Glycerol Kinase: Synthesis of Dihydroxyacetone Phosphate, *sn*-Glycerol-3-phosphate, and Chiral Analogues[1]

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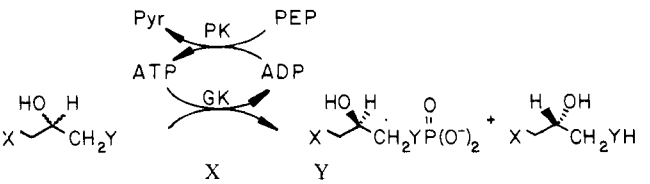
Abstract: Glycerol kinase (E.C. 2.7.1.30, ATP: glycerol-3-phosphotransferase from *S. cerevisiae*) catalyzes the phosphorylation by ATP of glycerol, dihydroxyacetone, and certain structural analogues of glycerol. Phosphorylation of racemic mixtures produces chiral organic phosphates with enantiomeric excess (ee) >90–95% in yields of 75–95% (based on the quantity of that enantiomer present in the starting racemic mixture). The unphosphorylated enantiomers can be recovered from the reaction mixtures in 30–40% yields and 80–90% ee. This paper illustrates the use of glycerol kinase for preparative-scale (10–500 mmol) phosphorylations and for kinetic resolutions. Products produced include D-3-chloropropane-1,2-diol-1-phosphate, D-3-mercaptopropane-1,2-diol-1-phosphate, D-3-aminopropane-1,2-diol-3-phosphate, D-3-methoxypropane-1,2-diol-1-phosphate, and D-butane-1,2,4-triol-1-phosphate. Convenient preparations of *sn*-glycerol-3-phosphate (1 mol) and dihydroxyacetone phosphate (0.4 mol) are also described. Regeneration of ATP in situ is accomplished using either phosphoenol pyruvate and pyruvate kinase or acetyl phosphate and acetate kinase.

Introduction

Glycerol kinase (GK, E.C. 2.7.1.30, ATP: glycerol-3-phosphotransferase) catalyzes the phosphorylation of glycerol, dihydroxyacetone, and a number of analogues of these substances by ATP.[2,3] The reaction is highly enantioselective.[2,3] The enzyme is inexpensive and stable in immobilized form. This enzyme-catalyzed phosphorylation thus provides a practical method for the preparation of *sn*-glycerol-3-phosphate,[4,5] dihydroxyacetone phosphate,[6] and chiral analogues of *sn*-glycerol-3-phosphate.[2] This manuscript illustrates the use of GK in syntheses and provides experimental details for representative procedures in the 10–500-mmol scale.

Our interest in this enzyme stems from three types of applications in synthesis. First, *sn*-glycerol-3-phosphate is a precursor to synthetic phospholipids;[7,8] the configuration of the compound produced by glycerol kinase catalyzed phosphorylation of glycerol is that found in naturally occurring phospholipids.[7] Second, dihydroxyacetone phosphate is a valuable starting material for aldolase-catalyzed reactions.[6,9] Third, chiral analogues of glycerol-3-phosphate are potentially interesting as starting materials for the preparation of phospholipid analogues,[10] and as chiral synthons for other synthetic applications. We focused on two questions in this work. First, what quantities of products can be made using these GK-catalyzed phosphorylations? Second, what enantioselectivities can be obtained in resolutions based on them?

We were particularly interested in the latter question (eq 1). The schemes for kinetic resolution examined here were based on limiting the extent of reaction by limiting the quantity of PEP added during the course of the reaction: typically, 0.50 equiv of PEP was used per equiv of racemic starting material. The



	X	Y		
DL-1	Cl	O	D-1-P	L-1
DL-2	HO	HN	D-2-P	L-2
DL-3	HS	O	D-3-P	L-3
DL-4	CH_3O	O	D-4-P	L-4
DL-5	$HOCH_2$	O	D-5-P	L-5
DL-6	Br	O	D-6-P	L-6
DL-7	CH_3CH_2	O	D-7-P	L-7
8	HO	O	*sn*-Gly-3-P	

(1)

phosphorylated product was easily separated from unphosphorylated material, usually by selective precipitation of the organic phosphate as its barium salt. We were able to isolate the phosphorylated products in 75–95% of their theoretical yields. Unphosphorylated materials were experimentally more difficult to isolate, and isolated yields were typically only 20–40% of the theoretical. ATP was recycled using PEP as the ultimate phosphate donor.[11] This regeneration scheme was chosen rather than one based on acetyl phosphate[12] or methoxycarbonyl phosphate[13] in order to minimize the production of free phosphate in solution by spontaneous hydrolysis of the phosphate,[14] and thus to minimize difficulties in isolations. For reactions run on larger scales than those described here, acetyl phosphate might be useful because it is easily obtained in large amounts.[12] Pyruvate was easily separated from other products as its crystalline sodium salt.[15]

Glycerol kinase is commercially available from a number of microbial sources.[2] In this work, we used the glycerol kinase from *S. cerevisiae* exclusively. The specific activity appears to be similar for all of the commercially available enzymes.[2] The substrate specificity is somewhat wider for glycerol kinase from *S. cerevisiae* and *C. mycoderma* than for glycerol kinase from *E. coli* and *B. stearothermophilus*. The enzyme was used in immobilized form in order to facilitate its recovery from reaction mixtures and to

(1) Supported by the National Institutes of Health, Grant GM 30367.
(2) Crans, D. C.; Whitesides, G. M. *J. Am. Chem. Soc.*, preceeding paper in this issue.
(3) Thorner, J. W.; Paulus, H. In "The Enzymes"; Boyer, P. D., Ed.; Academic Press: New York, 1973; Vol. 8, pp 487–508.
(4) The IUPAC chemical name for *sn*-glycerol-3-phosphate is D-propane-1,2,3-triol-1-phosphate. It is known as *syn*-glycerol-3-phosphate (or L-glycerol-3-phosphate) in the biochemical literature. The analogues of *sn*-glycerol-3-phosphate will be referred to by their systematic chemical names in this paper. We use DL nomenclature rather than *RS* nomenclature to indicate absolute configuration. The names of the following substrates for glycerol kinase illustrate our choice of nomenclature system: D-propane-1,2-diol ((*R*)-propane-1,2-diol), D-3-fluoropropane-1,2-diol ((*S*)-3-fluoropropane-1,2-diol), D-3-aminopropane-1,2-diol ((*R*)-3-aminopropane-1,2-diol), and D-3-mercaptopropane-1,2-diol ((*S*)-3-mercaptopropane-1,2-diol).
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(13) Kazlauskas, R. J.; Whitesides, G. M. *J. Org. Chem.* in press.
(14) The half-lives for spontaneous hydrolysis of the phosphoryl group donors at pH 7.5, 25 °C, are as follows (phosphoryl group donor, $t_{1/2}$): acetyl phosphate, 21 h; phosphoenolpyruvate, $\sim 10^3$ h; methoxycarbonyl phosphate, 0.3 h.
(15) "Beilsteins Handbuch der Organischen Chemie"; Prager, B., Jacobsen, P., Eds.; Deutschen Chemischen Gesellschaft, Verlag von Julius Springer: Berlin, 1921; Vol. 3, pp 608–23.

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Table I. Kinetic Resolutions of Glycerol Analogues N (Eq 1)

compd no. N	scale[a] (mmol)	D-N-P yield, %	D-N-P purity %[b] enz	D-N-P purity %[b] ^{31}P NMR	D-N-P ee, %[c]	L-N yield, %	L-N $[\alpha]_D$,[d] deg	L-N ee(%) rotation	L-N ee(%) shift reagent
1	50	94	84	86	>94[e]	38	−6.4[f]	85	88
	500	93	92	90	>94[e]	31	−6.8[f]	92	94
2	50	75	79	81	94				
3	10	82	84	83	90				
4	10	82	84	83	90				
5	25	98		95		29	−26.6	92	
6	1	95	90	88	90				
7	1	82		82					

[a] The scale refers to the quantity of racemic starting material. All reactions were carried out at 27 °C. Other experimental conditions are given in the Experimental Section. [b] The enzymatic assay for purity was based on measuring the quantity of D-N-P which reacted with NAD in reaction catalyzed by GDH. [c] Unless otherwise indicated, the enantiomeric excesses were determined in ref 2. The accuracy of the method is ±3%. [d] $[\alpha]^{22}_D$ (*c* 5, H_2O). [e] The enantiomeric purity was determined using Eu(hfc)$_3$. The accuracy of this method is ±3%. [f] The rotation of D-**1** produced from D-^{1}-P by hydrolysis catalyzed by alkaline phosphatase was $[\alpha]^{25}_D$ +7.1 (*c* 5, H_2O).

improve its stability in aqueous solution.[2] The stability of the soluble enzyme is low in solutions which do not contain glycerol as a stabilizing agent;[3] less than 20% activity was observed after 24 h at pH 7.5 and 4 °C for glycerol kinase from *S. cerevisiae* in glycerol-free solution. Immobilization of glycerol kinase in PAN gel[16] increases the stability of the enzyme; immobilized glycerol kinase from *S. cerevisiae* lost no activity on storage for 6 months at 4 °C.

The two simplest synthetic applications of glycerol kinase are the phosphorylation of glycerol and dihydroxyacetone. For comparison with the kinetic resolutions by phosphorylation of unnatural substrates which form the basis for the major part of this paper, we include in this paper improved preparations of *sn*-glycerol-3-phosphate and dihydroxyacetone phosphate on 1-mol scales. In these syntheses, ATP was regenerated in situ using acetyl phosphate and acetate kinase.[12,17]

An accompanying paper describes studies of the substrate specificity of the several commercially available glycerol kinases and characterizes these enzymes kinetically.[2] This paper also reviews published background information relevant to applications of GK in organic synthesis.

Results

All GK-catalyzed reactions were carried out using similar procedures. One equivalent of racemic substrate was allowed to react with ~0.05 equiv of ATP in the presence of a regeneration system consisting of pyruvate kinase (PK, E.C. 2.7.1.40, ATP: pyruvate 2-*O*-phosphotransferase) and 0.50 equiv of PEP. The reaction was allowed to proceed to completion. The course of typical phosphorylations is summarized in Figure 1. The organic phosphate formed was isolated by precipitation as its barium salt. If desired, unphosphorylated species were recovered from the supernatant which remained after precipitation of the organic phosphate. Residual salt and sodium pyruvate were separated from the concentration supernatant by precipitation with ethanol. Results are summarized in Table I.

Yields and enantiomeric purities of products were established using several technique. Enantiomeric purities are based on an enzymatic assay using glycerol-3-phosphate dehydrogenase (GDH, E.C. 1.1.1.8, *syn*-glycerol-3-phosphate: NAD 2-oxidoreductase, eq 2)[18,19] that determined the content of organic phosphate which

(2)

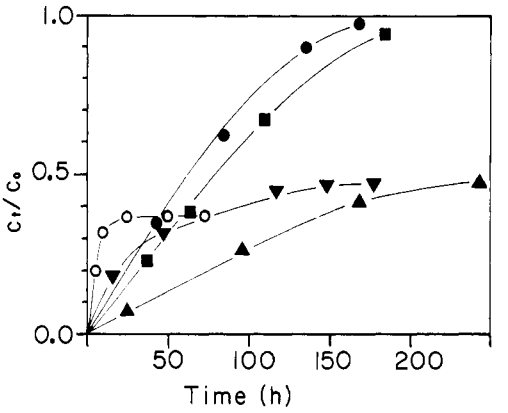

Figure 1. Extent of reaction in the GK-catalyzed phosphorylation of glycerol analogues as a function of time where C_t = the concentration of product at time t and C_0 = the concentration of original starting material. Substrates examined were glycerol (●), ethylene glycol (■), DL-3-chloropropane-1,2-diol (▼), DL-butane-1,2,4-triol (▲), and DL-3-methoxypropane-1,2-diol (○).

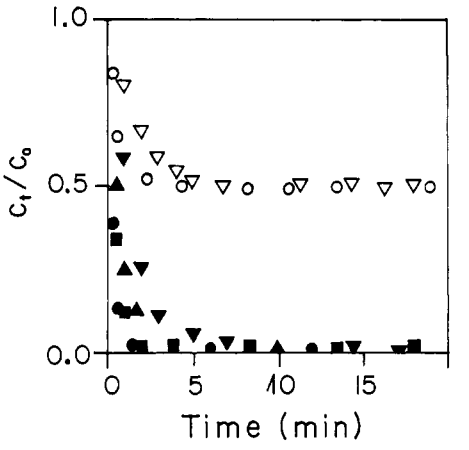

Figure 2. Reaction of phosphorylated species with NAD catalyzed by GDH: *syn*-glycerol-3-phosphate, 50 mM (●); D-3-chloropropane-1,2-diol-1-phosphate, 73 mM (■); D-3-aminopropane-1,2-diol-3-phosphate, 41 mM (▼); D-3-mercaptopropane-1,2-diol-1-phosphate, 28 mM (▲); DL-glycerol-1-phosphate, 25 mM (○); DL-3-aminopropane-1,2-diol-3-phosphate, 32 mM (▽).

contained the configuration found for *sn*-glycerol-3-phosphate,[18,19] and comparison of this number with a quantitative ^{31}P NMR assay that determined the total yield of organic phosphate (called here the "chemical yield").[20] The GDH assay measured the quantity of NADH produced on oxidation catalyzed by GDH. All of the

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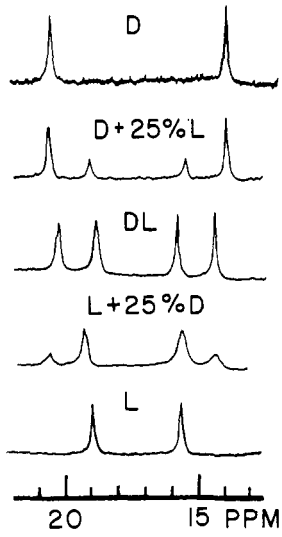


Figure 3. The ^{1}H NMR spectra of the hydroxyl protons of D, L, and DL-3-chloropropane-1,2-diol in the presence of Eu(hfc)$_3$.

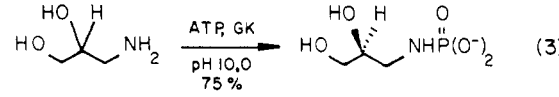
phosphorylated products described in this paper (with the exception of L-**5**-P) were substrates for GDH. The quantitative ^{31}P NMR assay measured the integrated area of the signal of the product relative to that of an internal standard.[21] The two procedures were in good agreement (±3%). Enantiomeric excesses of the phosphorylated products were estimated by straightforward extensions of the enzymatic techniques used to assay *sn*-glycerol-3-phosphate. GDH has been demonstrated to be highly selective for one enantiomer of DL-glycerol-3-phosphate and two of its analogues (eq 2).[18,19] Reactions used to assay the enantiomeric excesses reported in Table I were carried out using a 10-fold excess of NAD at a concentration ([NAD]$_0$ = 0.8 mM) slightly above the Michaelis constant ($K_{m,NAD}$ = 0.38 mM).[18] We did not explicitly measure values of K_m for the analogues of *sn*-glycerol-3-phosphate with GDH. Kinetic plots (Figure 2) for the reactions showed, however, that the consumption of organic phosphate reached a plateau at around ~97% of the total organic phosphate calculated to be present on the basis of NMR spectroscopy. No evidence for unreacted organic phosphate or for slowly reacting components was observed in the GDH assays. In the case of D-3-chloropropane-1,2-diol-1-phosphate the enantiomeric purity was established independently by the use of chiral shift reagents.[22] The phosphate ester group was cleaved by treatment with alkaline phosphatase and the phosphate ion removed by precipitation. The NMR spectrum of the resulting diol was examined in the presence of Eu(hfc)$_3$[23] in acetonitrile. This technique differentiated the hydroxyl groups of the two enantiomers of the vicinal diols. Acetonitrile rather than conventional solvents was chosen for this assay for three reasons. First, the ^{1}H NMR chemical shifts of the two hydroxyl groups of the two enantiomers are clearly distinguished in the presence of Eu(hfc)$_3$ in this solvent (Figure 3). Second, the solubility of the vicinal diols in question (3-chloropropane-1,2-diol, 3-aminopropane-1,2-diol, etc.) is greater in acetonitrile than in solvents normally used with shift reagents. Third, the diols are hydrophilic and difficult to obtain in anhydrous form; the use of acetonitrile minimizes or elminates the interfering precipitates caused by the reaction of the europium salts with small amounts of contaminating water.

The unphosphorylated products of eq 1 were isolated only in *two* cases: L-3-chloropropane-1,2-diol (L-**1**) and L-butane-1,2,4-triol (L-**5**). In both instances, chemical recoveries were low; the 38% yield for **1** reported in Table I, for example, represents 38% of the theoretical 0.5 equiv of L enantiomer present in the DL mixture. These low chemical yields reflect the difficulty of isolating diols and triols from relatively dilute aqueous solutions.[24] Enantiomeric excesses for these compounds were estimated primarily by comparison of optical rotations with reported literature values. In the case of L-**1**, Eu(hfc)$_3$ provided an independent estimate.

The procedures leading to phosphorylated products give, in general, good yields and high enantiomeric excesses. The results for unphosphorylated species are less satisfactory. Although the enantiomeric excesses are adequate in the two cases examined, in general they are, as expected, lower than for the phosphorylated products. The use of 0.50 equiv of PEP per equiv of racemic substrate limits overphosphorylation and facilitates isolation of the phosphorylated product by ensuring that no PEP remains unreacted at the conclusion of the reaction. These considerations are especially important for compound **2** since L-**2**-P is labile and generates **2** in decomposing. If, however, any ATP, PEP, or phosphorylated product hydrolyzes, the regeneration system contains insufficient phosphoryl donor to phosphorylate the D enantiomer completely. In this circumstance, the enantiomeric excess of the remaining (nominally L) enantiomer is lowered, but the D (phosphorylated) enantiomer still has an enantiomeric excess determined only by the enantioselectivity of the enzyme-catalyzed reaction.

Certain properties of compounds listed in Table I deserve brief mention. Compound D-**1**-P, 3-chloropropane-1,2-diol-1-phosphate, is stable in solution for months at room temperature and pH 7. In strongly acidic solution (pH <1) ^{31}P NMR spectroscopy indicates that phosphate equilibrates between the C-1 and C-2 hydroxyl groups; the equilibrium mixture appears to contain ~80% C-1 and ~20% C-2 phosphorylated substances.[25] Both positional isomers are substrates for alkaline and acid phosphatase. In basic solution (pH >14) D-**1**-P converts to D-glycidol phosphate in high (~quantitative) yield. The facile reaction of D-**1**-P with strong nucleophiles is illustrated by its quantitative conversion to D-3-aminopropane-1,2-diol on treatment with aqueous ammonia.

The product obtained on phosphorylation of DL-3-aminopropane-1,2-diol was the corresponding phosphoramidate (eq 3). The preparation and isolation of the phosphoramidate can be complicated by its reactivity. Kühne has demonstrated that



(3)

aqueous phosphoramidates react in aqueous triethanolamine buffer (pH 7–10) and generate *O*-phosphorylated products derived from phosphorylation of the buffer.[26] Our reactions were carried out at pH 10.0 in water containing no buffer. D-**2**-P is labile ($t_{1/2}$ = 3 h, pH 7.0) at neutral pH and stable at alkaline pH ($t_{1/2}$ = 14 days, pH 10). The presence of magnesium ion further decreases the stability of the amidate. It was thus necessary to restrict manipulations of this compound to values of pH about 9.5. By working at these high values, however, several problems arise. The stability and activity of the glycerol kinase are reduced under these conditions (to 30 and 50% of the stability and activity at pH 7),[5] and magnesium ion required for enzymatic activity is removed by precipitation as magnesium hydroxide and magnesium phosphate. The N-phosphorylated derivative of D-3-aminopropane-1,2-diol is a substrate for both alkaline phosphatase and GDH.

(21) The internal standard had been standardized against DL-glycerol-3-phosphate (Glonek, T. *J. Am. Chem. Soc.* **1976**, *98*, 7909–2. Glonek, T.; Van Wazer, J. R. *J. Phys. Chem.* **1976**, *80*, 639–43.

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(25) Phosphorylation of DL-3-chloropropane-1,2-diol with phosphorus oxytrichloride yields a 1:1 mixture of primary and secondary phosphorylated alcohols (according to the procedure described in the Experimental Section for DL-3-aminopropane-1,2-diol-3-phosphate). When the reaction product is hydrolyzed under acidic conditions at 0 °C, pH <1, 2 h, the organic phosphate rearranges and the isolated product contains 80% primary and 20% secondary phosphate. Prolonged reaction time (24 h) did not alter the ratio between primary and secondary phosphates.

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The preparation of D-3-P was straightforward, except that the thiol group was sensitive to autoxidation. Oxidation of this substance to its disulfide dimer was, however, readily reversed by reaction with sodium borohydride or 2-mercaptoethanol. Alkaline phosphatase accepted both the thiol and the disulfide as substrate.

Compound **4** was notable only in that it induced varying degrees of ATPase activity in both immobilized and soluble glycerol kinase. This ATPase activity was highest for the soluble enzymes at high concentrations of **4** (>250 mM) and low pH (pH <7).

The synthesis of compound **5** illustrates the manipulations necessary to phosphorylate a poor substrate ($V_{max} \simeq 0.02V_{max,glycerol}$). The reaction was straightforward, although it required large quantities of enzyme. The product, D-**5**-P, was not a substitute for GDH. The absolute configuration of the product from the GK-catalyzed phosphorylation was established by two pieces of evidence. First, chiral D- and L-butane-1,2,4-triol were prepared chemically (by reduction of D- and L-malic acids[27]) and tested for activity with GK. Only the triol derived from D-malic acid showed substrate activity. Second, the optical rotation of the recovered unphosphorylated enantiomer is that corresponding to L-butane-1,2,4-triol.[28]

Discussion

The utility of glycerol kinase as a catalyst in organic synthesis is determined by three factors: first, the range of structures which glycerol kinase will accept as substrate; second, the enantioselectivity of the phosphorylations glycerol kinase catalyzes; third, the practicality of the operations (the cost of the enzyme, the volume of solution required to produce a given quantity of product, the stability of the product under the reaction conditions, and related matters) involved in conducting reactions catalyzed by glycerol kinase. A previous paper has explored structural features required for useful activity. In brief, the conclusion from these studies was that glycerol kinase accepts a range of substituents at the C-3 position of the nominal glycerol moiety (that is, the terminal position that is not phosphorylated),[4] limited substitution at C-2, and little or no substitution at C-1 (the terminal position that is phosphorylated).[4] GK-catalyzed phosphorylation is clearly the best preparative route to *sn*-glycerol-3-phosphate and dihydroxyacetone phosphate.[5,6,9,17] For the unnatural substrates summarized in Table I, the scale and practicality of reaction is limited primarily by V_{max}, and thus by the cose of the quantity of enzyme required to achieve useful reaction velocities. For glycerol kinase, product inhibition and deactivation of the enzyme by high concentrations of reactants and products are not limiting factors in the concentration range in which we have conducted reactions (0.1–0.5 M).[29] Based on experience with the compounds in Table I, we found that it is practical to carry out syntheses of up to 10 g of product in convenient laboratory-scale reactions using substrates having activity as low as 1% that of glycerol.

We believe that the phosphorylation reaction is enantiospecific,[2,3] and that the phosphorylated products listed in table I are enantiomerically pure (that is, as enantiomerically pure as is *sn*-glycerol-3-phosphate, a compound which is presumed to be produced by GK-catalyzed phosphorylation of glycerol in 100% ee[2,3]). This conclusion is based on the observation that these phosphorylated products are substrates for glycerol-3-phosphate dehydrogenase—itself a highly enantiospecific enzyme[18,19]—and that they appear to be completely consumed in reaction. The chemical purities (as determined by quantitative ^{31}P NMR) were in agreement with the enantiomeric purities (±~3%) determined by the assay based on glycerol-3-phosphate dehydrogenase. Further, most of the compounds had been prepared previously

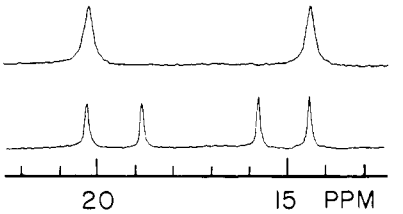

Figure 4. The ^1H NMR spectrum of the hydroxyl protons in the presence of Eu(hfc)$_3$ of D-3-chloropropane-1,2-diol generated by enzymatic hydrolysis of D-3-chloropropane-1,2-diol-1-phosphate produced by GK-catalyzed phosphorylation (a) and DL-chloropropane-1,2-diol (b).

on a 0.1–0.5-mmol scale using soluble enzymes. The chemical purities of these preparations are often 97–100% (±3%) as determined by GDH assay. It hereby follows that a maximum of 3% of the L enantiomer can be present in the product and that the minimum enantiomeric excess if 94%. We believe the values of ee are considerably greater than this estimate, and note that D-3-chloropropane-1,2-diol-1-phosphate generated in 84% purity, yielded, after enzymatic hydrolysis of the organic phosphate, D-3-chloropropane-1,2-diol. Analysis of the % ee of this substance by chiral shift reagent also showed no detectable L enantiomer (<3%, >94% ee, Figure 4). The unphosphorylated L enantiomers were, not surprisingly, obtained with lower enantiomeric excesses than the phosphorylated products. The stratagem used in carrying out these reactions (that is, to use exactly 0.50 equiv of phosphoenol pyruvate and ~0.05 equiv of ATP in order to limit the number of phosphate-containing organic species present in completed reaction solution, and thus to simplify purifications) will not give complete phosphorylation of the D enantiomer if significant hydrolysis of product, ATP, or PEP occurs during the course of the reaction. The residual D enantiomer present at the end of the reaction will, of course, lower the enantiomeric excess of the isolated unphosphorylated L enantiomer. In the event that the L enantiomer is desired in high enantiomeric purity, using more than 0.55 equiv of phosphoryl donors and allowing the reaction to proceed to completion should yield L enantiomer in higher enantiomeric excess. This conceptual stratagem was tested experimentally with DL-3-methoxypropane-1,2-diol and DL-3-aminopropane-1,2-diol. The phosphorylation of DL-3-methoxypropane-1,2-diol is complicated by concurrent ATPase activity in the glycerol kinase. We hoped the use of 0.65 equiv of PEP would substantially increase the extent of phosphorylation of D-3-methoxypropane-1,2-diol. In fact, we observed only an insignificant increase (2%) in yield of phosphorylated product and a large increase in the quantity of inorganic phosphate produced. We therefore conclude that only modest increases in the extent of phosphorylation of the D enantiomer (1–5%) and in the enantiomeric purity of the L enantiomer can be achieved by this method for the particular case of 3-methoxypropane-1,2-diol. Nonetheless, we recommend the use of a slight excess of PEP (0.51–0.52 equiv) when the L enantiomer is desired in high enantiomeric purity. The lability of D-3-aminopropane-1,2-diol-3-phosphate required a different approach. The amidate was isolated when its decomposition became appreciable in the reaction, and after recovering the amine the phosphorylation was continued in consecutive reactions using the recovered enzymes. The extent of phosphorylation of D-3-aminopropane-1,2-diol was considerably increased (from 69 to 85%) by two consecutive reactions.

Alkaline phosphatase catalyzes hydrolysis of the phosphorylated D enantiomer and provides a route to enantiomerically pure unphosphorylated material. This transformation was demonstrated on a 2-mmol scale with D-3-chloropropane-1,2-diol-1-phosphate using soluble phosphatase. The % ee of the D-3-chloropropane-1,2-diol recovered from this reaction was >94% (see Table I).

Several of the compounds prepared in this study are of specific interest. The conversion of **2** to its N-phosphorylated product, D-**2**-P, at high pH provides an example of the use of an enzyme as a catalyst under nonphysiological conditions. The corresponding O-phosphorylated material, D-3-aminopropane-1,2-diol-1-phosphate, was prepared from D-3-chloropropane-1,2-diol-1-phosphate

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and ammonia. The acid lability of D-2-P is such that it will probably be of only specialized interest as a starting material for the preparation of phospholipid analogues; the O-phosphorylated isomer should, however, provide entry to useful, nonhydrolyzable amide analogues of phospholipids. Other compounds listed in Table I, especially D-3-chloropropane-1,2-diol-1-phosphate and D-3-mercaptopropane-1,2-diol-1-phosphate, are interesting for their relevance as antifertility agents.[30] DL-3-Chloropropane-1,2-diol has potent activity as an antifertility agent in several mammals and is currently sold as a rat poison (Epibloc).[30] The mechanism of action is poorly understood, and three distinct metabolic routes with three active metabolites have been proposed.[30] D-3-Chloropropane-1,2-diol-1-phosphate was suggested as one of these active metabolites and glycerol kinase was proposed to be the enzyme that catalyzed the conversion of D-3-chloropropane-1,2-diol to the corresponding organic phosphate.[31] This suggestion was rejected on the basis of evidence[32] that was interpreted as demonstrating that D-3-chloropropane-1,2-diol was not a substrate for glycerol kinase.[32,33] Our evidence indicates that this interpretation was incorrect. Labeled D-3-chloropropane-1,2-diol has subsequently been shown to be converted in vivo to labeled β-chloroacetic acid and to labeled β-chlorolactaldehyde.[34] This oxidative pathway is currently believed to be that reponsible for its biological activity. Our demonstration that D-3-chloropropane-1,2-diol is a substrate for glycerol kinase is not relevant to the correctness of this proposal for the primary mode of action of the antifertility agent. It may, however, explain some of the additional biological effects associated with administration of DL-3-chloropropane-1,2-diol.[30,32]

We conclude from this study that glycerol kinase is a practical catalyst for the enantioselective phosphorylation of a number of unnatural analogues of glycerol. The phosphorylated products are potentially useful as chiral synthons, both for phospholipids[8] and, after removal of the phosphate group, for other chiral substances.[35,36] Synthetic routes from *sn*-glycerol-3-phosphate to phospholipids are well developed (i.e., for glycerol phosphatidic acid derivatives,[8] glycerol phosphorylcholine derivatives,[37] glycerol phosphorylserine derivatives,[38] and glycerol phosphorylethanolamine derivatives[39]). Similar routes should work with many of the *sn*-glycerol-3-phosphate analogues described here.[8,40]

The question of whether glycerol kinase catalyzed phosphorylation provides the *best* route to any of the compounds in Table I remains to be established. It seems unlikely that other routes will compete in ease and efficiency for enantiospecific phosphorylation of glycerol itself. Several of these compounds could, however, be prepared by alternative routes. For example, the unphosphorylated precursors might be resolved independently (by conventional chemical resolution,[41] by lipase-[42,43] or esterase-catalyzed[44,45] enantioselective hydrolysis, or by chiral reduction[46]) or prepared by chiral synthesis (from, for example, chiral epoxides[47]). Chemical phosphorylation might then provide simpler routes to the phosphorylated species discussed here. Without carrying out analysis and comparisons of applicable synthetic techiques on a case-by-case basis, it is impractical to judge the relative merits of possible synthetic techniques. In fact, it is improbable that any single synthetic technique will prove best in all instances.

Experimental Section

General. Chemicals were reagent grade and were used without further purification. Phosphoenolpyruvate[11] and acetyl phosphate[12] were prepared as described previously. Glycerol kinase from *S. cerevisiae* was obtained from Genzyme, Inc. (Boston, MA). Other enzymes and biochemicals were obtained from Sigma unless otherwise specified. Water was distilled twice, the second time from glass. Spectrophotometric measurements were performed at 25 °C using a Perkin-Elmer Model 552 spectrophotometer equipped with a constant-temperature cell. Phosphorus NMR measurements were routinely carried out at ~30 °C on a 40.48-MHz (23-T) Varian instrument in 12-mm NMR tubes. Samples (nominally 0.05 M) contained 20% deuterium oxide as an internal lock. Chemical shifts for ^{31}P are reported relative to external H_3PO_4; downfield shifts are positive. Accumulation parameters used for assays were: pulse angle, 20°; pulse delay, 1.5 s. These conditions gave adequate spectra after 100 transients. Quantitative ^{31}P NMR measurements were carried out using a 90° pulse angle and 2-min pulse delays,[21] and in internal standard of known concentration (sodium phosphate).[21] Carbon-13 NMR spectroscopy was routinely performed on a 67.8-mHz (63-T) Jeol spectrometer in a 5-mm NMR tube. Samples (100–200 mg/mL) were dissolved in deuterium oxide and ^{13}C chemical shifts were reported relative to external sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). The spectra were completely proton decoupled; a pulse angle of 90° and a pulse delay of 1.45 s were used.

Immobilizations of enzymes in PAN gel were carried out following the procedures described previously for GK [E.C. 2.7.1.30], PK [E.C. 2.7.1.40], and AK [E.C. 2.7.2.1].[16]

The enzymatic reactions were carried out in three-necked, round-bottomed flasks, which were modified to accommodate a pH electrode. The pH of the reaction mixtures was controlled when necessary with a Horizon pH controller (Ecology Co., Chicago) coupled to an LKB 10200 peristaltic pump. Prior to the addition of the immobilized enzymes, all solutions were deoxygenated with argon using a gas dispersion tube. The enzymatic reactions were conducted at room temperature under an argon atmosphere unless otherwise specified. At the end of each reaction the solutions containing the enzyme-containing gels were transferred to 250-mL centrifuge bottles, and the gels were compacted by low-speed (3000 rpm) centrifugation. The supernatant was decanted and the gels were washed with deoxygenated water and compacted by centrifugation.

Determination of Chemical Purity by ^{31}P NMR Spectroscopy. The phosphorus-containing compounds were assayed quantitatively by integration of their ^{31}P NMR signals and comparison of the observed intensities with that of an internal standard of known concentration.[20] The ^{31}P NMR spectra were recorded in 20% deuterium oxide (pH 7–9) using a pulse angle of 90° and a pulse delay of 2 min.[20] The data were processed twice and the average was reported. A representative sample solution was prepared as follows. D-3-Chloropropane-1,2-diol-1-phosphate barium salt (155 mg) was dissolved in water (3 mL) by addition of excess sodium sulfate (225 mg). The precipitated barium sulfate was removed by centrifugation, and the precipitate was washed with deuterium oxide (0.8 mL). After precipitate was removed from the deuterium oxide, the supernatants were combined in a 12-mm NMR tube and a ^{31}P NMR spectrum was recorded. An internal standard, inorganic phosphate, was added (in the case of DL-3-chloropropane-1,2-diol-1-phosphate, 0.5 mL of 0.4 M monosodium phosphate solution), and another ^{31}P NMR spectrum was recorded. The intensities in the ^{31}P NMR spectra were measured and the purity of the organic phosphate was calculated based on the inorganic phosphate used as internal standard. The inorganic phosphate used as internal standard had previously been calibrated against DL-glycerol-1-phosphate.[21] The sensitivity of the method was examined by adding increasing amounts of inorganic phosphate to a sample solution containing known amounts of DL-glycerol-1-phosphate disodium salt. This assay was able to detect inorganic phos-

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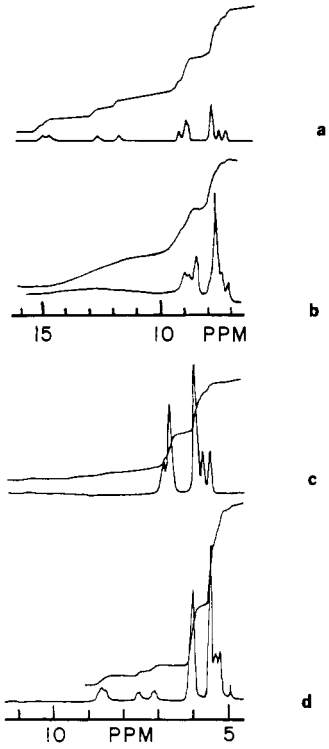

Figure 5. Assignments in the ^1H NMR spectrum of DL-3-chloropropane-1,2-diol used for determinations of enantiomeric excess: (a) DL-3-chloropropane-1,2-diol (reference sample); (b) with acetic acid added to the reference sample; (c) with DL-3-chloropropane-1,2-diol-d_2 substituted for DL-3-chloropropane-1,2-diol; (d) with DL-3-chloropropane-1,2-diol was added to (c).

phate at a concentration equal to 2 mol % of the major phosphate component (DL-glycerol-3-phosphate). Impurities were therefore detected at the level of 2 mol %, but the overall accuracy of the quantitative determinations was not higher than 5%.

Determinations of the Enantiomeric Excesses by Assays Based on Glycerol-3-phosphate Dehydrogenase (GDH). The procedure for quantitative determinations of *sn*-glycerol-3-phosphate was based on the glycerol-3-phosphate dehydrogenase catalyzed reduction of NAD. Since most of the *sn*-glycerol-3-phosphate analogues are substrates for glycerol-3-phosphate dehydrogenase, the procedure for assay of *sn*-glycerol-3-phosphate was expanded to include these analogues. The assay was carried out as described previously with minor modifications.[18,19] A representative assay for D-3-aminopropane-1,2-diol-3-phosphate follows. A buffer containing hydrazine and glycine (2.00 mL, 0.4 M hydrazine, 0.5 M glycin, pH 9.8) and NAD (0.1 mL, 31 mM) (20 μL, 20 U) was placed in a 3-mL cuvette. The cuvette was equilibrated for 2–4 min at 25 °C; glycerol-3-phosphate dehydrogenase was added. The absorbance at 340 nm was recorded (ϵ_{NADH} = 6220 M^{-1} cm^{-1}). When a steady absorbance level (baseline) was reached, a small volume (0.1 mL) of the sample solution was added to the assay solution and the increase in absorbance was recorded. After 5 to 10 min no further increase was observed. The sample solution contained D-3-aminopropane-1,2-diol-3-phosphate barium salt (32 mg) dissolved in water (25 mL). The assays were run in duplicates (or triplicates if more than 3% variation was observed in the first two determinations). The absorbance at 340 nm was measured against a blank, although this control appeared not to be critical for the determinations. In the blank, water was substituted for the sample solution.

Determination of Enantiomeric Excess of D- and L-3-Chloropropane-1,2-diol Using Europium(III) Shift Reagents. The enantiomeric excess of 3-chloropropane-1,2-diol was conveniently determined using tris[3-(heptafluoropropylhydroxymethylene)-*d*-camphorato]europium(III) ($Eu(hfc)_3$) in CD_3CN.[20,21] We employed a 2:1 ratio of shift reagent to diol. Enantiomers of substituted[48] vicinal diols were found to be clearly differentiated without any chemical modifications. The shifts of *OH* signals and the integration of the peaks suggests the signals demonstrating the enantiomeric excess are the hydroxyl protons. This assignment was supported by the following two observations (Figure 5). First, replacement of the hydroxyl protons in DL-3-chloropropane-1,2-diol with deuterium removes the observed signals from the NMR spectra. Addition of DL-3-chloropropane-1,2-diol to the above sample reintroduces the four hydroxyl signals. Second, the signals from protons of the hydroxyl group disappear after addition of acetic acid; the signals became so broad they were barely distinguished from the baseline.

A representative determination of enantiomeric excess is as follows. Dry DL-3-chloropropane-1,2-diol (5 μL, a commercial sample dried overnight at 1 torr over $CaSO_4$) was dissolved in 0.6 mL of acetonitrile-d_3 in a 5-mm NMR tube (the commercial solvents were used without further manipulations). A ^1H NMR spectrum was recorded. Approximately 40–100 mg of $Eu(hfc)_3$ was added directly as a solid to the NMR tube. The shift reagent was dissolved by shaking the NMR tube and a ^1H NMR spectrum was recorded; the integration of the hydroxyl group peaks measures the relative amounts of D and L enantiomers.

***sn*-Glycerol-3-phosphate (*sn*-Gly-3-P).**[17] A 2-L aqueous solution of glycerol (92.1 g, 1 mol), ATP (4.4 g, 8.0 mmol), magnesium chloride (6.1 g, 30 mmol), and 2-mercaptoethanol (0.5 mL, 7 mmol) was adjusted to pH 7.0 and deoxygenated. A suspension of immobilized glycerol kinase (600 U) and acetate kinase (800 U) was added and the mixture was stirred magnetically at ambient temperature under argon. Disodium acetyl phosphate (1.2 L, 1.1 mol) kept at 0 °C was added via a peristaltic pump over 5 days. The pH was kept at 7.0 (±0.5) using a pH controller with automatic addition of 4 M sodium hydroxide solution. The reactor was left for 2 days after completing the addition of acetyl phosphate. At this time enzymatic assay showed 97% conversion of glycerol to *sn*-glycerol-3-phosphate. The solution was separated from the enzym-containing gel by decantation followed by centrifugation. The enzymes were washed with 200 mL of deoxygenated water and centrifuged; the supernatant was added to the reaction solution. The solution was passed through charcoal (~50 g) to remove ATP and concentrated at 1 torr to 0.5–1 L. A saturated solution of barium chloride dihydrate (48.8 g, 0.200 mol) was added, and the precipitate, consisting primarily of barium phosphate, was separated by filtration. Additional barium chloride dihydrate (244 g, 1.00 mol) and ethanol (3 L) were added to precipitate the barium salt of *sn*-glycerol-3-phosphate. The precipitate was allowed to consolidate for 2 days to facilitate filtration. After drying at 1 torr over $CaSO_4$, a total of 0.92 mol (92%) of *sn*-glycerol-3-phosphate was obtained (314 g of solid containing 90% barium *sn*-glycerol-3-phosphate as determined by enzymatic assay): ^{13}C NMR (D_2O, pH ~12) δ (DSS) 76.8 (d, $^3J_{CCOP}$ = 6.2 Hz), 73.1 (d, $^2J_{COP}$ = 3.5 Hz), 68.9 (s); ^{31}P NMR (D_2O, pH ~12) δ 4.2 (t, $^3J_{POCH}$ = 6.4 Hz); $[\alpha]^{25}_D$ −1.2° (*c* 2.5, H_2O, pH ~0). The turnover number for ATP during the synthesis was 115, and the activities of enzymes recovered in the gel were: GK 95%; AK 83%.

Dihydroxyacetone Phosphate (DHAP).[17] The reaction was repeated for three consecutive runs of approximately 0.32 mol. A total of 0.95 mol of DHAP was generated. To a 1 L of deoxygenated solution containing dihydroxyacetone (36.0 g, 0.400 mol), ATP (2.2 g, 4.0 mmol), magnesium chloride (1.6 g, 8.0 mmol), and 2-mercaptoethanol (0.3 mL, 4 mmol) were added PAN-immobilized glycerol kinase (1500 U, determined with glycerol as substrate) and PAN-immobilized acetate kinase (1700 U). To this mixture a deoxygenated solution of acetyl phosphate (410 mL, 0.45 mol) was added using a peristaltic pump over 16 h. The mixture was stirred at room temperature under argon, and the pH was automatically controlled at 6.7–7.0 by addition of aqueous NaOH (4 M) through a peristaltic pump. The reaction was stopped when enzymatic assay indicated 98% conversion (16 h). After the reaction solution was separated from the enzyme-containing gel, the solution was filtered through charcoal (~25 g). Quantitative ^{31}P NMR was used to determine the concentration of inorganic phosphate in the reaction mixture, and a solution of barium chloride dihydrate (14.6 g, 60 mmol) was added at pH 6 to precipitate the phosphate. After the precipitate was removed by filtration, the dihydroxyacetone phosphate was precipitated by addition of barium chloride dihydrate (98 g, 0.40 mol) and 3 volumes of ethanol. Drying at 1 torr over $CaSO_4$ resulted in 116 g of product (0.33 mol, 87% pure, calculated as Ba·DHAP by enzymatic assay, overall yield 83%): ^{13}C NMR (D_2O, pH 2–3) δ (DSS) 213.5 (d, $^3J_{CCOP}$ = 8.8 Hz), 98.8 (d, $^3J_{CCOP}$ = 8.8 Hz), 72.2 (d, $^2J_{COP}$ = 4.4 Hz), 71.1 (d, ^2J = 4.4 Hz), 69.6 (s), 68.0 (s); ^{31}P NMR (D_2O, pH 9.5) δ 4.2 (t, $^3J_{POCH}$ = 6.3 Hz), 3.8 (t, $^3J_{POCH}$ = 7.2 Hz). The turnover number for ATP was 83 and the activities for the enzymes recovered at the conclusion of the three runs were: GK, 62%; AK, 30%.

D-3-Chloropropane-1,2-diol-1-phosphate (D-1-P). PEP^-K^+ (10.9 g, 50.0 mmol), ATP (1.6 g, 2.5 mmol), magnesium chloride (1.5 g, 7.5 mmol), 2-mercaptoethanol (100 μL, 1.4 mmol), and DL-3-chloro-

(48) This technique was found to work for substituted vicinal diols when the substituent is larger than a methyl group. DL-Propane-1,2-diol did not show sufficiently large shifts to completely separate the two enantiomers in this technique. One hydroxyl group can be substituted with an amine group or a carboxylic acid group. No separation was observed when one hydroxyl group was substituted with an ester group.

propane-1,2-diol (8.4 mL, 100 mmol) were dissolved in 130 mL of water. The pH of the solution was adjusted to 7.5 with 4 N NaOH. The reaction was started by the addition of immobilized GK (1300 U, determined with glycerol as substrate) and PK (500 U). The reaction was monitored by ^{31}P NMR; aliquots (3-mL) were removed from the reactor and added to 1.5–2.0 mL of deuterium oxide in a 12-mm NMR tube, and a ^{31}P NMR spectrum was recorded. After 5 days the reaction was complete. The supernatant was separated from the immobilized enzymes by centrifugation and decantation, and the enzyme-containing gel particles were washed once with 50 mL of deoxygenated water. The wash solution and the supernatant were combined and passed through charcoal (~25 g) to remove ADP and ATP. Barium chloride dihydrate (13.4 g, 55.0 mmol) was added, and a fine precipitate formed. To this suspension was added 5 volumes of 95% ethanol. The precipitate was allowed to settle, separated by filtration, and dried at 1 torr overnight over $CaSO_4$. A total of 18.1 g of white solid was isolated. The purity was 84% (that is, it contained 84% D-**1**-P·Ba) as determined by enzymatic assay and by quantitative ^{31}P NMR spectroscopy; this purity implies the isolation of 47 mmol of D-**1**-P·Ba, and a 94% yield based on D-3-chloropropane-1,2-diol: ^1H NMR (D_2O, pH 9.5) δ (DSS) 4.0–4.1 (1 H, m), 3.6–3.9 (4 H, m); ^{13}C (D_2O, pH 9.5) δ (DSS) 75.2 (d, $^3J_{CCOP}$ = 5.5 Hz), 69.7 (d, $^2J_{COP}$ = 3.6 Hz), 50.8 (s); ^{31}P NMR (D_2O, pH 9.5) δ 4.4 (t, $^3J_{POCH}$ = 6.8 Hz); $[\alpha]^{25}_D$ +3.3° (*c* 2.5, H_2O, pH ~0). The turnover number for ATP during the synthesis was 20. The enzymatic activities recovered in the gel were: GK, 75%; PK, 82%.

D-3-Chloropropane-1,2-diol-1-phosphate (D-1-P) (Large Scale). PEP^-K^+ (127 g, 0.600 mol), ATP (4.0 g, 7.0 mmol), magnesium chloride (15.3 g, 75.0 mmol), 2-mercaptoethanol (0.2 mL, 3 mmol), and DL-3-chloropropane-1,2-diol (133 g, 1.20 mol) were dissolved in 2 L of deoxygenated water. The pH of the solution was adjusted to 7.2 with NaOH (~30 g of solid NaOH and 4 M NaOH). The reaction was started by the addition of immobilized GK (10 000 U determined with glycerol as substrate) and PK (1000 U). The reaction was monitored by ^{31}P NMR: aliquots (3 mL) were removed from the reactor and added to 1.0–2.0 mL of deuterium oxide in a 12-mm NMR tube. After 7 days the reaction was complete, but the reaction was continued for 2 additional days. The supernatant was separated from the immobilized enzymes by centrifugation and decantation, and the enzyme-containing gels were washed twice with 200-mL portions of deoxygenated water. The wash solutions and the supernatant were combined (total 2.6 L) and passed through charcoal (~100 g) to remove ADP and ATP. Barium chloride dihydrate (159 g, 0.65 mol) was added and a precipitate formed. Ethanol (95%, 8 L) was added to this suspension and the precipitate was allowed to settle overnight. The solid was isolated by filtration and dried at 1 torr overnight over $CaSO_4$. A total of 203 g of white solid was isolated. The solid contained 92% of D-**1**-P·Ba as determined by enzymatic assay (90% D-**1**-P·Ba as determined by quantitative ^{31}P NMR) and 3% inorganic phosphate. The yield was 93% based on D-3-chloropropane-1,2-diol: ^1H NMR (D_2O, pH 7) δ (DSS) 4.0–4.1 (1 H, m), 3.6–3.9 (4 H, m); ^{13}C NMR (D_2O, pH 7) δ (DSS) 75.1 (d, $^3J_{CCOP}$ = 7.3 Hz), 69.6 (d, $^2J_{COP}$ = 3.6 Hz), 50.7 (s); ^{31}P NMR (D_2O, pH 7.2) δ 3.9 (t, $^3J_{POCH}$ = 6.6 Hz); $[\alpha]^{25}_D$ +3.3° (*c* 2.5, H_2O, pH ~0). The turnover number for ATP during the synthesis was 85. The recovered enzyme activities were as follows: GK, 76%; PK, 81%.

D-Glycidol Phosphate. D-**1**-P·Ba (500 mg, 1.6 mmol) was dissolved in 4 mL of deuterium oxide at 0 °C by addition of Dowex 50W-X8 resin. The Dowex 50W-X8 resin was removed by filtration, and the pH was increased to ≳14 with 4 N NaOH. After ~15 min at room temperature all the D-**1**-P was converted to D-glycidol phosphate. The pH was then immediately reduced ($t_{1/2}$ ~1 h at pH ≥ 14) to ~7.5 by addition of solid $NaHCO_3$ (to buffer the solution) followed by 2 N HCl. The aqueous D-glycidol phosphate was stored at 4 °C until use. No other product was observed by ^{31}P NMR: ^1H NMR (D_2O, pH >14) δ (DSS) 4.4–4.55 (1 H, m), 3.65–3.85 (1 H, m), 3.3–3.45 (1 H, m), 2.7–2.85 (1 H, dd, *J* = 5 Hz), 2.5–2.65 (1 dd, *J* = 4, 5 Hz); ^{13}C NMR (D_2O, pH ~14) δ (DSS) 65.3 (d, $^2J_{POC}$ = 4.3 Hz), 53.5 (D, $^3J_{POCC}$ = 7.7 Hz), 46.3 (s); ^{31}P NMR (D_2O, pH ~14) δ 4.1 (t, $^3J_{POCH}$ = 6.1 Hz).

D-3-Aminopropane-1,2-diol-1-phosphate. D-3-Chloropropane-1,2-diol-1-phosphate barium salt (600 mg, 1.75 mmol) was added to water (5 mL) and cooled in an ice bath. Dowex 50W-X8 was added until the organic phosphate dissolved (pH <2). The Dowex 50W-X8 beads and the faint yellow color of the solution were removed by passing the solution through charcoal. The Dowex 50W-X8 resin was washed by passing an additional 20 mL of water through the charcoal. The D-3-chloropropane-1,2-diol-1-phosphate solution was added dropwise to a stirred solution of ammonium hydroxide (25 mL) placed in an ice bath. After the end of this addition the solution was stirred for 30 min at 0 °C, the ice bath was removed, and the reaction solution was stirred for 1 h at ambient temperature. The excess ammonia and water are removed by evaporation at 40–50 °C, and the resulting oil was dissolved in 2 mL of water. Barium chloride dihydrate (0.44 g, 1.8 mmol) and ethanol (20 mL, 95%) were added to precipitate the D-3-chloropropane-1,2-diol-1-phosphate monobarium salt. The suspension was stirred for 15 min at 0 °C, and the solid was separated by centrifugation. The supernatant was discarded. The solid was washed with ethanol (10 mL, 95%), and, after centrifugation, the solid was dried over $CaSO_4$ at 1 torr overnight. The resulting barium D-3-aminopropane-1,2-diol-1-phosphate (0.50 g, 94%) was more than 95% pure as determined by quantitative ^{31}P NMR (no other phosphate-containing compound was observed): ^{13}C NMR (D_2O, pH 13) δ (DSS) 76.8 (d, ^3J = 5.5 Hz), 70.5 (d, ^2J = 3.7 Hz), 47.6 (s); ^{31}P NMR (D_2O, pH 10) δ 4.5 (t, ^3J = 6.3 Hz).

A similar preparation was carried out for DL-3-chloropropane-1,2-diol-1-phosphate on a 1-mmol scale. The isolated DL-3-aminopropane-1,2-diol-1-phosphate barium salt (0.28 g) was more than 95% pure (no other phosphate-containing compound was observed) as determined by quantitative ^{31}P NMR (88% reaction yield).

D-3-Aminopropane-1,2-diol-3-phosphate (D-2-P). PEP^-K^+ (11 g, 50 mmol), ATP (1.6 g, 2.5 mmol), magnesium chloride (1.5 g, 7.5 mmol), 2-mercaptoethanol (0.1 mL, 1.4 mmol), and DL-3-aminopropane-1,2-diol (9.1 g, 7.8 mL, 100 mmol) were dissolved in approximately 100 mL of water and the pH was adjusted to 10.5 with 4 N NaOH. Immobilized GK (3500 U, determined with glycerol as substrate) and PK (500 U) were added, and the volume was adjusted to approximately 250–300 mL. The reaction was monitored by ^{31}P NMR; aliquots (3-mL) were removed from the reactor and added to 1.5–2.0 mL of deuterium oxide in a 12-mm NMR tube, and a ^{31}P NMR spectrum was recorded. After 2 days 55% of the PEP had been converted into product, a precipitate had formed and the pH had dropped to 9.5. The pH was adjusted to 10 with 4 N NaOH and magnesium chloride (1.5 g) was added. After 2 more days the reaction suspension contained 69% product, but inorganic phosphate was forming (by decomposition of phosphoramidate) at the same rate as product was being produced. The reaction was therefore stopped at this point and worked up. The enzyme-containing gels were separated by centrifugation; the supernatant was removed and combined with the first wash of enzymes. After passing the solution through charcoal, the inorganic phosphate was precipitated by addition of barium chloride dihydrate (1.0 g, 4 mmol). The barium phosphate was removed by filtration, and more barium chloride dihydrate (7.6 g, 31 mmol) and 95% ethanol (1.5 L) were added to precipitate the amidate. The precipitate was allowed to settle and the amidate was collected in a Büchner funnel and dried at 1 torr over $CaSO_4$. The unreacted amine was recovered from the supernatant by evaporation of solvent (ethanol and water). The amine was redissolved in 50 mL of water, and ATP (0.78 g, 1.3 mmol), magnesium chloride (0.75 g, 3.7 mmol), and PEP monopotassium salt (3.5 g, 17 mmol) were added. The pH was adjusted above 9.5 with 4 N NaOH. This reaction was continued for 4 days until no additional increase in amidate concentration was observed. The solution was then worked up as before. The solid fractions were combined to give 13.9 g of solid product, which by enzymatic assay and quantitative ^{31}P NMR contained 81% of D-**2**-P·Ba and 18% of PEP·Ba. The overall yield of the chiral amidate was therefore 75% (37 mmol of D-**2**-P·Ba): ^1H NMR (D_2O pH 11) δ (DSS) 3.7–3.8 (1 H, m), 3.5–3.65 (2 H, m), 2.75–2.90 (2 H, m); ^{13}C NMR (D_2O pH 12) δ (DSS) 76.9 (d, $^3J_{CCNP}$ = 7.3 Hz), 68.1 (s), 49.0 (d, $^2J_{CNP}$ = 1.6 Hz); ^{31}P NMR (D_2O, pH 11) δ 9.4 (t, $^3J_{PNCH}$ = 8.9 Hz); $[\alpha]^{25}_D$ +1.2° (*c* 2.5, H_2O, pH 12). The recovered activities of the immobilized enzymes were: GK, 29%; PK, 38%.

D-3-Mercaptopropane-1,2-diol-1-phosphate (D-3-P). PEP^-K^+ (2.2 g, 10 mmol), ATP (0.48 g, 0.75 mmol), magnesium chloride (0.45 g, 2.3 mmol), and DL-3-mercaptopropane-1,2-diol (1.7 mL, 20 mmol) were dissolved in 100 mL of water, and the pH was adjusted to 7.5. Immobilized GK (500 U, determined with glycerol as substrate) and PK (200 U) were added to the solution. The reactor was stirred at room temperature under an argon atmosphere. The reaction progress was monitored by ^{31}P NMR, and after 7 days no PEP remained. The reaction suspension was transsferred by forced siphon through a stainless steel cannula into 250-mL centrifuge bottles, and the enzyme-containing gels were compacted by centrifugation. The gels were washed with 50 mL of deoxygenated water. The supernatant was combined with the wash and the solution passed through charcoal (~25 g). The ^{31}P NMR spectrum at this point indicated the presence of inorganic phosphate (~1 mmol). The inorganic phosphate was precipitated by the addition of barium chloride dihydrate (0.24 g, 1 mmol), and barium phosphate was removed by filtration. The organic phosphate was precipitated by addition of additional barium chloride dihydrate (2.4 g, 10 mmol) and 500 mL of deoxygenated 95% ethanol. The precipiate was allowed to settle to facilitate filtration, and after drying at 1 torr over $CaSO_4$ the two fractions containing the following: fraction 1 (0.43 g), mainly inorganic phosphate monobarium salt (as determined by ^{31}P NMR); fraction 2 (2.69 g), 90% D-**3**-P·Ba (as determined by enzymatic assay) and 2% inorganic phosphate (as determined by quantitative ^{31}P NMR). The

yield of D-3-P·Ba was 84% (8.4 mmol): ^{1}H NMR (D_2O, pH 9.0) δ (DSS) 4.0–4.2 (1 H, m), 3.6–4.0 (2 H, m), 2.6–2.9 (2 H, m); ^{13}C NMR (D_2O, pH 9.5) δ (DSS) 79.4 (d, $^3J_{CCOP}$ = 5.5 Hz), 71.9 (d, $^2J_{COP}$ = 3.6 Hz), 32.5 (s); ^{31}P NMR (D_2O, pH 8.5) δ 4.4 (t, $^3J_{POCH}$ = 6.4 Hz); $[\alpha]^{25}_D$ +9.7° (*c* 2.5, H_2O, pH ~0). The recovered enzyme activities were the following: GK, 79%; PK, 83%.

Contact with air during the GK-catalyzed reaction (or isolation procedure) resulted in oxidation of the thiol to the disulfide. The thiol can conveniently be regenerated by reduction of the disulfide with sodium borohydride[42] or 2-mercaptoethanol. A representative procedure using 2-mercaptoethanol is as follows. D-3-Mercaptopropane-1,2-diol-1-phosphate (and disulfide impurities) (1.0 g, 2.5 mmol) was dissolved in 10 mL of deoxygenated water by addition of Dowex 50W-X8 resin. The Dowex 50W-X8 resin was removed by filtration and washed with 5 mL of deoxygenated water. The pH of the solution was increased to 9.0 with deoxygenated 4 N NaOH. 2-Mercaptoethanol (0.70 mL, 10 mmol) was added to the solution and stirred for 1 h at room temperature under argon. Barium chloride dihydrate (0.73 g, 3.0 mmol) and 50 mL of deoxygenated ethanol are added to the solution. The resulting solid was separated by centrifugation, the supernatant was discarded, and the solid was washed with 50 mL of deoxygenated ethanol. After drying overnight at 1 torr over $CaSO_4$, 0.90 g of solid was recovered.

D-3-Methoxypropane-1,2-diol-1-phosphate (D-4-P). PEP^-K^+ (2.2 g, 10 mmol), ATP (0.48 g, 0.75 mmol), magnesium chloride (0.45 g, 2.3 mmol), and DL-3-methoxypropane-1,2-diol (2.1 g, 20 mmol) were dissolved in 50 mL of water and the pH was adjusted to 7.5. The reaction was started by addition of immobilized GK (160 U, determined with glycerol as substrate) and PK (83 U), and the total volume was adjusted to 150 mL. The reaction was monitored by ^{31}P NMR. In the late stages of the reaction inorganic phosphate formed simultaneously with product. After 4 days the reaction was complete (no PEP remained). The enzymes were separated from the reaction mixture by centrifugation and decantation, and the enzyme-containing gels were washed once with 50 mL of deoxygenated water. The wash solution and reaction mixture were combined and passed through charcoal (25 g). Barium chloride dihydrate (0.73 g, 3 mmol) was added to precipiate the inorganic phosphate. After the precipiate was removed by filtration, a second batch of barium chloride dihydrate (2.0 g, 8 mmol) was added. To this suspension were added 2 volumes of 95% ethanol and 3 volumes of ice-cold acetone to precipitate the organic phosphate. The precipitate was allowed to settle and filtered. The white solid (3.1 g) was dried at 1 torr overnight over $CaSO_4$. The purity was 84% as determined by enzymatic assay (corresponding to 8.1 mmol of D-4-P·Ba, 81% yield). The major impurity was inorganic phosphate (15%) as determined by quantitative ^{31}P NMR: ^{1}H NMR (D_2O, pH 7.0) δ (DSS) 4.0–4.05 (1 H, m), 3.75–3.85 (2 H, m), 3.6–3.5 (2 H, S), 3.4 (3 H, s); ^{13}C NMR (D_2O, pH 7.0) δ (DSS) 77.8 (s), 74.1 (d, $^3J_{CCOP}$ = 7.3 Hz), 70.1 (d, $^2J_{COP}$ = 5.5 Hz), 63.1 (s); ^{31}P NMR (D_2O, pH 7.0) δ 3.3 (t, $^3J_{PNCH}$ = 6.6 Hz). The recovered enzyme activities were the following: GK, 95%; PK, 84%.

D-Butane-1,2,4-triol-1-phosphate (D-5-P). PEP^-K^+ (5.43 g, 25.0 mmol, ATP (0.96 g, 1 mmol), magnesium chloride (1.8 g, 9.0 mmol), and DL-butane-1,2,4-triol (5.31 g, 50.0 mmol) were dissolved in 130 mL of water and the pH was adjusted to 7.5 with 4 N NaOH. The reaction was started by addition of immobilized GK (1300 U, determined with glycerol as substrate) and PK (70 U). The reaction was followed by ^{31}P NMR, and after 9 days the reaction was completed (no PEP remained). The reaction mixture was centrifuged and the supernatant was removed by decantation. The enzymes were washed once with 50 mL of deoxygenated water, and the enzyme wash was combined with the supernatant of the reaction mixture and passed through charcoal (~25 g). Barium chloride dihydrate was added (0.24 g, 1.0 mmol) to precipitate the inorganic phosphate. After the precipitate was removed by filtration, additional barium chloride dihydrate (25 mmol, 6.1 g) was added. The organic phosphate precipitated after the addition of 500 mL of 95% ethanol. The precipitate was filtered and dried over $CaSO_4$ at 1 torr overnight. A total of 7.44 g of white solid was isolated. The purity was 95% as determined by ^{31}P NMR and corresponds to the isolation of 24 mmol of D-5-P·Ba (97% yield): ^{1}H NMR (D_2O, pH 7.0) 3.75–3.8 (1 H, m), 3.5–3.7 (3 H, m), 1.55–1.7 (2 H, m); ^{13}C NMR (D_2O, pH 7.0) δ (DSS) 72.9 (d, $^3J_{CCOP}$ = 5.5 Hz), 72.6 (d, $^2J_{COP}$ = 3.7 Hz), 63.0 (s), 39.3 (s); ^{31}P NMR (D_2O, pH 7.0) δ 4.3 (t, $^3J_{POCH}$ = 6.5 Hz); $[\alpha]^{25}_D$ +2.5° (*c* 2.5, H_2O, pH ~0). The recovered enzyme activities were as follows: GK, 80%; PK, 85%.

D-3-Bromopropane-1,2-diol-1-phosphate (D-6-P). DL-3-Bromopropane-1,2-diol (0.15 mL, 1.5 mmol), PEP^-K^+ (110 mg, 0.53 mmol), and ATP (20 mg, 0.03 mmol) were dissolved in 4 mL of triethanolamine buffer (0.1 M) containing magnesium chloride (1.0 mM) and adjusted to pH 7.8. Soluble GK (20 U, determined withhglycerol as substrate) and PK (10 U) were added to the solution. After the reaction mixture had been incubated at ambient temperature for 24 h, its ^{31}P NMR showed no remaining PEP or ATP. The solution was passed through charcoal to remove ATP and enzymes. Barium chloride dihydrate (0.24 g, 1.0 mmol) and 25 mL of ice-cold acetone were added to the solution. The resulting white precipitate of D-6-P·Ba was separated by centrifugation and washed once with 50 mL of ice-cold acetone before it was dried over $CaSO_4$ at 1 torr overnight. The resulting solid (230 mg, 0.53 mmol) was 90% pure by enzymatic assay (88% pure by quantitative ^{31}P NMR). The yield was 95% based on combined phosphoryl group donors (ATP and PEP): ^{1}H NMR (D_2O, pH 7.0) δ (DSS) 3.95–4.05 (1 H, m) 3.80–3.88 (2 H, m), 3.45–3.65 (2 H, m); ^{13}C NMR (D_2O, pH 7.0) δ (DSS) 75.0 (d, $^3J_{CCOP}$ = 6.7 Hz), 70.8 (d, $^2J_{COP}$ = 4.4 Hz), 39.8 (s); ^{31}P NMR (D_2O, pH 7.0) δ 3.4 (t, $^3J_{POCH}$ = 6.7 Hz).

D-Butane-1,2-diol-1-phosphate (D-7-P). DL-Butane-1,2-diol (100 μL, 1.1 mmol), PEP^-K^+ (42 mg, 0.2 mmol), and ATP (20 mg, 0.03 mmol) were dissolved in 4 mL of triethanolamine buffer (0.1 M) containing magnesium chloride (10 mM) and the pH was adjusted to 7.5. GK (150 U, determined with glycerol as substrate) and pK (10 U) were added to the solution. After incubation at ambient temperature for 42 h, ^{31}P NMR showed no remaining PEP or ATP. The solution was passed through charcoal to remove ADP and the enzymes. The D-7-P·Ba precipitated on addition of barium chloride dihydrate (0.12 g, 0.5 mmol) and 4 volumes of 95% ethanol. The solid was isolated by centrifugation and decantation of the supernatant. The solid was washed once with 10 mL of 95% ethanol and dried over $CaSO_4$ at 1 torr overnight. The 66 mg of white solid, approximately 85% pure (containing 9% inorganic phosphate), corresponded to a yield of 82% based on combined ATP and PEP: ^{1}H NMR (D_2O, pH 10) δ (DSS) 3.6–4.0 (3 H, m), 1.35–1.6 (2 H, m), 0.85–0.95 (3 H, m); ^{13}C NMR (D_2O, pH 10) δ (DSS) 77.3 (d, $^3J_{CCOP}$ = 7.3 Hz), 72.4 (d, $^2J_{COP}$ = 3.7 Hz), 30.0 (s), 13.9 (s); ^{31}P NMR (D_2O, pH 10) δ 4.3 (t, $^3J_{POCH}$ = 6.1 Hz).

L-3-Chloropropane-1,2-diol (L-1) (Small Scale). L-3-Chloropropane-1,2-diol was recovered from the supernatant of the precipitation of barium D-3-chloropropane-1,2-diol-1-phosphate in the 50-mmol scale reaction (2.4 L). The solution was concentrated to ~500 mL by rotary evaporation, added to 500 mL of ethanol (95%), and concentrated to ~200 mL. The precipitate (mainly sodium pyruvate) was removed by filtration and washed with absolute ethanol. This procedure was repeated four times and a yellow oil remained. The oil was purified by distillation (bp 96 °C, 1 torr) to yield 2.2 g of colorless liquid (20 mmol, 39% yield) [lit.[47] bp 213 °C] ^{1}H NMR (D_2O) δ (DSS) 3.85–4.0 (1 H, m), 3.5–3.7 (4 H, m); ^{13}C NMR (D_2O) δ (DSS) 75.7 (s), 67.1 (s), 50.4 (s); $[\alpha]^{20}_D$ −6.4 (*c* 5, H_2O). The rotation corresponds to an enantiomeric excess of ~85%.[43] The enantiomeric excess was also determined to be 88% using $Eu(hfc)_3$ in CD_3CN.

L-3-Chloropropane-1,2-diol (L-1) (Large Scale). L-3-Chloropropane-1,2-diol (L-**1**) was recovered from the supernatant from the large-scale synthesis of D-**1**-P·Ba. The supernatant (10 L) was concentrated by evaporation to ~1 L. To the solution was added ~2 L of 95% ethanol and the resulting solid was removed by filtration and washed with absolute ethanol. The solution was concentrated to ~200 mL and added to ~1 L of ethanol. The precipitate was again removed by filtration and the solution was concentrated to dryness. The resulting oil was dissolved in 200 mL of absolute ethanol and the precipitate was removed by filtration. After removing most of the ethanol by evaporation, the residual oil was distilled [bp 98 °C, 1 torr (lit.[47] bp 213 °C)]. This procedure yield 21 g of colorless liquid (190 mmol, 31% yield): ^{1}H NMR (D_2O) δ (DSS) 3.85–4.0 (1 H, m), 3.5–3.7 (4 H, m); ^{13}C NMR (D_2O) δ (DSS) 75.7 (s), 67.1 (s), 50.4 (s); $[\alpha]^{22}_D$ −6.8° (*c* 5, H_2O). The enantiomeric excess was also determined to be 94% using $Eu(hfc)_3$ in CD_3CN.

D-3-Chloropropane-1,2-diol (D-1). Barium D-3-chloropropane-1,2-diol-1-phosphate (D-**1**-P) (0.80 g, 2.1 mmol) was reconstituted in 6 mL of water by addition of Dowex-50 until the pH fell below 4. After removal of the resin, the pH of the solution was raised to ~9 by addition of sodium carbonate. Alkaline phosphatase (20 U) was added in soluble form, and the solution was left at ambient temperature. After 30 min the solution was cloudy (due to inorganic phosphate). Hydrolysis was complete by the following day as observed by ^{31}P NMR. The inorganic phosphate was removed by first adding barium chloride dihydrate (0.5 g, 2 mmol) to the solution and then adding 20 mL of absolute ethanol. The precipitate was removed by centrifugation and the supernatant was concentrated to ~5 mL. Another 20 mL of absolute ethanol was added and the precipitated solid was removed by centrifugation. After concentrating the supernatant to ~0.5 mL, 1 mL of absolute ethanol was added, the solid was removed by centrifugation, and the supernatant concentrated. D-3-Chloropropane-1,2-diol was not further purified. The 132 mg of colorless liquid corresponded to a yield of 57%: ^{1}H NMR (acetonitrile-d_3) 3.8–4.0 (1 H, m), 3.5–3.7 (4 H, m); $[\alpha]^{22}_D$ +7.1° (*c* 5, H_2O)[47] (corresponding to ~95% ee). The enantiomeric purity was determined using $Eu(hfc)_3$ in CD_3CN and found to be >97% ee (no L enantiomer was observed).

L-(−)-**Butane-1,2,4-triol (L-5)** was recovered from the supernatant of the precipitation of barium D-1,2,4-butanetriol-1-phosphate on a 25-mmol scale (1.1 L). The solution was concentrated to ~400 mL by evaporation and 500 mL of 95% ethanol was added to the solution. After the solution was concentrated to ~200 mL, another 800 mL of 95% ethanol was added and the solid (mainly sodium pyruvate) was removed by filtration. The solid was washed with ethanol and the combined solution concentrated. The removal of sodium pyruvate by filtration was continued until no further precipitate formed by evaporation and a slightly yellow oil remained. The crude product (0.77 g) corresponded to a yield of ~29%: ^{1}H NMR (D_2O) δ (DSS) 3.5–3.8 (3 H, m), 3.2–3.5 (2 H, m), 1.4–1.6 (2 H, m); ^{13}C NMR (D_2O) δ (DSS) 73.3 (s), 70.0 (s), 62.8 (s), 39.2 (s); $[\alpha]^{22}_D$ −26.6° (*c* 5, H_2O).[26]

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^{1}H NMR Analyses, Shielding Mechanisms, Coupling Constants, and Conformations in Steroids Bearing Halogen, Hydroxy, Oxo Groups, and Double Bonds

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Abstract: The ^{1}H NMR analyses of 16 5α-androstanes and one progesterone analogue furnish shifts and coupling constants for the basic steroid skeleton and substituent-induced shifts (SIS) for oxo, hydroxy, and halogen groups as well as for a Δ^5 double bond. It is shown how a single 2D experiment complemented by a NOE difference spectrum can lead to complete assignments even with the most complicated spin systems comprising, e.g., 29 strongly coupled protons within only 1 ppm; the accuracy of information from 2D techniques is evaluated by comparison to some 1D and computer-simulated spectra. On the basis of up to six simultaneously observable couplings, a special approach is used to scan the conformational space of particularly flexible parts. Intermediate conformations between half-chair and twist are obtained with a torsional C14–C15–C16–C17 angle of $\phi \cong 20°$ for the D ring with a sp^2 (17-oxo) carbon and of $\phi \cong 10°$ with only sp^3 carbon atoms; the observed flat profiles, however, allow also for mixtures of different conformations, which is supported by MM2 calculations. For the Δ^4-3-oxo A ring, a sofa conformation is favored compared to a half-chair geometry. The observed shielding effects of heterosubstituents are partially at variance with the few earlier observations, which were mostly based on polysubstituted compounds. Classical shielding mechanisms were evaluated with the program SHIFT, based on force-field-minimized structures. Steric-induced shielding dominates in the hydrocarbon, leading to upfield shifts increasing with the number of 1,3-diaxial interactions. Linear electric-field effects predict, e.g., the shielding difference between equatorial and axial protons vicinal to C–Hal bonds and the deshielding observed for diaxial C–Hal/C–H bond arrangements. A combination of anisotropy and electric-field effects explains all shifts observed in the ketones with the exception of protons vicinal to C=O; a multilinear regression analysis leads to $\Delta\chi_1^{C=O} = -36$ (−27) and $\Delta\chi_2^{C=O} = -24$ (−21) (10^{-3} cm^3/molecule, old ApSimon values in parentheses); it is, however, demonstrated, that an analysis on the basis of NMR shifts alone leads to broad ranges of parameters. Parallels between ^{1}H and ^{13}C NMR shifts are drawn, particularly at γ and ϑ positions to C–Hal bonds.

Several aspects make steroids a particularly attractive challenge for the application of modern ^{1}H NMR methods: (i) the technical problems to be surmounted with these probably most complicated spin systems; (ii) the largely undiscovered wealth of information regarding the relation between chemical shifts, coupling constants, and molecular structure; (iii) the change of biological activity with structural variation. The combination of high magnetic fields, computer-aided PFT, and in particular 2D NMR spectroscopy has already been used by several workers for the ^{1}H assignments of steroids, in which the presence of functionalities and double bonds has led to spectral simplification.[1,2] Trying to find the most economical approach with these techniques, we analyzed 17 steroids including the basic skeleton androstane, which comprises 29 nonequivalent and strongly coupled protons over a range of only 1 ppm. The spectacular progress of spectral techniques finds the chemists rather unprepared for the intelligent digestion of the many newly accessible data. There has been very little progress in the quantitative analysis of NMR shielding parameters after the classical studies of Zürcher,[3] ApSimon,[4] and co-workers, who were forced to limit themselves largely to the observation of few time-averaged methyl signals in steroids. A very useful number of ^{1}H-shielding effects in cyclohexanes have been isolated by Anteunis and other workers;[5] the scrutinization in terms of shielding mechanisms is largely lacking, and the unique ability of steroids to provide many experimental data in a geometrically

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