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JANOLUSIMIDE, A LIPOPHILIC TRIPEPTIDE TOXIN FROM THE NUDIBRANCH MOLLUSC *JANOLUS CRISTATUS*

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Abstract.- The structure of janolusimide, a lipophilic tripeptide toxic to mice isolated from the nudibranch mollusc Janolus cristatus, has been established by spectroscopic and chemical means.

Numerous investigations in recent years[1] have shown that nudibranch molluscs owe their survival to various toxic or unpleasant chemical constituents. Although the nature of these chemicals appears to be extremely variable, the majority of them are of terpenoidic origin.

We report now that the major organic constituent of the Mediterranean[2] nudibranch *Janolus cristatus* Delle Chiaje (Nudibranchia, Arminacea) is the unusual tripeptide *1*, named janolusimide, which is toxic to mice.

Approximately 300 specimens of *J. cristatus* (average length 35 mm) were extracted with acetone. Removal of the solvent and sequential partition of the residual water with diethyl ether and n-butanol furnished the principal metabolite in the n-butanol fraction. Chromatography on LH-20 (MeOH) and then on silica gel ($CHCl_3$--MeOH, 8:2) yielded 129 mg of *1* as a colorless amorphous solid, $[\alpha]_D^{25}$- 10.3°(C, 2.5; $CHCl_3$); ν_{max} ($CHCl_3$) 3340, 1775, 1736, 1703, 1665.

EI mass spectrometry gave the molecular ion at m/z 383, accompanied by a strong M+1 ion whose intensity was found to be variable from run to run. The FAB mass spectrum (Sulfolane; $NaBF_4$) gave the molecular ion at m/z 384 $[M+H]^+$,supported by an ion at m/z 406 $[M+Na]^+$. An accurate mass measurement on the molecular ion in the EI mass spectrum, 383.2372 (calculated 383.2420), showed the empirical formula to be $C_{19}H_{33}N_3O_5$, which was substantiated by the n.m.r. spectral data.

The peptide nature of janolusimide was suggested by the ^{13}C-n.m.r. spectrum (Table) which contained four carbonyl carbon signals, three of which resonating in the 170-180 ppm region.

Informations on the gross structure of janolusimide were obtained by an extensive use of mono-dimensional and two-dimensional (2D) n.m.r. methods. The results, reported in the Table, can be summarized as follows. A COSY experiment revealed the presence of three partial structures, $-NH-CH(CH_3)-CH(X)-CH(CH_3)-$, $\geq CH-CH_3$, $\geq CH-CH(CH_3)_2$, linked to quaternary carbons and/or to heteroatoms. The ^{13}C chemical shift values were assigned by a 2D $^1H-^{13}C$ heteronuclear correlation, leaving some uncertainty on the attribution of the values to several methyl groups because of the close proximity of the signals in the ^1H-n.m.r. spectrum. A $^1H-^{13}C$ heteronuclear long range correlation was of particular value for establishing the presence of a N-methylalanine residue in the molecule since the N-methyl protons at δ 2.52 (C-1) were found to be long range coupled to the methine carbon at δ 59.6 (C-2). A relevant information was also the coupling exhibited by the carbonyl carbon at δ 177.3 (C-7) with the methine proton at δ 3.77 (C-10). Finally a NOESY experiment, showing a nuclear Overhauser effect of the C-2 proton at δ 3.35 with both the N-methyl protons at δ 2.52 and the amide proton at δ 7.62, allowed the sequencing of the N-methylalanine and 4-amino-3--hydroxy-2-methylvaleric acid as depicted in *1*. This evidence leaves undetermined the structure of the pyrrolidinedione moiety substituted by an isopropyl and by two methyl groups on a quaternary carbon atom.

Janolusimide (*1*) was found to be sensitive to both dilute acid and alkali. Treatment of *1* with methanolic bicarbonate solution (1% $NaHCO_3$; 3:1 $MeOH-H_2O$; 2 h; r.t.), removal of the methanol and solvent partition afforded the $CHCl_3$-soluble pyrrolidinedione (*2*) and the n-butanol-soluble dipeptide methyl ester (*3*). The structure of *3* was established from spectral data[3] while the structure of *2* $[\alpha]_D^{25}$ - 38.3° (C, 0.7; $CHCl_3$), was deduced from spectral data[4] and by $NaBH_4$ reduction to a mixture of two diasteroisomeric alcohols *4* which were separated by silica gel column chromatography. The ^1H-n.m.r. spectrum of the major diastereomer[5] showed that the methine bearing the isopropyl substituent was coupled with the newly formed methine carrying the hydroxy group. The ^{13}C chemical shift values of the ring carbons in the intact molecule *1* (Table) as well as in *2*[4] and in *4*[5] univocally determined the structure of the pyrrolidine-2,4-dione moiety.

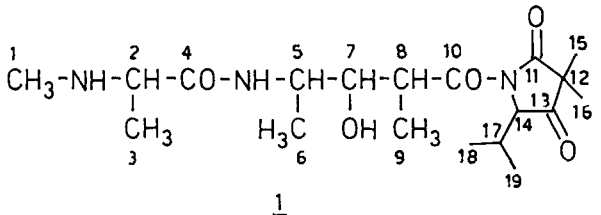
Hydrolysis of janolusimide with 6N HCl (8 h; reflux) and chromatography on cellulose of the resulting mixture (eluant: n-BuOH-AcOH-H_2O, 60:15:25) afforded N-methyl-L-alanine, identified from its spectral properties and by comparison of its chromatographic behaviour on Chiralplate® with authentic samples of N-methyl--D,L-alanine and N-methyl-L-alanine[13], and two new aminoacids (*5* and *6*).

The structure of *5*, $[\alpha]_D^{25}$ - 11.7° (C, 0.3; H_2O), a minor component of the hydrolysis mixture clearly arising from deydration of *6* in the reaction conditions, was argued from the ^1H-n.m.r. spectrum. The *Z* stereochemistry around the double bond was established by synthesis of the *E* isomer *7*. Wittig reaction of N-t-BOC-L--alaninal[7] with ethyl 2-(triphenyl-phosphoranylidene)-propionate (C_6H_6; r.t.; 1 h) followed by removal of the protecting groups (1N KOH, CH_3OH-H_2O, 1:1; 4N HCl,

TABLE - NMR data of Janolusimide (1)[a]

Position	δ ^{1}H (multiplicity;J,Hz)	δ ^{13}C
1	2.52 (s)	33.7
2	3.35 (q;6.9)	59.6
3	1.42 (d;6.9)	18.4
4		172.2
5	4.15 (m)	46.3
6	1.28 (d;6.9)	18.2[d]
7	3.77 (dd;2.3,9.4)	77.4
8	3.95 (dq;9.4,6.7)	44.1
9	1.18 (d;6.7)	19.1[e]
10		177.3
11		177.6
12		49.2
13		210.0
14	4.54 (d;3.6)	69.4
15	1.24 (s)[b]	19.0[d]
16	1.26 (s)[b]	22.3[d]
17	2.50 (m)	29.9
18	0.84 (d;7.0)[c]	17.0
19	1.17 (d;7.0)[c]	14.0[e]
NH(amidic)	7.62 (d;8.6)	

[a]129 mg in 0.5 ml $CDCl_3$; Bruker WM 500.
[b-e]Values with identical superscript within each column may be interchanged.



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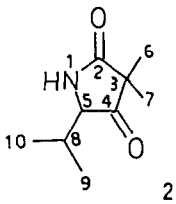

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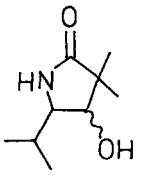

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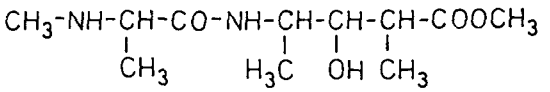

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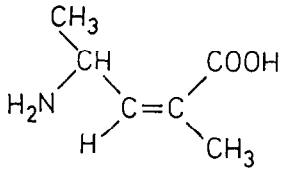

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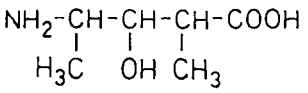

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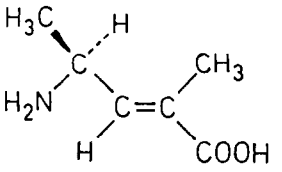

7

dioxane) afforded *7*, $[\alpha]_D^{25}$ - 11.9° (C, 3.0; H_2O), having the *S* stereochemistry at the unique chiral centre. Comparison of the ^{1}H-n.m.r. spectra of *5*[6] and *7*[8] established the *Z* stereochemistry for *5* and the *E* stereochemistry for *7*.

The ^{1}H- and ^{13}C-n.m.r. spectra of *6*[9], $[\alpha]_D^{25}$ + 10° (C, 0.8; H_2O), together with the spectral and chemical evidence arising from *1*, *3* and *5*, suggested the 4-amino-3-hydroxy-2-methylvaleric acid structure for this compound. (2*S*, 3*S*, 4*R*)--4-amino-3-hydroxy-2-methylvaleric acid is an aminoacid component of the antitumor antibiotic bleomycin[10]; however its reported ^{1}H-[11] and ^{13}C-n.m.r.[12] spectra are somewhat different from those of *6* suggesting that the two compounds must be diastereoisomers. Work is in progress for establishing the stereochemistry of *6* as well as of *2*.

Janolusimide is an unusual tripeptide since, besides N-methyl-L- alanine, it contains two rather unusual constituents. From a biogenetic point of view the aminoacid *6* could derive by condensation of alanine and propionate while the pyrroli-

dine-2,4-dione *2* could be imagined deriving by coupling of valine and isobutyrate.

Janolusimide is toxic to mice (LD 5 mg/Kg; i.p.); at lower concentrations its neurotoxic action is antagonized by atropine suggesting that Janolusimide affects the acetylcholine receptors. An account of the pharmacological effects of janolusimide will be reported elsewhere.

ACKNOWLEDGEMENTS. - *Janolus cristatus* was collected in the bay of Naples by G. Villani. We are grateful to Dr. V. Politi, Polifarma, Roma, for the *in vivo* testing of janolusimide. Thanks are due to A. Crispino, C. Faruolo and D. Ricciardi for technical assistance. Mass spectral data were provided by "Servizio di Spettrometria di Massa del C.N.R. e dell'Università di Napoli"; the assistance of the staff is gratefully acknowledged.

REFERENCES AND NOTES

1.- G. Cimino, S. De Rosa, S. De Stefano, R. Morrone and G. Sodano, *Tetrahedron 41*, 1093 (1985); K. Gustafson and R. J. Andersen, *Tetrahedron 41*, 1101 (1985); D. J. Faulkner and M. T. Ghiselin, *Mar. Ecol. Prog. Ser. 13*, 295 (1983); G.R.Schulte and P. J. Scheuer, *Tetrahedron 38*, 1857 (1982).

2.- L. Schmekel and A. Portmann, "Opisthobranchia des Mittelmeeres", Springer-Verlag, Heidelberg (1982).

3.- EIMS, m/z: 246 (M^+), 215, 199, 159, 129. ^{1}H-n.m.r.; δ($CDCl_3$): 7.35 (1H, d, J 9.1; amidic NH), 4.15 (1H, m; H-4); 3.71 (3H, s; $-OCH_3$), 3.67 (1H, dd, J 2.2 and 9.0; H-7), 3.08 (1H, q, J 6.8; H-2), 2.50 (1H, dq, J 9.0 and 7.2; H-8), 2.40 (3H, s; CH_3-1), 1.28 (3H, d, J 6.8; CH_3-3), 1.24 (3H,d, J 6.8;CH_3-6), 1.20 (3H, d, J 7.2; CH_3-9).

4.- EIMS, m/z: 169 (M^+), 141, 127, 126, 98, 73, 71. IR ν_{max} ($CHCl_3$) 1769 (weak), 1701 cm^{-1}.^{1}H-n.m.r. δ($CDCl_3$): 6.88 (1H, b; H-1), 3.91 (1H, d, J 4.1;H-5), 2.19 (1H, m; H-8), 1.25 (3H, s), 1.21 (3H,s), 1.04 (3H,d, J 6.9), 0.89 (3H, d, J 6.8).^{13}C-n.m.r. δ($CDCl_3$): 214.1, 178.5, 66.6, 46.7, 30.4, 21.9, 19.3, 19.1,17.1.

5.- EIMS, m/z: 171(M^+), 128, 114. IR ν_{max} ($CHCl_3$) 1699 cm^{-1};^{1}H-n.m.r. δ($CDCl_3$): 6.10 (1H,b; H-1), 3.91 (1H,d, J 4.0;H-4),3.27 (1H,dd, J 4.0 and 10.3; H-5), 2.03 (1H,m; H-8),1.20 (3H,s), 1.17 (3H,s), 1.03 (3H,d, J 6.5), 1.01 (3H,d, J 6.5).^{13}C-n.m.r. δ(CD_3OD) 77.5, 64.5, 41.3, 28.3, 23.0, 20.7, 19.2, 18.1. Due to the minute amount of material the signal of the carbonyl carbon was not detected.

6.- ^{1}H-n.m.r. δ(D_2O): 6.11 (1H, dq, J 9.5 and 1.4), 4.25 (1H, dq, J 9.5 and 6.6), 1.83 (3H, d, J 1.4), 1.36 (3H, d, J 6.6).

7.- Y. Hamada and T. Shioiri, *Chem. Pharm. Bull. 30*, 1921 (1982).

8.- ^{1}H-n.m.r. δ(D_2O): 6.56 (1H, d, J 9.5), 4.30 (1H, dq, J 9.5 and 6.5) 1.83 (3H, s), 1.35 (3H, d, J 6.5).

9.- EIMS, m/z: 147 (M^+), 129, 114. ^{1}H-n.m.r. δ(D_2O): 3.56 (1H, dd, J 4.1 and 7.2; H-3), 3.41 (1H, m; H-4), 2.60 (1H, dq, J 4.1 and 7.1;H-2), 1.32 (3H, d, J 6.6; 4-CH_3), 1.22 (3H, d, J 7.1; 2-CH_3).^{13}C-n.m.r. δ(D_2O):15.4,15.9,43.5,51.4,75.2,182.4.

10.- T. Takita, Y. Muraoka, T. Yoshioka, A. Fuyii, Y. Umezawa, H. Naganawa, H. Umezawa, *J. Antibiot. (Tokyo) 31*, 801 (1978).

11.- R. M. Di Pardo and M. G. Bock, *Tetr. Letters 24*, 4805 (1983).

12.- H. Naganawa, Y. Muraoka, T. Takita and H. Umezawa, *J.Antibiot. (Tokyo) 30*, 388 (1977).

13.- Eluant: CH_3OH, H_2O, CH_3CN, 50:50:30; N-methyl-L-alanine, R_f 0.65; N--methyl-D-alanine, R_f 0.70.

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