Indoleamine 2,3-Dioxygenase Inhibitors from the Northeastern Pacific Marine Hydroid *Garveia annulata*

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Crude extracts of the marine hydroid *Garveia annulata* show potent inhibition of indoleamine 2,3-dioxygenase (IDO). Fractionation of the extract led to the identification of the new polyketides annulin C (**1**), 2-hydroxygarveatin E (**4**), garveatin E (**5**), and garvin C (**9**). Annulins A (**2**), B (**3**), and C (**1**) were found to be submicromolar inhibitors of IDO.

Immune escape plays an important role in cancer progression[1−5] and fetal development.[6] Although the mechanism is still not completely understood, it has been proposed that indoleamine 2,3-dioxygenase (IDO) plays a central role in evasion of T-cell-mediated immune rejection.[4] IDO catalyzes the oxidative cleavage of the 2,3 bond of tryptophan, which is the first and rate-limiting step in the kynurenine pathway of tryptophan catabolism in mammalian cells.[5] T-cell lymphocytes are extremely sensitive to tryptophan shortage, which causes them to undergo cell cycle arrest in G1. Degradation of tryptophan by IDO expressed by tumors or by the placenta inhibits T-cell proliferation and, as a result, prevents immunological rejection of the tumor or fetus. Expression of IDO is known to be constitutively activated in a large number of human cancers.[3] It has recently been shown that the IDO inhibitor 1-methyltryptophan potentiates the efficacy of DNA-damaging chemotherapeutic agents in the inhibition of tumor growth in mouse models.[7] In addition, IDO present in the ocular lens has been implicated as a key factor in the development of senile cataracts.[8] Specifically, the long-term accumulation of tryptophan degradation products and the ability of these compounds to modify the major lens protein, crystallin, have been identified by Truscott and colleagues as a major mechanism of lens opacification.[9]

Most of the known IDO inhibitors are tryptophan analogues, which are active only at concentrations of ~10 μM and greater, making them marginal drug candidates.[7,10] As part of a program designed to find more potent IDO inhibitors belonging to new structural classes, we have screened a library of marine invertebrate extracts for their ability to inhibit human IDO in vitro. A crude MeOH extract of the Northeastern Pacific hydroid *Garveia annulata* showed promising inhibitory activity in the IDO assay. *G. annulata* has previously been shown to be an extremely rich source of highly functionalized anthracene- and naphthalene-based polyketides.[11−14] IDO assay[15] guided fractionation identified the new polyketide annulin C (**1**) and the known compounds annulin A (**2**) and annulin B (**3**) as the most potent IDO inhibitors in the *G. annulata* crude extract. A second group of compounds that included the new compounds 2-hydroxygarveatin E (**4**) and garveatin E (**5**) as well as the known compounds garveatin A (**6**), garveatin C (**7**), and 2-hydroxygarvin A (**8**) were found to be less potent IDO inhibitors. Garvin C (**9**), an inactive *G. annulata* metabolite with a new carbon framework, was isolated during the course of this investigation. Details of the isolation and structure elucidation of the new metabolites **1**, **4**, **5**, and **9** along with a discussion of the IDO inhibitory activities of the *Garveia* polyketides are presented below.

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Samples of *G. annulata* were collected by hand using scuba in Barkley Sound, British Columbia. Freshly collected animals were exhaustively extracted with MeOH, and the combined extracts were concentrated in vacuo to give a deep orange, gummy residue. The residue was added to water, and the resulting suspension was sequentially extracted with hexanes, CH_2Cl_2, and EtOAc. Only the CH_2Cl_2-soluble material inhibited IDO. Silica gel flash chromatography followed by both normal- and reversed-phase HPLC was used to generate pure samples of annulins A (**2**), B (**3**), and C (**1**), garveatins A (**6**), C (**7**), and E (**5**), 2-hydroxygarveatin E (**4**), 2-hydroxygarvin A (**8**), and garvin C (**9**). The known compounds annulin A (**2**),[13] annulin B (**3**),[13] garveatin A (**6**),[11] garveatin C (**7**),[12] and 2-hydroxygarvin A (**8**)[12] were identified by comparison of their spectroscopic data with the literature values.

Annulin C (**1**) was isolated as a yellow oil that gave an $[M]^+$ ion at *m*/*z* 374.1367 in the HREIMS, appropriate for a molecular formula of $C_{20}H_{22}O_7$. The 1D and 2D NMR data obtained for annulin C (**1**) showed a strong resemblance to the data obtained for annulin A (**2**),[11] indicating that the molecules were closely related. The major difference in the ^{1}H NMR spectra of the two compounds was the absence in the spectrum of annulin C (**1**) of a resonance that could be assigned to the hemiketal OH present in the spectrum of **2** (δ 4.85) and its replacement by a methyl singlet at δ 3.45. This methyl resonance showed an HMBC correlation to a carbon resonance at δ 106.8, assigned to a ketal, suggesting that the hemiketal at C-2 in annulin A (**2**) had been replaced by a methyl ketal in annulin C (**1**). The MS data and the remainder of the NMR data were consistent with the structure **1** for annulin C. Annulin C (**1**) may be an isolation artifact in which the C-8 methyl ketal has been formed by reaction of the co-occurring metabolite annulin A (**2**) with the MeOH extraction solvent.

2-Hydroxygarveatin E (**4**), isolated as a yellow oil, gave a $[M + Na]^+$ ion at *m*/*z* 379.1160 in the positive ion HRESIMS, consistent with a molecular formula of $C_{20}H_{20}O_6$ (calcd for $C_{20}H_{20}O_6$-Na, 379.1158), requiring 11 sites of unsaturation. The ^{1}H NMR spectrum of **4** contained resonances that could be assigned to an ethyl fragment (δ 1.12, t, J = 7.7 Hz, Me-1; 2.67, q, J = 7.7 Hz, H-2/H-2′), four methyl groups (δ 1.44, s, Me-19; 1.56, s, Me-18; 1.76, s, Me-20; 2.21, s, Me17), an aromatic methine (δ 7.72, s, H-13), and a phenol OH (δ 13.37, s, OH-6), accounting for 19 of the 20 protons in the molecule. HMBC correlations observed between the methyl resonance at δ 1.13 (Me-1) and a carbon resonance at δ 149.2 (C-3); between the methylene resonance at δ 2.67 (H-2/H-2′) and carbon resonances at δ 144.3 (C-16), 149.2 (C-3) and 188.6 (C-4); and between the methyl resonance at δ 2.21 (Me-17) and carbon resonances at δ 144.3 (C-16), 149.2 (C-3), and 183.5 (C-15) suggested that the methyl and ethyl residues were vicinal substituents on a *para* quinone substructure. The aromatic

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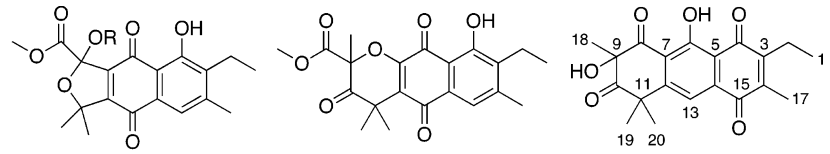
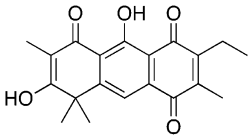
Chart 1



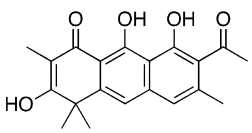
1 R = Me Annulin C
2 R = H Annulin A

3 Annulin B

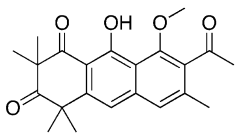
4 2-Hydroxygarveatin E



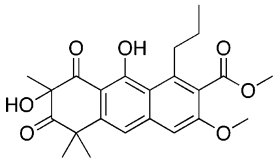
5 Garveatin E



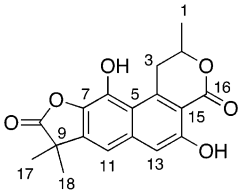
6 Garveatin A



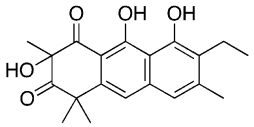
7 Garveatin C



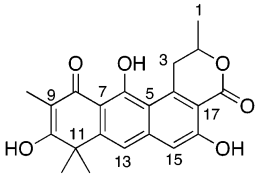
8 2-Hydroxygarvin A



9 Garvin C



10 2-Hydroxygarveatin B



11 Garvin B

methine resonance at δ 7.72 (H-13) showed an HMBC correlation to the quinone carbonyl resonance at δ 183.5 (C-15), which situated the aromatic methine carbon vicinal to the carbonyl. Additional HMBC correlations between the aromatic methine (δ 7.72) and carbon resonances at δ 114.5 (C-5) and 122.3 (C-14) and between the phenolic OH resonance at δ 13.27 (OH-6) and carbon resonances at δ 114.5 (C-5), 122.3 (C-14), and 160.4 (C-6) were consistent with placement of the phenol-bearing carbon (C-6) vicinal to the second quinone carbonyl (C-4). The two methyl resonances at δ 1.44 (Me-19) and 1.76 (Me-20) showed HMBC correlations to carbon resonances at δ 48.4 (C-11), 154.5 (C-12), and 207.0 (C-10), which demonstrated the methyls were geminal substitutents on a quaternary carbon (C-11) attached on one side to an aromatic ring and on the other side to a saturated ketone. HMBC correlations from the methyl resonance at δ 1.56 (Me-18) to carbon resonances at δ 83.9 (C-9), 194.3 (C-8), and 207.0 (C-10) showed that the methyl was attached to a nonprotonated carbon (C-9) bearing an oxygen, which was in turn flanked by two ketones. The oxygen atom on C-9 had to be part of a hydroxyl functionality to account for the molecular formula of **4**. There were two possible ways to attach the *gem*-dimethyl-bearing carbon (C-11) and ketone termini (C-8) of the aliphatic fragment to the unsatisfied valences of the napthaquinone fragment (C-7 and C-12). The HMBC data had shown that the *gem*-dimethyl-bearing carbon was linked to a carbon with a chemical shift of δ 154.5. This deshielded carbon (C-12) could only be *meta* to the phenol (C-6), leading to the structure **4** for 2-hydroxygarveatin E, which is a C-4/C-15 *para* quinone analogue of the previously described metabolite 2-hydoxygarveatin B (**10**).

Garveatin E (**5**), a very minor component of the extract, was obtained as a pale yellow oil that gave an $[M + Na]^+$ ion at *m*/*z* 363.1200 in the HRESIMS, consistent with a molecular formula of $C_{20}H_{20}O_5$ (calcd for $C_{20}H_{20}O_5Na$, 363.1208), which differed from the molecular formula of 2-hydroxygarveatin E (**4**) simply by loss of one oxygen atom. Analysis of the NMR data obtained for **5** indicated that it differed from 2-hydroxygarveatin E (**4**) simply by loss of the hydroxyl functionality at C-9. The ^{1}H NMR spectrum of **5** contained resonances that could be assigned to an ethyl (δ 1.14, t, J = 7.6 Hz, Me-1; 2.66, q, J = 7.6 Hz; H-2/H-2′) and two olefinic methyl (δ 2.21, s, Me-17; 1.95, s, Me-18) groups, an aromatic methine (δ 7.76, s, H-13), and a pair of geminal methyls (δ 1.46, s, Me19/Me-20). HMBC correlations between the methylene proton resonance at δ 2.66 (H-2/H-2′) and a carbonyl resonance at δ 188.9 (C-4) and between the methyl resonance at δ 2.21 (Me-17) and a carbonyl resonance at δ 183.3 (C-15) confirmed that the ethyl and one of the methyl residues in **5** were also vicinal substituents on a *para* quinoine, as in **4**. The olefinic methyl resonance at δ 1.95 (Me-18) showed HMBC correlations to carbon resonances at δ 200.0 (C-8), 113.3 (C-9), and 160.6 (C-10), and the aromatic methine at δ 7.76 (H-13) showed an HMBC correlation to the quaternary carbon at δ 48.3 (C-11), all in agreement with the proposed structure **5**.

Garvin C (**9**) was isolated as a pale yellow solid that gave a $[M - H]^-$ ion at *m*/*z* 327.0866 in the negative ion HRESIMS, appropriate for a molecular formula of $C_{18}H_{16}O_6$ (calcd for $C_{18}H_{15}O_6$ 327.0869), requiring 11 sites of unsaturation. Detailed analysis of the NMR data collected for **9** showed that it was closely related to the known metabolite garvin B (**11**). In particular, the COSY, HMQC, and HMBC data (Figure 1) confirmed the presence of δ-lactone (C-1 to C-4, C-15, and C-16) and dihydroxy naphthalene (C-4 to C-6 and C-11 to C-15) substructures in **9** that were identical to the corresponding substructures in **11**. Two long-range HMBC correlations not shown in Figure 1, between the aromatic methine at δ 7.15 (H-13) and the ester carbonyl resonance at δ 169.9 (C-16) and between the 14-OH resonance at δ 10.94 and the aromatic carbon resonance at δ 136.8 (C-12), both ascribed to *W* coupling pathways, provided additional support for the substitution pattern on the naphthalenic substructure. HMBC correlations observed

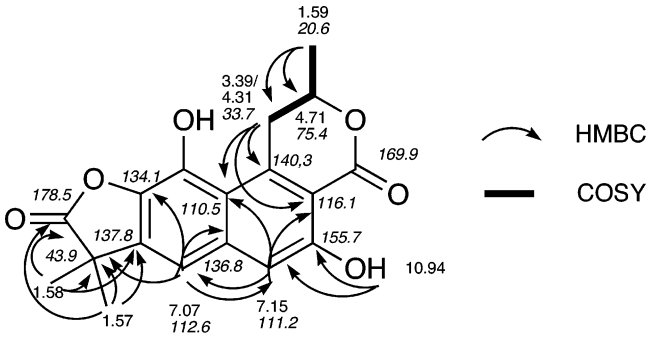


Figure 1. Selected COSY and HMBC correlations observed for garvin C (**9**).

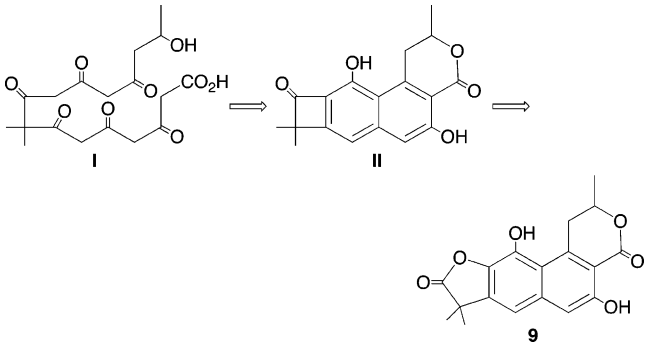


Figure 2. Possible biogenesis of garvin C (**9**) from an octaketide precursor.

between methyl resonances at δ 1.57 (Me-17) and 1.58 (Me-18) and carbon resonances at δ 43.9 (C-9), 137.8 (C-10), and 178.5 (C-8) identified a quaternary carbon bearing a pair of geminal methyl groups that was linked to an ester/acid carbonyl as one of the two remaining substituents at C-7 or C-10 of the naphthalenic fragment in **9**. In order to account for the molecular formula of **9**, the carbonyl functionality had to be part of a γ-lactone fused to the naphthalenic fragment. An HMBC correlation between the aromatic methine at δ 7.07 (H-11) and the quaternary carbon resonance at δ 43.9 (C-9) demonstrated that the quaternary carbon was *ortho* to H-11, as shown in **9**, which was consistent with the relatively shielded chemical shift observed for the oxygenated aromatic carbon C-7 (δ 134.1), which was part of the γ-lactone.

The new metabolites annulin C (**1**), 2-hydroxygarveatin E (**4**), and garveation E (**5**) represent minor structural variations of the known compounds annulin A (**2**) and garveatin B. Garvin C (**9**) on the other hand, while obviously related to garvin B (**11**), has a new carbon framework not previously found among *Garveia* polyketides. The garvin C skeleton might arise from degradation of garvin B (**11**), which requires excision of the equivalent of one polyketide residue comprising C-8/C-9 along with the associated subsituents. Alternatively, as shown in Figure 2, garvin C (**9**) might arise from a dimethylated octaketide **I** instead of the putative nonaketide precursor to garvin B. Cyclization of the octaketide **I** to give a naphthalenic core fused to a δ-lactone on one end and a cyclobutanone on the other (i.e., **II**), followed by a biological Baeyer Villager reaction on the cyclobutanone, would lead directly to garvin C (**9**).

Table 1 lists the K_i values for in vitro inhibition of IDO by the *G. annulata* polyketides as determined by continuous spectrophotometric analysis.[16] The most active compounds are annulin B (**3**), annulin C (**1**), and annulin A (**2**), which have a common 5-hydroxy-6-ethyl-7-methyl-1,4-naphthoquinone core substructure. The annulins are all significantly more active than 1-methyltryptophan ($K_i \approx 6.6\ \mu$M), which is one of the most potent IDO inhibitors reported to date in the literature.[10] Annulins A, B, and C were all inactive in a recently developed yeast-based IDO inhibition assay,[17] suggesting that they may not pass through the yeast cell wall. The potency of the annulins should make them useful tools to investigate the mechanism of action of IDO in vitro, but further studies on human cells will be required to determine if the annulins have potential as drug leads or cell biology tools.

Table 1. In Vitro Inhibition of IDO by *G. annulata* Metabolites

	K_i (μM)
annulin C (**1**)	0.14
annulin A (**2**)	0.69
annulin B (**3**)	0.12
2-hydroxygarveatin E (**4**)	1.4
garveatin E (**5**)	3.1
garveatin A (**6**)	3.2
garveatin C (**7**)	1.2
2-hydroxygarvin A (**8**)	2.3
1-methyltryptophan[10]	6.6

Experimental Section

General Experimental Procedures. NMR spectra were recorded on a Bruker Avance 400 and a Bruker Avance 600 equipped with a Cryoprobe, at 400 and 600 MHz, respectively, in $CDCl_3$-*d*. NMR spectra were referenced to $CDCl_3$ (^{1}H δ 7.24 ppm; ^{13}C δ 77.0 ppm). EIMS spectra were obtained with Kratos MS-50, Micromass LCT, and Bruker Esquire-LC mass spectrometers. Silica gel (Silicycle, 230−400 mesh) was used for column chromatography; precoated Si gel plates (Merck, Kieselgel 60 F_{254}, 0.25 mm) were used for TLC analysis. A Waters 1500 Series pump system, equipped with a Waters 2487 dual λ absorbance detector and an Alltech Econosil silica 5 μm or a C18 5 μm column, was used for HPLC.

Animal Material. The hydroid *Garveia annulata* (Nuttig, 1901; phylum Cnidaria; class Hydrozoa; order Anthomedusae; suborder Filifera; family Bougainvilliidae) was collected by hand using scuba in Barkley Sound, British Columbia, at a depth of 10−20 m. Samples were stored in a freezer for 3 days until extraction.

Extraction and Isolation. Collected animals were extracted with MeOH, and the combined extracts concentrated in vacuo to give an orange residue (0.36 g). The residue was added to H_2O and partitioned with hexanes, CH_2Cl_2, and EtOAc. The CH_2Cl_2-soluble IDO-active fraction (0.13 g) was subjected to Si gel CC (20% EtOAc/hexanes), followed by normal phase HPLC (15% EtOAc/hexanes), to afford the new analogue annulin C (**1**) (0.3 mg) and the known metabolite annulin B (**3**) (0.6 mg). A second batch of *G. annulata* specimens was processed as above to generate 2.5 g of active CH_2Cl_2 extract. Si gel gradient CC, followed by gradient (20% CH_3CN/H_2O to 83% CH_3CN/H_2O) or isocratic (70% CH_3CN/H_2O) reversed-phase HPLC, yielded the new garveatin E (**5**) (0.5 mg) and the previously reported 2-hydroxygarveatin B (**10**) (1.1 mg), annulin A (**2**) (0.5 mg), garveatins A (**6**) (34 mg) and C (**7**) (0.5 mg), and 2-hydroxygarvin A (**8**) (3.7 mg). Additional purification of IDO-active fractions by reversed-phase HPLC (70% CH_3OH/H_2O) yielded the new compounds 2-hydroxygarveatin E (**4**) (0.4 mg) and garvin C (**9**) (0.3 mg). Due to limited amounts of compounds isolated, all ^{13}C NMR data were derived from HMQC and HMBC spectra.

Enzyme Activity Assays. The effect of extracts and purified compounds on IDO activity was determined with the use of recombinant human IDO expressed in *E. coli*[17] and purified by a method similar to that used by others.[18,19] Assays of IDO activity used for screening inhibitory capability of extracts were performed by the end-point method of Takikawa et al.[15] Inhibitory constants (K_i values) were determined with a continuous spectrophotometric activity assay[16] performed in sodium phosphate buffer (100 mM, pH 6.5, 25 °C) with [IDO] of 50 nM and [L-Trp] of 0.1 mM. The uncertainty in K_i values determined in this work is 10%.

Annulin C (1): yellow oil; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 12.2 (1H, s, OH-4), 7.44 (1H, s, H-15), 3.82 (3H, s, H-10), 3.45 (3H, s, H-18), 2.75 (2H, q, J = 7.5 Hz, H-2), 2.41 (3H, s, H-17), 1.68 (3H, s, H-19), 1.62 (3H, s, H-20), 1.12 (3H, t, J = 7.5 Hz, H-1); ^{13}C NMR ($CDCl_3$) δ 167.2 (C-9), 160.3 (C-4), 154.3 (C-12), 145.3 (C-16), 140.0 (C-3), 121.5 (C-15), 106.8 (C-8), 88.3 (C-11), 52.5 (C-10), 51.7 (C-18), 26.4 (C-19), 26.4 (C-20), 19.7 (C-17), 19.4 (C-2), 12.8 (C-1); HREIMS *m/z* 374.1367 (calcd for $C_{20}H_{22}O_7$, 374.1366).

Garveatin E (5): pale yellow oil; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 14.6 (1H, s, OH-10), 10.15 (1H, s, OH-5), 7.76 (1H, s, H-13), 2.66 (2H, q, J = 7.6 Hz, H-2), 2.21 (3H, s, H-17), 1.95 (3H, s, H-18), 1.46 (3H, s, H-19), 1.46 (3H, s, H-20), 1.14 (3H, t, J = 7.6 Hz, H-1); ^{13}C NMR ($CDCl_3$) δ 200.0 (C-8), 188.9 (C-4), 183.3 (C-15), 160.6 (C-10), 148.9 (C-3), 145.3 (C-16), 122.2 (C-14), 117.5 (C-13), 114.4 (C-12), 113.3 (C-9), 48.3 (C-11), 30.0 (C-19), 27.7 (C-20), 19.5 (C-2), 12.5 (C-1), 12.2 (C-17); HRESIMS *m*/*z* 363.1200 (calcd for $C_{20}H_{20}O_5$-Na, 363.1208).

2-Hydroxygarvin E (4): yellow oil; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 13.3 (1H, s, OH-6), 7.72 (1H, s, H-13), 2.67 (2H, q, J = 7.7 Hz, H-2), 2.21 (3H, s, H-17), 1.76 (3H, s, H-20), 1.56 (3H, s, H-18), 1.44 (3H, s, H-19), 1.12 (3H, t, J = 7.7 Hz, H-1); ^{13}C NMR ($CDCl_3$) δ 207.0 (C-10), 194.3 (C-8), 188.6 (C-4), 183.5 (C-15), 160.4 (C-6), 154.5 (C-12), 149.2 (C-3), 144.3 (C-16), 122.3 (C-14), 115.2 (C-13), 114.5 (C-5), 83.9 (C-9), 48.4 (C-11), 30.0 (C-19), 26.4 (C-18), 26.2 (C-20), 19.8 (C-2), 12.7 (C-1), 12.3 (C-17); HRESIMS *m*/*z* 379.1160 (calcd for $C_{20}H_{20}O_6$Na, 379.1158).

Garvin C (9): pale yellow oil; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 10.94 (1H, s, OH-14), 7.15 (1H, s, H-13), 7.07 (1H, s, H-11), 4.71 (1H, m, H-2), 4.31 (1H, dd, J = 17.9, 4.3 Hz, H-3a), 3.39 (1H, dd, J = 18.2, 3.4, H-3b), 1.59 (3H, d, J = 6.36 Hz, H-1), 1.57 (3H, s, H-17), 1.58 (3H, s, H-18); ^{13}C NMR ($CDCl_3$) δ 178.5 (C-8), 169.9 (C-16), 155.7 (C-14), 140.3 (C-4), 137.8 (C-10), 136.8 (C-12), 134.1 (C-7), 116.1 (C-15), 112.6 (C-11), 111.2 (C-13), 75.4 (C-2), 43.9 (C-9), 33.7 (C-3), 25.1 (C-17), 25.1 (C-18), 20.6 (C-1); HRESIMS *m*/*z* 327.0866 (calcd for $C_{18}H_{15}O_6$Na, 327.0869).

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