Anti-AIDS Agents, 6. Salaspermic Acid, an Anti-HIV Principle from Tripterycium wilfordii, and the Structure-activity Correlation with its Related Compounds

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J. Nat. Prod., **1992**, 55 (3), 340-346• DOI:
10.1021/np50081a010 • Publication Date (Web): 01 July 2004

Journal of Natural Products
Vol. 55, No. 3, pp. 340-346, March 1992

ANTI-AIDS AGENTS, 6[1]. SALASPERMIC ACID, AN ANTI-HIV PRINCIPLE FROM *TRIPTERYGIUM WILFORDII*, AND THE STRUCTURE-ACTIVITY CORRELATION WITH ITS RELATED COMPOUNDS

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ABSTRACT.—Salaspermic acid [**1**], an inhibitor of HIV reverse transcriptase and HIV replication in H9 lymphocyte cells, was isolated from the roots of *Tripterygium wilfordii* for the first time. The structure of **1** derived from spectral data was established unequivocally by an X-ray analysis of crystals of the monohydrate. A structure-activity correlation of **1** with ten related compounds indicated that the acetal linkage in ring A and the carboxyl group in ring E of **1** may be required for the anti-HIV activity.

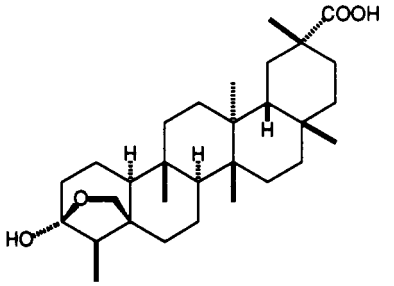
Tripterygium wilfordii Hook. (Celastraceae) is a liana that is distributed in southern China. Because of its high toxicity, it was used as an insecticide in the folklore. This plant was recently found to possess anti-inflammatory, anti-tumor, and immunosuppressive activity (2,3). Its main toxicity is present in the root bark. Purification of the extract of its roots, after removal from the bark, furnished a fraction which is known commercially as "Lei-gong-teng duo-dai" (LGTDD). LGTDD has been used for the treatment of various diseases such as dermatitis, rheumatoid arthritis, systemic acne rosacea, and nephritis, with good results and without hormonal side effects (4).

As a result of our continuing search for novel bioactive natural products as anti-AIDS agents, LGTDD was found to show significant anti-HIV activity. Bioassay-directed fractionation of LGTDD has now led to isolation and characterization of salaspermic acid [**1**] as the anti-HIV principle from the $CHCl_3$-soluble fraction.

RESULTS AND DISCUSSION

Compound **1** was isolated as prisms (mp >320°). Hrms measurement established

[1]For Part 5, see Kashiwada *et al.* (1).



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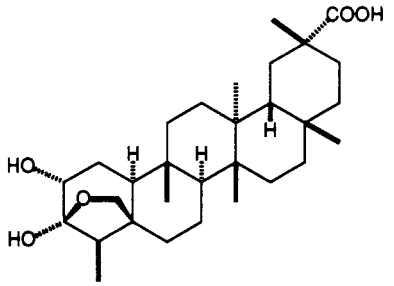

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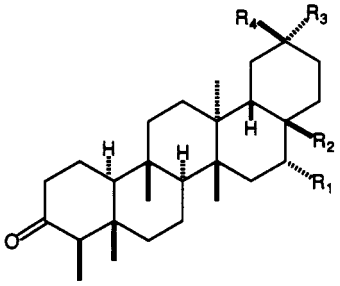
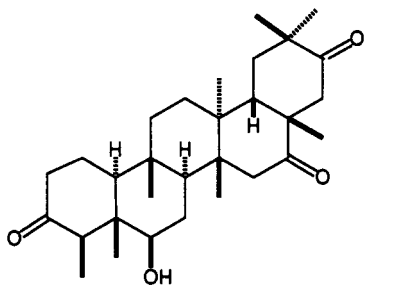


3 R_1=H, R_2=R_4=CH_2OH, R_3=Me
4 R_1=H, R_2=R_3=CH_2OH, R_4=Me
5 R_1=H, R_2=CH_2OH, R_3=R_4=Me
6 R_1=H, R_2=COOH, R_3=R_4=Me
7 R_1=OH, R_2=R_3=R_4=Me
8 R_1=H, R_2=R_4=Me, R_3=COOH
9 R_1=H, R_2=R_3=Me, R_4=COOH
10 R_1=H, R_2=OOH, R_3=R_4=Me



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its molecular formula at $C_{30}H_{48}O_4$. Ir spectral data indicated that **1** possessed hydroxy and carboxy groups. The presence of five tertiary methyl groups and one secondary methyl group in addition to an oxymethylene moiety in **1** was revealed by its ^{1}H-nmr spectrum. Lack of any unsaturation indicated that **1** had a friedelane skeleton. The foregoing evidence, coupled with mass spectral data, led to identification of **1** as salaspermic acid, which had been previously isolated from *Salacia macrosperma* by Viswanathan (5) and Zhang *et al.* (6).

X-ray analysis of crystals of the monohydrate of **1** confirmed the structure and provided details of the molecular geometry. The crystal structure was solved by direct methods. Fractional atomic coordinates for the non-hydrogen atoms are listed in Table 1.[2] A view of the solid-state conformation is provided in Figure 1. Although strain is evident in a number of bonds involving quaternary centers, the bond lengths overall agree well with expectations (7). [Bond lengths ($\sigma \pm 0.003$ Å) ≥ 1.560 Å are: C-5–C-10 = 1.564, C-8–C-9 = 1.564, C-8–C-14 = 1.563, C-9–C-10 = 1.571, C-13–C-14 = 1.573, C-13–C-18 = 1.573, C-17–C-18 = 1.576, C-20–C-21 = 1.560.] Rings A, B, and C have chair conformations, with that of ring A more puckered than normal around C-4 and flattened somewhat around C-1 due to the presence of the

[2]Atomic coordinates for salaspermic acid [**1**] monohydrate have been deposited at the Cambridge Crystallographic Data Centre, and can be obtained on request from Dr. Olga Kennard, University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW, UK.

TABLE 1. HIV Inhibition by Salaspermic Acid and Related Compounds.

Compound	IC_{50}		ED_{50}	
	μg/ml	μM	μg/ml	μM
1	25	53	5	10
2	31	64	50	103
3	9	20	6	13
4	7	15	5	11
5	8	18	2	4
6	10	22	7	15
7	3.5	8	0.9	2
8	20	44	30	66
9	20	44	27	59
10	17	38	17	38
11	50	106	>50	>106

oxymethylene bridge between C-3 and C-5. [Endocyclic torsion angles (ω_{ij} ± 0.2–0.4°) about the ring bonds between atoms *i* and *j* are: $\omega_{1,2}$ 44.8, $\omega_{2,3}$ −61.3, $\omega_{3,4}$ 73.8, $\omega_{4,5}$ −72.2, $\omega_{5,10}$ 61.7, $\omega_{10,1}$ −46.2 in ring A; $\omega_{5,6}$ 45.8, $\omega_{6,7}$ −56.5, $\omega_{7,8}$ 65.0, $\omega_{8,9}$ −61.4, $\omega_{9,10}$ 51.9, $\omega_{10,5}$ −44.8 in ring B; $\omega_{8,9}$ 49.3, $\omega_{9,11}$ −49.1, $\omega_{11,12}$ 57.4, $\omega_{12,13}$ −58.3, $\omega_{13,14}$ 54.6, $\omega_{14,8}$ −53.7 in ring C; $\omega_{13,14}$ −66.5, $\omega_{14,15}$ 29.4, $\omega_{15,16}$ 23.4, $\omega_{16,17}$ −39.3, $\omega_{17,18}$ 1.0, $\omega_{18,13}$ 51.0 in ring D; and $\omega_{17,18}$ −7.0, $\omega_{18,19}$ −48.8, $\omega_{19,20}$ 53.3, $\omega_{20,21}$ −1.7, $\omega_{21,22}$ −54.5, $\omega_{22,17}$ 58.6 in ring E; $\omega_{3,4}$

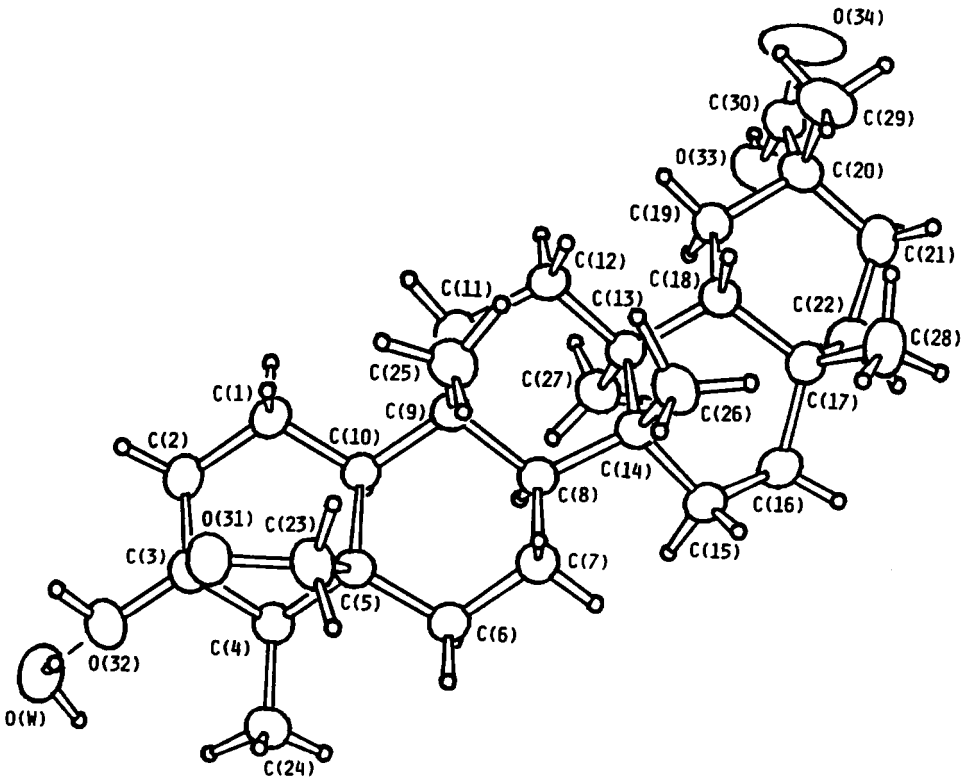

FIGURE 1. ORTEP diagram showing the atom numbering scheme and solid-state conformation of salaspermic acid [**1**] in crystals of the monohydrate; small circles represent hydrogen atoms.

-43.1, $\omega_{4,5}$ 44.9, $\omega_{5,23}$ -32.2, $\omega_{23,31}$ 6.1, $\omega_{31,3}$ 23.8 in the tetrahydrofuran ring.] The tetrahydrofuran ring has an envelope form with C-4 as the out-of-plane atom. In the *cis*-decalin D/E system, ring E approximates to a boat form whereas ring D is distorted towards a 1,3-diplanar form in order to minimize severe α-face transannular interactions between the H-16α and the methyl group at C-13. Thus, the conformation found here constitutes an additional example of the stretched (D and E boat-like) conformation that is more prevalent in the solid state than the folded (D and E deformed chairs) form for friedelane derivatives despite the fact that force-field calculations indicate the latter conformation to be slightly more stable for friedelin (8).

Salaspermic acid [**1**] inhibited HIV replication in H9 lymphocytes with an EC_{50} value of 5 μg/ml (10 μM), and it inhibited uninfected H9 cell growth with a IC_{50} value of 53 μM (Table 1).

Compound **1** also showed an inhibitory effect against HIV-1 recombinant reverse-transcriptase-associated reverse transcriptase activity. Interestingly, this effect appeared to be template-primer dependent, so that different EC_{50} values could be estimated using different template-primers (Table 2). This particular behavior could be due to changes of the conformation of binding position for the inhibitor as the result of the interaction between reverse transcriptase and templates. Unpublished results (E. Tramontano and Y.C. Cheng, unpublished data) showed that also other non-nucleoside-inhibitors, such as TIBO derivatives or BIRG-587, have template-dependent patterns of inhibition, but these were different from the pattern observed in the inhibition by **1**. In fact, the enzyme was more sensitive to **1** having Poly(rC)·oligo(dG) or Poly(rA)·oligo(dT) as template-primer, less sensitive having Poly(rU)·oligo(dA), and almost insensitive, at a drug concentration of 100 μg/ml, using Poly(rG)·oligo(dC) as template-primer.

TABLE 2. Inhibition of HIV-1 Reverse Transcriptase by Salaspermic Acid [**1**].

Associated Activity	Template-primer	EC_{50} (μg/ml)
Reverse Transcriptase	Poly(rA)·oligo(dT)	16
	Poly(rC)·oligo(dG)	15
	Poly(rU)·oligo(dA)	>100[a]
	Poly(rG)·oligo(dC)	>100[b]
DNA Polymerase	Poly(dC)·oligo(dG)	75

[a]63% of control at 100 μg/ml.
[b]96% of control at 100 μg/ml.

Compound **1** revealed an inhibitory effect also against HIV-1 reverse-transcriptase-associated DNA polymerase activity, but in this case only one template-primer was tested. It might be possible that even this enzyme function will show a template-primer dependence. However, comparing the inhibitory effect against HIV-1 reverse-transcriptase-associated reverse transcriptase and DNA polymerase activities using Poly(rC)·oligo(dG) and Poly(dC)·oligo(dG), respectively, as template-primers, compound **1** appeared to have more efficacy against reverse transcriptase activity. On the contrary, compound **1** showed no inhibitory effect upon the HIV-1 reverse-transcriptase-associated RNAse H activity when tested at a concentration of 100 μg/ml with Poly(dT)·[3H]Poly(rA) as substrate.

The behavior of **1** was also tested against HIV-2 recombinant reverse-transcriptase-associated reverse transcriptase activity, but the degree of inhibition appeared to be lower [EC_{50} = 100 μg/ml with Poly (rC)·oligo(dG) as template-primer]. However, to

establish whether the anti-viral action of **1** against HIV-1 in H9 lymphocytes is the result of the HIV-1 reverse transcriptase will require further studies.

In order to investigate structure-activity relationships, the HIV-replication-inhibitory effect of **1**-related compounds, such as **2** (13), **3** (14), **4** (15), **5** (16), **6** (16), **7** (17), **8** (18), **9** (18), **10** (19), and **11** (20), isolated from various plant sources, was evaluated. The results are summarized in Table 1.

Compounds **5** and **7** showed marginal anti-HIV activity, whereas **3**, **4**, and **6** were active only at toxic concentrations. Compound **2** is structurally quite similar to **1**, except for the difference of an additional α-hydroxy group at C-2, and we therefore expected that **2** should show anti-HIV activity. However, it failed to demonstrate an effect against HIV replication at non-toxic concentrations. The inactivity of **8** would suggest that the acetal ring in **1** may be required for the anti-HIV activity. Modification of the C-20 carboxylic acid moiety by replacing it with Me (e.g., **3**, **5**–**7**, **9**, and **10**) or CH_2OH (e.g., **4**) gave rise to less active or more toxic compounds. A five-fold difference between the values of ED_{50} and IC_{50} is considered to be active. Further evaluation for **5** and **7** as anti-HIV agents is in progress.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—The melting point was taken on a Fischer-Johns melting point apparatus and is uncorrected. The ir spectrum was recorded on a Perkin-Elmer 1320 spectrophotometer. The ^{1}H-nmr spectrum was obtained on a Bruker AC 300 nmr spectrometer; all chemical shifts are reported in ppm from TMS. Mass spectral analysis was performed on a VG 70-250 SEQ mass spectrometer. Analytical tlc was carried out on Kieselgel 60F-254 (0.2 mm thickness, Merck). Aldrich Si gel 60 (5–25 μ) was used for cc.

PLANT MATERIAL.—*T. wilfordii* used for this investigation was collected in Fujian Province, China. "Lei-gong-teng duo-dai" (LGTDD) was produced by Hongqi Pharmaceutical Company of Shanghai Medical University. A voucher specimen of *T. wilfordii* is available for inspection at the School of Pharmacy, Shanghai Medical University.

ISOLATION OF SALASPERMIC ACID.—The roots of *T. wilfordii*, after removal from the bark, were extracted with MeOH, and the extract was purified by cc to obtain LGTDD. A solution of LGTDD (10 g) in $CHCl_3$ was flash-chromatographed on Si gel and eluted with hexane-$CHCl_3$ (1:2) to afford 14 fractions (ca. 200 ml per fraction). Fraction 4 was rechromatographed on Si gel employing hexane-Et_2O (1:1) as eluent to yield 5 mg of **1** after recrystallization.

Salaspermic acid [**1**].—Colorless prisms (Me_2CO): mp > 320°; R_f 0.28 [C_6H_6-Me_2CO (5:2)]; hrms calcd for $C_{30}H_{48}O_4$, 472.3552, found *m/z* [M]$^+$ 472.3537; ir (KBr) ν max 3400, 3300, 2650–2500, 1685, 1450, 1380, 1220, 1135, 1050, 995, 875 cm^{-1}; ^{1}H nmr (C_5D_5N) δ 4.20 (1H, d, J = 8.0 Hz), 3.65 (1H, d, J = 8.0 Hz), 2.63 (1H, d, J = 15.0 Hz), 2.48 (1H, br d, J = 14.0 Hz), 2.31 (1H, td, J = 14.0, 4.0 Hz), 2.13 (1H, dd, J = 10.5, 4.0 Hz), 1.38, 1.26, 1.08, 0.86, 0.78 (each 3H, s), 1.21 (3H, d, J = 7 Hz); ^{13}C nmr (C_5D_5N) δ 181.38 (s), 106.03 (s), 73.11 (t), 57.43 (d), 54.02 (d), 47.22 (s), 44.85 (d), 40.76 (s), 39.64 (s), 39.36 (s), 39.20 (t), 37.71 (s), 37.50 (t), 36.76 (t), 34.88 (t), 34.11 (t), 32.33 (q), 32.14 (q), 30.97 (t), 30.55 (t), 30.52 (s), 29.74 (t), 29.58 (t), 20.59 (t), 19.64 (t), 18.13 (q), 16.95 (q), 16.80 (q), 8.59 (q); ms *m/z* [M]$^+$ 472, 454, 426, 395, 370, 318, 302, 289, 287, 259, 249, 235, 207, 203, 189, 150, 125, 109.

X-RAY CRYSTAL STRUCTURE ANALYSIS OF SALASPERMIC ACID MONOHYDRATE [**1**]·H_2O.—*Crystal data*.—$C_{30}H_{48}O_4 \cdot H_2O$, MW = 490.73, monoclinic, space group $P2_1$ (no. 4) from the systematic absences 0*k*0 when *k* is odd and **1** is chiral, a = 7.323 (1) Å, b = 30.128 (2) Å, c = 6.446 (1) Å, β = 113.30 (1)° (from 25 orientation reflections, 40° < θ < 45°), V = 1306.2 (6) Å^3, Z = 2, D_c = 1.248 g·cm^{-3}, μ(CuKα radiation, λ = 1.5418 Å) = 6.2 cm^{-1}; crystal size = 0.16 × 0.20 × 0.30 mm.

Preliminary unit-cell parameters and space group information were obtained from oscillation and Weissenberg photographs. Intensity data (+*h*, +*k*, ±*l*; θ_{max} = 75°, 2733 non-equivalent reflections) were recorded on an Enraf-Nonius CAD-4 diffractometer [CuKα radiation, graphite monochromator; ω–2θ scans, scanwidth (0.80 + 0.14 tan θ)°]. The intensities of four reference reflections, monitored every 2 h during data collection, showed no significant variation (<1%). The data were corrected for the usual Lorentz and polarization effects. A total of 2289 reflections with $I > 3.0\sigma(I)$ were retained for the analysis.

The crystal structure was solved by direct methods (MULTAN11/82). Initial carbon and oxygen

TABLE 3. Non-hydrogen Atom Fractional Coordinates and Equivalent Isotropic Thermal Parameters for Salaspermic Acid [1] Monohydrate, with Estimated Standard Deviations in Parentheses.

Atom	x	y	z	B_{eq} (Å^2)
C-1	0.3997(3)	−0.07959(9)	0.4024(4)	2.92(5)
C-2	0.4490(4)	−0.03933(9)	0.2868(5)	3.13(5)
C-3	0.2663(3)	−0.01054(8)	0.1749(4)	2.51(4)
C-4	0.1020(3)	−0.03641(8)	−0.0067(4)	2.46(4)
C-5	0.0352(3)	−0.06690(8)	0.1458(4)	2.29(4)
C-6	−0.1670(3)	−0.08868(8)	0.0120(4)	2.75(5)
C-7	−0.2170(3)	−0.12708(8)	0.1358(4)	2.80(5)
C-8	−0.0509(3)	−0.16209(7)	0.1994(4)	2.18(4)
C-9	0.1482(3)	−0.14236(8)	0.3737(4)	2.25(4)
C-10	0.2030(3)	−0.10214(8)	0.2541(4)	2.19(4)
C-11	0.3110(3)	−0.17823(8)	0.4246(4)	2.55(4)
C-12	0.2516(3)	−0.22393(8)	0.4820(4)	2.43(4)
C-13	0.0618(3)	−0.24266(7)	0.2943(4)	2.10(4)
C-14	−0.1135(3)	−0.20899(7)	0.2518(4)	2.21(4)
C-15	−0.2974(3)	−0.22526(9)	0.0456(5)	2.95(5)
C-16	−0.3157(4)	−0.27560(9)	0.0047(5)	3.27(5)
C-17	−0.2061(3)	−0.30642(8)	0.2105(4)	2.63(4)
C-18	0.0048(3)	−0.28838(8)	0.3705(4)	2.31(4)
C-19	0.1704(3)	−0.32313(8)	0.4090(4)	2.63(4)
C-20	0.1242(3)	−0.36995(8)	0.4751(4)	2.79(5)
C-21	−0.0806(4)	−0.38616(9)	0.2983(5)	3.48(6)
C-22	−0.1822(4)	−0.35177(9)	0.1146(5)	3.19(5)
C-23	0.0293(4)	−0.03196(9)	0.3169(4)	2.93(5)
C-24	−0.0594(4)	−0.00632(9)	−0.1670(5)	3.56(6)
C-25	0.1408(4)	−0.12876(10)	0.6010(4)	3.13(5)
C-26	−0.1729(3)	−0.20597(9)	0.4565(4)	2.90(4)
C-27	0.1078(3)	−0.24887(9)	0.0810(4)	2.76(4)
C-28	−0.3427(4)	−0.31332(10)	0.3399(5)	3.93(6)
C-29	0.1294(5)	−0.36792(12)	0.7136(5)	4.41(7)
C-30	0.2826(4)	−0.40167(8)	0.4679(5)	3.04(5)
O-31	0.1826(3)	0.00000(−)[a]	0.3390(3)	3.08(3)
O-32	0.3087(3)	0.02940(6)	0.0919(3)	3.34(4)
O-33	0.3035(3)	−0.40248(8)	0.2750(4)	4.12(4)
O-34	0.3727(4)	−0.42630(10)	0.6219(5)	7.01(7)
O-W	0.4600(3)	0.03276(8)	−0.2506(5)	4.14(4)

[a]The *y* coordinate of O-31 was held constant throughout the analysis to define the space group origin in this direction.

atom coordinates were derived from an *E*-map. A series of difference Fourier syntheses, evaluated following several rounds of full-matrix least-squares adjustment of positional and thermal parameters for these atoms (at first isotropic, then anisotropic), yielded hydrogen atom positions. With the inclusion of hydrogen atom positional and isotropic thermal parameters, and latterly an extinction correction (g), as variables in the subsequent least-squares iterations, the refinement converged (max, shift, ESD = 0.03) at $R = \Sigma||F_o| - |F_c||/\Sigma|F_o| = 0.036$, $R_w = [\Sigma w(|F_o| - |F_c|)^2/\Sigma w|F_o|^2]^{1/2} = 0.048$, $g = 2.4(4) \times 10^{-6}$, $GOF = [\Sigma w(|F_o| - |F_c|)^2/(N_{observations}-N_{parameters})]^{1/2} = 1.12$. A final difference Fourier synthesis contained no unusual features (max. $\Delta\rho = 0.19$ e/Å^3).

Crystallographic calculations were performed on PDP11/44 and MicroVAX computers by use of the Enraf-Nonius Structure Determination Package (SDP). For structure-factor calculations, neutral atom scattering factors and their anomalous dispersion corrections were taken from the literature (9). During the least-squares iterations, $\Sigma w\Delta^2[w = 1/\sigma^2(|F_o|), \Delta = (|F_o| - |F_c|)]$ was minimized.

HIV INHIBITION ASSAY.—Inhibition assays were conducted as previously described (10). H9 lymphocytes (3.5×10^6 cells/ml) were incubated both in the presence and absence of HIV-1 (HTLV-IIIB, 0.01-0.1 $TCID_{50}$/cell) for 1 h at 37°. Cells were washed thoroughly to remove unadsorbed virions and resuspended at 4×10^5 cells/ml in the culture medium. Aliquots were placed in the wells of 24-well culture

plates containing an equal volume of test compound (diluted in the culture medium). After incubation of 3 days at 37°, the cell density of uninfected cultures was determined by counting cells in a Coulter counter to assess toxicity of the test compound. A p24 antigen capture assay was used to determine the level of virus released in the medium of the HIV-infected cultures. The medium effective (EC_{50}) and inhibitory (IC_{50}) concentrations (for anti-HIV activity and cytotoxicity, respectively) were estimated graphically. The percent inhibition (for infected and uninfected cultures) was plotted versus concentration for each compound, and the 50% inhibition value was read from the graph.

PREPARATION OF ENZYME.—HIV-1 reverse transcriptase was purified using *Escherichia coli* JM 109 containing pKRT 2 kindly provided by Summers (Yale University) (11). HIV-2 reverse transcriptase was purified using *E. coli* bearing the plasmid pASRT 2 purchased from Boehringer Ingelheim.

ENZYME ASSAY.—All reactions were developed in a total volume of 50 μl containing 50 mM Tris HCl pH 7.8, 2 mM $MgCl_2$, 100 μg/ml nuclease-free BSA, 1 mM dithiothreitol, 0.5 $O.D._{260}$ unit/ml of template-primer, and 330 nM of [^{3}H]dNTP according to Cheng *et al.* (12).

ACKNOWLEDGMENTS

This investigation was supported by grant A1-25697 from the National Institute of Allergies and Infectious Diseases awarded to K.H. Lee, Y.C. Cheng, and A.J. Bodner.

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Received 29 July 1991