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ISOLATION AND STRUCTURE IDENTIFICATION OF AN ACTIVE DNA STRAND-SCISSION AGENT, (+)-3,4-DI-HYDROXY-8,9-METHYLENEDIOXYPTEROCARPAN

SWAPAN K. CHAUDHURI, LI HUANG, FEKADU FULLAS, DANIEL M. BROWN, MANSUKH C. WANI, MONROE E. WALL,*

Chemistry and Life Sciences Group, Research Triangle Institute, P.O. Box 12194, Research Triangle Park, North Carolina 27709

JOHN C. TUCKER,

Box 69, Maple Texas 79344

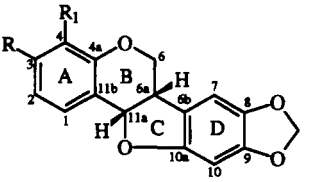
CHRISTOPHER W.W. BEECHER, and A. DOUGLAS KINGHORN

Program for Collaborative Research in the Pharmaceutical Sciences, Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, Chicago, Illinois 60612

ABSTRACT.—A new pterocarpan, (+)-3,4-dihydroxy-8,9-methylenedioxypterocarpan [**1**], was isolated from the flowers of *Petalostemon purpureus* by a DNA strand-scission assay-guided fractionation procedure. Compound **1** demonstrated activity in a standard in vitro DNA strand-scission assay, and cytotoxicity toward a KB tumor cell line. Two other related pterocarpans [**2**, **3**] isolated from same plant were found to be moderately active for KB cells, but were inactive in the DNA strand-scission assay. (+)-4-Hydroxy-3-methoxy-8,9-methylenedioxypterocarpan [**2**] has not been reported previously as a natural product, while (+)-maackiain [**3**] has been isolated only as an optically inactive racemate along with its optical antipode, the (−)-isomer.

Petalostemon purpureus Rydb. (Leguminosae) is a species that grows in the United States. Biological studies of this plant showed that a saline extract has a hemagglutinin effect (1). No phytochemical study on this plant has been reported previously. However, studies on other species of the genus *Petalostemon* have revealed the presence of flavonoids (2,3).

In the course of our continuing efforts to discover natural products with antineoplastic activity, we investigated an extract of the flowers of this plant and found that it possessed DNA strand-scission activity. Initial fractionation of a 20% MeOH/$CHCl_3$ extract by Si gel cc yielded an active fraction. Further fractionation of this active fraction by prep. tlc yielded three pterocarpan derivatives, (+)-3,4-dihydroxy-8,9-methylenedioxypterocarpan [**1**], (+)-4-hydroxy-3-methoxy-8,9-methylenedioxypterocarpan [**2**], and (+)-3-hydroxy-8,9-methylenedioxypterocarpan [**3**]. The structures of compounds **1–3** were determined by ^{1}H- and ^{13}C-nmr spectral analysis as well as by comparison with the previously reported data on their optical isomers and related compounds (4–6). Further structural confirmation of **1** was achieved using HMQC and HMBC experiments. The absolute configurations of **1–3** were established by comparing their optical rotations with those of the corresponding known optical isomers (4,5).



1 R=R_1=OH
2 R=OMe, R_1=OH
3 R=OH, R_1=H

A high-resolution eims study of compound **1** indicated a chemical composition of $C_{16}H_{12}O_6$ (M^+ *m/z* 300.0636). The ^{1}H-nmr spectrum showed a characteristic set of peaks corresponding to a pterocarpan skeleton as suggested by Mizuno *et al.* (7) and Pachler *et al.* (8): δ

3.52–3.53 (1H, m, H-6a), δ 3.70 (1H, dd or t, J=11 Hz, H-6_{ax}), δ 4.31 (1H, dd, J=11 and 5.0 Hz, H-6_{eq}), and δ 5.51 (1H, d, J=7.0 Hz, H-11a). The ^{1}H-nmr spectrum also showed a methylenedioxy function indicated by a two-proton double-doublet at δ 5.92 and two D_2O-exchangeable phenolic protons at δ 5.41 and 5.35. In the aromatic region, there were two singlets (δ 6.72, 6.44) and two mutually coupled doublets (δ 6.98, 6.68, J=8.5 Hz), which suggested that compound **1** has a methylenedioxy moiety in the aromatic ring D and a catechol unit in ring A. The exact substitution pattern was subsequently ascertained by the close agreement of the ^{1}H-nmr chemical shifts of the aromatic protons of **1** and those reported for (−)-3,4-diacetoxy-8,9-methylenedioxypterocarpan (4). Accordingly, the two coupled doublets at δ 6.98 and 6.68 were assigned to H-1 and H-2, respectively, while the two singlets at δ 6.72 and 6.44 were in turn assigned to H-7 and H-10. Thus, the structure of **1** was established as 3,4-dihydroxy-8,9-methylenedioxypterocarpan. The assignment of ^{13}C-nmr chemical shifts of **1** was based on further analysis of its HMQC and HMBC spectra and by comparison with previously reported data of related compounds (6,7). In addition, the positive optical rotation ([α]D +154°, c=1.36) indicated that **1** is a new pterocarpan, while the reported compound is optically negative. Moreover, it is clear that the known (−)-3,4-dihydroxy-8,9-methylenedioxypterocarpan has an *R,R* configuration at C-6a, C-11a. Thus, compound **1** must have the *S,S* configuration at C-6a and C-11a (4).

Compound **2** was isolated as an optically active, [α]D +122° (c=0.56), white solid. Like compound **1**, the ^{1}H-nmr spectrum indicated a pterocarpan skeleton. A comparison of its ^{1}H-nmr chemical shifts with those reported for known pterocarpans (4) indicated that **2** has the structure 4-hydroxy-3-methoxy-8,9-methylenedioxypterocarpan. The optical rotation suggested that it had the same configuration as **1**, and was thus an enantiomer of the reported (−)-4-hydroxy-3-methoxy-8,9-methylenedioxypterocarpan (4).

The structure of compound **3** was also established through ^{1}H- and ^{13}C-nmr analysis and by comparison with previously reported (−)- and (±)-maackiain (5,9). The positive optical rotation ([α]D +169°, c=0.55) indicated that it is (+)-maackiain.

Although these compounds belong to a structurally related group, only compound **1** showed DNA strand-scission activity, while compounds **2** and **3** were inactive. Compounds **1–3** showed modest activity against the KB cell line. A literature search indicated that all three dextro-pterocarpans have never been reported before as natural products. It appears that the levo-8,9-methylenedioxypterocarpan derivatives are more commonly distributed in plant species than their dextro enantiomers. The (−)-isomers of compounds **1** and **2** have been isolated previously from *Dalbergia spruceana* (4). The (−)-isomer of compound **3**, known as maackiain, was isolated from a number of plants (5). The optically inactive (±)-maackiain was also isolated as a racemic mixture but there has been no previous report on (+)-maackiain [**3**] (9). In the DNA strand-scission assay, compound **1** exhibited activity, while compounds **2** and (+)-maackiain [**3**] showed no activity. Because all these compounds have the same pterocarpan skeleton with only different substitution in ring A, an apparent structure-activity relationship can be suggested through simple structural comparison. The ortho phenolic functions seem to play a role in the DNA strand-scission activity, inasmuch as the absence of the ortho phenol groups resulted in inactive compounds as shown in **2** and **3**, each having a single phenolic group. In a contribution from Hecht's laboratory (10) it was stated that chelation of Cu(II) ions

by catechols may be responsible for the increase in DNA strand-scission activity noted for such compounds.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Nmr experiments were performed on a Bruker AMX 500 or a Bruker AMX 250 MHz spectrometer operating at 500 or 250 MHz for proton, and 125 or 62.5 MHz for carbon, respectively. $CDCl_3$ was used as solvent and TMS as internal standard. Optical rotations were measured with a Perkin-Elmer Model 141 polarimeter. Uv and ir spectra were recorded on a Varian 2290 uv-vis spectrometer and a Shimadzu IR-460 spectrometer, respectively. Hreims was measured on a VG-ZAB magnetic sector instrument. Cc was carried out on Si gel 60 (70–230 mesh, E. Merck, Darmstadt, Germany). Fractions were monitored by tlc (Silica 60 F_{254}, 0.25 mm thick) with visualization under uv light (254 and 365 nm) and by using phosphomolybdate spray reagent (5% phosphomolybdic acid in EtOH). Prep. tlc was carried out on Merck Si gel F_{254} plates (1-mm thickness).

PLANT MATERIAL.—The flowers of *Petalostemon purpureus* were collected in July 1992 at Maple, Texas. A voucher specimen (AA1873) is on deposit in the John G. Searle Herbarium of the Field Museum of Natural History, Chicago, Illinois.

EXTRACTION AND ISOLATION.—Dried and ground flowers of *Petalostemon purpureus* (273 g) were extracted with 50% MeOH/$CHCl_3$ (2×1 liter) in a Soxhlet apparatus. Extracts were concentrated *in vacuo* at 40° and partitioned between 20% MeOH/$CHCl_3$ and H_2O. The organic portion was concentrated *in vacuo* and the residue partitioned between 90% MeOH/H_2O and hexane.

The 90% MeOH/H_2O extract (1.27 g) was chromatographed over Si gel (120 g) using $CHCl_3$ and increasing percentages of MeOH in $CHCl_3$. A total of 925 fractions of 10–12 ml each was collected and combined to produce 14 pooled fractions, based on the tlc profile.

The fraction (95 mg) which eluted with 0.4–0.5% MeOH/$CHCl_3$ showed activity in the DNA strand-scission assay. This active fraction was further fractionated over Si gel and further purified by repeated prep. tlc which afforded a DNA strand-scission-active compound, (+)-3,4-dihydroxy-8,9-methylenedioxypterocarpan [**1**] (9 mg), along with two related but inactive compounds, (+)-4-hydroxy-3-methoxy-8,9-methylenedioxypterocarpan [**2**] (1.7 mg) and (+)-3-hydroxy-8,9-methylenedioxypterocarpan [**3**] (10 mg).

(+)-3,4-Dihydroxy-8,9-methylenedioxypterocarpan [**1**].—Amorphous; $[\alpha]^{24}D$ +155° (c=1.36, $CHCl_3$); uv λ max (EtOH) (log ε) 310 (3.68) nm; ir ($CHCl_3$) ν max 3540, 1626, 1498, 1474, 1142 cm^{-1}; ^1H nmr (500 MHz) δ 6.99 (1H, d, J=8.5 Hz, H-1), 6.72 (1H, s, H-7), 6.68 (1H, d, J=8.5 Hz, H-2), 6.44 (1H, s, H-10), 5.92 (2H, each d, J=13.0 Hz, OCH_2O), 5.41, 5.35 (1H, each s, OH), 5.51 (1H, d, J=7.0 Hz, H-11a), 4.31 (1H, dd, J=11.0 and 5.0 Hz, H-6α), 3.69 (1H, dd, J=11.0 Hz, H-6β), 3.51 (1H, ddd, J=11.0, 7.0, and 5.0 Hz, H-6a); ^{13}C-nmr data, see Table 1; eims m/z $[M]^+$ 300 (100), 162 (48), 175 (7), 150 (12); hreims m/z $[M]^+$ 300.0636 ($C_{16}H_{12}O_6$ requires 300.0633).

(+)-4-Hydroxy-3-methoxy-8,9-methylenedioxypterocarpan [**2**].—Amorphous; $[\alpha]^{23}D$ +122° (c=0.56, $CHCl_3$); uv λ max ($CHCl_3$) (log ε) 311 (3.65) nm; ir ($CHCl_3$) ν max 3525, 1639, 1530, 1466, 1139, 1100 cm^{-1}; ^1H nmr (250 MHz) δ 7.03 (1H, d, J=8.5 Hz, H-1), 6.74 (1H, s, H-7), 6.67 (1H, d, J=8.5 Hz, H-2), 6.44 (1H, s, H-10), 5.91 (2H, each d, J=15.0 Hz, OCH_2O), 5.52 (1H, d, J=7.0 Hz, H-11a), 5.48 (1H, s, OH), 4.34 (1H, dd, J=11.0 and 4.5 Hz, H-6α), 3.91 (3H, s, OMe), 3.70 (1H, t, J=11.0 Hz, H-6β), 3.53 (1H, ddd, J=11.0, 7.0, and 5.0 Hz, H-6a); ^{13}C-nmr data, see Table 1; eims m/z $[M]^+$ 314 (100), 299 (52), 162 (45), 281 (20), 175 (7); hreims m/z $[M]^+$ 314.0793 ($C_{17}H_{14}O_6$ requries 314.0790).

(+)-3-Hydroxy-8,9-methylenedioxypterocarpan [**3**].—Amorphous; $[\alpha]^{23}D$ +169° (c=0.55, $CHCl_3$); uv λ max ($CHCl_3$) (log ε) 311 (3.53) nm; ir ($CHCl_3$) ν max 3580, 1618, 1498, 1460, 1156, 1114 cm^{-1}; ^1H nmr (250 MHz) δ 7.35 (1H, d, J=8.5 Hz, H-1), 6.54 (1H, dd, J=8.5 and 2.5 Hz,

TABLE 1. ^{13}C-Nmr Spectral Data of Compounds **1–3**.

Carbon	Compound		
	1	**2**	**3**
C-1	121.7	121.0	132.1
C-2	109.5	105.3	109.8
C-3	144.4	143.2	157.1
C-4	131.5	133.9	103.6
C-4a	143.0	147.3	156.6
C-6	66.9	66.8	66.4
C-6a	40.3	40.2	40.1
C-6b	117.4	117.7	117.9
C-7	104.7	104.8	104.7
C-8	141.8	141.7	141.7
C-9	148.2	148.1	148.1
C-10	93.9	93.8	93.8
C-10a	154.2	154.2	154.2
C-11a	78.3	78.3	78.5
C-11b	112.5	113.9	112.5
-OCH_2O-	101.3	101.3	101.3
OCH_3	—	56.3	—

H-2), 6.72 (1H, s, H-7), 6.43 (1H, s, H-10), 6.40 (1H, d, J=2.5 Hz, H-4), 5.91 (2H, each d, J=14.0 Hz, OCH_2O), 5.47 (1H, d, J=7.0 Hz, H-11a), 4.21 (1H, dd, J=11.0 and 5.0 Hz, H-6α), 3.64 (1H, t, J=11.0 Hz, H-6β), 3.45 (1H, ddd, J=11.0, 7.0, and 5.0 Hz, H-6a); ^{13}C-nmr data, see Table 1; eims m/z [M] 284 (100), 162 (20), 267 (15), 175 (13); hreims m/z $[M]^+$ 284.0683 ($C_{16}H_{12}O_5$ requires 284.0685).

DNA STRAND-SCISSION ASSAY.—The DNA strand-scission assay was adapted from the procedure described by Sugiyama *et al.* (11). Briefly, samples were dissolved in DMSO at a concentration of 2 mg/ml, and assayed at a final concentration of 25 μg/ml. The assay reaction mix contained 25 mM cacodylate buffer pH 7.0, 0.3 mM $CuCl_2$, and 500 ng covalently closed circular pBR322 (≥90% supercoiled) as a substrate. The assay was initiated by addition of the sample, incubated for 30 min at room temperature while protected from light, and terminated by addition of a stop mix (7.8% Ficoll, 7 mM EDTA, 0.8% sodium dodecylsulfate, 0.25% bromophenol blue, 0.25% xylene cyanol). The reaction mixture was analyzed by electrophoresis at 55 volts overnight (15 h) on a 1% agarose (Gibco) gel prepared in 0.5X TBE buffer (45 mM Tris-borate, 1 mM EDTA). The DNA was imaged by ethidium bromide fluorescence which was photographed on Polaroid type 57 film. The fluorescence of the relaxed, supercoiled, and linear pBR322 bands was measured through the use of a PDI scanning densitometer with Quantity One software.

Each experiment included DMSO and 0.1 μg/ml bleomycin sulfate (Sigma)/Fe^{++} samples as negative and positive controls, respectively. The result of each assay was calculated as the difference between the relative percent of relaxed and supercoiled pBR322. This result was normalized to the response of the negative and positive controls according to the following equation:

$$\text{ratio to control} = \frac{(\Delta_S - \Delta_-)}{(\Delta_+ - \Delta_-)}$$

where Δ_S, Δ_-, and Δ_+ correspond to the difference values of the sample, and the negative and positive controls, respectively. Thus, an experimental value of 1.0 means that the response for that sample at 25 μg/ml is equivalent to the response of 0.1 μg/ml bleomycin sulfate.

In order to compare the potency of pure compounds, the response from a sample dilution series was compared to that of a bleomycin sulfate dilution series, and the midpoints of the response curves were determined. The midpoint for **1** was 1.9 μg/ml, while the midpoint for bleomycin sulfate was 0.05 μg/ml. This indicated that bleomycin is approximately 38 times more potent than **1** on a weight basis. Correcting for the mol wts of **1** and bleomycin sulfate (1510 g/mol), the DNA strand-scission activity of bleomycin is approximately 190 times more potent than **1** on a molar basis.

CYTOTOXICITY ASSAY.—The KB cell line was purchased from the American Type Culture Collection (Rockville, MD). The assay was carried out in a 96-well microtiter plated as described previously (12). Compounds **1–3** showed moderate cytotoxicity with ED_{50} values of 0.9, 4.0, and 5.6 μg/ml, respectively.

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