Phytochemistry, Vol. 26, No. 8, pp. 2377–2379, 1987.
Printed in Great Britain.

0031-9422/87 $3.00 + 0.00

FLAVONOL AND SECOIRIDOID GLYCOSIDES FROM *COUTOUBEA SPICATA*

DANIEL SCHAUFELBERGER,* MAHABIR P. GUPTA† and KURT HOSTETTMANN

Institut de Pharmacognosie et Phytochimie, Ecole de Pharmacie de l'Université de Lausanne, 2, rue Vuillermet, CH-1005 Lausanne, Switzerland; †Pharmacognosy Research Unit (FLORPAN), College of Pharmacy and Specialized Institute of Analysis, University of Panama, Republic of Panama

(*Received* 22 *December* 1986)

Key Word Index—*Coutoubea spicata*; Gentianaceae; secoiridoid glycosides; acylated flavonols; clovin; 4″-*trans-p*-coumaroylclovin; chemotaxonomy.

Abstract—Two flavonol glycosides, unusual for the Gentianaceae, clovin and the new quercetin 3-*O*-rhamnosyl-(1 → 6)-(4″-*trans-p*-coumaroyl)galactoside 7-*O*-rhamnoside (4″-*trans-p*-coumaroylclovin) have been isolated from the aerial parts of *Coutoubea spicata*. The bitter principles of *C. spicata* were identified as gentiopicrin and swertiamarin. Minor polyphenolic glycosides were characterized by high pressure liquid chromatography with photodiode array detection (HPLC-UV/Vis).

INTRODUCTION

Coutoubea spicata Aubl. (Gentianaceae) is a tropical herbaceous plant found in Central and South America. The aerial parts have been used in Brazil as a gastrointestinal stimulant [1]. The chemistry of the Gentianaceae from temperate zones has been described in more than 100 publications so far. Tropical members of the family, however, have rarely been studied and as such represent an interesting field for phytochemical investigations. The present work was undertaken in the course of our systematic screening of Gentianaceae for xanthones with possible monoamino oxidase (MAO) inhibition activity [2, 3]. However, xanthones could neither be isolated, nor detected by HPLC–UV/Vis. On the other hand, we obtained quercetin 3-*O*-robinobioside 7-*O*-rhamnoside (clovin) **1** and its previously undescribed *p*-coumaric ester: 4″-*trans-p*-coumaroylclovin **2**. The secoiridoid glycosides gentiopicrin and swertiamarin were found to be responsible for the bitter taste of *C. spicata*.

RESULTS AND DISCUSSION

The aerial parts of *C. spicata* were extracted successively with petrol, chloroform and methanol. HPLC was applied to the separation of the methanolic extract, after purification over Polyamide (see Experimental). A photodiode array detector [4] allowed the characterization of compounds **1**–**7** by their UV/Vis spectra [5, 6]. Compound **1** is characterized by a UV spectrum typical of flavonoids [7]. The absorption maxima of **2** correspond to those observed for flavonoids esterified with cinnamic acid derivatives (i.e. an additional band near 300 nm) [8]. The minor constituents **3**–**7** have spectra similar to those of **1** (**3**, **4**) or **2** (**5**–**7**). HPLC of the bitter principles was reported previously [9].

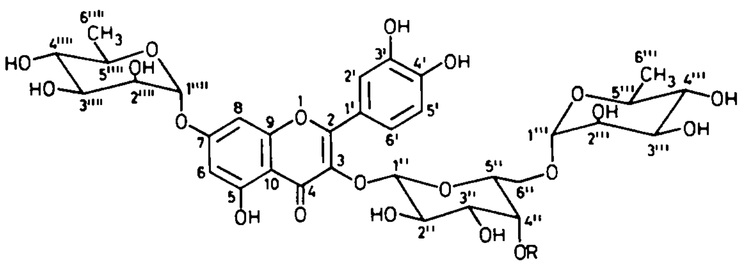
Compound **1** is a glycoside which releases quercetin, galactose and rhamnose upon acid hydrolysis (UV, TLC) [7]. Comparison of the UV spectra of both flavonoids before and after acid hydrolysis, indicates that the sites of glycosylation are at the 3 and 7 positions. The underivatized glycoside was submitted to fast atom bombardment mass spectroscopy (FAB-MS) and to desorption chemical ionization MS (D/CI MS) [10]. The FAB-MS of **1** shows signals corresponding to a quasimolecular ion at *m/z* 755 $[M-H]^-$ and its sodium chloride complex at *m/z* 813 $[(M-H)+NaCl]^-$. The signals found at *m/z* 609 $[(M-H)-146]^-$, 447 $[(M-H)-308]^-$ and 301 $[(M-H)-454]^-$ indicate the successive elimination of rhamnose, galactose, and of a second unit of rhamnose, respectively. The D/CI MS (reactant gas NH_3) did not show any quasimolecular ions but provided evidence of a rhamnogalactosyl unit as indicated by the signal at *m/z* 326 $[308+NH_4]^+$.

Hydrogen peroxide oxidation [8] of **1** yielded a disaccharide (TLC) identical to that obtained when robinin is treated under the same conditions. Compound **1** was not affected by enzymatic hydrolysis with β-galactosidase under standard conditions [8]. The interglycosidic linkage and the positions of glycosylation were finally determined by ^{1}H and ^{13}C NMR spectroscopy. Chemical shifts of the sugar moiety of **1** correspond to those reported for kaempferol 3-*O*-α-L-rhamnosyl-(1 → 6)-β-D-galactoside 7-*O*-α-L-rhamnoside (robinin) [11, 12]. Carbons at C-3, C-7 and their neighbours show chemical shifts typical of flavonol 3-*O*-glycosyl 7-*O*-rhamnosides [13, 14]. Thus, the structure of **1** is quercetin 3-*O*-α-L-rhamnopyranosyl-(1 → 6)-β-D-galactopyranoside 7-α-L-rhamnopyranoside. This compound was first isolated from *Vinca minor* [15] and later from the flowers of *Melilotus alba* (white sweet clover) where it was named clovin [16].

The UV spectrum of **2** (Fig. 1) shows maxima at 256, 268 (sh), 315 and 365 (sh) nm. Alkaline hydrolysis with aq. NaOH 2% [17] afforded *p*-coumaric acid (by comparison with an authentic sample) and a glycoside which was

---

*Present address: Cancer Research Institute, Arizona State University, Tempe, AZ 85287, U.S.A.



1 R = H

2 R = *trans-p*-coumaroyl

found to be identical with **1** (TLC, UV, HPLC-UV/Vis, acid hydrolysis). The FAB-MS spectrum of **2** shows a quasimolecular ion at *m/z* 901 $[M-H]^-$. A signal at *m/z* 755 $[(M-H)-146]^-$ can be attributed to the elimination of either a *p*-coumaroyl or a rhamnosyl moiety. Peaks of lower mass units correspond to those observed in the spectrum of compound **1**. The ^{1}H NMR spectrum of **2** shows all signals observed for compound **1** and additional protons from the *p*-coumaroyl unit. The olefinic proton H-8 at 6.50 ppm (*d*, $J = 16$ Hz) suggests the presence of a *trans-p*-coumaroyl unit (for *cis-p*-coumaroyl moieties: $J = 11$ Hz [18]). The site of esterification was determined by partial hydrolysis and by ^{13}C NMR. Glycoside **2** was treated with an excess of β-galactosidase. Interestingly, under these conditions, the enzyme also cleaves α-rhamnose units. After incubation, the mixture was analysed directly by HPLC-UV/Vis. Compound **2** and three degradation products **2a**–**2c** could be detected. Their UV/Vis spectra indicated that they all carried the ester moiety (acylated sugars are usually not affected by enzymatic hydrolyses [8]). The HPLC separation was repeated and shift reagents added by means of a post-column derivatization kit [5]. The UV/Vis spectra of **2a** and **2c** showed a bathochromic shift of band II when a weak base was added to the column eluate. This shift can be attributed to the presence of a free OH group at position 7 of the aglycone, thus indicating that *p*-coumaric acid must be linked to the sugar moiety located in position 3. The ^{13}C NMR spectrum of **2** is identical to that of **1** as far as the aglycone and the rhamnosyl moieties are concerned, but nine additional signals appear between 114 and 166 ppm (*trans-p*-coumaric acid). Different chemical shifts are observed for the galactosyl carbons: C-4 is moved downfield (+1.5 ppm) whereas C-3 (−1.3 ppm) and C-5 (−1.5 ppm) appear at higher fields compared to the non-acylated compound. This indicates that *p*-coumaric acid is attached at position 4 of the galactose. Compound **2** is therefore the new quercetin 3-*O*-α-L-rhamnopyranosyl-(1 → 6)-(4''-*trans-p*-coumaroyl)-β-D-galactopyranoside 7-*O*-α-L-rhamnopyranoside (4''-*trans-p*-coumaroylclovin).

Coutoubea spicata contains gentiopicrin and swertiamarin, two typical bitter principles of the Gentianaceae [9]. Its content in polyphenolic glycosides, on the other hand, is unique. It has been shown by HPLC-UV/Vis that the aerial parts of *C. spicata* are free of xanthones and flavone glycosides, but contain non-acylated and acylated flavonols. Quercetin 3-*O*-robinobioside 7-*O*-rhamnoside **1** (clovin) and its new *p*-coumaric ester **2** are reported for the first time from a species of the Gentianaceae. Flavonols are rare constituents of this family and their occurrence in *C. spicata* could be of chemotaxonomic interest. HPLC-UV/Vis, with optional post-column derivatization, was found to be very helpful in the characterization of complex mixtures of polyphenolics and can be regarded as the method of choice for the screening of flavonoids esterified with aromatic acids.

EXPERIMENTAL

Plant material. C. spicata was collected in July 1983 in Panama by M.P.G. and a voucher specimen was deposited at the University of Panama.

General techniques. TLC was carried out on pre-coated silica gel 60-F254 aluminium sheets (Merck) with EtOAc–MeCOEt–HOAc–H_2O (50:30:10:10) (system 1) and on pre-coated cellulose F254 aluminium sheets (Merck) with HOAc 5% (system 2). CC was achieved on Polyamide SC 6, particle size $< 70\,\mu$m (Macherey-Nagel) with MeOH–H_2O in different concns and on Sephadex LH-20 (Pharmacia) using MeOH as eluent. Acid hydrolyses and recording of the UV spectra with the usual shift reagents were made according to standard procedures [7, 8]. Sugars were analysed by TLC on silica gel with EtOAc–H_2O–MeOH–HOAc (13:3:3:4) and visualized by spraying with *p*-anisidine phthalate. Alkaline hydrolysis was carried out by dissolving 2 mg of the glycoside in 1 ml of aq. NaOH 2%. After 24 hr (room temp., N_2) the mixture was neutralized with Amberlite 200, acidic form, (Merck) and extracted with ether and butanol [17]. Enzymatic hydrolysis with β-galactosidase (Sigma) was carried out under standard conditions (30°, 24 h, pH 4.6) and under modified conditions: 2 mg of glycoside **2** in 2 ml of acetate buffer (pH 4.6) were incubated with 30 mg β-galactosidase (Sigma G-7138 grade XI) for 48 hr at 30°. The mixture was evaporated to dryness and dissolved in 1 ml MeOH. The filtered solution was directly analysed by HPLC without further purification. HPLC with photodiode array detection (HPLC-UV/Vis) was carried out on RP-8 Hypersil 5 μm, 100 × 4.6 mm I.D. columns (Hewlett Packard) with MeOH–H_2O as the mobile phase. To avoid tailing of phenolic compounds, phosphoric acid was added to the solvents (0.3 ml/l). The mobile phase was delivered by a SP 8700/SP 8750 pump (Spectra Physics). The chromatograms and the UV/Vis spectra (210–450 nm) were recorded with a photodiode array detector HP 1040A (Hewlett Packard). Details for the post-column derivatization with shift reagents are summarized in ref. [5]. The crude methanolic extract was purified prior to HPLC analysis: 10–20 mg in 1 ml of aq. MeOH 20% were introduced onto a small Polyamide SC 6 column (30 × 10 mm I.D.) and washed with aq. MeOH 20% (2 ml). The fraction containing the polyphenolic glycosides was then eluted with 2 ml aq. MeOH

90% and 10 μl of this solution were injected onto the HPLC column. ^{1}H NMR and ^{13}C spectra were recorded in DMSO-d_6 with TMS as int. standard. FAB-MS spectra were produced on a ZAB-IS spectrometer (VG Instruments). The matrix was thioglycerol. D/CI MS spectra were recorded on a Ribermag R 10-10B quadrupole spectrometer with a SIDAR data system.

Isolation. The dried plant material (34 g) was extracted successively with petrol (b.p. 80–95%), $CHCl_3$ and MeOH (under reflux). The crude methanolic extract (3 g) was separated by CC on Polyamide SC 6 with aq. MeOH 20–90%. The isolation of the bitter glycosides from the fraction eluted with MeOH 20% (1970 mg) is described in ref. [9]. The fraction containing **1** (120 mg) was separated by reversed-phase liquid chromatography (RP-8, 40–63 μm, 310 × 25 mm I.D; Lobar column size B, Merck) using a linear gradient of aq. MeOH 25–50% in 150 min. The mobile phase was delivered at a flow rate of 5 ml/min by two HPLC pumps M-6000 with a gradient controller M-720 (Waters). The fraction containing **2** (100 mg) was treated in the same way, using a gradient of aq. MeOH 25–70% in 300 min. The two glycosides were finally purified over Sephadex LH-20 (MeOH), yielding 14 mg of **1** and 11 mg of **2**.

Quercetin 3-*O*-α-L-rhamnopyranosyl-(1 → 6)-β-D-galactopyranoside-7-*O*-α-L-rhamnopyranoside (clovin) **1**: yellow powder; $C_{33}H_{44}O_{20}$, M_r: 756.73, mp 195–197°; R_f = 0.10 (system 1); acid hydrolysis: quercetin, galactose and rhamnose; UV λ_{max} nm: (MeOH) 257, 265 sh, 358; ($AlCl_3$) 274, 300 sh, 330 sh, 437; ($AlCl_3$/HCl) 268, 302 sh, 365 sh, 403; (NaOAc) 258, 290 sh, 370, 415 sh; (NaOMe) 269, 395; (H_3BO_3/NaOAc) 260, 295 sh, 380. MS data are discussed above. ^{1}H NMR (DMSO-d_6): δ12.73 (1H, *s*, OH on C-5), 7.80 (1H, *dd*, H-6′), 7.70 (1H, *d*, H-2′), 6.96 (1H, *d*, H-5′), 6.89 (1H, *d*, *J* = 2 Hz, H-8), 6.56 (1H, *d*, *J* = 2 Hz, H-6), 5.67 (1H, *br s*, H-1⁗), 5.50 (1H, *d*, *J* = 8 Hz, H-1″), 4.54 (1H, *br s*, H-1‴), 1.26, 1.18 (3H, *d*, 2 × Me rhamnose). ^{13}C NMR (DMSO-d_6): δ177.6 (C-4), 161.6 (C-7), 160.6 (C-5), 156.9 (C-2), 155.9 (C-9), 148.7 (C-4′), 144.9 (C-3′), 133.7 (C-3), 122.1 (C-6′), 120.9 (C-1′), 116.1 (C-5′), 115.2 (C-2′), 105.5 (C-10), 101.6 (C-1″), 99.9 (C-1‴), 99.4 (C-6), 98.4 (C-1⁗), 94.4 (C-8), 73.6 (C-5″), 73.0 (C-3″), 71.7 (C-4‴)^a, 71.6 (C-4⁗)^a, 71.1 (C-2″), 70.6 (C-3⁗)^b, 70.4 (C-3‴)^b, 70.2 (C-2‴)^c, 70.1 (C-2⁗)^c, 69.8 (C-5⁗), 68.3 (C-4″), 68.0 (C-5‴), 65.0 (C-6″), 17.9 (C-6‴, C-6⁗).

Quercetin 3-*O*-α-L-rhamnopyranosyl-(1 → 6)-(4″-*trans*-*p*-coumaroyl)-β-D-galactopyranoside 7-*O*-α-L-rhamnopyranoside **2**: yellow powder, $C_{42}H_{46}O_{22}$, M_r: 902.88, mp 214–216°; R_f 0.23 (system 1); acid hydrolysis: quercetin, galactose, rhamnose, alkaline hydrolysis: **1** and *p*-coumaric acid (UV λ_{max} nm: (MeOH) 288, 310 sh; R_f 0.41 (system 2); EIMS: *m/z* 164); UV λ_{max} nm: (MeOH) 256, 268 sh, 295 sh, 315, 365 sh; ($AlCl_3$) 275, 300 sh, 438; ($AlCl_3$/HCl) 274, 300 sh, 400; (NaOAc) 263, 315, 372, 420 sh; (NaOMe) 270 (dec), 365. MS data are discussed above. ^{1}H NMR (DMSO-d_6): δ12.76 (1H, *s*, OH on C-5), 7.85–6.60 (9H, aromatic protons and H-7 coum), 6.50 (1H, *d* 16 Hz, H-8 coum), 5.69 (1H, *br s*, H-1⁗), 5.61 (1H, *d* 8 Hz, H-1″), 4.50 (1H, *br s*, H-1‴), 1.26, 1.01 (3H, *d*, 2 × Me rhamnose) ^{13}C NMR (DMSO-d_6): δ177.5 (C-4), 166.0 (C-9 coum), 161.6 (C-7), 160.9 (C-5), 159.8 (C-4 coum), 157.0 (C-2), 155.9 (C-9), 149.2 (C-4′), 145.1 (C-7 coum)^d, 144.9 (C-3′)^d, 133.4 (C-3), 130.3 (C-2 and C-6 coum), 125.1 (C-1 coum), 121.8 (C-6′), 120.7 (C-1′), 116.4 (C-5′), 115.9 (C-3 and C-5 coum), 115.2 (C-2′), 114.2 (C-8 coum), 105.5 (C-10), 101.8 (C-1″), 100.3 (C-1‴), 99.4 (C-6), 98.4 (C-1⁗), 94.4 (C-8), 71.9 (C-5)^e, 71.7 (C-3″)^e, 71.6 (C-4⁗)^e, 71.4 (C-4‴)^e, 71.0 (C-2″), 70.4 (C-3‴, C-3⁗), 70.2 (C-2‴)^f, 70.1 (C-2⁗)^f, 69.8 (C-4″, C-5⁗) 68.4 (C-5‴), 64.7 (C-6″), 18.0 (C-6‴)^g, 17.8 (C-6⁗)^g. Assignments bearing the same superscript might be interchanged.

HPLC-UV/Vis: spectra recorded in (MeOH–H_2O–H^+), λ_{max} nm: **1**: 253, 268 sh, 352: **2**: 256, 268 sh, 316, 365 sh; **3**: 266, 319 sh, 350; **4**: 252, 277 sh, 297 sh, 340; **5**: 258, 270 sh, 312, 360 sh; **6**: 266, 316, 360 sh; **7**: 258, 270 sh, 290 sh, 315, 365 sh; post-column derivatization: R_t time (min): **2** (9.7), **2a** (10.3), **2b** (10.9), **2c** (11.5); UV λ_{max} nm: (MeOH–H_2O–H^+) **2**, **2a**–**2c**: 255, 267 sh, 295 sh, 315, 365 sh; (Na_2HPO_4) **2**, **2b**: 257, 266, 296 sh, 315, 370 sh; **2a**, **2c**: 275, 315, 377.

Acknowledgement—Financial support by the Swiss National Science Foundation is gratefully acknowledged. We thank Dr W. Richter, Ciba–Geigy AG, Basel (FAB MS), Mr D. Chollet, Zyma SA, Nyon (D/CI MS) and Spectrospin AG, Fällanden (NMR) for spectral measurements. Thanks are also due to the Organisation of American States for the support of the project FLORPAN in Panama through its Regional Scientific and Technological Development Program.

REFERENCES

1. Peckholt, T. (1899) *Ber. Deutsch. Pharm Ges.* **9**, 222.
2. Suzuki, O., Katsumata, Y., Oya, M., Chari, V. M., Klapfenberger, R., Wagner, H. and Hostettmann, K. (1978) *Biochem. Pharmacol.* **27**, 2075.
3. Suzuki, O., Katsumata, Y., Oya, M., Chari, V. M., Klapfenberger, R., Wagner, H. and Hostettmann, K. (1980) *Planta Med.* **39**, 1.
4. Miller, J. C., George, S. A. and Willis, B. G. (1982) *Science* **218**, 241.
5. Hostettmann, K., Domon, B., Schaufelberger, D. and Hostettmann M. (1984) *J. Chromatogr.* **283**, 137.
6. Hostettmann, M., Gafner, F., Marston, A. Schaufelberger, D. and Hostettmann, K. (1986) *Bull. Liaison Groupe Polyphenol* (Narbonne) **13**, 121.
7. Mabry, T. J., Markham, K. R. and Thomas, M. B. (1970) *The Systematic Identification of Flavonoids.* Springer, Berlin.
8. Markham, K. R. (1982) *Techniques of Flavonoid Identification.* Academic Press, London.
9. Schaufelberger, D. and Hostettmann, K. (1985) *J. Chromatogr.* **346**, 396.
10. Domon, B. and Hostettmann, K. (1985) *Phytochemistry* **24**, 757.
11. Moon, C. K., Park, K. K., Park, K. S. and Soo, Y. C. (1983) *Seoul Univ. J. Pharm. Sci.* **8**, 28.
12. Wenkert, E. and Gottlieb, H. E. (1977) *Phytochemistry* **16**, 1811.
13. Markham, K. R. and Ternai, B. (1976) *Tetrahedron* **32**, 2607.
14. Markham, K. R., Ternai, B., Stanley, R., Geiger, H. and Mabry, T. J. (1978) *Tetrahedron* **34**, 1389.
15. Harborne, J. B. and Williams, C. A. (1975) *The Flavonoids* (Harborne, J. B., Mabry, T. J. and Mabry, H. eds) pp. 376–442. Chapman & Hall, London.
16. Nicollier, G. F. and Thompson, A. C. (1982) *J. Agric. Food Chem.* **30**, 760.
17. Romussi, G., Cafaggi, S. and Bignardi, G. (1982) *Pharmazie* **37**, 738.
18. Romussi, G., Parodi, B. and Sancassan, F. (1984) *Liebigs Ann. Chem.* 1867.