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Lepadiformine, a new Marine Cytotoxic Alkaloid from *Clavelina lepadiformis* Müller

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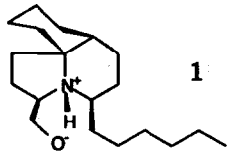
Abstract : A cytotoxic alkaloid, lepadiformine **1**, with a new heterocyclic skeleton, has been isolated from the ascidian *Clavelina lepadiformis*. The structure of **1** was established on the basis of chemical properties and by spectroscopic means : it includes a unique zwitterionic-like moiety.

Ascidians are a rich source of nitrogen compounds with a wide range of biological activities[3,4]. Recently, alkaloids with unusual structures have been recovered from the *Clavelinidae* family: clavepictines A and B[5] (cytotoxic quinolizidines), pictamine[6] (a quinolizidine), piclavines[7] (antimicrobial indolizidines) from *Clavelina picta*, lepadin A[8] (decahydroquinoline) from *Clavelina lepadiformis*, wakayin[9] (a cytotoxic pyrrolo-iminoquinone) from *Clavelina sp.*



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During our ongoing program to isolate novel cytotoxic and nitrogenous marine natural products, we have found that extracts from the tunicate *Clavelina lepadiformis* Müller (*Chordata, Aplousobranchia, Clavelinidae*) contains a TLC-Dragendorff-positive product that exhibits moderate *in vitro* activity against KB cells (IC_{50} = 16.8 µg/mL). *Clavelina lepadiformis* was collected during summer near Tabarka (North-West of Tunisia) using SCUBA at a depth between 15-25 m. It was identified by Dr. F. Monniot at the Muséum d'Histoire Naturelle, Paris. The fresh tunicate (1500 g) was exhaustively extracted by 95% ethanol. The crude extract was treated by solvent partition (dichloromethane *vs* water). The dichloromethane-soluble fraction was suspended in aqueous N HCl and the suspension was filtered. The filtrate was washed by diethyl ether and extracted by dichloromethane. The crude alkaloid was purified by elution through a Sephadex LH-20 column. The main fractions contain two minor, but inseparable, by-products in addition to lepadiformine. They convert into lepadiformine on evaporation of an acidic solution (methanol:aqueous N HCl ; 99:1) of the mixture, affording lepadiformine **1** as a colorless oil ($5.3.10^{-2}$% of ascidian dry weight).

Structural elucidation : The molecular formula was determined by FAB-MS (M^++1 : 294, 100 %) and high resolution EI-MS (m/z 293.271850) corresponding to $C_{19}H_{35}NO$ (calc. 293,271848, Δ 2.05 mmu). As the ^1H NMR spectrum does not display any signal at low field, due to olefinic protons, such a molecular formula requires the presence of three rings. The base peak (EI-MS, m/z 262.2530, Δ 0.46 mmu) at M^+-31 suggests the presence of either -OCH_3 or -CH_2OH as a substituant. Actually, the J-modulated ^{13}C spectrum[10], shows the signal of an oxygenated methylene (δ 59.96) but no methoxyl group. In addition to this, the

^{13}C NMR spectrum shows the signals for one quaternary, three methine, thirteen methylene and one methyl carbons.

Since the high field region (δ 2.10 - 0.80) of the ^{1}H NMR spectrum exhibits 28 out of the 35 proton signals in spite the use of a 500 MHz spectrometer to record it, this region was so crowded that the conventional 2D heteronuclear experiments (HMQC[11], HMBC[12]) could not lead to the total elucidation of the structure. However, the ^{13}C NMR INADEQUATE[13] unambiguously gave us the complete carbon skeleton of the molecule as shown in Figure 1 (no overlapping signals). Thus, the assignment of the correct chemical shift to every ^{1}H atom could be easily made by the HMQC and the HMBC (J = 7.2 Hz) spectra in which all the ^{13}C signals were unequivocally known (Table 1).

C atom	^{13}C	HMQC/^{1}H	H atom	COSY	HMBC
1	59.96	3.56 (d, J=13.4Hz)	1	4.10	24.30, 63.54
		4.10 (d, J=13.4Hz)		3.56, 3.61	24.30, 63.54
2	63.54	3.61	2	1.95, 2.32, 4.10	-
3	24.30	1.95	3	1.75, 2.32, 3.61	76.58
		2.32		1.75, 1.95, 3.61	-
4	26.42	1.75	4	1.95, 2.00, 2.32	24.30, 33.77, 76.58
		2.00		1.75	24.30, 33.77, 76.58
5	76.58		-		
6	33.77	2.10	6 (2H)	1.25-1.35, 1.63, 1.75	23.19, 24.85, 26.28, 36.19, 76.58
7	23.19	1.25-1.35	7	1.75, 2.10	24.85, 33.66
		1.75		1.25-1.35, 1.63, 2.10	24.85, 30.66, 33.66, 76.58
8	24.85	1.25-1.35	8	1.00, 1.63	30.66, 36.19
		1.63		1.00, 1.25-1.35	23.19, 30.66
9	30.66	1.00	9	1.25-1.35, 1.63, 2.00	36.19
		1.63		1.00, 1.25-1.35, 2.00	23.19, 76.58
10	36.19	2.00	10	1.00, 1.30-1.40, 1.63, 1.75	22.49, 26.28, 30.66, 76.58
11	22.49	1.30-1.40	11	1.48, 1.75, 2.10	19.17, 30.66, 36.19, 58.71
		1.75		1.30-1.40, 1.48, 1.89, 2.00	19.17, 30.66, 36.19, 76.58
12	19.17	1.48	12	1.30-1.40, 1.75, 1.89, 3.61	-
		1.89		1.48, 1.75	22.49, 36.19
13	58.71	3.61	13	1.20-1.30, 10.00	-
14	29.83	1.20-1.30	14	2.42, 3.61	19.17, 26.28, 58.71
		2.42		1.20-1.30, 1.25-1.35	26.28, 58.71
15	26.28	1.25-1.35	15 (2H)	2.42	28.89, 29.83
16	28.89	1.30-1.40	16 (2H)	-	22.32, 31.51
17	31.51	1.20-1.30	17 (2H)	-	22.32, 28.89
18	22.32	1.20-1.30	18 (2H)	0.82	13.82, 31.51
19	13.82	0.82 (t, J=6.5Hz)	19 (3H)	1.20-1.30	22.32, 31.51
		10.0 (broad)	NH	3.61	

Table 1 : ^{1}H and ^{13}C NMR Data of Lepadiformine **1** ($CDCl_3$, 500 MHz)

Clearly, from these spectra, the position of heteroatoms (O and N) are those indicated on the planar structure of lepadiformine in figure 2.

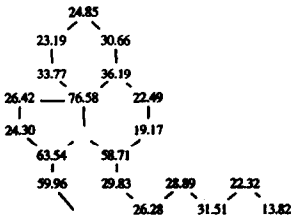

Fig. 1

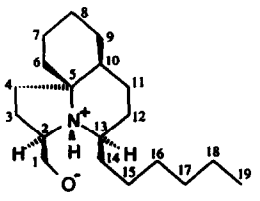

Fig. 2

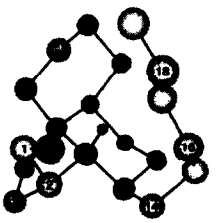

Fig. 3

However, an important choice had to be made between the apparently more classical neutral form (tertiary amine and primary alcohol) and the zwiterionic one as depicted here. The latter structure was finally proposed as a result of further spectroscopic evidences and also of chemical properties that could be summarized as follows :

• The rather low chemical shifts of the carbons (C-2, C-5 and C-13) directly bonded to the nitrogen

and also the important shielding of C3 and C12 (both at a β-position with respect to nitrogen) are better explained by the protonated ammonium structure[14].

• The "acidic" proton appears, as a broad singlet, in the ^1H NMR spectrum at a much too low field ($\delta \approx 10$ ppm) to be the one of either an aliphatic alcohol or of an alternative secondary amine. Even though the signal for this proton was quite broad ($w_{\frac{1}{2}} = 18$ Hz) and because the solubility in $CDCl_3$ was very high (250 mg in 0.5 mL), we could run an heteronuclear ^1H-^{15}N HMQC NMR experiment at the natural isotopic abundance. The optimized signal was acquired without decoupling for $J \approx 75$ Hz. The only intense correlation at the ^{15}N frequency (δ -302 ppm, $CH_3{}^{15}NO_2$ as an external reference at δ 0 ppm), made of a doublet ($J = 75.3$ Hz) centered on the ^1H frequency of this proton, was the best proof for such a predominant form, even though we cannot totally exclude its equilibrium with the neutral one (not seen in the spectra !).

• Thus, the numerous sharp bands observed between 2350 and 2700 cm^{-1} in the FT-IR spectrum originate from this function.

• In order to chemically prove the presence of these elements in lepadiformine **1**, we have tried many reaction conditions in which they should have been acylated or alkylated (THF/NaH or nBuLi, then Ac_2O or $(CF_3CO)_2$ or CH_3I), but only starting material was recovered in any attempt. However, the N-H proton was proved by NMR to be slowly exchangeable in D_2O. Thus, either the strong base has no access to abstract hydrogen or the only acceptable electrophile in size is proton itself.

These behaviours taken all together speak in favor of a "cage-like" structure, with in its core the polar part of it (a proton) hidden in lipophilic surroundings. A molecular model[15] of it is presented (figure 3)[16] that takes into account the correct relative configuration[17] according to NOESY[18] correlations (mainly H1-H6, NH-H6) and strong shielding of some carbon atoms (C-12) by a γ-effect with the protonated nitrogen lone pair).

Biological activities : Lepadiformine has a moderate cytotoxic activity : IC_{50} : KB 9.20 μg/mL, HT29 0.75 μg/mL, P388 3.10 μg/mL, P388 doxorubicin-resistant 6.30 μg/mL, NSCLC-N6 (non-small-cell lung carcinoma[19]) 6.10 μg/mL.

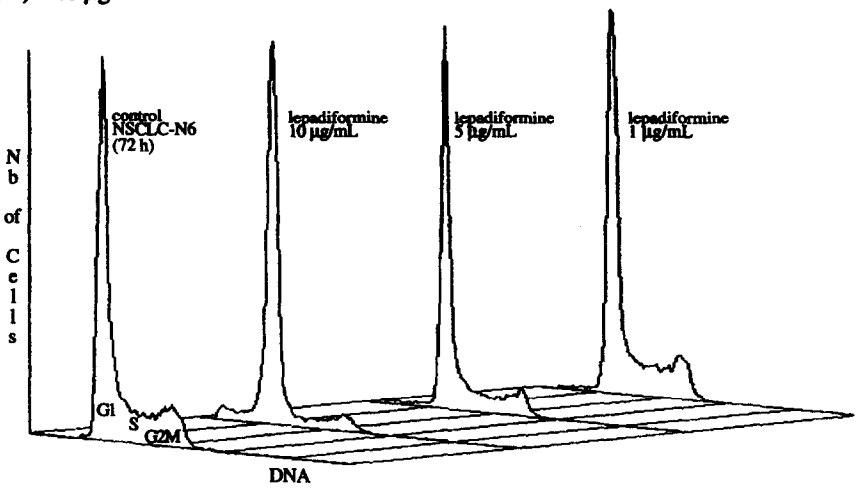

Figure 4 : Histogram of the DNA amount of NSCLC-N6 Cells Cultured for 72 h in the Presence of Various Concentrations of Lepadiformine **1**.

In vitro study of the effects on the NSCLC-N6 cells according to concentration shows that **1** has cycle-dependent and phase-dependent properties (Figure 4 and Table 2). A partial, dose-dependent, G1 phase blockade of the cells was noted after 72h of growth in a continuous drug exposure experiment. Simultaneously, the percentage of S phase cells decreased and cellular debris appeared. This appearance of debris could be

related to mortality of the first cells blocked in G1 phase.

		Lepadiformine		
Phase	Control	10 µg/mL	5 µg/mL	1 µg/mL
G1	62.8	84.2	70.7	62.6
S	31.1	11.0	25.3	32.5
G2	6.1	4.0	4.1	4.9

Table 2 : Percentage of NSCLC-N6 cells in G1, S and G2 phases of the cell cycle in the presence of various concentrations of lepadiformine **1** (control : untreated cells)

Conclusion : To the best of our knowledge, this is the first example of a decahydro-1H pyrrolo-[2,1-*j*] quinoline as a natural compound. The carbon skeleton of **1**, unique in Nature, is partially related to those of other alkaloids of *Clavelina* genus, like the already cited clavepictines and pictamine. Therefore, this genus continues to be a fruitful source of structurally new alkaloids. Due to its moderate cytotoxicity and effects on cell cycle, lepadiformine seems to be of little interest as regards in antitumoral activity. However, the special zwiterionic structure may be of strong interest in terms of biological "proton-transfer mechanisms" since it seems to be a natural example of models recently described ("*cis*-decalin amino acid" derivatives[20]) having such important properties.

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16. All the hydrogen atoms but the one on the nitrogen are hidden for more clarity.
17. Even though the measured specific rotation is zero (C=1, $CHCl_3$), it does not mean that lepadiformine is racemic.
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