COMPARISON OF BALANOL FROM *Verticillium balanoides* AND OPHIOCORDIN FROM *Cordyceps ophioglossoides*

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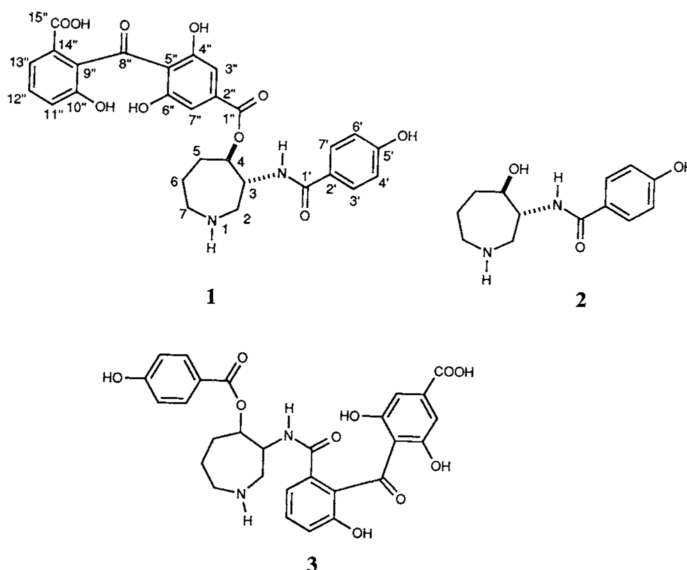
Recently, we reported the isolation of the potent protein kinase C inhibitor balanol (**1**) from the fungus *Verticillium balanoides*. In an earlier study, König *et al.* reported the isolation of ophiocordin (**3**), a structural isomer of **1**, from the fungus *Cordyceps ophioglossoides*. The present study was designed to clarify whether or not balanol and ophiocordin are different compounds. The results indicated that the two fungi produced the same compound, the structure being that assigned to balanol. In addition, a thirty-fold increase in the production of balanol from *V. balanoides* was observed when the culture medium was changed from cornmeal/tomato paste to soy meal/glycerol.

In our search for protein kinase C (PKC) inhibitors from microbial sources, we recently disclosed the isolation and structure elucidation of the potent inhibitor balanol (**1**) from the fungus *Verticillium balanoides*.[1)] Balanol represents a novel class of PKC inhibitors and is the most potent inhibitor reported since the discovery of staurosporine.[2~4)] Human PKC enzymes α, βI, βII, γ, δ, ε, and η were inhibited by **1** with IC_{50} values of 4~9 nM. The structure of balanol was unambiguously determined by 1D and 2D NMR data and chemical degradation. The absolute stereochemistry of **1** was assigned by X-ray diffraction of a *para*-bromobenzoyl derivative of **2**.[1)] Very recently, the structure of balanol was confirmed by total



synthesis (unpublished results). During the course of the structure elucidation of **1**, a literature search revealed the occurrence of a related compound ophiocordin (**3**)—a weak antifungal antibiotic isolated from the fungus *Cordyceps ophioglossoides*.[5,6] Ophiocordin has a molecular formula and structural components identical to those of balanol. Moreover, the ^1H NMR spectra of **1** and **3**[5] in CD_3OD/D_2O appeared strikingly similar, suggesting that the two compounds may be identical. Unfortunately, authentic **3** was unavailable for direct comparison, and we therefore obtained the vouchered fungus *C. ophioglossoides* from the American Type Culture Collection (ATCC). We herein report the fermentation, isolation and structure elucidation of the secondary metabolite from *C. ophioglossoides*.

Results and Discussion

Taxonomy

Verticillium balanoides (Drechsler) Dowsett *et al.* is a Deuteromycete characterized by phialides producing unicellular, hyaline, cuneiform conidia and has no known teleomorphic (sexual) state. *V. balanoides* has been isolated on numerous occasions from leaf litter, where it is a predator of nematodes. The producing strain was isolated from a yellow rhizomorph in *Pinus palustris* needle litter located in Hoffman, North Carolina. The microhabitat contained numerous fruiting bodies of *Elaphomyces* sp.

Cordyceps ophioglossoides (Ehrenberg ex Link) Fries is an Ascomycete producing typically solitary stromata containing perithecia with eight-spored asci and has no known anamorphic (asexual) state. *C. ophioglossoides* is a parasite of *Elaphomyces* fruiting bodies. The producing strain (TÜ 276)[5] was obtained from the American Type Culture Collection, Rockville, Maryland, U.S.A. (ATCC 36865).

Fermentation

The yield of **3** from *C. ophioglossoides* grown in a soy meal/glycerol medium was reported to be approximately 185 mg/liter.[5] This far exceeded our production of **1** from *V. balanoides* grown in a cornmeal/tomato paste medium (0.3 mg/liter).[1] In order to determine the effects of culture medium on PKC activity, four 150 ml cultures using both fungi and the two media were prepared, and the methanol extracts of each were assayed for *in vitro* inhibition of mixed PKC isoenzymes isolated from rat brain. The results are summarized in Table 1.

Both fungi produced more highly-inhibitory extracts when cultured in the soy meal/glycerol medium, indicating a higher production of active compound. It is interesting to note that while *V. balanoides* produced moderately active extract when grown in the cornmeal/tomato paste medium, *C. ophioglossoides* extract from that medium was completely inactive. For these reasons, the large-scale fermentations of both organisms were carried out in the soy meal/glycerol medium. Yields of active compound from 5-liter soy meal/glycerol cultures of *C. ophioglossoides* were ~10 mg/liter and from *V. balanoides* were ~9 mg/ liter. The latter represented a thirty-fold increase in production over fungus grown in the cornmeal/tomato paste medium.[1]

Table 1. Effect of culture medium on inhibition of rat brain PKC.

Organism	Medium	PKC inhibition IC_{50} (μg/ml)
Verticillium balanoides	Corn meal/tomato paste	12
	Soy meal/glycerol	3.5
Cordyceps ophioglossoides	Corn meal/tomato paste	>50
	Soy meal/glycerol	2.5

Isolation and Structure Elucidation

Both the mycelia and lyophilized broth from a total of 10 liters of *C. ophioglossoides* culture were

extracted with MeOH. This extract after solvent partitioning was chromatographed over C_{18} (vacuum-liquid chromatography using water and MeOH) with final purification by C_{18} HPLC (water - acetonitrile - MeOH). Approximately 100 mg of compound was isolated and characterized by ^{1}H and ^{13}C NMR spectroscopy (see experimental); FAB-MS *m/z* 551 $(M+H)^+$; and optical rotation $[\alpha]_D$ $-127.8°$ (*c* 0.27, MeOH); in all cases, it was similar to **1**.[1] As further confirmation, 2D NMR correlation experiments were employed to determine the relative connectivities of the three major fragments readily deduced from the ^{1}H NMR spectrum—an hexahydroazepine ring with amide and ester substituents on C-3 and C-4, respectively; a *para*-hydroxybenzoic acid; and a benzophenone moiety composed of 1,2,3-trisubstituted and 1,2,3,5-tetrasubstituted benzene rings. ^{1}H-^{13}C heteronuclear multiple-quantum coherence spectroscopy (HMQC) was used to assign the direct carbon-proton connections, and analysis of the ^{1}H-^{13}C heteronuclear multiple-bond correlations (HMBC) unambiguously provided the arrangement of the aromatic fragments on the central hexahydroazepine ring. The critical correlations are shown in Fig. 1.

The amide linkage was located on C-3 of the hexahydroazepine since H-3 (4.36 ppm) was coupled to the amide proton (8.37 ppm), H-4 (5.20 ppm), and the AB pair H-2a and H-2b adjacent to the ring nitrogen (3.13 and 3.09 ppm). Additionally, in the HMBC experiment, the correlation of the C-1′ carbonyl (165.9 ppm) to H-3 (4.36 ppm) supported this assignment. C-1′ also correlated to the amide proton (8.37 ppm) and the degenerate H-3′, H-7′ of the *para*-hydroxybenzoic acid (7.63 ppm). Thus, the *para*-hydroxybenzamide moiety was definitively positioned at C-3 of the hexahydroazepine. Similarly, the ester linkage was fixed at C-4 by a correlation

Fig. 1. Selected ^{1}H-^{13}C HMBC (600 MHz, DMSO-d_6) data for the *Cordyceps ophioglossoides* isolate.

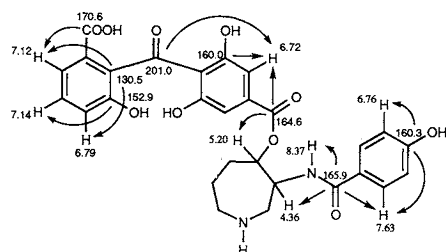
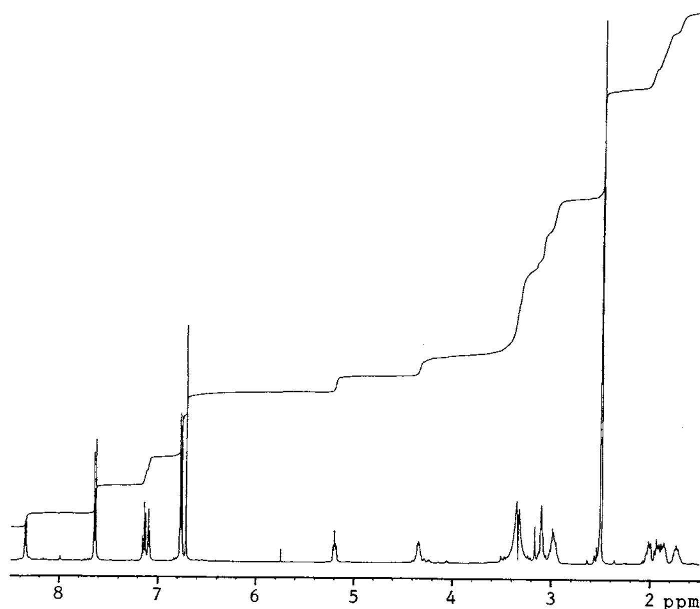


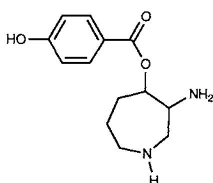
Fig. 2. ^{1}H NMR spectrum (600 MHz, DMSO-d_6) of balanol from *Verticillium balanoides* mixed with *Cordyceps ophioglossoides* isolate. The water peak at 3.35 ppm has been suppressed.



between the C-1″ carbonyl (164.6 ppm) and H-4 (5.20 ppm). C-1″ also showed a correlation to the degenerate H-3″, H-7″ (6.72 ppm) which in turn correlated to the ketone C-8″ (201.0 ppm). Other correlations supported the assignments of the quaternary carbons, especially of the carbonyls whose correct assignments were crucial in determining the proper connections of the amide and ester. The above results, including the specific rotation, suggested that the substance isolated from *C. ophioglossoides* was the same as balanol. However, the ^{1}H NMR spectra of the two samples were not superimposable. We attributed the differences in certain resonances to concentration-dependence, since a 1 : 1 mixture of the isolates from both fungi gave only one set of resonances (Fig. 2).

Multiple growths of *C. ophioglossoides* provided the same results and led to the inescapable conclusion that the fungus was producing balanol (**1**). How then to explain the reported isolation of ophiocordin (**3**) from the same organism? The two compounds share identical structural features with only two exceptions: **3** has the C-3 amide and C-4 ester linkages involving the benzophenone and *para*-hydroxybenzoic acid, respectively—this arrangement being the opposite of that in **1**; and the benzophenone of **3** is linked through the acid of the trisubstituted benzene ring rather than through the acid of the tetrasubstituted benzene ring as in **1**. In the reported structure elucidation of ophiocordin,[6] the location of the aromatic moieties was determined on the basis of degradation with 3 N HCl at 100°C, subsequent isolation of fragments, and characterization by NMR and MS data. The isolation of fragment **4** was used to assign the *para*-hydroxybenzene portion to an ester linkage at C-4 of the azepine. However, under such severe hydrolysis conditions, it is not unreasonable to suggest that transesterification could have readily occurred such that the benzamide moiety was converted to a benzoate ester. Similarly, fragment **5** was formed by treatment of **3** with 1.5 N methanolic HCl (room temperature) and then 3 N HCl (100°C) followed by silylation and was characterized by MS data. This also does not conclusively prove that the ester or amide linkage is through the acid of the trisubstituted benzene ring; again, ester scrambling is quite possible under such harsh conditions.

Since analysis of degradation products was the only method used to determine the locations of the ester and amide, it is possible that these assignments were incorrect. However, the irrefutable differences in the pyridine-d_5 ^{1}H NMR spectra[6] and the opposite optical rotations of balanol and ophiocordin must be addressed. The reported isolation of ophiocordin differs significantly from that of **1** (see experimental) in that it utilizes HCl acidification of the culture broth to pH 2.5 prior to *n*-BuOH extraction.[5] We speculated that the hydrochloride salt may have been isolated by KNEIFEL *et al.*,[5] and that this could account for the differences observed in the ^{1}H NMR spectra. Treatment of **1** with HCl-saturated MeOH at room temperature gave the hydrochloride salt, and a ^{1}H NMR spectrum in 3 : 1 CD_3OD/D_2O was recorded. This was nearly identical (Table 2) to the published spectrum of ophiocordin recorded in CD_3OD/D_2O,[5] the two spectra differing only slightly in the chemical shifts of the aromatic H-11″, H-12″, and H-13″ protons. Deshielding of the H-2 and H-7 methylenes α to the hexahydroazepine nitrogen are



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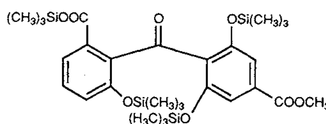

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Table 2. Comparison of ^1H NMR chemical shifts for balanol hydrochloride and ophiocordin in CD_3OD/D_2O and pyridine-d_5. Coupling constants are reported in Hz.

	3:1 CD_3OD/D_2O		Pyridine-d_5	
	Balanol hydrochloride	3[5]	Balanol hydrochloride	3[6]
H-2a, H-2b	3.44 (2H, m)	~3.5 (2H, m)	3.27 (1H, dd) J=13.8, 1.8 3.20 (1H, dd) J=13.8, 5.0	3.85 (2H, AB) J=13.2
H-3	4.52 (1H, m)	~4.6 (1H, m)	4.80 (1H, m)	5.32 (1H, m)
H-4	5.39 (1H, m)	~5.4 (1H, m)	5.73 (1H, dt) J=10.4, 6.5	6.04 (1H, m)
H-5a, H-5b	1.97~2.32 (4H, m)	~2.0~2.3 (4H, m)	2.02 (2H, m)	2.16~2.42 (4H, m)
H-6a, H-6b	1.97~2.32 (4H, m)	~2.0~2.3 (4H, m)	1.52~1.76 (2H, m)	2.16~2.42 (4H, m)
H-7a	3.33 (obscured)	Obscured	2.94 (1H, dd) J=11.8, 5.9	3.28 (1H, m)
H-7b	3.33 (obscured)	Obscured	2.80 (1H, m)	3.55 (1H, m)
CONH	—	—	8.81 (1H, d) J=7.8	9.9 (1H, d) J=8.7
H-3′, H-7′	7.62 (2H, d) J=8.7	7.64 (2H, d) J=8.8	8.21 (2H, d) J=8.6	8.51 (2H, d)
H-4′, H-6′	6.83 (2H, d) J=8.7	6.85 (2H, d) J=8.8	7.13 (2H, d) J=8.6	7.13 (2H, d)
H-3″, H-7″	6.93 (2H, s)	6.94 (2H, s)	7.49 (2H, s)	Obscured
H-11″	6.88 (1H, dd) J=7.6, 1.6	7.2 (1H, dd) J=8, 1	7.34 (1H, d) J=8.0	Obscured
H-12″	7.24 (1H, t) J=7.6	7.34 (1H, t) J=8	7.40 (1H, dd) J=8.0, 7.2	7.58 (1H, s)
H-13″	7.29 (1H, dd) J=7.6, 1.6	7.45 (1H, dd) J=8, 1	8.07 (1H, d) J=7.2	8.1 (1H, d)

typical for an amine salt and were observed in both spectra. When the spectrum was recorded in pyridine-d_5, however, there were still many significant differences (Table 2) when compared to the published spectrum of ophiocordin in pyridine-d_5.[6] Again, these differences may be due to concentration dependence.

Optical rotation of the hydrochloride salt of **1** was also obtained (−98.2° *c* 0.11, MeOH); the direction was again the opposite of that reported for ophiocordin (+70° *c* 1, MeOH).[6] Optical rotation of **1** itself could not be obtained in MeOH at a concentration of 1 g/100 ml due to incomplete solubility; however, addition of water allowed complete dissolution, and a rotation of −103.5° (*c* 1.14, MeOH-water) was obtained, indicating that an unusual concentration-dependent reversal of rotation is not occurring. One possible way to explain the opposite rotations is to assign ophiocordin as the enantiomer of balanol. The absolute stereochemistry of **3** had been proposed as 3*R*,4*R* by gas chromatographic analysis of the N-(L-α-chloroisovaleryl-O-trimethylsilyl)-derivatives of the hexahydroazepine fragment and comparison of retention times with those of other such derivatized aminoalcohols.[6] This is not considered to be a rigorous proof, and since the 3*R*,4*R* absolute stereochemistry of **1** was unambiguously determined by X-ray diffraction, there was still room to suggest that the two compounds were enantiomers. Recently, however, KÖNIG's group reported the enantiospecific synthesis of the (3*R*,4*R*)-3-amino-4-hydroxyhexahydroazepine and found it to be identical to the hexahydroazepine portion obtained by degradation of **3**.[7]

The results of our non-destructive NMR structural analyses of **1** have recently been corroborated by the total synthesis of balanol. The total synthesis of **3** has thus far been elusive, and unless an authentic sample of **3** can be produced, no rigorous comparison with **1** can be made. In our laboratories, *C.*

ophioglossoides and *V. balanoides* produced the same compound, and we are inclined to conclude that ophiocordin and balanol are identical.

A further aspect of this study is the possibility that *C. ophioglossoides* and *V. balanoides* are conspecific, the former being the teleomorph and the latter the anamorph. Both are parasitic fungi which can be recovered from the same habitat, and we have shown that both produce the same secondary metabolite, namely, balanol (**1**). However, cultural and genetic studies are necessary to determine whether they are, in fact, a single species.

Experimental

General

TLC was performed on precoated silica gel plates (Merck HPTLC Silica Gel 60 F_{254}, 0.25 mm) eluting with 4 : 1 : 1 *n*-BuOH - water - AcOH. Visualization was with UV (λ=254 nm) and 0.1% ninhydrin - EtOH spray reagent followed by heating. HPLC was performed on two systems: a Waters 600E system with a Waters 486 tunable absorbance detector (λ=254 nm) using a Waters μBondapak C_{18} column (7.8 × 300 mm, flow rate 3 ml/minute) and water - acetonitrile gradient elution; and a Rainin SD-1 system with an UV-1 absorbance detector (λ=254 nm) using a Dynamax 60A C_{18} column (41.4 × 250 mm, flow rate 25 ml/minute) and water - 37.5% acetonitrile - MeOH gradient elution. NMR spectra were recorded on Varian Unity (^{1}H: 600 MHz) and Gemini (^{1}H: 300 MHz, ^{13}C: 75 MHz) spectrometers. FAB-MS were obtained through M-Scan, Inc., using a VG Analytical ZAB 2-SE high-field mass spectrometer. Optical rotations were determined on a Perkin-Elmer model 241 polarimeter at ambient temperatures.

Fermentation

Verticillium balanoides (Drechsler) Dowsett *et al.* was isolated from a yellow rhizomorph in *Pinus palustris* Mill (Pinaceae) needle litter located in a U.S. Forest Service *P. palustris* forest near Hoffman, North Carolina, U.S.A. *Cordyceps ophioglossoides* (Ehrenberg *ex* Link) Fries (strain TÜ 276)[5] was obtained from American Type Culture Collection, Rockville, Maryland, U.S.A. (ATCC 36865). Both *V. balanoides* and *C. ophioglossoides* were maintained on malt extract 1% and agar 1.8%. The organism was transferred to a Corning 50 ml conical centrifuge tube containing 6 ml of YePD broth consisting of yeast extract (Difco) 1.0%, peptone (Sigma) 2.0%, and dextrose 2.0%. The 6 ml cultures were incubated for 7 days at 21°C (Labline 3590 orbital shaker, 200 rpm) before being transferred to 75 ml YePD broths in 250 ml Erlenmeyer flasks and further incubated for 7 days. These growths were then either directly harvested for the small-scale medium optimization analyses or used to inoculate six or seven 75 ml broths consisting of either (a) cornmeal 2%, tomato paste 2%, yeast extract 1%, and mineral salts solution 1 ml ($FeSO_4 \cdot 7H_2O$ 5 mg, $MnSO_4 \cdot H_2O$ 2 mg, $MgSO_4 \cdot 7H_2O$ 1 mg, $CuCl_2 \cdot 2H_2O$ 2.5 mg, $ZnSO_4 \cdot 7H_2O$ 2 mg, $CaCl_2 \cdot 2H_2O$ 1 mg, H_3BO_3 0.5 mg, $(NH_4)_6Mo_4O_{24} \cdot 4H_2O$ 0.2 mg, and $CoCl_2$ 2 mg in 1 liter HPLC grade water) or (b) 8/16 or 20/80 soy grits (Carsoy, soaked with water for 2 hours and filtered before use) 2% and glycerol 1.6%, pH adjusted to 6.0 with 0.5 N NaOH. These cultures were shaken for three weeks at 17 ~ 21°C and then filtered to separate the mycelia from the broth. 5 liter cultures were obtained by pooling sixty-six 75 ml cultures grown simultaneously and inoculated from the same set of seed cultures.

Isolation

Mycelia from 5 liters of *C. ophioglossoides* culture were filtered from the broth which was then lyophilized. The mycelial cake was broken up and extracted with MeOH (3 × 2 liters) which was concentrated *in vacuo* to a brown residue. This was dissolved in water (200 ml) and extracted with hexanes (2 × 200 ml) and EtOAc (2 × 200 ml). The organics were extracted with brine (1 × 50 ml each), and the brine was combined with the aqueous layer which was then extracted with *n*-BuOH (3 × 200 ml, 1.1 g). The lyophilized broth was extracted with MeOH (4 × 1 liter), and the residue obtained after concentration *in vacuo* was partitioned between water (250 ml) and *n*-BuOH (4 × 200 ml, 1.4 g). The TLC profiles of the *n*-BuOH extracts of the mycelia and the broth were similar, and the extracts were combined to give a dark brown residue (2.5 g). The combined material was chromatographed by vacuum-liquid chromatography

(Bakerbond Octadecyl (C_{18}) silica, 40 μm, 391 g in a 600 ml fritted glass funnel height 8.8 cm × diameter 9.6 cm) using water - MeOH step-gradient elution. F13 (MeOH, 1 liter) and F14 (CH_2Cl_2 rinse, 500 ml) were combined after TLC analysis to yield 730 mg of material. Reversed-phase HPLC (C_{18}, water - 37.5% acetonitrile - MeOH gradient elution) gave pure compound (24 mg) eluting in 30 ~ 35% organic mobile phase. Similar treatment of 5 liters of *V. balanoides* culture yielded 77 mg of **1** ~ 80% pure by ^{1}H NMR.

Physico-chemical Characteristics

FAB-MS *m/z* 573 $(M+Na)^+$, 551 $(M+H)^+$; $[\alpha]_D$ −127.8° (*c* 0.27, MeOH); ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.37 (1H, d, J=8.3 Hz, CONH), 7.63 (2H, d, J=8.8 Hz, 3′, 7′-H), 7.13 (1H, d, J=6.8 Hz, 13″-H), 7.13 (1H, t, J=6.8 Hz, 12″-H), 6.78 (1H, dd, J=6.8, 2.0 Hz, 11″-H), 6.76 (2H, d, J=8.8 Hz, 4′,6′-H), 6.72 (2H, s, 3″, 7″-H), 5.20 (1H, ddd, J=8.3, 7.8, 3.4 Hz, 4-H), 4.36 (1H, m, 3-H), 3.13 (1H, dd, J=14.2, 3.9 Hz, 2a-H), 3.09 (1H, dd, J=14.2, 7.3 Hz, 2b-H), 3.00 (1H, dd, J=13.2, 5.4 Hz, 7a-H), 2.95 (1H, ddd, J=13.2, 8.3, 4.9 Hz, 7b-H), 2.01 (1H, m, 5a-H), 1.92 (1H, m, 5b-H), 1.87 (1H, m, 6a-H), 1.73 (1H, m, 6b-H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 201.5 (C-8″), 170.6 (C-15″), 165.9 (C-1′), 164.6 (C-1″), 160.3 (C-5′), 160.0 (C-4″, C-6″), 152.9 (C-10″), 137.6 (C-14″), 134.5 (C-2″), 130.5 (C-9″), 129.2 (C-3′, C-7′), 128.7 (C-12″), 124.7 (C-2′), 119.0 (C-13″), 117.6 (C-5″), 117.1 (C-11″), 114.8 (C-4′, C-6′), 107.6 (C-3″, C-7″), 76.4 (C-4), 52.4 (C-3), 47.4 (C-2), 46.6 (C-7), 28.4 (C-5), 21.3 (C-6).

Preparation of the Hydrochloride Salt of 1

1 (14 mg, ~80% pure) was suspended in MeOH (1 ml), and MeOH saturated with HCl (0.75 ml) was added dropwise. The reaction was stirred for 4 hours concentrated, and chromatographed by HPLC (C_{18}, water - acetonitrile gradient elution) to give the hydrochloride salt (7 mg) eluting at 40% organic mobile phase. FAB-MS *m/z* 551 $(M+H)^+$; $[\alpha]_D$ −98.2° (*c* 0.11, MeOH); ^{1}H NMR (300 MHz, CD_3OD) δ 7.62 (2H, d, J=8.8 Hz, 3′,7′-H), 7.32 (1H, dd, J=7.7, 1.1 Hz, 13″-H), 7.20 (1H, t, J=7.9 Hz, 12″-H), 6.92 (2H, s, 3″,7″-H), 6.85 (1H, dd, J=8.2, 1.1 Hz, 11″-H), 6.77 (2H, d, J=8.8 Hz, 4′,6′-H), 5.36 (1H, dt, J=8.6, 3.4 Hz, 4-H), 4.37 (1H, dt, J=8.6, 5.6 Hz, 3-H), 3.37 (2H, d, J=5.6 Hz, 2a, 2b-H), 3.30 ~ 3.06 (2H, m, 7a, 7b-H), 2.20 ~ 1.80 (4H, m, 5a, 5b, 6a, 6b-H).

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