Anti-HIV-1 Activity of Poly(mandelic acid) Derivatives

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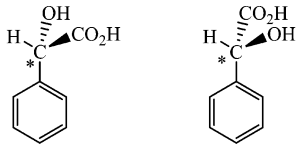
Received February 22, 2007; Revised Manuscript Received September 7, 2007

Homo- and heterochiral poly(mandelic acid)s (PMDAs) were synthesized under strongly acidic, mildly acidic, and nonacidic conditions. The water-soluble fractions of these polymers were evaluated with respect to their inhibitory activity against the human immunodeficiency virus (HIV-1). Polymers were prepared via a step-growth mechanism, yielding linear polyesters. The polymers were characterized by CHS elemental microanalysis, X-ray fluorescence (XRF), and FT-IR spectroscopy. Polymers prepared by the three methods have different structures. Both elemental microanalysis and XRF indicated the presence of S in those polymers prepared by treatment with concentrated H_2SO_4, which were the only ones exhibiting inhibitory and virucidal activity against HIV-1, mediated by their binding to cellular co-receptor binding sites on the virus envelope glycoprotein gp120. Additionally, FT-IR spectroscopy indicated the complete absence of C═O functionality in the H_2SO_4-prepared PMDA.

Introduction

Mandelic acid (MDA), Figure 1, is a simple chiral compound that has been employed as a reagent facilitating the resolution of chiral alcohols.[1] In addition, it has been demonstrated that MDA can be polymerized by both radical (under γ-irradiation conditions)[2] and step-growth mechanisms. The step-growth polymerizations can be acid catalyzed,[3,4] employing species such as concentrated sulfuric acid, *p*-toluenesulfonic acid (*p*-TolSO_3), or Nafion. Alternatively, PMDA can be prepared under simple bulk melt conditions.[4,5] Given the presence of the chiral carbon (denoted by the star in Figure 1), one additionally has the option of preparing homochiral or heterochiral (co)polymers, that is, polymers derived from either the *R* or the *S* enantiomer, a nonstoichiometric mixture of the *R* and *S* species, or the racemate.[4]

MDA monomer has long been known to possess therapeutic properties. In particular, it has been demonstrated to be a highly effective treatment for urinary tract infections and to possess certain advantages over more conventional antibiotics. For example, methenamine mandelate is marketed in the U.S. under the name Mandelamine.[6] Recently, PMDA has attracted attention as a viable candidate in various biomedical applications. For example, Zaneveld et al.[7] have described the contraceptive and antimicrobial activity of PMDA synthesized via the concentrated sulfuric acid treatment of mandelic acid. Additionally, Herold and co-workers[8,9] reported the application of PMDA as a novel microbicide to prevent the sexual transmission of both human immunodeficiency virus (HIV-1) and herpes simplex virus (HSV). However, the exact structure of the tested materials has not been established. They are prepared in the presence of concentrated H_2SO_4 and referred to as SAMMA (sulfuric acid-modified mandelic acid), yet the materials tested by Zaneveld and Herold are apparently different from the expected structures (vide infra). According to the patented procedure of Zaneveld, the PMDA is prepared from the racemate by heating in concentrated H_2SO_4, yielding a "light burnt orange" powder with significant water solubility. The authors reported that elemental microanalysis of the final product indicated the absence of sulfur. Indeed, the absence of detectable sulfur in either the sulfate or the sulfonate functional forms is highlighted as one of the novel features of these materials.[9]



S-Mandelic acid *R*-Mandelic acid

Figure 1. Chemical structures of *S*- and *R*-mandelic acid.

Table 1. Synthesis of Poly(mandelic acid) with Concentrated H_2SO_4

stereoisomer and sample ID	monomer added (g)	H_2SO_4 (mL)	polymer recovered (g)	yield (%)
R, MW-1-13-9a	5.00	5.00	3.03	60.6
S, MW-1-13-10a	5.00	5.00	2.67	53.4

Concentrated H_2SO_4 is a well-documented sulfonating agent of aromatic rings,[10,11] as well as an oxidizing agent capable of reacting with alcohols to form alkyl sulfonate esters, and thus provides at least two potential pathways by which sulfur-containing functional groups could be incorporated into the polymer structure. Therefore, we decided to conduct model experiments aimed at probing the structure−biological activity relationships of PMDA prepared under both acid-catalyzed and noncatalyzed conditions. We describe herein the preparation of PMDA via a step-growth mechanism under three different synthetic conditions: (i) those described by Zaneveld et al.;[3] (ii) under mild acid catalysis with *p*-TolSO_3; and (iii) in the absence of an added acid catalyst, that is, under simple melt conditions. Additionally, we have prepared hetero- and homo-

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10.1021/bm070221y CCC: $37.00
Published on Web 10/20/2007

Table 2. Synthesis of Poly(mandelic acid) with *p*-Toluenesulfonic Acid (*p*-$TolSO_3$)

stereoisomer and sample ID	monomer added (g)	*p*-$TolSO_3$ (g)	benzene added (mL)	polymer recovered (g)	yield (%)
R,*S*, MW-1-25-15a	10.00	0.0125	50.0	3.61	36.1
R,*S*, MW-1-33-19a	10.00	0.0125	50.0	3.29	33.0
R, MW-1-16-11a	10.00	2.26	300.0	9.54	95.4
R, MW-1-27-17a	7.00	0.009	50.0	0.764	10.9
S, MW-1-17-12a	10.0	2.26	300.0	10.0	100
S, MW-1-29-18a	7.00	0.009	50.0	1.42	20.2

Table 3. Synthesis of Poly(mandelic acid) under Melt Conditions

stereoisomer and sample ID	monomer added (g)	polymer recovered (g)	yield (%)
R,*S*, MW-1-25-13a	10.00	7.81	78.1
R,*S*, MW-1-39-20a	10.00	6.48	64.8
R, MW-1-25-14a	10.00	7.51	75.1
R, MW-1-39-21a	5.00	3.24	64.9
S, MW-1-26-16a	10.0	5.28	52.8
S, MW-1-39-22a	5.00	3.36	67.2

chiral products using the three different techniques in an effort to probe the effect, if any, of stereochemistry on the properties of the resulting materials. Water-soluble fractions from these materials were evaluated for their virus inhibitory and virucidal activities against HIV-1 IIIB (a virus utilizing CXCR4 as a cellular co-receptor = X4 virus) and HIV-1 BaL (a virus utilizing CCR5 as a cellular co-receptor = R5 virus). R5 viruses are more frequently transmitted sexually.

Experimental Section

DL-Mandelic acid was purchased from Sigma, St. Louis, MO. *R*-(−) and *S*-(+) mandelic acids and polyethylene glycol (PEG) 8000 NF, respectively, were from Spectrum, New Brunswick, NJ.

Recombinant proteins employed were HIV-1 IIIB gp120, biotinyl-HIV-1 IIIB gp120, CD4, and biotinyl-CD4 (ImmunoDiagnostics, Inc., Woburn, MA); and HIV-1 IIIB BaL gp120 and FLSC (a full length single chain protein consisting of BaL gp120 linked with the D1D2 domains of CD4 by a 20 amino acid linker) (produced in transfected 293T cells).[12] Monoclonal antibodies (mAb) 9284, specific for the gp120 V3 loop, were from NEN Research Products, DuPont, Boston, MA. Monoclonal antibodies NC-1 were raised against the HIV-1 IIIB gp41 six-helix bundle[13] and were shown to react also with six-helix bundles from HIV-1 BaL.[14] Rabbit antibodies against the gp41 six-helix bundle were prepared as described.[15] Pelletted, 1000-fold concentrates of HIV-1 IIIB (6.8×10^{10} virus particles/mL) and BaL (2.47×10^{10} virus particles/mL) were from Advanced Biotechnologies, Inc., Columbia, MD. MT-2 cells, HeLa-CD4-LTR-β-gal, and U373-MAGI-CCR5E cells (both contributed by Dr. Michael Emerman) and Cf2Th/synCCR5 cells (contributed by Dr. Tajib Mirzabekov and Dr. Joseph Sodroski) were obtained from the AIDS Research and Reference Reagent Program operated by McKesson BioServices Corp., Rockville, MD.

Biotinyl-CD4, horseradish peroxidase (HRP)-labeled streptavidin, and HRP-labeled goat anti-rabbit IgG were from ImmunoDiagnostics Inc. (Woburn, MA), Zymed (South San Francisco, CA), and Southern Biotechnology Associates, Inc. (Birmingham, AL), respectively. The Galacto-Light Plus System chemiluminescence reporter assay for quantitation of β-galactosidase was obtained from Applied Biosystems, Foster City, CA. Dulbecco's modified Eagle medium (DMEM) was from GIBCO Invitrogen Corp., Carlsbad, CA.

All other chemicals were purchased from Aldrich Chemical Co., Milwaukee, WI, and were used as received unless stated otherwise. Poly(sodium 4-styrenesulfonate) was obtained from Alco Chemical Co., Chattanooga, TN. Phosphate buffer was prepared as follows: In a 1 L volumetric flask were added potassium dihydrogen phosphate (68.0 g, 0.5 mol) and disodium hydrogen phosphate heptahydrate (134.0 g, 0.5 mol). The salts were then dissolved in deionized water (850 mL). After complete dissolution, the pH was adjusted to 6.98 by the addition of sodium hydroxide (NaOH). The solution was then fully diluted to 1 L with deionized water.

The buffer used to lyse and inactivate HIV-1 ("lysis buffer") was 1% Nonidet P40 (NP40), 100 ug/mL bovine serum albumin (BSA) in phosphate buffered saline, pH 7.2 (PBS).

Polymerization of Mandelic Acid in Concentrated H_2SO_4. Polymers were prepared according to the method of Zaneveld et al.[3] Below is a typical procedure for the polymerization of *R*-mandelic acid.

To a 100 mL round-bottomed flask equipped with a magnetic stir bar was added concentrated sulfuric acid (5.0 mL). The flask was then immersed in an oil bath at room temperature. *R*-Mandelic acid (5.0 g, 33.0 mmol) was then added to the concentrated sulfuric acid with stirring over a period of ~20 min. During the addition, the temperature was allowed to rise to ~55 °C. Subsequently, the solution was heated at 80 °C for 40 min. The polymerization was terminated by the addition of ice-cold ethanol (90.0 mL). The *S*- and *R*,*S*-mandelic acids were polymerized in an identical fashion.

Polymerization of Mandelic Acid in the Presence of *p*-Toluenesulfonic Acid (*p*-$TolSO_3$). Below is a typical procedure for the acid-mediated polymerization of *R*-mandelic acid:

To a 500 mL round-bottomed flask equipped with a Dean−Stark trap, magnetic stir bar, and water condenser were added *p*-$TolSO_3$ (2.26 g, 11.9 mmol), *R*-mandelic acid (10.0 g, 65.7 mmol), and benzene (300 mL). The flask was immersed in a preheated oil bath at 106 °C, and the reaction was allowed to proceed for 4 h. After this time, the hot solution was filtered to remove the cyclic mandelide dimer[16] that had formed as a byproduct of the polymerization. The solution was transferred to a rotary evaporator where the benzene was removed in vacuo. The isolated solid was then dried in a vacuum oven overnight. *S*-Mandelic acid was polymerized in a similar fashion.

Melt Polymerization of Mandelic Acid. Below is a typical procedure for the melt polymerization of *R*-mandelic acid: To a 300 mL beaker was added *R*-mandelic acid (10.0 g, 65.7 mmol). The beaker was then immersed in a pre-heated oil bath at 180 °C. The polymerization was allowed to proceed for 3 h.

Isolation of Water-Soluble Poly(mandelic acid) (PMDA) Fractions. Water-soluble fractions of the polymers prepared in concentrated sulfuric acid were isolated according to the patent procedure described by Zaneveld et al.[3] For those polymers prepared under melt conditions or in the presence of *p*-$TolSO_3$, a different procedure was adopted: To a 250 mL separatory funnel were added finely ground PMDA (~1.0 g), 0.5 M phosphate buffer (~13.0 mL), and ethyl acetate (10.0 mL). After thorough mixing, the aqueous and organic phases were collected. Water-soluble PMDA species were subsequently isolated by freeze-drying. The concentration of PMDA sequestered into the phosphate buffer was determined by UV spectroscopy.

General Analysis. UV−vis spectra were recorded on a Shimadzu UV-2401 PC spectrophotometer using a 1 cm quartz cuvette. CHS elemental microanalysis was performed by Quantitative Technologies Inc., Whitehouse, NJ. Size exclusion chromatographic (SEC) analysis was conducted in tetrahydrofuran at room temperature at a flow rate

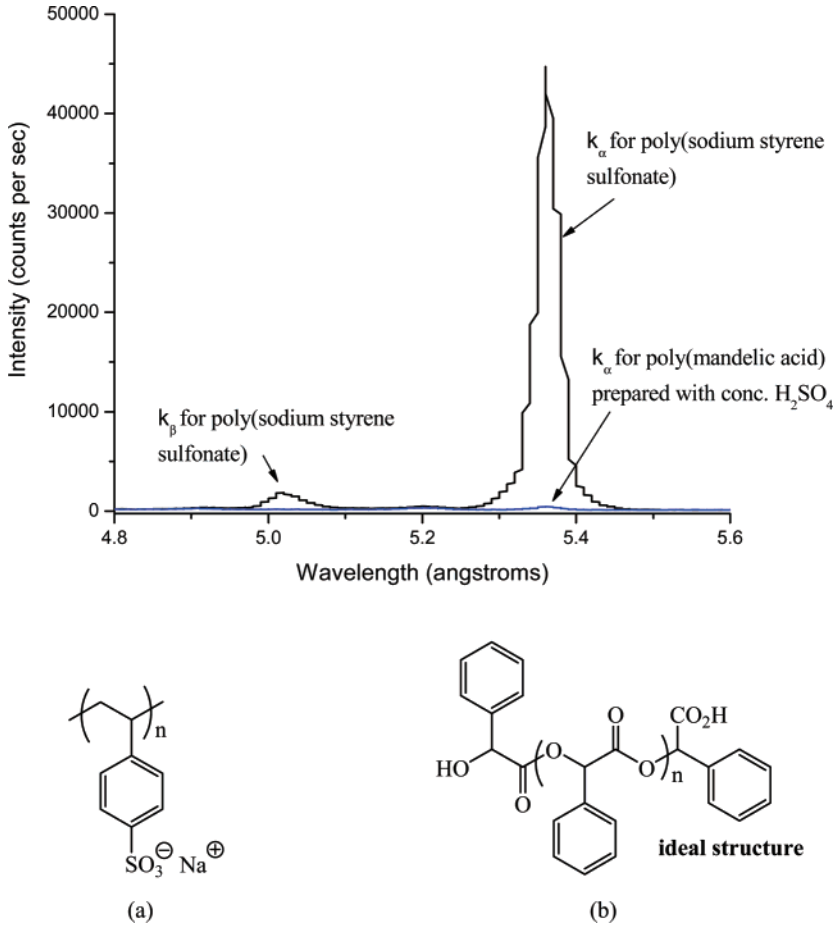


Figure 2. Experimentally determined XRF spectra for poly(sodium styrenesulfonate) (a) used as a standard and poly(mandelic acid) (b) prepared with concentrated H_2SO_4.

Table 4. Summary of the Theoretical and Experimentally Determined CHS Elemental Microanalysis Results for PMDAs Prepared in the Presence of H_2SO_4, *p*-TolSO_3, and under Melt Conditions

	H_2SO_4-prepared PMDAs			pTolSO_3-prepared PMDAs			melt-prepared PMDAs		
	% C	% H	% S	% C	% H	% S	% C	% H	% S
theory[a]	69.31	4.73	0.00	69.31	4.73	0.00	69.31	4.73	0.00
found[b]	46.96	5.01	0.55	68.03	4.45	0.00	62.70	4.97	0.00

[a] Assuming the ideal structure shown in Figure 2a with a number average degree of polymerization (DP_n) = 2. [b] An average of two measurements.

of 0.3 mL/min. Analysis was performed on a Waters instrument: Waters U6K' injector, a Waters 410′ HPCL pump, a Styragel 6MWE′ column (in a column heater set at 35 °C), and a Waters 510′ refractive-index detector. The column was calibrated with polystyrene standards of molecular weights ranging from 3250 to 3 040 000. FTIR spectra were recorded on a Thermo Nicolet Nexus 470 FTIR spectrometer equipped with a Smart Orbit.

X-ray Fluorescence (XRF). One gram of finely powdered sample was mounted into a modified Rigaku S-Max spectrometer, and a wavelength dispersive spectrogram was obtained using rhodium X-rays as the incident radiation source. The fluorescing X-rays from the sample were focused onto a PET crystal monochromator ($d = 4.375$ Å) and dispersed by scanning from $2\theta = 8.00°$ to 149.00° and converted from 2θ to its k_α wavelength by $\lambda = 2 \times 4.375$ Å/[sin(2θ/2)]. The intensity at each angular increment ($\Delta 2\theta = 0.05°$) was measured in counts per second.

Measurements of HIV-1 Infectivity. Two-fold serial dilutions of SAMMA treated HIV-1 IIIB separated from unbound SAMMA by precipitation with 3% PEG and control virus (100 μL), respectively, were added to HeLa-CD4-LTR-β-gal cells, which had been plated a day before infection in 96-well plates at 1×10^4 cells/well in 100 μL of DMEM medium containing 10% fetal bovine serum (FBS). After incubation at 37 °C for 48 h, the culture supernatant fluids were removed and the cells washed once with PBS. Subsequently, 50 μL of lysis buffer from the Galacto-Light Plus kit was added to the wells for 1 h at 20 °C. Aliquots (20 μL) of the cell lysates were transferred into wells of 96-well microplates, and β-galactosidase was quantitated using the Galacto-Light Plus System chemiluminescence reporter assay in a Microlight ML 2250 luminometer (Dynatech Laboratories, Inc., Chantilly, VA). The infectivity of treated and control HIV-1 BaL was measured by the same method except that MAGI-CCR5 cells were used.

Inhibition of HIV-1 Infection by SAMMA. Equal volumes of serially diluted SAMMA in DMEM medium were mixed with an equal volume of HIV-1 IIIB and BaL, respectively, and the mixtures were added to the reporter cell lines described above. After incubation for 48 h at 37 °C, β-galactosidase was quantitated.

Enzyme-Linked Immunosorbent Assays (ELISA). CD4-HIV-1 gp120 binding and its inhibition were measured by ELISA. Wells of 96-well polystyrene plates (Immulon II, Dynatech Laboratories, Inc., Chantilly, VA) were coated with 100 ng/well of gp120 IIIB and post-coated as described.[17] Dilutions of SAMMA in 0.14 M NaCl, 0.01 M Tris, 0.02% sodium merthiolate, pH 7.0 (TS), containing 100 μg/mL BSA were added to the wells for 1 h at 37 °C. The wells were washed five times with TS. Biotinyl-CD4 (1 μg/mL) in TS-1% gelatin was

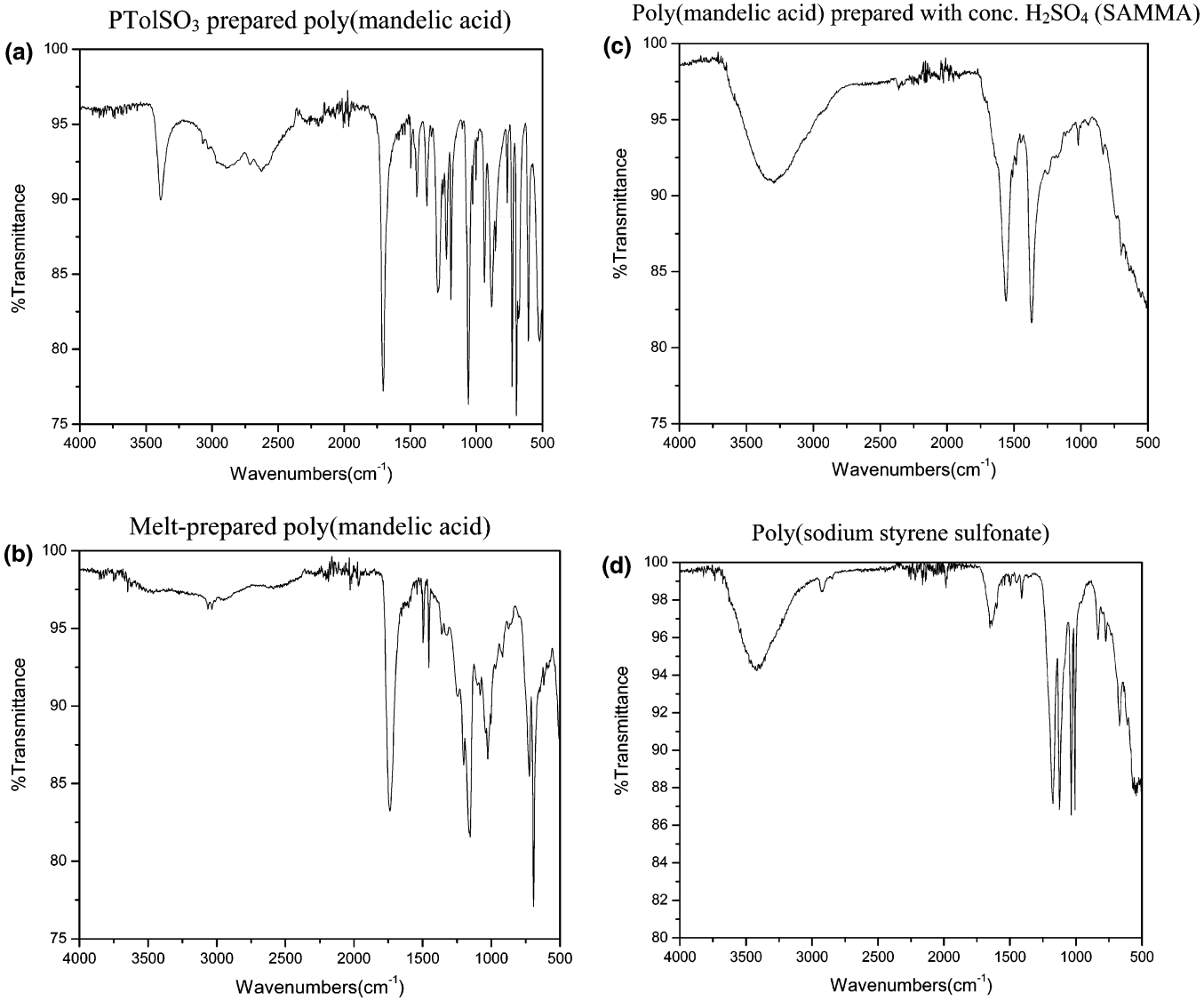


Figure 3. FT-IR spectra of PMDA prepared in the presence of *para*-tolenesulfonic acid (a), under melt conditions (b), in the presence of concentrated sulfuric acid (c), and a spectrum of poly(sodium styrenesulfonate) (d).

added to the wells for 5 h at 37 °C. After washing one time with TS−0.1% Tween 20 and five times with TS, HRP-streptavidin (0.625 μg/mL; Amersham, Arlington Heights, IL) in TS−2% gelatin−0.05% Tween 20 was added. After 30 min at 37 °C, the wells were washed four times with TS−0.1% Tween 20 and two times with TS. Bound HRP was detected using a kit from Kirkegaard and Perry Laboratories Inc. (Gaithersburg, MD), and the absorbance read 450 nm. To measure binding to gp120 of mAb 9284, the mAb (1 μg/mL) was diluted in a mixture of FBS and goat serum (9:1) containing 0.1% Tween 20 (pH 8.0) and added to gp120 wells. Bound IgG was detected with HRP-labeled anti-mouse IgG (Sigma, St. Louis, MO; 1 μg/mL in TS−10% goat serum−0.1% Tween 20). A cell-based ELISA was used to measure the blocking of CCR5 binding sites on HIV-1 BaL gp120 by SAMMA.[12] Briefly, FLSC (125 ng/mL) in the absence or presence of graded amounts of SAMMA was added to Cf2Th/synCCR5 cells fixed with 5% formaldehyde in wells of 96-well plates. After 1 h at 37 °C, bound FLSC was detected with mAb M-T441 (125 ng/mL; Ancell, Bayport, MN) specific for the CD4 D2 domain, followed sequentially by biotinylated anti-mouse IgG and HRP-streptavidin.

The sandwich ELISA for gp41 six-helix bundles was performed as described.[15] SAMMA treated and control virus preparations were incubated with lysis buffer for 30 min at 20 °C and then added to wells coated with rabbit polyclonal antibodies to the gp41 six-helix bundles. After incubation at 4 °C overnight, binding of six-helix bundles was determined from subsequent binding of mAb NC-1, which was added at 1 μg/mL in PBS/1% BSA/1% gelatin (100 μL/well) for 1 h at 37 °C. Subsequently, the wells were washed three times with PBS/0.05% Tween 20, and biotin-labeled anti-mouse IgG (100 μL/well; 125 ng/mL diluted in PBS containing 1% dry fat-free milk) was added. After incubation for 1 h at 37 °C, the wells were washed as described above, and HRP-streptavidin (125 ng/mL in PBS containing 10% goat serum; 100 μL/well) was added. After incubation for 1 h at 37 °C, the wells were washed six times with PBS/0.05% Tween 20, and HRP was quantitated.

To measure SAMMA binding to gp120, wells of 96-well polystyrene plates were incubated with a solution of SAMMA (1 mg/mL) in 0.14 M NaCl, 0.01 M Tris, pH 8.8, for 2 h at 20 °C. The wells were washed and post-coated with 1% BSA−0.25% gelatine. Control wells were post-coated only. The wells were washed with PBS. Serial dilutions of gp120 in 0.14 M NaCl, 0.01 M Tris, pH 7.2, containing 100 μg/mL BSA were added to the wells for 6 h at 20 °C. The wells were washed, and 1000-fold diluted rabbit anti-gp120 serum (produced in the Biochemical Virology Laboratory) was added. After incubation overnight at 20 °C, the wells were washed and probed with HRP-labeled anti-rabbit IgG. HRP was quantitated using a kit from Kirkegaard & Perry Laboratories, Inc.

Results

Synthesis. Homo- and heterochiral PMDAs have been prepared under three different sets of condensation conditions: in concentrated sulfuric acid, in the presence of *p*-$TolSO_3$, and under melt conditions.

PMDA esters have been prepared from the *R*, *S*, and *R*,*S* substrates. Tables 1−3 summarize the polymerization conditions and yields of the PMDAs prepared under the three different sets of conditions.

Isolation of Water-Soluble Fractions. Water-soluble fractions from the resulting polymers were isolated according to the procedure outlined in the experimental section. In the case of the polymers extracted into the phosphate buffer, the final PMDA content, after lyophilization, was determined by UV spectroscopy. The molar absorptivity, ϵ, as determined via the Beer−Lambert Law, for mandelic acid was found to be 204 L/mol/cm.

Water-insoluble PMDA, prepared by condensation with concentrated sulfuric acid, was tittered with 1 N NaOH. To bring the final pH to ∼7.0, 5 mmol of NaOH per gram of polymer was needed. The polymer dissolved at pH ≥ 4.07.

Elemental Microanalysis. CHS elemental microanalysis was performed on PMDA samples prepared via the three different polymerization procedures outlined above. The results are summarized in Table 4.

X-ray Fluorescence (XRF). The XRF spectrum of poly(sodium 4-styrenesulfonate), a model polymer used as a reference for analysis of the PDMA samples, indicated a large sulfur k_α peak at 5.37 Å (44 575 cps) and the corresponding sulfur k_β peak at 5.03 Å. The XRF spectrum of the PMDA prepared via the concentrated H_2SO_4 protocol contains a small sulfur k_α peak (457 cps), while the corresponding k_β is not discernible; see Figure 2.

FT-IR Analysis. FTIR spectra were recorded of a poly(mandelic acid) prepared via each of the three routes as well as a sample of poly(sodium 4-styrenesulfonate), Figure 3a−d. Several features are worth noting when directly comparing the spectra of the three PMDA samples. The most striking difference relates to the carbonyl (C═O) absorption. The C═O absorbance in clearly evident in Figure 3a and b at ca. 1750 cm^{-1} but is apparently completely absent in SAMMA. In the case of the SAMMA sample, there are only three distinct bands. The broad absorbance centered at ca. 3400 cm^{-1} is characteristic of inter-/intramolecularly H-bonded alcohols, the band at just above 1500 cm^{-1} is typical of a monosubstituted aromatic species, while the band at ca. 1400 cm^{-1} is in the range consistent with a C−C stretch (in ring) for aromatics and also for the C−O stretch associated with alcohols and ethers (ether linkages are also possibly formed as described below). Interestingly, no evidence of −OH groups can be seen in the spectra of the melt-prepared PMDA or the $PTolSO_3$-catalyzed-prepared PDMA. The remaining absorptions in Figure 3a and d are more difficult to interpret given that the bands appear in the fingerprint region. As a comparison, Figure 3d shows the FTIR spectrum for poly(sodium styrenesulfonate). The key signals are, again, the broad signal centered around 3500 cm^{-1} and the peaks between ca. 1250 and 1000 cm^{-1} that are consistent with a disubstituted benzene ring.

Biological Activity of Poly(mandelic acid)s (PMDAs) against HIV-1. Among PMDAs only the polymer prepared by condensation with concentrated sulfuric acid (to be further designated as SAMMA) inhibited infection by both HIV-1 IIIB (an X4 virus) and BaL (an R5 virus), respectively, Figure 4. In contrast, mandelic acid oligomers *R*,*S*, *S*, and *R*, prepared by other methods, inhibited infection of HIV-1 IIIB by 24%, 19%, and 28%, respectively, at a concentration of 1500 ug/mL, while

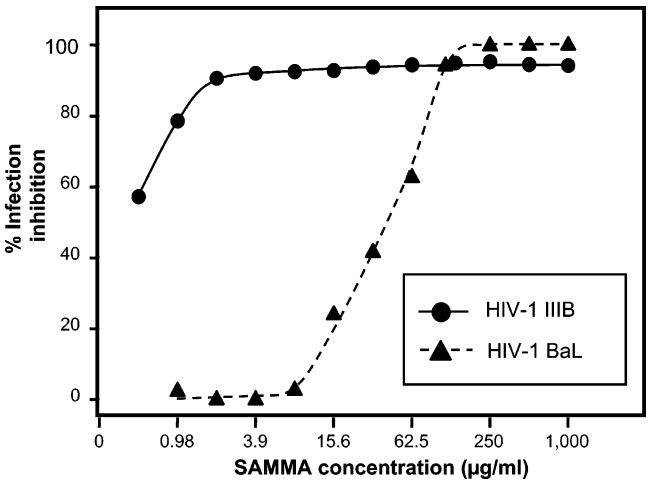


Figure 4. Inhibitory effect of sulfuric acid-modified mandelic acid (SAMMA) polymer on infection of cells by HIV-1 IIIB (●) and HIV-1 BaL (▲), respectively. HIV-1 IIIB is one of the X4 viruses that utilize the cellular co-receptor CXCR4 in initiation of infection. HIV-1 BaL is one of the R5 viruses that utilize the cellular co-receptor CCR5 in initiation of infection. Serial 2-fold dilutions of SAMMA and constant amounts of either virus (providing luminescence readouts of ≤70 in the absence of SAMMA) were added to indicator cell lines producing β-galactosidase upon infection. The enzyme was quantitated 48 h after infection. Percentages of inhibition were calculated for each SAMMA concentration by comparing readouts corresponding to virus additions in the presence and absence of SAMMA, respectively.

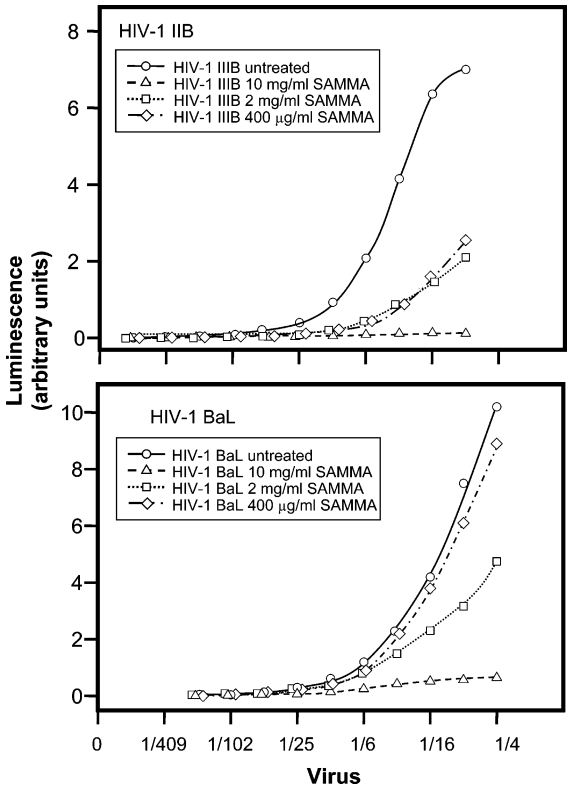


Figure 5. Inactivation by SAMMA of HIV-1 IIIB and BaL, respectively. Aliquots of the respective virus suspensions were mixed with equal volumes of SAMMA solutions to result in final concentrations indicated in the insets on the figures. The mixtures were incubated for 5 min at 37 °C. Virus and SAMMA were separated from each other by precipitation with 3% PEG 8000. After centrifugation, the viruses in the pellets were resuspended, and serial 2-fold dilutions were added to cells. Infection was monitored as described in Figure 3.

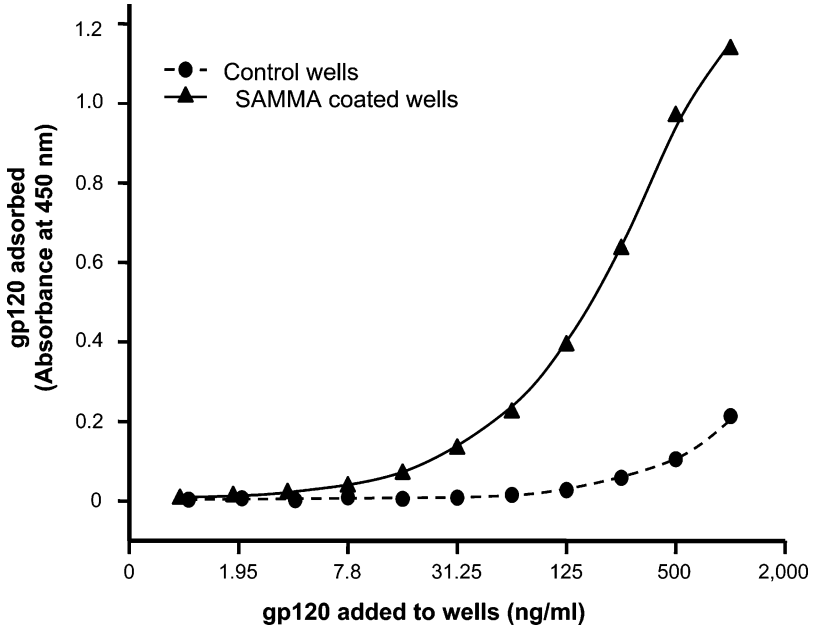


Figure 6. Binding of recombinant HIV-1 IIIB gp120 to wells of 96-well polystyrene plates precoated with SAMMA. Graded quantities of gp120, indicated on the abscissa, were added to SAMMA-coated and control wells. Bound gp120 was quantitated by ELISA utilizing biotinyl-rabbit anti-gp120 followed by horseradish peroxidase (HRP)-labeled anti-rabbit IgG. Enzymatic activity was measured by optical absorbance at 450 nm.

lower concentrations (corresponding to serial 2-fold dilutions) had no inhibitory effects, data not shown. The shift of inhibition curves indicates that SAMMA concentrations required to inhibit HIV-1 BaL are ~120-fold higher that those needed for equal inhibition of HIV-1 IIIB. This differential effect is in general agreement with observations for other negatively charged anti-HIV-1 microbicides.[18,19] SAMMA concentrations required for virus inactivation were by at least 2 orders of magnitude higher than those sufficient for complete inhibition of virus infection. A concentration of 10 mg/mL was sufficient to inactivate HIV-1 IIIB within 5 min at 37 °C, but residual infectious HIV-1 BaL was still detectable under these conditions, Figure 5.

Further studies were carried out to establish the mechanism of anti-HIV-1 activity of SAMMA. The binding of recombinant HIV-1 IIIB envelope glycoprotein gp120 to SAMMA immobilized on wells of 96-well polystyrene plates, Figure 6, suggested that gp120 on the surface of virus particles is the target site for SAMMA. SAMMA inhibited the binding between gp120 and recombinant soluble CD4 only at relatively high concentrations (50% inhibition at ~1 mg/mL, while oligomers *R,S*, *S*, and *R*, respectively, prepared by methods other than sulfuric acid treatment, had no inhibitory activity at a concentration of 3 mg/mL; data not shown), suggesting that the primary target site for SAMMA on gp120 is not the binding site for the virus cell receptor, CD4.[20] However, SAMMA blocked the binding to gp120 IIIB and BaL, respectively of monoclonal antibodies 9284, specific for the V3 loop region of gp120,[21] Figure 7. This region is involved in HIV-1 binding to cellular co-receptors CXCR4 and CCR5, respectively.[22,23] In agreement with this, SAMMA inhibited the binding of a gp120 BaL-CD4 fusion protein (designated as FLSC) to cells expressing CCR5, as determined by a cell-based ELISA,[12] Figure 8. In conclusion, SAMMA is an HIV-1 entry inhibitor targeted to the co-receptor binding sites on the gp120 envelope glycoproteins.

Discussion

Recently, PMDAs prepared under step-growth conditions in concentrated H_2SO_4 have been reported to exhibit significant biological activity against both HIV-1 and HSV. In an attempt to elucidate the structure−activity relationships, we have examined the effect of synthesis conditions as well as stereochemistry on the activity of the PMDA polymers against HIV-1. Given the reported efficacy of PMDAs (also referred to in the literature as SAMMA) prepared by step-growth mechanisms as topical microbicides, it is imperative that the active species be defined. There is confusion in the reported literature regarding the structure of the materials that have thus far been evaluated. For example, PMDA prepared by the method of Zaneveld et al.,[3] that is, in concentrated H_2SO_4, would, in the ideal case, yield a polymer with the structure shown in Figure 9a (neglecting stereochemistry and assuming only the occurrence of condensation reactions). However, Herold et al.[8] prepared a sample of material, which was also referred to as SAMMA, via the "condensation" of methyl mandelate with concentrated H_2SO_4 and claimed that the "polymer", isolated after treatment with base, was the disodium salt of 2,2′-diphenyl-2,2′-oxidiacetic acid; see Figure 9b.

It is apparent that these are two very different materials and yet appear to be referred to in the literature as being one and the same. Figure 9a shows an example of a linear polyester where *n* denoted the number of repeat units on average in each polymer chain, whereas Figure 9b represents a simple low molecular mass ether; that is, it is not polymeric.

PMDAs can be prepared via a number of different routes. Step-growth procedures offer the most convenient route via a polyesterification pathway. Such methods may be catalyzed (by acid) or may be performed under noncatalyzed conditions via a simple melt procedure. As described above, one can use a strong acid catalyst such as concentrated sulfuric acid or a weaker species such as *p*-$TolSO_3$. There are pros and cons to each of these methods. In the case of the concentrated H_2SO_4 method, the polymerization is straightforward. However, one cannot dismiss the occurrence of undesirable side reactions such as sulfonation of the aromatic ring, carbocation formation via the protonation of the benzylic −OH and subsequent loss of H_2O, and protonation of the C═O group

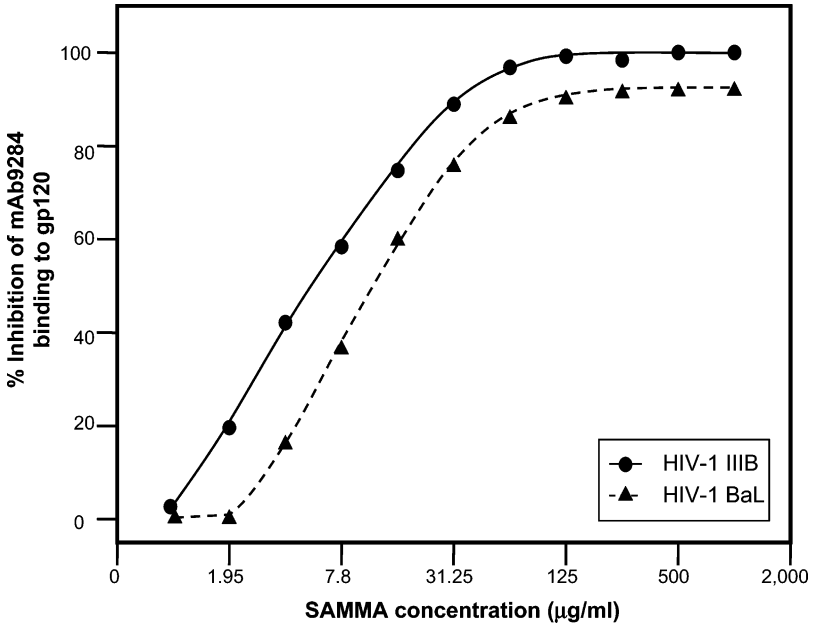


Figure 7. Inhibition by SAMMA of binding to gp120 IIIB and BaL, respectively, of mouse monoclonal antibody (mAb) 9284 specific for the V3 loop region of gp120. Graded quantities of SAMMA and constant amounts of mAb 9284 were added to gp120-coated wells of 96-well polystyrene plates. Subsequently, bound mAb was quantitated by ELISA using HRP-labeled anti-mouse IgG. Binding in the presence and absence of SAMMA, respectively, was compared, and the percentage of inhibition was calculated.

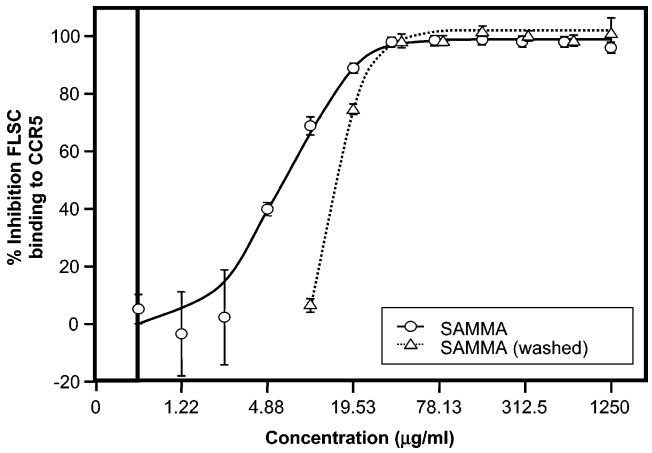


Figure 8. Inhibition by SAMMA of binding to CCR5 of a chimeric recombinant protein consisting of gp120 BaL linked with the D1D2 domain of CD4, designated as FLSC. The D1D2 domain of CD4 encompasses the HIV-1 gp120 binding sites on the CD4 molecule, the primary receptor for HIV-1 (Esser et al. AIDS Research and Human Retroviruses 16:1845−1854, 2000). SAMMA in graded quantities, indicated on the abscissa, and constant amounts of FLSC were added to Cf2Th/synCCR5 cells, expressing CCR5. FLSC binding in the presence and absence of SAMMA was quantitated by a cell-based ELISA, and the percentage of binding inhibition was calculated.

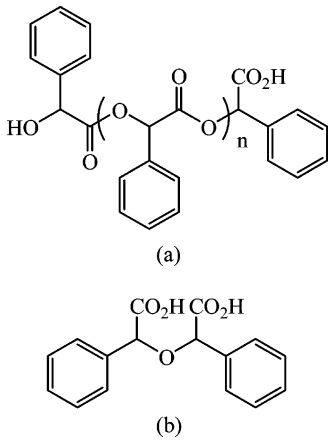
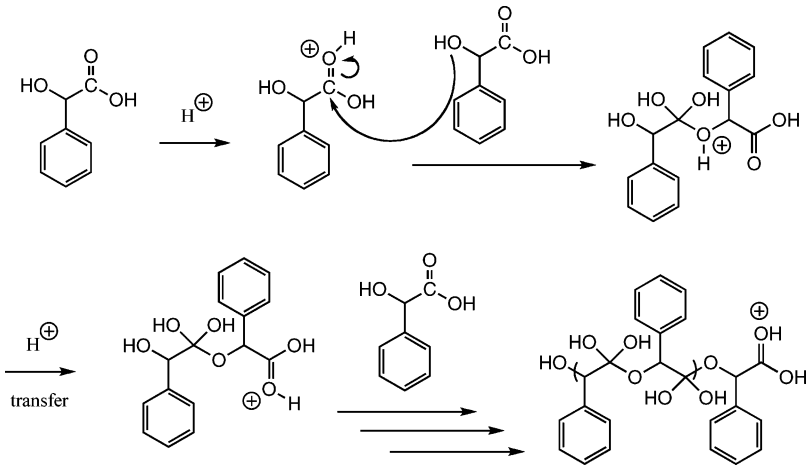


Figure 9. Chemical structures of poly(mandelic acid) (a) and 2,2′-diphenyl-2,2′-oxidiacetic acid (b).

followed by nucleophilic attack. Indeed, the polymer prepared by treatment with H_2SO_4 has a distinct orange color, whereas materials prepared via the other routes yield white products. In the case of the *p*-TolSO_3-prepared polymers, the synthesis is also simple. However, in this case one must ideally remove residual catalyst, or at the very least ensure that it is non-active, that is, inert, in any proposed end application. The melt polymerizations are the simplest ones to conduct and should yield near "pristine" PMDA (at least material that is free of undesirable functionality). However, a possible side reaction in the case of melt polymerizations is decarboxylation. Such a reaction has been demonstrated by Pojman and co-workers.[24]

Structure of H_2SO_4-Prepared Poly(mandelic acid)s (PMDAs). One of the primary motivations for this study was based on the reported observations of Zaneveld et al.[3] regarding the absence of detectable levels of sulfur in mandelic acid-based polyesters prepared in concentrated H_2SO_4. However, in our hands, and following the procedure described by Zaneveld and co-workers, both elemental microanalysis and XRF indicated that the water-soluble fractions do, in fact, contain detectable levels of sulfur in some functional form (we have not attempted to determine the actual S-containing functional groups). Additionally, FTIR analysis indicates the absence of a key functional group and the presence of non-expected species. In the case of CHS elemental microanalysis of PMDAs prepared via the concentrated H_2SO_4 route, two features are immediately apparent. First, S is present in some functional form with approximately 0.55% S being detected. This may be covalently or noncovalently bound S. Given the sulfonating and oxidizing

Scheme 1. Proposed Formation of a Polymer Derived from H_2SO_4 Treatment of Mandelic Acid To Yield a Material Free of C═O Functional Groups and with a High Concentration of −OH Functionality



abilities of concentrated H_2SO_4, it is certainly possible that S is covalently attached either to the aromatic ring or as part of the polymeric backbone. However, it must be noted that this finding is in contrast to that reported by Zaneveld, who claimed the absence of detectable S in PMDAs prepared via this route. Second, there is an extremely large discrepancy between the theoretical and experimentally determined carbon contents with theoretical %C = 69.13 and found %C = 46.96. At this point, we cannot explain such a large difference. Clearly, these elemental analysis results would seem to indicate that the PMDA resulting from the treatment of mandelic acid monomer with concentrated H_2SO_4 does not have the expected structure shown in Figure 9a. To confirm the presence of S as determined by elemental microanalysis, the same sample was subjected to analysis by XRF. XRF is a technique in which the ground-state electrons of an atom are ejected to produce excited atomic states. As the atom de-excites, electromagnetic radiation is emitted, and the energy of the emitted photon is the difference between the energies of the excited and ground atomic states of the atom.[25] The resulting electromagnetic radiation is characterized either by its wavelength (λ) or by its energy (E).[25,26] When the electron is ejected from the 1s orbital, the electromagnetic radiation is termed the X-ray k-series, and the wavelength of the emitted photons is given by $1/\lambda = K \times [Z - \alpha]$, where K is a grouping of fundamental constants, Z is the atomic number of the element, and α is the electron density screening constant for the element. The wavelengths of the characteristic K-series X-rays emitted by each of the elements are well-known.[25,26] The measured intensity for the k_α photons of the peak for element Z, $I(Z)$, is related to the abundance, C_z, of that element in a sample by:

$$C_Z = I(Z) \times Q \times M \times P$$

where Q is the instrumental calibration constant, M is the matrix effect of the entire sample, and P represents specimen effects.[27,28] The product $Q \times M \times P$ may be eliminated by selection of a series of "standard mixtures", which contain both the analyte Z and the other elements in the sample(s) to be analyzed.[29,30] Thus, $C_z^* = I^*(Z) \times [C^\circ(Z)/I^\circ(Z)$, where $C^\circ(Z)/I^\circ(Z)$ is the relationship between the abundance of analyte Z in reference samples of known composition and the measured analyte intensity from the WDXRS of each reference. Because each is composed predominantly of carbon and hydrogen and each contains a small amount of sulfur, PMDA and poly(sodium 4-styrenesulfonate) fit this relationship.

Given that the intensity of the S peak is related to the abundance of S in each sample and also that sodium poly(sodium 4-styrenesulfonate) is, to a first approximation, an appropriate polymer for comparative purposes, we can directly ratio the k_α peaks to determine the relative abundance of S present in the H_2SO_4-prepared PMDA polymers. Poly(sodium 4-styrenesulfonate) (prepared via the free radical polymerization of sodium 4-styrenesulfonate monomer) contains an $-SO_3Na$ functional group on every repeat unit, and as such contains approximately 16% S. A ratio of the k_α peak for poly(sodium 4-styrenesulfonate) with the k_α peak for a sample of PMDA prepared with H_2SO_4 yields a value of 0.01. Given the theoretical value of 16% S in the standard, this implies that there is approximately 0.16% S present in the PMDA sample. This value is in good agreement (same order of magnitude) with that determined by CHS elemental microanalysis of the same sample, which yielded an S content of 0.56%.

The FT-IR spectrum of the concentrated sulfuric acid-prepared PMDA (SAMMA) is not consistent with pristine PMDA. In particular, the absence of a clear and identifiable C═O band and the presence of a broad band associated with inter-/intramolecularly bonded OH groups are troubling. PMDA formed by transesterification alone will yield a polymer with the structure shown in Figure 9a. In this species, a C═O group is present in every repeat unit and as a possible end group. Additionally, the only −OH group is a terminal species and as such may be difficult to detect. The expected high concentration of C═O functional groups, and the fact that such functional groups typically give strong, intense bands, would seem to suggest that SAMMA does not have the structure claimed in the literature. Scheme 1 outlines a possible reaction pathway by which a polymer may be formed that is free of C═O groups but rich in −OH functional groups.

Initial protonation of the C═O group followed by nucelophilic attack by −OH from a second MDA species yields an intermediate "diol" derived from the C═O group. Subsequent proton transfer from the trivalent oxygen to the C═O group of the carboxylic acid on the second MDA monomer reactivates it toward further nucleophilic attack by −OH on a third MDA substrate. In the presence of excess monomer, a polymer results without carbonyl functional groups, a high concentration of −OH groups, and that also contains ether linkages. While this is consistent with the FT-IR analysis, both XRD and elemental microanalysis also indicate the presence of S, and as such this particular side reaction, or rather proposed mechanism of

polymerization, is likely one of several that is occurring under treatment of mandelic acid with concentrated sulfuric acid.

Biological Activity against HIV-1. Water-soluble fractions of the PMDAs synthesized via the three general routes described above were tested with respect to their efficacy as HIV-1 inhibitors and virucides, respectively. Only the S-containing polymers were active. They inhibited a representative both of X4 viruses, HIV-1 IIIB, and of R5 viruses, HIV-1 BaL, and inactivated these viruses. This is in reasonable agreement with results published by Zaneveld et al.[3] but disagrees with findings of Keller et al.[9] claiming that SAMMA is not directly virucidal. The HIV-1 inhibitory and virus-inactivating activities of the S-containing polymer can be ascribed to its strong binding to the co-receptor binding sites on the gp120 envelope glycoprotein of the viruses. It seems possible that a portion of SAMMA remains bound to the viruses even after removal of excess polymer from virus particles by precipitation with PEG (see Experimental Section) and blocks virus binding to cellular co-receptors, thus preventing infection. On the other hand, gp120 binding to soluble CD4 was not inhibited. Unlike some other polymers [cellulose acetate 1,2-benzenedicarboxylate and poly-(naphthalenesulfonate)],[19] the effect of SAMMA seemed to be restricted to the envelope glycoprotein gp120 because it did not elicit the formation of envelope glycoprotein gp41 "dead-end" six-helix bundles (data not shown).

The HIV-1 IIIB inhibitory activity of SAMMA is similar to that of other anionic polymeric candidate microbicides,[31] but its anti-HIV-1 BaL activity is $\leq 1/8$ of that of other microbicides. Nevertheless, because of the ease of synthesis and low cost, SAMMA is a compound to be considered for the development of microbicides to prevent sexual transmission of HIV-1.

Summary/Conclusions

Step-growth polymerization of mandelic acid can be accomplished by acid catalysis using *p*-$TolSO_3$ and/or concentrated H_2SO_4. Alternatively, poly(mandelic acid) (PMDA) can be prepared via a simple melt procedure. While we have not attempted to fully elucidate the structure of the materials prepared by each method, it appears that SAMMA, in particular, while active against HIV-1, does not possess the structure claimed in the literature and is quite likely not a polyester. The inhibitory activity of PMDA synthesized under the three distinct conditions against infection in vitro by HIV-1 was studied. Surprisingly, only PMDA prepared via catalysis with concentrated H_2SO_4 (designated SAMMA) had biological activity.

Acknowledgment. A.R.N. wishes to thank Dr. Qian Zhao for experiments with the full length single chain protein consisting of HIV-1 BaL gp120 linked with the D1D2 domains of CD4 (FLSC), and Veronica Kuhlemann for assistance and editorial help in preparing the manuscript and for the production of graphs. Financial support for this research was provided by the Marilyn M. Simpson Charitable Trust, the Glickenhaus Foundation, and an NIH grant (PO1HD41761).

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BM070221Y