Four New Steroids from Two Octocorals†

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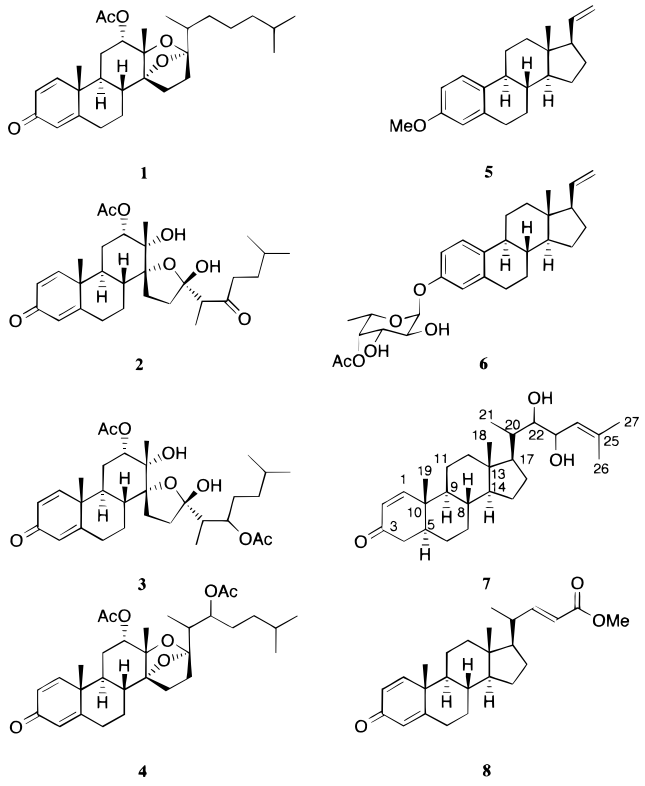
In search of analogues of isogosterones A−D (**1**−**4**), a group of antifouling 13,17-*seco*-steroids found in octocorals of the order Alcyonacea, we have isolated four new steroids possessing aromatic, enone, or dienone A-rings from two octocorals, *Alcyonium gracillimum* and *Dendronephthya* sp. These compounds, 3-methoxy-19-norpregna-1,3,5(10),20-tetraene (**5**), 3-(4-*O*-acetyl-6-deoxy-*β*-galactopyranosyloxy)-19-norpregna-1,3,5(10),20-tetraene (**6**), 22,23-dihydroxycholesta-1,24-dien-3-one (**7**), and methyl 3-oxochola-4,-22-dien-24-oate (**8**), showed no antifouling activity against barnacle (*Balanus amphitrite*) larvae, but lethality to barnacle larvae at a concentration of 100 μg/mL (LD_{100}).

Many marine organisms defend themselves from ubiquitous fouling organisms by possessing antifouling agents.[1] In the course of our program designed to discover new antifouling substances from marine benthic invertebrates,[2] we have recently reported isolation of four new 13,17-*seco*-steroids, isogosterones A−D (**1**−**4**),[3] from an octocoral *Dendronephthya* sp. (order Alcyonacea, family Nephtheidae) collected off the Izu Peninsula, Japan, as antifouling substances against barnacle larvae. Since these compounds possess a unique D-*seco*-steroid skeleton as well as considerable antifouling activity, we further examined extracts of several octocorals of the order Alcyonacea. This work resulted in the isolation of four new steroids from *Alcyonium gracillimum* and *Dendronephthya* sp. The structures of these compounds, as determined from spectral data, are 3-methoxy-19-norpregna-1,3,5(10),20-tetraene (**5**), 3-(4-*O*-acetyl-6-deoxy-*β*-galactopyranosyloxy)-19-norpregna-1,3,5(10),20-tetraene (**6**), 22,23-dihydroxycholesta-1,24-dien-3-one (**7**), and methyl 3-oxochola-4,22-dien-24-oate (**8**) (Chart 1). These compounds showed no antifouling activity against barnacle (*Balanus amphitrite*) larvae, but lethality to the barnacle larvae at a concentration of 100 μg/mL (LD_{100}). In this paper, we report the isolation and structural elucidation of these steroids.

Chart 1



1 5 2 6 3 7 4 8

Results and Discussion

The methanolic extract of the octocoral *A. gracillimum* (40 g; wet weight) was partitioned between Et_2O and water, and the Et_2O layer was chromatographed on silica gel successively with toluene, $CHCl_3$, 10% MeOH/$CHCl_3$, and MeOH. The $CHCl_3$ eluate was purified by reversed-phase HPLC to afford a 3-methoxysteroid (**5**; 10.2 mg) and a steroidal saponin (**6**; 1.1 mg), while the 10% MeOH/$CHCl_3$ eluate was similarly purified to yield a dihydroxyketosteroid (**7**; 0.8 mg). On the other hand, the methanolic extract of an octocoral *Dendronephthya* sp. (68 g; wet weight) was similarly processed to afford a steroid with an enone ring-A (**8**; 5.6 mg).

Compound **5** had a molecular formula of $C_{21}H_{28}O$ as established by HREIMS, indicating 8 degrees of unsaturation. The ^1H and ^{13}C (including DEPT) NMR spectra implied the presence of a singlet methyl (δ_H 0.62/δ_C 12.9), a methoxyl (δ_H 3.76/δ_C 55.2), and a terminal vinyl group (δ_H 4.97 (2H)/δ_C 114.6 and 5.78/139.8) (Table 1). Coupling constants of three aromatic protons at δ_H 6.62 (d, J = 2.5 Hz), 6.69 (dd, J = 2.5, 8.5 Hz), and 7.19 (d, J = 8.5 Hz), characteristic of a 1,2,4-trisubstituted benzene ring, together with two shielded aromatic protons and a methoxyl group, resulted in partial structure **a** (Figure 1). These structural features accounted for 5 of the 8 degrees of

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Table 1. ^{13}C NMR Data for Compounds **5**, **6**, **7**, and **8** in $CDCl_3$[a]

carbon	5	6	7	8
1	126.3 (d)	126.5 (d)	158.5 (d)	155.9 (d)
2	111.4 (d)	113.8 (d)	127.4 (d)	127.4 (d)
3	157.4 (s)	154.4 (s)	200.0 (s)	186.3 (s)
4	113.8 (d)	116.6 (d)	41.0 (t)	123.8 (d)
5	138.0 (s)	138.5 (s)	44.3 (d)	169.0 (s)
6	29.9 (t)	29.8 (t)	27.6 (t)	32.9 (t)
7	27.9 (t)	27.8 (t)	31.2 (t)	33.6 (t)
8	38.9 (d)	38.8 (d)	35.7 (t)	35.5 (d)
9	44.1 (d)	44.1 (d)	49.9 (d)	52.3 (d)
10	133.0 (s)	135.3 (s)	39.0 (s)	43.3 (s)
11	26.3 (t)	26.3 (t)	21.3 (t)	22.6 (t)
12	37.5 (t)	37.5 (t)	39.8 (t)	38.5 (t)
13	43.9 (s)	43.8 (s)	42.5 (s)	42.6 (s)
14	54.7 (d)	54.7 (d)	56.3 (d)	55.1 (d)
15	24.5 (t)	24.5 (t)	24.0 (t)	24.1 (t)
16	27.3 (t)	27.3 (t)	27.9 (t)	27.5 (t)
17	55.5 (d)	55.5 (d)	52.6 (d)	55.8 (d)
18	12.9 (q)	12.9 (q)	12.0 (q)	12.3 (q)
19			13.0 (q)	18.7 (q)
20	139.8 (d)	139.8 (d)	36.8 (d)	39.9 (d)
21	114.6 (t)	114.6 (t)	12.5 (q)	20.0 (q)
22			77.0 (d)	155.3 (d)
23			70.3 (d)	118.7 (d)
24			123.8 (d)	163.6 (s)
25			138.5 (s)	
26			18.6 (q)	
27			26.0 (q)	
CH_3O	55.2 (q)			51.4 (q)
1′		97.5 (d)		
2′		69.5 (d)		
3′		70.0 (d)		
4′		72.8 (d)		
5′		66.0 (d)		
6′		16.2 (q)		
CH_3COO		20.8 (q)		
CH_3COO		171.2 (s)		

[a] Multiplicities (in parentheses) were determined by DEPT and HMQC experiments.

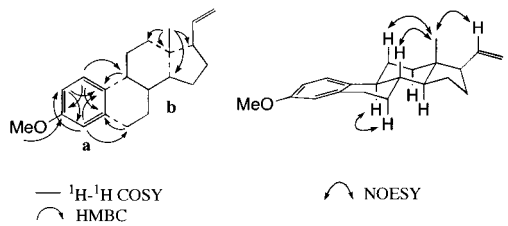

Figure 1. Key COSY, HMBC, and NOESY correlations in **5**; **a** and **b** are partial structures.

unsaturation, thus suggesting the tetracyclic nature of **5**. Interpretation of $^1H-^1H$ COSY correlations readily led to partial structure **b** (Figure 1). The planar structure of **5** was assigned as a norpregnane-type steroid from HMBC correlations between H-6/C-5, H-9/C-10, and Me-18/C-12, C-13, C-14, C-17 (Figure 1). The stereochemistry of **5** was inferred from NOESY correlations between Me-18/H-8, H-11β, H-20, and H-7α/H-9 as well as by typical ^1H and ^{13}C chemical shift values of 18-Me for C/D *trans*-fused steroids.[4] Thus, **5** is a 3-methoxy-19-norpregna-1,3,5(10),-20-tetraene.

Compound **6** had a molecular formula of $C_{28}H_{38}O_6$ as established by HREIMS. The ^{13}C NMR spectrum was consistent with the presence of a sugar at C-3 instead of a methoxyl group as in **5** (Table 1). The sugar moiety was readily assigned to be a 4-*O*-acetyl-6-deoxy-β-galactopyranose (4-*O*-acetyl-β-fucose) by interpretation of $^1H-^1H$ COSY data together with an HMBC cross-peak H-4′/CH_3COO, coupling constants of H-2′ (J = 4, 10 Hz), and NOESY correlations H-3′/H-4′, H-5′ and H-4′/H-5′. The

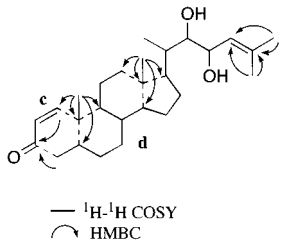

Figure 2. Key COSY and HMBC correlations in **7**; **c** and **d** are partial structures.

HMBC correlation between H-1′ (δ_H 5.53) and C-3 (δ_C 154.4) secured the final structure of **6** as a 3-(4-*O*-acetyl-6-deoxy-β-galactopyranosyloxy)-19-norpregna-1,3,5(10),20-tetraene.

Norpregnane-type steroids possessing an aromatic A-ring have been reported from a soft coral of the genus *Capnella*[4] and the marine sponge *Cribrochalina olemda*.[5] Although a 3-hydroxyl analogue of **5** was isolated from a Tasmanian soft coral of the genus *Capnella*[4] and **5** was chemically derived from a 3-methoxy-19-norpregna-1,3,5(10)-trien-20-one,[6] this is the first isolation of 3-methoxy-19-norpregna-1,3,5(10),20-tetraene (**5**) as a natural product. Among pregnane-3-glycosides, 6-deoxyhexose[5,7] and 2,6-dideoxyhexose derivatives[7,8] are common, thus 4-*O*-acetyl-6-deoxy-β-galactopyranose in **6** adds another example to this group.

Compound **7** had a molecular formula of $C_{27}H_{42}O_3$ as established by HREIMS. The ^1H NMR spectrum revealed the presence of two olefinic methyls (δ_H 1.73 and 1.75), two hydroxyl groups (δ_H 3.45 and 4.25), and a trisubstituted olefin (δ_H 5.03). The presence of an α,β-unsaturated carbonyl group (partial structure **c** in Figure 2) was straightforward from NMR signals at δ_H 5.83 (1H, d, J = 10 Hz)/δ_C 127.4 (d), 7.11 (1H, d, J = 10 Hz)/158.5 (d), and 200.0 (s) (Table 1), as well as from an IR absorption at 1675 cm^{-1}. The 1D NMR data could account for 3 of the 7 degrees of unsaturation, suggesting the tetracyclic nature of **7**. Twenty-seven carbons including five methyls suggested that **7** was an analogue of cholesterol. Interpretation of the $^1H-^1H$ COSY spectrum led to partial structure **d** (Figure 2). HMBC correlations between Me-26/C-24, C-25 and Me-27/C-24, C-25 placed a double bond between C-24 and C-25, and the two olefinic methyls at C-26 and C-27 (Figure 2). Rings A and B were elucidated on the basis of HMBC cross-peaks between Me-19/C-1, C-5, C-9, C-10 and H-4/C-3, whereas rings C and D were completed based on HMBC correlations between Me-18/C-12, C-13, C-14, C-17. Comparison of ^{13}C NMR chemical shift values of **7** with those of five cholest-1-en-3-ones reported from the octocoral *A. gracillimum*[9] inferred normal stereochemistry of the ring junctures of **7**. Less than 0.6 ppm differences were observed between the ^{13}C chemical shft values for the steriodal skeleton of **7** and those of cholest-1-ene-3,22-dione. A paucity of material hampered the elucidation of the C-22 and C-23 stereochemistry. Thus, **7** is a 22,23-dihydroxycholest-1,24-dien-3-one, reminiscent of brassinosteroids which are a class of plant growth regulators in a wide variety of higher plants.[10]

Compound **8** had a molecular formula of $C_{25}H_{34}O_3$ as determined by HREIMS. The presence of an α,β-unsaturated methyl ester was readily inferred from 1D NMR signals resonating at δ_H 3.71 (3H, s)/δ_C 51.4 (q), 5.75 (1H, d, J = 15.7 Hz)/118.7 (d), 6.82 (1H, dd, J = 9.9, 15.7 Hz)/155.3 (d), and 163.6 (s) (Table 1) together with an IR absorption at 1720 cm^{-1}. The UV maximum at 243 nm was characteristic of a cross-conjugated cyclohexadienone functionality which was substantiated by an IR band at 1660

cm^{-1}. The 1D NMR data could account for 5 of the 9 degrees of unsaturation, suggesting the tetracyclic nature of **8**. Interpretation of the $^1H-^1H$ COSY spectrum resulted in a cholane-type steroidal skeleton. HMBC correlations between H-23/C-24 and MeO/C-24 placed the methyl ester at the end of the side chain. Comparisons of ^{13}C NMR chemical shift values of **8** with those of chola-1,4-dien-3-ones reported from a soft coral *Minabea* sp.[11] disclosed the expected all-*trans* stereochemistry at the ring junctures of **8**. Thus, **8** is a methyl 3-oxochola-1,4,22-trien-24-oate. An analogue of **8**, 3-oxochola-4,22-dien-24-oic acid is known from the dorid nudibranch *Aldisa sanguinea cooperi*.[12]

Perhaps the methyl ether **5** and methyl ester **8** are artifacts arising during extraction with MeOH. However, we could not detect either the corresponding free alcohol or acid in the extracts which were prepared in relatively short times. These facts may support that these methylated metabolites are natural products.

The new steroids **5**−**8** were lethal to cyprids of *B. amphitrite* at 100 μg/mL (LD_{100}), but did not inhibit larval settlement of *B. amphitrite* at 50 μg/mL. Since isogosterones **1**−**4**, which have the same enone A-ring as **8** show considerable antifouling activity against cyprids (EC_{50}: 2.2 μg/mL), a hemiacetal or an acetal moiety in a steroidal skeleton may be important for antifouling activity. It also appears that neither a conjugated cyclohexenone nor an aromatic A-ring is essential for antifouling activity.

Experimental Section

General Experimental Procedure. IR spectra were recorded on a JASCO IR-700 spectrometer. UV spectra were determined with a Hitachi U-2000 spectrometer. Measurements of high-resolution EI mass spectra (HREIMS) were performed with a JEOL SX-102A mass spectrometer. ^1H and ^{13}C NMR spectra were recorded on a BRUKER ARX-500 spectrometer in $CDCl_3$ at 500.14 and 125.77 MHz at 300 K. Chemical shifts were reported using residual $CHCl_3$ (δ 7.24), $CDCl_3$ (δ 77.0) as internal standards. Optical rotations were measured with a JASCO DIP-1000 polarimeter. Bioassay experiments for settlement of barnacle larvae were performed as reported previously.[2,3]

Animal Materials. Specimens of *A. gracillimum* Kukenthal, 1907 were collected at a depth of 15 m off Atami in the Gulf of Sagami, Japan (35° 06′ N, 139° 06′ E). A voucher specimen (C8403) was deposited at Wakayama Prefectural Museum of Natural History. Specimens of the second octocoral were collected at a depth of 10 m off the Kii Peninsula, Japan (33° 27′ N, 135° 46′ E). Although this octocoral was very similar to *Dendronephthya habereiri* Kukenthal, the specimen differed in the size of its polyps and the arrangement of spicules in the polyps. At present our specimen cannot be identified at the species level. A voucher specimen (C 95-004) was also deposited at Wakayama Prefectural Museum of Natural History.

Isolation of 5, 6, and 7. The frozen octocoral (40 g; wet wt) of *A. gracillimum* was extracted with MeOH (2 × 0.5 L). The extract was concentrated under reduced pressure and partitioned between H_2O and Et_2O (3 × 0.25 L). The Et_2O layer (131 mg) was fractionated by silica gel column chromatography (Wakogel C-200, Wako Purechemical Industries, Tokyo, Japan) with toluene, $CHCl_3$, 10% MeOH/$CHCl_3$, and MeOH, successively. The fraction eluted with $CHCl_3$ was purified by reversed-phase HPLC [YMC R−ODS-5 (S-5 120A ODS), YMC Co., Kyoto, Japan] with 90% MeOH/H_2O to afford **5** (10.2 mg, 2.6×10^{-2} % on wet wt basis) and **6** (1.1 mg, 2.8×10^{-3} %). The fraction eluted with 10% MeOH/$CHCl_3$ from the silica gel column was similarly purified to afford **7** (0.8 mg, 2.0×10^{-3} %).

Isolation of 8. The frozen sample (68 g) of *Dendronephthya* sp. was extracted and partitioned in the same way as above. The concentrated Et_2O layer (250 mg) was fractionated by silica gel column chromatography with toluene, $CHCl_3$, 10% MeOH/$CHCl_3$, and MeOH, successively. The fraction eluted with $CHCl_3$ was purified by reversed-phase HPLC (90% MeOH/H_2O) to afford **8** (5.6 mg, 8.2×10^{-3} %).

Compound 5: $[\alpha]^{22}_D$ +19.9 (*c* 0.21, $CHCl_3$); IR (neat) 3070, 2915, 1730, 1605, 905 cm^{-1}; UV (MeOH) λ_{max} 222 (ε 12 000), 278 (ε 1700), 287 nm (ε 1500); ^1H NMR ($CDCl_3$) δ 7.19 (1H, d, *J* = 8.5 Hz, H-1), 6.69 (1H, dd, *J* = 2.5, 8.5 Hz, H-2), 6.62 (1H, d, *J* = 2.5 Hz, H-4), 2.83 (2H, m, H-6), 1.38 (1H, m, H-7α), 1.89 (1H, m, H-7*β*), 1.41 (1H, m, H-8), 2.20 (1H, m, H-9), 2.27 (1H, m, H-11α), 1.47 (1H, m, H-11*β*), 1.26 (1H, m, H-12α), 1.83 (1H, m, H-12*β*), 1.25 (1H, m, H-14), 1.80 (1H, m, H-15α), 1.28-(1H, m, H-15*β*), 1.81 (1H, m, H-16α), 1.59 (1H, m, H-16*β*), 2.04 (1H, m, H-17), 0.62 (3H, s, H-18), 5.78 (1H, m, H-20), 4.97 (2H, m, H-21), 3.76 (3H, s, C*H*$_3$O); ^{13}C NMR ($CDCl_3$), see Table 1.; HREIMS *m*/*z* 296.2144 (calcd for $C_{21}H_{28}O$, Δ +0.4 mmu).

Compound 6: $[\alpha]^{22}_D$ −96.5 (*c* 0.055, $CHCl_3$); IR (neat) 3355, 2920, 1735, 1605, 970 cm^{-1}; UV (MeOH) λ_{max} 215 (ε 19 000), 222 (ε 15 000), 229 (ε 11 000), 274 (ε 2100), 283 nm (ε 1500); ^1H NMR ($CDCl_3$) δ 7.21 (1H, d, *J* = 8.5 Hz, H-1), 6.85 (1H, dd, *J* = 2.5, 8.5 Hz, H-2), 6.79 (1H, d, *J* = 2.5 Hz, H-4), 2.84 (2H, m, H-6), 1.34 (1H, m, H-7α), 1.78 (1H, m, H-7*β*), 1.39 (1H, m, H-8), 2.20 (1H, m, H-9), 2.25 (1H, m, H-11α), 1.48 (1H, m, H-11*β*), 1.26 (1H, m, H-12α), 1.83 (1H, m, H-12*β*), 1.23 (1H, m, H-14), 1.80 (1H, m, H-15α), 1.28 (1H, m, H-15*β*), 1.81 (1H, m, H-16α), 1.59 (1H, m, H-16*β*), 2.04 (1H, m, H-17), 0.61 (3H, s, H-18), 5.77 (1H, m, H-20), 4.99 (2H, m, H-21), 5.53 (1H, d, *J* = 4 Hz, H-1′), 3.91 (1H, dd, *J* = 4, 10 Hz, H-2′), 4.14 (1H, dd, *J* = 3.5, 10 Hz, H-3′), 5.26 (1H, dd, *J* = 0.5, 3.5 Hz, H-4′), 4.18 (1H, dq, *J* = 0.5, 6.5 Hz, H-5′), 1.12 (3H, d, *J* = 6.5 Hz, H-6′), 2.17 (3H, s, C*H*$_3$COO); ^{13}C NMR ($CDCl_3$) see Table 1; HREIMS *m*/*z* 470.2661 (calcd for $C_{28}H_{38}O_6$, Δ −0.8 mmu).

Compound 7: $[\alpha]^{22}_D$ −40.9 (*c* 0.13, $CHCl_3$); IR (neat) 3400, 2920, 1730, 1675 cm^{-1}; UV (MeOH) λ_{max} 229 nm (ε 15 000); ^1H NMR ($CDCl_3$) δ 7.11 (1H, d, *J* = 10 Hz, H-1), 5.83 (1H, d, *J* = 10 Hz, H-2), 2.20 (1H, m, H-4α), 2.34 (1H, m, H-4*β*), 1.91 (1H, m, H-5), 1.42 (2H, m, H-6), 1.42 (1H, m, H-7α), 1.72 (1H, m, H-7*β*), 1.43 (1H, m, H-8), 0.97 (1H, m, H-9), 1.72 (1H, m, H-11α), 1.41 (1H, m, H-11*β*), 1.24 (1H, m, H-12α), 1.98 (1H, m, H-12*β*), 1.09 (1H, m, H-14), 1.61 (1H, m, H-15α), 1.10 (1H, m, H-15*β*), 1.23 (1H, m, H-16α), 1.90 (1H, m, H-16*β*), 1.52 (1H, m, H-17), 0.66 (3H, s, H-18), 0.99 (3H, s, H-19), 1.47 (1H, m, H-20), 0.88 (3H, d, *J* = 6.6 Hz, H-21), 3.45 (1H, br d, *J* = 8.7 Hz, H-22), 4.25 (1H, t, *J* = 8.7 Hz, H-23), 5.03 (1H, d, *J* = 8.7 Hz, H-24), 1.73 (3H, s, H-26), 1.75 (3H, s, H-27); ^{13}C NMR ($CDCl_3$), see Table 1; HREIMS *m*/*z* 414.3161 (calcd for $C_{27}H_{42}O_3$, Δ +2.7 mmu).

Compound 8: $[\alpha]^{22}_D$ +53.6 (*c* 0.28, $CHCl_3$); IR (neat) 3425, 2935, 2860, 1720, 1660, 930 cm^{-1}; UV (MeOH) λ_{max} 215 nm (ε 21 000), 243 nm (ε 16 000); ^1H NMR ($CDCl_3$) δ 7.00 (1H, d, *J* = 10 Hz, H-1), 6.19 (1H, dd, *J* = 2, 10 Hz, H-2), 6.04 (1H, t, *J* = 2 Hz, H-4), 2.44 (1H, m, H-6α), 2.32 (1H, m, H-6*β*), 1.00 (1H, m, H-7α), 1.90 (1H, m, H-7*β*), 1.55 (1H, m, H-8), 0.98 (1H, m, H-9), 1.65 (2H, m, H-11α), 1.57 (2H, m, H-11*β*), 0.99 (1H, m, H-12α), 1.74 (1H, m, H-12*β*), 0.95 (1H, m, H-14), 1.62 (1H, m, H-15α), 1.14 (1H, m, H-15*β*), 1.86 (1H, m, H-16α), 1.29 (1H, m, H-16*β*), 1.23 (1H, m, H-17), 0.69 (3H, s, H-18), 1.19 (3H, s, H-19), 2.24 (1H, m, H-20), 0.96 (3H, d, *J* = 6.7 Hz, H-21), 6.82 (1H, dd, *J* = 10, 15.7 Hz, H-22), 5.75 (1H, d, *J* = 15.7 Hz, H-23), 3.71 (3H, s, C*H*$_3$COO); ^{13}C NMR ($CDCl_3$), see Table 1; HREIMS *m*/*z* 382.2508 (calcd for $C_{25}H_{34}O_3$, Δ +0.2 mmu).

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