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3,7-Isoquinoline quinones from the ascidian tunicate *Ascidia virginea*

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Abstract: A new isoquinoline quinone system and its iodinated derivatives were isolated from the ascidian tunicate *Ascidia virginea* Müller 1776 (Phlebobranchia: Ascidiidae). Structures were elucidated by spectroscopic methods and derivatization reactions. Ascidine A (3,7-dihydro-1,8-dihydroxy-4-(4′-hydroxyphenyl)isoquinoline-3,7-dione (**1**), ascidine B (3,7-dihydro-1,8-dihydroxy-4-(4′-hydroxy-3′-iodophenyl)isoquinoline-3,7-dione (**2**), and ascidine C (3,7-dihydro-1,8-dihydroxy-4-(4′-hydroxy-3′,5′-diiodophenyl)isoquinoline-3,7-dione (**3**) represent a novel type of tyrosine-derived alkaloids.

Keywords: *Ascidia virginea*; iodinated dihydroisoquinoline-3,7-dione; isoquinoline quinone; structure elucidation; tunicate.

Dedication: In memoriam Lothar Jaenicke

1 Introduction

Ascidians have been shown to produce a wide variety of biologically active natural products [1]. The majority of the ascidian species studied so far are of tropical, subtropical, or Mediterranean origin, whereas only few species from the Northeastern Atlantic have been investigated [2, 3]. To the best of our knowledge, (*R*)-2,6-dimethylheptyl sulfate, which is relatively wide spread among Ascidians, is the only secondary metabolite known from *A. virginea* (http://www.marinespecies.org/aphia.php?p=taxdetails&id=103717). Here we report on the isolation and structure elucidation of three unprecedented isoquinoline quinones isolated from the red colored *Ascidia virginea*, an ascidian frequently found in the Norwegian fjords [4].

2 Results and discussion

Frozen specimens of *A. virginea* were homogenized and subsequently extracted with acetone. The resulting extracts were combined and concentrated to give an inhomogeneous aqueous residue, which was extracted with dichloromethane. The ^{1}H NMR spectrum of the crude, deep red-colored extract revealed the presence of relatively large amounts of unsaturated and/or aromatic compounds. Fractionation over Sephadex LH-20, followed by HPLC separation, revealed the red-colored fraction to contain two major components, **1** and **2**, and a minor component **3**, which we named ascidine A, B, and C, respectively. The UV–vis spectra of these compounds showed maxima at 485 and 322 nm.

Mass spectra were acquired using atmospheric pressure and chemical ionization in negative ion mode (NI-APCI-MS). The mass spectrum of the crude extract (Figure 1) was dominated by three intense signals at *m/z* 283, *m/z* 409, and *m/z* 535, which were assigned to ascidines A, B, and C (**1**, **2**, and **3**). HPLC/HR-MS revealed their elemental composition to be $C_{15}H_9NO_5$ (*m/z* 283, **1**), $C_{15}H_8INO_5$ (*m/z* 409, **2**), and $C_{15}H_7I_2NO_5$ (*m/z* 535, **3**). These data suggested **1–3** to form molecular ions $[M]^-$ by electron capture (EC) rather than pseudo-molecular ions $[M-H]^-$ via deprotonation. EC is well known to occur with halogenated compounds but is also characteristic for carbonyl compounds such as quinones [5]. The easiness of **1–3** to undergo EC was helpful for structure assignments of these compounds. Since the nonhalogenated component **1** was no less susceptible to EC than the iodinated compounds

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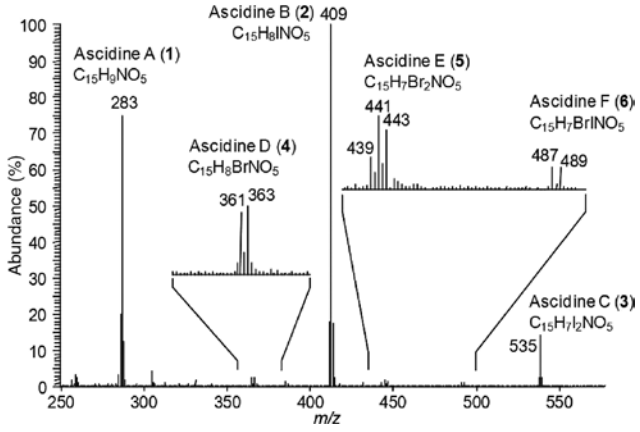

Figure 1: NI-APCI-mass spectrum of the crude extract of *A. virginea*.

2 and **3**, the presence of a quinoid substructure seemed likely, which was supported by the relatively high number of oxygens in all three compounds as well as by their remarkably intense red color.

In addition to ascidines A–C (**1–3**), some trace components (**4–6**) were detected by NI-APCI-MS. The isotopic patterns and masses of molecular ions at *m/z* 361/363, *m/z* 439/441/443, and *m/z* 487/489 suggested the formulas $C_{15}H_8BrNO_5$ (**4**), $C_{15}H_7Br_2NO_5$ (**5**), and $C_{15}H_7BrINO_5$ (**6**), presumably corresponding to brominated derivatives of **1–3**. However, owing to their low abundance, the elemental compositions of **4–6** could not be determined by HR-MS.

Compounds **1** and **2** could be isolated by preparative HPLC in quantities sufficient for NMR-spectroscopic analysis. The low number of hydrogen atoms as determined by high-resolution MS corresponded to very simple ^{1}H NMR-spectra (see Tables 1 and 2). Ascidine A (**1**) showed three singlets (8.69, 8.93, and 9.90 ppm), each representing one proton, one pair of doublets, and an aromatic AA′BB′ system. Using HMQC and HMBC experiments, the doublets at 6.72 and 7.34 ppm were shown to represent a *cis*-substituted double bond, which was likely conjugated with a carbonyl group. The AA′BB′ system at 6.89 and 7.20 ppm indicated the presence of a *para*-substituted phenol. Subtracting this phenol unit (C_6H_4OH) from the overall molecular formula $C_{15}H_9NO_5$, left a $C_9H_4NO_4$ core to be characterized.

The ^{13}C NMR spectrum of ascidine A (**1**) displayed 13 signals for the 15 carbon atoms. Four signals (114.91, 122.63, 133.11, and 158.40 ppm) were assigned to the six carbon atoms of the *p*-hydroxyphenyl substituent. Of the remaining nine signals, two sp^2-methines (129.64, 138.93 ppm) represented the aforementioned *cis*-double-bond while the remaining seven signals belonged to sp^2-quarternary carbons (92.46, 126.58, 136.94, 148.58, 161.28, 162.33, and 179.80 ppm). HMBC cross peaks of the protons at 7.20 ppm of the *p*-hydroxyphenyl unit to carbon atoms at 161.28, 126.58, and 136.94 ppm allowed the connection of this substituent to the $C_9H_4NO_4$ core (Figure 2A).

The proximity of the *cis*-double bond to the attachment site of the hydroxyphenyl substituent was evident from HMBC cross peaks of the protons at 6.72 and 7.34 ppm, which are part of the $C_9H_4NO_4$ unit. In addition, NOESY cross peaks between the proton at 7.34 ppm

Table 1: NMR data of ascidine A (**1**).[a]

Position	^{13}C δ [ppm]	^{1}H δ [ppm]	COSY90	HMBC	NOESY
1	162.33				
3	161.28				
4	126.58				
4a	136.94				
5	138.93	7.34, 1H, d, J = 10.0 Hz	H-6	C-1, C-4, C-4a, C-7, C-8, C-8a, C-6[a]	H-6
6	129.64	6.72, 1H, d, J = 10.0 Hz	H-5	C-3, C-4a, C-7, C-8, C-8a	H-5
7	179.80				
8	148.58				
8a	92.46				
1′	122.63				
2′/6′	133.11	7.20, 2H, d, J = 8.5 Hz	H-3′/5′	C-3[a], C-4, C-4a[a], C-2′/6′, C-4′, C-3′/5′	H-3′/5′
3′/5′	114.91	6.89, 2H, d, J = 8.5 Hz	H-2′/6′	C-4[a], C-1′, C-2′/6′, C-3′/5′, C-4′	H-2′/6′, 4′-OH
4′	158.40				
1-OH	–	8.69, 1H, s(b)		C-7, C-8a	8-OH[b]
8-OH	–	8.93, 1H, s(b)		C-7	1-OH[b]
4′-OH	–	9.90, 1H, s	–	C-2′/6′, C-3′/5′, C-4′	H-3′/5′

[a]Recorded in DMSO-d_6, ^{1}H: 500 MHz, ^{13}C: 100.6 MHz.

Table 2: NMR data of ascidine B (**2**).[a]

Position	^{13}C δ [ppm]	^{1}H δ [ppm]	COSY90	HMBC	NOESY
1	162.6[a]				
3	?				
4	125.0				
4a	137.4				
5	138.7	7.32, 1H, d, J = 10.0 Hz	H-6	C-1, C-4, C-4a, C-7, C-8a	H-6, H-2′
6	130.0	6.72, 1H, d, J = 10.0 Hz	H-5, OH-8	C-4a, C-8	
7	180.0				
8	148.9				
8a	92.6				
1′	125.0				
2′	141.6	7.69, 1H, d, J = 2.1 Hz	H-6′	C-1′, C-3′, C-4′, C-6′	
3′	84.2				
4′	157.7				
5′	114.2	6.99, 1H, d, J = 8.3 Hz	H-6′	C-1′, C-3′, C-4′	
6′	133.0	7.20, 1H, dd, J = 2.1 Hz, 8.3 Hz	H-2′, H-5′	C-1′, C-2′, C-4′	
1-OH	–	8.74, 1H, s(b)			8-OH[b]
8-OH	–	8.97, 1H, s(b)		C-7	1-OH[b]
4′-OH	–	10.82, 1H, s(b)			

[a]Recorded in DMSO-d_6, ^{1}H: 500 MHz, ^{13}C: 100.6 MHz.

of the *cis*-double bond and the protons at 7.20 ppm of the *p*-hydroxyphenyl substituent supported the partial structure shown in Figure 2B. The NOESY and HMBC spectra of **1** are shown as supporting material.

The remaining two protons, which appeared as broad singlets at 8.69 and 8.93 ppm in the ^{1}H NMR spectrum, were assigned to two phenolic hydroxy groups. A positive NOESY cross peak between these two hydroxy groups indicated spatial proximity (Figure 2C). The remarkably low chemical shift of 92.46 ppm – unusual for an sp^2-hybridized carbon atom – suggested this carbon to be spatially close to the two phenolic hydroxy groups. C–H-long-range correlations of the *cis*-double bond protons (6.72 and 7.34 ppm) and of the OH groups (8.69 and 8.93 ppm) to the keto group (179.80 ppm) as well as to the quarternary carbon at 92.46 ppm allowed to complete the structure. The placement of C-1 was based on HMBC cross peaks from H-5 (Figure 2C). The position of the nitrogen as part of an imide is in agreement with the ^{13}C chemical shifts of the adjacent carbons (C-1 and C-3) and was supported by intense IR-absorption bands at 1617, 1598, and 1587 cm^{-1} [6]. Thus, the $C_9H_4NO_4$ core was assigned to be a 3,7-dihydro-1,8-dihydroxyisoquinolin-3,7-dione and, consequently, compound **1** (ascidine A) was identified as 3,7-dihydro-1,8-dihydroxy-4-(4′-hydroxyphenyl)isoquinolin-3,7-dione.

The ^{1}H NMR spectrum of the iodinated **2** differed from that of **1** by showing a three-proton spin system corresponding to a 1,3,4-trisubstituted phenyl group, which replaced the AA′BB′ system found in **1**. ^{1}H,^{1}H-COSY, and HMBC data revealed the iodine substituent to be in *ortho*-position to the hydroxy group of the hydroxyphenyl substituent. The ^{1}H and ^{13}C signals of the remaining $C_9H_4NO_4$ core were almost identical to those of **1**, and consequently, ascidine B was identified to be 3,7-dihydro-1,8-dihydroxy-4-(4′-hydroxy-3′-iodophenyl)isoquinolin-3,7-dione. The 1D and 2D NMR data of the compound are given in Table 2.

Having the structures of ascidine A and B assigned, the structures of the minor compound **3** and the trace components **4–6**, which could not be isolated or fully characterized, were proposed. The doubly iodinated **3** (ascidine C) was likely to have both iodine substituents placed in *ortho*-position to the hydroxy group of the hydroxyphenyl substituent – a structural motif well known from other iodinated aromatic natural products [7]. To scrutinize this assumption, ascidine A (**1**) was treated with the mild iodination reagent bis(pyridine)iodonium tetrafluoroborate [8, 9], which is known to transform phenols into their *ortho*-mono- and diiodo derivatives. As expected, the crude reaction mixture contained both ascidine B (**2**) and a doubly iodinated compound that was identical with **3** with respect to HPLC retention time and mass spectrometric properties. Thus, the structure of **3** was assigned to be the 3′,5′-diiodo derivative of **1**. Furthermore, based on mass spectroscopic evidence, we propose the trace components **4–6** (ascidines D, E, and F) to be halogenated derivatives of **1**, namely **4** is 3′-bromo-**1** and **5** is 3′,5′-dibromo-**1**, whereas **6** is 5′-bromo-3′-iodo-**1**.

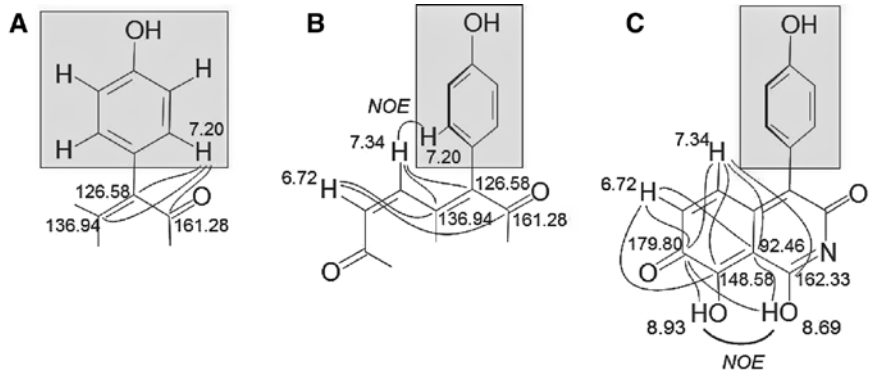

Figure 2: Selected HMBC and NOE correlations in ascidine A (**1**).

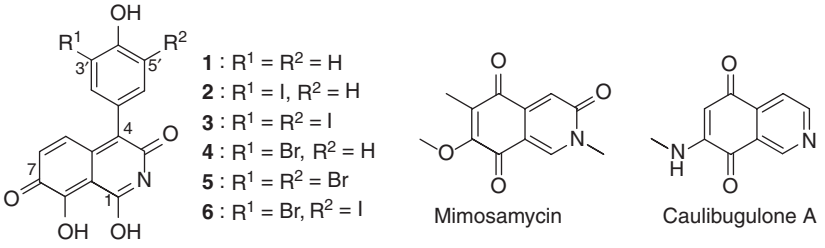

Figure 3: Stuctures of ascidines, mimosamycin, and caulibugulone A.

Oxygenated isoquinoline antibiotics are widespread natural products. Several 5,8-dihydroisoquinoline-5,8-diones such as the yellow mimosamycin, isolated from a *Streptomyces* strain [10, 11], or the cytotoxic caulibugulone A and its 6-halogenated derivatives, constituents of the marine bryozoan *Caulibugula* intermis [12] (for structures, see Figure 3), as well as structurally related 5,8-isoquinoline quinones have been described from marine sponges [13, 14] or nudibranchs [15]. However, in contrast to all these compounds, the newly discovered ascidines represent an unprecedented suite of 3,7-isoquinolone quinones. Nevertheless, mimosamycin shows an oxygenation pattern similar to that of the new natural products. The discovery of these secondary metabolites, featuring an intensively red chromophore, raises the question of their physiological or ecological function in the tunicate. To date nothing is known about predators of *A. virginea* or its need to control bacterial or fungal overgrowth. However, it is remarkable that in contrast to several closely related species found in the same habitat, such as *Ascidia mentula*, sampled specimens of *A. virginea* never appeared to be damaged by feeders and did not show any signs of infestation by microorganisms. Given their unusual structures, it is likely that the novel ascidines play a significant role in the chemical ecology of this ascidian. Investigations on the biological properties of the ascidines are in progress.

3 Experimental section

3.1 General experimental procedures

IR spectra were recorded with a Thermo Nicolet® Avatar 370 FT IR spectrometer. NMR spectra were recorded with Bruker DRX 500 (500 MHz) and AMX 400 (400 MHz) as well as Varian Inova 600 MHz spectrometers. Low- and high-resolution mass spectrometry was performed on a ThermoQuest® Finnigan MAT 95XL mass spectrometer, equipped with an APCI source. Analytical and preparative HPLC separations were carried out with Merck-Hitachi® HPLC systems, using an L-4500 diode array detector for the acquisition of UV spectra.

3.2 Animal material

Specimens of *Ascidia virginea* were collected in the Korsfjord area of Bergen, Norway. Sampling was performed

with a triangular dredge from the research vessel "Hans Brattström" at 60 m depth. The samples were frozen and stored at –30 °C until extraction.

3.3 Extraction and purification

Wet frozen material (170 g) was homogenized and extracted three times with 100-mL acetone (Merck, Supra-Solv® grade). The total acetone extract was concentrated, and the aqueous residue first extracted with pentane to remove lipophilic material and subsequently with dichloromethane. The dichloromethane layer was dried over $MgSO_4$ and concentrated. Chromatography of the crude natural products was performed over a column packed with Sephadex LH-20 using acetone–dichloromethane 1:1, which was monitored visually via the red color of components. The red-colored fractions containing the ascidines were submitted to HPLC, using a Kromasil® RP-18 column (21.2×250 mm, 5 µm particle size) and acetonitrile–water 1:1. Ascidine A (**1**) eluted before its 3′-iodo derivative **2**.

Ascidine A (=3,7-dihydro-1,8-dihydroxy-4-(4′-hydroxyphenyl)isoquinoline-3,7-dione, **1**). Dark red amorphous powder (acetone). UV–vis (acetonitrile-water 1:1): λ_{max} = 322 nm, 485 nm. IR (KBr): 3530, 3365, 3276, 1734, 1707, 1617, 1598, 1587, 1375, 1310, 1285, 1238, 1188, 1162, 1117, 1094, 1027, 982, 843, 638 cm^{-1}. NMR: see Table 1. HR-APCI-MS: *m/z* 283.0474 (calcd for $C_{15}H_9NO_5$, 283.0481).

Further details concerning NMR-spectra of Ascidine A are provided in the Supplement.

Ascidine B (=3,7-dihydro-1,8-dihydroxy-4-(4′-hydroxy-3′-iodophenyl)isoquinoline-3,7-dione, **2**). Red amorphous powder (acetone or dichloromethane). UV–vis (acetonitrile–water 1:1): λ_{max} = 322 nm, 485 nm. IR (KBr): ν 3422, 3318, 1735, 1686, 1585, 1384, 1311, 1288, 1233, 1140, 1094, 985, 841, 802 cm^{-1}. NMR: see Table 2. HR-APCI-MS: *m/z* 408.9441 (calculated for $C_{15}H_8INO_5$, 408.9447).

Further details concerning NMR-spectra of Ascidine B are provided in the Supplement.

Ascidine C (=3,7-dihydro-1,8-dihydroxy-4-(4′-hydroxy-3′,5′-diiodophenyl)isoquinoline-3,7-dione, **3**). HR-APCI-MS: *m/z* 534.8416 (calcd for $C_{15}H_7I_2NO_5$, 534.8414).

3.4 Conversion of ascidine A into ascidines B and C

A sample of **1** (~50 µg) was dissolved in dry dichloromethane (1–2 mL) and treated with a solution of bis(pyridine)iodonium tetrafluoroborate (~130 µg IPy_2BF_4 in 130-µL dichloromethane, two equivalents). After stirring for 20 min at room temperature, the crude reaction mixture was submitted to HPLC/DAD and APCI-MS analysis.

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