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# PLANT ANTICANCER AGENTS XLIV. CYTOTOXIC CONSTITUENTS FROM *STIZOPHYLLUM RIPARIUM*[1]

CHANG-YIH DUH, JOHN M. PEZZUTO, A. DOUGLAS KINGHORN,* SAI L. LEUNG,[2] and NORMAN R. FARNSWORTH[3]

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

ABSTRACT.—Bioactivity-guided fractionation of a $CHCl_3$ extract of *Stizophyllum riparium* has afforded six new compounds, namely, the triterpene esters, 3β-hydroxy-24-*trans*-ferulyloxyurs-12-en-28-oic acid [**1**], 3β-hydroxy-24-*cis*-ferulyloxyurs-12-en-28-oic acid [**2**], 3β,19-dihydroxy-24-*trans*-ferulyloxyurs-12-en-28-oic acid [**3**], and the pregnane derivatives, 2α,3β,12β-trihydroxypregna-4,7,16-trien-20-one [**4**], 2α,3β,12β-trihydroxypregna-4,7-dien-20-one [**5**], and 16α-methoxy-2α,3β,12β-trihydroxypregna-4,7-dien-20-one [**6**]. The structures of these isolates were established by spectroscopic and chemical methods. Compounds **1**, **2**, and **4** exhibited cytotoxic activity against the P-388 lymphocytic leukemia test system in cell culture.

*Stizophyllum riparium* (H.B.K.) Sandw. (Bignoniaceae) is one of three members of a small genus native to continental tropical America (2). No previous phytochemical or biological studies appear to have been performed on extracts of this species.

As part of a continuing search for tumor inhibitors from plant sources, *S. riparium* was selected for study when a $CHCl_3$ extract of the entire plant was found to display significant activity against the P-388 lymphocytic leukemia test system in cell culture, when assessed using standard protocols (3). Bioactivity-guided chromatographic fractionation has led to the isolation and characterization of two new cytotoxic triterpene esters, 3β-hydroxy-24-*trans*-ferulyloxyurs-12-en-28-oic acid [**1**] and its isomer, 3β-hydroxy-24-*cis*-ferulyloxyurs-12-en-28-oic acid [**2**], as well as a new cytotoxic pregnane derivative, 2α,3β,12β-trihydroxypregna-4,7,16-trien-20-one [**4**]. An additional four compounds were isolated in the course of this investigation that were devoid of cytotoxic activity, namely, ursolic acid, and the new compounds, 3β,19-dihydroxy-24-*trans*-ferulyloxyurs-12-en-28-oic acid [**3**], 2α,3β,12β-trihydroxypregna-4,7-dien-20-one [**5**], and 16α-methoxy-2α,3β,12β-trihydroxypregna-4,7-dien-20-one [**6**].

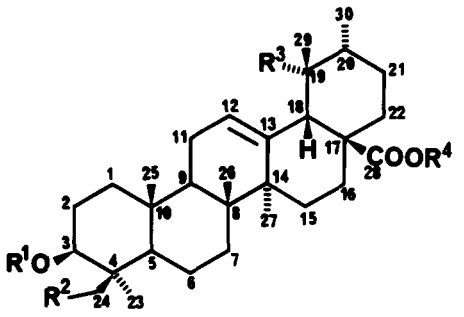
## RESULTS AND DISCUSSION

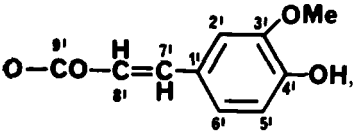
The molecular formula of **1**, the most abundant triterpene ester constituent obtained in this investigation, was determined as $C_{40}H_{56}O_7$ by hrms. In its ir spectrum, **1** exhibited characteristic signals at 3400 (OH), 1705 (carboxylic acid), 1693 (αβ-unsaturated ester), and 1628 and 1595 $cm^{-1}$ (aromatic ring). The presence of significant fragment peaks in the mass spectrum of this compound at *m/z* 248 and *m/z* 207, resulting from *retro*-Diels-Alder cleavage of ring C under electron impact, suggested that **1** was a $\Delta^{12}$-unsaturated triterpene (4-6). Comparison of the methyl signals in the $^1H$-nmr spectrum of **1** (Table 1), as well as its $^{13}C$-nmr data (Table 2) with published values, allowed the assignment of the carbon skeleton of this isolate as $\Delta^{12}$-ursene (5-14). Spectroscopic evidence indicated that **1** possessed three functionalities, namely, a carboxylic acid unit, a secondary hydroxy group, and a *trans*-ferulic acid (3-methoxy-4-hy-

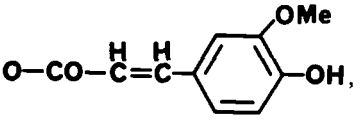
[1]This article commemorates the 50th year of publication of the *Journal of Natural Products* (formerly *Lloydia*). For the previous paper in this series, see Hamburger *et al.* (1).

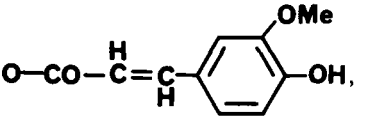
[2]On leave from the National University of Singapore, Singapore.

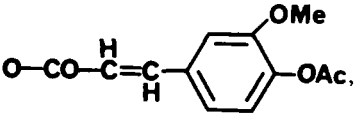
[3]Member of the Editorial Advisory Board of the *Journal of Natural Products* (*Lloydia*) since 1961.



**1** $R^1$=H, $R^2$=  $R^3$=H, $R^4$=H

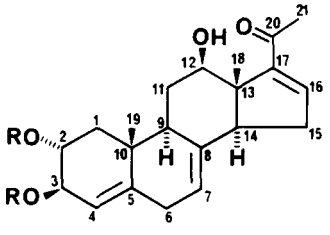
**2** $R^1$=H, $R^2$=  $R^3$=H, $R^4$=H

**3** $R^1$=H, $R^2$=  $R_3$=OH, $R^4$=H

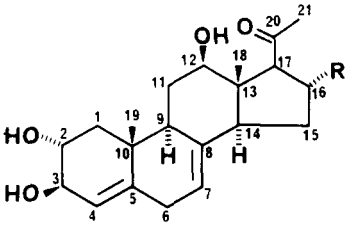
**7** $R^1$=Ac, $R^2$=  $R^3$=H, $R^4$=H

**8** $R^1$=H, $R^2$=OH, $R^3$=H, $R^4$=$CH_3$

**9** $R^1$=Ac, $R^2$=OAc, $R^3$=H, $R^4$=$CH_3$



**4** R=H
**10** R=Ac



**5** R=H
**6** R=OMe

droxycinnamic acid) moiety esterified at a hydroxymethyl substituent. The observation of a characteristic doublet at δ 2.18 ppm ($J$=11 Hz) for the allylic 18β-H, pointed to **1** being a $\Delta^{12}$-ursene derivative substituted in rings D and E with a free COOH group at C-28 (4,6,7,14). Comparison with the $^1$H-nmr data of known ursolic acid derivatives with 3α- or 3β-OH substituents (15-18) permitted the tentative assignment of the doublet of doublets that was centered at δ 3.32 in the $^1$H-nmr spectrum of **1**, as being due to a 3α- (axial-) substituted proton. Acetylation of **1** to produce **7** led to the observation in the $^1$H-nmr spectrum of angular methyl signals at δ 0.98, 1.08, and 0.77 (C-23, -25, and -26, respectively), and a 3α-H resonance at δ 4.60, which were consistent

TABLE 1. $^1$H-nmr Chemical Shifts (δ) and Coupling Constants (Hz, in parenthesis) of the Triterpenoids **1-3**, **7**, and **8**[a]

| Proton | Compounds | | | | |
|---|---|---|---|---|---|
| | **1** | **2** | **3** | **7** | **8** |
| 3α | 3.32 dd (9,8) | 3.27 dd (10,6) | 3.32 dd (9,8) | 4.60 m | 3.44 dd (11,4) |
| 12 | 5.23 t (3) | 5.23 t (3) | 5.35 t (3) | 5.23 br s | 5.23 t (4) |
| 18β | 2.18 d (11) | 2.18 d (11) | 2.54 s | 2.18 (11) | 2.21 d (12) |
| 23 | 0.98 s | 0.93 s | 0.93 s | 0.98 s | 0.86 s |
| 24 | 4.27 d (12) | 4.17 d (12) | 4.26 d (12) | 4.18 d (12) | 3.33 d (11) |
| | 4.47 d (12) | 4.37 d (12) | 4.48 d (12) | 4.60 d (12) | 4.20 d (11) |
| 25 | 1.08 s | 1.07 s | 1.21 s | 1.08 s | 1.07 s |
| 26 | 0.75 s | 0.74 s | 0.71 s | 0.77 s | 0.71 s |
| 27 | 1.20 s | 1.12 s | 1.22 s | 1.09 s | 1.23 s |
| 29 | 0.85 d (6) | 0.84 d (6) | 1.26 s | 0.85 d (6) | 0.85 d (5) |
| 30 | 0.94 d (6) | 0.93 d (6) | 0.95 d (6) | 0.97 d (6) | 0.94 d (6) |
| 2′ | 6.88 d (1) | 7.68 d (2) | 6.91 d (2) | 7.07 m | |
| 5′ | 6.85 d (8) | 6.88 d (8) | 6.86 d (8) | 7.07 m | |
| 6′ | 6.97 dd (8,1) | 7.13 dd (8,2) | 7.00 dd (8,2) | 7.07 m | |
| 7′ | 7.50 d (16) | 6.80 d (13) | 7.52 d (16) | 7.60 d (16) | |
| 8′ | 6.18 d (16) | 5.78 d (13) | 6.19 d (16) | 6.35 d (16) | |
| 3′-OMe | 3.83 s | 3.91 s | 3.85 s | 3.87 s | |
| 28-OMe | | | | | 3.59 s |
| -OAc | | | | 2.00 s, 2.33 s | |

[a]360 MHz, $CDCl_3$, δ-scale, relative to TMS.

with **7** being a 3β,24-disubstituted $\Delta^{12}$-ursene derivative (14). The $^{13}$C-nmr chemical shifts C-23 and C-24 in **1** (δ 22.2 and 65.5 ppm, respectively) were indicative of the ferulic acid substitution in this compound occurring at C-24, since these data are comparable with analogous chemical shifts of known 24-*O*-substituted ursene derivatives (8,14).

Hydrolysis of **1** with 1% KOH in MeOH at room temperature afforded several major products after methylation. One of these products, **8**, was identified as methyl 3β,24-dihydroxyurs-12-en-28-oate, and in its $^1$H-nmr spectrum (Table 1) the C-24 methylene protons exhibited a greater chemical shift difference (δ 3.33 vs. 4.20) than observed for **1**, probably because of hydrogen bond formation between the 3β- and 24-hydroxy groups. Acetylation of **8** afforded the known compound 3β,24-diacetoxyurs-12-en-28-oic acid methyl ester [**9**], and this identification was confirmed by direct comparison of the $^1$H-nmr spectrum of **9** with those of the authentic compound and its isomer, methyl 3β,23-diacetoxyurs-12-en-28-oate, that were obtained in a previous investigation on *Hedyotis lawsoniae* (DC.) Wright & Arn. (Rubiaceae) (14). A second major product obtained in this investigation was *trans*-ferulic acid methyl ester, which was identical in all respects (uv, ir, $^1$H nmr, ms, tlc) to a methylated commercial sample of *trans*-ferulic acid. Compound **1** was, therefore, assigned as 3β-hydroxy-24-*trans*-ferulyloxyurs-12-en-28-oic acid.

Compound **2** was obtained in lower yield than its isomer **1**, and the two compounds exhibited closely comparable spectroscopic data. However, in the $^1$H-nmr spectrum of **2**, *cis*-conjugated olefinic protons (δ 6.80 and 5.78 ppm, $J$=13 Hz) were apparent, in contrast to the analogous *trans*-conjugated signals (δ 7.50 and 6.18 ppm, $J$=16 Hz) observed in the $^1$H-nmr signal of **1**. In addition, the aromatic ring protons at C-2′ and C-6′ occurred at lower field in the $^1$H-nmr spectrum of **2** than those of **1** (Table 1). Similar chemical shift and coupling constant differences have been described in the literature for *cis*- and *trans*-*p*-coumaryl esters of another triterpene alcohol (19). Therefore,

TABLE 2. $^{13}$C-nmr Chemical Shifts of the Triterpenoids **1-3**, **7**, and **8**[a]

| Carbon | Compounds | | | | |
|---|---|---|---|---|---|
| | **1** | **2** | **3** | **7** | **8** |
| 1 | 38.5 | 38.9 | 38.3 | 38.3 | 38.2 |
| 2 | 27.1 | 27.9 | 27.2 | 23.4 | 27.5 |
| 3 | 79.4 | 79.6 | 79.5 | 80.1 | 80.7 |
| 4 | 42.3 | 42.5 | 42.3 | 41.8 | 42.5 |
| 5 | 55.8 | 56.3 | 55.8 | 55.8 | 55.7 |
| 6 | 18.7 | 19.1 | 18.7 | 19.2 | 18.3 |
| 7 | 32.9 | 33.4 | 32.7 | 33.2 | 33.0 |
| 8 | 39.3 | 39.7 | 39.8 | 39.3 | 39.3 |
| 9 | 47.4 | 47.9 | 47.1 | 47.4 | 47.4 |
| 10 | 36.7 | 37.1 | 37.4 | 36.7 | 36.5 |
| 11 | 23.9 | 24.3 | 23.7 | 23.9 | 23.4 |
| 12 | 125.4 | 125.8 | 129.0 | 125.3 | 125.2 |
| 13 | 137.7 | 138.2 | 137.7 | 137.8 | 137.6 |
| 14 | 41.7 | 42.2 | 42.3 | 41.2 | 41.8 |
| 15 | 27.8 | 28.2 | 28.0 | 27.8 | 27.9 |
| 16 | 23.3 | 23.6 | 25.1 | 23.3 | 24.0 |
| 17 | 47.8 | 48.2 | 47.6 | 47.8 | 47.9 |
| 18 | 52.3 | 52.8 | 52.6 | 52.4 | 52.7 |
| 19 | 38.8[b] | 39.3[b] | 72.9 | 38.9 | 38.9[b] |
| 20 | 38.7[b] | 39.1[b] | 40.9 | 38.8 | 38.7[b] |
| 21 | 30.4 | 30.8 | 25.8 | 30.4 | 30.5 |
| 22 | 36.6 | 36.9 | 36.8 | 36.5 | 36.5 |
| 23 | 22.2 | 22.6 | 22.2 | 22.5 | 22.4 |
| 24 | 65.5 | 65.7 | 65.5 | 65.2 | 64.4 |
| 25 | 15.7 | 16.1 | 15.6 | 15.4 | 15.8 |
| 26 | 16.8 | 17.0 | 16.5 | 16.5 | 16.7 |
| 27 | 23.5 | 23.8 | 24.4 | 23.4 | 23.4 |
| 28 | 183.5 | 183.8 | 183.8 | 183.1 | 177.9 |
| 29 | 16.9 | 17.3 | 27.3 | 16.9 | 16.9 |
| 30 | 21.1 | 21.4 | 16.1 | 21.2 | 21.1 |
| 1′ | 126.6 | 125.8 | 126.6 | 133.1 | |
| 2′ | 109.2 | 109.6 | 109.0 | 111.1 | |
| 3′ | 146.2[c] | 146.3[c] | 146.5[c] | 151.2 | |
| 4′ | 147.9[c] | 147.8[c] | 147.9[c] | 141.3 | |
| 5′ | 114.5[d] | 113.1[d] | 114.5[d] | 120.9 | |
| 6′ | 114.9[d] | 114.2[d] | 115.0[d] | 118.4 | |
| 7′ | 145.0 | 144.7 | 145.0 | 143.7 | |
| 8′ | 123.1 | 116.7 | 123.1 | 123.1 | |
| 9′ | 166.4 | 166.6 | 167.0 | 166.5 | |
| -$OCH_3$ | 56.2 | 56.3 | 56.0 | 55.9 | 51.4 |
| -OAc | | | | 170.6, 168.6<br>20.6, 20.6 | |

[a]Chemical shifts were determined at 90.8 MHz in $CDCl_3$. The δ values are in ppm downfield of TMS.
[b-d]Signals may be interchanged.

compound **2** was accorded the structure 3β-hydroxy-24-*cis*-ferulyloxyurs-12-en-28-oic acid.

In the mass spectrum, compound **3** ($C_{40}H_{56}O_8$) exhibited a series of fragment peaks 16 mass units greater than analogous fragment peaks in the mass spectrum of compound **1**, and those at *m/z* 264 and 252 were consistent with there being an extra hydroxy group located in either ring D or ring E in **3** (5). Comparison of the $^1$H-nmr spectra of **3** and **1** indicated that the doublet due to the C-18 proton of the latter compound at δ 2.18 ppm was replaced by a singlet resonating at δ 2.54 ppm. In addition,

the C-29 methyl signal appeared downfield as a singlet at δ 1.26 ppm in the $^1$H-nmr spectrum of **3**, an observation consistent with this compound being a 19α-hydroxyurs-12-en-28-oic acid derivative (6-8,13,14). Confirmation of the assignment of the structure of **3** as 3β,19α-dihydroxy-24-*trans*-ferulyloxyurs-12-en-28-oic acid was obtained by the close comparison of the C-19, -20, -21, -22, -29, and -30 chemical shifts of the compound (Table 2) with published $^{13}$C-nmr data for known 19α-hydroxylated urs-12-en-28-oic acid derivatives (8,13). The chemical shifts of the remaining carbon atoms of **3** (Table 2) closely resembled those of **1**.

The functional groups of compound **4** ($C_{21}H_{28}O_4$) were determined as one or more hydroxy groups (3390 $cm^{-1}$) and an αβ-unsaturated carbonyl (1643 $cm^{-1}$) from an analysis of its spectrum. Furthermore, the $^1$H-nmr signals (Table 3) at δ 0.77 (18-

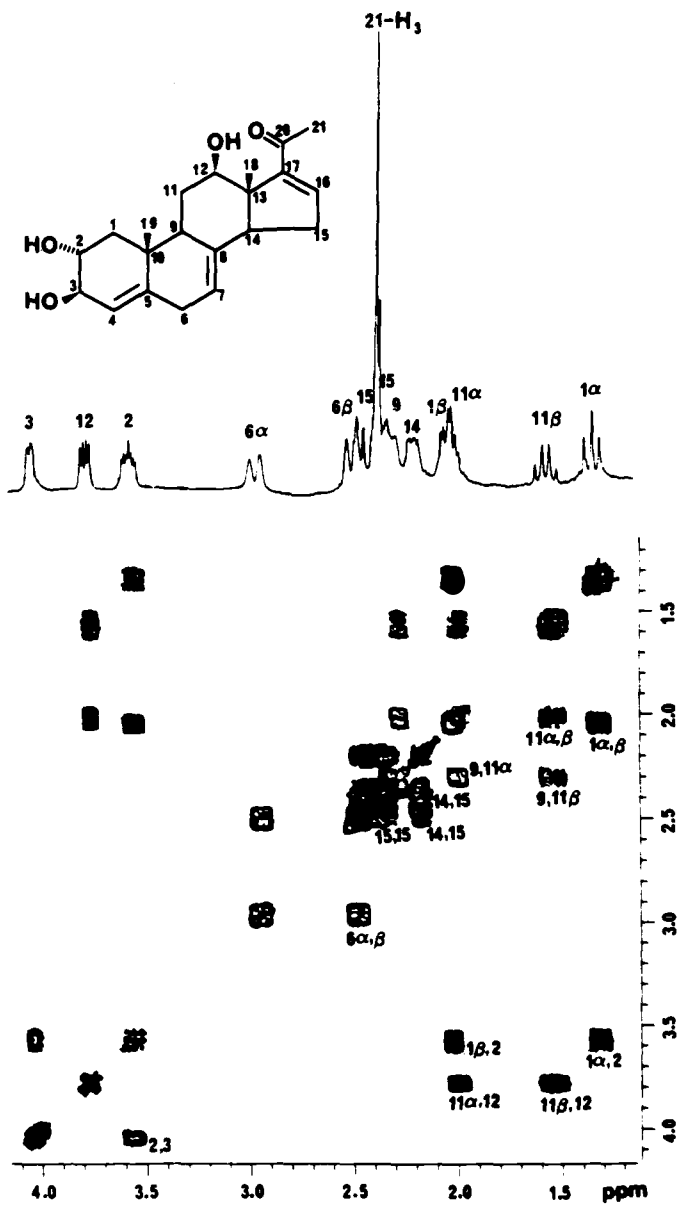



FIGURE 1. Upfield region of $^1$H-$^1$H homonuclear shift correlated nmr spectrum of **4**

TABLE 3. $^1$H-nmr Chemical Shifts (δ) and Coupling Constants (Hz, in parenthesis) of the Pregnane Derivatives **4-6** and **10**[a]

| Proton | Compounds | | | |
|---|---|---|---|---|
| | **4** | **5** | **6** | **10** |
| 1α | 1.32 dd (13,12) | 1.35 dd (13,13) | 1.35 dd (14,12) | 1.48 dd (14,12) |
| 1β | 2.05 dd (13,4) | 2.05, m | 2.05 dd (14,4) | 2.06 dd (14,4) |
| 2β | 3.58 ddd (12,8,4) | 3.57 m | 3.56 ddd (12,8,4) | 4.98 ddd (12,8,4) |
| 3α | 4.06 dd (8,2) | 4.03 dd (8,2) | 4.05 m | 5.35 m |
| 4 | 5.24 bs | 5.19 bs | 5.23 bs | 5.16 bs |
| 6α | 2.98 d (18) | 2.93 d (18) | 2.94 d (18) | 3.00 d (18) |
| 6β | 2.48 m | 2.47 d (18) | 2.49 m | 2.48 m |
| 7 | 5.35 m | 5.25 m | 5.24 m | 5.33 m |
| 9α | 2.32 m | 2.25 m | 2.24 m | 2.37 m |
| 11α | 2.04 m | 1.75 m | 1.90 ddd (13,7,5) | 2.00 m |
| 11β | 1.54 ddd (13,12,10) | 1.44 ddd (13,12,12) | 1.40 ddd (13,12,11) | 1.53 ddd (13,12,10) |
| 12α | 3.79 dd (10,5) | 3.58 m | 3.64 dd (12,5) | 3.80 dd (10,5) |
| 14α | 2.20 m | 1.79 m | 2.09 m | 2.21 m |
| 15α,β | 2.40 m | 1.70 m | 1.83 m | 2.34 m |
| | 2.48 m | 1.71 m | 1.81 m | 2.45 m |
| 16 | 7.00 dd (3,2) | 2.00 m (α or β) | 4.11 ddd (8,7,2) (β) | 6.97 dd (3,2) |
| | | 2.22 m (α or β) | | |
| 17 | | 2.57 dd (10,10) | 2.51 d (7) | |
| 18 | 0.77 s | 0.59 s | 0.58 s | 0.79 s |
| 19 | 1.06 s | 1.02 s | 1.02 s | 1.10 s |
| 21 | 2.37 s | 2.22 s | 2.34 s | 2.38 s |
| $-OCH_3$ | | | 3.35 s | |
| -OAc | | | | 2.01 s |

[a]360 MHz, $CDCl_3$, δ-scale, relative to TMS.

methyl), 1.06 (19-methyl), 2.37 (methyl group affixed to hydrogen-bonded αβ-unsaturated ketone), 3.79 (methine proton of 12β-hydroxyl), and 7.00 ppm (16-H) indicated the skeleton of **4** to be 12β-hydroxypregn-16-en-20-one (20-23). Two additional olefinic protons were observed in the $^1$H-nmr spectrum of **4** (δ 5.24 and 5.35 ppm) (Table 3), while three olefinic double bonds were apparent from $^{13}$C-nmr shifts at δ 120.2, 143.2, 118.0, 135.2, 148.7, and 154.4 ppm (Table 4). The absence of a conjugated diene system in the molecule of **4** was apparent from the lack of a uv maximum at a higher wave length than 241 nm. This factor, as well as coupling patterns in the $^1$H-nmr spectrum of **4** determined by $^1$H-$^1$H homonuclear shift-correlated experiments (24) (Figure 1), suggested that the two extra double bonds of **4** should be placed between carbons 4 and 5 and carbons 7 and 8.

The configuration of the hydroxy groups affixed to C-2 and C-3 in **4** was assigned as 2α-equatorial and 3β-equatorial for several reasons. The chemical shifts of the angular 19-methyl group of **4** and its diacetate **10** did not occur appreciably downfield in their $^1$H-nmr spectra (Table 3). Had the acetoxy group of **10** been in the 2β-axial position, the 19-methyl group chemical shift at δ 1.10 would have been expected to be significantly deshielded (25). In addition, the resonance at δ2.05 in the $^1$H-nmr spectrum of **4** was assigned to the 1β-equatorial proton, due to the proximity of the C-19 methyl group. The coupling constant of 4 Hz between 1β-H and the 2-proton of **4** suggested that the latter was affixed in a β-axial manner, and the 12 Hz $J$ value between the 2β- and 1α-protons was indicative of axial/axial coupling. It is further deduced that the 2β- and 3α-protons were also arranged *trans*-diaxially ($J$=8 Hz). Additional information for the structure proposed for **4** came on the acetylation of the C-2 hydroxyl group in

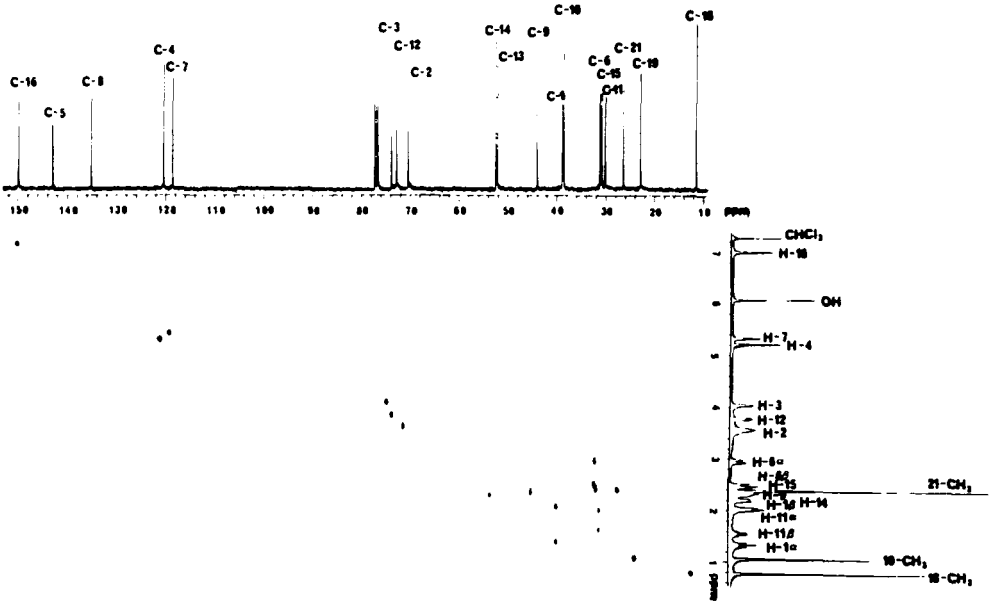



FIGURE 2. $^{1}H$-$^{13}C$ Heteronuclear shift correlated nmr spectrum of **4**

**10**, which demonstrated selective deshielding of the 1α-proton (δ 1.48 in **10** vs. δ 1.32 in **4**).

The stereochemistry of **4** between the ring junctions was confirmed by a two-dimensional nOe experiment (24), in which nOe enhancements were observed between the 9- and 12- protons, thus implying an α-orientation of H-9, as well as between H-9 and H-14, which suggested a C/D-ring *trans*-junction. Similarly, nOe effects were observed between the 18- and 19-angular methyl groups and between both of these functionalities and H-11β. The assignment of the $^{13}C$-nmr chemical shifts of **4** was achieved by the application of a $^{1}H$-$^{13}C$ heteronuclear shift correlated two-dimensional experiment (26), the results of which are shown in Figure 2.

Compound **4** was, therefore, assigned the structure 2α,3β,12β-trihydroxypregna-4,7,16-trien-20-one, and is the first pregnane derivative to have been found in a plant in the family Bignoniaceae. It may be pointed out that **4** formed only a diacetate, **10**, when acetylated under normal conditions, presumably because of steric hindrance of the 12β-hydroxy group and/or hydrogen bonding between this functionality and the carbonyl group at C-20.

Compound **5** ($C_{21}H_{30}O_4$) was obtained as a further steroidal isolate from *S. riparium*, and spectral evidence suggested that this compound was a reduced form of **4** in which one of the three double bonds of the parent compound was hydrogenated. The observation in the ir spectrum of an absorbance at 1690 $cm^{-1}$ indicated the presence of a nonconjugated, hydrogen-bonded carbonyl group in **5**. In the $^{1}H$-nmr spectrum of this compound, the olefinic 16-proton of **4** resonating at δ 7.00 was replaced by two proton signals at δ 2.00 and 2.22, and the C-21 methyl group signal of **5** (δ 2.22) was no longer deshielded by the 16,17-double bond as in **4** (δ 2.37). Other upfield shifts consistent with a less electronegative environment in ring D of **5**, when compared to **4**, were expressed in the $^{1}H$-nmr and $^{13}C$-nmr spectra (Tables 3 and 4, respectively) of the reduced compound. Since the $^{1}H$- and $^{13}C$-nmr data for **4** and **5** were closely comparable in other regions of the molecule, **5** was assigned as 2α,3β,12β-trihydroxypregna-4,7-dien-20-one.

Compound **6** ($C_{22}H_{32}O_5$) was a further derivative of **4** and showed a higher molecu-

TABLE 4. $^{13}$C-nmr Chemical Shifts of the Pregnane Derivatives **4**-**6** and **10**[a]

| Carbon | Compounds | | | |
|---|---|---|---|---|
| | **4** | **5** | **6** | **10** |
| 1 | 38.9 | 39.0 | 38.9 | 38.3 |
| 2 | 70.8 | 71.2 | 71.2 | 69.4 |
| 3 | 74.1 | 74.4 | 74.4 | 72.5 |
| 4 | 120.2 | 119.8 | 119.9 | 118.1 |
| 5 | 143.3 | 143.8 | 143.6 | 145.7 |
| 6 | 31.3 | 31.5 | 31.4 | 31.3 |
| 7 | 118.0 | 119.2 | 119.3 | 116.0 |
| 8 | 135.2 | 136.2 | 135.6 | 135.5 |
| 9 | 44.2 | 44.6 | 44.5 | 44.4 |
| 10 | 38.9 | 38.6 | 38.6 | 36.0 |
| 11 | 30.3 | 30.6 | 30.3 | 30.2 |
| 12 | 72.8 | 76.9 | 76.4 | 72.5 |
| 13 | 52.3 | 48.3 | 48.6 | 52.4 |
| 14 | 52.6 | 52.2 | 49.8 | 52.5 |
| 15 | 30.9 | 22.3 | 29.0 | 30.8 |
| 16 | 148.7 | 24.6 | 82.0 | 149.4 |
| 17 | 154.4 | 63.5 | 62.8 | 154.5 |
| 18 | 11.5 | 7.8 | 9.2 | 11.6 |
| 19 | 23.0 | 23.3 | 23.2 | 22.7 |
| 20 | 199.1 | 214.2 | 212.9 | 198.9 |
| 21 | 26.5 | 30.2 | 29.7 | 26.6 |
| -OMe | | | 52.7 | |
| -OAc | | | | 170.6, 170.2<br>21.1, 21.1 |

[a]90.8 MHz, $CDCl_3$, δ-scale, relative to TMS.

lar weight by 32 mass units. As with **5**, the $^1$H-nmr (Table 3) and $^{13}$C-nmr (Table 4) chemical shifts of **6** were very similar to those of **4**, except in ring D. It was apparent that the 16,17-double bond of **4** was substituted by a methoxy group affixed to C-16 in **6**, as evidenced by the replacement of the C-16 olefinic proton at δ 7.00 in **4** by a signal at δ 3.35 that integrated for three protons. The configuration of the methoxyl group at C-16 was established as α by the observation of a negative molecular rotation difference (21,27) between compounds **5** and **6**. The structure of **6** was, therefore, affirmed as 16α-methoxy-2α,3β,12β-trihydroxypregna-4,7-dien-20-one. There is a possibility that **6** may be artifactual, as a result of Michael addition of MeOH (a solvent used in the plant extraction) to the ring-D αβ-unsaturated ketone of **4**, since this phenomenon has been noted for the withanolide withaferin A (28). However, an attempt to generate **6** by refluxing **4** overnight with MeOH was unsuccessful.

The triterpene esters **1** and **2** were both cytotoxic against P-388 cultured cells ($ED_{50}$ 1.2 and 1.0 μg/ml, respectively). However, compound **3**, which differed from **1** only by the presence of a 19α-hydroxy group, was inactive in this regard. The most highly cytotoxic compound obtained in this investigation on *S. riparium* was **4**, for which we propose to give the trivial name, stizophyllin. Stizophyllin [**4**] exhibited an $ED_{50}$ of 0.07 μg/ml, and, because its cytotoxic activity was abrogated on reduction of the double bond at position 16 when **5** was tested, the αβ-unsaturated carbonyl moiety appears to be the site of **4** responsible for this type of biological activity. A more detailed study of the mechanism of action of **4** against P-388 cells is in progress. While triterpene esters of the ursene type do not appear to have been reported to possess

cytotoxic activity previously, a number of other plant-derived pregnane derivatives are known to be inhibitors of cell growth (29-32).

## EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.— Melting points were determined using a Kofler hot-stage instrument and are uncorrected. Optical rotations were measured with a Perkin-Elmer 241 polarimeter. The uv spectra were obtained on a Beckman DB-G grating spectrophotometer and the ir spectra measured on a Nicolet MX-1 FT-ir (AgCl) interferometer. $^1$H-nmr spectra were recorded in $CDCl_3$, using TMS as internal standard, employing a Nicolet NT-360 instrument (360 MHz). $^{13}$C-nmr spectra were recorded in $CDCl_3$ with a Nicolet NT-360 instrument operating at 90.8 MHz. Low resolution mass spectra were obtained with a Varian MAT 112S instrument operating at 70 eV.

PLANT MATERIAL.—*S. riparium* whole plant parts were collected in Peru in August 1981, by staff members of the Economic Botany Laboratory, Agriculture Research Service, BARC-East, USDA, Beltsville, Maryland. Voucher specimens are in deposit at the herbarium of the National Arboretum, Washington, DC.

EXTRACTION AND FRACTIONATION.—The air-dried, milled plant material (15.7 kg) was extracted sequentially with petroleum ether (bp 60-80°) and MeOH. After removal of solvent in vacuo, the MeOH-soluble residue was partitioned between $H_2O$ and $CHCl_3$. The dried $CHCl_3$ extract (306 g) was found to exhibit activity against the P-388 lymphocytic leukemia system in vitro (3) with an $ED_{50}$ of 5.3 μg/ml.

Column chromatography of a portion (280 g) of the $CHCl_3$ extract over silica gel 60 (4 kg; Merck, Darmstadt, W. Germany) was undertaken using $CHCl_3$ and $CHCl_3$/MeOH mixtures of increasing polarity. A total of 96 fractions (2 liters each) was collected. Elution by $CHCl_3$-MeOH (49:1) afforded a fraction containing ursolic acid (19.7 g) from which this triterpene was crystallized using MeOH. Further elution of the chromatographic column by $CHCl_3$-MeOH (49:1) and $CHCl_3$-MeOH (9:1), respectively, led to the isolation of crude mixtures of triterpene esters **1-3** (16.2 g) and pregnane derivatives **4-6** (3.5 g). The cytotoxic triterpene esters were further purified by sequential column chromatography over Florisil (700 g) and Sephadex LH-20 (100 g), to yield active fractions of 3.7 g and 0.7 g that were eluted, respectively, with $CHCl_3$-MeOH (49:1) and hexane-$CH_2Cl_2$ (2:1). Final purification of these compounds was performed by preparative tlc on Analtech GHLF (Analtech, Newark, DE), using $C_6H_6$-EtOH (10:1) as eluent. This stage was carried out in a fumehood, and compounds **1-3** exhibited Rf values of 0.55, 0.60, and 0.39, respectively. The cytotoxic fraction containing the pregnane derivatives was purified, in turn, by column chromatography over Sephadex LH-20 (100 g) and Florisil (10 g), after which activity was concentrated in fractions weighing 0.53 g and 0.41 g, respectively, that were eluted with hexane-$CH_2Cl_2$ (1:1) and $CHCl_3$-MeOH (19:1). Final purification of compounds **4-6** was achieved by preparative tlc with $CHCl_3$-MeOH (9:1) as solvent. The Rf values of compounds **4** through **6** in this system were 0.36, 0.27, and 0.28. Multiple development was necessary to separate compounds **5** and **6**. Additional quantities of the most abundant pregnane derivative, **4**, were isolated in a similar manner from other cytotoxic fractions obtained from the $CHCl_3$ extract of the plant material.

CHARACTERIZATION OF 3β-HYDROXY-24-*TRANS*-FERULYLOXYURS-12-EN-28-OIC ACID [**1**].—This isolate was obtained as an amorphous solid (205 mg, 0.00141% w/w), mp 200-202°, $[\alpha]^{25}D$ +28.2° (*c* 0.28, $CHCl_3$) and exhibited the following spectral data: uv (MeOH) λ max (log ε) 234 (4.23), 297 (4.28), 322 nm (4.42); ir (AgCl) ν max 3400, 2970, 2962, 2944, 2928, 2881, 2878, 1705, 1693, 1628, 1516, 1269, 1173, 938, 713 cm$^{-1}$; $^1$H nmr (360 MHz, $CDCl_3$) see Table 1; $^{13}$C nmr (90.8 MHz, $CDCl_3$) see Table 2; ms *m/z* 648 ($M^+$, 2%) (found 648.4019, calcd for $C_{40}H_{56}O_7$, 648.4026), 602 (1), 454 (1), 436 (1), 400 (7), 248 (40), 236 (1), 207 (7), 194 (33), 177 (100), 149 (6), 147 (8), 119 (20).

CHARACTERIZATION OF 3β-HYDROXY-24-*CIS*-FERULYLOXYURS-12-EN-28-OIC ACID [**2**].—This isolate was obtained as an amorphous solid (20 mg, 0.00013% w/w), mp 178-180°, $[\alpha]^{25}D$ +33.3° (*c* 0.69, $CHCl_3$) and exhibited the following spectral data: uv (MeOH) λ max (log ε) 230 (4.09), 303 (4.14), 323 nm (4.26); ir (AgCl) ν max 3400, 2390, 1712, 1691, 1630, 1600, 1460, 1449, 1380, 1227, 1180, 1170, 1030, 830, 758 cm$^{-1}$; $^1$H nmr (360 MHz, $CDCl_3$) see Table 1; $^{13}$C nmr (90.8 MHz, $CDCl_3$) see Table 2; ms *m/z* 648 ($M^+$, 1%) (found 648.4025, calcd for $C_{40}H_{56}O_7$, 648.4026), 602 (1), 454 (1), 436 (1), 400 (5), 248 (36), 236 (1), 207 (5), 194 (28), 177 (100), 149 (6), 147 (6), 119 (18).

CHARACTERIZATION OF 3β,19-DIHYDROXY-24-*TRANS*-FERULYLOXYURS-12-EN-28-OIC ACID [**3**].—This isolate was obtained as an amorphous solid (7 mg, 0.00004% w/w), mp 195-197°, $[\alpha]^{25}D$ +1.58° (*c* 0.38, $CHCl_3$) and exhibited the following spectral data: uv (MeOH) λ max (log ε) 243 (3.89), 298 (4.02), 324 nm (4.17); ir (AgCl) ν max 3300, 2950, 1702, 1691, 1630, 1600, 1515, 1461, 1390, 1267, 1070, 1025, 760 cm$^{-1}$; $^1$H nmr (360 MHz, $CDCl_3$) see Table 1; $^{13}$C nmr (90.8 MHz, $CDCl_3$) see

Table 2; ms *m/z* 664 ($M^+$, 1%) (found 664.3970, calcd for $C_{40}H_{56}O_8$, 664.3975), 618 (1), 470 (0.4), 452 (1), 400 (2), 264 (1), 252 (1), 207 (4), 194 (34), 177 (100), 149 (10), 147 (15), 119 (21).

CHARACTERIZATION OF 2α,3β,12β-TRIHYDROXYPREGNA-4,7,16-TRIEN-20-ONE [**4**].—This amorphous isolate (**4**, 400 mg, 0.0027% w/w) exhibited the following data: mp 194-197°, $[\alpha]^{25}D$ +60.5° (*c* 0.54, $CHCl_3$); uv (MeOH) λ max (log ε) 241 (3.97), 219 nm (3.73); ir (AgCl) ν max 3390, 1643, 1584, 1377, 1372, 1241, 1058, 1015, 753, 668 $cm^{-1}$; $^1H$ nmr (360 MHz, $CDCl_3$) see Table 3; $^{13}C$ nmr (90.8 MHz, $CDCl_3$) see Table 4; ms *m/z* 344 ($M^+$, 5%) (found 344.1986, calcd for $C_{21}H_{28}O_4$, 344.1987), 326 (8), 308 (40), 197 (13), 123 (16), 97 (18), 91 (18), 83 (22), 56 (65), 54 (45), 43 (100).

CHARACTERIZATION OF 2α,3β,12β-TRIHYDROXYPREGNA-4,7-DIEN-20-ONE [**5**].—This amorphous isolate (**5**, 20 mg, 0.0001% w/w) exhibited the following data: mp 115-118°, $[\alpha]^{25}D$ +16.5° (*c* 0.33, $CHCl_3$); uv (MeOH) λ max (log ε) 200 nm (4.06); ir (AgCl) ν max 3303, 2960, 1690, 1471, 1461, 1451, 1059, 1037, 854 $cm^{-1}$; $^1H$ nmr (360 MHz, $CDCl_3$) see Table 3; $^{13}C$ nmr (90.8 MHz, $CDCl_3$) see Table 4; ms *m/z* 346 ($M^+$, 1%) (found 346.2145, calcd for $C_{21}H_{30}O_4$, 346.2144), 328 (3), 267 (3), 225 (3), 211 (3), 197 (3), 145 (7), 133 (6), 107 (11), 105 (13), 93 (12), 91 (18), 81 (20), 55 (20), 43 (100).

CHARACTERIZATION OF 16α-METHOXY-2α,3β,12β-TRIHYDROXYPREGNA-4,7-DIEN-20-ONE [**6**].—Compound **6**, obtained as an amorphous solid (6 mg, 0.00003% w/w), exhibited the following data: mp 90-92°, $[\alpha]^{25}D$ +1.6° (*c* 0.25, $CHCl_3$); uv (MeOH) λ max (log ε) 282 (3.08), 213 nm (4.06); ir (AgCl) ν max 3350, 2924, 1702, 1461, 1450, 1360, 1094, 1053, 1028, 860, 757 $cm^{-1}$; $^1H$ nmr (360 MHz, $CDCl_3$) see Table 3; $^{13}C$ nmr (90.8 MHz, $CDCl_3$) see Table 4; ms *m/z* 376 ($M^+$, 2%), 361 (1), 358 (5), 344 (4) (found 344.1987, calcd for $C_{21}H_{28}O_4$, 344.1978), 326 (4), 315 (5), 283 (17), 265 (6), 145 (6), 143 (9), 131 (8), 129 (6), 123 (6), 105 (10), 101 (10), 91 (12), 55 (13), 43 (100).

ISOLATION OF URSOLIC ACID.—This isolate was recrystallized from $CHCl_3$/MeOH to afford 2.1 g of ursolic acid (0.014% w/w), mp 295-298°, identical with an authentic sample ($^1H$ nmr, ms, tlc) obtained previously in our laboratory (33).

ACETYLATION OF COMPOUND **1**.—Compound **1** (10 mg) was treated with $Ac_2O$-pyridine (1:1, 1 ml) overnight at room temperature. Work-up in the usual manner afforded an amorphous diacetate (**7**, 12 mg) that exhibited: $[\alpha]^{25}D$ +40.0° (*c* 0.14, $CHCl_3$); uv (MeOH) λ max (log ε) 305 (4.07), 278 (4.32), 249 (3.93), 232 (4.19), 224 (4.21), 211 (4.32); ir (AgCl) ν max 1950, 1760, 1727, 1712, 1695, 1644, 1600, 1511, 1466, 1460, 1454, 1365, 1258, 1247, 1194, 1151, 1123, 1032 $cm^{-1}$; $^1H$ nmr (360 MHz, $CDCl_3$) see Table 1; $^{13}C$ nmr (90.8 MHz, $CDCl_3$) see Table 2; ms *m/z* 690 ($M^+$-42, 0.5%), 689 (2), 644 (1), 442 (1), 436 (1), 249 (4), 248 (23), 203 (21), 188 (18), 177 (48), 145 (17), 133 (24).

HYDROLYSIS OF COMPOUND **1**.—Compound **1** (10 mg) was saponified with 1% KOH-MeOH at room temperature for 24 h and then methylated (Diazald Kit, Aldrich Chemical Co., Milwaukee, Wisconsin) for 2 min. The resulting mixture of products was purified by column chromatography over silica gel, using $CHCl_3$-MeOH in the polarity range 99:1 to 19:1 as eluting solvent, to afford three compounds, 3β,24-dihydroxyurs-12-en-28-oic acid methyl esters (**8**, 3 mg), *trans*-ferulic acid methyl ester (1 mg), and 3,4-dimethoxy-*trans*-cinnamic acid methyl ester (1 mg).

3β,24-Dihydroxyurs-12-en-28-oic acid methyl ester [**8**] exhibited the following data: mp 235-237°, $[\alpha]^{25}D$ +45.8° (*c* 0.12, $CHCl_3$); uv (MeOH) λ max (log ε) 200 nm (3.42); ir (AgCl) ν max 3360, 2950, 1722, 1458, 1381, 1376, 1248, 1237, 1227, 1200, 1171, 1144, 1044, 756 $cm^{-1}$; $^1H$ nmr (360 MHz, $CDCl_3$) see Table 1; $^{13}C$ nmr (90.8 MHz, $CDCl_3$) see Table 2; ms *m/z* 486 ($M^+$, 4%) (found 486.3708, calcd for $C_{31}H_{50}O_4$, 486.3709), 453 (1), 428 (3), 395 (5), 262 (100), 224 (8), 204 (21), 203 (82), 189 (17), (176 (12), 175 (22), 133 (41), 119 (12), 95 (14).

Compound **8** (2 mg) was treated with $Ac_2O$-pyridine (1:1, 0.5 ml) at room temperature overnight to give methyl 3β,24-diacetoxyurs-12-en-28-oate (**9**, 2 mg), mp 178-180°, $[\alpha]^{25}D$ +40.0° (*c* 0.08, $CHCl_3$) [lit. mp 180-182°, $[\alpha]^{22}D$ +50.8° (*c* 0.12, $CHCl_3$) (14)]. This derivative exhibited closely comparable spectroscopic (uv ir, $^1H$ nmr, ms) data to literature values for methyl 3β,24-diacetoxyurs-12-en-28-oate [**9**] obtained from a study on *H. lawsoniae* (14). This identification was confirmed by comparison with the $^1H$-nmr spectrum of an authentic sample and that of its isomer, methyl 3β,23-diacetoxyurs-12-en-28-oate (14).

The acid moiety of compound **1** was confirmed as *trans*-ferulic acid by comparison on methylation with an authentic sample (Sigma Chemical Co., St. Louis, Missouri) that was methylated by treatment with $CH_2N_2$ (Diazald Kit) for 2 min. Methylation of both the hydrolyzate of **1** and the standard compound produced a mixture of *trans*-ferulic acid methyl ester (resinous) and the less polar 3,4-dimethoxy-*trans*-cinnamic acid methyl ester (mp 62°). Both pairs of methyl esters (experimentally obtained and authentic) exhibited closely comparable spectroscopic (uv, ir, $^1H$ nmr, ms) and chromatographic (tlc) data.

ACETYLATION OF COMPOUND **4**.—Compound **4** (10 mg) was treated with $Ac_2O$-pyridine (1:1, 1

ml) at room temperature overnight. Work-up in the usual manner afforded an amorphous diacetate (**10**, 12 mg), $[\alpha]^{25}D$ +135° (*c* 0.38, $CHCl_3$); uv (MeOH) λ max (log ε) 239 nm (4.22); ir (AgCl) ν max 3400, 3280, 2934, 2845, 1743, 1649, 1580, 1450, 1428, 1370, 1241, 1055, 1029, 753 $cm^{-1}$; $^1H$ nmr (360 MHz, $CDCl_3$) see Table 3; $^{13}C$ nmr (90.8 MHz, $CDCl_3$) see Table 4; ms *m/z* 428 ($M^+$, 9%), 377 (13), 368 (28), 350 (39), 328 (13), 308 (39), 294 (29), 293 (49), 275 (30), 264 (26), 183 (22), 95 (16), 89 (17), 79 (24), 45 (100).

CYTOTOXIC ACTIVITY.—The isolates from *S. riparium* were evaluated for cytotoxic activity against the P-388 lymphocytic leukemia cell culture system (3). Compounds **1**, **2**, and **4** were found to exhibit $ED_{50}$ values of 1.2, 1.0, and 0.07 μg/ml, respectively. An isolate is considered active in this system if it shows an $ED_{50}$ of ≤4.0 μg/ml.

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