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Sequencing of Cyclosporins by Fast Atom Bombardment and Linked-scan Mass Spectrometry

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The mass spectrometric sequence determination of amino acid residues in cyclosporins using fast atom bombardment, collisionally activated dissociations in the first field-free region and linked *B/E* scan is described. The general fragmentation scheme was derived from the spectra of cyclosporins A, B, C, D, F, G, L and [DH-MeBmt^1]CS. The main fragmentation pathways start by primary splitting between amino acids 2–3, 1–11 and 5–6. The corresponding *N*-terminal b-type ions are common fragment types in the mass spectra. The 1–11 splitting can be enhanced by the introduction of a lactone group into the peptide ring by conversion of cyclosporins into iso-cyclosporins. The fragmentation scheme was used for amino acid sequence determination in four new natural cyclosporins, [MeLeu^1]CS, [Leu^4]CS, [Ile^4]CS and [Leu^5]CS.

INTRODUCTION

Cyclosporins represent a group of interesting fungal neutral, cyclic undecapeptides, which exhibits a number of interesting biological activities.[1] The most important representative cyclosporin A (CS A, Fig. 1) is a potent immunosuppressant widely used in human medicine to prevent rejection of transplanted organs such as kidney, liver, heart and bone marrow.[2] The multifunctional enzyme system is most probably involved in the biosynthesis of cyclosporin in fungi.[3] This non-ribosomal pathway is less specific when compared with the ribosomal pathway with respect to possible amino acids used as substrates for biosynthesis, which leads to a number of very similar cyclosporins produced.[4] The fact that even a small change in the cyclosporin structure substantially influences its biological activity demands new methods for the identification of new cyclosporins and their metabolites in man.

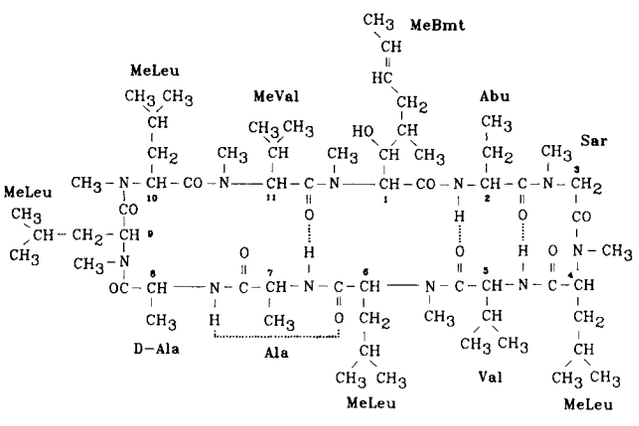

Figure 1. General structure of cyclosporin A.

Up to now, the contribution of mass spectrometry to the structure elucidation of novel cyclosporins has usually been based on the determination of their molecular mass and of the type of amino acid substituents only. Molecular mass information is provided by chemical ionization,[5] fast atom bombardment (FAB),[4,6–14] field desorption[4] and laser desorption[15] mass spectra. The presence of (4*R*)-4-[(*E*)-but-2-enyl]-4, *N*-dimethyl-L-threonine (MeBmt) in the cycle was deduced[4] from the occurrence of characteristic fragment ions $[M + H - C_7H_{13}O]^{+\cdot}$.

Hartman and co-workers[11,12] described a method involving the total and/or partial hydrolysis of cyclosporin metabolites, the derivatization of the amino acids formed and finally the gas chromatographic/mass spectrometric analysis of the resulting mixture (electron impact ionization). However, this time-consuming method can provide only limited information for sequence analysis.

Orlando *et al.*[9] used ammonia for low-energy collision with $[M + H]^+$ ion of CS A generated by FAB. Their idea was to study the neutralization reaction of protonated peptide molecules as a function of the endothermicity of this proton-transfer reaction and thus to predict the site of peptide protonation. The observed low endothermicity of the process with CS A establishes

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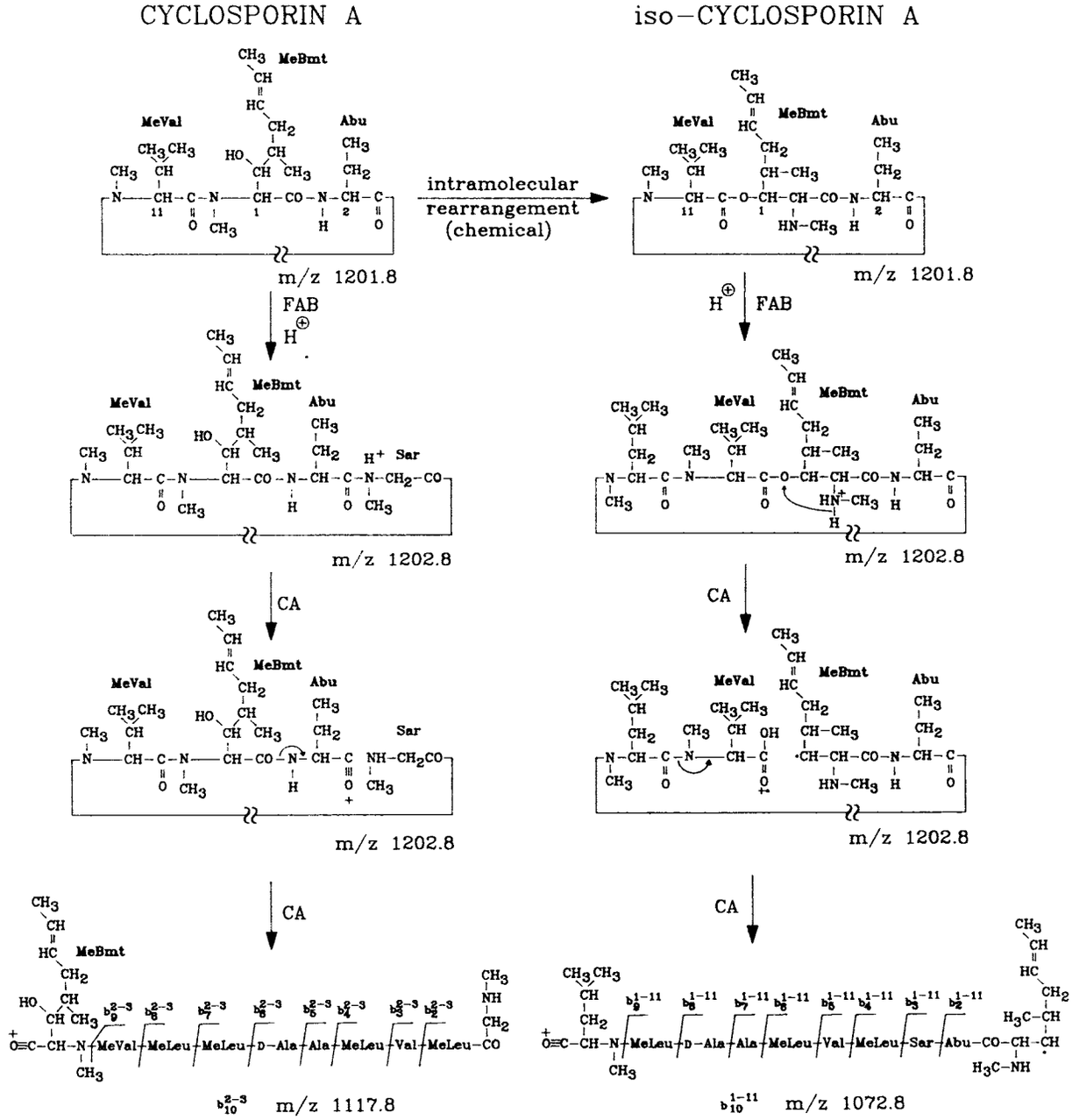
the amide groups as the only protonation sites. The variations in the basicity of amide groups can be provided by *N*-methyl substituents only.

The use of techniques of linked-scan or tandem mass spectrometry (MS/MS) in the sequence determination of cyclosporins has not been reported. The reason might be the complexity of the spectra caused by the expected non-selective ring-opening mechanism of cyclosporins due to the absence of a constituent with a basic residue or hydrophilic side-chain. The aim of this study was to characterize natural cyclosporins by FAB ionization and collisionally activated dissociation (CAD) in comparison with isocyclosporins, which can be prepared from corresponding cyclosporins by intramolecular rearrangement[16] (see Scheme 1). The specific position of lactone and secondary amine groups in isocyclosporins is expected to improve the selectivity of fragmentation under FAB and CAD conditions.

EXPERIMENTAL

Investigated cyclosporins and related compounds

Cyclosporins A, B, C, D, F, G, L and [6',7'-dihydro-MeBmt[1]] cyclosporin ([DH-MeBmt[1]]CS) were obtained from Galena (Opava-Komárov, Czech Republic) and their identities were established by comparison of their ^{1}H and ^{13}C NMR spectral data with literature.[4,16,17] The isolation and complete characterization of the new natural cyclosporins [MeLeu[1]]CS (CS J), [Leu[4]]CS, [Ile[4]]CS and [Leu[5]]CS, isolated from the fungus *Tolypocladium terricola* is described in detail elsewhere.[18] All cyclosporins used for the study were of better than 98% purity (HPLC grade). The amino acid sequences of investigated cyclosporins are listed in



Scheme 1. Proposed mechanisms of dominant cleavages in cyclosporin A and isocyclosporin A under FAB/CAD conditions.

Table 1. The structures of the four new cyclosporins were elucidated by ^1H and ^{13}C NMR spectroscopy (400 and 100 MHz). NMR spectra were recorded on a Varian VXR-400 spectrometer. Spin systems of the individual amino acids were found by COSY, delayed-COSY and RELAY experiments. A check was provided by ^{13}C NMR spectra including signal multiplicity determination by APT and DEPT. Sequence information was obtained from NOESY or ROESY spectra (cross peaks between H-α and N-H or N-CH_3).

NMR data in $CDCl_3$ at 25 °C (distinct amino acid only)

The abbreviations used below are s = singlet, d = doublet, t = triplet, q = quartet and m = multiplet.

[MeLeu^1]CS. ^1H NMR spectrum: 1.014 d (3 H, J = 6.5 Hz, H-δ), 1.027 d (1 H, J = 6.5 Hz, H-δ′), 1.180 m (1 H, H-β), 1.410 m (1 H, H-γ), 1.994 m (1 H, H-β′), 3.354 s (3 H, N-CH_3), 5.126 dd (1 H, J = 9.0 Hz, 6.5, H-α). ^{13}C NMR spectrum: 23.78 q (C-δ), 23.83 q (C-δ′), 24.37 d (C-γ), 31.73 q (N-CH_3), 33.88 t (C-β), 55.33 d (C-α).

[Leu^4]CS. ^1H NMR spectrum: 0.913 d (3 H, J = 6.5 Hz, H-δ), 0.920 d (1 H, J = 6.4 Hz, H-δ′), 1.480 m (1 H, H-β), 1.650 m (1 H, H-γ), 1.980 m (1 H, H-β′), 4.490 ddd (1 H, J = 9.8 Hz, 7.2, 3.7, H-α), 6.090 d (1 H, J = 9.8 Hz, N-H). ^{13}C NMR spectrum: 21.11 q (C-δ), 23.20 q (C-δ′), 24.98 d (C-γ), 34.86 t (C-β), 51.97 d (C-α).

[Leu^5]CS. ^1H NMR spectrum: 0.967 d (3 H, J = 6.6 Hz, H-δ), 0.976 d (1 H, J = 6.5 Hz, H-δ′), 1.780 m (1 H, H-γ), 2.116 m (1 H, H-β), 2.141 m (1 H, H-β′), 4.855 m (1 H, H-α), 7.456 d (1 H, J = 8.3 Hz, N-H). ^{13}C NMR spectrum: 18.46 q (C-δ), 20.93 q (C-δ′), 26.03 d (C-γ), 34.84 t (C-β), 55.55 d (C-α).

[Ile^4]CS. ^1H NMR spectrum: 0.939 t (3 H, J = 7.3 Hz, H-γ), 1.013 d (3 H, J = 6.7 Hz, β-CH_3), 1.251 m (1 H, H-χ), 1.315 m (1 H, H-γ′), 1.482 m (1 H, H-β), 4.191 dd (1 H, J = 10.3 Hz, 4.9, H-α), 6.098 d (1 H, J = 10.3, N-H). ^{13}C NMR spectrum: 12.13 q (C-δ), 19.76 q (β-CH_3), 23.78 d (C-β), 24.06 t (C-χ), 55.42 d (C-α).

Rearrangement of cyclosporins to isocyclosporins

Isocyclosporins A, D, G, [Leu^4]CS and [Ile^4]CS were prepared from the corresponding cyclosporins according to a modified procedure:[16] cyclosporin (100 μg–1 mg), distilled dioxane (200 μl) (Lachema, Brno, Czech Republic) and methanesulphonic acid (1.5 μl, 98%) (Aldrich, Milwaukee, WI, USA) were sealed in a glass ampoule and kept for 24 h at 50 °C. The reaction mixture was then partitioned between diethyl ether (5 ml) and 0.1 M sodium phosphate buffer (pH 6, 5 ml). The organic layer was evaporated, dissolved in methanol–water (4:1, v/v) (100 μl) and applied to a reversed-phase column (250 × 8 mm I.D.) packed with SGX RPS (7 μm) from Tessek (Prague, Czech Republic). Isocratic elution was carried out at 50 °C with methanol-water mixture (4:1, v/v) containing 0.2% of trifluoroacetic acid. With this chromatographic system, isocyclosporins were eluted at relative retention times approximately half those of the corresponding cyclosporins. Fractions containing isocyclosporins (as their CF_3COOH salts) were pooled and evaporated. Final purification of the isocyclosporins was carried out on silica gel 60 (Merck, Darmstadt, Germany) with the chloroform–methanol (95:5, v/v) as the eluent. For the preparative isolation of isocyclosporins, the same protocol was applied with 100 mg of cyclosporin, 2 ml of dioxane and 15 μl of methanesulphonic acid. The yield was 30–80%.

Lactone formation was confirmed by the characteristic downfield shift (4–6 ppm) of the C-1β resonance in the ^{13}C NMR spectrum.

Mass spectrometric measurements

All mass spectra were recorded on a Finnigan MAT 90 double-focusing instrument (Finnigan MAT, Bremen, Germany) of BE geometry. The standard saddle field FAB gun (Ion Tech, Teddington, UK) was operated at 2 mA current and 6 kV energy, using xenon as a bombarding gas [1×10^{-5} mbar (1 bar = 10^5 Pa)]. *m*-Nitrobenzyl alcohol (Aldrich Chemie, Steinheim, Germany) was used as a matrix. The liquid nitrogen baffle was mounted on the ion source to cool the FAB volume during operation. Calibration was performed

Table 1. Amino acid sequences of investigated cyclosporins and related compounds (residues in italics express the modification when compared with cyclosporin A)

Cyclosporin	Amino acid 1	2	3	4	5	6	7	8	9	10	11	Molecular mass	Composition
CS A	MeBmt	Abu	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	MeVal	1201.8	$C_{62}H_{111}N_{11}O_{12}$
[DH-MeBmt^1]CS	*DH-MeBmt*	Abu	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	MeVal	1203.8	$C_{62}H_{113}N_{11}O_{12}$
CS B	MeBmt	*Ala*	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	MeVal	1187.8	$C_{61}H_{109}N_{11}O_{12}$
CS C	MeBmt	*Thr*	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	MeVal	1217.8	$C_{62}H_{111}N_{11}O_{13}$
CS D	MeBmt	*Val*	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	MeVal	1215.9	$C_{63}H_{113}N_{11}O_{12}$
CS F	*3′-Deoxy-MeBmt*	Abu	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	MeVal	1185.8	$C_{62}H_{111}N_{11}O_{11}$
CS G	MeBmt	*Nva*	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	MeVal	1215.9	$C_{63}H_{113}N_{11}O_{12}$
CS L	*Bmt*	Abu	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	MeVal	1187.8	$C_{61}H_{109}N_{11}O_{12}$
[MeLeu^1]CS	*MeLeu*	Abu	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	MeVal	1145.8	$C_{59}H_{107}N_{11}O_{11}$
[Leu^4]CS	MeBmt	Abu	Sar	*Leu*	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	MeVal	1187.8	$C_{61}H_{109}N_{11}O_{12}$
[Ile^4]CS	MeBmt	Abu	Sar	*Ile*	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	MeVal	1187.8	$C_{61}H_{109}N_{11}O_{12}$
[Leu^5]CS	MeBmt	Abu	Sar	MeLeu	*Leu*	MeLeu	Ala	D-Ala	MeLeu	MeLeu	MeVal	1215.9	$C_{63}H_{113}N_{11}O_{12}$

with Ultramark 1600F (PCR, Gainesville, FL, USA) as a standard.

The products of CAD in the first field-free region of the instrument were analysed by fragment-ion linked scan (*B/E* constant) using the manufacturer's software. The collision gas (helium) pressure was adjusted for 50% attenuation of the primary ion beam, with the collision cell voltage maintained at the ground potential. The mass range was scanned at a rate of 40 s per 100 u and the conventional resolution of the instrument was adjusted to 3000 (10% valley). Data acquisition was performed without accumulation or smoothing and single scans are shown in all figures.

RESULTS AND DISCUSSION

Magnetic scan FAB mass spectra of CS A and iso-CS A are not significantly different. In the positive-ion mode (Fig. 2), both contain *N*-methylleucine (MeLeu) immonium ion (*m/z* 100.1) as the base peak and abundant $[M + H]^+$ (*m/z* 1202.8) and $[M + Na]^+$ (*m/z* 1224.8) ions. In contrast to the iso-CS A mass spectrum, the spectrum of CS A exhibits more extensive fragmentation in the high-mass region. Special attention should be paid to the peak at *m/z* 1089.7 (i.e. $[M + H - 113]^+$), revealing the side-chain loss in MeBmt. A similar situation is encountered in the negative-ion mode (Fig. 3). Whereas in the spectrum of iso-CS A the $[M - H]^-$ ion dominates in the high-mass region, with cyclosporin the ion at *m/z* 1089.0 is more abundant than $[M - H]^-$. The low-mass regions of both CS A and iso-CS A exhibit peaks corresponding to the matrix only (denoted by asterisks). Fragment ions of the deprotonated molecule of iso-CS A exhibit an abundant *m/z* 1090.0 ion with weak further fragmentation.

Figure 4 compares the *B/E* CAD mass spectra of $[M + H]^+$ ions of CS A (top) and iso-CS A (bottom) as representatives of the investigated compounds. The fundamental question is whether the primary cleavage of the peptide ring occurs randomly, or whether there are sites on the primary $[M + H]^+$ cyclic ions with preferential cleavage. In the high-mass region of the fragment-ion mass spectrum of $[M + H]^+$ of CS A, there are four dominating peaks at *m/z* 1089.9, 934.9, 821.8 and 694.7. The peaks at *m/z* 934.9, 821.8 and 694.7 may be attributed to the splitting off of MeBmt-Abu, MeVal-MeBmt-Abu and MeLeu-MeVal-MeBmt-Abu fragments after the primary cleavage between sarcosine and aminobutyric acid, i.e. between amino acid residues 2 and 3 (see Fig. 1). According to the Roepstorff nomenclature,[19] these can be assigned as b_9^{2-3}, b_8^{2-3} and b_7^{2-3}, respectively; we have introduced the superscript 2–3 for the characterization of a primary cleavage of cyclic peptides with the origin of a defined linear peptide sequence (see Scheme 1). The ion at *m/z* 1089.9 may be attributed to several fragmentation pathways: it might be caused by the loss of the MeBmt side-chain; however, alternatives might also be the loss of MeVal (the corresponding fragment would be labelled b_{10}^{1-11} (e.g $[MH - C_6H_{11}NO]^+$). Another possibility for the ion at *m/z* 1089.9 is a_{10}^{2-3}. The complicated fragment-ion spectrum of the *m/z* 1089.7 ion in CS A (Fig. 5, top) supports this conclusion. Also other peaks corresponding to b_{10}^{1-11}, b_9^{1-11}, b_8^{1-11}, a_8^{1-11}, b_6^{1-11} or b_i ions of the 5–6 series can be observed in spectra of all the investigated cyclosporins (Table 2). Since these peaks with corresponding mass shifts have beeen observed not only with cyclosporins with different amino acid residues at positions 1 and 2, but also in positions 4 and 5, the validity of the suggested peak interpretation may be general. Whereas the b_i fragments of 2–3 and 1–11 cleavages are the most prominent in the high-mass region, the 5–6 series is significant especially in the middle-mass range.

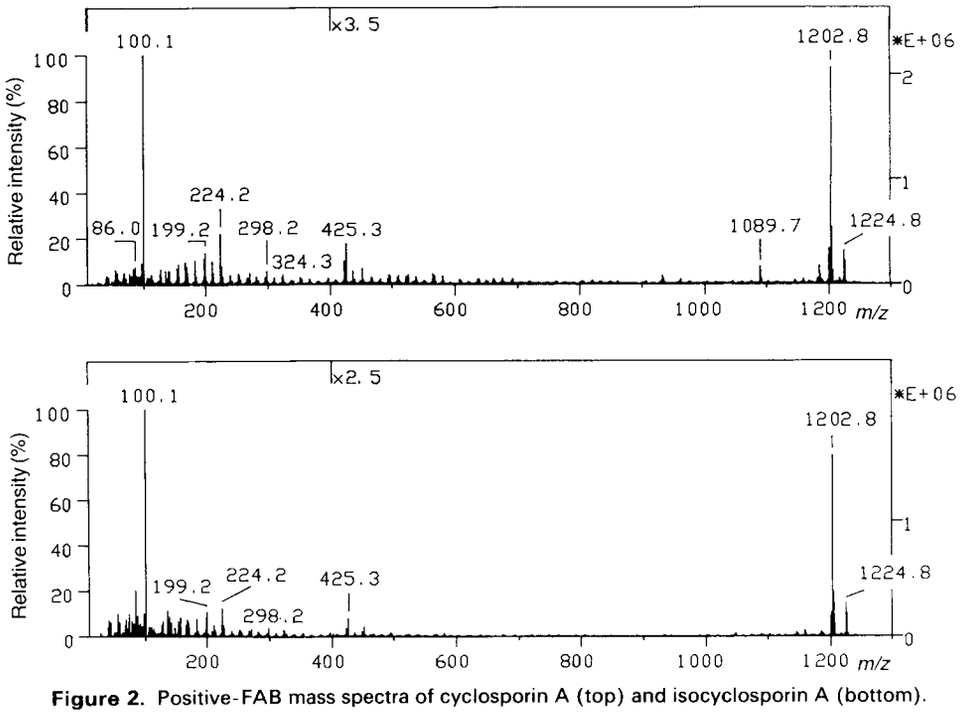


Figure 2. Positive-FAB mass spectra of cyclosporin A (top) and isocyclosporin A (bottom).

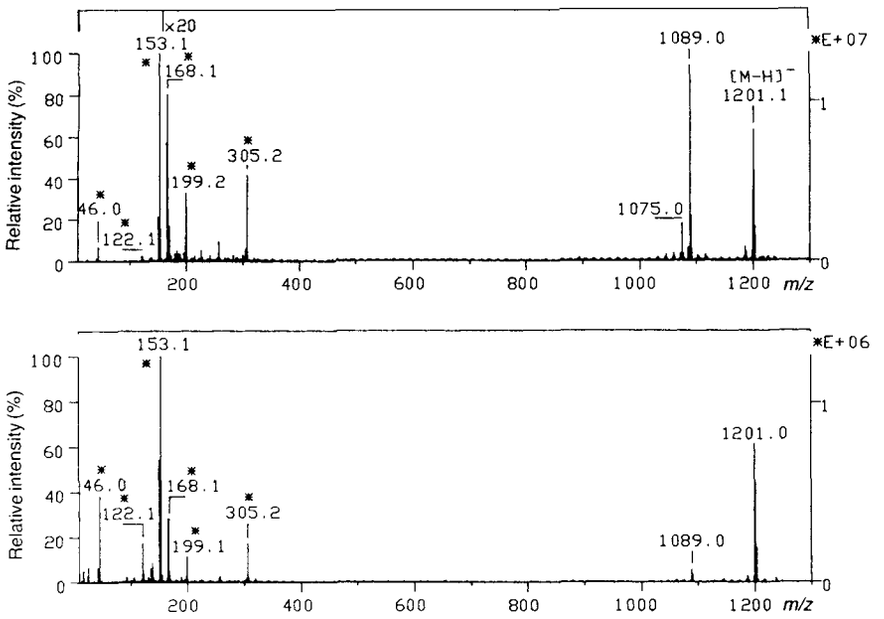


Figure 3. Negative-ion FAB mass spectra of cyclosporin A (top) and isocyclosporin A (bottom).

Elimination of 113 u from the $[M + H]^+$ ions was observed in all cyclosporins including [DH-MeBmt[1]] CS, CS F and [MeLeu[1]]CS that cannot provide an $[MH - C_7H_{13}O]^{+\bullet}$ fragment. This is evidence for the hypothesis that $[MH - 113]^+$ peaks are built by several isobaric ions of different constitutions and structures. Their occurrence in the fragment-ion mass spectra of $[M + H]^+$ ions cannot be used as proof of the presence of MeBmt or Bmt in the molecule. On the other hand, the absence of $[MH - OH]^{+\bullet}$ ions in the spectra is significant for the absence of a hydroxylated amino acid residue in the cyclosporin molecule. The loss of MeLeu is ubiquitous for all cyclosporins studied.

In the *B/E* CAD spectra of $[M + H]^+$ ions of iso-CS A, all pathways typical of cyclosporins, corresponding to 2–3, 1–11 and 5–6 cleavages were observed, superimposed by a new type of 1–11 cleavage characteristic of the isocyclosporins only. It should be noted that the mentioned signal overlap of series 2–3, 5–6, and 'old' 1–11 may also originate from partial *in situ* rearrangement of isocyclosporin to cyclosporin[3] taking place under high-energy collision. In iso-CS A, the novel 1–11 fragmentation involves ions at *m/z* 1072.8, 945.8 and 676.3 (see Fig. 4, bottom spectrum) and can be completed by the ions at *m/z* 548.7, 449.8, etc., after measuring the fragment ions of *m/z* 1072.8 (Fig. 5, bottom). This ion arises by the splitting off the whole *N*-methylvaline at position 11 of the cyclosporin ring. For this reason, all fragments of the new 1–11 cleavage of isocyclosporins differ from those of cyclosporins by

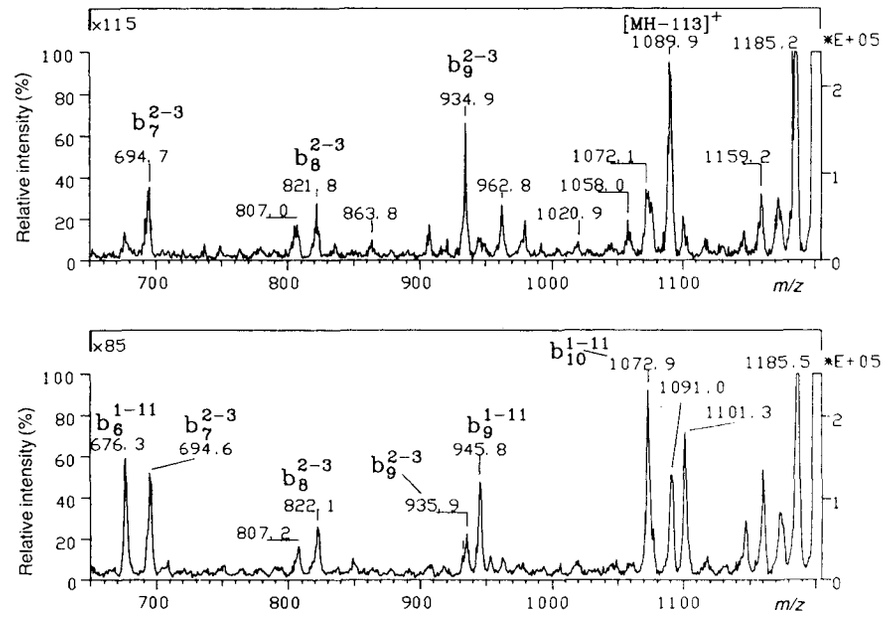


Figure 4. Comparison of *B/E* FAB CAD mass spectra of $[M + H]^+$ ions of cyclosporin A (top) and isocyclosporin A (bottom)

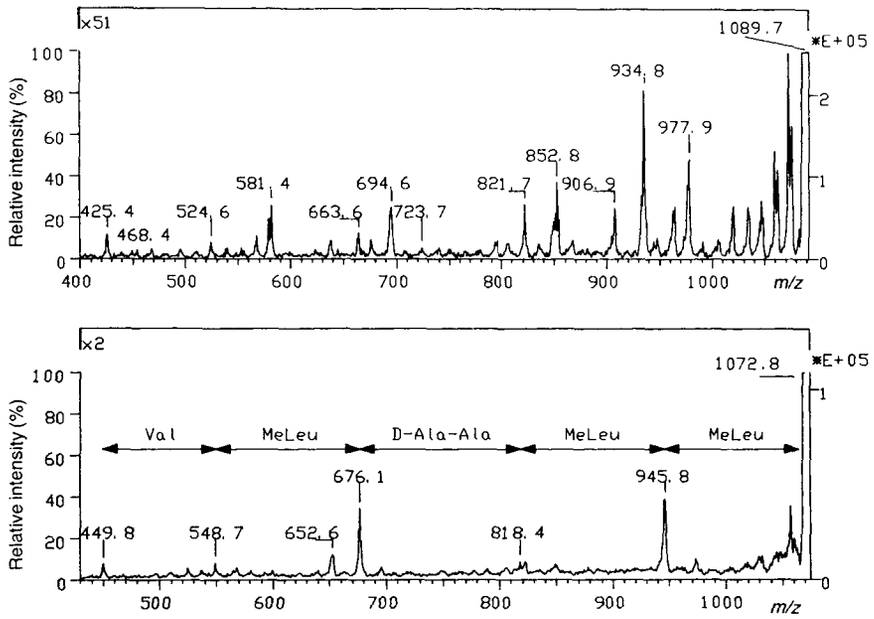


Figure 5. Fragment ion spectrum of *m/z* 1089.7 ion obtained from cyclosporin A (top) and fragment ions of *m/z* 1072.8 from isocyclosporin A (bottom) under FAB/CAD conditions.

17 u, i.e. by a hydroxyl group increment. To verify the general validity of the domination of the novel 1–11 pathway in isocyclosporins, a series of five analogues were synthesized. Corresponding mass spectral data are summarized in Table 3. Typical intense triplets appertaining to eliminations of 101, 113 and 130 u from the protonated molecules were observed. Whereas the $[MH - 113]^+$ ions correspond to the b_{10}^{1-11} fragments of the 1–11 cleavage of cyclosporins, the elimination of 130 u (*N*-methylvaline) is typical of the new 1–11 cleavage typical of isocyclosporins only. The $[MH - 101]^+$ peak may be attributed to the loss of isovaleric acid formed from *N*-methylvaline at position 11 of the isocyclosporin ring. Two pairs of isocyclosporins, iso-CS D, iso-CS G ($[Val^2]$CS and $[Nva^2]$CS) and iso-$[Leu^4]$CS, iso-$[Ile^4]$CS, reveal almost identical fragment-ion mass spectra, as expected.

Using the suggested fragmentation scheme (Table 2, Scheme 1), the amino acid sequences of several natural cyclosporins were established and verified by 2D NMR

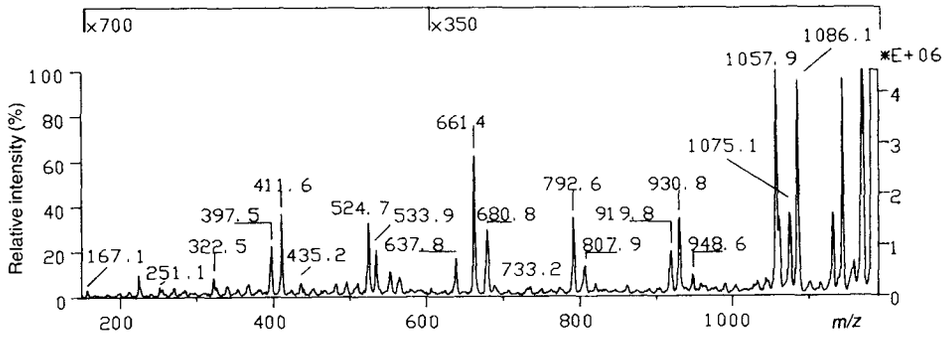


Figure 6. Fragment ions of $[M + H]^+$ ion of $[Leu^4]$CS.

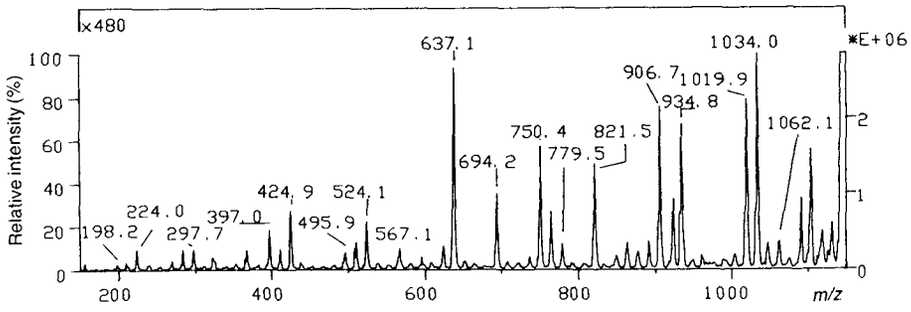


Figure 7. Fragment ions of $[M + H]^+$ ion of $[MeLeu^1]$CS.

Table 2. Dominant *N*-terminal fragment ions (*m/z*) observed in *B/E* CAD mass spectra of $[M + H]^+$ ions of cyclosporins

Cyclosporin		Fragment									
		$[MH - OH]^{+\cdot}$	$[MH - C_7H_{13}O]^{+\cdot}$	$[MH - MeLeu]^+$	b_{10}^{2-3}	b_9^{2-3}	b_8^{2-3}	b_7^{2-3}	b_6^{2-3}	b_5^{2-3}	b_4^{2-3}
CS A	Calc.	1185.8	1089.7	1075.7	1117.8	934.7	821.6	694.5	567.4	496.3	425.3
	Meas.	1185.2	1089.9	1075.2	1117.1	934.9	821.8	694.7	567.6	496.6	425.7
[DH-MeBmt^1]CS	Calc.	1187.9	1089.7	1077.7	1119.8	934.7	821.6	694.5	567.4	496.3	425.3
	Meas.	1187.2	Absent	1076.0	1120.0	934.9	821.8	695.8	567.6	496.6	426.0
CS B	Calc.	1171.8	1075.7	1061.7	1117.8	934.7	821.6	694.5	567.4	496.3	425.3
	Meas.	1171.5	1076.4	1062.3	Absent	935.2	822.0	694.9	567.8	496.8	426.1
CS C	Calc.	1201.8	1105.7	1091.8	1117.8	934.7	821.6	694.5	567.4	496.3	425.3
	Meas.	1201.3	1107.2	1091.5	1116.2	935.0	822.9	694.8	567.8	496.8	426.1
CS D	Calc.	1199.8	1103.7	1089.8	1117.8	934.7	821.6	694.5	567.4	496.3	425.3
	Meas.	1199.3	1105.0	1089.9	1115.1	934.6	821.5	694.1	566.8	496.7	425.0
CS F	Calc.	1169.8	1073.8	1059.8	1101.8	934.7	821.6	694.5	567.4	496.3	425.3
	Meas.	Absent	Absent	1060.3	1102.4	935.2	821.9	694.9	567.7	496.8	426.2
CS G	Calc.	1199.8	1103.7	1089.8	1117.8	934.7	821.6	694.5	567.4	496.3	425.3
	Meas.	1199.7	1103.7	1090.5	1117.6	935.3	821.2	695.0	567.9	496.9	426.1
CS L	Calc.	1171.8	1075.7	1061.7	1103.8	934.7	821.6	694.5	567.4	496.3	425.3
	Meas.	1171.4	1076.0	1062.2	1105.0	934.9	821.6	694.4	567.0	495.9	425.3
[MeLeu^1]CS	Calc.	1129.8	1033.7	1019.7	1061.8	934.7	821.6	694.5	567.4	496.3	425.3
	Meas.	Absent	Absent	1019.9	1062.1	934.8	821.5	694.2	567.1	495.9	424.9
[Leu^4]CS	Calc.	1171.8	1075.7	1061.7	1103.8	920.7	807.6	680.5	553.4	482.3	411.3
	Meas.	1171.5	1076.4	1062.3	1104.5	920.2	808.1	679.9	552.8	482.7	411.9
[Ile^4]CS	Calc.	1171.8	1075.7	1061.7	1103.8	920.7	807.6	680.5	553.4	482.3	411.3
	Meas.	1170.8	1075.6	1061.5	Weak	920.6	807.3	679.3	552.4	482.6	411.2
[Leu^5]CS	Calc.	1199.8	1103.7	1089.8	1131.8	948.7	835.6	708.5	581.4	510.4	439.3
	Meas.	1199.5	1104.4	1090.3	1132.4	949.2	836.0	707.8	581.7	510.8	440.1

Cyclosporin		b_3^{2-3}	b_2^{2-3}	b_{10}^{1-11}	b_9^{1-11}	b_8^{1-11}	a_8^{1-11}	b_6^{1-11}	b_6^{5-6}	b_5^{5-6}	b_4^{5-6}
CS A	Calc.	298.2	199.1	1089.8	962.7	835.6	807.6	693.5	637.5	524.4	397.3
	Meas.	298.4	198.9	1089.9	962.8	835.6	807.0	694.7	637.6	524.8	397.4
[DH-MeBmt^1]CS	Calc.	298.2	199.1	1091.8	964.8	837.7	809.6	695.5	637.5	524.4	397.3
	Meas.	298.1	198.1	1090.9	965.7	837.8	808.9	695.8	638.0	524.6	397.5
CS B	Calc.	298.2	199.1	1075.8	948.7	821.6	793.6	679.5	637.5	524.4	397.3
	Meas.	298.7	199.5	1076.4	949.2	822.0	793.0	679.9	637.7	524.8	397.9
CS C	Calc.	298.2	199.1	1105.8	978.7	851.6	823.6	709.5	637.5	524.4	397.3
	Meas.	298.9	199.2	1107.2	979.1	851.8	822.9	709.8	637.7	524.8	397.8
CS D	Calc.	298.2	199.1	1103.8	976.7	849.6	821.6	707.5	637.5	524.4	397.3
	Meas.	297.6	198.3	1105.0	976.7	849.4	821.5	707.2	637.1	524.0	396.5
CS F	Calc.	298.2	199.1	1073.8	946.7	819.6	791.6	677.5	637.5	524.4	397.3
	Meas.	298.8	199.5	1074.3	946.7	820.0	791.0	677.9	637.7	524.8	397.8
CS G	Calc.	298.2	199.1	1103.8	976.7	849.6	821.6	707.5	637.5	524.4	397.3
	Meas.	298.8	199.4	1103.7	977.3	849.5	821.2	708.0	638.0	525.0	397.9
CS L	Calc.	298.2	199.1	1075.8	948.7	821.6	793.6	679.5	637.5	524.4	397.3
	Meas.	297.6	199.7	1076.0	948.8	821.6	792.5	679.8	637.2	524.0	396.9
[MeLeu^1]CS	Calc.	298.2	199.1	1033.7	906.6	779.5	751.6	637.5	637.5	524.4	397.3
	Meas.	297.7	198.2	1034.0	906.7	779.5	750.4	637.1	637.1	524.1	397.0
[Leu^4]CS	Calc.	284.2	185.1	1075.8	948.7	821.6	793.6	679.5	637.5	524.4	397.3
	Meas.	283.9	186.4	1076.4	949.2	822.0	793.0	679.9	637.8	524.7	397.7
[Ile^4]CS	Calc.	284.2	185.1	1075.8	948.7	821.6	793.6	679.5	637.5	524.4	397.3
	Meas.	283.0	184.0	1075.6	948.4	821.3	792.4	679.3	637.3	524.2	397.1
[Leu^5]CS	Calc.	312.2	199.1	1103.8	976.7	849.6	821.6	707.5	637.5	524.4	397.3
	Meas.	312.7	198.0	1104.4	977.2	849.8	821.0	707.8	637.8	524.9	397.8

spectroscopy. One of them is [Leu^4]CS, formerly found as a CS A metabolite in human bile or urine[20] and denoted AM4N(M21). The complete *B/E* mass spectrum of the $[M + H]^+$ ion of its lactone is presented in Fig. 6. All mass-labelled peaks in Fig. 6 were assigned in Tables 2 and 3 to corresponding *N*-terminal fragment ions.

In spite of the usefulness of the b_i^{1-11} fragment series in isocyclosporins, in cases when MeBmt of Bmt are not present in the molecule it is necessary to characterize an intact cyclosporin. Thus the structure of a new atypical naturally occurring cyclosporin, containing MeLeu instead of MeBmt at position 1, was relatively easily determined (for illustration see Fig. 7 and Table 2). The relatively high abundance of the *m/z* 637.1 ion (in comparison with other cyclosporins) may be attributed to the existence of two isobaric species, b_6^{1-11} and b_6^{5-6}.

Unfortunately, it is not possible to distinguish between series of isobaric amino acid residues, e.g. Leu, Ile or Nva, Val. Our future work will be focused on the

Table 3. Principal fragment ions (m/z) observed in B/E CAD mass spectra of $[M + H]^+$ ions of isocyclosporins iso-CS A, D, G, [Leu⁴]CS and [Ile⁴]CS

Isocyclosporin		Fragment												
		$[MH - OH]^{+\cdot}$	$[MH - C_4H_8COOH]^+$	$[MH\text{-}113]^+$	$[MH\text{-}130]^+$	b_5^{1-11}	$b_8^{1\ 11}$	$b_7^{1\ 11}$	b_6^{1-11}	$b_5^{1\ 11}$	b_4^{1-11}	$b_3^{1\ 11}$	$b_2^{1\ 11}$	$b_1^{1\ 11}$
Iso-CS A	Calc.	1185.8	1101.8	1089.8	1072.8	945.7	818.6	747.5	676.5	549.4	450.3	323.2	252.2	167.1
	Meas.	1185.5	1101.1	1091.0	1072.9	945.8	818.4	748.6	676.1	548.7	449.8	323.7	251.9	167.1
Iso-CS D	Calc.	1199.8	1115.8	1103.8	1086.8	959.7	832.6	761.5	690.5	563.4	464.3	337.2	266.2	167.1
	Meas.	1199.3	1115.1	1104.1	1086.0	958.8	831.1	761.2	689.2	562.9	464.7	335.7	265.3	167.0
Iso-CS G	Calc.	1199.8	1115.8	1103.8	1086.8	959.7	832.6	761.5	690.5	563.4	464.3	337.2	266.2	167.1
	Meas.	1199.4	1115.1	1104.2	1086.1	958.8	833.5	761.4	689.2	562.0	462.9	335.5	264.7	167.4
Iso-[Leu⁴]CS	Calc.	1171.8	1087.8	1075.8	1058.8	931.7	804.6	733.5	662.5	535.4	436.3	323.2	252.2	167.1
	Meas.	1171.2	1086.1	1075.1	1057.9	930.8	805.0	733.2	661.4	533.9	435.2	322.1	251.1	167.1
Iso-[Ile⁴]CS	Calc.	1171.8	1087.8	1075.8	1058.8	931.7	804.6	733.5	662.5	535.4	436.3	323.2	252.2	167.1
	Meas.	1171.6	1086.4	1076.0	1058.3	931.0	804.0	733.0	662.0	534.9	436.3	323.1	252.3	166.8

discrimination among isobaric residues with respect to the presence or absence of *N*-methyl groups.

CONCLUSION

In spite of the fact that cyclosporins do not contain basic residues or hydrophilic side-chains, it has been shown that fragment-ion mass spectra of $[M + H]^+$ ions generated by FAB and CAD in the first field-free region are relatively simple. They can be almost completely interpreted on the basis of primary ring cleavages between amino acid residues 2–3, 1–11 and 5–6. This was assumed for cyclosporin A and proved using the series of cyclosporins containing different amino acid residues. The general validity of the fragmentation pattern was confirmed by sequence determination of four new natural cyclosporins, [MeLeu¹]CS, [Leu⁴]CS, [Ile⁴]CS and [Leu⁵]CS. Isocyclosporins may be useful for enhancing the 1–11 primary splitting, which is here a dominant process. The characterization of unknown cyclosporins using the suggested general fragmentation scheme is much faster (10 min) in comparison with NMR spectroscopy, not to mention the sample amounts required for the analysis and usually being available only after tedious chromatographic separation in the latter case.

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