c = 0.4); ^{1}H NMR δ 7.63-7.55 (m, 2 H, aromatic protons), 7.43-7.29 (m, 3 H, aromatic protons), 6.36 (bs, 1 H, NH), 5.40 (bs, 1 H, NH), 3.09 (s, 1 H, OH), 2.21 (m, 2 H, CH_2), 0.98 (t, 3 H, J = 7.4, CH_3).

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Supplementary Material Available: MS, IR, and ^{13}C NMR data for **2-32** and ^{1}H NMR spectra of **17-32** (23 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

Structural Investigation and Anti-HIV Activities of High Molecular Weight ATA Polymers

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Aurintricarboxylic acid (ATA) was prepared from salicylic acid, formaldehyde, sulfuric acid, and sodium nitrite using a prolonged reaction time in order to favor the production of high molecular weight polymers. The resulting material was fractionated on the basis of molecular weight into nine fractions by equilibrium dialysis and ultrafiltration. Comparison of the NMR spectral data of these polymer fractions with the NMR spectra of low molecular weight ATA components provided a revised schematic representation of the structures of the ATA polymers used in the present study. The low molecular weight components were previously obtained from an ATA sample prepared using a limited reaction time. The anti-HIV activities of the ATA fractions were also investigated. The assays included prevention of the cytopathic effect of HIV-1 and HIV-2 in MT-4 cells, prevention of the cytopathic effect of HIV-1 in CEM cells, cytotoxicity in MT-4 cells, inhibition of syncytium formation between MOLT-4 cells and either HIV-1- or HIV-2-infected HUT-78 cells, and inhibition of HIV-1 binding to MT-4 cells. The potencies of the fractions in preventing the cytopathic effects of HIV-1 and HIV-2 increased as the average molecular weights of the fractions increased up to a dialysis molecular weight range of 7000-12 000 Da, which had weight average and number average molecular weights (M_w and M_n) of approximately 2937 and 2547, respectively. Further increase in the molecular weight beyond this point did not result in a further increase in potency, and the highest molecular weight fractions displayed decreased potencies for prevention of the cytopathic effects of HIV-1 and HIV-2 in MT-4 cells as well as for inhibition of syncytium formation between MOLT-4 cells and HIV-1 or HIV-2-infected HUT-78 cells.

Aurintricarboxylic acid (ATA) is a heterogeneous, polymeric substance that forms when a mixture of salicylic acid and formaldehyde is treated with sulfuric acid and sodium nitrite.[1-3] Interest in ATA has been stimulated by the observation that it prevents the cytopathic effect of HIV-1 in ATH8, MT-4, and HUT-78 cell cultures.[4,5] We recently fractionated ATA by a combination of equilibrium dialysis, ultrafiltration, and gel permeation chromatography and established a correlation between the average molecular weights of the ATA fractions and their potencies in preventing the cytopathic effects of HIV-1 and HIV-2 in several different cell cultures.[6] The molecular weights also correlated with activities in a number of related assays, including inhibition of syncytium formation between HIV-1- or HIV-2-infected HUT-78 cells and uninfected MOLT-4 cells, prevention of the binding of the OKT4A monoclonal antibody to the CD4 receptor, inhibition of binding of anti-gp120 monoclonal antibody to gp120, inhibition of the attachment of HIV-1 virions to cells, and inhibition of HIV-1 reverse transcriptase.[6] In all of these assays, the higher the average molecular weight, the higher the activity.[6]

The ATA sample used in these previous studies was prepared at 0 °C for 15 min, conditions which favor the formation of lower molecular weight material. In addition, the highest molecular weight cutoff value of the dialysis tubing used was 12 000 Da. An obvious and important question posed by these results is how far the correlation of molecular weight with activity would hold if fractions were obtained having higher average molecular weights. In order to obtain these higher molecular weight fractions for the present study, the polymerization reaction used to prepare ATA was performed for 64 h instead of 15 min, and additional dialysis membranes were used having molecular weight cutoff values greater than 12 000 Da. The potencies of the fractions in preventing the cytopathic effects of HIV-1 in CEM cell cultures as well as the cy-

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Table I. Weight Distributions, Viscosities, and Visible Absorptions of ATA Fractions

fraction no.	dialysis range (Da)	wt %	viscosity[a] (centistokes)	absorpn (λ 520 nm)	$(E^{1cm}_{1\%})$ (λ 700 nm)
1	>300 000	8.1	1.372	103.80	24.22
2	100 000–300 000	7.1	1.278	98.61	10.90
3	30 000–100 000	8.8	1.246	93.42	4.84
4	12 000–30 000	15.8	1.226	57.09	2.98
5	7000–12 000	11.8	1.224	55.01	2.77
6	3500–7000	7.9	1.218	29.41	2.08
7	2000–3500	7.2	1.216	34.60	3.81
8	1000–2000	5.8	1.202	18.17	1.90
9	<1000	27.5		8.65	0

[a] The viscosities of aqueous solutions of the ATA fractions in ammonium salt form (2.000 g/mL) were determined on a Cannon-Ubbelohde viscometer (size No. 25, International Research Glassware).

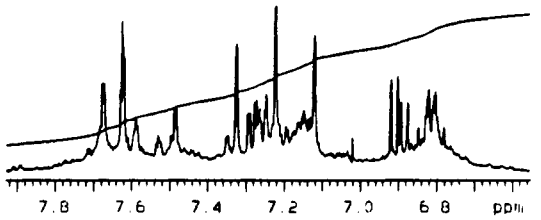


Figure 1. Partial 500-MHz ^{1}H NMR spectrum of the 2000–3500 ATA dialysis fraction.

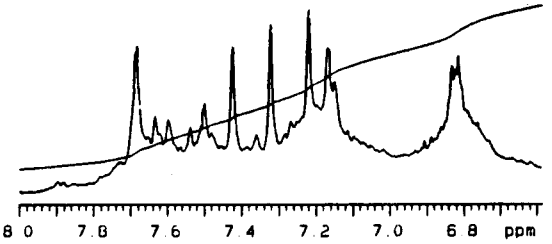


Figure 2. Partial 500-MHz ^{1}H NMR spectrum of the 3500–7000 ATA dialysis fraction.

topathic effects of HIV-1 and HIV-2 in MT-4 cell cultures were determined. The activities of the fractions were also examined in preventing syncytium formation between MOLT-4 cells cocultured with HIV-1 and HIV-2-infected HUT-78 cells, and the abilities of the fractions to prevent HIV-1 binding to MT-4 cells were determined. Furthermore, the ATA fractions were examined and characterized by ^{1}H and ^{13}C NMR spectroscopy and by visible absorption spectrophotometry.

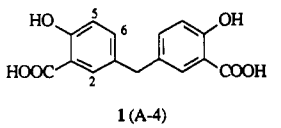
Results

The ammonium salt of ATA (aluminon) was prepared by a modification of the procedure published by Heisig and Lauer.[7] The material was then fractionated by equilibrium dialysis and ultrafiltration as outlined in Scheme I. The weight distributions of the fractions and the viscosities of their 2% (w/v) aqueous solutions are listed in Table I.

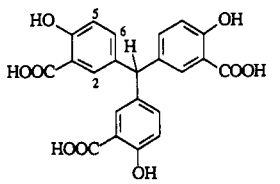
The nine fractions were characterized by ^{1}H NMR spectroscopy. Although the information about the structures of the components is obviously limited by the heterogeneity of the fractions, some insight is made possible by a prior study in which the structures of low molecular weight components were determined after isolation from ATA prepared using a 15-min reaction time.[8] The ^{1}H NMR chemical shift values of the aromatic protons of several of these compounds **1** (A-4), **2** (A-8), **3** (A-12), **4** (A-13), and **5** (A-15) are listed in Table II. The ^{1}H NMR and TLC data of the material obtained after acidification of fraction 9 with HCl indicated that it was almost entirely A-13.

Table II. ^{1}H NMR Chemical Shift Values of ATA Oligomers

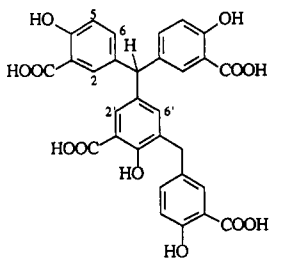
	H2	H5	H6	H2′	H6′	H2″	H5″	H6″
1 (A-4)	7.6	6.9	7.4					
2 (A-8)	7.5	6.9	7.2					
3 (A-12)	7.48	6.92	7.21	7.36	7.22	7.60	6.83	7.28
4 (A-13)	7.6	6.93	7.3					
5 (A-15)	7.64	6.92	7.27	7.50	7.28	7.50	6.83	7.25



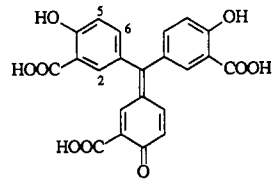
1 (A-4)



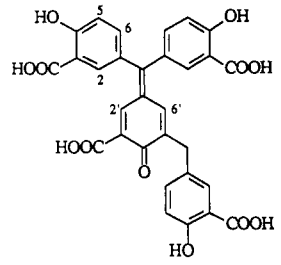
2 (A-8)



3 (A-12)



4 (A-13)



5 (A-15)

The 500-MHz ^{1}H NMR data for components A-12 and A-15 (Table II) indicate that the aromatic protons H5 ortho to the phenolic hydroxyl groups on branched ends, as at the top of structure **3** (A-12), are centered at δ 6.92, whereas those of H5″ on the unbranched end, as at the bottom of structure **3** (A-12), occur upfield at δ 6.83. As the average molecular weights of the ATA fractions increased, the intensities of the lower field peaks at δ 6.92 decreased relative to those of the upfield peaks at δ 6.83 (compare the spectra of fractions 7 and 6 in Figures 1 and 2). This suggests that as the average molecular weights of the ATA fractions increase, the ratio of unbranched to

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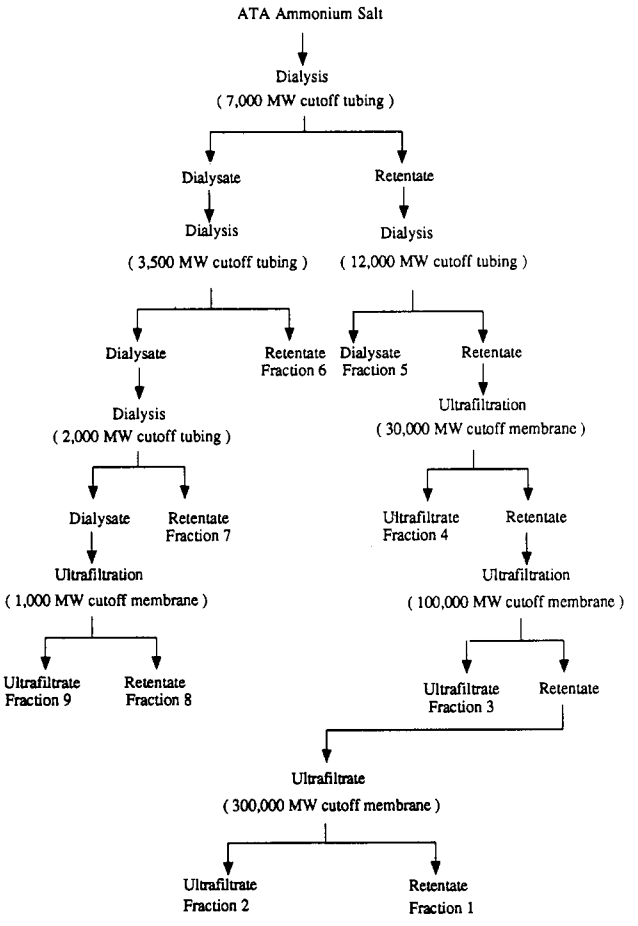
Scheme I



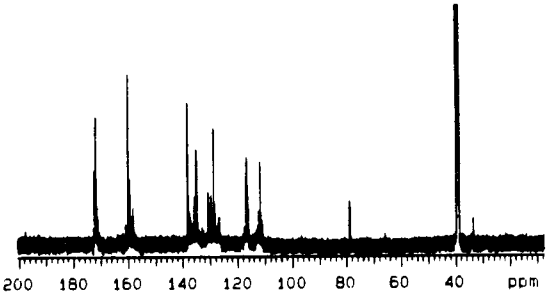


Figure 3. 125.7-MHz ^{13}C NMR spectrum of the 2000–3500 ATA dialysis fraction.

branched end units increases.

The data from ^{13}C NMR spectroscopy of the fractions and the purified components provided additional information about the linkages between the aromatic rings. The ^{13}C NMR spectra of components A-12 and A-15 displayed signals for the methylene carbons at δ 33.9, while the signals assigned methine carbons of A-8 and A-12 appeared at δ 52.3 and those of the central quinone methide carbons of A-13 and A-15 were at δ 79.0.[8] In all of the ATA fractions, peaks were found at δ 33.9 and 79.0, although in the higher molecular weight fractions the peaks corresponding to the methylene carbons appeared with decreased intensity. There were no signals corresponding to methine carbon in any of the fractions. These trends can be observed in Figures 3 and 4, corresponding to fractions 7 and 2, respectively.

The fractions were also subjected to 2D COSY NMR analysis. In the lower molecular weight fractions, obtained using the 1000–7000 MW cutoff dialysis membranes, distinct cross peaks were observed between the signals at δ 6.83 and 7.20, 6.92 and 7.28, 7.18 and 7.50, and 7.18 and 7.70 (e.g., Figure 5). These cross peaks result from trisubstituted salicylic acid rings. In the higher molecular weight fractions, cross peaks were not observed (e.g., Figure 6). The absence of cross peaks between these signals in the higher molecular weight fractions may reflect increased heterogeneity or peak broadening due to increased rotational correlation time.

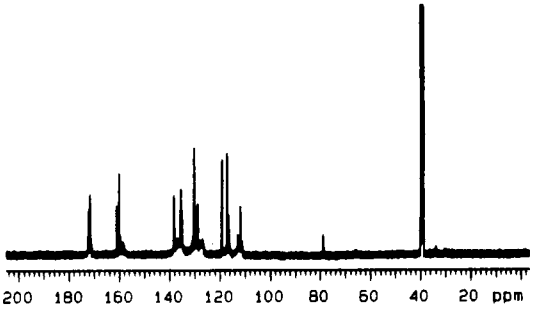


Figure 4. 125.7-MHz ^{13}C NMR spectrum of the 100 000–300 000 ATA dialysis fraction.

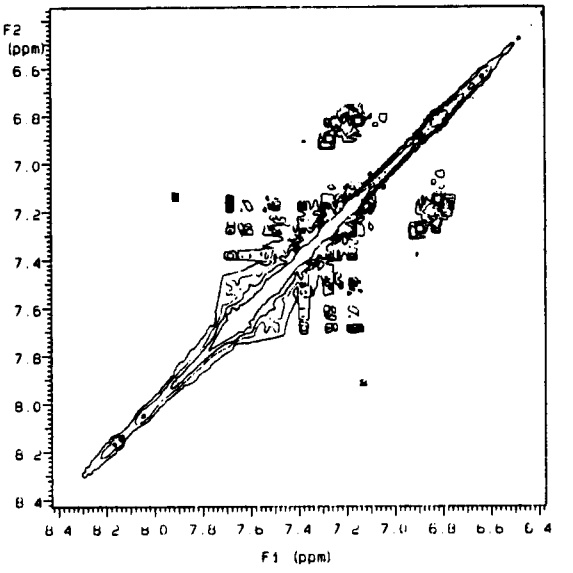


Figure 5. Partial 2D COSY NMR spectrum of the 3500–7000 ATA dialysis fraction.

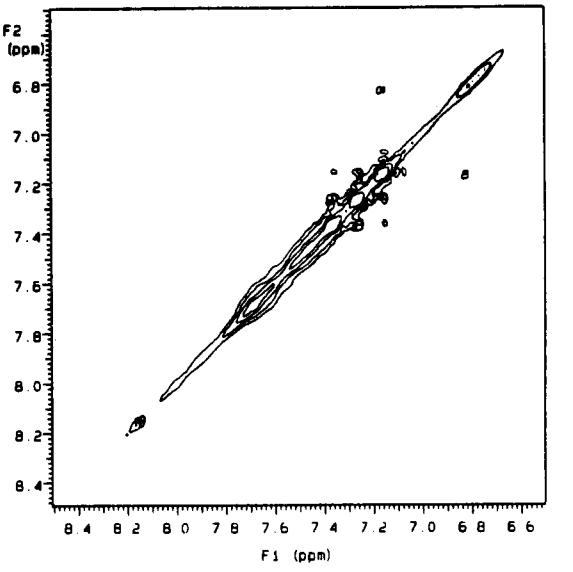


Figure 6. Partial 2D COSY NMR spectrum of the 30 000–100 000 ATA dialysis fraction.

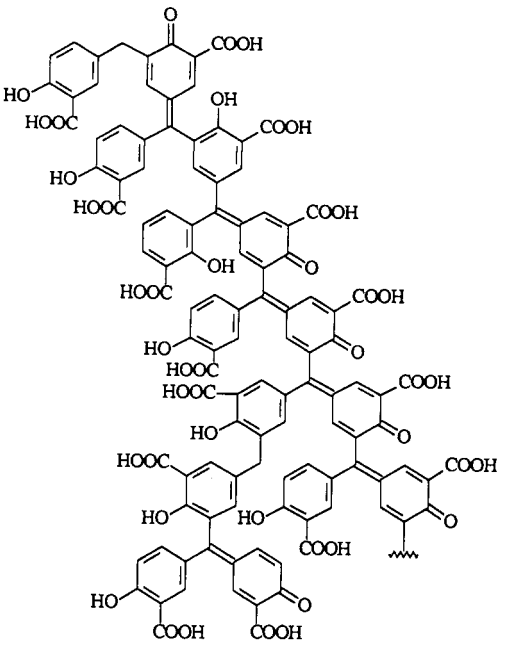
Table III. ^{13}C NMR Chemical Shifts of the One-Carbon Units Connecting the Aromatic Rings of ATA Polymer Fractions and Low Molecular Weight ATA Components

substance	δ	assignment
7[a]	82.2	trisalicylcarbinol in equilibrium with quinone methide[b]
	68.9	tetrasalicylcarbinol
	54.8	methine
	40.4	*p,p*-methylene[c]
	35.1	*o,p*-methylene
	29.8	*o,o*-methylene
1 (A-4)	38.72	*p,p*-methylene
2 (A-8)	52.32	methine
3 (A-12)	52.42	methine
	33.86	*o,p*-methylene
4 (A-13)	79.00	quinone methide
5 (A-15)	79.07	quinone methide
	33.95	*o,p*-methylene
8 (A-2)	28.42	*o,o*-methylene
9	80.59	trisalicylcarbinol
10 (A-10)	191.79	ketone
	33.62	*o,p*-methylene
6[d]	79.00	quinone methide
	33.99	*o,p*-methylene

[a] These ^{13}C NMR chemical shift values were reported for an unfractionated sample of ATA which was assigned the schematic structure 7 as reported in ref 2. [b] This assignment was offered in ref 2. Our subsequent studies have shown that trisalicylcarbinol 9 and quinone methide 4 can be observed separately by ^{13}C NMR spectroscopy.[8] [c] The designations *p,p*-methylene, *o,p*-methylene, and *o,o*-methylene refer to linkages relative to the phenolic hydroxyl groups of the attached aromatic rings. [d] These ^{13}C NMR chemical shift values were observed in ATA fractions having equilibrium dialysis MW cutoff values of 2000 and above.

The visible absorption spectra of the fractions were also recorded in aqueous solution and are reported in Table I. As the molecular weights of the fractions increased, the absorption values also increased, indicating more extended conjugation in the higher molecular weight fractions.

A schematic representation of the present ATA preparation is proposed as structure **6**. This structure is consistent with the spectroscopic data as well as the prior study on the structures of low molecular weight ATA oligomers prepared using a shorter reaction time.[8] To aid comparison, the ^{13}C NMR chemical shift values of the one



6

carbon units connecting the aromatic rings of the ATA polymer fractions and low molecular weight components are presented together in Table III. The data indicate the presence of quinone methide units and the absence of trisalicylcarbinol and trisalicylmethane units. The disalicylmethane units are linked ortho–para and not ortho–ortho or para–para relative to the phenolic hydroxyl groups.

The biological activities of the nine fractions were determined and the results are listed in Table IV. The assays included prevention of the cytopathic effect of HIV-1 and HIV-2 in MT-4 cells, prevention of the cytopathic effect of HIV-1 in CEM cells, cytotoxicity of the compounds in MT-4 cells, inhibition of syncytium formation between MOLT-4 cells and either HIV-1- or HIV-2-infected HUT-78 cells, and inhibition of HIV-1 binding to MT-4 cells.

Table IV. Anti-HIV Activities of the ATA Ammonium Salt (Aluminon) Fractions Listed in Table I[a]

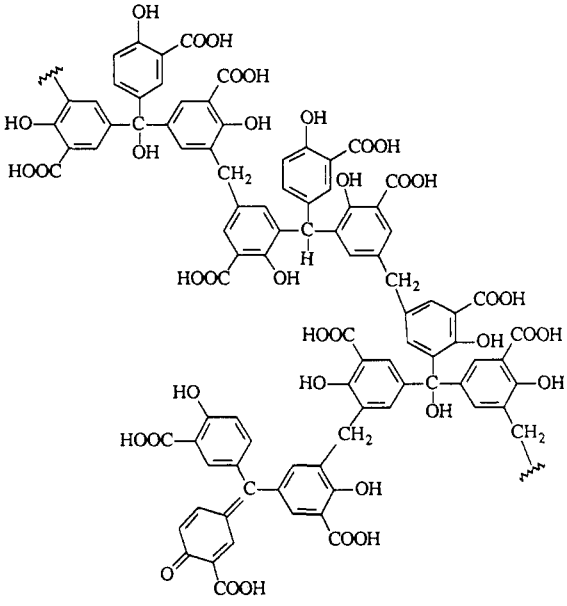
	IC_{50} (μg/mL)						
	HIV cytopathicity				giant cell assay[b] MOLT-4 cells co-cultured with		
fraction no. (dialysis range)	HIV-1 MT-4 cells	HIV-2 MT-4 cells	HIV-1 CEM cells	cell viability[c]	HIV-1 infected HUT-78 cells	HIV-2 infected HUT-78 cells	inhibitory index of HIV-1 binding to MT-4 cells (II_{VB})[d]
1 (>300 000)	9.2	9.1	0.6	35	5	17	0.76
2 (100 000–300 000)	3.9	8.3	0.6	48	2	10	1.00
3 (30 000–100 000)	2.8	5.9	0.4	74	1	7	0.91
4 (12 000–30 000)	2.7	6.7	0.5	62	4	3	0.91
5 (7000–12 000)	2.5	7.1	0.5	49	8	1	0.88
6 (3500–7000)	6.8		5.1	45	30	1	0.72
7 (2000–3500)	9.9	8.4	8.0	47	30	7	0.85
8 (1000–2000)	>40	10.3	16.5	38	30	9	0.63
9 (<1000)	>40	>40	>50	41	>100	40	0.91

[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 at least 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%. [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. [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: $II_{VB} = 1 - (MF_{VC} - MF_C3c)/(MF_V - MF_C)$, whereby MF_{VC} is the mean fluorescence (MF) with a given concentration of the compound in HIV-1-inoculated cells, MF_C3c 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).

Discussion

The equilibrium dialysis molecular weight ranges listed in Tables I and IV are derived using unbranched polysaccharides and therefore are not a valid indication of the actual molecular weights of branched polymers like ATA. The weight average and number average molecular weights of branched polymers like ATA are significantly lower than indicated by the dialysis ranges, since branched polymers cannot worm through the pores in the dialysis membranes like unbranched polysaccharides. As part of our prior study of the fractionation of an ATA sample obtained using a 15-min reaction time, the weight average molecular weights (M_W) were determined for the lower dialysis ranges as follows: >12 000 (M_W 3336); 7000–12 000 (M_W 2326); 3500–7000 (M_W 1609); 2000–3500 (M_W 1437); 1000–2000 (M_W 1149); 500–1000 (M_W 475); <500 (M_W 328). These values provide an approximation of the actual weight average molecular weights of fractions 5–9 listed in Tables I and IV. The actual weight average molecular weights of fractions 1–9 in Tables I and IV were not determined for the ATA fractions used in the present study.

The representation **6** proposed here for ATA differs from structure **7** previously proposed by Gonzáles, Blackburn, and Schleich.[2] An obvious difference is the absence of



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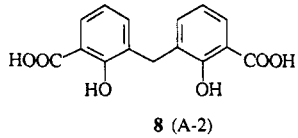
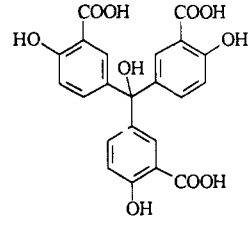


8 (A-2)



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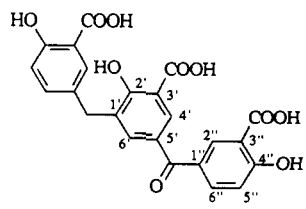
methine carbons in **6**, which were clearly present in the ATA sample studied previously, as indicated by an intense peak at δ 54.8 in the ^{13}C NMR sample of ATA prepared using ^{13}C enriched formaldehyde.[2] We recently isolated the trisalicylmethane **2** (A-8) from a low molecular weight fraction of ATA, isolated from an ATA sample prepared using a 15-min reaction time, and determined that the central, methine carbon of **2** (A-8) appears at δ 52.83.[8] The absence of any signal in this region of the ^{13}C NMR spectra of the present ATA fractions is therefore noteworthy. The ATA studied by Gonzáles et al. was an unfractionated sample prepared by the procedure of Heisig and Lauer, which utilizes a reaction time of "a few minutes".[7] The methine carbon signal which they observed in the ^{13}C NMR spectrum of their sample might therefore be due to the presence of the low molecular weight trisalicylmethane **2** (A-8), as well as compound **3** (A-12) and related low molecular weight materials which were factored into their ATA polymer structure. It is likely that the absence of methine carbons observed in the present ATA fractions results from extensive oxidation of methine to quinone carbons during the 64-h reaction time employed to favor production of high molecular weight ATA polymers containing extended conjugation.

An additional distinction between our ATA fractions and the ATA studied by Gonzáles et al. is the presence of intense signals at δ 40.4, 35.1, and 29.8 in their preparation, attributed to methylene carbons linked para–para, ortho–para, and ortho–ortho to phenols, whereas our fractions contained only a weak signal at δ 33.99. Since their ATA preparation used for NMR studies was unfractionated, the three methylene signals may have been due to the three possible methylenedisalicylic acids. Indeed, we previously isolated two of the three possible methylenedisalicylic acids, compounds **1** (A-4) and **8** (A-2), from the ATA prepared using the 15-min reaction time.[8] The methylene carbons of **1** and **8** appeared at δ 38.72 and 28.42, respectively.[8] The weak signal at δ 33.99 in the present ATA fractions (Figures 3 and 4) appears to be due to methylenes linked ortho–para to phenolic groups.

Another difference between the schematic representations **6** and **7** is the absence of trisalicylcarbinol moieties in **6**. The low molecular weight quinone methide component **4** (A-13) has been isolated from the ATA sample prepared using a 15-min reaction time and its conversion to the covalent hydrate **9** in acetone containing water has been studied.[8] The trisalicylcarbinol **9** has also been synthesized and characterized.[3] The results have indicated that the trisalicylcarbinol **9** dehydrates to the quinone methide **4** in the presence of acid, and that the quinone methide **4** hydrates to the trisalicylcarbinol **9** under neutral or basic conditions. The carbinol carbon of **9** has been assigned to a signal at δ 80.59 in its ^{13}C NMR spectrum, while the central carbon of the quinone methide **4** (A-13) has been assigned to a peak at δ 79.00. The ^{13}C NMR spectra of all of the ATA fractions obtained in the present study displayed only one signal in this region at δ 79 (Figures 3 and 4). This indicates that although the quinone methide **4** (A-13) is hydrated to the trisalicylcarbinol **9** in the presence of water, the trisalicylmethane units in the ATA polymer obtained after precipitation from aqueous HCl exist in the dehydrated, quinone methide form when observed in DMSO-d_6. These dehydrated, quinone methide moieties allow extended conjugation and may therefore be energetically favored in the polymer relative to the trisalicylcarbinol forms.

We also previously obtained a ketone-containing oligomer **10** (A-10) from the ATA sample prepared using the 15-min reaction time.[8] The ketone carbon appeared in



10 (A-10)

the ^{13}C NMR spectrum of this material at δ 191.79.[8] The

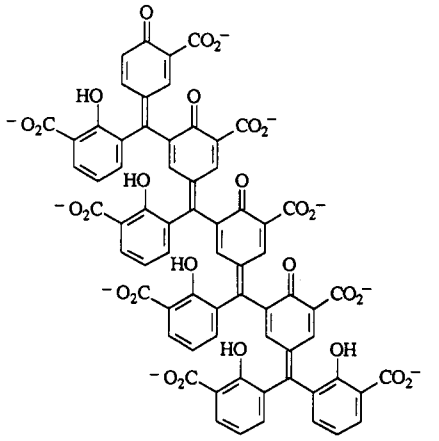
absence of signals in this region of the ^{13}C NMR spectra of the ATA fractions prepared using the 64-h reaction time shows that similar ketones are not present. This indicates that although benzophenone moieties do not occur in the present ATA polymer, substituted benzophenones are involved as intermediates in the polymerization reaction pathway leading to ATA.

Overall, the spectral data for the present ATA fractions obtained using the 64-h reaction time are unexpectedly simple and indicate a much more regular schematic structure 6 for the polymer as opposed to the more complicated prior representation 7. In contrast, ATA prepared with a short reaction time contains a variety of lower molecular weight substances that are more structurally diverse.[8]

As mentioned above, the ATA sample previously obtained with a 15-min reaction time was separated into fractions corresponding to 5–9 in Tables I and IV.[6] The antiviral activities of those fractions increased as the weight average molecular weights increased. One of the main reasons for undertaking the present investigation was to determine how far that relationship could be pushed before a plateau in potency is reached. The results in Table IV show quite clearly that there is no further increase in potency as the molecular weights are increased further beyond fraction 5, and that there is in fact an eventual decrease in potency for the highest molecular weight fractions studied. A similar profile of potencies as a function of molecular weights of ATA fractions was reported recently for inhibition of shear stress-induced, von Willebrand factor (vWF)-mediated platelet aggregation.[9]

Besides inhibiting the cytopathic effects of HIV-1 and HIV-2 in cell cultures, various ATA preparations also inhibit a variety enzymes that process oligonucleotides, including DNA[10-12] and RNA[12-16] polymerases, reverse transcriptase,[5,17] aminoacyl-*t*RNA synthetase,[18] ribonucleotide reductases,[19] and ribonucleases.[15,20-23] They also block the attachment of mRNA to ribosomes in cell free systems[24-26] and bind to the polynucleotide domains of the dihydroxyvitamin D_3[27] and glucocorticoid receptors.[28] We have also recently demonstrated that a variety of ATA fractions obtained by equilibrium dialysis, ultrafiltration, and gel permeation chromatography, as well as several ATA monomers and monomer analogues, inhibit the HIV-1 integration protein.[29] The available evidence indicates that various ATA preparations inhibit these protein nucleic acid interactions by binding to the oligonucleotide binding sites on the proteins instead of the nucleic acids.[13,21] The recognition of ATA by oligonucleotide processing proteins may result from the resemblance of its polymeric and polyanionic nature of the phosphodiester backbone of oligonucleotides. The distance between the carboxylate anions of ATA at physiological pH may approximate the distance between the negatively charged phosphates in nucleic acids. In addition, the salicylic acid units branching at regular intervals from the ATA "backbone" may stack and resemble the bases present in nucleic acids. In this sense, ATA could be functioning as a metabolically stable oligonucleotide mimetic.

Molecular modeling experiments were therefore undertaken to determine the conformation of an idealized ATA oligomer and measure the distances between the carboxylate anions. An ATA tetramer 11 was constructed



11

using the ChemNote application of Quanta (Polygen/Molecular Simulations, 796 North Pastoria Avenue, Sunnyvale, CA 94086). The residue topology file created by ChemNote was amended to maintain the quinone rings in a planar conformation by adding additional improper dihedral interactions similar to those used in aromatic ring systems. The tetramer was then manipulated using the torsions option into a conformation in which the quinoid "backbone" formed a slight helix and the salicylate groups were stacked nearly orthogonal to the quinoid "backbone" axis. A 15-Å equilibrated solvent sphere of TIPS3P water molecules was created around the tetramer and then the entire system minimized with CHARMm using a distance-dependent dielectric constant and a conjugate gradient minimization algorithm. The resulting conformation of the tetramer was a right-hand helix in which the average distance between the carbonyl carbons of the carboxylate anions on the ATA "backbone" was 6.1 Å, compared with the average distance between the phosphorus atoms of ApApApAp of 5.6 Å for the A-form (RNA) and 6.5 Å for the B-form (DNA) generated in Quanta using the Bio-

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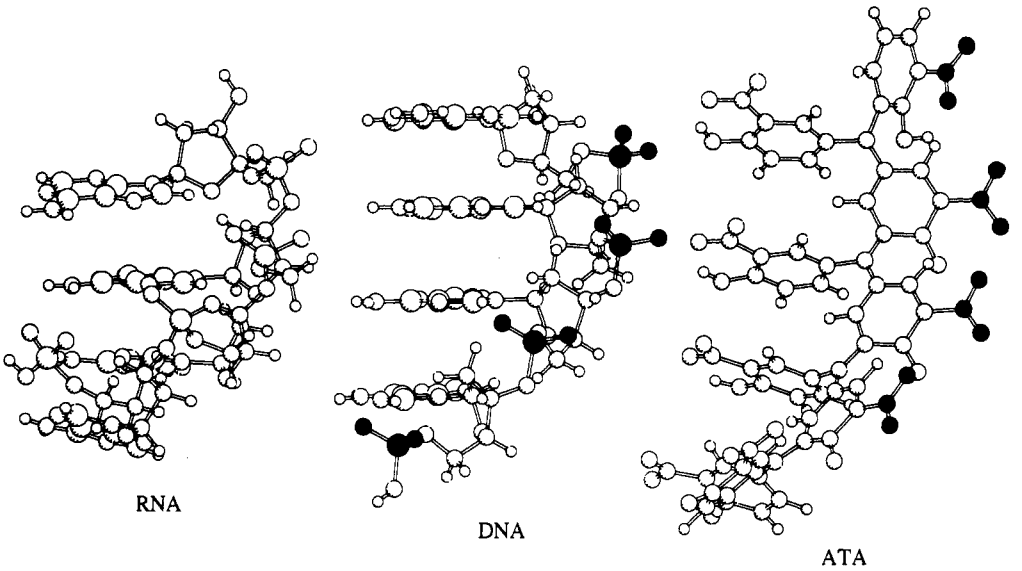

Figure 7. Comparison of the structures of RNA, DNA, and ATA tetramers as shown by molecular modeling. The PO_2^- groups of DNA and the backbone CO_2^- groups of ATA are shown in black.

polymer Build application (Figure 7). An average angle of 32° between the least-square planes of successive quinone rings indicates approximately 11 quinone rings of ATA per turn of the helix compared with 11 bases (A-form) or 10 bases (B-forms) for RNA and DNA, respectively. This preliminary molecular modeling data strongly supports the idea that ATA may be functioning as a metabolically stable oligonucleotide mimetic.

Experimental Section

^1H NMR Spectra were recorded at 500 MHz, and ^{13}C NMR spectra were obtained at 125.7 MHz. All NMR spectra were obtained on an NMR spectrometer equipped with a 5-mm, variable-temperature, indirect detection probe. The ^1H COSY spectra were obtained using a standard Varian pulse sequence. The ATA samples used in the NMR studies were obtained by acidification of aqueous ATA ammonium salt solutions with HCl. The resulting solids were dissolved in DMSO-d_6. The NMR samples were referenced to TMS as an internal standard.

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 during 30 min. The reaction flask was removed from the ice bath and allowed to stir at room temperature for 10 min. 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. The reaction mixture was allowed to stir slowly at 0 °C for an additional 64 h. 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 reddish. After being stirred at room temperature for 30 min, the suspension was filtered. A red solid was recovered. About 300 mL of distilled water was added to this solid, and the mixture was stirred for 20 min and then filtered. The washing was repeated for three more times. Finally, dilute ammonium hydroxide 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 17 h at room temperature. A dark red powder (8.05 g) was recovered, which is the ammonium salt of ATA (aluminon).

Dialysis of Aluminon. Aluminon (6.95 g) was taken up in about 40 mL of distilled water. This solution was placed evenly into four dialysis bags (Spectrapor, MW cutoff 6000–8000 Da, Scientific Products Division). The bags were immersed in a 4-L beaker containing about 3800 mL of distilled water. Stirring was started. After about 4 h, the combined retentates were concentrated to about 40 mL and placed in the bags, and dialysis was continued for another 6 h. The retentates were then concentrated and dialized again for an additional 12 h. At this time, the retentate was concentrated again and dialized in 3800 mL of fresh distilled water for 24 h. The retentates were dialized again in 3800 mL of fresh water, and the process was repeated again for 24 h. The total dialysate was about 12 L and the dialysis time was about 70 h. The last 3800 mL of dialysate was quite light color, suggesting that almost all ATA molecules smaller than the apertures of the dialysis tubings had diffused out. Other dialysis tubings used were as follows: (1) Dialysis "Sacks", Sigma Chemical Co., MW cutoff 12000; (2) Spectrapor, MW cutoff 3500, Scientific Products Division; (3) Spectrum, MW cutoff 2000, Spectrum Medical Industries, Inc.

Ultrafiltration of Aluminon. An Amicon 8200 ultrafiltration apparatus with a 200-mL capacity cell and four membranes (Amicon Division, Danver, MA), Amicon YM2 (MW cutoff value 1000), YM30 (MW cutoff value 30000), YM100 (MW cutoff value 100000), XM300 (MW cutoff value 300000) were used. Aluminon samples were dissolved in about 180 mL of distilled water, and the solution was placed in the cell and run under 40 psi. When about 40 mL of solution was left in the cell, the ultrafiltration was stopped, fresh distilled water (140 mL) was added, and the ultrafiltration was continued. When the ultrafiltrate was very light color, the ultrafiltration was stopped. A total of 700 mL of ultrafiltrate was collected from five operations.

Giant Cell Assay. This assay is based on syncytium formation and destruction of $CD4^+$ cells (MOLT-4) cocultured with T cells (HUT-78) persistently infected with HIV as demonstrated by flow cytometry.[30]

Cell Viability Assay. Cell viability was quantified by a tetrazolium (MTT) colorimetric method in 96-well microtrays.[31]

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.[32]

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Cell Viability Assay in HIV-1 Infected and Uninfected CEM Cells. Cell viability was determined by a tetrazolium (XTT) assay.[33]

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Supplementary Material Available: Molecular modeling parameters for ATA structure 11 (1 page). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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Regioselective Introduction of Carbon-3 Substituents to 5-Alkyl-7-methoxy-2-phenylbenzo[*b*]furans: Synthesis of a Novel Adenosine A_1 Receptor Ligand and Its Derivatives[1]

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By maintaining the balance between the electronic requirements, the stereochemical restrictions as well as the kinetic and thermodynamic factors, the unprecedented regioselective electrophilic aromatic substitution of formyl and nitro groups to carbon-3 of 5-alkyl-7-methoxy-2-phenylbenzo[*b*]furans have been achieved. Subsequent transformation of the resulting formyl group into methyl, hydroxymethyl, 1-hydroxyethyl, and cyano groups are also described.

Introduction

Considerable attention has been devoted recently to the potential therapeutic properties of *Salvia miltiorrhiza* Bunge (Danshen), whose aqueous extracts have been used widely in China to treat acute myocardiac infarction and angina pectoris.[3] In the course of our own evaluation of the pharmacological profile of the aqueous extracts of Danshen, we observed a significant inhibition of [^{3}H]-phenylisopropyladenosine binding to the adenosine A_1 receptor on bovine cerebral cortex membranes.[1b] In the hope of identifying potential cardiovascular compounds,[4] we have initiated a program to isolate the active component from Danshen, being monitored by the adenosine A_1 radioligand binding assay.[5,6] A new compound, 5-(3-hydroxypropyl)-7-methoxy-2-(3′-methoxy-4′-hydroxyphenyl)benzo[*b*]furan-3-carbaldehyde (**1**), with an exceedingly high potency (IC_{50} = 17 nM), was isolated as a yellowish oil.[1b] It is noteworthy that one of the most potent A_1-selective adenosine antagonists,[7-10] namely 1,3-dipropyl-8-cyclopentylxanthine (CPX) (**2**), shows extremely high A_1 affinity (IC_{50} = 0.92 nM).[11,12] Another well-known adenosine antagonist is 4-amino-8-hydroxytriazoloquinazoline (**3**).[13,14]

Unlike **2** and **3**, which are nitrogen-containing compounds, compound **1** has a skeleton devoid of nitrogen but is strikingly similar peripherally to **2** and **3**. Compound **1** therefore provides a new kind of structure having a high A_1 receptor antagonist activity and is relatively soluble in water. We report here the total synthesis of **1** as well as

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