Kinetic Chiral Resolutions of 1,2-Diols and Desymmetrization of Glycerol Catalyzed by Glycerol Kinase

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Enantioselective phosphorylation catalyzed by glycerol kinase (EC 2.7.1.30) facilitated the kinetic chiral resolution of 3-chloro-1,2-propanediol, 3-fluoro-1,2-propanediol, 3-butene-1,2-diol, and 1,2,4-butanetriol. Both enantiomers of each compound were isolated in free diol or triol form, in excellent enantiomeric purity (91 to >99.5% ee) and in moderate to good yield (60−94% of theoretical). The enantioselectivities of glycerol kinase from different sources were compared, using 1,2,4-butanetriol as the substrate. The effect of elevated temperatures on enzymatic activity, stability, and enantioselectivity were studied, and procedures for the isolation of diol and triol products were optimized. Glycerol kinase-catalyzed phosphorylation facilitated the three-step chemoenzymatic conversion of glycerol to (*S*)-1,2-*O*-isopropylideneglycerol in 83% yield and >99.5% ee).

Introduction

Glycerol kinase[1] (EC 2.7.1.30) catalyzes the phosphorylation of glycerol to form (*R*)-glycerol 1-phosphate (*sn* or L-glycerol 3-phosphate). Because it is both regiospecific and completely stereoselective in the reaction it catalyzes, we felt it might be useful for the kinetic chiral resolution of 1,2-diols and the desymmetrization of 2-substituted 1,3-propanediols. In pioneering work by Crans and Whitesides,[2] 1,2-diols were resolved using glycerol kinase. However, the reactive enantiomer of most compounds was isolated as its phosphate ester and not as the free diol. The unreactive enantiomers were recovered in low yield and moderate enantiomeric purity. Furthermore, all of the resolutions were catalyzed by glycerol kinase from *Saccharomyces cerevisiae*, an enzyme which is no longer commerically available.

Previous work on the substrate specificity of glycerol kinase has shown that it accepts a number of substituents in place of the hydroxyl group of glycerol distal to the site of phosphorylation.[3,4] It is less tolerant, however, of structural changes at C-2, although 2-fluoro-1,3-propanediol,[3] 2-methylpropane-1,2,3-triol, and 2-ethylpropane-1,2,3-triol[5] are reported to be substrates. We report here on the kinetic chiral resolution of 3-chloro-1,2-propanediol, 3-fluoro-1,2-propanediol, 3-butene-1,2-diol, and 1,2,4-butanetriol, as well as on efforts to desymmetrize 2-substituted 1,3-propanediols.

Optically active 3-chloro-1,2-propanediol has been used as a chiral synthon in the synthesis of natural products[6] and drugs[7,8] and is readily converted into other chiral synthons, such as glycidol[9] and epihalohydrins.[10,11] Enantiomerically pure 3-chloro-1,2-propanediol has been prepared by chemical degradation of methyl 5-chloro-5-deoxy-α-L-arabinofuranoside and methyl 6-chloro-6-deoxy-α-D-glucopyranoside[12] and by chemical transformation of D-mannitol[13] via the intermediate, 2,3-*O*-isopropylideneglyceraldehyde.[14] 3-Chloro-1,2-propanediol of varying optical purity has been produced by lipase or protease-catalyzed resolution,[7,15,16] destructive fermentation of the racemate,[9,17] microbial transformation of 1,3-dichloro-2-propanol,[18] recrystallization of a diastereomeric derivative,[11] and asymmetric dihydroxylation of 3-chloropropene.[19] Optically active 3-butene-1,2-diol has served as a chiral synthon in the synthesis of oscillatoxin,[20] lipoxin B,[21] thienamycin,[22] and ethambutol.[22] It or a derivative thereof has been prepared by either enzymatic[16] or chemical[22,23] resolution, by Wittig methylidenation of (*R*)- or (*S*)-2,3-*O*-isopropylideneglyceraldehyde,[14] or by trans-

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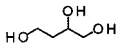
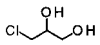
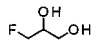
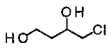
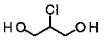
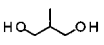
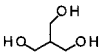
Table 1. Activity of Unnatural Substrates with Glycerol Kinase from Various Species

compound	relative activity,[a] %				
	C. mycoderma	*S. canus*	*Cellulomonas* sp.	*E. coli*	*Arthrobacter* sp.
	2.02	0.46	0.37 (0.25)	0.94 (0.33)	0.41
	0.4	0.35	0.19 (0.15)		
		0.12	0.46		
	*b*	0.01 (0.01)	*b* (*c*)	*b* (*b*)	*b*
	0.009	0.006		0.0048	0.0036
	*d*	*b*	*b* (*c*)	0.0053	0.003
	*e*	*e*	*e*	*e*	*e*
	*b*	*b*	*b*	*b* (*b*)	*b*
	*d*	*b*	*b*		

[a] Initial rates of phosphorylation of 20 mM racemic substrate determined by continuous spectrophotometric assay are reported as a percent of the rate of phosphorylation of 10 mM glycerol ($v = 100$). Activities reported in parentheses were determined by an end-point assay for ADP released. [b] Relative activity is <0.01%. [c] Relative activity is <0.003%. [d] Relative activity is <0.001%. [e] The relative activities observed with glycerol kinase from the five species listed above were 0.013, 0.072, 0.042, and 0.012, respectively. However, attempted preparative-scale phosphorylation of 2-chloro-1,3-propanediol using glycerol kinase from *S. canus* produced no diol phosphate and resulted in only hydrolysis of ATP.

formation of (*S*)-*O*-benzylglycidol[24] or diethyl (*R*,*R*)-(+)-tartrate.[21] Enantiomerically pure 1,2,4-butanetriol has been used in syntheses of pederin,[25] oscillatoxin A,[20] and araguspongine D[26] and is most commonly obtained by the reduction of (*R*)- or (*S*)-malic acid.[21,24−27]

Because glycerol kinase—especially that from bacterial sources—is a metabolic enzyme, we expected it to exhibit generally higher enantioselectivity than lipases and esterases, which are degradative enzymes. Because of the enantioselectivity displayed by glycerol kinase with its natural substrate, glycerol, we believed that one enantiomer of unnatural 1,2-diols would undergo glycerol kinase-catalyzed phosphorylation to give products analogous to (*R*)-glycerol 1-phosphate. We were further encouraged by the low Michaelis constant of glycerol kinase for glycerol ($K_m = 0.01$ and 0.035 mM for glycerol kinase from *Escherichia coli* and *Candida mycoderma*, respectively). Efficient binding of substrate makes it more likely that the reactive enantiomer will be completely consumed, leaving behind unreacted 1,2-diol that is essentially enantiomerically pure.

In this work, we compare the substrate specificities and enantioselectivities of commercially available glycerol kinases from a variety of biological sources. We examine the effect of temperature on enzymatic activity, stability, and enantioselectivity and report optimized conditions for the isolation of both enantiomers of resolved 1,2-diols in the free alcohol form. Both enantiomers of each compound were recovered in good yield and generally in 99 to >99.5% ee.

Results and Discussion

Activity of Glycerol Kinase with Unnatural Substrates. A continuous spectrophotometric assay was used to measure the activity of glycerol kinase with a number of short-chain diols and triols as potential substrates. In the assay, ADP generated from ATP during phosphorylation of substrate was rephosphorylated by phosphoenolpyruvate (PEP) and pyruvate kinase, and the pyruvate produced was reduced in situ by NADH and lactate dehydrogenase. Potential substrates were tested with glycerol kinases from *Candida mycoderma*, *Streptomyces canus*, *Cellulomonas* sp., *Escherichia coli*, and *Arthrobacter* sp., all of which are commercially available. These enzymes exhibited specific activities of 9−44 units mg^{-1} when assayed at 25 °C with 10 mM glycerol as substrate.

An end-point assay for ADP released during glycerol kinase-catalyzed phosphorylation was used to verify very low activities observed by the continuous spectrophotometric assay with some of the unnatural substrates. This assay allowed for larger quantities of glycerol kinase to be used without substantial interference from a background reaction.

Initial rates of phosphorylation of unnatural substrates (20 mM racemate) were determined relative to the initial rates observed with 10 mM glycerol (Table 1). All of the unnatural substrates tested exhibited relatively low activities. 1,2,4-Butanetriol, 3-chloro-1,2-propanediol, and 3-fluoro-1,2-propanediol exhibited the highest activities among the unnatural substrates. 3-Butene-1,2-diol, 3-butyne-1,2-diol, and 4-chloro-1,3-butanediol also showed some activity with glycerol kinase from at least some of the species tested. Among the symmetric 2-substituted

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1,3-propanediols tested, only 2-chloro-1,3-propanediol gave any indication of being a substrate. However, further investigation revealed that 2-chloro-1,3-propanediol only stimulated ATPase activity within glycerol kinase (see Desymmetrization of Glycerol below). 2-Methyl-1,3-propanediol and 2-(hydroxymethyl)-1,3-propanediol were not substrates of glycerol kinase. In general, glycerol kinases from different species displayed only small differences in activity with a given substrate.

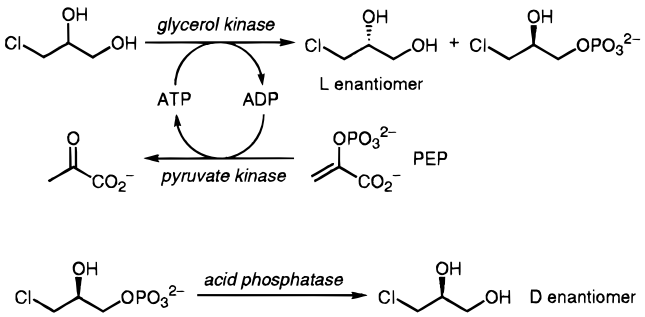
Kinetic Chiral Resolutions. To demonstrate the utility of glycerol kinase for the kinetic chiral resolution of analogues of glycerol, we subjected four compounds representing a two 100-fold range of reactivity (v_{rel} = 0.01−2.0% of that of glycerol) to enzymatic phosphorylation. 1,2,4-Butanetriol, 3-chloropropane-1,2-diol, 3-fluoropropane-1,2-diol, and 3-butene-1,2-diol were the substrates chosen for study. Issues of concern were how to maximize the effective catalytic activity of glycerol kinase with relatively unreactive substrates and how to recover both enantiomers of each compound in the free diol (or triol) form, in high chemical yield, and with high enantiomeric purity.

An initial substrate concentration of 200 mM racemic material was used for the resolutions. Catalytic ATP was regenerated using PEP and pyruvate kinase[28] in order to minimize the expense due to ATP and in order to prevent inhibition of glycerol kinase by the accumulation of ADP.[5] No inhibition of glycerol kinase by PEP was observed.[29] Both glycerol kinase and pyruvate kinase were stable in solution and retained at least 50% of their original activity during the course of resolutions. To minimize autoxidation of the enzymes, reaction solutions were sparged with nitrogen just before adding enzymes, and reactions were run in sealed flasks. The progress of each resolution was monitored by assaying for PEP and pyruvate at the beginning and periodically throughout the reaction.

When assays for PEP and pyruvate indicated that the progress of the resolution had leveled off, the reaction mixture was applied to a column of Dowex-1 (HCO_3^-). The phosphate ester and carboxylate anions were bound to the anion exchange column, and the unreacted substrate was eluted from the column with 50% aqueous ethanol. Carboxylate anions were eluted from the column with 65 mM ammonium bicarbonate in 50% aqueous ethanol, and the diol or triol phosphate was eluted with 200 mM aqueous ammonium bicarbonate. Hydrolysis of the phosphate ester was effected by either acid phosphatase (EC 3.1.3.2) or alkaline phosphatase (EC 3.1.3.1), thereby allowing the dephosphorylation of base or acid sensitive compounds, respectively. Both phosphatase enzymes exhibited significant inhibition by ammonium cation. However, removal of ammonium by cation exchange allowed the phosphatase reactions to proceed uneventfully.

Glycerol kinase phosphorylated the primary hydroxyl group of the 1,2-diol moiety of each substrate. The regiochemistry of each reaction was demonstrated by the changes in ^{13}C NMR chemical shift induced by phosphorylation and by the two- and three-bond coupling constants between phosphorus and the α- and β-carbon

Scheme 1



atoms of each diol, respectively. All were as they were expected to be. Upon phosphorylation, the primary carbon of each 1,2-diol moiety displayed a downfield shift in resonance of 1.2−4.1 ppm, relative to the unphosphorylated compound. Concurrently, the secondary carbon of each 1,2-diol moiety displayed an upfield shift in resonance of 1.1−1.6 ppm. In every case, the two-bond coupling constant between phosphorus and the primary carbon ($^2J_{CP}$) of the diol was approximately 5 Hz while the three-bond coupling constant between phosphorus and the secondary carbon ($^3J_{CP}$) was approximately 7 Hz.

Enantiomeric purities were determined by ^1H NMR of the derivatives formed by peracylation of each diol or triol with (*R*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetic acid (MTPA). If ^1H NMR showed no signal due to a diastereomeric impurity, the NMR sample was spiked with 0.5 mol % of the (+)-MTPA derivative of the appropriate racemic diol or triol. By this calibration, it was demonstrated in every case that a 0.25% diastereomeric impurity—corresponding to an ee for the diol or triol of 99.5%—could be detected. Small diastereomeric impurities in resolutions of 1,2,4-butanetriol were likewise calibrated by adding known quantities of the (+)-MTPA derivative of the racemic triol.

3-Chloro-1,2-propanediol was first resolved using glycerol kinase from *C. mycoderma* (Scheme 1).[30] Although a slight excess of PEP was used to ensure that all of the reactive D enantiomer was consumed, enzymatic assay indicated that the resolution proceeded to only 47% conversion. Unreacted L-3-chloro-1,2-propanediol was isolated in 42% yield but in only 70% ee.[31] The D enantiomer was isolated in 36% overall yield and >99.5% ee after dephosphorylation.

When 1,2,4-butanetriol was treated similarly with glycerol kinase from *C. mycoderma*, the reaction proceeded to 53% conversion. The unreactive L enantiomer was recovered in 48% yield and >99.5% ee, while the D enantiomer was recovered in 24% yield and only 92% ee. Partial transformation of the L enantiomer may have occurred through phosphorylation of the C-4 hydroxyl group, either with or without stereoselectivity for the L enantiomer. Stereoselectivity might result from the fact that the topology of L-1,2,4-butanetriol bound with its C-4 hydroxyl group oriented toward the ATP binding site of glycerol kinase would resemble that of glycerol productively bound in the same active site.

To minimize the phosphorylation of the L enantiomer, a two-stage resolution procedure was developed, in which

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(29) Chenault, H. K.; Mandes, R. F.; Hornberger, K. R. *J. Org. Chem.* **1997**, *62*, 331−336.

(30) Until recently, glycerol kinase from *C. mycoderma* was available from Biozyme Labratories for $10/kilounit.

(31) Yields are reported based on the mass of the original racemate. The maximum theoretical yield for a resolution is 50%. Thus, a 42% yield corresponds to 84% of the theoretical maximum for a single enantiomer.

Table 2. Two-Stage Resolutions of 1,2,4-Butanetriol Using Glycerol Kinase from Various Species[a]

species	units[c]	*t*,[d] h	2nd % conversion[e]	% yield[b] (% ee) D	% yield[b] (% ee) L
C. mycoderma	44	48	3.6	33 (95)	21 (>99.5)
S. canus	435	12	14.8	28 (92)	47 (91)
Cellulomonas sp.	210	12	9.7	28 (78)	27 (97)
E. coli	176	41	13.2	31 (91)	40 (91)
Arthrobacter sp.	221	20	18.5	30 (83)	43 (57)

[a]Resolutions were performed on 2 mmol of racemic substrate. [b]Yields are expressed as a percent of the mass of the original racemate. Maximum theoretical yield for a pure enantiomer is 50%. [c]Units of glycerol kinase used for the first stage of the resolution. [d]Time for the first stage of the resolution to reach 42.5% conversion of the racemate, on the basis of assay of PEP consumed. [e]Additional percent of original racemate consumed during the second stage of the resolution, on the basis of assay of PEP consumed.

the amount of PEP used was limited to 42.5 mol % of the racemic substrate. When all of the PEP was consumed, products were separated by anion exchange chromatography as described earlier. Unreacted material enriched in the L enantiomer was resubmitted to resolution conditions in order to consume the remaining D enantiomer. The two-stage resolution procedure improved the enantiomeric purity of the D-1,2,4-butanetriol produced to 95% ee. When the resolution was allowed to proceed to 48% conversion with glycerol kinase from *Cellulomonas* sp., however, and the unreacted enantiomer was resubmitted to resolution conditions, D- and L-1,2,4-butanetriols were obtained in 43 and 37% yields and in 93 and 60% ee, respectively.

At this point, glycerol kinases from different sources were examined to see if they differed substantially in their enantioselectivities with 1,2,4-butanetriol as substrate. All enzymes examined were commercially available and came from *C. mycoderma*, *S. canus*, *E. coli*, *Cellulomonas* sp., and *Arthrobacter* sp. The results are shown in Table 2. Glycerol kinase from *S. canus* was selected for further study because of its good enantioselectivity, relatively broad substrate specificity (Table 1), and ready availability.[32]

To utilize the enzyme's catalytic activity most efficiently, we investigated the effect of temperature on the activity and stability of glycerol kinase from *S. canus* (Figure 1). At 45 °C, the activity of glycerol kinase with 3-chloro-1,2-propanediol was 15 times greater than that observed at 25 °C. However, glycerol kinase also exhibited a sharp decrease in stability at temperatures of 40 °C and greater. At 35 °C, the enzymatic activity was 5 times greater than that at 25 °C, but the stability was essentially the same as that seen at 25 °C. Furthermore, the enantioselectivity of the enzyme remained outstanding at 35 °C. Resolution of 3-chloro-1,2-propanediol at either 25 or 35 °C yielded both enantiomers in >99.5% ee.

While studying the effect of temperature on enantiomeric purity, we found that 3-chloro-1,2-propanediol hydrolyzed to form glycerol in the presence of mixed-bed and strongly acidic ion-exchange resins. Although the enantioselectivities for the reactions discussed above were excellent, the yields of resolved enantiomers were quite low (11−20% for D and 30−38% for L). To avoid

(32) Glycerol kinase from *S. canus* is available from Genzyme for $29/kilounit.

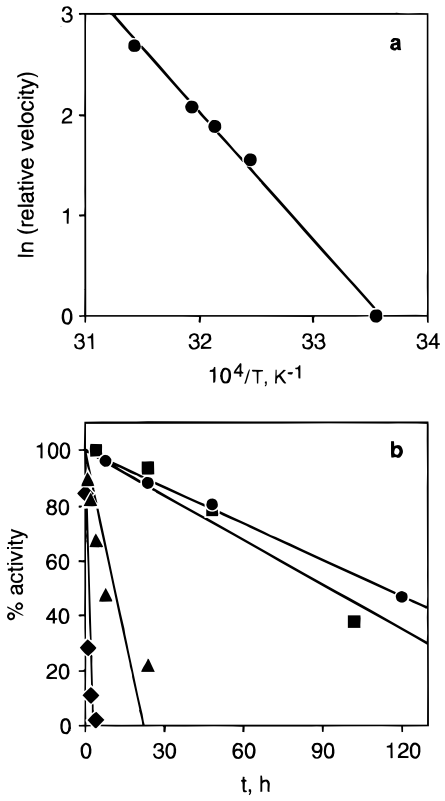


Figure 1. (a) Effect of temperature on the initial rate of phosphorylation of 3-chloro-1,2-propanediol catalyzed by glycerol kinase from *S. canus*. (b) Effect of temperature on the stability of glycerol kinase from *S. canus*. Residual activity with 3-chloro-1,2-propanediol at 25 °C was measured after the enzyme had been incubated at 25 °C (●), 35 °C (■), 40 °C (▲), and 45 °C (◆).

the problem of hydrolysis, the procedure for isolating products was modified. Instead of desalting and evaporating aqueous solutions of diol products, free diols were recovered from aqueous solution by continuous liquid−liquid extraction using ethyl acetate. Thus, after separating reactive and unreactive enantiomers by anion exchange chromatography, the solution containing the unreacted L enantiomer was extracted for 24 h with ethyl acetate. The D enantiomer was eluted from the anion exchange column, dephosphorylated with phosphatase, and then similarly extracted from solution with ethyl acetate.

An attempt to recover unreacted L-3-chloro-1,2-propanediol directly from the resolution reaction mixture by continuous liquid−liquid extraction led to migration of the phosphate ester of D-3-chloro-1,2-propanediol 1-phosphate, giving 20% conversion to 3-chloro-1,2-propanediol 2-phosphate. Although the regiochemistry of the phosphate group is irrelevant since it is eventually removed, this complication and the fact that yields were not improved by the new procedure led us to choose anion exchange, followed by liquid−liquid extraction, as the method for separating and recovering enantiomeric products from a resolution.

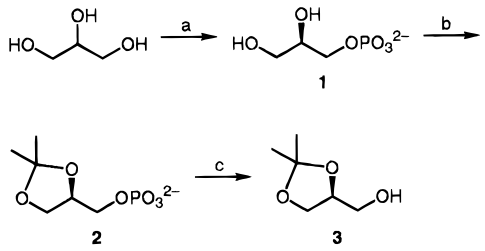
When the optimized resolution procedure was applied to the resolution of 3-chloro-1,2-propanediol (47% initial conversion), the yield of the D enantiomer was noticeably improved by the use of continuous liquid−liquid extraction (Table 3), and both enantiomers were recovered in excellent enantiomeric purity (>99.5% ee). Similarly,

Table 3. Kinetic Chiral Resolutions Using Glycerol Kinase from *Streptomyces Canus*

		D		L	
substrate	scale, mmol	% yield[a]	% ee	% yield[a]	% ee
3-chloro-1,2-propanediol	18	41	>99.5	38	>99.5
3-fluoro-1,2-propanediol	2.3	34	>99.5	30	>99.5
3-butene-1,2-diol	2.8	36	>99.5	38	99

[a] Yields are expressed as a percent of the mass of the original racemate. Maximum theoretical yield for a pure enantiomer is 50%.

Scheme 2[a]



[a] Reagents and conditions: (a) glycerol kinase, 2.9% ATP, PEP, pyruvate kinase; (b) Dowex 50W (H^+), then $(CH_3O)_2C(CH_3)_2$/CH_3OH, then KOH; (c) alkaline phosphatase.

resolutions of 3-fluoro-1,2-propanediol and 3-butene-1,2-diol gave both enantiomers of each compound in good chemical yield and excellent enantiomeric purity.

Desymmetrization of Glycerol. The use of enzymes to desymmetrize *meso* substrates is a particularly powerful tool in organic synthesis.[33] With an aim at desymmetrizing 2-substituted 1,3-propanediols, we first investigated the natural reaction catalyzed by glycerol kinase, the desymmetrization of glycerol. When enzymatically prepared (*R*)-glycerol 1-phosphate (**1**) was converted to its free acid and dissolved in a mixture of methanol and 2,2-dimethoxypropane (Scheme 2), the acidic phosphoryl group catalyzed the conversion of the diol into (*S*)-2,3-*O*-isopropylideneglycerol 1-phosphate (**2**). Compound **2** was unstable when isolated as its free acid but could be isolated in good yield after the pH of the reaction mixture had been adjusted to 10. Alkaline phosphatase catalyzed the dephosphorylation of **2** to give (*S*)-1,2-*O*-isopropylideneglycerol (**3**) in 83% overall yield and >99.5% ee. This method compares favorably with the preparation of other enantiomerically enriched derivatives of glycerol by lipase-catalyzed resolutions[34] and with the reduction of enantiomerically pure 2,3-*O*-isopropylideneglyceraldehyde obtained from D-mannitol[14,35] or ascorbic acid.[14,36]

Attempted desymmetrization of 2-chloro-1,3-propanediol (100 mM) using glycerol kinase from *S. canus* led only to the hydrolysis of ATP. Even when the initial concentration of ATP was increased to 50 mM, no phosphorylated diol was observed by ^{31}P NMR. All of the ATP was hydrolyzed to ADP. Although the pyruvate kinase in solution was not irreversibly inactivated, it failed to catalyze phosphoryl transfer from PEP to the accumulated ADP.

Scheme 3

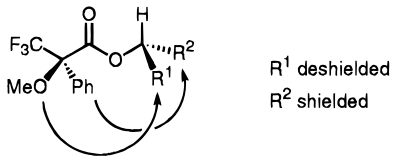


Table 4. ^1H NMR Data for (+)-MTPA Peresters of Enantiomeric 1,2-Diols

		δ, ppm	
diol	configuration	H-1,1′	H-3,3′
3-chloro-1,2-propanediol	D (*S*)[a]	4.91, 4.65	3.89, 3.78
	L (*R*)	4.77, 4.57	3.94, 3.85
3-fluoro-1,2-propanediol	D (*S*)[a]	4.92, 4.62	4.72, 4.59
	L (*R*)	4.80, 4.53	4.79, 4.66
3-butene-1,2-diol	D (*R*)[a]	4.80, 4.49	5.85
	L (*S*)	4.73, 4.45	5.96
1,2,4-butanetriol	D (*R*)[a]	4.78, 4.50	1.96-1.91
	L (*S*)	4.72, 4.40	2.07-1.92

[a] Enantiomer that reacts preferentially with glycerol kinase.

Absolute Configuration of Products. ^1H NMR of the (*R*)-(+)-MTPA peresters of the resolved diols and triols verified in every case that the enzymatically produced phosphate esters were stereochemically analogous to (*R*)-glycerol 1-phosphate.[37] Thus, glycerol kinase catalyzed the stereoselective phosphorylation of (*S*)-D-3-chloro-1,2-propanediol, (*S*)-D-3-fluoro-1,2-propanediol, (*R*)-D-1,2,4-butanetriol, and (*R*)-D-3-butene-1,2-diol. The absolute configurations of the products were determined from the NMR signals of the protons attached adjacent to each chiral secondary carbinol center. Correlations between absolute configuration and relative chemical shifts were made using an empirical model that assumes the structure shown in Scheme 3 as the predominant conformation in (+)-MTPA esters.[37] In this conformation, protons on substituent R^1 are deshielded by the methoxy group, and protons on substituent R^2 are shielded by the phenyl group. All of the bis- and tris-(+)-MTPA esters studied in this work complied with this empirical spectroscopic model (Table 4). Additional (+)-MTPA ester groups at C-1 or C-4 appear not to perturb the chemical shift behavior of the protons adjacent to C-2.

Conclusion

Glycerol kinase catalyzes the kinetic chiral resolution of three- and four-carbon 1,2-diols, providing both enantiomers in excellent enantiomeric purity and with predictable absolute configuration. Product recovery by anion exchange, treatment with phosphatase and continuous liquid−liquid extraction is both convenient and potentially scalable. In certain cases, resolutions using glycerol kinase may be preferable to lipase-catalyzed processes since the latter often proceed with only modest enantioselectivity and require adsorption chromatography to separate reactive and unreactive enantiomers. Although most unnatural substrates display relatively low specific activities with glycerol kinase, elevated reaction temperatures can be used to increases reaction rates without detriment to the enzyme's enantioselectivity.

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(34) (a) Theil, F.; Weidner, J.; Ballschuh, S.; Kunath, A.; Schick, H. *J. Org. Chem.* **1994**, *59*, 388−393. (b) Wang, Y. F.; Wong, C.-H. *J. Org. Chem.* **1988**, *53*, 3127−3129.
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Experimental Section

General Procedures. Glycerol kinase was purchased as an ammonium sulfate suspension from Genzyme (*S. canus*, *Arthrobacter* sp., *Cellulomonas* sp.), Sigma (*E. coli*), and Biozyme (*C. mycoderma*) and was used without further purification. PEP was either purchased as the cyclohexylammonium salt from Genzyme or prepared as the potassium salt according to a published procedure.[38] Glycerol kinase showed no dependence of activity or stability on the salt form of PEP. NADH was purchased from Genzyme. ATP (disodium salt), acid phosphatase (potato, Type IV−S), and alkaline phosphatase (bovine intestinal mucosa, Type I−S) were purchased from Sigma. Pyruvate kinase (rabbit muscle, PK3) and lactate dehydrogenase (rabbit muscle, LDHR2) were purchased from Biozyme Laboratories and were used without additional purification. Units of enzymatic activity refer to activity measured with the natural substrate of each enzyme. (*R*)-(+)-MTPA was purchased from Aldrich.

Unless otherwise noted, ^{1}H and ^{13}C spectra were acquired at nominal resonance frequencies of 400 and 101 MHz, respectively, using $CDCl_3$ as the solvent and residual $CHCl_3$ (δ 7.24) as the internal reference. Spectra obtained in D_2O were referenced internally to sodium 3-(trimethylsilyl)propanoate-*2,2,3,3-d*$_4$ (TSP, δ 0.00) unless otherwise noted. ^{31}P NMR spectra were acquired at a nominal resonance frequency of 162 MHz using D_2O as solvent and were referenced externally to 85% H_3PO_4 (δ 0.00).

Continuous Spectrophotometric Assay of Glycerol Kinase Activity. To a final volume of 3.00 mL of 0.10 M sodium phosphate buffer, pH 7.6, containing 2.9 units of pyruvate kinase, 0.13 units of lactate dehydrogenase, 10 mM substrate (20 mM if substrate was racemic), 3.0 mM ATP, 14 mM $MgCl_2$, 1 mM KCl, and 0.2 mM NADH, at 25 °C, was added 25 μL of a solution of glycerol kinase (either 174 or 0.174 units mL^{-1}). The absorbance at 334 nm was recorded continuously for 10 min, and the activity of glycerol kinase was calculated using $\epsilon_{334} = 6.18$ mM^{-1} cm^{-1} as the molar absorptivity of NADH. If no activity was observed with an unnatural substrate, glycerol was added to ensure that the glycerol kinase was active. Calibration by varying the amount of glycerol kinase used demonstrated that the assay was linear in its response over a range of activity from 2.2×10^{-3} to 85×10^{-3} μmol min^{-1}.

ADP End-Point Assay of Glycerol Kinase Activity. To a final volume of 500 μL of 0.10 M sodium phosphate buffer, pH 7.6, containing 10 mM substrate (20 mM if substrate was racemic), 4.7 mM ATP, 6 mM $MgCl_2$, and 10 mM KCl was added 25 μg (25 μL of a 1.0 mg mL^{-1} solution) or 1.0 mg of glycerol kinase. Aliquots (50 μL) were removed periodically and assayed for ADP as follows: To a final volume of 2.290 mL of 0.10 M sodium phosphate buffer, pH 7.6, containing 10 units of lactate dehydrogenase, 0.9 mM PEP, 33 mM $MgCl_2$, 118 mM KCl, and 0.2 mM NADH was added 50 μL of the sample to be tested for ADP content. The absorbance at 334 nm was recorded (A_o), and 1 unit (20 μL) of pyruvate kinase was added. After a few minutes, the final absorbance (A) was recorded, and the concentration of ADP was calculated from $\Delta A = A_o - A$ using $\epsilon_{334} = 6.18$ mM^{-1} cm^{-1} as the molar absorptivity of NADH. The assay for ADP was calibrated using known amounts of ADP and was found to be accurate over the range of 3.5×10^{-3}−3.5×10^{-1} μmol of ADP.

Temperature-Dependent Activity and Stability of Glycerol Kinase. Temperature-dependent activity of glycerol kinase was measured using the continuous spectrophotometric assay. Reactions were initiated by adding 25 μL of a solution of glycerol kinase (0 °C) to a 3.00-mL reaction volume previously equilibrated at the appropriate temperature. Temperature-dependent stability of glycerol kinase was measured by incubating glycerol kinase at the appropriate temperature in deoxygenated 100 mM phosphate buffer, pH 7.6, containing 1 mM 2-mercaptoethanol. Periodically, 25-μL portions were removed and assayed for glycerol kinase activity at 25 °C using the continuous spectrophotometric assay.

Assay for PEP. To a final volume of 2.98 mL of 0.10 M sodium phosphate buffer, pH 7.6, containing 10 units of lactate dehydrogenase, 4.9 mM $MgCl_2$, 9.1 mM KCl, 0.2 mM NADH, and 4.3 mM ADP was added 100 μL of the sample to be tested for PEP content. The absorbance at 334 nm was recorded (A_o), and 1 unit (100 μL) of pyruvate kinase was added. After a few minutes, the final absorbance (A) was recorded, and the concentration of PEP was calculated from $\Delta A = A_o - A$ using $\epsilon_{334} = 6.18$ mM^{-1} cm^{-1} as the molar absorptivity of NADH.

Assay for Pyruvate. To a final volume of 2.98 mL of 0.10 M sodium phosphate buffer, pH 7.6, containing 0.2 mM NADH was added 100 μL of the sample to be tested for pyruvate content. The absorbance at 334 nm was recorded (A_o), and 1 unit (100 μL) of lactate dehydrogenase was added. After a few minutes, the final absorbance (A) was recorded, and the concentration of pyruvate was calculated from $\Delta A = A_o - A$ using $\epsilon_{334} = 6.18$ mM^{-1} cm^{-1} as the molar absorptivity of NADH.

General Procedure for One-Stage Resolutions. Racemic substrate (0.200−2.00 g, 185 mM) was combined with ATP (5.4 mM), $MgCl_2 \cdot 6H_2O$ (10 mM), KCl (11 mM), NaN_3 (1.5 mM), 2-mercaptoethanol (10 mM), and PEP (111 mM) in deionized water. Before the final volume was set, the solution was adjusted to pH 9.5 using aqueous potasssium hydroxide, and the solution was sparged with nitrogen. Glycerol kinase (about 0.5 units of activity measured with the unnatural substrate per mmol of racemate) and pyruvate kinase (about 15 units per mmol of racemic substrate) were added. Ammonium sulfate from the suspension of glycerol kinase lowered the pH of the reaction to 8.0. Periodically, the reaction was assayed for PEP and pyruvate.

When assays for PEP and pyruvate indicated that conversion had reached 50% or had leveled off, the reaction mixture was diluted with an equivalent volume of ethanol and applied to a column of Dowex-2 × 8-100 (HCO_3^-) resin that was twice the volume of the original reaction. The column was rinsed with 2.5 column volumes of 50% aqueous ethanol to remove the unphosphorylated substrate, eluted with 4 column volumes of 65 mM ammonium bicarbonate in 50% aqueous ethanol to remove carboxylate anions, and then eluted with 25 column volumes of 200 mM aqueous ammonium bicarbonate to remove the phosphorylated substrate. Cations were removed from the fraction containing unreacted substrate by adding Dowex 50W×8 (H^+) resin and filtering. The solvent was removed by rotary evaporation at 40 °C.

Fractions containing phosphorylated product were combined and concentrated by rotary evaporation at 40 °C. The residue was dissolved in enough water to make a solution approximately 100 mM, and the pH was adjusted to 4.8 with aqueous hydrogen chloride. The solution was sparged with nitrogen, and acid phosphatase (18 units per mmol of phosphate ester) was added. The progress of the reaction was monitored by examining aliquots by ^{1}H NMR. After the reaction was complete (~48 h), the solution was desalted with Dowex MR-3 resin and freed of solvent by rotary evaporation at 40 °C.

Determination of Enantiomeric Purity. Enantiomeric purities were determined by ^{1}H NMR analysis of the products formed by peracylation of the resolved diols and triols with (*R*)-(+)-MTPA.[37] To 25.8 mg (0.11 mmol) of (*R*)-(+)-MTPA, 24.8 mg (0.12 mmol) of dicyclohexylcarbodiimide, and 1.2 mg (0.01 mmol) of 4-(dimethylamino)pyridine in 750 μL of dry ether was added 0.05 mmol of diol or 0.03 mmol of triol. The reaction mixture was stirred overnight, diluted with 10 mL of ether, and filtered through glass wool. The ether was washed successively with water, 5% acetic acid, water, and brine. The ether was dried with $MgSO_4$ and concentrated under reduced pressure. If necessary, the crude product was dissolved in acetone and filtered through a small plug of silica gel. If ^{1}H NMR analysis revealed signals due to the (+)-MTPA derivative of only one enantiomer of diol or triol, the NMR sample was spiked with 0.5 mol % of the (+)-MTPA derivative of the appropriate racemic diol or triol. By this calibration, it was

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demonstrated in every case that a 0.25% diastereomeric impurity—corresponding to an ee for the diol or triol of 99.5%—could be detected.

General Procedure for Two-Stage Resolutions. Racemic substrate was phosphorylated using glycerol kinase from *S. canus* according to the General Procedure for One-Stage Resolutions, except that only 79−87 mM PEP was used. When all of the PEP had been consumed, the reaction was diluted and chromatographed on Dowex-2 × 8−100 (HCO_3^-) resin as described above. The fractions containing unphosphorylated substrate were resubjected to enzymatic phosphorylation conditions, except using only 11−28 mM PEP. When assays for PEP and pyruvate indicated that conversion had leveled off, the unreacted 1,2-diol was recovered by desalting and evaporating the solvent (isolation method A) or by continuous liquid−liquid extraction (isolation method B).

Fractions from the original phosphorylation reaction containing phosphorylated substrate were combined and adjusted to pH ≤ 3 using Dowex-50W × 8−100 (H^+). The solution was filtered, and the phosphate ester was hydrolyzed using either alkaline phosphatase (hydrolysis method A) or acid phosphatase (hydrolysis method B).

Isolation Method A. Dowex MR-3 resin (2 mol equiv of exchangeable ions) was added to the aqueous solution of 1,2-diol or triol obtained from the second stage of resolution or the hydrolysis of phosphate ester. The mixture was stirred with a stir bar for 30 min, filtered, and concentrated by rotary evaporation at 40 °C to yield the free 1,2-diol.

Isolation Method B. To the 12-mL aqueous solution of diol or triol, obtained from the second stage of resolution or from the hydrolysis of phosphate ester, was added 2 g of sodium chloride. The solution was extracted with 35 mL of ethyl acetate for 24 h, using a continuous liquid−liquid extractor. The ethyl acetate was dried ($MgSO_4$) and concentrated by rotary evaporation to yield the enantiomerically enriched product.

Resolution of 1,2,4-Butanetriol. Resolution of 1,2,4-butanetriol at 25 °C according to the General Procedure for Two-Stage Resolutions (42.5% initial conversion, isolation method A, and hydrolysis method A) gave D- and L-1,2,4-butanetriol as reported in Table 2. Resolution of 1,2,4-butanetriol (24 mmol) with glycerol kinase from *Cellulomonas* sp. (48% initial conversion) gave D- and L-1,2,4-butanetriol in 43 and 37% yield and 93 and 60% ee, respectively.

Resolution of 3-Chloro-1,2-propanediol. Resolution of 3-chloro-1,2-propanediol (18.1 mmol) at 25 °C according to the General Procedure for Two-Stage Resolutions (218 units, 47% initial conversion in 24 h, isolation method A, and hydrolysis method B) gave D- and L-3-chloro-1,2-propanediol as reported in Table 3. Resolution of 3-chloro-1,2-propanediol at 35 °C gave essentially identical results.

Resolution of 3-Fluoro-1,2-propanediol. Resolution of 3-fluoro-1,2-propanediol (2.26 mmol) at 35 °C according to the General Procedure for Two-Stage Resolutions (436 units, 47% initial conversion in 56 h, isolation method A, and hydrolysis method B), except using 50 mM ATP, gave D- and L-3-fluoro-1,2-propanediol as reported in Table 3.

Resolution of 3-Butene-1,2-diol. Resolution of 3-butene-1,2-diol (2.84 mmol) at 35 °C according to the General Procedure for Two-Stage Resolutions (522 units, 47% initial conversion in 25 h, isolation method B, and hydrolysis method B) gave D- and L-butene-1,2-diol as reported in Table 3.

(*R*)-Glycerol 1-Phosphate (1). In a 125-mL Erlenmeyer flask were combined 1.00 g (10.9 mmol) of glycerol, 60 mg (0.109 mmol) of ATP, 44 mg (0.218 mmol) of $MgCl_2 \cdot 6H_2O$, 40 mg (0.545 mmol) of KCl, 3.5 mg (0.054 mmol) of NaN_3, 3.80 μL (0.054 mmol) of 2-mercaptoethanol, 2.07 g (11.0 mmol) of PEP, and 45 mL of deionized water. The solution was adjusted to pH 8.0 with aqueous potasssium hydroxide, and the final volume was adjusted to 50 mL. The solution was sparged with nitrogen, and glycerol kinase (35 units) and pyruvate kinase (29 units) were added. Periodically, the reaction was assayed for PEP and pyruvate.

When all of the PEP had been consumed, the reaction mixture was diluted with 40 mL of ethanol and applied to a 100-mL column of Dowex-2 × 8-100 (HCO_3^-) resin. The column was rinsed with 100 mL of 50% aqueous ethanol, eluted with 400 mL of 65 mM ammonium bicarbonate in 50% aqueous ethanol to remove carboxylate anions, and then eluted with 500 mL of 400 mM aqueous ammonium bicarbonate to recover glycerol 1-phosphate. The glycerol 1-phosphate solution was adjusted to pH 2 by addition of Dowex-50W × 8 (H^+), filtered, and concentrated by rotary evaporation at 40 °C to yield 1.86 g (100%) of **1** as the free acid. ^{1}H NMR showed this material to be a 9:1 mixture of glycerol phosphate and pyruvic acid. It was thus 95% pure by weight.

(*R*)-2,3-*O*-Isopropylideneglycerol 1-Phosphate (2). To 1.86 g of **1** (free acid, 95% pure by weight) in 15 mL of dry methanol was added in portions 30 mL of dimethoxypropane. After 60 h, ^{1}H NMR analysis of an aliquot indicated 89% conversion. Additional portions of dimethoxypropane failed to further the conversion. Aqueous potassium hydroxide was added to the solution to raise the pH to 10 (as indicated by pH meter). Evaporation of the solvent under reduced pressure gave crude **2**: ^{1}H NMR (D_2O) δ 4.20 (m, 1H), 3.94 (dd, J = 6.8, 8.6 Hz, 1H), 3.62 (dd, J = 6.3, 8.6 Hz, 1H), 3.59−3.48 (m, 2H), 1.25 (s, 3H), 1.18 (s, 3H).

(*S*)-1,2-*O*-Isopropylideneglycerol (3). To the crude **2** prepared above was added 110 mL of H_2O and 22 mg (0.11 mmol) of $MgCl_2 \cdot 6H_2O$. The solution was sparged with nitrogen, and alkaline phosphatase (64 units) was added. The reaction was monitored by ^{1}H NMR analysis of 100-μL aliquots. After 24 h, the reaction was complete and was transferred to a continuous liquid−liquid extractor, where it was extracted with $CHCl_3$ for 13 h. The chloroform was dried ($MgSO_4$) and evaporated to give 1.18 g (87% from **1**): ^{1}H NMR δ 4.16−4.11 (m, 1H), 3.95 (dd, J = 6.6, 8.2 Hz, 1H), 3.68 (dd, J = 6.5, 8.2 Hz, 1H), 3.60 (dd, J = 4.0, 11.7 Hz, 1H), 3.50 (dd, J = 5.4, 11.6 Hz, 1H), 2.77 (br s, 1H), 1.34 (s, 3H), 1.28 (d, J = 0.5 Hz, 3H); ^{13}C NMR δ 109.2, 76.1, 65.6, 62.8, 26.5, 25.1.

Supporting Information Available: Selected ^{1}H, ^{13}C, and ^{31}P NMR spectra of 1,2,4-butanetriol, 3-chloro-1,2-propanediol, 3-fluoro-1,2-propanediol, 3-butene-1,2-diol, their 1-phosphate esters, and their (+)-MTPA perester derivatives, with peak assignments. Selected ^{1}H, ^{13}C, and ^{31}P NMR spectra of **1**, **2**, **3**, and the (+)-MTPA ester of **3**, with peak assignments (3 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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