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SECOIRIDOIDS AND ANTIFUNGAL AROMATIC ACIDS FROM *GENTIANA ALGIDA*

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Key Word Index—*Gentiana algida*; Gentianaceae; secoiridoids; 2′-(*o*,*m*-dihydroxybenzyl)sweroside; sterols; aromatic acids; anofinic acid; fomannoxin acid; antifungal activity.

Abstract—Fractionation of an aqueous acetone extract of the whole herb of *Gentiana algida* gave one new [2′-(*o*,*m*-dihydroxybenzyl)sweroside] and five known secoiridoids, together with anofinic acid, fomannoxin acid, sitosterol, daucosterol, stigmasterol, oleanolic acid, orientin and gentianose. The structures were determined by spectral methods and a few chemical transformations. Anofinic acid and fomannoxin acid were found to be active against *Cladosporium cucumerinum*, a plant pathogenic fungus. Preliminary structure–activity studies indicated that the presence of carboxylic moieties in these acids was presumably a precondition for activity, whereas their methyl esters, inactive to the fungus, were active against the human pathogenic yeast *Candida albicans*. The chemotaxonomic significance of the isolates is discussed briefly.

INTRODUCTION

Species of the Gentianaceae are receiving growing attention owing to their biological importance [1, 2] and chemical diversity [3, 4]. As an extension of our previous investigations of these plants common in South America and Africa [1, 5, 6], we have reported the isolation and structure determination of a novel type of acylated secoiridoid glycoside from *Gentiana rhodentha* (native to southwestern China), a traditional Chinese drug with a long history of use in the treatment of pneumonia, bronchitis, tuberculosis, and inflammations of the gallbladder and liver [3]. The chemical constituents of the whole plant of *G. algida* Pall. (growing in northwestern China), also a folk medicine utilized since ancient times for the treatment of pneumonia [7], remain uncertain although some phenolic compounds were detected in the same species collected in Russia where geographic and climatic conditions are similar to those in the northwestern region of China [8, 9]. However, the secoiridoids gentiopicroside and deglucosytrifloroside were characterized previously from *G. algida* collected in the USA [10]. Bioassay and on-line liquid chromatography (LC)–UV–mass spectrometry (MS) screening of the crude extract showed the presence of antifungal principle(s) and secoiridoids of high molecular weight carrying aromatic unit(s). These observations prompted us to reinvestigate this species collected in northwestern China, and the results are discussed in this paper.

RESULTS AND DISCUSSION

The powdered whole herb of *G. algida* was extracted twice with aqueous acetone at room temperature. The crude extract was screened chemically by LC–UV and LC–MS as described before [3] (Fig. 1). Special attention was paid to constituents which exhibited UV absorption bands near 237, 255 and 325 nm, and showed the elimination of a neutral fragment of 136 mass units in the on-line thermospray (TSP) mass spectra, as these spectral features were found to be typical of secoiridoids with aromatic residues [4]. The extract was separated by flash chromatography on silica gel. Column chromatography fractions were subjected to LC–UV analyses. Further fractionations were accomplished by gel filtration on Sephadex LH-20, medium pressure liquid chromatography (MPLC) and semi-preparative HPLC to afford the widespread plant sterols sitosterol, daucosterol and stigmasterol, the trisaccharide gentianose, the flavone *C*-glycoside orientin, the triterpene oleanolic acid, the new (**6**) and known (**1**–**5**) secoiridoids as well as anofinic acid (**7**) and fomannoxin acid (**8**).

The structure of **1** was established from its spectral data. The ^{1}H and ^{13}C NMR spectra were similar in part

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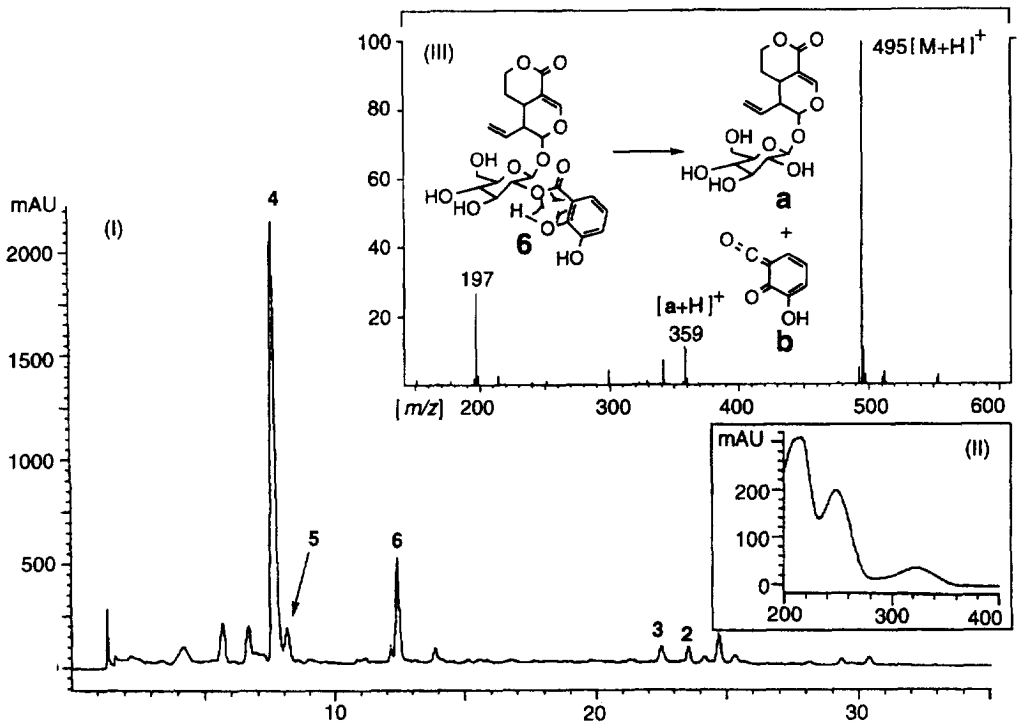
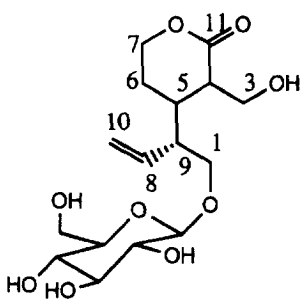


Fig. 1. The LC–MS and LC–UV analyses of the aqueous acetone extract of *G. algida*. (I) The HPLC chromatograph of the crude extract; (II) the on-line UV spectrum of compound **6**; (III) the on-line TSP mass spectrum and fragmentation of compound **6**.

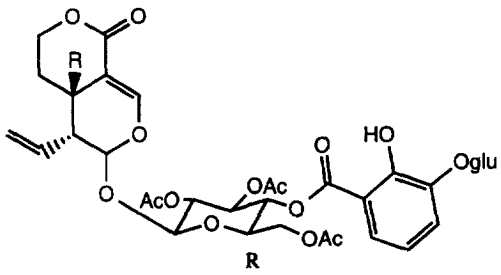
to those of sweroside (**5**) [3]. The proton coupling sequence could be deduced from the ^1H–^1H COSY spectrum, which indicated that the H-4 and H-9 signals were coupled with different oxygenated methylene functions. This observation, along with the evidence from the DEPT pulse sequence and the D/CI mass spectrum, led to the structure of secoiridoid **1**, identical with 1-*O*-β-D-glucopyranosylamplexine recently characterized from *Anthocleista amplexicaulis* (Loganiaceae) [11].

The UV spectra of **2** and **3** were nearly identical (UV absorption maxima for both were at 238, 255 and 323 nm). However, the TSP mass spectra showed that the quasi-molecular ion of **3** (at *m/z* 816) was 16 mass units higher than that of **2** (at *m/z* 800). These findings, combined with the ^1H and ^{13}C NMR spectral data, established the identities of **2** and **3** as trifloroside and rindoside, respectively, the bitter principles characterized previously from *G. gelida* [12] and *G. scabra* var. *buergeri* [13]. Secoiridoid glycosides **4** and **5** possessed very similar polarity, but strikingly different UV spectra. The mass spectrum of **4** gave a molecular weight which was 2 a.m.u. higher than that of **5**, indicating **4** to be the corresponding dehydro-derivative. This assumption was confirmed by the ^1H and ^{13}C NMR data, which resulted in the identification of **4** and **5** as gentiopicroside and sweroside, respectively, both being widespread in the Gentianaceae [14].

The UV spectrum of the new compound **6** (Experimental), similar to those of **2** and **3** (see above), suggested that it was also a secoiridoid glycoside bearing an aromatic nucleus. Furthermore, the TSP mass spectrum exhibited an intense quasi-molecular ion at *m/z* 495 $[M + H]^+$. The ^{13}C NMR spectrum of **6**, edited by DEPT pulse sequences, afforded a total of 23 resonance lines consisting of four methylene (two oxygenated C at δ 69.36 and 62.57), 13 methine (eight oxygen-bearing C at δ 97.49, 97.10, 78.17, 75.54, 75.37, 71.87, 69.40 and 62.68) and six quaternary (two ester carbonyl C at δ 171.30 and 167.23) carbons. Summarizing these data, a molecular formula of $C_{23}H_{26}O_{12}$ was determined. In the ^1H NMR spectrum of **6**, a set of 1,2,3-trisubstituted benzene signals at δ 7.30 *dd* (J = 8.0 and 1.8 Hz), 7.01 *dd* (J = 8.0 and 1.8 Hz) and 6.72 *t* (J = 8.0 Hz) could be assigned to a 2,3-dihydroxybenzoyl group whose presence was reinforced by the typical carbon resonances at δ 113.57 (C-1″), 151.26 (C-2″), 147.13 (C-3″), 122.31 (C-4″), 120.51 (C-5″), 120.94 (C-6″) and 171.30 (C-7″) [4]. Except for the aromatic proton signals, the ^1H NMR spectrum of **6** was very close to that of **5**. All proton signals could be assigned from the ^1H–^1H COSY spectrum and by comparison with those of sweroside (**5**) [3] and its *m*-hydroxybenzoates [15]. H-1′ and H-2′ resonances (Experimental) were seen downfield from those of sweroside by *ca* 0.3 and 1.2 ppm, respectively [3]. Accordingly, **6** was most likely a 2′-(*o*,*m*-dihydroxy)benzoyl derivative of sweroside. To confirm this hypothesis, sweroside was liberated by the mild alkaline hydrolysis of **6** with 0.5 M NaOH followed by neutralization with acetic acid. Moreover, the position of attachment of the aromatic moiety to the sweroside nucleus was established from the

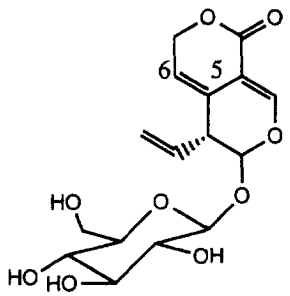


1



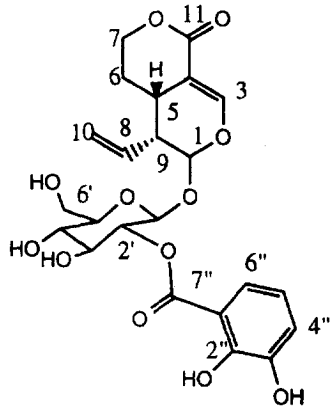
R

2 H
3 OH

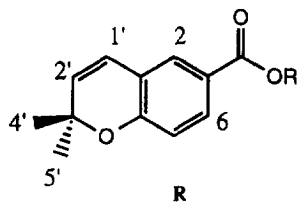


4

5 5β,6-dihydro

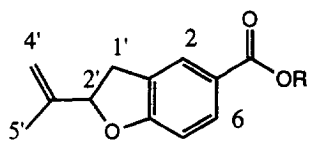


6



R

7 H
7a Me



R

8 H
8a Me

^{13}CNMR spectra of sweroside and its *m*-hydroxybenzoyl esters. Benzoylation at the sugar residue is known to cause a downfield shift for α-carbon and upfield shifts for β-carbons. A similar observation was made between the ^{13}CNMR spectra of depresteroside and its desbenzoyl product [4]. The chemical shifts of sugar carbons of **6**, which were closely similar to those of desacetylcentapicrin [14], showed the expected shift variations of C-1′ to C-3″ (if compared to those of sweroside [3] and decentapicrins A–C [15]), showing the *o,m*-dihydroxybenzoyl group to be attached at C-2′. Finally, this structure was confirmed by its TSP mass spectrum in which the intense peak at *m/z* 359 corresponded to a typical protonated fragment (**a** + H^+) produced through a rearrangement, leading to the elimination of a neutral molecule (**b**) (Fig. 1). A similar fragmentation pattern, confirming the presence of *o*-hydroxybenzoyl-like substituent(s), was also observed in the desorption/chemical ionization mass spectra of deglucosyltrifloroside and 2-hydroxybiphenylcarbonyl derivatives of sweroside [16].

The spectral data for **7** showed that it was anofinic acid, first isolated from *Anodendron affine* (Apocynaceae) [17] and reisolated from the fungi *Lactarius deliciosus* (Russulaceae) [18] and *Curvularia fallax* (Hyphomycetes) [19]. Its methyl ester was characterized from *Piper hostmannianum* (Piperaceae) [20]. Compound **8** was identified as fomannoxin acid (2-isopropenyl-2,3-dihydrobenzofuran-5-carboxylic acid) by comparing its spectral data with those previously reported [21] and those for fomannoxin [22]. This acid was previously found to be a metabolite of a pathogenic basidiomycete *Heterobasidion annosum* (Syn. *Fomes annosus*) [23]. This is the first isolation of this acid from plant origin.

All constituents isolated were tested for their biological activities against *Cladosporium cucumerinum* and *Candida albicans* by the established methods [24, 25]. Compounds **7** and **8**, the actual antifungal principles in the crude extract, showed growth inhibition of *Cladosporinum cucumerinum* at quantities of 50 and 5 μg, respectively, on a TLC plate (Table 1). The esters **7a** and

Table 1. Antifungal activities of compounds **7**, **8**, **7a** and **8a**

Compounds	Activity against *Cladosporium cucumerinum* (μg)	Activity against *Candida albicans* (μg)
7	50*	> 200
8	5	> 200
7a	> 200	50
8a	> 200	25
Miconazole		0.01
Propiconazole	0.001	

*Minimum amount of compound needed to inhibit fungal growth on TLC plates.

8a, prepared for a structure–activity relationship evaluation, inhibited weakly the growth of *C. cucumerinum*, but were active against *Candida albicans*. The minimum quality of **7a** and **8a** required to inhibit the growth of the yeast on TLC were 50 and 25 μg, respectively (Table 1). Acids **7** and **8** showed no growth inhibition against *C. albicans*. Accordingly, it could be concluded that the carboxylic groups in **7** and **8** are associated with the antifungal activity against *C. cucumerinum* while the ester functions in **7a** and **8a** are needed for the growth inhibition of the yeast *C. albicans*.

The present phytochemical investigation of *G. algida* shows its relationships to other *Gentiana* species such as *G. gelida* [12], *G. scabra* var. *buergen* [13], *G. asclepiadea* [26], *G. macrophylla* [27, 28] and *G. triflora* var. *japonica* [29]. However, an increase in the chemical diversity of Gentianaceae has been achieved by the characterization of amplexine glycoside (**1**), anofinic acid (**7**) and famannoxin acid (**8**), since constituents of these types were detected in Gentianacae for the first time.

EXPERIMENTAL

General. Mps: uncorr. ^{1}H and ^{13}C NMR spectra were measured in CD_3OD and/or $CDCl_3$ at 200.06 and 50.30 MHz, respectively with TMS or solvent signals used as int. standard. D/CIMS and EIMS spectra were recorded on a TSQ 700 Finnigan MAT mass spectrometer. TLC was performed on pre-coated Silica gel 60 F_{254} aluminium sheets, RP-8 and RP-18 WF254S HPTLC plates. Semi-prep. HPLC was conducted with LiChrosorb RP-18 (7 μm, 25 × 1.6 cm i.d.) at a flow rate of 10 ml min^{-1}. The HPLC–UV analyses of the extract and the purity check of the isolates prior to spectral analyses were accomplished on a Hewlett-Packard-1090 (Series II) instrument equipped with a photodiode array detector using a Nova-Pak RP-18 column. An MeCN–H_2O gradient (1:19 → 13:7) (containing 0.1% TFA) over 50 min was applied at a flow rate of 1 ml min^{-1}. The HPLC column was packed with Nucleosil RP-18 (5 μm, 12.5 × 0.5 cm i.d.) for selecting solvent systems for MPLC and semi-prep. HPLC, and with Nova-Pak RP-18 (5 μm, 15 × 0.39 cm i.d.) for other analyt. purposes.

TSP LC–MS analysis. HPLC configuration: Waters 600MS solvent delivery system, on-line UV Waters 490 MS multiwavelength detector and Waters 590 MS pump for post-column addition of buffer. Chromatographic conditions were identical to those for HPLC–UV. An aq. soln of NH_4OAc was added post-column (0.5 M, 0.2 ml min^{-1}) to promote ionization. A Thermospray 2 (Finnigan MAT) interface was used with the following parameters: source temp. 280°, vaporizer 90°, aerosol 322°, filament off mode. MS detection was carried out on a Finnigan MAT TSQ 700 triple stage quadrupole instrument. Spectra (150–1200 amu) were recorded every 1.2 sec.

Plant material. The whole herb of *G. algida*, collected in August 1994 in Pingliang County, Gansu Province, P. R. China, was identified by Dr L. X. Zhang, Department of Biological Sciences & Technology, Nanjing University. A voucher specimen (94-815-LPY) was deposited in the herbarium of the department.

Extraction and isolation. The air-dried and well chopped plant material (1 kg) was extracted (× 2) at room temp. with Me_2CO containing *ca* 2% H_2O. The dried extract (28 g) was subsequently subjected to flash CC (silica gel, 60–230 μm, 800 g), eluting first with $CHCl_3$ and then with a gradient of $CHCl_3$–MeOH (20:2 → 1:9). Seven frs were collected (F-1: 3.8 g, F-2: 4.7 g, F-3: 2.3 g, F-4: 1.2 g, F-5: 2.9 g, F-6: 6.7 g, F-7: 4.3 g). F-3 was sepd into 4 parts (f-3/1, f-3/2, f-3/3, f-3/4) on silica gel (120 g) using a $CHCl_3$–MeOH gradient (25:1 → 1:8). CC of f-3/1 with a petrol–Me_2CO gradient yielded sitosterol (41 mg) and stigmasterol (52 mg) while fractionation of f-3/2 in the same fashion gave oleanolic acid (87 mg). CC of f-3/3 with CH_2Cl_2 with increasing amounts of MeOH afforded oleanolic acid (14 mg) and a mixt. which supplied daucosterol (viz. sitosterol-β-D-glucopyranoside, 24 mg) by gel filtration on Sephadex LH-20 with $CHCl_3$–MeOH (1:1). CC of f-3/4 using a $CHCl_3$–MeOH gradient (15:1 → 1:1) gave mainly a mixt. showing antifungal activity, which afforded **7** (8 mg) and **8** (3 mg) after semi-prep. HPLC with H_2O–MeCN (53:47). F-4 was further subjected to CC, eluting with a gradient of $CHCl_3$–MeOH (10:1 → 1:1) to yield 3 parts (f-4/1, f-4/2, f-4/3). Gel filtration of f-4/1 gave brown pigments and a mixt. which, by semi-prep. HPLC with H_2O–MeOH (12:13), afforded **2** (8.5 mg) and **3** (7.4 mg). CC of f-4/2 using $CHCl_3$–MeOH (10:1 → 1:9) yielded further daucosterol (123 mg) and a black mixt. of no interest. Gel filtration of f-4/3 with MeOH–H_2O (9:1) gave **4** (22 mg) and a complex mixt. of pigments. MPLC of F-5 afforded 3 parts (f-5/1, f-5/2, f-5/3) with H_2O–MeOH system (0 → 90 min, 3:1, 90 → 180 min, 13:7). Semi-prep. HPLC of f-5/2 with H_2O–MeOH (17:8) afforded again **4** (18 mg) and a mixt. (9 mg) of **4** and **5**, while f-5/1 was combined with F-6 after HPLC comparison. Gel filtration of f-5/3 gave further **4** (21 mg) and daucosterol (45 mg). MPLC of F-6 with the same solvent system used for F-5 (see above) gave 3 parts (f-6/1, f-6/2, f-6/3). Semi-prep. HPLC of f-6/1 with H_2O–MeOH (37:63) afforded

6 (7 mg). Gel filtration of f-6/2 using MeOH–H_2O (9:1) supplied **1** (11 mg). MPLC of F-7 combined with f-6/3 gave gentianose (165 mg) and two mixts (f-7/1 and f-7/2). Gel filtration of f-7/1 with MeOH–H_2O (9:1) afforded mainly orientin, while f-7/2 gave **1** (7 mg).

Bioassays. Bioautography with *Cladosporium cucumerinum* and *Candida albicans* for evaluating biological activity of the pure products was performed by the methods described [24, 25], and the results are given in Table 1.

2′-(o,m-*Dihydroxybenzoyl*)*sweroside* (**6**). Yellow amorphous powder, mp: 101–103° $[\alpha]_D^{20}$ − 86° (MeOH; *c* 6.349); UV λ_{max}^{MeOH} nm (log ε) 237 (4.23), 255 (3.11), 325 (1.12). IR ν_{max}^{KBr} cm^{-1}: 3510–3320, 1710, 1690, 1605, 1485, ^{1}H NMR (CD_3OD): δ 5.49 (1H, *d*, *J* = 1.4 Hz, H-1), 7.25 (1H, *d*, *J* = 1.8 Hz, H-3), 3.22 (1H, *m*, H-5), 1.75 (1H, *m*, H-6a), 1.61 (1H, *m*, H-6b), 4.30 (1H, *m*, H-7a), 3.98 (1H, *m*, H-7b), 5.58 (1H, *ddd*, *J* = 16.5, 9.5, 7.8 Hz, H-8), 2.77 (1H, *m*, H-9), 5.35 (1H, *m*, H-10a), 5.26 (1H, *m*, H-10b), 4.93 (1H, *d*, *J* = 7.6 Hz, H-1′), 5.01 (1H, *dd*, *J* = 8.9, 7.6 Hz, H-2″), 3.61–3.85 (3H, *m*, H-3′, H-4′ and H-6′a), 3.46 (1H, *m*, H-5′), 3.91 (1H, *dd*, *J* = 12.1, 1.3 Hz, H-6′b), 7.01 (1H, *dd*, *J* = 8.0, 1.8 Hz, H-4″), 6.72 (1H, *t*, *J* = 8.0 Hz, H-5″), 7.30 (1H, *dd*, *J* = 8.0, 1.8 Hz, H-6″); ^{13}C NMR (CD_3OD, multiplicities by DEPT experiments): δ97.39 (*d*, C-1)[a], 153.39 (*d*, C-3), 105.72 (*s*, C-4), 28.75 (*d*, C-5), 25.68 (*t*, C-6), 69.36 (*t*, C-7), 132.75 (*d*, C-8), 43.31 (*d*, C-9), 121.08 (*t*, C-10), 167.23 (*s*, C-11)[b]; 96.76 (*d*, C-1′)[a], 78.74 (*d*, C-2′), 75.13 (*d*, C-3′)[c], 71.78 (*d*, C-4′), 75.49 (*d*, C-5′)[c], 62.57 (*t*, C-6′), 131.57 (*s*, C-1″), 151.26 (*s*, C-2″), 147.13 (*s*, C-3″), 122.31 (*d*, C-4″), 120.51 (*d*, C-5″)[d], 120.94 (*d*, C-6″)[d], 171.30 (*s*, C-7″)[b] (a,b,c,d: interchangeable assignments).

Hydrolysis of **6** *with* 0.5 *M NaOH.* A soln of **6** (5 mg) in MeOH (0.5 ml) was kept overnight with 0.5 M NaOH (0.2 ml) at room temp. The reaction mixt., after neutralization with HOAc, was purified by prep. TLC with $CHCl_3$–MeOH (7:1) to afford a secoiridoid (*ca* 3 mg) which was identical with sweroside in every respect (co-TLC, co-HPLC and UV spectra).

Fomannoxin acid (**8**). Gum; IR, EIMS and ^{1}H NMR spectral data were identical with those reported [21, 22]. ^{13}C NMR ($CDCl_3$): δ121.63 (*s*, C-1), 128.72 (*d*, C-2), 127.19 (*s*, C-3), 164.56 (*s*, C-4), 109.00 (*d*, C-5), 131.84 (*d*, C-6), 171.81 (*s*, C-7), 33.90 (*t*, C-1′), 86.96 (*d*, C-2′), 143.30 (*s*, C-3′), 112.61 (*t*, C-4′), 17.08 (*q*, C-5′). Treatment of **8** (and **7**) with CH_2N_2 followed by usual work-up gave the Me ester (**8a**), a transparent oil: EIMS *m/z* (rel. int): 218 $[M]^+$ (100), 203 $[M - Me]^+$ (78), 171 (23), 159 (64), 158 (43), 144 (39); ^{1}H NMR ($CDCl_3$): δ7.86 (1H, *d*, *J* = 2.0 Hz, H-2), 6.81 (1H, *d*, *J* = 8.1 Hz, H-5), 7.88 (1H, *dd*, *J* = 8.1, 2.0 Hz, H-6), 3.38 (1H, *dd*, *J* = 15.8, 9.6 Hz, H-1′a), 3.05 (1H, *dd*, *J* = 15.8, 8.1 Hz, H-1b′), 5.26 (1H, *dd*, *J* = 9.6, 8.1 Hz, H-2′), 5.09 (1H, *br s*, H-4′a), 4.93 (1H, *br s*, H-4′b), 1.76 (3H, *br s*, H-5′); ^{13}C NMR ($CDCl_3$): δ122.63 (*s*, C-1), 126.64 (*d*, C-2), 127.05 (*s*, C-3), 163.79 (*s*, C-4), 108.87 (*d*, C-5), 131.15 (*d*, C-6), 166.94 (*s*, C-7), 33.99 (*t*, C-1′), 86.80 (*d*, C-2′), 143.39 (*s*, C-3′), 112.55 (*t*, C-4′), 17.08 (*q*, C-5′), 51.85 (*q*, OMe).

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