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STRUCTURAL STUDIES ON THE PEPTIDE MOROIDIN FROM LAPORTEA MOROIDES†

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Abstract - By a combination of chemical, ^{1}H and ^{13}C NMR, and mass spectrometric studies, two alternative structures for Moroidin, from Laportea moroides, have been elucidated. Moroidin, which may be responsible at least in part for the toxicity of Laportea moroides, is a tricyclic octapeptide containing an unusual C-N linkage between tryptophan and histidine residues.

Introduction

Laportea moroides is a bush of the genus Urticaceae[1]. It is covered with stinging hairs and grows profusely in rain forests in Eastern Australia. Cutaneous contact with the hairs results in intense pain, piloerection, arteriolar dilatation and local sweating. In extreme cases, unconsciousness and death have been reported. It is regarded as a menace to stock and particularly to horses which, if severely stung, become violent and have had to be shot. Men, too, become frantic if badly stung and timber workers and bushmen are the main sufferers. Although there is no record of the death of any human victims from the sting of Laportea moroides, a human fatality in New Guinea[2] following the sting of a similar species Laportea condata, has been reported.

The severity and long duration (lasting as long as 30 h) of the sting of the Laportea species have been known since the middle of the nineteenth century. Robertson and Macfarlane's work[3] on Laportea moroides found 0.01-0.05 μg acetylcholine-like, 0.025-0.05 μg histamine-like and 0.001 μg 5-hydroxytryptamine-like activities per stinging hair. However, when acetylcholine, histamine and 5-hydroxytryptamine were injected together intradermally in human subjects, very little effect was produced compared with that of extracts of Laportea hairs. They are not predominantly responsible for the painful sting. There must be at least one other extremely active substance in Laportea hair; however, its nature is still unknown. Here we report the purification, and structural studies on bicyclic octapeptide, which is biologically active, isolated from Laportea moroides.

Extraction and Purification

Crude material was isolated from the frozen leaves and leaf atallus of Laportea moroides by solvent extraction and column chromatography (by a modification

† This publication is dedicated to Ralph A Raphael on the occasion of his 65th birthday.

of Oelrichs and Robertson's method[4]). Fractions were monitored by TLC and bioassay and the active fractions were collected. TLC showed that the active fraction contained a complex mixture of UV absorbing compounds. They were subjected to reversed-phase HPLC analysis (see experimental) and one major compound was detected. This compound, termed Moroidin, was isolated by preparative HPLC. It has a concentration of 2.0-2.4 mg per 100 g of the frozen leaves of Laportea moroides.

Structural Studies

Moroidin showed a λ_{max} at 215 nm and 275 nm in the UV spectrum. It gave a positive test with chlorine-starch-iodine suggesting that it might be a peptide. Amino acid analysis showed Moroidin to contain (molar ratio in parentheses): Glu(1), Gly(1),Val(1), Leu(1), His(1) and Arg(1). Moroidin gave no reaction with Pauly's reagent (sulphanilamide/HCl; $NaNO_2/H_2O$). This test is positive only if an imidazole ring has a free imino group and a proton attached at the 2-, 4- or 5-position.

The molecular weight of Moroidin was found to be 986 daltons by FABMS, but in neither the FAB mass spectrum nor the EI mass spectrum of the permethylated Moroidin were sequence ions observed. Further FABMS experiments showed that Moroidin was N-terminally blocked (no acetylation with Ac_2O/H_2O), contained one carboxyl group (esterified once with MeOH/HCl), and no alcohol groups (no acetylation with Ac_2O/pyridine).

The C-terminus of Moroidin was determined by selective deuteration (a modified tritium-labelling method[5]) of the C-terminal amino acid residue through racemisation, via the oxazolone intermediate, using acetic anhydride in a medium containing D_2O and pyridine. The deuterium labelled C-terminal amino acid was then characterised by negative ion mode CI-GCMS after total acid hydrolysis (6M HCl, 105°C, 24 h) and derivatisation (N-trifluoroacetyl-isopropyl ester derivatives). It was found that deuterium was incorporated only in histidine by the observation of an increase in the $(M-H)^-$ ion of histidine by one mass unit.

Further evidence was obtained by recording ^1H NMR spectra of Moroidin in d_6-DMSO with varying amounts of added acid. A limiting downfield shift of 0.2 ppm on the α-proton of the histidine residue was observed.

A 500 MHz ^1H NMR spectrum of Moroidin in d_6-DMSO solution at 333K is reproduced in Figure 1. Assignment of the amino acids found by amino acid analysis was achieved by using decoupling difference, two-dimensional ^1H-^1H COSY and variable temperature studies. The COSY spectrum is shown in Figure 1 and the data derived from the decoupling difference and COSY spectra are summarised in Table 1.

The ^1H NMR spectrum of Moroidin indicated that the β-protons of the glutamic acid residue were very non-equivalent (δ 1.76 and 2.26 ppm) and their γ-CH_2 signal was very complex compared to the simple triplet expected for a normal glutamic acid residue[6]. This indicated very restricted rotation and so it seemed likely that Moroidin contained a pyroglutamyl residue rather than a normal glutamyl residue. This conclusion was supported by partially hydrolyzing the Moroidin with 6M HCl at 110°C for 10 min after which addition of water was detected by FABMS. Acetylation (Ac_2O/H_2O) and esterification (MeOH/HCl) showed the product to contain an amino group and two carboxyl groups. Loss of 129 daltons (ie Glu) from the $(M+H)^+$ ion was observed after one cycle of Edman degradation. Furthermore, loss of 111 daltons was found when the FAB spectrum of Moroidin was re-run using a higher kinetic energy (9.5 KeV) of xenon atoms (Figure 2). The blocked N-terminus was thus found to be an N-terminal pyroglutamic acid residue. However, attempts to cleave or open the pyroglutamic acid residue by pyroglutamyl aminopeptidase[7] (an enzyme which cleaves pyroglutamic acid specifically), or by selective methylamine cleavages[8], failed.

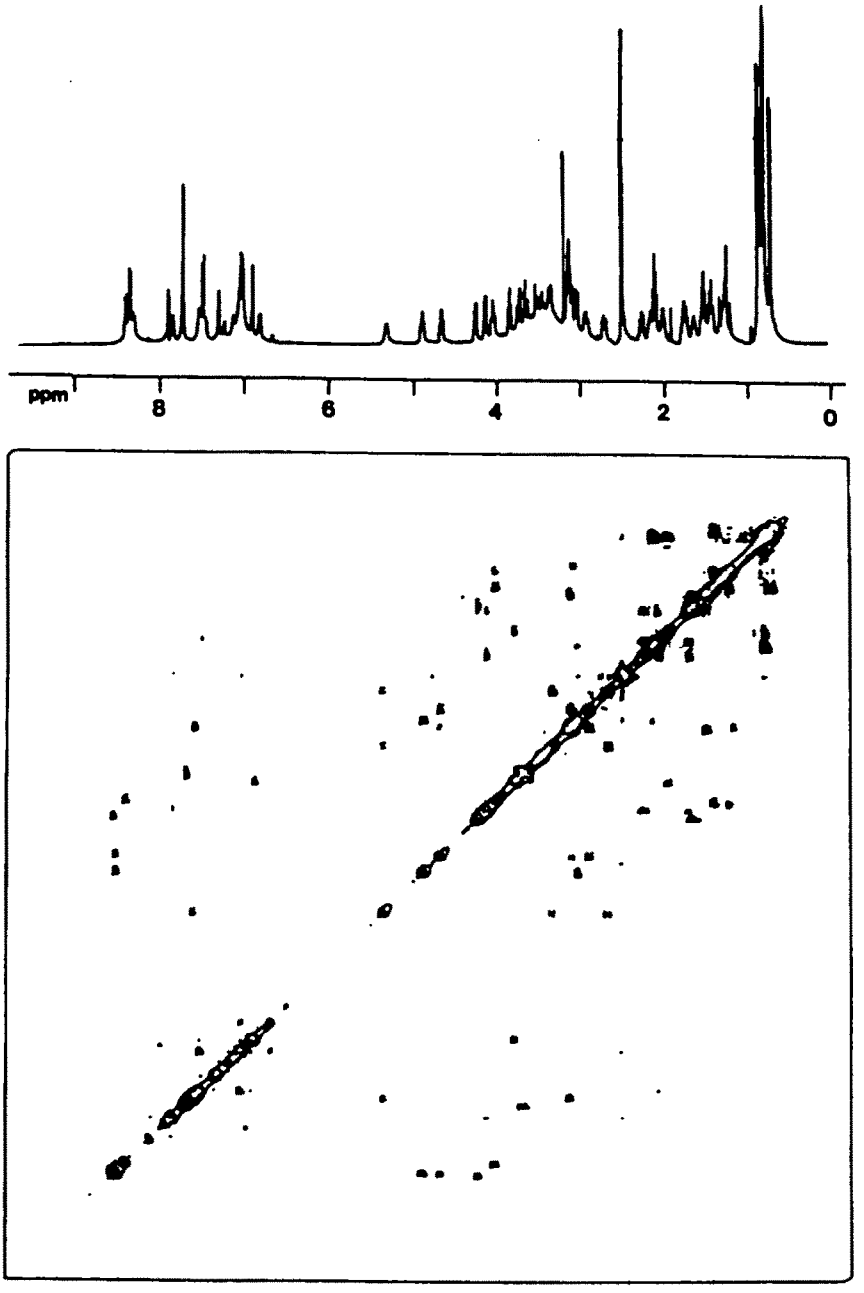


Figure 1 - ^{1}H NMR spectrum of Moroidin (~ 14 mM) at 500 MHz in d_6-DMSO solution at 333 K. The COSY spectrum obtained at 400 MHz is shown in the lower half of the figure.

Table 1 : ^{1}H NMR assignment for Moroidin[a]

Amino acid		δobserved[b]	J[b]	δliterature[c]
Glycine	NH	7.53	4.8	8.15
	αCH	3.63	4.7, 15.8	3.76
		3.73	4.7, 15.8	3.76
Valine	NH	6.81	8.2	7.88
	αCH	3.84	8.2	4.26
	βCH	2.01	o	1.97
	γCH_3	0.83	7.0	0.92
		0.86	7.0	0.98
Leucine	NH	8.31	10	7.95
	αCH	4.04	dt	4.37
	βCH_2	1.26	O	n.c.
	γCH	1.46	m	n.c.
	δCH_3	0.72	6.5	0.85
		0.81	6.6	0.89
pyroGlu	NH	7.73	s	n.c.
	αCH	4.14	4.1, 8.8	4.34
	βCH	1.76	m	1.87
		2.26	m	1.87
	γCH_2	2.10	m	2.26
Arginine	NH	8.41	7.1	8.07
	αCH	4.25	q	4.38
	βCH	1.64	sextet	1.56
		1.76	m	1.56
	γCH_2	1.53	quintet	1.56
	δCH_2	3.13	O	3.17
	εNH	7.41	O	-
Histidine	NH	8.36	9.1	8.31
	αCH	4.66	dt	4.66
	βCH	2.98	10, 15.3	2.96
		3.13	O	3.22
	C_2H	7.73	s	8.35
	C_4H	7.31	br.s	7.35
β^6Leu[d]	NH	8.36	d	7.95
	αCH	4.89	9.4	4.37
	βCH	3.04	3.5, 11.7	1.26
	γCH	2.16	m	1.46
	δCH_3	0.80	7.0	0.85
		0.87	7.0	0.89
Tryptophan	NH	7.53	br.s	8.09
	αCH	5.32	br.s	4.58
	βCH	2.71	8.5, 15.3	2.93
		3.35	6.4, 15.3	3.16
	C_4H	7.49	8.2	7.65
	C_5H	7.03	8.2	7.01
	C_7H	6.91	s	7.35
	indole NH	11.5	s	10.76

(a) Reported values measured at 333K in d_6-DMSO, 500MHz.
(b) δobserved, in ppm; J, in Hz.
(c) δliterature, in ppm , from the protected linear tetrapeptide F_3C-CO-Gly-Gly-X-Ala-OCH_3 where X stands for the different amino acid[11].
(d) Abbreviations used : s=singlet, d=doublet, dd=doublet of doublets, dt=doublet of triplets, m=multiplet, br.=broad, O=not measured due to overlap.

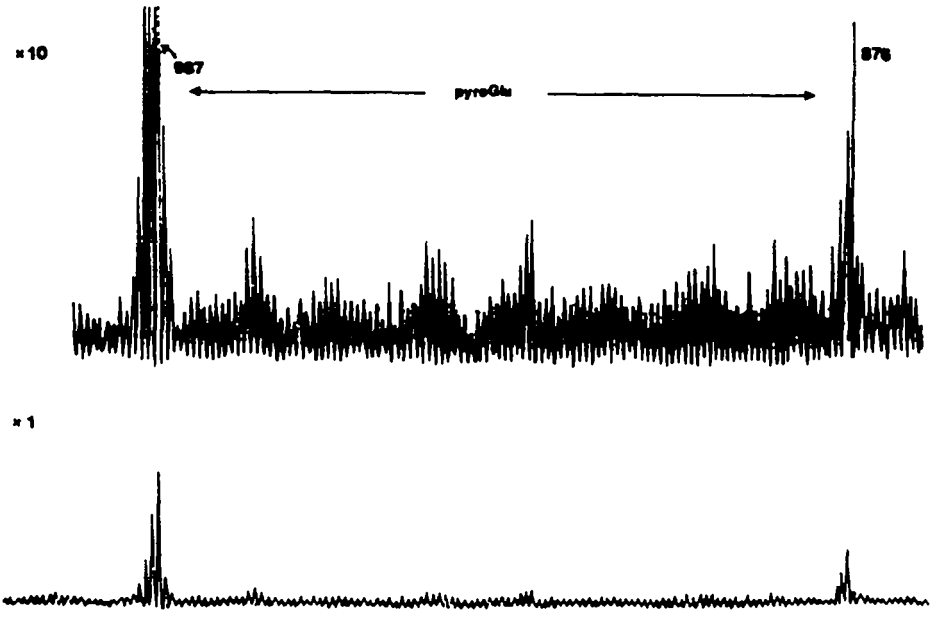


Figure 2 - High mass region of the positive ion FAB mass spectrum of Moroidin obtained using 9.5 KeV xenon atoms.

Partial Sequence of Moroidin

Moroidin was found to be resistant to enzymic cleavage with chymotrypsin, carboxypeptidase Y, thermolysin, papain and Staphylococcus protease. However, it could be opened by trypsin (which cleaves specifically at the C-terminal side of Arg and Lys). Addition of 18 daltons was then observed by FABMS. Acetylation and esterification showed that this product contained an amino and two carboxyl groups. After one Edman degradation cycle[9] on the tryptic digest of Moroidin, the FAB mass spectrum of the remaining peptide showed that N-terminal glycine has been cleanly removed. The second Edman cycle failed as evidenced by the increase in the molecular weight of the product by 135 daltons, as determined by FABMS. The increase in mass was due to the coupling of the peptide with PITC. This showed that the next residue is not a standard amino acid, or alternatively that the linkage may be stereochemically hindered so that cyclisation cannot occur.

In order to reveal the nature of the next amino acid residue, the first Edman cycle was repeated on the product of tryptic digestion, and this product was then protected with dansyl chloride (4-N,N-dimethyl-amino-1-naphthalene sulphonyl chloride). Upon total hydrolysis of this material (6M HCl, 105°C, 24 h), the N-terminal residue was identified as histidine by the use of TLC on polyacrylamide plates; the dansylated N-terminal residue showed the same chromatographic properties as the authentic dansyl-histidine. Since trypsin cleaves specifically at the carboxyl end of arginine, it is obvious that the newly generated C-terminus in the tryptic digest is arginine. Therefore, the partial sequence of Moroidin is:

-Arg-Gly-His(OH)-.

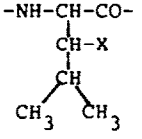
It became obvious that the histidine was linked in the intact molecule through its imidazole moiety. The negative reaction with Pauly's reagent indicates that the histidine's imidazole ring could be substituted at the NH, C_2 or C_4 position(s).

The ^{1}H NMR spectrum of Moroidin showed the presence of the C_2 and C_4 protons of histidine (δ 7.73 and 7.35 ppm) by comparison of the observed chemical shifts with the literature values (δ 8.35 and 7.35 ppm), deuterium exchange[10] and pH dependence studies[11]. In addition, no resonance assignable to an imidazole NH was found. It could thus be concluded that the histidine residue of Moroidin is substituted at the imidazole NH.

Nature of the Additional Fragments

FABMS showed that the molecular weight of Moroidin was larger than the sum of the masses of the amino acids found by amino acid analysis. By peak matching, an exact mass of 986.50718 (average value) daltons was obtained for Moroidin using Substance P (4-11), Dynorphin (1-8) and Oxytocin as references. This determination gave a molecular formula of $C_{47}H_{66}O_{10}N_{14}$ (calculated=986.5086) for Moroidin and indicated that the mass and molecular formula of the additional fragments are 296.1399 and $C_{17}H_{18}O_2N_3$ respectively. The structures of the fragments giving rise to this mass difference were partially revealed by NMR spectroscopy.

The ^{1}H NMR spectrum of Moroidin appeared to show the presence of two novel amino acids. One of them gave rise not only to an α-proton signal (δ 4.89 ppm) coupled to amide (δ 8.36 ppm) and β-proton (δ 3.04 ppm) resonances, but to a γ-proton (δ 2.16 ppm) coupled to the same β-proton and to two methyls (δ 0.80 and 0.87 ppm). This was assigned to a β-substituted leucine residue (A) in which the substituent (X) could be either an aromatic system or an electron withdrawing group.



(A)

The other amino acid was found to be a substituted tryptophan residue. Evidence for this was as follows. First, a λ_{max} at 275 nm was observed in the UV spectrum of Moroidin. Second, the ^{1}H NMR spectrum contained an α-proton signal (δ 5.32 ppm; Table 1) coupled to an amide resonance (δ 7.53 ppm) and two non-equivalent protons (δ 2.98 and 3.35 ppm, similar to those of the histidine residue). The degree of non-equivalence of the β-protons suggested that they were attached to an aromatic system. Third, three aromatic protons were found in addition to those assigned to histidine; two of these were coupled to one another with a coupling constant of 8.5 Hz. Fourth, an exchangeable proton signal at δ 11.5 ppm was found which gave an NOE to the third aromatic singlet at δ 6.91 ppm in the ^{1}H NMR spectrum of Moroidin. Fifth, the ^{13}C NMR spectrum showed Moroidin to contain eight additional aromatic carbons. Three of these were shown to be methine carbons by ^{13}C DEPT experiments[12]. Comparison of the literature values of ^{13}C chemical shifts[11] of aromatic amino acids with the observed ^{13}C shifts showed they were consistent with the presence of a substituted tryptophan residue. Agreement for the indole moiety was good (± 3 ppm; Table 2) except for the C_2 and C_6 carbons (see Fig 3 for numbering of the indole nucleus) which showed a downfield shift of 9 and 7.7 ppm respectively. This suggested that the tryptophan residue was substituted at these positions.

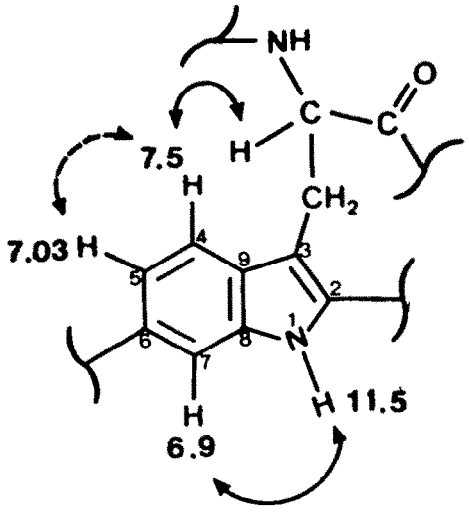
Substitution at the C_2 position of the proposed tryptophan residue was shown by the negative result obtained with Ehrlich's reagent (p-dimethylaminobenzaldehyde/HCl), which reacts with pyrrole derivatives having one intact CH group. Substitution at the C_6 position was shown by NOE and decoupling difference experiments.

The α-proton of the tryptophan residue showed an NOE with one of a pair of aromatic doublets (J_{HH}=8.5Hz). This together with the NOE seen between the singlet at δ 6.9 ppm and the NH at δ 11.5 ppm confirmed the substitution at the C_6 position. The results are summarised in Figure 3 with coupling and NOEs between two protons indicated by (⌒) and (⌒) respectively.

Table 2 : Comparison of the aromatic ^{13}C NMR signals of Moroidin with literature values for tryptophan[11].

Assignment	Chemical Shift/ppm δobserved	δliterature[a]
C_2	135.0	126.0
C_3	105.3	108.4
C_4	121.6	120.3
C_5	123.0	122.9
C_6	127.0	119.3
C_7	121.0	118.7
C_8	137.5	137.3
C_9	128.0	127.5

(a) Measured in protected linear tetrapeptide[83], TFA-Gly-Gly-X-Ala-OCH_3, solvent used was D_2O.



⌒ indicates coupling between two protons.

⌒ indicates NOE between two protons.

Figure 3 - Structure of the 2,6-disubstituted tryptophan residue deduced from UV, NMR and chemical evidence.

The presence of the β-substituted leucine and 2,6-disubstituted tryptophan residues accounted for the measured molecular weight and formula of Moroidin exactly. Altbough attempts to isolate the two novel amino acids by alkaline[13] (4M NaOH, 106°C, 24 h) and p-toluenesulphonic acid hydrolysis[14] (4M, 105°C, 24 h) failed, we consider evidence for their presence using ^1H and ^{13}C NMR to be satisfactory.

Determination of the Full Amino Acid Sequence using NOE experiments

Taking the chemical information in conjunction with the amino acid composition,

it was clear that Moroidin must be bicyclic, with a partial sequence of -Arg-Gly-His(OH)-. However, no further sequence information could be obtained from either selective enzymic or acid cleavages. Therefore, classical methods or mass spectrometric studies could not readily deal with this problem. In solving this problem, both one-dimensional NOE difference and two-dimensional NOE spectroscopy (NOESY), which give information on relative interproton distances, were used. All NOE experiments were done at room temperature (293K) using d_6-DMSO as solvent for the sample. The study of NOEs was concentrated primarily on the NH and α-protons to deduce the sequence of the amino acids. The data are tabulated in Table 3 and are summarised in Figure 4. The NOEs between two protons are indicated by the arrows (⌒).

Table 3 : Nuclear Overhauser effects observed in Moroidin.

Proton	NOE observed
Trp C_4	Trp C_5, Trp αCH
Trp C_5	Trp C_4, β^aLeu αCH
β^aLeu αCH	Trp C_5, Leu NH
Leu NH	β^aLeu αCH, Val NH
Trp αCH	Trp C_4, Arg $NH^{(a)}$, Trp βCH
Trp NH	Val αCH
Gly NH	Gly αCH, Arg αCH
Gly αCH	Gly NH, His $NH^{(a)}$
pyroGlu αCH	β^aLeu $NH^{(a)}$
β^aLeu βCH	Trp C_7, β^aLeu $NH^{(a)}$
His αCH	His C_4
Trp NH_{indole}	Trp C_7

(a) Revealed after acid titration.

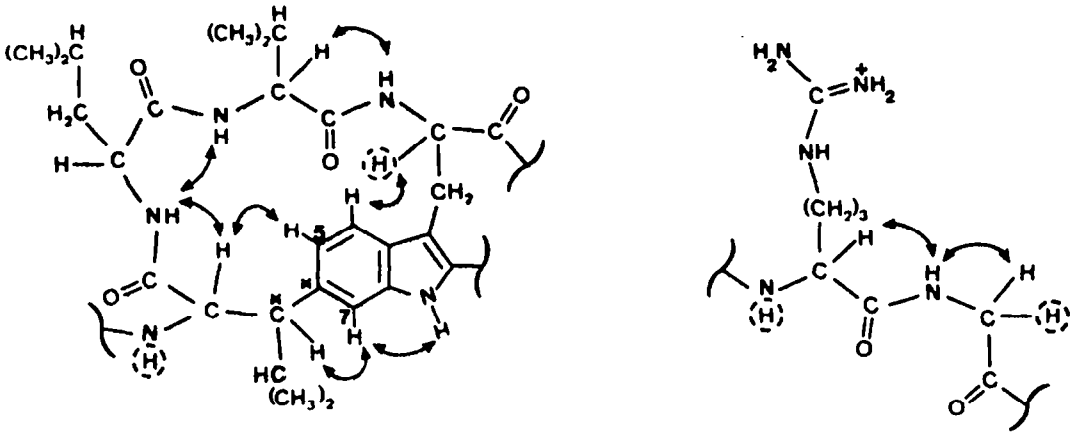


Figure 4 - Partial amino acid sequences of Moroidin derived from proton NOEs (indicated by ⌒). The sequence -Arg-Gly- is independently available from enzymic digestion and mass spectrometric experiments (see text).

The observed NOEs were shown to be direct (that is not arising via spin diffusion) by studying their rate of growth. This was done by measuring the % NOE growth as a function of the preirradiation time (0.2, 0.4, 0.8, 1.6, 3.2, 6.4 s). This showed that the time dependences of the observed NOEs were exponential with characteristic $t_{\frac{1}{2}}$'s (the time taken for the intensity changes to reach half their maximal values). The $t_{\frac{1}{2}}$'s are proportional to r^{-6}, where the values of r are the various interproton distances. These allowed us to estimate the relative distances between protons. This gave a sequence of:

-β^SLeu-Leu-Val-Trp-

(where β^SLeu represents the β-substituted leucine unit) which was then linked to form a cyclic structure through a C^*-C^* bond (Figure 4). Evidence for this linkage was the observation of fast growing NOEs given by the C_5 and C_7 protons of the tryptophan residue to the α- and β-protons of the β-substituted leucine, respectively. The structural conclusion derived in the left hand portion of Figure 4 is supported by the subsequent demonstration (see later) that the NH of the β-substituted leucine residue is attached to the available carbonyl group of pyroglutamic acid.

The sequence of -Arg-Gly- had already been found by tryptic digestion and mass spectrometric studies as discussed above. In addition, NOEs were also observed for the protons encircled (Figure 4) to the NH protons at δ 8.2-8.3 ppm. These amide protons (β^SLeu, Arg and His) overlapped with one another and so linkages were ambiguous at this stage.

The connections of all the amino acids were more clearly established by recording a combined COSY-NOESY spectrum of Moroidin under acidic conditions so that the above mentioned amide protons were well-separated. The derived connections are shown in Figure 5 and are represented by the symbol •——•.

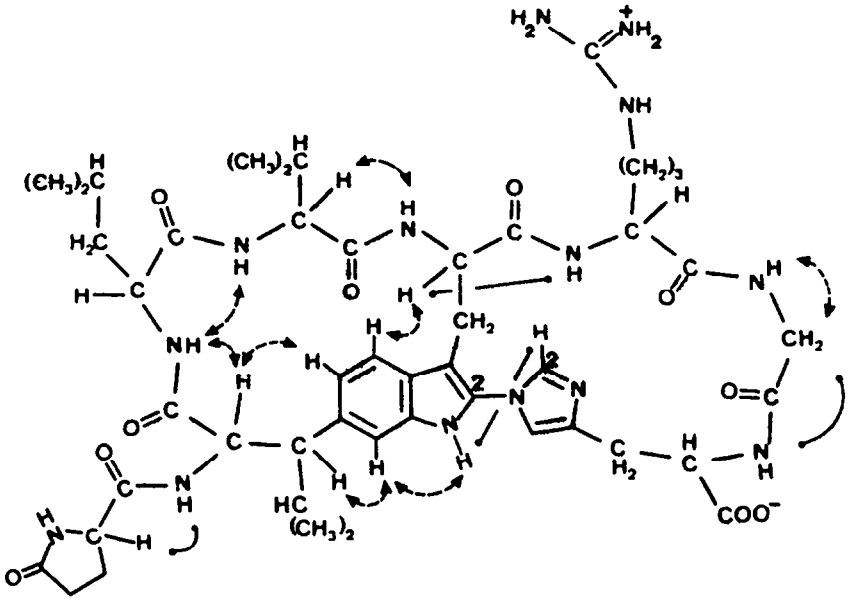


Figure 5 - Covalent structure of Moroidin. The other N atom of the imidazole may equally be attached to C-2 of the indole.

An important feature of Moroidin not yet discussed is the C-N linkage between the histidine and tryptophan residues (Figure 5) which completes the bicyclic structure of Moroidin. This was suggested by the NOE observed between the C_2 proton of the histidine residue and the indole NH of the tryptophan residue. The other N atom of the imidazole may equally be attached to C-2 of the indole.

Oxidation of Moroidin

The C-N linkage was demonstrated chemically by oxidizing Moroidin with bis-trifluoroacetoxy-iodobenzene (TIB). TIB is normally used for Hofmann degradation to convert an amide function to an amine. It contains trivalent iodine which makes it a potential oxidizing agent. Moroidin was allowed to react with TIB in CH_3CN/H_2O (1:1) at room temperature in the dark. The product was analysed by FABMS. FABMS showed that the product was a mixture of components with molecular weights

of 1054 and 1020 daltons. The mixture was then analysed by HPLC and one major product (OPI) was isolated and characterised by FABMS, UV and ^{1}H NMR spectroscopy.

FABMS showed the molecular weight of OPI to be 1020 daltons. Accurate mass measurement gave an exact mass of 1020.5151 and a molecular formula of $C_{47}H_{68}O_{12}N_{14}$ (calculated=1020.5142) using [Val^5]-Angiotensin II (M_R=1031) and Oxytocin (M_R=1006) as references. This indicated that the extra mass of OPI compared to Moroidin was due to the addition of two hydrogen and two oxygen atoms. Acetylation (Ac_2O/H_2O) and esterification (MeOH / HCl) showed the oxidation product contained no additional amino or carboxyl groups.

In the positive ion mode FAB mass spectrum, sequence ions which appeared as doublets separated by 16 mass units were seen at m/z 883, 826 and 670 from the pseudomolecular ion of OPI (Figure 6). Consideration of the mass difference between the sequence ions and comparison of these differences with those arising from the peptide fragmentations commonly occurring in FAB mass spectra[15] gave the NA sequence of -Arg-Gly-His-OH. Doublets separated by 16 mass units are not commonly observed in NA sequence ions. Their appearance may possibly be due to the oxidized nature of OPI. The C-terminal histidine was confirmed by performing a carboxypeptidase Y digestion. Loss of 137 daltons was observed in FAB mass spectrum after the digestion. This suggested that the imidazole NH of histidine which was absent in the original Moroidin was generated during the reaction. This was further supported by the observation of an NH resonance at δ 18.4 ppm in the ^{1}H NMR spectrum of OPI. Furthermore, a positive reaction with Pauly's reagent was observed with OPI.

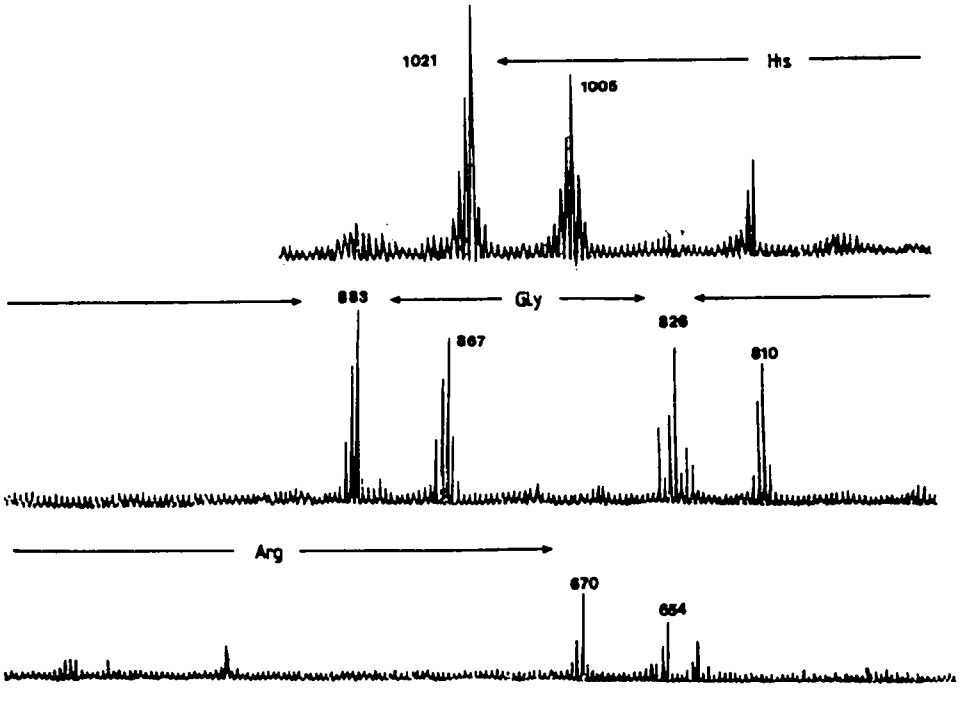


Figure 6 - High mass region of the positive ion FAB mass spectrum of OPI, obtained from Moroidin by oxidation with bis-trifluoroacetoxyiodobenzene (TIB).

When the oxidation reaction of Moroidin was repeated in a deuterated medium (CD_3CN/D_2O), it was found that no deuterium was incorporated as no change in molecular weight was observed by FABMS. This implied that the two added hydrogens in OPI are exchangeable and are not carbon-bound protons.

In the ^{1}H NMR spectrum of OPI (Figure 7) all the resonances of Moroidin were

found. Most shifts agreed to within 0.3-0.5 ppm, except those of the signals due to the amide protons and the β-protons of tne tryptophan residue. Any newly generated protons and those that suffered a relative chemical shift of ± 0.15 ppm or more on the oxidation of Moroidin to OPI are shown with the shift (in ppm) adjacent in Figure 8, where no stereochemical detail is given.

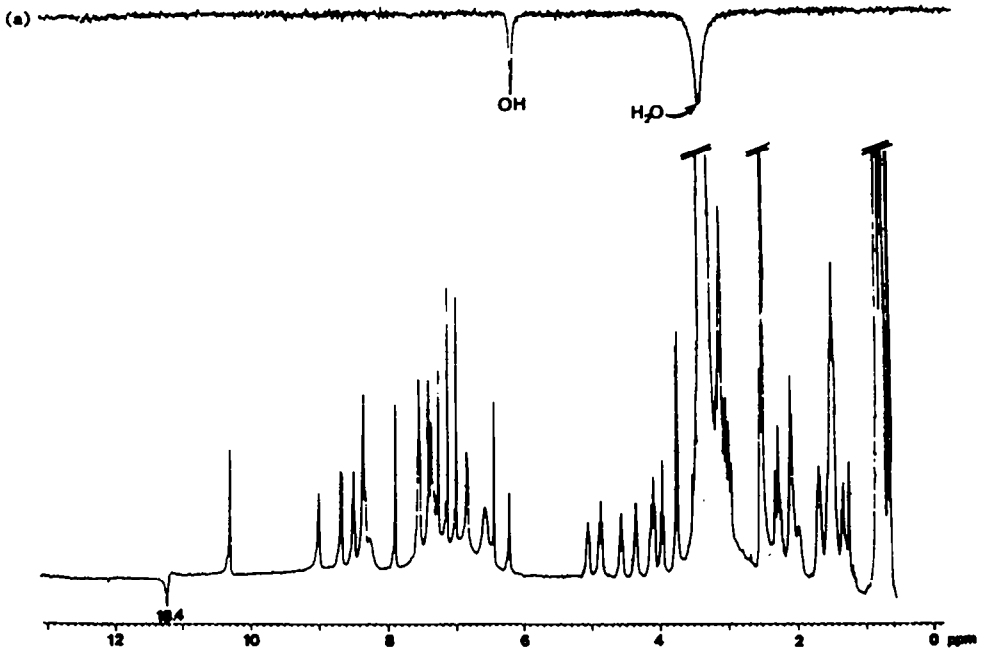


Figure 7 - ^{1}H NMR spectrum of OPI in d_6-DMSO. The superimposed trace (a) is a difference spectrum obtained by subtraction of the spectrum obtained upon irradiation of the water peak from the normal spectrum.

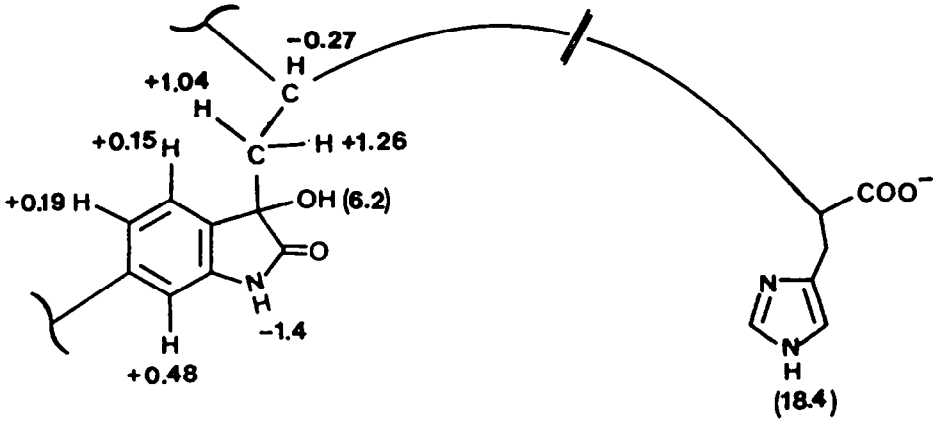


Figure 8 - Modified structural units deduced to be present in OPI (relative to Moroidin). Chemical shifts of newly generated protons are indicated in parentheses. Changes in chemical shifts ≥ 0.15 ppm are also indicated; a '+' sign indicates an upfield chemical shift.

The β-protons of the tryptophan residue showed a significant upfield shift (ie from δ 2.71 and 3.35 ppm to δ 1.45 to 2.29 ppm) suggesting that part of the aromatic system of the indole moiety had been destroyed. This was further supported by the UV absorption of OPI at 269 nm (compared with 275 nm for the intact Moroidin). The

hypsochromic shift of the λ_{max} was found to be consistent with the conversion of an indole to an oxindole. The presence of a hydroxyl group was revealed by a ^1H NMR saturation transfer difference experiment performed in d_6-DMSO. The difference spectrum is shown in Figure 7(a). The water signal, which was saturated in the experimental data set, appears as a negative peak, as does the hydroxyl proton which has become saturated, or partially saturated, by chemical exchange with the water in the solvent.

Attempts to acetylate the hydroxyl group of OPI with either acetic anhydride/pyridine or acetic anhydride/dimethylaminopyridine[16] failed (the latter probably due to solubility problems). Since the hydroxyl group is not coupled to any protons as observed by decoupling difference experiments, we conclude that it is tertiary. Taking all this information, it became clear that the oxidation product, OPI, of Moroidin was (B), which is consistent with the mechanistic scheme shown in Figure 9. It is proposed that reaction is occurring through oxidation of the indole ring, followed by a nucleophilic attack of water, to give the product OPI.

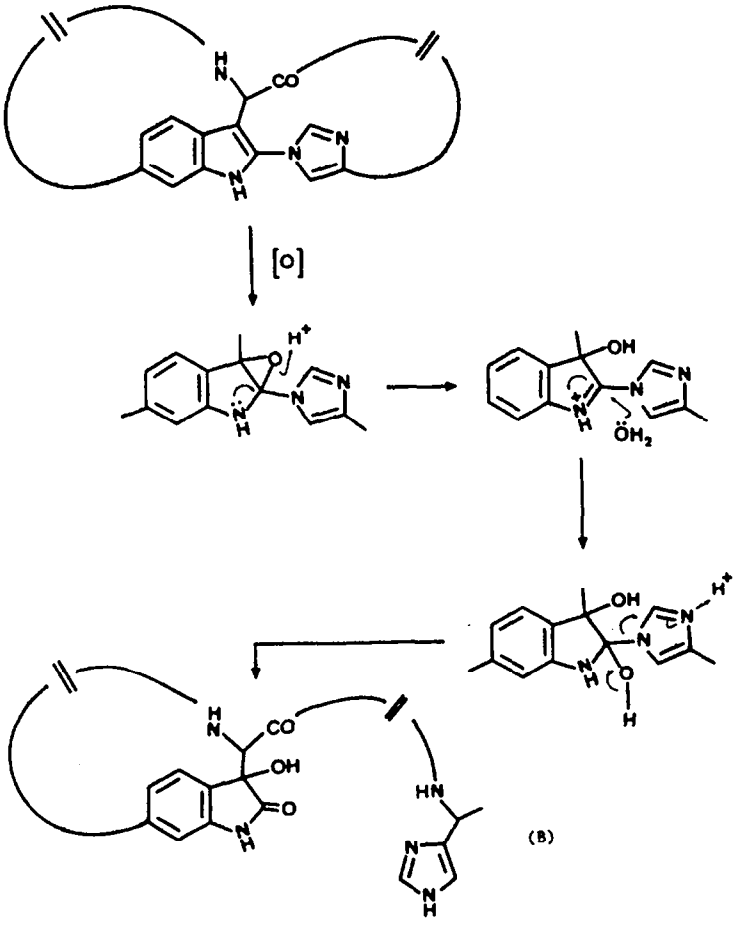


Figure 9 - Mechanism for the production of OPI from Moroidin.

Stereochemistry of the Amino Acids

From the enzymic digestion of Moroidin by trypsin and the carboxypeptidase Y cleavage of OPI, we knew that arginine and histidine must possess the L absolute configuration. The configurations of the other amino acids, except glycine (which is not chiral), ß-substituted leucine and tryptophan, were determined by GC analysis on a capillary column coated with a chiral stationary phase (Chirasil-Val)[17]. This phase has been designed to resolve complex mixtures of amino acids

and, at the same time, to resolve the D- and L- enantiomers of each amino acid. Moroidin was hydrolysed into its amino acid components, and these were derivatised to their N-trifluoroacetyl-isopropyl esters (isopropanol/HCl; $(CF_3CO)_2O/CH_2Cl_2$), and detected, after passage through the GC column, by flame ionization. Co-injection with derivatives of authentic D- and L-amino acids showed that the configurations of pyroglutamic acid, leucine and valine are L.

The stereochemistries of the β-substituted leucine and tryptophan residues have not yet been determined since these amino acids could not be isolated. Figure 10 shows the stereochemistry of 6 of the 8 amino acid residues of Moroidin.

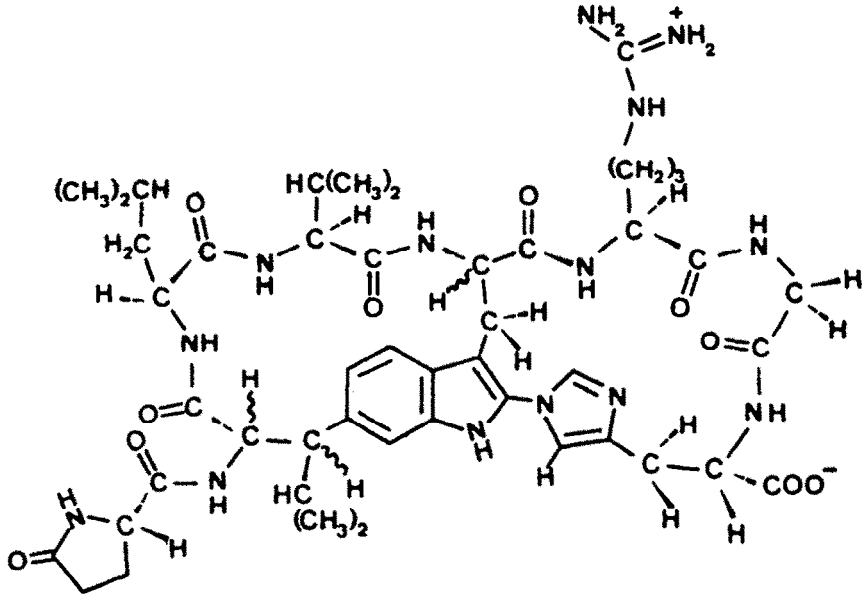


Figure 10 - One of two possible structures for Moroidin, indicating available stereochemistry.

Biological Activity and Discussion

The method used for testing the stinging activity of Moroidin was developed by Macfarlane[1] and involves injecting dilute saline solutions of the sample intradermally into human forearms. Local responses (like pain, redness and duration) were observed and were rated on a scale of 1-5. 5 μg of pure Moroidin was found to give a moderate reaction of activity rating 2 and about 10 μg gave a severe reaction of activity rating 4 on injection into human forearms. It showed that Moroidin is less active than the crude material of which about 1 μg produces a severe and long lasting reaction (rating 5). It is difficult to measure the stinging activity of Moroidin quantitatively using the above method and the results can only be used as a rough guide. Until a more accurate test is developed, the activity of Moroidin cannot be quantified. However, it seems sure that Moroidin is not the only active

component in the extract of Laportea moroides. For example, one fraction from column chromatography which does not contain Moroidin also produces severe reaction on human forearms. This fraction has not been investigated in our laboratory and it may be worth studying in the future.

The mechanism of the effects of the extracts of Laportea moroides when injected intradermally into human skin have not been analysed fully. Observation of the immediate pain sensation, vasodilation, piloerection and local sweating suggested that that the injected material stimulates the sweat gland directly and could be in contact with pain nerves. Rubbing of the extract into unbroken skin produced none of the responses associated with its injection suggesting activation could possibly take place in the skin.

EXPERIMENTAL

The active principle was isolated from the frozen leaves and leaf stalks of Laportea moroides by solvent extraction, and column chromatography using a modification of Oelrichs and Robertson's method. The method involved initial extraction of frozen (-30°C) leaves and leaf stalks (500 g) with 50% aqueous methanol, concentration of the extract to a syrup followed by extraction (x 6) with n-butanol. The n-butanol extract was dried, and the residue purified on 2 consecutive silicic acid columns (each 100 g) using in the first the lower phase of $CHCl_3$/MeOH/H_2O (65:35:10) and in the second a gradient of MeOH in $CHCl_3$. Removal of the active $CHCl_3$ principle from the second column was facilitated by adding 20% AcOH in MeOH to the final fractions. The active fraction was further purified on a column of micronized xAD-2 resin (50 g) using a gradient of n-propanol in water. The active fraction was subjected to HPLC analysis and one major compound termed Moroidin was detected, and isolated via preparative HPLC. For all isolations, HPLC was performed using a reversed-phase column of Microbondapack MCH-5 (analytical) or Zorbax 5 ODS (preparative) on a Varian 5000 machine. The solvent was 10-35% CH_3CN in 0.1% aqueous trifluoroacetic acid.

UV spectra were determined on a Unicam SP800 spectrometer.

FAB Mass spectra were recorded on a Kratos MS50 instrument fitted with a standard FAB source and a high field magnet. The sample (ca. 1 nmole) was dispersed in a few μl of α-thioglycerol:diglycerol (1:1, v/v) matrix and bombarded with a 6-8 KeV beam of Xenon atoms (except where stated). The high resolution peak matching measurement was made using Substance P(4-11), Dynorphin(1-8), Oxytocin and [Val^5]-Angiotensin II as reference masses at a resolving power of approximately 10,000.

Electron impact (EI) mass spectra were recorded on an AEI MS902 or MS30 (with data system DS30) instrument.

GLC was performed using a fused silica capillary column of SE54 and a capillary column of "Chirasil-Val" on a Carlo Erba 6130 chromatograph.

GCMS was performed using a column of SE54 on a Finnigan 4000 instrument operating in the negative ion (ammonia) chemical ionization mode.

^1H NMR spectra were usually obtained using a modified Bruker WH-400 or AM-500 spectrometer operating in the Fourier transform mode. Solutions were approximately 14 mM in d_6-DMSO, prepared from samples previously dried in vacuum over P_2O_5, sometimes after lyophilization from D_2O. Spectra were obtained over the temperature range from ambient to 70°C.

Typical data accumulations were made using a spectral width of 4000-6000 Hz acquisition in 8 K data points, and quadrature detection and phase alternation. For the measurement of chemical shifts and coupling constants, appropriate Gaussian multiplication of the FID was used. Decoupling and NOE difference experiments were performed using standard microprograms.

The NOE difference experiments were performed using an irradiation time of 0.2 s. In studies of the rates of build-up of NOEs, the irradiation time, τ_1, was set at 0.2, 0.4, 0.8, 1.6, 3.2 and 6.4 s. A minimum of 128 transients were recorded for each irradiation time and frequency, and there was a 5 s delay between the end of one acquisition and the start of the next τ_1. The time $t_{\frac{1}{2}}$, taken to reach half the maximum NOE values was measured from the graph of h_t vs t, where h_t is the % growth of the peak in the difference spectrum for an irradiation time t.

COSY experiments were acquired at 400 MHz with sweep widths of 4000 Hz (2 K data points) in ω_2 and ±2000 Hz (~200 t_1 values zero filled to 1 K before FT) in ω_1. A 2 second relaxation delay was used and 224 transients were accumulated for each t_1 value. NOESY experiments were recorded at 500 MHz using a mixing time of 0.2 s and a relaxation delay of 2 s. 256 transients were performed for each t_1 value in a data matrix of 5000 Hz (2 K data points) in ω_2 and ±5000 or 2500 Hz (~300 t_1 values, zero filled to 1 K before FT) in ω_1.

The combined COSY-NOESY experiments were performed at 400 or 500 MHz using the same parameters as the NOESY experiments.

^{13}C spectra were obtained at 100.13 MHz on a Bruker WH-400 instrument. Spectra were obtained at ambient temperature by using either d_6-DMSO or D_2O (traces of d_4-CH_3OH), as solvent. Accumulations were made with spectral width of 20,000 Hz and acquisition of 8 K data points. The DEPT spectra were recorded with the delay, ($\frac{1}{2}J$), set at 3.7 ms.

Partial Hydrolysis of Moroidin

Moroidin (0.1 mg) was hydrolysed with 6M HCl at 105°C in a sealed tube for 10 min. After cooling, the unsealed contents were freeze-dried. Four fractions were collected by HPLC, which gave compounds having molecular weight of 1022 (2 NH_2, 3 COOH) 1004 (1 NH_2, 2 CO_2H), 986 (starting material) and 809 (1 NH_2, 1 CO_2H), as determined by FABMS. One cycle of subtractive Edman degradation showed Glu was removed completely from all of the above mentioned compounds.

Selective Deuteration

Moroidin (0.1 mg) was treated with an acetic anhydride-pyridine-D_2O mixture following the procedure of Matsuo *et al.*[5], except D_2O was added rather than H_2O. The product was hydrolysed (6M HCl, 105°C, 24 h) and the resulting amino acids were derivatised as their N-trifluoroacetyl isopropyl ester derivatives and analysed by GCMS. The temperature gradient used was 4°C min^{-1} from 70° to 300°C with 2 min holds at 70° and 300°C. Deuterium was found to be incorporated in histidine only, (M-H) = 293.

Tryptic Digestion of Moroidin

Moroidin (0.1 mg) was dissolved in 1 cm^3 of 50 mM ammonium acetate, pH 8.5, containing 0.5 mM calcium chloride. Trypsin was added at an enzyme/substrate ratio of 75/1 (w/w) and the solution was incubated at 37°C for 4-8 h. The progress of digestion was monitored by HPLC. After completion, the hydrolysates were freeze-dried and characterised by FABMS.

Dansylation of Peptides

The peptide (2 nmol) or amino acid was dissolved in 200 µl of lithium carbonate solution (40 mM, pH 9.5). Dansyl chloride (500 µl, 1.5 mg cm^3 of CH_3CN) was added and was gently shaken for 2 min. It was then allowed to stand at room temperature for another 30 min in the dark.

2-Dimensional TLC on Dansyl-amino Acids

The product from above was subjected to standard conditions for acid hydrolysis (6M HCl, 105°C, 24 h). The unknown dansyl amino acid of the mixture (in 1 µl of 50% aq pyridine) was applied as a spot on one side of a double sided 5 x 5 cm^2 polyacrylamide plate. On the other side of the plate, another dansyl amino acid(s) was applied. Chromatography was performed in solvent I (1.5% v/v aqueous formic acid) until the solvent front just reached the top of the plate. The plate was dried and chromatography was performed in the second dimension in solvent II (toluene/acetic acid, 10:1 v/v), then in solvent III (pyridine/acetic acid/water/ethanol), 1:2:100:34 v/v) to resolve the basic amino acid after drying.

Oxidation of Moroidin with I,I-(bistrifluoroacetoxy)iodobenzene (TIB)

Moroidin (0.1 mg) was dissolved in 50% aqueous acetonitrile (40 µl) and an equimolar amount (100 nmol, 0.044 mg) of TIB dissolved in ca 10 µl of the same solvent was added. The mixture was stirred at room temperature for 5 h in the dark and 0.01 M HCl (ca 150 µl) was then added. The mixture was freeze-dried and purified by HPLC and characterised by FABMS, UV, NMR.

Carboxypeptidase Y Digestion on the Oxidative Product of Moroidin (OPI)

OPI (10 nmol) was dissolved in 200 µl of ethyl morpholine acetate (7.4 mM, pH 7). Carboxypeptidase Y was added at an enzyme/substrate ratio of 75/1 w/w and the solution was incubated at 37°C for 24 h. The reaction was monitored by FABMS. After completion, the sample was freeze-dried.

Absolute Configuration of the Amino Acids of Moroidin

Moroidin (0.1 mg) was hydrolysed (6M HCl, 105°C, 72 h) in a sealed tube. The resulting amino acids were then heated with propan-2-ol (1 cm^3) containing HCl

(1.25 M) at 100°C for 30 min in a Teflon-lined screw-cap Wheaton vial. Excess reagent and solvent were then removed by evaporation under reduced pressure. The residue was dissolved in dichloromethane (1 cm^3), and trifluoroacetic anhydride (200 µl) was added and the solution was heated at 100°C for 10 min. Excess reagents were removed with a gentle stream of dry nitrogen at 0°C. The amino acid derivatives were dissolved in about 50 µl of ethyl acetate and were subjected to GC analysis on a "Chirasil-Val" column (25 m x 0.5 mm ID). The temperature gradient used was 4°C min^{-1} from 80° to 180°C, with 4 min holds at 80° and 180°C. The carrier gas was hydrogen, maintained at a flow rate of 1.5 cm^3 min^{-1} and the injection and detection blocks were held at 250°C. Detection was by flame ionization. Co-injection with derivatised standard L- and D-amino acids established the conclusions given in the text.

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