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New bioactive metabolites isolated from sponge *Hamigera hamigera*

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ABSTRACT

24 ethyl-5α-cholest-7-en-3α-ol (**1**), trans-P-hydroxycinnamaldehyde (**2**), para-hydroxybenzaldehyde (**3**), hamigeramine A (new alkaloid, **4**) and Hamiguanasinol A (new nucleoside, **5**) were isolated from the sponge *Hamigera hamigera* and their structures were established on the basis of UV data, NMR analysis and mass spectra. The extracts showed activity in fish feeding assay and were inactive in brine shrimp and antimicrobial assay.

Key words: *Hamigera hamigera*, sterols, nucleoside, alkaloids brine shrimp and fish feeding assay.

INTRODUCTION

Several classes of bioactive metabolites such as tripeptide, dimeric peptide, alkaloids, sulfer containing compounds and chlorine containing phenolic compounds have been isolated from family *Anchinoidae*[1-3], many of these metabolites exhibit very interesting biological activities such as antifungal and cytostatic[4-5]. The antiviral and anticancer arabinosyl nucleoside, spongothymidine, spongosine and sponguridine were isolated from marine sponges[6-8], they have provided the lead compounds for the development of the therapeutically used Ara-C for treatment of leukaemia, in addition to the virustatic agent Ara-A (Vidarabin), which was introduced to the market and used therapeutically against *Herpes encephalitis* since the late of 1970[9]. Because of the well known number of biologically active compounds isolated from genus hamigera family *Anchinoidae* as brominated and debrominated compounds which have been isolated from sponge *Hamigera tarangaensis*[10,11], Bisformamidodiphenylbutadiene from a sponge – associated fungus *Hamigera avellanea*[12] and new nuclosides, sterol, indole and phenolic compounds were also isolated from *Hamigera hamigera* sponge[13]. And as a part of our continuing studies on marine organisms we have recently described the isolation of two new compunds 4 and 5 in addition to three known compounds 1, 2 and 3 from sponge *Hamigera hamigera*. In this paper we report the structural elucidation of the isolated compounds

MATERIAL AND METHODS

Sponge Material

Specimens of sponge *Hamigera hamigera* Schmidt (order *Poecilosclerida*, family *Anchinoidae*) were collected at a depth of 15 to 21 ft. along the coasts of Elba Island (Mediterranean Sea), the sponge was identified by Prof. Van Soest (Amsterdam) and a voucher specimens have been deposited in the Zoological Museum Amsterdam under registration number ZMA POR 14397.

Fish Material

Mediterranean fish *Blennius sphinx* obtained from Elba Island (Mediterranean Sea) were used.

Apparatus

UV spectra were measured in methanol on a Perkin-Elmer UV/Vis lambda spectrophotometer, while ^{1}H (1D, 2D, COSY) and ^{13}C (1D, 2D, HMBC) NMR spectra were recorded on Bruker AM 300 and ARX 400 NMR spectrometers. Mass spectra recorded on Finningan MAT TSQ-7000 mass spectrometer, Solvents were distilled prior to use, and spectral grade solvents were used for spectroscopic measurements. TLC was performed on pre-coated TLC plates with silica gel F_{254} (Merck, Darmestadt, Germany). Sephadex LH-20 column was used for isolation of the compounds using methanol as eluent. RP-18 was also used as stationary phase. Semi-preparative HPLC was performed on HPLC system (Merck, Darmestadt, Germany) coupled with UV detector L7400 (UV detection at 280 nm), the separation column (8 x 250 mm) pre-packed with Eurosphere C18 (Knauer, Berlin, Germany). The compounds were eluted with solvent system of MeOH/H_2O containing 1% TFA for improved separation, at flow rate of 5 ml/min.

Extraction and Purification

The freeze-dried sponge (2 kg wet weight, 350 g dry weight) was extracted several times with methanol. The combined extracts were concentrated under reduced pressure to yield 9.5 g. the residue was suspended in aqueous methanol followed by extraction several times with cyclohexane, then ethyl acetate and finally with n-BuOH. The cyclohexane soluble material of *Hamigera hamigera* (129 mg) was chromatographed on a silica gel column. The column was eluted with Hexan: EtOAc (9: 1) to give compound **1**. The EtOAc soluble material (364 mg) was subjected to sephadex column (100% MeOH as eluant) followed by silica gel column eluted with (CH_2Cl_2: MeOH; NH_4OH, 70: 30: 1%) and then to semi-preparative HPLC to yield the compounds **2**, **3** and **4**. The aqueous extract (3.8 g) was subjected to reversed phase silica gel column eluted by MeOH: H_2O: TFA (50: 50: 1%), followed by semi-preparative HPLC to yield the compound **5**.

Compound 1 was obtained as white amorphous powder, 4.5 mg (0.0125 %), EIMS spectrum showed the molecular ion peak at m/z (rel. abu.) 414 $[M]^+$ (52%), and anther fragments at m/z 399 (8%), 371 (4%), 356 (4%), 273 (16%), 255 (25%), 234 (14%), 201(7%), 121 (25%), 107 (46%), 95 (53%), and 43 (100%). ^{1}H- NMR (400 MHz, $CDCl_3$) showed the following signals at α_H 0.54 (s, 3H-18), 0.79 (s, 3H-19), 0.94 (d, 6.3 Hz, 3H-21), 0.80 (d, 6.5Hz, 3H-26), 0.83 (d, 6.5Hz, 3H-27), 086 (t, 7.1 Hz, 3H-29), 3.6 (m, H-3) and 5.15 (t, 7.1 H-7). ^{13}C- NMR and DEPT (400 MHz, $CDCl_3$) showed the following signals at δ_C 37.3 (t, C-1), 31.6 (t, C-2), 71.2 (d, C-3), 38.1 (t, C-4), 40.3 (t, C-5), 29.7 (t, C-6), 117.3 (d, C-7), 139.7 (s, C-8), 49.5 (d, C-9), 34.1 (s, C-10), 21.6 (t, C-11), 39.7 (t, C-12), 43.4 (s, C-13), 55.1 (d, C-14), 23.0 (t, C-15), 28.8 (t, C-16), 56.2 (d, C-17), 11.9 (q, C-18), 19.1 (q, C-19), 36.2 (d, C-20), 19.1 (q, C- 21), 33.9 (t, C-22), 26.6 (t, C-23), 26.6 (t, C-24), 29.0 (t, C-25), 19.1 (q, C-26), 19.6 (d, C- 27), 23.1(q, C-28) and 11.9 (q, C-29).

Compound 2 was obtained as a white amorphous powder, 5.0 mg (0.01 38 %); it has UV absorbances at λ_{max} 325 nm. The EIMS showed the molecular ion peak at m/z 148 $[M]^+$; ^{1}H NMR (400 MHz, DMSO-d6) Table 1.

Compound 3 was obtained as a pale yellowish white amorphous powder, 3.5 mg (0.0097 %); it has UV absorbances at λ_{max} 221.6 and 283.8 nm. The EIMS showed the molecular ion peak at m/z 122 $[M]^+$ and anther fragment at m/z 93 $[M^+$- COH] ; ^{1}H and ^{13}C- NMR (400 MHz, MeOH and DMSO-d6) Table 2.

Compound 4 was obtained as a white amorphous powder, 3.0 mg (0.0083 %); it has UV absorbances at λ_{max} 230.6 and 352.2 nm. The positive ESIMS showed the molecular ion peak at m/z 204 $[M^+$+ H] and the negative ESIMS showed the molecular ion peak at m/z 202 $[M^+$- H], 159 $[M^+$- 43]; ^{1}H and ^{13}C-NMR (400 MHz, MeOD and DMSO-d6) Table (2).

Compound 5 was obtained as pale yellowish white amorphous powder, 2.5 mg (0.0069 %); it has UV absorbances at λ_{max} 254.6. ESIMS spectrum showed the molecular ion peak at m/z 330 $[M^+$**+**H] and at m/z 659 $[2M^+$+H]**,** m/z 179 (20%). ^{1}H NMR (400 MHz, DMSO-d6) Table 3.

Biological assay

Fish feeding assay[13]

The feeding experiments for crude

Table 1: NMR data of compound 2 and 3 (CD_3OD) and DMSO-d6 400MHZ

S. No	Compound 2 δH (DMSO)	δH (CD_3OD)	Compound 3 δC	COSY
1	-	-	128.4	-
2	6.92 (d, 8.5 Hz)	7.8 (d, 8.8 Hz)	7.7 (d, 7.7 Hz)	132.3	3
3	6.60 (d, 8.5 Hz)	6.8 (d, 8.5 Hz)	6.8 (d, 7.7 Hz)	115.8	2
4	9.25 (s)	10.6 (s)		163.3	-
5	6.60 (d, 8.5 Hz)	6.8 (d, 8.5 Hz)	6.8 (d, 7.7 Hz)	115.8	6
6	6.92 (d, 8.5 Hz)	7.8 (d, 8.8 Hz)	7.7 (d, 7.7 Hz)	132.3	5
7	6.20 (1 H, d, 14.8 Hz)	9.75 (s)	9.9 (s)	190.9	-
8	6.85 (1 H, dd, 14.5 and 10.8 Hz)	-	-	-	-
9	10.00 (d, 10.8 Hz)	-	-	-	-

Table 2: NMR data of compound 4 (CD_3OD) and DMSO-d6 400MHZ

S. No	δH (DMSO)	δH (CD_3OD)	δC*	HMBC (CD_3OD)
1	-	-	124.8	-
2	7.62 (7.3)	7.55 (d, 8.8 Hz).	132.0	4
3	6.80 (8.2)	6.85 (d, 8.5 Hz).	117.7	1, 7
4	10.1 (br. s, OH)	-	160.0	-
5	6.80 (8.2)	6.85 (d, 8.8 Hz).	117.5	1, 7
6	7.62 (7.3)	7.55 (d, 8.5 Hz).	132.0	4
7	6.50 (s)	6.85 (s)	116.0	2, 6, 8, 9
8	-	-	132.5	-
9	-	-	167.0	-
10	11.9 (br. s NH)	-	-	-
11			183.0	-
12		2.68 (s)	35.0	-

Carbon signals was obtained from HMBC correlation

Table 3: ^{1}HNMR data and COSY correlation of compound (5) (DMSO-d_6, 500 MHz)

No.	δH	*COSY*
1	-	-
2 (NH_2)	6.50 (s, 2H)	-
3	-	-
4	-	-
5	-	-
6	10.70 (br. s)	-
7	-	-
8	7.90 (s)	-
9	-	-
1´	5.72 (d, 5.4 Hz)	2´ (H)
2´ (H)	4.60 (t, 6.3 Hz)	3´(H)
2´ (O*H*)	5.53 (t, 7.8 Hz)	2´ (H)
3´(H)	4.10 (m)	2´, 4´
3´(O*H*)	5.37 (br. t)	3´(H), 4´
4´ (H)	4.20 (m)	3´, 5´
5´	3.25 (d, 6.9 Hz)	5´ B, 4´
6´	2.60 (s, 3H)	5´A, 4´

extracts, total, hexane, ethyl acetate, butanol and aqueous extracts were investigated Table 4.

Anti-microbial assay[13]

Agar plate diffusion technique was used, susceptibility test discs 5mm in diameter were impregnated with 20 µl containing 1 mg of total, hexane, ethyl acetate, butanol and aqueous extracts and placed on agar plates inoculated with Gram positive bacteria *Bacillus subtilis*, Gram negative bacteria *Echerichia coli* and two fungal strains: *Cladosporium herbarum* and Cladosporium *cucumerinum* were examined.

- Brine Shrimp Assay[13]

Brine shrimp assay is in vivo lethality test involving the whole body of a tiny crustacean, brine shrimp (*Artemia salina* Leach, Table 5.

Table 4: Results of fish-feeding assay of different extracts of *Hamigera hamigera*

Extract	% of eaten food	
	Experiment	Blank
Total extract	13.5	86.5
Hexane Ext.	38.6	61.4
EtOAc Ext.	31.4	68.6
H_2O Ext.	16.0	84.0
Butanol Ext.	46.2	53.8

Table 5: Results of Brine Shrimp Assay

Substance	Conc.	Died shrimps	Live shrimps
Hexane extract	1 mg/ml	8	12
	0.5 mg/ml	7	13
P-hydroxycin-	1 mg/ml	3	17
namaldehyde	0.5 mg/ml	2	18

RESULTS AND DISCUSSION

Compound 1 showed the molecular ion peak at m/z 414 in the EIMS which was compatible with the molecular composition of $C_{29}H_{48}O$. The fragmentation at m/z 371 (M^+- isopropyl), m/z 356 (M^+- isopropyl - Me) to loss of isopropyl and one methyl group, fragments at m/z 273 (M^+ -$C_{10}H_{21}$) and the fragment at m/z 255 (M^+-side chain - H_2O) confirmed the presence of mono-hydroxylated steroidal compound with saturated C_{10} side chain[14-16]. The fragment at m/z 43 confirmed the presence of terminal ispopropyl group[14-16]. The ^{1}H-NMR spectrum showed signals for six methyl groups, one of them is triplet at ä 0.86 with coupling constant 7.1 Hz corresponded to the presence of terminal ethyl group (C_2H_5). Further signals at δ_H 0.80 (3H-d, 6.5 Hz) and 0.83 (3H- d, 6.5 Hz) were assigned for CH_3-26 and CH_3-27 of the isopropyl group, the signal of methyl group at δ_H 0.94 (d, 6.3 Hz) is typical for C-21. The 1H-NMR signals at δ_H 3.6 (m, H-3) and 5.15 (br.s) are diagnostic feature for the presence of Δ^7-3β-monohydroxylated steroidal nucleus[14-15]. The ^{13}C-NMR resonances confirmed the above suggestion where it showed signals for six methyl groups, three douplet, two singlet and one triplet, in addition to the signals at δ 71.2, 117.3 and 139.7 for one hydroxylated and two olefinic carbons respectively. All the above data when compared with literatures it was found that compound **1** is 24 ethyl -5α-cholest-7-en-3β-ol[14-16]. Regarding to the stereochemistry of this compound the configuration of C-24 was clear as in literatures[14,16, 17]. This is the first isolation of this compound from *Hamigera hamigera*.

Compound 2 was obtained as white amorphous powder; it has UV absorbances at λ_{max} 228.6 and 293.8 nm. The EIMS spectrum showed the molecular ion peak at m/z 148 [M^+] which corresponds to the molecular formula $C_9H_8O_2$.The ^{1}H-NMR spectrum (Table 1) showed signals corresponding to p- hydroxyl benzene ring. The excess two proton signals at δ_H 6.20 (1 H, d, 14.8 Hz) and 6.85 (1 H, dd, 14.8 and 10.4 Hz) indicate the presence of trans-olefinic protons one of them coupled with aldehydic proton [2, 18]. From the above data and through the comparison with literatures[19,20] compound **2** was concluded to be p-hydroxy-cinnamaldehyde and this is the first isolation of this compound from *Hamigera hamigera* sponge.

Compound 3 was obtained as white amorphous powder; it has UV absorbances at λ_{max} 221.6 and 283.8 nm. The EIMS spectrum showed the molecular ion peak at m/z 122 [M^+] which corresponding to the molecular formula $C_7H_6O_2$, in addition the fragment at m/z 93 [M^+- CHO]. The ^{1}H-NMR spectrum (Table 2) showed AA2 BB2 spin system for 1, 4 disubstituted benzene ring [6.85 (2 H, d, 8.5 Hz). and 7.8 (2 H, d, 8.8 Hz)]. The ^{13}C-NMR signals at 128.4 (C-1), 132.0 (C-2, C- 6), 115.8 (C-3, C-5) and 163.3 (C-4) confirmed the AA2 BB2 spin system, the phenolic ring is deduced from the carbon signal at 163.3 in addition to the proton signal at δ_H 10.6 (br. s) for the hydroxyl group. The singlet proton signal at 9.60 which did not exchange in methanol indicated the aldehydic nature of the proton[19,20]. Carbon signal at 190.0 confirmed the above suggestion. From the above mentioned data compound **3** was P- Hydroxybenzaldehyde**.** This is the first isolation of this compound from *Hamigera hamigera*.

Compound 4 was obtained as white amorphous powder; it has UV absorbances at λ_{max} 230.6 and 352.2 nm. ESIMS spectrum showed the molecular ion peak at m/z 204 [M^+**+**H] and at m/z 202 [M^+**-**H]**,** m/z 159 [M^+**-**43] which revealed the loss of CO-NH moiety from the heterocyclic ring[21]. The ^{1}H-NMR spectrum (Table 2) showed AA2 BB2 spin system for 1, 4 disubstituted benzene ring [7.55 (2H, d, 8.8 Hz) and 6.85 (2 H, d, 8.8 Hz)]. The ^{13}C-NMR signals at 124.8 (C-1), 132 (C-2, C- 6), 117.5 (C-3, C-5) and 160 (C-4) confirmed the presence of AA2 BB2 spin system, the phenolic ring is deduced from the carbon signal at 160 in addition to the proton signal at 10.1 (br. s) for the hydroxyl group. The proton signal at 6.88 (s) which showed long range correlation to C-2, C-6 confirmed the presence of CH=C moiety connected with the phenolic ring, Long range correlation of H-7 with C-9 and C-12 in addition the correlation of H-12 with C-9 and presence of proton signals at 11.9 (br. s for NH) and at 2.6 (2H, s) for methylene group with further carbon signals at 167.0 and 183.0 for two carbonyl group confirmed the presence of the heterocyclic ring system [(22-24)]. All the above data confirmed the structure of the new compound **4** as hamigeramine A.

Compound 5 was obtained as pale yellowish white amorphous powder, it has UV absorbances at λ_{max} 254.6 which exactly identical to that of hamiguanasinol[13]. ESIMS spectrum showed the molecular ion peak at m/z 330 [M^+**+**H] and at m/z 659 [2M^++H] which corresponding to the molecular formula $C_{11}H_{15}N_5O_5S$, the fragment at m/z 179 (20%) for methyltiooxyribosyl moiety instead of 163 for methyl thioribsyl in hamiguanasinol[13] showed 16 mass unit difference revealed that compound **5** is the oxygenated analogue of Hamiguanasinol[13]. The presence of guanine nucleus was estimated from the mass fragment at m/z 151 (100%) while the subsequent fragment at m/z 97 indicated the presence of SCH3 moiety. The ^{1}H-NMR signals at δ5.72 (d, 5.4 Hz), 4.60 (t, 6.3 Hz), 5.53 (t, 7.8 Hz), 5.37 (br. t), 4.10 (m) and 4.20 (m) indicated the presence of a β–ribosyl moiety. The remaining ^{1}H-NMR resonances at δ_H 10.70 (1H, br. s), 7.90 (1H, s) and 6.50 (2H, br.s) were assigned to the 2-amino purine nucleus. For the guanine nucleus, the presence of a hydroxyl group at C-6 instead of a keto group was confirmed from the resonance at δ_H 10.57. The presence of CH_2OSCH_3 moiety was deduced from the two proton NMR signals at δ_H 3.25 (2H, d, 6.9 Hz) and 2.60 (3H, s). From the above mentioned results and through the comparison with literatures[12,13, 19,25,26] compound **5** was concluded to be the new compound, Hamiguanasinol A or chemically as [2-(2-amino-6-hydroxy-purin-9-yl)-5´-methylsulfoymethyl-tetrahydro-furan-2´, 3´-diol].

Biological assay [27]:

- Fish feeding assay[27]

The feeding experiments for crude extracts, total, hexane, ethyl acetate, butanol and aqueous extracts were investigated. As we see in (table 4). The total extract exhibited strong activity and the aqueous fraction is responsible for this activity.

- Anti-microbial assay[27]

All the tested extracts showed no activity in this assay.

- Brine Shrimp Assay[27]

In this test the hexane extract showed moderate activity where it led to death of 40 % of the shrimp in 1 mg/ml concentration.

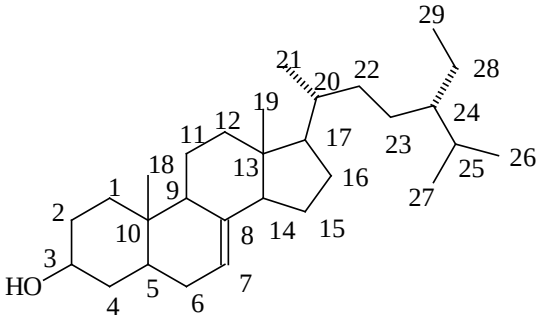
Cytotoxicity Assay

Preliminary bioactive screening was carried out to compounds **2** (P-Hydroxycinamaldehyde) and **3** (P-Hydroxybenzaldehyde) and we can not do it for compounds **1**, **4** and **5** due to the low yield.

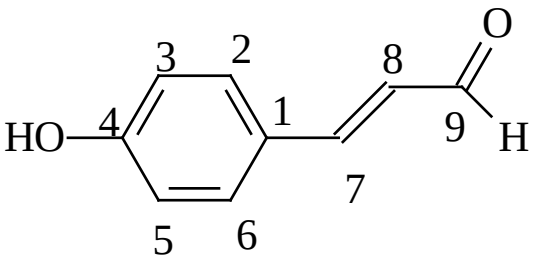
In this assay the two compounds **2** and **3** showed no activity as cytotoxic agent against Hela (Human cervix cancer) and L5178Y (mouse lymphoma) cells.

Conclusion

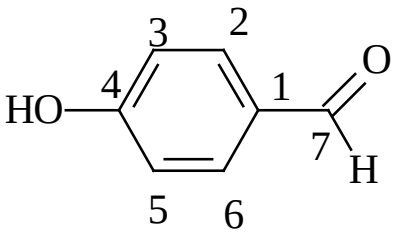
To best of our knowledge, many marine sterols have recently been isolated and most of them exhibit biological activities such as reversing multidrug resistance[28], cytotoxic[29], antiplasmodial[30] and immune-suppressive effects[31]. On the other hand nucleoside compounds are known for their antiviral, antiretroviral and anticancer activities and the first notable discovery of biologically active marine metabolites was actually unusual nucleosides. They were first isolated from a Caribbean sponge in the 1950s, and later served



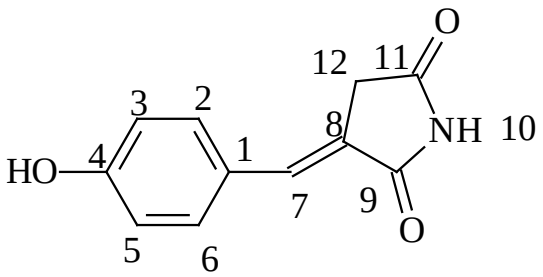
Compound 1



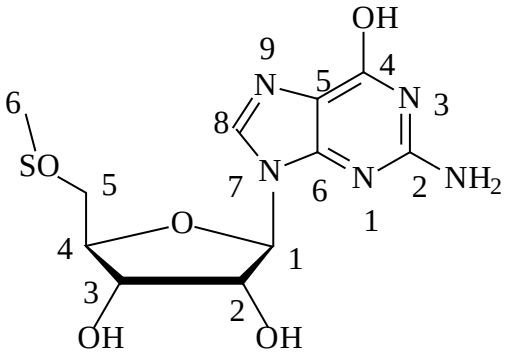
Compound 2



Compound 3



Compound 4 (new)



Compound 5 (new)

Compounds isolated from *Hamigera hamigera* sponge

as lead structures for the development of the important anti-viral drugs such as Ara-A known commercially as acyclovir[13, 32-34]. The phenolic compound (**3**) isolated in this work is reported to have antimalarial action[35].

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