	*d*	9.7
2α	42.80	5.260	*dq*	9.7, 6.9
2β	17.48	1.230	*d*	6.9
N-CH_3	39.52	3.380	*s*	
3α	50.44	4.733	*d*	13.913.9
		3.210	*d*	
N-CH_3	30.00	3.123	*s*	
4α	55.64	5.357	*dt*	11.1, 4.1
4β	36.72	1.619	*m*	
		2.013	*m*	
4γ	24.62	1.362	*m*	
4δ_u	21.11	0.870	*d*	6.7
4δ_d	24.06	0.948	*d*	6.7
5-NH	—	7.344	*d*	8.6
5α	55.34	4.814	*dd*	8.7, 8.6
5β	31.28	2.464	*dqq*	8.7, 6.9, 6.6
5γ_u	22.84	0.977	*d*	6.9
5γ_d	19.70	1.110	*d*	6.6
N-CH_3	31.95	3.271	*s*	
6α	57.23	4.709	*dd*	10.1, 5.3
6β	38.27	1.418	*m*	
		1.999	*m*	
6γ	25.26	1.713	*m*	
6δ_u	22.47	0.917	*d*	6.6
6δ_d	18.65	0.983	*d*	6.3
7-NH	—	8.408	*d*	9.1
7α	48.79	4.626	*dq*	9.1, 7.4
7β	18.16	1.210	*d*	7.4
8-NH	—	7.803	*d*	6.2
8α	48.87	4.735	*dq*	6.2, 6.7
8β	18.53	1.335	*d*	6.7
N-CH_3	31.46	3.138	*s*	
9α	49.40	5.683	*dd*	10.6, 5.2
9β	39.04	1.305	*m*	14.8, 10.6, 3.2
		2.124	*ddd*	
9γ	24.65	1.428	*m*	
9δ_u	21.30	0.864	*d*	6.7
9δ_d	23.39	0.967	*d*	6.6
N-CH_3	29.09	2.871	*s*	
10α	57.77	5.093	*dd*	7.2, 7.1
10β	38.92	1.527	*m*	13.9, 7.2, 7.2
		1.852	*ddd*	
10γ	24.89	1.433	*m*	
10δ_u	23.37	0.970	*d*	6.5
10δ_d	22.96	0.976	*d*	6.4
11-NH	—	7.058	*d*	9.7
11α	52.40	4.929	*t*	9.7, 9.7
11β	32.83	1.643	*m*	
11γ_u	17.34	0.652	*d*	6.7
11γ_d	19.10	0.875	*d*	6.7

C=O: 168.47, 169.75, 169.86, 170.31, 171.37 (2 C), 171.65, 173.42, 173.70, 174.35, 174.57.



1 $R^1 = R^2 =$ methyl (cyclosporin A)
2 $R^1 = R^2 = H$ ([Ala^2, Val^{11}]cyclosporin)

Mycelium sterilae MS 2929 is a cyclosporin A high-producing strain obtained from the natural isolate of entomopathogenic fungus isolated from the larva of *Culex pipiens* by a combination of UV-light and nitrosoguanidine treatment [9]. *Mycelium sterilae* MS 2929 is deposited in the Czech Collection of Microorganisms of Masaryk Univerzity Brno under the collection number CCM 8184. Submerged cultivation of *Mycelium sterilae* MS 2929 has been described elsewhere [9].

Isolation of [Ala^2,Val^{11}]-cyclosporin (**2**)

A crude extract of cyclosporins was obtained by the extraction of separated mycelia (*ca.* 100 kg) with MeOH. The resulting extract was roughly fractionated by LC on silica gel using a stepwise MeOH-CH_2Cl_2 gradient (up to 10% vol. of MeOH in CH_2Cl_2). Fractions corresponding to cyclosporin A (**1**) were pooled and evaporated. The crude extract (100 g) was crystallized twice from Me_2CO-petroleum ether (200 ml and 800 ml, respectively). The crystalline part (14.1 g) was purified by LC on a silica gel (Merck, 40–63 μm, 800 g) with EtOAC as a mobile phase, yielding a concentrate of a new cyclosporin (**2**, 1.2 g). Finally, the pure compound was obtained by reversed-phase chromatography on two coupled columns filled with Biospher Si C-18 and Biospher Si SPR, respectively, (250 × 21 mm I.D.), using isocratic elution with MeCN-*tert*-butylmethyl ether-H_2O (12:1:7), at 70°C, UV detection at 234 nm (yield 100 mg). Recrystallization from Me_2CO-petroleum ether afforded 80 mg of white crystalline [Ala^2,Val^{11}]-cyclosporin. Identification: MS; Principal b_i-fragment ions generated by PSD of the $[M + Na]^+$ ion [*m/z* (assignment)]: 2-3 series: 943.0 (b_9), 844.7 (b_8), 715.9 (b_7), 588.4 (b_6), 518.2 (b_5), 447.0 (b_4), 322.1 (b_3); 1–11 series: 844.7 (b_8), 772.4 (b_7), 702.5 (b_6), 574.3 (b_5); for the NMR data see Table 1.

Acknowledgement—This work was in part supported by grants No. VS 96085 of Ministry of Education and 203/97/0623 of the Grant Agency of the Czech Republic.

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