Phosphate Triester 32. Treatment of compound **25** (400 mg, 1.28 mmol) with phosphoramidite **28** (650 mg, 1.28 mmol) and 1*H*-tetrazole (127 mg, 1.8 mmol) following the procedure described for **29** gave triester **31** (725 mg, 79%) as a colorless foam after chromatography (eluent CH_2Cl_2-Et_3N-MeOH, 96:3:1). The ^{31}P NMR showed peaks at δ 140.80, 140.91. Compound **31** (350 mg, 0.49 mmol) was converted to **32** (278 mg, 80%) by oxidation with I_2/H_2O following the procedure described for the preparation of compound **26**: UV (MeOH) λ_{max} 230, 262, 304, nm; ^1H NMR ($CDCl_3$) δ (italicized protons are assigned to the thymidyl moiety) 1.26 (0.7 × 3 H, br s, 5-C*H*$_3$), 1.73 (0.3 × 3 H, br s, 5-C*H*$_3$), 2.27 (1 H, m, 2′-*H*), 2.64 (1 H, m, 2′-*H*), 3.80 (0.7 × 3 H, d, J_{HP} = 11.4 Hz, $POCH_3$), 3.81 (0.3 × 3 H, d, J_{HP} = 11.4 Hz, $POCH_3$), 4.30 (2 H, m, 5′-H), 4.48–4.72 (4 H, m, 3′-*H*, 4′-*H*, 5′-*H*), 5.13 (1 H, m, 4′-H), 6.16 (1 H, m, 2′-H), 6.25–6.31 (2 H, m, 1′-*H*, 3′-H), 7.05 (0.3 × 1 H, m, 1′-H), 7.16 (0.7 × 1 H, m, 1′-H), 7.40–7.62 (7 H, m, C_6H_5, 5-H), 7.87 (1 H, m, 6-*H*), 7.99 (4 H, m, C_6H_5), 8.66 (0.7 × 1 H, br s, NHCO), 8.72 (0.3 × 1 H, NHCO), 8.83 (1 H, br s, NH); ^{31}P NMR ($CDCl_3$) δ 0.06; MS (FAB) *m/z* 736 (M + 1), 737 (M + 2). Anal. ($C_{34}H_{34}N_5O_{12}P\cdot0.5H_2O$) H, N, P; C: calcd, 54.84; found, 54.20.

Phosphate Diester 17. Compound **32** (240 mg, 0.35 mmol) was deprotected by following the procedure described for the preparation of **16** to give **17** (145 mg, 82%) as a colorless solid: UV (H_2O) λ_{max} 268 nm; ^1H NMR (D_2O) δ (italicized protons are assigned to the thymidyl moiety) 1.90 (3 H, br s, 5-C*H*$_3$), 2.18 (1 H, quintet, 2′-H), 2.43 (1 H, ddd, *J* = 14.1, 6.3, 3.0 Hz, 2′-H), 3.70 (1 H, dd, *J* = 12.6, 4.8 Hz, 5″-H), 3.78 (1 H, dd, *J* = 12.6, 3.6 Hz, 5′-H), 3.98–4.14 (3 H, m, 4′-*H*, 5′-H), 4.65 (1 H, m, 3′-H), 5.10 (1 H, m, 4′-H), 5.98 (1 H, app dt, *J* = 6.0, 1.8 Hz, 2′-H), 6.03 (1 H, d, *J* = 7.5 Hz, 5-H), 6.20 (1 H, t, *J* = 6.3 Hz, 1′-*H*), 6.48 (1 H, app dt, *J* = 6.0, 1.7 Hz, 3′-H), 6.96 (1 H, m, 1′-H), 7.62 (1 H, m, 6-*H*), 7.79 (1 H, d, *J* = 7.5 Hz, 6-H); ^{31}P NMR (D_2O) δ −0.60; ^{13}C NMR(D_2O) δ (thymidyl carbons are italicized) 12.44 (5-*C*H$_3$), 38.43 (d, *J* = 3.3 Hz, *C*-2′), 61.83 (*C*-5′), 66.77 (d, *J* = 5.4 Hz, C-5′), 75.77 (d, *J* = 5.3 Hz, *C*-3′), 85.82 (*C*-1′), 86.36 (d, *J* = 6.4 Hz, *C*-4′), 86.55 (d, *J* = 9.3 Hz, C-4′), 91.67 (C-1′), 96.66 (C-5), 112.20 (*C*-5), 126.45 (C-2′), 134.68 (C-3′), 138.07 (*C*-6), 143.96 (C-6), 152.17 (*C*-2), 157.83 (C-2), 166.24 (*C*-4), 167.10 (C-4); MS (FAB) *m/z* 530 (M^+), 514 (M + 1, for free acid), 513 (M^+, free acid). Anal. ($C_{19}H_{27}N_6O_{10}P\cdot H_2O$) C, H, N, P.

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The Calanolides, a Novel HIV-Inhibitory Class of Coumarin Derivatives from the Tropical Rainforest Tree, *Calophyllum lanigerum* [1]

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Eight new coumarin compounds (1–8) were isolated by anti-HIV bioassay-guided fractionation of an extract of *Calophyllum lanigerum*. The structures of calanolide A (1), 12-acetoxycalanolide A (2), 12-methoxycalanolide A (3), calanolide B (4), 12-methoxycalanolide B (5), calanolide C (6) and related derivatives 7 and 8 were solved by extensive spectroscopic analyses, particularly HMQC, HMBC, and difference NOE NMR experiments. The absolute stereochemistry of calanolide A (1) and calanolide B (4) was established by a modified Mosher's method. Calanolides A (1) and B (4) were completely protective against HIV-1 replication and cytopathicity (EC_{50} values of 0.1 μM and 0.4 μM, respectively), but were inactive against HIV-2. Some of the related compounds also showed evidence of anti-HIV-1 activity. Studies with purified bacterial recombinant reverse transcriptases (RT) revealed that the calanolides are HIV-1 specific RT inhibitors. Moreover, calanolide A was active not only against the AZT-resistant G-9106 strain of HIV-1 but also against the pyridinone-resistant A17 strain. This was of particular interest since the A17 virus is highly resistant to previously known HIV-1 specific, non-nucleoside RT inhibitors (e.g., TIBO; BI-RG-587; L693,593) which comprise a structurally diverse but apparently common pharmacologic class. The calanolides represent a substantial departure from the known class and therefore provide a novel new anti-HIV chemotype for drug development.

Introduction

The National Cancer Institute is actively acquiring and screening extracts from diverse plant, marine, and microbial sources for anti-HIV activity.[2] Stemming from these efforts, HIV-inhibitory compounds have thus far been isolated and identified from plants in the families Euphorbiaceae,[3,4] Ancistrocladaceae,[5] Combretaceae,[6] and Piperaceae.[7] In this continuing program, an organic ex-

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§ ABL-Basic Research Program.

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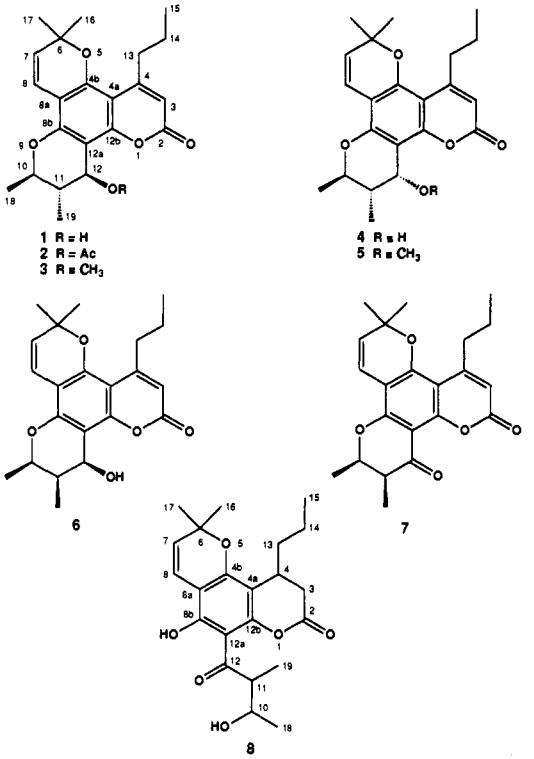
tract of the tropical rainforest tree *Calophyllum lanigerum* Miq. var. *austrocoriaceum* (T.C. Whitmore) P.F. Stevens (Guttiferae) has been found to inhibit the cytopathic effects of in vitro HIV infection. We report here the results of our bioassay-directed fractionation of the extract, and the chemical and biological characterization of active constituents. Previous phytochemical studies of the genus *Calophyllum* had revealed it to be a rich source of secondary metabolites. Xanthones,[8] steroids,[9] triterpenes,[10] coumarins,[11] and benzopyrans[12] were among the compounds reported earlier from *Calophyllum* species; however, no antiretroviral activity had previously been associated with this genus.

Chemistry

The 1:1 CH_2Cl_2/MeOH extract of *C. lanigerum* fruit and twigs showed confirmable anti-HIV activity in the initial screen.[13] A sequential solvent/solvent partitioning protocol[14] provided hexane- and CCl_4-soluble fractions in which the HIV-inhibitory activity was concentrated. Repeated vacuum liquid chromatography on silica gel, eluting with mixtures of hexane/EtOAc, provided fractions which contained a series of coumarin derivatives which we have named the calanolides. The anti-HIV activity of all fractions was assessed at each chromatographic step and active fractions were pooled together according to their ^1H NMR and TLC profiles. On silica TLC plates, the coumarin derivatives charred upon treatment with vanillin/H_2SO_4 to give blue spots which intensified in color over 24 h to a deep ink-blue appearance. Final purification of the individual constituents was achieved by silica HPLC for calanolide A (1), calanolide B (4), and compound 7. Reversed-phase C_{18} HPLC using MeOH/H_2O mixtures provided 12-acetoxycalanolide A (2), 12-methoxycalanolide A (3), 12-methoxycalanolide B (5), calanolide C (6), and compound 8.



Calanolide A. This compound (1) was isolated as an optically active oil, $[\alpha]_D = +60°$, which gave a HREIMS parent ion at m/z 370.1736 Da, indicating a molecular formula of $C_{22}H_{26}O_5$. The mass spectrum contained significant fragment ions for $M^+ - CH_3$ (m/z 355, 100%), $M^+ - CH_3 - H_2O$ (m/z 337, 12%) and $M^+ - C_5H_{11}$ (m/z 299, 29%). The infrared spectrum showed bands corresponding to hydroxyl (3300 cm^{-1}) and carbonyl (1735 cm^{-1}) groups. Resonances for 11 sp^2 carbons in the ^{13}C NMR spectrum revealed a conjugated ester (δ 160.4), a disubstituted olefin conjugated to a phenyl group (δ 126.9 [1 H] and 116.5 [1 H]), a trisubstituted olefin conjugated to a carbonyl (δ 158.9 and 110.1 [1 H]), and a fully substituted benzene ring bearing three oxygen moieties (δ 154.5, 153.1, 151.1, 106.3, 106.4, and 104.0). Taking into account the number of double bond equivalents implicit in the molecular formula, calanolide A (1) had to be tetracyclic. The ^1H NMR spectrum contained two methyl singlets (δ 1.49 and 1.44), two methyl doublets (δ 1.44 and 1.13), and a methyl triplet

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Table I. 500-MHz ^{1}H NMR Data for Compounds 1–8[a]

proton no.	1	2	3	4	5	6	7	8
3	5.92, t, J = 1.0 Hz	5.93, t, J = 1.0 Hz	5.94, t, J = 1.0 Hz	5.93, t, J = 1.0 Hz	5.92, t, J = 1.0 Hz	5.94, t, J = 1.0 Hz	5.98, t, J = 1.0 Hz	2.67, dd, J = 6.5, 15.0 Hz 2.81, dd, J = 9.0, 15.0 Hz
4								3.67, br m
7	5.52, d, J = 9.5 Hz	5.52, d, J = 10.0 Hz	5.50, d, J = 10.0 Hz	5.51, d, J = 10.0 Hz	5.51, d, J = 10.0 Hz	5.56, d, J = 10.5 Hz	5.61, d, J = 11.0 Hz	5.45, d, J = 10.0 Hz
8	6.60, d, J = 9.5 Hz	6.61, d, J = 10.0 Hz	6.61, d, J = 10.0 Hz	6.61, d, J = 10.0 Hz	6.60, d, J = 10.0 Hz	6.83, d, J = 10.5 Hz	6.78, d, J = 11.0 Hz	6.58, d, J = 10.0 Hz
10	3.90, dq, J = 9.0, 6.5 Hz	4.16, quin, J = 6.5 Hz	4.26, ddq, J = 1.3, 3.5, 6.5 Hz	4.24, dq, J = 10.5, 6.5 Hz	4.27, dq, J = 11.0, 6.0 Hz	4.32, dq, J = 2.5, 7.0 Hz	4.69, dq, J = 3.0, 6.5 Hz	4.49, dq, J = 3.5, 7.0 Hz
11	1.91, ddq, J = 9.0, 8.0, 6.5 Hz	2.09, ddd, J = 6.0, 6.5, 7.5 Hz	2.23, ddq, J = 3.5, 3.7, 7.5 Hz	1.73, ddq, J = 10.5, 3.3, 6.5 Hz	1.69, ddq, J = 11.0, 2.5, 6.0 Hz	2.22, ddq, J = 2.5, 6.0, 7.0 Hz	2.61, dq, J = 3.0, 7.0 Hz	2.52, dq, J = 3.5, 6.5 Hz
12	4.70, d, J = 8.0 Hz	5.97, d, J = 6.0 Hz	4.31, dd, J = 3.7, 1.3 Hz	4.95, d, J = 3.3 Hz	4.54, d, J = 2.5 Hz	5.06, dd, J = 6.0, 1.5 Hz		
13,13′	2.87, m	2.86, m	2.80, ddd 2.92, ddd	2.87, m	2.88, m	2.88, ddd 2.79, ddd	2.85, m	1.50, m 1.80, m
14,14′	1.63, m	1.63, sext, J = 7.0 Hz	1.63, m	1.63, sext, J = 7.0 Hz	1.64, sext, J = 7.0 Hz	1.60, sext, J = 7.0 Hz	1.63, sext, J = 7.0 Hz	1.14, m (2 H)
15	1.01, t, J = 7.5 Hz	1.01, t, J = 7.0 Hz	1.01, t, J = 7.5 Hz	1.01, t, J = 7.5 Hz	1.01, t, J = 7.5 Hz	0.98, t, J = 7.5 Hz	1.01, t, J = 7.5 Hz	0.84, t, J = 7.0 Hz
16	1.44, s	1.46, s	1.47, s	1.46, s	1.46, s	1.46, s	1.50, s[b]	1.41, s
17	1.49, s	1.49, s	1.45, s	1.47, s	1.47, s	1.46, s	1.50, s[b]	1.45, s
18	1.44, d, J = 6.5 Hz	1.43, d, J = 6.5 Hz	1.45, d, J = 7.0 Hz	1.41, d, J = 6.0 Hz	1.38, d, J = 6.5 Hz	1.41, d, J = 7.0 Hz	1.42, d, J = 7.0 Hz	1.35, d, J = 7.0 Hz
19	1.13, d, J = 6.5 Hz	1.05, d, J = 7.5 Hz	1.00, d, J = 7.5 Hz	1.12, d, J = 7.0 Hz	1.13, d, J = 6.5 Hz	1.06, d, J = 7.5 Hz	1.14, d, J = 7.5 Hz	1.12, d, J = 6.5 Hz
OR	3.55, br s (OH)	2.10, s (OAc)	3.59, s (OMe)	2.43, br s (OH)	3.58, s (OMe)	3.64, d, J = 1.5 Hz (OH)		12.4, s (OH)

[a] Spectra obtained in $CDCl_3$. Compounds 1, 7, and 8 were assigned by HMQC, HMBC, COSY and difference NOE experiments. Assignments for compounds 2–6 were made by analogy. [b] Two peaks separated by 0.04 Hz.

Table II. 125-MHz ^{13}C NMR Data for Compounds 1–8[a]

carbon no.	1	2	3	4	5	6	7	8
2	160.4	160.0	160.9	160.9	160.8	160.8	160.0	178.6
3	110.1	110.9	110.7	110.3	110.3	111.1	111.4	38.5
4	158.9	157.7	158.2	158.7	158.5	158.6	158.1	30.5
4a	104.0	101.1[b]	102.7	103.5	103.2	103.5[b]	102.7	108.9
4b	151.1	151.7	151.6	151.4	151.4	150.6	157.6	157.3
6	76.6	77.8	77.6	77.7	77.6	78.8	78.9	78.2
7	126.9	126.8	126.6	126.7	126.6	126.9	128.2	125.6
8	116.5	116.4	116.6	116.5	116.6	115.7	115.0	115.6
8a	106.3	104.1[b]	104.1	106.1[b]	104.7[b]	102.9[b]	104.1	102.6
8b	153.1	152.6	151.6	153.9	153.8	152.6	160.0	160.0[f]
10	77.7	76.6	73.8	73.0	73.4	75.6	77.4	76.1
11	40.5	38.6[c]	35.1	38.6[c]	38.66	35.1	45.7	44.2
12	67.2	67.1	77.6	61.9	70.8	65.9	192.9	201.0
12a	106.3	106.2[b]	106.4	106.2[b]	106.0[b]	109.2[b]	106.8	101.2
12b	154.4	154.4	155.1	153.1	153.1	154.6	154.3	160.0[f]
13	38.7	38.1[c]	38.6	38.2[c]	38.65	38.9	38.9	35.4
14	23.3	23.3	23.3	23.3	23.3	23.2	23.0	20.7
15	14.0	14.0	14.0	14.0	14.1	14.0	13.9	14.0
16	27.4	27.8	27.7	27.8	27.8	28.2	28.0	28.1
17	28.0	28.0	27.9	27.7	27.9	28.4	28.1	28.5
18	18.9	19.2	19.5	18.9	19.2	16.8	15.9	16.2
19	15.1	15.3	17.0	12.5	13.3	7.2	9.0	9.3
		170.7[d]	57.6[e]		59.4[e]			
		21.2[d]						

[a] Spectra recorded in $CDCl_3$ and attached protons determined by the DEPT pulse sequence. [b,c] Resonances within a column may be interchangeable. [d] Acetyl resonances. [e] Methoxy resonance. [f] In CD_3OD these signals appeared as doubled peaks at δ 161.20, 161.16 and δ 161.10, 161.03.

(δ 1.01). Additional proton signals included those of an allylic methylene (δ 2.87 [2 H], m), an aliphatic methylene (1.63 [2 H], m), and three olefin protons (δ 6.60 d, J = 9.5 Hz; 5.92 t, J = 1.0 Hz; 5.52 d, J = 9.5 Hz). These data suggested that calanolide A (1) was a coumarin derivative related to costatolide (9), a metabolite from *Calophyllum costatum*.[15]

One-bond and long-range proton-detected heteronuclear correlation experiments (HMQC and HMBC) allowed the complete assignment of both the ^{1}H NMR (Table I) and ^{13}C NMR spectra (Table II) of calanolide A (1). Key correlations included those between H8 and carbons 4b, 6, 8a and 8b, which helped establish the position of the 2,2-dimethylchromene system. Placement of the *n*-propyl group at C4 was aided by a 1.0-Hz allylic coupling between the C13 allylic methylene protons and the C3 olefin proton, and by three-bond heteronuclear correlations from the C13 methylene protons to C3 and C4a. The remaining substitution pattern about the coumarin nucleus was defined by correlations between H12 and carbons 8b, 10, 11, 12a, 12b, and 19. This confirmed that calanolide A (1) had the same skeleton as costatolide (9)[15] and apparently differed from 9 only in the relative stereochemistry of the sub-

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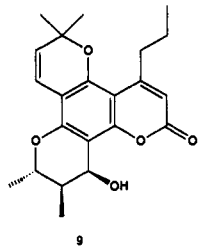
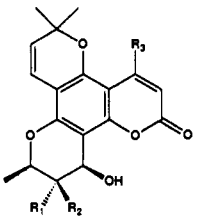
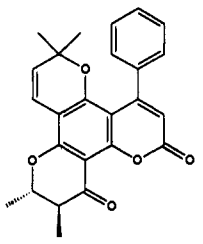
stituents about the 2,3-dimethylchromanol ring.



9



10 $R_1 = CH_3$, $R_2 = H$, $R_3 = C_6H_5$
11 $R_1 = CH_3$, $R_2 = H$, $R_3 = CH_3$
12 $R_1 = H$, $R_2 = CH_3$, $R_3 = C_6H_5$



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In the ^1H NMR spectrum of compound 1, the H12 benzylic carbinol proton showed an 8.0-Hz coupling to H11, which revealed that these two protons had a trans-diaxial orientation. A 9.0-Hz coupling between H11 and H10 established that H10 also was axial. This assignment was supported by NOE enhancements (3%) observed between the diaxial H10 and H12 protons. Calanolide A (1) was thus a diastereomer of costatolide (9), which showed J_{10-11} and J_{11-12} of 10.5 and 3.5 Hz, respectively.[15] Two related coumarin derivatives, inophyllum B (10)[16] and cordatolide A (11)[17] reportedly had the same relative stereochemical features about the chromanol ring as those found in 1, but differed in their C4 substituents. The J_{10-11} and J_{11-12} coupling constants observed in calanolide A (1) were in very close agreement with those reported for both compounds 10[16] and 11.[17]

12-Acetoxycalanolide A. Compound 2, $[\alpha]_D = +20°$, gave a parent ion by HREIMS at m/z 412.1825 Da corresponding to a molecular formula of $C_{24}H_{28}O_6$. Significant fragment ions were observed for $M^+ - CH_3$ (m/z 397, 41%), $M^+ - AcOH$ (m/z 352, 30%) and $M^+ - AcOH - CH_3$ (m/z 337, 100%). The presence of an acetate group was suggested by a sharp 3 H singlet in the ^1H NMR spectrum at δ 2.10 and ^{13}C NMR resonances at δ 21.2 (3 H) and 170.7. The remaining ^1H and ^{13}C NMR signals for compound 2 were very similar to those recorded for calanolide A (1), except that the H12 resonance in 2 was shifted downfield to δ 5.97. This suggested that compound 2 was the 12-acetoxy derivative of calanolide A (1). The J_{10-11} (6.5 Hz) and J_{11-12} (6.0 Hz) couplings in 2 supported a pseudoaxial orientation of the chromanol ring protons. The slightly diminished chromanol proton couplings in 2 conceivably resulted from a slight twisting of the flexible chromanol ring. Further evidence for the proposed substituent configuration was provided by difference NOE enhancements of 2% measured between H10 and H12.

12-Methoxycalanolide A. This compound (3), $[\alpha]_D = +32°$, showed an HREIMS parent ion at m/z 384.1924 Da, corresponding to a molecular formula of $C_{23}H_{28}O_5$. Significant fragment ions observed for $M^+ - CH_3$ (m/z 369, 12%), $M^+ - CH_3OH$ (m/z 352, 9%) and $M^+ - CH_3OH - CH_3$ (m/z 337, 100%) suggested the presence of a methoxyl group, which was confirmed by a ^1H NMR singlet (δ 3.59, 3 H) and a corresponding carbon resonance at δ 57.6. The ^1H and ^{13}C NMR spectra revealed that compound 3 had the same skeleton as calanolide A (1). However, important differences were observed in the signals for some of the chromanol ring substituents. In addition to the vicinal couplings of J_{10-11} (3.5 Hz) and J_{11-12} (3.7 Hz), a W coupling of 1.3 Hz was observed between H10 and H12 in compound 3. The W coupling required a pseudodiequatorial configuration for the C10 and C12 protons. Significant NOE enhancements between H11 and both the C10 methyl group (3.5%) and the C12 methoxyl group (3.5%) indicated that H11 was cis to these two substituents and therefore had an equatorial orientation about the chromanol ring. It appeared that in 12-methoxycalanolide A (3) the preferred conformation of the chromanol ring was inverted relative to calanolide A (1). Thus, while H10, H11, and H12 were oriented α, β, α, respectively, in both compounds, in calanolide A (1) all three protons were axial, and in 12-methoxycalanolide A (3) they were all equatorial.

Calanolide B. This compound (4), $[\alpha]_D = +8°$, was isomeric to calanolide A (1), as it also showed a HREIMS parent ion at m/z 370.1747 Da, corresponding to $C_{22}H_{26}O_5$. The ^1H and ^{13}C NMR spectra of calanolide B (4) were virtually identical to those from calanolide A (1), with the exception of some variations in signals from the chromanol ring. It was clear from the spectral data that compound 4 differed from 1 only in the stereochemical disposition of the chromanol ring substituents. Proton–proton coupling constant analysis showed a 10.5-Hz J_{10-11} coupling and a 3.3-Hz J_{11-12} coupling. Thus, H10 and H11 were trans-diaxial while H11 and H12 were in a cis configuration with H12 in a pseudoequatorial orientation. Calanolide B (4) had the same relative stereochemistry as costatolide (9) but its optical rotation was opposite in sign to that reported for 9;[15] therefore, compounds 4 and 9 were enantiomeric.

12-Methoxycalanolide B. This compound (5), $[\alpha]_D = +34°$, provided a HREIMS parent ion at m/z 384.1890 Da appropriate for a molecular formula of $C_{23}H_{28}O_5$. Additional fragment ions were seen for $M^+ - CH_3$ (m/z 369, 12%), $M^+ - CH_3OH$ (m/z 352, 13%) and $M^+ - CH_3OH - CH_3$ (m/z 337, 100%). The ^1H and ^{13}C NMR spectra of 5 were virtually identical to those recorded for compound 4 with the addition of a sharp 3 H singlet at δ 3.58 and a corresponding carbon resonance at δ 59.4. These data indicated that compound 5 was the 12-methoxyl derivative of calanolide B (4). This assignment was confirmed by acid hydrolysis of 5 using 6 N HCl at room temperature in THF/H_2O for 48 h to provide compound 4 as the only product.

Calanolide C. Compound 6, $[\alpha]_D = +68°$, also was isomeric with calanolide A (1), since it showed a similar HREIMS parent ion at m/z 370.1695 Da, consistent with a molecular formula of $C_{22}H_{26}O_5$. Fragment ions were found at $M^+ - CH_3$ (m/z 355, 100%), $M^+ - CH_3 - H_2O$ (m/z 337, 25%) and $M^+ - C_5H_{11}$ (m/z 299, 35%). Again, the only notable differences between the ^1H and ^{13}C NMR spectra of 6 and those recorded for compound 1 were the resonances associated with the chromanol ring. The J_{10-11}

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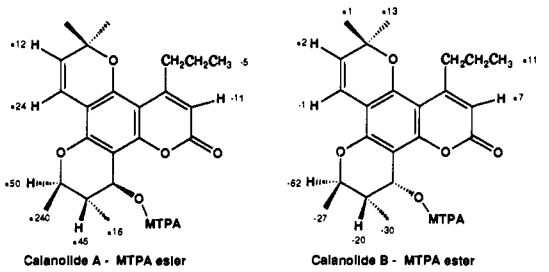
in 6 was 2.5 Hz while J_{11-12} was 6.0 Hz. These coupling constants were insufficient to define the relative stereochemistry of carbons 10, 11, and 12. However, the C12 hydroxyl proton exhibited a 1.5-Hz coupling to H12, which suggested that the rate of exchange of the OH proton was reduced due to hydrogen bonding to O1. Hydrogen bonding to O1 would require an equatorial OH at C12. A 5% NOE enhancement between H10 and H12 confirmed that these protons each had axial orientations. Therefore, H11 had to be equatorial because of the small J_{10-11} coupling. Calanolide C (6) was thus the C11 epimer of calanolide A (1) and had the same substitution pattern and relative stereochemistry about the chromanol ring as the coumarin derivative inophyllum A (12).[16] The J_{10-11} (3.3 Hz) and J_{11-12} values (5.4 Hz) reported for 12[16] were in good agreement with the respective couplings observed in calanolide C (6).

Compound 7. This compound, $[\alpha]_D = +60°$, provided a HREIMS molecular ion at m/z 353.1352 Da appropriate for a molecular formula of $C_{22}H_{24}O_5$. This indicated that 7 had one additional unsaturation equivalent relative to calanolide A (1). The infrared spectrum, with bands at 1734 and 1697 cm^{-1}, suggested the presence of an additional carbonyl group. Heteronuclear correlation experiments allowed the complete assignment of the ^1H and ^{13}C NMR spectra of 7. While the ^{13}C NMR spectrum of compound 7 was quite similar to that of calanolide A (1), the C12 peak in 7 was shifted downfield to δ 192.9, indicative of an α,β-unsaturated ketone functionality. A shift of the C11 proton resonance in 7 to δ 2.61 supported its placement α to a ketone carbonyl.

The small coupling measured between H10 and H11 (J_{10-11} = 3.0 Hz) indicated that at least one of these protons was equatorial. The oxidation product (13) of soulattrolide[16b] contains a similar 2,3-dimethylbenzopyranone ring system. In compound 13, H10 and H11 are trans and a J_{10-11} coupling of 11.0 Hz was recorded.[16b] This indicated that when the H10 and H11 protons were trans, the ring adopted a conformation with these two protons in a diaxial orientation. Therefore, the relative stereochemistry of the H10 and H11 protons in calanolide D (7) had to be cis. The absolute stereochemistry at C10 and C11 has not been determined and therefore both of the corresponding methyls have been drawn arbitrarily as β. The ketone 7 could be cleanly converted to calanolide C (6) by reduction with $NaBH_4$; the compounds were identical in all respects.

Compound 8. This compound, $[\alpha]_D = +30°$, had a molecular formula of $C_{22}H_{28}O_6$, as indicated by the HREIMS parent ion at m/z 388.1890 Da. Fragment ions appropriate for $M^+ - CH_3$ (m/z 373, 100%), $M^+ - C_3H_7$ (m/z 345, 3%), $M^+ - CO_2CH_3$ (m/z 329, 5%), $M^+ - C_3H_7O_2$ (m/z 313, 3%) and $M^+ - COCHCH_3CHOHCH_3$ (m/z 287, 3%) were also observed. The complete ^1H and ^{13}C NMR spectra of 8 were assigned with information provided from NOE experiments and heteronuclear correlations. The ^{13}C NMR spectrum contained signals for an unsaturated ketone (δ 201.0), a saturated ester (δ 178.6), a disubstituted olefin (δ 125.6 [1 H] and 115.6 [1 H]), and a fully substituted benzene ring bearing three oxygens (δ 160.0 [2 C], 157.3, 108.9, 102.6, and 101.2). Therefore, 8 had only three of the four rings found in the other members of the calanolide series. In contrast to compounds 1–7, which gave vivid blue spots on TLC when charred with vanillin/H_2SO_4, 8 gave a brownish-green spot.

The ^1H and ^{13}C NMR spectra of 8 showed some resonances that corresponded closely to the coumarin and 2,2-dimethylchromene ring systems of compounds 1–7. However, the C3/C4[18] double bond in 1–7 was fully saturated in 8. This resulted in a methylene (δ 2.81 dd, J = 15.0, 9.0 Hz and 2.67 dd, J = 15.0, 6.5 Hz) α to the lactone carbonyl that was coupled to the C4 benzylic methine (δ 3.67 m). The C4 proton also showed heteronuclear correlations to C2, C3, C4a, C12b, C13, and C14, which supported the presence of a 3,4-dihydrocoumarin skeleton with an n-propyl substituent at C4. Heteronuclear correlations, including those between H8 and C4b, and C6 and C8b, confirmed the placement of the chromene functionality on the coumarin ring system. This suggested that the chromanol ring system present in 1–7 was open in 8.



Calanolide A - MTPA ester Calanolide B - MTPA ester

Figure 1. ^1H NMR $\Delta\delta$ values ($\Delta\delta = \delta_S - \delta_R$ in Hertz at 500 MHz) for (R)- and (S)-MTPA esters of calanolide A (1) and calanolide B (4).

The position of the phenol on C8b was established by heteronuclear correlations from the phenolic proton to C8a, C8b and C12a and an NOE interaction with H8. Since the remaining ketone group in the molecule was unsaturated, it had to be located on C12. In this position it could accept a hydrogen bond from the C8b phenol proton, which appeared as a sharp singlet at δ 12.40. The downfield shift of H11 (δ 2.52 dq, J = 3.5, 6.5 Hz) was appropriate for a methine located α to a ketone. The C10 carbinol methine proton (δ 4.49 dq, J = 7.0, 3.5 Hz) showed vicinal coupling to H11 and to the C18 methyl group. All heteronuclear correlations, including those from the C19 methyl protons to C10, C11, and C12, were fully consistent with the assigned structure for 8.

Stereochemistry. The absolute stereochemistry of calanolide A (1) and calanolide B (4) was determined by a modified Mosher's method.[19] The technique utilized anisotropic shifts induced in the ^1H NMR spectra of α-methoxy-α-(trifluoromethyl)phenylacetic (MTPA) esters of secondary alcohols to define the absolute stereochemistry. Both (+)-(R)- and (−)-(S)-MTPA esters of compounds 1 and 4 were prepared and $\Delta\delta$ values (Figure 1) from their 500-MHz ^1H NMR spectra were calculated ($\Delta\delta = \delta_S - \delta_R$). By this method, the absolute configuration of C12 was determined to be 12S in calanolide A (1) and 12R in calanolide B (4). As established earlier, calanolide A (1) [10R, 11R, 12S] and calanolide B (4) [10R, 11R, 12R] were C12 epimers.

Esterification of calanolide A (1) occurred slowly (24 h reflux for 1 vs 5 h for 4) and by ^1H NMR analysis esterification appeared to cause a change in the conformation of the chromanol ring. The methyls and the ester group flipped from equatorial to axial positions in the MTPA

(18) For clarity of discussion, calanolide E (8) has been numbered in a scheme that is analogous to the numbering in the other members of the calanolide series (1–7).

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Table III. Comparative Evaluation of Anti-HIV-1 Activity of Compounds 1–8 in the Primary Screening Assay

compd	maximum protection (%)	EC_{50} (μM)[a]	IC_{50} (μM)[a]	TL
1	100	0.1	20	200
2	100	2.7	13	5
3	*b*	*b*	21	*b*
4	100	0.4	15	37
5	35	*b*	19	*b*
6	30	*b*	30	*b*
7	*b*	*b*	27	*b*
8	*b*	*b*	25	*b*

[a] Averages of triplicate determinations; standard errors averaged ≤10% of the respective means. [b] Not measurable.

ester of 1, as J_{10-11} = 2.5 Hz (previously 9.0 Hz) and J_{11-12} = 2.5 Hz (previously 8.0 Hz). In addition, a four-bond W coupling of 1.5 Hz between H10 and H12 could also be observed. Similar changes in the conformation of the chromanol ring were previously noted for compound 3. The anisotropic shifts induced in the MTPA esters indicated that the bulky MTPA group was sterically repulsed by the coumarin ring lactone. Thus, the plane which divided the molecule's proton resonances into $\Delta\delta$-positive and $\Delta\delta$-negative did not cleanly bisect the dihydropyran ring through C12 and O9. In calanolide A (1) the dividing plane seemed to come closer to C6 and in calanolide B (4) to C8b. Due to the 1,3-diaxial orientation of the C10β-methyl and the C12β-ester group in the MTPA ester of 1, the former methyl group was very strongly influenced by the ester ($\Delta\delta$ = +240 in comparison to +16 measured for the C11α-methyl).

Biological Activity

Structure–Activity Comparisons. The pure compounds 1–8 were comparatively evaluated for anti-HIV activity in the primary screening assay[13] (Table III). Over a broad concentration range (<0.1–>10μM), calanolide A (1) provided 100% protection against the cytopathic effects of HIV-1 infection and essentially halted HIV-1 replication in human T-lymphoblastic (CEM-SS) cells (EC_{50} = 0.1 μM, IC_{50} = 20 μM). The ester derivative, 12-acetoxycalanolide A (2), also was active, albeit less potent (EC_{50} = 2.7 μM, IC_{50} = 13.0 μM), while 12-methoxycalanolide A (3) showed no detectable antiviral activity in the primary screening assay. Calanolide B (4), the C12 epimer of 1, was fully active against HIV-1 and was more comparable to 1 in potency (EC_{50} = 0.4 μM, IC_{50} = 15.0 μM). The 12-methoxycalanolide B (5) and the calanolide C (6) showed very weak but detectable activity in the primary screen, while compounds 7 and 8 were inactive. The apparent in vitro therapeutic indices (TI) for compounds 1, 2, and 4 were 200, 5, and 37, respectively. While the absolute stereochemistry of calanolide C (6) was not determined, the markedly diminished activity of 6 suggested that the stereochemical orientation of both the hydroxyl and also the methyl substituents about the chromanol ring significantly influenced the activity in this series of compounds.

Correlative Assays. For a more definitive demonstration of the anti-HIV-1 activity of the pure calanolides 1 and 4, a battery of interrelated assays was performed on individual wells of 96-well microtiter plates.[20] Cellular viability, in the presence and absence of the calanolides, was estimated in uninfected and HIV-1-infected cells, both by an adaptation[20] of the published XTT-tetrazolium method,[8] and by means of the fluorescent probe 2′,7′-bis(carboxyethyl)-5-carboxyfluorescein 6-acetoxymethyl ester (BCECF),[21] a nonfluorescent molecule which enters viable cells where it is hydrolyzed by cellular esterases to a fluorescent compound. Total cellular DNA content was measured with the dye, 4′,6-diamidino-2-phenylindole (DAPI),[22] which fluoresces when intercalated at A-T specific sites in chromatin. Supernatant viral reverse transcriptase (RT), viral p24 antigen (p24), and syncytium-forming units (SFU) were assayed as described.[20]

As illustrated in Figure 2a–d, calanolide A was capable of complete inhibition of all of the indices of cytopathic effects of HIV-1 and of HIV-1 replication in CEM-SS human lymphoblastoid target cells in vitro (EC_{50} 0.06–0.08 μM); direct cytotoxicity of the compound upon the target cells was apparent only at 200-fold or greater concentrations (IC_{50} 12–16 μM). Calanolide A also strikingly inhibited the production of RT, p24 core protein, and infectious virus from HIV-1-infected CEM-SS within these same inhibitory effective concentrations, indicating a cessation of viral replication. Similar results (data not shown) as those depicted for calanolide A in graphs in Figure 2a–d were also obtained with calanolide B, although requiring approximately 4-fold higher concentrations of the latter for equivalent cytoprotection, as consistent with the earlier comparative testing in the primary screen.

Activity of Calanolides against HIV-1 Reverse Transcriptase. Calanolide A was also tested against several strains of HIV-2 and found inactive (data not shown). This raised the possibility that the calanolides might fall into an emerging group of structurally-diverse, non-nucleoside, HIV-1-specific inhibitors which act at a common site on HIV-1 reverse transcriptase.[23–28] As an initial step to examine this question, we tested calanolides A and B against bacterial recombinant HIV-1 and HIV-2 reverse transcriptases using a poly(rA)-p$(dT)_{10}$ homopolymer template.[29–31] Both compounds selectively in-

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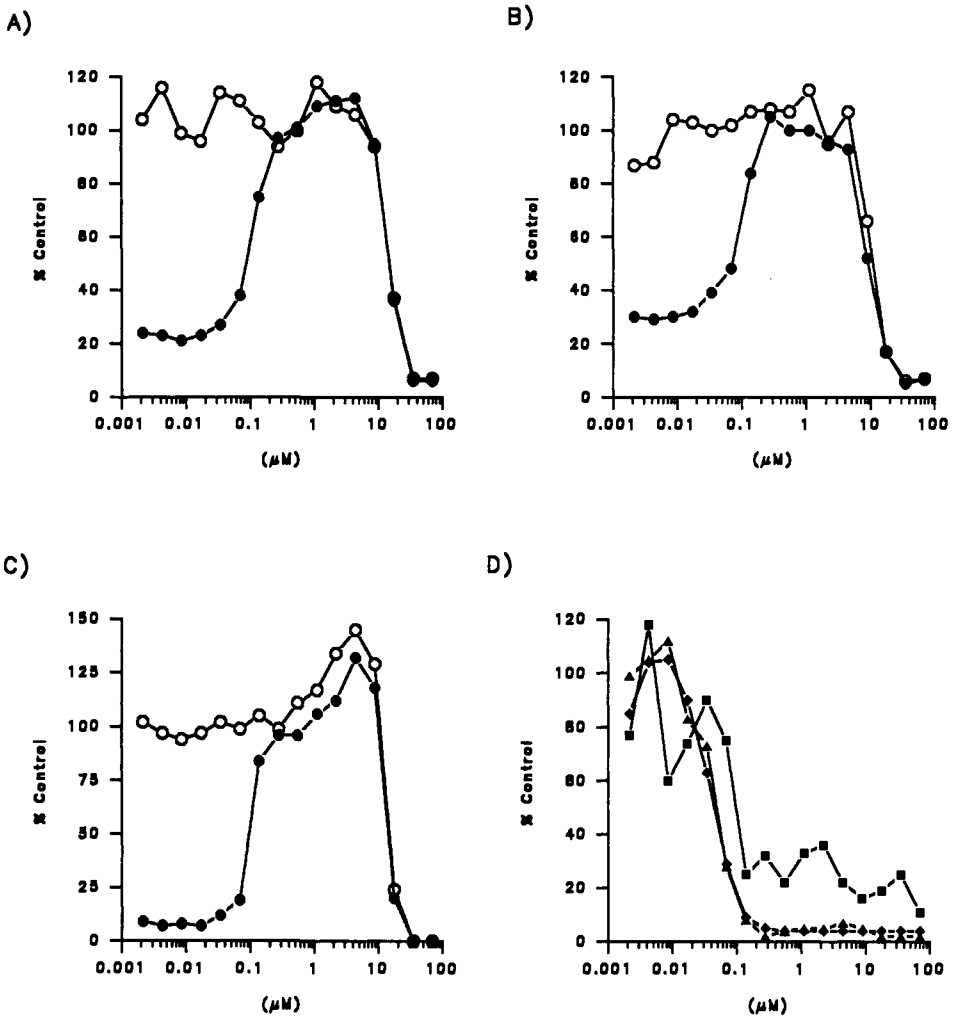

Figure 2. Graphs A, B, and C show the effects of a range of concentrations of calanolide A upon uninfected CEM-SS cells (○) and upon HIV-1 infected CEM-SS cells (●), as determined after 6 days in culture. Graph A depicts the relative numbers of viable CEM-SS cells as assessed by the BCECF assay; graph B depicts the relative DNA content of the respective cultures; graph C depicts the relative numbers of viable CEM-SS cells, as assessed by the XTT assay. Graph D shows the effects of a range of concentrations of calanolide A upon indices of infectious virus or viral replication; these indices include viral reverse transcriptase activity (▲), production of viral core protein p24(♦), and syncytium-forming units (■). In graphs A, B, and C, the data points are represented as the percent of the respective uninfected, nondrug treated control values. In graph D, the data points are represented as the percent of the respective infected, nondrug treated control values. In all graphs, each point was derived from the mean of at least quadruplicate determinations; standard errors averaged ≤10% of the respective means.

hibited the HIV-1 reverse transcriptase (Figure 3). While the apparent IC_{50}'s for HIV-1 RT inhibition were 5–10-fold higher than for the HIV-1 anticytopathic EC_{50}'s, this could be explained by the use of the recombinant enzymes and/or the homopolymer template in the assays.[32] Therefore as a followup, calanolide A was tested against HIV-1 RT using a ribosomal RNA template;[32] the resulting

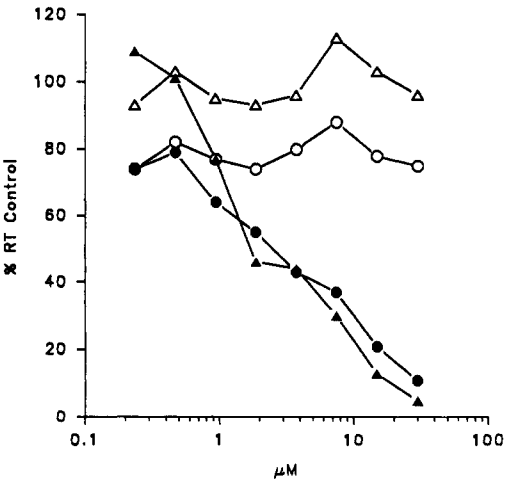

Figure 3. Effects of calanolide A (●,○) and calanolide B (▲,△) on HIV-1 RT (closed symbols) and HIV-2 RT (open symbols). Each point represents the mean of triplicate determinations; standard errors averaged ≤15% of the respective means.

IC_{50} value (0.07 μM; data not shown) corresponded closely to the antiviral EC_{50} values (Figure 2).

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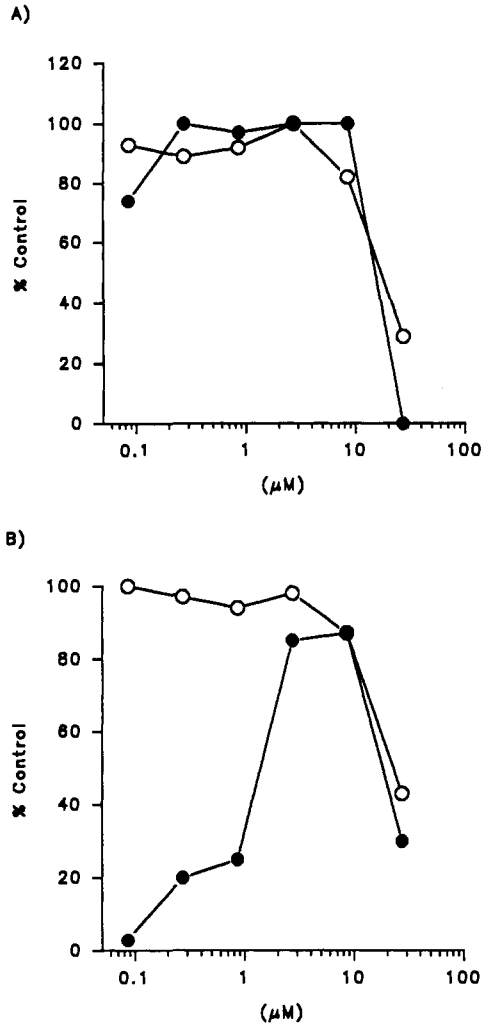

Figure 4. The effect of calanolide A on uninfected MT-2 cells (○) and on infected MT-2 cells (●) with (A) AZT-resistant HIV-1 strain g9106 or (B) pyridinone-resistant HIV-1 strain A17. Each point represents the mean of triplicate determinations; standard errors averaged ≤15% of the respective means.

Calanolide A Activity against Drug Resistant Strains of HIV-1. Experiments were performed with calanolide A against MT-2 target cells infected with the AZT-resistant HIV-1 strain, G-9106,[33] or with the pyridinone-resistant HIV-1 strain, A17.[27] The AZT-resistant virus is inhibited by other reported non-nucleoside HIV-1 specific inhibitors; however, the pyridinone-resistant virus is highly cross-resistant to all of these structurally diverse compounds (including TIBO, BI-RG-587, and L963,593) which appear to comprise a common pharmacologic class.[23-28] It is currently surmised that this group of compounds interacts with the same binding site on HIV-1 RT and that cross-resistance to the group results, at least in part, from a mutation at amino acid position 181.[27] It was therefore of particular interest in the present study that calanolide A was capable of fully protecting MT-2 target cells from the cytopathic effects of not only the AZT-resistant virus but also the A17 strain of HIV-1 (Figure 4). This suggested that the calanolides represent a new pharmacologic class of non-nucleoside, HIV-1 specific antiviral RT inhibitor. More detailed analyses of the molecular pharmacology of RT inhibition by the calanolides have solidified this view; these results will be presented in detail in a separate manuscript.

Conclusion

Nucleoside derivatives, such as AZT and DDI, currently represent the only commercially available, known clinically active anti-HIV agents used widely in the therapy of AIDS. While these agents are useful but not curative, the search for new, non-nucleoside agents which do not share cross-resistance, or which have different mechanisms of action or nonoverlapping toxicity profiles is an urgent priority. Combination chemotherapy with rationally selected drugs representing diverse anti-HIV chemotypes will likely be required for major advances in the effective treatment of AIDS.[34]

The calanolides represent a novel anti-HIV chemotype distinct from any previously known pharmacologic class. Moreover, the relative structural simplicity of the class may make synthesis feasible as a source for further study, as well as offer the opportunity for synthetic or semisynthetic structure–activity optimization. The calanolides merit careful consideration as a priority class for drug development.

Experimental Section

General. A description of the equipment and instrumentation used in these studies has been provided in previous reports.[4,7] The paucity of the individual compounds available from the extracts and the urgent need for detailed biological evaluation precluded the use of combustion analyses. Composition and purity were assessed by high resolution mass spectrometry and NMR analyses (see supplementary material).

Collection and Extraction. Fruit and twig samples of *Calophyllum lanigerum* were collected in Sarawak, Malaysia, as part of an NCI plant collection contract. Voucher specimens for this collection are maintained for botanical reference at the Smithsonian Institution Museum of Natural History, Botany Department. Dried plant material (763 g) was stored at −20° until it was ground, percolated in 1:1 CH_2Cl_2/MeOH and washed with 100% MeOH. Removal of the solvent under reduced pressure provided 72.5 g of organic extract.

Chromatographic Separation. A 10-g portion of the organic extract was subjected to a solvent/solvent partitioning protocol[14] which concentrated the anti-HIV activity in the hexane (770 mg) and CCl_4 (590 mg) soluble fractions. The active fractions were individually separated by vacuum liquid chromatography (VLC) on 10 g of silica using mixtures of hexane/EtOAc. The active constituents eluting with 10–25% EtOAc were combined on the basis of TLC and ^1H NMR profiles to provide two active fractions. The individual fractions were further separated by VLC on silica using gradual step gradient elution with hexane/EtOAc mixtures. Final purification was achieved by silica HPLC using 7:3 hexane/EtOAc for calanolide A (1) (11.7 mg), calanolide B (4) (5.0 mg) and calanolide D (7) (12.5 mg). Purification of the other components was effected by C_{18} HPLC with 9:1 MeOH/H_2O to give 12-acetoxycalanolide A (2) (7 mg), 12-methoxycalanolide A (3) (5 mg), 12-methoxycalanolide B (5) (16 mg), calanolide C (6) (4 mg) and calanolide E (8) (11 mg).

Calanolide A (1): $[\alpha]_D$ +60° ($CHCl_3$, *c* 0.7); UV λ_{max} (MeOH) 325 (ε 13,700), 284 (ε 22,800), 228 (ε 22,200) nm; IR (film) ν_{max} 3439, 2966, 1735, 1713, 1583, 1111 cm^{-1}; HREIMS obsd *m/z* 370.1764, calcd for $C_{22}H_{26}O_5$, 370.1780; LRMS *m/z* 370 (38%), 355 (100%), 337 (12%), 299 (29%).

12-Acetoxycalanolide A (2): $[\alpha]_D$ +20° ($CHCl_3$, *c* 0.5); IR (film) ν_{max} 2960, 1738, 1585, 1376, 1230, 1138 cm^{-1}; HREIMS obsd *m/z* 412.1825, calcd for $C_{24}H_{28}O_6$, 412.1886; LRMS *m/z* 412 (13%), 397 (41%), 352 (30%), 337 (100%), 299 (8%).

12-Methoxycalanolide A (3): $[\alpha]_D$ +32° ($CHCl_3$, *c* 0.8); IR (film) ν_{max} 2960, 1731, 1584, 1380, 1137 cm^{-1}; HREIMS obsd *m/z*

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384.1924, calcd for $C_{23}H_{28}O_5$, 384.1937; LRMS m/z 384 (5%), 369 (12%), 352 (9%), 337 (100%).

Calanolide B (4): $[\alpha]_D$ +10° (acetone, c 1.0); UV λ_{max} (MeOH) 325 (ϵ 13,700), 284 (ϵ 22,800), 228 (ϵ 22,200) nm; IR (film) ν_{max} 3470, 2970, 1732, 1587, 1464, 1376, 1137 cm^{-1}; HREIMS obsd m/z 370.1747, calcd for $C_{22}H_{26}O_5$, 370.1780; LRMS m/z 370 (3%), 355 (100%), 337 (13%), 300 (5%), 299 (20%).

12-Methoxycalanolide B (5): $[\alpha]_D$ +34° ($CHCl_3$, c 0.5); IR (film) ν_{max} 2966, 1734, 1716, 1700, 1558, 1540, 1506, 1457 cm^{-1}; HREIMS obsd m/z 384.1890, calcd for $C_{23}H_{28}O_5$, 384.1937; LRMS m/z 384 (4%), 369 (12%), 352 (13%), 337 (100%).

Calanolide C (6): $[\alpha]_D$ +68° ($CHCl_3$, c 0.7); IR (film) ν_{max} 2960, 1729, 1620, 1582, 1120 cm^{-1}; HREIMS obsd m/z 370.1695, calcd for $C_{22}H_{26}O_5$, 370.1780; LRMS m/z 370 (52%), 355 (100%), 337 (25%), 299 (35%).

Compound 7: $[\alpha]_D$ +60° ($CHCl_3$, c 0.5); IR (film) ν_{max} 2960, 1734, 1697, 1684, 1575, 1558 cm^{-1}; HREIMS obsd m/z 368.1213, calcd for $C_{22}H_{24}O_5$, 368.1624; LRMS m/z 368 (25%), 353 (100%), 297 (68%).

Compound 8: $[\alpha]_D$ +30° ($CHCl_3$, c 0.5); IR (film) ν_{max} 2960, 1706, 1644, 1625, 1442, 1131 cm^{-1}; HREIMS obsd m/z 388.1890, calcd for $C_{22}H_{28}O_6$, 388.1886; LRMS m/z 388 (19%), 373 (100%), 345 (3%), 329 (5%), 313 (3%), 287 (3%).

Preparation of the (*R*)- and (*S*)-MTPA Esters of Calanolide A (1) and Calanolide B (4). A solution of (*R*)-α-methoxy-α-(trifluoromethyl)phenylacetic acid chloride (2.5 mg in 50 μL of benzene) was added to 3 mg of calanolide A (1) dissolved in 3 mL of dry benzene. A 0.03-mg aliquot of (dimethylamino)pyridine and 10 μL of triethylamine were added, and the reaction mixture was refluxed. After 3 h, a second 2.5-mg portion of (*R*)-MTPA chloride was added, and the reaction was refluxed for an additional 21 h. When the mixture had cooled, 10 mL of benzene was added and the organic phase was successively washed with 10% HCl, 1 N $NaHCO_3$, and H_2O. The solution was dried over Na_2SO_4, evaporated to dryness, and then quickly chromatographed on a short plug (1 × 2 cm) of silica, eluting with mixtures of hexane/EtOAc. A compound which appeared to be an elimination product eluted first with 5% EtOAc while the desired (*R*)-MTPA ester eluted with 12% EtOAc. The same procedure was repeated with (*S*)-MTPA chloride to give the (*S*)-MTPA ester. The (*R*)- and (*S*)-MTPA esters of calanolide B (4) were prepared in an identical fashion, with the exception that after the second addition of the MTPA chloride, the reaction mixture was refluxed for only an additional 2 h (total time of reflux = 5 h).

Hydrolysis of 12-Methoxycalanolide B (5) To Give Calanolide B (4). A solution of 2 mg of compound 5 in 400 μL of 2:1 THF/H_2O was treated with 8 μL of 6 N HCl. The reaction mixture was maintained at room temperature for 48 h, at which time no more starting material remained as judged by TLC. Ten milliliters of $CHCl_3$ were added, and the organic phase was washed with 1 N $NaHCO_3$, dried over Na_2SO_4, and evaporated to dryness to give calanolide B (4) as the only product.

Antiviral Assays. The XTT-tetrazolium primary NCI screen for anti-HIV activity was performed as described previously.[3,4,13,20] The confirmatory assays were also performed as described elsewhere.[20] The CEM-SS human lymphocytic target cell line used in the antiviral assays was maintained in RPMI 1640 medium (Gibco, Grand Island, NY) without phenol red and supplemented with 5% fetal bovine serum, 2 mM L-glutamine and 50 μg/mL gentamicin (complete medium). Exponentially growing cells were pelleted and resuspended at a concentration of 2.0×10^5 cells/mL in complete medium. The Haitian variant of HIV, $HTLV\text{-}III_{RF}$ (3.54×10^6 SFU/mL) was used throughout. Frozen virus stock solutions were thawed immediately before use and resuspended in complete medium to yield 1.2×12^5 SFU/mL. The appropriate amounts of the pure compounds for anti-HIV evaluations were dissolved in 100% DMSO and then diluted in complete medium to the desired initial concentration (and with final DMSO content not exceeding 1%). All serial drug dilutions, reagent additions, and plate-to-plate transfers were carried out with an automated Biomek 1000 Workstation (Beckman Instruments, Palo Alto, CA).

For the experiments yielding the data depicted in Figure 2, additional details are as follows. Uninfected CEM-SS cells were plated at a density of 1×10^4 cells in 50 μL of complete medium. Diluted HIV-1 virus was then added to appropriate wells in a volume of 50 μL to yield a multiplicity of infection of 0.6. Appropriate cell, virus, and drug controls were incorporated in each experiment; the final volume in each microtiter well was 200 μL. Quadruplicate wells were used for virus-infected cells, and duplicate wells were used for uninfected cells. Plates were incubated at 37 °C in an atmosphere containing 5% CO_2 for 6 days. Subsequently, aliquots of cell-free supernatant were removed from each well using the Biomek, and analyzed for reverse transcriptase activity, p24 antigen production, and synthesis of infectious virions as described.[20] Cellular growth or viability then was estimated on the remaining contents of each well using the XTT,[13,20] BCECF,[21] and DAPI[22] assays as described.[20]

Antiviral analysis was also performed with calanolide A in MT-2 target cells infected with the AZT-resistant strain of HIV, G-9106,[33] or the pyridinone-resistant HIV strain, A17.[27] The viruses were grown and the antiviral assays performed in these cells as previously described.[35]

Reverse Transcriptase Assay. Compounds were tested for their inhibitory activity against recombinant HIV-1 and HIV-2 reverse transcriptases which were prepared and assayed as previously described[29-31] with certain modifications. Briefly, test compounds were diluted in a virus disruption buffer (VDB) containing 50 mM Tris, pH 7.8, 0.15 mg/mL dithiothreitol (DTT), and 0.1% Triton X-100. Serial dilutions of candidate drugs were added to the individual well of a 96-well V-bottom plate. Recombinant enzyme was diluted in VDB and added to appropriate wells (50 μL/well). RT control wells received only VDB. Aliquots of 10 μL from each well were added to 30 μL of cocktail containing 2 μL of 1 mM Tris, pH 7.8, 1 μL of 3 M KCl, 5 μL of 3 mg/mL DTT, 5 μL of 0.1 M magnesium acetate, 10 μL of Poly(rA)-$p(dT)_{50}$ (2 units/mL), 6.5 μL of distilled water, 0.5 μL of 10% Triton X-100, and 10 μL of [^{3}H]dTTP (16.56 Ci/mmol). Samples were incubated for 30 min at 37 °C, harvested onto DE81 ion-exchange paper, and allowed to absorb with distilled water. Pads were then dried and counted in a liquid scintillation counter.

In an additional experiment, a solution containing 16S and 23S *Escherichia coli* ribosomal RNA, obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN) was used for the primer in an anti-HIV-1 RT assay. The preparation of the enzyme and the other methods used in this assay have been described elsewhere.[32]

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Supplementary Material Available: 500-MHz ^{1}H NMR spectra of compounds 1-8 (8 pages). Ordering information is given on any current masthead page.

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