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SYNTHESIS OF THE COVALENT HYDRATE OF THE INCORRECTLY ASSUMED STRUCTURE OF AURINTRICARBOXYLIC ACID (ATA)

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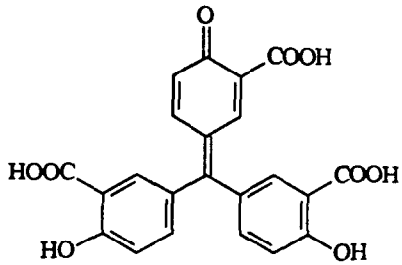
Abstract. The triphenylcarbinol **6**, which is the covalent hydrate of the generally accepted, but incorrect structure of aurintricarboxylic acid (ATA), has been synthesized and found to protect against the cytopathic effect of HIV-1 in CEM-V lymphocyte cell culture The synthesis involved the first preparation of pure methylene disalicylic acid (**5**) The triphenylcarbinol **6** provides a novel lead, apart from (polymeric) ATA, for the development of potential anti-AIDS agents The central carbinol oxygen of **6** did not exchange in the presence of excess oxygen-18 labelled water at room temperature even after seven days, indicating that there was no equilibration between **6** and **1** under these conditions

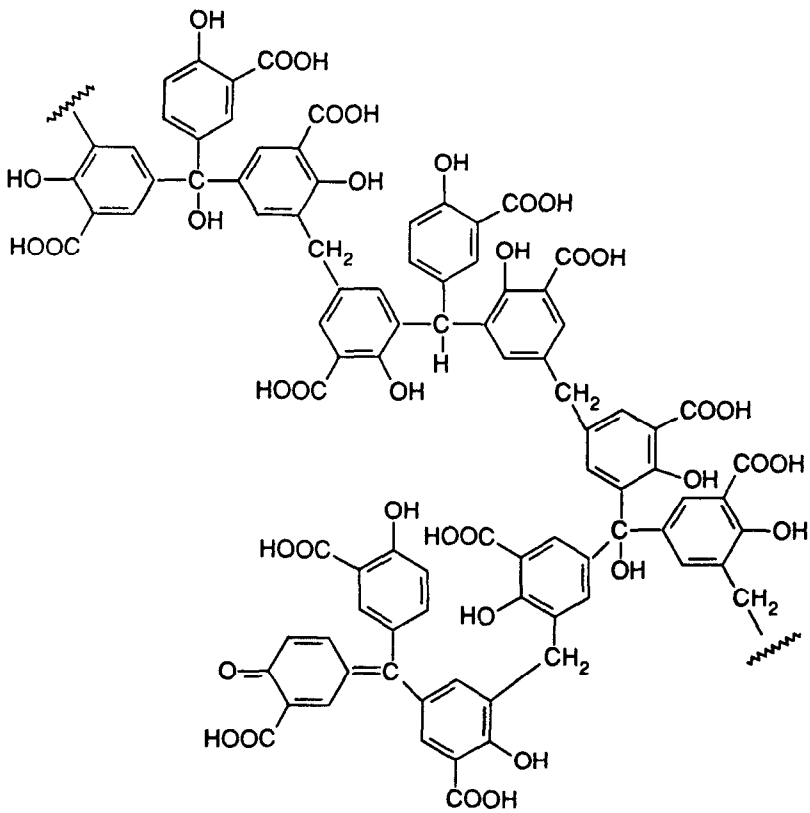
INTRODUCTION

Treatment of a mixture of salicylic acid and formaldehyde with sulfuric acid and sodium nitrite results in the formation of a solid substance known as aurintricarboxylic acid (ATA) [1] This material was originally believed to have the triphenylmethane dye structure **1**, and this structure has persisted in the current literature even though studies reported by González, Blackburn, and Schleich seem to indicate quite clearly that ATA is actually a heterogeneous mixture of polymers which they represented schematically as structure **2** [2]

ATA is a potent inhibitor of cellular processes that depend on the binding of nucleic acids to proteins This inhibition of the binding of nucleic acids to proteins may be due to the fact that the polymeric and polyanionic nature of ATA resembles the structures of oligonucleotides To the extent that ATA occupies the oligonucleotide binding sites of a wide variety of proteins that normally act on oligonucleotides, it can be regarded as an oligonucleotide mimetic Examples include the inhibition of protein synthesis by blocking the attachment of mRNA to the ribosome[3] and inhibition of aminoacyl-*t*RNA synthetase [4] ATA is also known to reduce the affinity of the DNA primer for reverse transcriptase,[5] and to interfere with the activities of DNA[6] and RNA[7] polymerases Ribonucleotide


O
COOH
HOOC
COOH
HO
OH

1


OH
COOH
COOH
HO
OH
HOOC
OH
CH2
OH
COOH
OH
COOH
C
H
HOOC
OH
CH2
COOH
OH
COOH
OH
C
OH
HOOC
HOOC
OH
HO
CH2
O
C
CH2
HOOC
OH
COOH

2

reductases from a variety of sources are inhibited uniformly by ATA,[8] as are an array of ribonucleases [9] ATA has recently been shown to block DNA-cellulose binding to progesterone, estrogen, glucocorticoid, and 1,25-dihydroxyvitamine D_3 receptors, suggesting that steroid hormone receptors and nucleic acid polymersases may have similar polynucleotide binding sites [10]

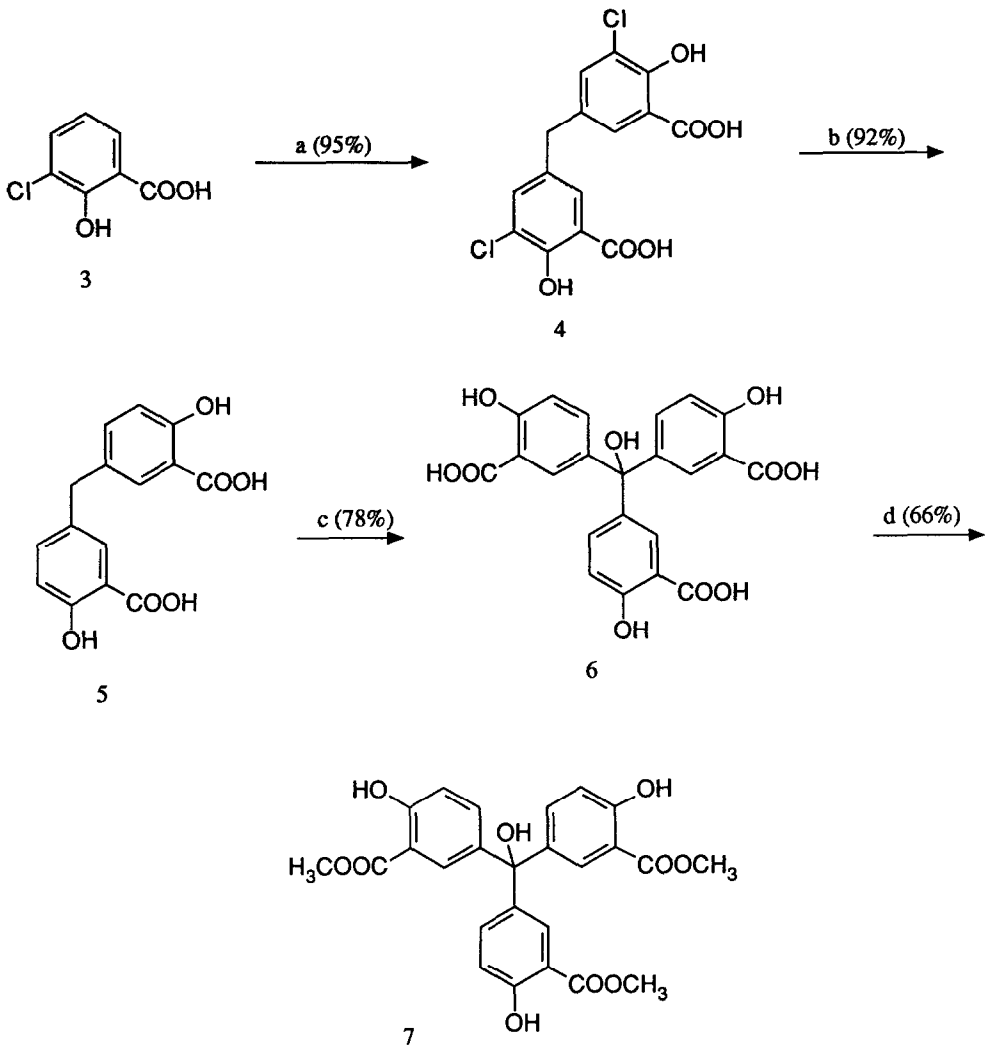
It was recently demonstrated that ATA prevents the cytopathic effect of HIV-1 in ATH8 cell culture as well as the expression of p24 in H9 cells infected with HIV-1, and it has been suggested that this effect may be due to the inhibiton of HIV-1 reverse transcriptase[11a] and/or the blockade of the HIV/CD4 cell receptor [11b] In addition, it has been reported that the triphenylmethane dye fuchsin acid, as well as ATA, inhibit the cytopathic effect of HIV-1 in MT-4 cell culture [12] A variety of authentic triphenylmethane dyes sharing the same skeletal structure as fuchsin acid, along with ATA, have also been reported to inhibit Raucher leukemia virus reverse transcriptase, *E coli* RNA polymerase, and protein biosynthesis [7b] These observations seem to contradict the conclusions of other workers "that aurintricarboxylic acid in the form of the commonly accepted structure (**1**) is ineffective as an inhibitor of protein nucleic acid interactions, and that the inhibitory activity of an aurintricarboxylic acid preparation is proportional to the degree of polymerization" [2] It seems legitimate to ask, if the latter conclusions are true, then why do non-polymeric, authentic triphenylmethane dyes inhibit protein nucleic acid interactions?[7b] Assuming that the known inhibiton of HIV-1 reverse transcriptase by both ATA (**2**) and fuchsin acid is indeed responsible for the observed anti-HIV activity,[11,12] a related question is whether or not a compound having the commonly accepted, incorrect structure **1** of ATA would have any potential as an anti-AIDS agent. In order to answer the latter question, as well as to gain further insight into the true chemical nature of ATA, a synthesis of the compound having structure **1** was attempted

RESULTS

A three step synthesis that gave the triphenylcarbinol **6** was devised as depicted in Scheme I This is the covalent hydrate of the hypothetical structure **1**, which we could not obtain under various conditions This route relied heavily on the use of chlorine as a protecting group in order to control the regiochemistry during the first step and to prevent the further reaction of the diphenylmethane in the presence of formaldehyde and acid to form polymers of the phenol-formaldehyde type Thus, 3-chlorosalicylic (**3**) acid was treated with formaldehyde and sulfuric acid to give the diphenylmethane **4** Both of the chlorine atoms present in **4** were then removed by hydrogenolysis over palladium on charcoal to afford methylene disalicylic acid (**5**) Reaction of intermediate **5** with salicylic acid in the presence of sulfuric acid and sodium nitrite then gave the triphenylcarbinol **6**, which is the covalent hydrate of structure **1** The triester **7** was also prepared by treatment of **6** with diazomethane

The triphenylcarbinol structure **6** of the product was distinguished from the quinone methide **1** by FAB mass spectroscopy run in the negative ion mode, using KCl in a glycerol matrix [13] The spectrum displayed a molecular ion at *m/e* 439 ($M - H^+$) In contrast, the FAB mass spectrum run in the positive ion mode displayed an ion at *m/e* 423, corresponding to the protonated, dehydrated form The ^1H NMR spectrum showed that the three aromatic protons in each ring were equivalent, resulting in signals at δ 7 88 (d, J = 2 Hz), 7 46 (dd, J = 9 and 2 Hz), and 6 92 (d, J = 9 Hz), which is also in agreement with the triphenylcarbinol structure **6** as opposed to **1** However, the NMR data is not as convincing for structure **6** as the FAB mass spectral data, since a rapid tautomerization of **1** might also result in equivalent aromatic protons An attempt was also made to demonstrate the

Scheme I



[a]H_2SO_4, CH_2O, -5 °C to 0 °C (2 h), room temperature (24 h) [b]H_2, Pd/C (10 %), EtOH, Et_3N, room temperature (48 h) [c]Salicylic acid, H_2SO_4, NaONO, room temperature (24 h) [d]Diazomethane, Et_2O, 5 °C (48 h)

presence of the quinone methide **1** in equilibrium with the triphenylcarbinol **6** Thus, compound **6** was dissolved in acetone-d_6 in the presence of a large excess of $H_2{}^{18}O$ and the ^{13}C NMR spectrum monitored for seven days in order to detect the upfield shift in the central carbinol carbon that would result from the incorporation of ^{18}O [14] No change in chemical shift was detected, indicating that equilibration between **6** and **1** was not taking place In order to corroborate these results, the FAB mass spectrum was run in the negative ion mode on the sample that had been subjected to $H_2{}^{18}O$ for seven days The results (*m/e* 439, M - H^+) confirmed that there was no ^{18}O present

In preliminary screening[15] at the National Cancer Institute, Bethesda, MD, USA, compound **6** was found to afford high levels of protection (up to 89% at 0 37 mM, or 167 μg/mL) against the cytopathic effect of HIV-1 in CEM cells in culture, with little or no evidence of cytotoxicity to the cells In comparison, ATA polymer **2** afforded complete protection at *ca* 10 μg/mL, with little or no accompanying cytotoxicity Both the trimethyl ester **7** and methylenedisalicylic acid **5** were inactive Synthesis and comparison of biologically active structural analogs of **6** is in progress and will be reported

DISCUSSION

Many syntheses of supposedly pure methylene disalicylic acid (**5**) have been reported in the literature [16] However, all of them involve the treatment of salicylic acid with formaldehyde under acidic conditions and they all give heterogeneous mixtures of multimeric forms [6b] The synthesis of intermediate **5** outlined in Scheme I therefore represents the first true synthesis of pure methylenedisalicylic acid There are actually two methods for the synthesis of ATA in the literature One involves the previously mentioned treatment of salicylic acid with formaldehyde, sulfuric acid, and sodium nitrite The other involves the oxidative condensation of supposedly pure methylenedisalicylic acid with salicylic acid, which was claimed by Smith *et al* to give pure ATA as structure **1** [16c] However, the methylenedisalicylic acid used by Smith was later shown to be a mixture of 6-8 components,[17] and a resynthesis of ATA by the method of Smith was shown to give at least 8 products [6b] The present work demonstrates that the oxidative condensation of methylenedisalicylic acid in fact gives the covalent hydrate of the incorrect structure of ATA It also points out the fact that the incorrect structure **1** of ATA is not only incorrect in the sense that it does not represent the true structure of ATA, but it is also incorrect in the sense that the substance does not exist in the quinone methide form **1**, it exists as the covalent hydrate **6**

The present work is also in agreement with the polymeric structure of ATA as indicated by the previous work of González, Blackburn, and Schleich.[2] Comparison of the properties of **6** with those of ATA verifies that they are not the same substance Our preliminary work on the gel permeation chromatography (gpc) of ATA definitely shows that it is a very complex, heterogeneous mixture of polymers, and that there is very little, if any, triphenylcarbinol **6** present in ATA, which is also in agreement with the previously reported study [2] Further work on the fractionation of ATA is in progress The ^{13}C NMR spectroscopic data on the triphenylcarbinol **6** may eventually prove to be of value in the investigation of the structure of the ATA polymer

The fact that both ATA (**2**) and fuchsin acid are reported to inhibit HIV-1 reverse transcriptase suggests that the triphenylcarbinol **6** may be functioning similarly [12] It is also possible that compound **6**, like ATA, is blocking the CD4 receptor [11b] However, the pharmacological mechanism of action of compound **6** which is responsible for its prevention of the cytopathic effect of HIV-1 in cell culture remains to be established It is considerably less potent than the ATA polymer **2**, and may conceivably operate by a distinct mechanism. In any case, the

riphenylcarbinol **6** represents a new lead, distinct from ATA polymer, for the development of potential anti-HIV igents

EXPERIMENTAL SECTION

Melting points were determined on a Thomas-Hoover Unimelt melting point apparatus and are uncorrected ^{1}H NMR spectra were recorded on a Chemagnetics A-200 spectrometer IR spectra were recorded on a Beckman R-33 spectrophotometer Low resolution chemical ionization mass spectra (CIMS) were determined on a Finnegan 4000 spectrometer using 2-methylpropane as the reagent gas Low resolution fast atom bombardment mass spectra (FABMS) were obtained on a Kratos MS50 spectrometer Microanalyses were performed by the Purdue Microanalytical Laboratory

3,3'-Dichloro-5,5'-dicarboxy-4,4'-dihydroxydiphenylmethane (4). 3-Chloro-salicylic acid (1 72 g, 10 mmol) was dissolved in methanol (10 mL) Water (2 5 mL) was added and the flask was cooled to -5 °C in an ice-salt bath Concentrated sulfuric acid (30 mL) was added during 20 min while the temperature was maintained at -5 to 0 °C The reaction mixture was then stirred at this temperature for 1 h while an aq solution of 37% formaldehyde (4 mL) was added The temperature was then maintained at 0 °C for 1 h before the reaction mixture was left at room temperature for 24 h The mixture was poured into crushed ice (150 g) and the precipitate was filtered and dried to give the product **4** (1 7 g, 96%) as a solid The analytical sample was recrystallized from chloroform-methanol (2 1) mp 302 °C, UV (ethanol) 316 nm (ε 8,300), IR (KBr) 3400-2850, 1680, 1610, 1460, 1290, 1235, 1170, 1370, 780 cm^{-1}, ^{1}H NMR (200 MHz, acetone-d_6) δ 7 68 (s, 2 H), 7 47 (s, 2 H), 3 87 (s, 2 H), ^{13}C NMR (DMSO-d_6) δ 171 53, 155 23, 135 57, 132 29, 128 77, 120 64, 114 32, 37 89, CIMS *m/e* (relative intensity) 357 (MH^+, 93), 339 (100) Anal Calcd for $C_{15}H_{10}Cl_2O_6$ C, 50 42, H, 2 80 Found C, 50 11, H, 2 72

3,3'-Dicarboxy-4,4'-dihydroxydiphenylmethane (5). Compound **4** (1 78 g, 5 mmol) was dissolved in ethanol (30 mL) and triethylamine (15 mL) Pd/C (10%, 500 mg) was added to the solution and the mixture was stirred under an atmosphere of hydrogen for 48 h The catalyst was filtered off, the solvent was evaporated, and water (100 mL) was added to the residue The solution was cooled and acidified by addition of conc hydrochloric acid (5 mL) The white precipitate was filtered and dried to give the product **5** (1 32 g, 92%) as a solid The analytical sample was recrystallized from chloroform-methanol (2 1) 268-269 °C, UV (ethanol) 312 nm (ε 7,530), IR (KBr) 3400, 3250-2980, 1680, 1620, 1590, 1490, 1445, 1280, 1299 cm^{-1}, ^{1}H NMR (200 MHz, acetone-d_6) δ 10 95 (s, 2 H, exchangeable with D_2O), 7 73 (d, 2 H, J = 2 Hz), 7 33 (dd, 2 H, J = 8 and 2 Hz), 6 88 (d, 2 H, J = 8 Hz), 3 89 (s, 2 H), ^{13}C NMR (DMSO-d_6) δ 171 85, 159 55, 136 13, 132 05, 129 79, 117 28, 112 71, 38 72, CIMS *m/e* (relative intensity) 289 (MH^+, 100), 271 (37) Anal Calcd for $C_{15}H_{12}O_6$ C, 62 50, H, 4 16 Found C, 62 67, H, 3 98

3,3',3''-Tricarboxy-4,4',4''-trihydroxytriphenylcarbinol (6). Powdered sodium nitrite (0 276 g, 4 mmol) was added with vigorous stirring to concentrated sulfuric acid (3 mL) A mixture of compound **5** (0 576 g, 2 mmol) and salicylic acid (0 276 g, 2 mmol) was added in portions The mixture was stirred until it was homogeneous and was then poured into a solution of sodium nitrite (0 276 g, 4 mmol) in sulfuric acid (3 mL) Stirring was then continued at room temperature for an additional 18 h The mixture was then poured into crushed ice (100 g) with stirring The orange precipitate was filtered and dried to give the product **6** (0 686 g, 78 %) mp 236-238 °C (dec), UV (ethanol) 312 (ε 9 935), 412 (ε 3,425), 553 nm (ε 10,460), IR (KBr) 3400, 3200-2840, 1670, 1610, 1575, 1480, 1430, 1350, 1290, 1200, 1130, 1070 cm^{-1}, ^{1}H NMR (200 MHz, acetone-d_6) δ 11 12 (s,

1 H, exchangeable with D_2O), 7 88 (3 H, d, J = 2 Hz), 7 46 (3 H, dd, J = 9 and 2 Hz), 6 92 (d, 3 H, J = 9 Hz), 5 58 (brs, exchangeable with D_2O), ^{13}C NMR (acetone-d_6) δ 172 65, 161 99, 139 16, 136 45, 130 17, 117 60, 112 28, 80 59, FABMS (negative ion mode) *m/e* (relative intensity) 515 [(M - $2H^+ + 2K^+)_{salt}$ - H^+, 23], 477 [(M - $H^+ + K^+)_{salt}$ - H^+, 74], 439 (M - H^+, 100)

3,3',3''-Trimethoxycarbonyl-4,4',4''-trihydroxytriphenylcarbinol (7). A solution of diazomethane in ether (20 mL, prepared from 0 824 g of nitrosomethylurea) was added to a solution of **6** (0 440 g, 1 mmol) in ether (20 mL) at 5 °C and the mixture was kept at this temperature for 48 h A few drops of acetic acid were added to the solution, the solvent was evaporated, and the product was purified by flash chromatography over silica gel (60-200 mesh, 40 g) The analytical sample was recrystallized from hexane-methylene chloride (1 1) mp 189-190 °C (dec), UV (ethanol) 312 nm (ε 11,365), IR (KBr) 3460, 3180, 1680, 1610, 1590, 1490, 1445, 1340, 1295, 1210, 1080 cm^{-1}, ^1H NMR (200 MHz, acetone-d_6) δ 10 82 (s, 3 H, exchangeable with D_2O), 7 78 (d, 3 H, J = 2 Hz), 7 29 (dd, 3 H, J = 9 and 2 Hz), 6 93 (d, 3 H, J - 9 Hz), 3 89 (s, 9 H), ^{13}C NMR δ 170 22, 160 58, 137 38, 135 15, 128 46, 117 18, 111 57, 80 17, 52 16 cm^{-1}, CIMS *m/e* (relative intensity) 483 (MH^+, 54), 465 (100), 331 (16) Anal Calcd for $C_{25}H_{22}O_{10}$ C, 62 24, H, 4 56 Found C, 61 88, H, 4 60

Subjection of Compound 6 to $H_2{}^{18}O$ in Acetone-d_6. Compound **6** (50 mg) was dissolved in acetone-d_6 (0 4 mL) in a 5 mm NMR tube and $H_2{}^{18}O$ (0 230 g) was added The ^{13}C NMR spectrum was recorded after 6 h, 48 h, and 7 days No change in the ^{13}C NMR chemical shift value of the central carbinol carbon was observed The FABMS (negative ion mode, KCl in glycerol matrix) run of the solution after 7 days showed the molecular ion at *m/e* 439 (M - H^+)

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