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An unusual side chain C-C cleavage at the MeBmt amino acid in cyclosporin A

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Summary. The mixture of products obtained by alkaline treatment of cyclosporin A was analyzed by HPLC-continuous-flow-FAB/MS. The changes involve the atypical amino acid (*4R*)-4-((*E*)-2-butenyl)-4,*N*-dimethyl-*L*-threonine (MeBmt) without affecting the cyclic structure. The main degradation pathway is dehydration producing all four possible anhydro-MeBmt containing cyclosporins. A new cyclosporin, [Sar[1]]CS, resulting from the side chain cleavage of MeBmt has been isolated and characterized.

Keywords: Amino acids – Cyclosporins – HPLC-continuous-flow-FAB/MS – Side chain cleavage

Introduction

Cyclosporin A (Fig. 1) is the cyclic undecapeptide produced by some imperfect fungi (Jegorov et al., 1990). It is widely used nowadays to prevent transplanted organ rejection and for the treatment of various autoimmune diseases. Cyclosporin A differs from the most of peptides by the presence of several N-methylated amino acids and by the presence of an unusual amino acid MeBmt (von Rügger et al., 1976). Due to its cyclic structure and lipophilic character, cyclosporin A is considerably stable in organic solvents under wide range of experimental conditions. Only diluted mineral acids cause the N-O rear angement (Havlíček et al., 1993; Oliyai and Stella, 1992; von Rügger et al., 1976), affording iso-cyclosporin A. At higher temperatures, the cyclic structure is nonspecifically hydrolysed to smaller linear peptides (Magni et al., 1994). Acid hydrolysis of cyclosporin A is accompanied by the cyclization of MeBmt to 2-(5-ethyl-3-methyl-tetrahydrofuryl-2)-sarcosine (von Rügger et al., 1976) The same reaction has also been observed *in vivo* (Maurer et al., 1989). However, the stability of cyclosporin A towards alkali is limited and complex mixture of degradation products is formed. To get some insight into the character of these degradation pathways, the analysis of

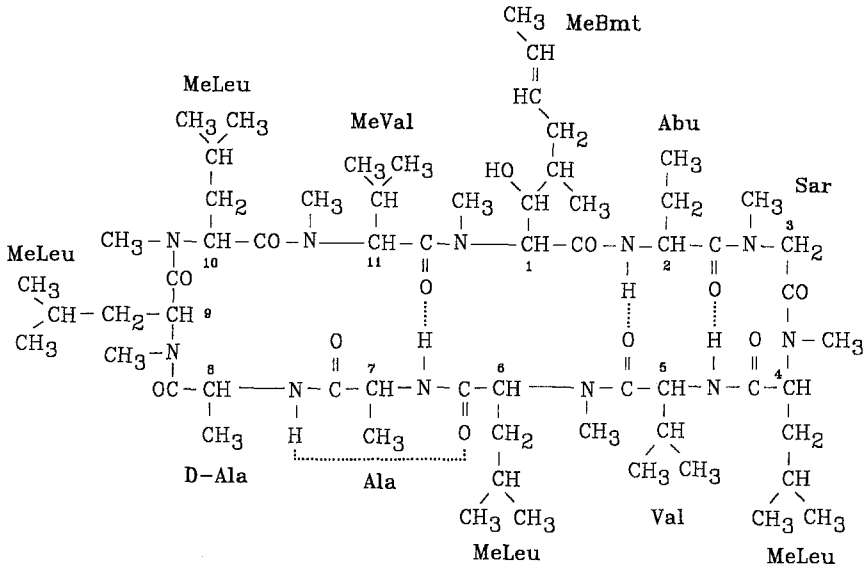

Fig. 1. Structure of cyclosporin A

alkaline hydrolysis products of cyclosporin A has been performed by HPLC-continuous-flow-FAB/MS.

Materials and methods

Alkaline hydrolysis of cyclosporin A

Aqueous NaOH (0.5 g, 5 ml of water) was added under stirring to the solution of cyclosporin A (1 g, 99.5%, Galena Co., Czech Republic) in 95 ml of methanol. The solution was maintained at 35°C for 10 h and then was partitioned between water and dichloromethane. The degradation products were obtained after solvent removal in vaccuo.

HPLC-continuous-flow-FAB/MS

Spectra-Physics isocratic pump, manual injection with Rheodyne valve with a 10 ml loop. Column SGX C-18 250 × 25 mm I.D (Tessek, Prague, Czech Republic), isocratic elution with CH_3CN/H_2O = 73:27 (v/v), 0.8 ml/min, column thermostated at 70°C, detector set at 228 nm. HPLC-MS interface: splitter 4:1, than FAB matrix was added by the T tube in the 3:1 ratio (mobile phase/α-monothioglycerol, v/v) by Pharmacia isocratic pump. The second splitter was constructed as a T-connection (Scientific Glass Engineering Pty. Ltd. Kilton Keynes, U.K.) with a micro needle valve (stem length 50 mm, 1 mm I.D., 1.6 mm O.D.). The split ratio was adjusted experimentally by the length of capillary to provide a constant flow about 2 μl/min to the frit of the CF-FAB target. The residual matrix was continuously exhausted by absorption material mounted at the bottom part of the CF-FAB target. Positive-ion FAB mass spectra were recorded on a Finnigan MAT 90 double-focusing instrument (Finnigan MAT, Bremen, Germany) of BE geometry using a standard continous-flow FAB probe. The saddle field FAB gun (IonTech, Teddington, UK) was operated at 2 mA current and 6 keV energy, using xenon as a bombarding gas (1×10^{-5} mBar). Positive-ion FAB mass spectra were recorded on a Finnigan MAT 90 double-focusing instrument (Finnigan MAT, The matrix used was α-monothioglycerol (Sigma, St. Louis, MO, U.S.A.). A liquid nitrogen baffle was mounted on the ion source

to cool the FAB volume during the operation. Calibration was performed with Ultramark 1600F (PCR, Gainesville, FL, U.S.A.) as a standard.

Preparative isolation and identification of cyclosporins

Preparative HPLC: SGX C-18 250 × 25 mm I.D. column (Tessek, Prague, Czech Republic), isocratic elution with methanol/water (7:3, v/v), 60° C, 6 ml/min, detector set at 245 nm. Fractions were analyzed on a SGX RPS (250 × 4 mm I.D.) column (Tessek, Prague, Czech Republic), isocratic elution acetonitrile/water (75:25, v/v) 70°C, 0.7 ml/min, detector set at 214 nm.

For the sequence determination of [Sar^1]CS the static FAB ionization in combination with collisionally activated diagnostic ions was applied. The products of collisionally-induced dissociations in the first field-free region of the instrument were analysed by the daughter-ion scan (B/E constant) using the manufacture's software. The collision gas (He) pressure used attenuated primary ion beam by 50%. The matrix used was 3-nitrobenzylalcohol (NOBA) (Aldrich Chemie, Steinheim, Germany).

All isolated compounds were also characterized by NMR spectrocopy (Varian VXR-400, 400 MHz for ^{1}H, 100 MHz for ^{13}C, $CDCl_3$). Experiments performed: ^{1}H, ^{13}C, APT, DEPTGL, HOM2DJ, COSY, delay-COSY, ROESY, double and triple RELAY, HETCOR, long-range HETCOR (J = 5 and 10 Hz).

Results

Cyclosporin A decomposes under alkaline conditions yielding a complex mixture of products even at 35°C. By the HPLC-continuous-flow FAB/MS analysis of the degradation products, cyclosporin derivatives have been detected at three molecular masses: 1202.8, 1184.7, and 1090.7. Trace at m/z 100.2 (methylleucine immonium ion, the most abundant ion in FAB mass spectra of cyclosporins) can be used for the quantitative purposes (Fig. 2). Trace at m/z 1202.8 corresponds to the protonated molecule of unreacted cyclosporin A and to its analogues having some amino acids racemized. Trace at m/z 1184.7 should be attributed to a mixture of [anhydro-MeBmt^1] cyclosporins formed by usual β-elimination (Manning et al., 1989). Attemps to isolate a pure compound and to characterize it by NMR failed. NMR analysis of even apparently single compound afforded a complex spectra, indicating the presence of derivatives containing all possible isomers of [anhydro-MeBmt^1]CS.

The polar compound corresponding to m/z 1090.7 was isolated by HPLC on a reversed phase column. The presence of four N-H doublets and seven N-methyl singlets in the ^{1}H NMR spectrum placed this compound among the "normal" series of cyclosporins (Table 1). Following spin systems were found by COSY, delay-COSY, double- and triple-RELAY experiments: 2 × Ala, Abu, 2 × Sar, Val, MeVal, 2 × MeLeu. Upon assigning the N-methyls to their amino acid systems by delay-COSY, the amino acid sequence was determined by ROESY. Cross-peaks between 2-NH and H-1α, 5-NH and H-4α, 7-NH and H-6α, 8-NH and H-7α, 3-NMe and H-4α, 4-NMe and H-3α_d, 6-NMe and H-5α, 9-NMe and H-8α, 11-NMe and H-10α, and 1-NMe and H-11α were found. Taken together with a cross-peak between H-9α and H-10α (significant for a *cis*- amide bond at this position), they complete the structure to cyclo(-Sar^1-Abu^2-Sar^3-MeLeu^4-Val^5-Leu^6-Ala^7-D-Ala^8-MeLeu^9-MeLeu^{10}-

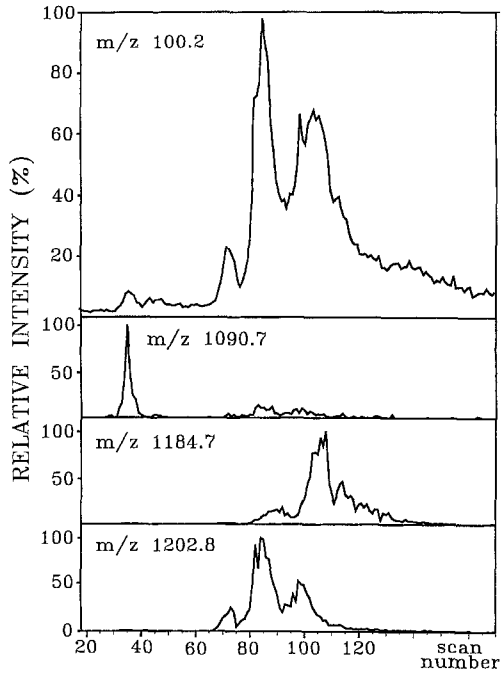

Fig. 2. Reconstructed ion chromatogram (HPLC/MS) of the reaction mixture resulting from the basic hydrolysis of cyclosporin A (for details see Experimental)

MeVal[11]-), i.e., [Sar[1]]CS, (Fig. 3). The sequence of [Sar[1]]CS can be also unambiguously determined from MS/MS spectral data (Havlíček et al., 1993, 1995). The main fragmentation pathways observed in CID mass spectra of the protonated molecule start by primary splitting between amino acids 2–3, 1–11 and 5–6 of the cycle. The corresponding N-terminal acylium fragment ions (for the MS ion nomenclature see Roepstorff and Fohlman, 1989) are the most prominent in the daughter ion FAB mass spectrum (Fig. 4).

Discussion

When the peptides with unknown constituents are to be analyzed, the decomposition accompanying the acidic or alkaline hydrolysis of proteins sometimes complicates the analysis and identification of some amino acids. Whereas the chemical instability processes including e.g. racemization, oxidation, deamidation, β-elimination, or disulfide exchange at the protein amino acids are well documented (Manning et al., 1989), the C-C cleavage at the amino acid side chains is quite unexpected. In the particular case of cyclosporin A, C-C cleavage at MeBmt can be detected at relatively very mild conditions with the retention of the cyclopeptide structure. At 35°C, the conversion to [Sar[1]]CS corresponds to several relative percents only (Fig. 2). With the increased temperature the reaction becomes more pronounced. Due to the simultaneous racemization of some amino acids, however, resulting epimeric derivatives make the isolation of pure [Sar[1]]CS more difficult. It can be

Table 1. [Sar[1]]CS: ^{1}H and ^{13}C NMR data (399.95 and 100.577 MHz, $CDCl_3$)

	Group	δ_C	δ_H	m.	J [Hz]
Sar[1]	1α	39.19	3.458	s	
	CH_3-N	52.01	5.044	d	15.4
			3.827	d	15.4
n-Abu[2]	N-H	–	8.426	d	9.5
	2α	49.21	4.921	ddd	9.5, 7.4, 7.4
	2β	25.43	1.684	m	7.4, 7.4
	CH_3 (γ)	9.78	0.864	t	7.4
Sar[3]	CH_3-N	38.97	3.445	s	
	3α	49.92	4.718	d	13.9
			3.174	d	13.9
MeLeu[4]	CH_3-N	29.63	3.119	s	
	4α	55.29	5.308	dd	11.7, 4.0
	4β	36.09	1.990	m	
			1.621	m	
	4γ	24.92	1.438	m	
	CH_3 (δ)	23.47	0.940	d	6.7
	CH_3 (δ')	21.92	0.881	d	6.7
Val[5]	N-H	–	7.323	d	8.9
	5α	54.70	4.757	dd	9.0, 8.9
	5β	31.36	2.329	dqq	9.0, 6.5, 6.5
	CH_3 (γ)	19.19	1.021	d	6.5
	CH_3 (γ')	18.56	0.861	d	6.5
MeLeu[6]	CH_3-N	31.11	3.255	s	
	6α	53.85	5.098	dd	7.3, 6.0
	6β	37.44	1.828	m	
			1.563	m	
	6γ	24.43	1.630	m	
	CH_3 (δ)	22.49	0.819	d	6.4
	CH_3 (δ')	21.69	0.783	d	6.5
Ala[7]	N-H	–	7.776	d	7.1
	7α	48.35	4.418	dq	7.1, 7.2
	CH_3 (β)	15.03	1.351	d	7.2
D-Ala[8]	N-H	–	7.407	d	8.1
	8α	44.76	4.836	dq	8.1, 7.0
	CH_3 (β)	17.75	1.259	d	7.0
MeLeu[9]	CH_3-N	31.39	3.150	s	
	9α	48.08	5.660	dd	10.9, 4.3
	9β	39.19	2.102	m	
			1.210	m	
	9γ	24.67	1.323	m	
	CH_3 (δ)	23.70	0.942	d	6.6
	CH_3 (δ')	21.21	0.861	d	6.5
MeLeu[10]	CH_3-N	29.86	2.692	s	
	10α	57.21	5.110	dd	6.0, 5.8
	10β	40.89	2.132	m	
			1.210	m	
	10γ	24.60	1.512	m	
	CH_3 (δ)	23.90	1.005	d	6.6
	CH_3 (δ')	23.55	1.021	d	6.6
MeVal[11]	CH_3-N	29.58	2.654	s	
	11α	57.48	5.125	d	11.0
	11β	29.12	2.163	dqq	11.0, 6.5, 6.5
	CH_3 (γ)	19.81	0.808	d	6.5
	CH_3 (γ')	19.53	0.934	d	6.5

Carbonyls: 173.74, 173.27, 173.12, 172.15, 171.75, 171.08, 170.96, 170.65, 170.43, 169.73, 168.75.

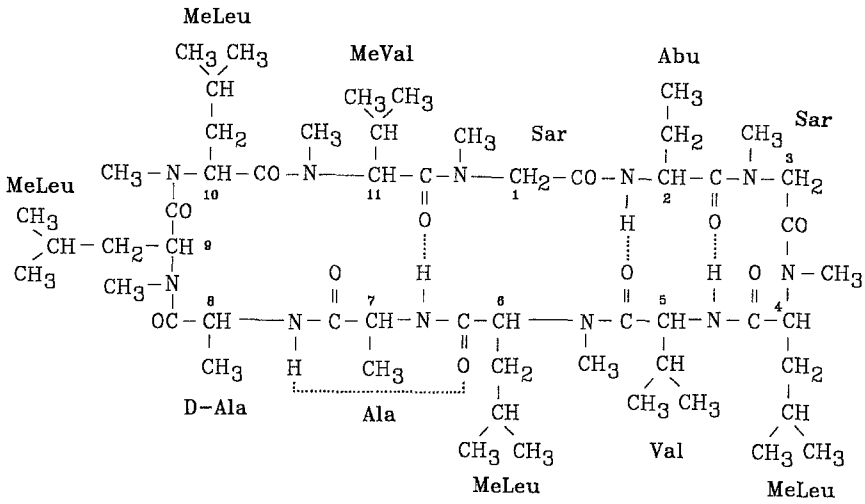

Fig. 3. Structure of [Sar^1]CS

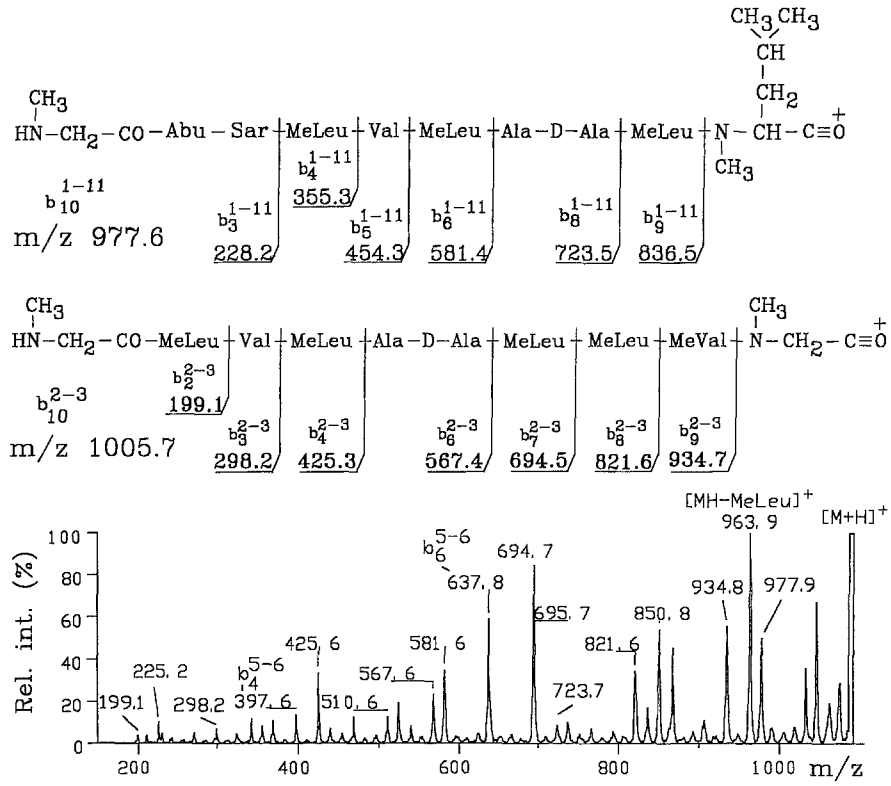

Fig. 4. Fragment ion CID FAB mass spectrum of the protonated molecule of [Sar^1]CS (bottom). For the nature of the most abundant linear acylium ions see the top of the figure

concluded, that MeBmt is very sensitive amino acid decomposing both at acidic and alkaline conditions even before the hydrolysis of the peptide bonds. Thus, MeBmt cannot be recovered from cyclosporins neither by acidic or alkaline hydrolysis.

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