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FLAVONOIDS AND BIOACTIVE COUMARINS OF *TORDYLIUM APULUM*

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Key Word Index—*Tordylium apulum*; Apiaceae; dihydro-/furanocoumarins; flavonols; antifungal activity.

Abstract—A new flavonol diglycoside, quercetin-3-*O*-[3,4 diacetyl-α-L-rhamnopyranosyl-(1-6)β-D-glucopyranoside] and an antifungal dihydrofuranocoumarin, 2′(*S*),3′(*R*)-2′-acetoxyisopropyl-3′-acetoxy-2′,3′-dihydroangelicin, together with four other known flavonoids and seven known bioactive coumarins, were isolated from the aerial parts of *Tordylium apulum* and their structures elucidated by NMR and mass spectroscopy.

INTRODUCTION

Tordylium apulum is an annual herb widely used as a spice in Greece. Apart from its essential oil composition [1], it has not been so far investigated phytochemically. Based on preliminary bio-assays for the isolation of potentially new antifungal compounds, a series of bioactive phenolic- [umbelliferone (**3**)], furano- [isopimpinellin (**2**), xanthotoxin (**5**), isobergapten (**8**)] and dihydrofurano-coumarins [columbianetin acetate (**1**), diacetyldihydroangelicin (**4**), cnidiadin (**6**) and columbianetin (**7**)] were isolated, as well as flavonol glycosides [kaempferol-3-*O*-neohesperidoside (**9**), quercetin-(3‴,4‴diacetyl) rhamnosyl-glucoside (**10**), kaempferol-3-*O*-rutinoside (**11**), isorhamnetin-3-*O*-rutinoside (**12**) and quercetin-3-*O*-rutinoside (**13**)] from the aerial parts of this species. We report herein, the isolation of a coumarin (**4**), which was fully elucidated for the first time, and a novel flavonoid (**10**) [2].

RESULTS AND DISCUSSION

The UV spectrum of **4** in MeOH indicated the presence of a 7-hydroxylated, 8- (or 6-) substituted coumarin (see Experimental) and, especially, of a dihydrofurano- (or dihydropyrano-) coumarin due to absorption of the shoulders at 241 and 253 nm [3]. Addition of alkali did not result in any shift of the phenolic absorption implying the absence of any free phenolic group. The coumarin nucleus was further confirmed by the ^{1}H NMR spectrum (200 MHz) in $(CD_3)_2CO$. ^{1}H NMR spectrum integration, in combination with ^{1}H-^{1}H COSY, assigned the multiplet at 6.9–7.0 ppm to the chemical shift of two protons, namely H-6 (in *ortho*-coupling with the H-5 doublet) and to another proton (doublet, 6.6 Hz) coupling with the proton at 5.2 ppm (also a doublet, 6.6 Hz). This AX-spin system together with the upfield signals at 1.6 and 1.7 ppm for the methyl protons are typical of a diacylated 2′(*S*),3′(*R*)-2′-hydroxyisopropyl-3′-hydroxy-2′,3′-dihydro angelicin with esterified dihydrofurano- and *iso*propyl-hydroxy groups [4].

The *cis*-configuration was assigned to H-2′–H-3′ based on the coupling constant (J = 6–7 Hz), generally considered to be consistent with *cis*-configuration of the dihydrofuran protons of 8,9-dihydroselol derivatives [5], the *trans*-configuration having J = 3 Hz. Since the 8(*S*) configuration has been established for 8(*S*)-9-hydroxy-8,9-dihydroselol [5], the relative configuration of 2′(*S*),3′(*R*)-2′-acetoxy*iso*propyl-3′-acetoxy-2′,3′ dihydroangelicin is assigned to compound (**4**), which was further reconfirmed by the DCI-MS spectrum (m/z 346 $[M+18]^+$). The El-mass spectrum, besides the $[M]^-$ at m/z 364, revealed two other fragments, m/z 244 and 229, which are typical of a 2′-(2-hydroxy) *iso*-propyldihydrofuranocoumarin. Furthermore, fragments at m/z 286 $[M-RH_2COOH]^+$ and m/z 244 $[286-OC{=}CHR]^+$, confirmed that the acetyl group was eliminated first from the 2′,3′diacetyl-substituted dihydroangelicin, followed by the OC=CHR group

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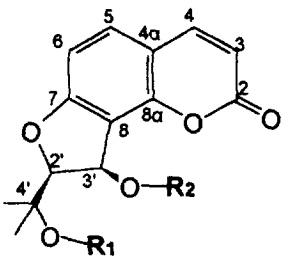
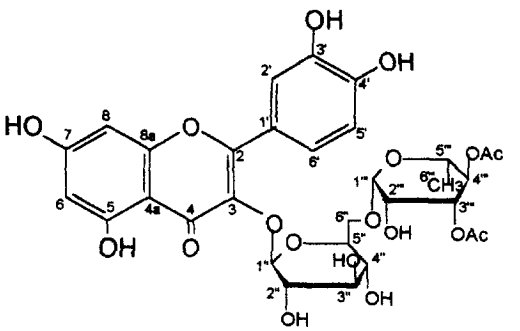


R_1	R_2	
CH_3CO-	H	**(1)**
CH_3CO-	CH_3CO-	**(4)**



(10)

[6, 7]. Of the two acetylated hydroxyl groups in dihydrofuranoangelicin, the acetyl group with the longest side-chain is eliminated first [6, 8].

The m.p. of (**4**) was 135° substantially different from that recorded for vaginidiol diacetate (123°), a compound isolated from *Ligusticum pyrenaicum* [9], whose structure based on ^{1}H NMR (100 MHz) and EI-mass spectrometric data is identical to that of compound (**4**). Vaginidiol diacetate was separated from a complex mixture of 2′,3′-dihydroangelicin esters by preparative silica gel TLC (Et_2O–petrol and CCl_4–CH_2Cl_2), which in our opinion can hardly achieve a satisfactory separation as a first step, and the only one in the case of *L. pyrenaicum*. The different m.p.s between (**4**) and vaginidiol diacetate, the complexity of the original mixture, together with the few available spectroscopic data for vaginidiol diacetate, prompted us to further examine compound (**4**) by ^{13}C NMR (DEPT) and HETCOR, which proved unequivocally that isolated compound (**4**) has indeed the structure of vaginidiol diacetate suggested by Bohlmann in 1969 [9]. The ^{13}C NMR spectrum, together with the DEPT experiment confirmed the carbon atoms included in methine and methyl groups. ^{1}H NMR spectral assignments of the 6.9–7.0 ppm-2H multiplets were reconfirmed by HETCOR results which proved the C-6-H-6 and C-3′-H-3′ couplings. Besides these couplings, the *iso*propyl moiety as a whole, together with the ester moieties were further reconfirmed by a FLOCK experiment (COLOC), by verifying the proton-carbon couplings over three consecutive bonds, namely H-6–C-4a, N-6–C-8 and H-3′–C-7. This experiment originally applied to the structure of a dihydrofuranoangelicin ester, defined unequivocally the structure of **4** and may serve as a useful tool for all acylated dihydrofuran angelicins.

The UV spectrum of (**10**) in MeOH was indicative of a 3-*O*-glycosylated flavonol [10] and UV-spectrometry using MeOH and shift reagents (MeONa, NaOAc–H_3BO_3 and $AlCl_3$–HCl) confirmed the structure of a 5,7,3′,4′-tetrahydroxyflavonol-3-*O*-glycoside (quercetin-3-*O*-glycoside). TSP-mass spectrometry revealed the $[M+H]^+$ at *m/z* 695 and the ion *m/z* 303 corresponding to quercetin [aglycone + H]$^+$. The fragment *m/z* 465 is indicative of a hexose linked directly to the quercetin 3-hydroxy group (465 − 303 = 162), whereas that at *m/z* 230 indicated the presence of a substituted sugar moiety, probably acylated desoxyhexose (695 − 465 = 230), with an acyl-mass of 86 (230 − 144). The 3,5,7,3′,4′-pen-

tahydroxyflavone (quercetin) structure was further confirmed for **10**, based on ^{1}H NMR in DMSO-d_6, which revealed H-2′–H-6′ *meta*-coupling (1.8 Hz) at 7.7 ppm, H-6′–H-5′ *ortho*-coupling (8.5 Hz) at 7.7 ppm and 6.8 ppm, respectively, and H-8–H-6 *meta*-coupling (1.8 Hz) at 6.4 ppm and 6.2 ppm, respectively. As far as the disaccharide moiety is concerned, the doublet (7 Hz) at 5.3 ppm is assigned to the anomeric proton of 3-*O*-β-D-glucopyranose and the doublet (1.7 Hz at 4.4 ppm, together with the doublet (7 Hz) at 1.0 ppm, to the anomeric and methyl group protons of α-L-rhamnopyranose [11]. The nature of the sugars and the way they are linked to the aglycone as deduced by ^{1}H NMR, were confirmed by mass spectrometry for the quercetin 3-*O*-acylated diglycoside.

Furthermore, the double-doublet (2.5 Hz) at 4.6 ppm and signals at −2.0 ppm are indicative of an acylated rhamnose, which causes the acylated carbon-proton to shift upfield in relation to the proton of non-acylated sugar [11]. Specifically, the 2‴- or 4‴-non-acetylated carbon-proton of a 3‴,4‴- or 2‴,3‴-diacetylated rhamnose normally resonate at ca 1.5 ppm downfield in relation to the proton of a non-substituted rhamnose. By comparison between the ^{1}H NMR spectrum of (**10**) to spectral reference data of diacylated rhamnose derivatives [12, 13], the 4.6 ppm double-doublet was assigned to H2‴ (J = 2.5 Hz) of a 3‴,4‴-diacetylated rhamnose. The diacetylated rhamnose-structure was further confirmed by the mass spectral fragmentation pattern and the ion m/z 230 (144 + 43 + 43) and reconfirmed together with the acylation site from ^{13}C NMR/DEPT experiments. When compared with the ^{13}C NMR/DEPT spectrum of quercetin 3-*O*-rutinoside, the structure of quercetin 3-*O*-[(acetylated)α-L-rhamnopyranosyl(1‴–6″)β-D-glucopyranoside] was assigned to (**10**). The DEPT data showed the methylene signal of glucose at 67.3 ppm, which confirmed the 6″–1‴ interglycosidic bond of glucose (directly linked to aglycone) to rhamnose [14]. Rhamnose acetyl-groups were further confirmed by their carbonyl-carbon shifts at 171.7 and 175.2 ppm, and the shifts of their methyl groups at 21 and 25 ppm, respectively [7, 12, 13, 15–18]. Acetylated carbons were assigned by comparison of ^{13}C NMR spectrum of **10** with that of the corresponding quercetin 3-*O*-rutinoside for the sugar methine spectrum-region (60–80 ppm). Only three signals within this region were not identical to that of the non-acylated rutinoside, at 68.5, 70.1 and 73.7 ppm, which were assigned to the 2-,3- and 4- rhamnose carbons, two hydroxyl groups of which were acetylated according to ^{1}H /^{13}C NMR and mass spectrometric data. Sugar carbons with acetylated groups shift downfield (by ca 2 ppm) and those next to the acylation site, shift slight upfield (by ca 1–2 ppm) in relation to the non-substituted ones [9, 10, 14]. Therefore, the signals at 70.1 and 73.7 ppm, were assigned to C-3‴ and C-4‴, which resonate downfield, whereas that at 68.5 ppm was assigned to C-2‴, which resonates upfield (likewise C-5‴) in relation to free rhamnose carbons. Thus, the structure of quercetin-3-*O*-[3,4-diacetyl-α-L-rhamnopyranosyl-(1-6)β-D-glucopyranoside] was assigned to **10**, which is a new natural product.

Plant extract fractions exhibiting antibacterial properties according to the bio-assays used, were further purified in order to obtain the pure bioactive compounds. All the isolated coumarins, except **7**, exhibited antifungal properties against *Cladosporium cucumerinum*. Among them, **8**, **5** and **3** were the most active with inhibition zones of 13, 12.4 and 9 mm, respectively, followed by **1** (7 mm), **2** (5.5 mm), **4** (5 mm) and **6** (4 mm) when spotted on TLC at a concentration of 0.001%. All the isolated coumarins and flavonol glycosides (**1**–**13**) were inactive against both *Candida albicans* and *Bacillus subtilis*.

EXPERIMENTAL

General

M.p.s: uncorr. EI-MS, D/CI-MS and FAB: triple stage quadrupole instrument. ^{1}H and ^{13}C NMR: Varian VXR-200 in DMSO-d_6, $CDCl_3$, CD_3OD and $(CD_3O)_2CO$, with TMS as int. standard. LC-UV: diode-array detection. LC-MS: UV-detector coupled with thermospray module (TSP).

Plant material

The plant material was collected from the Attica region, Athens, Greece, in April 1992, and a voucher specimen is deposited at the Herbarium of the Division of Pharmacognosy, School of Pharmacy, University of Athens (ATPH 294).

Extraction and isolation

Dried aerial parts (4 kg) were ground and extracted successively at room temp. with solvents of increasing polarity, yielding Et_2O (63 g), Me_2CO (43 g) and MeOH (438 g) extracts.

Isolation of coumarins

Et_2O and Me_2CO extracts were combined and defatted with petrol. The crude coumarin residue (50 g) was further fractionated by VLC (*n*-hexane–EtOAc and $CHCl_3$–MeOH gradient systems), affording fractions (I–VIII). Compounds **1** and **2** were obtained from fr. III (934 mg) and separated by MPLC on silica gel (petrol–EtOAc, 4:1); **2** was further purified by HPLC on RP-Silica (MeOH–H_2O, 1:1). Fr. IV (675 mg), was further subjected to low-pressure LC on a diol column (hexane–EtOAc, 3:1) and compound **4** was obtained in a pure crystalline state (56 mg), separately from **5** and **3**, which were further purified by LC on a diol column (hexane–EtOAc, 3:1) and subsequently by CC on silica gel (petrol–$CHCl_3$–MeOH, 8:4:0.2). Compounds **7** and **8** present in fr. V

Table 1. ^{1}H (200 MHz) and ^{13}C NMR (50 MHz) spectroscopic data of compound **4** in $(CD_3)_2CO$ (*J* in Hz)

Position	^{1}H NMR	Position	^{13}C NMR	Position	^{13}C NMR
3	6.2 *d* (9.5)	2	159.8	2′	89.1
4	7.9 *d* (9.5)	3	113.6	3′	69.2
5	7.6 *d* (8.5)	4	144.8	4′	81.2
6	6.9 *d* (8.5)	4a	114.3	gem-C<u>H</u>$_3$	21.2
2′	5.2 *d* (6.6)	5	132.8		22.1
3′	7.0 *d* (6.6)	6	108.1	3′,4′ O<u>C</u>(O)—	169.5
gem-C<u>H</u>$_3$	1.6 *s*,	7	164.3		170.6
	1.7 *s*	8	113.2	3′,4′OC(O)—C<u>H</u>$_3$	22.7
3′,4′OC(O)—C<u>H</u>$_3$	1.9 *3Hs*	8a	152.6		25.8
	2.0 *3H-s*				

Table 2. ^{1}H (200 MHz) and ^{13}C NMR (50 MHz) spectroscopic data of compound **10** in DMSO-d_6 (*J* in Hz)

Position	^{1}H NMR	Position	^{13}C NMR	Position	^{13}C NMR
2′6′	7.7 *2H-dd* (8.5, 1.8)	2	156.4	1″	101.4
3′,5′	6.8 *d* (8.5)	3	133.4	2″	74.0
6	6.2 *d* (1.8)	4	177.3	4″	76.4
8	6.4 *d* (1.8)	4a	103.9	4″	68.9
1″	5.3 *d* (8)	5	161.2	5″	76.0
1‴	4.4 *d* (2)	6	98.6	6″	67.3
		7	164.1	1‴	101.3
		8	93.5	2‴	68.5
		8a	156.4	3‴	70.1
		1′	121.6	4‴	73.7
		2′	115.3	5‴	67.9
		3′	144.7	6‴	17.1
		4′	148.4	3‴,4‴O<u>C</u>(O)—	171.7
		5′	116.2		175.2
		6′	121.2	3‴,4‴OC(O)—C<u>H</u>$_3$	22.1
					25.0

(943 g) were separated by MPLC on a diol column (petrol–EtOAc, 5:1).

Isolation of flavonoids

A part of the MeOH residue (60 g) was subjected to a CC on Sephadex LH-20 (MeOH). From the two main frs, 18.41 g and 7.14 g, respectively, containing the flavonoid complex, the first yielded compound **9** by CPC. The second fr. was further subjected to centrifugal partition chromatography ($CHCl_3$–MeOH–*n*-BuOH–H_2O, 7:6:3:4, lower phase), yielding flavonols **10**, **13** and a mixt. of **11** and **12**. Compound **10** (148 mg), was obtained by Sephadex LH-20 gel filtration (MeOH). Flavonols **11** and **12** were separated on silica RP-18 (MeCN–H_2O, 3:17); **11** was finally purified by HPLC on silica RP-18.

2′(2-acetoxy)-isopropyl-3′acetoxy-2′(*S*),3′(*S*)-dihydroangelicin (*vaginidiol diacetate*) (**4**). White needles, m.p. 134–135° (from MeOH). UV λ_{max}^{MeOH} nm: 207, 320 (shoulders: 265, 253, 241). EI-MS *m/z* (rel. int.): 346 $[M]^+$ (86), 286 (22), 271 (12), 244 (68), 229 (100), 187 (11) and 158 (13). DCI-MS *m/z*: 364 $[M+18]^+$. ^{1}H and ^{13}C NMR: Table 1.

2′(2-acetoxy-)isopropyl-2′(*S*),3′-dihydroangelicin (*columbianetin acetate*) (**1**). M.p. 131–132° (from Me_2CO-MeOH). UV λ_{max}^{MeOH} nm: 209, 326 (shoulders: 269, 256, 246). ^{1}H NMR (200 MHz, $(CD_3)_2CO$): δ 6.2 (1H, *d*, $J = 9.5$ Hz, H-3), 7.9 (1H, *d*, $J = 9.5$ Hz, H-4), 7.4 (1H, *d*, $J = 8.3$ Hz, H-5), 6.7 (1H, *d*, $J = 8.3$

Hz, H-6), 5.2 (1H, *dt*, $J = 2.8$ Hz, H-2′), 3.3 (2H, *dd*, $J = 2.8$ Hz, H-3′), 1.6 (1H, *s*, CH_3), 1.5 (1H, *s*, CH_3) and 1.9 (3H, *s*, $COCH_3$). ^{13}C NMR (50.1 MHz, $(CD_3)_2CO$): δ 160.6 (C-2), 112.7 (C-3), 145.0 (C-4), 114.2 (C-4a), 130.0 (C-5), 107.0 (C-7), 164.7 (C-7), 113.9 (C-8), 152.3 (C-8a), 89.9 (C-2′), 27.8 (C-3′), 82.4 (C-4′), 21.2 (CH_3), 21.9 (CH_3), 22.1 ($COCH_3$), 170.4 ($OCOCH_3$). EI-MS *m/z* (rel. int.): 288 $[M]^+$ (63), 228 (24), 213 (100), 187 (22), 176 (20). DCI-MS *m/z* (rel. int): 306 $[M^+ + 18]^+$ (100), 289 $[M^+ + 1]^+$ (19).

Quercetin-3-*O*-(3‴,4‴ diacetyl-α-L-rhamnopyranosyl-(1‴–6″)β-D-*gluco pyranoside* (**10**). UV λ_{max}^{MeOH} nm: 255, 349; with MeONa: 271, 406 plus 328; with NaOAc: 273, 390 and after addition of H_3BO_3: 260, 377; with $AlCl_3$: 275, 433 and $AlCl_3$ + HCl): 270, 402 and 364; TSP-MS *m/z* (rel. int.): 695 $[M+H]^+$ (69), 465 [303 + Glu]$^+$ (22), 303 [aglycone: M − (Glu + diacylRha]$^+$ (100). ^1H and ^{13}C NMR: Table 2.

Isopimpinellin (**2**), powder, m.p. 149–150° (from MeOH), xanthotoxine (**5**), yellow powder, m.p. 147–148° (from MeOH), isobergaptene (**8**), powder, m.p. 221–222° (from *n*-hexane), columbianetin (**7**), white powder, m.p. 162–163° (from *n*-hexane), umbelliferone (**3**), white crystals, m.p. 222–223° (from *n*-hexane), cnidiadin (**6**), powder, m.p. 145–146° (from *n*-hexane). Spectral data identical to lit. [3, 22, 23]. Kaempferol-3-*O*-neohesperidoside (**9**), kaempferol-3-*O*-rutinoside (**11**), isorhamnetin-3-*O*-rutinoside (**12**), quercetin-3-*O*-rutinoside (**13**). Spectral data identical to lit. [24, 25].

Bioassays. Crude plant extracts, as well as frs of pure substances, were tested by direct bioautography on TLC (silica gel developed with $CHCl_3$–MeOH mixts) at 0.001%, for their activity against *Cladosporium cucumerinum*. Clear inhibition zones were observed after 3 days incubation [20]. Activity against *Candida albicans* and *Bacillus subtilis* was tested by agar overlay bioautography [21].

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