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New Aromatic Inhibitors of EPSP Synthase Incorporating Hydroxymalonates as Novel 3-Phosphate Replacements

Ajit Shah,[a] Jose L. Font,[a,†] Michael J. Miller,[a] Joel E. Ream[a] Mark C. Walker[a] and James A. Sikorski[b]*

[a]*Ceregen and* [b]*Monsanto Corporate Research, Units of Monsanto Company, 700 Chesterfield Parkway North, St. Louis, MO 63198, U.S.A.*

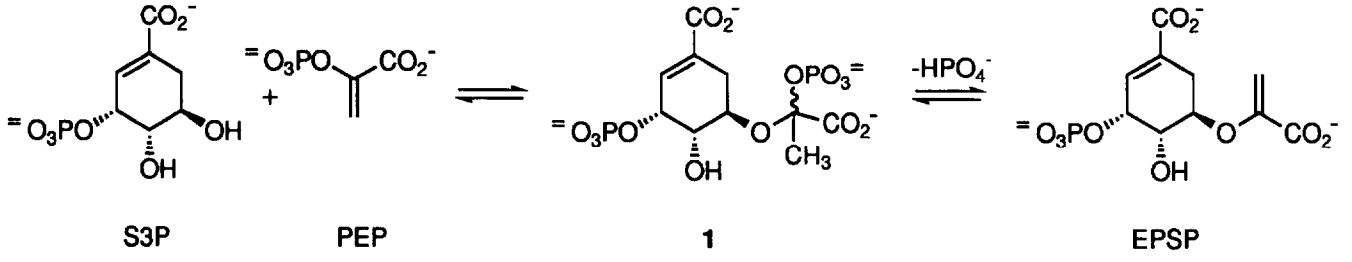
Abstract—A new, aromatic analogue of the EPSP synthase enzyme reaction intermediate **1** has been identified, which contains a 3-hydroxymalonate moiety in place of the usual 3-phosphate group. This simplified inhibitor was readily prepared in five steps from ethyl 3,4-dihydroxybenzoate. The resulting tetrahedral intermediate mimic **9** is an effective, competitive inhibitor versus S3P with an apparent K_i of 0.57 ± 0.06 μM. This result demonstrates that 3-hydroxymalonates exhibit potencies comparable to aromatic inhibitors containing the previously identified 3-malonate ether replacements and can thus function as suitable 3-phosphate mimics in this system. These new compounds provide another example in which a simple benzene ring can be used effectively in place of the more complex shikimate ring in the design of EPSP synthase inhibitors. Furthermore, the greater potency of **9** versus the glycolate derivative **10** and the 5-deoxy-analog **11**, again confirms the requirement for multiple anionic charges at the dihydroxybenzoate 5-position in order to attain effective inhibition of this enzyme.

Introduction

The enzyme 5-enolpyruvoylshikimate-3-phosphate (EPSP) synthase (EPSPS, EC 2.5.1.19) represents a central step in the biosynthesis of aromatic amino acids,[1] and as the biological target for the commercially successful herbicide, glyphosate, has been an important target for rational inhibitor design.[2] EPSPS catalyzes the unusual transfer of the carboxyvinyl moiety from phosphoenolpyruvate (PEP) to the 5-hydroxyl of shikimate-3-phosphate (S3P), via the single, kinetically competent, tightly bound, tetrahedral intermediate **1** (Scheme 1).[3] Highly potent EPSPS bisubstrate inhibitors, such as **2** and **3**, have been reported as shikimate-based analogues of **1**.[4] The potency of these inhibitors is thought to be a direct consequence of their ability to occupy simultaneously both the S3P and PEP subsites. Spectroscopic investigations conducted in these laboratories have also determined that the shikimate ring in enzyme-bound S3P and EPSP adopts an unusually flattened conformation.[5] As a result, the aromatic tetrahedral intermediate mimic **4** has recently been identified as a potent EPSPS inhibitor, where the simplified benzene ring functions as a suitable substitute for the more highly functionalized shikimate ring.[6]

These inhibitors contain either allylic or phenolic 3-phosphate groups, which are readily susceptible to hydrolytic cleavage. The 3-phosphate group in S3P contributes more than 8 kcal/mol in binding energy to EPSPS.[7] Inhibitors lacking this important group typically exhibit little significant interaction with this enzyme.[8–10] A search for a more stable 3-phosphate replacement moiety unexpectedly uncovered the 3-malonate ether group, which serves as a suitable 3-phosphate mimic in 4,5-dideoxy-S3P inhibitors.[8] Similarly, **5**, the corresponding analogue of S3P where the 3-phosphate is replaced by a 3-malonate ether, undergoes enzyme-catalyzed conversion to an EPSP-like product.[8] While the kinetic and mechanistic details of this conversion have yet to be investigated, one

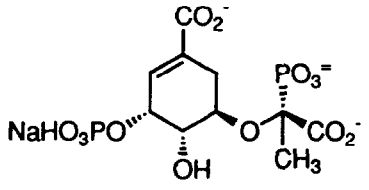
†Present Address: Astra Arcus USA Inc., P.O. Box 20890, Rochester, NY 14602, U.S.A.



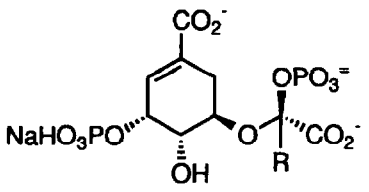
Scheme 1. The reaction catalyzed by EPSP synthase.

might assume that this conversion proceeds through the tetrahedral intermediate **6**, and that close structural analogues of **6** would also be potent EPSPS inhibitors. As a result, the symmetrical 3,5-bismalonate ether[11] **7**, and the unsymmetrical 5-phosphonoacetoxy analogue[12] **8** were recently identified as new aromatic tetrahedral intermediate mimics which incorporate a 3-malonate ether in place of this 3-phosphate group.

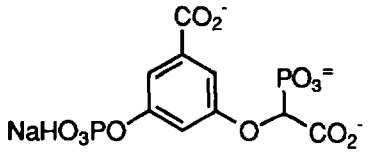
While **7** and **8** were effective low micromolar inhibitors of EPSPS, their potencies failed to match the low nanomolar levels frequently observed with 3-phosphate-based inhibitors, such as **2**–**4**. Consequently, we sought an alternative replacement group for the 3-phosphate moiety in these aromatic systems. Here we report the design, synthesis and biochemical evaluation of the 4,5-dihydroxybenzoate analogue **9** as an easily



R-**2**
$K_{i(app)} = 5 \pm 1$ nM



3a R = CH_2F
R-**3b** R = CHF_2 $K_{i(app)} = 4 \pm 0.3$ nM
R-**3c** R = CF_3 $K_{i(app)} = 32 \pm 3$ nM



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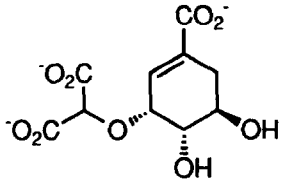

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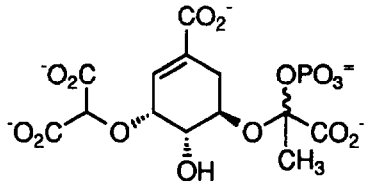

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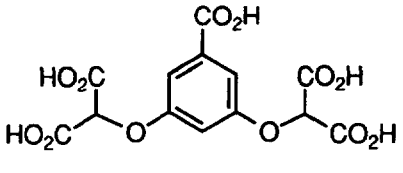

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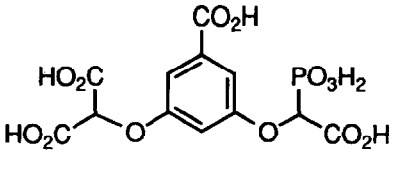

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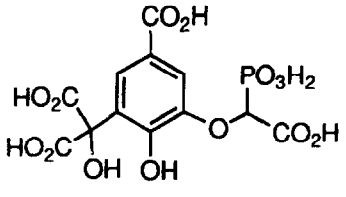

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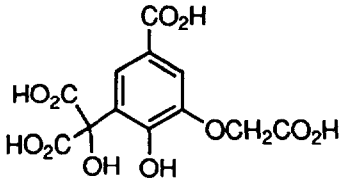

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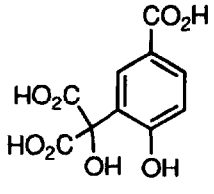

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accessible aromatic tetrahedral intermediate mimic which utilizes a 3-hydroxymalonate group as a novel 3-phosphate replacement.

Results and Discussion

Design of EPSPS inhibitor 9

Calorimetry studies[13] and kinetic evaluations[7] have clearly demonstrated that the recognition of both substrates and inhibitors by this enzyme is largely driven by electrostatic interactions through multiple anionic functionalities. Recent studies on tRNA synthetase[14] have also established that similar charged group interactions between enzyme and substrate are far more important contributors to binding and catalysis than interactions involving only hydrogen bonding. The 3-phosphate group in S3P is known to be a critical recognition element for enzymatic turnover as well as to achieve potent bisubstrate inhibition.[7,9b,10] Consequently, a molecular modeling comparison of representative aromatic inhibitors versus **1** focused on optimizing interaction at this subsite.

At least two different binding modes could be considered to compare the benzene-based inhibitor **4** with **1**. Force fitting the benzene ring in **4** to overlap maximally with the shikimate ring in **1** displaces the angular aromatic 3-phosphate group from achieving optimal overlap with the presumably axial 3-phosphate moiety in **1**. This is unlikely to occur since more extended shikimate 3-phosphate homologues bearing either O_3PCH_2O or O_3POCH_2—linkages[8,15] bind much less effectively to the S3P subsite. These results suggest that the 3-phosphate subsite is limited by a maximum steric constraint. On the other hand, maximizing overlap between the two 3-phosphate moieties will likely limit overlap of the two ring systems.

With these constraints in mind, we sought to develop a predictive three-dimensional model for **1** which could be used to design new EPSPS inhibitor classes with improved metabolic stability at the 3-phosphate site. A pharmacophore model was developed for **1** using the active analogue approach.[16,17] The anionic carboxylate and phosphate functionalities in **1** were treated computationally in their neutral, protonated states to simplify the calculations and more carefully probe different conformational binding modes. Recognition at each of the four anionic (two phosphates and two carboxylates) centers was assumed to occur with a single point on the enzyme. These ionic interactions with enzyme were represented by four dummy atoms, symmetrically positioned 3 Å away from the phosphorus and carboxylate carbon atoms, as shown in Figure 1.

The 3-phosphate group was fixed in an axial orientation, as previously indicated from transfer NOE NMR studies of S3P and EPSP.[5] The experimental distances between individual protons obtained from these NOE experiments were then used as constraints in a ring conformational analysis at 5° torsional increments. A

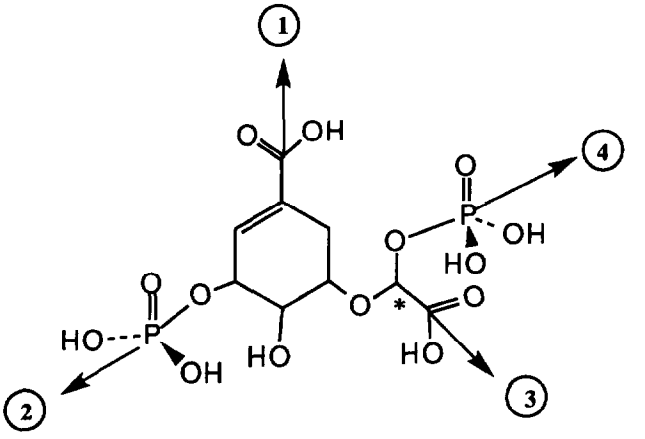


Figure 1. The pharmacophore starting point for modeling compound **1** with Sybyl. Dummy atoms (1–4) were symmetrically positioned 3 Å away from the center atom in each of the anionic functional groups.

single conformational family was obtained, and the midpoint conformation was chosen to represent the family. This midpoint conformation was then used to generate all of the possible conformations for compounds **1**, **2** and **4**. An analysis of the resulting final set of conformations for each of these compounds indicated that they all belong to a single conformational family and could therefore be represented by a single conformation. The representative conformations were fitted against each other using the Multifit option in Sybyl using the four anionic functionalities as anchors.

Figure 2 shows the results of this comparison between **1** and the corresponding aromatic 3-phosphate analogue **4**.[9] As depicted in Figure 2, the C—O—P bond angle in **4** differs significantly from that which accompanies the axial 3-phosphate in **1**. As a result, the benzene ring orientation in **4** becomes sharply skewed relative to the shikimate ring as overlap between the two 3-phosphate groups is maximized. The strain introduced by this twisted ring orientation might partially account for the lack of potency in these aromatic systems, since the overlap with the other critical anionic recognition sites appears to be largely maintained.

Other known[4] shikimate inhibitors (**3a–c**) were incorporated into this model and essentially maintained the shikimate ring conformation depicted for **1** in Figure 2. Then various aromatic inhibitors were evaluated using this model to probe the orientation of the benzene ring versus the shikimate ring conformation in **1**, while maintaining maximum overlap at the 3-phosphate subsite. Interestingly, a smaller distortion in ring orientation is observed with the shortened aromatic phosphonate analogue **12a** (Fig. 2). This result suggests that aromatic inhibitors incorporating slightly shortened 3-phosphate mimics might be more effective EPSPS inhibitors. However, a previous kinetic evaluation of **12b** demonstrated that this shortened phosphonate analogue had little significant interaction with enzyme.[11] This suggests that the bridging oxygen

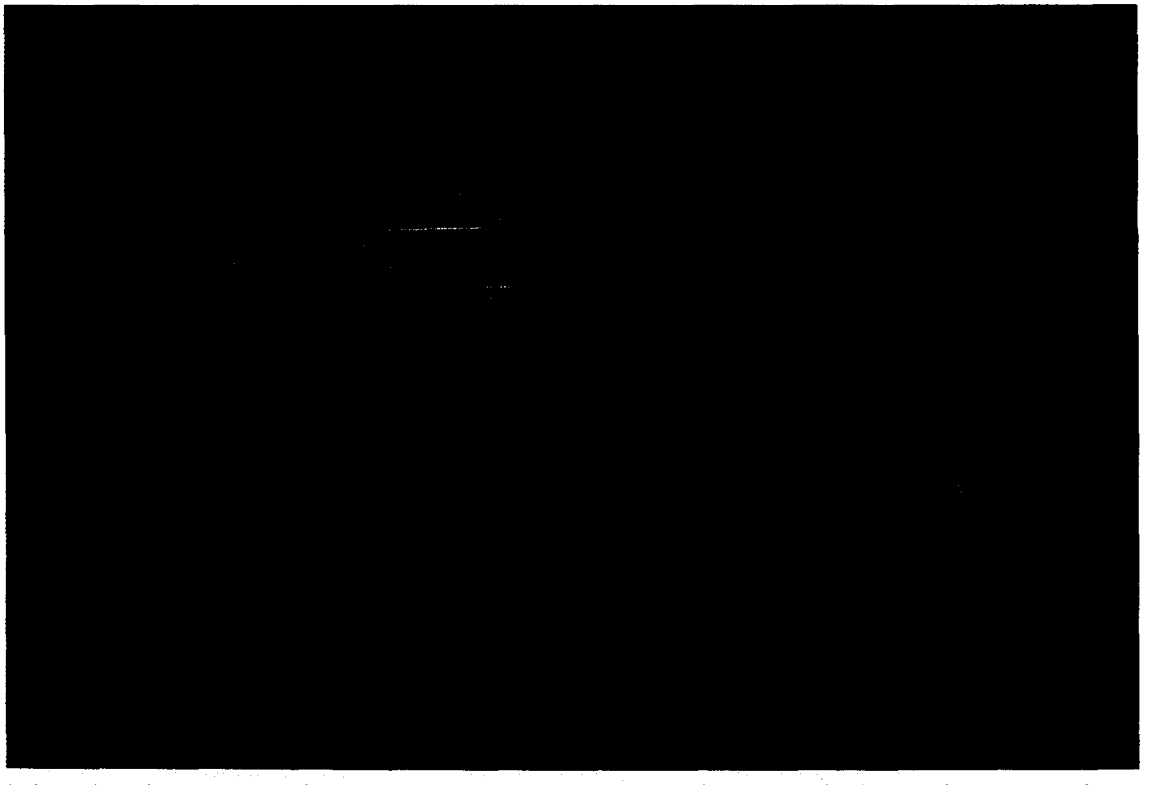
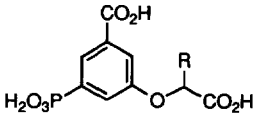


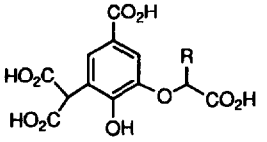
Figure 2. A three-dimensional representation of the single conformation developed for compound **1** (red) at the top versus the corresponding aromatic 3-phosphate **4** (green) at the bottom and the shortened aromatic phosphonate **12a** (white) in the middle.

in the 3-phosphate group plays a critical role in enzyme recognition, as observed previously with a series of 4,5-dideoxy shikimate 3-phosphate analogues.[8]

This smaller distortion in ring orientation was also observed when the shortened aromatic 3-malonate **13a** was modeled versus **1** (Fig. 3). In this case, analogue **13a** achieved almost the same ring orientation as observed previously with the shortened phosphonate **12a**. Some literature precedent has been established for using malonates as phosphate mimics. Related dicarboxylates function effectively as mimics of cyclic monophosphates in the naturally occurring griseolic acids,[18a,b] and the polycarboxylates contained in the zaragozic acid natural products have recently been identified as pyrophosphate replacement groups.[18c] However, the need to satisfy the enzyme's interaction with a bridging oxygen atom led us to conclude that the shortened 3-malonate analogues **13a** and **b** by themselves would be ineffective as an EPSPS inhibitor. Clearly, an alternative solution was needed.



12a R = -PO_3H_2
12b R = -H



13a R = -PO_3H_2
13b R = -H

The critical interaction between enzyme and the bridging oxygen atom in the 3-phosphate group might be restored by adding an alcohol functionality to the 3-malonate group. Since this enzyme is better able to recognize aromatic inhibitors incorporating 3-hydroxymethylphosphonates than those with simple 3-methylphosphonates,[11] we incorporated an alcohol moiety into **12a** to devise the novel 3-hydroxymalonate derivative **9**. A modeling comparison of **9** versus **1** is virtually identical to Figure 3 and suggests that the less skewed orientation displayed by **13** can be essentially maintained by **9** in this system. Based upon these modeling comparisons, hydroxymalonates appeared to offer a more compact alternative to 3-malonate ethers, which occupy a larger total volume than a typical phosphate group.[19] Since there were no literature reports describing the hydroxymalonate moiety as a stable phosphate mimic for inhibitor design, we set out to evaluate 3-hydroxymalonates as 3-phosphate replacement groups in this system. Consequently, we sought to develop an efficient synthesis of the aromatic tetrahedral intermediate mimic **9**.

Synthesis of 3-hydroxymalonate inhibitors 9–11

While the reaction between activated phenols or anilines with diethyl ketomalonate (DEKM) has been known since 1907 as a viable route to aryl hydroxymalonate esters,[20,21] there are no known examples of hydroxymalonates attached to a benzoate ring system. Indeed, the primary reference articles in the area strongly suggest that the available synthetic methodo-

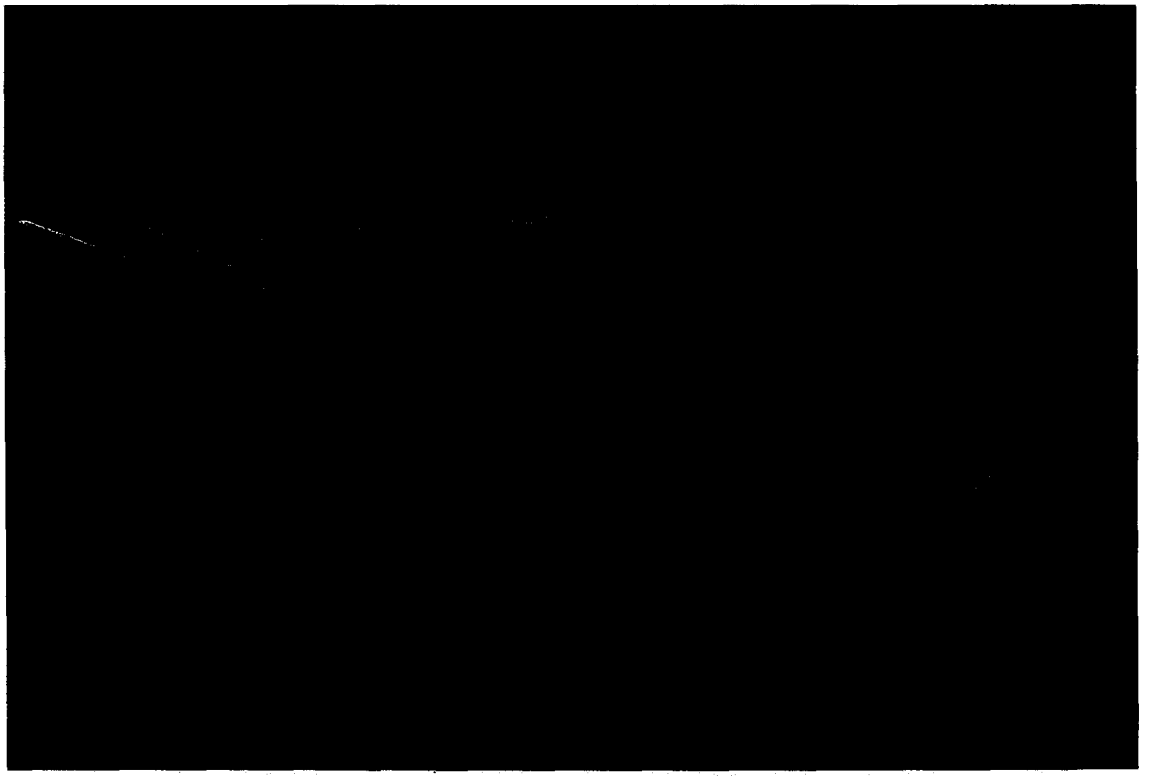
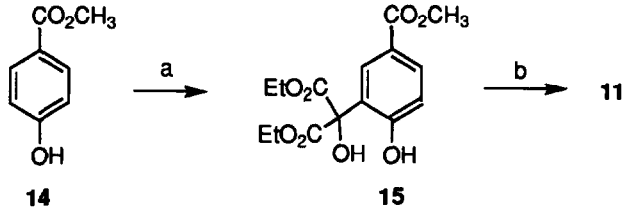


Figure 3. A three-dimensional representation of the single conformation developed for compound **1** (red) at the top versus compound **9** (white) at the bottom.

logy would not be compatible with an electron-withdrawing carboxylate ester.[22,23] Consequently, this approach would only provide a new potential series of EPSPS inhibitors if suitable synthetic methodology could be developed to access the required intermediates.

The incorporation of a 3-hydroxymalonate moiety into a representative model system (methyl *p*-hydroxybenzoate, **14**) was surprisingly efficient using a modified Friedel–Crafts alkylation procedure.[23] The reaction proceeds cleanly and in good yield using $SnCl_4$ as a catalyst in methylene chloride at room temperature to give the desired product **15**. Subsequent deprotection under standard conditions[6,12] produces the 5-deoxy analogue **11**. Compound **11** can be isolated in stable form as either its triethylammonium or sodium salts after ion-exchange chromatography. Careful acidification of these salts with Dowex (H^+) resin will produce the corresponding free carboxylic acid, which is somewhat susceptible to decarboxylation upon prolonged standing at room temperature (see Scheme 2).



Scheme 2. Reagents and conditions: (a) DEKM, $SnCl_4$, CH_2Cl_2, 0 °C to room temperature, 36 h (80%); (b) NaOH, H_2O; DEAE Sephadex chromatography.

With these encouraging results in hand we turned our attention to the unsymmetrically substituted targets, **9** and **10**. The synthesis of these materials began from a common intermediate derived from commercially available ethyl 3,4-dihydroxybenzoate **16**. As shown in Scheme 3, alkylation of **16** with benzyl bromide produced a complex mixture of mono- and di-substituted benzyl ethers. GC analysis of the crude mixture and subsequent spectroscopic characterization of the isolated materials indicated that the desired *para*-mono-substituted product **17** predominated (50%) in the crude mixture over either the corresponding *meta* isomer **18** (5%) or the dibenzyl ether **19** (30%). Pure **17** could then be isolated in low yield (32%) by selective crystallization from cold ethyl acetate and hexane. Subsequent purification of the mother liquor by HPLC provided additional amounts of **17** as well as an analytical sample of **18**. While **17** and **18** were remarkably similar by ^1H NMR, they could be easily distinguished by ^{13}C NMR. An unambiguous structural assignment could therefore be made on the basis of HOESY and HETCOR spectroscopy of each purified sample.

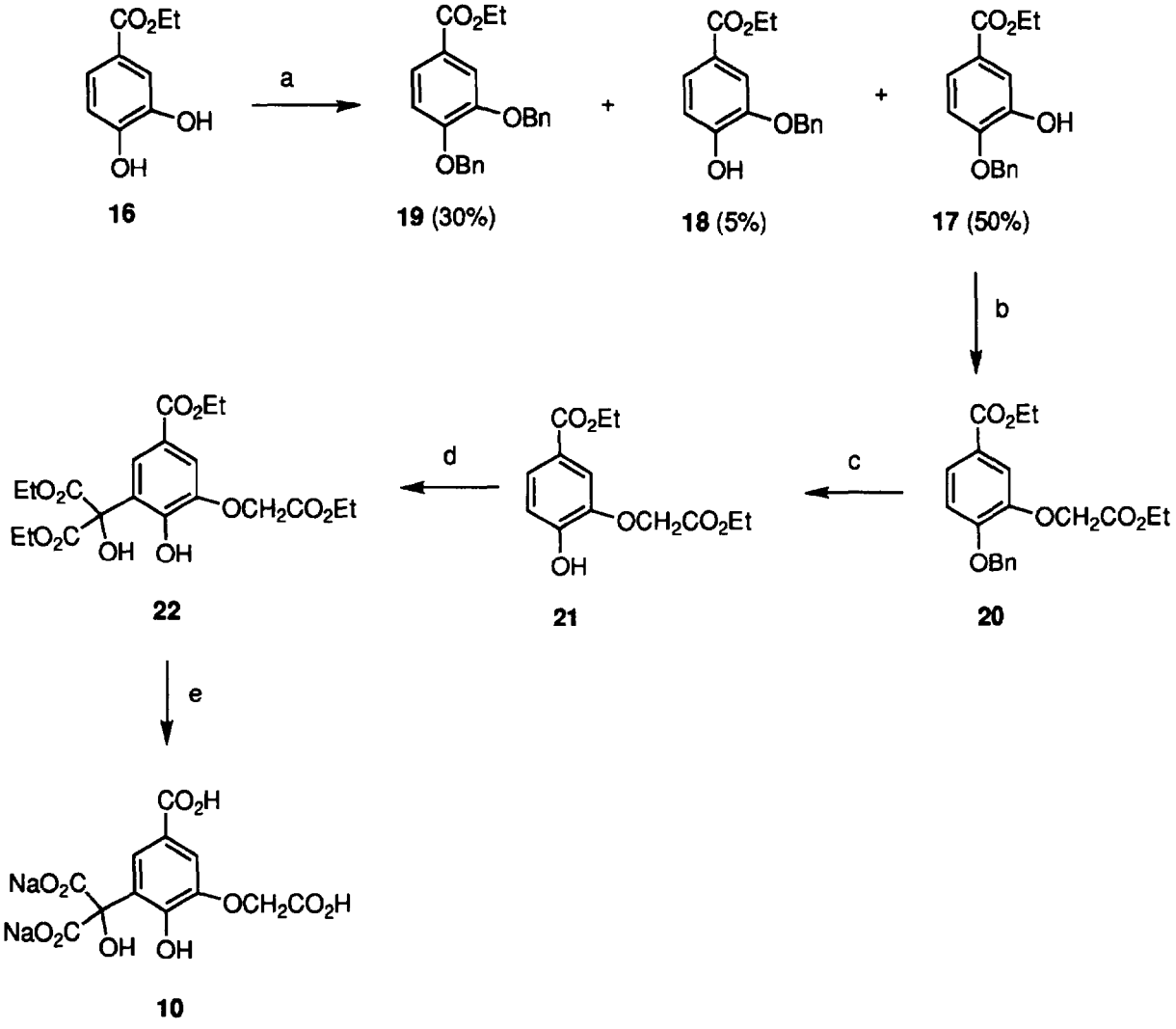
All attempts to incorporate the hydroxymalonate moiety directly into **17** failed under the usual conditions. Consequently, an alternative strategy was employed. The synthesis of **10** proceeded from **17** by subsequent alkylation with ethyl iodoacetate and potassium carbonate in acetone to form the protected glycolate **20**. Removal of the benzyl ether protecting group could then be accomplished in good yield by hydrogenolysis to give the deprotected 4-phenol derivative **21**.

Treatment of **21** with DEKM and stannic chloride under the standard conditions proceeded cleanly to introduce the desired 3-hydroxymalonate functionality producing **22** in good isolated yield. Careful saponification with aqueous base in tetrahydrofuran removed all four of the ester protecting groups. Subsequent neutralization with AG 50W-X8 resin (pH = 5.3) then gave the desired deprotected product **10** in excellent yield (90%) as a trisodium salt.

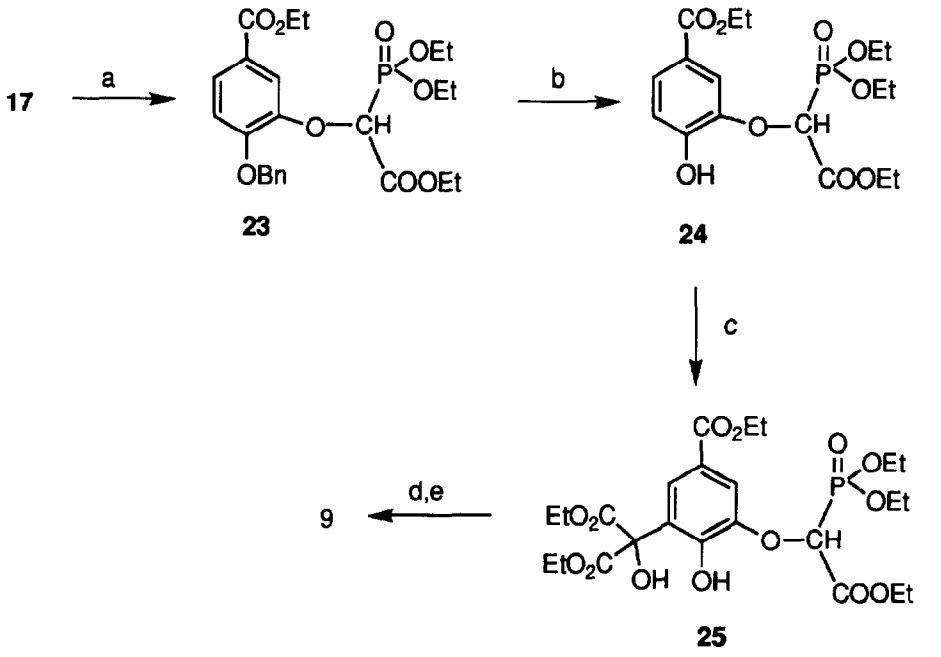
As shown in Scheme 4, the synthesis of **9** proceeded from **17** by first incorporating the requisite phosphonoacetate ether at the 5-position. Rhodium acetate-catalysed[6,12,24] coupling of **17** with triethyl diazophosphonoacetate[25] gave **23** in good yield. Hydrogenolysis of this benzyl ether proceeded smoothly to give the deprotected 4-phenol **24**. Subsequent reaction with diethyl ketomalonate and stannic chloride produced a modest yield of the protected chain-shortened 3-hydroxymalonate intermediate **25**. Cleavage of the phosphonate esters with TMSBr and base hydrolysis of the remaining carboxylate ethyl esters provided **9** in analytically pure form as a mixture of triethylammonium salts after ion-exchange chromatography. The spectral characterizations of the purified products **9–11** using ^{1}H, ^{13}C, and ^{31}P NMR as well as elemental analyses were all consistent with their assigned structures. All of these isolated materials were >95% pure by analytical ion-exchange chromatography.

Biochemical evaluation of hydroxymalonates 9–11

Compounds **9–11** were evaluated for inhibition of *Escherchia coli* EPSP synthase[26] by monitoring the conversion of ^{14}C-PEP or ^{14}C-S3P to ^{14}C-EPSP.[7,27] The resulting IC_{50} values are shown in Table 1. For comparison purposes, the IC_{50} for product inhibition of the forward reaction of EPSPS is also shown in Table 1. Our results demonstrate that the aromatic 3-hydroxymalonate ethers display significant inhibition of EPSPS, with an increase in enzyme affinity proceeding from the 5-deoxy-S3P analogue **11**, to the glycolate analogue **10**, and finally to the tetrahedral intermediate mimic **9**. This increase in binding parallels the trend



Scheme 3. Reagents and conditions: (a) BnBr, K_2CO_3, 2-butanone, 5 h, reflux; (b) ICH_2CO_2Et, K_2CO_3, acetone, 25 h, reflux (80%); (c) H_2, 10% Pd/C, EtOH, 50 psi, (75%); (d) DEKM, $SnCl_4$, CH_2Cl_2, 0 °C to room temperature, 36 h (56%); (e) NaOH, THF (85%).



Scheme 4. Reagents and conditions: (a) $Et_2O_3PC(=N_2)CO_2Et$, $Rh_2(OAc)_4$, benzene, reflux (68%); (b) H_2, 10% Pd/C, EtOH, 50 psi (95%); (c) DEKM, $SnCl_4$, CH_2Cl_2, 0 °C to room temperature, 36 h (30%); (d) TMSBr, C_5H_5N, CH_2Cl_2; (e) NaOH, H_2O; DEAE Sephadex chromatography (20%).

observed previously for the analogous shikimate and aromatic 3-phosphate[6] or aromatic 3-malonate ether series[12] 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.

A more complete kinetic characterization of **9** was conducted at varying S3P concentrations which demonstrated competitive inhibition (Fig. 4) with an apparent K_i of 0.57 ± 0.06 μM.[28] The potency observed for **9** is comparable to that recently reported for the symmetrical 3,5-bismalonate ether **7**[11] and the unsymmetrical 3-malonate ether **8**.[12] This result demonstrates for the first time that 3-hydroxymalonates are suitable 3-phosphate replacements in these aromatic systems.

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

Compound	IC_{50} (mM)[a]	$K_{i,\,apparent}$ (μM)[b]
4	0.02[c]	0.16 ± 0.04[c]
7	0.21[d]	2.5 ± 0.40[d]
8	0.07[e]	1.3 ± 0.22[e]
9	0.04	0.57 ± 0.06
10	3.0	
11	20	
EPSP	0.18	$(K_d = 1.0 \pm 0.01)$[f]

[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, 50 mM KCl, pH 7.0.
[b] Apparent K_is versus S3P were determined with [PEP] fixed at 100 μM.
[c] Reference 6.
[d] Reference 11.
[e] Reference 12.
[f] Reference 26b.

In addition, these results again show that a simple benzene ring is an acceptable substitute for the more complex shikimate ring in the design of EPSP synthase inhibitors.

The apparent K_i obtained for the three-hydroxymalonate **9** is about 3-fold weaker than that reported for the corresponding 3-phosphate **4**. Similarly, the micromolar potency observed for **9** is considerably weaker than the low nanomolar potency obtained with various shikimate-based[4] bisubstrate inhibitors possessing a 3-phosphate group. Since the enzymatic product EPSP also has affinity for free enzyme in the low micromolar

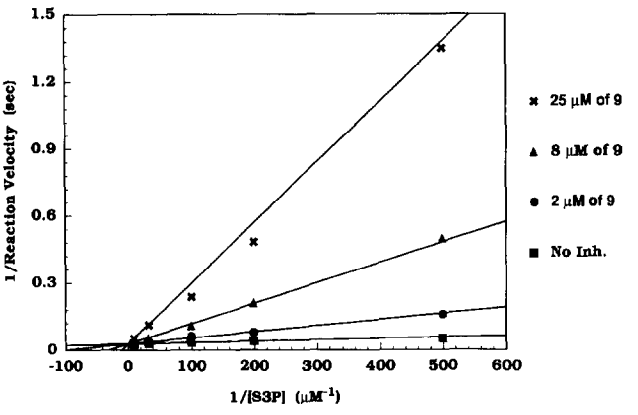


Figure 4. Inhibition of EPSP synthase by compound **9**. Double reciprocal plot of reaction velocity versus S3P concentration obtained at varying concentration of inhibitor **9**. The lines drawn were obtained from a nonlinear least-squares fit to the observed data using eq. (1) which corresponds to a kinetic model for competitive inhibition. Fitting to eq. (2), corresponding to a kinetic model for mixed inhibition, gave a very poor fit. The data, therefore, is best interpreted in terms of competitive inhibition versus S3P.

range,[26b] it is tempting to speculate that this potential bisubstrate inhibitor **9** containing a 3-hydroxymalonate moiety simply functions as a substrate-analogue inhibitor and is consequently not able to access the optimum enzyme conformation which stabilizes **1**. A 3-hydroxymalonate may still be limited in its ability to function as a more effective 3-phosphate replacement in this system by the inherently larger size of the hydroxymalonate group or because the six-membered benzene ring still imposes some twisted conformational constraint preventing better overlap with **1**.

Conclusions

In conclusion, we have now shown in several series of aromatic inhibitors that the structural requirements for effective inhibition of EPSP synthase can be dramatically simplified by taking advantage of information about the conformation of the reaction substrate and product. Reasonable potency can be maintained in aromatic inhibitors which eliminate all three stereocenters from the shikimate ring. We have also demonstrated the utility of the 3-hydroxymalonate group as a 3-phosphate mimetic which may find application for inhibitors of other enzyme systems with phosphate-based substrates. Our results suggest that the 3-hydroxymalonate moiety represents a less than ideal solution as a spatial mimic of the more labile 3-phosphate group in this system. Efforts to identify a more spatially effective 3-phosphate mimic which would be more generally applicable to EPSP synthase inhibitors are ongoing.

Experimental Section

EPSPS kinetic assays

EPSPS was isolated[25a] from an over-expressing *E. coli* strain pMON6001 and was purified[26b] using published procedures. Enzyme turnover was monitored as previously described, and the observed values for K_m and V_{max} in the absence of inhibitor were consistent with previous values.[7,27] Inhibition was measured by following the conversion of ^{14}C-S3P or ^{14}C-PEP to ^{14}C-EPSP as determined by HPLC with on-line radioactive flow detection. 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.[28] Fitting was performed using the following steady-state rate equations corresponding to competitive and mixed inhibition, respectively, in a single substrate system:

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

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

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 (corresponding to slope and intercept effects in reciprocal Lineweaver–Burk plots).

Synthesis: general

Anhydrous CH_2Cl_2 was Aldrich anhydrous grade solvent. Reactions requiring anhydrous conditions were performed in oven-dried glassware under a positive pressure of nitrogen. Benzene for rhodium-catalyzed diazo coupling reactions was generally dried in the reaction flask immediately prior to use by distillation of a few milliliters of benzene/water azeotrope. Purified H_2O was obtained from a Barnstead Nanopure II purification unit. Melting points were obtained in unsealed capillaries and are uncorrected. Elemental analyses for carbon and hydrogen were performed by Atlantic Microlabs, Inc. (Norcross, GA).

^{1}H NMR spectra were recorded at 400, 360 or 300 MHz. Chemical shifts are reported in ppm (δ) using either internal tetramethylsilane (TMS) or the residual protons of the deuterated solvent as standard. ^{13}C NMR spectra (proton decoupled) were recorded at 100, 90 or 75 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 and are referenced to external 85% H_3PO_4 or to the internal deuterium lock reference.

Chromatography

Flash chromatography was performed using Merck Kieselgel 60 (#9385), 230–400 mesh.

Preparative ion-exchange chromatography was performed at 4 °C in a 5.5 × 60 cm glass column packed with DEAE Sephadex A-25 anion-exchange resin (Pharmacia). Columns were eluted at 3–4 mL/min with a 5–8 L linear gradient of triethylammonium bicarbonate (TEAB) buffer. Fractions containing pure product (determined by analytical ion-exchange chromatography, with 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 their sodium salts by slow passage of an aqueous solution of the salt through a column of AG 50W-X8 (Na^+) cation exchange resin. Analytical ion exchange chromatography was performed on Synchropak AX-100 columns (4.6 mm × 25 cm at 1.5 mL/min or 10 mm × 25 cm at 4–6 mL/min) using isocratic elution with pH 3.5 NaH_2PO_4 buffer (0.25–1.5 M).

Diethyl [2-hydroxy-5-(methoxycarbonyl)phenyl]hydroxypropanedioate (15). To an ice-cooled, magnetically stirred solution of methyl 4-hydroxybenzoate (10 g, 66 mmol) and diethyl ketomalonate (14 g, 80 mmol) in

CH_2Cl_2 (250 mL), stannic chloride (21.35 g, 82 mmol) was added dropwise over 5 min. The mixture was stirred for 10 min, the ice bath was removed, and stirring was continued for an additional 3 h at 20 °C, during which time the reaction mixture turned to a solid mass. This solid was combined with a mixture of aqueous HCl in crushed ice, and the resulting mixture was extracted with ether (3 × 50 mL). The combined ether extracts were washed with water (2 × 20 mL), dried ($MgSO_4$), filtered and concentrated in vacuo to afford 21.3 g of a white powder. This powder was crystallized from ethyl acetate and hexane to afford 17.1 g (80%) of analytically pure **15** as a white solid, mp 113–114 °C: ^{1}H NMR ($CDCl_3$): δ 8.50 (s, 1H), 8.07 (d, J=2 Hz, 1H), 7.90 (dd, J=9, 2 Hz, 1H), 6.90 (d, J=9 Hz, 1H), 4.75 (s, 1H), 4.31 (q, J=7 Hz, 4H), 3.81 (s, 3H), 1.28 (t, J=7 Hz, 6H); ^{13}C NMR ($CDCl_3$): δ 163.04, 153.73, 125.03, 123.49, 119.40, 114.09, 109.81, 73.33, 55.72, 45.46, 7.84, 7.75. Anal. calcd for $C_{15}H_{18}O_8$: C, 55.21; H, 5.55; found: C, 55.15, H; 5.54%.

Hydroxy-[2-hydroxy-5-(carboxy)phenyl]propanedioic acid (11)

(a) Diethylethanamine salts. To a stirred solution of **15** (652 mg, 2 mmol) in 10 mL of THF was added 6 mL (15 mmol) of 2.5 N NaOH. Distilled water was added until the solution was homogeneous, and the resulting solution was allowed to stir at room temperature for 27 h. The clear, yellow solution was then cooled (0 °C), and the pH was adjusted to 8.4–8.5. The yellow solution was then concentrated cold under reduced pressure to give a yellowish white paste, which was purified by ion-exchange chromatography to provide a mixture of triethylammonium salts of **11** as a colorless glass (102 mg): ^{1}H NMR (D_2O): δ 8.05 (d, J=2 Hz, 1H), 7.77 (dd, J=7, 2 Hz, 1H), 6.81 (d, J=7 Hz, 1H), 3.0 (q, J=7 Hz, 20H), 1.15 (t, J=7 Hz, 30H); ^{13}C NMR (D_2O): δ 179.45, 177.47, 161.24, 132.34, 130.40, 130.16, 128.67, 119.55, 98.97, 48.44, 48.36, 10.52, 10.48. Anal. calcd for $C_{10}H_8O_8 \cdot 2.4Et_3N \cdot 2.4H_2O$: C, 54.05; H, 8.87; N 6.28; found: C, 54.06, H, 9.05; N, 6.19%.

(b) Monosodium salt. To a stirred solution of **15** (2.3 g, 7.05 mmol) in 50 mL of THF was added 70 mL (70 mmol) of 1 N NaOH. Distilled water was added until the solution was homogeneous, and the resulting solution was allowed to stir for 27 h. The clear, yellow solution was then cooled (0 °C), the pH was adjusted to 8.4–8.5, and then the solution was concentrated under reduced pressure to yield 7.84 g of a yellow paste. A small portion of this crude product (0.6 g) was dissolved in 10 mL of distilled water. The yellow solution thus obtained was cooled in an ice-bath, and acid resin AG 50W-X8 was slowly added until a pH of 5.31 was achieved. The solution was immediately filtered and concentrated under vacuum to afford the monosodium salt of **11** as a yellow solid (0.3 g): ^{1}H NMR (D_2O): δ 8.08 (d, J=2.4 Hz, 1H), 7.84 (dd, J=8.4, 2.4 Hz, 1H), 6.94 (d, J=2.4 Hz, 1H); ^{13}C NMR (D_2O): δ 179.36, 175.24, 161.69, 134.12, 131.98, 130.56, 126.22, 120.08, 84.71. ESMS (M-2H + Na): 277.2.

Ethyl 3-hydroxy-4-(phenylmethoxy)benzoate (17). A mixture of ethyl 3,4-dihydroxybenzoate (25 g, 132 mmol, Aldrich), benzyl bromide (25.83 g, 151 mmol) and anhyd K_2CO_3 (28.4 g, 206 mmol) in 300 mL of 2-butanone was heated to 100 °C for 3 h. After cooling to room temperature, the reaction mixture was filtered and concentrated in vacuo affording 40 g of a brownish paste. This crude material was dissolved in 50 mL of hot ethanol, and the solution was kept in the refrigerator overnight. When no crystallization was observed the next day, the mass was scratched and left in the refrigerator. After 1 h a brownish solid separated, which was filtered and recrystallized from ethanol to yield 12.3 g (25%) of the dibenzyl analogue **19** as a white solid. The remaining mother liquor was concentrated in vacuo, and the residue was crystallized from ethanol–hexane to yield 8.1 g (21%) of ethyl 3-hydroxy-4-(phenylmethoxy)benzoate (**17**) as white crystalline needles, mp 75–76 °C. ^{1}H NMR ($CDCl_3$): δ 7.58 (m, 2H), 7.39 (m, 5H), 6.91 (d, J=8 Hz, 1H), 5.67 (s, 1H), 5.14 (s, 2H), 4.30 (q, J=7 Hz, 2H), 1.34 (t, J=7 Hz, 3H); ^{13}C NMR ($CDCl_3$): δ 165.89, 149.09,145.07, 135.25, 128.47, 128.29, 127.49, 123.74, 122.26, 115.46, 110.84, 70.78, 60.41, 13.98. Anal. calcd for $C_{16}H_{16}O_4$: C, 70.57; H, 5.92; found: C, 70.65, H; 5.98%.

The remaining ethanol/hexane mother liquor was concentrated in vacuo, and the resulting residue was purified by gradient flash chromatography (10% ethyl acetate in hexane–35% ethyl acetate in hexane) to yield an additional 4.2 g (11%) of **17** as a white solid (total yield 32%) along with 1.2 g (3.2%) of the isomeric product, ethyl 4-hydroxy-3-(phenylmethoxy)-benzoate (**18**) as a white solid; mp 74–75 °C. ^{1}H NMR ($CDCl_3$): δ 7.7 (m, 2H), 7.45 (m, 5H), 6.97 (d, J=8 Hz, 1H), 6.05 (s, 1H), 5.18 (s, 2H), 4.38 (q, J=7 Hz, 2H), 1.4 (t, J=7 Hz, 3H); ^{13}C NMR ($CDCl_3$): δ 166.46, 150.32, 145.50, 135.91, 128.76, 128.55, 128.07, 124.44, 122.56, 114.45, 113.35, 71.26, 60.85, 14.40.

Ethyl 3-(2-ethoxy-2-oxoethoxy)-4-(phenylmethoxy)benzoate (20). A stirred suspension of **17** (2.72 g, 10 mmol), ethyl iodoacetate (2.56 g, 12 mmol) and K_2CO_3 (1.727 g, 12.5 mmol) in 100 mL of dry acetone was refluxed for 25 h. The mixture was cooled, diluted with acetone, filtered and concentrated under vacuum to yield a yellowish solid, which on crystallization from ethyl acetate/hexane gave 2.86 g (80%) of the desired product **20** as white needles, mp 53–54 °C: ^{1}H NMR ($CDCl_3$): δ 7.3–7.75 (m, 7H), 7.0 (d, J=10 Hz, 1H), 5.38 (s, 2H), 4.79 (s, 2H), 4.39 (q, J=7.2 Hz, 2H), 4.31 (q, J=7.2 Hz, 2H), 1.30–1.45 (m, 6H); ^{13}C NMR ($CDCl_3$): δ 168.68, 166.02, 152.72, 147.44, 136.35, 128.62, 128.05, 127.22, 124.82, 123.46, 115.86, 113.43, 70.94, 66.65, 61.30, 60.81, 14.34, 14.14. Anal. calcd for $C_{20}H_{22}O_6 \cdot 0.25\ H_2O$: C, 66.20; H, 6.31; found: C, 66.50; H, 6.27%.

Ethyl 3-(2-ethoxy-2-oxoethoxy)-4-hydroxybenzoate (21). A positive pressure of H_2 (50 psi) was maintained for 2 h over a vigorously stirred suspension of 1.79 g (5 mmol) of benzyl ether **20** and 10% palladium on

carbon in 50 mL of anhyd ethanol. The slurry was carefully filtered and concentrated affording a white solid. The white solid was further purified by gradient elution flash chromatography (50% ethyl acetate–hexanes to 100% ethyl acetate) to provide 1.0 g (75%) of **21** as a white solid, mp 60–61°C: ^{1}H NMR ($CDCl_3$): δ 7.65 (dd, J=8.5 Hz, J=1.8 Hz, 1H), 7.53 (d, J=1.8 Hz, 1H), 6.91 (d, J=8.5 Hz, 1H), 4.63 (s, 2H), 1.32 (t, J=7 Hz, 3H), 1.24 (t, J=7 Hz, 3H); ^{13}C NMR ($CDCl_3$): δ 170.25 166.06, 151.90, 145.55, 126.18, 122.49, 117.10, 115.87, 68.21, 62.01, 60.79, 14.31, 14.02. Anal. calcd for $C_{13}H_{16}O_6 \cdot 0.34H_2O$: C, 56.20; H, 6.01; found: C, 56.25, H; 6.19%.

Diethyl [5-(ethoxycarbonyl)-3-(2-ethoxy-2-oxoethoxy)-2-hydroxyphenyl]-hydroxy-propanedioate (22). To an ice-cooled and magnetically stirred solution of **21** (3.0 g, 11.2 mmol) and diethyl ketomalonate (1.95 g, 11.2 mmol) in CH_2Cl_2 (5 mL) was added dropwise $SnCl_4$ (3.64 g, 14.0 mmol) over 5 min. The mixture was stirred for 10 min, the ice bath was removed, and stirring was continued for an additional 36 h at 20 °C. The reaction mixture was then poured into a mixture of crushed ice and HCl and extracted with ether (3 × 50 mL). The combined ether extracts were washed with saturated brine (2 × 20 mL), dried ($MgSO_4$), filtered, and concentrated in vacuo to afford a yellow oil. The yellow oil was further purified by crystallization from a solution of 30% ethyl acetate in 70% hexanes to provide 2.80 g (56.5%) of pure **22** as a white solid, mp 82–83 °C: ^{1}H NMR ($CDCl_3$): δ 8.18 (s, 1H), 7.79 (d, J=1.8 Hz, 1H), 7.63 (d, J=1.8 Hz, 1H), 4.67 (s, 2H), 4.54 (s, 1H), 4.2 (m, 8H), 1.22 (m, 12 H); ^{13}C NMR ($CDCl_3$): δ 171.78, 170.75, 166.86, 151.68, 147.47, 126.33, 126.01, 123.24, 119.88, 80.47, 70.37, 64.28, 63.46, 62.23, 15.58, 15.29, 15.16. Anal. calcd for $C_{20}H_{26}O_{11}$: C, 54.29; H, 5.92; found: C, 54.14; H, 5.93%.

Trisodium [5-carboxy-3-(carboxymethoxy)-2-hydroxyphenyl]hydroxypropanedioate (10). To a stirred solution of **22** (885 mg, 2 mmol) in 10 mL of THF was added 12 mL (12 mmol) of 1 N NaOH. Water (distilled) was added until the solution was homogeneous, and the resultant solution was allowed to stir for 27 h. The clear, yellow solution was then cooled to 0 °C, and the pH was adjusted to 8.4–8.5. The yellow solution was cooled in an ice-bath and acid resin (AG 50W-X8) was slowly added until a pH of 5.31 was achieved. The solution was immediately filtered and concentrated in vacuo to afford 0.75 g (90%) of analytically pure **10** as a hygroscopic yellow solid: ^{1}H NMR (D_2O): δ 7.21 (d, J=2.0 Hz, 1H), 7.03 (d, J=2.0 Hz, 1H), 4.23 (s, 2H); ^{13}C NMR (D_2O): δ 181.84, 180.62, 179.41, 163.58, 151.36, 132.05, 126.14, 120.88, 115.51, 88.90, 70.51. Anal. calcd for $C_{12}H_7O_{11}Na_3 \cdot 3.60H_20$: C, 31.26; H, 3.11; found: C, 31.16, H, 2.72%.

Ethyl 3-[1-(diethoxyphosphinyl)-2-ethoxy-2-oxoethoxy]-4-(phenylmethoxy)benzoate (23). In a 100 mL three-necked flask fitted with a reflux condenser and 2 rubber septa was placed, 3.32 g of **17** (12 mmol), 40 mL of benzene, and 0.108 g of rhodium(II) diacetate (0.24 mmol). The mixture was heated to reflux, and then triethyl diazo-phosphonoacetate[25] (4.58 g, 18 mmol) in 20 mL of benzene was added with a syringe pump over a 2 h period. The mixture was then allowed to cool to room temperature. The mixture was concentrated in vacuo, then filtered through a pad of silica gel with ethyl acetate. The eluent was concentrated in vacuo, and purified by gradient elution flash chromatography (50% ethyl acetate in hexanes to 100% ethyl acetate) to provide 4.1 g (68%) of analytically pure **23** as a white solid, mp 72–74 °C: ^{1}H NMR ($CDCl_3$): δ 7.67 (dd, J=8.5, 2 Hz, 1H), 7.54 (d, J=2 Hz, 1H), 7.25–7.40 (m, 5H), 6.91 (d, J=8.5 Hz, 1H), 5.06 (s, 2H), 5.05 (d, J=18 Hz, 1H), 4.19–4.29 (m, 8 H), 1.15–1.32 (m, 12H); ^{13}C NMR ($CDCl_3$): δ 166.20, 165.75, 153.14, 146.67 (d, J=12.3 Hz), 135.86, 128.53, 128.25, 127.88, 125.93, 123.26, 118.04, 112.96, 75.85 (d, J=155.5 Hz), 70.89, 64.20, 64.11, 62.01, 60.81, 16.29, 16.23, 14.31, 14.02; ^{31}P NMR ($CDCl_3$): δ 12.44. Anal. calcd for $C_{24}H_{31}O_9P \cdot 0.5\ H_2O$: C, 57.31; H, 6.40; found: C, 57.31; H, 6.42%.

Ethyl 3-[1-(diethoxyphosphinyl)-2-ethoxy-2-oxoethoxy]-4-hydroxybenzoate (24). A positive pressure of H_2 was maintained for 2 h over a vigorously stirred suspension of 4.1 g (8.3 mmol) of **23** and 10% palladium on carbon in 50 mL of anhyd ethanol. The slurry was carefully filtered and concentrated affording a white solid. The white solid was further purified by gradient elution flash chromatography (50% ethyl acetate–hexanes to 100% ethyl acetate) to provide 3.2 g (95%) of analytically pure **24** as a white solid, mp 92–93 °C: ^{1}H NMR ($CDCl_3$): δ 7.68 (m, 2H), 6.92 (d, J=9 Hz, 1H), 4.73 (d, J=19 Hz, 1H), 4.25 (m, 8H), 1.30 (m, 12H); ^{13}C NMR ($CDCl_3$): δ 168.20, 166.97, 154.43, 147.49 (d, J=9 Hz), 129.02, 123.70, 122.29, 118.12, 80.03 (d, J=158.36 Hz), 65.87 (d, J=7 Hz), 65.64 (d, J=7 Hz), 63.99, 61.95, 17.57 (d, J=3 Hz), 17.52 (d, J=3 Hz), 15.53, 15.22; ^{31}P NMR ($CDCl_3$): δ 11.69. Anal. calcd for $C_{17}H_{25}O_9P$: C, 50.49; H, 6.23; found: C, 50.23; H, 6.31%.

Diethyl [3-[1-(diethoxyphosphinyl)-2-ethoxy-2-oxo-ethoxy]-5-(ethoxycarbonyl)-2-hydroxyphenyl]hydroxypropanedioate (25). To an ice-cooled and magnetically stirred solution of **24** (3.0 g, 7.4 mmol) and diethyl ketomalonate (1.42 g, 8.2 mmol) in CH_2Cl_2 (10 mL) was added dropwise $SnCl_4$ (2.41 g, 9.3 mmol) over 5 min. The mixture was stirred for 10 min, the ice bath was removed, and stirring was continued for an additional 36 h at 20 °C. The reaction mixture was then combined with a crushed ice–HCl mixture and extracted with ether (3 × 50 mL). The combined ether extracts were washed with saturated brine (2 × 20 mL), dried ($MgSO_4$), filtered, and concentrated in vacuo to afford a yellow oil. The yellow oil was further purified by gradient elution flash chromatography (50% ethyl acetate–hexanes to 100% ethyl acetate) to provide 1.28 g (30%) of analytically pure **25** as a white solid, mp 82–84 °C: ^{1}H NMR ($CDCl_3$): δ 7.75 (d, J=2 Hz, 1H), 7.68 (d, J=2 Hz, 1H), 4.68 (d, J=18 Hz, 1H), 4.20 (m,

12H), 1.22 (m, 18H); ^{13}C NMR ($CDCl_3$): δ 170.66, 170.64, 168.23 (d, $J=1.9$ Hz), 166.60, 152.76, 148.08 (d, $J=9.5$ Hz), 127.46, 127.37, 123.14, 123.05, 80.53, 80.47 (d, $J=158$ Hz), 66.01 (d, $J=6.5$ Hz), 65.73 (d, $J=6.5$ Hz), 64.18, 62.18, 17.63, 17.59, 15.56, 15.26, 15.15: ^{31}P NMR ($CDCl_3$): δ 12.51. Anal. calcd for $C_{24}H_{35}O_{14}P$: C, 49.82; H, 6.06; found: C, 49.77, H, 6.07%.

Propanedioic acid, [5-(carboxy)-3-[(carboxyphosphono)-methoxy]-2-hydroxyphenyl]-hydroxypropanedioate (9). To the phosphonate **25** (0.5 g, 0.87 mmol) in 25 mL of dry CH_2Cl_2 at 0 °C was added a solution of TMSBr (0.384 g, 2.51 mmol) and pyridine (0.14 mL, 1.7 mmol) in 5 mL of CH_2Cl_2 slowly by syringe. The mixture was warmed to room temperature, stirred for 3 h, then treated with 15 mL of cold water and stirred for 3 min, at which time, 6 mL of 1 N NaOH was added. The layers were separated, and the organic layer was extracted twice with water. The combined aqueous layers were treated with an additional 2.5 mL of 1N NaOH and allowed to stir overnight for 18 h at room temperature. The reaction mixture was neutralized to pH 7.5 with 1 N HCl and diluted to 280 mL (conductance 980). This solution was applied to a 5×50 cm column of DEAE Sephadex A-25 anion-exchange resin equilibrated with 0.3 M TEAB. Elution with a linear TEAB gradient (0.3–1.2 M) afforded a major band which gave, after very careful concentration at ca. 10 °C, 0.12 g (20%) of **9** as a white paste: ^1H NMR (D_2O) δ 7.4 (d, $J=2$ Hz, 1H), 7.16 (d, $J=2$ Hz, 1H), 4.44 (d, $J=17$ Hz, 1H), 2.99 (q, $J=7$ Hz, 36H), 1.12 (m, 54H); ^{13}C NMR (D_2O): δ 180.95 (d, $J=1.8$ Hz), 179.68, 177.99, 150.72, 150.3 (d, $J=9.2$ Hz), 131.34, 129.47, 124.99, 116.32, 84.25 (d, $J=193.5$ Hz), 84.5, 49.38, 44.93, 13.48, 11.62. ^{31}P NMR (D_2O): δ 9.6. Anal. calcd for $C_{12}H_{11}O_{14}P\cdot2.4\ Et_3N\cdot1.0H_2O$: C, 47.35; H, 7.36; found: C, 47.33; H, 7.75%.

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