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Functionalized 3,5-Dihydroxybenzoates as Potent Novel Inhibitors of EPSP Synthase

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Abstract—Aromatic analogues of the EPSP synthase enzyme substrate (S3P), reaction intermediate (**1**), and product (EPSP) were synthesized from 3,5-dihydroxybenzoic acid and were evaluated as inhibitors of *E. coli* EPSP synthase. These simple, synthetically accessible aromatic analogues are highly effective competitive inhibitors versus S3P with an apparent K_i for the tetrahedral intermediate analogue **4** of 160 ± 40 nM. This demonstrates that a simple benzene ring is a quite suitable substitute for the complex shikimate ring in the design of EPSP synthase inhibitors.

Introduction

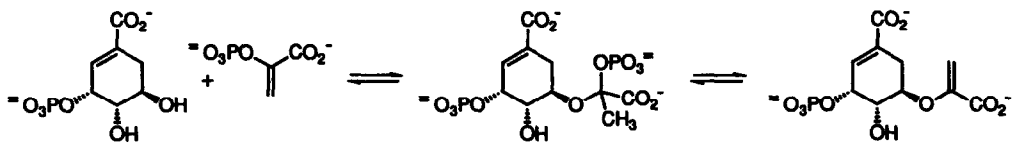
The enzyme 5-enolpyruvoylshikimate-3-phosphate (EPSP) synthase (EC 2.5.1.19) occupies a central position in aromatic amino acid biosynthesis[1] and as such represents an important target for rational inhibitor design.[2] EPSP synthase catalyzes the remarkable transfer of the carboxyvinyl moiety of phosphoenolpyruvate (PEP) to the 5-hydroxyl of shikimate-3-phosphate (S3P), via the key tetrahedral intermediate **1**[3] (Scheme I). The design of shikimate-based analogues of **1** has been successfully implemented for the development of potent EPSP synthase inhibitors.[4] However, the construction of such complex molecules represents a formidable synthetic undertaking, and we sought an alternative approach to effective yet simple and more readily accessible inhibitors.

Spectroscopic investigations conducted in these laboratories have determined that the shikimate ring in enzyme-bound S3P and EPSP adopts an unusually flattened conformation.[5] It occurred to us that aromatic rings might effectively mimic this conformational state and thus serve as targets for the synthesis of novel, highly potent EPSP synthase inhibitors. We now report the successful realization of this idea and describe a series of aromatic analogues (**2**–**4**, Scheme II) of the enzyme substrate (S3P), product (EPSP), and intermediate **1** that are readily elaborated from 3,5-dihydroxybenzoic acid. These simple aromatic analogues function as highly effective EPSP synthase inhibitors, while the tetrahedral intermediate analogue **4** is one of the most potent aromatic inhibitors of the shikimate pathway identified to date.[6]

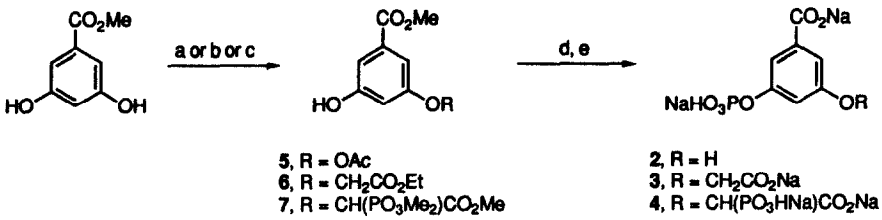
Results and Discussion

The aromatic phosphates **2**–**4** were conveniently prepared from methyl 3,5-dihydroxybenzoate (Scheme II) via intermediates **5**–**7** bearing the desired C-5 functional groups in protected form. As an alternative to hydroxyl group differentiation in the symmetrical bis-phenol, we generally found it easier to separate product mixtures formed directly from the starting material. The glycolyl intermediate **6** was expeditiously prepared by sodium iodide-catalyzed alkylation of the unprotected dihydroxybenzoate with ethyl bromoacetate followed by chromatographic separation. In this case, even the robust *t*-butyldimethylsilyl group was ineffective as a protecting group under the conditions required for successful phenol alkylation. By contrast, rhodium acetate-catalyzed coupling[7] of monosilylated 3,5-dihydroxybenzoate with trimethyl diazophosphonoacetate[8] smoothly provided the key intermediate **7** after desilylation with tetrabutylammonium fluoride.

Synthetic intermediates **5**–**7** were easily converted to the desired inhibitors **2**–**4** by reaction of the sodium phenoxides with tetrabenzyl pyrophosphate,[9] followed by phosphorus ester cleavage (TMSBr), saponification (NaOH), anion exchange chromatography (DEAE



Scheme I. The reaction catalyzed by EPSP synthase.



Scheme II. Reagents and conditions: (a) Preparation of **5**: Ac_2O, pyridine, DMAP; (b) Preparation of **6**: $BrCH_2CO_2Et$, K_2CO_3, NaI, acetone, reflux; (c) Preparation of **7**: TBDMSCl, imidazole, CH_2Cl_2; $Me_2O_3PC(=N_2)CO_2Me$, $Rh_2(OAc)_4$, benzene, reflux; TBAF, THF; (d) NaH, $Bn_4P_2O_7$, THF; (e) TMSBr, Et_3N, CH_2Cl_2; NaOH, H_2O; DEAE Sephadex chromatography; AG 50W-X8 (Na^+).

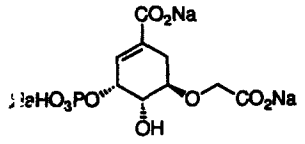
Sephadex, triethylammonium bicarbonate), and cation exchange to the sodium salts. The aromatic phosphates are stable as dry solids, although slow phosphate ester hydrolysis is observed in solution below pH 7.

The aromatic analogues **2–4** were evaluated for inhibition of *E. coli* EPSP synthase[10] by monitoring the conversion of [^{14}C]-PEP or [^{14}C]-S3P to [^{14}C]-EPSP.[11] As a first approximation, IC_{50} values were determined and are shown in Table 1. For comparison purposes, the IC_{50} for EPSP-mediated product inhibition of the forward reaction of EPSP synthase is shown in Table 1, as well. Our results demonstrate that the aromatic phosphates display significant inhibition of EPSP synthase, with a striking increase in enzyme affinity proceeding from the S3P analogue **2** to the tetrahedral intermediate analogue **4**. This increase in binding closely parallels the trend observed for the analogous shikimate series of compounds and demonstrates that appropriate ionic functional groups incorporated at the C-5 position can access the enzymatic PEP binding site and thus markedly enhance inhibition.

Table 1. Inhibition of *E. coli* EPSP Synthase

Compound	IC_{50} (mM)[a]	$K_{i,\ apparent}$ (μM)
2	7.9	
3	1.4	
4	0.020	0.16 ± 0.04
8	0.10	1.53 ± 0.26
EPSP	0.18	(K_d = 1.0 ± 0.01)[b]

[a] Concentration of inhibitor necessary to provide 50 % inhibition at fixed concentrations of S3P and PEP of 100 μM at 30 °C in 100 mM HEPES/KOH, 50mM KCl, pH 7.0.
[b] Reference 10b.



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When the EPSP analogue **3** is compared with the shikimate glycolyl analogue **8**[12] or to EPSP, it is evident that these aromatic inhibitors bind to EPSP synthase with affinities approximately one order of magnitude less than those for their corresponding shikimate counterparts. It is likely that much of this attenuation can be attributed to the lack of a C-4 hydroxyl group in the present aromatic series.[13] A more complete kinetic characterization of the most active compound **4** was conducted at varying S3P concentrations demonstrating competitive inhibition versus S3P (Figure 1) with an apparent K_i of 160 ± 40 nM.[14] This enhanced level of inhibition is comparable to that displayed by the well known EPSP synthase inhibitor glyphosate (K_d = 150 ± 30 nM).[15]

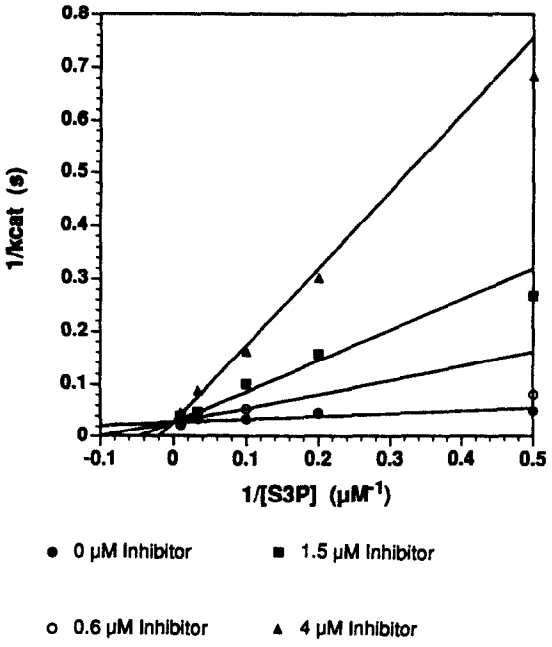

Figure 1. Inhibition of EPSP synthase by compound **4**. Double reciprocal plot of reaction velocity versus S3P concentration obtained at varying concentration of inhibitor **4**. The lines drawn were obtained from a nonlinear least squares fit to the observed data using equation (1) which corresponds to a kinetic model for competitive inhibition. Fitting to equation (2), corresponding to a kinetic model for mixed inhibition, gave an equivalent 'goodness of fit' as fitting to equation (1), however, the uncompetitive contribution to the overall inhibition was insignificant (i.e., $K_{ii} >> K_{is}$). The data, therefore, is best interpreted in terms of competitive inhibition versus S3P.

In conclusion, we have shown that the structural requirements for effective inhibitors of EPSP synthase can be dramatically simplified by taking advantage of information about the conformation of the reaction substrate and product. Strong inhibition can be maintained in aromatic inhibitors streamlined by elimination of all three stereocenters from the shikimate ring. Our results thus clearly demonstrate that simple benzene rings can be substituted for the highly complex shikimate moiety previously thought to be required for effective inhibition of EPSP synthase. This discovery provides new opportunities in the design of EPSP synthase inhibitors and is likely to have broad impact in other inhibitor design

strategies. Aromatic compounds also provide a simple synthetic framework for the incorporation and investigation of stable mimics of the somewhat labile 3-phosphate group in EPSP synthase inhibitors.[16]

Experimental Section

Enzyme assays. General

Triethylammonium bicarbonate buffer (TEAB) was prepared by bubbling CO_2 through an aqueous solution of freshly distilled triethylamine of appropriate concentration, with constant stirring at 4 °C, until the solution pH reached 7.5. Buffers and reagents were obtained from commercial sources. Barnstead Nanopure water was used throughout the study.

EPSP synthase, over-expressed in *E. coli* (strain pMON6001),[10a] was purified by the procedure described previously.[10b] EPSP synthase stock solutions were stored at -80 °C in 25 mM Tris-HCl, pH 7.5, containing 25 mM KCl, 5 mM β-mercaptoethanol, and 10 % (w/v) glycerol. The specific activity of protein preparations varied from 55 to 70 μmoles min^{-1} mg^{-1}. Protein concentrations were determined from the absorbances at 280 nm, using an extinction coefficient of 35,200 M^{-1} cm^{-1}.[17]

Unlabeled S3P was synthesized from shikimic acid as previously described.[18] Similarly, EPSP was prepared enzymatically from S3P and PEP by incubation with EPSP synthase. [U-^{14}C]-S3P was synthesized from [U-^{14}C]-shikimic acid using a partially purified preparation of shikimate kinase.[19] [U-^{14}C]-shikimic acid (50 μCi, 19.7 mCi/mmol, New England Nuclear) was dried under nitrogen, resuspended in 1 mL of distilled water, neutralized with TEAB, and incubated at a final concentration of 1 mM with 2 mM ATP, 3 mM magnesium acetate, 0.2 mM sodium tungstate, 100 mM HEPES/KOH (pH 7.0), 50 mM potassium chloride, 1 mM dithiothreitol, 10 % (w/v) glycerol, and 0.2 mL shikimate kinase in a final volume of 2.43 mL. Progress of the reaction was monitored by HPLC using isocratic elution of shikimic acid and S3P from an anion exchange column (Partisil-10 SAX, Whatman Inc., Clifton, New Jersey) with 150 mM potassium phosphate (pH 5.5) at a flow rate of 1 mL/min. A 25 min incubation at 23 °C yielded 98 % conversion of [U-^{14}C]-shikimic acid to [U-^{14}C]-S3P.

[U-^{14}C]-S3P was purified by anion exchange chromatography over a 1.5 x 16 cm bed volume of DEAE Sephadex A-25 using a 300 mL linear gradient from 0.2 to 0.8 M TEAB. The major peak fractions identified by liquid scintillation counting were pooled and concentrated by rotary evaporation. The dried product was resuspended in 100 % ethanol and dried two times before a final resuspension in distilled water. Purity of the final product (3.0 mL, 35.9 mCi) was determined by HPLC to be 99 % and 88 % by ^{14}C and UV (220 nm) monitoring, respectively. The sample was stored at -80 °C in a stoppered serum vial, thawed for each experiment and then refrozen. This sample was stable for several months and numerous freeze/thaw cycles.

EPSP synthase kinetic assays

For kinetic assays, stock solutions of EPSP synthase were prepared and stored at -80 °C as single-use aliquots in 20 mM HEPES/KOH, pH 7.0, containing 2 mM dithiothreitol and 40 % (w/v) glycerol. Activity assays were performed as generally described by Padgette *et al.*[11] in 100 mM HEPES/KOH, pH 7.0, 50 mM KCl. For the determination of IC_{50}, substrate concentrations of 100 μM S3P and 100 μM PEP were used varying the inhibitor concentration. For analyzing competitive inhibition of EPSPS with respect to S3P, the [S3P] was varied from 2 to 100 μM while [PEP] was held constant at 100 μM. Approximately 32,000 dpm of either [1-^{14}C]-PEP (Amersham) or [U-^{14}C]-S3P was included in order to monitor the conversion of substrates to product. Test compounds to be examined as potential inhibitors were prepared as 5X-molar stock solutions in 150 mM TEAB, pH 7–8.5, and included in the incubation solution before enzyme addition. The final concentration of TEAB in the assay mixture was 30 mM.

Enzyme assays were initiated by the addition of EPSP synthase stock solution to temperature-equilibrated buffer containing substrates and inhibitors. In order to maintain steady state conditions over the time course of the reaction, the final enzyme concentration was adjusted to ensure that less than 25 % of labeled substrate was converted to product. The assay solution was incubated at 30 °C for 4 min, and was then quenched by the addition (1:1 v/v) of 10 % 1.0 M potassium acetate, pH 4.5, in ethanol. The percent conversion of labeled substrate to [^{14}C]-EPSP was determined by HPLC over a 25 cm x 4.6 mm Synchropak AX-100 anion exchange column (Synchrom, Inc.). Elution was performed isocratically with 240 mM potassium phosphate (pH 6.5) at a flow rate of 1 mL/min. S3P, PEP, and EPSP typically eluted at 6.0, 9.3 and 16.8 min, respectively, under these conditions. Peaks containing radioactivity were detected and integrated using an in-line radioactive flow detector (Flow-One Beta, Radiomatic Instruments and Chemical Co., Inc.) with Floscint IV scintillation cocktail (Radiomatic Inst., Meriden, CT) at a flow rate of 4 mL/min. Enzyme activity was calculated by multiplying the percent turnover of labeled substrate to [^{14}C]-EPSP by the initial amount of labeled substrate and dividing by the incubation time and assay protein concentration. Enzyme activity was observed to be linear throughout the incubation period (i.e. up to 25 % substrate turnover).

Data obtained from the examination of enzyme activity as a function of substrate concentration in the presence of fixed concentrations of inhibitor were analyzed using the commercial software GraFit.[14] Fitting was performed using the following steady state rate equations corresponding to competitive and mixed inhibition, respectively, in a single substrate system:

$$\upsilon = V_{max}A / [K_m (1 + I/K_{is}) + A] \quad (1)$$
$$\upsilon = V_{max}A / [K_m (1 + I/K_{is}) + A(1 + I/K_{ii})] \quad (2)$$

where υ represents the observed reaction velocity expressed as Turnover Number with units of reciprocal seconds, V_{max} represents the theoretical maximal velocity, A represents the concentration of S3P, K_m represents the apparent Michaelis constant for S3P at a fixed concentration of 100 μM PEP, I represents the concentration of the inhibitor, and K_{is} and K_{ii} represent the apparent inhibition constants for competitive and uncompetitive contributions, respectively, to the overall inhibition versus S3P (i.e., slope and intercept effects in reciprocal Lineweaver–Burk plots).

Synthesis. General

Anhydrous CH_2Cl_2 and THF were Aldrich anhydrous grade solvents. Reactions requiring anhydrous conditions were performed in oven-dried glassware under a positive pressure of nitrogen. Unless otherwise indicated, reaction mixtures after aqueous workup were dried over $MgSO_4$ and concentrated on a rotary evaporator.

Melting points were obtained in unsealed capillaries and are uncorrected. Elemental analyses for carbon and hydrogen were performed by Atlantic Microlabs, Inc. Analyses for sodium and phosphorus were performed by the Monsanto Physical Sciences Center (inductively coupled argon plasma) or by Galbraith Laboratories, Inc.

^1H NMR spectra were recorded at 399.95 MHz or 360.13 MHz . Chemical shifts are reported in units of δ using either TMS as internal standard or the residual protons of the deuterated solvent as standard. ^{13}C NMR spectra (proton decoupled) were recorded at 100.58 MHz or 90.56 MHz. Chemical shifts are reported in ppm downfield from TMS using internal TMS, the deuterated solvent, or, for D_2O as solvent, the internal instrument lock as standard. ^{31}P NMR spectra (proton decoupled) were recorded at 121.4 MHz or 40.5 MHz and are referenced to external 85 % H_3PO_4 or to the internal deuterium lock reference.

Chromatography

Analytical reverse phase liquid chromatography (RPHPLC) was performed on a μBondapak C_{18} column with acetonitrile/H_2O or acetonitrile/3 mM aqueous H_3PO_4 (for acidic compounds) as mobile phase at a flow rate of 1 mL/min. In general, a 15 min gradient from 10 % acetonitrile to 100 % acetonitrile was run followed by a 5 min hold. Detection was by UV absorbance at 254 nm. Analytical ion exchange HPLC was performed on Synchropak AX-100 columns (4.6 mm x 25 cm at 1.5 mL/min or 10 mm x 25 cm at 4–6 mL/min) using isocratic elution with pH 3.5 NaH_2PO_4 buffer (0.25–1.5 M); or on Mono Q 5/5 columns using isocratic pH 8 ammonium bicarbonate buffer (0.5–1.0 M) or a KCl gradient in pH 7.5 Tris buffer.

Preparative normal phase chromatography was performed on a Waters Associates Prep 500A Chromatograph using Prep-Pak 500 silica gel cartridges or on a medium pressure system (MPLC) using Lichroprep Si60 columns, 15 mL/min flow rate. Flash chromatography was performed using Merck Kieselgel 60 (#9385), 230–400 mesh. Preparative reverse phase chromatography was performed on a medium pressure system using Lichroprep RP-8 columns, 15 mL/min flow rate.

Preparative ion exchange chromatography was performed at 4 °C in a 5.5 x 60 cm glass column packed with DEAE Sephadex A-25 anion exchange resin (Pharmacia). Columns were eluted at 3–4 mL/min with a 5 to 8 L linear gradient of TEAB buffer. Before application to the column, mixtures were adjusted to pH 7–8 with aq. HCl or NaOH and diluted with water to an ionic strength less than or equal to that of the starting buffer. Fractions containing pure product (determined by analytical ion exchange chromatography, UV detection at 254 nm) were combined and evaporated and the residue was concentrated twice from EtOH to remove traces of TEAB. The resulting triethylammonium salts were converted to the sodium salts by slow passage of an aqueous solution of the salt through a column of AG 50W-X8 (Na^+) cation exchange resin. The final aromatic phosphates were generally found to be somewhat unstable to hydrolysis in aqueous solutions, particularly below pH 7. Thus, fractions containing pure product were worked up as quickly as possible completely to isolation of a solid sodium salt keeping temperatures (rotary evaporator bath, etc.) low (< 28 °C) throughout.

Preparation of 2

Methyl 3-acetyloxy-5-hydroxybenzoate (5). To 10.0 g (59.5 mmol) of methyl 3,5-dihydroxybenzoate in 40 mL of pyridine at 0 °C was added with stirring 6.5 mL (7.0 g, 69 mmol) of acetic anhydride and a catalytic amount of 4-(dimethylamino)pyridine. The mixture was then allowed to warm to rt, stirred for 18 h, and partitioned between CH_2Cl_2 (150 mL) and 10 % aq. HCl (100 mL). The organic layer, combined with a CH_2Cl_2 extract of the aqueous layer, was washed with 10 % aq. HCl, dried, and concentrated to 11.0 g of orange oil. Chromatography (Prep 500A, 25:75 EtOAc:cyclohexane, 225 mL/min) effected poor separation of the two-component mixture yielding the desired monoacetate (6.38 g, 45 %) in 89 % purity (contaminated with 11 % of the diacetate by RPHPLC). Two recrystallizations of a small amount of this material from benzene/pet ether gave pure **5** as a white solid, mp 120–121 °C (lit.[20] 118–121 °C): ^1H NMR ($CDCl_3$) δ 7.38 (unresolved dd, 1H), 7.30 (unresolved dd, 1H), 6.85 (br s, 1H), 6.82 (dd, appears as t, 1H, J = 2.2 Hz), 3.90 (s, 3H), 2.31 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 169.9, 166.6, 157.0, 151.3, 131.9, 114.7, 114.4, 114.1, 52.5, 21.1. Anal. Calcd for $C_{10}H_{10}O_5$: C, 57.14; H, 4.80. Found: C, 57.00, H, 4.85.

Methyl 3-(acetyloxy)-5-[[bis(phenylmethoxy)phosphinyl]-oxy]benzoate. To 4.3 g (18.2 mmol) of methyl 3-

(acetyloxy)-5-hydroxybenzoate (89 % by RPHPLC), in 150 mL of dry THF at 0 °C was added 0.90 g (22.5 mmol) of NaH (60 % oil dispersion) portionwise. After 15 min (H_2 evolution had ceased), 9.4 g (17.5 mmol) of tetrabenzyl pyrophosphate[9] in 150 mL of dry THF was added over 3–4 min. The mixture was stirred at 0 °C for 10 min, then warmed to rt whereupon an unstirrable solid mass resulted. After setting for 6 h, sat. aq. $NaHCO_3$ was added until a granular solid resulted. The solid was removed by filtration, and the filtrate was partitioned between H_2O and CH_2Cl_2. The organic layer, combined with a back-extract of the aqueous layer was washed with H_2O (2x), dried ($MgSO_4$), and evaporated to 8.7 g of oil containing a small amount of solid. The oil was dissolved in EtOAc:cyclohexane, filtered to remove the solid, and chromatographed on silica gel (Prep 500A, 30:70 EtOAc:cyclohexane, 200 mL/ min). Three cleanly separated bands eluted. The first and third bands were identified as the diacetate and bis(dibenzylphosphate), respectively, resulting from scrambling of the acetate groups during the reaction. Fractions containing the second band were evaporated and pumped under high vacuum to afford 3.7 g (43 %) of the desired monophosphate as a viscous oil: ^1H NMR ($CDCl_3$) δ 7.64 (m, 5 lines, 1H), 7.60 (m, 5 lines, 1H), 7.33 (s, 10H), 7.15 (m, 6 lines, 1H), 5.13 (d, 4H, J = 9.7 Hz), 3.90 (s, 3H), 2.29 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 168.6 (Q), 165.1 (Q), 151.1 (Q), 150.8 (d, J = 5.8 Hz) (Q), 135.1 (d, J = 6.9 Hz) (Q), 132.4 (Q), 128.7 (CH), 128.6 (CH), 128.1 (CH), 119.7 (CH), 118.66 (d, J = 5.3 Hz) (CH), 118.55 (d, J = 4.5 Hz) (CH), 70.3 (d, J = 6.0 Hz) (CH_2), 52.4 (CH_3), 20.9 (CH_3); ^{31}P NMR ($CDCl_3$) δ -7.41. Anal. Calcd for $C_{24}H_{23}O_8P$: C, 61.28; H, 4.93. Found: C, 60.89; H, 5.11.

3-Hydroxy-5-(phosphonooxy)benzoic acid, disodium salt (2). To 3.00 g (6.38 mmol) of methyl 3-(acetyloxy)-5-[[bis(phenylmethoxy)phosphinyl]oxy]benzoate in 20 mL of dry CH_2Cl_2 at 0 °C was added with stirring a cold solution of 2.0 mL (2.3 g, 15. mmol) of TMSBr and 1.0 mL (1.0 g, 13. mmol) of pyridine in 15 mL of dry CH_2Cl_2. The mixture was stirred at 0 °C for 30 min, then at rt for 40 min. Cold water (30 mL) was added followed immediately by 32 mL (32 mmol) of cold 1 N NaOH. After stirring for 1 min, the aqueous layer was separated and maintained at 0 °C for 110 min resulting in a light yellow, cloudy solution. Preparative anion exchange chromatography (DEAE Sephadex A-25, 0.2 to 1.0 M TEAB linear gradient, 6.0 L) gave two major bands at 0.65–0.74 M TEAB and 0.82–0.88 M TEAB. From the earlier band, was obtained 1.05 g (46 %) of unhydrolyzed ester, methyl 3-hydroxy-5-(phosphonooxy)benzoate, as the triethylammonium salt. Evaporation of fractions containing the second band and passage through a 30 mL (51 meq) column of AG 50W-X8 (Na^+) resulted in a glass which slowly solidified under high vacuum over 3 days to 0.68 g (34 %) of **2** as an off-white solid, mp >180 °C (dec): ^1H NMR (D_2O) δ 7.05 (m, 1H), 6.98 (m, 1H), 6.75 (m, 1H); ^{13}C NMR (D_2O) δ 176.9, 158.8, 155.8 (d, J = 7.6 Hz), 141.1, 115.6 (d, J = 4.5 Hz); 113.8, 113.0 (d, J = 3.8 Hz); ^{31}P NMR (D_2O) δ -2.02. Anal. Calcd for [$C_7H_5O_7Na_2P$ + $C_7H_6O_7NaP$ (82:18)] · 2.3 H_2O: C, 26.90; H, 3.06; Na, 13.3; P, 9.9. Found: C, 27.01; H, 2.82; Na, 13.3; P, 9.5.

Preparation of 3

Methyl 3-(2-ethoxy-2-oxoethoxy)-5-hydroxybenzoate (6). Methyl 3,5-dihydroxybenzoate (10.0 g, 59.5 mmol), ethyl bromoacetate (12.9 g, 77.3 mmol), NaI (1.6 g, 10.6 mmol, 18 mol %), and anhydrous K_2CO_3 (16.2 g, 117 mmol) were stirred in 150 mL of acetone at reflux for 12 h. The resulting suspension was worked up with water and CH_2Cl_2 to provide, after evaporation of the CH_2Cl_2 layer, 15.0 g of oil consisting of a mixture of two products and unreacted starting material as indicated by TLC [R_f = 0.17 (starting material), 0.25, 0.34; 30:70 EtOAc:cyclohexane]. Half of this material was purified by flash chromatography (300 mL of silica gel, 9:1 to 4:1 to 3.3:1 hexane:EtOAc, 1 L each). Isolation of the first-eluted component afforded 2.94 g (29 %) of the bis(carboxymethoxy)benzoate. Fractions containing the desired component were rechromatographed (flash chromatography, 250 mL of silica gel, 8:1 to 6:1 to 4.5:1 to 4:1 hexane:EtOAc, 600 mL each) providing 1.68 g (22 %) of **6** as an oil which solidified on pumping under high vacuum, mp 77.5–78.5 °C: ^1H NMR ($CDCl_3$) δ 7.20 (dd, 1H, J = 1.4, 2.2 Hz), 7.08 (dd, 1H, J = 1.4, 2.2 Hz), 6.67 (dd, appears as t, 1H, J = 2.2, 2.2 Hz), 6.64 (br s, 1H), 4.64 (s, 2H), 4.28 (q, 2H, J = 7.2 Hz), 3.88 (s, 3H), 1.30 (t, 3H, J = 7.2 Hz); ^{13}C NMR ($CDCl_3$) δ 169.1, 166.9, 158.8, 157.2, 132.0, 110.6, 107.7, 106.9, 65.3, 61.7, 52.3, 14.1. Anal. Calcd for $C_{12}H_{14}O_6$: C, 56.69; H, 5.55. Found: C, 56.58; H, 5.58.

Methyl 3-[[bis(phenylmethoxy)phosphinyl]oxy]-5-(2-ethoxy-2-oxoethoxy)benzoate. To a solution of **6** (2.46 g, 9.68 mmol) in 120 mL of dry THF at 0 °C was added portionwise 0.416 g (10.4 mmol, 1.07 eq) of a 60 % oil dispersion of NaH. After stirring for 30 min (H_2 evolution had ceased) a solution of tetrabenzyl pyrophosphate[9] (5.34 g, 9.93 mmol) in 80 mL of THF was added by means of a cannula. The solution was allowed to warm to rt and after 15 min turned to a white gelatinous mass. Addition of 100 mL of additional THF did not break up the mass significantly; however, after setting for 5.5 h, the mixture was once again stirrable. Water was then added, the pH of the resulting solution was adjusted to 11–12 with Na_2CO_3 solution, and the mixture was extracted with 500 mL of CH_2Cl_2. The organic layer was washed with water and sat. aq NaCl, dried, and evaporated to 5.23 g of oil containing a small amount of solid. Flash chromatography of the oil (100 mL of silica gel, 1:99 iPrOH:CH_2Cl_2) provided 3.25 g (65 %) of material suitable for further transformation. An additional 0.77 g (60 % pure by RPHPLC) was obtained and purified further by reverse phase MPLC (LiChroprep RP-8, Size B, 65:35 CH_3CN:H_2O, 10 mL/min). Fractions containing pure material were combined, evaporated, taken up in CH_2Cl_2 and evaporated once more to give 0.50 g (10 %, total yield = 75 %) of

viscous, colorless oil: ^{1}H NMR ($CDCl_3$) δ 7.44 (m, 1H), 7.37 (m, 1H), 7.33 (s, 10H), 6.92 (m, 1H), 5.12 (d, 4H, *J* = 8.8 Hz), 4.57 (s, 2H), 4.27 (q, 2H, *J* = 7.1 Hz), 3.89 (s, 3H), 1.30 (t, 3H, *J* = 7.1 Hz); ^{13}C NMR ($CDCl_3$) δ 168.0, 165.6, 158.6, 151.2 (d, *J* = 6.6 Hz), 135.2 (d, *J* = 6.7 Hz), 132.4, 128.7, 128.6, 128.1, 114.6 (d, *J* = 5.1 Hz), 112.1, 112.0 (d, *J* = 5.2 Hz), 70.1 (d, *J* = 6.0 Hz), 65.5, 61.5, 52.3, 14.1; ^{31}P NMR ($CDCl_3$) δ -8.35. Anal. Calcd for $C_{26}H_{27}O_9P$: C, 60.70; H, 5.29. Found: C, 60.82; H, 5.33.

3-(Carboxymethoxy)-5-(phosphonooxy)benzoic acid, disodium salt (3). To a stirred solution of 3.25 g (6.32 mmol) of the dibenzylphosphate, above, in 40 mL of dry CH_2Cl_2 at 0 °C was added 2.65 mL (1.92 g, 19.0 mmol) of Et_3N followed by 2.50 mL (2.90 g, 18.9 mmol) of TMSBr. The resulting clear solution was stirred for 30 min then warmed to rt and stirred for 80 min. Cold water (20 mL) was added and after stirring for 3 min, 20 mL (20 mmol) of 1 N NaOH was added. The layers were separated, the organic layer was washed with water and the combined aqueous layers were treated with an additional 20 mL (20 mmol) of 1 N NaOH and set aside for 5 h at rt. After pH adjustment to 7.5 and dilution to 350 mL, the mixture was chromatographed on a DEAE Sephadex A-25 column (6 L linear gradient, 0.5–1.1 M TEAB). Ion exchange HPLC analysis of the major band that eluted from the column indicated that a minor impurity (maximum of 1.6 %) had virtually coeluted with the desired product. The early fractions in the band were uncontaminated, however, and were evaporated, taken up twice in EtOH and evaporated, then passed through a 25 mL (42.5 meq) column of AG 50W-X8 (Na^+). Evaporation and drying at high vacuum afforded 0.436 g [1.98 g (85 %) calculated total yield] of white solid **3** as a 79:21 mixture of di- and trisodium salts, respectively: ^{1}H NMR (D_2O) δ 7.25 (m, 1H), 7.15 (m, 1H), 6.87 (m, 1H), 4.46 (s, 2H); ^{13}C NMR (D_2O) δ 178.5, 174.2, 161.1, 155.6 (d, *J* = 7.2 Hz), 137.6, 116.9 (d, *J* = 4.7 Hz), 113.8 (d, *J* = 4.7 Hz), 113.4, 69.4; ^{31}P NMR (D_2O) δ -3.55. Anal. Calcd for [$C_9H_7O_9Na_2P$ + $C_9H_6O_9Na_3P$ (79:21)] · 1.5 H_2O: C, 29.4; H, 2.68; Na, 13.8; P, 8.4. Found: C, 29.4; H, 2.71; Na, 13.8; P, 8.5.

Preparation of 4

Methyl 3-[[(1,1-dimethylethyl)dimethylsilyl]oxy]-5-hydroxybenzoate. To 20.64 g (0.123 mol) of methyl 3,5-dihydroxybenzoate in 75 mL of DMF at 0 °C was added 18.5 g (0.123 mol) of solid *tert*-butyldimethylsilyl chloride and 8.8 g (0.13 mol) of imidazole. The resulting solution was stirred for 23 h at rt, then partitioned between EtOAc and H_2O. The aqueous layer was washed with EtOAc and the combined organics were washed with water (2x), dried, and evaporated to a mixture of oil and solid. The oil was dissolved in cyclohexane and the white solid was removed by filtration to afford 4.48 g (22 %) of recovered starting material. Evaporation of the filtrate gave 30.5 g of oil consisting of the mono- and disilyloxybenzoates. Purification by chromatography on silica gel (Prep 500A, two runs, 15:85 EtOAc: cyclohexane) afforded 8.27 g (22 % based on recovered starting material) of disilyl material and 16.95 g (62 %) of the desired monosilyl compound as a colorless oil which slowly solidified. Recrystallization from hexanes of a sample of this material provided white platelets, mp 77–78.5 °C: ^{1}H NMR ($CDCl_3$) δ 7.15 (dd, 1H, *J* = 1.3, 2.4 Hz), 7.08 (dd, 1H, *J* = 1.4, 2.2 Hz), 6.54 (t, 1H, *J* = 2.3 Hz), 6.4–6.5 (br s, 1H), 3.88 (s, 3H), 0.97 (s, 9H), 0.20 (s, 6H); ^{13}C NMR ($CDCl_3$) δ 167.5, 156.94, 156.91, 131.6, 113.7, 112.4, 109.9, 52.4, 25.6, 18.1, -4.5. Anal. Calcd for $C_{14}H_{22}O_4Si$: C, 59.54; H, 7.85. Found: C, 59.61; H, 7.91.

Methyl 3-[1-(dimethoxyphosphinyl)-2-methoxy-2-oxoethoxy]-5-[[(1,1-dimethylethyl)dimethylsilyl]oxy]benzoate. To the silyloxyphenol (3.40 g, 12 mmol), above, in 100 mL of benzene (dried by removing 20 mL of benzene/water azeotrope) was added rhodium acetate dimer (0.080 g, catalytic) and trimethyl diazophosphonoacetate[8] (3.4 g, 16.3 mmol). The mixture was heated at reflux for 6 h, then additional diazophosphonoacetate (3.0 g, 14.4 mmol) and rhodium acetate (0.040 g) was added. After 12 h further reflux, the brown mixture was filtered through a 1.5 inch pad of silica gel eluting with 250 mL each of 20 % and 60 % EtOAc/cyclohexane. Evaporation and purification by MPLC (Lichroprep Si60 column, size C, 75 % EtOAc/cyclohexane) gave 3.19 g (57 %) of slightly yellow viscous oil: ^{1}H NMR ($CDCl_3$) δ 7.20 (dd, 1H, *J* = 1.5, 2.5 Hz), 7.19 (dd, 1H, *J* = 1.5, 2.5 Hz), 6.65 (dd, 1H, *J* = 2.3, 2.3 Hz), 5.10 (d, 1H, *J* = 20 Hz), [3.93, 3.93 (two overlapping d, 3H, 3H, *J* = 11.0 Hz, *J* = 10.9 Hz)], 3.90 (s, 3H), 3.86 (s, 3H), 0.99 (s, 9H), 0.21 (s, 6H); ^{13}C NMR ($CDCl_3$) δ 166.5, 166.2, 158.3 (d, *J* = 13.4 Hz), 156.9, 132.3, 115.8, 112.2, 108.5, 74.2 (d, *J* = 160 Hz), 54.6 (d, *J* = 6.7 Hz), 54.4 (d, *J* = 6.4 Hz), 53.2, 52.3, 25.5, 18.1, -4.5; ^{31}P NMR ($CDCl_3$) δ 12.23. Anal. Calcd for $C_{19}H_{31}O_9PSi$: C, 49.34; H, 6.76. Found: C, 49.53; H, 6.86.

Methyl 3-[1-(dimethoxyphosphinyl)-2-methoxy-2-oxoethoxy]-5-hydroxybenzoate (7). The silyl ether (2.86 g, 6.18 mmol), above, in 50 mL of THF was stirred with 6.2 mL (6.2 mmol) of a 1M solution of tetra-*n*-butylammonium fluoride in THF at 0 °C for 2 h. The mixture was then partitioned between CH_2Cl_2 and H_2O, the aqueous layer was washed with CH_2Cl_2, and the combined organics were washed with H_2O and dried ($MgSO_4$). Purification by MPLC (Si60, size C, 100 % EtOAc) gave **7** as a colorless oil (1.93 g, 90 %) which slowly crystallized, mp 112.5–115 °C: ^{1}H NMR ($CDCl_3$) δ 8.02 (br s, 1H), 7.23 (dd, 1H, *J* = 1.5, 2.4 Hz), 7.04 (dd, 1H, *J* = 1.5, 2.4 Hz), 6.85 (dd, 1H, *J* = 2.4, 2.4 Hz), 5.23 (d, 1H, *J* = 19.4 Hz), [3.94, 3.94 (two overlapping d, 3H, 3H, *J* = 11.0 Hz, *J* = 10.9 Hz)], 3.88 (s, 3H), 3.81 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 166.5, 166.4 (d, *J* = 0.9 Hz), 158.4, 158.3 (d, *J* = 13.8 Hz), 132.2, 111.9, 107.9, 106.2, 73.7 (d, *J* = 161 Hz), 54.9 (d, *J* = 6.6 Hz), 54.8 (d, *J* = 6.6 Hz), 53.2, 52.2; ^{31}P NMR ($CDCl_3$) δ 12.45. Anal. Calcd for $C_{13}H_{17}O_9P$: C, 44.84; H, 4.92. Found: C, 45.46; H, 5.07.

Methyl 3-[bis(phenylmethoxy)phosphinyloxy]-5-[1-(dimethoxyphosphinyl)-2-methoxy-2-oxoethoxy]benzoate. To the phenol **7** (1.16 g, 3.33 mmol) in 90 mL of dry THF at 0 °C was added NaH (60 % oil dispersion, 0.18 g, 4.5 mmol) in two portions. When hydrogen evolution ceased (20 min), tetrabenzyl pyrophosphate[9] (1.98 g, 3.68 mmol) in 40 mL of dry THF was added by syringe, and the mixture was warmed to room temperature and stirred overnight. The resulting suspension was filtered to remove the solids, and the filtrate was partitioned between CH_2Cl_2 and water. The organic layer was combined with a CH_2Cl_2 wash of the aqueous layer, washed with water and dried. Evaporation gave an oil and a small amount of solid which was taken up in CH_3CN containing a small amount of water, filtered to remove the solid, and chromatographed by reversed phase MPLC (Lichroprep RP-8, size C, 60 % CH_3CN/H_2O, 15 mL/min) to give a viscous colorless oil, 1.70 g (84 %): ^1H NMR ($CDCl_3$) δ 7.47 (ddd, 1H, J = 1.2, 1.2, 2.4 Hz), 7.37 (m, 1H), 7.33 (m, 10H), 6.95 (ddd, 1H, J = 1.0, 2.4, 2.4 Hz), 5.12 (two closely spaced d, 4H, J = 8.8 Hz, J = 8.8 Hz), 5.08 (d, 1H, J = 19.0 Hz), 3.91 (d, 6H, J = 11.2 Hz), 3.89 (s, 3H), 3.83 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 166.0, 165.3, 158.1 (d, J = 13.5 Hz), 151.3 (d, J = 6.7 Hz), 135.1 (d, J = 6.6 Hz), 132.7, 128.7, 128.5, 128.0, 115.5 (d, J = 5.1 Hz), 112.4, 112.2 (d, J = 5.1 Hz), 74.1 (d, J = 159 Hz), 70.2 (two overlapping d, J = 6.1 Hz, J = 6.1 Hz), 54.6 (d, J = 6.5 Hz), 54.4 (d, J = 6.7 Hz), 53.2, 52.4; ^{31}P NMR ($CDCl_3$) δ 11.79, -9.14. Anal. Calcd for $C_{27}H_{30}O_{12}P_2 \cdot 0.3\ H_2O$: C, 52.83; H, 5.02. Found: C, 52.77; H, 5.12.

3- (Carboxyphosphonomethoxy) -5- phosphonooxybenzoic acid, trisodium salt (4). To the dibenzyl phosphate (1.20 g, 1.97 mmol), above, in 25 mL of dry CH_2Cl_2 at 0 °C was added a solution of TMSBr (1.50 mL, 1.74 g, 11.4 mmol) and pyridine (0.32 mL, 0.31 g, 4.0 mmol) in 5 mL of dry CH_2Cl_2 slowly by syringe. The mixture was warmed to rt, stirred for 3 h, then 15 mL of cold H_2O was added, stirring was continued for 3 min after which 1N NaOH (12 mL, 12 mmol) was added. The layers were separated and the organic layer was washed with two 30 mL portions of H_2O. The combined aqueous solutions were treated with additional 1N NaOH (8 mL, 8 mmol) and were allowed to set for 18 h at rt. Preparative anion exchange chromatography (DEAE Sephadex A-25, 0.3–1.2 M TEAB linear gradient) and cation exchange provided the trisodium salt (0.40 g, 40 %) as an off-white glassy solid: ^1H NMR (D_2O) δ 7.23 (m, 1H), 7.14 (m, 1H), 6.86 (m, 1H), 4.72 (d, 1H, J = 18.1 Hz); ^{13}C NMR (D_2O) δ 176.5, 174.7, 161.6 (d, J = 13.7 Hz), 155.6 (d, J = 6.7 Hz), 138.3, 117.1 (d, J = 4.4 Hz), 113.9 (d, J = 4.6 Hz), 113.5, 79.7 (d, J = 142 Hz); ^{31}P NMR (D_2O) δ 10.65, -3.45. Anal. Calcd for [$C_9H_7Na_3O_{12}P_2$ + $C_9H_6Na_4O_{12}P_2$ (83:17)] · 3.6 H_2O: C, 21.41; H, 2.75; Na, 14.50; P, 12.24. Found: C, 21.39; H, 2.69; Na, 14.49; P, 10.68.

An approximately equal amount of material having an unhydrolyzed methyl ester at the phosphonoacetate carboxylate was isolated from an earlier eluting band in the ion exchange chromatography.

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