Computer-Assisted Structure Determination. Structure of the Peptide Moroidin from *Laportea moroides*

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A combination of NMR and molecular modeling techniques has been used to fully assign the stereochemical relationships in the bicyclic peptide moroidin. The structure proposed results from a peptide backbone constructed exclusively from L amino acids. An unusual C–N linkage between the tryptophan and histidine residues has been found to be between C_2 of tryptophan and N_1 of the imidazole moiety of histidine.

Introduction

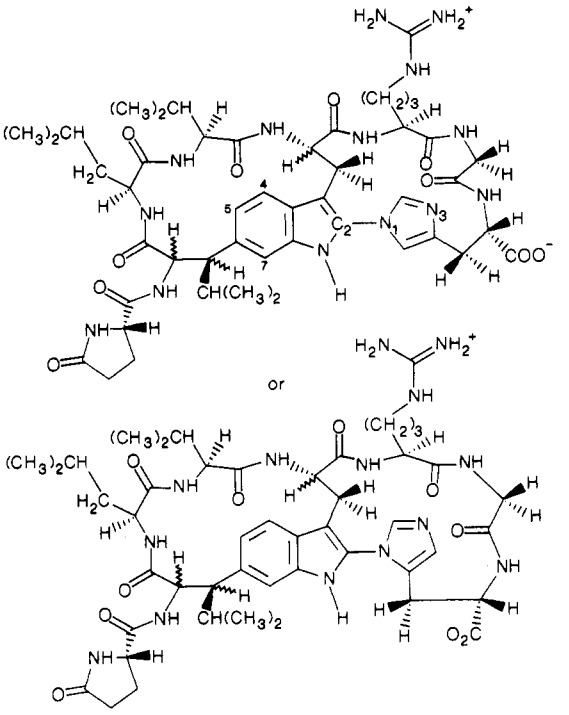
Determination of the structures of peptides containing nonstandard amino acids is made considerably more complex by the problem of establishing the absolute configuration of the unusual amino acid residues. In contrast with most proteinogenic amino acids, "unusual" residues are frequently destroyed by standard hydrolytic techniques, and reference amino acids of known absolute configuration are seldom available.

In the absence of suitable crystals for X-ray diffraction studies, NMR is the technique of choice for determining the covalent structures of such peptides.[1] When the peptide displays no preferred conformation, a time-averaged spectrum is obtained, and little stereochemical information can be obtained by NMR techniques. However, if the molecule is constrained in some manner such that one preferred conformation is significantly populated, the intramolecular nuclear Overhauser effect can provide useful information about relative stereochemistries.[2] If the same molecule contains a center of defined absolute configuration, knowledge of relative stereochemistries leads logically to the assignment of absolute stereochemistries. A case in point is presented herein for the octapeptide moroidin, where the required conformational preference is provided by the constraint of a bicyclic structure.

The isolation and structure determination of moroidin, a novel bicyclic octapeptide, was recently reported.[3] The structure as described left undefined the absolute configuration of three asymmetric centers, as well as the point of attachment of an imidazole ring. The coupled application of available computational and two-dimensional NMR techniques has allowed these ambiguities to be resolved, attesting to the utility of this general methodology. Described herein is the structure of moroidin in complete configurational and stereochemical detail.

Moroidin (1) is a bicyclic octapeptide isolated from the leaves and leaf stalks of the bush *Laportea moroides*, which grows in the Eastern Australian rain forests. The bush is covered with stinging hairs, and skin contact with these hairs results in pain, piloerection, arteriolar dilatation, and local sweating.[4] As previously described, pure moroidin can induce these symptoms to some extent but does not appear to be the only active component in the extract of *Laportea moroides*.

Moroidin displays several distinctive structural features that center around an unusually substituted tryptophan residue. This residue is coupled at C_2 to an imidazole-ring nitrogen of L-histidine and at C_6 to the β-carbon of a substituted leucine residue. Previous studies[3] had not allowed assignment of the configuration of the α-carbons



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of the substituted tryptophan and leucine residues, nor did they distinguish between structures in which *either* imidazole nitrogen was bonded to the C_2 of tryptophan.

Examination of CPK molecular models reveals that structures in which the tryptophan (Trp) and β-substituted leucine (β^SLeu) α-carbons are either *R,R* or *S,S* (but not *R,S* or *S,R*) cannot be eliminated with any certainty by consideration of the observed NOEs. Similarly, either configuration of the imidazole ring appeared consistent with the available spectroscopic data. The configuration of the β-carbon of the β^SLeu residue (i.e., *S*) was defined by the observation of a large NOE between the β^SLeu and Trp_{C_7H}, which is incompatible with the alternative configuration at this center in a structure that maintains reasonable nonbonded repulsions.

Unfortunately, using CPK models as a guide to three-dimensional structure has been shown to be seriously misleading in at least one previous case,[5] suggesting that

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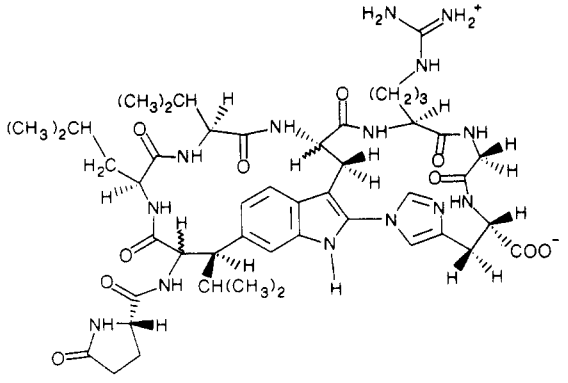
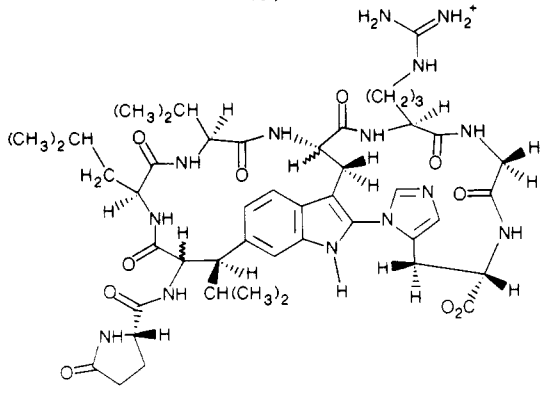
an alternative means of examining the putative structures of moroidin would be useful. We were thus led to explore the possibility of using molecular mechanics techniques as a tool to aid in the determination of the structures of unknown compounds. The philosophy behind this decision was to test whether computational techniques offer any useful advantages over more traditional model-building methods in allowing one to ascertain which of a set of possible structures is most compatible with available data. Although molecular mechanics is now a well-established technique for predicting preferred molecular conformations,[6] its use as a tool for structure determination has yet to be fully exploited.[7]

Methods

The previous uncertainties in the structural assignment of moroidin concerned the absolute configuration of the α-carbons of the 2,6-disubstituted tryptophan and the β-substituted leucine residues, in addition to the site of attachment of the tryptophan indole to the imidazole ring of histidine. Four model constructs were therefore employed: (**1a**) *S*-($C_\alpha\beta^S$Leu), *S*-(C_αTrp), C_2–N_1 imidazole linkage; (**1b**) *R*-($C_\alpha\beta^S$Leu), *R*-(C_αTrp), C_2–N_1 imidazole linkage; (**1c**) *S*-($C_\alpha\beta^S$Leu), *S*-(C_αTrp), C_2–N_3 imidazole linkage; and (**1d**) *R*-($C_\alpha\beta^S$Leu), *R*-(C_αTrp), C_2–N_3 imidazole linkage; with the remaining chiral centers assigned the *S* configuration.[3]

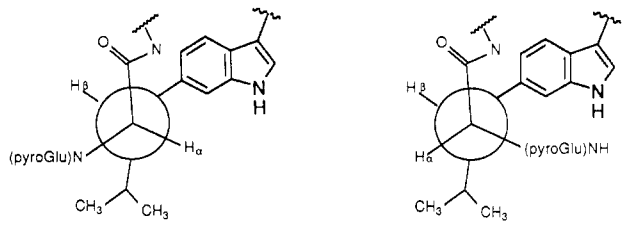


1a, b



1c, d

Model structures **1a–d** were energy minimized with the COSMIC[8] empirical force field, until the root mean squared (RMS) force on all atoms was less than 0.25 mN, and the change in energy for five successive optimization cycles did not exceed 0.05 kcal·mol^{-1}. In all cases, partial atomic charges were assigned to each atomic center by using the method of Abraham.[9] Because the calculations were performed in vacuo, all charged residues, i.e., arginine and histidine, were modeled as their neutral forms. This precaution was adopted in order that charged side-chain–side-chain interactions (Arg^+–His^-) would not distort the bicyclic skeleton, due to an overemphasized electrostatic attraction resulting from a dielectric of unity.



1a, 1c **1b, 1d**

Figure 1. Newman projection down the C_α–C_β bond of the β-substituted leucine in model moroidin structures.

In all cases, multiconformer searches were employed that generated several test structures for each of the models constructed (i.e., **1a–d**), many of which were clearly incompatible with the NOE results. The structures reported herein are those that best fit the constraints imposed by the NMR data and do not necessarily correspond to the calculated global minima for the individual forms.

Molecular dynamics simulations, again utilizing the COSMIC force field, were performed in order to assess the flexibility of the bicyclic assembly. As with the aforementioned molecular mechanics calculations, all simulations were carried out in the absence of solvent, on model structures without any charged residues.

Approximate interproton coupling constants and NOEs were calculated for the model structures by using an automated procedure. Vicinal[10] and amide-CH_α coupling constants[11] were calculated via Karplus-type relationships that take into account the effect of substituents on the participating atomic centers. Geminal coupling constants were estimated by using data reported by Sternhell[12] and Günther,[13] from the structural relationships of model systems.

A sample of crude moroidin extract (kindly supplied by Dr. P. B. Oelrichs) was purified by reversed-phase HPLC as described previously.[3] ^{1}H NMR spectra were recorded at 330 K, on Bruker AM500 and AM400 spectrometers operating at 500 MHz and 400 MHz, respectively. Solutions were approximately 10 mM in d_6-DMSO, prepared from samples dried under vacuum over P_2O_5. For spectra run under acidic conditions, 1 μL of neat trifluoroacetic acid was added to the NMR sample. For the purpose of this study, all NH–C_α, C_α–C_β, and C_β–C_β (geminal) coupling constants were remeasured and are summarized in Table I.

Double quantum filtered phase-sensitive COSY[14] and phase-sensitive NOESY[15] spectra were recorded at 500 MHz by using a spectral width of 5000 Hz (2K data points) in F_2 and 512 t_1 values in F_1 (zero-filled to 1K data points before FT). Suitable apodization functions were applied in both F_1 and F_2 before FT. Mixing time for the NOESY experiment was 400 ms, subjected to a 20 ms z-filter. CAMELSPIN[16] spectra were recorded at 400 MHz with a 3-kHz spin-lock field applied for 200 ms. Simultaneous use of both NOESY and CAMELSPIN techniques al-

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Table I. Proton-Proton Coupling Constant Data for Moroidin and Moroidin Model Structures

residue	coupled protons	obsd J values/Hz	model structure 1a	1b	1c	1d
β^sLeu	$^3J_{\alpha-\beta}$	11.7	12.3	1.2	12.4	1.3
	$^3J_{\alpha-NH}$	9.4	3.9	8.6	3.7	8.8
pyroGlu	$^3J_{\alpha-NH}$	br s	2.1	1.9	2.3	2.0
Leu	$^3J_{\alpha-NH}$	10.5	9.4	7.6	9.3	7.1
Val	$^3J_{\alpha-NH}$	8.2	8.9	6.2	8.9	6.7
Trp	$^3J_{\alpha-NH}$	br s	4.6	4.0	4.1	3.9
	$^3J_{\alpha-\beta}$	6.4, 8	1.9, 11.3	4.5, 10.2	1.8, 12.1	4.2, 10.2
	$^2J_{\beta}$	15.3	~16	~14	~14	~15
Arg	$^3J_{\alpha-NH}$	7.3	9.2	7.3	6.9	6.6
Gly	$^3J_{\alpha-NH}$	5, 5.3	3.6, 5.6	2.0, 8.5	3.1, 7.4	2.5, 7.7
	$^2J_{\alpha}$	15.8	~16	~13	~16	~16
His	$^3J_{\alpha-NH}$	9	9.4	7.0	3.7	7.3
	$^3J_{\alpha-b}$	10	2.3, 13.7	2.7, 13.4	1.6, 12.9	2.6, 2.8
	$^2H_{\beta}$	15.5	~16	~14	~16	~16

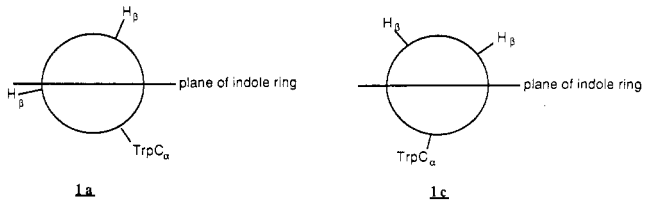

Figure 2. Newman projection down the C_β–C_2 (indole) bond of the 2,6-disubstituted tryptophan in model moroidin structures.

lowed clear distinction between genuine direct nuclear Overhauser enhancements and those observed via the indirect pathway of spin diffusion.

Results and Discussion

The measured interproton scalar couplings and the values calculated for **1a–d** by using the Karplus-type relationship proposed by Gandour[10] are collected in Table I. Comparison of the experimental and calculated values reveals some striking differences between the model structures. Of particular note, **1a** and **1c** (all *S* chiral centers) are predicted to have a trans arrangement of the α and β protons of the β-substituted leucine, in contrast to forms **1b** and **1d**, which have ^3J values indicative of a gauche relationship. Furthermore, as demonstrated in Figure 1, the gauche assignment of $^3J_{\alpha-\beta}$ (β-substituted leucine) in **1b** and **1d** is independent of the absolute stereochemistry at C_β; conversion of C_β from an *S* to an *R* configuration (exchange of H_β with the isopropyl side chain) maintains a similar gauche relationship. The experimentally determined $^3J_{\alpha-\beta}$ (β-substituted leucine) value of 11.7 Hz is typical of a trans relationship between vicinal protons,[17] thus arguing strongly against forms **1b** and **1d**. Therefore, independent of the indole$_{Trp}$–imidazole$_{His}$ linkage (i.e., **1b,d**), *R* configurations at C_α of β^SLeu and Trp cannot be accommodated with the experimental findings and must be eliminated as possible candidates for moroidin. Hence, the peptide backbone of moroidin is composed of *S* stereocenters exclusively, i.e., L amino acids.

An interesting conformational distinction between the remaining model moroidin structures **1a** and **1c**, differing only in the identity of the imidazole nitrogen attached to C_2 of the tryptophan indole, is revealed in Figure 2. In **1a**, the tryptophan C_β protons span both sides of the planar indole ring system, whereas in **1c**, both of the geminal protons are situated on the same side of the aromatic ring. Theoretical considerations, supported by experimental observations, suggest that the former situation (**1a**) should give rise to a larger geminal coupling constant than the alternative arrangement (**1c**), by advent of orientational effects upon hyperconjugative electron shifts.[12,13] Unfortunately, the variety of substituent-related influences on ^2J values, and the large spread in the observed experimental values, have precluded formulation of reliable empirical models for the prediction of the effects of adjacent π-systems on geminal coupling constants.[13] Nevertheless, consideration of the angular dependence of $^2J_{C_\beta H}$(Trp) for **1a** and **1c** yields values of ~16 Hz and ~13 Hz, respectively, neither of which can be distinguished with any certainty from the *average* value reported for toluene (i.e., 14.8 Hz).[18] The observed value of 15.3 Hz for $^2J_{C_\beta H}$(Trp) in moroidin, while more consistent with structure **1a** than **1c**, is in the median range where the two forms are not distinguishable within experimental uncertainties.[19]

Table II. Observed NOEs for Moroidin and Proton-Proton Distances[a] in Moroidin Model Structures

obsd NOE[b]	model structure 1a	1b	1c	1d
Trp_{C_4}–Trp_{C_5}	2.4	2.4	2.4	2.4
Trp_{C_4}–$Trp_{\alpha CH}$	2.4	2.6	2.3	2.4
Trp_{C_4}–$Trp_{\beta CH}$	2.3, 3.8	2.6, 2.7	2.2, 3.8	2.4, 2.7
Trp_{C_5}–$\beta^s Leu_{\alpha CH}$	2.6	4.5	2.6	4.4
$Trp_{\alpha CH}$–Arg_{NH}	2.4	3.6	2.1	3.6
$Trp_{\alpha CH}$–$Trp_{\beta CH}$	2.6, 3.0	1.8, 2.9	2.6, 3.0	2.2, 2.9
$Trp_{\beta CH}$–His_{C_5}	1.9	>4.5	>4.5	>4.5
$Trp_{\beta CH}$–Arg_{NH}	3.0, 3.8	3.6, 4.0	3.8, 4.1	3.6, 3.9
Trp_{NH}–$Val_{\alpha CH}$	2.2	2.4	2.2	2.4
$Trp_{NH_{indole}}$–Trp_{C7}	2.7	2.6	2.7	2.7
$\beta^s Leu_{\alpha CH}$–Leu_{NH}	2.2	3.6	2.2	3.6
$\beta^s Leu_{\beta CH}$–Trp_{C_7}	3.6	3.8	3.6	3.8
Leu_{NH}–Val_{NH}	3.7	2.7	3.6	2.6
Gly_{NH}–$Gly_{\alpha CH}$	2.3, 2.9	2.6, 3.0	2.4, 3.0	2.7, 3.0
Gly_{NH}–$Arg_{\alpha CH}$	3.6	3.6	2.2	3.6
Gly_{NH}–Arg_{NH}	3.0	2.0	>4.5	1.8
$Gly_{\alpha CH}$–His_{NH}	2.6, 3.6	2.3, 3.5	2.5, 3.5	2.6, 3.6
$His_{\alpha CH}$–His_{C_5}	4.2–4.6[c]	3.5	2.1	4.1
$His_{\alpha CH}$–$His_{\beta CH}$	2.4, 3.1	2.4, 3.1	2.6, 3.0	2.3, 2.7
$His_{\alpha CH}$–His_{NH}	3.0	2.9	2.8	2.9

[a] In angstroms. [b] Other observed NOEs: Trp_{C_5}–$\beta^s Leu_{methyl}$; Trp_{C_7}–$\beta^s Leu_{methyl}$; $\beta^s Leu_{\beta CH}$–$\beta^s Leu_{\gamma CH}$;β^s–Leu_{NH}–$\beta^s Leu_{\alpha CH}$; $\beta^s Leu_{NH}$–$\beta^s Leu_{\beta CH}$; $\beta^s Leu_{NH}$–$\beta^s Leu_{\gamma CH}$; Leu_{NH}–$Leu_{\beta CH}$; Leu_{NH}–$Leu_{\gamma CH}$; $Val_{\alpha CH}$–$Val_{\beta CH}$; $pyroGlu_{\alpha CH}$–$\beta^s Leu_{NH}$; $pyroGlu_{NH}$–$pyroGlu_{\alpha CH}$. [c] This portion of the molecule was found to be flexible. The range shown was calculated by using molecular dynamics simulations.

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Approximate interproton distances (Table II) found for the model structures provide further information to clarify the structure of moroidin. While all model structures discussed are necessarily static representations of dynamic systems, the positions of the individual atoms should be indicative of "average" internuclear separations and thus should correlate with proton-proton NOEs. As concluded earlier, structures **1b** and **1d** are inconsistent with the experimental data presented herein, and this is highlighted again by the data in Table II. Neither structure would be expected to exhibit NOEs between the proton pairs Trp_{C_5}-$\beta^S Leu_{\alpha CH}$ and $Trp_{\beta CH}$-His_{C_5} (and in **1d**, $His_{\alpha CH}$-His_{C_4}), contrary to the experimental findings.

The distinction between structures **1a** and **1c** is apparent upon examination of their three-dimensional structure and is also revealed by the measured interproton distances in Table II. Topologically, the two structures are essentially identical over the "western" peptide ring containing the pyroGlu-β^SLeu-Leu-Val-Trp residues. They differ dramatically, however, over the "eastern" peptide ring formed by the Arg-Gly-His-Trp residues. These differences are no doubt a consequence of the $indole_{Trp}$-$imidazole_{His}$ attachment, although it is interesting that a change in this linkage affects only the conformation of the ring in which it is contained-no effect is observed in the ring containing the Val-Leu-pyroGlu-β^SLeu residues. In the case of **1c**, this conformational change causes the Trp_{C_β} and His_{C_5} protons to become separated by more than 4.0 Å, a distance inconsistent with the strong NOE found between these protons. Furthermore, **1c** does not account for the NOE observed between Gly_{NH} and Arg_{NH}, although here molecular dynamics simulations indicate that the flexibility in the eastern ring could possibly result in a flip of the Gly_{NH} amide linkage allowing the closer approach of Gly_{NH} and Arg_{NH} in model structure **1c**. In **1a**, Gly_{NH} bisects the plane spanned by the Arg_{NH} and C_α protons, in accord with the observed NOE to both these atoms.

Consideration of the NOE and coupling constant data, in conjunction with the molecular modeling studies, leads to the conclusion that moroidin possesses structure **1a**. Negative evidence consistent with the conclusion is the absence of any NOE between His_β and Trp_β, which in structure **1c** would be expected to be large, but is not predicted in structure **1a**.

In order to test the validity of the calculations on structure **1a**, this form was also energy minimized by using the CHARMM force field.[22] The results obtained were entirely compatible with those found by using the COSMIC force field, confirming that the result was not dependent on the particular force field used.

Conclusions

Using a combination of NMR and molecular modeling techniques, the structure of moroidin is shown to be composed entirely of L amino acids. Consideration of scalar proton-proton coupling constants eliminates structures having an *R* configuration at either the β-substituted leucine or tryptophan residues. Intramolecular NOEs further define the linkage between C_2 on the tryptophan indole and N_1 on the imidazole ring of histidine. Structure **1a** therefore represents the correct structure of moroidin. Molecular modeling via molecular mechanics and molecular dynamics using the COSMIC force field has been shown to be a highly efficient means of correlating available experimental data with several possible structural variations of moroidin.

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Registry No. 1, 104041-75-0; **1a**, 119528-95-9; **1b**, 119616-50-1; **1c**, 119528-96-0; **1d**, 119616-51-2.

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N-Chloroazasteroids: A Novel Class of Reactive Steroid Analogues. Preparation, Reaction with Thiols, and Photochemical Conversion to Electrophilic *N*-Acyl Imines

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N-Chloroazasteroids **2**, **5**, **7**, **10**, **12**, **15**, **18**, and **22** were easily prepared by the reaction of *N*-chlorosuccinimide with the parent azasteroid lactams **1**, **4**, **6**, **9**, **11**, **14**, **17**, and **21**, respectively. The products reacted with the model thiols benzenethiol and L-cysteine ethyl ester to afford the corresponding *N*-thiolactams **3**, **8**, **13**, **16**, and **23**, as well as the parent lactams and disulfides, via sulfenyl chloride intermediates. The reaction of benzenethiol with *N*-chloro-17β-hydroxy-4-aza-3-androstanone (**2**) resulted in the anomalous formation of the stable sulfenate and sulfinate esters **24a** and **24b**. Photolyses of *N*-chloroazasteroids in methanol resulted in the formation of enamides **27**, **28**, and **30**, or the carbinol amide methyl ethers **32** and **34**. These products were formed by the isomerization or solvent trapping of reactive *N*-acyl imine intermediates. The ability of *N*-chloroazasteroids to react covalently with thiols and to generate electrophilic *N*-acyl imines suggests potential biological applications in affinity labeling and enzyme inhibition and for use as antihormonal agents.

Steroid analogues that contain alkylating or other reactive functional groups have several potentially useful applications. When such species mimic natural steroid hormones with respect to recognition by their respective

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