Dicarboxylic Acid Bis(methyl phosphates): Anionic Biomimetic Cross-Linking Reagents

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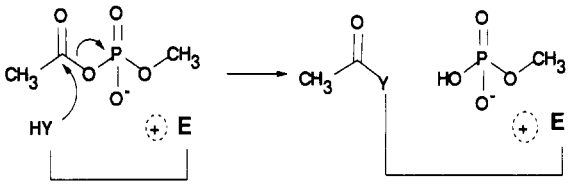
Monoesters of acyl phosphates are anionic electrophiles and their difunctional analogues are expected to function as site-directed cross-linking reagents. Dimethyl phosphate anhydrides of mono- and dicarboxylic acids are conveniently prepared by the reaction of an acyl chloride with sodium dimethyl phosphate in tetrahydrofuran. These are converted to monomethyl acyl phosphates by reaction with sodium iodide. Methyl acetyl phosphate and a representative group of symmetrical difunctional bis(methyl phosphates) (**1a–5a**) were prepared by this route. Hemoglobin is cross-linked in its reactions with difunctional methyl acyl phosphates.

Acyl phosphates (mixed anhydrides of carboxylic and phosphoric acids) are species that occur widely as intermediates in biochemical processes.[1] These intermediates transfer either the acyl group or phosphate residue to an acceptor.[2,3] In nonenzymic reactions unesterified acyl phosphates react at phosphorus in neutral solutions while monoesters of acyl phosphates transfer the acyl group to nucleophiles.[4,5] The monoesters are relatively stable in neutral aqueous solutions,[4,6] and methyl acetyl phosphate (MAP) has been shown to acetylate amino groups located in cationic regions of proteins.[6-9] Kern et al. found that an aminoacyladenylate (which is a monoester of an acyl phosphate) reacts with ϵ-amino residues of lysines during its production by an aminoacyl t-RNA synthetase.[10] Thus, the synthetic acyl phosphate monoesters are potentially biomimetic agents (see Scheme I).

Based on the reaction patterns of MAP,[7-9] it is reasonable to expect that dicarboxylic acid bis(acyl phosphate monomethyl esters) will have the specific reaction properties of the functional group while being able to produce cross-links in proteins.[11] Such properties are particularly desirable for the development of materials that will stabilize tetrameric hemoglobins for the production of synthetic oxygen transport systems[12] as well as for the modification of enzymes. However, we found that routes used for synthesis of specific monofunctional acyl phosphate monoesters[13,14] did not produce dicarboxylic acid bis(methyl phosphates). We therefore have developed an alternative route in which sodium iodide[15] is used to convert a dimethyl acyl phosphate[16-18] to the monomethyl compound. Our key finding is that the dimethyl acyl phosphate can be prepared in a convenient reaction from the corresponding diacid chloride. The utility of anionic acylating agents in cross-linking hemoglobin is well documented.[19]

Scheme I. Reaction of Methyl Acetyl Phosphate and a Protein



Experimental Section

Materials and Methods. Commercial reagents were utilized without further purification. Solvents were dried prior to use. Acetone was dried over magnesium sulfate for 5 min and then filtered immediately before making solutions of sodium iodide. Tetrahydrofuran was dried by distillation from sodium benzophenone ketyl. Sodium dimethyl phosphate was prepared by the reaction of trimethyl phosphate with sodium iodide in acetone.

Deuterated solvents were from MSD Isotopes Ltd. and Aldrich Chemical Co. Organic reagents and solvents were purchased from BDH and Caledon Laboratories Ltd. Inorganic materials were purchased from BDH Canada Ltd. or Fisher Scientific. Human hemoglobin, cross-linked bovine hemoglobin (doubly crystallized, dialyzed, lyophilized), and proteins used as molecular weight standards for gel electrophoresis were from the Sigma Chemical Co.

Purity of Materials. The purity of samples of newly synthesized materials was assessed by a combination of NMR spectroscopy and analytical thin-layer chromatography. Elemental analyses were conducted Galbraith Laboratories, Knoxville, Tn. We found that the elemental analyses of the bis(dimethyl phosphate) salts in particular were not readily reproducible and variations were outside the normally acceptable range. Therefore, we developed a kinetic titration method that permits the integrity of a sample to be evaluated. This is especially useful for testing samples that may have undergone decomposition during storage. The amount of base necessary to completely saponify a sample with observed first-order kinetics is an accurate measure of purity (taken together with spectroscopic data) since each anhydride cleaved consumes 2 equiv of base. Freshly prepared samples were

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routinely >98% pure. Although the compounds are stable in neutral solution, they react with hydroxide so the kinetic titration is performed at pH 10.5, 40 °C, as described in the following section.

Kinetics. pH-Stat Kinetics. The rates of hydrolysis reactions of acyl phosphate monoesters were determined with a Radiometer TTT11 titrator and ABU13 autoburet as a pH-stat, interfaced to a Commodore PET microcomputer which was used to collect data. Temperature was maintained with a Neslab circulating bath through a glass-jacketed reaction vessel (20 mL) that was kept under argon and stirred magnetically. The titrant was 0.1 N sodium hydroxide, and the reaction medium was 1.0 M potassium chloride. The end point of each reaction is the extrapolated infinity value of the pseudo-first-order reaction. For difunctional acyl phosphates, 4 equiv of base is required per equivalent of bisanhydride hydrolyzed.

NMR Kinetics. This method was used to assess the long-term stability of samples in neutral solution. The dicarboxylic acid bis(methyl phosphate) (60 mM) in 1:1 D_2O:0.200 M Tris-HCl (pH 7.4) was incubated at 37 °C, and the ^{31}P NMR spectrum was recorded at 0.5-h intervals during a 9-h period and then at 24-h intervals.

Spectra. Proton NMR spectra were recorded on a Varian T-60 (60 MHz) spectrometer or a Varian Gemini (200 MHz) spectrometer. Phosphorus spectra were recorded on a Varian XL-200 spectrometer. ^{13}C NMR spectra were recorded on the Varian Gemini spectrometer. Infrared spectra were recorded on a Nicolet 5DX FTIR spectrometer.

Molecular mechanics calculations were performed with an Intel 80386 (25 MHz) microprocessor-based microcomputer with an 80387 mathematics coprocessor using the program ALCHEMY II from Tripos Associates, St. Louis, MO. The program uses standard force fields (described in the published manual) for bond bending, bond stretching, out-of-plane distortions (for coplanar functional groups), torsional energies, and van der Waals repulsions. Minimization is iterative. The starting conformation for the analysis had the molecule fully extended.

Cross-Linking of Hemoglobin. Solutions (2 mL) of stilbene-4,4'-dicarbonyl bis(methyl phosphate) at concentrations of 0.3, 3.0, 30, and 300 mM were prepared in 0.100 M Tris-HCl buffer, pH 7.4. A control solution was prepared and contained only the buffer. To each of these freshly prepared solutions, 40 mg of human hemoglobin was added so that the resulting solution was 0.3 mM in hemoglobin. The reaction mixtures were then stirred vigorously and incubated at 37 °C. After 4 h the solutions were dialyzed against 0.100 M sodium phosphate buffer, pH 7.0, for 20 h at 4 °C to remove the unreacted acyl phosphates. The samples were then lyophilized and analyzed by SDS-polyacrylamide gel electrophoresis. Fumaroyl bis(methyl phosphate) was reacted at concentrations of 0.3, 3.0, and 30 mM with hemoglobin as above.

Polyacrylamide gel electrophoresis of the reacted hemoglobin was carried out to assess the extent of intersubunit cross-linking.[20] Prior to electrophoresis, proteins (hemoglobin samples, cross-linked hemoglobin, and molecular weight standards) were denatured by boiling for 15 min in 0.11 M sodium phosphate buffer, pH 7.0, which contained 1% sodium dodecyl sulfate, 1% 2-mercaptoethanol, 36% urea, and 0.015% bromphenol blue. The final protein concentrations were 2 mg/mL, and 10–20 μL was loaded on the gel. The process was conducted with a Bio-Rad Mini-Protein II dual-slab cell apparatus. The extent of cross-linking of the hemoglobin could be estimated by visual comparison of the resolved electrophoretic bands after fixation followed by staining with Coomassie Brilliant Blue R. The results were recorded on photographs.

Syntheses. Dimethyl Acetyl Phosphate: From Acetyl Bromide. This material has previously been prepared by extended refluxing of a solution of acetyl chloride and trimethyl phosphate.[16,17] The reaction is accomplished much more rapidly by using acetyl bromide in place of acetyl chloride. Thus, acetyl dimethyl phosphate was prepared by dropwise addition of acetyl bromide (4 g, 32 mmol) to trimethyl phosphate (10 g, 71 mmol) at 50 °C over a period of 15 min. After an additional 15 min, the reaction solution was distilled at 0.30 Torr through a 20 × 1 cm vacuum-jacketed column. An initial low-boiling fraction of the excess trimethyl phosphate was followed by the product at 55–60 °C; yield, 4.0 g, 75%. The proton NMR spectrum was identical with that of material prepared from acetyl chloride and trimethyl phosphate[18] (in CCl_4, relative to tetramethylsilane, δ 2.2 (3 H, d, J_{P-H} = 1.5 Hz), 3.75 (6 H, d, J_{P-H} = 11 Hz)).

Dimethyl Acetyl Phosphate: From Sodium Dimethyl Phosphate and Acetyl Chloride. A suspension of sodium dimethyl phosphate (14.8 g, 0.1 mol, from trimethyl phosphate and sodium iodide) and acetyl chloride (7.8 g, 0.1 mol) in dry tetrahydrofuran (80 mL) was stirred for 2 days at room temperature in a flask fitted with a drying tube. The reaction solution was filtered and the tetrahydrofuran was removed under reduced pressure. The resulting colorless liquid was Kugelrohr distilled (Aldrich Kugelrohr apparatus, 55–60 °C, 0.30 Torr) to give 14.2 g (80%) of dimethyl acetyl phosphate.

Sodium Methyl Acetyl Phosphate (MAP). This was prepared as previously reported from dimethyl acetyl phosphate.[6] A solution of sodium iodide (2.4 g, 16 mmol) in dry acetone (15 mL) was added to a solution of acetyl dimethyl phosphate (2 g, 16 mmol) in dry acetone (10 mL). The pale yellow solution was allowed to stand overnight at room temperature. The precipitate was collected by filtration on a sintered-glass funnel and washed with dry acetone followed by methylene chloride. The resulting white powder was dried under vacuum and recrystallized from hot 2-propanol to give 2.2 g (80%) of the sodium salt of methyl acetyl phosphate. The ^1H NMR spectrum was identical with that of material produced by the previously reported procedure[6] (in D_2O, relative to DSS, δ 2.18 (3 H, J_{P-H} = 1.4 Hz), 3.67 (3 H, d, J_{P-H} = 11.6 Hz)).

Fumaroyl Bis(dimethyl phosphate) (1). A suspension of sodium dimethyl phosphate (6.9 g, 47 mmol) and fumaroyl chloride (3.6 g, 24 mmol) was stirred in dry tetrahydrofuran (60 mL) under nitrogen at room temperature for 2 days. The solution was filtered through a sintered-glass funnel and solvent was removed, leaving the product as a solid. Recrystallization from benzene and ether yielded the product as white flakes (4.7 g, 61%): mp 76–77 °C; IR (KBr) C=O 1743 cm^{-1}; ^1H NMR ($CDCl_3$) δ 6.93 (HC=C, 2 H, s), 4.05 (OCH_3, 12 H, d, J_{P-H} = 11.6 Hz); ^{13}C NMR ($CDCl_3$) δ 158.12 (d, J_{P-C} = 7.9 Hz), 134.39 (d, J_{P-C} = 9.4 Hz), 55.39 (d, J_{P-C} = 5.9 Hz); ^{31}P NMR ($CHCl_3$) δ −6.2 (sept, J_{P-H} = 11.9 Hz). The reaction was repeated with addition of 0.01 and 0.1 equiv of 18-crown-6 (in order to increase the extent of dissolution of sodium phosphate). The yield was lower in both cases than when the crown ether was absent.

Isophthaloyl bis(dimethyl phosphate) (2) was prepared from isophthaloyl dichloride (4.83 g, 23 mmol) and sodium dimethyl phosphate (6.9 g, 47 mmol) in dry tetrahydrofuran (50 mL) as above to produce a colorless oil in 83% yield: IR (film) C=O 1754 cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.72 (1 H, t, J = 1.7 Hz), 8.35 (2 H, dd, J = 1.7, 7.8 Hz), 7.68 (1 H, t, J = 7.8 Hz), 4.02 (12 H, d, J = 11.7 Hz); ^{13}C NMR ($CDCl_3$) δ 160.60 (d, J_{P-C} = 7.0 Hz), 137.39 (d, J_{P-C} = 11.5 Hz), 136.94, 133.43, 130.39, 56.23 (d, J_{P-C} = 4.8 Hz); ^{31}P NMR ($CHCl_3$) δ −4.7 (sept, J_{P-H} = 11.7 Hz).

Terephthaloyl bis(dimethyl phosphate) (3) was prepared from terephthaloyl dichloride (4.83 g, 23 mmol) and sodium dimethyl phosphate (6.9 g, 47 mmol) in dry tetrahydrofuran (50 mL) as above to give a white solid. Recrystallization from benzene with addition of ether gave crystals (91% yield): mp 81–82 °C, IR (KBr) C=O 1743 cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.10 (4 H, s), 4.00 (12 H, d, J_{P-H} = 12 Hz); ^{13}C NMR ($CDCl_3$) δ 160.00 (d, J_{P-C} = 7.9 Hz), 133.10 (d, J_{P-C} = 8.6 Hz), 130.90, 55.48 (d, J_{P-C} = 4.7 Hz); ^{31}P NMR ($CHCl_3$) δ −4.6 (sept, J_{P-H} = 11.8 Hz).

Conversion of bis(dimethyl phosphates) to bis(methyl phosphates) was accomplished by reaction with sodium iodide in acetone. The products were identified as symmetrical monomethyl phosphates by analysis of proton-coupled ^{31}P NMR spectra and proton NMR spectra. The proton-coupled ^{31}P NMR spectrum of a bis(dimethyl phosphate) consists of a single phosphorus signal that is a septet due to coupling of two equivalent

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phosphorus nuclei to equivalent sets of six protons (from the two methyl groups). Cleavage of one methyl group from each end converts the material to one whose phosphorus NMR signal is a quartet. Integration of the signal of the methoxy protons in the proton NMR spectrum, relative to that of the remaining protons in the molecule, shows that cleavage of half of the ester groups has occurred.

Fumaroyl Bis(sodium methyl phosphate) (1a). A solution of sodium iodide (0.9 g, 6 mmol) in dry acetone (6 mL) was added to an acetone (6 mL) solution of fumaroyl bis(dimethyl phosphate) (1 g, 3 mmol) in a 25-mL flask. The solution was shaken and the flask was left for 12 h, during which time the product precipitated as a pale yellow powder. Filtration followed by washings with dry acetone and methylene chloride resulted in an off-white powder that was dried under vacuum. One gram of the material was recrystallized by dissolving in 20 mL of methanol. Then 40 mL of 1:1 ethanol:2-propanol was added and the solution was allowed to stand for 30 min. The resulting crystals were collected and dried in vacuo (93% yield): mp >200 °C; IR (KBr) C=O 1714 cm^{-1}; ^1H NMR (D_2O) δ 6.85 (HC=C, 2 H, d, J = 2 Hz), 3.65 (OCH_3, 6 H, d, J_{P-H} = 12 Hz); ^{13}C NMR (D_2O) δ 163.0 (d, J_{P-C} = 8 Hz), 135.6 (d, J_{P-C} = 7.6 Hz), 54.76 (d, J_{P-C} = 6.4 Hz); ^{31}P NMR (D_2O) δ -6.2 (q, J_{P-H} = 11.3 Hz). Anal. Calcd for $C_6H_8O_{10}P_2Na_2$: C, 20.69; H, 2.32; P, 17.80. Found: C, 20.46; H, 2.38; P, 17.39.

Isophthaloyl bis(sodium methyl phosphate) (2a) was prepared in 95% yield from isophthaloyl bis(dimethyl phosphate) (1.82 g, 4.8 mmol) and sodium iodide (1.44 g, 9.6 mmol) as above: mp >200 °C; IR (KBr) C=O 1720 cm^{-1}; ^1H NMR (D_2O) δ 8.52 (1 H, t, J = 1.8 Hz), 8.17 (2 H, dd, J = 1.8, 7.9 Hz), 7.52 (1 H, t, J = 7.9 Hz), 3.56 (6 H, d, J_{P-H} = 11.4 Hz); ^{13}C NMR (D_2O) δ 166.40 (d, J_{P-C} = 8.1 Hz), 138.72, 137.95 (d, J_{P-C} = 10.0 Hz), 134.89, 132.66, 56.91 (d, J_{P-C} = 6.2 Hz); ^{31}P NMR (D_2O) δ -6.2 (q, J_{P-H} = 11.4 Hz).

Terephthaloyl bis(sodium methyl phosphate) (3a) was prepared in 95% yield from terephthaloyl bis(dimethyl phosphate) (1 g, 2.6 mmol) and sodium iodide (0.78 g, 5.2 mmol) in acetone as above: mp >200 °C; IR (KBr) C=O 1715 cm^{-1}; ^1H NMR (D_2O) δ 7.93 (4 H, s), 3.56 (6 H, d, J_{P-H} = 11.5 Hz); ^{13}C NMR (D_2O) δ 166.36 (d, J_{P-C} = 8.1 Hz), 136.63 (d, J_{P-C} = 7.0 Hz), 133.35, 56.72 (d, J_{P-C} = 2.6 Hz); ^{31}P NMR ($CHCl_3$) δ -3.2 (q, J_{P-H} = 11.6 Hz). Anal. Calcd for $C_{10}H_{10}O_{10}P_2Na_2$: C, 30.17; H, 2.53; P, 15.56. Found: C, 30.15; H, 2.62; P, 15.67.

Stilbene-3,3′-dicarboxylic acid and stilbene-4,4′-dicarboxylic acid were prepared by the method of Toland et al.[21]

Stilbene-3,3′-dicarbonyl Dichloride. The dicarboxylic acid (4.9 g, 18 mmol), thionyl chloride (50 mL), and a catalytic amount of dry dimethylformamide (10 drops) were refluxed for 12 h. Excess thionyl chloride (20 mL) was distilled off and the product crystallized as yellow needles. The crystals (2.53 g, 8.3 mmol, 46%) were collected by filtration, washed carefully with ether, and pumped dry. Recrystallization from toluene gave pure material (mp 179–181 °C (lit.[5] mp 179–181 °C)); ^1H NMR ($CDCl_3$) δ 3.68 (16 H, s), 7.07 (2 H, s), 7.20–7.51 (8 H, m).

Stilbene-3,3′-dicarbonyl Bis(dimethyl phosphate) (4). Stilbene-3,3′-dicarbonyl dichloride (2.4 g, 7.9 mmol) and sodium dimethyl phosphate (2.3 g, 15.8 mmol) were stirred at room temperature in dry tetrahydrofuran for 48 h under nitrogen. The reaction mixture was filtered and solvent evaporated to give a solid. This was recrystallized from benzene/ether (2.62 g, 5.4 mmol, 68% yield): mp 114 °C; ^1H NMR ($CDCl_3$) δ 8.19 (1 H, t, J = 1.6 Hz), 7.99 (1 H, dt, J = 1.6, 7.7 Hz), 7.95 (1 H, dt, J = 1.6, 7.7 Hz), 7.52 (1 H, t, J = 7.7 Hz), 4.02 (12 H, d, J = 11.7 Hz); ^{13}C NMR ($CDCl_3$) δ 161.00 (d, J_{P-C} = 8.3 Hz), 137.60, 132.50, 130.00, 129.33, 128.91, 128.74, 128.56 (d, J_{P-C} = 8.5 Hz), 55.35 (d, J_{P-C} = 5.4 Hz); ^{31}P NMR ($CHCl_3$) δ -5.2 (sept, J_{P-H} = 11.8 Hz); TLC R_f 0.29 (silica plates, 1:1 dichloromethane:ethyl acetate).

Stilbene-3,3′-dicarbonyl bis(sodium methyl phosphate) (4a) was prepared from stilbene-3,3′-dicarbonyl bis(dimethyl phosphate) (3.52 g, 7.3 mmol) and sodium iodide (2.19 g, 14.6 mmol) in acetone as above in 93% yield: mp >200 °C; IR (KBr) C=O 1714 cm^{-1}; ^1H NMR (D_2O) δ 7.51 (2 H, d, J = 7.9 Hz), 7.47 (2 H, s), 7.22 (2 H, d, J = 7.9 Hz), 7.10 (12 H, t, J = 7.9 Hz), 6.56 (s, 2 H), 3.62 (6 H, d, J = 11.4 Hz); ^{13}C NMR (D_2O) δ 167.00 (d, J_{P-C} = 8.3 Hz), 140.01, 134.88, 132.30, 132.08, 131.94, 131.76, 130.05 (d, J_{P-C} = 8.1 Hz), 56.95; ^{31}P NMR (D_2O) δ -3.2 (q, J_{P-H} = 11.2 Hz); TLC R_f 0.39 (silica plates, ethanol).

Stilbene-4,4′-dicarbonyl Dichloride. A suspension of stilbene-4,4′-dicarboxylic acid (5 g, 18.6 mmol), thionyl chloride (60 mL), and a catalytic amount of dry dimethylformamide (0.25 mL) was refluxed for 24 h. The dicarboxylic acid did not dissolve but was converted directly into the diacid chloride, which was also largely insoluble in the reaction medium. The reaction solution was refrigerated for 12 h. The yellow crystalline product was collected by filtration, washed sparingly with acetone, and pumped to dryness (5.2 g, 17 mmol, 92% yield): mp 238 °C; ^1H NMR (dimethyl-d_6 sulfoxide) δ 7.95 (4 H, d, J = 10 Hz), 7.73 (4 H, d, J = 10 Hz), 7.48 (2 H, s).

Stilbene-4,4′-dicarbonyl Bis(dimethyl phosphate) (5). Stilbene-4,4′-dicarbonyl dichloride (2.6 g, 8.5 mmol) and sodium dimethyl phosphate (3.1 g, 21.3 mmol) were stirred in dry THF at 50 °C under nitrogen for 48 h. The reaction mixture was filtered through a sintered-glass funnel and the solvent removed by evaporation to give the crude product. Recrystallization from tetrahydrofuran/ether gave pure material (1.19 g, 23%): mp 176–177 °C; ^1H NMR ($CDCl_3$) δ 8.08 (4 H, d, J = 8.5 Hz), 7.65 (4 H, d, J = 8.5 Hz), 7.29 (2 H, s), 4.03 (12 H, d, J = 11.7 Hz); ^{13}C NMR ($CDCl_3$) δ 160.5 (d, J_{P-C} = 8.1 Hz), 142.5, 131.1, 127.1, 127.0, 55.2 (d, J_{P-C} = 5.9 Hz); ^{31}P NMR ($CHCl_3$) δ -5.4 (sept, J_{P-H} = 11.7 Hz); thin-layer chromatography, R_f 0.27 (silica plates, 1:1 dichloromethane:ethyl acetate).

Stilbene-4,4′-dicarbonyl bis(sodium methyl phosphate) (5a) was prepared from stilbene-4,4′-dicarbonyl bis(dimethyl phosphate) and sodium iodide in acetone as above: mp >200 °C; IR (KBr) C=O 1715 cm^{-1}; ^1H NMR (D_2O) δ 7.88 (4 H, d, J = 8.1 Hz), 7.27 (4 H, d, J = 8.1 Hz), 6.77 (2 H, s), 3.80 (6 H, d, J = 11.3 Hz); ^{13}C NMR (D_2O) δ 166.7 (d, J_{P-C} = 8.3 Hz), 144.8, 133.5, 132.7, 130.8 (d, J_{P-C} = 8.1 Hz), 129.7, 56.6; ^{31}P NMR (D_2O) δ -2.4 (sept, J_{P-H} = 11.3 Hz).

Results and Discussion

General Route to Dimethyl Acyl Phosphates and Methyl Acyl Phosphates. The reaction of acid chlorides with trimethyl phosphate under reflux, in analogy to the published method for producing dimethyl acetyl phosphate,[16,17] did not succeed with succinyl chloride or fumaroyl chloride. However, we found that the synthesis of dimethyl acetyl phosphate can be achieved efficiently through the reaction of acetyl bromide and trimethyl phosphate. The reaction with acetyl bromide is complete at room temperature in a few hours instead of the week at reflux required with acetyl chloride. This did not extend to diacids, however.

A potential route for the production of a dimethyl acyl phosphate is the reaction of an acyl halide with a dimethyl phosphate salt. Hata and co-workers reported that tributyltin diethyl phosphate, which is prepared from sodium diethyl phosphate, reacts with acid chlorides in carbon tetrachloride to give diethyl acyl phosphates.[18] These workers reported that the esters decomposed upon distillation.

In our attempts to extend this procedure, we found that the products of the coupling reaction contain a large amount of contaminating alkyltin derivatives that are not readily separated and that interfere with the distillation. However, there is a convenient and superior alternative. We find that sodium dimethyl phosphate can be used in a direct reaction with acid chlorides in tetrahydrofuran (the reaction presumably occurs through a small amount of sodium dimethyl phosphate that dissolves and reacts with the acid chloride) to give the dimethyl acyl phosphate. The insoluble byproduct is sodium chloride and efficient distillation or chromatography permits isolation of the desired product in high yield (see Scheme II).

Scheme II. General Synthesis of Acyl Phosphate Dimethyl Esters

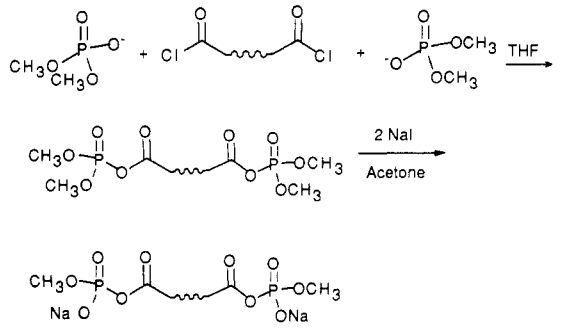
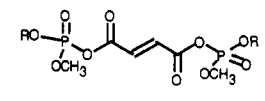


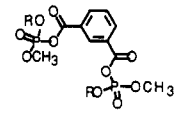
Table I. Estimated Distance between Amino Groups That Can Be Cross-Linked with Diacyl Bis(methyl phosphates) As Calculated from the Span of the Diamide Derived from the Dicarboxylic Acid

diacyl bis(methyl phosphate)	span of amide, Å
fumaroyl (**1a**)	6.1
isophthaloyl (**2a**)	7.2
terephthaloyl (**3a**)	7.4
3,3′-stilbenedicarbonyl (**4a**)	13.7
4,4′-stilbenedicarbonyl (**5a**)	13.9

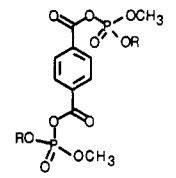
We have used this process followed by reaction of the resulting neutral ester with sodium iodide in acetone to prepare MAP as well as difunctional materials (**1–5**, **1a–5a**).



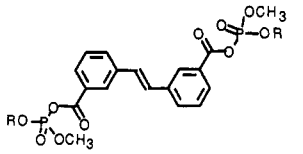
1, R = CH_3; **1a**, R = Na



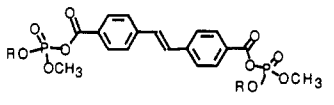
2, R = CH_3; **2a**, R = Na



3, R = CH_3; **3a**, R = Na



4, R = CH_3; **4a**, R = Na



5, R = CH_3; **5a**, R = Na

Design of Cross-Linking Agents: Molecular Mechanics. The choice of the difunctional materials was based on calculations of their size relative to the distance for cross-linking amino groups in the diphosphoglycerate binding site of human hemoglobin.[12,19] Crystallographic studies of human hemoglobin show that the site that binds the anionic regulator species 2,3-diphosphoglycerate is highly cationic.[24] The site contains the amino group of the terminal valine of the β-polypeptide chain as well as a number of lysyl side chains. The distances between amino groups in the diphosphoglycerate binding site are thus accurately known. Manning and co-workers have shown that methyl acetyl phosphate acetylates amino groups exclusively in this region of the protein,[8,9] presumably due to the anionic state of the material, as had been proposed for the reaction of the same reagent with D-3-hydroxybutyrate dehydrogenase.[7]

We calculated the structure of carboxamide derivatives of the acyl phosphates as models for the products that would result from the reaction of amino groups on the protein with the acyl phosphates. In Table I we present the calculated distances in the carboxamides.

Stability of Bis(methyl acyl phosphates). Since the diacyl bis(methyl phosphates) are electrophiles, it is important to test if they react with water or buffers during the course of the reaction with a protein. Thus, the hydrolysis of **1a**, **4a**, and **5a** in 0.100 M Tris-HCl buffer, pH 7.4, was followed by integrating signals for the reactant and the phosphorus-containing product, methyl phosphate, in the ^{31}P NMR spectrum. Production of methyl phosphate was observed as an indication of hydrolysis or of acylation of the buffer. The half-life of the fumaroyl compound, **1a**, was 36 h under these conditions. The half-lives of the two stilbene derivatives were over 100 h. This indicates that the reagents do not decompose rapidly in neutral solution and that they do not react rapidly with buffer, in agreement with earlier reports on the reactivity of acyl phosphate esters.[4] The hydrolysis is expected to be base catalyzed,[4] and the rate of reaction at pH 10.5 (40 °C) was sufficiently rapid (half-times range from 2 to 22 min for all compounds in the study) to use quantitative saponification as a measure of the number of acylating equivalents in a sample.

Hemoglobin Modification. Two of the bis(methyl acyl phosphates) were tested for their ability to cross-link hemoglobin as a general test of the hypothesis of this study. Thus, reaction of hemoglobin with stilbene-4,4′-dicarbonyl bis(methyl phosphate) and with fumaroyl bis(methyl phosphate) produced dimeric and tetrameric species as indicated by SDS gel electrophoresis. The lanes containing the hemoglobin modified with these reagents show bands corresponding to the dimer (32 000), trimer (48 000), and tetramer (64 000) (compared with the Sigma Dalton Mark VII-L standard cross-linked bovine hemoglobin). As well, there are trace bands of higher molecular weight. Bands for unreacted hemoglobin indicate that this material is fully dissociated into monomers. Control experiments with hemoglobin that has been reacted with MAP and with stilbenedicarboxylic acid gave materials that are monomeric according to the gel patterns. These results indicate that cross-linking reactions are taking place only in the presence of the difunctional acyl phosphate esters. On the basis of the known specificity of the reactions of monofunctional acyl phosphate esters with hemoglobin,[8,9] it is likely that the cross-linking reaction occurs in the region of the protein that binds 2,3-diphosphoglycerate. Recently, Jones and co-workers have found that the sites of reaction of human hemoglobin with fumaroyl bis(methyl phosphate) are in that site.[25] In the absence of 2,3-diphosphoglycerate, reaction of fumaroyl bis(methyl phosphate) produces material that is cross-linked between β subunits (Val-1 to Lys-82) and between the same residues in a single β subunit as well as a cross-link between α subunits involving Lys-99 in each subunit. In the presence of 2,3-diphosphoglycerate, only the α–α cross-link is

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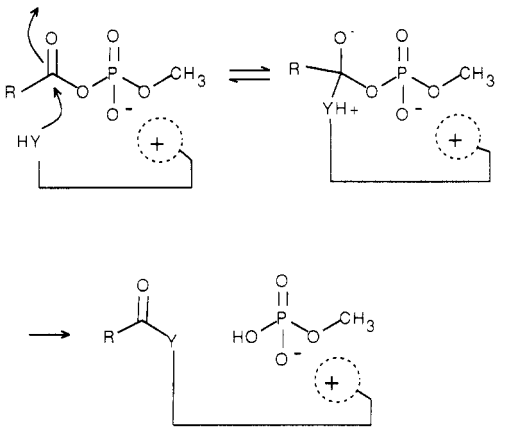
Scheme III. Mechanism of Acylation Reaction



formed. Detals of that work will be published when that study is completed.

Generalization. The reaction of an acyl chloride and dimethyl phosphate followed by ester cleavage provides a convenient means of preparing monomethyl esters of a wide variety of acyl phosphates. Since the coupling reaction is independent of the alkyl group, it is likely that the method can be extended to give materials in which other alkyl and or aryl ester functions are present. In addition, cleavage of a single ester group by the reaction with sodium iodide can be accomplished with other primary alkyl or benzyl side chains. Since the reagents appear to recognize a general class of site on a protein and react with amino groups in that site, they also should function as general inactivators of enzymes with similar active sites. A preliminary survey reveals that fumaroyl bis(methyl phosphate) irreversibly inactivates enzymes that bind NADH.[29]

Mechanism of the Reaction of Acyl Phosphates with Nucleophiles. The addition of a nucleophile to an acyl phosphate ester is expected to form a tetrahedral addition intermediate that can revert to reactants or proceed to give the acylated nucleophile (Scheme III). The initial tetrahedral adduct can revert to starting materials or expel methyl phosphate. Since the leaving group, methyl phosphate, is moderately basic, it will leave less readily than very weakly basic groups such as water, alcohols, or halide ions, and the reagent will manifest its selectivity even after adduct formation has occurred.

The half-time for hydrolysis of phenyl acetyl phosphate is approximately 80 h at 39 °C ($k = 2.50 \times 10^{-6}$ s^{-1}) while the second-order rate constant for the reaction of phenyl acetyl phosphate and glycine is 41 M^{-1} s^{-1} (at 39 °C).[4] Thus, in contrast to their stability in water, acyl phosphate monoesters react rapidly with amine nucleophiles and are selective. When these molecules associate with a protein, they should react with adjacent nucleophiles, as is observed. On the other hand, dialkyl acyl phosphates are extremely reactive toward nucleophiles and are unstable in water.[17] The leaving group (dimethyl phosphate in the case of dimethyl acetyl phosphate) is the conjugate base of a strong acid and thus leaves readily from the tetrahedral intermediate.

Conclusions

We have shown that acyl phosphate monomethyl esters can be prepared by a route that should permit almost any carboxylic acid to be converted to the corresponding acyl phosphate monomethyl ester or diester. The combination of a negative charge and electrophilic reactivity will make these materials candidates for trials as site-directed reagents for protein modification. The difunctional analogues can be tested as site-directed cross-linking agents. Combination of the acyl phosphate monoester functional group with other selective electrophiles in a heterodifunctional molecule should provide reagents that will give further types of specificity.

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Chelating Ligands Functionalized for Facile Attachment to Biomolecules. A Convenient Route to 4-Isothiocyanatobenzyl Derivatives of Diethylenetriaminepentaacetic Acid and Ethylenediaminetetraacetic Acid

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The title compounds were synthesized by alkylation of the monoenolates of DTPA and EDTA permethyl esters **3** and **4** with benzyl bromide. The benzylated esters **5** and **6** were nitrated, hydrogenated, and converted to their isothiocyanate derivatives **13** and **14** in good yields. The Gd(III) complex of 4-(isothiocyanatobenzyl)-DTPA **16**, a potential contrast-enhancing agent for magnetic resonance imaging (MRI), was prepared.

Poly(amino carboxylate) chelates of metal ions are widely used as probes of protein structure,[1] as contrast-enhancing agents for magnetic resonance imaging (MRI),[2] and as radiopharmaceuticals.[3] Recent novel applications of these chelates involve their covalent attachment to drugs, antibodies, or other biomolecules. For example, Meares and co-workers[4] have prepared a bleomycin–EDTA

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