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Full Papers

Paracelsin; characterization by NMR spectroscopy and circular dichroism, and hemolytic properties of a peptaibol antibiotic from the cellulolytically active mold *Trichoderma reesei*. Part B[1,2]

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Summary. Paracelsin, a hemolytic and membrane active polypeptide antibiotic of the peptaibol class which is excreted by the mold *Trichoderma reesei,* was obtained by a simplified and rapid isolation procedure utilizing hydrophobic adsorber resins. Investigation by ^{13}C nuclear magnetic resonance spectroscopy and circular dichroism revealed considerable helical portions in solution, and the very recently accomplished sequence determination of paracelsin allows the discussion of the results with regard to the closely related analogues, alamethicin and suzukacillin. A selective cleavage of the peptide was achieved by careful treatment with various acids, and a buffer of pH 8.25 and of high ionic strength made possible the quantitative determination of the C-terminal phenylalaninol released by means of ion-exchange chromatography. The significance of the production of paracelsin and related mycotoxins of the peptaibol class, exhibiting various kinds of biological activity, is discussed with respect to the extensive effort being made towards biotechnological applications of species, strains and cellulolytically highly active mutants of the fungus *Trichoderma.*

Key words. Trichoderma reesei; molds; paracelsin; ^{13}C NMR spectroscopy; circular dichroism; antibiotics, polypeptide; mycotoxins.

Introduction

In a previous communication we reported the isolation of paracelsin[14], a fungal metabolite of the peptaibol class in which a high proportion of the uncommon α-aminoisobutyric acid (Aib, $H_2N-C(CH_3)_2$-COOH) and a C-terminal bond amino alcohol are common features. The mold *Trichoderma reesei* Simmons[53] QM 9414 (= ATCC 26921, IMI 192656) which produces paracelsin was obtained by irradiation of conidia of the parent wild strain *Trichoderma reesei* (formerly *viride*) QM 6a with high energy electrons from a linear accelerator and screening the survivors for cellulase production. A strain thus obtained, QM 9123, showed an increase in enzyme production, and repetition of the procedure created the mutant QM 9414 with a cellulolytic activity nearly 4 times higher than that of the wild strain[37,39]. The polypeptide paracelsin was isolated from both the mycelium and culture broth of the fungus and, although crystalline and found to be uniform when investigated by thin-layer chromatography (TLC), it could be resolved by reversed-phase high-performance liquid chromatography (HPLC) into 3 main components.

This more sensitive method was also demonstrated to be a powerful tool for the detection of microheterogeneities due to amino acid exchange in the peptaibol antibiotics samarosporin (= stilbellin, emerimicin)[18], alamethicin, suzukacillin, trichotoxin and hypelcin (fig. 1). Furthermore, repetitive HPLC allowed the isolation of homogeneous components from heterogeneous peptaibol mixtures of both paracelsin and trichotoxin up to about 100 mg amounts[20].

The application of the recently developed techniques of field desorption (FD) and fast atom bombardment (FAB) mass spectrometry[49] to these peptides, together with a sophisticated combination of highly efficient solvent (matrix) systems and a selective acidolytic cleavage step in situ, made possible the rapid and complete sequence analyses of paracelsin and related peptaibol antibiotics[20,50]. Therefore, this combined application of HPLC, FD and FAB mass spectrometry was demonstrated to be most valuable for the separation, quantitative correlation and determination of primary structures of this class of fungal metabolites.

In part A[14] we described the isolation, composition, antibiotic activity and influence on rumen fermentation of paracelsin. In the present paper we describe an improved isolation procedure for paracelsin or hydrophobic relatives and the ascertainment of the conformation of paracelsin by ^{13}C nuclear magnetic resonance (NMR) spectroscopy and circular dichroism (CD). Furthermore a method for the selective cleavage and determination of the C-terminal bonded amino alcohol and the action of paracelsin on the membrane of human erythrocytes is described.

Material and methods

Circular dichroism spectra were taken on a Jasco J-500 A spectropolarimeter equipped with a Tektronix 4025 data system and 4662 digital plotter. The measurements have been checked by means of a solution of epiandrosterone in dioxane and a molar ellipticity $[\Theta]_{304\ nm}$ of 10925 deg cm $dmol^{-1}$ was assumed as standard. The circular dichroism data have been calculated from the spectra as molecular ellipticities Θ. Molecular masses employed were 1963 for alamethicin I and 1605 for antiamoebin I. For paracelsin an average molecular

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

1 Ac-Aib-Ala-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Aib-Aib-Pro-Val-Aib-Aib-Gln-Gln-Pheol (A)
Ac-Aib-Ala-Aib-Ala-Aib-Ala-Gln-Aib-Leu-Aib-Gly-Aib-Aib-Pro-Val-Aib-Aib-Gln-Gln-Pheol (B)
Ac-Aib-Ala-Aib-Ala-Aib-Aib-Gln-Aib-Val-Aib-Gly-Aib-Aib-Pro-Val-Aib-Aib-Gln-Gln-Pheol (C)
Ac-Aib-Ala-Aib-Ala-Aib-Aib-Gln-Aib-Leu-Aib-Gly-Aib-Aib-Pro-Val-Aib-Aib-Gln-Gln-Pheol (D)

2 Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Glu-Gln-Pheol (I)
(Aib) (II)

3 Ac-Aib-Ala-Aib-Ala-Aib-Ala-Gln-Aib-Aib-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Gln-Gln-Pheol
(Aib) (Leu) (Iva)

4 Ac-Aib-Gly-Aib-Leu-Aib-Gln-Aib-Aib-Aib-Ala-Ala-Aib-Pro-Leu-Aib-Aib-Gln-Valol (E)
Ac-Aib-Gly-Aib-Leu-Aib-Gln-Aib-Aib-Ala-Ala-Ala-Aib-Pro-Leu-Aib-Iva-Gln-Valol (F)
Ac-Aib-Gly-Aib-Leu-Aib-Gln-Aib-Aib-Aib-Ala-Ala-Aib-Pro-Leu-Aib-Iva-Gln-Valol (G)

5 Ac-Aib-Gly-Aib-Leu-Aib-Gln-Aib-Aib-Aib-Ala-Aib-Aib-Pro-Leu-Aib-Iva-Glu-Valol
(Ala) (Ala) (Aib)

6 Ac-Phe-Aib-Aib-Aib-Val-Gly-Leu-Aib-Aib-Hyp-Gln-Iva-Hyp-Ala-Pheol (III)
(Aib) (IV)

7 Ac-Phe-Aib-Aib-Aib-Iva-Gly-Leu-Aib-Aib-Hyp-Gln-Iva-Hyp-Aib-Pro-Pheol (I)
(Pro) (II)

Figure 1. Sequences of paracelsin A–D[20,50] **1** and related peptaibol antibiotics alamethicin I(II)[24,45,47] (= F-30) **2**: suzukacillin[32] **3**; trichotoxin A-50 **4**, main components[20,50] E (13.9%), F (17.5%) and G (35.7%); trichotoxin A-40[4,16,19] **5**; emerimicin[44] (= samarosporin, stilbellin[18]) **6** and antiamoebin I(II)[18,46] **7**. Aib, α-aminoisobutyric acid (2-methylalanine); Iva, isovaline (2-ethylalanine); Pheol, Phol, phenylalaninol; Valol, valinol. All chiral constituents are of the L(S)-configuration with the exception of D(R)-Iva[10].

mass of 1921 was used, calculated from the percentage (%) occurence of components A (28.1), B (40.8) and C* (31.1) = C (16.1) and D (15.0) (C and D calculated from the relative intensities of MH^+ ions of FD spectra[20].

^{13}C NMR Fourier transform spectra were recorded on a Bruker WM 250 NMR spectrometer (62.89 MHz for ^{13}C). 100 mg crystalline paracelsin were dissolved in 0.5 ml ^{12}C-enriched and deuterated methanol (^{12}C-methanol-d_4, 99.5% for NMR-spectroscopy, Merck no. 10682). With proton broad band decoupling 25000 interferograms were accumulated on a 32 K storage (Bruker computer Aspect 2000). The pulse width used was 5 μs and the pulse interval was 1.15 sec. The chemical shifts were printed as δ-values in ppm and referred to the 0.5% methanol-d_4 in the solvent. CH, CH_2 and CH_3 resonances were distinguished by distortionless enhancement by polarization transfer (DEPT)[21].

High-performance liquid chromatography (HPLC) was carried out on a liquid chromatograph series 3B (Perkin-Elmer, Überlingen, FRG) with 2 dual-head reciprocating pumps, a recorder 561, integrator M3B and a variable wavelength spectrophotometer LC-75. Samples were introduced by a model 7120 loop injection valve (Rheodyne, Cotati, CA, USA).

Thin-layer chromatography (TLC) was carried out on preformed silica gel plates (Merck, silica gel 60 F_{254}, 0.2 mm thickness). The following solvent systems were used (v/v):

I chloroform/methanol/water/acetic acid (65:25:4:3),
II chloroform/methanol/water (65:25:2),
III chloroform/methanol/aq. ammonia, 17% (70:35:10),
IV 1-butanol/acetic acid/water (30:10:10)
V 1-butanol/pyridine/acetic acid/water (42:24:4:30),
VI 2-butanol/acetic acid/water (67:10:23).

Glass chambers coated with filter paper and equilibrated at ambient temperature were used. Usually 2 μl of 1% solutions of the peptides in methanol were subjected to TLC. The distance from the starting point to the solvent front was 10 cm. Peptaibols were made visible on the same plate by careful spraying with water and, after drying, with chlorine/o-tolidine or chlorine/4,4-bis(dimethyl)diphenyl-methane[18]. Boc-L-Ala-OH was used as an internal standard: R_f(I) 0.70, R_f(II) 0.32, R_f(III) 0.26, R_f(IV) 0.72, R_f(V) 0.58, R_f(VI) 0.73.

Phenylalaninol was selectively cleaved and determined as follows. 23.03 mg (12 μmol) of paracelsin were dissolved in 2 ml 6 N HCL/dioxane, 1:1, v/v, and incubated at 37 °C in a closed vessel (c = 6 mmol peptide/l). At suitable intervals 0.1-ml aliquots were transferred to 0.9 ml pH 8.25 buffer (see below) to stop the reaction and the Pheol released determined on the short column (1.2 × 6.5 cm, AA-27 sulfonated polystyrene resin) of a Beckman Unichrom analyzer using the following special elution buffer: 97.32 g (0.3 mol) tri-potassium citrate trihydrate, 74.56 g (1 mol) potassium choride, 1 g phenol and 100 ml n-propanol are dissolved to a final volume of 1 l and adjusted to pH 8.25 by the addition of 2 N KOH.

The *hemolytic assay* was carried out by a modification of a method published elsewhere[27] and is described in detail in the following. For the preparation of a standard of erythrocytes 2 ml of human venous blood in 18 ml 3.8% sodium citrate were centrifuged at 1000 rpm for 2 min. The supernatant was discarded and the remaining precipitate washed twice with 20 ml of sodium phosphate buffer (5 mmol NaH_2PO_4, 5 mmol Na_2HPO_4 and 154 mmol NaCl dissolved in 1 l of water and adjusted to pH 7.0 by addition of 0.1 N NaOH). The procedure was repeated twice and the erythrocytes finally suspended in 18 ml of the phosphate buffer. To 2.7 ml of this suspension 0.1 ml methanol and about 2 mg digitonin (Sigma, St. Louis, USA) were added to give complete hemolysis of the erythrocytes. After centrifugation as described above, 2 ml of that solution, 0.1 ml of 0.1% $K_3[Fe(CN)]_6$ and, after 5 min, 0.2 ml of 0.1% KCN were admixed and the extinction measured at 546 nm against a blank prepared without addition of digitonin (model 34 Beckman spectralphotometer, using disposible polystyrene cuvettes, path length 1 cm). The standard was defined as having an extinction (E) of

exactly 0.500; therefore the final volume of the original suspension of erythrocytes had to be adjusted by addition (or removal) of some phosphate buffer. The standard was not stored for longer than 24 h at +4 °C. For comparison of the hemolytic activities of peptaibols 2.7 ml phosphate buffer, 0.1 ml methanolic solutions of the respective peptaibols, covering the range from 0 to 100% hemolysis, and 0.2 ml of a standard suspension of erythrocytes were admixed, incubated at 37 °C for 1 h and centrifuged at 1000 rpm for 2 min. To 2.0 ml of the supernatant $K_3[Fe(CN)]_6$ and KCN were added as described above and the amount of hemolysis measured against a blank obtained from a mixture of 2.7 ml phosphate buffer, 0.1 ml methanol and 0.2 ml of the standard suspension of erythrocytes. The percentage (%) of hemolysis was calculated from the extinction measured (e.g. E 0.500 = 100%, E 0.250 = 50%) and is expressed as a function of the logarithm of the molar concentration (c) of the respective peptaibol. The molar concentration of peptaibols causing 50% hemolysis (c_{50}) were compared with one another and sodium dodecyl sulphate (SDS) was used as an external standard.

Fermentation of *Trichoderma reesei* and improved isolation of paracelsin: Lyophilized spores of *Trichoderma reesei* QM 9414 (obtained from the Quartermaster collection, Department of Botany, University of Massachusetts, Amherst, Mass., USA) were suspended in sterile water and, after soaking for 15 min, transferred to potato dextrose agar slants. After 7 days at 25 °C the sporulation and germination was completed and eight 250 ml Erlenmeyer flasks containing 50 ml Raulin-Thom medium[51] were each inoculated with 1 ml of a spore suspension (approximately 10^6 spores/ml) prepared by suspending spores from the above agar slants in sterile bidistilled water and incubated on a rotary shaker at 26 °C and 90 rpm for 8 days. The cultures were transferred to eight 1-l Erlenmeyer flasks containing 400 ml Raulin-Thom medium and grown for a further 8 days as described above. The combined contents (3.2 l) of these flasks was used for the seeding of 100 l of Raulin-Thom medium in a 150-l fermenter (Bioengineering AG, Wald, Switzerland) and the fermentation was carried out as described previously[14]. The antibiotic production in both shake-flasks and fermenter could be monitored by extraction with organic solvents[14] or by the following method. 20 ml aliquots of filtered culture broth were pressed through cartridges packed with alkylsilica (Sep-Pak® C_{18} cartridges, 10 × 10 mm, Waters Associates, Milford, MA 01757, USA) using a 30 ml syringe equipped with a Luerlok® connection, with subsequent washing with 5 ml of water and elution of the components adsorbed with 3 ml methanol. After evaporation to dryness the remaining residue was dissolved in 0.5 ml of methanol and the amount of paracelsin determined qualitatively by TLC, or quantitatively after total hydrolysis of the residue and determination of Aib and Pheol by automatic analysis.

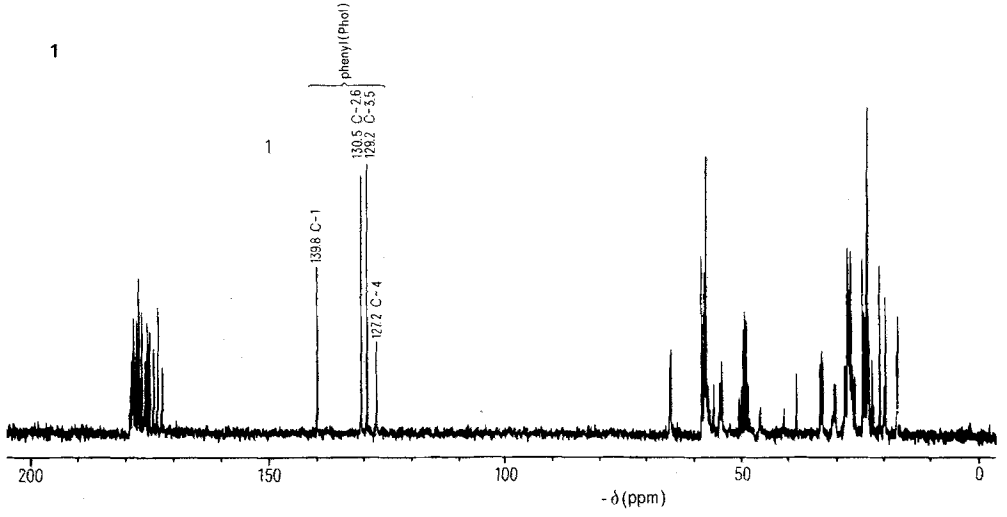
Paracelsin was isolated from the filtered culture broth of the fermentation process[14]. However, remarkable amounts of the peptide remained during the purification procedure in the mother liquors of precipitations from organic solvents and, furthermore, could be obtained as a crude extract from the mycelium as is described in part A of the present paper[14].

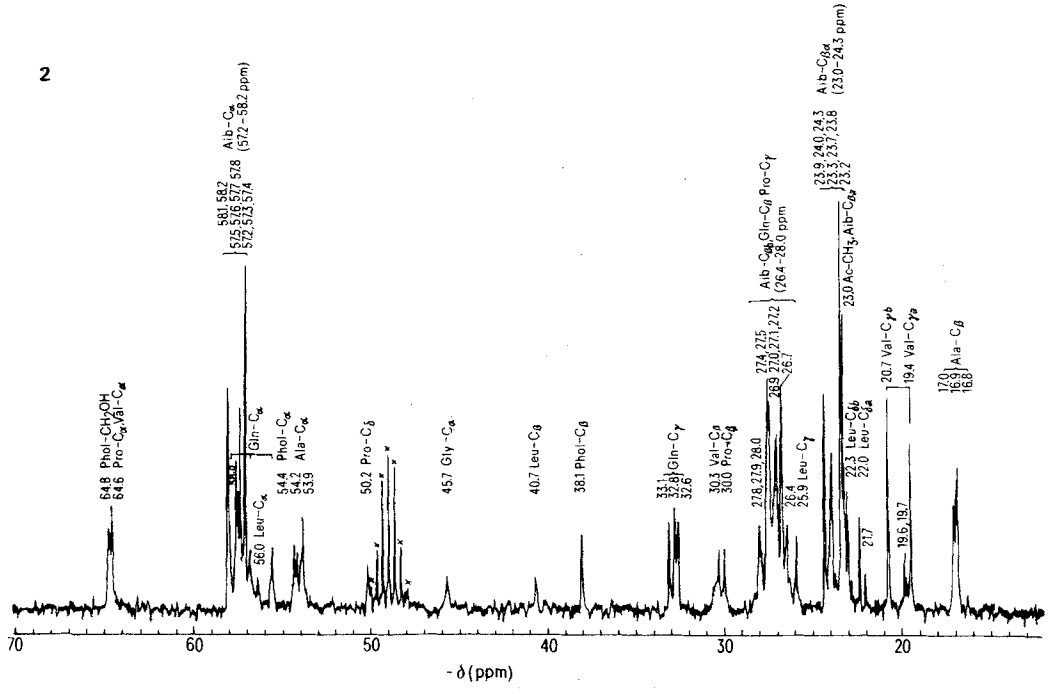
Therefore, to optimize the yield of the isolation procedures, both mother liquors (ML, 240 g) and mycelial extract[14] (ME, 66 g) were dissolved in methanol (ML, 300 ml and ME, 100 ml respectively) and diluted with about equal volumes of water and passed through a column (4 × 25 cm) packed with Servachrom XAD-2 adsorber resin (100–200 µm, Serva, Heidelberg, FRG). After rinsing with 200 ml of water the peptides were eluted with 400 ml 50% aqueous aceton and, after repetition of the above procedure, with a linear gradient 75% to 100% methanol. The elution was monitored by TLC (system I) and appropriate fractions combined and evaporated to dryness, yielding 6.2 g (ML) and 6.3 g (ME) brown solid. This simple method of the adsorption of peptaibols by XAD resins and desorption by means of organic solvents was also found to be applicable directly to the filtered culture broth from fermentations. The peptides were dissolved in 50 ml chloroform/methanol 7:3 and subjected to silica gel column chromatography (column 2.4 × 80 cm, silica gel 200–400 mesh, Merck no. 7734) with an eluent of the same composition. Appropriate fractions as indicated by TLC were combined yielding 2.4 g (ML) and 2.7 g (ME) brown solids. Finally the peptides were dissolved in 15 ml methanol and subjected to Sephadex LH-20 column chromatography to remove yellow impurities (column 5 × 90 cm, eluent methanol) yielding 1.6 g (ML) and 1.8 g (ME) pale brownish solid. Crystallization was achieved from 40 ml boiling 50% aqueous methanol yielding 1.3 g (ML) and 1.7 g (ME) colorless crystals (m.p.253–255 °C; $[\alpha]_D^{21} = -19.48°$, c = 2, methanol; R_f(I) 0.36, R_f(II) 0.29, R_f(III) 0.39, R_f(IV) 0.45, R_f(V) 0.58, R_f(VI) 0.38.

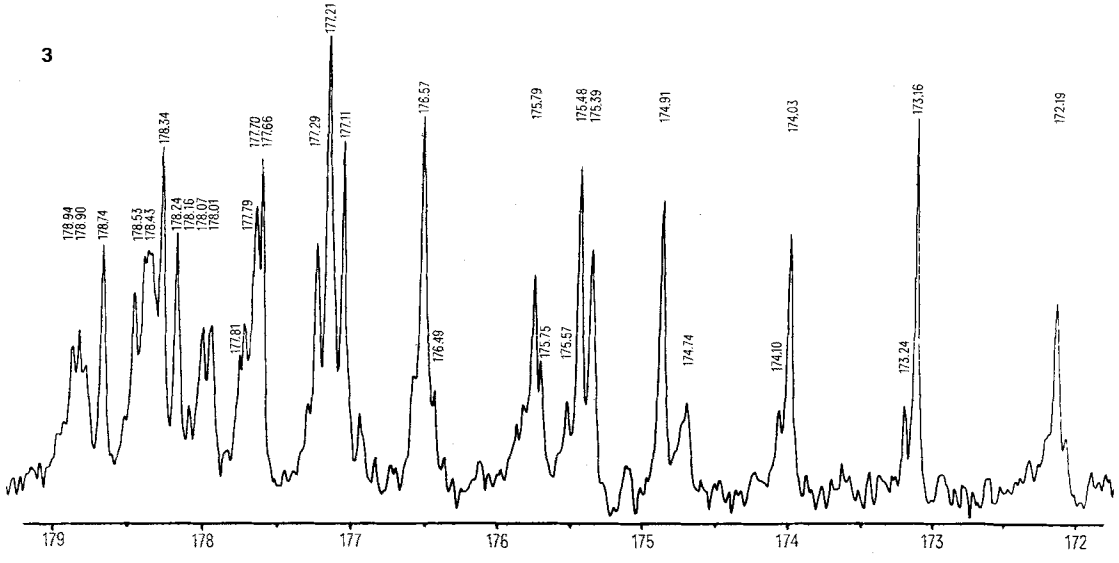
Results and discussion

Isolation of paracelsin

Because of the extraction process employed with both culture broth and mycelium, the procedures as described above had to be applied. However, the adsorption of peptaibols by non-specific adsorber resins based on crosslinked polystyrene (XAD-2), polyacrylic acid ester (XAD-7) or related types of adsorber resins, and subsequent elution of polypeptides, can also be applied directly to the culture broth from fermentations. Therefore, for convenience, one may even abandon completly the extraction by organic solvents and discard the mycelium. Following adsorption chromatography additional purification procedures, e.g. silica gel chromatography and Sephadex LH-20 and precipitation or crystallisation operations are usually necessary, but in the end HPLC is essential for obtaining single peptaibol components from microheterogeneous mixtures. Furthermore, a rapid, specific and quantitative detection of Aib-containing peptides is achieved by an adsorption technique employing cartridges packed with reversed-phase alkylsilica. After desorption of hydrophobic components by methanol, peptaibols and related compounds are easiliy detectable by TLC or, after total hy-


1
phenyl(Phol)
130.5 C-2,6
129.2 C-3,5
139.8 C-1
127.2 C-4
200
150
100
50
0
-δ(ppm)


2
Aib-Cα (57.2-58.2 ppm)
Aib-Cβα (23.0-24.3 ppm)
Aib-Cβ, Gln-Cβ Pro-Cγ (26.4-28.0 ppm)
64.8 Phol-CH2OH
64.6 Pro-Cα, Val-Cα
56.0 Leu-Cα
54.4 Phol-Cα
54.2 Ala-Cα
53.9
50.2 Pro-Cδ
45.7 Gly-Cα
40.7 Leu-Cβ
38.1 Phol-Cβ
33.1 Gln-Cγ
32.8
32.6
30.3 Val-Cβ
30.0 Pro-Cβ
23.0 Ac-CH3, Aib-Cβα
22.3 Leu-Cδb
22.0 Leu-Cδa
20.7 Val-Cγb
19.4 Val-Cγa
17.0 Ala-Cβ
70
60
50
40
30
20
-δ(ppm)


3
177.21
178.57
175.79
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174.91
174.03
173.16
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177.66
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177.11
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178.90
178.74
178.53
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178.07
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drolysis, by utilizing the uncommon α,α-dialkyl α-amino acids and amino alcohols as specific markers.

Circular dichroism, ^{13}C NMR spectroscopy and conformation

The CD spectra of paracelsin are almost identical to those of alamethicin[30] and suzukacillin[31] taking into account the somewhat different primary structure, microheterogeneity and precision of measurements. By analogy, the spectra show negative Cotton effects typical for right-handed helical portions in peptides, i.e. the n,π^*-transition at 222 nm and the π,π^*-transition at 207 nm (an additional positive absorbtion at 192 nm cannot be seen in the spectra presented) (fig. 2).

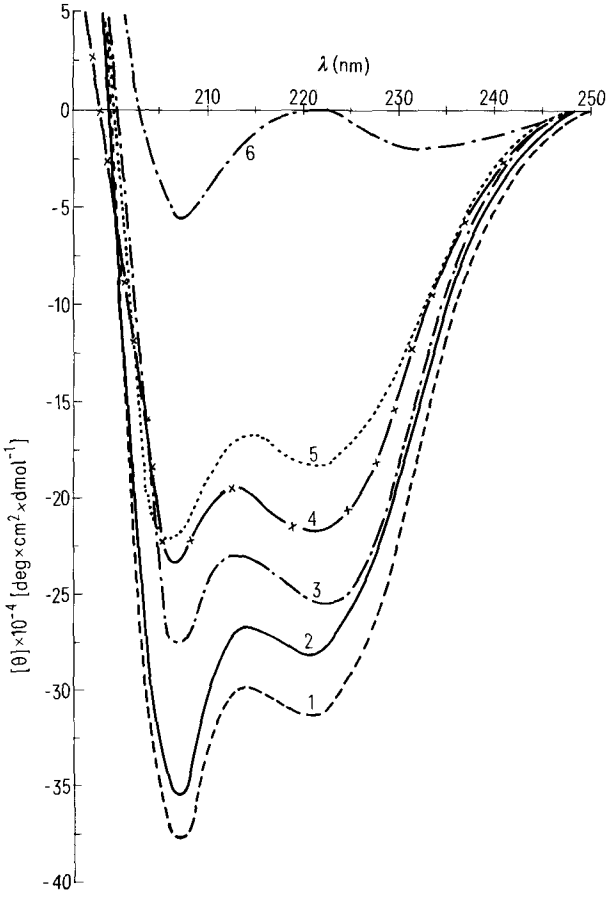

Figure 2. Solvent-dependent circular dichroism and molar ellipticities $[\theta]_\lambda$ of peptaibol antibiotics alamethicin F-30, methanol **1**; paracelsin, methanol **2**, methanol 80% **3**, methanol, 50% **4**, trifluoroethanol **5** and antiamoebin, methanol **6**. T, 20 °C, concentration, 0.25 mg/ml

		$[\theta]_{207\,nm}$	$[\theta]_{222\,nm}$	$\frac{[\theta]_{222\,nm}}{[\theta]_{207\,nm}}$
Alamethicin	**1**	− 378102	− 312967	0.83
Paracelsin	**2**	− 354977	− 276093	0.78
	3	− 273216	− 255323	0.93
	4	− 233989	− 216784	0.93
	5	− 219608 ($[\theta]_{206\,nm}$)	− 182549	0.83
Antiamoebin	**6**	− 56471	− 18824 ($[\theta]_{232\,nm}$)	0.33

In solution the molar ellipticity of paracelsin and analogues is dependent on the solvent, and the helical content decreases strongly on the addition of water to methanolic solutions, or when hydrogen-bond-forming 1,1,1-trifluoroethanol is used as a solvent. The values of the molar ellipticities found for alamethicin agree very well with those reported previously when corrected for the subsequently-discovered components Pheol and N-terminal N-acetyl-α-aminoisobutyric acid. The ratio of intensities $\Theta_{222}/\Theta_{207}$ is < 1 in organic solvents, approaches 1 with the addition of water and becomes > 1 in predominantly aqueous solutions. For comparison the CD spectrum of the hexadecapeptide antiamoebin (cf. fig. 1) was taken and found to be quite different from those of the eicosapeptides paracelsin and alamethicin. The spectrum shows only weak negative absorption at 207 nm and a red-shift from 222 to 232 nm compared to paracelsin, reflecting a deviating, nonhelical structure of antiamoebin.

This is independently supported by ^{13}C NMR spectroscopy of antiamoebin by the drastically reduced magnetic nonequivalence (MNE) of the geminal Aib-*pro*-S-C_β (Aib-$C_{\beta a}$) and Aib-*pro*-R-C_β[28] (Aib-$C_{\beta b}$) methyl groups (Δ MNE $\cong 2$ ppm; [^{12}C,^2H]methanol; 20.115 MHz) compared to the mainly helical peptaibols alamethicin, suzukacillin, trichotoxin and paracelsin (Δ MNE 3–4 ppm, fig. 3). The complete assignment of the ^{13}C NMR resonances of the latter was facilitated by comparison with spectra of alamethicin[30] and natural and synthetic sequences of trichotoxin[13, 16, 28]. The signals observed are in full agreement with those one expects from the constituents of paracelsin A–D and with regard to its microheterogeneity (cf. fig. 1).

Further evidence for a deviating secondary structure is the inability of antiamoebin and its homologue emerimicin (= samarosporin, = stilbellin)[18] and trichotoxin[17] to form well resolved pore-state conductances in black lipid bilayer membranes comparable to those of paracelsin[14] and alamethicin[8].

Relationship of paracelsin, suzukacillin and alamethicin

The recently-elucidated primary structures of paracelsin[20, 50] and suzukacillin[32] allow them to be compared with one another and their analogue alamethicin (cf. fig. 1 for sequences). Because of a nonribosomal mechanism of enzymatic biosynthesis[33, 52] HPLC revealed these peptaibols to be microheterogeneous mixtures of closely related polypeptides, distinguished only by an exchange

Figure 3. Fourier transform ^{13}C NMR spectrum (62.89 MHz) of paracelsin with assignment of the aromatic region **1**, an expanded plot and assignment of the aliphatic region **2** and an expanded plot with the carbonyl resonances **3**. Spektra are taken in [^{12}C,^2H] methanol; concentration 100 mg/0.5 ml; temperature, 25 °C.

of a few amino acids, or just one. Suzukacillin, in contrast to alamethicin and paracelsin, contains components with isovaline residue (it is uncertain in which of the peaks shown in fig. 4, 1), and both paracelsin and suzukacillin contain only one proline residue, whereas alamethicin contains two.

Comparison of crystalline suzukacillin and paracelsin by HPLC (fig. 4) demonstrates a pronounced microheterogeneity of the former, and spiking of suzukacillin with paracelsin revealed an identical elution behavior of paracelsin components A+B and C+D and suzukacillin components 1 and 3, respectively. From this results the identity of some sequences of these peptides is suspected, but it has not been established in which of the peaks shown in figure 4.

Selective cleavage and quantitative determination of phenylalaninol

Action of a mixture of aqueous HCl and dioxane released C-terminal bonded Pheol selectively from paracelsin (50% in about 9 h), with a cleavage rate that is comparable to those of valinol from the C-terminal hexapeptide of trichotoxin[16] (50% in about 10 h). Difficulties arose in the quantification of Pheol because it is too alkaline to be eluted under standard conditions from the strongly acidic, sulphonated polystyrene resins commonly used by amino acid analyzers; a property that originally prevented the detection of Pheol as a constitutent of alamethicin[43,47]. Therefore a special buffer of high ionic strength and an enhanced pH of 8.25 (see material and methods) was developed which enabled the determination of the cleavage rate of the amino alcohol within 40 min.

Action of trifluoroacetic acid on paracelsin does not split off Pheol but N-terminal N-acetyl-α-amino-isobutyric acid, which is readily detectable using TLC and GLC-MS methods described previously[15]. Moreover, trifluoroacetolysis yields a limited number of cleavage fragments which are separated by TLC and made visible by successive spraying of the plates with water, ninhydrin and TDM-reagent or isatin[15,16,18]. Application of the latter reagent yielded intensive blue spots of identical R_f-value both from paracelsin and alamethicin F-50 (distinguished from alamethicin F-30 mainly by an exchange of Glu against a Gln residue, cf. fig. 1), indicating the identity of the C-termini of these peptides with the sequences Pro[14]-Val-Aib-Aib-Gln-Gln-Pheol[20].

This conspicuous sensitivity against acidic conditions was used and studied thouroughly during the sequencing of trichotoxin[13,15,19] and invariably all peptaibols were found to be cleaved very rapidly at the carboxy side of α,α-dialkyl α-amino acids (Aib, Iva) when linked to secondary amino acids Pro and Hyp, and with reduced cleavage rates when bonded to primary α-amino acids. Aqueous HCl and mixtures with organic solvents, e.g. alcohols and cyclic ethers, solutions of hydrogen chloride or perchloric acid in acetic acid, trifluoroacetic acid and mixtures with organic solvents have been found very useful for acidolyses. This particular acidolytic sensitivity was also used as a sophisticated proof of the α-linkage of valinol to glutamic acid in trichotoxin[16] and an in situ acidolytic procedure was, in addition to the utilization of efficient matrix (solvent) systems, essential for obtaining the complete sequence determination by FAB mass spectrometry of components of paracelsin and trichotoxin, isolated by HPLC[20,50].

Hemolytic properties

Hemolysis of erythrocytes is a common characteristic of the peptaibols alamethicin, suzukacillin and trichotoxin[27] and is also to be found in paracelsin. The in vitro hemolytic activity of paracelsin ($c_{50} = 3.7 \times 10^{-5}$ mol l^{-1}) is lower than that of trichotoxin ($c_{50} = 2.1 \times 10^{-5}$ mol l^{-1}) and alamethicin F-50 ($c_{50} = 1.6 \times 10^{-5}$ mol l^{-1}), but all of them are more active than sodium dodecyl sulphate ($c_{50} = 5.1 \times 10^{-5}$ mol l^{-1}). Again antiamoebin shows deviation in behavior; it is 2½ times less active ($c_{50} = 1.25 \times 10^{-4}$ mol l^{-1}) than SDS and is considered to be just on the borderline of hemolytic ability (fig. 5). In vivo hemolysis is also suspected to be the reason why the lethal doses is about 5 mg/kg

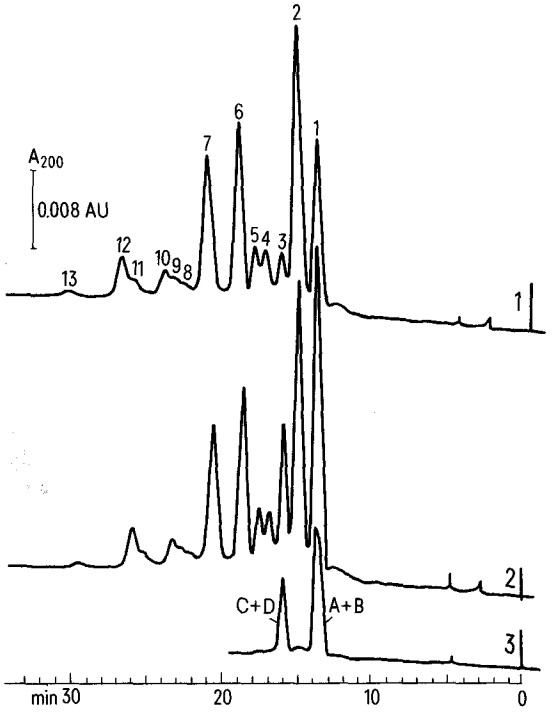

Figure 4. HPLC of suzukacillin **1**, a mixture of suzukacillin and paracelsin **2** and paracelsin **3**. Conditions: Vertex column, 250 × 16 mm and guard column 30 × 16 mm (Knauer, Bad Homburg, FRG), both packed with Nucleosil RP-18, 5 µm (Macherey-Nagel, Düren, FRG); eluent, methanol-water 85:15, v/v; isocratic operation; flow rate 7 ml min^{-1}; pressure 18 MPa; temperature, ambient; sensitivity recorder, 10 mV; chart speed, 0.5 cm min^{-1}; injection, 250 µg peptide 1 in 5 µl methanol **1**, 150 µg 1 and 50 µg 3 in 10 µl methanol **2** and 50 µg 3 in 5 µl methanol **3**. Relative peak area (%) **1**: 1 (13.3), 2 (22.9), 3 (5.0), 4 (5.2), 5 (4.7), 6 (16.1), 7 (16.2), 8+9+10 (7.2), 11+12 (8.1), 13 (1.4); AU, absorption units; A, absorption (nm).

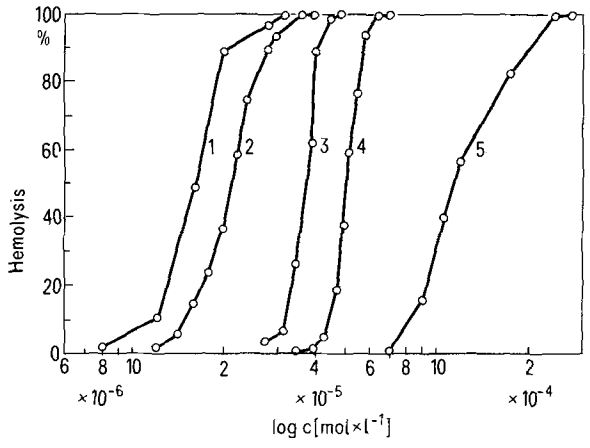

Figure 5. Concentration dependence of the hemolytic activity of peptaibol antibiotics alamethicin **1**, trichotoxin A-40 **2**, paracelsin **3** and antiamoebin **5**, compared to sodium dodecyl sulphate **4**.

mice when paracelsin is administered intraperitoneally. The drastically reduced hemolytic ability of the hexadecapeptide antiamoebin as compared to the eicosapeptides paracelsin or alamethicin and the octadecapeptide trichotoxin is influenced rather by the shorter chain length than the deviating secondary structure, as is evident from the inability of helical, N-terminal dodecapeptides isolated from trichotoxin to cause hemolysis[29].

Conclusions

The new polypeptide antibiotic paracelsin from *Trichoderma reesei* is demonstrated to be a close analogue of alamethicin and suzukacillin, each distinguished from the other only by a limited exchange of a few amino acids. Suzukacillin exhibits a pronounced microheterogeneity which has made it difficult until now to correlate single peptide sequences with the HPLC elution pattern. However, as demonstrated above, it might be possible that the suzukacillin complex and paracelsin have certain components in common – a conclusion that is not in contradiction with the lack of isovaline in paracelsin.

The methods outlined for the detection, isolation, purification and selective cleavage of paracelsin, together with those elaborated for the rapid sequence determination by field desorption, fast atom bombardment mass spectrometry[18, 20, 49, 50] and gas chromatography mass spectrometry (GC/MS)[4, 15, 19] are applicable to all members of the peptaibol group and will be very valuable for the discovery and characterization of additional fungal metabolites of this kind in the future[58]. In particular, the adsorption of these hydrophobic peptide antibiotics on unspecific adsorbents (Servachrom XAD resins or related types) is considered to be most advantageous for isolation procedures in attaining optimal yields and in avoiding troublesome extraction of culture broths with organic solvents, in particular when large scale production is concerned.

The increase of the digestion of starch and cellulose in rumen liquor by the addition of *low* concentrations of paracelsin[14] should be considered in the discussion of the association of metabolites from *Trichoderma* with the ill-thrift of pasture fed ruminants[11, 12], and may be explained by the suppression of various feed rivals of the cellulolytic activ microorganisms.

With regard to the most intensive efforts for a biotechnological application of *Trichoderma*[25, 26, 35, 36, 55-57] – both in submerged and solid-substrate fermentation[14, 40] – for the enzymatic conversion of cellulosic materials used for animal feed or human food and, moreover, the contribution of these microfungi and their relatives[18] to the moldiness, spoilage and deterioration of organic matter, the excretion of paracelsin and related metabolites should be seriously taken into account. These mycotoxins exhibit a great variety of biological activity[6-8, 22, 27, 29, 34, 41, 48, 54] and are sometimes produced in extraordinarily high amounts[38, 42]. Finally, in this discussion it should also be emphasized that several commercially-available and widely used cellulase preparations are isolated from various species and mutants[5, 39] of *Trichoderma* and the utilization of its antagonistic properties for a biological control of fungal attack has been discussed.

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Direct observation of enzyme substrate complexes by stopped-flow fluorescence: mathematical analyses

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Summary. The fluorescence changes which occur upon the interaction of enzyme and substrate under stopped-flow conditions can provide a sensitive means to directly observe ES complexes. The interconversion of the intermediates during catalysis causes changes in fluorescence, signaling directly their existence, and allowing their quantitation. We have studied extensively an approach which measures radiationless energy transfer (RET) between enzyme tryptophanyl residues and a fluorescent peptide or ester substrate. Our studies of a number of proteolytic enzymes have validated the approach, which is sensitive and applicable to a variety of enzymes under a wide range of experimental conditions, including subzero temperatures. Direct excitation of fluorescent substrates can also be used to observe ES complex formation and breakdown and is complementary to the RET approach. Here we review both the RET and direct excitation kinetic approaches, with particular emphasis on the mathematical foundations we have developed which are critical to the successful interpretation of these or any other spectroscopic approach which yields a signal that is unique to the ES complex.
Key words. Enzyme-substrate complexes; stopped-flow fluorescence, proteolytic enzymes; radiationless energy transfer; enzyme kinetics.

1. Introduction

The interaction between enzyme and substrate is the characteristic feature of enzyme catalysis, and mechanistic studies are greatly facilitated by the direct visualization of reaction intermediates. This obviously desirable goal has proven elusive, because the inherently short lifetimes and low concentrations of ES complexes make particularly demanding requirements of available techniques. While stopped-flow equipment and methodology, now generally available, have done much to advance the field, visualization of ES complexes is not an inherent feature of this technique. Thus spectral systems that can directly signal the ES complex must be designed to be compatible with rapid mixing instrumentation.

Our studies of a number of proteolytic enzymes have demonstrated that measurement of radiationless energy transfer (RET) between enzyme tryptophanyl residues and a fluorescent dansyl-peptide or ester provides a sensitive means to observe ES complexes at both steady-state and pre-steady-state conditions[4, 6, 13, 22, 23]. The interconversion of ES complexes during catalysis causes changes in fluorescence, signaling directly their existence, and allowing their quantitation.

The direct observation of ES complexes using fluorescent substrates is also possible using an alternative approach in which the substrate fluorophore is excited directly, and is thus always fluorescent. On binding to the enzyme surface, the intrinsic fluorescence of the substrate is often greatly enhanced due to changes in the fluorophore environment. While less sensitive than the RET method, since there is always background fluorescence, this technique is complementary to the RET approach, and has been used effectively by Fruton and co-worders using N-mansyl peptide substrates for proteolytic enzymes[24, 25, 28].

The mathematical foundations critical to the successful interpretation of the observed fluorescence changes have received little attention, presumably because of the inherent difficulty in direct visualization of ES complexes. Here we review the RET kinetic approach and illustrate its applicability under a variety of experimental conditions, with particular emphasis on the theoretical framework we have developed for its interpretation. In addition, we extend the mathematics to the interpretation of stopped-flow fluorescence signals obtained upon direct excitation of fluorescent substrates. Moreover, we emphasize that this theoretical