# Inhibition of EPSP Synthase by Analogues of the Tetrahedral Intermediate and of EPSP

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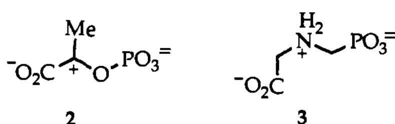
**Abstract:** A number of analogues of the intermediate (**1**) and product of the enzyme EPSP synthase are described. Inert analogues that show high affinity for the enzyme from *Petunia hybrida* are produced by replacing the labile ketal phosphate moiety of this intermediate with a phosphonate (**4**) or by stabilizing the ketal phosphate against ionization through introduction of fluorine substituents (**5** and **6**). The most potent inhibitor is the *R*-stereoisomer of the difluoromethyl derivative, (*R*)-**6**, with a $K_i$ value of 4 nM. The uncertain stereochemical preference exhibited by the enzyme for the side-chain stereoisomers of these tetrahedral analogues and for the reduced derivatives of EPSP itself (**8**) suggests that the methyl binding site is indistinct and that the phosphate and carboxylate moieties may interchange binding sites without undue penalty. Fluorine or methyl substitution at the 9-*Z* position of EPSP reduces the affinity of these compounds by 1 order of magnitude only, but it abolishes their ability to act as substrates. The analogues were synthesized by a general strategy (Chart I) that involves introduction of the C-5 side chain onto a 3,4 protected shikimate, lactonization to facilitate separation and assignment of the diastereomers and to allow selective introduction of the allylic phosphate, and final deprotection. Novel synthetic methods developed in this connection include the multistep, single-flask generation of the ketal phosphates (viz. **13a** → **18** → **19** → **20** → **14c**) and an improvement in the procedure for double deprotection of bis(*p*-nitrophenethyl) phosphate triesters under basic conditions. A model study involving pyruvate ketal phosphates substituted with a single electron-withdrawing substituent demonstrated the stability of this functional group and helped to define methods for its construction.

## Introduction

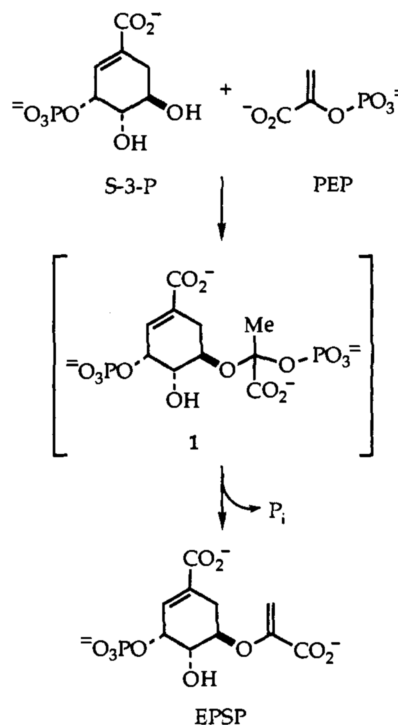
Occupying a key position in a pathway replete with unusual transformations, 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase (EC 2.5.1.19) is distinguished as the subject of long-standing mechanistic investigation and the target of extensive inhibitor development. The enzyme catalyzes transfer of a carboxyvinyl group from phosphoenol pyruvate (PEP) to the 5-hydroxyl group of shikimate 3-phosphate (S3P) as depicted in Scheme I.

Sprinson and his co-workers first proposed this scheme, including tetrahedral adduct **1**, during their pioneering investigations of the enzyme.[1] The mechanism was confirmed by the isolation and characterization of **1** by Anderson et al.[2] Anderson and Johnson and their associates have determined kinetic parameters for all individual steps of association, chemical transformation, and dissociation of substrates and products[2,3] and demonstrated an ordered sequence of substrate binding (S3P followed by PEP) and product release ($P_i$ prior to EPSP). It remains to be determined conclusively whether C–H and C–O bond formation and cleavage in the addition and elimination steps occur synchronously or in a stepwise manner with formation of oxocarbonium ion intermediates. However, Abeles and his co-workers[4] and recently Pansegrau et al.[5] have found that enzyme-catalyzed exchange of the vinyl hydrogens of PEP does not require nucleophilic addition by a shikimate 5-hydroxyl group, implying that **1** is formed via oxocarbonium ion **2**.




Knowles and Floss and their co-workers first showed that formation of EPSP from a suitably labeled form of PEP occurs with net retention of configuration at the double bond.[6] This observation requires that the addition and elimination steps proceed with opposite stereochemistry, but it does not define which step is syn and which anti. Moreover, the absolute configuration of the extracyclic stereocenter in **1** has yet to be proven. In a preliminary report, we suggested that this center has the *R*-configuration (vide infra);[7] however, the *S*-configuration has also been inferred[3c] from the stereochemistry of a decomposition product of **1** identified by Sammons et al.[8]

Scheme I




† Current address: Ciba-Geigy AG, CH-4002 Basel, Switzerland.

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(2) Anderson, K. A.; Sikorski, J. A.; Benesi, A. J.; Johnson, K. A. *J. Am. Chem. Soc.* **1988**, *110*, 6577–6579.
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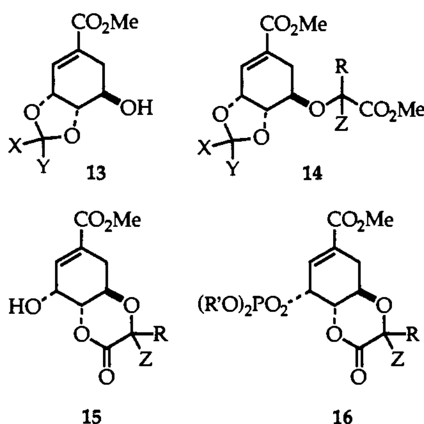
Chart I




| | X,Y | Z | R | R' |
|---|---|---|---|---|
| a: | $Me_2$ | $PO_3Bn_2$ | H | -- |
| b: | $Me_2$ | $PO_3Bn_2$ | Me | Bn |
| c: | $Me_2$ | $OPO_3(NPE)_2$ | $CF_3$ | NPE |
| d: | H,OMe | $OPO_3(NPE)_2$ | $CHF_2$ | NPE |
| e: | $Me_2$ | H | Me | NPE |
| f: | -- | H | Me | Bn |
| g: | $Me_2$ | H | H | NPE |
| h: | $Me_2$ | $CO_2Me$ | H | -- |
| i: | $Me_2$ | $CO_2Me$ | $CHF_2$ | Bn |
| j: | $Me_2$ | $PO_3Me_2$ | H | -- |
| k: | $Me_2$ | [Z,R = (=CHMe)] | | NPE |

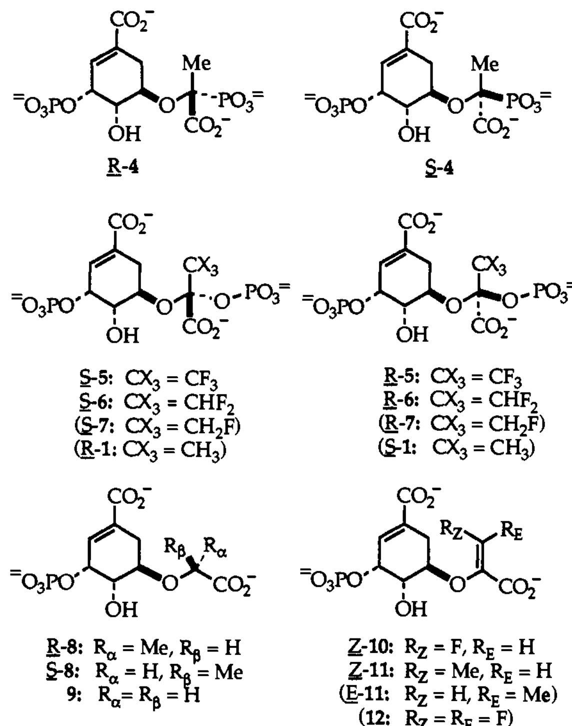
(Note: not all combinations are represented; NPE = p-nitrophenethyl)

While mechanistic studies have sparked interest in developing inhibitors for EPSP synthase, such efforts have been fueled by the realization that the important herbicide glyphosate [*N*-(phosphonomethyl)glycine, 3] derives its activity through inhibition of this enzyme.[9] Glyphosate competes with PEP for binding to EPSP synthase and, like that substrate, binds to the enzyme·S3P complex; the dissociation constant for the equilibrium

lies between 0.2 and 0.4 $\mu$M for the enzyme from *Petunia hybrida* (pH 7.5, 25 °C; $K_m$ for PEP under these conditions is 8.4 $\mu$M).[3a,10] The structural and electronic similarities between oxocarbonium ion **2** and zwitterion **3** form the basis for the generally accepted notion that the factors that facilitate formation of **2** in the enzyme active site serve to stabilize its complex with **3**. However, it could be argued that this notion would predict enhanced binding of *N*-methylglyphosate, which is not the case.[3e] Indeed, extensive alteration to this simple structure has failed to improve upon the binding affinity significantly.[3e,9b] These results as well as other considerations have led Anderson et al. to a different view of the basis for glyphosate binding and of the importance of the cationic species **2** in the reaction pathway.[3a,b]

The design of analogues of the tetrahedral intermediate **1** is an alternative approach to the invention of potent inhibitors of EPSP synthase.[7,11] Analogues of **1** not only incorporate the binding determinants of both substrates but may also take advantage of any stabilization that the intermediate enjoys in the active site. Estimates of the affinity of EPSP synthase for **1** diverge (ranging from 0.05[3d] to 0.2–3 nM[12]); however, as an example from our initial work, the phosphonate analogue (*R*)-**4** binds to the *P. hybrida* enzyme with an inhibition constant of 15 nM.[7]

In extending our studies on EPSP synthase inhibitors, we had several goals: to devise robust analogues of the labile ketal phosphate moiety, to scrutinize further the question of side-chain configuration, and to determine if the estimated affinity of **1** could be approached with a suitable analogue. In this report, we describe the synthesis and evaluation of the trifluoro- and difluoromethyl derivatives **5** and **6**, the dihydro- and nor-EPSP analogues **8** and **9**, and the (*Z*)-9-fluoro- and (*Z*)-9-methyl-EPSP derivatives (*Z*)-**10** and (*Z*)-**11**. We also report our approaches to the monofluoromethyl analogue **7**.




## Synthesis

**General Considerations.** The general strategy for synthesis of EPSP derivatives is outlined in Chart I. The readily available shikimate ketal **13a**[13] or orthoester **13d**[14] is functionalized at the C-5 position, either via carbenoid insertion or hemiketal formation, to introduce the appropriate side-chain moiety (**14**). Deprotection of the ketal or orthoester unveils the C-3 and C-4 hydroxyls, which are then differentiated by lactonization from the side chain (**15**). In addition to masking the C-4 hydroxyl selectively, incorporation of the side chain in a rigid system facilitates separation of the diastereomers and determination of their configuration through 2D NMR techniques. Introduction of the C-3 phosphate group (**16**) and global deprotection then provide the target compound.

In addition to this general strategy, the syntheses outlined below led to the development of a mild method for the conversion of stably protected phosphate triesters to the monoesters. The presence of acid-labile enol ethers or ketal phosphates in the final products argued against acidic conditions for deprotection, and the allylic phosphate group complicated attempts to use esters that are cleaved by hydrogenolysis or nucleophilic attack. We have

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(10) Padgette, S. R.; Huynh, Q. K.; Borgmeyer, J.; Shah, D. M.; Brand, L. A.; Re, D. B.; Bishop, B. F.; Rogers, S. G.; Fraley, R. T.; Kishore, G. M. *Arch. Biochem. Biophys.* **1987**, *258*, 564–573.
(11) Sikorski, J. A., personal communication.
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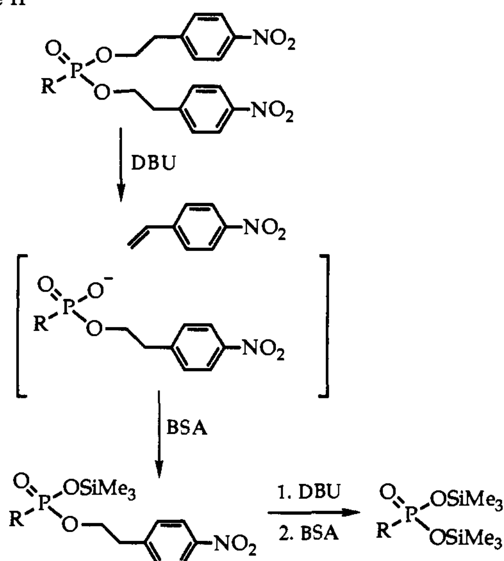
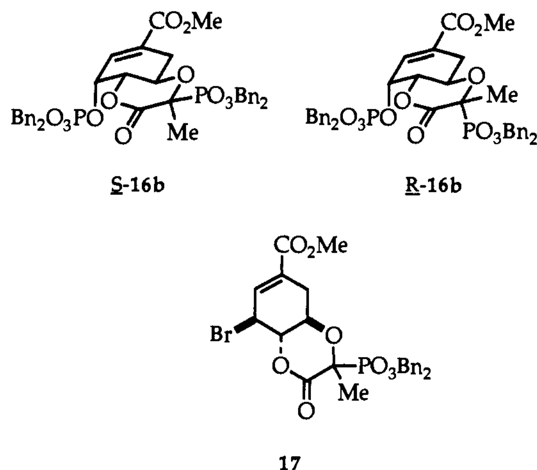
(13) Fischer, H. O. L.; Dangschat, G. *Helv. Chim. Acta* **1935**, *18*, 1206–1213.
(14) Zamir, L. O.; Luthe, C. *Can. J. Chem.* **1984**, *62*, 1169–1175.

Scheme II




in some instances used *O*-benzyl protection of phosphonates and phosphates in these[7] and related compounds,[15] relying on the faster rate of trimethylsilyl bromide (TMS-Br) induced cleavage of benzylic phosphorus esters in comparison to the C-3 allylic P–O bond.[16] However, selectivity is not complete, so we turned to the protection strategy developed by Pfleiderer et al., which is based on use of *p*-nitrophenethyl (NPE) and related esters.[17] Deprotection is accomplished under basic, nonnucleophilic conditions by treatment with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in pyridine, with formation of *p*-nitrostyrene. However, this method presents problems for sensitive substrates, since prolonged reaction times are required for deprotection to the dianion. Not surprisingly, the first elimination step proceeds more rapidly than the second (hours vs days at room temperature), since the dianion is much worse as a leaving group than the monoanion. We have found that the second step can be made as fast as the first through the simple expedient of including bis(trimethylsilyl)acetamide (BSA) in the reaction mixture. The intermediate anion from the first elimination is converted to the silyl ester, which as a neutral triester loses the second NPE moiety as easily as the first (Scheme II). The product of the elimination reaction is the bis(trimethylsilyl) ester, which undergoes hydrolysis spontaneously on exposure to water at workup.

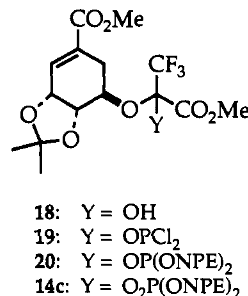
**Synthesis of Phosphonates (*R*)-4 and (*S*)-4.** Preparation of the diastereomeric phosphonates (*R*)-**4** and (*S*)-**4** according to the general procedure of Scheme II has been described in a preliminary communication.[7] C-5 functionalization was accomplished by rhodium-catalyzed carbenoid insertion[18] of methyl (dibenzylphosphono)diazoacetate, to give **14a**, and methylation of the anion to give **14b**. After formation of the corresponding lactone and phosphorylation with tetrabenzyl pyrophosphate,[15,19] the diastereomers of **16b** were separated and assigned by 2D ROESY spectroscopy.[20] For the more polar isomer, NOE interaction between the methyl group and H-5 indicates their cis




relationship and the *S* side-chain configuration. In turn, for the less polar *R*-isomer, an NOE cross-peak between the methyl and H-4 reflects their cis relationship.

Cleavage of the phosphonate esters of **16b** can be accomplished with reasonable selectivity with TMS-Br in the presence of pyridine[16] if the reaction is monitored closely by $^1$H or $^{31}$P NMR. On prolonged reaction with an excess of reagent, formation of the allylic bromide **17** is observed.[21] The tetra(trimethylsilyl) esters are not isolated, but treated directly with an excess of NaOH to hydrolyze the methyl and silyl esters and open the lactones to give (*R*)-**4** and (*S*)-**4**. After ion-exchange chromatography, these diastereomers are isolated in 45% and 81% yields from the fully protected derivatives (*R*)-**16b** and (*S*)-**16b**, respectively. Interestingly, the *S*-diastereomer is more resistant to lactone hydrolysis and shows a significant tendency to relactonize if allowed to stand in neutral aqueous solution.

**Synthesis of Trifluoromethyl Ketal Phosphates (*R*)-5 and (*S*)-5.** The ketone carbonyl of methyl 3,3,3-trifluoropyruvate[22] is strongly electron deficient and adds alcohols readily. The resultant hemiketals are not derivatized with typical phosphorylating reagents, however. Reaction of the isopropyl alcohol adduct with dibenzyl phosphorochloridate[23] or dibenzyl *N,N*-diethylphosphoramidite,[24] or treatment of the corresponding lithium or sodium salts with tetrabenzyl pyrophosphate,[19] was unsuccessful. However, an effective, one-pot alternative was devised after the observation that the hemiketal does react with $PCl_3$. Thus, combination of acetonide **13a** with 1.5 equiv of methyl 3,3,3-trifluoropyruvate in THF at room temperature serves to form the hemiketal adduct **18** quantitatively. This solution is cooled to −78 °C, and 10 equiv



**18**: Y = OH
**19**: Y = $OPCl_2$
**20**: Y = $OP(ONPE)_2$
**14c**: Y = $O_2P(ONPE)_2$

of pyridine and 1 equiv of $PCl_3$ are added to form the dichlorophosphite **19**. After 1 h, a solution of 2 equiv of *p*-nitrophenethyl alcohol in THF is transferred by cannula and formation of the

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(21) This side reaction proved to be particularly troublesome during early stages of this work, in which the phosphonate dimethyl ester moiety was employed (e.g., **14** (R = $CH_3$, Y = $PO_3Me_2$, R′ = Bn)).

(22) Pasetti, A.; Tarli, F.; Sianesi, D. *Gazz. Chim. Ital.* **1968**, *98*, 277. Kunyants, I. L.; Shokina, V. V.; Tyuleneva, V. V. *Dokl. Akad. Nauk.* **1966**, *169*, 594; *Proc. Acad. Sci. USSR, Chem. Sect.* **1966**, *169*, 594.

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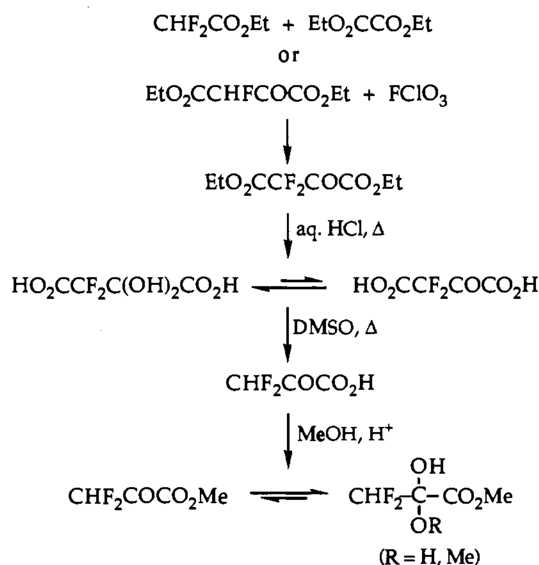
Scheme III




trialkyl phosphite **20** is allowed to proceed as the solution is warmed to 0 °C over 2 h. Finally, 1.5 equiv of *m*-chloroperoxybenzoic acid (mCPBA) is added to oxidize the phosphite to the phosphate **14c**. The yield of purified material from this four-step procedure ranges from 60% to 80%.

Because of the strongly electron-withdrawing trifluoromethyl group, ketal phosphate **14c** is stable to the acidic conditions for removal of the acetonide moiety and lactonization to **15c** (Dowex 50W-X8 resin in methanol at 60–65 °C for 3 days, followed by *p*-toluenesulfonic acid in refluxing benzene). In this series, separation of diastereomers is achieved prior to phosphorylation at C-3, which is accomplished with bis(*p*-nitrophenethyl) *N*,*N*-diisopropylphosphoramidite and tetrazole, followed by oxidation with mCPBA.[25]

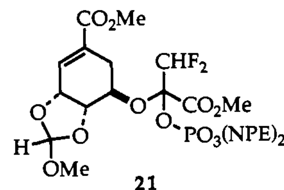
The configurations of (*R*)- and (*S*)-**16c** were assigned by $^1H$–$^{19}F$ heteronuclear Overhauser enhancement spectroscopy (HOESY).[26] A single cross-peak was present in the spectrum for each isomer, representing interaction between the $CF_3$ group and either H-5 or H-4, respectively.

Treatment of the fully protected derivatives with an excess of DBU and BSA in acetonitrile at room temperature for 4 h, followed by alkaline hydrolysis and ion-exchange purification, affords the trifluoroketal phosphates (*R*)-**5** and (*S*)-**5** in 60–70% yield.

**Synthesis of Difluoromethyl Ketal Phosphates (*R*)-6 and (*S*)-6.** Synthesis of the difluoromethyl analogues (*R*)-**6** and (*S*)-**6** was strictly parallel to that of the trifluoromethyl derivatives. However, the requisite starting material, difluoropyruvic acid or its esters, had not been described previously. A synthesis of methyl difluoropyruvate is outlined in Scheme III. Diethyl difluorooxaloacetate can be prepared via Claisen condensation[27] of diethyl oxalate and ethyl difluoroacetate or by fluorination of diethyl fluorooxaloacetate with perchloryl fluoride.[28] The latter route affords product that is easier to purify, and the difficulties of handling pure $FClO_3$[29] are avoided by preparing it as a dilute mixture with nitrogen and using it as formed.[30] Thus, $FClO_3$ generated from heating a mixture of $KClO_4$ and $FSO_3H$ is swept with a stream of nitrogen into a slurry of sodium diethyl fluorooxaloacetate in THF at 0 °C. From 2 to 2.7 equiv of $KClO_4$, yields of distilled product on the order of 60–75% were realized on a 5–15-g scale.

Diethyl difluorooxaloacetate is hydrolyzed in boiling 3 N HCl to afford the corresponding diacid as the crystalline monohydrate.[28] As noted previously,[28a] this material is extraordinarily resistant to decarboxylation, presumably reflecting the difficulty in cracking the hydrate and the destabilizing influence of the fluorine substituents on enol formation.[31] Although decarboxylation is effected after 2 days in concentrated HCl at 130 °C, fluorine-containing byproducts are evident in the product and most of the material is deposited as a polymer. The diacid can be distilled (bulb-to-bulb, 230 °C/20 mmHg) without loss of $CO_2$! Fortunately, we found that decarboxylation proceeds cleanly in DMSO at 100–105 °C over a period of 2 h (or at 75–80 °C overnight). Separation of difluoropyruvic acid from DMSO was not attempted; rather, the solution was treated with *p*-toluenesulfonic acid in refluxing methanol to form the methyl ester. After evaporation of the methanol and separation of DMSO by partitioning between ether and water, a mixture of methyl difluoropyruvate and the corresponding hydrate and methyl hemiacetal was isolated in 35% yield by distillation from $P_2O_5$. Repeated distillation from $P_2O_5$ removed the methanol but failed to dehydrate the ketone completely; however, the mixture of methyl difluoropyruvate and its hydrate (ca. 7:3 ratio) proved satisfactory for the subsequent step.

At this stage in our work, the orthoester **13d**[14] was the preferred protected form of shikimate for the condensation reaction, since this moiety is removed under milder conditions than is the acetonide. Adduct **21** is formed from this material and the prepa-




ration of methyl difluoropyruvate by stirring in THF overnight in the presence of 4-Å molecular sieves. After sequential addition of $PCl_3$, *p*-nitrophenethyl alcohol, and mCPBA as described above, the ketal phosphate **14d** is isolated in 64% yield. Cleavage of the orthoester (Dowex resin, $H^+$ form, 1% aqueous methanol at room temperature) and lactonization (*p*-toluenesulfonic acid in benzene at reflux) provide the diastereomeric lactones (*R*)-**15d** and (*S*)-**15d** in 26% and 30% yields, respectively, after chromatographic separation. Their configurations were assigned unambiguously by $^1H$–$^{19}F$ HOESY NMR, as described above for the trifluoromethyl analogues. The procedures developed in that series for introduction of the allylic phosphate moiety as the bis(NPE) ester and for global deprotection were again employed for the conversion of the **15d** diastereomers to (*R*)-**6** and (*S*)-**6**.

**Model Studies for the Monofluoro Analogue 7 and Intermediate 1.** In contrast to the trifluoro- and difluoropyruvates, methyl fluoropyruvate[32] cannot be induced to undergo the one-pot condensation/phosphorylation with a shikimate alcohol derivative. At least, if the product is formed, it is not stable to the usual isolation conditions. However, condensation can be effected in an intramolecular context, i.e., via a lactone–hemiketal, as demonstrated with *trans*-1,2-cyclohexanediol as a model system. Acid-catalyzed condensation of this diol with fluoropyruvic or bromopyruvic acid or with the corresponding esters affords the adducts **22a,b**. The phenylsulfonyl analogue **22c** can be prepared in the same manner, although it is more readily obtained by displacement of the bromide ion of **22b** with benzenesulfinate, presumably via the open-chain isomer. Condensation of the diol with pyruvic acid itself leads to an equilibrium mixture of comparable amounts of the cyclic lactone–hemiketal **22d** and the

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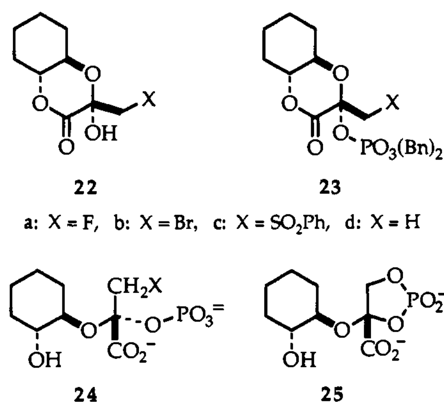
(32) Blank, I.; Mayer, J.; Bergmann, E. D. *J. Chem. Soc.* **1955**, 2190.



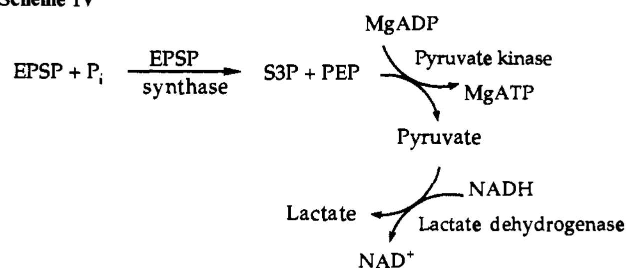
open-chain keto ester. In the absence of acid or base catalysis, equilibration between these species is slow, and the hemiketal can be isolated in pure form by crystallization from the mixture. However, although we explored a variety of phosphorylating agents, we were unable to isolate a ketal phosphate derivative of this tautomer (e.g., **23d**), either because of the instability of this product or because of preferential reaction by the open-chain isomer. In contrast, the substituted analogues can all be phosphorylated with tetrabenzyl pyrophosphate to give the hemiketal phosphates **23a–c**. In each case, a predominant isomer is formed (ratios 5–10:1), to which we assign the axial configuration based on the anomeric effect and steric considerations.

Hydrogenolysis of the benzyl esters and alkaline hydrolysis of the lactone moiety afford the model structures **24a–c**, which have been identified by $^1H$, $^{31}P$, and $^{19}F$ NMR. The fluoro and sulfonyl derivatives are stable at alkaline pH and can be purified by ion-exchange chromatography and isolated and characterized as the lithium salts after lyophilization. However, under similar deprotection conditions, the bromide **23b** cyclizes to the ketal phosphate **25**.

These model studies provided assurance that the pyruvate–ketal phosphates bearing a single electron-withdrawing substituent are stable enough to warrant further investigation; however, this method of assembly poses significant problems for synthesis of the corresponding shikimate 3-phosphate derivatives (e.g., **7**). There is an obvious regiochemical question that arises with an unsymmetrical diol as substrate for the initial condensation. In addition, the strong preference of the hydroxy and phosphoryloxy groups for the axial position in the lactone ketals means that only one of the side-chain diastereomers would be readily accessible by this route. Although the benzenesulfonyl derivative **22c** was prepared in the hope that conditions could be found for reductive cleavage of this substituent, our initial attempts to do so in the model system have not been successful.

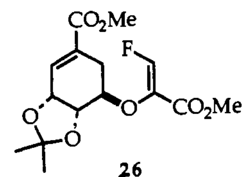
**Synthesis of Lactyl and Glycolyl Analogues 8 and 9.** The *S*-diastereomer of 5-lactylshikimate lactone **15e** has been described by Ganem et al., who prepared it by acid-catalyzed condensation of shikimate acetonide **13a** and ethyl diazopropionate, followed by deketalization and lactonization.[33] Walsh and Berchtold and their co-workers[34] have introduced both the lactyl and glycolyl side chains into a chorismate precursor by rhodium-catalyzed insertion[18] of methyl diazopropionate[35] and methyl diazoacetate.[36] Using this approach, we obtained the lactyl and glycolyl ethers **14e** and **14g** in 60% and 86% yields, respectively, from **13a**. Acid-catalyzed ketal hydrolysis and subsequent cyclization then provide the hydroxy lactones **15e,g**. By minimizing the time for which the lactyl analogue is subjected to the acidic conditions, we were able to preserve the 1:1 ratio of diastereomers that arises from the diazo insertion reaction. These isomers were separated by reversed-phase HPLC and assigned by $^1H$ NMR after conversion to the bis(*p*-nitrophenethyl) phosphates (*R*)- and (*S*)-**16e**; the NOE interaction between H-5 and the axial methyl identified the less stable *R*-isomer.

Scheme IV




Although it is not easily discerned by spectroscopic examination of the product, enzymatic analysis[37] of the dihydro-EPSP isomers (*R*)-**8** and (*S*)-**8** obtained on cleavage of the NPE groups from **16e** and lactone hydrolysis indicated that epimerization of the side chain occurs under these conditions. As a result, benzyl esters were used for phosphate protection (**16f**), with cleavage effected by TMS-Br in pyridine.[16] This procedure provides the pure diastereomers of (*R*)-**8** and (*S*)-**8**. No such complications were presented in the case of the glycolyl ether **15g**, which was converted to **9** via the bis(NPE) phosphate **16g**.

**Synthesis of (*Z*)-9-Fluoro- and (*Z*)-9-Methyl-EPSP, (*Z*)-10 and (*Z*)-11.** The synthesis of fluoro analogue (*Z*)-**10** was achieved in four steps from the malonyl ether **14h**, which is an intermediate in the synthesis of EPSP.[38] Reaction of the sodium salt of the malonate with difluorocarbene generated in situ from chlorodifluoromethane[39] gave the difluoromethyl adduct **14i** in modest yield, accompanied by a small amount of the vinyl fluoride **26**



from hydrolysis and decarboxylative fragmentation. Conversion of **14i** to the hydroxy lactone **15i**, formation of the dibenzyl phosphate **16i**, and TMS-Br cleavage were carried out as described above for related systems. Alkaline hydrolysis of the malonyl lactone is accompanied by loss of both $CO_2$ and fluoride ion to give (*Z*)-9-fluoro-EPSP ((*Z*)-**10**) in 60% overall yield from the carbene adduct **14i**.

That a single isomer is formed in the hydrolytic fragmentation is precedented in work in related systems.[40,41] Evidence for the *Z*-configuration of (*Z*)-**10** was obtained by partial isomerization of a derivative[42] to the *E*-isomer on irradiation. The downfield shift of the enol ether hydrogen in the starting material, relative to the photoisomer, is consistent with a trans relationship to the ether oxygen, based on precedent with the fluoro-PEP isomers.[41]

The 9-methyl derivative of EPSP was prepared from the known phosphonate **14j**[7] using Horner–Emmons olefination with acetaldehyde. This approach has been used by Lesuisse and Berchtold to prepare the related analogues of chorismic acid.[43] In analogy to their findings, we obtained a 5:1 mixture of *E*/*Z* isomers **14k** as the kinetic product. After ketal hydrolysis and base-catalyzed lactonization ($K_2CO_3$ in acetonitrile), the now 3:1 *E*/*Z* mixture

(33) Muralidharan, V. B.; Wood, H. B.; Ganem, B. *Tetrahedron Lett.* **1990**, *31*, 183–184.
(34) Walsh, C. T.; Erion, M. D.; Walts, A. E.; Delany, J. J., III; Berchtold, G. A. *Biochemistry* **1987**, *26*, 4734–4745.
(35) Searle, N. E. *Org. Synth.* **1956**, *36*, 25–28.
(36) Takamura, N.; Mitzoguchi, T. *Tetrahedron* **1975**, *31*, 227–230.
(37) Performed with chorismate synthase: Lauhon, C. T.; Bartlett, P. A., unpublished work.
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(40) McDonald, I. A.; Lacoste, J. M.; Bey, P.; Palfreyman, M. G.; Zreika, M. *J. Med. Chem.* **1985**, *28*, 186–193.
(41) Duffy, T. H.; Nowak, T. *Biochemistry* **1984**, *23*, 661–670.
(42) Hydrolysis and cyclization of byproduct **26** provides the lactone **15** (Z,R = (*Z*)=CHF): $^1H$ NMR δ 7.15 (*J* = 75); irradiation of this compound in $C_6D_6$ with a 450-W Hanovia Hg arc lamp leads to formation of the isomeric material **15** (Z,R = (*E*)=CHF): $^1H$ NMR δ 6.59 (*J* = 75).
(43) Lesuisse, D.; Berchtold, G. A. *J. Org. Chem.* **1988**, *53*, 4992–4997.

Table I. Inhibition of EPSP Synthase by Analogues of the Tetrahedral Intermediate[a]

| compd | $K_i$ (nM) vs EPSP[b] | $K_i$ (nM) vs $P_i$[c,d] |
|---|---|---|
| (*R*)-**4** | 15 ± 1 | 113 ± 15 |
| (*S*)-**4** | 1130 ± 70 | 2100 ± 20 |
| (*R*)-**5** | 32 ± 3 | 53 ± 4 |
| (*S*)-**5** | 26 ± 2 | 81 ± 6 |
| (*R*)-**6** | 4.0 ± 0.3 | nd |
| (*S*)-**6** | 75 ± 7 | nd |
| (*R*)-**8** | 71000 ± 10000 | nd |
| (*S*)-**8** | 52000 ± 8000 | nd |
| **9** | 1800 ± 200 | nd |
| (*Z*)-**10** | 13000 ± 2000 | nd |
| (*Z*)-**11** | 40000 ± 3000 | nd |
| EPSP | ($K_m$ = 2100) | |
| S3P | ($K_m$ = 6400)[e] | |
| glyphosate (**3**) | | 400[e,f] |

[a] Determined at 25 °C in potassium-HEPES buffer, pH 7.5. [b] [$P_i$] = 50 mM. [c] [EPSP] = 50 μM. [d] nd, not determined. [e] Reference 10. [f] Glyphosate competes with PEP for the E·S3P form of the enzyme.

of **15k** could be separated by normal-phase HPLC. Introduction of the phosphate moiety (NPE diester) and removal of the protecting groups provide the thermodynamically more stable isomer (*Z*)-**11** in pure form in 65% yield from the lactone (*Z*)-**15k**. Unfortunately, a pure sample of the *E*-isomer has so far eluded us; using either NPE or benzyl protection for the phosphate results in significant isomerization to the *Z*-isomer on deprotection.

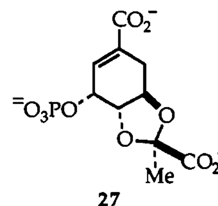
## Inhibition of EPSP Synthase

**Method of Assay.** The compounds described above were evaluated as inhibitors of EPSP synthase from *P. hybrida*[10] by following the reverse reaction (EPSP + $P_i$ → PEP + S3P) using a coupled assay with pyruvate kinase and lactate dehydrogenase (Scheme IV).[44] Formation of PEP is detected by eventual conversion to lactate with oxidation of NADH to $NAD^+$ ($\Delta\epsilon_{340}$ = 6220 $M^{-1}$ $cm^{-1}$). Inhibitors were evaluated by varying EPSP at saturating phosphate (50 mM, $K_m$ = 1.4 mM) and, for the phosphonates (*R*)-**4** and (*S*)-**4** and trifluoromethyl analogues (*R*)-**5** and (*S*)-**5**, by varying phosphate at saturating EPSP (50 μM, $K_m$ = 2.3 μM). The inhibition constants determined for the various analogues are listed in Table I.

**Analogues of the Tetrahedral Intermediate.** The phosphonate and phosphate inhibitors are all competitive with EPSP, as is fully expected in view of the kinetic mechanism of the reverse reaction (EPSP binds first).[3b,45] Of greatest importance are the magnitude of the inhibition constants and their dependence on the configuration of the side chain. With the exception of (*S*)-**4**, the tetrahedral mimics are bound 2–3 orders of magnitude more tightly than the substrate EPSP or S3P. Indeed, the difluoromethyl analogue (*R*)-**6**, with a $K_i$ value of 4 nM, is the most tightly bound inhibitor so far reported.

Anderson et al.[3d] and Cleland[12] have taken different approaches to predicting the dissociation constant, $K_d$, of the actual intermediate **1**. Anderson et al. determined rate constants for association and dissociation of the phosphonate mimics (*R*)-**4** and (*S*)-**4** with EPSP synthase and found it to be the former that distinguishes them ($k_{on(R)-4} = 1.5 \times 10^5$ $M^{-1}$ $s^{-1}$, $k_{on(S)-4} = 2.7 \times 10^3$ $M^{-1}$ $s^{-1}$), rather than the latter ($k_{off(R)-4} = 2.2 \times 10^{-3}$ $s^{-1}$, $k_{off(S)-4} = 3.1 \times 10^{-3}$ $s^{-1}$). With the assumption that the actual intermediate **1** dissociates from the enzyme at the same rate as the phosphonates and from $k_{on(1)} = 5 \times 10^7$ $M^{-1}$ $s^{-1}$, Anderson et al. estimated $K_{d(1)}$ = 0.05 nM.[3d] In an alternative approach, Cleland estimated the equilibrium constant, $K_{eq}$, for S3P + PEP ↔ **1** to be 25–250, which leads to a prediction for $K_{d(1)}$ of 0.25–2.5 nM. With these disparate methods yielding estimates of $K_{d(1)}$ within 2 orders of magnitude, the fact that the difluoromethyl analogue (*R*)-**6** ($K_i$ = 4 nM) is at the upper edge of the range suggests that the two fluorine atoms which represent the difference between **6** and **1** do not perturb the interaction between enzyme and ligand to a significant degree. Synthesis of the monofluoromethyl analogues **7** continues to be an important goal, since their behavior in comparison to the trifluoromethyl and difluoromethyl derivatives would help to resolve the assumptions made in estimating $K_{d(1)}$.

The influence of the side-chain configuration on the relative binding affinities of the diastereomeric inhibitors is also of interest, since the configuration of the actual intermediate **1** is still unknown. When we reported our initial results with the methyl phosphonates **4**,[7] we took the 70-fold advantage of the *R*-diastereomer to imply a similar three-dimensional disposition of functional groups in the intermediate **1**. Our implicit assumption was that the carboxylate, methyl, and shikimate 3-phosphate moieties occupy their usual binding sites and that the shorter phosphonate binds in the site normally occupied by the phosphate group. The alternative assignment of the configuration of **1** has been offered by Leo, Sikorski, and Sammons, as a result of their identification of bicyclic ketal **27** as a decomposition product of



**27**

the intermediate.[8] Ketal **27** is formed as a single diastereomer whose *R*-configuration has been assigned unambiguously by 2D NMR; since the most likely process for stereospecific conversion of **1** to **27** would involve inversion at the ketal center, this logical sequence suggests that **1** has the *S*-configuration.

Because they incorporate a ketal phosphate moiety instead of a truncated phosphonate, the fluorine-substituted analogues **5** and **6** are in some respects closer mimics of intermediate **1** than is the phosphonate **4**. However, the discrimination by EPSP synthase between the diastereomers of **5** and **6** is significantly reduced (Table I). Indeed, there is no discrimination for the isomers of the trifluoromethyl analogue **5**, and the 20-fold preference for (*R*)-**6** over (*S*)-**6** is opposite in stereochemical sense to that shown for the isomers of **4**. In comparing the stereoisomers of phosphonate **4** and the actual intermediate **1** with the fluoro derivatives **5** and **6**, it is important to note that the fluorine substituents alter the Cahn–Ingold–Prelog priorities about the side-chain stereocenters. Thus, the (*R*)-trifluoromethyl- and -difluoromethyl ketal phosphates (*R*)-**5** and (*R*)-**6** correspond in three-dimensional arrangement of functional groups to methyl phosphonate (*S*)-**4** and to the *S*-configuration of the intermediate (*S*)-**1**. From the preference for (*R*)-**6** over (*S*)-**6**, one could now make the argument that intermediate **1** has the *S*-configuration, based on the logical premise that the carboxylate, phosphate, and shikimate 3-phosphate moieties occupy their usual binding sites and that the difluoromethyl group binds in the site normally occupied by the methyl. It is clear that this issue will be resolved more satisfactorily when the fluoromethyl analogues **7** or the intermediate itself becomes available, or when the structure of an appropriate enzyme/inhibitor complex is solved.[47]

**Analogues of EPSP.** The EPSP analogues **8**–**11** display inhibition constants in the micromolar range, comparable to S3P


(44) Mousdale, D. M.; Coggins, J. R. *Planta* **1984**, *160*, 78–83. Boocock, M. R.; Coggins, J. R. *FEBS Lett.* **1983**, *154*, 127–133.

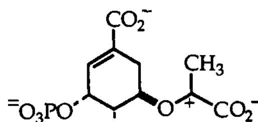

(45) In the evaluation with phosphate as the varied substrate, competitive behavior was observed for (*S*)-**4**, the most weakly bound of the tetrahedral mimics. Such behavior is not expected in view of the fact that this inhibitor and phosphate do not compete for the same form of the enzyme. However, as noted previously,[7] if equilibria among the various enzyme complexes is rapid,[46] the ability of phosphate to pull the equilibrium E·EPSP + $P_i$ → E·EPSP·$P_i$ can produce this result. In view of the saturating levels of EPSP employed in the assays with phosphate as varied substrate ([EPSP] = 50 μM, $K_m$ = 2.1 μM), the distinction between competitive and noncompetitive kinetic behavior is small in any event and is unlikely to have a significant bearing on the mode of binding or mechanism of inhibition by these compounds.


(46) Segel, I. H. *Enzyme Kinetics*; John Wiley and Sons: New York, 1975; pp 320–329.

(47) Stallings, W. C.; Abdel-Meguid, S. S.; Lim, L. W.; Shieh, H. S.; Dayringer, H. E.; Leimgruber, N. K.; Stegeman, R. A.; Anderson, K. S.; Sikorski, J. A.; Padgette, S. R.; Kishore, G. M. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 5046–5050.

and EPSP itself (see Table I). The phosphate and phosphonate moieties in the side chain of the tetrahedral mimics thus contribute on average 3 orders of magnitude in binding affinity to those compounds. Interestingly, the methyl group does not appear to be important for recognition: there is little discrimination between the *R*- and *S*-diastereomers of the dihydro-EPSP analogue **8**, and neither is bound as tightly as nor-EPSP (**9**). This latter observation is reminiscent of the loss of binding on N-methylation of glyphosate[9] (**3**) and calls into question the existence of a specific methylene or methyl binding site, or suggests that the phosphate group plays a role in its definition. The loss of only 1 order of magnitude of binding affinity of the methyl analogue (*Z*)-**11** (based on comparison of $K_i$ with $K_m$ of EPSP) is also consistent with this view. The stereochemical tolerance generally exhibited by the enzyme for side-chain diastereomers suggests that the binding subsites, to the extent that they are defined, are not absolute in their specificity but permit interchange of the phosphate and carboxylate groups, for example.

**Fluorinated Analogues as Substrates for EPSP Synthase.** The difluoromethyl analogues **6** are at least formally able to undergo elimination of phosphate with formation of 9,9-difluoro-EPSP (**12**) and could be potential substrates for EPSP synthase. However, if the enzyme-catalyzed elimination of **1** is analogous to its formation, i.e., if it proceeds via cationic intermediate **28** from




**28**

ionization of phosphate prior to loss of the proton, the fluorine substituents of **6** would strongly deactivate this compound as a substrate.[48] For example, (*Z*)-3-fluoro-PEP is unable to substitute for PEP in the forward reaction catalyzed by EPSP synthase,[49] presumably because protonation to give the fluorine analogue of cation **2** is disfavored. The effect of EPSP synthase on the stability of the difluoromethyl analogues **6** was studied at pH 7.5 using a 30-fold higher concentration of enzyme than is typically introduced into an assay solution. The release of phosphate from decomposition of **6** was slow, amounting to ca. 5% of the starting inhibitor over a 72-h period, and it was not accelerated by EPSP synthase.

The fluorinated analogue of EPSP, (*Z*)-**10**, proved to be a modest inhibitor of the enzyme, with an affinity reduced 6-fold in comparison with EPSP itself. More importantly, the fluorine substitution makes (*Z*)-**10** inert as a substrate: by HPLC assay, there is no discernible formation of S3P over a 48-h period in the presence of enzyme and inorganic phosphate, under conditions where cleavage at 0.2% of the rate of EPSP could have been detected. The inert nature of this analogue is also consistent with the postulate of cationic intermediates in the enzymatic mechanism. Less obvious is why the methyl-substituted derivative (*Z*)-**11** is also inert. There is no compelling electronic reason why this compound cannot be converted to the corresponding tetrahedral intermediate, and the additional methyl group appears to be accommodated sterically, reducing the affinity of the EPSP analogue by only a factor of 20. The unreactivity of this analogue may simply stem from misalignment in the active site.

## Conclusions

The interplay between organic synthesis and the investigation of enzyme mechanism has been important at a number of points along the shikimate pathway, and EPSP synthase is no exception. Mechanistic postulates have pointed the way to the design of potent inhibitors, and these in turn have provided insight into details of the reaction as well as useful structural models for elusive intermediates.

## Experimental Section

**Synthesis. General Procedures.**[50] Methyl 3,3,3-trifluoropyruvate was a generous gift of Dr. Paul Resnick of E. I. Du Pont de Nemours, Inc. Bis(*p*-nitrophenethoxy)*N*,*N*-diisopropylphosphoramidite was prepared by the literature method.[25] The synthesis of (*R*)-**4** and (*S*)-**4** has been described in the supplementary material to ref 7. Spectral details and analytical data indicated at the end of each procedure are provided in the supplementary material to this paper.

**Representative Procedures: Conversion of Ketal 12a to the Trifluoromethyl Analogues (*R*)-5 and (*S*)-5. Methyl [3a(*R*)-(3a*α*,7*β*,7a*α*)]-7-[1-[[Bis[2-(4-nitrophenyl)ethoxy]phosphinyl]oxy]-1-(trifluoromethyl)-2-methoxy-2-oxoethoxy]-2,2-dimethyl-3a,6,7,7a-tetrahydro-1,3-benzodioxole-5-carboxylate (14c).** A mixture of 2.65 g (11.61 mmol) of acetonide **13a**, 2.47 g (15.83 mmol) of methyl trifluoropyruvate, and 300 mg of crushed 4-Å molecular sieves in 115 mL of THF was stirred at room temperature for 2 h. The mixture was cooled to −78 °C and 9.4 mL (116 mmol) of pyridine followed by 1.02 mL (11.7 mmol) of $PCl_3$ was added. After 70 min, 3.90 g (23.3 mmol) of *p*-nitrophenethyl alcohol was added via cannula as a precooled (0 °C) solution in 10 mL of THF; after 1.5 h, the mixture was warmed to 0 °C and stirred for an additional 30 min. To this mixture was added 3.4 g (16 mmol) of mCPBA (80–85%). After 1 h at 0 °C, the mixture was diluted with ether and filtered through a pad of Celite. The filtrate was washed once with 10% $Na_2S_2O_5$, twice with saturated $NaHCO_3$, and once with brine. The organic phase was separated and dried over $MgSO_4$, the solvent was removed under reduced pressure, and the yellow residue was purified by flash chromatography (8:1 $CHCl_3$/THF) to provide 5.40 g of ketal phosphate **14c** as a pale yellow oil (61% yield, ca. 60:40 mixture of diastereomers): $^1$H NMR; $^{13}$C NMR; $^{19}$F NMR; HRMS (FAB); Anal.

**Methyl [3(*R*)-4a(*R*)-(4a*β*,8*α*,8a*α*)]-3-[[Bis[2-(4-nitrophenyl)ethoxy]phosphinyl]oxy]-2,3,4a,5,8,8a-hexahydro-8-hydroxy-2-oxo-3-(trifluoromethyl)-1,4-benzodioxin-6-carboxylate ((*R*)-15c) and the 3(*S*) Diastereomer ((*S*)-15c).** A mixture of 549 mg (0.720 mmol) of **14c** and 281 mg (1.43 mequiv) of Dowex 50W-X8 resin ($H^+$ form) in 35 mL of methanol was stirred at 60–65 °C for 63 h. The mixture was filtered through a pad of Celite, and the solvent was removed under reduced pressure to afford 521 mg of the diol (about 20% of the diol had been converted to lactone **15c**). The yellow residue was treated with 14 mg (0.074 mmol) of *p*-toluenesulfonic acid in 20 mL of gently refluxing benzene (residual $H_2O$ and about 5 mL of benzene were removed with a Dean–Stark trap). After 5.5 h, the solution was cooled to room temperature, washed twice with saturated $NaHCO_3$ and once with brine and dried over $Na_2SO_4$. The solvent was removed in vacuo to afford 430 mg of a yellow oil. The two diastereomers were separated by reversed-phase HPLC on a Whatman Partisil $C_{18}$ preparative reversed-phase column (22.1 mm × 50 cm), eluting with a concave gradient of $CH_3CN$/water (50:50 → 70:30) with a flow rate of 10 mL/min and monitoring at 368 nm. The separation provided 176 mg of (*R*)-**15c** ($t_R$ 93.7 min) and 93 mg of (*S*)-**15c** ($t_R$ 96.8 min) (54% overall yield) as amorphous solids. The diastereomeric purity was determined by analytical HPLC on a Rainin Microsorb $C_{18}$ reversed-phase column (5 μm, 100-Å pore size, 4.6 mm × 25 cm), eluting with $CH_3CN/H_2O$ (55:45) at a flow rate of 1 mL/min, monitoring at 270 nm. (*S*)-**15c** was contaminated with <0.3%

(48) Poulter, C. D.; Argyle, C. J.; Mash, E. A. *J. Biol. Chem.* **1978**, *253*, 7227–7233.

(49) Ife, R. J.; Ball, L. F.; Lowe, P.; Haslam, E. *J. Chem. Soc., Perkin Trans. 1* **1976**, 1776–1783.

(50) Melting points are uncorrected. Unless otherwise noted, starting materials were commercially available and used without further purification. Tetrahydrofuran (THF), diethyl ether (ether), and benzene were distilled from sodium/benzophenone. Dichloromethane, pyridine, diisopropylamine and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) were distilled from calcium hydride. Chromatography refers to the method of Still, Kahn, and Mitra[51] using silica gel 60 (E. Merck, Darmstadt) and the indicated solvent. All reactions were carried out under a nitrogen atmosphere unless specified otherwise. TBK buffer refers to aqueous triethylammonium bicarbonate prepared by passing a stream of $CO_2$ through an aqueous solution of 1 M triethylamine until the desired pH (8.5) is obtained. IR spectra were obtained in $CHCl_3$, unless otherwise indicated. $^1$H, $^{13}$C, and $^{31}$P NMR spectra were obtained in $CDCl_3$ (unless otherwise indicated) at 250 or 400, 62.5, and 81 MHz, respectively. $^1$H and $^{13}$C NMR spectra are reported as ppm downfield from internal tetramethylsilane (multiplicity, number of hydrogens, coupling constants in hertz). NMR spectra of salts were obtained in $D_2O$; $^1$H and $^{13}$C NMR spectra in $D_2O$ are referenced to internal $CH_3OH$ at 3.39 and 49.9 ppm, respectively, or to residual HOD at 4.63 ppm. $^{31}$P chemical shifts are reported in ppm downfield from external trimethyl phosphate at 3.086 ppm. $^{19}$F NMR spectra were obtained at 235 or 376 MHz and are reported in ppm relative to external trifluoroacetic acid at −78.5 ppm. Mass spectra were performed by the UCB Mass Spectrometry Laboratory and are tabulated as *m/z* (intensity expressed as percent of total ion current). Elemental analyses were performed by the Microanalytical Laboratory operated by the College of Chemistry, UC–Berkeley.

(51) Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978**, *43*, 2923–2925.

of the *R*-diastereomer, while (*R*)-**15c** contained <1.2% of the *S*-diastereomer. The stereochemistry of the side chain of each isomer was determined by 2D NMR after conversion to the corresponding phosphates **16c** (see below). (*R*)-**15c**: IR; $^1$H NMR; $^{13}$C NMR; $^{31}$P NMR; $^{19}$F NMR; Anal. (*S*)-**15c**: IR; $^1$H NMR; $^{13}$C NMR; $^{31}$P NMR; $^{19}$F NMR; Anal.

**Methyl [3(*R*)-4a(*R*)-(4a$\beta$,8$\alpha$,8a$\alpha$)]-3-[[Bis[2-(4-nitrophenyl)ethoxy]phosphinyl]-8-[[bis[2-(4-nitrophenyl)ethoxy]phosphinyl]oxy]-2,3,4a,5,8,8a-hexahydro-2-oxo-3-(trifluoromethyl)-1,4-benzodioxin-6-carboxylate ((*R*)-16c).** A solution of 367 mg (0.532 mmol) of allylic alcohol (*R*)-**15c**, 320 mg (0.690 mmol) of bis(*p*-nitrophenethyl) *N,N*-diisopropylphosphoramidite, and 112 mg (1.60 mmol) of tetrazole in 4 mL of THF was stirred under a dry $N_2$ atmosphere at room temperature for 1.5 h. The mixture was cooled to 0 °C, and 180 mg (0.83 mmol) of mCPBA (80–85%) was added as a solution in 4 mL of $CH_2Cl_2$. After 10 min, the solution was warmed to room temperature and allowed to stir for 30 min. The mixture was diluted with ethyl acetate and washed with 10% aqueous $Na_2S_2O_5$, twice with saturated $NaHCO_3$, with 0.5 M phosphate buffer (pH 7.0), and with brine. The organic phase was separated and dried over $MgSO_4$. Removal of the solvent under reduced pressure afforded 642 mg of crude (*R*)-**16c**, which was taken on without further purification: $^1$H NMR; $^{31}$P NMR; $^{19}$F NMR; HRMS (FAB).

**Methyl [3(*S*)-4a(*R*)-(4a$\beta$,8$\alpha$,8a$\alpha$)]-3-[[Bis[2-(4-nitrophenyl)ethoxy]phosphinyl]oxy]-8-[[bis[2-(4-nitrophenyl)ethoxy]phosphinyl]oxy]-2,3,4a,5,8,8a-hexahydro-2-oxo-3-(trifluoromethyl)-1,4-benzodioxin-6-carboxylate ((*S*)-16c).** In a similar manner, 144 mg (0.208 mmol) of allylic alcohol (*S*)-**15c** was converted to 260 mg of crude (*S*)-**16c**, which was carried on without further purification: $^1$H NMR; $^{31}$P NMR; $^{19}$F NMR; HRMS (FAB).

**$^1$H–$^{19}$F HOESY 2D NMR Spectra of Diastereomers (*R*)-16c and (*S*)-16c.** HOESY (heteronuclear Overhauser effect spectroscopy) spectra of (*R*)-**16c** and (*S*)-**16c** were acquired on a Bruker AM 400 spectrometer with the pulse sequence of Yu and Levy[52] without $^1$H–$^{19}$F decoupling. The spectra were acquired in magnitude mode with a mixing time of 1 s. The $^1$H spectra width was 3600 Hz, and the $^{19}$F spectral width was 3760 Hz. A total of 256 1K spectra were recorded and zero filled in $\omega_1$ to 512*w* to give a final 512 × 512 matrix. Sixty-four scans were recorded per $t_1$ point, and a $\pi/2$ sine bell window function was employed in both dimensions.

**3(*R*)-[3$\alpha$,4$\alpha$,5$\beta$(*R*)]-5-[1-Carboxy-1-(phosphonooxy)-2,2,2-trifluoroethoxy]-4-hydroxy-3-(phosphonooxy)-1-cyclohexene-1-carboxylate, Hexasodium Salt ((*R*)-5).** To a solution of 642 mg crude (*R*)-**16c** in 5 mL of dry $CH_3CN$ was added 745 $\mu$L (3.01 mmol) of bis(trimethylsilyl)acetamide (BSA), followed by 900 $\mu$L (6.01 mmol) of DBU. After 4.5 h at room temperature, the brown reaction mixture was diluted with $CHCl_3$ and the excess BSA was quenched with a few drops of water. The mixture was partitioned between 10 mL of 2 M NaOH and 10 mL of $CHCl_3$, and the aqueous phase was separated, washed twice with $CHCl_3$, and allowed to stand at room temperature for 4 h. The solution was acidified to pH 7.3 with Dowex 50W-X8 resin ($H^+$ form) and lyophilized to afford 792 mg of a yellow solid. The residue was purified by anion-exchange chromatography on DEAE Sephadex A-25 ($HCO_3^-$ form), eluting with a linear gradient of triethylammonium bicarbonate buffer (0–0.8 M, pH 8.1). Fractions absorbing at 240 nm were combined and lyophilized to afford 296 mg of the product as the triethylammonium salt. Cation exchange on Dowex 50W-X8 resin ($Na^+$ form) gave, after lyophilization, 223 mg of (*R*)-**5** (69% yield from alcohol (*R*)-**15c**): $^1$H NMR $\delta$ 6.58 (dd, 1, $J$ = 2.1, $J_{HCCOP}$ = 2.1), [H-3 is obscured by the HOD peak], 4.61 (ddd, 1, $J$ = 5.6, 6.8, 8.3), 3.98 (dd, 1, $J$ = 4.2, 8.3), 2.83 (dd, 1, $J$ = 5.6, 18.3), 2.39 (dd, 1, $J$ = 6.8, 18.3); $^{13}$C NMR $\delta$ 172.23, 167.89 (d, $J_{CCOP}$ = 4.1), 133.21 (d, $J_{CCOP}$ = 3.4), 132.15, 121.19 (qd, $J_{CF}$ = 287.9, $J_{CCOP}$ = 6.3), 96.99 (qd, $J_{CCF}$ = 31.3, $J_{COP}$ = 7.8), 72.05, 70.53 (d, $J_{COP}$ = 5.1), 67.98 (d, $J_{CCOP}$ = 4.3), 28.24; $^{31}$P NMR $\delta$ 4.92, −0.77; $^{19}$F NMR $\delta$ −79.24. Anal. Calcd for $C_{10}H_7F_3Na_6O_{14}P_2 \cdot 5H_2O$: C, 17.20; H, 2.46; P, 8.87; Na, 19.76. Found: C, 18.42; H, 3.04; P, 8.85, Na, 12.4.

**3(*S*)-[3$\alpha$,4$\alpha$,5$\beta$(*R*)]-5-[1-Carboxy-1-(phosphonooxy)-2,2,2-trifluoroethoxy]-4-hydroxy-3-(phosphonooxy)-1-cyclohexene-1-carboxylate, Hexasodium Salt ((*S*)-5).** In a procedure similar to that described above for the *R*-diastereomer, 260 mg of (*S*)-**16c** was converted to 87 mg of (*S*)-**5** (69% yield from alcohol (*S*)-**15c**): $^1$H NMR $\delta$ 6.63 (dd, 1, $J$ = 1.7, $J_{HCCOP}$ = 1.6), 4.95 (m, 1, partially obscured by HOD), 4.69 (ddd, 1, $J$ = 4.7, 5.0, 7.5), 4.14 (dd, 1, $J$ = 3.8, 7.5), 2.87 (dd, 1, $J$ = 4.7, 18.5), 2.55 (dd, 1, $J$ = 5.0, 18.5); $^{13}$C NMR $\delta$ 173.73, 168.22 (d, $J_{CCOP}$ = 3.8), 134.42, 131.10 (d, $J_{POCC}$ = 2.3), 122.40 (qd, $J_{CF}$ = 288.4, $J_{CCOP}$ = 8.4), 98.30 (qd, $J_{CCF}$ = 31.3, $J_{COP}$ = 7.6), 71.97, 70.36 (d, $J_{COP}$ = 5.4), 68.79 (d, $J_{CCOP}$ = 5.3), 30.20; $^{31}$P NMR $\delta$ 1.11, −4.52; $^{19}$F NMR $\delta$ −81.22. Anal. Calcd for $C_{10}H_7F_3Na_6O_{14}P_2 \cdot 5H_2O$: C, 17.20; H, 2.46; P, 8.87; Na, 19.76. Found: C, 19.03; H, 2.85; P, 9.31; Na, 14.30.

**Synthesis of the Difluoromethyl Analogues (*R*)-6 and (*S*)-6. Diethyl Fluorooxalacetate, Sodium Salt.** A modification of the procedure of Bergman and Shahak[53] was used to prepare this enolate. Thus, 34.8 g (0.735 mol) of a 50% suspension of NaH in mineral oil was washed twice with anhydrous hexane and once with benzene to remove the mineral oil. The solid was suspended in 400 mL of benzene and cooled to 0 °C, and 50 mL (0.852 mol) of absolute ethanol (distilled from Mg) was added dropwise over a 2-h period with vigorous stirring. After $H_2$ evolution had ceased, 108 mL (0.795 mol) of diethyl oxalate (distilled from $CaH_2$) was added, followed by the dropwise addition of 70 mL (0.72 mol) of ethyl fluoroacetate over 30 min. After the addition was complete, the mixture was stirred at room temperature for 2 days. The thick slurry was diluted with 350 mL of ether, cooled to 0 °C, and filtered. The solid was rinsed with a large amount of ether until the washings were colorless. The white solid was dried under high vacuum to afford 114 g (69% yield) of the sodium enolate, which was used without further purification: $^1$H NMR (methanol-$d_4$, 400 MHz, relative to residual $CHD_2OD$ at 3.30 ppm) $\delta$ 4.19 (q, 2, $J$ = 7.2), 4.09 (q, 2, $J$ = 7.1), 1.31 (q, 3, $J$ = 7.2), 1.22 (q, 3, $J$ = 7.1); $^{19}$F NMR $\delta$ −188.32.

**Diethyl Difluorooxalacetate.** In a modification of the procedure of Kun et al.,[28] a slurry of 6.40 g (0.028 mol) of the sodium salt of diethyl fluorooxalacetate in 300 mL of THF was stirred and cooled to 0 °C. A stream of dilute $FClO_3$ gas in a $N_2$ carrier was passed through this solution over 6 h. The $FClO_3$ gas was generated from $FSO_3H$ and $KClO_4$ by the method of Barth-Wehrenalp.[54] Thus, a three-neck 300-mL round-bottom flask equipped with a $N_2$ inlet line and a reflux condenser was charged with 10.4 g (0.075 mol) of $KClO_4$ and 58 mL (1.01 mol) of $FSO_3H$ (Aldrich, triple distilled). This mixture was stirred gently and heated slowly to about 140 °C, with a slow stream of $N_2$ flowing through the apparatus. When the temperature reached about 60 °C, $FClO_3$ gas began to evolve. The gas, carried by the flow of $N_2$, was passed out the top of the reflux condenser, over a stirring solution of 10% NaOH/5% $Na_2S_2O_5$, then through such a solution, and finally through solid KOH before bubbling through a glass frit into the THF/sodium enolate slurry (only glass and Teflon tubing were used). The $KClO_4/FSO_3H$ mixture, beginning as a clear, colorless solution, gradually yellowed then darkened to orange, red, and then finally a dark brown color before gradually lightening again to a colorless solution; it was at this point that heating was discontinued. The reaction flask containing diethyl difluorooxalacetate and THF was allowed to stir overnight at room temperature, open to the atmosphere. Most of the THF was removed in vacuo, and the residue was diluted with 300 mL of ether and washed with $H_2O$ and brine. The combined aqueous extracts were extracted with ether (2 × 50 mL), and the combined ethereal layers were dried over $MgSO_4$. Solvent was removed in vacuo, and the residue was distilled under reduced pressure [bp 64–67 °C/0.5 mmHg (Lit.[28a] bp 64 °C/1 mmHg; lit.[28b] 65–66 °C/0.5 mmHg)] to afford 4.7 g (74%) of diethyl difluorooxalacetate: IR (film) 3480, 2990, 2950, 2920, 1765, 1745, 1475, 1450, 1380, 1330, 1310, 1270, 1205, 1195, 1180, 1160, 1140, 1100, 1020, 980, 860, 755 cm$^{-1}$; $^1$H NMR $\delta$ 4.42 (q, 2, $J$ = 7.1), 4.41 (q, 2, $J$ = 7.1), 1.40 (q, 3, $J$ = 7.1), 1.36 (q, 3, $J$ = 7.1); $^{13}$C NMR $\delta$ 177.28 (t, $J_{CCF}$ = 28.9), 159.82 (t, $J_{CCF}$ = 30.6), 157.32, 106.80 (t, $J_{CF}$ = 261.4), 63.89, 63.82, 13.41, 13.39; $^{19}$F NMR $\delta$ −116.71.

**Difluorooxalacetic Acid Monohydrate.** Difluorooxalacetic acid was prepared by the method of Kun et al.[28] A mixture of 12.78 g of diethyl difluorooxalacetate and 85 mL of 3 N HCl was boiled for 1.25 h. The mixture was concentrated in vacuo to a thick syrup. About 15 mL of trifluoroacetic acid was added to the residue, and the flask was scratched with a glass rod to induce crystallization. A white solid was isolated and dried under a high vacuum to afford 7.09 g of the diacid. The mother liquor provided two more batches of crystals for a total yield of 9.10 g (86%): mp 119–122 °C (Lit.[28a] mp 119–120 °C); $^{19}$F NMR ($D_2O$) $\delta$ −120.21; LRMS (negative FAB) *m/z* 185 (M − H)$^-$, 167 (M − H − $H_2O$)$^-$.

**Methyl Difluoropyruvate.** A solution of 7.68 g (41.2 mmol) of difluorooxalacetic acid monohydrate in 20 mL of DMSO was heated to 100–105 °C for 2.25 h to provide difluoropyruvate as a DMSO solution: $^1$H NMR (400 MHz, DMSO/DMSO-$d_6$, relative to DMSO at 2.49 ppm) $\delta$ 5.83 (t, $J_{HCF}$ = 55.0); $^{19}$F NMR ($CD_3CN$, relative to internal trifluoroacetic acid at −78.0 ppm) $\delta$ −137.05 (t, $J_{FCH}$ = 54.8). To this DMSO solution were added 175 mL of MeOH and 460 mg (2.29 mmol) of *p*-toluenesulfonic acid. The mixture was refluxed at 80–85 °C for 3 days (the progress of the reaction was conveniently followed by $^{19}$F NMR). The solution was cooled to room temperature, 500 mg of $Na_2HPO_4$ was added, and the MeOH was removed by distillation. The residue was dissolved in 350 mL of ether, and the resulting solution was

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(54) Barth-Wehrenalp, G. *J. Inorg. Chem.* **1956**, *2*, 256.

washed with 50 mL of saturated $NaHCO_3$ and twice with 50 mL of $H_2O$. The combined aqueous layers were extracted twice with 75 mL of ether; all organic layers were combined and washed once with 50 mL of $H_2O$ and dried over $Na_2SO_4$. The ether was removed, and the residue was distilled from $P_2O_5$ (bulb-to-bulb, 100 °C at 15 mmHg) to provide 2.28 g of a clear colorless oil (35% yield from the diacid). Despite distillation from $P_2O_5$, this material proved to be a mixture of the hydrate (68%), the hemiketal adduct with MeOH (27%), and only a trace of the free ketone (5%). Repeated bulb-to-bulb distillations from large amounts of $P_2O_5$ failed to dehydrate this material completely (after a second distillation only a trace of the hemiketal remained but a mixture of hydrate (27%) and ketone (63%) persisted), so it was carried on as this mixture: $^1$H NMR free ketone, $\delta$ 6.40 (t, 1, $J_{HCF}$ = 52.7), 3.99 (s, 3); hydrate, $\delta$ 5.88 (t, 1, $J_{HCF}$ = 54.7); methyl hemiketal, $\delta$ 5.81 (dd, 1, $J_{HCF}$ = 54.8, 54.8); $^{19}$F NMR ($CDCl_3$) free ketone, $\delta$ −132.90 (d, $J_{FCH}$ = 52.7); hydrate, $\delta$ −137.54 (d, $J_{FCH}$ = 54.7); methyl hemiketal, $\delta$ −132.33 (dd, $J_{FCH}$ = 54.8, $J_{FCF}$ = 298.9), −139.82 (dd, $J_{FCH}$ = 54.8, $J_{FCF}$ = 298.9); HRMS (FAB) $MH^+$ calcd for $C_4H_4F_2O_3$ $m/z$ 138.0129, found $m/z$ 138.0134.

**Methyl [3a(*R*)-(3a$\alpha$,7$\beta$,7a$\alpha$)]-7-[[Bis[2-(4-nitrophenyl)ethoxy]phosphinyl]oxy]-1-(difluoromethyl)-2-methoxy-2-oxoethoxy]-2-methoxy-2,3a,6,7,7a-pentahydro-1,3-benzodioxole-5-carboxylate (14d).** A mixture of 1.70 g (7.38 mmol) of orthoformate **13d**,[14] 0.93 g (6.4 mmol) of methyl difluoropyruvate (63:27 mixture of the free ketone and hydrate), 2.0 g of crushed 4-Å molecular sieves, and 2.5 mL of dry THF was stirred at room temperature. After 15 h, an additional 15 mL of THF was added and the slurry was cooled to −78 °C. To the cold slurry was added 6.2 mL (77.0 mmol) of dry pyridine followed by 0.675 mL (7.74 mmol) of $PCl_3$. The mixture was stirred for 30 min and then 2.97 g (17.77 mmol) of 4-nitrophenethyl alcohol was added via cannula as a precooled (0 °C) solution in 8 mL of THF. After being stirred for 15 min, the thick slurry was warmed to 0 °C and stirred for 30 min, and 2.0 g (9.5 mmol) of mCPBA (80–85%) was added as a solution in 25 mL of $CH_2Cl_2$. The mixture was warmed to room temperature over 30 min, diluted with ether, and filtered through a pad of Celite. The filtrate was further diluted with 150 mL of ether and washed once with 10% aqueous $Na_2SO_3$, twice with saturated $NaHCO_3$, once with $H_2O$, and finally with brine. The ethereal layer was dried over $MgSO_4$, concentrated in vacuo, and purified by flash chromatography (8:1 ether/ethyl acetate and then 3:1 ether/ethyl acetate) to afford 3.08 g (64% yield) of **14d** as mixture of four diastereomers (7:10:33:50): IR; $^1$H NMR; $^{19}$F NMR; $^{31}$P NMR; HRMS (FAB).

**Methyl [3(*R*)-4a(*R*)-(4a$\beta$,8$\alpha$,8a$\alpha$)]-3-[[Bis[2-(4-nitrophenyl)ethoxy]phosphinyl]oxy]-2,3,4a,5,8,8a-hexahydro-8-hydroxy-2-oxo-3-(difluoromethyl)-1,4-benzodioxin-6-carboxylate ((*R*)-15d) and the 3(*S*) Diastereomer ((*S*)-15d).** A mixture of 2.66 g (3.558 mmol) of orthoformate **14d** and 1.40 g (7.14 meq) of Dowex 50W-X8 resin ($H^+$ form) in 170 mL of 1% $H_2O$ in MeOH was stirred vigorously for 28 h, then filtered through a pad of Celite, and concentrated to provide the diol. This material was treated with 45 mg (0.24 mmol) of *p*-toluenesulfonic acid in 40 mL of gently refluxing benzene (residual $H_2O$ and about 10 mL of benzene were removed via a Dean–Stark trap) for 6.5 h. The mixture was cooled to room temperature, diluted with 50:50 ethyl acetate/ether, washed with saturated $NaHCO_3$ and brine, and dried over $Na_2SO_4$. The solvent was removed under reduced pressure to afford 2.16 g of a viscous yellow oil. The diastereomers were separated by medium-pressure liquid chromatography (Lobar prepacked column, size C 440 mm × 37 mm LiChroprep silica gel 60 (40–63 $\mu$m), E. Merck, Darmstadt; eluted with 7:1 $CHCl_3$/THF) to afford 635 mg of (*R*)-**15d** (high $R_f$ diastereomer, TLC 7:1 $CHCl_3$/THF), 711 mg of (*S*)-**15d** (low $R_f$ diastereomer), and 51 mg of a mixture of the two which was not separated (total yield 1.40 g, 58% yield from orthoester **14d**). (*R*)-**15d**: $^1$H NMR; $^{13}$C NMR; $^{19}$F NMR; $^{31}$P NMR; Anal. (*S*)-**15d**: $^1$H NMR; $^{13}$C NMR; $^{19}$F NMR; $^{31}$P NMR; Anal.

**$^1$H–$^{19}$F HOESY 2D NMR Spectra of Diastereomers (*R*)-15d and (*S*)-15d.** HOESY spectra of (*R*)-**15d** and (*S*)-**15d** were acquired on a Bruker AM 400 spectrometer with the pulse sequence of Yu and Levy[52] without $^1$H–$^{19}$F decoupling. The spectra were acquired in magnitude mode with a mixing time of 0.3 s. For (*R*)-**15d**, the $^1$H spectral width was 4237 Hz and the $^{19}$F spectral width was 6493 Hz. A total of 256 1K spectra were recorded and zero filled in $\omega_1$ to 512*w* to give a final 512 × 512 matrix. Sixty-four scans were recorded per $t_1$ point, and a $\pi/2$ sine bell window function was employed in both dimensions. For (*S*)-**15d**, the $^1$H spectral width was 2564 Hz and the $^{19}$F spectral width was 4201 Hz. A total of 416 1K spectra were recorded and zero filled in $\omega_1$ to 1K to give a final 1K × 1K matrix. Thirty-two scans were recorded per $t_1$ point and a $\pi/2$ sine bell window function was employed in both dimensions.

**Methyl [3(*R*)-4a(*R*)-(4a$\beta$,8$\alpha$,8a$\alpha$)]-3-[[Bis[2-(4-nitrophenyl)ethoxy]phosphinyl]oxy]-8-[[bis[2-(4-nitrophenyl)ethoxy]phosphinyl]oxy]-2,3,4a,5,8,8a-hexahydro-2-oxo-3-(difluoromethyl)-1,4-benzodioxin-6-carboxylate ((*R*)-16d).** As described above for the phosphorylation of (*R*)-**15c**, 443 mg (0.659 mmol) of difluoromethyl lactone (*R*)-**15d** was converted to 832 mg of (*R*)-**16d** as a yellow oil. This material was taken on without further purification: $^1$H NMR; $^{19}$F NMR; $^{31}$P NMR; LRMS.

**Methyl [3(*S*)-4a(*R*)-(4a$\beta$,8$\alpha$,8a$\alpha$)]-3-[[Bis[2-(4-nitrophenyl)ethoxy]phosphinyl]oxy]-8-[[bis[2-(4-nitrophenyl)ethoxy]phosphinyl]oxy]-2,3,4a,5,8,8a-hexahydro-2-oxo-3-(difluoromethyl)-1,4-benzodioxin-6-carboxylate ((*S*)-16d).** In a similar manner, 422 mg (0.627 mmol) of (*S*)-**15d** was converted to 750 mg of the lactone phosphate (*S*)-**16d** as a thick yellow oil, which was taken on without further purification: $^1$H NMR; $^{19}$F NMR; $^{31}$P NMR; HRMS.

**3(*R*)-[3$\alpha$,4$\alpha$,5$\beta$(*R*)]-5-[1-Carboxy-1-(phosphonooxy)-2,2-difluoroethoxy]-4-hydroxy-3-(phosphonooxy)-1-cyclohexene-1-carboxylate, Hexasodium Salt ((*R*)-6).** As described above for deprotection of the trifluoromethyl analogue (*R*)-**16c**, 832 mg of crude lactone bisphosphate (*R*)-**16d** was converted to 247 mg of the sodium salt (*R*)-**6** as a light yellow solid (63% yield from alcohol (*R*)-**15d**): $^1$H NMR $\delta$ 6.29 (dd, 1, $J$ = 1.5, $J_{HCCOP}$ = 1.5), 6.19 (dd, 1, $J_{HCF}$ = 54.5, 54.6), 4.73 (m, 1), 4.23 (ddd, 1, $J$ = 5.2, 5.3, 7.6), 3.95 (dd, 1, $J$ = 3.9, 7.6), 2.55 (dd, 1, $J$ = 5.2, 18.5), 2.19 (dd, 1, $J$ = 5.3, 18.5); $^{13}$C NMR $\delta$ 175.70, 171.50 (d, $J_{CCOP}$ = 8.0), 136.12, 131.38 (d, $J_{CCOP}$ = 3.5), 113.82 (dd, $J_{CF}$ = 249.9, 249.9), 99.09 (ddd, $J_{COP}$ = 9.4, $J_{FCC}$ = 24.5, $J_{CCF}$ = 26.8), 72.75, 71.49 (d, $J_{COP}$ = 5.1), 69.77 (dd, $J_{CCOP}$ = 4.5), 30.51; $^{19}$F NMR $\delta$ −132.63 (dd, $J_{FCH}$ = 54.6, $J_{FCF}$ = 284.3), −134.72 (dd, $J_{FCH}$ = 54.5, $J_{FCF}$ = 284.3); $^{31}$P NMR ($D_2O$) $\delta$ 1.46, −4.28. Anal. Calcd for $C_{10}H_8F_2O_{14}P_2$: C, 20.36; H, 1.37; P, 10.50; Na, 23.38. Found: C, 19.34; H, 2.72; P, 9.52; Na, 14.5.

**3(*S*)-[3$\alpha$,4$\alpha$,5$\beta$(*R*)]-5-[1-Carboxy-1-(phosphonooxy)-2,2-difluoroethoxy]-4-hydroxy-3-(phosphonooxy)-1-cyclohexene-1-carboxylate, Hexasodium Salt ((*S*)-6).** In a similar manner, 750 mg of lactone bisphosphate (*S*)-**16d** was converted to 270 mg of the sodium salt as a light yellow solid (73% yield from alcohol (*S*)-**15d**): $^1$H NMR $\delta$ 6.31 (dd, 1, $J$ = 2.0, $J_{HCCOP}$ = 1.9), 6.13 (dd, 1, $J_{HCF}$ = 54.8, 54.8), H-3 is obscured by HOD signal, 4.24 (ddd, 1, $J$ = 5.3, 6.0, 8.1), 3.79 (dd, 1, $J$ = 3.9, 8.1), 2.66 (dd, 1, $J$ = 5.3, 18.4), 2.34 (dd, 1, $J$ = 6.0, 18.4); $^{13}$C NMR $\delta$ 176.47, 172.35 (d, $J_{CCOP}$ = 6.9), 136.78, 131.43 (d, $J_{CCOP}$ = 2.4), 114.06 (ddd, $J_{CCOP}$ = 4.1, $J_{CCF}$ = 248.9, 250), 99.07 (ddd, $J_{COP}$ = 8.5, $J_{CCF}$ = 23.9, 27.1), 72.55, 70.81 (d, $J_{CCOP}$ = 4.9), 70.69 (d, $J_{COP}$ = 5.1), 32.63; $^{19}$F NMR $\delta$ −132.74 (dd, $J_{FCH}$ = 54.8, $J_{FCF}$ = 283.4), −133.66 (dd, $J_{FCH}$ = 54.8, $J_{FCF}$ = 283.4); $^{31}$P NMR $\delta$ 3.38, 2.31. Anal. Calcd for $C_{10}H_8F_2O_{14}P_2$: C, 20.36; H, 1.37; P, 10.50; Na, 23.38. Found: C, 18.70; H, 2.67; P, 8.99; Na, 18.1.

**Synthesis of Lactyl Analogues (*R*)-8 and (*S*)-8. Methyl [(1*R*),1$\alpha$,5$\beta$,6$\alpha$]-5-(1-Methyl-2-methoxy-2-oxoethoxy)-8,8-dimethyl-7,9-dioxabicyclo[4.3.0]non-2-ene-3-carboxylate (14e).** To a solution of 2.41 g (10.6 mmol) of alcohol **13a** and 1.45 g (12.7 mmol) of methyl 2-diazopropanoate in 150 mL of dry benzene was added 28 mg (0.06 mmol) of $Rh_2(OAc)_4$. The yellow-green mixture evolved gas immediately and for an additional 15 min, at which time the solution turned clear with a green suspension. An addition of 0.1 equiv of the diazo compound was made to complete the conversion. After stirring for 30 min, the solvent was removed in vacuo and the residue was purified by chromatography (ether/hexane, 1:8–1:4) to give 2.84 g (86%) of **14e** as a clear oil and ca. 1:1 mixture of diastereomers: IR; $^1$H NMR; $^{13}$C NMR; LRMS; Anal.

**Methyl (3*R*)-[(4a*R*),4a$\beta$,8$\alpha$,8a$\alpha$]-2,3,4a,5,8,8a-Hexahydro-2-oxo-3-methyl-8-hydroxy-1,4-benzodioxin-6-carboxylate ((*R*)-15e) and the (3*S*)-Isomer ((*S*)-15e).** As described above for the deketalization and lactonization of **14c**, 2.83 g (9.0 mmol) of lactyl ether **14e** was converted to 1.77 g (81%) of **15e** as a 1.3:1 mixture of diastereomers after purification by chromatography (ether/hexane, 9:1). The isomers were separated by HPLC using a 5-$\mu$m Whatman Partisil column (EtOAc/hexane, 1:5, with 2% (v/v) THF) to give each diastereomer as a white solid. (*R*)-**15e**: mp 160–161 °C; IR; $^1$H NMR; $^{13}$C NMR; LRMS; Anal. (*S*)-**15e**: mp 136–137 °C (Lit.[33] mp 128–129 °C; Lit.[55] mp 133–135 °C); IR; $^1$H NMR; $^{13}$C NMR; LRMS; Anal.

**Methyl (3*R*)-[(4a*R*),4a$\beta$,8$\alpha$,8a$\alpha$]-2,3,4a,5,8,8a-Hexahydro-2-oxo-3-methyl-8-[[bis(phenylmethoxy)phosphinyl]oxy]-4-benzodioxin-6-carboxylate ((*R*)-16f).** As described above for the phosphorylation of (*R*)-**15c**, 90 mg (0.37 mmol) of lactone (*R*)-**15f** was converted to (*R*)-**16f** as a clear oil. This material was generally used without purification but could be purified by short-column chromatography (ether) to give a clear oil: $^1$H NMR; $^{13}$C NMR; $^{31}$P NMR; HRMS.

**Methyl (3*S*)-[(4a*R*),4a$\beta$,8$\alpha$,8a$\alpha$]-2,3,4a,5,8,8a-Hexahydro-2-oxo-3-methyl-8-[[bis(phenylmethoxy)phosphinyl]oxy]-4-benzodioxin-6-carboxylate ((*S*)-16f).** This isomer was prepared from the (*S*)-hydroxy lactone (*S*)-**15f** in the same manner: $^1$H NMR; $^{13}$C NMR; $^{31}$P NMR; HRMS.

(55) Isogai, A.; Washizu, M.; Murakoshi, S.; Suzuki, A. *Agric. Biol. Chem.* **1985**, *49*, 167.

**(3*R*)-[3α,4α,5β(*R*)]-5-[1(*R*)-1-carboxyethoxy]-4-hydroxy-3-(phosphonooxy)-1-cyclohexene-1-carboxylate ((*R*)-8).** A solution of 0.45 mmol of crude (*R*)-**16f** in 2 mL of acid-free $CHCl_3$ was transferred via cannula to a dry NMR tube equipped with a rubber septum. To this solution was added 289 μL (3.6 mmol) of pyridine and 118 μL (0.9 mmol) of bromotrimethylsilane via syringe, and the tube was inverted several times. The reaction progress was then monitored by $^{31}P$ NMR. After 40 min, an additional 10 μL of bromotrimethylsilane was added via syringe to complete the conversion. The reaction mixture was added to 5 mL of $H_2O$, and the aqueous phase was washed twice with 5 mL of $CHCl_3$. To the aqueous phase at 0 °C was then added 1.4 mL of 1 M NaOH, and this mixture was stirred for 2 h. The reaction mixture was then diluted to 10 mL and loaded onto a column of DEAE Sephadex A-25 ($HCO_3^-$ form), eluting with a linear gradient of triethylammonium bicarbonate (0–0.8 M, pH 7.3, 400 mL). Fractions absorbing at 240 nm were pooled, and the solvent was removed in vacuo to give the tetrakis(triethylammonium) salt of the (*R*)-lactyl ether. This material was converted to the tetrasodium salt by passage through a short column of Dowex X-8 cation-exchange resin ($Na^+$ form) to give 100 mg (65% from hydroxy lactone **14e**) of the tetrasodium salt of (*R*)-**8**: $^1H$ NMR δ 6.40 (d, 1, $J$ = 1.0), 4.75 (m, 1), 4.15 (q, 1, $J$ = 6.75), 3.95 (dd, 1, $J$ = 4.0, 7.4), 3.74 (q, 1, $J$ = 4.0), 2.68 (dd, 1, $J$ = 4.0, 18.0), 2.23 (dd, 1, $J$ = 5.5, 18.0), 1.28 (d, 3, $J$ = 6.75); $^{13}C$ NMR δ 183.1, 176.5, 136.1, 132.0 (d, $J_{CCOP}$ = 4.0), 77.7, 75.7, 70.8 (d, $J_{CCOP}$ = 2.9), 70.3 (d, $J_{COP}$ = 4.7), 31.3, 19.6; $^{31}P$ NMR δ 4.77; $\epsilon_{240}$ = 950 $M^{-1}$ $cm^{-1}$.

**3(*R*)-[3α,4α,5β(*R*)]-5-[1(*S*)-1-carboxyethoxy]-4-hydroxy-3-(phosphonooxy)-1-cyclohexene-1-carboxylate ((*S*)-8).** This compound was prepared from 166 mg (0.33 mmol) of (*S*)-**16f** in the same manner as the corresponding *R*-isomer, to give 64 mg (47% yield) of the tetrasodium salt of (*S*)-**8**: $^1H$ NMR δ 6.44 (t, 1, $J$ = 1.8), 4.75 (m, 1), 4.08 (q, 1, $J$ = 6.75), 3.92 (dd, 1, $J$ = 4.1, 7.7), 3.78 (m, 1), 2.63 (dd, 1, $J$ = 4.8, 18.1), 2.22 (dd, 1, $J$ = 5.75, 18.1), 1.28 (d, 3, $J$ = 4.75); $^{13}C$ NMR δ 182.8, 176.5, 135.8, 132.4 (d, $J_{CCOP}$ = 2.6), 76.5, 75.5, 70.9 (d, $J_{CCOP}$ = 3.4), 70.0 (d, $J_{COP}$ = 4.7), 30.0, 19.9; $^{31}P$ NMR δ 4.71; $\epsilon_{240}$ = 1055 $M^{-1}$ $cm^{-1}$.

**Synthesis of Glycolyl Analogue 9. Methyl [(1*R*),1α,5β,6α]-5-[(Methoxycarbonyl)methoxy]-8,8-dimethyl-7,9-dioxabicyclo[4.3.0]non-2-ene-3-carboxylate (14g).** To a solution of 1.0 g (4.38 mmol) of alcohol **13a** in 50 mL of dry benzene was added an excess of a solution of methyl diazoacetate in 15 mL of $CH_2Cl_2$ via cannula. To this solution was added 12 mg (0.03 mmol) of $Rh_2(OAc)_4$; the mixture turned yellow-green and gas was evolved. After stirring 1 h at room temperature, the solvent was removed in vacuo, and the residue was purified by chromatography (ether/hexane, 1:1) to give 770 mg (60%) of glycolate ether **14g** as a clear oil. Starting alcohol could also be recovered: IR; $^1H$ NMR; $^{13}C$ NMR; LRMS; Anal.

**Methyl [(4a*R*),4aβ,8α,8aα]-2,3,4a,5,8,8a-Hexahydro-2-oxo-8-hydroxy-1,4-benzodioxin-6-carboxylate (15g).** As described above for the deketalization and lactonization of **14c**, 158 mg (0.53 mmol) of glycolate ether **14g** was converted to 60 mg (50%) of lactone **15g** as a white solid after purification by chromatography (EtOAc/hexane, 3:1): mp 118–120 °C; IR; $^1H$ NMR; $^{13}C$ NMR; LRMS; Anal.

**Methyl [(4a*R*),4aβ,8α,8aα]-2,3,4a,5,8,8a-Hexahydro-2-oxo-8-[[bis-[2-(4-nitrophenyl)ethoxy]phosphinyl]oxy]-1,4-benzodioxin-6-carboxylate (16g).** As described above for the phosphorylation of (*R*)-**15c**, 237 mg (1.04 mmol) of hydroxy lactone **15g** was converted to 700 mg of **16g** as a clear yellowish oil. This material was carried on without further purification but could be purified by reversed-phase HPLC (Whatman Partisil, 10-μm particle size, eluting with $CH_3CN/H_2O$, 55:45, 10 mL/min, flow rate, $t_R$ = 40 min, 100 mg/injection) to give **16g** as a hygroscopic white solid: IR; $^1H$ NMR; $^{13}C$ NMR; $^{31}P$ NMR; Anal.

**3(*R*)-[3α,4α,5β(*R*)]-5-Carboxymethoxy-4-hydroxy-3-(phosphonooxy)-1-cyclohexene-1-carboxylate (9).** As described above for deprotection of (*R*)-**16c**, 49 mg (0.08 mmol) of phosphate **16g** was converted to 26 mg (65%) of the tetrasodium salt of glycolate **9** as a white solid: $^1H$ NMR ($D_2O$) δ 6.51 (d, 1, $J$ = 2), 4.85 (m, 1), 4.06 (q, 2, $J$ = 16), 4.00 (m, 1), 3.87 (dd, 1, $J$ = 6, 13), 2.78 (dd, 1, $J$ = 5, 18), 2.29 (dd, 1, $J$ = 6, 18); $^{13}C$ NMR ($D_2O$) δ 178.6, 173.8, 134.4, 133.4 (d, $J_{CCOP}$ = 2.9), 76.7, 71.5 (d, $J_{COP}$ = 5.4), 69.8 (d, $J_{CCOP}$ = 4.8), 69.2, 29.9; $^{31}P$ NMR ($D_2O$) δ 1.26; $\epsilon_{240}$ = 970 $M^{-1}$ $cm^{-1}$.

**Synthesis of Fluoro-EPSP Analogue (*Z*)-10. Methyl [(1*R*),1α,5β,6a]-5-[2-Bis(methoxycarbonyl)-1,1-difluoroethoxy]-8,8-dimethyl-7,9-dioxabicyclo[4.3.0]non-2-ene-3-carboxylate (14i).** To a three-neck round-bottom flask equipped with a balloon and stopcock adapter was added 258 mg (2.68 mmol) of sodium *tert*-butoxide and 5 mL of THF. To the stirring suspension was added a solution of 534 mg (1.49 mmol) of malonate **14h** in 10 mL of THF, and the resulting mixture was stirred at room temperature for 30 min. HMPT (0.5 mL) was added to the mixture, which was then placed in a water bath at 45 °C and saturated with a rapid stream of chlorodifluoromethane until the balloon had fully expanded. After being stirred for 1 h at 45 °C, the reaction was quenched with water and extracted with ether (2 × 40 mL). The combined extracts were dried ($MgSO_4$), and the solvent was removed to give a mixture which was combined with the crude products of three similar scale reactions. The combined mixture (from 2.26 g of malonate) was purified by flash chromatography (ether/hexane, 30:70) to yield 685 mg (28%) of pure **14i**, 239 mg (10%) of the vinyl fluoride **26**, and 145 mg (5%) of starting malonate. For **14i**: IR; $^1H$ NMR; $^{13}C$ NMR; $^{19}F$ NMR; LRMS; Anal. For **26**: IR; $^1H$ NMR; $^{13}C$ NMR; $^{19}F$ NMR; LRMS.

**Dimethyl [(4a*R*),4aβ,8α,8aα]-2,3,4a,5,8,8a-Hexahydro-2-oxo-3-(difluoromethyl)-8-hydroxy-1,4-benzodioxin-3,6-dicarboxylate (15i).** As described above for the deketalization and lactonization of **14c**, 254 mg (0.622 mmol) of **14i** was converted to 207 mg (99%) of essentially pure **15i** as a clear oil. $^1H$ NMR shows this material to be a 60:40 mixture of diastereomers which were not separable by TLC: IR; $^1H$ NMR; $^{13}C$ NMR; $^{19}F$ NMR; LRMS; Anal.

**Dimethyl [(4a*R*),4aβ,8α,8aα]-2,3,4a,5,8,8a-Hexahydro-2-oxo-3-(difluoromethyl)-8-[[bis(phenylmethoxy)phosphinyl]oxy]-1,4-benzodioxin-3,6-dicarboxylate (16i).** As described above for the phosphorylation of (*R*)-**15c**, 209 mg (0.62 mmol) of lactone **15i** was converted to 385 mg of dibenzyl phosphate **16i** with dibenzyl *N,N*-diisopropylphosphoramidite. This material was carried on without purification. $^{31}P$ NMR shows the crude product to be ~80% pure in phosphorus content with the major impurity being dibenzyl phosphite. $^1H$ NMR shows the product to be a 2:1 mixture of diastereomers. One diastereomer could be purified by chromatography (ether) for spectral analysis: $^1H$ NMR; $^{13}C$ NMR; $^{19}F$ NMR; $^{31}P$ NMR; LRMS; HRMS.

**(−)-5-(*Z*)-Fluoroenolpyruvylshikimate 3-Phosphate ((*Z*)-10).** The crude dibenzyl phosphate **16i** described above was dissolved in 1.0 mL of acid-free $CDCl_3$ (by passing through a plug of basic alumina) and transferred via cannula to an NMR tube sealed with a rubber septum. To this solution was added 269 μL (3.31 mmol) of pyridine followed by 164 μL (1.66 mmol) of bromotrimethylsilane. The tube was inverted and shaken vigorously, and the reaction progress was followed by $^{31}P$ NMR. After completion of the reaction, the mixture was added to 5 mL of $D_2O$ at 0 °C, and the organic phase was extracted with an additional 5 mL of $D_2O$. To the combined aqueous extracts at 0 °C was added 2.07 mL of 1 N NaOH in $D_2O$, and the reaction was stirred for 30 min at 0 °C. The mixture was then purified by anion-exchange chromatography using Sephadex A-25-120 resin, eluting with a linear gradient of 0–0.8 M triethylammonium bicarbonate. Fractions absorbing at 240 nm are combined and the solvent was removed in vacuo, with final evaporation from methanol, to give the tetrakis(triethylammonium) salt of 9-fluoro-EPSP. This material was passed through a column of Dowex 50X8-100 cation-exchange resin ($Na^+$ form) to yield 80 mg (45% from hydroxy lactone **15i**) of the tetrasodium salt of (*Z*)-F-EPSP ((*Z*)-**10**): $^1H$ NMR δ 7.35 (d, 1, $J$ = 75), 6.51 (t, 1, $J$ = 2.25), 4.91 (m, 1), 4.45 (m, 1), 4.12 (dd, 1, $J$ = 5.5, 8.75), 2.69 (dd, 1, $J$ = 6.0, 22.0), 2.39 (dd, 1, $J$ = 6.0, 24.0); $^{13}C$ NMR δ 176.0, 170.7, 148.7 (d, $J_{CF}$ = 269), 137.0, 136.0, 131.4, 78.3, 71.3 (d, $J_{COP}$ = 5.0), 69.6, 29.6; $^{19}F$ NMR δ −145.4 (d, $J$ = 75); $^{31}P$ NMR δ 3.92; $\epsilon_{240}$ = 1620 $M^{-1}$ $cm^{-1}$.

**Synthesis of (*Z*)-9-Methyl-EPSP (*Z*-11). Methyl [(1*R*),1α,5β,6α]-5-[[1-(Methoxycarbonyl)propenyl]oxy]-8,8-dimethyl-7,9-dioxabicyclo[4.3.0]non-2-ene-3-carboxylate (14k).** To a solution of 582 μL (2.76 mmol) of hexamethyldisilazane in 10 mL of THF at 0 °C was added 1.65 mL (2.64 mmol) of a 1.6 M solution of *n*-butyllithium in hexanes via syringe. After being stirred for 10 min at 0 °C, the mixture was added via cannula to a solution of phosphonate **14j**[7] in 40 mL of THF at −78 °C. This mixture was stirred at −78 °C for 15 min and then an excess of acetaldehyde was added via cannula. The resulting yellow mixture was allowed to warm to room temperature and stirred for 12 h, when it was partitioned between aqueous $NH_4Cl$ and 60 mL of $CH_2Cl_2$. The organic layer was separated, and the aqueous phase was extracted with 20 mL of $CH_2Cl_2$. The combined organic extracts were dried ($MgSO_4$), and the solvent was removed in vacuo to give a yellow oil. This material was purified by chromatography (ether/hexane, 70:30) to give 630 mg (80%) of alkene **14k** as a clear oil. $^1H$ NMR showed this material to be a 5:1 *E*/*Z* mixture of isomers which were not separated: IR; $^1H$ NMR; $^{13}C$ NMR; Anal.

**Methyl [3*E*,(4a*R*),4aβ,8α,8aα]-2,3,4a,5,8,8a-Hexahydro-3-ethylidene-2-oxo-8-hydroxy-1,4-benzodioxin-6-carboxylate ((*E*)-15k) and the (3*Z*)-Isomer ((*Z*)-15k).** A solution of 320 mg (0.99 mmol) of Wittig adduct **14k** and 10 mg (0.05 mmol) of *p*-toluenesulfonic acid in 60 mL of 20% aqueous $CH_3CN$ was heated at reflux for 12 h, and the solvent was removed by lyophilization. The residue was dissolved in benzene, the solvent was removed in vacuo (twice), and the residue was dissolved in 80 mL of dry acetonitrile. To this solution was added 14 mg (0.1 mmol) of anhydrous $K_2CO_3$, and the mixture was stirred at 50 °C for 2 h. TLC (ether/hexane, 4:1) showed complete conversion to lactone.

The mixture was diluted with 50 mL of $CH_2Cl_2$ and washed with saturated aqueous $NH_4Cl$. The organic layer was dried ($MgSO_4$), and the solvent was removed in vacuo to give 239 mg (95%) of a 3:1 (*E*:*Z*) mixture of lactones as a white solid. The isomers could be partially separated by chromatography (ether/hexane, 3:1) or completely separated by HPLC (silica, Rainin, 10-μm particle size; flow rate 2 mL/min, EtOAc/hexane, 1:4) to give 100 mg (40%) of (*E*)-**15k** and 40 mg (16%) of (*Z*)-**15k**. (*E*)-**15k**: IR; $^1$H NMR; $^{13}$C NMR; LRMS; Anal. (*Z*)-**15k**: mp 135–137 °C; IR; $^1$H NMR; $^{13}$C NMR; LRMS; Anal.

**Methyl [3*Z*,(4a*R*),4a*β*,8*α*,8a*α*]-2,3,4a,5,8,8a-Hexahydro-3-ethylidene-2-oxo-8-[[bis[2-(4-nitrophenyl)ethoxy]phosphinyl]oxy]-1,4-benzodioxin-6-carboxylate ((*Z*)-16k).** As described above for phosphorylation of (*R*)-**16c**, 39 mg (0.15 mmol) of (*Z*)-**15k** was converted to 96 mg of (*Z*)-**16k** as a light yellow oil, which was normally carried on without purification. A small amount could be purified by a single injection on a reversed-phase HPLC column, as described above for the glycolate **16g**, to give pure (*Z*)-**16k** as a clear oil: IR; $^1$H NMR; $^{13}$C NMR; $^{31}$P NMR; LRMS; HRMS.

**3(*R*)-[3*α*,4*α*,5*β*(*R*)]-5-[[1(*Z*)-Carboxypropenyl]oxy]-4-hydroxy-3-(phosphonooxy)-1-cyclohexene-1-carboxylate, (−)-(*Z*)-Methyl-EPSP ((*Z*)-11).** In a procedure analogous to that described for deprotection of (*R*)-**16c**, 56 mg (0.09 mmol) of phosphate (*Z*)-**16k** was converted to 24 mg (63%) of (*Z*)-**11** as the tetrasodium salt: $^1$H NMR δ 6.48 (br s, 1), 6.04 (q, 1, *J* = 7.0), 4.89 (m, 1), 4.34 (m, 1), 4.18 (t, 1, *J* = 4.7), 2.38 (dd, 1, *J* = 1.7, 16.9), 2.28 (ddd, 1, *J* = 1.7, 1.7, 16.9), 1.54 (d, 3, *J* = 7.0); $^{13}$C NMR δ 176.4, 172.5, 148.3, 134.8, 133.8, 122.5, 76.7, 70.2 (d, $J_{COP}$ = 4.7), 69.2, 27.4, 11.8; $^{31}$P NMR δ 4.58; $\epsilon_{240}$ = 2430 M$^{-1}$ cm$^{-1}$.

**3-(Fluoromethyl)-3-hydroxy-2-oxo-2,3,4a,5,6,7,8,8a-octahydro-1,4-benzodioxin (22a).** To a solution of 146 mg (1 mmol) of sodium 3-fluoropyruvate monohydrate and 195 mg (1.02 mmol) *p*-toluenesulfonic acid monohydrate in 1 mL of water was added a solution of 116 mg (1 mmol) of *trans*-1,2-cyclohexanediol in 40 mL of benzene. The two-phase reaction mixture was heated under reflux, and the water was removed azeotropically with a Dean–Stark trap. After most of the water had been removed, a precipitate of sodium *p*-toluenesulfonate formed. After 3 h, the mixture was cooled and filtered through a pad of Celite, and the clear, colorless solution was evaporated. The white, crystalline residue (209 mg) was recrystallized from benzene/hexane to give 153 mg (75% yield) of compound **22a** as white crystals: mp 105–107 °C; IR; $^1$H NMR; Anal.

**3-[[(Dibenzyloxy)phosphinyl]oxy]-3-(fluoromethyl)-2-oxo-2,3,4a,5,6,7,8,8a-octahydro-1,4-benzodioxin (23a).** To a cold solution (−78 °C) of 408 mg (2.0 mmol) of fluorohydrin **22a** in 10 mL of dry THF under nitrogen was added 1.5 mL (2 mmol) of a 1.37 M solution of *tert*-butyllithium in pentane. After 20 min, a solution of 1.65 g (3 mmol) of tetrabenzyl pyrophosphate in 4 mL of dry THF was added in one portion. After 30 min at −78 °C, the clear solution was allowed to warm to room temperature and a white precipitate of lithium dibenzylphosphate began to form. After 2 h, the mixture was poured into dilute, ice-cold $NaHCO_3$ and extracted twice with ether. The ether solutions were washed once with ice-cold dilute $NaHCO_3$ and twice with brine, dried over $MgSO_4$, filtered, and evaporated to leave 1.41 g of a yellow oil. Flash chromatography (hexane/ethyl acetate, 2:1) afforded 763 mg of a colorless oil, which was crystallized from ether/hexane to give 560 mg (60% yield) of dibenzyl ester **23a** as colorless crystals: mp 76–78 °C; IR; $^1$H NMR; $^{31}$P NMR; Anal.

**3-Fluoro-2-[(2-*trans*-hydroxycyclohexyl)oxy]-2-(phosphoryloxy)-propanoate (24a).** To a solution of 92.9 mg (0.2 mmol) of dibenzyl phosphate **23a** in 5 mL of THF were added 53 mg (0.6 mmol) of finely powdered $NaHCO_3$ and 5 mg of 9% Pd/C. The mixture was cooled in an ice bath, and the reaction flask was evacuated and flushed with hydrogen from a balloon. This latter process was repeated three more times and then the mixture was stirred for 30 min under hydrogen; 1 mL of water was added and stirring was continued for another 15 min. The mixture was removed by filtration through a Millipore filter, the flask and catalyst were washed twice with water, and the combined filtrates were lyophilized to leave 81 mg of a white solid, which contained the partially deprotected lactone as the major component: $^1$H NMR (500 MHz, $D_2O$) δ 4.69 (dd, 1, *J* = 9.2, 45.9, partly obscured by HOD), 4.60 (dd, 1, *J* = 9.2, 45.8), 4.40–4.34 (m, 1), 4.31–4.25 (m, 1), 2.14–1.95 (m, 2), 1.80–1.68 (m, 2), 1.55–1.20 (m, 4); $^{13}$C NMR δ 90.14 (d, *J* = 176.9), 88.59, 77.65, 34.36, 33.95, 27.96, 27.76, (tertiary C atoms are missing); $^{31}$P NMR δ ($D_2O$) 0.2.

This solid was dissolved in 2 mL (2 mmol) of 0.1 N NaOH solution and left at room temperature for 45 min. The solution was lyophilized, and the crude reaction product was dissolved in 2 mL of 50 mM pH 8.5 TBK buffer and applied to a 2.0 × 11 cm DEAE Sephadex column. The column was first eluted with 25 mL of 50 mM pH 8.5 TBK buffer, and then a linear gradient with 0.5 M pH 8.5 TBK buffer (200 mL each) was applied. Six-milliliter fractions were collected, and those giving a positive reaction to Hanes reagent[56] were combined and lyophilized to afford 136 mg of a very hygroscopic white solid. This material was dissolved in 2 mL of water and passed through a 2 × 6 cm column of Dowex 50 in the $Li^+$ form. Fractions showing a positive reaction to the Hanes reagent[56] were collected and lyophilized to give 51 mg (80% yield) of compound **24a** as a white slightly hygroscopic solid: $^1$H NMR (500 MHz, $D_2O$) δ 4.82 (dd, 1, *J* = 9.6, 45.6), 4.64 (dd, 1, *J* = 9.6, 45.6), 3.62–3.55 (m, 1), 3.45–3.35 (m, 1), 2.22–2.12 (m, 1), 1.92–1.82 (m, 1), 1.6–1.5 (m, 2), 1.3–1.05 (m, 4); $^{31}$P NMR (81.75 MHz, $D_2O$) δ −1.61 (s); $^{13}$C NMR (125.76 MHz, $D_2O$) δ 175.2, 100.17 (dd, *J* = 8.0, 24), 84.21 (dd, *J* = 4.0, 171), 79.61, 73.76, 33.18, 32.73, 24.27, 23.81; IR (KBr) 3420 (br), 2940, 2860, 1655 (sh), 1640, 1455, 1425, 1255, 1155, 1130, 1120, 1040, 980 cm$^{-1}$; HRMS (FAB) calcd for $C_9H_{13}FO_8PLi_3$ *m/z* 321.0890 (M + 1), found *m/z* 321.0893. The synthesis and characterization of the bromo- and benzenesulfonyl analogues, **22b–23b** and **22c–24c**, respectively, are described in the supplementary material.

**4-Carboxy-4-[(2-*trans*-hydroxycyclohexyl)oxy]-2-oxido-1,2,3-dioxaphospholane 2-Oxide (25).** To a solution of 53 mg (0.1 mmol) of the bromomethyl dibenzyl phosphate **23b** in 4 mL of dry methanol were added 26 mg (0.3 mmol) of finely powdered $NaHCO_3$ and 5 mg of 9% Pd/C. The mixture was cooled in an ice bath, evacuated, and flushed with hydrogen from a balloon. This latter process was repeated three more times, and the mixture was stirred under a hydrogen atmosphere for 20 min. The mixture was then filtered, the flask and catalyst were washed with water, and the clear solution was lyophilized to afford 38 mg of an approximately 1:1 mixture of compounds **24b** and **25**: $^1$H NMR ($D_2O$) δ 4.65–4.40 and 4.38–4.25 (m, 1), 4.22 (dd, ca. 0.5, *J* = 10.1, 19.5), 4.19 (dd, ca. 0.5, *J* = 10.1, 3.1), 3.89 (d, ca. 0.5, *J* = 10.1), 3.85 (d, ca. 0.5, *J* = 10.5), 3.58–3.40 (m, ca. 1), 2.3–1.15 (m, 8); $^{31}$P NMR ($D_2O$) δ 18.11, −0.83; $^1$H-coupled: 18.11 (dd, *J* = 19.5, 3.1), −0.83 (s).

This mixture was dissolved in 2 mL of water, left to stand at room temperature for 2 h, and then lyophilized to give 44 mg of a white solid. Half of the above material was purified by preparative HPLC on a reversed-phase column (Whatman ODS-3 Partisil 10) using a 8:2 mixture of 0.1 N TBK buffer at pH 7.5 and methanol as the eluent. The triethylammonium salt thus obtained was dissolved in water and applied to a 1.5 × 4 cm column of Dowex 50 in the $Li^+$ form, to afford 10 mg (55% yield) of compound **25** as a white solid: HRMS (FAB) calcd for $C_9H_{13}O_8PLi_2$ *m/z* 295.0746 (M + 1), found *m/z* 295.0738; $^1$H NMR (500 MHz, $D_2O$) δ 4.216 (dd, 1, *J* = 10.1, 19.5), 4.19 (dd, 1, *J* = 10.1, 3.1), 3.48 (m, 2), 2.3–2.2 (m, 1), 2.0–1.9 (m, 1), 1.7–1.55 (m, 2), 1.4–1.2 (m, 4); $^{31}$P NMR ($D_2O$) δ 18.07; $^1$H-coupled δ 18.07 (dd, *J* = 19.5, 3.1); $^{13}$C NMR ($D_2O$) δ 173.08, 104.04, 80.94, 72.84, 72.34, 32.42, 32.00, 23.39, 22.98.

**3-Hydroxy-3-methyl-2-oxo-2,3,4a,5,6,7,8,8a-octahydro-1,4-benzodioxin (22d).** To a cold solution (0 °C) of 4.36 g (37.6 mmol) of *trans*-1,2-cyclohexanediol and 3.23 mL (37.6 mmol) of dry pyridine in 30 mL of dry THF was added 3.58 g (33.6 mmol) of freshly prepared pyruvyl chloride.[57] After 20 min, pyridinium hydrochloride began to precipitate and the mixture was warmed to room temperature for 30 min. The mixture was filtered, diluted with 50 mL of $CH_2Cl_2$, and washed with ice-cold 0.1 N HCl. The aqueous layer was extracted with $CH_2Cl_2$, and the combined organic layer was filtered through cotton wool and evaporated under reduced pressure. The oily residue was chromatographed ($CH_2Cl_2$/ether, 4:1) to give 2.26 g (34%) of a 1:1 mixture of *trans*-2-hydroxycyclohexyl pyruvate and the corresponding cyclic hemiketal **22d**.

A 6.2-g sample of material from another preparation was dissolved in 20 mL of ether and 20 mL of hexane, and the emulsion was warmed until homogeneous. On being cooled in the refrigerator, the solution formed two layers, from the lower of which the pure hemiketal **22d** began to crystallize after 2 days. The colorless crystals were removed, the mother liquor was evaporated to dryness, and the procedure was repeated with smaller quantities of solvents. After five subsequent crystallizations, 3.0 g of analytically pure hemiketal **22d** was obtained: mp 104 °C; $^1$H NMR δ 3.73 (ddd, 1, *J* = 4.6, 9.3, 10.9), 3.59 (ddd, 1, *J* = 4.5, 9.3, 11.4), 3.46 (br s, 1), 1.80–1.60 (m, 2), 1.61 (s, 3), 1.22–0.55 (m, 6); $^{13}$C NMR (acetone-$d_6$) δ 168.78, 96.34, 83.40, 71.45, 30.81, 30.26, 26.65, 24.29, 23.93. Anal. Calcd for $C_9H_{14}O_4$: C, 58.05; H, 7.57. Found: C, 58.18; H, 7.65.

**Enzyme Assays. General Procedures.** EPSP was generously supplied by Dr. Alan R. Rendina from Chevron Chemical Co.; it was also prepared by the method of Chouinard and Bartlett.[15b] EPSP concentration was obtained by acid hydrolysis of the enolpyruvyl side chain of EPSP and subsequent end point assay of the pyruvate released using lactate dehydrogenase and NADH. The concentrations of solutions of the tet-

(56) Stanley, C. W. *J. Chromat.* **1964**, *16*, 467.
(57) Ottenheijm, H. C. J.; DeMan, J. H. M. *Synthesis* **1975**, 163.

rahedral analogues **4–6** were determined by dilution of a solution prepared from a precisely weighed sample of inhibitor and correction for the percent phosphorus determined by elemental analysis; in the case of (*R*)-**6**, the concentration was adjusted to account for ca. 5% of an impurity. The inhibitors are more stable at high pH, so stock solutions were adjusted to pH 11 with KOH. Concentrations of the other inhibitor solutions were obtained by treatment with alkaline phosphatase and quantitation of the inorganic phosphate released using the method of Hess and Derr as modified by Lanzetta et al.[58] These values were compared to that obtained with EPSP using the same method, as well as to a prepared standard curve.

EPSP synthase from *Petunia hybrida* was obtained as a generous gift from Dr. Ganesh Kishore at the Monsanto Co. The enzyme was stored at −80 °C in a storage buffer containing 50% glycerol, 50 mM potassium HEPES (pH 7.5), 70 mM KCl, and 2.2 mM dithiothreitol. Immediately prior to assays, a working stock solution was prepared by dilution of the enzyme into a buffer with final concentrations of 30% glycerol, 50 mM potassium-HEPES (pH 7.5), 5.2 mM dithioerythritol, 20 mM KCl, and 1 mg/mL bovine serum albumen and kept on ice during the assay period.

Unless otherwise noted, all UV assays were performed on a Cary 219 UV–vis spectrophotometer or a Uvikon 860 (Kontron) spectrophotometer using 1-cm path-length cells with a thermostated cell holder, and circulating water bath at 25.0 °C. For kinetic studies, EPSP synthase activity was coupled to pyruvate kinase (PK) and lactate dehydrogenase (LDH). The assay mixture consisted of 100 mM potassium-HEPES (pH 7.5), 2.5 mM ATP, 4 mM $MgCl_2$, 50 mM potassium phosphate, 150 μM NADH, 22 units of LDH, 30 units of PK, and varying amounts of EPSP and EPSP synthase, all in a total volume of 1.0 mL. The reaction was initiated by addition of EPSP synthase, and the absorbance at 340 nm was monitored for oxidation of NADH. Rates were obtained by analyzing the linear portion of the initial 10% of each reaction curve, and kinetic parameters were obtained using the program ENZFITTER[59] as well as the COMP and NCOMP programs of Cleland.[60]

**Determination of $K_m$ for EPSP and $K_i$ Values of Tetrahedral Analogues 4–6 with EPSP as Varied Substrate.** These experiments were performed in 10-cm path-length cells in a total volume of 3.5–4.5 mL with 50 mM (=$50K_m$) potassium phosphate (pH 7.5), 25–150 μM NADH, 22–100 units of LDH, and 30–140 units of PK. Five inhibitor concentrations were examined for each compound, and for each inhibitor concentration, five concentrations of EPSP were used (2.0–30.0 μM), with at least two determinations carried out at each concentration. All rates measured were corrected for a small background rate observed when the assay was run without added EPSP. The Michaelis and inhibition constants are given in Table I.

**Determination of $K_m$ for Phosphate and $K_i$ Values for Analogues 4 and 5 with Phosphate as Varied Substrate.** These assays were carried out in a total volume of 1 mL with 1-cm path length cells. The reaction solutions were as described above but with constant (50 μM, =$25K_m$) EPSP and variable (0.75–5 mM) potassium phosphate. The data were fit to both the competitive and noncompetitive models using the equations $Y = V[S]/(K(1 + [I]/K_i) + [S])$ and $Y = V[S]/(K_m(1 + [I]/K_{is}) + [S](1 + [I]/K_{ii}))$ respectively, using COMP and NCOMP.[60] For (*S*)-**4**, the data fit the competitive model best; for (*R*)-**4**, (*R*)-**5**, and (*S*)-**5**, the data fit both models, with a better fit for noncompetitive inhibition. The concentration ranges examined and the computed $K_i$ values for the inhibitors are as follows: (*S*)-**4** (0–2.6 μM, competitive) $K_i = 2.1 \pm 0.02$ μM; (*R*)-**4** (0–160 nM, noncompetitive) $K_{i(slope)} = 0.113 \pm 0.015$ μM, $K_{i(intercept)} = 0.16 \pm 0.06$ μM; (*S*)-**5** (0–180 nM, noncompetitive) $K_{i(slope)} = 0.081 \pm 0.006$ μM, $K_{i(intercept)} = 1.0 \pm 0.2$ μM; (*R*)-**5** (0–500 nM, noncompetitive) $K_{i(slope)} = 0.053 \pm 0.004$ μM, $K_{i(intercept)} = 1.4 \pm 0.2$ μM.

**Determination of Phosphate Release from (*R*)-6 and (*S*)-6 in the Presence and Absence of Enzyme.** The sensitive malachite green assay[56] was used to measure phosphate. The assay mixture contained 30 mM potassium-HEPES buffer (pH 7.5), 5.32 or 5.48 mM (*R*)-**6** or (*S*)-**6**, respectively, and ca. 0.34 nanounit of EPSP synthase (1 unit is defined as 1 μmol of phosphate released/min at 25 °C), about 30 times the amount used in the standard coupled assays described above, in a total volume of 1.5 mL. Two controls were run. One contained all of the above but without (*R*)-**6**; in the other, the EPSP synthase was omitted. The assay mixtures were kept at 25 °C for 3 days. The release of phosphate was followed by removing 100-μL aliquots of the above mixtures, adding 800 μL of malachite green reagent and, after 1.5 min, 100 μL of 34% sodium citrate. After developing for 40 min, the $A_{690}$ was measured and the amount of phosphate was determined from a standard curve. In all experiments, less than 5% of the total phosphate was released as inorganic phosphate over the 3-day period and there was no increase in the rate of release in the presence of enzyme.

**Determination of the $K_i$ Values of Substrate Analogues 8–11 with EPSP as Varied Substrate.** These assays were performed with 1-cm path length cells in a total volume of 1 mL, using the assay method described in the general section. The dihydro analogue (*S*)-**8** was analyzed and found to show competitive inhibition against EPSP. The $K_i$ value for this inhibitor was found using Cleland's COMP program.[60] The $K_i$ values for the other analogues were determined by varying inhibitor concentration at a substrate concentration equal to $K_m$ and fitting the data to the equation $V_0/V_i = [I](1/K_i)(K_m/(K_m + [S])) + 1$ using a Dixon plot of $V_0/V_i$ vs [I].[46,61] The inhibition constants are given in Table I.

**Determination of Substrate Activity for (*Z*)-10 and (*Z*)-11 with EPSP Synthase.** An HPLC assay was used in which the reverse reaction could be monitored for the appearance of S3P. The separation method employed a MonoQ 10/10 FPLC anion-exchange column (Pharmacia) eluted with a linear gradient of 0.2–1.0 M TBK at a flow rate of 1.0 mL/min, monitored at 240 nm. Reaction mixtures contained 50 mM Tris-HCl (pH 7.5), 25 mM of potassium phosphate, 1 milliunit of EPSP synthase, and 1 mM substrate. Aliquots of 50 μL were injected onto the column and analyzed for product formation. Under these conditions, the detection of 5 mol % S3P was readily achieved. Both (*Z*)-**10** and (*Z*)-**11** failed to show any detectable S3P formation after 48 h, while the reaction with EPSP reached equilibrium within 1.5 h (ca. 2:1 EPSP/S3P). These results show that both analogues are turned over at a rate less than 0.2% that of EPSP.

**Acknowledgment.** This work was supported by the National Institutes of Health (Grant GM-28965), by Ciba-Geigy AG, and through fellowship support to D.G.A. from General Electric and Chevron and to A.F. from the Swiss National Foundation. We also express our appreciation to Dr. Ganesh Kishore (Monsanto Co.) for providing us with EPSP synthase, to Dr. Alan Rendina (Chevron Chemical Co.) for a gift of EPSP, to Dr. Paul Resnick (E. I. Du Pont de Nemours) for a generous gift of methyl trifluoropyruvate, and to Dr. John Bushweller and Dr. Felicia Etzkorn for assistance with the 2D NMR spectra.

**Supplementary Material Available:** Spectral and analytical characterization of synthetic intermediates, synthesis and characterization of model compounds **22a**, **23a**, and **22b–24b**, and kinetic plots for determination of $K_i$ values (15 pages). Ordering information is given on any current masthead page; also available with the archival edition in many libraries.

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