Park (Bastrop, TX). BDF1 mice weighing 18–20 g were purchased from Charles River Breeding Laboratories, Inc. (Wilmington, MA).

In vivo antitumor activity was assessed against L1210/0 and L1210/cisplatin leukemia. Groups of six B6D2F1 mice weighing 20–25 g were inoculated with 10^6 cells (ip) on day 0. Treatment started on day 1 using the same route used for tumor inoculation. Two different treatment schedules were used: a single dose on day 1 or once daily doses on days 1, 5, and 9. The following doses were administered 12.5, 25, and 50 mg/kg. The optimal dose was defined as the dose that resulted in a higher % T/C without causing toxic deaths. Toxic deaths were defined as those deaths occurring during the first 10 days in animals without ascites or liver involvement by tumor at autopsy.

In the experiments performed using the L1210/cisplatin cell line, 10^6 cells were inoculated ip on day 0. Treatment was given (ip) on days 1, 5, and 9. Results were expressed as median survival of treatment animals divided by median survival of control animals multiplied by 100 (% T/C). All experiments were designed to be terminated on day 60. Long term survivors were rarely seen. They were not excluded from the calculations of % T/C since we used the median survival time rather than the mean survival time.

The optimal doses of the different drugs were used in all antitumor activity studies. For L-Pt complexes the optimal doses for single injection on day 1 are in the range of 25–50 mg/kg, and for cisplatin, is 10 mg/kg. Results presented are the mean of three experiments in each case.

For days 1, 5, and 9 the optimal doses for L-Pt 1, 7, and 9 are 25 mg/kg and for cisplatin is 5 mg/kg. Results presented are the average of two experiments in each case.

The free platinum complexes could not be used as controls in the non-liposomal form due to their lack of aqueous solubility.

Subacute Toxicity Studies. Groups of six to eight CD1 Swiss mice weighing 20–25 g each received iv doses of the L-Pt complexes. Animals were observed, and deaths were recorded on a daily basis. The LD_{50} dose was calculated from the curve obtained by plotting the logarithm of the dose and the % survival on day 15.

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Registry No. 1, 130197-73-8; 2, 130272-75-2; 3, 130197-74-9; 4, 130272-76-3; 5, 130197-75-0; 7, 130197-76-1; 8, 130272-77-4; 9, 130197-77-2; 10, 130272-78-5; L-NDDP, 114488-24-3; K_2PtCl_4, 10025-99-7; cisplatin, 15663-27-1; dach, 694-83-7; dichloro(dach)platinum(II), 61848-66-6; aqua(dach)sulfatoplatinum(II), 123122-57-6; sodium 2,2-dimethyloctanoate, 68298-98-6.

Preparation and Anti-HIV Activities of Aurintricarboxylic Acid Fractions and Analogues: Direct Correlation of Antiviral Potency with Molecular Weight

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Aurintricarboxylic acid (ATA) was fractionated by a combination of dialysis, ultrafiltration, and gel permeation chromatography. The number average and weight average molecular weights of the ATA fractions were determined by the universal calibration method. The sulfonic acid analogue of ATA was prepared and separated in high and low molecular weight fractions. The phosphonic acid analogue of ATA was also synthesized. All of the ATA fractions were tested for prevention of the cytopathic effect of HIV-1 and HIV-2 in MT-4 cell culture as well as against HIV-1 in CEM cell culture. The abilities of the fractions and analogues to inhibit syncytium formation between HIV-1- and HIV-2-infected HUT-78 cells and uninfected MOLT-4 cells were evaluated. In addition, the fractions and analogues were tested for cytotoxicity in mock-infected MT-4 cells, prevention of the binding of the OKT4A monoclonal antibody to the CD4 receptor, inhibition of the binding of anti-gp120 monoclonal antibody to gp120, inhibition of attachment of HIV-1 virions to MT-4 cells, and inhibition of HIV-1 reverse transcriptase. In all of these assays except cytotoxicity, there was a correlation of potency with molecular weight. The higher the molecular weight, the higher the activity. Several of the lower molecular weight fractions of ATA, which bound to gp120 but not to CD4, prevented HIV-1 and HIV-2 cytopathicity. A similar profile was observed for the phosphonic acid analogue of ATA and the lower molecular weight fraction of the sulfonic acid analogue. The results on the ATA fractions indicate that the binding of ATA to gp120 in the absence of CD4 binding is sufficient for anti-HIV activity. The active compounds bind more avidly to gp120 than to CD4. The anti-HIV activity of the ATA fractions is due to inhibition of virus binding due to an interference with the gp120-CD4 interaction.

Aurintricarboxylic acid (ATA) is a heterogeneous mixture of polymers that forms when salicylic acid is treated with formaldehyde, sulfuric acid, and sodium nitrite.[1-3] ATA is often incorrectly represented as a triphenylmethane dye 1 rather than as a polymer as schematically portrayed in structure 2. Recent interest in ATA has resulted from the finding that ATA inhibits the cytopathic effect of HIV-1 in ATH8, MT-4, and HUT-78 cell cultures.[4,5] ATA selectively prevents the binding of the OKT4A/Leu-3a monoclonal antibody to the CD4 receptor and it inhibits the attachment of HIV-1 particles to MT-4 cells.[6] ATA also prevents the staining of membrane-bound gp120 by a monoclonal antibody against it.[7]

The inhibition of protein nucleic acid interactions by ATA in cell-free systems has been known for a long time. The evidence indicates that ATA binds to the nucleotide

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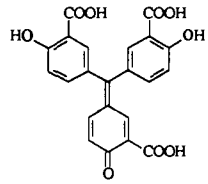

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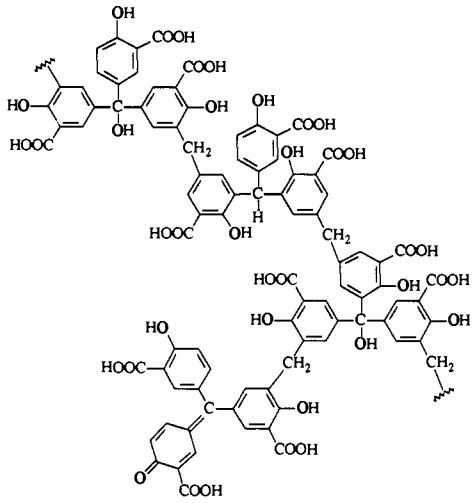

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binding sites of a wide variety of enzymes that process nucleotides, including DNA[8-10] and RNA[10-14] polymerases, reverse transcriptase,[5,15] aminoacyl-*t*RNA synthetase,[16] ribonucleotide reductases,[17] and ribonucleases.[13,18-22] ATA also blocks the attachment of mRNA to ribosomes in cell-free systems,[23-25] and it binds to the polynucleotide domains of the dihydroxyvitamine D_3[26] and glucocorticoid receptors.[27]

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Scheme I. Flow Chart of ATA Ammonium Salt Fractionation

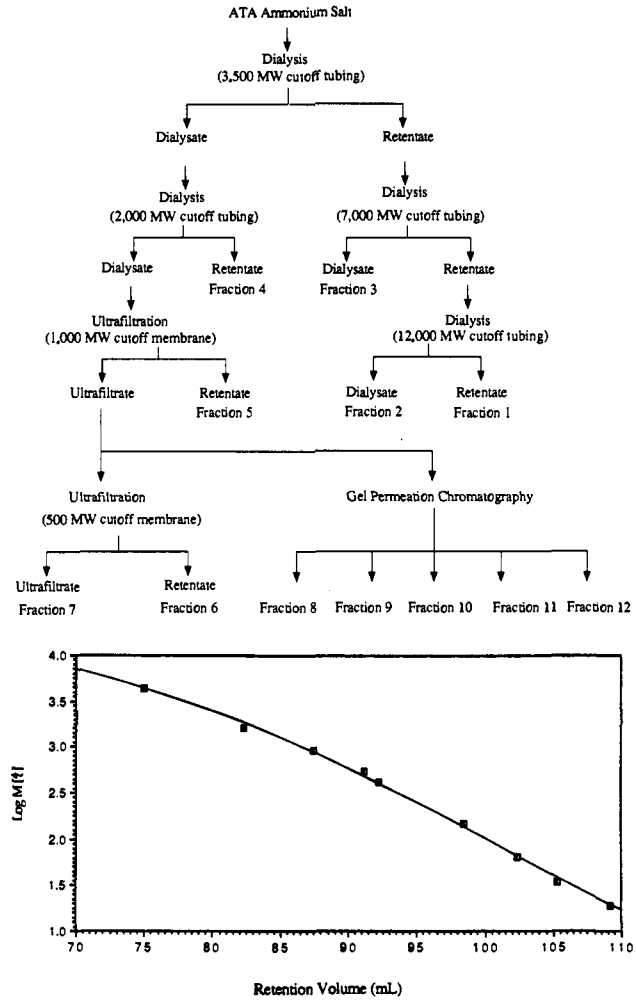

Figure 1. Universal calibration curve obtained from plotting the products $M[\eta]$ of polystyrene standards having molecular weights of 950, 1250, 1700, 2450, 4136, 5050, 7000, 11 600, and 22 000 vs retention volumes on a 2 × 50 cm Shodex HF-2003 semipreparative GPC column with an exclusion limit of 70 000 daltons and 12 000 theoretical plates.

In a prior study of the relationship between the average molecular weights of ATA fractions and their abilities to inhibit protein nucleic acid interactions, it was found that there was a direct correlation of molecular weight with potency in preventing DNA–poly-L-lysine complex formation.[2] A direct correlation was also observed between average molecular weights and ability of ATA fractions to inhibit both polyribouridylic acid binding to the ribosomal S1 protein and polyribouridylic acid binding to 70S ribosomes.[2] These facts raise the important question of whether or not there would be a correlation between the average molecular weights of ATA fractions and their anti-HIV activities, which might be expected since this activity appears to also be mediated through binding to the proteins constituting the CD4 and/or gp120 receptors. Also of interest is the possible correlation of molecular weights of ATA fractions with the inhibition of HIV-1 reverse transcriptase in cell-free systems.[5] In order to answer these questions, ATA has been fractionated and the activities of the fractions have been determined in a variety of appropriate assay systems.

Results

Chemistry. The ammonium salt of ATA (aluminon) was prepared twice by a modification of the method re-

Table I. Molecular Weights, Weight Distributions, and Retention Times from the First Fractionation of the Ammonium Salt of ATA According to Scheme I

dialysis and ultrafiltration					GPC analyses[a]		
fraction no.	no. av MW (M_n)[b]	wt av MW (M_w)[b]	M_w/M_n	wt, %	no of peaks	retention time, min	peak area, %
1	2411	3336	1.4	8.4	1	27.9	100
2	1353	2326	1.7	4.4	1	29.0	100
3	1059	1609	1.5	8.0	1	29.6	100
4	971	1437	1.5	4.0	1	29.8	100
5	841	1149	1.4	6.4	1	29.9	100
6	352	475	1.4	38.2	3	30.9	84.5
						31.4	15.3
						32.4	0.2
7	250	328	1.3	32.6	3	31.4	21.4
						32.4	56.5
						34.4	22.1

[a] The gel permeation chromatography analyses were performed on a 2 × 50 cm Shodex HF-2003 semipreparative column with an exclusion limit of 70 000 daltons and 12 000 theoretical plates. A 0.8 × 5 cm precolumn was employed and THF was used as the mobile phase with a flow rate of 3.5 mL/min. [b] These molecular weights were calculated with the aid of the calibration curve in Figure 2.

Table II. Molecular Weights, Weight Distributions, and Retention Times from the Second Fractionation of the Ammonium Salt of ATA According to Scheme I

dialysis and ultrafiltration							GPC analyses[b]		UV analyses	
fraction no.	MW[c]	no. av MW (M_n)[d]	wt av MW (M_w)[d]	M_w/M_n	wt, %	intrinsic viscosity (η)	retention vol, mL	retention time, min	λ_{max}	$E^{1cm}_{1\%}$
1	5460	3511	4579	1.3	6.8	0.041	96.3	27.5	316	240
2	3228	2547	2937	1.2	4.0	0.039	99.1	28.3	315	197
3	2489	1767	2275	1.3	5.7	0.035	101.2	28.9	316	230
4	2231	1759	2408	1.4	3.0	0.034	101.5	29.0	315	168
5	1554	1103	1495	1.4	8.7	0.033	103.3	29.5	315	543
8		740	927	1.3	4.8		105.0	30.0	315	215
9	738	485	623	1.3	27.0	0.031	107.8	30.8	312	432
10[e]	424	369	409	1.1	23.2		110.3	31.5	312	241
11[e]	288	288	288	1.0	13.8		113.4	32.4	310	271
12[e]	138	138	138	1.0	2.2		120.4	34.4	304	237

[a] The intrinsic viscosities of the ATA samples were determined on a Cannon-Ubbelohde viscometer (size No. 25, International Research Glassware) in THF solvent. [b] The gel permeation chromatography analyses were performed on a 2 × 50 cm Shodex HF-2003 semipreparative column with an exclusion limit of 70 000 daltions and 12 000 theoretical plates. A 0.8 × 5 cm precolumn was employed and THF was used as the mobile phase with a flow rate of 3.5 mL/min. [c] These molecular weights were determined by the universal calibration method. [d] These molecular weights were calculated with the aid of the calibration curve in Figure 2. [e] The molecular weights of these fractions were determined by chemical-ionization mass spectrometry.

ported by Heisig and Lauer.[28] The material from each preparation was then fractionated separately by a combination of equilibrium dialysis, ultrafiltration, and gel permeation chromatography according to the procedure outlined in Scheme I. During the first fractionation, the final step involved ultrafiltration using a 500 MW cutoff membrane, whereas in the second fractionation, the lower molecular weight components were fractionated by gel permeation chromatography. The gel permeation chromatography retention times of the fractions obtained from each sample were determined on a Shodex HF-2003 semipreparative column (exclusion limit 70 000 daltons) using THF as the mobile phase. These retention times are listed in Tables I and II, along with the weight distributions. Fractions 1–5 showed single peaks in their gel permeation chromatograms, and those of fractions 6 and 7 (Scheme I) had multiple shoulders and peaks, as indicated in Table I. However, all of the fractions listed in Table II produced single peaks in their gel permeation chromatograms. The ATA ammonium salts did not lose nitrogen either when being dried in vacuo or when subjected to prolonged dialysis.

The molecular weights of ATA fractions were determined by the universal calibration method described by Lau, Kirkland, and Bly.[29] The narrow molecular weight distribution standards were polystyrene samples (Shodex Standard, Showa Denko K.K.) with MW 950, 1250, 1700, 2450, 4136, 5050, 7000, 11 600, and 22 000. Viscosities of polystyrene standards and ATA fractions obtained from the second fractionation were measured in THF at 25 °C. The results are listed in Table II. Intrinsic viscosities $[\eta]$ were determined by extrapolating values of η_{sp}/c obtained at different concentrations to zero concentration, where η_{sp} is the specific viscosity and c is weight concentration. The logarithm of the product $M[\eta]$ of the polystyrene standards plotted against the GPC retention volume provided the universal calibration curve shown in Figure 1. According to the universal calibration method, a plot of $M[\eta]$ versus retention volume V_R for all polymers should be identical, where M is the molecular weight. At any given retention volume, the hydrodynamic volume of polystyrene standards and ATA fractions are equal, e.g.

$$\log M_S[\eta]_S = \log M_A[\eta]_A \quad (1)$$

$$M_A = M_S[\eta]_S/[\eta]_A \quad (2)$$

where M_S is the molecular weight of the standard, $[\eta]_S$ is the intrinsic viscosity of the standard, M_A is the molecular weight of the ATA fraction, and $[\eta]_A$ is the intrinsic viscosity of the ATA fraction.

After the intrinsic viscosities of polystyrene standards and ATA fractions are measured (Table II), the molecular weights of the ATA fractions can be calculated by eq 2 and Figure 1. On the basis of the molecular weight data of the ATA fractions, the gel permeation column was calibrated

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Table III. Anti-HIV Activities of the ATA Fractions Listed in Table I and the ATA Analogues[a]

fraction (M_w) or analogue	IC_{50}, μg/mL: HIV cytopathogenicity: HIV-1 MT-4 cells	HIV-2 MT-4 cells	HIV-1 CEM cells	cell viability[c]	giant cell assay:[b] MOLT-4 cells cocultured with: HIV-1-infected HUT-78 cells	HIV-2-infected HUT-78 cells	inhibitory index of HIV-1 binding to MT-4 cells (II_{VB})[d]
1 (3336)	0.81	0.75	0.8	312	4	4	0.98
2 (2326)	1.9	1.1	2.0	277	8	7	0.89
3 (1609)	3.0	2.4	2.9	261	15	7	0.89
4 (1437)	5.8	3.1	6.4	268	31	11	0.79
5 (1149)	6.7	3.3	12.5	237	40	12	0.70
6 (475)	15.1	10.6	18.4	305	60	20	0.31
7 (328)	>100	>100	53.0	243	>100	40	0.00
ATPA	10.3	8.0	27.3	180	60	8	0.89
ATSAH	0.99	0.89	8.9	177	5	5	0.97
ATSAL	5.7	4.5	3.8	193	20	12	0.84
5	>100	>100	122.7	>100	>100	>100	0.00
ATA[e]	1.1	0.85	2.1	280	4	4	0.99

[a] The data in MT-4 cells are the mean values for at least three experiments. The data in CEM cells are the mean values of from two to four experiments. IC_{50} is the 50% inhibitory concentration for cytopathicity of HIV-1 ($HTLV\text{-}III_B$) or HIV-2 ($LAV\text{-}2_{ROD}$) in MT-4 cells or in CEM cells as specified. The viral dose was 100–500-fold higher than that required to infect 50% of the MT-4 cells. The compounds were supplied as the free acids. [b] IC_{50} for the giant cell formation between HIV-1- or HIV-2-infected HUT-78 cells and uninfected MOLT-4 cells corresponds to the 50% inhibitory concentration that reduced the number of giant cells by 50%.[36] [c] IC_{50} for viability is the 50% cytotoxic dose in mock-infected MT-4 cells. Viability was quantified by a tetrazolium (MTT) colorimetric method in 96-well microtrays.[37] [d] All of the fractions were tested at a concentration of 25 μg/mL. The inhibitory index for virus binding (II_{VB}) was calculated according to the following formula: $II_{VB} = 1 - (MF_{VC} - MF_{CC})/(MF_V - MF_C)$, whereby MF_{VC} is the mean fluorescence (MF) with a given concentration of the compound in HIV-1 inoculated cells, MF_{CC} is the mean fluorescence for the control cells (not exposed to HIV-1) treated with compound, MF_V is the mean fluorescence for the HIV-1-inoculated cells (not treated with any compound), and MF_C is the mean fluorescence for the control cells (not exposed to HIV-1 and not treated with any compound). [e] This is unfractionated ATA that was obtained from Aldrich Chemical Co., Inc.

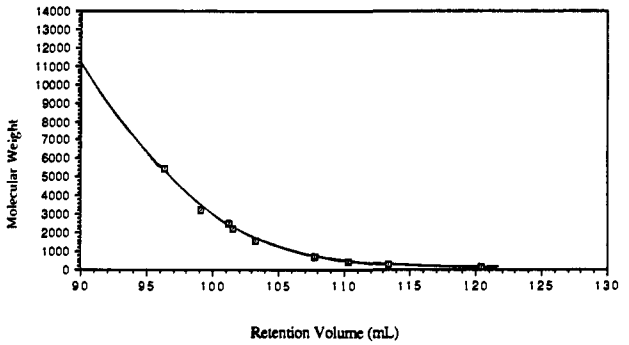

Figure 2. Calibration curve obtained by plotting the molecular weights of the ATA fractions vs retention volume on a 2 × 50 cm Shodex HF-2003 semipreparative GPC column with an exclusion limit of 70 000 daltons and 12 000 theoretical plates.

by using the peak position calibration method, which is valid because these ATA fractions had narrow molecular weight distributions. The results are shown in Figure 2. The number and weight average molecular weights of the ATA fractions were then calculated.[29] The molecular weights of ATA fractions 10–12 from Table II were determined by mass spectrometry.

Several analogues of ATA were also synthesized in which the carboxylic acid groups were replaced with sulfonic acid or phosphonic acid moieties. The ammonium salt of aurintriphosphonic acid (ATPA) was synthesized by polymerizing 2-hydroxybenzenephosphonic acid (**3**)[30] under the same conditions used to make ATA.[28] The ammonium salt of 2-hydroxybenzenesulfonic acid (**4**) was also prepared by a known procedure[31] and reacted with formaldehyde, sulfuric acid, and nitrous acid to give aurintrisulfonic acid. This material was then fractionated into a high molecular weight fraction (ATSAH) and a low molecular weight fraction (ATSAL) by equilibrium dialysis and ultrafiltration. The low molecular weight compound **5** was prepared as part of a separate effort to synthesize ATA monomer analogues,[32] and was tested for anti-HIV activity in the assays employed in the present study. No effort was made to determine accurately the molecular weight distributions of these sulfonic and phosphonic acid analogues of ATA.

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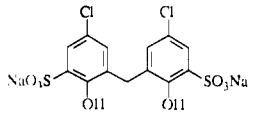

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Biological Evaluation. Some of the anti-HIV activities of the fractions listed in Table I are provided in Table III. In addition, the activities of the aurintriphosphonic acid and aurintrisulfonic acid analogues are listed in Table III. The fractions and analogues were tested for prevention of the cytopathic effect of HIV-1 and HIV-2 in MT-4 cells and HIV-1 in CEM cells. The cytotoxicities of the ATA fractions and analogues in mock-infected MT-4 cells were also determined. The abilities of the fractions and ATA analogues to inhibit syncytium formation between HIV-1- or HIV-2-infected HUT-78 cells and uninfected MOLT-4 cells were evaluated.

The capacities of the fractions to inhibit the binding of HIV-1 virions to MT-4 cells were also determined by fluorescence intensity measurements. The results are shown graphically in Figure 3 and are also listed quanti-

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Table IV. Anti-HIV Activities of the ATA Ammonium Salt (Aluminon) Fractions Listed in Table II

	IC_{50}, μg/mL						
	HIV cytopathogenicity				giant cell assay:[b] MOLT-4 cells cocultured with		
fraction (M_W)	HIV-1 MT-4 cells	HIV-2 MT-4 cells	HIV-1 CEM cells	cell viability[d]	HIV-1-infected HUT-78 cells	HIV-2-infected HUT-78 cells	HIV-1 RT inhibition[c]
1 (4579)	0.2	1.0	0.6	>100	2	4	0.008 ± 0.002
2 (2937)	0.6	1.0	5.0	>100	4	4	0.028 ± 0.002
3 (2275)	1.5	1.0	0.7	>100	4	4	0.078 ± 0.004
4 (2408)	1.5	1.4	2.5	>100	4	4	0.103 ± 0.014
5 (1479)	4.4	5.4	10.1	>100	20	14	1.40 ± 0.043
8 (927)	9.3	8.3	28.4	>100	40	14	2.30 ± 0.10
9 (623)	>100	>100	34.5	66	>100	>100	26.4 ± 3.68
10 (409)	>100	>100	49.7	72	>100	>100	43.00 ± 11.1
11 (288)	>100	>100	71.2	92	>100	>100	>100
12 (138)	>100	>100	>42.8	83	>100	>100	>100
ATA[e]	0.5	1.3	1.2	>100	4	4	

[a] The data in MT-4 cells are the mean values for at least three experiments. The data in CEM cells are mean values for two or three experiments. IC_{50} is the 50% inhibitory concentration for cytopathicity of HIV-1 ($HTLV\text{-}III_B$) or HIV-2 ($LAV\text{-}2_{ROD}$) in MT-4 cells or in CEM cells as specified. The viral dose was 100–500-fold higher than that required to infect 50% of the MT-4 cells. The compounds were supplied as ammonium salts. [b] IC_{50} for the giant cell formation between HIV-1- or HIV-2-infected HUT-78 cells and uninfected MOLT-4 cells corresponds to the 50% inhibitor concentration that reduced the number of giant cells by 50%. [c] Inhibition of HIV-1 reverse transcriptase. Fractions were dissolved in water and diluted for each experiment. The results represent the mean ± SEM of three experiments each carried out in triplicate. [d] IC_{50} for viability is the 50% cytotoxic dose in mock-infected MT-4 cells. Viability was quantified by a tetrazolium (MTT) colorimetric method in 96-well microtrays. [e] This was tested as the free acid instead of the ammonium salt.

tatively in Table III. All of the fractions listed in Table III were tested as free acids, and the aurintriphosphonic acid and aurintrisulfonic acid analogues were tested as their ammonium salts.

The anti-HIV activities of the ATA ammonium salt (aluminon) fractions listed in Table II are given in Table IV. The assays involved are similar to those in Table III, except that these fractions were tested for inhibition of HIV-1 reverse transcriptase instead of inhibition of HIV-1 binding to MT-4 cells.

The ATA fractions in Table I and the ATA phosphonic and sulfonic acid analogues were also evaluated for their abilities to inhibit the binding of fluorescein isothiocyanate labeled OKT4A monoclonal antibody to the CD4 receptor in MT-4 cells and for their abilities to inhibit the binding of anti-gp120 monoclonal antibody to HIV-1-infected HUT-78 cells. The results are given in Tables V and VI. Calculations were based on mean fluorescence intensity, because this is more sensitive than the percentage of fluorescent cells.

Discussion. Samples of ATA obtained from different sources have been observed to vary considerably, both with regard to chemical composition[2] and potency in inhibiting protein nucleic acid interactions.[33] The method employed in the present study is a controlled polymerization which gives lower molecular weight material than that obtained from the commercial samples of ATA that have been fractionated previously.[2] It is also apparent that the actual molecular weights of the fractions listed in Tables I and II are lower than those which might be anticipated from the dialysis and ultrafiltration membrane cutoffs given in Scheme I. However, the cutoff values are derived from work with linear polysaccharides, which can worm through smaller pore sizes than more branched polymers like ATA. In any case, the procedure outlined in Scheme I did in fact fractionate the ATA so that the molecular weights are in the overall order expected. Fractions 1–5 in Tables I and II have different number average and weight average molecular weights, which have evidently resulted from unspecified differences in the synthesis and/or fractionation procedures between the two runs. The polymerization process appears to be sensitive to small variations in reaction conditions. Therefore, when conducting this type of polymerization reaction, every effort should be made to control the rate of addition of formaldehyde, stirring speed, reaction time, reaction temperature, exposure to atmospheric moisture, and exact quantities of the reagents employed.

By far the most impressive result of this study is the direct correlation of activity of the ATA fractions with molecular weight in every test except that for host cell viability. The higher the molecular weight, the higher the potency. This correlation of molecular weight with anti-HIV potency also extended to the high and low molecular weight fractions of the sulfonic acid analogues (ATSAH and ATSAL). A similar trend was previously reported in the inhibition of several different protein nucleic acid interactions by ATA fractions.[2] All of the evidence documented in the ATA literature indicates quite clearly that the ability of ATA to prevent protein nucleic acid interactions results from its binding to protein, not nucleic acid. This agrees with the results presented here which indicate that the larger ATA molecules bind more avidly to the proteins constituting the CD4 receptor and gp120. This is reflected in the greater potency of the higher molecular weight fractions of ATA in inhibiting the cytopathic effect of HIV-1 and HIV-2 in cell culture. Although the molecular weights of the ATA fractions also correlated with ability to inhibit HIV-1 reverse transcriptase, it seems unlikely that this inhibition is responsible for the anti-HIV activity of ATA, because prior studies have shown that ATA does not inhibit cellular polymerases, presumably because of an inability of the polymer to get into cells.[4]

The data presented in Tables III and IV show that the ATA fractions are slightly more active against HIV-2 than HIV-1. This was reflected in both prevention of the cytopathic effect and inhibition of syncytium formation. Several other antiviral agents are more active against HIV-2 than HIV-1, including pentosan sulfate,[34] dextran sulfate,[34] soluble CD4,[35] glycosylation inhibitors (i.e. castanospermine),[36] and low molecular weight (i.e. 1000–2000)

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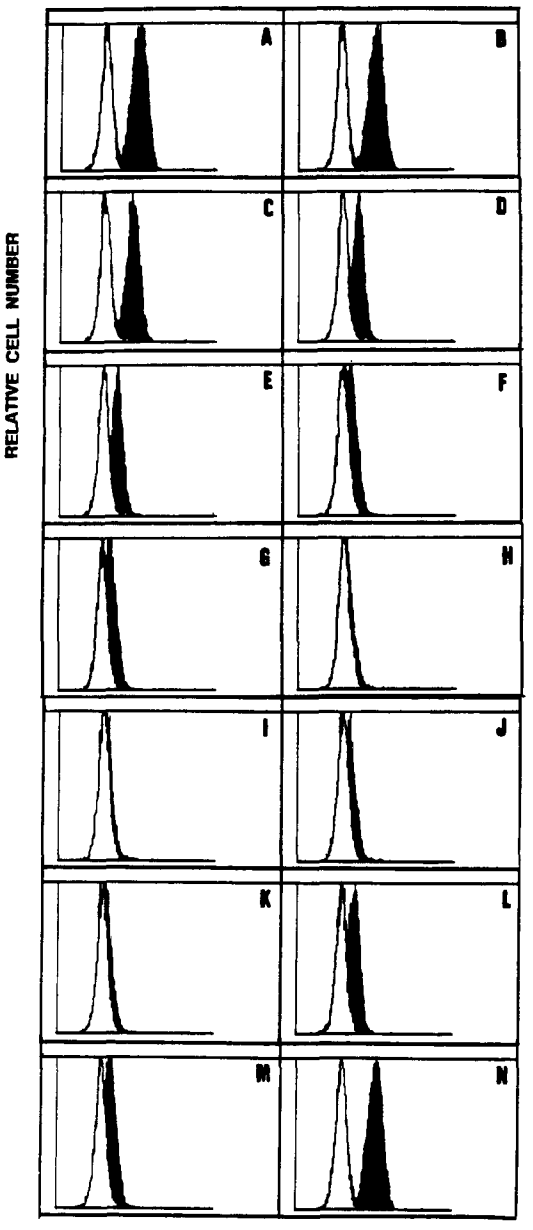

Figure 3. Inhibitory effect of the compounds on HIV-1 binding to MT-4 cells.[38] The white histograms represent the fluorescence of MT-4 cells which were not exposed to HIV-1 virions. The black histograms represent the fluroescence of MT-4 cells which were exposed to HIV-1 virions. The MT-4 cells were exposed or not exposed to HIV-1, in the absence of compound (panel A), or in the presence of the following ATA fractions listed in Table I: fraction 7 (panel B), fraction 6 (panel C), fraction 5 (panel D), fraction 4 (panel E), fraction 3 (panel F), fraction 2 (panel G), fraction 1 (panel H), unfractionated ATA from Aldrich (panel I), dextran sulfate (MW 5000, Sigma) (panel J), ATSAH (panel K), ATSAL (panel L), ATPA (panel M), and compound **5** (panel N). All compounds were used at 25 μg/mL. The mean fluorescence values were as follows: 23 (A), 22 (B), 23 (C), 18 (D), 25 (E), 22 (F), 20 (G), 23 (H), 23 (I), 23 (J), 22 (K), 20 (L), 21 (M), and 20 (N) for the nonspecific fluorescence and 124 (A), 123 (B), 93 (C), 48 (D), 47 (E), 32 (F), 32 (G), 25 (H), 24 (I), 29 (J), 24 (K), 37 (L), 32 (M) and 125 (N) for the specific fluorescence.

sulfated polysaccharides.[37] On the other hand, TIBO derivatives[38] and HEPT derivatives[39] are active against HIV-1 and inactive against HIV-2.

ATA fraction 1 and the commercial sample of ATA were highly effective in inhibiting CD4 expression, even at a concentration of 4 μg/mL. Their IC_{50} values could be estimated at about 2 μg/mL. For fraction 2, the IC_{50} was about 20 μg/mL, whereas the ATA fractions of lower molecular weight were essentially inactive in inhibition CD4 expression (Table V). However, these lower molecular weight fractions still retained activity in preventing the cytopathic effect of HIV-1 and HIV-2 and they also re-

Table V. Effects of the ATA Fractions in Table I and ATA Analogues on CD4 Expression in MT-4 Cells[a]

fraction (M_W) or analogue	concn, μg/mL	II_{OKT4A}	% OKT4A positive cell
1 (3336)	100	0.96	2.5
	20	0.98	1.3
	4	0.90	9.9
	0.8	0.30	99.1
	0.16	0.00	99.6
2 (2326)	100	0.95	3.8
	20	0.77	45.1
	4	0.20	99.5
3 (1609)	100	0.66	80.2
	20	0.26	99.2
	4	0.4	
4 (1437)	100	0.20	99.5
	20	0.10	
	4	0.02	
5 (1149)	100	0.16	99.7
	20	0.08	
6 (475)	100	0.07	99.6
	20	0.03	
7 (328)	100	0.02	99.6
	20	0.01	
	4	0.01	
ATPA	100	0.05	99.5
	20	0.02	
	4	0.01	
ATSAH	100	0.75	52.7
	20	0.19	99.6
	4	0.01	
ATSAL	100	0.06	99.6
	20	0.02	
	4	0.01	
5	100	0.00	99.7
ATA[b]	100	0.97	2.7
	20	0.98	1.8
	4	0.91	9.2
	0.8	0.26	99.2
	0.16	0.00	99.5

[a] MT-4 cells (200 000 cells/100 μL of PBS) were incubated with the test compounds at the indicated concentrations at 20 °C for 10–20 s, stained with anti-OKT4A-FITC for 20 min at 4 °C, washed, fixed in 0.37% formaldehyde, and analyzed cytofluorometrically. Measurements were based on the percentage of cells showing fluorescence intensity greater than the control cells stained with normal mouse IgG-FITC to monitor nonspecific immunoglobulin labeling. The inhibitory index for OKT4A mAb binding inhibition (II_{OKT4A}) was calculated according to the following formula: $II_{OKT4A} = 1 - (MF_{OKTAX} - MF_C)/(MF_{OKT4} - MF_C)$, whereby MF_{OKT4A} is the mean fluorescence (MF) for the cells incubated only with OKT4A mAb, MF_{OKT4AX} is the MF for the cells incubated with test compound and OKT4A mAb and MF_C is the mean fluorescence of the cells incubated with IgG-FITC. [b] This is unfractionated ATA that was obtained from Aldrich Chemical Co., Inc.

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Table VI. Effects of ATA Fractions in Table I and ATA Analogues on the Binding of Anti-gp120 mAb to Persistently HIV-1-Infected HUT-78 Cells As Detected by FACS Analysis[a]

fraction (M_W) or analogue	concn, μg/mL	II_{gp120}
1 (3336)	100	0.98
	20	1.00
	4	0.78
	0.8	0.73
	0.16	0.66
2 (2326)	100	0.99
	20	0.99
	4	0.85
	0.8	0.56
	0.16	0.00
3 (1609)	100	0.94
	20	0.93
	4	0.55
	0.8	0.00
4 (1437)	100	0.84
	20	0.75
	4	0.09
5 (1149)	100	0.93
	20	0.78
	4	0.61
6 (475)	100	0.76
	20	0.65
	4	0.00
7 (328)	100	0.00
	20	0.00
ATPA	100	0.91
	20	0.92
	4	0.92
	0.8	0.06
ATSAH	100	0.98
	20	0.99
	4	0.81
	0.8	0.63
ATSAL	100	0.95
	20	0.76
	4	0.05
	0.8	0.06
5	100	0.18
ATA[b]	100	1.00
	20	0.98
	4	0.88
	0.8	0.87
	0.16	0.55

[a] HIV-1-infected HUT-78 cells (200 000 cells) in 100 μL of RPMI with 10% FCS were washed twice, incubated with the compounds at the indicated concentrations at 20 °C for 5 min, and then stained with anti-sp120 mAb (9284, du Pont de Nemours, Brussels, Belgium) for 45 min at 37 °C, washed twice, incubated with fluorescein isothiocyanate-conjugated $F(ab')_2$ fragments of rabbit anti-mouse immunoglobulin antibody [RaM-IG-$F(ab')_2$-FITC] (Prosan, Ghent, Belgium) for 45 min at 37 °C, washed twice with phosphate-buffer saline (PBS), resuspended in 0.5 mL of 0.37% paraformaldehyde in PBS, and analyzed with the fluorescence-activated cell sorter (FACSTAR, Becton Dickinson). The threshold of positivity for green fluorescence intensity was arbitrarily established on the basis of the control sample of infected HUT-78 cells incubated only with RaM-IG-$F(ab')_2$-FITC. The inhibitory index for anti-gp120 mAb binding inhibition (II_{gp120}) was calculated according to the formula: $II_{gp120} = 1 - (MF_{gp120x} - MF_C/(MF_{gp120} - MF_C)$, whereby MF_{gp120} is the mean fluorescence (MF) for the cells incubated only with anti-gp120 mAb, MF_{gp120x} is the MF for the cells incubated with test compound and anti-gp120 mAb, and MF_C is the mean fluorescence of the cells incubated with RaM-IG-F-$(ab')_2$-FITC.[7] [b] This is unfractionated ATA that was obtained from Aldrich Chemical Co., Inc.

tained the ability to bind to gp120. This indicates that at least some of the prevention of cytopathicity of HIV-1 and HIV-2 by ATA must be due to gp120 binding and that it cannot be due exclusively to CD4 binding. This is consistent with the fact that the phosphonic acid analogue (ATPA) does not bind to CD4, but it binds to gp120 and retains some ability to prevent the cytopathic affects of HIV-1 and HIV-2. A similar profile was observed for the lower molecular weight sulfonic acid analogue (ATSAL). The observation that the lower molecular weight fractions of ATA bind to gp120 and not CD4 has clear implications for further drug development.

The fact that the molecular weights of the ATA fractions correlate with anti-HIV activity and not cytotoxicity indicates that different mechanisms may be involved for antiviral activity and the cytotoxic effect. It also indicates that an increase in the therapeutic index is possible by increasing the molecular weights of the ATA fractions. Further work is indicated so that the limits of this effect can be clearly delineated.

The results presented here provide us with a picture of the structure–activity relationships of the ATA fractions and their mechanism of action (inhibition of virus binding due to an interference with the gp120-CD4 interaction). As demonstrated by using monoclonal antibodies to gp120 and CD4, the active compounds interact more avidly with gp120 than CD4. This may explain the previous observation that concentrations of serum that bind ATA and therefore prevent its interaction with CD4 are less effective in inhibiting the binding of HIV-1 virions to MT-4 cells.[6] In other words, components of ATA may be able to bind to gp120 in the presence of concentrations of serum proteins that prevent binding of ATA to CD4. The binding of ATA to gp120 clearly plays a role in prevention the cytopathic effect of HIV-1 and HIV-2, but the function that CD4 binding plays is less certain.

In conclusion, the fractionation of ATA has demonstrated that it is possible to separate binding to gp120 as opposed to CD4 binding and also that prevention of HIV cytopathicity can be differentiated somewhat from cytotoxicity. An attractive feature for further drug development based on ATA is the fact that it is extremely inexpensive. It can be prepared in one step from salicylic acid, formaldehyde, sulfuric acid, and sodium nitrite, all of which are readily available commercially at low cost. There is presently a need for an affordable anti-AIDS agent.

Experimental Section

IR spectra were recorded on a Beckman IR-33 spectrophotometer. The results in CEM cells were obtained under the auspices of the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Rockville, MD. Studies on the inhibition of HIV-1 reverse transcriptase were performed at Merck Sharp and Dohme Research Laboratories, West Point, PA. All of the other biological tests were done at the Rega Institute for Medical Research, Katholiecke Universiteit, Leuven, Belgium.

Aurintricarboxylic Acid (ATA) Ammonium Salt (Aluminon). Concentrated H_2SO_4 (35 mL) was added to a 500 mL, three-necked round-bottomed flask fitted with a mechanical stirrer. The flask was immersed in an ice bath and allowed to stir for 10 min. Sodium nitrite (Mallinckrodt, 5.0 g, 72.4 mmol) was added in small portions with stirring during 10 min. Vigorous stirring was then continued for an additional 10 min. Salicylic acid (10.0 g, 72.4 mmol) was added slowly over 30 min. The reaction flask was removed from the ice water bath and allowed to stir at room temperature for 10 min. Following this, the reaction mixture was again cooled to 0 °C. Formaldehyde solution (37% w/w, 1.96 g, 24.15 mmol) was added dropwise to the cooled, stirred solution at such a rate that the addition took 10 min. On this addition the mixture went from brownish-orange to reddish-purple. After the mixture was stirred an additional 15 min at 0 °C, crushed ice (about 50 g) was added to the reaction flask along with 2–3 mL of ethyl ether to control foaming. Ice water (about 200 mL) was added and the mixture became bright orange. After stirring at room temperature for 2.5 h, the solution was filtered. A red solid was recovered. This solid was washed in distilled water (5 × 300 mL), each time for 5 min. Dilute ammonia solution (60 mL, 1 part concentrated NH_4OH to 2 parts water) was passed

over the filter cake. This converted the solid ATA into its ammonium salt (in the form of a dark purple solution). The NH_3 and water were removed by evaporation, and the residue was dried under reduced pressure (1.5 mm) for 24 h at room temperature. A dark red powder (8.19 g) was recovered, which is the ammonium salt of ATA (aluminon).

Dialysis of Aluminon. Aluminon (3.0 g) was taken up in 30 mL of distilled water. This solution was evenly placed into two dialysis bags (Spectrapor, MW cutoff 3500 daltons). The bags were immersed in a 4-L beaker containing ~3800 mL of distilled water. Stirring was started. After 12 h, the combined retentates were concentrated to their original volumes, and dialysis was continued for another 12 h. At this time, the retentate was concentrated again and dialized in 3800 mL of fresh distilled water. After 24 h, the retentate was concentrated and dialized in fresh distilled water for the last 24 h. The total dialysate was 12 L, and the dialysis time was 72 h. The last 3800 mL of dialysate was almost colorless, suggesting that all ATA molecules smaller than the apertures of dialysis tubing had diffused out. Other dialysis tubings used were (1) Dialysis "Sacks", Sigma Chemical Co., MW cutoff 12000, (2) Spectrapor, MW cutoff 6000–8000, (3) Spectrum, wet cellulose dialysis tubing, MW cutoff 2000. The solid ammonium salts were isolated by evaporating the water on a rotary evaporator (bath 45 °C) under reduced pressure and then drying the residues under vacuum (1.5 mm) at room temperature for 12 h. The solid carboxylic acids were isolated by concentrating the fractions to 100 mL and acidifying with 1.2 N HCl until the filtrates were colorless when the solutions were passed through a sintered glass frit of medium porosity. After standing for an additional 30 min, the solid acids were isolated by filtration through a glass frit.

Gel Permeation Chromatography (GPC). GPC was carried out using a 2 × 50 cm Shodex GPC HF-2003 semipreparative column with an exclusion limit of 70000 daltons and 12000 theoretical plates. A 0.8 × 5 cm precolumn was employed. The mobile phase consisted of 100% THF, the flow rate was 3.5 mL/min, and the elution was followed by UV detection at a wavelength setting of 254 nm. Before being used as the GPC mobile phase, THF (Aldrich, HPLC grade) was purified by rotary evaporation in the presence of ferrous sulfate to remove any impurities and organic peroxide.

Aurintriphosphonic Acid (ATPA) Ammonium Salt. Concentrated sulfuric acid (3.6 mL) was placed in a two-necked flask equipped with a mechanical stirrer. The flask was cooled in a dry ice–acetone–water bath at 0–10 °C and sodium nitrite (0.52 g, 7.47 mmol) was added in small portions with vigorous stirring. 2-Hydroxybenzenephosphonic acid[29] (1.30 g, 7.47 mmol) was then added in small portions with stirring. The reaction mixture became a brown solution. Formaldehyde (0.23 g, 37% aqueous solution, 2.8 mmol) was added dropwise to the flask with vigorous stirring, while the temperature of the bath was kept at −5 °C. After the addition was complete, the reaction mixture was stirred at 0–5 °C for 20 min and then at room temperature for another 10 min. Crushed ice (10 g) was added to the reaction mixture. This resulted in a dark red solution. The solution was basified with conc ammonium hydroxide to pH 8 (Hydrion paper). This solution was then placed in a dialysis tube with a molecular weight cutoff of 3500. The dialysis tube was placed in stirred water. The dialysate was replaced with fresh water several times until there was only a very faint color in the dialysate. The water was evaporated from the retentate on a rotary evaporator. The residue was dried in a drying pistol overnight to afford a dark red solid (290 mg): IR (KBr) 3160, 1569, 1393, 1232, 1124, 1073, 1018, 878 cm^{-1}.

Synthesis and Fractionation of Aurintrisulfonic Acid into High and Low Molecular Weight Fractions (ATSAH and ATSAL). Concentrated sulfuric acid (7.4 mL) was placed in a 50-mL three-necked flask equipped with a mechanical stirrer, a thermometer, and a nitrogen gas inlet. The flask was cooled in an ice bath while sodium nitrite (1.06 g, 15.3 mmol) was added with vigorous stirring. Sodium 2-hydroxybenzenesulfonate[30] (3.00 g, 15.3 mmol) was added to the solution in small portions with stirring. The flask was then cooled in a dry ice–acetone–water bath. Formaldehyde (0.60 g of 37% solution, 7.4 mmol) was added to the flask with vigorous stirring while the temperature of the reaction mixture was kept below 5 °C. After the addition of the formaldehyde was complete, the reaction mixture was stirred at room temperature for another 30 min and then poured into crushed ice (10 g). The solution was neutralized with concentrated ammonium hydroxide. The resulting solution was placed in a dialysis tube having a molecular weight cutoff of 3500. The dialysis tube was suspended in water which was agitated with a magnetic stirrer. The dialysate was replaced with fresh water several times until there was only a very faint color present in the dialysate. The retentate was evaporated on a rotary evaporator. The residue was dried in a drying pistol for 18 h to afford a dark red solid of ATSAH (400 mg): IR (KBr) 3448, 3158, 1401, 1210, 1033, 633 cm^{-1}. All of the dialysates were collected and subjected to ultrafiltration using an Amicon pressure cell fitted with a Diaflo YC05 membrane (molecular weight cutoff = 500). The retentate was rotary evaporated and the residue dried in a drying pistol to afford a red glass (330 mg). This solid was dissolved in water and subjected to another ultrafiltration with a Diaflow YM2 membrane (molecular weight cutoff = 1000). Virtually all of the solution passed through this membrane. The ultrafiltrate was rotary evaporated and the residue was dried in a drying pistol to afford ATSAL as a red glass (323 mg).

Elemental Analyses. The ATA fractions listed in Table II, with the exception of fraction 8, were subjected to elemental analysis and the following results were obtained: Fraction 1: found C, 56.01; H, 4.09. Fraction 2: found C, 55.47; H, 4.25; N, 6.92. Fraction 3: found C, 56.39; H, 4.07; N, 6.29. Fraction 4: found C, 56.38; H, 4.06. Fraction 5: found C, 56.49; H, 4.06. Fraction 9: found C, 60.25; H, 4.04; N, 6.54. Fraction 10: C, 57.56; H, 3.98. Fraction 11: found C, 56.10; H, 3.85. Fraction 12: found C, 58.93; H, 4.27. ATSAH: found C, 38.61; H, 4.48; N, 5.42; S, 13.19. Unfractionated ATA ammonium salt (aluminon) after drying for 12 h at room temperature (0.3 mm): C, 54.51; H, 5.19; N, 6.70. Unfractionated ATA ammonium salt (aluminon) after drying for 36 h at room temperature (0.3 mm): C, 56.11; H, 5.40; N, 6.88. Unfractionated ATA ammonium salt (aluminon) after dialysis using a 3500 molecular weight cutoff membrane for 72 h and drying for 12 h at room temperature (0.3 mm): C, 54.87; H, 5.16; N, 6.56.

HIV-1 Reverse Transcriptase Inhibition Assay. Recombinant HIV-1 reverse transcriptase (NY-5 isolate) was expressed in *Escherichia coli* following IPTG induction.[40] Subsequent to bacterial cell lysis, 50% ammonium sulfate precipitation and dialysis, the dialysate was purified by DE-52 and phosphocellulose chromatography.[41] HIV-1 RT assays, in vitro, were carried out in Skatron tube strips using a buffer composed of 55 mM Tris·HCl pH 8.2, 80 mM KCl, 1 mM DTT, 50 μM EGTA, 2.5 μg/mL rA·dT, 0.5 μCi[^{3}H]TTP, 10 μM TTP, and 0.005% Triton X-100 (50 μL final volume). After addition of HIV-1 reverse transcriptase, reactions were carried out for 45 min at 37 °C. At the completion of the reaction, the racks were chilled, and 200 μL of 13% trichloroacetic acid/10 mM sodium phosphate was added to each tube. After 30 min, acid precipitable cDNA was collected on filters using a Skatron cell harvester. The radioactivity present on the filters was determined by liquid scintillation spectroscopy. Experiments were carried out in triplicate and the results expressed as the mean ± SEM of at least three experiments.

Giant Cell Assay. This assay is based on syncytium formation and destruction of bystander $CD4^+$ cells cocultured with T cells persistently infected with HIV as demonstrated by flow cytometry.[42]

Cell Viability Assay. Cell viability was quantified by a tetrazolium (MTT) colorimetric method in 96-well microtrays.[43]

Anti-gp120 mAb Binding Assay. The assay of binding of anti-gp120 mAb to persistently HIV-1-infected HUT-78 cells was performed by FACS analysis.[7]

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Assay of HIV-1 Virion Binding to MT-4 Cells. Virion binding to the cell membrane was determined by an indirect immunofluorescence assay using human anti-HIV 1 serum, rabbit anti-human-IG-F(ab')-fluorescein isothiocyanate, and flow cytometry.[44]

Assay of CD4 Expression. MT-4 cells were incubated with the test compound, stained with anti-OKT4A-FITC, and analyzed cytofluorometrically.[6]

Cell Viability Assay in HIV-1-Infected and Uninfected CEM Cells. Cell viability was determined by a tetrazolium (XTT) assay.[45]

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Registry No. 4, 130246-99-0; 5, 33206-58-5; ATA, 569-58-4; ATPA, 129777-62-4; 2-hydroxybenzenephosphonic acid, 53104-46-4; Salicylic acid, 69-72-7; reverse transcriptase, 9068-38-6; aurintrisulfonic acid, 129608-01-1; sodium 2-hydroxybenzenesulfonate, 51368-26-4.

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Synthesis and Anti-HIV Activities of Low Molecular Weight Aurintricarboxylic Acid Fragments and Related Compounds

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Several compounds corresponding to fragments of the schematic representation of the polymeric structure of aurintricarboxylic acid (ATA) have been prepared and tested for prevention of the cytopathic effect of HIV-1 and HIV-2 in MT-4 cell culture and HIV-1 in CEM cell culture. Both the triphenylcarbinol **3** as well as the triphenylmethane **5** were found to afford protection against the cytopathogenicity of HIV-2 in MT-4 cells and HIV-1 in CEM cells, but they were inactive against HIV-1 in MT-4 cells. Both substances were also found to inhibit syncytium formation when MOLT-4 cells were cocultured with HIV-2-infected HUT-78 cells, but were inactive in this assay against HIV-1-infected cells. When observed, the activity is generally moderate in degree of protection and requires concentrations in the 10^{-4} molar range. In contrast to ATA, both of these substances were inactive when tested for prevention of the binding of the OKT4A monoclonal antibody to the CD4 receptor and also for inhibition of HIV-1 reverse transcriptase. These substances therefore appear act by a mechanism that is distinct from that of polymeric ATA. Several active and inactive structural analogues of **3** and **5** were also synthesized. The anti-HIV activity in this series seems to depend on the presence of anionic carboxylate groups, since the methyl esters **4**, **6**, and **12** were uniformly inactive. The diphenylmethanes **8**, **14**, **18**, and **19** also reproducibly inhibited the cytopathic effect of HIV-1 in CEM cell culture.

Aurintricarboxylic acid (ATA) is a polymeric, solid substance that forms when a mixture of salicylic acid and formaldehyde is treated with sulfuric acid and sodium nitrite.[1] ATA is known to bind to the nucleotide binding sites of a variety of proteins that normally process nucleotides. Examples include the inhibitory effects of ATA on cell-free DNA[2-4] and RNA[4-8] polymerases, reverse transcriptase,[9,10] aminoacyl-*t*RNA synthetase,[11] ribonucleotide reductases,[12] and ribonucleases.[7,13-17] ATA inhibits cell free protein synthesis by blocking the attachment of mRNA to the ribosome,[18-20] and it also binds to the polynucleotide domains of the dihydroxyvitamin D_3[21] and glucocorticoid receptors.[22]

Recent interest in ATA has resulted from the reports that it inhibits the cytopathic effect of HIV in cell cultures

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