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Further syphonosides from the sea hare *Syphonota geographica* and the sea-grass *Halophila stipulacea*

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Abstract

The unusual structural features of syphonoside (**1**), recently reported from the marine mollusc *Syphonota geographica* and its prey *Halophila stipulacea*, stimulated further investigations on the minor secondary metabolites of both organisms. The three novel macrocyclic glycoterpenoids **2**–**4**, structurally related to the main co-occurring metabolite **1**, have been isolated and chemically characterized mainly by NMR spectroscopic techniques and degradation methods. Compounds **2** and **3** were found only in the mollusc whereas compound **4** was isolated in trace quantities exclusively from the sea-grass.

Keywords: Marine natural products; *Halophila stipulacea*; *Syphonota geographica*; Molluscs; Macrocycles; Glycoterpenoids

1. Introduction

In the course of our investigations on natural products from marine opisthobranchs,[1–12] we have recently analyzed the secondary metabolite content obtained from a Mediterranean population of the Lessepsian anaspidean mollusc *Syphonota geographica*, collected along the Greek coasts.[9,12] A number of degraded sterols, structurally related to those found in other anaspidean molluscs,[13–15] characterized the skin metabolite pattern of *S. geographica*[9] whereas the main secondary metabolite of the viscera was found to be the glycoterpenoid macrocycle syphonoside (**1**).[12] This compound was also found to be the main component of the butanol extract of the invasive sea-grass *Halophila stipulacea*, collected together with the mollusc.

The presence of syphonoside (**1**) in both organisms supported the trophic relationship between the mollusc and the sea-grass,[12] previously suggested by the detection of *H. stipulacea* fragments in the stomach content of *S. geographica*.[9] From the chemical point of view, syphonoside (**1**) is particularly interesting because it has an unusual framework in which a diterpene moiety is incorporated in a macrocycle along with a residue of 3-hydroxy-3-methyl glutaric acid and a glucopyranosyl moiety. The structure and the absolute stereochemistry of compound **1** were determined by a combination of spectroscopic techniques, chemical degradation methods, and conformational analysis.[12] In a preliminary cytotoxicity evaluation, **1** inhibited high density induced apoptosis in selected human and murine cancer cell lines,[12] suggesting a possible involvement in the regulation of the cell survival and death under specific conditions.

The intriguing structural features of syphonoside (**1**) prompted us to study the minor components of the glycoterpenoid fractions from the extracts of *S. geographica* and *H. stipulacea*. Herein we report the results of this investigation that led to the isolation and the chemical characterization of three novel glycoterpenoid macrocycles, compounds **2**–**4**,

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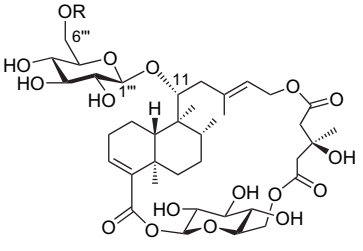
structurally related to the main metabolite **1**. Compounds **2** and **3** were isolated from the diethyl ether extract of the mollusc, whereas compound **4** co-occurred with the main metabolite **1** in the butanol extract of the sea-grass.

2. Results and discussion

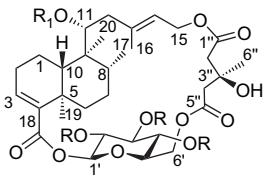
The diethyl ether soluble portion (589 mg) of the acetone extract, obtained as already described from the viscera of seven frozen *S. geographica* individuals,[9,12] was analyzed by TLC. Some polar components that were absent in the mantle of the mollusc were revealed by reaction with cerium sulfate. The extract was submitted to a chromatographic purification on a LH-20 Sephadex column, to afford fractions A and B. Preliminary ^{1}H NMR spectroscopic analysis of these fractions showed that both of them contained glycosyl compounds structurally related to syphonoside (**1**), previously isolated from the *n*-BuOH soluble portion of the same acetone extract.[12] Fractions A and B were further purified on preparative silica gel TLC plates ($CHCl_3$/MeOH eluent system) to obtain syphonosideol (**2**, 12.0 mg), the main component of the ether extract, and a mixture of syphonoside fatty acid ester derivatives **3** (5.4 mg).

A selected fraction obtained by LH-20 Sephadex chromatography of an aliquot (500 mg) of *n*-BuOH layer from the acetone extract of the sea-grass sample[12] was found to contain an additional syphonoside related metabolite as revealed by TLC and ^{1}H NMR analysis. This fraction was further purified by preparative silica-gel TLC chromatography ($CHCl_3$/MeOH eluent system) to yield the syphonoside acetyl derivative **4** (8.3 mg).

The new compounds **2**–**4** possessed the same macrocycle structure as the co-occurring syphonoside (**1**) and differed in the nature of the substituent at C-11 in the lateral chain of the diterpenoid unit. The structural elucidation of these molecules is described starting from syphonosideol (**2**), which was the main component of the diethyl ether extract from the viscera of the mollusc.



1 R = H
4 R = -Ac



2 R = R_1 = H
3 R = H, R_1 = $CH_3(CH_2)_{14}CO$- or $CH_3(CH_2)_{16}CO$-
5 R = R_1 = Ac

Syphonosideol (**2**) displayed a pseudo-molecular peak at *m/z* 647 in the HRESI-MS spectrum, indicating the molecular formula $C_{32}H_{48}O_{12}$, which was consistent with a structure containing one glucose unit less than **1**. Consistently, the ^{1}H NMR spectrum of **2** contained only one set of glycosyl signals resonating at δ 3.49–4.62 along with a doublet at δ 5.51 (J=8 Hz) due to the anomeric sugar proton (H-1′) involved in an ester linkage. Analogously with compound **1**, the proton NMR spectrum of syphonosideol (**2**) displayed five methyl signals at δ 1.78 (3H, br s, H_3-16), 1.39 (3H, s, H_3-6″), 1.26 (3H, s, H_3-19), 1.02 (3H, d, J=7 Hz, H_3-17), and 0.95 (3H, s, H_3-20); two olefinic multiplets at δ 5.36 (1H, m, H-14) and 6.54 (1H, t, J=3 Hz, H-3); a methylene at δ 4.56 (1H, dd, J=9 and 13 Hz, H-15a) and 4.70 (1H, m, H-15b) and a methine at δ 4.00 (1H, dd, J=2 and 8 Hz, H-11) both linked to an oxygen atom (Table 1). The ^{13}C NMR spectrum of **2** showed the presence of 25 sp^3 carbon signals (five CH_3, nine CH_2, eight CH and three C as deduced by the DEPT sequence), and 7 sp^2 carbon signals corresponding to two double bonds and three ester carbonyl groups (Table 1), as in syphonoside (**1**). A careful analysis of the 1D and 2D NMR spectra of compound **2** indicated the close similarity to **1**, due to the presence of the same macrocyclic carbon framework constituted by a clerodane acyl moiety oxidized at C-11, C-15, and C-18, a glucopyranosyl unit esterified at both H-1′ and H_2-6′ and a 3-hydroxy-3-methyl-glutaric acyl residue. Similarly to **1**, the α,β-unsaturated acyl function at C-18 of the clerodane moiety esterifies the hydroxyl group at the anomeric position H-1′ of the β-glucose unit, the primary hydroxyl group (6′-OH) of which is in turn linked to the carboxyl group (C-5″) of 3-hydroxy-3-methyl glutaric acid, further esterified at C-1″ with a primary hydroxyl group (15-OH) of the same clerodane, as reported in formula **2**. In fact, diagnostic HMBC correlations were observed between C-18 (δ 167.5) and H-1′ (δ 5.51), between C-5″ (δ 170.6) and H_2-6′ (δ 4.30 and 4.62) as well as between C-1″(δ 172.4) and H_2-15 (δ 4.56 and 4.70). Thus it was suggested that the only difference between compounds **1** and **2** was in the hydroxyl group at C-11, which was free in syphonosideol (**2**) and glycosylated by an additional glucose moiety in **1**.

In order to confirm the proposed structure and the relative stereochemistry of **2**, some degradation and derivatization reactions were performed. Initially, a sample of syphonosideol (**2**) was acetylated to give the tetra-acetyl derivative **5**, which was fully characterized (Table 1). Diagnostic acylation shifts were observed for the protons of the three carbinol groups (H-2′, H-3′, and H-4′) of the glucose unit as well as for H-11 of the clerodane portion, confirming the presence of the free hydroxyl at C-11 in compound **2**.

Analogously with syphonoside (**1**), the alkaline hydrolysis (Na, MeOH anhyd) of compound **2** was very informative