POTENTIAL ANTITUMOR AGENTS. XVII. PHYSALIN B AND 25,26–EPIDIHYDROPHYSALIN C FROM *WITHERINGIA COCCOLOBOIDES*

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ABSTRACT.—An investigation of *Witheringia coccoloboides* (Dammer) A. T. Hunziker [*Capsicum fuscoviolaceum* (Cufodontis) Mort. and Standl.] roots has resulted in the isolation of two cytotoxic compounds: physalin B (**1**) and a novel physalin, which was characterized by spectral analysis as 25,26-epidihydrophysalin C (**2**). Both compounds have demonstrated cytotoxic activity in 9KB and 9PS tumor cells (*in vitro*). Physalin B (**1**) has demonstrated moderate activity against the 3PS mouse leukemia (*in vivo*).

In our program of investigation of higher plants with antitumor activity, an alcohol extract of the roots of *Witheringia coccoloboides* (Dammer) Hunz. (Solanaceae) showed promising activity in Eagles 9KB nasopharyngeal carcinoma and 9PS mouse lymphocytic leukemia (*in vitro*) (1). These observations prompted a detailed study of this plant, the results of which are described in this paper.

The powdered roots of *W. coccoloboides* were extracted with 95% ethanol and further triturated with chloroform and then water to yield three fractions: chloroform solubles, water solubles, and residue (chloroform/water insolubles). The 9KB and 9PS activity was found to concentrate in the chloroform solubles and the residue (chloroform/water insoluble fraction, table 1). Further trituration of the chloroform fraction with first 10% aqueous methanol and then petroleum ether gave three fractions: aqueous methanol solubles, petroleum ether solubles, and residue (aqueous methanol/petroleum ether insolubles). This last fraction was developed on a silica gel column with a gradient elution of chloroform-methanol. The fractions were monitored by tlc and combined accordingly. Two major cytotoxic compounds, **1** and **2**, (table 1) were isolated by this method and were further purified by fractional crystallization with acetone and chloroform.

TABLE 1. Cytotoxic activity of fractions from *W. coccoloboides*.

Fraction[a]	9KB ED$_{50}$ µg/ml	9PS ED$_{50}$ µg/ml
95% ethanol solubles	1.9 x 10°	1.1 x 10¹
water solubles	3.3 x 10¹	3.5 x 10¹
chloroform-solubles	2.3 x 10°	2.6 x 10°
chloroform/water insolubles	1.5 x 10°	2.2 x 10°
aqueous methanol solubles	1.2 x 10°	4.9 x 10¹
aqueous methanol/petroleum ether insolubles	7.5 x 10⁻²	5.0 x 10⁻¹
petroleum ether solubles	1.9 x 10¹	1.6 x 10°
Physalin B[a]	3.1 x 10°	L 1.0 x 10⁻²
25,26-Epidihydrophysalin C	1.2 x 10¹	8.9 x 10⁻¹
5α,6α-Epoxyphysalin B[b]	4.3 x 10⁻¹	1.6 x 10°

[a]Physalin B (NSC-Number 287088) showed activity in 3PS (137% T/C at 300 mg/kg) and is undergoing further evaluation in the NCI tumor panel. All other fractions were inactive in 3PS.
[b]Prepared synthetically from physalin B.

TABLE 2. NMR spectra of Physalins.

	Physalin B[1] (published) (in CDCl3)	Physalin B[5] (in CDCl3)	Physalin B[3] (published) (in DMSO-d6)	Physalin B[3] (in DMSO-d6)	25,26-Epidihydrophysalin C (in CDCl3)	25,26-Epidihydrophysalin C (in DMSO-d6)	Physalin C[6] (published) (in DMSO-d6)	Tetrahydrophysalin C[6] (published) (in DMSO-d6)
H-2	5.96 dq	5.91 (ddd, 10.1, 3.1, 1.2)	5.80 (d, 10)	5.79 (d, 10)	5.87 (ddd, 10.1, 2.1, 1.1)	5.81 (bd, 10.8)	5.82 (bd, 9)	—
H-3	6.85 dq	6.79 (ddd, 10.1, 4.6, 2.7)	6.88 (dt, 10, 3)	6.88 (dt, 9.9)	6.77 (ddd, 10.1, 4.8, 2.7)	6.91 (bd, 10)	6.91 (dm, 9)	—
H-6	5.65 m	5.57 (dm, 6.2)	5.62 (m)	5.61 (m)	5.61 (dm, 6.4)	5.62 (m)	5.62 (m)	5.50 (m)
H-22	4.60 m	4.55 (dd, 3.5, 2.3)	4.57 (m)	4.57 (m)	4.56 (dd, 4.7, 1.3)	4.46 (dd, 4, 2.6)	4.55 (m)	4.45 (m)
H-26	—	3.76 (dd, 13.4, 1.3), 4.52 (dd, 13.6, 4.8)	3.60 (d, 13), 4.28 (dd, 13, 4)	3.59 (d, 12.9), 4.28 (dd, 13, 3.9)	1.3 (d, 7.3)	1.23 (d, 7.9)	5.57 (b..), 6.39 (bs)	1.10 (d, 8.0)
H-19	1.27 (s)	1.26 (s)	1.19 (s)	1.16 (s)	1.18 (s)	1.06 (s)	1.07 (s)	1.07 (s)
H-21	2.00 (s)	1.96 (s)	1.81 (s)	1.78 (s)	1.91 (s)	1.74 (s)	1.80 (s)	1.74 (s)
H-28	1.21 (s)	1.21 (s)	1.12 (s)	1.09 (s)	1.33 (s)	1.11 (s)	1.55 (s)	1.42 (s)
OH-13	—	4.06 (s)	6.26 (s)	6.27 (s)	—	6.12 (s)	6.13 (s)	6.07 (s)
OH-14	—	—	—	—	—	6.40 (s)	5.90 (s)	6.38 (s)

[1]Proton assignment for Physalin B (reference 3); [3]Reference 2; [3,4]Isolated Physalin B and 25,26-Epidihydrophysalin C recorded on Nicolet NTC 360; [6,7]Isolated Physalin in DMSO-d6 recorded on Varian FT-80; [6]Reference 4.

Spectroscopic analysis of compound **1** indicated that it was physalin B, a compound which had been previously reported in a number of *Physalis* species (2, 3). This assignment was confirmed by comparison with published physical data as well as with ir and nmr spectra of authentic physalin B (2, 3).

The ms of physalin B showed a strong molecular ion at 510. The ms of data a swell as with ir and nmr spectra of authentic physalin B (2, 3).
a strong peak at 494 (M^+–18). High resolution ms of this peak at 494.191 (M^+–18, calculated for $C_{28}H_{30}O_8$, 494.194) indicated, together with elemental analysis, a molecular formula of $C_{28}H_{32}O_9$ for compound **2**.

The uv spectrum of compound **2** showed λ max at 222 nm ($\epsilon = 7100$) which suggested the presence of a conjugated enone system. The ir spectra of **1** and **2** were very similar in the carbonyl region, but did show differences in the C–O stretching and CH_3– bending regions. The presence of one extra hydroxyl group was indicated by the appearance of an extra broad absorption band at 3240 cm^{-1} and was further confirmed by ^{1}H nmr (DMSO-d$_6$) (table 2).

Further examination of the ^{1}H nmr spectra of **1** and **2** in CDCl$_3$ and DMSO-d$_6$ led to the structure of compound **2**. In contrast to **1**, compound **2** showed a signal at δ 1.3 (d, 7.3 Hz) (CDCl$_3$) [δ 1.23 (d, *J* 7.9) (DMSO-d$_6$)], assigned to a secondary methyl group at C–25, which was accompanied by the disappearance of the AB quartet assigned to the C–26 methylene protons in **1**.

Comparison of the ^{1}H nmr of **2** with that of physalin C, **3** (table 2; 4), showed that the signals closely match except for two broad singlets at δ 5.57 and δ 6.39; these were assigned to the two olefinic protons in physalin C whose signals were replaced by the doublet assigned to the secondary methyl group at C–25 in **2**.

Again, examination of stereomodels of both compounds showed that the *tert*-Me at C–24 of physalin C is oriented in such a way with respect to the C–25,26-double bond as to experience a deshielding effect, hence the downfield shift of the signal by comparison with the corresponding group in **2** (table 2; 5).

The signals reported for the *tert*-Me and *tert*-OH groups of a semisynthetic compound, tetrahydrophysalin C (4) (table 2), closely matched the corresponding signals of **2**, with the exception of the 24-methyl signal at 1.14 ppm (in DMSO-d$_6$). The corresponding signal in tetrahydrophysalin C, (4) was δ 1.42 (s) (table 2). This difference may suggest that the secondary methyl group at C–25 in **2** has a β-configuration. Based on this observation, compound **2** is proposed to be 25,26-epidihydrophysalin C. This assignment was further substantiated by the difference in the *tert*-Me signals at C–24 observed in closely related semi-synthetic physalins with an α- or β-configuration of the secondary methyl group at C–25 (**5,6**, table 3, (2)). In all these cases the highest signal was assigned to the methyl at C–10 (H–19), the most shielded group due to its location perpendicular to the enone system. The lowest signal was assigned to the α-methyl at C–20 (H–21). This signal was not influenced by any shielding effect from neighboring groups and was deshielded by a neighboring oxygen atom.

Two unassigned singlets for the *tert*-OH at C–13 and C–14 were reported for physalin C (δ 6.13, δ 5.90) and tetrahydrophysalin C (δ 6.07, δ 6.38) (4). In epidihydrophysalin C, a pair of singlets at δ 6.12 and δ 6.40 was observed. Examination of a stereomodel of these compounds showed that, whereas the C–14 *tert*-OH was close enough to C–25 and C–26 to be influenced by reduction of the olefinic bond, the C–13 *tert*-OH was more distant. Accordingly, the singlet at δ 6.13 was ascribed to the *tert*-OH at C–13 of physalin C and the singlet at δ 6.07 assigned to the *tert*-OH at C–13 of tetrahydrophysalin C. Likewise the singlet at
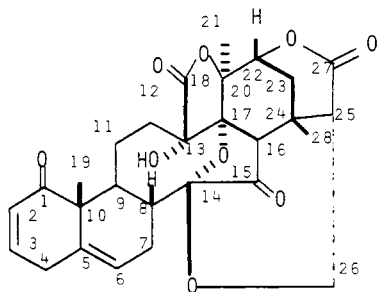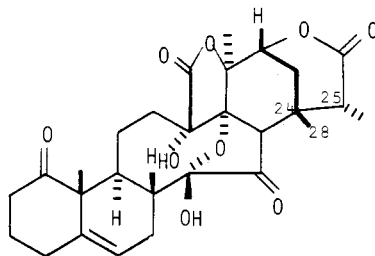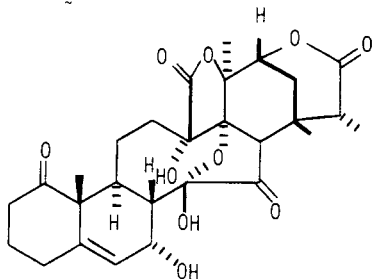
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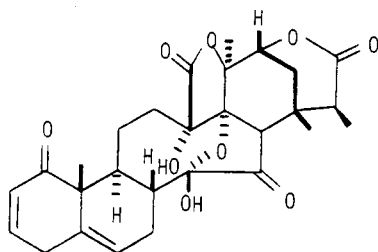
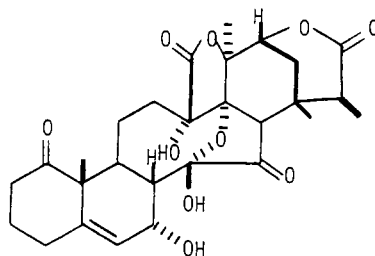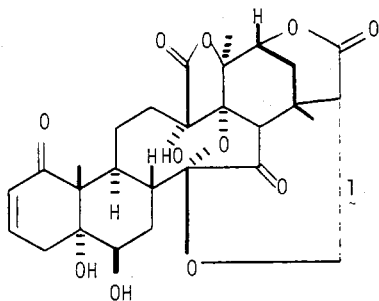2, 25,26-Epidihydrophysalin C
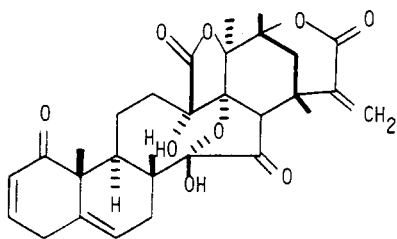
3, Physalin C

4, Tetrahydrophysalin C

5, Tetrahydrophysalin A

6, Epitetrahydrophysalin A

7, Physalin D

TABLE 3.   Nmr data of methyl groups of physalins with an α- or β-
secondary methyl at C–25 in DMSO-d_6.

Compound	H–19	H–21	H–28	H–26
2 (β)–Me	1.06 (s)	1.74 (s)	1.14 (s)	1.23 (d, J 7.9)
4 (α)–Me	1.07 (s)	1.74 (s)	1.42 (s)	1.10 (d, J 8.0)
5 (α)–Me	1.09 (s)	1.67 (s)	1.46 (s)	1.11 (d, J 8.0)
6 (β)–Me	1.12 (s)	1.69 (s)	1.12 (s)	1.26 (d, J 6.0)

2: 25,26-epidihydrophysalin C; **4**: tetrahydrophysalin C (reference 4);
5: tetrahydrophysalin A; **6**: epitetrahydrophysalin A (reference 2).

δ 6.12 was assigned to the C–13 *tert*-OH in epidihydrophysalin C.   The resonance
of the second *tert*-OH at δ 5.90 in physalin C compared to the signal at δ 6.40 for
epidihydrophysalin C and at δ 6.38 in tetrahydrophysalin C can be logically
explained as being a result of the shielding effect of the π-electrons on the C–25,26-
double bond resulting in the upfield shift of the C–14 *tert*-OH in physalin C by
comparison to epidihydrophysalin C and tetrahydrophysalin C.

The physalins are biosynthetically closely related to the withanolides, a group
of compounds with established cytotoxic and antitumor activity (6).   The
physalins have been less studied.   Only physalin D, (7) has been evaluated previ-
ously; it shows moderate activity in B16 melanocarcinoma (7).   It has been
found that the 5,6-epoxide ring of withanolides is partially responsible for their
antitumor activity (6).   Derivatives of physalin B with a 5α,6α-epoxide ring
(physalin J) and a 5β,6β-epoxide ring (physalin F) have both been found to
occur in *Physalis angulata* (8).

A synthesis of the mixed physalin B epoxides (**8, 9**) was done, and 5α,6α-
epoxyphysalin B was separated and found to be more cytotoxic in 9KB and less
toxic in 9PS than physalin B (table 1).   The epoxide mixture was submitted
for the B16 antitumor screen in conjunction with physalin B, which is currently
being investigated in the NCI animal tumor panel.
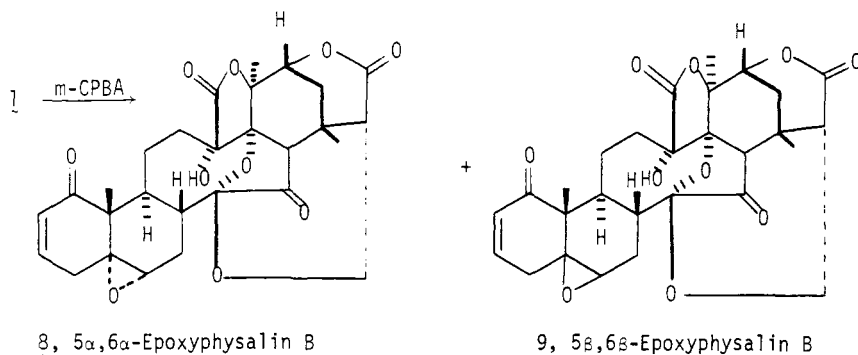
8, 5α,6α-Epoxyphysalin B   **9**, 5β,6β-Epoxyphysalin B

EXPERIMENTAL[1]

PLANT MATERIAL.—*W. coccoloboides* was collected in Costa Rica, by Dr. Jose Saenz-Renauld, University of Costa Rica, and authenticated by the Economic Botany Laboratory, USDA, Beltsville, Maryland, through which voucher specimens are preserved (B-822773, PR 49575).

EXTRACTION AND FRACTIONATION.—The dried, powdered root (20 kg) was percolated with 95% ethanol. The ethanol extract was then flash-evaporated, concentrated under vacuum, and finally freeze-dried to give 670 g of extract. The ethanol extract was fractionated by trituration and sonication with 2 liters of chloroform. The chloroform extract was then filtered through glass wool and evaporated under vacuum; 150 g of chloroform soluble material was obtained. The residue was then extracted in an identical manner with water to give 260 g of water solubles; 160 g of insoluble material remained.

The chloroform extract was further triturated with 1 liter of 10% aqueous methanol and then 1 liter of petroleum ether; 59 g of methanol solubles, 46 g of petroleum ether solubles and residue weighing 39 g resulted.

The seven fractions thus obtained were tested against 9KB and 9PS (*in vitro*) and in 3PS (*in vivo*) in accordance with established protocol (1) (table 1).

ISOLATION OF PHYSALINS.—The aqueous methanol-petroleum ether insoluble fraction was partially purified by successive treatment with petroleum ether, benzene, ethyl acetate, and 10% aqueous methanol. The insoluble residue was dissolved in chloroform, adsorbed onto 40 g MN–Kieselgel 60 (0.05–0.2 mm), and developed on a 500 g column of the same grade of silica gel.

Elution of the column was carried out with 9.5 liters chloroform; 6.0 liters chloroform-methanol (99.5:0.5); 4.75 liters chloroform-methanol (99:1); 2.0 liters chloroform-methanol (98:2); 2.0 liters chloroform-methanol (96:4); 1.0 liter chloroform-methanol (92:8), and, finally, 1.5 liters methanol. Fractionation was monitored by silica gel tlc with chloroform-methanol (95:5). The fractions were combined to form six major fractions as follows: 1–17.6 liters; 2–1.2 liters; 3–1.9 liters; 4–2.0 liters; 5–0.8 liters, and 6–1.7 liters.

The second fraction was found to be mainly physalin B (0.04% w/w). Tlc of the third and fourth fractions gave a major spot which was later identified as 25,26-epidihydrophysalin C (0.03% w/w). Both compounds were purified by repeated recrystallization from chloroform, acetone, or methanol.

IDENTIFICATION OF THE ISOLATED COMPOUNDS.—*Physalin B* (**1**)—This compound crystallized as colorless needles from methanol, mp 266–270° [lit. 269–272° (2)]. The compound was recrystallized from acetone; $[\alpha]D-124°$ (c 0.054 in EtOH) [lit. $-150°$ c 0.18 (2)]; ir ν max (KBr) 3400, 1780, 1755, 1740 and 1650 cm^{-1} (indistinguishable from the ir spectrum of authentic physalin B); uv λ max 222 nm ($\epsilon=7800$) [lit. 10,000 (2)]; ms: m/e 510 (M$^-$), 495 (M-CH$_3$) and 492 (M-H$_2$O); high resolution ms M$^+$ 510.192 (calc. C$_{28}$H$_{30}$O$_9$, M$^-$ 510.188).

25,26-EPIDIHYDROPHYSALIN C (**2**).—This compound crystallized from methanol mp 230–234° (white needles) (Found: C, 64.22; H, 6.50; C$_{28}$H$_{32}$O$_9$·CH$_3$OH calcd. C, 64.00; H, 6.60); ir ν max (CHCl$_3$) 3480 br (OH), 3240 br (OH), 1780 (γ-lactone), 1750 (cyclopentenone), 1720 (δ-lactone), 1660 (conjugated ketone), 1600 (carbonyl) cm^{-1}; uv: λ max (EtOH) 222 nm ($\epsilon=7100$); $[\alpha]D-114°$ (c, 0.057 in EtOH); high resolution ms M$^-$-H$_2$O 494.191 (calc. C$_{28}$H$_{30}$O$_8$, M$^-$-H$_2$O, 494.194).

EPOXIDATION OF PHYSALIN B.—This procedure was done according to the published method (3) as follows: m-chloroperbenzoic acid (250 mg) was added to a solution of physalin B (**1**) (500 mg) in chloroform (75 ml); the mixture was kept for 24 hrs at room temperature then washed successively with 2% NaHCO$_3$, 1N HCl and H$_2$O, dried over anhydrous Na$_2$SO$_4$, and the solvent was then removed under vacuum; 400 mg of the epoxide mixture was obtained, which on silica tlc in ethyl acetate-benzene (4:1), showed two spots, the one with lower Rf being the 5α,6α-epoxyphysalin B, the high Rf spot being the 5β,6β-epoxyphysalin B (**3**); 2 mg of 5α,6α-epoxyphysalin B was separated by preparative tlc and submitted for 9KB and 9PS testing (table 1). The mixture of epoxides (390 mg) was submitted to the National Cancer Institute for testing in B16.

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