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seco-Iridoids from *Calycophyllum spruceanum* (Rubiaceae)

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Dedicated to the memory of Professor Jeffrey B. Harborne

Abstract

Three *seco*-iridoids 7-methoxydiderroside, 6′-*O*-acetyldiderroside and 8-*O*-tigloyldiderroside, were isolated from the wood bark of *Calycophyllum spruceanum* together with the known iridoids loganetin, loganin and the *seco*-iridoids secoxyloganin, kingiside and diderroside. Their structures were elucidated by means of NMR and MS spectral data analysis. Using NOE correlations and coupling constants, the relative stereochemistry of the new derivatives was established. 7-Methoxydiderroside, 6′-*O*-acetyldiderroside and the known secoxyloganin and diderroside showed in vitro activity against trypomastigote forms of *Trypanosoma cruzi*, with IC_{50} values of 59.0, 90.2, 74,2 and 84.9 μg/mL, respectively and were compared to the standard gentian violet (IC_{50} 7.5 μg/ml).
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Keywords: *Calycophyllum spruceanum*; Rubiaceae; Iridoids; *seco*-Iridoids; Antitrypanosomal activity

1. Introduction

Calycophyllum spruceanum DC. (Rubiaceae), a huge endemic tree from the Amazon, has been traditionally used for the treatment of numerous human ailments including mycoses, influenza, diverse infections, cancer and skin diseases throughout "legal Amazonia," which includes Brazil, Peru, Bolivia and Colombia (Correa, 1974; Rizzini, 1978; Garcia, 1975). In Peruvian Amazon, a decoction of the bark is used against "sarna negra", a subcutaneous infection caused by an arachnid (Schultes and Raffauf, 1990). In Brazilian Amazon, this species is known to treat stomach diseases, skin inflammation and uterus tumors (Garcia, 1975). As part of our continuing efforts to study Brazilian Rubiaceae, mainly those with medicinal uses (Carbonezi et al., 1998), *C. spruceanum* DC was collected near Manaus, Amazon State, and submitted to a detailed chemical study. Three new *seco*-iridoids were obtained from the ethanolic extract of *C. spruceanum* wood bark: 7-methoxydiderroside (**1**), 6′-acetyl-β-D-glucopyranosyldiderroside (**2**) and 8-*O*-tigloyldiderroside (**3**), and the known compounds loganetin (Houghton and Ming, 1985), loganin (El-Naggar and Beal, 1980; Garcia and Chulia, 1986), kingiside (Inouye et al., 1974), secoxyloganin (Calis and Sticher, 1984) and diderroside (Adeoye and Waigh, 1983). Several natural products and synthetic compounds, e.g. sesquiterpenelactones from Asteraceae species (Chiari et al., 1991), lignans from *Virola surinamensis* (Myristicaceae) (Lopes et al., 1998) and quinoline derivatives (Nakayama et al., 2001) have been evaluated for antitrypanosomal activity against *T. cruzi*, the parasite of Chagas disease, which affects ca. 18 million people in Latin America and ca. 100,000 people in the United States. Besides prophylaxis, i.e. the elimination of vector insect, specific therapeutic agents are needed in order to replace gentian violet, currently in use, owing to the mutagenic and

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carcinogenic potentials of this dye, as well as to consumer resistance to this treatment since the patient turns blue after transfusion. In this paper, the isolation and structure elucidation of the new *seco*-iridoids **1–3** along with the known compounds are presented. Additionally, the biological evaluation of all isolates against the trypomastigote forms (Y form) of *T. cruzi* was undertaken. The taxonomic significance of these *seco*-iridoids in *C. spruceanum*, is also discussed.

2. Results and discussion

The ethanolic extract of dried and powdered wood bark of *C. spruceanum* was successively fractionated over a silica gel column and finally purified by reversed phase HPLC (see Experimental), affording the new *seco*-iridoids **1–3**. *seco*-Iridoid **1** was isolated as an amorphous powder that gave a $[M+H]^+$ ion at *m/z* 479.4539 in the high-resolution TOF-ESMS compatible with the molecular formula $C_{20}H_{30}O_{13}$. The UV maximum at 230 nm and IR absorptions at 3450, 1695, 1635 and 1280 cm^{-1} suggested that **1** had a conjugated double bond and an enol ether, which is characteristic of the chromophore –OCO–C=CH–O– of iridoids. This assumption was supported by the NMR signal observed at δ 7.44 (1H, *s*) characteristic of H-3 from iridoids. Besides this signal, the ^{1}H NMR spectrum (Table 1) showed three doublets at δ 5.66 (1H, $J=4.5$ Hz), 4.54 (1H, $J=7.5$ Hz) and 1.26 (3H, $J=6.5$ Hz), one double quadruplet at δ 4.97 (1H, $J=4.0$; 6.5 Hz) and several signals compatible with a glucosyl unit. In addition, the signals at δ 1.92 (3H, *s*), 3.54 (3H, *s*) and 3.58 (3H, *s*) analyzed with ^{13}C NMR spectroscopic data (Table 2), revealed the presence of an acetyl and two methoxyl groups. The ^{13}C NMR spectroscopic spectrum exhibited twenty signals ($4CH_3$, $2CH_2$, 10CH, and 4C) whose chemical shift values and multiplicities obtained from the DEPT-135° experiment strongly support the *seco*-iridoid skeleton for **1**, especially the signals of C-5, C-6, C-7, C-8, C-9 and C-10 at δ 28.6, 34.7, 172.0, 68.6 and 42.2 respectively. The structure of **1** was confirmed by 2D-NMR spectral analysis including ^{1}H–^{1}H COSY, HMQC and HMBC experiments. In the HMBC experiment, long-range correlations were observed between the following protons and carbons (H-1 and C-3, 5, 8, 1′; H-3 and C-5, 11; H-5 and C-3, 7, 1; H-8 and C-1, 5, 10, carbonyl of acetate) as shown in Fig. 1. The relative configuration of the stereogenic centers C-1, C-5, C- 8 and C-9 was established by analyzing coupling constant values, NOESY experiments and comparison with literature data (Adeoye and Waigh, 1983). The above evidence indicated **1** as the new derivative 7-methoxydiderroside (**1**).

Table 1
^{1}H NMR spectral data for *seco*-iridoids **1–3** (500 MHz) in DMSO-d_6[a]

H	1	2	3
1	5.66 *d* (4.5)	5.58 *d* (6.5)	5.76 *d* (6.5)
3	7.44 *s*	7.42 *d* (0.5)	7.42 *s*
4	–	–	–
5	3.10 *m*	3.28 *m*	3.20 *m*
6	2.48 *dd* (8.9, 16.0)2.85 *dd* (7.5, 16.0)	2.34-2.76 *m*	2.34 *dd* (8.0, 16.0)2.78 *dd* (7.2, 16.0)
7	–	–	–
8	4.97 *dq* (4.0, 6.5)	5.00 *dq* (4.5, 7.0)	5.09 *dq* (3.5, 6.5)
9	2.08 *ddd* (4.0, 4.5, 9.5)	2.12 *ddd* (4.5, 6.5, 9.0)	2.16 *ddd* (3.5, 6.5, 8.5)
10	1.26 *d* (6.5)	1.27 *d* (7.0)	1.30 *d* (6.5)
11	–	–	–
1′	4.54 *d* (7.5)	4.59 *d* (8.0)	4.59 *d* (8.0)
2′	3.18 *dd* (7.5, 11.2)	3.41 *dt* (8.0, 9.5)	2.99 *m*
3′	3.16 *m*	3.21 *m*	3.18 *m*
4′	3.12 *m*	3.01 *m*	3.01 *m*
5′	3.06 *m*	3.14 *ddd* (2.0, 6.5, 9.0)	3.20 *m*
6′	3.68 *dd* (5.0, 12.4)3.72 *dd* (5.7, 12.4)	4.26 *dd* (3.2, 12.0)4.06 *dd* (6.5, 12.0)	3.70 *dd* (5.6, 11.5)3.98 *dd* (6.8, 11.5)
OMe-7	3.58 *s*	3.60 *s*	3.59 *s*
OMe-11	3.54 *s*	–	–
AcO-8	1.92 *s*	1.92 *s*	–
AcO-6′	–	2.0 *s*	–
1″	–	–	–
2″	–	–	–
3″	–	–	6.79 *dq* (1.5, 7.2)
4″	–	–	1.76 *d* (1.5)
5″	–	–	1.74 *br s*

[a] Chemical shifts (relative to TMS) are in (δ) ppm, multiplicities and coupling constants in Hz in parentheses. Assignments were aided by ^{1}H–^{1}H COSY and HMQC.

Table 2
^{13}C NMR spectral data for *seco*-Iridoids **1-3** (δ in ppm, DMSO-d_6 solutions (δ_H, δ_C, 125 MHz)

C	1	2	3
1	94.9	95.0	94.1
3	152.5	152.1	152.1
4	108.7	109.1	109.1
5	28.6	28.0	28.0
6	34.7	34.6	34.4
7	172.0	173.0	173.0
8	68.6	68.5	68.6
9	42.2	42.0	42.1
10	18.7	18.6	18.8
11	166.3	166.2	166.2
1′	98.9	99.1	98.4
2′	73.3	73.0	73.2
3′	76.7	76.3	77.3
4′	70.1	70.0	70.1
5′	77.2	73.6	76.7
6′	61.4	63.4	61.3
OMe-7	51.4	–	–
OMe-11	51.1	50.9	50.9
AcO-8	20.9	20.8	–
	169.8	169.3	
AcO-6′	–	20.6	–
		170.3	
1″	–	–	166.3
2″	–	–	128.1
3″	–	–	137.4
4″	–	–	14.1
5″	–	–	11.8

The ^1H and ^{13}C NMR spectral signals (Tables 1 and 2) for compound **2** were found to be similar to those observed for **1**, except for the lack of one methoxyl group and one additional acetyl group. The change from one methoxyl to one acetyl group was also observed by HRTOF-ESMS, which showed a molecular ion peak at m/z $[M+H]^+$ 507.4725. In fact, when the ^{13}C NMR spectral data of compound **2** were compared with those of diderroside, the only difference observed was the presence of one additional acetyl group inferred by the chemical shift values at δ 20.6 (correlating with the singlet at δ 2.00, in the HMQC spectrum), and δ 170.3. It was also observed that the signals attributed to the hydroxymethylene carbon and hydrogens in the glucosyl unit are deshielded [δ 63.4; 4.26 (*dd*, $J=3.2$, 12.0 Hz) and 4.06 (*dd*, $J=6.5$, 12.0 Hz)], when compared with the values observed in a typical glucose moiety present in iridoids (El-Naggar and Beal, 1980; Boros and Stermitz, 1990; Boros and Stermitz, 1991). From the HMBC spectrum of **2** (Fig. 1), the diagnostic long-range correlations were observed, including those from H-8 to the carbonyl at δ 169.3; from H-10 to C-8 and C-9; and from H-6 to the carbonyl at δ 173.0. In addition, the correlation of H-6′ to C-4′ and C-5′ as well as to the carbonyl at δ 170.3, established the position of the acetyl group at C-6′. The cross peak observed for the methyl at δ 2.00 to the carbonyl at δ 170.3, was definitive to indicate that this acetyl was linked to hydroxymethylene C-6′ of the glucosyl moiety and establish the structure of **2** as being 6′-acetyl-β-D-glucopyranosyldiderroside.

Compound **3** was obtained as a brown amorphous solid. The molecular formula, $C_{22}H_{32}O_{13}$ was established by HRTOF-ESMS (m/z 527.4797 $[M+Na]^+$). This compound also presented a sulphuric vanillin coloration identical to those of **1** and **2** suggesting that it was an iridoid. Its UV spectrum (λ_{max}^{MeOH} 230, 297 nm), as well as its ^1H and ^{13}C NMR spectral data indicated that

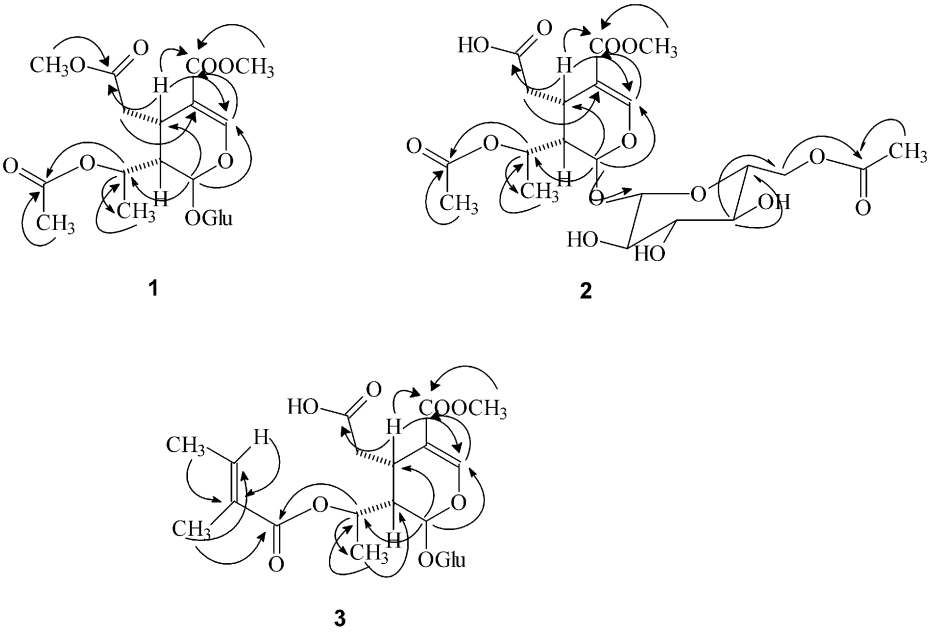

Fig. 1. Some selected HMBC correlations for *seco*-iridoids **1**–**3**.

3 consisted of a diderroside-like structure. The ^{1}H NMR spectrum (Table 1) displayed one signal for hydrogen H-3 at δ 7.42 (1H, *s*), which suggested the presence of an iridoid skeleton, a doublet at δ 4.59 (1H, $J = 8.0$) and several signals ranging from δ 2.99 to 3.70 attributed to the anomeric, methine and methylene hydrogens of the glucosyl moiety. The signals at δ 5.09 (1H, *dq*, $J = 3.5$, 6.5 Hz), and 1.30 (3H, *d*, $J = 6.5$ Hz) were compatible with those from diderroside skeleton type for this compound, as in **1** and **2**. However, additional NMR spectral signals at δ 6.79, 1.76 and 1.74, and the signals at δ 137.4, 14.1 and 11.8, indicated that this compound exhibited significant differences from the signals observed in diderrosides **1** and **2**. The ^{1}H–^{1}H COSY spectrum from **3** showed a cross peak between the signal at δ 6.79 (1H, *dq*, $J = 1.5$, 6.5) and the signal at δ 1.76 (3H, *d*, $J = 1.5$). In the HMQC experiments the signals at δ 1.74 (3H, *br s*), 1.76 (3H, *d*, $J = 1.5$) attributed to H-5″ and H-4″ respectively and δ 6.79 (1H, *dq*, $J = 1.5$, 6.5), correlated with the carbons at δ 11.8 (C-5″), 14.1 (C-4″) and 137.4 (C-3″), respectively. The combined analysis from these data and the presence of a sp^2 carbon at δ 128.1 suggested the existence of a tigloyl unit in this molecule, which was further determined to be linked at C-8 (δ 68.6). The HMBC correlations between the signal for H-8 at δ 5.09 with carbons C-1″ (δ 166.3), C-10 (δ 18.8) and C-1 (δ 94.1); H-3 at δ 7.42 with carbons C-11 (δ 166.2), C-4 (δ 109.1); methoxyl hydrogens with C-11; H-3″ at δ 6.79 with C-4″ (δ 14.1), C-2″ (δ 128.1) and C-1″ (δ 166.3) corroborated the proposed structure for **3** as 8-*O*-tigloyldiderroside.

This is the first report on the chemical composition of *C. spruceanum,* a species included in Cinchonoideae, which revealed the presence of iridoid and *seco*-iridoids, a chemical profile quite different from those previously described (Bolzani et al., 1984). This sub-family is characterized by indole alkaloids, which are considered as their chemical markers (Young et al., 1996). A comparison of the chemical composition of *Calycophyllum* with two species morphologically related such as *Chimarrhis* (accumulates indole alkaloids) and *Bathysa* (accumulates triterpenes and phenolics), revealed deep differences on the main isolated secondary metabolites. These species are often misplaced in their genera (Delprete, 1996), so further chemical studies on *Calycophyllum* and allies will be useful to establish the boundaries of each genera, and will contribute to the taxonomic classification of Rubiaceae, which is considered a very complex taxon (Robbrecht, 1993).

All compounds were assayed in vitro against the trypomastigote forms (Y form) of *T. cruzi*. The new compounds **1**, **2** and the known secoxyloganin and diderroside displayed activity with IC_{50} values of 59.0, 90.2, 74.2 and 84.9 μg/ml, respectively, when compared with the standard gentian violet (IC_{50} 7.5 μg/ml). Although these compounds have showed weak activities, to our knowledge this is the first report of the antitrypanosomal activity by *seco*-iridoids.

3. Experimental

3.1. General

Optical rotations were measured in MeOH using a Perkin-Elmer polarimeter with a sodium lamp operating at 598 nm and 25 °C. UV spectra were recorded on Shimadzu UV-2401 PC spectrophotometer. IR spectra were run on a Perkin-Elmer FT-IR 600 spectrophotometer. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectra were recorded on VARIAN DRX-500 spectrometer, using TMS as internal standard. ES-MS was conducted on a VG Platform Fisons instrument and HRTOF-ESMS was performed using a Q-Tof (Micromass) (40 eV). CC was carried out on silica gel 230–400 mesh (Merck), XAD-2 (Sigma-Aldrich) and Sephadex LH 20 (Pharmacia), respectively. TLC was performed using Merck silica gel 60 (> 230 mesh) and precoated silica gel 60 PF_{254} plates. Spots on TLC were visualized under UV light and/or by spraying with anisaldehyde-H_2SO_4 reagent followed by heating at 120 °C. Prep HPLC was performed on Waters Prep LC 4000 system (Waters) using C-18 (250 mm × 21.20 mm, Phenomenex) columns.

3.2. Plant material

Authenticated *C. spruceanum* plant material was collected in Manaus, Brazil, in July and August 1998. A voucher specimen was identified by Dr. J. A. da S. Cabral and deposited at the Herbarium of the Instituto Nacional de Pesquisas da Amazônia (INPA), Manaus (Voucher No 1998-182.249).

3.3. Bioassay screening

In vitro assay using trypomastigote forms (Y strain) from *T. cruzi* was performed according to the protocol described in the literature (Bastos et al., 1999).

3.4. Extraction and isolation

Pulverized dried wood bark from *C. spruceanum* (1.7 kg) was extensively extracted using MeOH at room temp. The combined extracts were concentrated in vacuo to give a dark solid (230 g), which was partitioned between *n*-BuOH and H_2O affording an *n*-BuOH (64 g) and an aqueous (107 g) soluble fraction. The *n*-BuOH fraction was suspended in aqueous MeOH 80% and successively partitioned with hexane, $CHCl_3$ and EtOAc. The EtOAc fraction (20.8 g) was subjected to XAD-2 chromatograph using H_2O with increasing proportions of MeOH (20–100%). Fractions I and II, obtained from this procedure, were further submitted to CC using Sephadex LH 20 as stationary phase and MeOH as isocratic mobile phase, yielding 21 fractions (F1–F21).

F-5 was further purified by means of HPLC coupled to a prep reversed phase column using MeOH/H_2O/HOAC (23:76.6:0.5) as mobile phase, UV detection set at 280 nm and a flow rate of 10 mL.min^{-1}, gave pure loganin (11.6 mg), diderroside (5.3 mg) and the new derivative **3** (11.6 mg). F-11 was purified with a similar chromatographic procedure, only varying the mobile phase MeOH/H_2O (27.5:72.5), giving diderroside (12.3 mg). Fraction F-18 was also subjected to HPLC purification using MeOH/H_2O/HOAc (27:72.5:0.5) as mobile phase, flow rate set at 8 mL.min^{-1} to afford secoxiloganin (5.7 mg), loganetin (2.0 mg), diderroside (2.1 mg) and the new compounds **1** (11 mg), **2** (13.6 mg) and **3** (1.2 mg).

3.4.1. *Diderroside methyl ester (**1**)*

Amorphous powder; $[\alpha]_D^{25}-36.0^\circ$ (MeOH; *c* 1.4); UV λ_{max} (CH_3OH) nm (log ε): 230 (4.6), 210.6 (4.2); IR ν_{max} (KBr) cm^{-1}: 3450, 2944, 1712, 1695, 1635, 1280. For ^1H and ^{13}C NMR see Tables 1 and 2; ES-MS: (70 eV) *m*/*z* (rel int.): $[M+H]^+$ 479 (65), 418 (100), 298 (56), 163 (38); HRTOF-ESMS: *m*/*z* 479.4539 $[M+H]^+$ (calc. for $C_{20}H_{30}O_{13}$, 479.4530).

3.4.2. *6′-Acetyl-β-D-glucopyranosyldiderroside (**2**)*

Amorphous powder; $[\alpha]_D^{25}-76.5^\circ$ (MeOH; *c* 1.0); UV λ_{max} (CH_3OH) nm (log ϵ): 238 (4.4), 210 (4.6); IR ν_{max} (KBr) cm^{-1}: 3455, 2940, 1715, 1690, 1630, 1263 cm^{-1}. For ^1H and ^{13}C NMR see Tables 1 and 2; ES-MS: (70 eV) *m*/*z* (rel int.): $[M+H]^+$ 507 (65), 286 (100), 205 (56); HRTOF-ESMS: *m*/*z* 507.4725 $[M+H]^+$ (calc.for $C_{21}H_{30}O_{14}$, 507.4727).

3.4.3. *8-O-Tigloyldiderroside (**3**)*

Amorphous powder; $[\alpha]_D^{25}-78.8^\circ$(MeOH; *c* 1.5); UV λ_{max} (CH_3OH) nm (log ε): 320 (4.0), 282 (4.5), 210 (4.1); IR ν_{max} (KBr) cm^{-1}: 3460, 2938, 1698, 1655, 1450, 1210 cm^1. For ^1H and ^{13}C NMR see Tables 1 and 2; ES-MS: (70 eV) *m*/*z* (rel int.): $[M+Na]^+$ 527 (15), 427 (10), 163 (100); HRTOF-ESMS: *m*/*z* 527.4797 $[M+Na]^+$ (calc. for $C_{22}H_{32}O_{13}$, 527.4802).

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