Structural elucidation of XR586, a peptaibol-like antibiotic from *Acremonium persicinum*

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A novel peptide, XR586, has been isolated from fermentations of *Acremonium persicinum* (Xenova culture collection number X21488). The structure of XR586 has been elucidated by means of NMR spectroscopy, electrospray and fast-atom bombardment MS, derivatization and enzymic digestion. It has been shown to be helical by CD measurements. XR586 shows many structural and conformational features in common with peptaibols, particularly the zervamicins. Peptaibol antibiotics are peptides, typically of 15–20 residues, containing a large proportion of α -aminoisobutyric acid (Aib) residues. These peptides adopt a helical conformation in solution and display anti-bacterial and toxic properties due to their ability to form pores in membranes.

However, while XR586 contains several Aib residues, it lacks a terminal phenylalaninol and terminates in the sequence Phe-Gly. The lack of reduction of the penultimate residue at the C-terminus may indicate that this step is normally at the end of the biosynthetic pathway of peptaibols and occurs with cleavage of Gly. The ^1H chemical shift assignments of XR586 are reported in Supplementary Publication SUP 50179 (3 pages), which has been deposited at the British Library Document Supply Centre, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in Biochem. J. (1996) 313, 9 ('Deposition of data').

INTRODUCTION

The peptide XR586 was isolated from a fungal culture, designated Xenova culture collection number X21488, originating from a single spore within a tropical soil sample collected during 1989. Structural studies revealed it to be a peptide with many similarities to the peptaibols, a class of peptide antibiotics, the distinctive feature of which is the presence of a large number of α -aminoisobutyric acid (Aib) residues [1]. In solution these peptides adopt helical conformations, often containing regions of 3_{10} as well as α -helix. These helices are amphipathic, i.e. they are hydrophilic on one side and hydrophobic on the other, thus allowing them to cluster together in membranes, forming voltage-gated ion channels [2,3]. This is the mechanism of the anti-bacterial activity and toxicity exhibited by the group.

EXPERIMENTAL

Fermentation

A cryovial containing 1 ml of conidia/mycelium suspended in a 10% glycerol solution was rapidly thawed and inoculated into a 250 ml baffled shake-flask containing 40 ml of seed medium (Xenova, Slough, Berks., U.K.). This flask was incubated at 25 °C on an orbital shaker at 240 rev./min, and transferred after 72 h into a 2 litre shake-flask containing 400 ml of seed medium and incubated under the same conditions. The contents of this flask were then used to inoculate a 14 litre fermenter containing 11 litres of production medium (malt extract, tryptone, phytic acid, CaCO_3 , Tween-80, pH 6.0). The production fermenter was stirred at 350 rev./min, aerated at 0.5 l/min and incubated at 25 °C. After 175 h the culture was harvested and centrifuged.

Isolation and purification of XR586

Diaion HP20 resin (500 ml; Mitsubishi Kasei Corp.) was added

to the supernatant and stirred for 1 h. The resin was then eluted with methanol (2 litres). After concentration, the methanol eluate was purified by chromatography on Sephadex LH20 (15 cm $\times$ 4.5 cm column) eluted with methanol. The fractions containing XR586 were then further purified by semi-preparative reverse-phase HPLC on two Prep Nova-Pak HR C_{18} radial compression cartridge columns (40 mm $\times$ 10 cm; 6 μm particle size; 60 Å pore size; Waters, WAT037704) connected in series in a PrepPak Holder Assembly with Extension (Waters) along with a prep Nova-Pak HR C_{18} Guard-Pak Insert (Waters, WAT037854). The columns were eluted with a linear 0.1 M aqueous ammonium acetate/acetonitrile gradient increasing, after a period of isocratic elution (5 min, 20% acetonitrile), from 20 to 100% acetonitrile over a period of 15 min at a flow rate of 40 ml/min. The eluate was monitored by its absorbance at 220 nm. XR586 was eluted as a sharp peak with a retention time of 15 min. The eluate containing this peak was collected for several injections and concentrated *in vacuo* to yield a white solid (12 mg).

Hydrolysis

XR586 (1 mg) was hydrolysed with 1 ml of 6 M HCl in a sealed tube heated overnight at 110 °C. The hydrolysate was lyophilized and redissolved in water (1 ml).

Amino acid analysis as *N,O*-(S)-*t*-butyldimethylsilyl (TBDMS) derivatives

Amino acid standards and the mixture derived from acidic hydrolysis of the peptide were each converted into their N, O(S)-TBDMS derivatives by the addition of acetonitrile (50 μl), dimethylformamide (10 μl) and *N*-methyl-*N*-*tert*-butyldimethyl-

Abbreviations used: Aib, α -aminoisobutyric acid; FAB-MS, fast-atom bombardment MS; GC, gas chromatography; HMBC, heteronuclear multiple bond correlation; HMQC, heteronuclear multiple quantum coherence; Iva, isovaline; OPA, o-phthalaldehyde; TBDMS, *N,O*-(S)-*t*-butyldimethylsilyl.

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silyltrifluoroacetamide (25–50 μ l; Pierce) and heating (120 °C, 2 h) in a 1 ml derivatization vial. After cooling, the samples were diluted with dichloromethane for gas chromatography (GC) and GC–MS analyses. GC was performed on a DB-5 column (30 m $\times$ 0.32 mm; 0.1 μ m film thickness; J&W) programmed from 50 to 120 °C at 20 °C/min, and then from 120 to 300 °C at 4 °C/min, with H₂ as carrier gas; samples were introduced by on-column injection and detected by flame ionization. GC–MS was performed on a VG Trio 3 MS instrument using electron-impact ionization; chromatographic conditions were similar to those described above, except that He replaced H₂ as carrier gas.

Amino acids were identified by co-chromatography with standards and/or by interpretation of their mass spectra. The relative retention times were similar to those previously reported [4]. After several days' storage at –20 °C, some degradation of the TBDMS derivatives of Pro and Hyp was noted; the decomposition products were not identified.

HPLC analysis of amino acid *o*-phthalaldehyde (OPA) derivatives

Aqueous solutions (50 μ l) of amino acid standards (1 mg/ml) or the peptide hydrolysate mixture were derivatized with 100 μ l of OPA-thiol reagent (Sigma) for 2 min at room temperature. Samples were then analysed by reverse-phase HPLC on a NovaPak C₁₈ cartridge column (8 mm $\times$ 10 cm; 4 μ m particle size; 60 Å pore size; Waters) contained in a Waters Radial Compression Module and eluted with a gradient formed from two eluents: eluent A (60 mM K₂HPO₄, pH 6.65) and eluent B, a mixture comprising eluent A/acetonitrile/methanol/propan-2-ol (46:18:18:18, by vol.). After 5 min at 10% B, the fraction of B was increased from 10 to 40% over a period of 12.5 min and then to 100% over the next 12 min, at a flow rate of 1 ml/min. Interference from reagent peaks was excluded by monitoring at 340 nm.

Amino acid stereochemistry was investigated by derivatization with a chiral mercaptan in the presence of OPA to form diastereomeric isoindoles, which can be resolved by reverse-phase HPLC [5]. The chiral derivatization reagent was prepared by dissolving *N*-acetyl-L-cysteine (3 mg; Sigma) in OPA reagent solution (3 ml). Aqueous solutions (100 μ l) of amino acid standards (1 mg/ml) and the peptide hydrolysate were each mixed with the chiral derivatizing reagent (400 μ l), and after 2 min the mixture was analysed by HPLC as described above.

NMR spectrometry

¹H and ¹³C NMR spectra were recorded at 308 K on a Bruker ACF400 spectrometer at 400 MHz and 100 MHz respectively. Standard Bruker pulse programs were used to obtain the distortionless enhancement by polarization transfer (DEPT), COSY-45, heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond correlation (HMBC) spectra.

Mass spectrometry

Fast-atom bombardment (FAB) mass spectra were acquired using a Kratos MS50 instrument fitted with an Antek Cs⁺ ion gun and a post-acceleration detector. The accelerating voltage was 8 kV. Data were typically collected with a scan rate of 10 s/decade. Calibration was achieved using CsI.

Electrospray mass spectra were obtained using a VG BioQ instrument, employing a source temperature of 70 °C and a capillary potential of 4 kV. The mobile phase used was acetonitrile/water (1:1, v/v) flowing at 4 μ l/min. Positive-ion mode was used for all samples except for those where acetylation

had been carried out and no protonation sites were expected. Typically, 5 μ l of a 50 μ M solution was injected and data collected over 30 scans.

Microderivatization reactions

Esterification

A 50 μ l aliquot of 0.1 M HCl in anhydrous methanol was added to approx. 25 nmol of peptide in a 0.3 ml vial. The mixture was left to stand for 24 h, after which excess reagents were removed under vacuum and the residue dissolved in acetonitrile/water (1:1, v/v) to give a 50 μ M solution for analysis by electrospray MS.

Acetylation I

A 50 μ l aliquot of a 1:1 (v/v) mixture of acetic anhydride and aqueous ammonium acetate, pH 8.4, was added to approx. 25 nmol of the peptide and the mixture sonicated for 2 h at room temperature. Excess reagents were removed under vacuum and a solution of the residue was prepared as above for analysis by electrospray MS.

Acetylation II

A 50 μ l aliquot of a 2:1 (v/v) mixture of pyridine and acetic anhydride was added to approx. 25 nmol of the peptide. The mixture was left at room temperature for 4 h, after which excess reagents were removed under vacuum and the residue analysed by electrospray MS.

Carboxypeptidase digestion

A 2 μ l aliquot of carboxypeptidase Y solution (1 mg/ml) was added to a 100 μ l aliquot of XR586 solution (1 mg/ml; 10% acetonitrile). The mixture was incubated at 37 °C; aliquots were removed at increasing time intervals and diluted with acetonitrile/water (1:1, v/v) to give a 50 μ M solution for analysis by electrospray MS.

α -Chymotrypsin digestion

A 2 μ l aliquot of a 5 mg/ml solution of α -chymotrypsin in ammonium acetate buffer, pH 7.8, was added to 200 μ l of a solution of XR586 (1 mg/ml; 20% acetonitrile). The mixture was incubated at 37 °C, and aliquots were removed periodically and analysed by electrospray MS as above.

Trifluoroacetic acid cleavage

A 100 μ l aliquot of trifluoroacetic acid was added to a small sample (50 nmol) of XR586. The mixture was left to stand at room temperature for 2 h, after which the acid was removed under vacuum. The residue was then diluted for analysis by electrospray MS.

RESULTS AND DISCUSSION

Taxonomy of fungus X21488

This fungus was designated as *Acremonium persicinum* Nicot (W. Gams) by comparison of its morphological characteristics with those given by Gams [6], following conidial inoculation on 2% malt extract agar. After 10 days the mainly white/pink, dense and compact mycelium reached 30–35 mm diameter at 24 °C and had an even margin of appressed hyphae. The aerial mycelium

was tufted with pale grey patches and the intensity of orange/pink pigmentation could be increased with near-UV irradiation. The unbranched, smooth, hyaline, thin-walled, awl-shaped phialides lacked collarettes and were usually borne on aerial hyphal bundles. Phialides were 15–35 μm long with a basal diameter of 1.5–2.5 μm , tapering to an apical diameter of approx. 0.5 μm . Conidia were mainly obovoid (3.0–4.5 μm $\times$ 1.5–2.5 μm), finely roughened at maturity and formed in heads or chains.

Physico-chemical properties and amino acid composition of XR586

Purified XR586 is a white powder with good solubility in polar organic solvents such as methanol and DMSO. Its UV–visible spectrum exhibited maxima at 222 and 281 nm, suggesting the presence of an indole-like chromophore. FAB-MS indicated an M_r of 1879, and initial inspection of ^1H and ^{13}C NMR spectra revealed that XR586 is peptidic in nature. The ^1H chemical shift assignments of XR586 are reported in Supplementary Publication SUP 50179 (2 pages), which has been deposited at the British Library Document Supply Centre, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in Biochem. J. (1993) **289**, 9.

The amino acid composition of XR586 was therefore investigated by hydrolysis followed by GC and HPLC. GC of amino acids as their *N,O*-(S)-TBDMS derivatives is a convenient means of analysis [4,7]. Accordingly, the amino acids released by hydrolysis of the peptide were converted into their TBDMS derivatives and subjected to GC and GC–MS analyses to reveal the presence of Gly, Aib, Leu, Ile, Pro, Thr, Phe, 4-Hyp and Glu residues. The area of the peak corresponding to Aib suggested the presence of more than one Aib residue in the peptide. An additional peak was found to elute just later than Val, which, by analogy with Leu and Ile, was suspected to be isovaline (Iva). This identification was supported by MS. Trp was not detected, as it is degraded under the hydrolysis conditions used, but its presence was suggested by the indole-like chromophore detected in the UV–visible spectrum.

HPLC of the amino acids present in the hydrolysate after derivatization with OPA-thiol reagent confirmed the identification of Gly, Aib, Leu, Ile, Thr, Phe and Glx. Analysis after derivatization in the presence of *N*-acetyl-L-cysteine indicated that Phe, Thr, Leu and Ile were present as the L-isomers.

Closer inspection of the ^1H , ^{13}C , COSY-45, HMQC and HMBC NMR spectra of XR586 revealed the presence of one residue each of Gly, Leu, Ile, Thr, Phe, 4-Hyp and Trp, two residues each of Iva, Pro and Glx and four residues of Aib, together with one acetyl group (Figure 1). The accurate mass of the peptide was determined to be 1879.03 by FAB-MS. The calculated mass for a linear peptide with the composition shown above, bearing one acetyl group and where the two Glx residues are Gln, is 1879.02. Thus the composition is supported by these values, which are accurate to 10 p.p.m. or 0.02 mass unit.

Functional group identification

To provide further evidence for the proposed amino acid composition, microderivatization reactions were performed. These esterification and acetylation reactions are high yielding and involve no tube-to-tube transfers. This enables the experiments to be performed on a very small scale, typically 10 nmol, which is ample for analysis by MS. Changes in mass allow the number of functional groups known to react with a particular reagent to be determined [8–10].

The underivatized peptide gave two peaks in its electrospray mass spectrum, at m/z 1903 and 963, due to $[\text{M.Na}]^+$ and

Figure 1 Portion of the HMBC spectrum of XR586 also showing ^{13}C and ^1H spectra

Analysis of this and other NMR spectra confirmed the amino acid composition derived by GC.

$[\text{M.Na}_2]^{2+}$. This corresponds to the expected average mass of 1880. When the peptide was treated with methanolic HCl, its mass increased by 14 mass units to 1894, indicating that one carboxylate group was present, consistent with the one expected carboxylate at the C-terminus of the peptide. Treatment with acetic anhydride in water results in selective acetylation of amino groups. This reaction caused no change in the mass of the peptide, indicating the presence of an acetyl group blocking the N-terminus. Finally, the peptide was reacted with acetic anhydride in pyridine, conditions under which hydroxy and amino groups are acetylated. This resulted in an increase of mass of 84 units, equivalent to two acetyl groups. The observed change in mass must be due to a reaction with hydroxy groups, given the absence of reaction with acetic anhydride in water. This was exactly as expected for a peptide containing one Thr and one Hyp residue.

Determination of sequence

FAB-MS provided an excellent method for obtaining sequence information on the peptide [11–13]. As well as the molecular ion, numerous fragment ions were observed (Figure 2). Many of these could be assigned as resulting from cleavage across peptide bonds to give a series of acylium-type ions. By considering the residue masses of the amino acids known to be present, a partial sequence was deduced. Not all possible fragment ions were observed, and so there remained two regions within which the precise order of amino acids was unknown. One of these was the C-terminal region, which was either Phe-Gly or Gly-Phe. The other was a central region of the sequence containing the three amino acids Pro, Aib and Gln. Also, the amino acids Hyp, Leu and Ile have the same mass, so their positions (residues 5, 8 and

Figure 2 FAB-MS of XR586
Fragments at stated integral masses arise from the loss of the indicated residues.

Ac	Trp	Iva	Gln	Aib	Ile	Thr	Aib	Leu	Aib	Pro	Gln	Aib	Hyp	Iva	Pro	Phe	Gly	Residue
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	Residue no.
•	•	•	•	•		•	•		•					•	•			FAB fragments
•	•	•	•	•		•	•		•					•	•	•	•	carboxypeptidase Y
•	•	•	•	•		•	•		•			•	•	•	•	•	•	acetylated fragments
•	•	•	•	•	•	•	•	•	•			•	•	•	•	•	•	α -chymotrypsin
•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	TFA-pulse hydrolysis

Figure 3 Sequence information revealed by the sequential application of structural tools
Solid circles indicate unambiguous assignment. FAB-MS gave a large part of the sequence, and the other techniques then completed the structure. TFA, trifluoroacetic acid.

13) could not be distinguished on the basis of the evidence so far (Figure 3).
The problem of the order of the C-terminal residues was tackled first. A small sample of the peptide was incubated with the enzyme carboxypeptidase Y. Samples were removed periodically for analysis by electrospray MS. As well as peaks at *m/z* 1903 and 963, corresponding to [M.Na]⁺ and [M.Na]₂²⁺ ions from the intact peptide, increasing amounts of peaks at *m/z* 1846 and 934.5 were also observed, corresponding to a peptide of mass 1823, i.e. 57 mass units lower than the intact peptide. This is consistent with the loss of Gly, which was therefore deduced to be the C-terminal residue. Later samples showed similar peaks corresponding to masses of 1676 and 1579, consistent with the loss of Phe plus Gly and of Pro plus Phe plus Gly, thus confirming that the C-terminal sequence is Pro-Phe-Gly (Figure 4). No further reaction was observed, probably because the next

residue is Iva, which lacks an α -proton and therefore does not have the correct geometry for reaction with the enzyme.
The position of the Hyp residue was established by examining the positive-ion FAB mass spectrum of the acetylated peptide. Treatment with acetic anhydride and pyridine acetylated the hydroxy groups of Thr and Hyp, increasing the residue mass of Thr and Hyp by 42 mass units. The mass of Hyp was therefore no longer the same as that of Leu/Ile, and its position could be deduced from the fragment masses of the acetylated peptide. In the FAB mass spectrum, the molecular ion showed the expected increase of 84 mass units, corresponding to the addition of two acetyl groups. However, the intense fragment ion peaks at *m/z* 1390 and 1080 were shifted by only 42 mass units, indicating that one acetyl group had been added after residue 12. This located the position of the Hyp as residue 13. Notably, the fragment ions continued to be 42 mass units higher than for the underivatized

Figure 4 Electrospray MS of the carboxypeptidase digest of XR586

The digestion time was 18 h. The peaks ($[M, Na_2]^{2+}$ ions) at 934.5, 861.0 and 812.6 arise from peptides from which Gly, Phe + Gly and Pro + Phe + Gly respectively have been cleaved. This confirms the C-terminal sequence as -Pro-Phe-Gly-OH.

peptide until mass 654. This corresponded to cleavage between Leu-5 (or Ile-5) and Thr-6, the position of the second acetylation. From this residue on, fragments were the same as for the underivatized peptide.

The positions of Ile and Leu were determined using the enzyme α -chymotrypsin. This enzyme cleaves peptides on the C-terminal side of aromatic residues. It also has similar activity at Leu, but not Ile [14]. The peptide was incubated with the enzyme and samples analysed by electrospray MS. A peak at m/z 928, arising from the sequence Aib-9 to Gly-17, confirmed Leu as residue 8. Several other peptides arising from cleavage at the aromatic amino acid residues, which confirm this conclusion, were also observed. As cleavage was not observed at Ile, its position must be residue 5 by elimination.

The remaining problem, the order of residues 10, 11 and 12, was resolved by treatment of the peptide with trifluoroacetic acid. This degradative procedure is known to preferentially cleave peptides of this type between Aib (or Iva) and Pro (or Hyp) [1,15,16]. Analysis of the hydrolysate by electrospray and

FAB-MS indicated the presence of the tripeptide Pro-Phe-Gly (m/z 320.1), which could arise from such an Iva-Pro cleavage. The hydrolytic fragments Hyp-Iva (m/z 231.0), (Pro-Aib-Gln)-Hyp-Iva (m/z 541.4) and (Pro-Gln-Aib) (m/z 329.0) were also observed. Residue 9 was already known to be Aib and residue 13 to be Hyp. This therefore suggested that residue 10 is Pro and residue 12 is Aib, as the same Aib(Iva)/Pro(Hyp) cleavage would then give rise to all these fragments. The assignment of residue 10 as Pro was supported by the FAB mass spectrum of the intact peptide, which showed a very intense fragment ion peak corresponding to cleavage between residues 9 and 10. Other peptaibols show similarly intense peaks for fragment ions resulting from cleavage between Aib and Pro [11]. The assignment of residue 12 as Aib was supported by examination of other dipeptide hydrolytic fragments observed in the mass spectrum. Many of these appeared to arise from cleavage at the C-terminal side of Aib or Iva. These included Leu-Aib and Thr-Aib. If the sequence had been Pro-Aib-Gln, a fragment corresponding to Pro-Aib might have been expected. However, no such fragment was seen. By contrast, a fragment corresponding to Pro-Gln-Aib was observed (m/z 329). It therefore seems that residues 11 and 12 are Gln and Aib respectively. The complete structure is shown in Figure 5.

This peptide clearly has many features in common with the peptaibols, particularly the zervamicins [11]. Figure 6 compares the sequences of several zervamicins with that of XR586. In particular, zervamicin ZII-3 shows a very similar structure, differing at only four positions. Three of these substitutions are for similar amino acids: Val to Iva, Hyp to Pro and Aib to Iva. However, there is a major difference between XR586 and peptaibol peptides. The C-terminal residue in peptaibols is always an amino alcohol [1,15–17], and in the zervamicins it is always phenylalaninol, the reduced form of Phe [11]. XR586 has an additional Gly residue after a normal Phe. This may well provide evidence for the route by which the peptaibols are biosynthesized, with cleavage of the Gly being associated with reduction of Phe. A very similar pathway operates in the C-terminal amidation of neuropeptides [18]. Here also, the peptide is expressed with an extra C-terminal Gly, and specific enzymes bring about amidation with concurrent cleavage of Gly. The difficulty of reducing a carboxylate anion might explain why such a mechanism is followed in the present case.

Figure 5 Structure of XR586

Z1A	Ac	Trp	Ile	Glu	Iva	Val	Thr	Aib	Leu	Aib	Hyp	Gln	Aib	Hyp	Aib	Pro	Phol
Z1C	Ac	Trp	Ile	Glu	Iva	Ile	Thr	Aib	Leu	Aib	Hyp	Gln	Aib	Hyp	Aib	Pro	Phol
ZII-3	Ac	Trp	Val	Gln	Aib	Ile	Thr	Aib	Leu	Aib	Hyp	Gln	Aib	Hyp	Aib	Pro	Phol
XR586	Ac	Trp	Iva	Gln	Aib	Ile	Thr	Aib	Leu	Aib	Pro	Gln	Aib	Hyp	Iva	Pro	Phe Gly

Figure 6 Sequences of XR586 and several zervamicins

Data for the zervamicins (Z1A, Z1C and ZII-3) were taken from [11]. Ac, acetyl; Phol, phenylalanol.

Figure 7 CD spectrum of XR586 in methanol/water (1:1, v/v)

Peptaibols are helical peptides which contain regions of 3_{10} as well as α -helix [2,3,17,19]. Lack of material prevented a detailed NMR analysis of the solution conformation of XR586, but CD measurements in methanol/water (1:1, v/v) showed a positive band below 200 nm and negative bands at 208 nm and 220 nm, as expected for a helical structure (Figure 7). It is not possible to distinguish a 3_{10} from an α -helix using CD, but it would seem likely that the regions are interspersed so as to give an amphipathic helix, with hydrophobic and hydrophilic faces, thus allowing membrane insertion and pore formation [2,3,19].

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