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A New Antitumor Isoquinoline Alkaloid from the Marine Nudibranch *Jorunna funebris*

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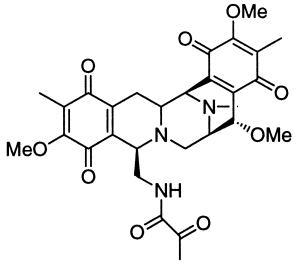
Abstract—A new dimeric isoquinoline alkaloid, jorumycin (**3**), has been isolated from the skin and the mucus of the Pacific nudibranch *Jorunna funebris*. The structure has been fully elucidated on the grounds of ESMS data and of an extensive 2D NMR analysis. The cytotoxicity of **3** was evaluated against various human cancer cell lines and was found to be slightly less potent than Et 743 (**2**).

Introduction

Dimeric isoquinoline alkaloids have been independently isolated from bacteria,[1–3] marine sponges,[4–6] and tunicates.[7,8] These metabolites have often revealed interesting antimicrobial and antitumor activities. The earliest example of this class of compounds has been saframycin C (**1**), the structure of which was determined by X-ray crystallographic analysis.[2] To date other saframycin-like products have been reported, including ecteinascidin 743 (Et 743, **2**), one of the most potent marine-derived antitumor agents.[9] In this paper we wish to report the isolation and structure elucidation of a new dimeric isoquinoline alkaloid, jorumycin (**3**), isolated from the mantle and mucus of the Pacific nudibranch *Jorunna funebris* (Mollusca: Nudibranchia: Doridina: Kentrodorididae). Although less potent than Et 743 (**2**), jorumycin (**3**) shares most of the promising antitumor properties.



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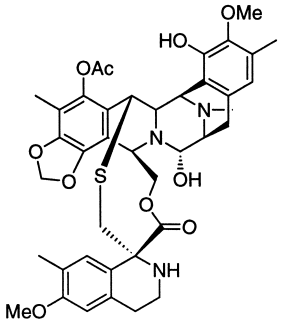

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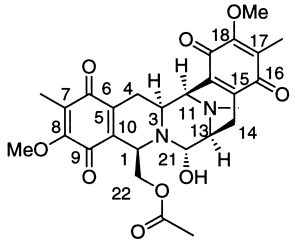

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Results and Discussion

The nudibranch *J. funebris* was collected in March 1998 together with a blue sponge. The biological material was immediately frozen. The nudibranchs (two specimens) and the mucus released by the animals were extracted with acetone. TLC analysis of these crude extracts indicated the presence of an UV-absorbing (254 nm) product, later characterized as **3**, localized in the mantle and the mucus of the molluscs. Although jorumycin (**3**) was the major constituent of extracts, its isolation and characterization was challenging because of the inherent instability of the molecule. The general procedure of purification involved two sequential Sephadex LH-20 columns, followed by preparative normal phase TLC and an HPLC purification step. The acquisition of the entire set of data needed for the chemical characterization and the evaluation of the biological activity has required several preparations of **3**.

Jorumycin (**3**) was obtained as an unstable pale-yellow powder. The ^{1}H NMR spectrum of this metabolite contained

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Table 1. NMR data ($CDCl_3$, 500 MHz) of jorumycin (**3**)

	^{1}H (δ, m, Hz)	^{13}C (δ, m)	^{13}C–^{1}H long-range correlation	NOEs correlation
1	4.36, bs	52.6, d	4.42	3.82
2				–
3	3.16, m	50.8, d		2.84 and 3.90
4	2.84, dd, 16.7 and 2.1	25.5, t		3.16 and 3.90
	1.24, dbd, 16.7 and 8.3			–
5	–	141.9, s	2.84 and 1.25	
6	–	185.5, s	1.93 and 2.84	
7	–	128.4, s	1.93	
8	–	155.7, s	1.93 and 3.98	
9	–	181.0, s	3.98	
10	–	141.7, s	2.84 and 4.36	
11	3.90, bs	54.4, d	2.26	3.16, 2.84 and 2.26
12				
13	3.18, m	57.7, d	3.18, 2.65 and 2.26	4.40, 2.65 and 2.26
14	2.65, dd, 20.1 and 7.3	20.6, t		3.18
	2.24, bd, 20.1			4.40
15	–	141.9, s	2.65 and 2.24	
16	–	185.7, s	1.96	
17	–	128.8, s	1.96	
18	–	155.1, s	4.00 and 1.96	
19	–	181.3, s	4.00	
20	–	137.2, s	2.65	
21	4.40, bs	83.0, d	2.65 and 2.24	3.18 and 2.24
22	4.42, dd, 11.2 and 3.7	64.2, t		–
	3.82, dd, 11.2 and 3.5			4.36
Me-7	1.93, s	8.7, q		
Me-17	1.96, s	8.7, q		
MeO-8	3.98, s	61.0, q		
MeO-18	4.00, s	61.0, q		
N-Me	2.26, bs	41.3, q		3.90 and 3.18
CH_3CO	–	170.0, s	4.42, 3.82 and 1.75	
CH_3CO	1.75, s	20.7, q		

only few well-resolved signals, including 6 methyl groups and 8 downfield resonances ranging between δ 3.10 and 4.50 (Table 1). This strongly contrasted with the complexity of ^{13}C NMR data that indicated 27 signals, most of which were assignable to aromatic or heteroatom-bearing carbons (Table 1). In particular, the NMR data suggested the presence of four quinone carbonyls (185.7, 185.5, 181.3 and 181.0 ppm), two arylmethyl groups (8.7 ppm), two methoxy groups (61.0 ppm), one *N*-methyl group (41.3 ppm) and an acetoxymethylene (64.2 ppm) (Table 1). Accordingly, the infrared spectrum showed an intense band at 1660 cm^{-1} (quinone carbonyl), whereas the UV spectrum had a maximum at 266 nm. The ES^+ MS analysis revealed a molecular ion at *m*/*z* 526 ($C_{27}H_{30}N_2O_9$) together with an intense peak at *m*/*z* 508 that was consistent with the molecular formula $C_{27}H_{28}N_2O_8$ due to the loss of water from **3**. Analysis on the same sample that was stored in a fridge for a week gave a new molecular cluster with two prominent peaks centered at *m*/*z* 528 (M+2) and 510 (M−H_2O+2) because of the reduction of a benzoquinone moiety.

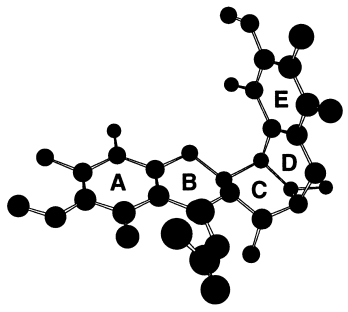


Figure 1. Computer generated structure of jorumycin (**3**).

COSY and HMQC experiments defined three isolated spin systems that can be interconnected by HMBC data (Table 1). The acetoxy group was linked at C-22 on the basis of the downshifted resonances of H-22 (δ 4.42 and 3.82), and the long-range ^{13}C–^{1}H correlation between the carboxylic group (170.0 ppm) and both H-22 signals.

The relative stereochemistry of **3** was assigned by NOESY experiments carried out at different mixing times (500, 700, and 1000 ms). Very diagnostic were the interactions between H-11 (δ 3.90) and H-4_{eq} (δ 2.84), as well as that between H-14 (δ 2.24, pseudo-equatorial) and H-21 (δ 4.40, axial). This required the structure to be folded in an L-shape with the aromatic ring E that approaches H-4_{ax} (δ 1.24), thus causing the large up-field shift of this last signal in the ^{1}H NMR spectrum (Fig. 1). A preliminary study based on restrained molecular dynamic calculations[10] suggested that the structure of **3** might be conformationally strained and very similar to the crystal structure of both Et 743 (**2**)[8] and the saframycins.[2] However, the relative stereochemistry at C-1 was doubtful. In fact, the intense homoallylic coupling between H-4_{ax} (δ 1.24) and H-1 (δ 4.36) required both protons to be orthogonal to the quinone plane. Assuming that, H-1 would have a pseudo-axial orientation. Two distinct conformers in which ring B adopts either a semi-chair (Fig. 2a) or twisted-boat (Fig. 2b) conformation allow such an arrangement. Attempts to measure NOEs that could confirm one of the two structures met with unsatisfactory results. The suggested relative stereochemistry at C-1 was

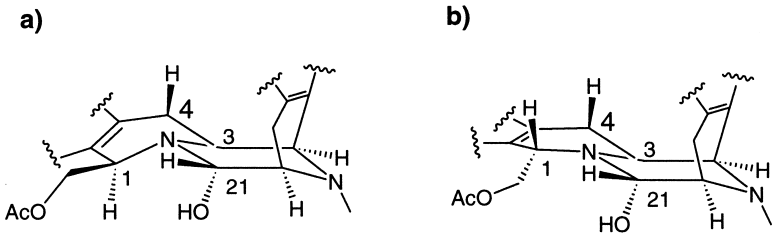
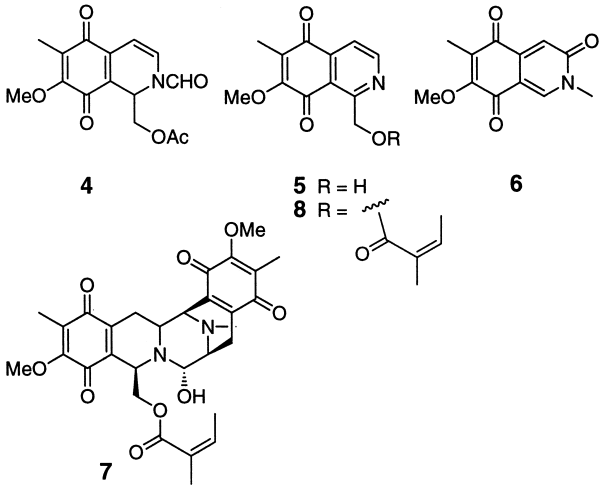


Figure 2. Possible conformations for the two alternative structures of jorumycin ring B: (a) semi-chair and (b) twisted-boat.

based on comparison of the NMR data with those reported in the literature for similar compounds.[2,3,6]

In a primary assay, jorumycin (**3**) showed very interesting activity against NIH 3T3 tumor cells (100% of inhibition at 50 ng/mL). A further investigation[11] demonstrated that **3** is cytotoxic (IC_{50} 12.5 ng/mL) against different tumor cell lines at very low concentrations (Table 2). However, due to the difficulty in obtaining pure stable preparation, the activity of **3** is suggested to be even more potent.

In a previous study of *J. funebris*[12], a series of isoquinoline-quinone metabolites, such as *N*-formyl-1,2-dihydrorenierol acetate (**4**), have been reported from the mollusc and its associated prey, the sponge *Xestospongia* sp. From another sponge, *Xestospongia calycedoi*, McKee and Ireland have described[13] two related alkaloids, renierol (**5**) and mimosamycin (**6**).[14] We have isolated compounds **5** and **6** from the sponge which *J. funebris* was grazing upon. No trace of jorumycin was found in the sponge. A second collection of both nudibranch and sponge yielded the same results, although a small amount of renierol (**5**) was isolated from the mollusc extract. This may confirm the prey–predator relationship between *J. funebris* and the blue sponge, but it does not clarify the origin of **3**. Further studies are needed on this point. Compounds **5** and **6** showed mild antimicrobial activity against Gram positive bacteria. On the other hand, jorumycin (**3**) inhibited the growth of various Gram positive bacteria (e.g. *Bacillus subtilis*, *Staphylococcus aureus*) at a concentration lower than 50 ng/mL (typically, 50 ng/mL gave an inhibition zone of 16 mm in 24 h). No inhibition was observed in *Escherichia coli* at the same concentration. The structure of jorumycin (**3**) resembles that of the unstable renieramycin E (**7**) isolated from the marine sponge *Reniera* sp.[5] The molecules differ only by the substituent at C-22. The authors report **7** underwent oxidative cleavage to give the "monomeric" isoquinolines **6** and **8**.[5] During our analysis we did not observe such a process, but it could explain the absence of **3** in the sponge.



4 **5** R = H **6**
8 R =
7

Table 2. Cytotoxic activity of jorumycin (**3**) (P388=mouse lymphoma; A549=human lung carcinoma; HT29=human colon carcinoma; MEL28=human melanoma)

Cell line	P388	A549	HT29	MEL28
IC_{50} (ng/mL)	12.5	12.5	12.5	12.5

Conclusions

Jorumycin (**3**) is a new dimeric isoquinoline related to the ecteinascidins and the saframycins, two well-known classes of promising antitumor and antimicrobial alkaloids. In particular jorumycin (**3**) shows a molecular structure very similar to that of renieramycin E (**7**), that has been isolated from the marine sponge *Reniera* sp. However, a full structure elucidation of **7**, as well as a discussion of its biological properties, have never been reported.[5] Carbinolamine-containing antibiotics, like Et 743 (**2**), are proposed to react with DNA to give covalent adducts with high sequence specificity.[15] The chemical reactivity of these agents has been supposed to reside in the iminium intermediate generated by dehydration of the carbinolamine moiety.[15,16] Although the mechanism of cytotoxic action of **3** has not been studied yet, it appears that the nudibranch alkaloid behaves similar to Et 743 (**2**) and the saframycins. On the other hand, the structural features of jorumycin (**3**), described in this paper, seems to confirm that even the lack of one of the isoquinoline groups in ET 743 results in only minor conformational modifications of the molecular scaffold. Finally, this is the first report of such a compound from marine molluscs. The presence of jorumycin (**3**) in the mucus and mantle of the nudibranchs leads us to suppose an ecological role for the alkaloid. The origin of the metabolite in the nudibranch is an intriguing question that, for the moment, can not be addressed. A more detailed conformational analysis of **3** is now underway.

Experimental

General methods

1D and 2D NMR spectra were recorded on Bruker AMX 500 and Bruker AMX 300. The $CHCl_3$ resonances at δ 7.26 and 77.0 were used as an internal reference. MS spectra

were obtained by 'Servizio di Spettrometria di Massa' of Naples. Infrared spectra were recorded by Bio-Rad FTS-7 FT/IR spectrophotometer. Optical rotations were determined by JASCO DIP-370 polarimeter. HPLC was performed by Waters liquid chromatography apparatus equipped with two 510 pump units.

Biological material

The sponge (*Oceanapia* sp.) and the mollusc *Jorunna funebris* (four specimens) were collected off Mandapam (India) in April 1998 and March 1999. A voucher specimen of the sponge (EM23SJ98) and nudibranch (EM23MJ98) are kept at NIO. Although the animals were immediately frozen, both sponge and nudibranch turned to darker colors. Sponge and nudibranchs of both collections were extracted in India and the frozen extracts transferred to Italy for further investigation.

Extraction of the biological material

The frozen nudibranchs were soaked in acetone for three times. These first fractions were filtered and the filtrates combined to give 46.2 mg of 'mantle' residue. Then, the animals were exhaustively extracted with acetone in a mortar to give 37.0 mg of 'inner organs' residue. Both extracts were separately partitioned between EtOAc and distilled H_2O to give after evaporation of the organic layers, 28.4 mg of '*mantle*' and 10.1 mg of '*body*' extracts. In the same way, partition of the mucus between EtOAc and H_2O gave 8.6 mg of lipophilic extract. Extraction of the nudibranchs collected in March 1999 (two specimens) gave 35.6 mg of EtOAc extract. The sponge from the earlier collection (28.2 g dry wt) was extracted with acetone. Removing the organic solvent gave an aqueous residue that was partitioned against EtOAc to give 585 mg of lipophilic extract. The sponge collected in March 1999 (21.1 g dry wt) yielded 339 mg of EtOAc extract.

Purification of 3

Several preparations of **3** were carried out. In a general procedure the oily extract of *J. funebris* was fractionated by Sephadex LH-20 chromatography ($CHCl_3$/MeOH 1:1). The elution of the components was monitored by SiO_2-TLC ($CHCl_3$/MeOH 95:5, R_f=0.4). Fractions containing the product of interest were recombined and chromatographed on LH-20 columns again. The final purification of **3** was achieved by SiO_2-TLC ($CHCl_3$/MeOH 95:5) and HPLC (Spherisorb S5W analytical column; isocratic elution with *n*-hexane/$CHCl_3$/TEA 90:10:10; detection with differential refractometer) to afford, in total, 4.6 mg of jorumycin (**3**).

Purification of 5 and 6

Renierol (**5**) and mimosamycin (**6**) were obtained from the sponge extracts by purification on LH-20 ($CHCl_3$/MeOH 1:1) followed by SiO_2 column. Spectroscopic data of **5** (6.1 mg) and **6** (5.0 mg) were identical to those reported in the literature.

Jorumycin (3)

Pale yellow powder ($C_{27}H_{30}N_2O_9$); $[\alpha]_D=-57$ (*c* 0.05, $CHCl_3$); IR (liquid film) 3310, 1731, 1660 cm^{-1}; UV (MeOH) 266 nm (ϵ=15 000); ^1H and ^{13}C NMR ($CDCl_3$) are reported in Table 1; ES^+ MS (*m*/*z*) 526 (20, M^+), 508 (100, $M-H_2O$), 494 (10, M−32).

Cytotoxicity assay

NIH 3T3 cells were grown in Dulbecco's modified Eagle medium (DMED) supplemented with 10% of heat inactivated fetal calf serum, 4 mM glutamine, 100 U/mL penicillin and 10 ng/mL streptomycin. All chemicals were dissolved in water at a concentration of 10 mM. Cells were plated at a dilution of 1×106 mm^2 culture dish and exposed to scalar concentrations of **3** in sterilized water for 48 h.

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