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NOVEL BIOACTIVE DITERPENOIDS FROM *AFRAMOMUM AULACOCARPOS*

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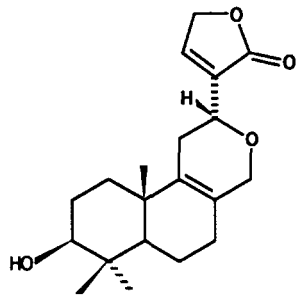
ABSTRACT.—Three new labdane diterpenes, 12α,17-epoxy-3β-hydroxy-8(9),13-labdadien-16,15-olide (aulacocarpinolide) [**1**], methyl 8β,17:14ζ,15-diepoxy-3β-hydroxy-12*E*-labden-16-oate (aulacocarpin A) [**2**], and methyl 8β,17:14ζ,15-diepoxy-3β,6β-dihydroxy-12*E*-labden-16-oate (aulacocarpin B) [**3**] have been isolated from the Cameroonian spice, *Aframomum aulacocarpos*, and their structures elucidated using spectroscopic techniques. The known bioactive diterpene dialdehyde, 8β,17-epoxy-12*E*-labdene-15,16-dial (aframodial) [**4**] was also isolated. Compounds **1–3** showed weak antimicrobial and cytotoxic activities.

In 1979, the hot-tasting diterpene dialdehyde, 8β,17-epoxy-12*E*-labdene-15,16-dial (aframodial [**4**]), was isolated in our laboratory from the seeds of *Aframomum daniellii* (Hook. f.) K. Schum. (Zingiberaceae) (1). Aframodial [**4**] has since been shown to be a non-toxic broad-spectrum antifungal agent (2), a cytotoxic molecule (2), and an anti-hypercholesterolemic principle (3). In an effort to discover other natural sources of this potential therapeutic agent we have investigated several species of *Aframomum* that grow in Cameroon. One of these, *A. aulacocarpos* Pellegr. ex. J. Koechlin, whose seeds are widely used as a food spice (4), has yielded in the present study three novel diterpenoids [**1–3**], in addition to aframodial [**4**]. In the structural elucidation of the new compounds, extensive use was made of high-field nmr spectroscopic techniques.

RESULTS AND DISCUSSION

Flash cc of the crude CH_2Cl_2 extract of the seeds of *A. aulacocarpos* followed by successive medium-pressure liquid chromatographic purification of the resulting fractions and recrystallization afforded four pure compounds, **1–4**. The structure of the known compound **4** was established by direct comparison (mmp and ir, ^{1}H-, and ^{13}C-nmr spectra) with an authentic sample.

Compound **1**, for which we propose the trivial name aulacocarpinolide, crystallized from an *n*-hexane/EtOAc mixture as colorless needles. Its elemental composition was deduced from elemental analysis and the ^{1}H- and ^{13}C-nmr data. The presence in the ir spectrum of intense absorption bands at ν max 3510, 1760, and 1660 cm^{-1} was indicative of hydroxyl, carbonyl, and olefinic functionalities, respectively. A ^{1}H-nmr signal at δ 3.23 and a ^{13}C-nmr methine signal at δ 78.73 revealed a secondary hydroxyl group. The ^{13}C-nmr spectrum confirmed the existence in **1** of two double bonds, one tetrasubstituted [δ 135.72 (s) and 134.88 (s)], and the other trisubstituted [δ 124.87 (s) and 144.86 (d)]. An examination of the ^{13}C-nmr carbonyl resonance at δ 172.22 in conjunction with the proton signals at δ 4.82 (t), 4.83 (d), and δ 7.39 suggested that the carbonyl group was part of an unsaturated lactone moiety. The chemical shifts of the latter signals and their splitting pattern are reminiscent of a 16,15-olide rather than a



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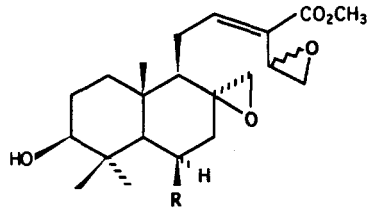
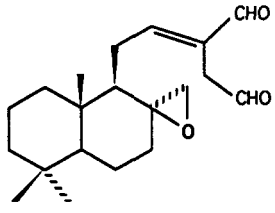


2 R=H
3 R=OH



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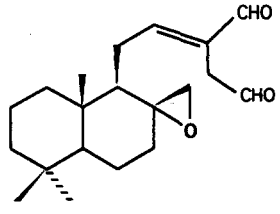

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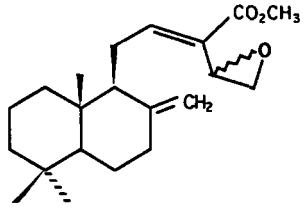

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15,16-olide. The above data account for the functionality of compound **1** which is, therefore, tetracyclic.

An analysis of the ^{13}C-nmr spectrum of **1** with the aid of DEPT 135, ^{1}H-^{13}C HETCOR and off-resonance decoupling techniques revealed the presence of 20 carbon atoms, which included three methyls, seven methylenes, four methines, and six quaternary carbons. A detailed analysis of the ^{1}H-^{1}H COSY spectrum in conjunction with one-bond heteronuclear correlation experiments established the presence of two isolated five-proton (-CH-CH_2-CH_2-) spin systems, a five-carbon (-CH_2-CH=C-CH-CH_2-) spin system, and an isolated CH_2 system. In the first spin system, the ^{1}H-^{1}H COSY spectrum showed correlations between the proton resonating at δ 3.23 (direct carbon 78.73 from HMQC; H-3) and two protons resonating at δ 1.71 (Hα-2) and 1.61 (Hβ-2). The protons with chemical shifts at δ 1.71 and 1.61, respectively, were in turn coupled to two protons resonating at δ 1.20 (Hα-1) and 1.72 (Hβ-1) which were also mutually coupled. In the ^{1}H-^{1}H COSY spectrum, the proton resonating farthest downfield at δ 7.40 (δ_C 144.86; H-14) was coupled to two protons occurring as a doublet and a triplet, respectively, at δ 4.82 and 4.83 (H_a-15, H_b-15). The downfield proton further displayed allylic coupling to another proton with a chemical shift of 4.24 ppm (direct carbon 69.95 ppm; H-12) which was in turn strongly correlated with two mutually coupled protons at δ 1.89 (H_a-11) and 2.42 (H_b-11). The protons on C-15 also

displayed weak homoallylic coupling with H_2-11. All the above correlations of the various spin systems were confirmed by HMQC-TOCSY experiments that further established a weak response from two protons constituting an AB pair centered at δ 4.06 and 4.03 (direct carbons δ 68.91; H_a-17 and H_b-17) to the proton resonating at δ 4.24 (δ 69.95; H-12). The response from C-17 was further extended to the protons at δ 1.89 (H_a-11) and 2.42 (H_b-11) when the mixing time of the HMQC-TOCSY experiment was increased to 54 msec. These spectral features, along with reports of labdane diterpenes (1,7) from the sister species, *A. daniellii,* and the co-occurrence of **1** with aframodial [**4**] in *A. aulacocarpos*, suggested that compound **1** was a labdane diterpene. On the basis of spectral data of model compounds (8) and some diagnostic heterocorrelations, the fragments were assembled to give the gross structure **1**. Direct and long-range ^{1}H-^{13}C HETCOR experiments were used to confirm structure **1** and to establish the functional group substitution. ^{1}H- and ^{13}C-nmr assignments made unambiguously with the aid of a combination of ^{1}H-^{13}C HETCOR, HMQC, ^{1}H-^{1}H COSY, HMQC-TOCSY, and ^{1}H-^{1}H decoupling experiments (Table 1), were also consistent with structure **1** for aulacocarpinolide.

The relative stereochemistries at C-3 and C-12 were deduced from the ^{1}H-nmr spectrum. The magnitude of the observed coupling constants of H-3 at δ 3.23, $J_{3,2ax}$=11.6 Hz and $J_{3,2eq}$=4.2 Hz indicates that the C-3 hydroxyl group must be axial and therefore β-oriented. Also, H-12 at δ 4.24 displayed two coupling constants with H-11, one large (9.8 Hz) and the other relatively smaller (1.3 Hz). Inspection of models revealed that these couplings required a conformation with an equatorial 16, 15-olide moiety. The values of the coupling constants compared well with those reported (5) for a closely related compound with the same stereochemistry at C-12. Aulacocarpinolide [**1**], to our knowledge, is the first naturally occurring labdane derivative with both a 12,17-ether linkage and a 16,15-olide residue at C-12.

Aulacocarpin A [**2**], obtained as colorless prisms, mp 102–103°, [α]D +11°, has the molecular formula $C_{21}H_{32}O_5$, as deduced from cims and ^{13}C nmr (Table 1). The broad ir

TABLE 1. Nmr Data (500 MHz) of Aulacocarpinolide [**1**].

Position	δ ^{13}C	δ ^{1}H, m, J in Hz	HMQC-TOCSY Correlations
1	33.69 t	1.20 (m), 1.72 (m)	C-2, C-3
2	27.32 t	1.61 (m), 1.77 (m)	C-1, C-3
3	78.73 d	3.23 (dd, J= 4.2 and 11.6)	C-1, C-2
4	36.96 s		
5	50.76 d	1.15 (dd, J=1.8 and 12.6)	C-6, C-7
6	17.89 t	1.52 (m), 1.76 (m)	C-5, C-7
7	26.93 t	1.89 (m)	C-5, C-6, C-12
8	135.72 s		
9	134.88 s		
10	38.67 s		
11	28.10 t	1.89 (m), 2.42 (m)	C-12, C-17
12	69.95 d	4.24 (dd, J=1.2 and 9.8)	C-15, C-12
13	124.87 s		
14	144.86 d	7.39 (tt, J=1.5 and 1.5)	
15	70.48 d	4.82 (t, J=1.5) 4.83 (d, J=1.8)	C-14, C-15
16	172.22 s		
17	68.91 t	4.06 and 4.03 (ABd, 15.3)	C-12, C-11
18	27.91 q	1.02 (s)	
19	18.98 q	1.02 (s)	
20	15.37 q	0.83 (s)	

absorption band at ν max 3500–3400 cm^{-1} was indicative of a hydroxyl group. Other strong ir absorptions at 1715 and 1680 cm^{-1} were in agreement with the presence of an α,β-unsaturated ester in **2**. Resonances for three sp^2 carbons in the ^{13}C-nmr spectrum revealed a conjugated methyl ester (δ 166.36) and a trisubstituted olefin [δ 127.41; (d); 149.81 (s)]. Taking into account the double-bond equivalents implicit in the molecular formula of aulacocarpin A, **2** had to be tetracyclic. The ^1H-nmr spectrum contained three methyl singlets. Additional proton signals included those of a vinyl methine [δ 6.78 (1H, t, J=7.1 Hz)], a monosubstituted epoxide [δ 3.59 (1H, br m), 2.98 (1H, dd, J=4.5 and 5.4 Hz), 2.79 (1H, dd, J=2.8 and 5.4 Hz)], a disubstituted epoxide [2.53 (d, J=4.0 Hz), 2.32 (d, J=4 Hz)], and an aliphatic methine-bearing oxygen [δ 3.25 (dd, J=4.6 and 11.5 Hz)]. These data suggested that aulacocarpin A [**2**] was a labdane diterpene derivative related to **6**, a metabolite isolated from *A. daniellii* in our laboratory (7). In fact, the ^1H-nmr spectral features of **2** were virtually identical with those of **6**, except for the absence of the resonances for the 8,17-exomethylene protons [δ 4.84 and 4.47 (both broad singlets)], which were replaced in **2** by the signals of the trisubstituted epoxide, strongly indicating epoxidation at these carbons. Another major difference between the two spectra was an extra aliphatic oxomethine proton signal in **2** suggesting further hydroxylation of **2** already implicit in its molecular composition. The magnitude of the chemical shift of this proton suggested the placement of the hydroxy group at either C-1 or C-3. Analysis of the ^1H-^1H COSY coupling network in **2** and heteronuclear correlation (HMQC, HETCOR) experiments, and comparison of ^1H- and ^{13}C-nmr shifts of **2** with those of **6** and other related labdane diterpenoids (8) enabled the hydroxy group to be placed at C-3. The C-3 hydroxyl, as in **1**, is equatorial since H-3 (δ 3.25) had one large (11.5 Hz) and one small coupling (4.6 Hz), indicating that it was axial. Full ^{13}C-nmr assignments for **2** (Table 1) were also consistent with structure **2**.

The β-orientation of the C-8, C-17 epoxide was deduced by comparison of the ^1H-nmr shifts of the epoxide protons H_a-17 and H_b-17 with those reported for aframodial [**4**] (1) and its synthetic 8α-isomer [**5**] (2). Protons H_a-17 and H_b-17 were observed at δ 2.53 and 2.32, respectively, in **2** and at δ 2.42 and 2.27 in **4**. In **5**, which has the opposite stereochemistry at C-8, the two protons were observed at δ 2.70 and 2.56, respectively. The ^{13}C-nmr resonances of C-8 and C-17 in **2** (δ 48.78, 57.30) also agreed better with those reported for **4** (δ 48.80, 57.60) than with those published for the synthetic 8α-isomer **5**, thus confirming the β-configuration for the 8,17-epoxide of aulacocarpin A.

No direct evidence was obtained for the stereochemistry of the 12, 13 double bond in **2** but the similarity of **2** with **4** and **6** with almost identical ^1H- and ^{13}C-nmr spectral features for H-12, C-12, and C-13 suggested the same *E* configuration for the three diterpenoids. Finally, the stereochemistry of the 14,15-epoxy group remains to be determined. Aulacocarpin A was therefore proposed as having structure **2**, methyl 8β,17:14ζ,15-diepoxy-3β-hydroxy-12*E*-labden-16-oate.

Aulacocarpin B [**3**], isolated as colorless needles, mp 140–141°, $[\alpha]_D$ +21°, analyzed for $C_{21}H_{32}O_6$ (elemental analysis). The ir spectrum showed close correspondence to the values recorded for **2**. Analysis of the ^1H- and ^{13}C-nmr spectral data of **3** indicated that it was closely related to aulacocarpin A [**2**]. In fact, the ^1H- and ^{13}C-nmr features were virtually identical except for the absence of one methylene ^{13}C-nmr signal and the appearance of a methine carbon resonance at δ 68.21 in the ^{13}C-nmr spectrum of **3**. The difference was also evident in the ^1H-nmr spectrum of **3** in which the methylene protons resonating upfield as a multiplet at δ 1.73–1.56 were replaced by a downfield doublet centered at δ 4.50. These spectroscopic data coupled with the molecular formula indicated that aulacocarpin B [**3**] has the same gross structure as aulacocarpin A [**2**] with

an additional secondary hydroxyl group. Comparison of the ^{13}C-nmr chemical shifts of **2** and **3** enabled the assignment of the hydroxyl group at C-6. This assignment was strongly supported by the highly shielded resonance of H-5 (δ 0.96) which is reminiscent of the H-5 protons of some 6β-hydroxylated *Scapania* labdane derivatives (9). It is known (9) that 6β-hydroxylation of the labdane skeleton causes strong shielding of H-5. An explanation for this high-field shift has been furnished by Huneck *et al.* (9). The C-6 hydroxy is axial and thus has the β-orientation since H-6 exhibits only a small coupling (2.3 Hz) to H-5. The stereochemistries at the other chiral centers in **3** are the same as those of compound **2** considering the similarities of their ^{1}H- and ^{13}C-nmr chemical shifts. Aulacocarpin may thus be defined as methyl 8β,17:14ζ,15-diepoxy-3β,6β-dihydroxy-12*E*-labden-16-oate.

Compounds **1–3** moderately inhibited the growth of the pathogenic bacterium *Bacillus subtilis* with MIC values of 25 μg/ml. The compounds were also weakly active against *Mucor miehei* (MIC 50 μg/ml). The three compounds were further evaluated for their cytotoxic potential against a small battery of cell lines. Aulacocarpinolide [**1**] and aulacocarpin B [**3**] showed ED_{50} values against L1210 cells at concentrations of 12.5 μg/ml and 25 μg/ml, respectively.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Mps were determined with a Kofler hot-stage apparatus and are uncorrected. Cims were recorded with a Nermag-R10-10C instrument. The reagent gas for cims was NH_3. Polarimetry was conducted using a Perkin-Elmer polarimeter model 241. Nmr spectra (1D and 2D) were recorded at room temperature in $CDCl_3$ with a Bruker AC 300 or a Bruker AMX 500 instrument (^{1}H, 500.13 MHz; ^{13}C, 125.6 MHz), equipped with an X32 data system. All 2D nmr experiments were performed employing the standard pulse sequences. ^{1}H- and ^{13}C-nmr chemical shifts in ppm were referenced to $CHCl_3$, resonating at δ 7.26 and 77.0 ppm, respectively. The DEPT spectra were obtained using polarization transfer pulses of 135°. Two-dimensional experiments were obtained using standard Bruker microprograms. Ir spectra were obtained with a Perkin-Elmer model 257 grating spectrophotometer. Medium-pressure liquid chromatography was performed using Baeckström Separo AB columns and Merck Si gel 60, 230–400 mesh. Tlc was performed with precoated Merck Kieselgel 60 F_{254} aluminum-backed plates, 0.2 mm thick. All solvents used for chromatography were redistilled before use.

PLANT MATERIAL.—The fruits of *Aframomum aulacocarpos* were collected at Bamenda, Cameroon, in November 1991 by one of us (J.F.A.). Voucher specimens were identified by P. Mezili of the National Herbarium, Yaoundé, Cameroon, where they have been deposited.

EXTRACTION AND ISOLATION.—The seeds (300 g) obtained from the fresh fruits of *A. aulacocarpos* were dried, ground into a fine powder, and extracted three times with $CHCl_3$ (2 liters×3). The solvent was evaporated *in vacuo* at about 50° to give 51 g of a brown residue. The residue was fractionated using three successive flash Si gel columns eluted with $CHCl_3$-MeOH (19:1). The resulting fractions, on further purification by mplc with Baeckstrom Separo AB columns (i.d., 25 mm and 30 mm, respectively), using a continuous gradient from pure *n*-hexane to pure EtOAc, yielded four pure compounds: **3**, **1**, **2**, and **4**, in order of decreasing polarity. The purity of the compounds was verified on precoated tlc plates with cyclohexane-EtOAc (3:2) and $CHCl_3$-MeOH (25:1). All the compounds were further purified by recrystallization in appropriate solvent systems before analysis.

Aulacocarpinolide [**1**].—Colorless needles from *n*-hexane/EtOAc (65 mg): mp 150–151° (dec); $[\alpha]^{20}_D$ +615° (*c*=0.02, MeOH); ir ν max (KBr) 3510, 2960, 2820, 1760, 1660 cm^{-1}; ^{1}H and ^{13}C nmr, see Table 1; cims *m/z* $[M+NH_4]^+$ 350 (20), $[M+H]^+$, 333 (22), 316 (100), 317 (300); *anal.* found C 72.18, H 8.56; $C_{20}H_{28}O_4$ requires C 72.26, H 8.49.

Aulacocarpin A [**2**].—Colorless prisms from *n*-hexane/EtOAc (860 mg): mp 102–103°; $[\alpha]^{20}_D$ +11° (*c*=0.43, MeOH); ir ν max (KBr) 3500–3400, 1715, 1680, 1465, 1440, 1286, 1050, 1010, 935, 920, 650 cm^{-1}; ^{1}H and ^{13}C nmr, see Table 2; cims, *m/z* $[M+NH_4]^+$ 382 (25), $[M+H]^+$ 365 (40); eims, *m/z* 364 (2), 346 (13), 328 (13), 314 (10), 298 (10), 268 (12), 204 (25), 186 (20), 134 (30), 121 (28), 104 (50), 94 (75), 92 (80), 90 (90), 80 (100), 78 (98); *anal.* found C 69.30, H 8.79; $C_{21}H_{32}O_5$ requires C 69.20, H 8.85.

Aulacocarpin B [**3**].—Colorless crystals from $CHCl_3$/EtOAc (3 g): mp 140–141°; $[\alpha]^{20}_D$ +21° (*c*=0.7, MeOH); ir ν max (KBr) 3500–3400, 1710, 1700, 1680, 1285, 1040, 940, 860, 650 cm^{-1}; ^{1}H and ^{13}C nmr,

TABLE 2. Comparison of Nmr Data of Compounds **2**, **3**, and **6** (500 and 125 MHz).

Position	Compound				
	2		**3**		**6**[a]
	δ ^{13}C	δ ^{1}H (m, *J* in Hz)	δ ^{13}C	δ ^{1}H (m, *J* in Hz)	δ ^{13}C
1	35.68	1.92 (m), 1.38 (m)	39.76	1.80 (m), 1.10 (dt, *J*=3.6 and 12.9)	39.2
2	27.09	1.73–1.56 (m)	27.21	1.71 (m)	19.4
3	78.55	3.25 (dd, *J*=4.6 and 11.5)	78.69	3.18 (dd, *J*=6.5 and 9.8)	42.1
4	38.95		39.58		33.6
5	55.08	1.01 (m)	55.80	0.96 (d, *J*=1.4)	55.5
6	19.64	1.73–1.56 (m)	68.21	4.50 (bd, *J*=2.3)	24.2
7	37.12	1.83 (dt, *J*=3.5 and 16.6)	43.67	2.22 (dd, *J*=3.6 and 14.6)	38.0
		1.12 (td, *J*=4.0 and 13.1)		1.56 (dd, *J*=2.7 and 14.6)	
8	57.30		58.62		148.4
9	52.55	1.55 (d, *J*=8.3)	52.50	1.61 (d, *J*=9.5)	56.9
10 ...	39.45		39.72		39.7
11 ...	20.83	2.34 (dd, *J*=6.1 and 17.5)	21.40	2.40 (dd, *J*=8.0 and 18.7)	
		2.04 (dt, *J*=17.5 and 8.2)		2.12 (dd, *J*=8.0 and 9.5)	23.6
12 ...	149.81	6.78 (t, *J*=6.7)	149.02	6.78 (t, *J*=6.6)	149.9
13 ...	127.41		127.84		127.3
14 ...	48.30	3.59 (m)	48.38	3.61 (m)	48.8
15 ...	47.57	2.98 (dd, *J*=4.5 and 5.4)	47.57	3.00 (dd, *J*=4.4 and 5.5)	47.7
		2.70 (dd, *J*=2.8 and 5.5)		2.77 (dd, *J*=2.8 and 5.5)	
16 ...	166.36		166.29		166.6
17 ...	48.78	2.53 (d, *J*=4.0)	47.10	2.56 (d, *J*=3.7)	
		2.32 (d, *J*=4.0)		2.32 (d, *J*=3.7)	107.7
18 ...	28.19	1.03 (s)	16.91	1.24 (s)	33.6
19 ...	15.26	0.84 (s)	28.01	1.27 (s)	21.8
20 ...	14.51	0.95 (s)	16.42	1.24 (s)	14.4
OCH_3	51.76	3.75 (s)	51.81	3.75 (s)	51.7

[a]Values from ref. (7).

see Table 2; cims *m/z* $[M+NH_4]^+$ 398 (20), $[M+H]^+$ 381 (16), 362 (100), 326 (48), 298 (12), 122 (15); cims, *m/z* 380 (2), 362 (15), 362 (15), 344 (18), 326 (10), 312 (10), 294 (8), 172 (20), 152 (20), 122 (48), 106 (50), 94 (70), 80 (100), 78 (92); *anal.* found C 66.38 H 8.39; $C_{21}H_{32}O_6$ requires C 66.30, H 8.48.

BIOASSAYS.—Antibacterial activity was tested using serial dilution assays with *Bacillus brevis* ATCC 9999, *B. subtilis* ATCC 6633, *Acinebacter calcoaceticus* DSM 30006, and *Micrococcus luteus* ATCC 381, using nutrient broth (Difco) as medium. For the measurement of antifungal activity, the following strains were used: *Mucor miehei, Penicillium notatum, Candida albicans,* and *Saccharomyces cerevisiae.* The medium was composed of 0.4% yeast extract, 1% malt extract, and 0.4% glucose. Yeasts were tested using serial dilution assays and filamentous fungi with the plate diffusion assay. Cytotoxic activity towards Ehrlich ascites tumor cells (ECA) and L1210 cells (lyphocytic leukemia mouse ATCC CCI 163) was measured as described earlier (10).

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