such studies and they will be reported later.

Hydrolysis of Methyl Hydroxyethyl Phosphate. The reaction almost certainly involves neighboring group participation to form ethylene phosphate and methanol (Scheme I). The pentavalent intermediate which forms in the initial intramolecular addition is a stereoisomer of the adduct formed by addition of lyate ion to methyl ethylene phosphate. In the case of the intermediate derived from methyl hydroxyethyl phosphate, the methoxyl group is axial and can leave directly to produce ethylene phosphate.[2,3] The intermediate derived from methyl ethylene phosphate must pseudorotate to give the same products, and the barrier to this is larger than the barrier to expulsion of methoxide.[2] However, since anionic ligands preferentially occupy equatorial positions, formation of the conjugate base of the intermediate will cause a rapid pseudorotation. Thus, the monoanionic intermediates from the two substrates are isomers which do not interconvert, but their conjugate bases do arrive at a common structure.

The Importance of Strain. The rate constant for the hydroxide-catalyzed hydrolysis of methyl ethylene phosphate is 10^6 times that for an acyclic analogue, trimethyl phosphate.[2] This factor is consistent with relief of enthalpic strain in the reaction of the cyclic ester of about 8 kcal/mol. Measurements of the enthalpic strain of methyl ethylene phosphate in 0.1 M NaOH yielded values of 7 to 9 kcal/mol.[1] A later report gave a lower value (5.5 kcal/mol),[20] but the acidity of the solution is unspecified. If significant exocyclic cleavage occurred under conditions in which the ethylene phosphate produced initially is slow to react, then a significant underestimation of enthalpy could have resulted. A more recent report suggests that the strain may indeed be as high as 9 kcal/mol.[7] Thus, the acceleration of hydrolysis in the triester is likely to result almost entirely from the relief of ring strain in the pentavalent transition state. Product patterns result from the apicophilicities of substituents[29] and the inherent energetics of pentacovalent intermediates.[30]

Conclusion

We have shown that methyl ethylene phosphate undergoes significant amounts of exocyclic cleavage in alkaline solution and that reports to the contrary are in error. A stereoelectronic theory, emphasizing the importance of antiperiplanar orbital interactions, strongly predicts that exocyclic cleavage of methyl ethylene phosphate will be highly disfavored relative to endocyclic cleavage. Therefore, the applicability of antiperiplanar lone-pair hypotheses to the reactions of cyclic phosphates appears to be doubtful.

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Structure of Trichorzianine A IIIc, an Antifungal Peptide from *Trichoderma harzianum*[1]

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Abstract: Trichorzianine A IIIc is the main component of a peptide mixture isolated from a sporulated culture of *Trichoderma harzianum* that inhibits the growth of some fungal plant pathogens. This novel peptide has been shown to consit of one acetylated N-terminal residue, 18 amino acids, and a tryptophanol C-terminal amino alcohol. Sequence assignment was based on positive-ion FAB mass spectrometry and high-field NMR data using two-dimensional NMR assignment techniques and proton NOE difference studies. Information on the conformation was derived from $J_{NH-C^{\alpha}H}$ coupling constants, solvent and temperature NH chemical shift dependence, and transfer of solvent saturation experiments. These data show the N-terminal part of the peptide to be ordered in a helix, the first turn of which is of the 3_{10} type.

Trichoderma are widespread soil fungi, some of which exhibit antagonistic properties against other microscopic fungi.[2] The remarkable antagonism of *T. harzanium* against the plant pathogen *Botrytis cinerea* has been shown to be due to diffusible chemical compounds by in vitro experiments.[3] We have studied the antifungal substances produced by this *Trichoderma* and have isolated a complex peptide mixture, containing what we call trichorzianines, that explains partly the observed activity.

Trichorzianines consist of two closely related groups of peptides: a main, neutral component, trichorzianine A (TA), and a minor acidic component, trichorzianine B (TB).

Since the ninhydrin test was negative and no methylation occurred upon treatment with diazomethane, TA is likely to have neither the N nor the C terminal free. The N-terminal group is acetyl protected and the C-terminal residue is an amino alcohol. Moreover, since trichorzianine A contains a high proportion of α-aminoisobutyric acid (Aib) and has a molecular weight near 2000, it belongs to the class of peptaibols.[4,5] This special class of antibiotic peptides includes alamethicin[6-8] and suzukacillin,[9,10]

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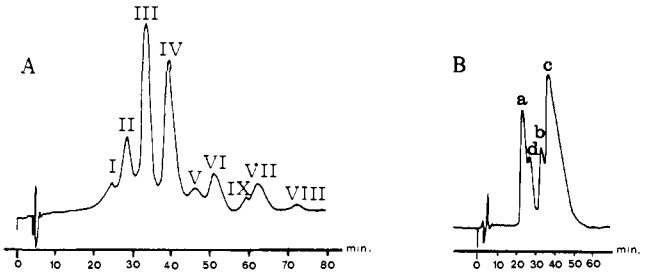

Figure 1. HPLC chromatograms of trichorzianines A. Absorption monitored at 225 nm. (A) Crude TA mixture on μBondapak C_{18} column (Waters Associates, 3.9 mm × 30 cm), flow rate 1 mL/min, eluent 60:40 EtOH/H_2O. (B) Purified TA III on SiO_2 μPorasil (Waters Associates, 7.8 mm × 30 cm), flow rate 4 mL/min, eluent 70:30 CH_2Cl_2/EtOH.

which have been shown to present remarkable pore-forming activity in lipid bilayer membranes.[11-13] We have observed similar modifications of membrane permeability with trichorzianine A.[14]

We report here on the isolation and sequence determination of trichorzianine A IIIc (TA IIIc), one of the major components of trichorzianine A.[15]

Isolation of Trichorzianine A IIIc. The crude trichorzianines were separated into the two components TA and TB by preparative open-column chromatography. TA was obtained as colorless crystals and was homogeneous by both TLC on silica and reversed-phase chromatography. But like alamethicin[7,16] and suzukacillin,[17] trichorzianine A appears to be a mixture of closely related peptides, the heterogeneity of which was deduced from HPLC.

The reversed-phase analytical HPLC performed on TA showed it to be composed of at least nine peptides (Figure 1A).

The major fraction TA III appeared to be still a mixture of four peptides when analyzed on silica (Figure 1B). As the sequence determination of heterogeneous peptides may lead to uncertain results, we purified the main component TA IIIc by semipreparative HPLC. TA IIIc was proved to be a single component by further analysis.

Amino Acid Composition and Absolute Configuration. The total acidic hydrolysate of TA IIIc (6 N HCl) gave eight amino acids: Aib (8), Ala (2), Glu (3), Ile (1), Leu (1), Ser (1), Pro (1), and Val (1). As TA IIIc does not contain any free carboxyl group, the three Glu residues in this hydrolysate arise from three Gln residues. Furthermore, the UV spectrum showed the presence of a tryptophanyl residue, which was identified as tryptophanol in the basic hydrolysis products.

We determined the L absolute configuration of the optically active amino acids in TA IIIc by employing gas chromatography techniques using a column packed with a chiral stationary phase (L-valine *tert*-butylamide on OV 225 silicone). The amino acids in the acidic hydrolysate were converted into the *n*-propyl esters of their *N*-trifluoroacetyl derivatives, the mixture was injected onto the column, and the retention times were compared with those of standards.

Molecular Formula. The ^{13}C NMR spectrum of TA IIIc exhibits 91 carbon atoms (Figure 2); 22 of them are carbonyl carbons, in agreement with previous results: 1 acetyl group and 18 amino acids, including 3 Gln. The aromatic carbons of the tryptophanol can be clearly recognized in the spectrum. If TA IIIc contains all these residues in a linear peptide, the molecular formula should be $C_{91}H_{149}N_{23}O_{24}$ and the molecular weight 1948 (^1H = 1.008). The negative-ion FAB mass spectrum of TA IIIc shows a quasi-molecular $(M - H)^-$ ion at 1947 amu, in concordance with the postulated molecular formula.[15]

In order to assign the sequence of amino acids, we used both mass spectrometry (MS) and ^1H NMR, the MS method being unable to distinguish between isomeric amino acids.

Sequence Determination. (A) Positive-Ion FAB Mass Spectrometry. We studied the positive-ion FAB mass spectrum of TA IIIc, from which we expected to see formation of a series of acylium ions: the sequence may be determined by the observation of such ions as ...CONHCHRC≡O$^+$ in the spectrum.[18] The spectrum of TA IIIc (Figure 3) shows the protonated molecular $(M + H)^+$ ion at the expected value 1949, the sodium adduct $(M + Na)^+$ at 1971, and useful structural information for masses lower than 1108. In the higher mass region no significative peak is observed.

In fact, an inspection of the spectrum reveals the presence of two acylium ion series. The series that defines the N-terminal oligopeptide (Nop) begins at 1108 and displays successive losses of Aib, Leu (or Ile), Ser, 3 Aib, Gln, 2 Aib, and 2 Ala, leaving the terminal *N*-acetylated Aib (*m/z* 128). The second series begins at *m/z* 841 and determines the shorter C-terminal oligopeptide (Cop) by successive losses of Trpol, 2 Gln, Ile (or Leu), and Aib. The residual fragment giving the peak *m/z* 197 is formulated as Pro-Val. The two complementary series Nop and Cop are formed from the entire molecule by a preferential breaking of the very labile Aib-Pro peptide bond.

Nop =
Ac-Aib-Ala-Ala-Aib-Aib-Gln-Aib-Aib-Aib-Ser-Leu-Aib
Ile

Cop = Pro-Val-Aib-Ile-Gln-Gln-Trpol
Leu

The sequence of TA IIIc may be obtained by connecting these two N and C oligopeptides, but some ambiguity remains related to the reciprocal position of the isomeric amino acids Leu and Ile.

We tried to solve this problem by a ^1H NMR approach of the sequence determination, the purpose being to get, in addition, some information about the three-dimensional structure.

(B) ^1H NMR. The sequence assignment of the peptide backbone relies on the study of nuclear Overhauser effects between amide protons and $C^\alpha H$ protons of neighboring residues. A prerequisite for such a strategy is the assignment to specific residues of the resonance lines in the ^1H NMR spectrum, especially those of the amide (NH) and $C^\alpha H$ protons.

A typical 500-MHz ^1H NMR spectrum of TA IIIc in Me_2SO-d_6 shows resonances from nearly all the protons to be well resolved (Figure 5). The spectral assignments were made with the help of homonuclear two-dimensional (2D) spectroscopy techniques.[19]

Figure 4 shows a contour plot of the 2D relayed correlated spectrum that exhibits all the connectivities. The results allowed us to assign the ^1H resonances to individual amino acids.

Measured coupling constants and chemical shifts are listed in Table I.

The $C^\alpha H$ protons (Figure 5B) are well resolved and most of the various multiplets can be easily analyzed. These protons occur between 3.3 and 4.4 ppm along with δ protons of proline, β-protons of serine, and the primary alcohol group of tryptophanol.

The amide protons (Figure 5C) appear between 7.0 and 8.5 ppm. As expected, the eight Aib amide protons form singlets due to the lack of α-protons, and the remaining amide protons show up as doublets. Most of the values of $^3J_{NH-C^\alpha H}$ coupling constants may be determined from the spectrum. In this part resonances of aromatic protons of Trpol and of primary carboxamide protons of the three Gln residues are also seen.

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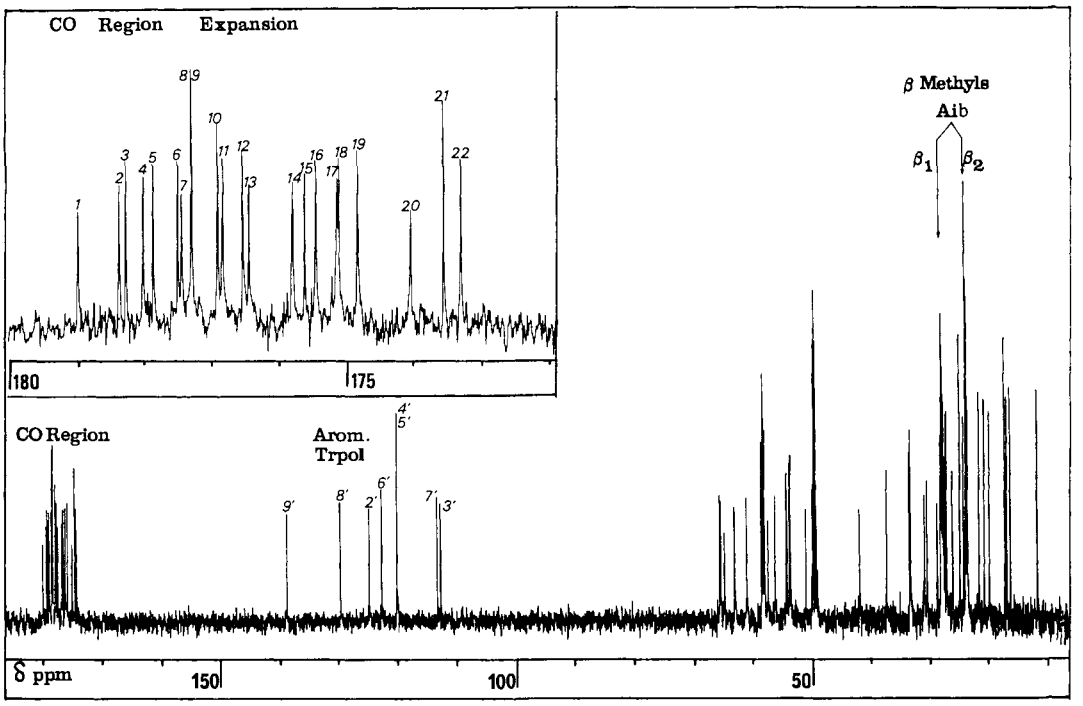

Figure 2. 125.7-MHz ^{1}H-decoupled ^{13}C NMR spectrum of TA IIIc ($^{12}CD_3OD$).

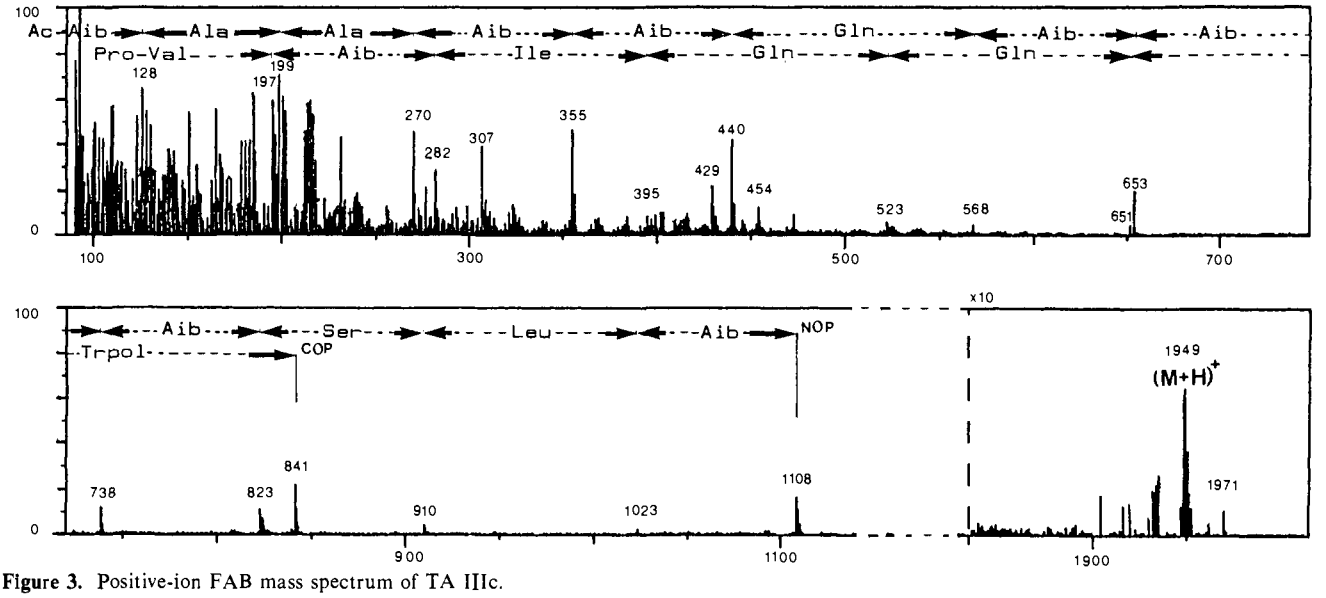

Figure 3. Positive-ion FAB mass spectrum of TA IIIc.

Connectivities between $C^{\alpha}H_i$ and NH_{i+1} as well as between NH_i and $C^{\alpha}H_{i-1}$ were established with one-dimensional (1D) NOE difference spectra (Table I).

This method enabled us to verify the sequence we had previously proposed from the MS study and to determine the specific location in the sequence of the isomeric and of the repetitive amino acids. Thus Ala A, Ala B, Gln A, Gln B, Gln C, Aib A, Aib B, and Aib H are located in position 2, 3, 6, 17, 18, 1, 12, and 15, respectively.

From this whole study the primary sequence of trichorzianine A IIIc appears to be

Ac-Aib-Ala-Ala-Aib-Aib-Gln-Aib-Aib-Aib-Ser-
1 2 3 4 5 6 7 8 9 10
Leu-Aib-Pro-Val-Aib-Ile-Gln-Gln-Trpol
11 12 13 14 15 16 17 18 19

Conformational Studies. Interesting results emerging from the ^{1}H NMR spectrum are the amide coupling constants $^3J_{NH-C^{\alpha}H}$ since they may provide a diagnostic for the secondary structure.[20] As we observed no discernible temperature dependence for these coupling constants, the secondary structure of TA IIIc was surmised to be quite rigid under the experimental conditions. Similar results were obtained from the spectrum of TA IIIc in CD_3OH (Figure 6), assignments of which were made previously.[15] Correlations of the sequential assignments between the two spectra were accomplished by solvent titration (Figure 7). In addition, the observed values of the $^3J_{NH-C^{\alpha}H}$ coupling constants do not differ greatly in an aprotic (Me_2SO-d_6) and a protic (CD_3OH) polar solvent. These results indicate the molecule to be highly ordered.

Tables I and II show the $^3J_{NH-C^{\alpha}H}$ to be divided into two sets of magnitude: they are less than 6 Hz for the N-terminal end of the peptide, whereas the C-terminal moiety has values in the range 7–9 Hz, suggesting that the peptide may be apportioned into two differently structured parts.

The values of 4–6 Hz are consistent with a helix structure as has been proposed for alamethicin and related peptides.[21-23] From

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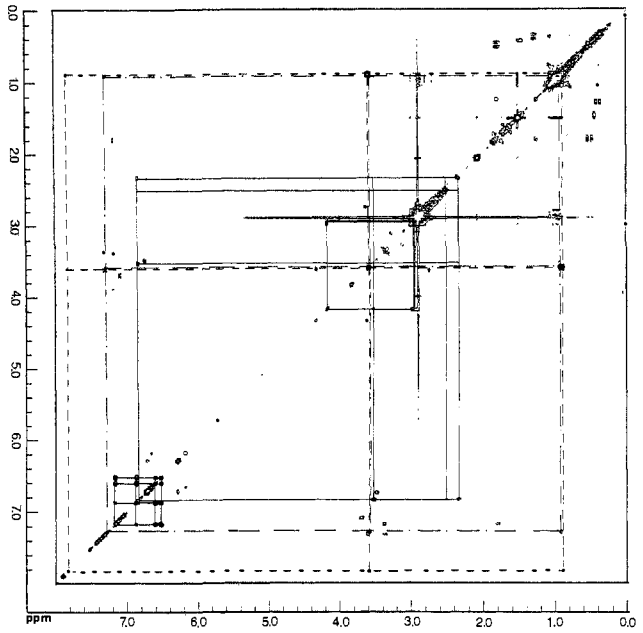


Figure 4. ^{1}H NMR 2D relayed COSY of TA IIIc (500.13 MHz, Me_2SO-d_6). As examples the cross peaks arising from J couplings between tryptophanol protons have been traced on the contour map (—) as well as those of Ala A (- - -) and Ala B (- · -).

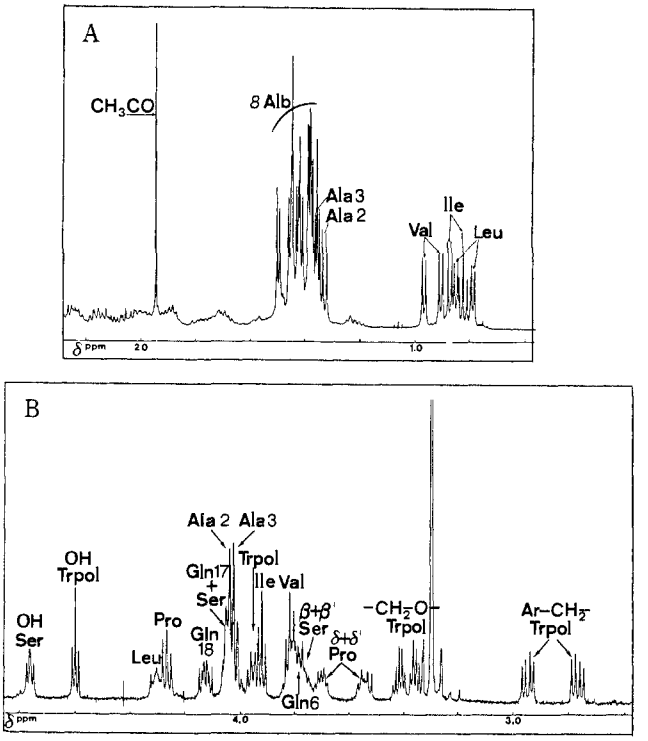
preliminary circular dichroism measurements (TFE solution), it may be estimated that 50%–60% of the TA IIIc backbone is in a right helix. The large magnetic nonequivalence observed for the geminal β-methyl groups of Aib in the ^{13}C NMR spectrum ($\Delta\delta \sim 4$, Figure 2) is indicative of a high content of preferred conformation[24–26] and may be also interpreted by their implication in a helix.

All these results are consistent with a structural conformation with a helical N-terminal end. It is noteworthy that most of the Aib units are located toward this part.

Two types of right-handed helical conformations are usually discussed for the hydrophobic peptides containing the C^α dialkylated Aib, which implies restriction of backbone conformation flexibility: the α (3.6_{13}) and the 3_{10} helix.[27–29] Nevertheless, as these two helices differ by small variations of ϕ and ψ angles (α helix: $\phi = -55°$, $\psi = -45°$; 3_{10} helix: $\phi = -60°$, $\psi = -30°$)[27] neither the $^3J_{NH\text{-}C^\alpha H}$ values, nor CD curves, nor magnetic nonequivalence can provide an unquestionable distinction between the two conformations.

But as an α helix is stabilized by intramolecular hydrogen bonds of the 5 → 1 type and a 3_{10} helix by H bonds of the 4 → 1 type,[27] a main distinction affects the first turn: the three NH protons of the first three amino acids of an α helix are not involved in the intramolecular hydrogen bonding and thus are exposed to the solvent, while only two NH protons are in such a situation in a 3_{10} helix. Therefore we have studied the exchange of the NH protons with the solvent.

A transfer of solvent saturation ^{1}H NMR experiment (CD_3OH) pointed out only the two amide hydrogens of the two N-terminal residues Aib-1 and Ala-2 undergo significant chemical exchange



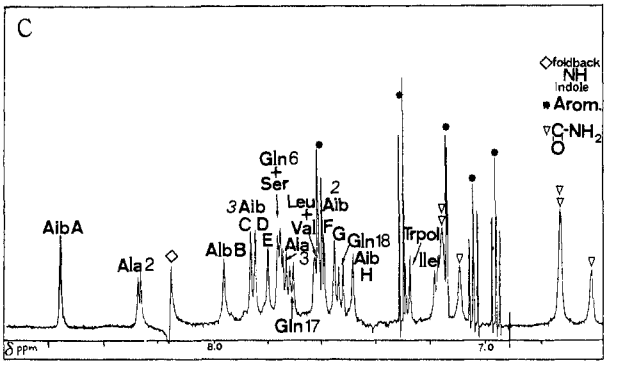


Figure 5. ^{1}H resolution-enhanced NMR spectrum of TA IIIc (500.13 MHz, Me_2SO-d_6): (A) methyl region; (B) $C^\alpha H$ region; (C) NH region. The data have been processed with a Lorentzian-to-Gaussian transformation to improve resolution (LB = −0.5, GB = 0.3).

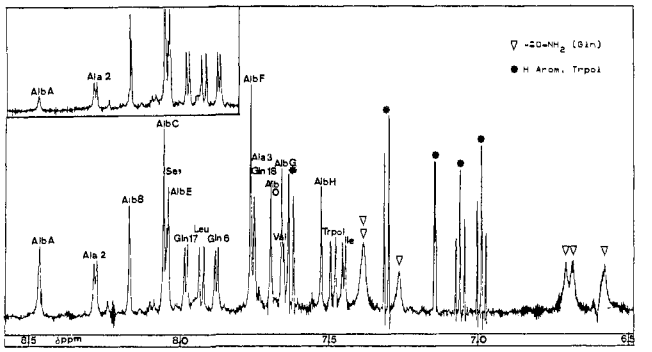


Figure 6. ^{1}H NMR spectrum of TA IIIc NH region (500.13 MHz, CD_3OH). Inset: transfer of solvent-saturation experiment.

with the solvent hydrogens (Figure 6).

We have measured the temperature dependence of the NH protons' chemical shifts in Me_2SO-d_6 and CD_3OH and observed a linear variation of $\Delta\delta$ with increasing or decreasing temperature (Figure 7). In such solvents high $\Delta\delta/\Delta T$ values (about 6×10^{-3} ppm/°C) correspond to solvated NH groups, whereas low values ($<2 \times 10^{-3}$ ppm/°C) are typical of NH groups involved in intramolecular H bonding.[30,31]

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Table I. ^{1}H NMR Spectrum (500.13 MHz, Me_2SO-d_6) of TA IIIc

amino acid	sequence assignt	NH δ	NH mult[a]	NH $^3J_{NH-C^\alpha H}$, Hz	NH NOE[b,c] with H^α of	NH $-\Delta\delta/\Delta T \times 10^3$	$C^\alpha H$ δ	$C^\alpha H$ mult[a]	$C^\alpha H$ J, Hz	$C^\alpha H$ NOE[h,c] with NH of	other groups[a] δ (J Hz)
Aib A	1	8.539	s		acetyl	4.67					
Aib B	12	7.946	s		Leu	1.22					
Aib C	7	7.847	s		Ala, Gln A	1.62					1.498, 1.488, 1.450, 1.446
Aib D		7.831	s			1.30					1.436, 1.436, 1.426, 1.418 } 16 CH_3^β
Aib E		7.785	s			1.40					1.414, 1.404, 1.384, 1.380
Aib F		7.579	s			1.03					1.375, 1.372, 1.365, 1.350
Aib G		7.541	s			1.70					
Aib H	15	7.472	s		Val	0.10					
Ala A	2	8.251	d	5.5		3.90	4.036	m			1.324 d (7.3) CH_3^β
Ala B	3	7.724	d	6.0	Ala A	0.22	4.024	m			1.349 d (7.3) CH_3^β
Gln A	6	7.744	d	4.3	Ser[e]	1.56[f]	3.790	m		Aib C, Aib H[d]	7.146 br s and 6.720 br s $CONH_2$
Gln B	17	7.698	d	6.6	Ile	1.70	4.036	m			7.146 br s and 6.720 br s } 2 $CONH_2$
Gln C	18	7.517	d	8.0	Gln B, Ser[d]	2.10	4.123	ddd	9.2, 8.0, 4.8	Trpol	7.080 br s and 6.609 br s
Ile	16	7.166	d	7.0		1.92	3.919	t	7.0	Gln B	0.869 d (6.8) CH_3^γ, 0.821 t (7.4) CH_3^δ
Leu	11	7.605	d	7.9	Ser, Val[d]	0.95[g]	4.301	m		Aib B	0.847 d (6.4) and 0.785 d (6.4) 2 CH_3^δ
Pro	13						4.262	t	7.7	Val	3.700 m and 3.562 m CH_2^δ
Ser	10	7.744	d	4.8	Gln A[d]	1.56[f]	4.036	m			3.790 m CH_2O^β, 4.760 dd (5.5, 7.0) OH
Val	14	7.605	d	7.3	Pro, Leu[e]	0.95[g]	3.815	t	7.3	Aib H, Aib C[d]	2.308 m CH^β, 0.964 d (6.6) and 0.902 d (6.8) 2 CH_3^γ
Trpol	19	7.272	d	8.4	Gln C	2.17	3.952	m			4.597 t (6.0) OH, 3.408 dt (11.5, 6.0), and 3.449 dt (11.5, 6.0) CH_2O, 2.94 dd (14.7, 6.3) and 2.766 dd (14.7, 7.1) CH_2Ar, 9.74 d (2.4) NH indole; Ar: 7.595 (H4'), 7.296 (H7'), 7.127 (H2'), 7.032 (H6'), 6.951 (H5')
acetyl							1.941	s			

[a] s = singlet, br s = broad singlet, d = doublet, t = triplet, and m = multiplet. [b] When NH is irradiated NOEs are observed with H^α of listed amino acids. [c] NOEs between protons within an amino acid are not indicated. [d,e] Some of the NOEs observed are not due to structural vicinity but to overlapping of $C^\alpha H^d$ or NH^e signals. [f,g] Signals overlap in the temperature range. [h] When $C^\alpha H$ is irradiated NOEs are observed with NH of listed amino acids.

Table II. ^{1}H NMR Spectrum of TA IIIc (500.13 MHz, CD_3OH)

δ	mult	$^3J_{NH-C^\alpha H}$,[a] Hz	assign	$-\Delta\delta/\Delta T \times 10^3$	δ	mult	$^3J_{NH-C^\alpha H}$,[a] Hz	assign	$-\Delta\delta/\Delta T \times 10^3$
8.471	s		Aib-1 (A)	5.75	7.691	s		Aib D	2.02
8.286	d	4.6	Ala-2	5.57	7.656	s		Aib G	3.00
8.167	s		Aib-12 (B)	3.55	7.638	d	7.4	Val-14	−0.58
8.055	s		Aib-7 (C)	3.55	7.521	s		Aib-15 (H)	−1.00
8.050	d	4.3	Ser-10	2.16	7.484	d	9.2	Trpol-19	3.55
8.040	s		Aib E	3.20	7.449	d	5.1	Ile-16	3.06
7.980	d	5.2	Gln-17	3.77	7.387	br s		$CONH_2$ Gln-6 & Gln	5.14
7.927	d	8.0	Leu-11	2.62	7.264	br s		$CONH_2$ Gln	6.32
7.876	d	5.5	Gln-6	2.70	6.707	br s		$CONH_2$ Gln-6	6.50
7.759	s		Aib F	1.42	6.686	br s		$CONH_2$ Gln	7.05
7.752	d	6.3	Ala-3	−0.75	6.579	br s		$CONH_2$ Gln	6.12
7.752	d	7.6	Gln-18	1.50					

[a] Some of the $^3J_{NH-C^\alpha H}$ coupling constants are slightly different from those determined in the 400-MHz spectrum[15] due to the better signal separation.

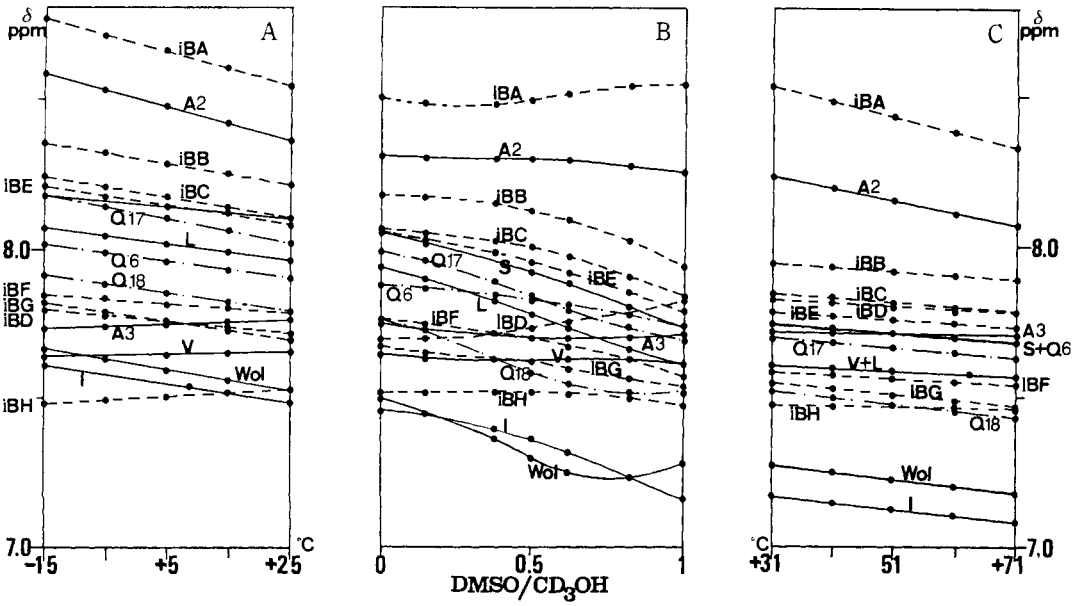

Figure 7. ^{1}H NMR spectrum of TA IIIc: temperature dependence of NH chemical shifts in CD_3OH (A) and Me_2SO-d_6 (C); solvent dependence of NH chemical shifts in CD_3OH/Me_2SO mixture (B) (32 °C). The usual amino acids are designated according to the one-letter code; Aib (---) is designated iB and Trpol is designated Wol.

The measured values of $\Delta\delta/\Delta T$ listed in Table I (Me_2SO-d_6) and Table II (CD_3OH) show those ratios to be separated into three classes: The class with the highest values includes the primary carboxamide groups of the Gln residues and the NH of Aib-1 and Ala-2, showing all these amide protons to be clearly solvent exposed. The class with the lowest values encomposses only the NH of Ala-3, Aib-15, and Val-14, the amide protons of which must be tied up in strong intramolecular hydrogen bonding. The remaining NH groups have moderate ratio values and appear to be partially solvent exposed.

These results show the third residue in the sequence, Ala-3, to be engaged in a strong intramolecular hydrogen bond: therefore the first turn of the N-terminal helical part of trichorzianine A IIIc must be of the 3_{10} type.

Experimental Section

General Remarks. The melting point is uncorrected and was determined with a Reichert melting point apparatus. Optical rotation was measured on a Perkin-Elmer Model 141 polarimeter. UV and CD spectra were obtained on a Beckman Acta C III spectrophotometer and a Jobin Yvon Mark III dichrograph, respectively.

Isolation of Trichorzianine A (TA). A solid sporulated culture (1 kg) of *Trichoderma harzianum* (ATCC 20672) furnished by Orsan Co. (Patent EP 124.388) was extracted 3 times with acetone (2 L) at room temperature with stirring for 2 days. The extracts were combined, and the solvent was removed under reduced pressure. The gummy residue (~33 g) was dissolved in diethyl ether. The ether-insoluble material was then dissolved in methanol and kept at room temperature for 15 h. A white precipitate mainly composed of mannitol was taken off by filtration, and the methanol-soluble fraction was concentrated and submitted to gel chromatography on Sephadex LH 20 with methanol as eluent. The crude mixture of trichorzianines (8.8 g) that eluted first was then chromatographed on silica gel with 65:24:4 methylene chloride/methanol/water as eluent and 100-mL fractions were collected. Trichorzianines A (5.1 g) were eluted in fractions 7–35.

HPLC Separation of Trichorzianine A IIIc (TA IIIc). High-performance liquid chromatography was carried out with a Waters liquid chromatograph (6000 A and M 45 pumps, a 702 solvent programmer, a WISP 701 automatic injector, and a 481 UV–vis detector).

The HPLC separation of TA IIIc from the TA mixture was performed in two steps. Separation of TA III from the TA mixture (see Figure 1A for analytical conditions) was accomplished on a semipreparative Waters μBondapak C_{18} column (7.8 mm × 30 cm) (eluent, 65:35 ethanol/water, flow rate 2 mL/min); R_f(TA III) =22 min. Separation of TA IIIc from the TA III mixture was accomplished on a semipreparative Waters μPorasil column (7.8 mm × 30 cm) (eluent 70:30 methylene chloride/ethanol, flow rate 4 mL/min) (Figure 1B); R_f(TA IIIc) = 38 min.

TA IIIc Characteristics. The TLC TA IIIc R_f value (SiO_2 Merck F_{254}, 6:2:2 butanol/acetic acid/water was 0.37. The spot was detected under UV light (254 nm); it gave no reaction with ninhydrin but became yellow upon H_2SO_4 spraying and turned purple upon heating.

TA IIIc was obtained as a white powder, very soluble in methanol, that crystallized from an acetonitrile/water mixture: mp 253–254 °C; $[\alpha]^{23}_D$ −25.0° (*c* 0.52, EtOH); UV λ_{max}^{EtOH}, (ε) 284 nm (5 400); circular dichroism (*c* 0.3 mM, 0.1-mm path length cells, $T \sim 25$ °C) λ ($[\theta]_M$ (deg·cm^2·dmol^{-1})) 191.2 (424 274), 206.6 (−287 715), 219.7 (−207 122).

Amino Acid Analysis. Total hydrolysis of TA IIIc was carried out according to the usual procedure for peptides (6 N HCl at 110 °C in sealed tubes for 18 h). Identification of the amino acids was accomplished with an amino acid analyzer,[15] TLC chromatography, and gas chromatography after derivatization.

(a) *N*-Trifluoroacetyl Isopropyl Ester Derivatives. The amino acids (10 mg) from the hydrolysate were heated at 100 °C for 20 min with an anhydrous solution of 3 N hydrogen chloride in 2-propanol. After evaporation of the reagents the residue was dissolved in dichloromethane (500 μL), and 100 μL of trifluoroacetic anhydride was added. The mixture was kept in a sealed tube at 150 °C for 5 min. The reagents were then evaporated, and the derivative was dissolved in dichloromethane for GC analysis.

(b) GC Analysis of *N*-Trifluoroacetyl Isopropyl Ester Derivatives. A 25 m × 0.25 mm glass capillary column of L-valine *tert*-butylamide on OV 225 silicone (Interchim) was used, and temperature was increased at the rate 5 °C/min over the range 50–190 °C. Retention times were compared to those of the derivatives of reference (D or L) amino acids and indicated all the amino acids of the hydrolysate to have the L-configuration. Retention times (min): Aib, 8.6; Ala, 10.7; Val, 4.7; Ile, 13.5; Ser, 14.2; Leu, 15.2; Pro, 16.1; Glu, 23.8.

Lower peaks corresponding to the D isomers were observed in the chromatogram. As their relative intensity increased with increasing time of acidic hydrolysis, they must be attributed to a partial racemization under these conditions.

(c) Tryptophanol Characterization. The peptide (10 mg), 65 mg of $Ba(OH)_2 \cdot 8H_2O$, and 1 mL of water were heated at 125 °C for 24 h in a sealed tube. The reaction mixture was then cooled and its pH adjusted to 6 with 2 N H_2SO_4. It was then heated to boiling and $BaSO_4$ was separated. The aqueous solution was concentrated and chromatographed on TLC (SiO_2, 6:2:2 butanol/acetic acid/water). Tryptophanol (R_f 0.58) was isolated and found to be identical with an authentic sample by cochromatography.

Positive-Ion FAB Mass Spectrometry. Measurements were made with a V.G. Analytical MM ZAB-HF mass spectrometer fitted with an Ion-Tech saddle-field primary atom gun using 8-keV xenon atoms. The sample was dissolved in α-monothioglycerol. The mass resolution was

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about 2500. m/z (relative intensity) 1971 $(M + Na)^+$ (2), 1949 $(M + H)^+$ (7), 1108 (17), 1023 (1), 910 (4), 841 (22), 823 (11), 738 (11), 653 (16), 651 (3), 568 (3), 523 (4), 454 (12), 440 (39), 429 (23), 410 (5), 395 (6), 355 (46), 307 (39), 282 (28), 270 (44), 232 (43), 215 (60), 202 (55), 199 (70), 197 (44), 128 (55), 93 (100). When NaCl is added to the matrix, the $(M + Na)^+$ peak becomes much more prominent than $(M + H)^+$.

NMR Spectroscopy. The 1D ^1H NMR spectra and the 2D experiments were performed on a WM 500 spectrometer (Bruker) equipped with an Aspect 2000 computer using DIS NMR P 830601 software. Internal Me_4Si was used as standard. Concentration for all the spectra was 0.02 M.

Solvent titration experiments were carried out by adding a peptide solution in Me_2SO-d_6 in aliquots to a CD_3OH solution, thus maintaining a fixed concentration of 0.02 M.

The applied pulse sequence allowing the ^1H–^1H relayed COSY experiment was (90°–t–90°–τ–180°–τ–90°–acquisition) (11-μs 90° pulse, 1.5-s relaxation delay, τ = 28 ms). The matrix was symmetrized. The spectral width in F_1 and F_2 was 5000 Hz, the number of data points in F_2 was 2048, and 512 increments of 32 scans were recorded. Before Fourier transformation the data were multiplied with an unshifted sine bell. Zero filling was applied only in F_1.

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Deuterium Isotope Effects on the ^{13}C NMR Spectra of 1-Methylcyclobutyl and Trishomocyclopropenyl Cations[1]

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Abstract: Saunder's deuterium isotopic perturbation technique using ^{13}C NMR spectroscopy has been applied to the 1-methylcyclobutyl and trishomocyclopropenyl cations **1** and **9**. The ^{13}C NMR chemical shift of the nondeuterated methylenes of α,α-dideuterio-1-methylcyclobutyl cation is shielded by 1.27 ppm at −50 °C to 1.41 ppm at −90 °C, implying an equilibrium isotope effect. The observed isotope effect between −50 and −90 °C is interpreted with the involvement of σ-delocalized cyclopropylcarbinyl cations **2**, which rapidly exchange methylene carbons in accordance with the previous ^{13}C NMR spectroscopic studies. However, at lower temperature (−154 °C), the observed spectrum shows no equilibrium isotope effect and closely resembles the spectrum obtained for the all-protio ion (except for a small intrinsic deuterium isotope effect), consistent with a symmetrical σ-bridged structure **6** and not a set of rapidly equilibrating bicyclobutonium ions **4**. Study of Winstein's trishomocyclopropenyl cation **9** upon deuterium substitution at the 3-position shows negligible isotope effect on methine signals (≤0.2 ppm), indicative of the static σ-bridged structure.

The isotopic perturbation method developed by Saunders and co-workers[2] to distinguish rapidly equilibrating systems with low energy barriers (double minima) from symmetric systems (single minimum) has become a versatile tool to study degenerate carbocation rearrangements. By asymmetric deuterium substitution, using ^{13}C NMR spectroscopy, the σ-bridged nature of several carbocations such as 2-norbornyl,[3] 2-bicyclo[2.1.1]hexyl,[4] cyclopropylcarbinyl,[5] and Coates's 9-pentacyclo[4.3.0.0^{2,4}.0.^{3,8}.0.^{5,7}]nonyl cations[3] has been demonstrated. Other rapidly equilibrating carbocationic ystems such as tetramethylethyl,[6] pentamethylethyl,[6] and dimethylcyclopenyl[7] cations, which are degenerate in solution even at −140 °C, were shown to be regular trivalent carbenium ions by this method. In fact, a recent solid-state cross-polarization magic-angle spinning ^{13}C NMR study[8] on the latter cations also indicates their regular trivalent carbenium nature. We now report preparation of deuterium-substituted 1-methylcyclobutyl and trishomocyclopropenyl cations **1** and **9** and their ^{13}C NMR spectroscopic study at low temperatures, which show no equilibrium isotope effect excluding equilibrating ions. Independent evidence for the σ-bridged nature of both cations **1** and **9** was obtained from ^{13}C NMR spectroscopic data.[9,10]

Results and Discussion

1-Methylcyclobutyl Cation. The structure of 1-methylcyclobutyl cation **1** under stable ion conditions has been investigated.[9,11–14] In the ^{13}C NMR spectrum at −80 °C, the ion **1** in SbF_5/SO_2ClF solution exhibits three absorptions; $\delta(^{13}C)$ 163.1 (s), 48.7 (t) and 25.4 (q). The observation of a single average absorption for the methylenes indicates a fast threefold degenerate rearrangement on the NMR time scale. The mechanism for such a process was rationalized[12,13] by the involvement of rapidly equilibrating bisected σ-delocalized 1-methylcyclopropylcarbinyl cations **2** exchanging

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