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GROSSULARINE-1 AND GROSSULARINE-2, CYTOTOXIC α-CARBOLINES FROM THE TUNICATE : *Dendrodoa grossularia*

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Summary.*Several indole derivatives were isolated from the tunicate: Dendrodoa grossularia among which two α-carbolines: grossularine-1 (revised structure) and grossularine-2. The structures were based upon spectral data, including X-ray analysis for grossularine-2. These compounds exhibit marked cytotoxicity toward murine and human tumor cells. They are the first example of naturally occurring α-carbolines.*

Dendrodoa grossularia (Styelidae), a solitary tunicate grows in dark red clusters along the coasts of Brittany, and inhabits preferentially caves partially discovered during very low tides, where specimens were collected.

In a preliminary screening, the dichloromethane extract of this tunicate exhibited a marked cytotoxic activity toward L1210 leukemia cell line, which stimulated the isolation of active compounds.

Fractionation of this extract was monitored by L1210 bioassay. A chromatographic separation on a silicagel column, eluted by chloroform with increasing amounts of acetone, led to complex fractions; three fractions retained significant cytotoxicity and were submitted to further purification in various systems, leading to pure compounds; some of which displayed cytotoxicity.

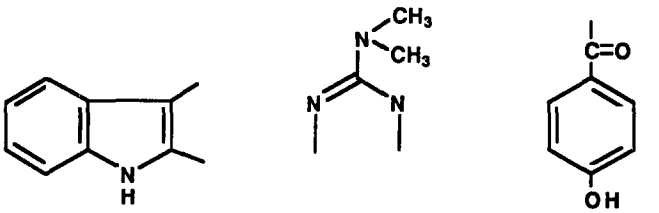
The less polar active fraction ($CHCl_3$-acetone 95/5) contained the previously described dendrodoine 1 (1), which showed moderate cytotoxicity for L1210 leukemia cells (ID_{50}:10 μg/ml). From a more polar fraction ($CHCl_3$-acetone 2/8), we isolated as the major component the imidazolone 2 (2), devoid of cytotoxicity.

The fraction eluted by $CHCl_3$-acetone 8/2 retained maximum activity and contained two yellow compounds of close R_f values on silicagel: grossularine-1 and grossularine-2. The separation of these two compounds could be achieved on preparative silicagel TLC ($CHCl_3$-acetone 7/3) or on alumina column: grossularine-1 is eluted by $CHCl_3$-acetone 7/3 while grossularine-2 is retained and finally recovered by exhaustive washing of alumina with acetone and methanol. The separation could also be performed by HLPC on reverse-phase C-18 using EtOH-H_2O (6/4) as eluent.

In a preliminary report (3) grossularine-1: ($C_{23}H_{18}N_6O$), was assigned structure 3 from spectral data. However, comparison of grossularine-1 and grossularine-2 led us to revise this structure (*vide infra*), and to assign structure 4.

Grossularine-2: a yellow pigment, m.p. 281-283 °C, was shown by HRMS to have the elemental composition: $C_{21}H_{17}N_5O_2$ (molecular ion M^+ 371.197). The ^1H NMR data (Table1) established the presence of a 2,3-disubstituted indole, a 1,4- disubstituted benzene ring, and a $N(CH_3)_2$ group. Selective irradiations allowed us to assign chemical shifts. The chemical shifts of the benzene protons suggest that the benzene ring bears a carbonyl and a hydroxyl group.

Treatment of this compound by acetic anhydride- pyridine (60°C) furnished a diacetylated derivative, for which ^{1}H NMR indicates : 1 NAc (δ 3.00 ppm), 1 OAc (2.36 ppm), a downfield shift for the H-8 indole proton, and for two benzene protons. These data correlated with ^{13}C NMR chemical shifts and MS analysis : presence of the fragment ions *m/e* 121 (hydroxy-bensoyl) and *m/e* 93 (hydroxy-phenyl) , allowed us to establish the following partial structures.



However as in the case of grossularine-1, it was difficult to confidently establish the structure of the molecule. Fortunately, grossularine-2 crystallized from a mixture of tetrahydrofuran-methanol and X-ray analysis gave the structure of a complex of grossularine-2 5 with tetrahydrofuran (4). Moreover this analysis showed that grossularine-2 is approximately planar owing to an hydrogen bonding occurring between the carbonyl (C-12) and the NH of the imidazole ring: the angle of the benzoyl group with the α–carboline was 21°.

The spectral data of grossularine-1 and -2 showed striking similarities. Of particular help was the ^{13}C NMR data listed in table 2. If we accept values for a carbonyl indole in grossularine-1 and of a 4-hydroxy-benzoyl group for grossularine-2 (5), the remaining signals appear approximately at the same chemical shifts and it is suggested that grossularine-1 also possess an α-carboline skeleton and has structure 4 rather than structure 3. Further proof of structure was gained by comparison of UV data (Fig.1) and MS fragmentation : both products exhibit a fragment ion *m/e*: 250 ($C_{14}H_{12}N_5$ from HRMS) which corresponds to the dimethylamino-2-imidazo-carboline moiety . Comparison of the ^{13}C NMR data of 4 and 5 with those of the unsubstituted α -carboline 6 (6) also helped us in the assignment of the chemical shifts.

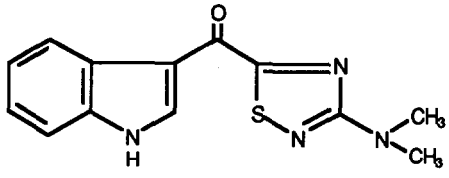
Table 1 - ^{1}H NMR (250 MHz) of grossularine-1: 4 and grossularine-2: 5 , δ ppm, DMSO-d_6,*J* (Hz)

4		5	
12.16	H 1'		
11.7 s	H 9	11.6 s	H 9
10.9 s	H 11	10.8	H 11
9.45 d	H 2'	8.27 d (8.7)	H 2',6'
8.56 m	H 5'		
8.24 br d (6.9)	H 5	8.25 br d(7)	H 5
7.56 m	H 8'	7.44 s	H 4'
7.5 br d (6.9)	H 8	7.48 br d (7)	H 8
7.42 dt (7;7;1)	H 7	7.42 dt (7;7;1))	H 7
7.29 m	H 6',7'		
7.23 dt (7;7;1)	H 6	7.21 dt (7;7;1)	H 6
		6.92 d (8.7)	H 3',5'
3.35 s	$N(CH_3)_2$	3.28 s	$N(CH_3)_2$

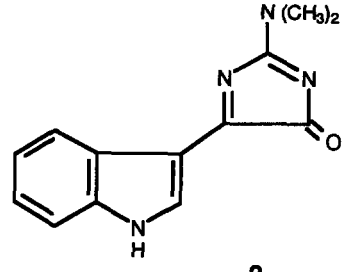
Table 2 - 13C NMR (20 MHz) of grossularine-1 4 , grossularine-2 5 and a -carboline 6 (DMSO-d 6, d ppm)

4		5		6	
186.8 s	C 12	184.7 s	C 12		
		162.7 s	C 4'		
159.4 s	C 10	159.8 s	C 10		
159.2 s	C 1a	159.7 s	C 1a	152.2 d	C 1a
146.8 s	C 2	147.1 s	C 2	146.4 d	C 2
145.7 s	C 4	146.7 s	C 4	129.3 d	C 4
139.3 s	C 8a	139.5 s	C 8a	139.3 s	C 8a
137.2 d	C 2'	133.4 d	C 2', 6'		
135.7 s	C 9'	128.2 s	C 1'		
132.9 s	C 4'				
127.1 s	C 5a	127.2 s	C 5a	121.5 s	C 5a
127.0 s	C 3	125.6 s	C 3	127.2 d	C 3
125.4 d	C 5'				
122.4 d	C 5	122.5 d	C 5	120.9 d	C 5
122.3 d	C 7	121.6 d	C 7	120.2 d	C 7
121.5 d	C 7'				
121.4 d	C 6'				
119.5 s	C 4a	119.6 s	C 4a	116.0 s	C 4a
118.6 d	C 6	118.8 d	C 6	115.6 d	C 6
114.3 s	C 3'	114.9 d	C 3', 5'		
111.8 d	C 8'				
110.3 d	C8	110.5 d	C 8	111.8 d	C 8
39.3 q	$N(CH_3)_2$	39.1 q	$N(CH_3)_2$		

Fig.1 - UV Spectra of grossularine-1 4 , grossularine-2 5 and α -carboline 6



1



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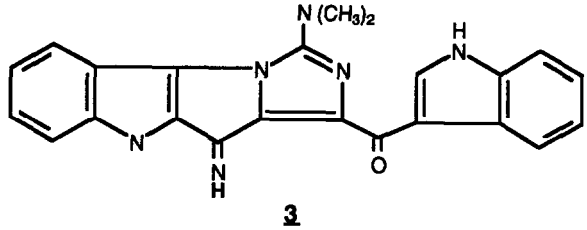

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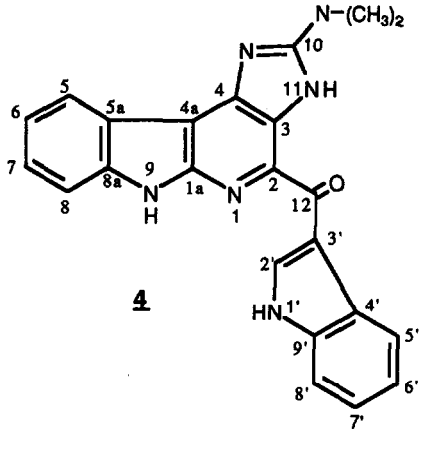

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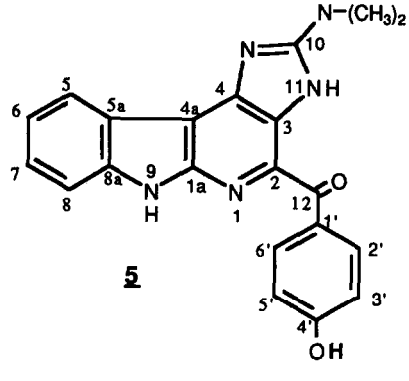

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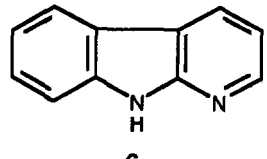

6

A literature survey has shown that 4 and 5 are, to the best of our knowledge, the first natural products possessing an α-carboline skeleton. The few α-carbolines described hitherto have been obtained from tobacco distillates as pyrolysis products (7) and are thus artefacts, or were produced by synthesis (8-11). They display antiviral (8), cytotoxic or antitumor activity (10,11).

Grossularine-1 4 and grossularine-2 5, are cytotoxic toward L1210 leukemia cells: ID_{50} respectively 6- and 4 µg/ml. A cell-flow cytofluorimetric analysis (12) performed on these cells revealed that 4 and 5 cause accumulation of cells in the G1 phase at concentrations of10 µg/ml for grossularine-1 and 1,5 µg/ml for grossularine-2. They are, however, more cytotoxic, 5 especially, toward solid human tumor cell lines: WiDr (colon) and MCF7 (breast), in a cloning system bioassay: they are active up to 10 ng/ml (13). Grossularine-2 5, appears to act on ADN as a mono-intercalating agent (14). Detailed biological studies will be given in separated papers (13,14).

The small amounts ofpurified compounds isolated precluded *in vivo* assays. Synthesis of these products is currently in progress in view of more intensive biological investigations.

Experimental section.

^1H and ^{13}C NMR spectra were recorded on Bruker WP 80, Bruker WM 200 and 500 instruments (TMS internal reference). Infrared and ultraviolet spectra were recorded on Perkin-Elmer 157 G and Uvikon 810 Kontron spectrophotometers, respectively. Low resolution mass spectra were recorded on a Thomson THN 208 mass spectrophotometer. High resolution mass spectra were supplied by a Kratos MS 50 spectrometer. C.I.M.S. on a Nermag U.3.0. All solvents were distilled prior to use. Melting points are uncorrected.

The specimens of *Dendrodoa grossularia* were collected in Brittany during low tides (Ile Callot) or by dredging.

Extraction: Method A: frozen animals were lyophilized (215g),ground and then extracted twice with dichloromethane (2x500 ml)) and twice with methanol(2500 ml). The methanolic extract was evaporated, and then extracted again by dichloromethane and ethyl acetate. The dichloromethane and ethyl acetate extracts were combined.

Method B: wet animals (5 kg were soaked twice in a mixture (1liter) of methanol-chloroform (1/1). The organic layer was decanted, the solvent evaporated and the aqueous suspension extracted with dichloromethane and ethyl acetate.The organic extracts were dried over sodium sulfate. We have noticed that method B is more convenient for the extraction of the polar compounds.

The dark gummy residue obtained after removal of solvent was chromatographed on a silicagel column (230-240 mesh) and eluted with chloroform, then chloroform with increasing amounts of acetone, and methanol.

Fractions eluted with chloroform-acetone 7/3 contained 4 and 5 in a complex mixture. Further purification was performed on a silicagel column (chloroform-acetone 8/2) followed by filtration on LH 20 (chloroform-methanol 6/4). Chromatography on alumina: chloroform-acetone 7/3 furnished 4, and subsequently, 5 was eluted with methanol. A further purification was achieved by HPLC (Whatman Partisil M 9 ODS-2), MeOH-H_2O: 6/4.

Grossularine-1, 4: amorphous yellow powder (0.003 % yield), m.p. 350°C. M^+ 394.153, calcd for $C_{23}H_{18}N_6O$: M^+ 394.1542; *m/e* (%): 394 (100), 365 (10), 350 (8), 277 (83) (M^+- indole- H), 250 (89)(250.109 : $C_{14}H_{12}N_5$, M^+-carbonyl5H12-3-indole), 249 (62), 234 (36)(250-CH_3-H), 197 (32), 144 (30)(C_9H_6NO, carbonyl-3-indole),116 (22)(indole). UV: λ_{max} (EtOH): 202, 235, 264 (sh), 340, 360 nm.^1H and ^{13}C NMR, see tables1 and 2.

Acetylation with Ac_2O-Py, 24 h, 60°C gave a diacetate, purified on silicagel: $CHCl_3$-acetone 9/1. M.p. 230°C, $[MH]^+$ 479 (C.I.); ^1H NMR (80 MHz) ($CDCl_3$) δ ppm : 10.0 (1H,br s); 8.60 (4H,m) (H-5, 5',8, 8'); 7.50 (4H,m, H-7, 6, 7,'6'); 3.25 (6H,s, $N(CH_3)_2$); 3.12 (3H,s); 2.72 (3H,s) two NAc (9,1').

Grossularine-2, 5 : amorphous yellow powder (0.003 % yield), crystallized from THF-MeOH. M.p. 281°C. M^+ 371.137, calcd for $C_{21}H_{17}N_5O_2$: M^+ 371.1382 , *m/e* (%): 371 (89),344 (19), 343 (22), 328(19), 262 (22), 250 (27)($C_{14}H_{12}N_5$, M^+- hydroxy-4-benzoyl group), 234 (53)(250-CH_3-H), 169 (61), 131 (100), 121 (83)(hydroxy-benzoyl), 119 (89),93 (80) (hydroxy-phenyl-).^1H and ^{13}C NMR, see tables1 and 2.

The diacetate was prepared as described above: m.p.152°C, $[MH]^+$ 456 (C.I.);^1H NMR (80 MHz, $CDCl_3$) δ ppm: 8.46 (4H,m); 7.42 (4H,m); 3.36 (6H,s, $N(CH_3)_2$; 3.00 (3H,s,9- NAc); 2.36(3H,s,4'- OAc).

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