Biochimica et Biophysica Acta, 507 (1978) 470—484

BBA 77932

TRICHOTOXIN A-40, A NEW MEMBRANE-EXCITING PEPTIDE

PART A. ISOLATION, CHARACTERIZATION AND CONFORMATION *

GERHARD IRMSCHER [a], GÜNTER BOVERMANN [a], GÜNTHER BOHEIM [b,**] and GÜNTHER JUNG [a]

[a] *Institut für Organische Chemie der Universität Tübingen, D-7400 Tübingen and* [b] *Fachbereich Biologie der Universität Konstanz, D-7750 Konstanz (G.F.R.)*

(Received July 6th, 1977)

Summary

The new polypeptide antibiotic trichotoxin A-40 is isolated by chloroform/methanol extraction from the dry mycelium of *Trichoderma viride* NRRL 5242. The lipophilic peptide is purified by chromatography on Kieselgel H-60 and reverse-phase chromatography on Lichrosorb RP-8. The new antibiotic differs in amino acid composition and various chemical and physicochemical properties from similar peptides such as trichotoxin A, the suzukacillins or alamethicins. The amino acid composition is $(Pro)_1$ $(Gly)_1$ $(Ala)_2$ $(Leu)_2$ $(Aib)_{10}$ $(Glx)_2$. (Aib, α-aminoisobutyric acid.) The antibiotic has a carboxyl group which can be esterified by diazomethane, which results in slightly enhanced membrane-modifying activities.

The peptide exhibits a right-handed α-helical conformation increasing about two-fold from aqueous to lipophilic media as shown by solvent-dependent circular dichroism measurements. Most of the ^{13}C-NMR resonances can be assigned unequivocally and amino acids situated in the α-helical part show characteristic shift differences from those in the non-helical regions. No β-phenylalaninol residue could be identified by ^{13}C-NMR and ultraviolet spectroscopy, as can be for alamethicins and suzukacillins. A pronounced hemolytic action is found on human erythrocytes, which develops at micromolar concentrations. Trichotoxin A-40 induces a voltage-dependent ionic conductance in bilayer lipid membranes and it can serve as a new pore-forming model system for structure/activity studies in membrane excitation by peptides.

---

* Published in part as a lecture note in Chemiker-Zeitung (1977) 101, 196—201.

** Present address: Department of Cell Physiology ND4, Ruhr-Universität Bochum, D-4630 Bochum, G.F.R.

Abbreviation: CD, circular dichroism.

Introduction

The problem of solving the complex molecular mechanism of the voltage-dependent pore formation of alamethicin [1—5], requires careful investigations on natural, synthetic and semisynthetic analogues in addition to conformational investigations on this peptide antibiotic itself [6]. We reported recently some results from structure/activity studies on suzukacillin A, a peptide antibiotic related to alamethicin [7,8]. Next we intended to isolate and investigate trichotoxin A, a peptide antibiotic that has been found in the mycelium of *Trichoderma viride*, strain NRRL 5242 [9]. This mycotoxin seemed to be very similar to alamethicin and suzukacillin since it has been reported to be cyclic and to contain one residue of the unusual amino acid 2-methylalanine, two proline, one glutamic acid, two glutamine, one glycine, three alanine and three leucine residues. However, we were not able to confirm this report in detail, and we found instead a previously unknown antibiotic that differs from the reported polypeptide in amino acid composition, R_F value, elemental analysis and various chemical and biophysical properties.

We soon detected that this new peptide could serve as a potent natural analogue of alamethicin, since it induces a voltage-dependent pore formation on artificial membranes [10] and it exhibits lytic properties on erythrocytes [11]. We call this new peptide trichotoxin A-40, and we report in the following details of the cultivation procedure, isolation, purification and characterization and some analytical and conformational studies. The information obtained from this investigation will be discussed in Part B [10] with respect to the membrane-modifying properties that have been found for trichotoxin A-40 in single-pore and multi-pore experiments on bilayer lipid membranes.

Materials and Methods

Cultivation of the Trichoderma viride strain and isolation of trichotoxin

Trichoderma viride NRRL 5242 was a gift of the Department of Agriculture, Peoria, Ill., U.S.A. A part of the lyophilized preparation was suspended in sterile water and transferred to agar slants that contained "seeding medium" [12] with 2% agar. After an incubation of 3 days at 27°C the germination, and after 5 days, the sporulation of the fungus was complete and the originally white mycelium had taken on a green colour. 15 Conical Fernbach flasks, (1800 ml) each containing 500 ml Raulin-Thom medium, were inoculated with the spores obtained from one agar slant. Each flask was inoculated with 1 ml suspension with a spore density of about $5 \cdot 10^8$ spores per ml. The mycelium plates obtained after an incubation of about 12 days at 27°C were lyophilized. The cultivation conditions correspond to those reported by Hou et al. [9]. The yield of about 105 g dry mycelium was powdered in a powder mill and extracted with 250 ml chloroform/methanol (1 : 1, v/v) under stirring for 12 h at room temperature. The suspension was separated by centrifugation and the sediment was washed twice with 250 ml methanol. The solutions were combined (Extract I, Fig. 1) and evaporated at water-pump pressure and 40°C bath temperature. The residual brown oil was dried for 12 h at 40°C and 0.2 bar. The resulting powder (16 g) was extracted with 3 × 50 ml light petroleum (b.p.

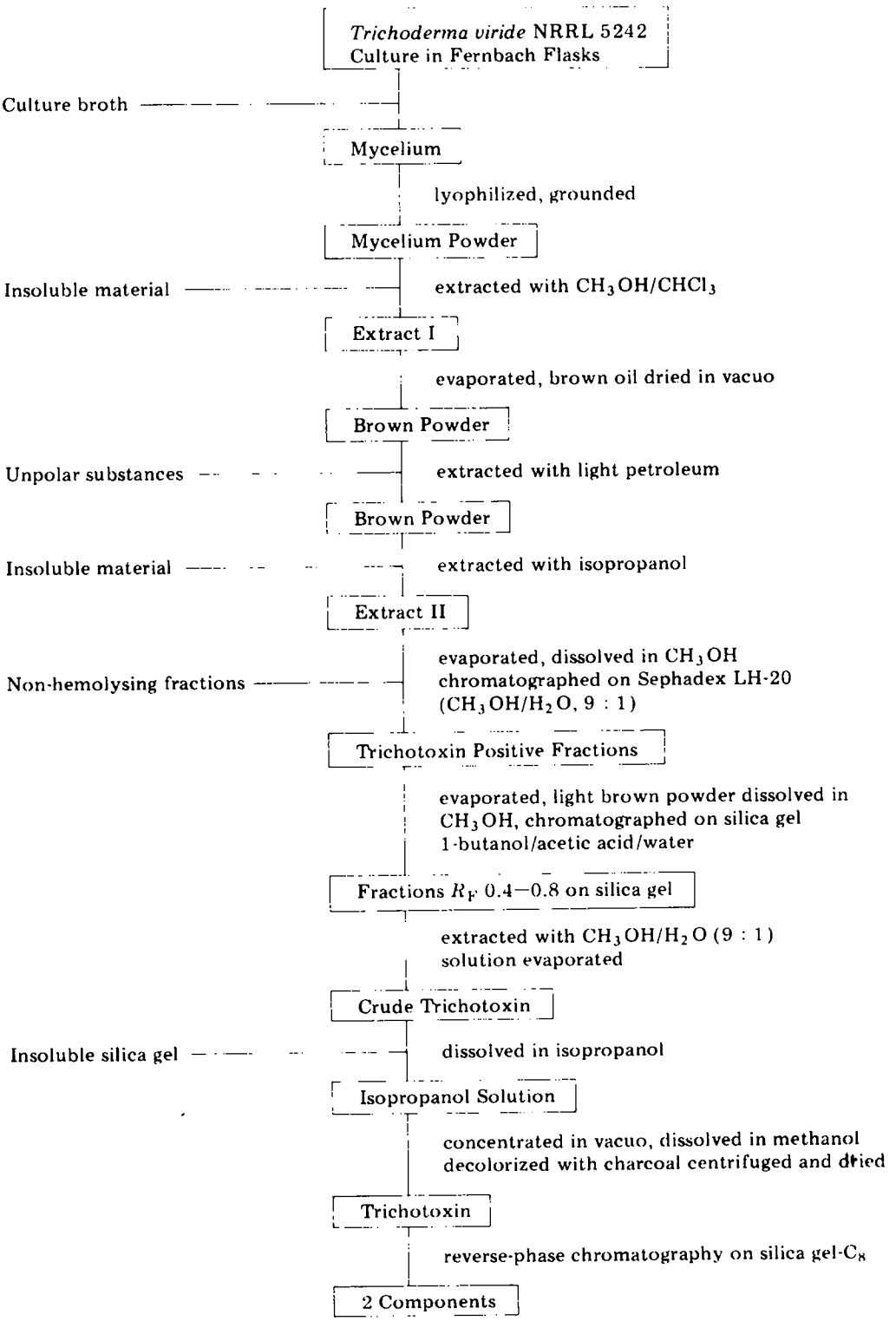


Fig. 1. Isolation and purification of trichotoxin A-40.

30—50°C) to remove non polar compounds. Then the residue (14 g) was extracted with 3 × 20 ml isopropanol (Extract II) and the isopropanol solution was evaporated. The residual brown powder was dissolved in 20 ml methanol and chromatographed in 5-ml portions on a Sephadex LH-20 column (4.5 × 50 cm) with methanol (1 ml per min). Crude trichotoxin was found by thin-layer chromatography and by tests of the hemolytic activity (see below) in the 10-ml fractions between fraction No. 21 and 24. These fractions were combined and evaporated to yield a brownish residue.

Purification of crude trichotoxin

The crude peptide antibiotic was purified by preparative thin-layer chromatography on 20 × 20 cm plates covered with 1 mm silica gel type 60 (E. Merck, Darmstadt). The brownish residue obtained (see above) was dissolved in 5 ml methanol and 1 ml of the solution was applied to each plate. The plates were chromatographed in the solvent system 1-butanol/acetic acid/water (3 : 1 : 1, v/v). After drying the silica gel layer, between R_F 0.4 and 0.8 was sampled and extracted with 3 × 50 ml 90% methanol. The combined extracts were evaporated to dryness and extracted with 5 ml dry isopropanol to remove traces of silica gel. After centrifugation of the suspension the clear, light brown supernatant solution was decanted, evaporated to dryness, redissolved in 3 ml methanol and shaken with charcoal (Darco G 60) sufficient to decolourize the solution. The charcoal was centrifuged down, the supernatant decanted and evaporated to dryness. The yield was 25 mg trichotoxin. These cultivation and isolation procedures were repeated ten times resulting in an overall yield of about 250 mg peptide antibiotic. This preparation was chromatographed in 100-mg portions on the reverse-phase Lichrosorb RP-8 in a prepacked column (Lobar® column size B, 25 × 310 mm, E. Merck, Darmstadt) using methanol/water (94.5 : 5.5, v/v) for elution (40 ml/h). The eluate was sampled in 5-ml fractions and trichotoxin-positive fractions were detected by thin-layer chromatography and by the hemolysis test [11]. Trichotoxin A-40 was eluated at first and well separated from its analogue A-50.

Characterization and chemical modification

For analytical thin-layer chromatography preformed silica gel plates (0.2 mm thickness, E. Merck) and the solvent system chloroform/methanol/water (13 : 5 : 0.8, v/v) were used. The main component of the purified peptide had an R_F value of 0.41 (trichotoxin A-40). A second component with R_F 0.48 (trichotoxin A-50) could be identified in amounts up to 30% in the crude peptide.

High-performance liquid chromatography on μ-Bondapak C-18 (Waters) using a methanol/water/isopropanol system (7 : 2 : 1, v/v) and thin-layer chromatography in the system chloroform/methanol/water (13 : 5 : 0.4) and chloroform/methanol/ammonia (17%, 14 : 7 : 2) separated both the analogues A-40 and A-50.

Trichotoxin A-50 was also active in lipid membranes and showed hemolysing properties (Boheim, G., Irmscher, G. and Jung, G., unpublished). The lipophilic peptides can be visualized as white spots after spraying the plate with water and slowly drying. The ninhydrin reaction is negative; however, this does not necessarily imply that such a peptide is cyclic as reported for trichotoxin A [9],

since the N-terminus may be acylated [13]. The chlorine/tolidine reaction is relatively weak and our peptide cannot be visualized by the anisaldehyde/sulfuric acid colour reaction as reported for trichotoxin A [9]. Trichotoxin A-40 decomposes at 158—160°C in the melting point glass capillary.

X-Ray photoelectron spectra were measured in order to detect the presence of inorganic material, atoms like Cl, Br, S, P, or metals. This technique is described elsewhere in detail and has been applied also for alamethicin and suzukacillin [7]. Except for very small traces of Na^+, Cl^-, Br^- (?), J^- (?) or Si^{4+} from silica gel nothing else could be detected in trichotoxin A-40.

Samples of purified peptide are hydrolysed with 6 M HCl in sealed ampoules at 110°C for 24, 48, 60 and 72 h and analyzed in a Beckmann-Unichrom amino acid analyzer. The 2-methylalanine content was determined by the aid of a standard solution. The low colour-yield of α-aminoisobutyric acid (Aib) has been occasionally overlooked [9,14]. The recovery of amino acids was optimal at a hydrolysis time of 48 h and the following composition was obtained for trichotoxin A-40: $(Glx)_2$ $(Pro)_1$ $(Gly)_1$ $(Ala)_2$ $(Aib)_{10}$ $(Leu)_2$. The composition of trichotoxin A-50 is the same except for 12 α-aminoisobutyric acid residues.

Trichotoxin A-40 was automatically titrated with $5 \cdot 10^{-3}$ M NaOH in water/methanol (1 : 1, v/v) and a pK value of 5.4 was determined, which is close to those of alamethicin R_F 30 (pK = 5.5) and suzukacillin-A (pK = 6.1) in the same solvent system. Trichotoxin A-40 was quantitatively methylated in methanol (concn., 10 mg/ml) by slow addition of a freshly prepared etheral diazomethane solution. On silica gel plates developed in $CHCl_3/CH_3OH/H_2O$ (13 : 5 : 0.8, v/v) methylated trichotoxin A-40 had an R_F of 0.64. Titration of methylated trichotoxin A-40 showed the absence of a dissociating group below pH 7 and circular dichroism revealed the same conformation as the untreated peptide. In contrast to trichotoxin A-40, both alamethicin F-50 [11] and suzukacillin A [7] could not be modified by diazomethane despite their similar behaviour in titration experiments. Alamethicin F-50 and suzukacillin A are peptide amides, whereas the alamethicin main component R_F 30 of Upjohn (see ref. 10) has a free carboxylate group.

The peptide chain of trichotoxin A-40 was lengthened by coupling with alanyl-alanine methylester as follows. 20 mg trichotoxin A-40, 1 mg H-L-Ala-Ala-OMe · HCl, 60 μl *N*-ethyl-morpholine and 40 μl of a dicyclohexylcarbodiimide solution (1 mol/l) were reacted in 1 ml dichloromethane for 18 h at 21°C. After evaporation of the solvent the residue was dissolved in 1 ml methanol and applied to silica gel thin-layer plates (20 × 10 cm, thickness 0.2 mm; E. Merck, Darmstadt) and chromatographed in chloroform/methanol/water (13 : 5 : 0.4). The new lipophilic zone (R_F = 0.65) containing the product was extracted with methanol. The solution was evaporated and the residue taken up in isopropanol, centrifuged to remove traces of silica gel, evaporated again and dried in vacuo; yield: 8 mg trichotoxin-alanyl-alanine methylester.

Attempts to identify the L-phenylalaninol residue found in alamethicin [6,13, 15] and suzukacillin A [7] in the structurally closely related peptide antibiotic trichotoxin A-40 by ultraviolet absorption and ^{13}C-NMR did not reveal an aromatic residue. Mild hydrolyses and subsequent thin-layer chromatography carried out in parallel runs with trichotoxin, alamethicin and suzakacillin yielded L-phenylalaninol only for alamethicin and suzukacillin. Partial hydrolyses by 3 M HCl in

methanol/water (3 : 1, v/v) [13] will split off phenylalaninol and the N-terminal *N*-acetyl-2-methylalanine from alamethicin. We found that these conditions damage various other peptide bonds too.

Circular dichroism, ultraviolet absorption and ^{13}C NMR measurements

Ultraviolet spectra were taken on a PMQ II spectrometer (Zeiss, Oberkochen). CD (circular dichroism) spectra were measured on Roussel-Jouan Dichrograph CD 185 and Jasco CD/ORD instruments. Samples were equilibrated at room temperature before the measurements. Solvents were Uvasole® or pure analytical grade (E. Merck). Hexafluoroacetone sesquihydrate and 2,2,3,3-tetrafluoro-1-propanol were products of PCR (Gainsesville, Fla., U.S.A.). Concentrations were 1.2 mg/ml corresponding to 0.6 mmol/l based on a theoretical molecular weight of 1670 for trichotoxin A-40. Each measurement was repeated twice. Molar ellipticities, $[\theta]$, and mean residue ellipticities, $[\theta]_M$, differ within the usual error of CD measurements (about 10%).

Fourier transform ^{13}C-NMR spectra were recorded on a Bruker HFX-90 multinuclear NMR spectrometer equipped with a 38 cm magnet (22628 MHz for ^{13}C). For the ^{13}C NMR measurements a sample of 120 mg trichotoxin was dissolved in 1.5 ml ^{12}C-enriched and deuterated methanol. With proton broad band decoupling 230 000 interferograms were accumulated on an 8K storage with an analogue digital resolution of 6 bits (Bruker Computer BNC-12). The pulse width used was 2.5 μs and the pulse interval was 0.7 s. ^{13}C NMR absorption spectra were obtained by Fourier transformation, and the chemical shifts were automatically read and printed as δ-values in ppm and referred to internal tetramethylsilane as standard (δ = 0 ppm). The sample cell had a temperature of 298 K.

pH-Dependence of the partition coefficients in octanol/water

As standards, the ultraviolet adsorption of solutions of 0.1—0.5 mg peptide antibiotics in 1 ml sodium acetate/acetic acid buffer (concn., 0.1 mol/l, pH 4), Na_2HPO_4/NaH_2PO_4 buffer (concn. 0.1 mol/l, pH 5—8) and 1-octanol was measured at 225 nm (for the acetate buffer at 230 nm). Partition coefficients were obtained as follows: 1.5 mg peptide was dissolved in 3 ml 1-octanol. After addition of 3 ml buffer the mixture was stirred for 60 min at room temperature and centrifuged for 10 min at 5000 rev./min. The extinctions of the clear aqueous and alcoholic phases were measured at 225 and 230 nm, respectively (path length, 1 cm; T, 20°C). The peptide concentrations were determined using the standard curve and the partition coefficient $\gamma = \text{concn.}_{\text{octanol}}/\text{concn.}_{\text{water}}$ was plotted versus the pH.

Results and Discussion

The new antibiotic trichotoxin A-40 is the third peptide exhibiting the particular characteristics of voltage-dependent pore formation and lytic properties in membranes. It differs from alamethicin [16,17] and suzukacillin [18] in lacking valine and L-phenylalaninol. More than 50% of the residues are 2-methylalanines and only one proline and two Glx residues are found. The potentiometric titration revealed a carboxylate group. Methylation by diazomethane revealed a

less polar product in the case of trichotoxin A-40, whereas alamethicin F-50 [11] and suzukacillin A [7] remained unchanged. Trichotoxin A-40 exhibits a higher solubility in less polar solvents when compared to alamethicin or suzukacillin.

Conformational behaviour

CD spectra show three Cotton effects around 221 nm (—), 207 nm (—) and 190 nm (+) characteristic for a right handed α-helical conformation (Fig. 2). The α-helix content has been estimated by comparison with poly(L-glutamic acid) [19] and by the method reported by Rosenkranz and Scholtan [20]. We are aware of the restrictions of these procedures when applied to short and unusual peptides and the strongly differing α-helix values (Table I) should be considered only as relative values. It should be pointed out that standards derived from proteins may not be applicable for the particular α-helices of the alamethicin analogues. Therefore, we are investigating at present suitable models such as the sequential peptides $(Aib\text{-}Ala)_n$ [21]. The α-helix content can be as high as approx. 40% in alcoholic media and less than 20% in phosphate buffer, pH 7, and in aqueous alcohol (Fig. 3). The high values are only possible assuming a helix as long as the alamethicin helix [6]. In contrast to alamethicin and suzukacillin, trichotoxin A-40 exhibits a ratio $[\theta]_{221}/[\theta]_{207} = 0.66 \pm 0.8$ in all solvents remaining below 1, even in aqueous solutions. This may be due to an aggregation behaviour which differs from that of its analogues both showing a reversible variation of this ratio on aggregation [6,7]. It may be possible that trichotoxin A-40 aggregates only to low molecular-weight micelles. In this context one should pay attention to the high content of α-helix-promoting amino acids such as 2-methylalanine and leucine and the

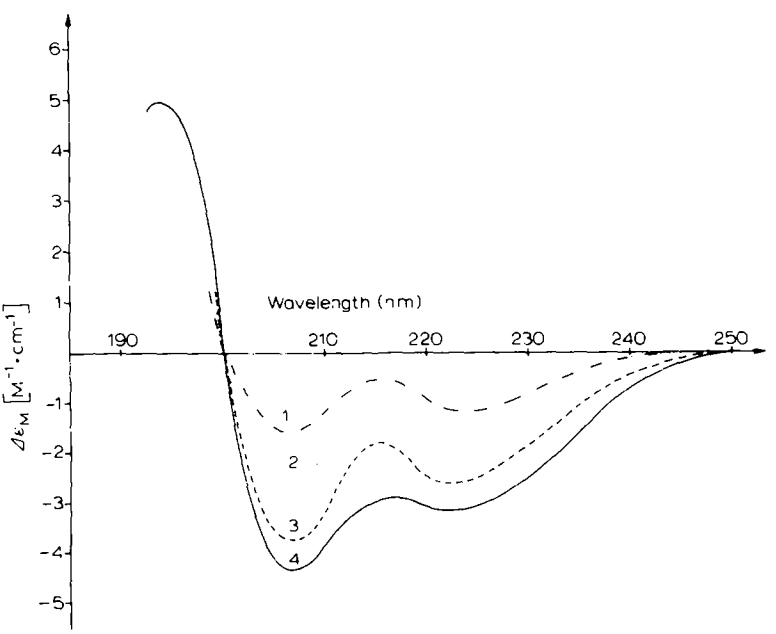


Fig. 2. CD spectra of trichotoxin A-40 in potassium phosphate (0.01 mol/l, pH 7) (1); Hexafluoracetone sesquihydrate (2); trifluorethanol (3); 1-butanol (4); (concn. $7.5 \cdot 10^{-4}$ mol/l; T, 23°C).

TABLE I

SOLVENT DEPENDENCE OF THE CIRCULAR DICHROISM SPECTRA OF TRICHOTOXIN A-40

Concn., 1.2 mg/ml, T, 20°C.

Solvent	λ = 221 nm		λ = 207 nm		$[\theta]_{221}/[\theta]_{207}$	α-helix (%)	
	$[\theta]$	$[\theta]_M$	$[\theta]$	$[\theta]_M$		(a)	(b) *
1-Octanol	−230 500	−12 800	−342 500	−19 050	0.66	29	44
1-Butanol	−223 900	−12 450	−368 800	−20 500	0.61	30	47
2-Propanol	−213 300	−11 800	−342 500	−19 050	0.62	30	44
Methanol	−220 000	−12 200	−303 000	−16 800	0.70	22	39
1,1,1-Trifluoroethanol	−135 300	−7 500	−234 100	−13 000	0.58	14	30
Hexafluoroacetone-sesquihydrate	−92 200	−5 100	−158 100	−8 800	0.58	5	20
Dioxane	−85 600	−4 750	−138 300	−7 700	0.62	—	18
Potassium phosphate (pH 7)	−79 000	−4 400	−118 600	−6 600	0.67	—	15
Ethanol 100%	−220 000	−12 200	−322 700	−17 950	0.67	23	42
95%	−192 100	−10 650	−292 400	−16 250	0.66	23	38
90%	−191 000	−10 600	−270 000	−15 000	0.70	17	35
80%	−184 400	−10 250	−263 500	−14 650	0.70	17	34
70%	−177 900	−9 900	−250 300	−13 900	0.71	12	32
60%	−177 800	−9 900	−256 900	−14 250	0.69	13	33
50%	−177 800	−9 900	−250 300	−13 900	0.71	13	32
40%	−164 700	−9 150	−223 900	−12 450	0.73	9	29
30%	−151 500	−8 400	−230 500	−12 800	0.66	7	30
20%	−138 300	−7 700	−197 600	−10 950	0.70	4	25
10%	−92 200	−5 100	−131 700	−7 300	0.70	—	17

* (a), Calculated according to Rosenkranz and Scholtan [20]; (b), estimated on the CD values of poly-(Glu) [19].

single helix-breaking proline and helix-weakening glycine residues in trichotoxin A-40. According to conformational calculations on the peptide models *N*-acetyl-amino-acid *N*-methylamides the energy minima region is reduced, replacing glycine by alanine and 2-methylalanine from 50 to 20 to 5% of the total region [22]. Therefore, the high content of 2-methylalanine should give rise to a pronounced reduction of the allowed conformations [22,23] for the amino acid residues of the peptide antibiotics from *Trichoderma viride*. The particularly high content (56%) of 2-methylalanine in trochotoxin A-40 imparts different solvent- and temperature dependencies of the conformations of the antibiotics, since alamethicin F-50 has a 2-methylalanine content of only 37% and suzukacillin A of 40%. Measurements of the solvent dependence on the dipole moment of alamethicin [24] revealed close relations between the change in α-helix content from hydrophilic to lipophilic media [6] and the effective length of the peptide. Comparative measurements of the dipole moments of suzukacillin and trichotoxin could be valuable for structure/activity studies since the dipole moment may be correlated with the voltage-dependent conductance in bilayer lipid membranes.

Assignments and discussion of the ^{13}C NMR spectrum of trichotoxin A-40

There is no phenylalaninol residue visible in the aromatic region of the spectrum (Fig. 4a). Trichotoxin contains two alanine and two leucine residues

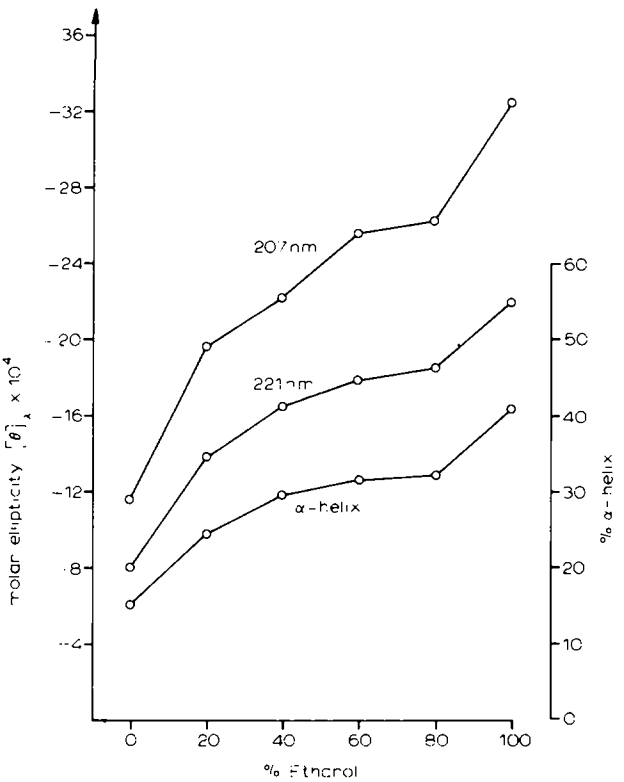
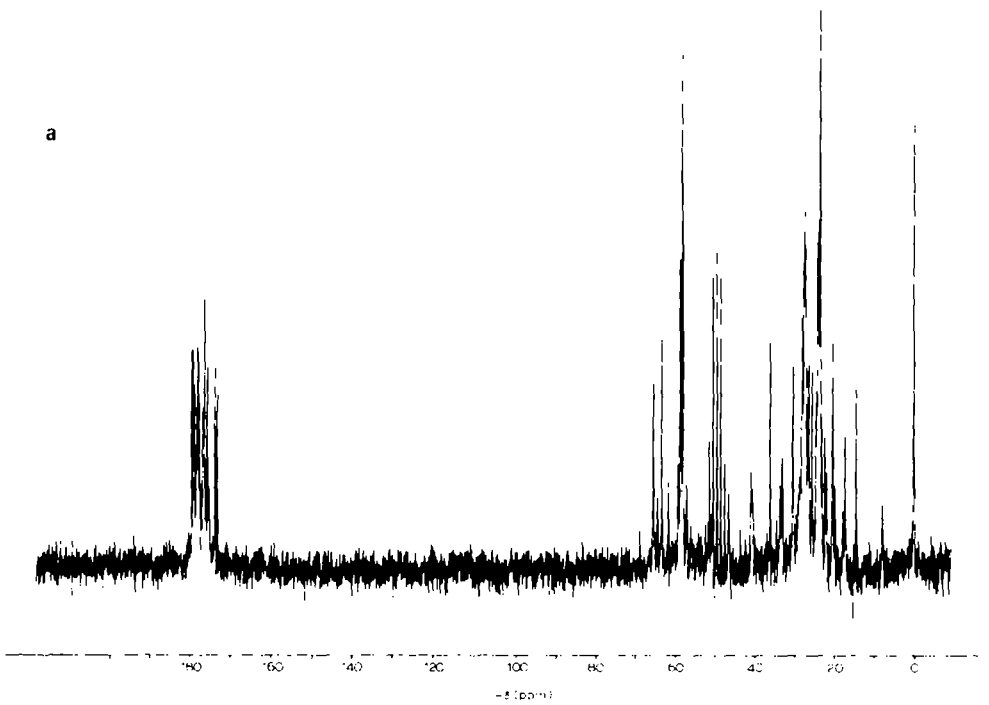


Fig. 3. Solvent dependence of the two negative Cotton effects at 207 nm and 221 nm of trichotoxin A-40 in ethanol/water mixtures (23°C, $7.5 \cdot 10^{-4}$ mol/l) and the estimation of α-helix content [19].

exhibiting six unequivocally assignable methyl signals (Fig. 4b). Since the methyl signals of the two alanines coincide, we can assume that their environments are similar and most probably located within the α-helical part adjacent to 2-methylalanine residues. This conclusion follows from the high field shifting of these methyl signals compared to small, randomly-oriented alanyl peptides. The methyl signals of the two leucines each differ by 0.4 ppm and are magnetically nonequivalent by 2 ppm compared to the usually observed 1.1—1.6 ppm. This could be caused by hindered rotation of the leucyl side chains. The strongest resonances are caused by coinciding signals of about twelve β-methyl groups of 2-methylalanine and about eight β-methyl groups corresponding to four α-aminoisobutyric acid residues 4 ppm downfield. We attribute the strong signal group at about 23.3 ppm to α-helical α-aminoisobutyric residues and that at about 27 ppm to non-helical residues [6]. The signal group at 23.3 ppm is relatively sharp due to faster rotation of the methyl groups bonded to the helical backbone. Furthermore, the signals of the methyl groups of each α-aminoisobutyric residue differ due to magnetic nonequivalence, because of neighbouring chiral amino acids (approx. 0—0.5 ppm), and to shift effects of the differently-substituted amino acid neighbours (approx. 0—1 ppm) [6].

In contrast to alamethicin there is only one proline residue in trichotoxin



For legend, see next page.

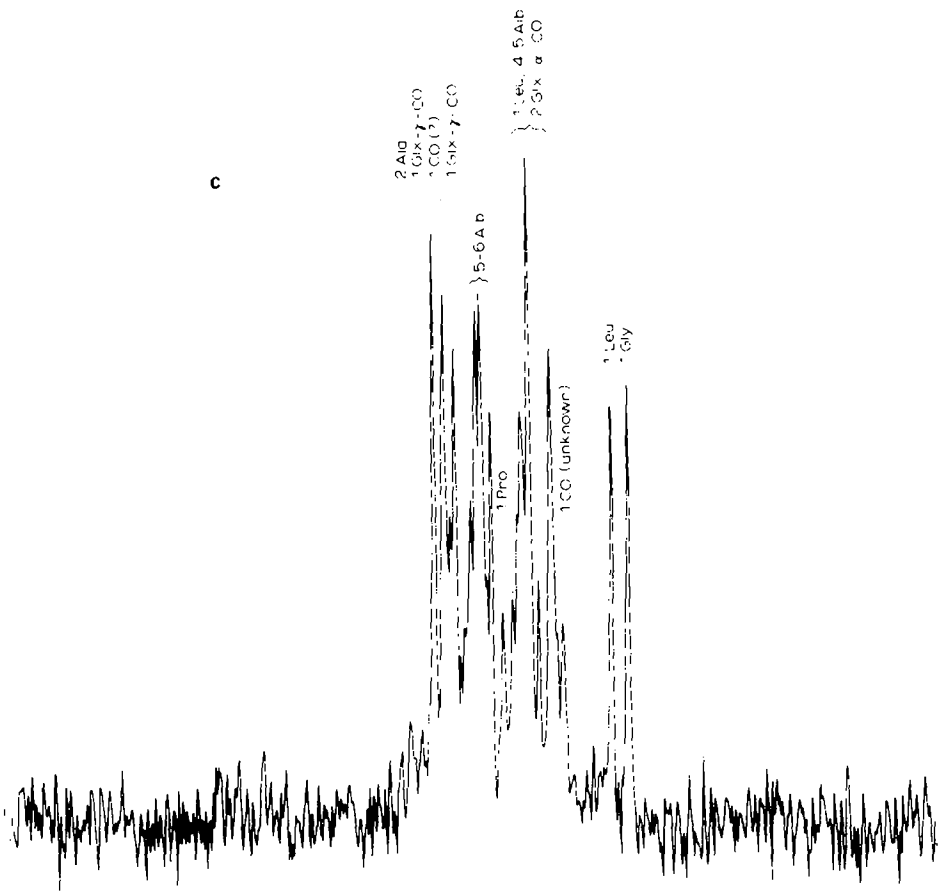


Fig. 4. a. Fourier transform ^{13}C NMR spectrum of trichotoxin A-40 in [^{12}C, ^{2}H]methanol (concn., 80 mg/ml; T, 298 K; 22 628 MHz; 230 000 scans). b. Aliphatic region of the ^{13}C NMR spectrum of trichotoxin A-40 (expanded plot). c. Carbonyl part of the ^{13}C NMR spectrum of trichotoxin A-40 (expanded plot).

A-40 to which we assign the C_γ signal at 24.2 ppm (Table II). We may attribute the signals at 25.3 and 26.1 to the C_γ of the two leucines, although the high-field signal is rather sharp compared to the other C_γ signal. The C_β of the single proline absorbs at 30.0 ppm and the two Glx C_γ absorb at 32.7 and 33.2 ppm. The two leucine C_β signals show only a minor shift difference. The single Gly C_α signal is found at 46.1 ppm. Partially covered by the solvent septett, the proline C_δ signal can be detected at 50.3 ppm.

The C_α region is dominated by two intense signals due to ten 2-methylalanine C_α carbons. The alanine C_α absorb at 56.5 ppm, whereas the two leucine C_α signals are hidden behind the Aib C_α signals. Compared to model peptides and the analogues [6,7] the leucine C_α resonances are unusually low-field shifted. The signals at 58.6 and 61.0 must be attributed to the Glx C_α carbons. The shift difference between these signals is larger than that in alamethicin and suzukacillin. The signal of the proline C_α is found at lowest field compared to all other C_α carbons. Several high-field signals are not compatible

TABLE II

CORRELATION OF THE ASSIGNMENTS OF THE ^{13}C NMR SIGNALS OF TRICHOTOXIN A-40 AND ALAMETHICIN

Trichotoxin A-40 in [^{12}C, ^{2}H]methanol				Alamethicin [6] in [^{12}C, ^{2}H]methanol			
δ (ppm)	Carbon atoms	δ (ppm)	Carbon atoms	δ (ppm)	Carbon atoms	δ (ppm)	Carbon atoms
7.7	1 CH_3 (unknown)	56.5	1—2 Ala-C_α	7.2	1 CH_3 (unknown)	55.5	1 Glx C_α
14.2	1-butanol C-4	57.4	10 Aib C_α	—		56.8	2 Glx C_α
17.0	2 Ala C_β	58.0	2 Leu C_α	17.0	2 Ala C_β	57.2	
19.6	1 Leu $C_{\delta a}$	58.6	1 Glx C_α	19.6	1 Val $C_{\gamma a}$	57.4	
20.0	1 Leu $C_{\delta a}$;	61.0	1 Glx C_α	20.3	1 Val $C_{\gamma b}$	57.6	7Aib C_α,
	1-butanol C-3	62.6	1-butanol C-1			58.0	1 pheol *
21.6	1 Leu $C_{\delta b}$			21.4	1 Leu $C_{\delta a}$		
22.0	1 Leu $C_{\delta b}$	63.7	1 Pro C_α	22.5	1 Leu $C_{\delta b}$	—	
22.8		64.7	CH_2OH (unknown)			63.2	1 Val C_α
23.2	12 Aib C_β	—		23.2	6 Aib C_β	63.9	2 Pro C_α,
23.5		172.7	1 Gly CO	23.5			1 pheol *-CH_2OH
23.8		173.2	1 Leu CO				
24.2	1 Pro C_γ	174.7	1 CO (unknown)	23.9	2 Pro C_γ		
25.3	1 Leu C_γ	—	—	25.6	1 Leu C_γ	—	—
26.1	1 Leu C_γ	175.1				64.9	1 Val C_α
26.8	8 Aib C_β	175.5	1 Leu CO	26.7		172.4	1 Gly CO
27.0	2 Glx C_β	175.8	4—5 Aib	27.1	8 Aib C_β	172.9	1 Val CO
27.7		176.1	2 Glx α-CO	27.4	3 Glx C_β	—	—
		176.3		27.9		174.0	1 Glu α-CO
30.0	1 Pro C_β	176.7	1 Pro CO	29.7	2 Pro C_β,	175.0	1 Val CO
32.7	1 Glx C_γ	177.0		30.4	2 Val C_β	175.2	1 Gln α-CO, 1 CO
33.2	1 Glx C_γ	177.3	5—6 Aib CO	31.8	1 Glx C_γ	175.5	1 Gln α-CO, 1 CO
35.7	1-butanol C-2	177.5		32.5	2 Glx C_γ	175.7	4 Aib CO
—		177.7		32.9		176.3	2 Pro
40.2	1 Leu C_β			—		177.0	
40.5	1 Leu C_β	178.2	1 Glx γ-CO	37.9	1 pheol *-CH_2-C_6H_5	177.2	
46.1	1 Gly C_α	178.4	1 CO (?)	--		177.6	3 Aib CO
50.3	1 Pro C_δ	178.5	1 Glx γ-CO	41.4	1 Leu C_β	177.8	2 Glx γ-CO
—		178.8	2 Ala CO	44.8	1 Gly C_α	177.9	
—				49.9	2 Pro C_δ	178.1	1 Glx γ-CO
				50.7			
				53.9	2 Ala C_α	178.4	1 CO (?)
				54.4	1 Leu C_α	178.8	2 Ala CO
						178.9	

* Pheol, β-phenylalaninol.

with the amino acid composition. There is a methyl signal at 7.7 ppm, which we have located already in the spectra of suzukacillin A (7.2 ppm) and of freshly prepared alamethicin. Near the proline and Glx C_α there are two signals assignable to CH_2OH groups. Solvent peaks at 14.2, 20.0, 35.7 and 62.6 ppm result from 1-butanol used in the final purification. Between 170 and 180 ppm there are signal groups corresponding to at least 20 CO carbons (Fig. 4c) in agreement with the number of peptide carbonyl groups derived from the 18 amino acids including two Glx γ-carbonyl groups (Table II).

Hemolytic properties and partition coefficient in water/octanol

The lytic action on human erythrocytes caused by trichotoxin A-40 resembles that of the alamethicins and suzukacillins and develops at concentrations of $2 \cdot 10^{-5}$ mol/l in phosphate buffer, pH 7, within an incubation time of 60 min at 37°C [11]. Alamethicin and its analogues constitute a group of cytolytic polypeptides, which develop their activity in the micromolar range between the more active bee-venom constituent melittin [25] and the less active detergent sodium dodecyl sulfate.

Suzukacillin A has the highest partition coefficient, γ, in the 1-octanol/water system at all pH values (Fig. 5) and it is the most active peptide in hemolytic

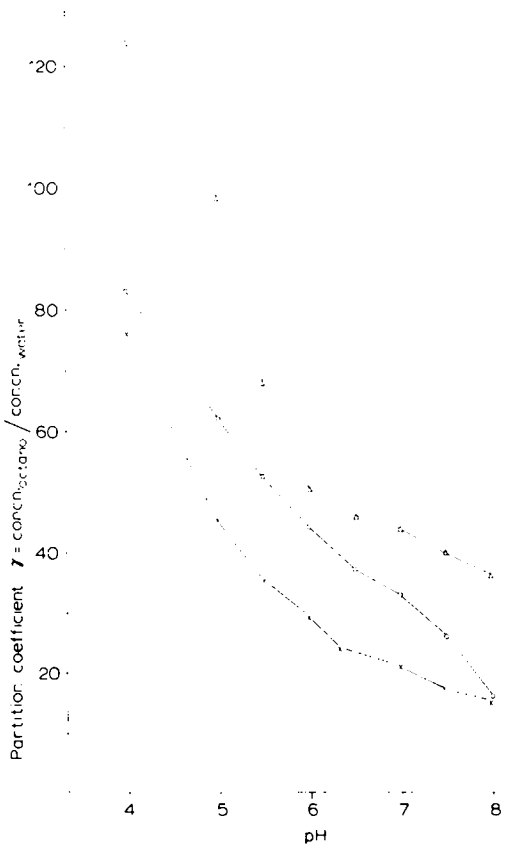


Fig. 5. Partition coefficients of trichotoxin A-40 (×), alamethicin F-50 (○) and suzukacillin A (△) on variation of the pH in the system 1-octanol/water.

experiments [11]. Lower partition coefficients are observed for alamethicin F-50 and the smallest values are found for trichotoxin A-40 (Fig. 5) despite its excellent solubility in lipophilic solvents. Particularly interesting is the strong decrease in γ in 1-octanol from pH 4 to pH 6 for all three peptides. The partition coefficient exhibits a particularly strong decrease for suzukacillin A in the region of pH 4—5.5 and a relatively smaller decrease for alamethicin. In the region of pH 6.5—8.0, suzukacillin A and trichotoxin A-40 exhibit a smaller decrease in γ, whereas in the case of alamethicin F-50, γ decreases more or less steadily in all pH regions investigated.

The numeric addition of the concentration values (%) of the aqueous and alcoholic phases gave only 90—95% of the applied material. This could be due to antibiotic adsorption at the water/octanol interphase.

Conclusion

A new polypeptide antibiotic has been found in the mycelium of a strain of *Trichoderma viride* which exhibits membrane modifying properties similar to those of alamethicin. The isolated material consists of two closely related analogues, trichotoxin A-40 and trichotoxin A-50. Trichotoxin A-40 is lacking the phenylalaninol component found in the alamethicins and suzukacillins and it shows an enhanced content of 2-methyl-alanine. Circular dichroism revealed a right-handed α-helix, a pronounced solvent- and temperature-inducable flexibility of its conformation and aggregation phenomena in aqueous media. Most of the ^{13}C-NMR signals are assignable to α-helical and nonhelical residues and the spectrum agrees well with the amino acid composition. The partition coefficient in 1-octanol/water shows a pH dependence which does not differ significantly from those of alamethicin F-50 and suzukacillin A. The main difference between trichotoxin A-40 and its natural analogues is the different aggregation behaviour of these peptides and the different flexibility of their α-helical conformation. The membrane-modifying properties seem to depend largely on these two prerequisite structural parameters. Relatively wide ranges of allowed sequential variations seem to be possible without loss of the lytic [11] and pore-forming [10] activities.

Acknowledgements

We gratefully acknowledge the support of this study by a grant of the Deutsche Forschungsgemeinschaft given to G.J. (SFB 76).

References

1 Mueller, P. and Rudin, D.O. (1968) Nature (Lond.) 217, 713—719
2 Eisenberg, M., Hall, J.E. and Mead, C.A. (1973) J. Membrane Biol. 14, 143—176
3 Boheim, G. (1974) J. Membrane Biol. 19, 277—303, and references cited therein
4 Baumann, G. and Mueller, P. (1974) J. Supramolec. Struct. 2, 538—557
5 Gordon, L.G.M. and Haydon, D.A. (1975) Phil. Trans. R. Soc. Lond. B 270, 433—447
6 Jung, G., Dubischar, N. and Leibfritz, D. (1975) Eur. J. Biochem. 54, 395—409
7 Jung, G., König, W.A., Leibfritz, D., Ooka, T., Janko, K. and Boheim, G. (1975) Biochim. Biophys. Acta 433, 164—181

8 Boheim, G., Janko, K., Leibfritz, D., Ooka, T., König, W.A. and Jung, G. (1975) Biochim. Biophys. Acta 433, 182—199
9 Hou, C.T., Ciegler, A. and Hesseltine, C.W. (1972) Appl. Microbiol. 23, 183—185
10 Boheim, G., Irmscher, G. and Jung, G. (1978) Biochim. Biophys. Acta 507, 485—506
11 Irmscher, G. and Jung, G. (1977) Eur. J. Biochem., 80, 165—174
12 Patentschrift der Upjohn Company, Nr. C 12d/A61 h, Paris
13 Martin, D.R. and Williams, R.J.P. (1976) Biochem. J. 153, 181—190
14 Ooka, T., Shimojima, Y., Akimoto, T., Takeda, I., Senoh, S. and Abe, J. (1966) Agric. Biol. Chem. 30, 700—702
15 Jung, G., Dubischar, N., Leibfritz, D., Ottnad, M., Probst, H. and Stumpf, D. (1974) Proc. 13th Euro. Peptide Symp. (Wolman, Y., ed.), pp. 345—354, John Wiley and Sons, New York
16 Reusser, F. (1967) J. Biol. Chem. 243, 243—247
17 Kleinkauf, H. and Rindfleisch, H. (1976) FEBS Lett. 62, 276—280
18 Ooka, T. and Takeda, I. (1972) Agric. Biol. Chem. 36, 112—119
19 Jirgensons, B. (1973) Optical Acitivty of Proteins and Other Macromolecules, 2nd edn., p. 74, Springer Verlag, Berlin
20 Rosenkranz, H. and Scholtan, W. (1971) Hoppe-Seyler's Z. Physiol. Chem. 352, 896—904
21 Jung, G., Dubischar, N., Irmscher, G., Mayr, W. and Oekonomopulos, R. (1977) Chemiker-Ztg. 101, 196—201
22 Burgess, A.W. and Leach, S.J. (1973) Biopolymers 12, 2691—2712
23 Johnson, M.E. and Wu, T.T. (1976) J. Theor. Biol. 60, 183—195
24 Yantorno, R.E., Takashima, S. and Mueller, P. (1977) Abstr. 21st Meet. of the Biophys. Soc., New Orlean, Biophys. J. 17, 87a
25 Habermann, E. (1972) Science 177, 314—322 and references cited therein