Tetrahedron Vol. 45, No. 18, pp. 5845 to 5852, 1989
Printed in Great Britain.

0040-4020/89 $3.00+.00

CLEROMYRINE I, A NEW CYCLOHEXAPEPTIDE FROM CLERODENDRUM MYRICOIDES

S. Bashwira[+], C. Hootelé*[+], D. Tourwé[#], H. Pepermans[#], G. Laus[&], G. Van Binst[#].
Service de Chimie Organique[+], Université Libre de Bruxelles, B-1050 Brussels; Eenheid Organische Chemie (ORGC)[#], Vrije Universiteit Brussel and Biorgan Brussels[&].

(*Received in Belgium* 12 *June* 1989)

Abstract
A cyclic hexapeptide was isolated from Clerodendrum myricoides. The amino acid composition was determined by chiral chromatography. The sequence c(Ala-Gly-Pro-Ile-Val-Phe). A proposal for the conformation is given.

Isolation and Structure Determination

In a recent communication, we reported the isolation and structure determination of two new macrocyclic spermidine alkaloids from Clerodendrum myricoides (Verbenaceae) (1). Chromatographic fractionation of a non-basic residue which was not investigated in the course of the alkaloid isolation procedure has now resulted in the isolation of two new homodetic cyclohexapeptides which we have named cleromyrine I and II. The structure of cleromyrine I is discussed in the present communication.

Cleromyrine I (0.03 % from the dried plant) was isolated as a crystalline compound mp. 159-160°C. (acetone-ether), $[\alpha]_D^{25} = -132°$ (c = 2.3, MeOH). The empirical formula $C_{30}H_{44}N_6O_6$ was established by high resolution mass spectrometry. Cleromyrine I was not affected by treatment with acetic anhydride in pyridine and the NMR spectra indicate the presence of five secondary and one tertiary amide groups. Hydrogenation over platinum yielded a hexahydroderivative which results from the saturation of the phenyl group present in cleromyrine I. As there is no indication of other multiple bonds, cleromyrine I has to be a monocyclic hexapeptide.

The amino acid composition and the chirality of the individual amino acids was determined after 6N DCl hydrolysis (2) of the peptide. Gas chromatography on a Chirasil-Val column (3) of the N-trifluoroacetyl-isobutylesters revealed the presence L-Ala, L-Val, Gly, L-Ile, L-Pro and L-Phe. Therefore, cleromyrine I belongs to the few naturally occuring homodetic cyclopeptides built exclusively of "natural" L-amino acids (4).

Sequence and Conformation

The 500 MHz ^1H NMR spectrum in $CDCl_3$ solution confirms the presence of the amino acid residues mentioned above. A complete signal assignment (Table

TABLE 1

^{1}H NMR Chemical Shifts and Coupling Constants of Cleromyrine I

($CDCl_3$, 300 K)

	NH	αH	βH	γH	δH
Ala	7.039 ppm (J=7.90 Hz)	4.48 ppm	1.330 ppm ($J_{\alpha\beta}$=6.27 Hz)		
Val	7.370 ppm (J=6.96 Hz)	3.262 ppm	2.41 ppm	0.844 ppm ($J_{\alpha\beta}$=6.60 Hz) 0.774 ppm ($J_{\alpha\beta}$=6.68 Hz)	
Gly	7.861 ppm	4.118 ppm (J=4.92 Hz) 3.512 ppm (J=5.98 Hz)			
Ile	7.159 ppm (J=8.87 Hz)	4.302 ppm ($J_{\alpha\beta}$=8.51 Hz)	2.25 ppm	1.44 ppm 1.23 ppm CH_3:0.931 ppm (J=6.70 Hz)	0.905 ppm (J=7.44 Hz)
Pro	-	4.468 ppm	2.22 ppm 2.00 ppm	2.12 ppm 2.04 ppm	3.98 ppm 3.59 ppm
Phe	7.22*	4.50 ppm	3.19 ppm	H(arom.) : 7.31-7.20 ppm	

* Signal buried under aromatic signals, $J_{\alpha\beta}$ not available.

TABLE 2

Carbon-13 Chemical Shifts (ppm) of Cleromyrine I

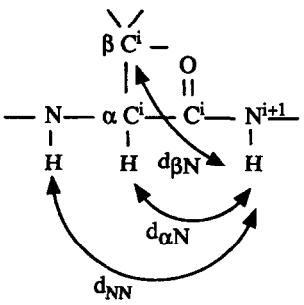
($CDCl_3$, 300 K)

C=O	173.8	173.3	172.6	171.9	171.8	169.4
	α	β	γ	δ	ε	ζ
Ala	49.1	17.3				
Val	64.8	27.8 19.3	19.6			
Gly	42.6					
Ile	61.5*	35.0	25.3 16.1	11.4		
Pro	58.1	30.0	24.8	47.8		
Phe	56.8*	37.4	136.8	128.9	129.4	127.3

* assignments can be interchanged

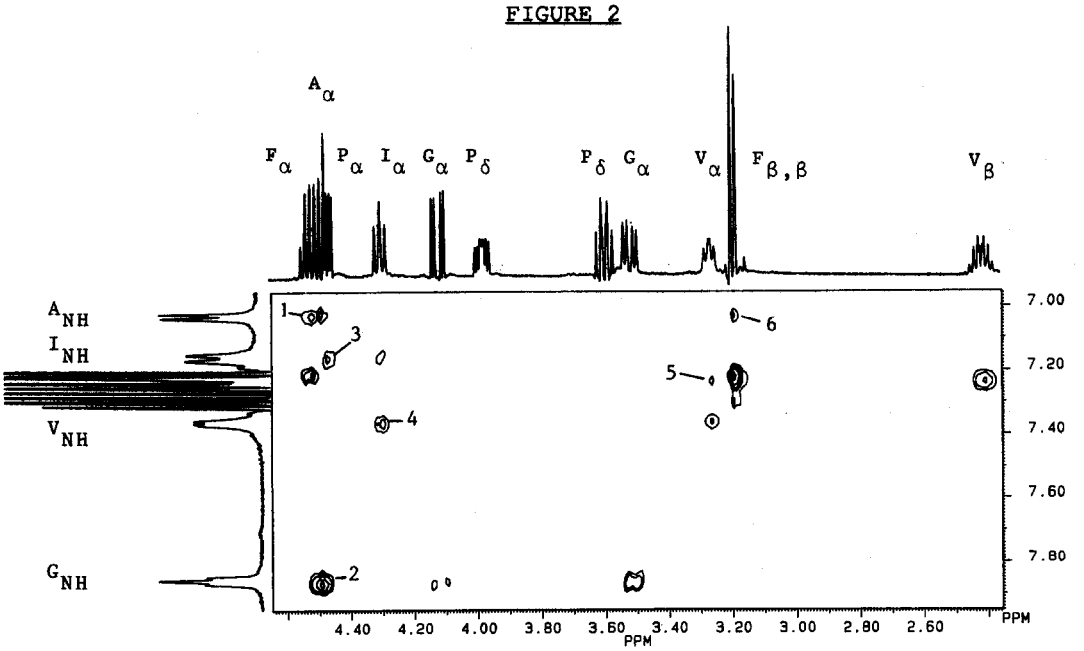
1) was possible by the use of a HOHAHA spectrum (5). In DMSO-d_6 solution however, the peptide occurs as mixture of at least three slowly interconverting conformers, which makes an interpretation of the spectrum very cumbersome. Such high-barrier conformational exchanges are usually observed for cis-trans isomerizations of the peptide bond, and can be expected to occur at Gly-Pro. For the third conformer it should occur at another position. However, we did not attempt to analyse the spectra in DMSO-d_6 solution, but further studies were performed in $CDCl_3$ solution, where only traces of minor conformers are present. The ^{13}C NMR spectrum is also in complete agreement with the proposed amino acid composition. By using a ^{1}H, ^{13}C correlated spectrum (6) combined with the multiplicities obtained by a DEPT experiment (7), the signals can be assigned as reported in Table 2. Since the α-protons of Phe, Ala, Pro and Ile strongly overlap at 250 MHz, the corresponding α-carbons could not be assigned. In order to be able to assign the sequence of the cyclic hexapeptide, one has to rely on the detection of spatial proximities between protons in different amino acid residues (8). The definition of these interresidue distances, according to Sherman is shown in Fig 1 (9).

Fig 1



The ROESY spectrum (Fig. 2) revealed all six $d_{\alpha N}$ connectivities, including the Gly-αCH_2 <---> Pro-δCH_2 cross peaks which can be considered of the same type (10) (Table 3). Of the four possible cross peaks between the two glycine α-protons and the two proline δ-protons, only two are observed. One of the glycine α-protons (δ = 4.118 ppm) shows cross peaks with both of the proline δ-protons, while the other Gly-αH (δ = 3.512 ppm) shows none. This is interpreted as the absence of the corresponding $d_{\beta N}$ connectivity (9) in Table 3, and contains important information on the conformation. Two $d_{\beta N}$ connectivities are observed. Due to the small chemical shift difference between the NH signals, possible δ_{NN} cross peaks are located too close to the diagonal to be detected, except for those involving Gly-NH, which are definitely absent.

FIGURE 2



ROESY spectrum of Cleromyrine I. Signal assignments are indicated using the one letter code for the amino acids (A : alanine, F : phenylalanine; G : glycine, I : isoleucine, P : proline, V : valine). The numbering of the ROESY cross peaks corresponds to the numbering in Table 3. Non-numbered cross peaks are due to intraresidual NOE-effects.

TABLE 3

Interresidual NOE effects

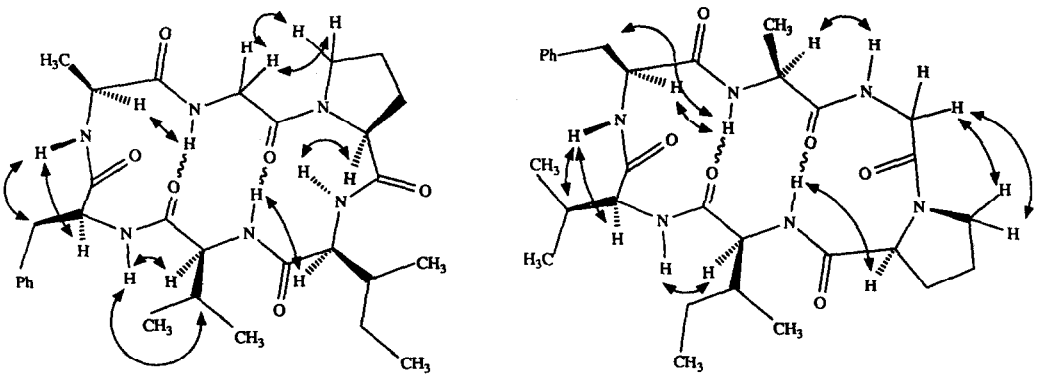
(the numbers correspond to those indicated in Fig. 2)

Residue	$d_{\alpha N}$	d_{NN}	$d_{\beta N}$
Phe			
	+(1)	?	+(6)
Ala			
	+(2)	-	-
Gly			
	+	-	-
Pro			
	+(3)	?	-
Ile			
	+(4)	?	-
Val			
	+(weak) (5)	?	+(strong) (7)
(Phe)			

Based on these NOE data, the sequence cyclo(Ala-Gly-Pro-Ile-Val-Phe) can unambiguously be deduced.

The observed NOE effects also contain information on the conformation of the cyclic hexapeptide. The frequent occurence of two β-turns linked by two antiparallel extended residues in cyclic hexapeptides (11-13), together with the known preference of the Pro residue for the i+1 position (12,13) and to a lesser extent for the i+2 positions of a β-turn (14), led us to examine two conformations I and II (Fig. 3).

FIGURE 3



I II

wavy lines indicate possible H-bonds
double arrows indicate observed NOE effects

The occurence of the NOE effect between the Pro-δCH_2 and one of the Gly-αCH_2's indicates a trans conformation for the Gly-Pro peptide bond (15). This is confirmed by the ^{13}C chemical shift of the proline γ-carbon (24.8 ppm) (16), as well as by the chemical shift difference between the β- and γ-carbons ($\Delta\delta$ = 5.2 ppm) (17).

In conformation I, both β-turns will preferentially be of type I because of the L-configuration of the amino acids (13). However, in cycloamanide A, with the very similar sequence cyclo(Ala-Gly-Pro-Val-Phe-Phe) (18) an unusual type II' β-bend around Phe-Ala is observed in the crystal. Therefore both a β_I, β_I and a β_{II}, β_I conformation I has to be considered. In a β_I turn no NOE should be observed between the αCH of the i+1 residue and the NH of the i+2 residue. The strong Pro-αCH <---> Ile-NH connectivity therefore excludes this conformation. This is further confirmed by the coupling $^3J_{\alpha H,NH}$ of the glycine residue. Indeed, in conformation I, the extended conformation of Gly should show one large and one small coupling (19). On the contrary, in conformation II the Gly NH bisects the α-CH_2's

in a $\beta_{II'}$ conformation, in agreement with two small and similar coupling constants. The occurence of an NOE between only one of the Gly-α protons and the Pro-δCH_2's is also in agreement with the $\beta_{II'}$ turn, as is the absence of any d_{NN} involving Gly-NH. The turn involving Val and Phe should preferentially be of type I. In agreement with this are the strong $d_{\beta N}$ connectivities between Val and Phe and between Ala and Phe. The $d_{\alpha N}$ connectivity between Val and Phe is however not consistent with this type of turn. The ROESY cross peak is however very weak (Fig. 2, cross peak 5), and may arise from a ROE/Hartman-Hahn relay PheNH/ValβH/ValαH (20).
Since it is known that the temperature dependence of NH chemical shifts in $CDCl_3$ does not give a clear indication of solvent exposure or shielding (21), and also since solvent titration gives rise to extensive conformational changes, no information about possible intramolecular hydrogen bonds is available. Nevertheless strong evidence exists for conformation II in which the Gly-Pro dipeptide occurs in a $\beta_{II'}$ conformation identical to the one found in the crystal structure of cleromyrine II (22).

Biological activities

The binding affinity of cleromyrine I was investigated in vitro in different radioligand receptor binding models for neurotransmitter-, ion channel-, peptide- and drug receptor binding sites in rat and guinea pig brain tissue (23). Up to 10^{-5}M, no binding was observed for cholinergic-muscarinic, dopamine-D_1 and -D_2, α_1- and α_2- adrenergic, β-adrenergic, 5-hydroxytryptamine- 1A and -2, neurotensin, substance P, μ-opiate, Ca^{2+}-channel, benzodiazepine and the monoamine release site receptors.
Also the potency of cleromyrine I to inhibit neurotransmitter uptake in rat brain synapstomoses was tested. No effect however was observed on GABA, dopamine, norepinephrine or 5-hydroxytryptamine uptake.

Experimental

Mass spectra were obtained with electron impact ionization on a Micromass 7070F spectrometer. Chiral amino acid analysis was performed on a Finnigan 3200 GC-MS system connected to an one-line Finnigan Incos data system. The sample was injected onto a Chirasil-Val (Alltech) capillary column, 50 m x 0.25 mm I.D. The operating conditions were as follows : temperature gradient 1°C/min, changing after 33.3 min to 4°C/min, from 80°C to 185°C.
^{13}C NMR spectra and ^{1}H, ^{13}C correlated spectra were recorded on a Bruker WM 250. ^{1}H NMR spectra were recorded on a Bruker AM500 spectrometer on a sample containing 2 mg of cleromyrine I in 0.4 ml $CDCl_3$ ("100 % d", CEA). Spectra were recorded on a Bruker AM 500 at 500 MHz and 300 K. The 1D spectrum consisted of 8 K of data points, multiplied by a Gaussian window (LB = -3, GB = 0.4), zero-filled up to 32 K, Fourier transformed, phase-

corrected, and baseline-corrected with a 4^{th} order polynominal. The ROESY and HOHAHA were recorded as 256 FID of 2 K data points. These were multiplied by a $\pi/3$-shifted sine-bell, Fourier transformed and phase corrected. The t_1-interferograms were multiplied by a $\pi/3$-shifted sine bell, zero-filled up to 2K, Fourier transformed and phase-corrected. The 1Kx1K absorption-absorption part was baseline corrected in both dimensions with 3^{rd} order polynomals. Spinlocking was realized using the decoupler, in CW mode at 2.5 kHz for ROESY and in MLEV17 mode at 10 kHz for HOHAHA.

Isolation of cleromyrine I and cleromyrine II

Clerodendrum myricoides (15 kg; collected near Bukavu, Zaïre) was extracted as described earlier[1]; fraction A_1 and A_2 were collected (18.5 g) and chromatographed on alumina (925 g). Elution with AcOEt yielded an oily residue which was not investigated; elution with a 7:3 mixture of AcOEt:MeOH furnished a mixture (5.5 g) of cleromyrine I and cleromyrine II. A further chromatography (alumina; $CHCl_3$:MeOH 85:15) allowed the separation of the two cyclopeptides; cleromyrine I (5 g) and cleromyrine II (0.29 g) were eluted successively and purified by crystallization from acetone-ether and from ethanol respectively.

Cleromyrine I : m.p. 159-160°C (acetone-ether), $[\alpha]_D^{25}$ = -132° (c=2.3, MeOH); MS : 584 ($M^{+\cdot}$, 100%, $C_{30}H_{44}N_6O_6$, found:584.3321, calc.:584.3322), 541(20), 540(22), 498(20), 493(37), 476(20), 472(20), 470(20), 469(26), 441(20), 422(54), 394(46), 359(33), 352(24), 351(87), 349(26), 324(20), 323(61), 310(22), 309(59), 295(31), 276(39).

Hexahydrocleromyrine I

Cleromyrine I (6 mg) was hydrogenated over platinumoxide for 48 hours (4 atm.) in MeOH in the presence of some drops of AcOH. After filtration of the solution and elimination of the solvent, hexahydrocleromyrine I was obtained quantitatively; oil. MS : $M^{+\cdot}$ at m/z 590; ^1H NMR : 8.0(t), 7.7(d), 7.6(d), 7.2(d) and 6.8(d) (5 NH).

Acknowledgements

One of us (S.B.) wishes to thank the "A.G.C.D." for the award of a fellowship; the authors thank Mrs. J.E. Leysen, Janssen Research Foundation (Beerse, Belgium) for the receptor binding assays. C. H. is a Research Associate and H. P. a Senior Research Assistant of the National Fund for Scientific Research (Belgium).

References

1. Bashwira, S.; Hootelé, C. Tetrahedron **1988**, 44, 4521-4526.
2. Liardon, R.; Ledermann, S. Anal. Chem., Symp. Ser. **1984**, 21, 7-17.
3. Nicholson, G.J.; Frank, H.; Bayer, E. J. High Resolut. Chromatogr. Chromatogr. Commun. **1982**, 5, 588-595.
4. Ovchinnikov, Y.A.; Ivanov, V.T. in "The Proteins", Neurath & Hill, 3rd Ed., Acad. Press **1982**, 5, 307-643.
5. Bax, A.; Davis, D.G. J. Magn. Reson. **1985**, 65, 355-360.
6. Ave, W.P.; Bartholdi, E.; Ernst, R.R. J. Chem. Phys. **1976**, 64, 2229-2246.
7. Bendall, M.R.; Doddrell, D.M.; Pess, D.T. J. Magn. Res. **1982**, 46, 43.
8. Billeter, M.; Braun, W.; Wüthrich, K. J. Mol. Biol. **1982**, 155, 321-346.
9. Sherman, S.A.; Adrianov, A.M.; Akhrem, A.A. J. Biomol. Struct. Dyn. **1987**, 4, 869-884.
10. Wagner, G.; Wüthrich, K. J. Mol. Biol. **1982**, 155, 347-366.
11. Ramakrishnan, C.; Paul, P.K.C.; Ramnarayan, K. Proc. Int. Symp. Biomol. Struct. Interactions, J. Biosci. **1985**, 8 (Suppl.), 239-251.
12. Smith, J.A.; Pease, L.G. CRC Crit. Revs. Biochem. **1980**, 8, 315-399.
13. Rose, G.D.; Gierasch, M.L.; Smith, J.A. Adv. Prot. Chem. **1985**, 37, 1-109.
14. Zimmerman, S.S.; Scheraga, H.A. Biopolymers **1977**, 16, 811-843.
15. Wüthrich, K. in "NMR of Proteins and Nucleic Acids", J. Wiley & Sons, **1986**, Chapters 7 and 8.
16. Deber, C.M.; Madison, V.; Blout, E.R. Acc. Chem. Res. **1976**, 9, 106-113.
17. Deslauriers, R.; Smith, I.C.P. in "Topics in Carbon-13 NMR Spectroscopy", Vol. 2, G.C. Levy (Ed), J. Wiley & Sons, **1976**, Chapter 1.
18. Chiang, C.C.; Karle, I.L.; Wieland, Th. Int. J. Pept. Prot. Res. **1982**, 20, 414-420.
    Karle, I.L.; Chiang, C.C. Acta Cryst. **1984**, C40, 1381-1386.
19. Bystrov, V.F. Prog. in NMR Spectrosc. **1976**, 10, 41-81.
20. Farmer, B.T. II; Macura, S.; Brown, L.R. J. Magn. Reson. **1987**, 72, 347-352.
21. Stevens, E.S.; Sugawara, N.; Bonara, G.C.; Toniolo, C. J. Am. Chem. Soc. **1980**, 102, 7048-7050.
22. Declercq, J.P.; Tinant, B.; Bashwira, S.; Hootelé, C. to be published.
23. Leysen, J.E.; Gommeren, W.; Eens, A.; de Chaffoy de Courcelles, D. Stoof; J.C. and Janssen, P.A. J. Pharmacol. Exp. Ther. **1988**, 247, 661-670.