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CHEMICAL STUDIES ON MEXICAN PLANTS USED IN TRADITIONAL MEDICINE, III: NEW 4-PHENYLCOUMARINS FROM *EXOSTEMA CARIBAEUM*[1,2]

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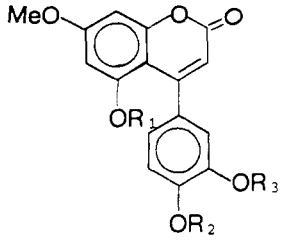
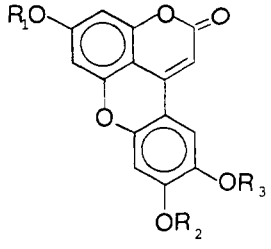
ABSTRACT.—Investigation of the MeOH extract of *Exostema caribaeum* (Rubiaceae) led to the isolation of three new 4-phenylcoumarins. Their structures, 5-*O*-β-D-galactosyl-7-methoxy-3′,4′-dihydroxy-4-phenylcoumarin [**1a**] 7,4′,5′-trihydroxy-4-phenyl-5,2′-oxido-coumarin [**2a**] and 7,4′-dimethoxy-5′-hydroxy-4-phenyl-5,2′-oxido-coumarin [**3a**] were elucidated by spectral methods and chemical transformations. It was also demonstrated that 4-phenylcoumarins undergo oxidative cyclization under basic conditions in the presence of air to give 4-phenyl-5,2′-oxido-coumarins.

Exostema caribaeum (Jacq.) Roem. et Schult. (Syn. *Cinchona caribaea*, Rubiaceae) is a tropical species found in the West Indies, México, and Costa Rica. The stem bark of this plant is used in folk medicine as a substitute for quinine (1-3). Previous phytochemical investigations resulted in the isolation of exostemin and mannitol (4,5). The present work describes the isolation and structure elucidation of three new 4-phenylcoumarins.

Dried stem bark of *E. caribaeum* was extracted with MeOH. The resulting extract was partitioned between EtOAc-MeOH-H_2O (12:1:3). From the EtOAc layer, compound **1a** crystallized. Si gel chromatography of the remaining EtOAc crude fraction allowed the isolation of compounds **2a** and **3a**.

Compound **1a** analyzed for $C_{22}H_{22}O_{11}$. Its H_2O solubility and a positive Molisch test suggested a glycoside (6). The ir absorption frequencies at 1711, 1613, and 817 cm^{-1}, as well as the uv λ max at 225 and 330 nm, were indicative of a coumarin-like structure (7). The ^{13}C-nmr spectra (Table 1) confirmed the presence of 22 carbons, six of which were readily assigned to β-D-galactose (8) and the one at δ 55.9 (q) to a





1a R_1=β-D-Gal, R_2=R_3=H
1b R_1=β-D-Gal, R_2=R_3=Me
1c R_1=β-D-2,3,4,6-tetraacetyl-Gal, R_2=R_3=Ac
1d R_1=R_2=R_3=H
1e R_1=R_2=R_3=Me
1f R_1=R_2=R_3=Ac
1g R_1=H, R_2=R_3=Me
1h R_1=Ac, R_2=R_3=Me

2a R_1=R_2=R_3=H
2b R_1=R_2=R_3=Me
3a R_1=R_2=Me, R_3=H
3b R_1=R_2=Me, R_3=Ac
4a R_1=Me, R_2=R_3=H

[1]For Part II, see Ma T. Reguero, R. Mata, G. Delgado, R. Bye, and E. Linares, *J. Nat. Prod.*, **50**, 315 (1987).

[2]Dedicated to the memory of the late Dr. Pierre Crabbé.

TABLE 1. ^{13}C-nmr Chemical Shifts of Compounds **1a**, **1d**, **3a** (50 MHz, DMSO-d_6, TMS as Internal Standard)

Carbon atom	Compounds		
	1a	**1d**	**3a**
2	159.52 s	160.00 s	160.90 s
3	112.20 d	110.64 d	100.51 d
4	156.30 s	156.81 s	153.70 s
4a	103.30 s	102.02 s	99.80 s
5a	155.44 s	156.20 s	150.49 s
6	98.50 d	98.30 d	96.41 d
7	162.70 s	162.70 s	163.02 s
8	95.22 d	92.90 d	93.12 d
8a	156.00 s	157.03 s	154.50 s
1′	130.44 s	130.44 s	107.14 s
2′	114.70 d	114.72 d	146.32 s
3′	144.10 s	144.20 s	96.00 d
4′	145.71 s	145.70 s	144.31 s
5′	115.51 d	115.60 d	141.00 s
6′	119.30 d	118.94 d	108.70 d
7-MeO	55.90 q	55.60 q	56.14 q
4′-MeO	—	—	56.14 q
1″	101.12 d	—	—
2″	70.13 d	—	—
3″	73.00 d	—	—
4″	68.10 d	—	—
5″	75.90 d	—	—
6″	60.43 d	—	—

methoxyl group; the remaining ^{13}C signals as well as the singlet at δ 6.20 in the ^{1}H-nmr spectrum (Table 2) strongly supported that **1a** was the galactoside of a 4-phenylcoumarin (9). Compound **1a** yielded a trimethylether (CH_2N_2), **1b**, and a hexaacetate (Ac_2O/pyridine), **1c**; acid (HCl 1N) and enzymatic hydrolysis (β-galactosidase) afforded β-D-galactose (tlc) and **1d**.

The aglycone **1d** showed important ions at *m/z* 300 (M^+, base peak), 272 (M^+-CO), and at 257 (M^+-CO-15) (10). The coupling pattern displayed by the aromatic protons (Table 2) and the multiplicities observed for the aromatic carbons in the ^{13}C nmr (Table 1) clearly indicated that one of the benzene rings was trisubstituted, while the other one was tetrasubstituted. The upfield methyl singlets at δ 3.48, 1.59, and 1.42 exhibited in the ^{1}H-nmr spectra of **1e**, **1f**, and **1h**, respectively, (Table 2) suggested that **1d** had a free hydroxyl group at C-5 (5). Because such a diamagnetic effect was not observed in any of the methyl groups of the -OMe or -OAc functionalities of **1b**, **1c**, and **1g**, it was obvious that the sugar portion was attached to position 5 in **1a**. Thereafter, the placement of the methoxyl group at C-7 in **1a** and **1d** was ascertained by the pyridine and acetylation induced shifts (Table 2) (11). The disposition of the hydroxyl functions at C-4′ and C-3′ was confirmed by comparison of the spectral and physical properties of **1e** with those previously reported (12), by the chemical shifts observed in the ^{13}C nmr (Table 1), and by the transformation of **1d** to **4a** by treatment with alkali in the presence of air (MeOH/KOH). Compound **4a** was previously isolated from *Hintonia latiflora* (Sesse et Moc. ex DC.) Bullock (13). Finally, the β configuration of the galactosyl unit was inferred by the coupling constant values for the anomeric proton (J=8 Hz) as well as for the anomeric carbon [^1J ^{13}C-H (1′)= 160 Hz] (14).

Compound **2a** was given the molecular formula $C_{15}H_8O_6$. Its eims showed the

TABLE 2. ^{1}H-nmr Chemical Shifts of Compounds **1a-1h**, **2a**, **2b**, **3a**, **3b**, and **4a** (80, MHz, TMS as Internal Standard)[a]

Compounds	H-3	H-6	H-8	H-2′	H-5′	H-6′	H-3′	OMe					OAc					H-1″	H-2″-H-6″-	OH
								C-7	C-5	C-3′	C-4′	C-5′	C-5	C-3′	C-4′	C-5′	C-2″-C-6″			
1a[b]	6.20 s	7.08 d (3)	6.57 d (3)	7.38 d (3)	7.20 d (8)	6.92 dd (8,3)	—	3.67 s	—	—	—	—	—	—	—	—	—	5.26 d (8)	4.0-4.5 m	3.50 bs
1b[c]	5.95 s	6.58 s	6.58 s		6.92 bs		—	3.85 s	—	3.85 s	3.90 s	—	—	—	—	—	—	4.64 d (8)	3.0-3.5 m	3.25 bs
1c[c]	6.15 s	6.65 d (3)	6.52 d (3)		7.10-7.25 m		—	3.85 s	—	—	—	—	—	2.28 s	2.28 s	—	1.85 s, 1.92 s 1.99 s, 2.18 s	5.05 d (8)	3.8-4.8 m	—
1c[b]	6.05 s	6.84 d (3)	6.68 d (3)	7.20 d (3)	7.50 d (8)	7.35 dd (8,3)	—	3.76 s	—	—	—	—	—	2.28 s	2.30 s	—	1.95 s, 2.00 s 2.10 s, 2.20 s	5.16 d (8)	4.3-5.7 m	—
1d[b]	6.06 s	6.58 d (3)	6.48 d (3)	7.24 d (3)	7.06 d (8)	6.90 dd (8,3)	—	3.70 s	—	—	—	—	—	—	—	—	—	—	—	9.30 bs
1d[d]	5.88 s	6.25 d (3)	6.40 d (3)	6.86 d (3)	6.87 d (8)	6.72 dd (8,3)	—	3.85 s	—	—	—	—	—	—	—	—	—	—	—	3.15 bs
1e[c]	6.00 s	6.23 d (3)	6.51 d (3)		6.74-6.91 m		—	3.86 s	3.48 s	3.92 s	3.86 s	—	—	—	—	—	—	—	—	—
1f[c]	6.07 s	6.82 d (3)	6.48 d (3)		7.10-7.30 m		—	3.83 s	—	—	—	—	1.59 s	2.30 s	2.30 s	—	—	—	—	—
1g[c]	5.96 s	6.27 d (3)	6.56 d (3)		6.87-7.03 m		—	3.84 s	3.90 s	3.90 s	—	—	—	—	—	—	—	—	—	—
1h[c]	6.00 s	6.87 m (3)	6.46 d (3)		6.51-6.93 m		—	3.87 s	3.88 s	3.93 s	—	—	1.42 s	—	—	—	—	—	—	—
2a[d]	5.91 s	6.44 s	6.44 s	—	—	7.21 s	6.73 s	—	—	—	—	—	—	—	—	—	—	—	—	10.30 bs
2b[d]	5.90 s	6.62 s	6.62 s	—	—	7.35 s	6.90 s	3.85 s	—	—	3.90 s	3.86 s	—	—	—	—	—	—	—	—
3a[b]	5.91 s	6.60 s	6.60 s	—	—	7.20 s	6.90 s	3.86 s	—	—	3.90 s	—	—	—	—	—	—	—	—	—
3a[b]	6.31 s	6.55 s	6.55 s	—	—	7.56 s	6.77 s	3.70 s	—	—	3.78 s	—	—	—	—	—	—	—	—	—
3b[b]	6.31 s	6.60s	6.60 s	—	—	7.75 s	6.86 s	3.74 s	—	—	3.80 s	—	—	—	—	2.24 s	—	—	—	—
4a[d]	5.97 s	6.60 s	6.60 s	—	—	7.22 s	6.73 s	3.86 s	—	—	—	—	—	—	—	—	—	—	—	10.35 bs 9.15 bs

[a]Coupling Constants (Hz) in parentheses.
[b]Solvent pyridine-d_5.
[c]Solvent $CDCl_3$.
[d]Solvent $CDCl_3$/DMSO-d_6.

molecular ion at *m/z* 284 (base peak) and fragment ions at *m/z* 256 (M^+-CO) and 228 (M^+-C_2O_2). Successive losses of 28 mass units from the molecular ion as well as the singlet at δ 5.91 in the ^{1}H-nmr spectrum (Table 2) and the ir and uv information suggested a 4-phenyl-5,2′-oxido-coumarin skeleton (13). The trimethylether, [**2b**], obtained by methylation with CH_2N_2 demonstrated the presence of three phenolic hydroxyl groups; the bathochromic shift observed in the uv spectrum upon addition of $AlCl_3$, reversible by HCl, indicated that two of these hydroxyls were in an *ortho* relationship (8). The two one proton singlets at δ 6.73 (H-3′) and δ 7.21 (H-6′), as well as the two protons broad singlet at δ 6.44 (H-6, H-8), were consistent with the locations of the hydroxyls at C-4′, C-5′, and C-7, respectively. Furthermore, the difference in chemical shift between the aromatic protons of **2b** and those of **2a** (ΔH_6-H_8=0.18, $\Delta H_{3'}$=0.17, and $\Delta H_{6'}$=0.14) and the fact that **2b** was identical to the methyl derivative of **4a** were consonant with the substitution pattern proposed.

The spectral properties of **3a** were similar to those of **2a** except that the former exhibited a molecular ion at *m/z* 312 (28 mass units more than **2a**) and showed resonances for two methoxyl groups (singlets at δ 3.70 and 3.78) in the ^{1}H-nmr spectrum in pyridine-d_5 (Table 2). Treatment with CH_2N_2 afforded **2b**; therefore, the substitution pattern was the same as those in **2a** and **4a**. The placement of the hydroxyl group in C-5′ was finally confirmed by the induced shift, upon acetylation, of one of the aromatic signals of **3a** from δ 7.56 (H-6′) to δ 7.75 in **3b**.

Compound **3a** was not toxic to brine shrimp (LC_{50} 1000 ppm, 99% by the IOCD confidence) (15); the biological activity of **1a** and **2a** is currently under investigation. Compound **1a** represents the first 4-arylcoumarin glycoside whose structure has been fully elucidated.

The coexistence of **2a**, **3a**, and **1a** is suggestive of their biogenetic interrelationship, as previously speculated by Bhanu *et al.* (16) during the course of their investigations of chemical transformation of 4-arylcoumarins into xanthones (16). The biosynthesis of **2a** or **3a** from **1a** might involve an oxidative phenol coupling after hydrolysis with a suitable enzyme.

Regarding the transformation of **1d** to **4a** it is important to point out that Bhanu *et al.* (16) achieved this kind of transformation by treatment of 5,2′ oxygenated 4-phenylcoumarins with boiling HI, and, in that case, the oxide ring formation involved the loss of H_2O between the hydroxyl groups located at positions 2′ and 5. However, under basic conditions and in the presence of air, it is not necessary to have any oxygenated substituent at 2′ to achieve cyclization. The reaction, thus, might proceed via an oxidative phenol coupling.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Melting points are uncorrected; ir spectra were recorded on a Nicolet FT-IR instrument, uv spectra were recorded on a Hitachi 220 S double beam spectophotometer with MeOH as solvent and ^{1}H-nmr spectra on a Varian FT spectrometer in $CDCl_3$, $CDCl_3$/DMSO-d_6, or pyridine-d_5 solutions, with TMS as internal standard; ^{13}C-nmr spectra were taken on a 50 MHz chemagnetics A-200 instrument; mass spectra were determined on a Hewlett-Packard 5985-B spectrometer. Si gel 60 (70-230 mesh) was used for column chromatography; tlc was done on Si gel 60 GF 254 plates (Merck), and the spots were visualized with uv radiation, 0.4 N H_2SO_4, or anisaldehyde reagent.

PLANT MATERIAL.—The stem bark of *E. caribaeum* was collected in Apaxtla, Guerrero, México, in May 1986. The botanical identification of this material was made by Dr. David Lorence, Instituto de Biología, UNAM. A voucher specimen was deposited at the National Herbarium, (Voucher 25V 1986 col: F. Calzada y E. Castro).

EXTRACTION AND PRELIMINARY FRACTIONATION.—The air-dried, shredded stem bark (4.5 kg) was defatted with hexane. The dried marc was then macerated 3 times with MeOH at room temperature for 3-day periods. The combined MeOH extracts were evaporated to yield a brown residue (1.3657 kg). A portion (382 g) of the MeOH extract was partitioned between EtOAc-MeOH-H_2O (12:1:3), using a continu-

ous liquid-liquid extractor. Evaporation of the organic solvent yielded a residue (104.5 g) that proved, as shown by tlc, to be a complex mixture of products.

ISOLATION OF 5-O-β-D-GALACTOSYL-7-METHOXY-3',4'-DIHYDROXY-4-PHENYLCOUMARIN [**1a**].—From the organic layer of the partition process, a solid precipitated spontaneously, and this solid, upon repeated recrystallizations from Me_2CO/EtOH, afforded 23.21 g of **1a** (1.84% yield), mp 228-231°; uv λ max (MeOH) 208, 255, 330 nm; ir (KBr) 3650, 3403, 1719, 1613, 1160, 1078, 1049 cm^{-1}. Anal. calcd for $C_{22}H_{22}O_{11}$: C, 57.14; H, 4.76. Found: C, 56.98; H, 4.89.

ISOLATION OF 7,4',5'-TRIHYDROXY-4-PHENYL-5,2'-OXIDO-COUMARIN [**2a**] AND 7,4'-DIMETHOXY-5'-HYDROXY-4-PHENYL-5,2'-OXIDO-COUMARIN [**3a**].—The concentrated organic residue (104.5 g), resulting from the partition process, was chromatographed in a glass column packed with Si gel (1 kg). The initial eluting solvent was $CHCl_3$ with the percentage of EtOH slowly allowed to increase with time. Fractions of 500 ml each were collected. From fractions 59-73, eluted with $CHCl_3$-EtOH (99:1), was obtained a yellow, crystalline material, which upon recrystallization with EtOH yielded 542 mg (0.14% yield) of **3a**, mp 273-274°; uv λ max (MeOH) 262, 308, 372, 388 nm; ir (KBr) 3330, 3400, 3175, 3160, 1677, 1618, 1523, 1458, 1289, 1205, 1169, 1120, 817 cm^{-1}; eims *m/z* (rel. int.) 312 (100), 284 (30.1), 269 (10), 256 (51.1). From fractions 328-346, eluted with $CHCl_3$-EtOH (90:10) was obtained 50 mg (0.013% yield) of **2a**, mp 350° (decomp.); uv λ max (MeOH) 262, 310, 350, 372, 390 nm; (+ $AlCl_3$) 325, 404 nm; (+ $AlCl_3$/HCl) 262, 310, 350, 372, 390 nm; ir ν max (KBr) 3425, 3250, 1690, 1630, 1560, 1460, 1298, 1170, 830 cm^{-1}; eims *m/z* (rel. int.) 284 (100), 256 (35), 241 (10), 228 (50).

HYDROLYSIS OF **1a**.—Compound **1a** (1 g) was refluxed for 1 h with 100 ml of 1 N HCl. A yellow powder precipitated from the acid solution, and, after washing repeatedly with H_2O and recrystallization from Me_2CO, 600 mg of **1d** was obtained; galactose was identified in the acid solution by comparison with an authentic sample by tlc. Compound **1d**, mp 138-140°; uv λ max (MeOH) 260, 330 nm; ir ν max (KBr) 3440, 1665, 1626, 1598, 1434, 1379, 1294, 1202, 1159, 1082 cm^{-1}; eims *m/z* (rel. int.) 300 (100), 272 (98), 257 (23), 127 (5).

PREPARATION OF METHYLETHER DERIVATIVES **1b**, **1e**, AND **2b**.—Compounds **1a**, **1d**, and **2a** (100 mg each) were dissolved in MeOH; to each solution was added an excess of CH_2N_2 in Et_2O, yielding derivatives **1b**, **1e**, and **2b**, respectively. Compound **1b**, 107 mg, mp 178-180°; ir ν max (KBr) 3400, 1710, 1620, 1430, 1360, 1270, 1258, 1212, 1180, 1080, 860 cm^{-1}. Compound **1e**, 90 mg, mp 166-168°, lit. mp 169-170° (12); uv λ max (MeOH) 252, 328 nm; ir ν max (KBr) 1730, 1620, 1520, 1470, 1420, 1360, 1110, 1020, 820 cm^{-1}; eims *m/z* (rel. int.) 342 (100), 314 (56.9), 299 (10). Compound **2b**, 80 mg, mp 265°, ir ν max (KBr) 1718, 1640, 1622, 1562, 1476, 1430, 1218, 1008, 845 cm^{-1}; eims *m/z* (rel. int.) 326 (100), 298 (45), 270 (60).

ACETYLATION OF **1a**, **1d**, **1g**, AND **3a**.—To separate solutions of **1a**, **1d**, **1g**, or **3a** (100 mg each) in 1 ml of pyridine was added 1 ml of Ac_2O; the mixtures were kept at room temperature for 24 h, and after usual work up, the acetyl derivatives **1c**, **1f**, **1h**, and **3b**, respectively, were obtained. Compound **1c**, 100 mg, mp 80-81°; ir ν max (KBr) 1753, 1616, 1506, 1433, 1370, 1219, 1168, 1079, 1055, 903 cm^{-1}. Compound **1f**, 90 mg, mp 169-170°; ir ν max (KBr) 1777, 1763, 1724, 1620, 1506, 1430, 1371, 1345, 1212, 1155, 1068, 908 cm^{-1}. Compound **1g**, oily, 90 mg; ir ν max (KBr) 1780, 1767, 1720, 1640, 1530, 1390, 1210, 1080, 900 cm^{-1}; eims *m/z* (rel. int.) 370 (42), 328 (100), 300 (58), 285 (15), 240 (5), 43 (32). Compound **3b**, 50 mg, oily; ir ν max (KBr) 1766, 1707, 1624, 1506, 1465, 1450, 1279, 1171, 1113 cm^{-1}.

CONVERSION OF **1d** TO **4a**.—Compound **1d** (25 mg) was treated with 5% KOH-MeOH (10 ml). The reaction mixture was left at room temperature for 3 h. The basic solution was neutralized with 1 N HCl, extracted with two 50-ml portions of EtOAc, and the resulting organic phase was washed twice with H_2O. The extract was concentrated affording an orange residue which upon recrystallization from EtOH yielded 14.7 mg of **4a**, mp 342° (decomp.), lit. mp 342-345° (13); ir ν max (KBr) 3450, 1700, 1640, 1525, 1480, 1313, 1250, 1180 cm^{-1}; uv λ max (MeOH) 260, 310, 375, 390 nm; (+ $AlCl_3$) 275, 325, 406 nm; (+ $AlCl_3$/HCl) 260, 310, 375, 390. Compound **1d** (50 mg) was dissolved in a solution of NaOMe (prepared by dissolving 15 mg of Na in 3 ml of anhydrous MeOH). The mixture was stirred at room temperature of 1 h. Evaporation of the solvent gave a residue which was recrystallized from EtOH to yield 15 mg of **4a** (ir and tlc).

CONVERSION OF **1b** TO **1g**.—Compound **1b** (300 mg) hydrolyzed in the same manner as previously described for **1a**. After work-up, 120 mg of **1g** was obtained; mp 185-187° (EtOH); ir ν max (KBr) 3400, 1700, 1620, 1360, 1160, 1100 cm^{-1}; eims *m/z* (rel. int.) 328 (100), 300 (43), 285 (15), 229 (10).

ENZYMATIC HYDROLYSIS OF **1a**.—Compound **1a** (5 mg) dissolved in H_2O was mixed with β-galactosidase (adequate amounts) and phosphate buffer (pH 4). The mixture was incubated for 12 h at 28°. The completeness of the hydrolysis was monitored by tlc. Galactose was readily identified by tlc in the hydrolysate.

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