Novel DNA-Damaging Tropolone Derivatives from *Goupia glabra*

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Dedicated to the memory of Professor Antonio González-González

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Two novel tropolone derivatives **1** and **2** have been isolated from *Goupia glabra*. Their structures were determined by extensive 1D and 2D NMR spectroscopic studies. Compound **2** constitutes the first example isolated from a natural source of a Diels–Alder adduct between a tropolone and a naphthalene derivative. Compounds **1** and **2** exhibit significant toxicity towards a panel of DNA damage checkpoint defective yeast mutants, and behave as genotoxins, which highlights their potential to be used as anticancer drugs.

Introduction

Tropolones and benzotropolones are aromatic compounds, frequently isolated from fungi[1] and rarely isolated from higher plants.[2] These compounds behave as dienes in Diels−Alder reactions, both with homo and heteroaromatic dienophiles,[3] and are reactive towards electrophilic reagents, but do not undergo Friedel−Crafts alkylations.[4] Some products containing a tropolone nucleus exhibit antitumour activity and there are several reports dealing with the preparation of tropolone derivatives and their corresponding biological evaluations. In this regard, the works carried out by Yamato et al.[5] and, more recently, by Crozet et al.,[6] are of special interest. In addition to the antitumour activity mentioned above, other reports describe different biological activities that are related to the presence of the tropolone nucleus. Some examples of these activities are antimalarial[7] and inhibitory ribonucleotide reductase activity.[6a] In addition, some tropolones extracted from a fungus induce erythropoietin gene expression, which is an activity of interest as a potential alternative treatment of patients with anemia resulting from chronic renal failure.[1a]

As part of an ongoing research programme aimed at isolating bioactive compounds from South American medicinal plants belonging to the Celastraceae family,[8] we studied *Goupia glabra* Aublet.[9] This species is distributed in the Amazonian region of Peru and is commonly known as "capricornia," "cupiuba", or "muena rifarillo". The inhabitants of these regions use it for the treatment of ocular diseases.[10] During the course of our research, a new report on the phylogenetic relationships within Celastraceae[11] has reassigned the genus *Goupia*. That study was mainly based on morphology, as well as the phytochrome B gene sequence. The new data reveal that the genus *Goupia*, which was questionably included within the Celastraceae, is more closely related to Corynocarpaceae and Linaceae than it is to Celastraceae. This conclusion is corroborated by our phytochemical study, because most of the derivatives isolated from this species are unusual when compared with other secondary metabolites from Celastraceae. In this regard, we isolated the new tropolone derivatives goupiolone A (**1**) and goupiolone B (**2**) from the EtOH extract of the aerial parts together with the known compounds squalene, 4-hydroxybenzaldehyde, cinnamic acid, vanillic acid, chlorophyll *b*, and blumenol A.[12]

Results and Discussion

Repeated chromatography on silica gel and Sephadex LH-20 of the EtOH extract from leaves of *G. glabra* yielded two new compounds (**1**, **2**, Figure 1), along with the other constituents of known structure mentioned above. Compound **1** was isolated as a yellow oil. Its IR spectrum reveals

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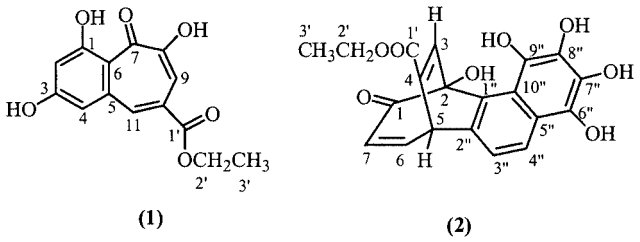

Figure 1. Structures of goupiolones A (**1**) and B (**2**)

the presence of hydroxyl groups (3400 cm^{-1}), carbonyl groups (1716 cm^{-1}), and an aromatic nucleus (1607, 1463 cm^{-1}). Compound **1** exhibits UV absorptions that are characteristic of a benzotropolone moiety (λ_{max} = 398 and 278 nm).[13] The molecular formula was determined by HREIMS to be $C_{14}H_{12}O_6$. The ^1H NMR spectrum of **1** (Table 1) displays signals attributable to four aromatic protons and a signal due to a strongly chelated hydroxyl proton at δ = 14.65 ppm. Two other singlets that are exchangeable with D_2O appear at δ = 6.56 and 8.18 ppm, which we assign to two hydroxyl groups. The remaining signals are a quadruplet at δ = 4.42 ppm (J = 7.1 Hz, 2 H) and a triplet at δ = 1.43 ppm (3 H, J = 7.1 Hz) that are attributable to an ethoxy group. The ^{13}C NMR spectrum displays the existence of an ethyl ester group and, in addition, the presence of a carbonyl carbon atom at δ = 184.0 ppm, in addition to ten aromatic carbon atoms. Among these aromatic carbon atoms, six are quaternary (δ =119.9, 124.3, 130.1, 147.6, 150.0, and 152.8 ppm) and the remaining four are unsubstituted (δ = 120.9, 128.5, 116.4, and 140.0 ppm). All these data suggest the presence of a benzotropolone nucleus having an ester substituent and three hydroxyl groups. The positions of these groups were established by the ^1H-^{13}C long-range correlations detected in the HMBC spectra, which are shown in Table 1. All of these data allow us to establish the structure of **1** as 1,3-dihydroxy-10-(ethoxycarbonyl)benzotropolone, which we have named goupiolone A.

Compound **2** was isolated as an oil having the molecular formula $C_{20}H_{16}O_8$. Its ^1H NMR spectrum ($CDCl_3$) displays signals attributable to an ethyl ester group [δ = 4.23 (m, 2 H); 1.30 (t, J = 7.0 Hz, 3 H) ppm], a doublet at δ = 5.03 ppm (J = 1.3 Hz, 1 H), a signal overlapped with that of $CHCl_3$ at δ = 7.26 ppm, two vinylic or aromatic AB systems (both with J = 8 Hz), and five broad singlets that are interchangeable with D_2O, which correspond to five hydroxyl groups. The IR and ^{13}C NMR spectra reveal the presence of two carbonyl groups at δ = 194.1 and 163.9 ppm and the presence of an aromatic nucleus. To elucidate the structure of this compound, it was essential to thoroughly analyse the two-dimensional COSY, HSQC, and HMBC NMR spectra. The COSY spectra exhibit a correlation between the doublet at δ = 5.03 ppm and the signal at δ = 7.26 ppm. A correlation between the protons of one of the AB systems is also detected. Specifically, we find a correlation between the doublet at δ = 6.77 ppm (J = 8.0 Hz, 1 H) and the doublet at δ = 6.97 ppm (J = 8.0 Hz, 1 H). The signals of the second AB system are very close to one another as well as to the diagonal peaks. Analysis of the HSQC spectra allows correlations to be made between the singlet at δ = 5.03 ppm and the signal at δ = 49.8 ppm and between the singlet at δ = 7.26 ppm and the signal at δ = 143.6 ppm. The two doublets at δ = 6.77 and 6.97 ppm

Table 1. NMR spectral data for goupiolone A (**1**) and goupiolone B (**2**) in $CDCl_3$ (* signal overlapped with that of $CHCl_3$)

	δ_H[a]	δ_C	HMBC[b]	δ_H[a]	δ_C	HMBC[b]
1	–	150.0 s	–	–	194.1 s	–
2	7.53 s	120.9 d	C-1, C-3, C-4, C-6	–	84.5 s	–
3	–	147.6 s	–	7.26*	143.6 d	C-4, C-5, C-1′
4	7.53 s	128.5 d	C-2, C-5, C-6	–	117.6 s	–
5	–	130.1 s	–	5.03 d (1.3)	49.8 d	C-7, C-1′, C-1′′, C-2′′, C-10′
6	–	119.9 s	–	6.84 d (8.0)	118.1 d	C-1, C-2′
7	–	184.0 s	–	6.82 d (8.0)	117.3 d	C-5
8	–	152.8 s	–	–	–	–
9	7.99 s	116.4 d	C-7, C-8, C-11, C-1′	–	–	–
10	–	124.3 s		–	–	–
11	8.42 s	140.0 d	C-4, C-6, C-9, C-10, C-1′	–	–	–
1′	–	165.4 s	–	–	163.9 s	–
2′	4.42 q (7.1)	62.2 t	C-1′, C-3′	4.23 m	67.2 t	C-1′, C-3′
3′	1.43 t (7.1)	14.3 q	C-2′	1.30 t (7.0)	14.1 q	C-2′
1′′	–	–	–	–	131.4 s	–
2′′	–	–	–	–	110.6 s	–
3′′	–	–	–	6.77 d (8.0)	114.3 d	C-1′′, C-4′′, C-10′′
4′′	–	–	–	6.97 d (8.0)	121.0 d	C-3′′, C-5′′, C-6′′, C-10′′
5′′	–	–	–	–	145.1 s	–
6′′	–	–	–	–	152.5 s	–
7′′	–	–	–	–	142.7 s	–
8′′	–	–	–	–	140.5 s	–
9′′	–	–	–	–	144.0 s	–
10′′	–	–	–	–	137.0 s	–

[a] Multiplicities; values of J (Hz) are given in parentheses. [b] Proton showing a long-range correlation to the indicated carbon atom.

are correlated with the signals at δ = 114.3 and 121.0 ppm, respectively, and, finally, the two doublets at δ = 6.84 and 6.82 ppm are correlated with the corresponding signals at δ = 118.1 and 117.3 ppm. The number of aromatic carbon atoms and the thirteen degrees of unsaturation present in the molecule lead us to propose the existence of two fused aromatic rings, which accounts for seven of the degrees of unsaturation. An additional four degrees of unsaturation could correspond to two carbonyl functions and two C=C double bonds, and the remaining two unsaturations could be the result of rings. The multiple correlations observed in the HMBC spectra (see Figures 2 and 3, and Table 1) for the signal at δ = 5.03 ppm (5-H) suggest the existence of a bridged bicyclic system (bridged naphthotropolone), with this hydrogen atom being in a bridgehead position. The locations of the different functional groups in the bridged bicyclic system were established by the detected HMBC, COSY and ROESY correlations. Thus, the position of the α,β-unsaturated carbonyl system through C-1, C-5, and C-6 was established by the HMBC correlations of 6-H to C-1 and 7-H to C-5. The position of the C-2−OH group was determined by the three-bond correlation of the hydroxyl proton to C-1. The position of the other unsaturated carboxyl system was established by the following correlations: 3-H/C-4, 3-H/C-1′, 3-H/C-5 and 5-H/C-1′. All of these correlations, and also the NOE effects detected in the ROESY spectrum, are consistent with the partial structure presented in Figure 2. The substitution pattern of the naphthalene unit was established by analysis of the COSY connectivities, the NOEs detected in the ROESY spectrum, and also the HMBC correlations. The long-range correlation between 5-H and 3′′-H, and also the NOEs observed from 5-H to 3′′-H and 4′′-H, are consistent with the presence of the aromatic AB system in ring A of the corresponding naphthalene unit. The hydroxylation pattern shown in Figure 3 is the only one that is compatible with the observed NOEs and also with the HMBC correlations detected.

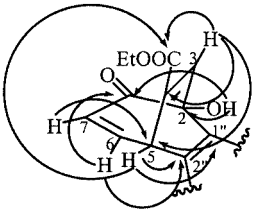

Figure 2. Selected HMBC correlations for compound **2**

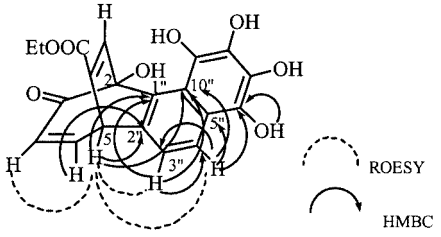

Figure 3. Selected ROESY and HMBC correlations for compound **2**

These data all indicate the structure of compound **2** to be as depicted in Figure 1. We could not determine the absolute configuration of **2** because we had isolated only a limited amount of this compound. Considering that EtOH was used in the extraction process, it seems probable that the metabolites in the plant are in carboxylic acid forms, rather than their corresponding ethyl esters.

Compound **2** is an adduct that appears to derive biogenetically from a Diels−Alder reaction between a tropolone unit and a benzyne intermediate formed from a naphthalene unit. In this hypothesis, the most electron-rich diene present in the tropolone intermediate reacts with a dienophile-type benzyne formed from naphthalene-1,2,3,4-tetraol. To the best of our knowledge, **2** constitutes the first recorded example of this type of adduct isolated from a natural source. In addition, this phytochemical study supports the reassignment of the genus *Goupia* as reported by Simmons et al.[11] since the metabolite profile of *G. glabra* does not relate to the usual compounds present in the Celastraceae.

Cancers exhibit and accumulate a large number of genetic changes during their progression towards malignancy because of an intrinsic genetic instability. These genetic alterations frequently affect DNA repair and cell cycle checkpoint pathways, which thereby increases the sensitivity of tumour cells towards DNA-damaging agents. Since these pathways are conserved throughout evolution, one can explore the therapeutic effects of molecules by using a panel of isogenic yeast strains with defined genetic alterations in DNA repair or checkpoint functions. Indeed, this approach has proven to be extremely useful in the analysis of well-known cytotoxic compounds that are currently used in cancer therapy.[14] Accordingly, we used a set of isogenic yeast strains that are defective for the G1/S and G2/M DNA damage checkpoints to detect the potential cytotoxicities of compounds **1** and **2** that are specific for these genetic backgrounds. Both compounds exhibit cytotoxicity in every checkpoint defective mutant strain that we tested, with no significant effect on the WT strain. The IC_{50} for each compound was quantified in the WT strain and the rad9 checkpoint mutant as representative of the strain panel. As shown in Table 2, compounds **1** and **2** were 50- and 16-times more cytotoxic for the rad9 mutant than for the WT. Neither compound **1** nor **2** exhibits significant toxicity towards the WT strain at concentrations of up to 100 μg/mL. Table 2 also includes the data obtained in the same assay for the antineoplastic agent, doxorubicin. These results suggest that **1** and **2** behave as genotoxins, i.e., they induce cell lethality by producing genomic DNA lesions,

Table 2. Values of IC_{50} (μg/ml) for **1**, **2**, and doxorubicin in WT strain and best yeast mutant strain Rad9

	1	**2**	Doxorubicin
Wild type	> 100	> 100	> 100
Rad9 mutant	2	6	12.5

which suggests a potential role for these molecules as anticancer drugs.

Conclusions

We have isolated two new tropolone derivatives from the leaves of *G. glabra*, along with the known compounds squalene, 4-hydroxybenzaldehyde, cinnamic acid, vanillic acid, chlorophyll *b*, and blumenol A. To the best of our knowledge, **2** constitutes the first example of a naphthotropolone Diels−Alder adduct isolated from a natural source. In addition, this phytochemical study supports the reassignment of the genus *Goupia* as reported by Simmons et al.[11] Compounds **1** and **2** exhibit significant toxicity towards a panel of DNA damage checkpoint defective yeast mutants — they behave as genotoxins — which suggests that these molecules are possible anticancer drugs.

Experimental Section

General Remarks: UV spectra were collected in absolute EtOH on a JASCO V-560 spectrophotometer. IR spectra were obtained using a Bruker IFS28/55 spectrophotometer. Optical rotations were measured with a Perkin−Elmer 241 automatic polarimeter. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ at 300 and 75 MHz, respectively, with TMS as the internal reference. The 2D NMR experiments were conducted on a Bruker WP-400 SY NMR spectrometer in $CDCl_3$ at 400 MHz. High- and low-resolution mass spectra were obtained on a VG Autospec spectrometer. Macherey−Nagel polygram Sil G/UV_{254} and preparative TLC Sil G-100UV254 foils were used for TLC. Silica gel (0.2−0.63 mm) and Sephadex LH-20 were used for column chromatography.

Plant Material: Leaves of *G. glabra* Aublet were collected at Loreto, Perú, in June 2001 and authenticated by Dr. V. Reyna (Department of Botany, Universidad Nacional de Ingeniería). Voucher specimens (voucher number 13652 USM) were deposited in the Herbarium at the Museo de Historia Natural de la Universidad Nacional Mayor de San Marcos, Lima, Perú.

Extraction and Isolation of Goupiolones A and B: Dried leaves of *G. glabra* (1.0 kg) were extracted with EtOH in a Soxhlet apparatus. The dried extract (0.3 kg) was treated with CH_2Cl_2/H_2O (1:1). The aqueous layer was separated and extracted several times with CH_2Cl_2. All the CH_2Cl_2 extracts were combined and then evaporated to dryness to yield a dark residue (71.3 g). This residue was chromatographed on silica gel using, as eluent, mixtures of *n*-hexanes/EtOAc of increasing polarity. Four fractions, A−D, were separated, studied, and chromatographed on Sephadex LH-20, eluting with *n*-hexanes/$CHCl_3$/MeOH (2:1:1). Some of the eluted products were separated by preparative TLC. Squalene[15] (1 g) was isolated from fraction A. Fraction B yielded 4-hydroxybenzaldehyde[16] (20 mg), cinnamic acid[17] (0.2 g), vanillic acid[18] (10 mg) and chlorophyll *b*[19] (0.3 g). Fraction C afforded **1** (5 mg). Blumenol A[12] (14.5 mg) and **2** (2.7 mg) were isolated from fraction D.

Goupiolone A (1): Yellow oil. IR ($CHCl_3$): $\tilde{\nu}_{max}$ = 3400, 3288, 2938, 2862, 1716, 1607, 1463, 1391, 1328, 1232, 1200 cm^{-1}. UV (EtOH): λ_{max} (logε) = 398.0 (4.0), 277.6 (4.3) nm. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ = 6.56 (br. s, 1H, C3-OH), 8.18 (br. s, 1H, C8-OH), 14.65 (br. s, 1H, C1-OH) ppm; for the remaining signals, see Table 1. ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): see Table 1. HMBC ($CDCl_3$, #-H⇄C-#): C-8−OH⇄C-7, C-8−OH⇄C-8; for the remaining correlations, see Table 1. EIMS: *m*/*z* (%) = 276 (100) $[M]^+$, 248 (15) $[M - CO]^+$, 231 (10) $[M - OC_2H_5]^+$, 220 (65), 203 (10), 175 (15). HREIMS: *m*/*z* = 276.0663 (calcd. for $C_{14}H_{12}O_6$, 276.0634), 248.0708 (calcd. for $C_{13}H_{12}O_5$, 248.0685), 231.0283 (calcd. for $C_{12}H_7O_5$, 231.0293).

Goupiolone B (2): Yellow oil. $[\alpha]_D^{20}$= −40 (*c* = 0.2, $CHCl_3$). IR ($CHCl_3$): $\tilde{\nu}_{max}$ = 3337, 2925, 2825, 2360, 2306, 1714, 1631, 1463, 1437, 1261, 1071 cm^{-1}. UV (EtOH): λ_{max} (logε) = 383.4 (3.0), 295.6 (3.4), 273.2 (3.4), 222.8 (4.1) nm. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ = 5.50 (br. s, 1 H, OH), 5.60 (br. s, 1 H, OH), 6.02 (br. s, 1 H, C2-OH), 7.66 (br. s, 1 H, OH), 9.18 (br. s, 1 H, OH); for the remaining signals, see Table 1. ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): see Table 1. HMBC ($CDCl_3$, #-H⇄C-#): C-2−OH⇄C-1, C-2−OH⇄C-2, C-6′′−OH⇄C-6′′; for the remaining correlations, see Table 1. MS: *m*/*z* (%) = 384 (45) $[M]^+$, 311 (100), 293 (30), 282 (10), 265 (20), 237 (10), 149 (15). HRMS: *m*/*z*: 384.0849 (calcd. for $C_{20}H_{16}O_8$, 384.1845).

Biological Assays. Yeast Strains: All the strains used in this study have the W3031A genetic background (MATa, ade2-1, can1-100, his3-11, leu2-3, trp11-1, ura3.1). The rad9, rad17, rad24, mec3, and tel1 strains, which harbour disruptions of the respective genes, have been described elsewhere.[20] The mec1-1 and rad53-11 yeast mutants have also been described previously.[20]

Yeast Growth Assays: Standard methods for yeast culture and manipulations were used.[21] To assess cell growth in the presence of increasing concentrations of each tested compound, mid-log cultures of each strain growing in liquid YPD medium were serially diluted by 10-fold and volumes of ca. 3 μL were applied with a stainless-steel replicator (SIGMA) on solid plates containing 2% Bacto-Agar (Difco) and YPD medium with 10-fold-increasing doses of each product. Growth was recorded after 2−3 days in all cases. For IC_{50} measurements, exponentially growing yeast cultures in YPD were diluted and dispensed into each well of flat-bottomed 96-well plates. Products were dispensed in seven twofold serial dilutions in 1% DMSO and aliquots were added in triplicate to the yeast-containing wells. Plates were incubated overnight at 30 °C, and the *A660* of the culture was read in a Multiskan Ascent microplate reader (lab systems).

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