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ANTI-AIDS AGENTS, 1.[1] ISOLATION AND CHARACTERIZATION OF FOUR NEW TETRAGALLOYLQUINIC ACIDS AS A NEW CLASS OF HIV REVERSE TRANSCRIPTASE INHIBITORS FROM TANNIC ACID

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ABSTRACT.—Four new tetragalloylquinic acids, 3,5-di-*O*-galloyl-4-*O*-digalloylquinic acid [**2**], 3,4-di-*O*-galloyl-5-*O*-digalloylquinic acid [**3**], 3-*O*-digalloyl-4,5-di-*O*-galloylquinic acid [**4**], and 1,3,4,5-tetra-*O*-galloylquinic acid [**5**], were isolated and characterized from a commercial tannic acid as a new class of human immunodeficiency virus (HIV) reverse transcriptase (RT) inhibitor. Compounds **2**, **3**, and **4** inhibit HIV RT activity 90, 89, and 84% at 100 μM and 73, 70, and 63% at 30 μM, respectively. Compounds **2–5** also inhibit the HIV growth in cells in the range of 61–70% with low cytotoxicity at 25 μM. The HIV cell growth inhibitory effects of these compounds at 25 μM and 6.25 μM (44–57%) are comparable to their effects against the HIV RT at 30 μM and 10 μM, respectively. The inhibitory effect of **3** against DNA polymerases indicates that the selective antiviral action of **3** is determined by more than its action with HIV RT.

Since the discovery of human immunodeficiency virus [HIV (HTLV-III/LAV)], much progress has been made in elucidating the genomic structure of HIV as well as the mechanism of HIV infection (1–3). The fact that the reverse transcriptase (RT) plays a very important role in controlling the replication of the HIV makes RT one of the most attractive targets in the development of anti-AIDS (acquired immunodeficiency syndrome) drugs. Some of the RT inhibitors include suramin, Evans blue, aurintricarboxylic acid, phosphonoformate (PFA), and 3′-azido-2′,3′-dideoxythymidine triphosphate (AZTTP), 2′,3′-dideoxycytidine triphosphate (DDCTP), ribavirin, and HPA-23 (2,4). Because some of these inhibitors, e.g., suramin, PFA, AZTTP, and DDCTP have value or potential value in the treatment of AIDS patients and their selective antiviral action was suggested to be associated with the unique behavior of RT (2), the use of RT as a prime target in the detection of chemotherapeutic agents against AIDS is certainly a logical approach.

In searching for natural products as potential anti-AIDS agents, the tannic acids were found to show potent inhibitory activity against HIV RT. Among the tannic acids investigated, which were obtained either from a commercial source or extracts prepared from both Turkish and Chinese galls (Table 1), the one from the commercial source showed the strongest inhibitory effect against HIV RT (74% at 100 μg/ml) (Table 1). Subsequent bioassay-directed fractionation of this tannic acid mixture resulted in the isolation of four new tetragalloylquinic acids. In this paper, we describe the isolation, characterization, and anti-AIDS effect of these new compounds.

RESULTS AND DISCUSSION

The EtOAc-soluble portion of tannic acid was fractionated using a column of Sephadex LH-20 to yield seven fractions. These fractions were separated according to

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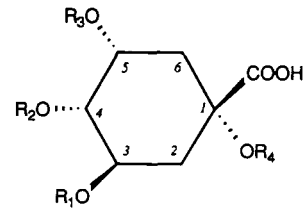
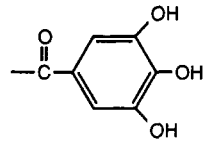


	R_1	R_2	R_3	R_4
1	galloyl	galloyl	galloyl	H
2	galloyl	digalloyl	galloyl	H
3	galloyl	galloyl	digalloyl	H
4	digalloyl	galloyl	galloyl	H
5	galloyl	galloyl	galloyl	galloyl

galloyl:



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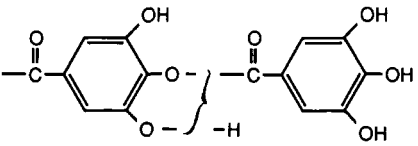

the degree of galloylation (5) and were composed of mono- to octagalloylated compounds. Among these, fraction G-4, which contained tetragalloylated compounds, showed the strongest inhibitory effect against HIV RT (Table 2). Subsequent repeated preparative hplc on reversed-phase columns yielded active compounds **2**, **3**, **4**, and **5**. Compound **1** was isolated from the less active fraction G-3.

A comparison of the physical constants and the ^{1}H- and ^{13}C-nmr spectra indicated that **1** is 3,4,5-tri-*O*-galloylquinic acid (6,7). The ^{1}H-nmr spectra of **2**, **3**, and **4** showed the signals from a depside galloyl group (doublets at δ 7.34 and 7.36 for **2**, 7.43 and 7.50 for **3**, and 7.35 and 7.42 for **4**) along with the singlets of galloyl groups and the signals of a 3,4,5-triacylated quinic acid moiety. The ^{13}C-nmr spectra of **2**, **3**, and **4** also indicated the presence of a depside galloyl group (signals at δ 114.7, 117.6, 143.7, and 151.2 for **2**, 114.7, 117.5, 143.2, and 151.2 for **3**, and 114.6, 117.5, 143.6, and 151.2 for **4**) (Table 3). After partial hydrolysis in boiling H_2O (8,9), **2**, **3**, and **4** gave gallic acid and 3,4,5-tri-*O*-galloylquinic acid [**1**], which were identified by

TABLE 1. Inhibitory Effect of Tannic Acids Against HIV Reverse Transcriptase.

Tannic Acid	Inhibition at 100 μg/ml (%)[a]
Commercial source	74 ± 7
Turkish galls extract	7 ± 8
Chinese galls extract	10 ± 14

[a]Positive control: AZT triphosphate at 100 nM 64 ± 6%, phosphonoformate 0.6 μM 63 ± 9%.

TABLE 2. Inhibitory Effect of the Fractions from Tannic Acids Against HIV Reverse Transcriptase.

Fraction	Inhibition at 100 μg/ml (%)[a]
G-1	0
G-2	10 ± 15
G-3	49 ± 4
G-4	83 ± 2
G-5	68 ± 5
G-6	56 ± 11
G-7	49 ± 17
G-8	48 ± 14

[a] Positive control: AZT triphosphate 100 nM 64 ± 6%, phosphonoformate 0.6 μM 63 ± 9%.

hplc. Therefore, **2**, **3**, and **4**, have a core structure of **1** with a depside galloyl group. The position of the depside galloyl group on **2**, **3**, and **4** was determined by comparison of their chemical shifts of the hydroxymethine carbons of quinic acid and moieties with those of **1**. In the spectrum of **2**, the signal due to C-4 of the quinic acid moiety was observed to be 0.4 ppm downfield shifted (Table 3) while those due to C-3 and C-5 of the same moiety remained unchanged. This implies that the depside galloyl group of **2** is attached to the galloyl group at the C-4 position (8,9). In the case of **3** and **5**, the signals due to C-5 and C-3 positions were observed to be 0.4 and 0.3 ppm downfield shifted, respectively, compared to those of **1**. Therefore, the structures of **2**, **3**, and **4** were characterized as the new 3,5-di-*O*-galloyl-4-*O*-digalloylquinic acid, 3,4-di-*O*-galloyl-5-*O*-digalloylquinic acid, and 3-*O*-digalloyl-4,5-di-*O*-galloylquinic acid, respectively.

The ^{1}H-nmr spectrum of **5** showed three acylated hydroxymethine protons due to a quinic acid moiety and four singlets of a galloyl group (δ 7.02, 7.07, 7.13, and 7.17). The ^{13}C-nmr spectrum also indicated that **5** is a tetragalloylquinic acid. There was no signal from the depside galloyl group in the ^{1}H- and ^{13}C-nmr spectra. Therefore, **5** is characterized as 1,3,4,5-tetra-*O*-galloylquinic acid.

There are many galloylquinic acids reported in the literature (6,7,10). Compounds **2**, **3**, and **4**, however, are the first examples of galloylquinic acids with depside galloyl groups.

The effects of **1** (3,4,5-GQA), **2** (3,5-G-4-diGQA), **3** (3,4-G-5-diGQA), **4** (3-diG-4,5-GQA), and **5** (1,3,4,5-GQA) against HIV RT isolated from infected cells and HIV-infected H9 lymphocytes are summarized in Table 4. Compounds **2**, **3**, **4**, and **5** showed 90, 89, 84, and 94% inhibitory activity, respectively, against HIV RT at 100 μM. Compounds **2**, **3**, and **4** exibited more than 50% inhibitory activity at 10 μM, and there was no significant difference in inhibitory activities among these three tetragalloylquinic acids. The reaction is linear with time with or without the presence of 6 μM of compound **3**. This suggested that these compounds acted as inhibitors rather than inactivators. The inhibitory activity of **1** was shown to be less than that of **2**, **3**, or **4**. This indicated that the depside galloyl group in the molecule of **2**, **3**, and **4** plays an important role in their inhibitory effects. In view of the difference of the dose response between **4** and **5**, the mode of inhibition of these two compounds could be different. This requires further investigation.

These compounds **1**–**5** also exhibited inhibitory activity against the growth of HIV in infected H9 lymphocytes (61–70% inhibition at 25 μM). At this level, these gal-

TABLE 3. ^{13}C-nmr Data of Compounds **1**–**5**.[a]

Moiety	Compound				
	1	**2**	**3**	**4**	**5**
Quinic acid moiety					
C-1	74.4	74.3	74.2	74.3	79.8
C-2	38.6	38.6	38.7	38.6	37.3
C-3	68.9	68.9	68.8	69.2(+0.3)	68.6
C-4	72.3	72.7(+0.4)	72.4	72.4	71.9
C-5	69.1	69.7	70.1(+0.4)	69.6	69.4
C-6	36.5	36.5	36.4	36.5	33.3
COOH	175.9	176.0	176.0	175.8	172.2
Gallic acid moiety					
C-2	121.2	120.7	120.5	120.6	121.2(2C)
	121.3	121.2	121.0	121.1	121.3(2C)
	121.9	121.4	121.3	121.3	
		121.9	121.8	121.9	
C-2,6	110.0(4C)	109.9	109.8	109.9	110.1(6C)
	110.2(2C)	110.0	110.0	110.0	110.3(2C)
		110.2	110.45	110.1	
		110.7	110.52	110.6	
		114.7	114.7	114.6	
		117.6	117.5	117.5	
C-3,5	145.9(6C)	143.7	143.2	143.6	145.6
		145.8	145.6	145.8	145.81
		145.9	145.8	145.95	145.85
		146.0	145.9	146.04	146.1
		146.7	146.5	146.8	
		151.2	151.2	151.2	
C-4	138.8	138.7	138.6	138.7	138.6
	138.9(2C)	138.9	139.7	138.9	138.9
		139.2	139.0	139.2	139.0
		139.4	139.2	139.4	139.1
		139.8	139.5	139.7	
-COO-	165.9	164.2	164.7	164.1	165.88
	166.0	164.9	164.8	164.7	166.93
	166.1	165.5	165.3	165.3	166.0
		165.6	165.5	165.97	166.2
		165.9	165.7	166.93	
		166.1	165.8		

[a]All spectra were measured in Me_2CO-d_6 using TMS as an internal standard.

loylquinic acids showed little cytotoxicity against the uninfected H9 cells (0-25%), and no cytotoxicity was observed at 6.25 and 1.25 μM. The growth of HIV was inhibited even at 6.25 μM (44-59%) and 1.25 μM (13–34%). It is interesting to note that the inhibition of HIV RT at 30 μM and HIV growth at 25 μM by **2**, **3**, **4**, was found to show good correlation. Good correlation was also found between HIV RT inhibition at 10 μM and HIV growth at 6.25 μM. Whether the anti-RT activity and anti-HIV activity are two independent events or this class of compounds inhibits HIV replication by acting on sites other than RT is not clear.

The inhibitory effect of **3** against DNA polymerase α, β, and γ, as well as HIV-RT, is shown in Figure 1. The sensitivity of these DNA polymerases against **3** was found to be quite different. DNA polymerase α is most sensitive to **3**. The ID_{50} of **3** for DNA polymerase α ($ID_{50} = 0.065 \pm 0.019$ μM) is about 1/450 of that for HIV RT

TABLE 4. Effect of Galloylquinic Acids on HIV Cell Growth and HIV Reverse Transcriptase (RT) Activity

Compound	HIV RT Activity[a] (% Inhibition)			HIV growth[b] (% Inhibition)		
	100 μM	30 μM	10 μM	25 μM[c]	6.25 μM	1.25 μM
3,4,5-TriGQA [**1**]	58 ± 6	48 ± 11	39 ± 10	70 ± 1[a] (14)[d]	59 ± 8	26 ± 1
3,5,-G-4-diGQA [**2**]	90 ± 3	73 ± 5	53 ± 3	71 + 1 (14)	57 ± 2	34 ± 5
3,4,-G-5-diGQA [**3**]	89 ± 5	70 ± 9	55 ± 11	63 ± 1 (0)	50 ± 3	28 ± 2
3-DiG-4,5-GQA [**4**]	84 ± 7	63 ± 6	50 ± 11	61 ± 1 (25)	44 ± 4	13 ± 2
1,3,4,5-TetraGQA [**5**] . . .	94 ± 2	84 ± 7	36 ± 6	66 ± 2 (25)	52 ± 1	15 ± 2

[a]The reverse transcriptase assay was described in the Experimental section.

[b]Growth inhibition assay was carried out according to procedure described in the Experimental section.

[c]The μM concentration of **1** was 30.8, 7.7, and 1.5 μM, respectively.

[d]The figures in parentheses indicate percent inhibition of uninfected H-9 cell growth in the presence of drug.

($ID_{50} = 29 \pm 18$ μM), and the sensitivities are in this order: DNA polymerase $\alpha > \gamma$ ($ID_{50} = 2.5 \pm 0.9$ μM) $> \beta$ (21 ± 11 μM) = HIV RT. However, compound **3** was much more potent against HIV growth than against cell growth. These results imply that there are other factors in uninfected cells which render them resistant to these compound. This is under current investigation.

Compounds **2**, **3**, and **4** appear to be the first examples of plant products that demonstrate potent inhibition of both HIV RT activity and HIV growth in culture. The structures of these compounds are unique compared to those of the other known HIV RT inhibitors. These compounds deserve further development as potential anti-AIDS drugs.

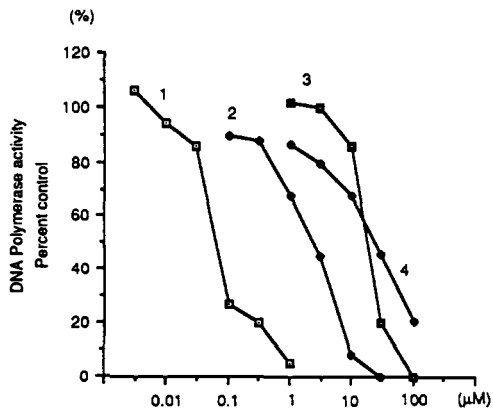

FIGURE 1. Inhibitory activity of compound **3** on DNA polymerase α, β, and γ and HIV reverse transcriptase: 1 = DNA polymerase α, 2 = DNA polymerase γ, 3 = DNA polymerase β, 4 = HIV RT.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—^{1}H- and ^{13}C-nmr spectra were measured on a JEOL JNM-GX400 spectrometer using TMS as an internal standard. Specific rotation was determined on a AUTOPOL (Rudolph Research) polarimeter. Hplc was performed on a Waters model 6000A chromatograph equipped with a model 440 absorbance detector, a U6K injecter, and a TSK-ODS 80Tm (4.6 mm i.d. × 150 mm, Toyo Soda) column for the reversed-phase hplc [mobile phase, MeCN-H_2O-HCO_2H (15:85:1)]; and a Porasil (3.9 mm i.d. × 300 mm, Waters) for the normal phase hplc [mobile phase, *n*-hexane–MeOH–THF–HCO_2H (55:33:11:1) containing oxalic acid 1 g/liter (5). Preparative hplc was performed on a Waters model 501 solvent delivery system using a μBondapak column (19 mm i.d. × 150 mm) and a Waters differential refractometer, which utilized a solvent system of MeCN-H_2O-HCO_2H (20:80:0.5, 15:85:0.5, and 13:87:0.5). Low pressure chromatography was performed with an FMI pump utilizing a Fuji-gel RQ-3 column.

ISOLATION.—The tannic acid purchased from Aldrich Chemical Company, Milwaukee, Wisconsin (lot no. 05720KM, 50 g) was partitioned with EtOAc and H_2O. The EtOAc layer (47.8 g) was chromatographed on a Sephadex LH-20 (4.5 mm i.d. × 45 cm) column, eluted with EtOH, EtOH/H_2O (increasing H_2O content of 10%, 20%, 30%, and 40%) and then EtOH/H_2O/Me_2CO (5). Each fraction (50–100 ml) was analyzed by normal phase hplc. Repeated chromatography yielded fractions containing mono- to heptagalloylated compounds. The lack of substantial increase of the specific anti-HIV RT in the peak fraction could be due to the presence of activity in most of the fractions isolated. The fraction that contained tetragalloyl compounds (G-4, 2.69 g) was further fractionated by low pressure chromatography on a Fuji gel RQ-3 column and preparative hplc to yield compounds **2** (48 mg), **3** (35 mg), **4** (30 mg), and **5** (48 mg) as amorphous powders. Compound **1** (186 mg) was isolated from the fraction containing trigalloyl moieties as amorphous powders by the same methods described above.

3,4,5-TRI-*O*-GALLOYLQUINIC ACID [**1**].—An amorphous powder: $[\alpha]_D$ −124° (c = 0.20, Me_2CO); ^{1}H-nmr (Me_2CO-d_6) δ 2.33 (1H, dd, J = 6.5 and 14.3 Hz, H-6), 2.44 (2H, m, H-2, -2′), 2.58 (1H, dd, J = 3.9 and 14.3 Hz, H-6′), 5.52 (1H, dd, J = 3.3 and 8.3 Hz, H-4), 5.81 (1H, m, W½ = 15 Hz, H-5), 5.83 (1H, m, W½ = 25 Hz, H-3), 7.06 (2H, s, galloyl), 7.09 (2H, s, galloyl), 7.17 (2H, s, galloyl); ^{13}C nmr see Table 3.

3,5-DI-*O*-GALLOYL-4-*O*-DIGALLOYLQUINIC ACID [**2**].—An amorphous powder: $[\alpha]_D$ −91° (c = 0.53, Me_2CO); uv (MeOH) λ max nm (log ε) 277 (4.54); ir (Nujol) ν max cm^{-1} 3150–3400 (OH), 1690–1705 (ester), 1600, 1535 (aromatic); ^{1}H-nmr (Me_2CO-d_6) δ 2.34 (1H, dd, J = 6.2 and 14.4 Hz, H-6), 2.43 (2H, m, H-2, -2′), 2.57 (1H, br d, H-6′), 5.54 (1H, dd, J = 3.0 and 8.4 Hz, H-4), 5.84 (2H, m, H-3, -5), 7.09 (s)*,[4] 7.17 (s)*, 7.24 (s)*, 7.34 (d, J = 2.0 Hz)*, 7.36 (d, J = 2.0 Hz)*, 7.10 (s)**,[4] 7.11 (s)**, 7.18 (s)** and 7.23 (s)**; ^{13}C nmr see Table 3. Calcd for $C_{35}H_{28}O_{22}\cdot H_2O\cdot EtOH$, C 51.40, H 4.20; found C 51.30, H 4.51%.

3,4-DI-*O*-GALLOYL-5-*O*-DIGALLOYLQUINIC ACID [**3**].—An amorphous powder: $[\alpha]_D$ −92° (c = 0.60, Me_2CO); uv (MeOH) λ max nm (log ε) 277 (4.54); ir (Nujol) ν max cm^{-1} 3150–3400, 1690–1750, 1600, 1535; ^{1}H nmr (Me_2CO-d_6) δ 2.33–2.45 (3H, m, H-2, -2′, 6), 2.58 (1H, dd, J = 3.5 and 14.5, H-6′), 5.52 (1H, dd, J = 3.5 and 8.7 Hz, H-4), 5.83 (1H, m, W½ = 15 Hz, H-5), 5.86 (1H, m, W½ = 25 Hz, H-3), 7.05 (s)*,[4] 7.07 (s)*, 7.28 (s)*, 7.43 (d, J = 2.0 Hz)*, 7.50 (d, J = 2.0 Hz)*, 7.05 (s)**,[4] 7.06 (s)**, 7.09 (s)**, 7.29 (s)**; ^{13}C-nmr see Table 3. Calcd for $C_{35}H_{28}O_{22}\cdot H_2O\cdot EtOH$, C 51.40, H 4.20; found C 51.62, H 4.42%.

3-*O*-DIGALLOYL-4,5-DI-*O*-GALLOYLQUINIC ACID [**4**].—An amorphous powder: $[\alpha]_D$ −106° (c = 0.48, Me_2CO); uv (MeOH) λ max nm (log ε) 277 (4.56); ir (Nujol) ν max cm^{-1} 3150–3400, 1690–1705, 1600, 1535; ^{1}H nmr (Me_2CO-d_6) δ 2.34 (1H, dd, J = 6.1 and 14.2 Hz, H-6), 2.40–2.48 (3H, m, H-2, -2′, -6′), 5.52 (1H, dd, J = 3.3 and 8.6 Hz, H-4), 5.81 (1H, m, W½ = 15 Hz, H-5), 5.86 (1H, m, W½ = 25 Hz, H-5), 7.04 (s)*, 7.17 (s)*, 7.26 (s)*, 7.35 (d, J = 2.0 Hz)*, 7.42 (d, J = 2.0 Hz)*, 7.06 (s)**,[4] 7.15 (s)**, 7.24 (s)**, 7.28 (s)**; ^{13}C nmr see Table 3. Calcd for $C_{35}H_{28}O_{22}\cdot H_2O\cdot EtOH$, C 51.40, H 4.20; found C 51.42, H 4.34%.

1,3,4,5-TETRA-*O*-GALLOYLQUINIC ACID [**5**].—An amorphous powder: $[\alpha]_D$ −72° (c = 0.20, Me_2CO); uv (MeOH) λ max nm (log ε) 277 (4.42), 296 (sh) (4.42); ir (Nujol) ν max cm^{-1} 3200–3400, 1690–1705, 1600, 1530; ^{1}H-nmr (Me_2CO-d_6) 2.47 (1H, dd, J = 7.8 and 13.7 Hz, H-6), 2.85–3.00 (3H, m, H-2, -6′), 5.61 (1H, dd, J = 3.5 and 8.6 Hz, H-4), 5.95 (1H, m, W½ = 15 Hz, H-5), 5.88

[4]* signals due to *m*-digalloyl group, ** signals due to *p*-digalloyl group.

(1H, m, W½ = 25 Hz, H-3), 7.06 (2H, s, galloyl), 7.07 (2H, s, galloyl), 7.13 (2H, s, galloyl), 7.17 (2H, s, galloyl), ^{13}C-nmr see Table 3. Calcd for $C_{35}H_{28}O_{22}\cdot 2H_2O$, C 50.24, H 3.86; found C 50.44, H 3.94%.

HIV RT ASSAY.—The HIV RT assay was performed according to the method described by Cheng *et al.* (4). The immuno-affinity purified enzyme from virions used was isolated from extracts of human T cells infected with HIV lymphotropic virus. Poly rA oligo dT_{10} (Pharmacia, Piscataway, New Jersey) was used as the template to measure the incorporation of [^{3}H] dTMP (20 μM). The percentage of inhibition was determined by comparing the RT activity of drug-containing assay to that of the drug-free control.

HIV GROWTH INHIBITION ASSAY.—This assay was performed by incubation of H9 lymphocytes (1×10^7 cells/ml) in the presence or absence of HIV-1 (HTLV-IIIB) for 1 h at 37°. Cells were washed thoroughly to remove unadsorbed virions and resuspended at 4×10^5 cells/ml in culture medium. Aliquots (1 ml) were placed in wells of 24-well culture plates containing an equal volume of test compound (diluted in test medium). After incubation for 3 days at 37°, cell density of uninfected cultures was determined to assess toxicity of the test compound. A p24 antigen capture assay was used to determine the level of HIV infection in HIV-treated cultures. The ability of test compounds to inhibit HIV replication was measured at four different concentrations of test compound relative to infected, uninfected cultures. Test compounds were considered to be active if p24 levels were less than 70% of infected, untreated cultures.

DNA POLYMERASE ASSAY.—Human DNA polymerases α (11), β (12), and γ (12) were purified as described previously (11). DNA polymerase α activity was assayed in 50 μl of reaction mixture containing 50 mM Tris buffer (pH 8.0), 1 mg/ml of bovine serum albumin, 6 mM $MgCl_2$, 1 mM dithiothreitol, 100 μg/ml of gapped duplex DNA (13), 10 μM [^{3}H]TTP (1 Ci/mmol), 50 μM each of dATP, dCTP and dGTP, and approximately 0.01 activity units of DNA polymerase. After incubation at 37° for 1 h, the DNA was precipated onto glass fiber filters with a 5% trichloroacetic acid/10 mM pyrophosphate solution and counted for radioactivity as previously described (11). DNA polymerases β and γ and HIV RT were assayed in the same manner except that 100 mM KCl was included in the reaction mixture.

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