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J. Nat. Prod., **1991**, 54 (6), 1543-1552• DOI: 10.1021/np50078a008 • Publication Date (Web): 01 July 2004

Journal of Natural Products
Vol. 54, No. 6, pp. 1543-1552, Nov-Dec 1991

PREPARATION, CHARACTERIZATION, AND ANTIVIRAL ACTIVITY OF MICROBIAL METABOLITES OF STEMODIN

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ABSTRACT.—Screening studies for microbial transformation products of stemodin [**2**] have identified a number of microbial metabolites. Scale-up fermentation with *Rhizopus arrhizus* ATCC 11145 and *Streptomyces* sp. NRRL 5691 have resulted in the production of five metabolites that have been characterized with the use of 2D nmr and X-ray techniques. These metabolites have been identified as 18-hydroxystemodin [**6**], 16,18-dihydroxystemodin [**7**], 8β-hydroxystemodin [**8**], 8β,18-dihydroxystemodin [**9**], and 7β,8β-dihydroxystemodin [**10**]. The antiviral activity and cytotoxicity of the isolated metabolites have been evaluated.

Stemodia maritima L. (Scrophulariaceae) has been known as a folk medicine for a long time by the people in the northern part of South America and in most of the Caribbean islands. For example, the plant has been used as a treatment for venereal diseases in traditional medicine in the Caribbean island of Curaçao (1). *Ste. maritima* has yielded a number of interesting diterpenes, of which stemodin [**2**] is the major stemodane diterpene (2,3). Stemodin [**2**] bears a close structural relationship to aphidicolin [**1**], a known antiviral compound (4,5).

In our studies, five metabolites of **2** were produced with two different microorganisms. Based on the spectroscopic and X-ray data, these metabolites have been identified as 18-hydroxystemodin [**6**], 16,18-dihydroxystemodin [**7**], 8β-hydroxystemodin [**8**], 8β,18-dihydroxystemodin [**9**], and 7β,8β-dihydroxystemodin [**10**].

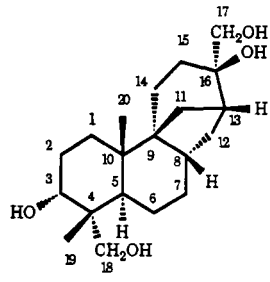
The isolation, structure elucidation, and antiviral evaluation of these metabolites are described herein.

RESULTS AND DISCUSSION

A total of 104 microorganisms were screened for their ability to biotransform stemodin [**2**]. *Rhizopus arrhizus* ATCC 11145 and *Streptomyces* sp. NRRL 5691 were selected for preparative scale transformation because no stemodin was detected in fermentation broths, and they produced different more polar metabolites as indicated by slower migration in tlc.

A preparative scale fermentation was performed with *R. arrhizus*, and four metabolites **3**, **4**, **6**, and **7** were isolated and purified. Two of these metabolites (**3** and **4**) were found to be identical with metabolites previously isolated by fermentation of stemodin by *Cunninghamella echinulata* (6).

The ei mass spectrum of **6** contained a peak at *m/z* 304 which was assigned to $[M-18]^+$ ($-H_2O$). The mol wt of **6** was proposed as 322, 16 mass units higher than that of **2**. This finding suggested that compound **6** was a monohydroxystemodin. The DEPTGL carbon multiplicity determination experiments showed the presence of three methyl carbons, nine methylene carbons, and four methine carbons, leaving four nonprotonated carbons. These results indicated that one methyl carbon of stemodin [**2**] had



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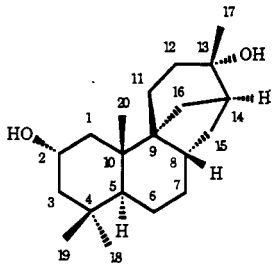

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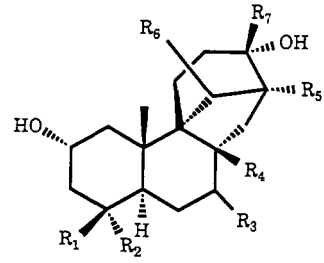

	R_1	R_2	R_3	R_4	R_5	R_6	R_7
3	Me	Me	α-OH	H	H	H	Me
4	Me	Me	β-OH	H	H	H	Me
5	Me	Me	H	H	OH	H	Me
6	Me	CH_2OH	H	H	H	H	Me
7	Me	CH_2OH	H	H	H	OH	Me
8	Me	Me	H	OH	H	H	Me
9	Me	CH_2OH	H	OH	H	H	Me
10	Me	Me	β-OH	OH	H	H	Me
11	CH_2OH	Me	H	H	H	H	Me
12	CH_2OH	Me	H	H	H	H	CH_2OH

been hydroxylated. To determine which methyl group in **2** had been hydroxylated, the chemical shifts of the quaternary (non-protonated) carbons of **2** at C-4 (where Me-18 and Me-19 are attached), C-10 (where Me-20 is attached), and C-13 (where Me-17 is attached) were studied. No significant changes were observed at C-10 and C-13, which indicated that both Me-17 and Me-20 were not hydroxylated. On the other hand, a significant change was observed at C-4 which led to the conclusion that either Me-18 or Me-19 of **6** was hydroxylated. The Me-18 and Me-19 groups of **2** had been assigned previously by chemical shift comparisons (7) and were confirmed by noting an nOe enhancement between H-2 and the protons of Me-19 (NOSEY experiment) (δ 34.8 and δ 23.8; C-18 and C-19, respectively). In the case of **6**, the signal at δ 34.8 (Me-19) was missing and the signal at δ 23.8 (Me-18) was shifted upfield to δ 21.3. This suggested that Me-18 of **2** was hydroxylated, and this hydroxylation resulted in a γ shift of the resonance signal for Me-19. The assignments of the various protons and carbons were consistent with an OH at Me-18. In the ^{13}C nmr of **6**, a new signal at δ 71.8 (methylene carbon) was assigned to Me-18. The ^1H nmr of **6** contained a pair of signals for the AB diastereotopic methylene protons (*CH2*-OH, Me-18) at δ_A 3.38 (1H, J = 10.5 Hz) and δ_B 3.66 (1H, J = 10.5 Hz). The HETCOR experiment on **6** correlated the carbon signal at δ 71.8 with the AB doublets of the diastereotopic methylene protons (H-18) at δ_A 3.38 (H, J = 10.5 Hz) and δ_B 3.66 (H, J = 10.5 Hz). The detailed carbon assign-

ments are shown in Table 1. Thus, compound **6** was assigned the structure 18-hydroxystemodin.

The eims of metabolite **7** contained a peak at *m/z* 302 which was assigned to $[M-36]^+$ ($-2\ H_2O$), suggesting that **7** is a dihydroxystemodin. The DEPTGL carbon multiplicity experiments for **7** indicated the presence of three methyl carbons, eight methylene carbons, and five methine carbons, leaving four non-protonated carbons, indicating that one methyl group and one methylene carbon had been hydroxylated. The same argument as that given for compound **6** is used in the analysis of **7**; the signal at 34.8 (Me-18 in stemodin [**2**]) was missing and the signal at 23.8 (Me-19 in [**2**]) was shifted upfield (γ effect) in the ^{13}C nmr of compound **7**. This situation indicated that in compound **7**, one of the two new hydroxyl groups had to be on Me-18. This conclusion was supported by noting new signals as AB doublets at δ_A 3.62 ($J = 10.1$ Hz) and δ_B 3.73 ($J = 10.1$ Hz), which were assigned to the diastereotopic methylene protons at C-18. Also, the ^{13}C nmr showed a new signal at δ 73.8 which was assigned to the hydroxylated methyl Me-18. The determination of which methylene group was hydroxylated from the eight possibilities (C-1, C-3, C-6, C-7, C-11, C-12, C-15, and C-16) in **2** was solved by noting the shifts of the methine carbons around each methylene carbon, since an OH on a methylene carbon would cause a downfield shift in the methine carbon(s) next to it. C-1, C-3, C-6, and C-7 were excluded because they did not show any significant changes to the methine carbons at C-2, C-5, and C-8. This situation left four possible methylene carbons in **2** for hydroxylation (C-11, C-12, C-15, and C-16). The methine carbon at C-8 of **7** did not show any significant changes, which suggested that C-15 was not hydroxylated. A significant shift was observed for the methine carbon at C-14 of **7**, which led to the conclusion that the most likely place for the second OH was at C-16. The comparative ^{13}C nmr assignments of **2** and **6** are shown in Table 1. The structure of **7** was proposed as 16,18-dihydroxystemodin. The

TABLE 1. ^{13}C-nmr Chemical Shift Assignments For Stemodin and Its Metabolites.[a]

Carbon	**2**	**6**	**7**	**8**	**9**	**10**
C-1	46.5(2)	46.6(2)	46.3(2)	47.3(2)	47.1(2)	46.5(2)
C-2	64.1(1)	64.4(1)	64.8(1)	64.1(1)	64.3(1)	63.9(1)
C-3	51.5(2)	45.5(2)	45.7(2)	51.7(2)	45.8(2)	51.8(2)
C-4	34.7(0)	39.6(0)	40.0(0)	34.8(0)	39.7(0)	34.7(0)
C-5	47.0(1)	39.6(1)	37.3(1)	44.7(1)	40.5(1)	45.3(1)
C-6	22.4(2)	22.3(2)	22.5(2)	20.2(2)	19.9(2)	32.5(2)
C-7	36.8(2)	36.6(2)	37.4(2)	48.5(2)	48.5(2)	77.9(1)
C-8	37.4(1)	37.5(1)	42.6(1)	79.0(0)	79.1(0)	81.3(0)
C-9	50.5(0)	50.7(0)	53.9(0)	51.8(0)	52.1(0)	52.6(0)
C-10	40.2(0)	40.3(0)	41.3(0)	40.7(0)	40.6(0)	40.7(0)
C-11	28.3(2)	28.5(2)	29.1(2)	24.5(2)	24.6(2)	24.9(2)
C-12	33.3(2)	33.4(2)	33.3(2)	34.3(2)	34.3(2)	34.3(2)
C-13	71.1(0)	71.1(0)	73.3(0)	71.7(0)	71.8(0)	71.7(0)
C-14	46.9(1)	47.9(1)	57.4(1)	45.0(1)	45.0(0)	44.9(1)
C-15	38.3(2)	38.5(2)	37.5(2)	44.2(2)	43.9(2)	44.9(2)
C-16	30.4(2)	30.6(2)	78.9(1)	32.5(2)	32.6(2)	29.0(2)
C-17	28.6(3)	28.8(3)	28.9(3)	28.8(3)	28.7(3)	28.8(3)
C-18	34.8(3)	71.8(2)	73.8(2)	35.1(3)	71.7(2)	33.9(3)
C-19	23.8(3)	21.0(3)	22.3(3)	23.9(3)	20.1(3)	22.9(3)
C-20	19.8(3)	20.1(3)	20.1(3)	18.0(3)	19.3(3)	18.4(3)

[a]The number in parentheses indicates the number of hydrogens attached to the corresponding carbon and was determined from DEPTGL experiments. Spectra recorded in pyridine-d_5 with TMS as internal standard.

stereochemistry of the OH at C-16 could not be solved by using spectroscopic techniques; consequently, X-ray analysis was considered. Several attempts were made to prepare a crystal for X-ray analysis but all were unsuccessful. Since only a very small quantity was available, the stereochemistry of the OH on C-16 of **7** was left undetermined.

A preparative scale fermentation of stemodin [**2**] with *Streptomyces* sp. led to the isolation of three metabolites **8**, **9**, and **10**. The eims of **8** contained a peak at *m/z* 304 which was assigned to $[M-18]^+$ ($-H_2O$). The mol wt of **8** was proposed as 322, 16 mass units higher than **2**. The DEPTGL carbon multiplicity experiments for **8** indicated the presence of four methyl carbons, eight methylene carbons, and three methine carbons, leaving five non-protonated carbons. These results suggested that one methine carbon (-CH) of **2** was converted to a quaternary oxygenated center in **8**. The new OH could be placed on any one of the four methine carbons (C-2, C-5, C-8, and C-14) of **2**. The possibility of the OH being on C-2 was ruled out because no significant changes were observed at C-1 and C-3; furthermore, the H-2 proton was clearly identified from the ^1H nmr as a multiplet at δ 4.1. C-5 was also eliminated due to the absence of any significant shift at C-4 and C-6. Thus, the two methine carbons at C-8 and C-14 in **2** were the only possibilities. C-14 was ruled out because C-13 of **8** was nearly unchanged from that in **2**. Therefore, C-8 was the only possible site for hydroxylation in **8**. This conclusion was further supported by noting downfield shifts of the methylene carbons at C-15 and C-7 and an upfield shift of C-6 of **8** when compared to **2**. The ^{13}C nmr of **8** contained a new signal at δ 79.9; this signal was assigned to an oxygenated quaternary carbon. Carbons C-7 and C-15 were shifted downfield to δ 48.5 and δ 44.2 (β effect) when compared to δ 36.8 and δ 38.3 for **2** at C-7 and C-15, respectively. At the same time C-6 and C-14 were shifted upfield to δ 45.0 and δ 20.2 (γ effect) when compared to δ 46.9 and δ 22.4 for **2** at C-6 and C-14, respectively. These data could only be accounted for if the new OH was placed at C-8. The stereochemistry at C-8 was assumed to have remained β, because if the OH groups had not be β, a completely different diterpene skeleton would have been observed. Therefore, the new OH at C-8 of **8** was assigned with the same stereochemistry (β) as in **2**. The detailed comparative ^{13}C-nmr assignments of compounds **8** and **2** are shown in Table 1. Thus, metabolite **8** was identified as 8-β-hydroxystemodin.

The eims of **9** contained a peak at *m/z* 302 which was assigned to $[M-36]^+$ ($-2H_2O$). The mol wt of **9** was proposed as 338, 32 mass units higher than **2**. The DEPTGL carbon multiplicity experiments of compound **9** indicated the presence of three methyl carbons, nine methylene carbons, and three methine carbons, leaving five non-protonated carbons. This situation suggested that **9** was a dihydroxystemodin. Also, DEPTGL experiments suggested that one methyl carbon of **2** was hydroxylated to a $-CH_2OH$ in compound **9** and one methine carbon of **2** was hydroxylated to an oxygenated quaternary carbon in compound **9**. To determine which methyl in **2** was hydroxylated in **9**, the same argument as that given for compound **6** was used. There was a significant shift for C-4 of **9**, where both Me-18 and Me-19 are attached. This shift suggested that the hydroxylated methyl in **9** was located at either C-18 or C-19. In **2**, Me-18 and Me-19 were assigned unambiguously (NOSEY) at δ 34.8 and δ 23.8, respectively. The ^{13}C nmr of **9** contained Me-19 (δ 23.8) shifted upfield at δ 20.1 while Me-18 (δ 34.8) was missing and replaced with a new signal at δ 71.7 which was assigned to CH_2OH (Me-18). There findings indicated that the Me-18 of **9** was hydroxylated. The HETCOR experiment supported this conclusion by correlating the carbon signal at δ 71.7 with the AB doublet protons (H-18) at δ_A 3.40 (1H, J = 10.5 Hz) and δ_B 3.56 (1H, J = 10.5 Hz). The remaining OH group of **6** was assigned to C-8 by comparison of **9** with **8**. Carbons C-7 and C-15 were shifted downfield (β effect), while C-6

and C-14 were shifted upfield (γ effect). These chemical shifts indicated that the other oxygenated center was at C-8. HETCOR and other nmr experiments were in agreement with this conclusion. The full carbon assignments are shown in Table 1. Thus, metabolite **9** was identified as 8β,18-dihydroxystemodin.

The DEPTGL carbon multiplicity experiments on compound **10** suggested that one of the methine carbons of stemodin [**2**] was converted to an oxygenated quaternary center and one methylene carbon was converted to a -CHOH. This situation suggested that **10** was a dihydroxystemodin. The chemical shifts of the ^{1}H and ^{13}C nmr of **10** were used to determine which one of the methine carbons in **2** [C-2, C-5, C-8, or C-14) was hydroxylated. The presence of the signal at δ 4.1 (H-2 in **2**) and the absence of any significant changes at either C-1 or C-3 were the reasons for excluding hydroxylation at C-2 from consideration. Methine carbon C-5 of **10** was also eliminated because C-4 and C-10 were almost unchanged when compared with C-4 and C-10 of **2**. No significant shift was observed at either C-13 or C-16, and this finding ruled out the possibility of having the OH on C-14. A significant shift was observed at C-9, which indicated that the new OH was most likely at C-8 of the eight methylene carbons of **2**. The two methylene carbons at C-1 and C-3 were excluded because the chemical shifts on the carbons around them were almost unchanged when compared to C-1 and C-3 of **2**. Using chemical shift arguments alone to assign which one of the remaining six methylene carbons of **10** was hydroxylated proved to be difficult. Compound **10** was crystallized several times from Et_2O/EtOAc until an appropriate crystal for X-ray analysis was obtained. The results of the single crystal X-ray analysis of **10** showed that the new oxygenated centers were at C-7 and C-8 with the same β stereochemistry for both OH. An ORTEP diagram (8) of **10** is shown in Figure 1. Three intermolecular and one intramolecular hydrogen bonds are observed for each molecule of **10**. The bonding parameters, the hydrogen bonding contact distances, and the crystal data are summarized in Tables 2–4.

Metabolites **6**, **7**, **8**, **9**, and **10** as well as **3**, **4**, **5**, **11**, and **12** (6) were tested for antiviral and cytotoxicity activities using an improved plaque-reduction assay for antiviral activity (9). Aphidicolin [**1**] was used as a positive control. The compounds were tested

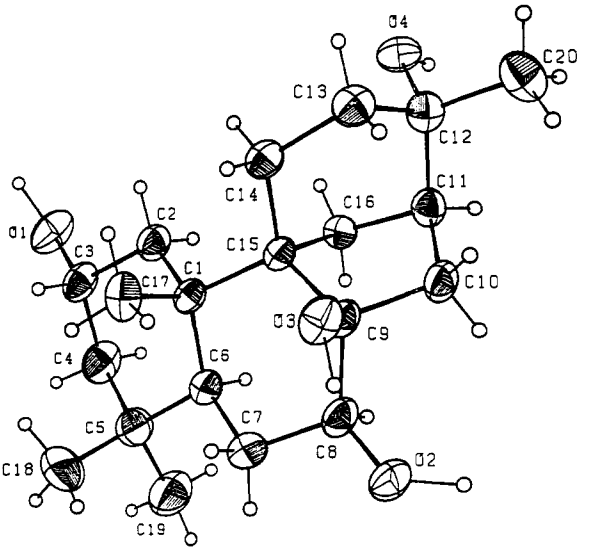

FIGURE 1. ORTEP diagram of compound **10**.

TABLE 2. Crystal Data and Summary of Intensity Data Collection and Structure Refinement of Compound **10**.

Compound	$C_{20}H_{34}O_4$
Color/shape	colorless/parallelepiped
Molecular weight	338.49
Space group	$P2_12_12_1$
Temperature, °C	20
Cell constants,[a] Å	
a	6.996(3)
b	14.465(5)
c	18.477(9)
Cell vol, Å^3	1870
Formula units/unit cell	4
D_c, g·cm^{-3}	1.20
μ calcd, cm^{-1}	0.88
Diffractometer/scan	Enraf-Nonius CAD-4/ω2θ
Radiation, graphite monochromator	MoKα (λ = 0.71073)
Max crystal dimensions, mm	0.13 × 0.23 × 0.43
Scan width	0.80 + 0.35 tan θ
Standard reflections	200; 060; 004
Decay of standards	+1%
Reflections measured	1926
2θ range, deg	2<2θ<50
Range of *h*, *k*, *l*	+8; +17; +22
Reflections observed [$F_o \geq 5\sigma(F_o)$][b]	1428
Computer programs[c]	SHELX-76 (11)
Structure solution	SHELXS (13)
No. of parameters varied	229
Weights	$[\sigma(F_o)^2 + 0.0008\,F_o^2]^{-1}$
GOF	1.68
$R = \Sigma\|\|F_o\|-\|F_c\|\|/\Sigma\|F_o\|$	0.046
R_w	0.060
Largest feature final difference map	0.2 e-Å^{-3}

[a]Least-squares refinement of $[(\sin\theta)/\lambda]^2$ values for 25 reflections θ>21°.

[b]Corrections: Lorentz-polarization.

[c]Neutral scattering factors and anomalous dispersion corrections from "International Tables for X-Ray Crystallography" (12).

against Herpes simplex type 1 (HS-1) grown on African green monkey kidney cells. Each compound exhibits some cytotoxicity. Compound **8** had the highest activity of all the compounds isolated in this study. It was able to reduce the number of plaques by 93% at a concentration of 0.1 mg/ml. Stemodin and several analogues exhibited weak antiviral activity, as shown in Table 5. On the other hand, compounds **4**, **9**, **10**, **11**, and **12** did not show any reduction in the number of plaques at the same concentration. Stemo-

TABLE 3. Contact Geometries of the Intermolecular and Intramolecular Hydrogen Bonds in Compound **10**.

Intermolecular contact	Distance (Å)	Angle (°)	Crystal coordinates
H-1 O-1 . . . O-4	1.76	159	$-\frac{1}{2}+x, -\frac{1}{2}-y, -1-z$
H-1 O-2 . . . O-1	1.82	153	$-\frac{1}{2}-x, -y, -\frac{1}{2}+z$
H-1 O-4 . . . O-3	1.89	156	$1+x, y, z$
Intermolecular contact			
H-1 O-3 . . . O-2	1.98	125	

TABLE 4. Final Fractional Coordinates for Compound **10**.

Atom	*x/a*	*y/b*	*z/c*	B(eqv)[a]
O-1	−0.2224(5)	−0.0537(2)	−0.3847(1)	2.78
O-2	−0.5643(4)	−0.0161(2)	−0.7920(1)	2.58
O-3	−0.5528(4)	−0.1801(2)	−0.7316(1)	2.13
O-4	0.1405(4)	−0.2823(2)	−0.6713(1)	2.12
C-1	−0.3955(5)	−0.0948(2)	−0.5776(2)	1.51
C-2	−0.2647(6)	−0.0986(3)	−0.5102(2)	1.89
C-3	−0.3445(7)	−0.0444(3)	−0.4470(2)	2.15
C-4	−0.3611(8)	0.0562(3)	−0.4668(2)	2.76
C-5	−0.4901(7)	0.0746(3)	−0.5328(2)	2.68
C-6	−0.4289(6)	0.0086(2)	−0.5967(2)	1.89
C-7	−0.5567(7)	0.0164(3)	−0.6640(2)	2.48
C-8	−0.4497(6)	−0.0220(3)	−0.7276(2)	1.98
C-9	−0.3859(5)	−0.1232(2)	−0.7187(2)	1.62
C-10	−0.2283(5)	−0.1434(3)	−0.7753(2)	2.01
C-11	−0.0444(5)	−0.1628(3)	−0.7325(2)	1.77
C-12	−0.0138(5)	−0.2661(3)	−0.7226(2)	1.85
C-13	−0.1936(6)	−0.3084(3)	−0.6914(2)	2.00
C-14	−0.2871(5)	−0.2515(2)	−0.6305(2)	1.75
C-15	−0.2950(5)	−0.1451(2)	−0.6434(2)	1.42
C-16	−0.0861(5)	−0.1172(2)	−0.6588(2)	1.55
C-17	−0.5832(6)	−0.1447(3)	−0.5591(2)	2.26
C-18	−0.7011(8)	0.0685(4)	−0.5113(3)	3.69
C-19	−0.453(1)	0.1747(3)	−0.5566(3)	4.09
C-20	0.0429(7)	−0.3140(3)	−0.7933(2)	2.79

[a]$B(eqv) = 4/3[a^2\beta_{11} + b^2\beta_{22} + c^2\beta_{33} + ab(\cos\gamma)\beta_{12} + ac(\cos\beta)\beta_{13} + bc(\cos\alpha)\beta_{23}]$.

din [**2**] reduced by 67% the number of plaques at 0.02 mg/ml. In spite of the fact that compound [**4**] failed to have any antiviral activity at 0.1 mg/ml, its epimer **3** had weak antiviral activity at this concentration, inhibiting 33% of the plaques. Compounds **5** and **6** reduced the number of plaques by 27% and 67%, respectively, at concentration of 0.1 mg/ml. Compounds **11** and **12** did not show antiviral activity up to 0.399 mg/ml. Also, compounds **11** and **12** did not exhibit any cytotoxicity toward Vero cells at

TABLE 5. Results of Antiviral/Cytotoxicity Testing.

Compound	% Reduction in number in plaques	Minimum antiviral concentration (mg/ml)	Cytotoxicity (CD_{50}) (mg/ml)
1[a]	100	0.005	0.20
2	67	0.02	0.04
3	33	0.1	0.20
4[b]	None	—	0.07
5[b]	27	0.1	0.15
6	67	0.1	0.20
7	None	—	0.07
8	93	0.1	0.30
9	None	—	0.66
10	None	—	0.04
11[b]	None	—	>0.399
12[b]	None	—	>0.399

[a]Aphidicolin [**1**] was used as a positive standard.
[b]These compounds are microbial metabolites obtained in a previous study (6).

concentrations up to 0.43 and 0.63 mg/ml respectively. All other compounds in the series exhibited cytotoxic activity in this system. Among compounds with both antiviral and cytotoxic activities, there was no significant correlation between the two activities ($r = 0.497$, $F = 1.312$, $p > 0.25$, analysis of variance). Among stemodin and its derivatives there was a very weak correlation between the two activities ($r = 0.817$, $F = 6.0$, $0.05, p \leq 0.1$, analysis of variance).

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Melting points were determined either on a Fisher-digital melting point analyzer model 355 or in open capillary tubes with Thomas-Hoover capillary melting point apparatus and are uncorrected. Ir spectra were recorded in KBr using a Perkin-Elmer 281 B infrared spectrophotometer.

^{1}H- and ^{13}C-nmr spectra were obtained on a VXR-300 spectrometer operating at 300 MHz and 75 MHz, respectively. The chemical shift values are reported in ppm units, and the coupling constants are in Hz. Abbreviations for nmr signals are as follows: s = singlet, d = doublet, t = triplet, q = quartet, dd = double doublet, m = multiplet, br = broad. Standard pulse sequences were used for COSY, HETCOR, DEPTGL, APT, and NOSEY experiments. Eims were obtained using an E.I. Finnigan model 3200 (70 eV ionization potential) with INCOS data system. High resolution mass spectra were carried out at the University of Utah, Salt Lake City. Low and high resolution fabms were carried out at the University of Kansas. Aphidicolin was purchased from Sigma Chemical Company.

CHROMATOGRAPHIC CONDITIONS.—Tlc chromatographic analyses were carried out on precoated Silica G-25 UV_{254} plates (Macherey-Nagel Duren). The adsorbent used for cc was Si gel 60 230–400 mesh (Macherey-Nagel Duren). Visualization of the tlc plates was performed using anisaldehyde-H_2SO_4 spray reagent. The developing system used for tlc was $CHCl_3$ Me_2CO (1:2).

MICROORGANISMS.—Cultures were obtained from the University of Mississippi, Department of Pharmacognosy; the American Type Culture Collection (ATCC), Rockville, Maryland; Northern Regional Research Laboratories (NRRL), Peoria, Illinois; or from Dr. John Rosazza, University of Iowa (UI cultures). The cultures used for preliminary screening were as follows: *Streptomyces punipalus* UI-3529 (+), *Calonectria decora* ATCC 14767 (+), *Cunninghamella echinulata* ATCC 9244 (+), *Cunninghamella blakesleeana* ATCC 8688a (+), *Rhizopus arrhizus* ATCC 11145 (+), *Aspergillus niger* UI-X-172 (+), *Dactylaria haptotyla* ATCC 28924 (+), *Doratomyces microsporus* ATCC 16225 (+), *Melanospora parasitica* ATCC 18055 (+), *Streptomyces* sp. NRRL 5691 (+), *Polyangium cellulosum* ATCC 29610 (+), *Cokeromyces recurvatus* ATCC 13568 (+), *Helicodendron triglintzienx* ATCC 16770 (+), *Streptomyces* sp. ATCC 5688 (+). The "+" indicates that these microorganisms produced one or more metabolites detectable by tlc.

MEDIA.—All preliminary screening and scale-up experiments were carried out in a medium consisting of dextrose 20 g, yeast extract 5 g, peptone 5 g, NaCl 5 g, K_2HPO_4 5 g, distilled H_2O 1000 ml. Stock cultures of fungi and bacteria were stored on slants of Mycophil (BBL) and Eugon (Difco) agar, respectively, at 4°.

FERMENTATION PROCEDURES.—Microbial transformation studies were carried out by incubating the cultures with shaking on the model G-10 Gyrotory shaker (New Brunswick Scientific Co., NJ), operating at 250 rpm, at 25°. Preliminary screening experiments were carried out in 125-ml stainless-steel-capped Delong culture flasks containing 25 ml of medium. The media were sterilized at 121° and 18 psi for 15 min. Fermentations were carried out according to a standard two-stage protocol (10). In general, the substrate was added to the incubation media 24 h after the incubation of the stage II cultures as a 10% solution in EtOH at a concentration of 0.2 mg/ml of stage II medium. Substrate controls were composed of sterile medium to which the substrate was added and incubated without microorganisms. Culture controls consisted of fermentation blanks in which the microogranisms were grown under identical conditions but without the substrate addition.

Stemodin [**2**] used in this study was isolated from *Ste. maritima* from collections from Curaçao (1).

MICROBIAL TRANSFORMATION OF STEMODIN [**2**] BY *R. arrhizus*.—Stemodin (700 mg) was dissolved in 7.0 ml of EtOH and distributed equally among seventeen 1-liter culture flasks, each containing 200 ml of 24-h-old *R. arrhizus* stage II culture. After 14 days, the entire incubation mixtures were combined and filtered. The cells and fermentation solids were extracted three times with EtOAc (1 × 3.4 liters, 2 × 1.7 liters). The organic layer was dried over anhydrous Na_2SO_4 and filtered, and the solvent was evaporated under reduced pressure to afford 850 mg of dark brown residue. This residue was purified on Si gel (150 g, 2.5 × 60 cm) using EtOAc as an eluent, and 20-ml fractions were collected.

ISOLATION AND CHARACTERIZATION OF COMPOUNDS **6** AND **7**.—Fractions 55–91 (a single spot with R_f 0.4) were combined to give 65 mg of **6** (9.3%). Crystallization from EtOAc gave colorless crystals: mp 219–221; $[\alpha]_D$ −3.7 (c = 1.0, pyridine); $[M-18]^+$ 304; hrms $[M]^+$ 322.25061, consistent with $C_{20}H_{34}O_4$ (calcd 322.2504); ir (Nujol) 3250, 3350 cm^{-1}; ^1H nmr 0.99 (3H, s, Me-20), 1.1 (1H, m, H-6), 1.12 (3H, s, Me-19), 1.2 (1H, m, H-15), 1.3 (3H, s, Me-17), 1.4 (2H, m, H-6 and H-11), 1.6 (3H, m, H-11 and H-12), 1.7 (3H, m, H-1, H-8, H-15), 1.9 (2H, m, H-7, H-16), 2.01 (1H, m, H-5), 2.1 (1H, dd, J = 6.9, 6.9 Hz, H-14), 2.4 (1H, dd, J = 12, 3 Hz, H-1), 3.38 (d, J = 10.2 Hz, H_A-18), 3.66 (d, J = 10.2 Hz, H_B-18), 4.2 (1H, m, H-2); ^{13}C nmr see Table 1.

Eluting the column with MeOH yielded a pure metabolite **7** (3 mg) (0.4%). Crystallization from EtOAc gave colorless crystals: mp 248–251; $[M-36]^+$ 302, hrms $[M-36]^+$ 302.2244, consistent with $C_{20}H_{30}O_2$ (calcd 302.2240); ^1H nmr 1.17 (3H, s, Me-20), 1.18 (3H, s, Me-19), 1.37 (3H, s, Me-17), 3.35 (1H, br d, J = 10.5 Hz, H-16), 3.62 (d, J = 11.7 Hz, H_A-18); 3.74 (d, J = 11.7 Hz, H_B-18), 4.3 (1H, m, H-2); ^{13}C nmr see Table 1.

MICROBIAL TRANSFORMATION OF STEMODIN [**2**] BY *STREPTOMYCES* SP.—Stemodin [**2**] (960 mg) was dissolved in 95% EtOH (9.6 ml) and added to stage II cultures of *Streptomyces* sp. After 14 days the broth was extracted three times with EtOAc (1 × 4 liters, 2 × 1.9 liters). The organic layer was dried over anhydrous Na_2SO_4, filtered, and evaporated under reduced pressure to afford 1.5 g brown residue. This residue was purified on Si gel (250 g, 2.5 × 90 cm), using hexane as an eluent and then using 100% EtOAc followed by increasing the polarity by adding increasing amounts of MeOH in EtOAc; finally the column was washed with 100% MeOH. Fractions 10–19 were combined and evaporated under vacuum at 40° to produce 120 mg of brown residue. This residue was purified on a Si gel column (10 g, 0.5 × 10 cm) using toluene as eluent with a gradually increased amount of EtOAc.

ISOLATION AND CHARACTERIZATION OF COMPOUNDS **8** –**10**.—Fractions 417–425 of the first column, which gave a single spot with R_f 0.75, were combined to give 46 mg of **8** (5%). Fractions 37–49 from the first column gave a single spot with R_f 0.3. These combined fractions gave 18 mg of **9** (2%). Fractions 22–25 of the second column, which gave one spot with R_f 0.7, were combined to give 9.1 mg of **10** (1.0%). Crystallization of **8** from EtOAc gave colorless crystals: mp 169–170; $[\alpha]_D$ −10.0 (c = 1.12, pyridine); $[M-18]^+$ 304; hrfabms $[M-Li]^+$ 329.2677, consistent with the formula $C_{20}H_{34}O_3$ (calcd 329.2666); ^1H nmr 0.97 (3H, s, Me-19), 0.98 (3H, s, Me-18), 1.3 (3H, s, Me-17), 1.4 (2H, m, H-3 and H-5), 1.5 (1H, m, H-1), 1.6 (1H, m, H-6), 1.64 (1H, m, H-16), 1.75 (1H, m, H-6), 1.78 (1H, m, H-15), 1.8 (1H, m, H-12), 1.98 (2H, m, H-7), 2.0 (1H, m, H-3), 2.1 (3H, m, H-11, H-14), 2.25 (1H, m, H-12), 2.3 (1H, m, H-1), 2.5 (1H, m, H-15), 2.6 (1H, br d, J = 13 Hz, H-16), 4.1 (1H, m, H-2); ^{13}C nmr, see Table 1.

Compound **9** was crystallized from EtOAc to give pure colorless crystals: mp 182–183; $[\alpha]_D$ −2.1 (c = 0.96, pyridine); $[M-36]^+$ 302; hrfabms $[M-H]^+$ 337.2389, consistent with the formula $C_{20}H_{34}O_4$ (calcd 337.2377); ^1H nmr 0.88 (3H, s, Me-19), 1.3 (3H, s, M-17), 1.5 (3H, s, Me-20), 1.51 (1H, m, H-1), 1.6–1.65 (2H, m, H-6 and H-12), 1.75 (1H, m, H-6), 1.9–1.98 (5H, m, H-3, H-7, H-15), 2.0 (1H, m, H-14), 2.1 (3H, m, H-5, H-15, H-16), 2.2 (2H, m, H-11), 2.3 (1H, H-12), 2.4 (1H, m, H-11), 2.45 (1H, m, H-1), 2.7 (1H, m, H-16), 3.4 (d, J = 105 Hz, H_A-18), 3.56 (d, J = 102 Hz, H_B-18), 4.3 (1H, m, H-2); ^{13}C nmr see Table 1.

Compound **10** was crystallized from EtOAc to give pure colorless crystals: mp 241–243; $[M-36]^+$ 302; hrfabms $[M-H]^1$ 337.2360, consistent with $C_{30}H_{34}O_4$; ir (Nujol) 3400 cm^{-1}; ^1H nmr 0.8 (3H, s, Me-19), 0.84 (3H, s, Me-18), 1.2 (3H, s, Me-20), 1.3 (3H, s, Me-17), 1.4 (2H, m, H-3, H-5), 1.5 (1H, m, H-11), 1.63 (1H, m, H-6), 1.78 (1H, m, H-15), 1.8 (1H, m, H-12), 1.85 (2H, m, H-16), 2.0 (1H, m, H-3), 2.1 (3H, m, H-11, H-14), 2.25 (1H, m, H-12), 2.3 (1H, m, H-1), 2.45 (1H, 2.45 (1H, m, H-6), 2.5 (1H, m, H-15), 3.7 (1H, m, H-7), 4.1 (1H, m, H-2); ^{13}C nmr see Table 1.

X-RAY DATA COLLECTION, STRUCTURE DETERMINATION, AND REFINEMENT OF COMPOUND **10**.[1]—A transparent single crystal of compound **10** was mounted on a pin and transferred to the goniometer. The space group was determined to be acentric $P2_12_12_1$. A summary of data collection parameters is given in Table 2.

Least-squares refinement with isotropic thermal parameters led to R = 0.114. The geometrically constrained hydrogen atoms were placed in calculated positions 0.95 Å from the bonded carbon atom and allowed to ride on that atom with B fixed at 5.5 $Å^2$. The methyl hydrogen atoms were included as a rigid group with rotational freedom at the bonded carbon (C-H = 0.95 Å, B = 5.5 $Å^2$). The alcoholic hydrogen

[1]Atomic coordinates for this structure have been deposited with the Cambridge Crystallographic Data Centre and can be obtained on request from Dr. Olga Kennard, University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW, UK.

atoms were located from a difference Fourier map and included with fixed contributions (B = 5.5 Å^2). Refinement of nonhydrogen atoms with anisotropic temperature factors led to the final values of R = 0.046 and R_w = 0.060.

ANTIVIRAL AND CYTOTOXICITY ASSAYS.—*Sample preparation.*—Antiviral and cytotoxicity assays were carried out essentially according to the method of Abou-Karam and Shier (9). Samples of test compounds were dissolved in 50 μl of DMSO and aliquoted into sterile culture medium at 0.4 mg/ml. These solutions were subdiluted to 0.02 mg/ml in sterile medium, and the two solutions were used as stocks for subdilution at 100, 50, 20, 10, 5, 2, and 1 μg/ml in triplicate in the wells of microtiter plates. Higher concentrations of some test compounds were required to determine the cytotoxic concentration (CD_{50}).

Cultures.—Herpes simplex type 1 (HS-1) was the gift of Dr. R.G. Hughes, Roswell Park Memorial Institute, Buffalo, NY. Working stocks of HS-1 that could conveniently be diluted to give about 30 plaques per culture well were prepared by titering virus by serial dilution in culture medium and assaying in triplicate on Vero monolayers in the wells of microtiter trays. Working stocks were stored at 4° until used. Vero African green monkey kidney cells (Viromed Laboratories, Minnetonka, MN) were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) calf serum (HyClone Laboratories, Ogden, UT), 60 μg/ml penicillin G, and 100 μg/ml streptomycin sulfate. All medium components were obtained from Sigma Chemical Co., St. Louis, Mo, unless otherwise indicated. Cells and viruses were cultured at 37° in a humidified atmosphere containing about 15% (v/v) CO_2 in air. Vero cultures for virus titration, antiviral assays, or cytotoxicity testing were grown to confluence in the wells of microtiter tray (Falcon Microtest III 96-wells trays, Becton Dickinson Labware, Lincolin Park, NJ) inoculated with Vero cells suspended at 2×10^4 cells per 200 μl culture in medium containing 10% (v/v) calf serum.

PROCEDURE.—Microtiter trays with confluent monolayer cultures of Vero cells were inverted, and the medium was shaken out and replaced with serial dilutions of sterile samples in triplicate in 100 μl of medium. Tray wells were inoculated with 30 plaque forming units of HS-1 virus in 100 μl medium containing 10% (v/v) calf serum. In each tray, the last row of wells were reserved for controls: Vero cells that were treated with virus but no test compounds or not treated with virus or test compound. The trays were incubated under normal culture conditions for 66 h. After incubation, the trays were inverted onto a pad of paper towels, and the remaining cells were rinsed carefully with medium and fixed with 3.7% formaldehyde in saline for at least 20 min. The fixed cells were rinsed with H_2O, stained with 0.5% crystal violet in 20% aqueous EtOH for 30 min, rinsed with H_2O, and examined visually. Antiviral activity was identified as confluent, relatively unaltered monolayers of stained Vero cells. Antiviral activity was estimated as the lowest concentration that caused the indicated per cent reduction in the number of virus plaques. Cytotoxicity was estimated as the concentration that caused approximately 50% loss of the monolayer from around the plaques caused by HS-1.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the help of Dr. Julia Morton, Morton Collectanea, University of Miami and Dr. John De Freitas, Stichting Carmobi, Carmobi Foundation, Curaçao for their help in providing large supplies of *Stemodia maritima* for isolation of stemodin used in this study.

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Received 11 March 1991