to give the dilithium salt of **1** (104 mg, 55%) (~90% pure by ^{1}H NMR): $[\alpha]_D$ +19.8° (*c* 0.50, H_2O), authentic[30] +18.6°; ^{1}H NMR (500 MHz, D_2O, pH 5.0) δ 1.80 (dd, J = 12, 13 Hz, 1 H), 2.19 (dd, J = 5, 3 Hz, 1 H), 3.53 (overlapping dd, J = 10 Hz), 3.82-3.87 (m, 1 H), 3.92-3.97 (m, 1 H), 4.07-4.17 (m, 1 H); ^{13}C NMR (125.7 MHz, D_2O, pH 5.0) δ 39.87, 64.91, 69.38, 71.14, 73.52 (d, J_{POC} = 7 Hz), 97.12, 177.17; ^{31}P NMR (121.47 MHz, D_2O, pH 5.0) δ 1.09.

A sample of **1** (5 mg) was applied to a column of Sephadex DEAE A-25 ion-exchange resin (HCO_3^- form, 10 mL) and eluted with a linear gradient to triethylammonium bicarbonate (150 mL of 100 mM to 150 mL of 350 mM). The DAHP containing fractions were pooled and lyophilized to give a white solid that was redissolved in water (10 mL) and passed down a column of AG 50W-X8 resin (H^+ form, 10 mL). Adjustment of the eluant to pH 5.0 with 0.1 M lithium hydroxide, followed by lyophilization, gave the dilithium salt of **1** (3 mg) (^{1}H NMR indicated ~95% purity). This sample was used for the assay with dehydroquinate synthase.

Assay of Synthetic DAHP (1) with Dehydroquinate Synthase. The assay procedure for DAHP used a coupled enzyme system of dehydroquinate synthase and dehydroquinase with subsequent monitoring of dehydroshikimate production. Assay solutions (1.00 mL) containing 50 mM MOPS buffer, pH 7.50, cobalt sulfate (50 μM), NAD^+ (15 μM), DAHP (500 μM), and 2 units of dehydroquinase were incubated at 20 °C in quartz cuvettes. The reaction was initiated by the addition of 800 milliunits of DHQ synthase, and the production of dehydroshikimate was monitored at 234 nm. Initial rates obtained from the first ~20 s after mixing were as follows: synthetic DAHP, 0.123 AU/min; authentic DAHP, 0.334 AU/min.

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Rabbit Muscle Aldolase as a Catalyst in Organic Synthesis[1]

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Abstract: D-Fructose-1,6-bisphosphate aldolase from rabbit muscle (RAMA, EC 4.1.2.13) is a synthetically useful catalyst for the stereoselective aldol condensation between dihydroxyacetone phosphate (DHAP, **1**) and aldehydes having a range of structures. This paper demonstrates that more than 50 aldehydes [in addition to its natural substrate, D-glyceraldehyde 3-phosphate, G-3-P (**2**)] are substrates for RAMA. Unhindered aliphatic, α-heteroatom-substituted, and differentially protected alkoxy and amino aldehydes are substitutes; sterically hindered aliphatic and α,β-unsaturated aldehydes are not. Aromatic aldehydes are either poor as substrates or they do not react. Phosphorylated aldehydes react more rapidly than their unphosphorylated counterparts, and aldehydes substituted in the α position with an electronegative group react more rapidly than unsubstituted aliphatic aldehydes. Permissible variations in the structure of the DHAP moiety are much more restricted; only two—1,3-dihydroxy-2-butanone 3-phosphate (**68**) and 1,4-dihydroxy-3-butanone-1-phosphonate (**69**)—of 11 DHAP analogues tested were substrates for RAMA. RAMA is stable under the reaction conditions used in synthetic applications: it withstands organic cosolvents (up to 20% DMSO or EtOH); it can be used in immobilized form, in soluble form, or enclosed within a membrane; it is also air-stable. Syntheses of 5,6-dideoxy-D-*threo*-2-hexulose 1-phosphate (**82**), 5-*O*-methyl-D-fructose (**85**), D-xylulose (**88**), and peracetylated D-*glycero*-D-*altro*-2-octulose (**91**) on scales of 4 mmol–1 mol demonstrate practical application of this catalytic synthetic methodology. In a limited study of kinetic diastereoselectivity, RAMA exhibits useful selectivity in two cases: with (±)-glyceraldehyde 3-phosphate [**2**; 20:1 ratio, the major isomer being D-fructose 1,6-bisphosphate (**3**)] and with (±)-2-(benzyloxy)propanal [**19**; 3:1 ratio, the major isomer being (*S,S,R*)-5-(benzyloxy)-3,4-dihydroxy-1-(phosphonooxy)-hexan-2-one (**91**)]. When (±)-3-hydroxybutanal (**100**) reacts with DHAP, high thermodynamic diastereoselectivity was observed: the ratio of the two stereoisomers (**103** to **104**) is 97:3 and the major isomer is 5,7-dideoxy-L-*gluco*-2-heptulopyranose 1-phosphate (**103**).

The development of methods for stereoselective formation of carbon–carbon bonds using the aldol reaction is a current focus of effort in organic synthesis.[7-12] Many successful strategies using chiral auxiliaries have been reported.[13-21] In principle, however, a catalytic asymmetric aldol reaction requiring minimal effort to prepare the catalyst would be preferable to methods requiring a stoichiometric equivalent of a chirotopic auxiliary. Previous reports[22] describe three nonbiological catalysts—Zn(II) complexes

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(4) Postdoctoral Fellow of Ciba-Geigy and the Government of Austria.
(5) Natural Science and Engineering Research Council of Canada, Predoctoral Trainee.
(6) Postdoctoral Fellow of the Deutsche Forschungsgemeinschaft, 1985-1986.
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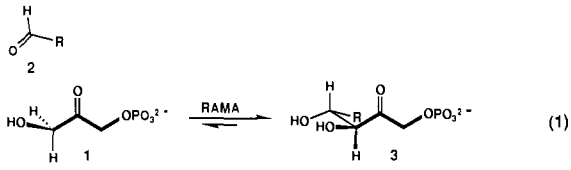
of amino acid esters,[23] Eu(DPPM)$_3$,[24] and a chirotopic ferrocenyl phosphine–gold(I) complex[25]—for the asymmetric aldol reaction. The chiral gold complex gives the highest stereoselectivities: 72–97% enantiomeric excess and 60–100% diastereomeric excess.

Enzymes often provide products with higher enantiomeric purity than do nonbiological catalysts.[26,27] This paper discusses the utility of the most readily available member of the class of proteins that catalyzes the aldol reaction,[28-32] rabbit muscle aldolase (RAMA, EC 4.1.2.13), as a catalyst for the asymmetric aldol reaction.[33]

Properties of RAMA.[34] In vivo, RAMA catalyzes the equilibrium condensation of dihydroxyacetone phosphate (DHAP, **1**) with D-glyceraldehyde 3-phosphate (G-3-P, **2**) to form D-fructose 1,6-bisphosphate (FDP, **3**). The equilibrium constant for this reaction is $K = [\mathbf{3}]/[\mathbf{1}][\mathbf{2}] = 10^4\ M^{-1}$ (eq 1).[31,32] Several



(1)

properties of RAMA make it a useful catalyst in organic synthesis. Commercially available preparations of the enzyme are inexpensive and have a useful specific activity (60 units/mg, 1 unit (U) = 1 μmol of product formed/min). Although product inhibition by unnatural substrates has not been explored thoroughly, product inhibition is not significant when FDP is a substrate.[30] The enzyme

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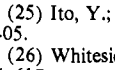
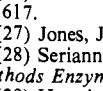
Table I. Relative Reactivities of Aldehydes (RCHO) with DHAP in RAMA-Catalyzed Aldol Condensations

R		V_{rel}[a]	substr class[b]	ref[c] or cosolv
$P_iOCH_2CH(OH)$	**2**	100[d]	+++	32
H	**4**	105	+++	
H_3C	**5**	120	+++	
CH_3CH_2	**6**	105	+++	
$CH_3CH_2CH_2$	**7**	43	+++	10% DMSO
$CH_3CH_2CH_2CH_2$	**8**	7	+	10% DMSO
$(CH_3)_2CH$	**9**	19	++	
$(CH_3)_2CHCH_2$	**10**	6	+	
$PhCH_2$	**11**	27	+++	20% DMSO
$CH_2=CH$	**12**	0	–	
$CH_3CH=CH$	**13**	0	–	
$(CH_3)_3C$	**14**	0	–	8% EtOH
$Ph(CH_3)CH$	**15**	1[e,f]	+	20% DMSO
$Ph(CH_3O)CH$	**16**	9	+	10% DMSO
$HOCH_2$	**17**	33	+++	
$CH_3CH(OH)$	**18**	10[e]	++	38
$CH_3CH(OBzl)$	**19**	20[e,f]	++	10% DMSO[46]
$CH_3CH_2CH(OH)$	**20**	10[d]	++	38
$HOCH_2CH(CH_3)$	**21**	7[d]	+	48
$HOCH_2CH(CH_2OH)$	**22**	34	+++	48
$HOCH_2CH(OH)$	**23**	15[e]	++	
$CH_3OCH_2CH(OH)$	**24**	15[d]	++	46
$HOCH_2CH(CH_3O)$	**25**	22[d]	++	46
$HOCH_2C(CH_3)(OH)$	**26**	2[e]	+	46
D-erythrose	**27**	<1	–	
D-erythrose-4-P_i	**28**	2[g]	+	
D-ribose	**29**	<1	–	39, 40, 44
D-ribose-5-P_i	**30**	5[g]	+	39, 40, 44
D-arabinose	**31**	<1	–	39, 40, 44
D-arabinose-5-P_i	**32**	1[g]	+	39, 40, 44
2-deoxy-D-glucose	**33**	<1	–	
2-deoxy-D-glucose-6-P_i	**34**	3[g]	+	39
D-glucose	**35**	<1	–	
D-glucose-6-P_i	**36**	1	+	39
$BzlOCH_2$	**37**	25	+++	49, 50
$THPOCH_2$	**38**	15	++	49, 50
$CH_3OCH_2CH(CH_3O)$	**39**	9	+	51
	**40**	10[d]	++	52
	**41**	7[d]	+	53
$CH_3SCH_2CH_2$	**42**	40	+++	54
OCH	**43**	12	++	
$OCH(CH_2)_2CH_2$	**44**	10	++	
CH_3CO	**45**	12[d]	++	
NaO_2C	**46**	4	+	
$ClCH_2$	**47**	340	+++	55
$BrCH_2$	**48**	400	+++	55
$ClCH_2CH_2CH_2$	**49**	160	+++	
Cl_3C	**50**	0	–	55
$BrCH_2CH_2$	**51**	0	–	
$NO_2CH_2CH_2$	**52**	0	–	
$NO_2CH_2CH_2CH_2$	**53**	170	+++	
$N_3CH_2CH_2$	**54**	0	–	46
$AcNHCH_2$	**55**	5	+	46
t-$BocNHCH_2$	**56**	2	+	46
$CbzNHCH_2$	**57**	1[g]	+	46
$AcNHCH(CH_3)$	**58**	6[e]	+	46
cyclopentyl	**59**	5[g]	+	46
N-Acetylpyrrolidinyl	**60**	4[g]	+	46
N-*t*-Boc-pyrrolidinyl	**61**	1	+	46
$BocNHCH(COOMe)CH_2$	**62**	5[g,h]	+	56
Ph	**63**	0	–	20% DMSO
2-pyrrolyl	**64**	<1	–	10% DMSO
2-furanyl	**65**	<1	–	10% DMSO
3-pyridyl	**66**	2	+	10% DMSO
2-pyridyl	**67**	1	+	10% DMSO

[a] Reactivities were measured in 0.2 M triethanolamine buffer (pH 7.0, 25 °C) containing both substrates at 50 mM concentration, unless indicated. [b] (+++) $V_{rel} > 25$; (++) $25 > V_{rel} > 10$; (+) $10 > V_{rel} > 1$; (–) $V_{rel} < 1$. [c] The references describe the preparation of aldehydes that were not commercially available. [d] V_{rel} is for the D stereoisomer. [e] A racemic mixture was assayed. [f] The kinetic diastereoselectivity of these aldehydes is discussed in the text. [g] Estimated from preparative-scale reactions. [h] Only the L-amino aldehydes were assayed.

is not particularly air-sensitive and may be used in immobilized[35] or soluble forms or enclosed in a dialysis membrane.[36]

Previous studies of the application of RAMA as a catalyst in organic synthesis by us,[37-39] Jones,[40] Wong,[41,42] and others[43-45] have focused mainly on the synthesis of carbohydrates. The qualitative conclusion of these and other biochemical studies[30] is that RAMA accepts a broad range of aldehydes as the electrophilic component of the aldol reaction, but accepts only DHAP or close analogues of DHAP as the nucleophilic component.

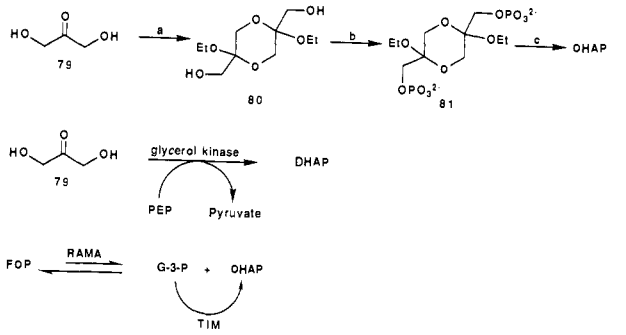
Objectives. The objectives of this work are (1) to suggest the range of synthetic utility of RAMA by establishing the structures of substrates accepted by the enzyme, (2) to provide representative experimental procedures for the production of 0.1–1.0-mol quantities of aldol adducts, and (3) to investigate the diastereofacial stereoselectivity of RAMA-catalyzed reactions with chiral aldehydes.

Results and Discussion

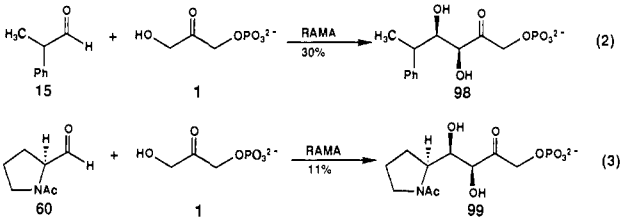
Reactivity of Aldehydes as Substrates. We describe elsewhere the preparation of aldehydes not available from commercial sources.[46] We found ozonolysis of α-olefins, when these precursors were available, to be the best route to the aldehydes used in the study. Table I lists aldehydes that have been tested as substrates with RAMA, together with estimates of the initial relative velocities (V_{rel}) expressed as a percentage of the initial velocity of the natural substrate D-G-3-P ($V_{50\,mM} = 100$). The substrates are grouped by reactivity into four classes: (1) $V_{rel} > 25$ (+++); (2) $25 > V_{rel} > 10$ (++); (3) $10 > V_{rel} > 1$ (+); (4) $V_{rel} < 1$ (−). This grouping provides a convenient, if qualitative, guide to applicability in synthesis. Standard concentrations used in the survey experiments were 50 mM for both aldehyde and DHAP.[47]

The observation of a slow disappearance of DHAP ($V_{rel} < 1$) in these experiments could be due to several factors: a slow enzyme-catalyzed reaction of the aldehyde substrate, a fast enzyme-catalyzed reaction with impurities present at low concentration in the aldehyde, or a slow non-enzyme-catalyzed decomposition or condensation. We have not confirmed that all of the more slowly reacting substrates listed in Table I do, in fact, produce aldol adducts with DHAP, because slowly reacting substrates are of intrinsically less interest in synthesis than are rapidly reacting ones. For several representative slowly reacting ($V_{rel} < 5$) aldehydes (**15**, **60**) we have, however, isolated the aldol products from the reaction, although in low yields (eq 2 and 3).

Scheme I. Enzymatic and Chemical Routes to DHAP **1**[a]



[a] Reagents: (a) $(EtO)_3CH$; (b) $POCl_3$–pyridine, H_2O–HCO_3^-–OH^-, $MgCl_2$–NH_4Cl, $BaCl_2$–EtOH; (c) Dowex 50W-X8 resin, H^+ form.



The studies summarized in Table I support the following generalizations concerning the substrate specificity of RAMA: (i) Aliphatic aldehydes are good substrates. Steric hindrance next to the aldehyde group reduces reactivity, but modest steric hindrance is tolerated; pivaldehyde (**14**), for example, is apparently not a substrate, but isobutyraldehyde (**9**) is a substrate. (ii) Most aldehydes that have unsaturation in conjugation with the carbonyl group are not accepted as substrates. α,β-Unsaturated aldehydes (**12**, **13**) do not react. Simple aromatic aldehydes either are poor substrates (**66**, **67**) or they do not react (**63**–**65**). In assay-scale experiments, the presence of 1 equiv of either crotonaldehyde (**13**) or benzaldehyde (**63**) did not inhibit the cleavage of FDP by RAMA; others have reported that benzaldehyde is an uncompetitive inhibitor of RAMA.[57] (iii) Aldehydes substituted with leaving groups in the position β to the carbonyl group [e.g., X–CH_2CH_2CHO; X = Br (**51**), NO_2 (**52**), N_3 (**54**)] are not useful substrates, probably because β-elimination under the reaction conditions forms α,β-unsaturated aldehydes. (iv) Molecules in which the aldehyde group is present predominantly as a hemiacetal or hydrate [e.g., CCl_3CHO (**50**)] are unreactive, with the exception of formaldehyde. (v) In contrast, some degree of activation of the carbonyl group by electron-withdrawing substituents increases reactivity, even though it must also increase the extent of hydration of the aldehyde group: $ClCH_2CHO$ (**47**) and $BrCH_2CHO$ (**48**) are more reactive than CH_3CHO (**5**) and CH_3CH_2CHO (**6**); $Cl(CH_2)_3CHO$ (**49**) is more reactive than $CH_3(CH_2)_3CHO$ (**8**). The balance between increased reactivity due to electron-withdrawing substituents and decreased reactivity due to hydration is not clear. (vi) Introduction of a phosphate group into the aldehyde-containing substrate increases reactivity: G-3-P (**2**) is a better substrate than glyceraldehyde (**23**); pentose 5-phosphates (**30**, **32**) are better substrates than unphosphorylated C_5 sugars (**29**, **31**). (vii) N-Protected primary and secondary α-amino aldehydes can be substrates; aldehydes derived from *N*-acetylglycine (**55**), *N*-*t*-Boc-glycine (**56**), *N*-acetylproline (**60**), and *N*-*t*-Boc-proline (**61**) all react at useful rates. The slight rate differences in this series are probably due to steric effects. These data also demonstrate that the enzyme tolerates several different protecting groups.

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(49) Danishefsky, S. J.; DeNinno, M. P. *J. Org. Chem.* **1986**, *51*, 2615. The THP protecting group was substituted for Bzl in the synthesis of **38**.
(50) Arndt, H. C.; Carroll, S. A. *Synthesis* **1979**, 202.
(51) Fedoronko, M.; Temkovic, P.; Mihalov, V.; Tvaroska, I. *Carbohydr. Res.* **1980**, *87*, 51.
(52) Schray, K. J.; O'Connell, E. L.; Rose, I. A. *J. Biol. Chem.* **1973**, *248*, 2214.
(53) Baer, E.; Fischer, H. O. L. *J. Biol. Chem.* **1939**, *128*, 463.
(54) Available from Kodak.
(55) Prepared by acid-catalyzed hydrolysis of the corresponding dimethoxyacetals: Reference 51.
(56) Turner, N. J.; Whitesides, G. M. submitted for publication in *J. Am. Chem. Soc.*
(57) Spolter, P. D.; Adelman, R. C.; Weinhouse, S. *J. Biol. Chem.* **1965**, *240*, 1327.
(58) Hartman, F. C. *Biochemistry* **1970**, *9*, 1776.
(59) Paterson, M. C.; Norton, I. L.; Hartman, F. C. *Biochemistry* **1972**, *11*, 2070.

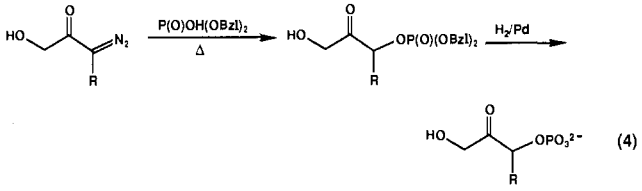
Table II. Relative Reactivities of DHAP and Analogues R_1COR_2 with G-3-P in RAMA-Catalyzed Aldol Condensations[a]

	R_1	R_2	V_{rel}	ref[b]
1	$HOCH_2$	CH_2OP_i	100	32
68	$HOCH_2$	$CH(CH_3)OP_i$	10[c]	46
69	$HOCH_2$	$CH_2CH_2PO_3H_2$	10	37
70	$HOCH_2$	CH_2SO_3H	<0.1	37
71	$HOCH_2$	CH_2OH	<0.1	37
72	H_3C	OCH_2OP_i	<0.1	37
73	N_3CH_2	CH_2OP_i	<0.1	46
74	$AcNHCH_2$	CH_2OP_i	<0.1	46
75	$HOCH(CH_3)$	CH_2OP_i	<0.1[c]	46
76	$ClCH_2$	CH_2OP_i	0	58, 59
77	$BrCH_2$	CH_2OP_i	0	59
78	ICH_2	CH_2OP_i	0	59

[a] Relative velocities were measured in 0.2 M triethanolamine buffer (pH 7.0, 25 °C) containing 50 mM substrates. [b] References are to preparation of the dihydroxyacetone substrate. [c] A mixture of D and L enantiomers was assayed.

We conclude that RAMA accepts a broad range of aldehydes. There is significant discrimination between enantiomers of chiral aldehydes in certain systems, and this discrimination may provide the basis for a useful method for controlling an additional stereogenic center. The issue of diastereoselectivity is discussed below.

Reactivity of Analogues of DHAP as Substrates. The syntheses of analogues of DHAP are described elsewhere.[46] In general, we found that reaction of diazoketones with dibenzyl phosphate was the best route to these compounds (eq 4). A potentially useful



(4)

route to analogues of DHAP proceeds via analogues of the dimer of DHAP;[45] Scheme I describes the preparation of DHAP by this method. We have not explored this route to analogues of DHAP in detail.

Other reports[30] describe RAMA as being highly selective for DHAP. Our limited studies (Table II) confirm this selectivity, but also establish that a limited variation in structure is possible but only at C-1. For example, $RCH(CH_3)OP_i$ (**68**) and $RCH_2CH_2PO_3H_2$ (**69**)[60] are substrates.

Synthesis of DHAP. DHAP can be prepared by three procedures (Scheme I): (1) from dihydroxyacetone (**79**) by phosphorylation using PEP and glycerol kinase (EC 2.7.1.30),[37] (2) from 1,3-dihydroxyacetone phosphate dimer **81** by chemical phosphorylation of dimer **80**,[37,45] and (3) from FDP using RAMA and triosephosphate isomerase (TIM, EC 5.3.1.1). Chemical phosphorylation of dihydroxyacetone with $POCl_3$[37] provides solutions of DHAP of lower purity (~60%) than do methods 1 and 2.

In our experience, the most convenient of these methods is that from FDP. RAMA converts FDP into DHAP and G-3-P; triosephosphate isomerase (TIM) in turn converts G-3-P into DHAP. In some cases, however, the presence of excess FDP complicates the isolation of products, especially when conversion of substrates to products is not complete. In addition, reactions using relatively pure preparations of DHAP (formed chemically or by using glycerol kinase) favor the formation of product; the equilibrium between products and FDP can in some cases be unfavorable. The advantage of the chemical method from dihydroxyacetone (**79**) is that it provides the cleanest preparation of DHAP and the barium salt of the dimer formed in the chemical route is stable to storage. The advantage of the route to DHAP using glycerol kinase is its simplicity and ease of use. Table III compares these methods.[61]

(60) Stribling, D. *Biochem. J.* **1974**, *141*, 725.

Table III. Comparison of Methods of Synthesizing DHAP[a]

	enzymatic (from **79**[37])	chemical (from **79**[45])	enzymatic (from FDP[37])
yield	83%	34%	in situ
purity	87%	95%[b]	NA
prepn (no. of steps)	1	8	1
convenience	++	+	+++

[a] Scheme I summarizes synthetic routes. [b] Purity analyzed by ^{31}P and ^1H NMR spectroscopy.

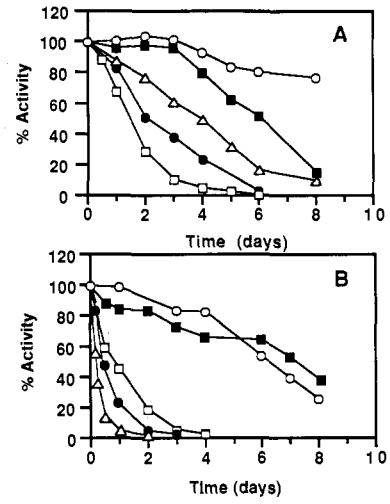


Figure 1. Stability of rabbit muscle aldolase (RAMA). (A) immobilized on PAN gel or dissolved in a homogeneous solution (RAMA 0.2 mg/mL in 50 mM TEA, pH 7.5, 25 °C): (○) PAN-immobilized and stored under air; (■) PAN-immobilized and stored with 2 mM 2-mercaptoethanol under nitrogen; (△) homogeneous solution stored under air; (●) homogeneous solution stored under nitrogen; (□) homogeneous solution stored with 2 mM 2-mercaptoethanol under air. (B) Membrane-enclosed RAMA in 50 mM TEA (pH 7.5 and 25 °C): (○) concentrated (50 mg/mL) RAMA; (■) concentrated (50 mg/mL) RAMA with 20 mg/mL BSA; (□) dilute (0.2 mg/mL) RAMA; (●) dilute (0.2 mg/mL) RAMA with 20 mg/mL BSA; (△) dilute (0.2 mg/mL) RAMA with 20 mg/mL BSA in a membrane pretreated with BSA. The activity of the enzyme was assayed by removing aliquots from the volume contained within the membrane.

Practical Issues

Stability. Although the potential of RAMA as a catalyst in organic synthesis rests on its breadth of applicability and on its stereoselectivity, a number of other issues—especially its cost and stability—bear on its practical utility. RAMA is a relatively inexpensive enzyme ($0.01/U), and in our experience, the most expensive component of the reaction mixtures is usually the aldehyde (which often must be synthesized) rather than the enzyme.

A number of factors determine the lifetime of the enzyme in use[35] including sensitivity to oxidation, to proteases, and to denaturation by adsorption at interfaces and by aggregation in solution. Figure 1 shows the stability of RAMA in a number of circumstances. We conclude that the enzyme is not sensitive to O_2 and that it should be used without adding reducing agents such as dithiothreitol or 2-mercaptoethanol. Either immobilization of RAMA on PAN gel[35] or confining it within a dialysis membrane (MEEC)[36] at a high concentration of protein (10 mg/mL) appreciably increases its operating lifetime; RAMA is unstable when enclosed in a dialysis membrane at low concentrations. Initial studies suggest that this instability is due to adsorption onto the

(61) Even if pure DHAP is used, FDP may still form in the reaction mixture. Triosephosphate isomerase contaminates most commercially available preparations of RAMA and converts DHAP to G-3-P; RAMA then rapidly forms FDP from these two substances. The formation of FDP can be an important competitive process in the case of slowly reacting substrates. This problem can be circumvented by the addition of 1-bromoacetone 3-phosphate, which inhibits the activity of TIM without interfering with that of RAMA: De La Mare, S.; Coulson, A. F. W.; Knowles, J. R.; Priddle, J. D.; Offord, R. E. *Biochem. J.* **1972**, *129*, 321.

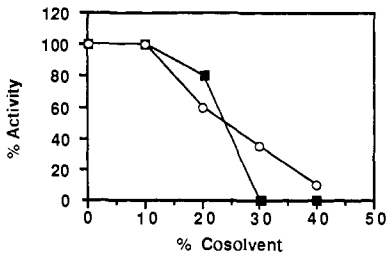


Figure 2. Activity of RAMA in a solution containing an organic cosolvent (v/v) in an aqueous TEA (80 mM, pH 7.0) buffer solution: (○) dimethyl sulfoxide (DMSO); (■) ethanol. The enzyme was exposed to the solution for 60 min before being sampled, diluted in buffer containing no added organic cosolvent, and assayed for residual activity.

surface of the membrane, although pretreatment of the membrane with bovine serum albumin (BSA) did not increase its stability.

It is practical (based on considerations of cost of enzyme and of reaction time) to use the enzyme in soluble form to catalyze aldol condensations involving substrates with a $V_{rel} > 1$ (Tables I and II) and a 1–10-mmol scale. Under ideal conditions, approximately 700 U of enzymatic activity is needed to produce 1 mol of product per day; the cost of the commercial enzyme in research quantities is \$60/5000 U. Thus, to make 0.1 mol of product in a reaction having $V_{rel} = 1$ over a 5-day interval would require RAMA costing approximately \$17.

Since deactivation of RAMA is fairly rapid in solution, the enzyme reactor should be recharged with fresh enzyme approximately every 48 h if soluble enzyme is used. Of course, under these conditions, the protein cannot be recovered from the reaction mixture and reused. The presence of soluble protein in the reaction mixture may, in some cases, also complicate the purification of products. The MEEC technique[36] circumvents this problem.

Organic Cosolvents. Some substrates are poorly soluble in water and the stability of RAMA toward organic cosolvents is important (Figure 2). It appears that RAMA tolerates 10–20% (v/v) concentrations of DMSO or ethanol with only slight (~20%) loss of activity compared with activity in aqueous triethanolamine buffer. As noted, we successfully used mixed water–organic solutions to obtain several of the data in Table I involving aldehydes that are poorly soluble in water.[62]

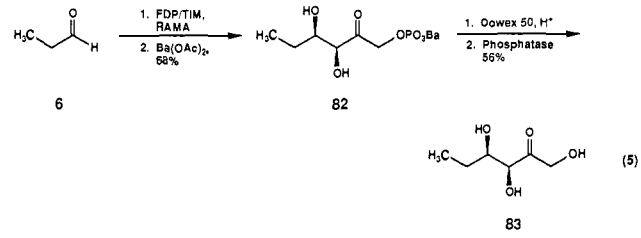
Removal of Phosphate Groups. We have explored several chemical and enzymatic methods for removing the phosphate group introduced into the product as a part of the DHAP moiety: acid-catalyzed hydrolysis using soluble acids or cation-exchange resins and hydrolysis catalyzed by acid phosphatase (EC 3.1.3.2) or alkaline phosphatase (EC 3.1.3.1). The most useful procedure uses acid phosphatase. In general, the nonenzymatic routes cause too much decomposition of the aldol adducts to be useful, although for certain acid-stable products this method can be used. The values of pH required (pH 8–9) for reasonable activity of alkaline phosphatase (EC 3.1.3.1) may also cause decomposition and reactions other than hydrolysis of phosphate in the case of adducts that are sensitive to base. Acid phosphatase (EC 3.1.3.2) operates in a pH range (pH 5–7) tolerated by most adducts; it accepts a wide range of substrates;[63] it is inexpensive; it is stable and easily manipulated. Acid phosphatase may be used in soluble form, but containment within a dialysis membrane is more convenient on a large scale[36] because separation of the protein from the reaction mixture is simplified.

Purification. The most useful methods—precipitation or ion-exchange chromatography—for purifying the adducts from aldolase reactions take advantage of the charged phosphate group: the presence of the phosphate group can thus be a significant advantage in product separation even when a nonphosphorylated product is ultimately required. After purification of the product, the phosphate group can easily be removed by enzymatic hydrolysis with phosphatase.

In cases where no excess of DHAP or FDP is present, precipitation of a barium or cyclohexylammonium salt of the organic phosphate is the most straightforward method of isolating the product. Products obtained in the form of barium or alkylammonium salts are seldom pure, but usually are satisfactory for use in other enzyme-catalyzed transformations: inorganic phosphate is normally the major impurity. A solution of the (essentially) barium-free organic phosphate is easily generated by treating a suspension of the barium salt with sulfuric acid, with concomittant formation of barium sulfate. In cases in which greater purity is desired or in which other phosphate-containing compounds are present in solution, purification by ion-exchange chromatography provides satisfactory results. The eluant may be a gradient of formic acid or, in the case of acid-sensitive compounds, triethylammonium bicarbonate solution (prepared by saturating a triethylamine–water solution with carbon dioxide until the pH is ≤ 8) or an ammonium bicarbonate solution.

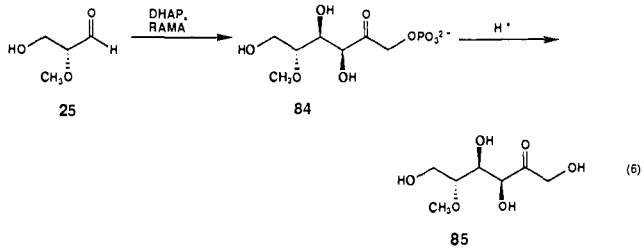
Analytical Techniques. Enzymatic assays[31] are precise but time-consuming methods for monitoring the progress of RAMA-catalyzed reactions. A more convenient qualitative technique for following reactions involves thin-layer chromatography on silica gel, eluting with solutions of 2-propanol–ammonium hydroxide–water (6:3:2) or 1-butanol–acetic acid–water (5:3:2). The blue-gray oxidized complexes are visualized with a ceric sulfate–ammonium molybdate stain.[64] For quantitative analysis, HPLC using ion-pairing reagents (such as the commercially available Waters PIC reagents) in acetonitrile–water solutions has proven useful. ^{31}P NMR spectroscopy also is a convenient and simple method for monitoring reactions.

Examples. The synthesis of 5,6-dideoxy-D-*threo*-2-hexulose 1-phosphate (**82**; eq 5) as the barium salt demonstrates the use



of RAMA in a synthesis carried out on a 1-mol scale; details are described in the Experimental Section. In this synthesis, TIM was used with RAMA to generate DHAP from FDP; excess aldehyde was used in the reaction to drive the equilibrium and to consume all the DHAP formed. To establish the stereochemistry of the adduct, we used acid phosphatase to convert **82** into 5,6-dideoxy-D-*threo*-2-hexulose (**83**). This triol had the same specific rotation as a sample prepared from D-fructose.[65]

The synthesis of 5-*O*-methyl-D-fructose (**85**) on a 4-mmol scale illustrates a procedure that uses DHAP synthesized from dihydroxyacetone (DHA, **79**) with glycerol kinase (eq 6). Acidic



hydrolysis dephosphorylates the aldol adduct **84** and gives the D sugar **85**, based on a comparison of the optical rotation of **85** to that of a compound prepared from D-fructose.[66] No epimerization

(62) A change in solvent can also occasionally be useful in shifting equilibrium constants, although the shifts are typically not large: Shih, Y.-S.; Whitesides, G. M. *J. Org. Chem.* **1977**, *42*, 4165.

(63) *The Enzyme Handbook*; Barman, T. E.; Ed.; Springer-Verlag: New York, 1969; Vol. II, p 523.

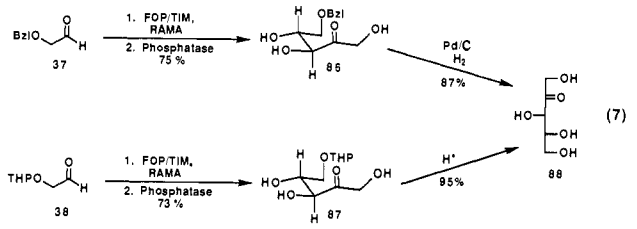
(64) The stain contained 24 g of ammonium molybdate and 1 g of cerium(III) sulfate in 500 mL of 10% (v/v) H_2SO_4.

(65) Gorin, P. A. J.; Hough, L.; Jones, J. K. N. *J. Chem. Soc.* **1955**, 2699.

(66) Heyns, K.; Heukeshoven, J. *Justus Liebigs Ann. Chem.* **1976**, 269.

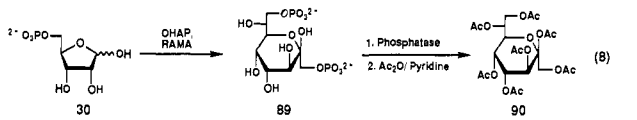
of aldehyde **25** or product **84** occurred during the aldolase reaction, nor during precipitation with barium chloride and dephosphorylation.

The synthesis of D-xylulose (**88**) illustrates the use of RAMA with substrates that require organic cosolvents (eq 7). In a



solution containing 20% DMSO (v/v), (benzyloxy)acetaldehyde (**37**) reacts with DHAP generated from FDP using TIM to form **86** in yields of ~80% on a 5–10 mmol scale following dephosphorylation using acid phosphatase, extraction of the product into ethyl acetate, and chromatography on silica gel. (Tetrahydropyranyloxy)acetaldehyde (**38**) reacts under similar conditions to give **87**. In this instance, the hydrophobic protecting groups facilitate the purification of the products by increasing their solubility in organic media; phosphates, sugars, and protein stay in the water layer. The conversion of **86** and **87** to D-xylulose (**88**) showed that both reactions produced only one stereoisomer; no arabinulose was detected.[67]

Chain Extension in Sugars. We believe that the condensation of a sugar phosphate with DHAP will be a broadly useful class of reactions for the preparation of complex monosaccharides.[39] The synthesis of D-*glycero*-D-*altro*-2-octulose 1-phosphate (**89**), characterized as peracetate **90**, from ribose 5-phosphate (**30**) illustrates this reaction (eq 8).[39] The key element in this synthetic



method is its ability to extend an unprotected pentose 5-phosphate or hexose 6-phosphate chain by three carbon atoms, while introducing two new stereogenic centers. We found that DHAP generated by phosphorylation of dihydroxyacetone with glycerol kinase and PEP gave higher yields than DHAP generated in situ by using FDP and TIM because the presence of residual FDP seriously complicated the purification of the products. The DHAP from dihydroxyacetone dimer **81** should also serve well. Analysis of the 500-MHz ^{1}H NMR spectrum of **90** established the stereochemistry of the aldol adduct. We conclude that the threo stereochemistry is retained during reactions of RAMA with sugar phosphates because, in this example, the small coupling constant between the C-3 and C-4 protons, 3.8 Hz, implies a trans diequatorial arrangement of these protons. A range of other examples supports this conclusion.[39]

Pentoses substituted at C-5 with phosphate and hexoses substituted at C-6 with phosphate react with DHAP in RAMA-catalyzed reactions faster than unphosphorylated sugars (for example, Table I, entries **29–34**). A similar difference in rate is also observed with glyceraldehyde and glyceraldehyde 3-phosphate: G-3-P reacts 7 times faster than glyceraldehyde with RAMA. This difference in rate between phosphorylated and nonphosphorylated aldehydes probably reflects an important interaction between the phosphate group and a positively charged group in the protein.

Diastereoselectivity Using Racemic Aldehydes. We have observed both kinetic and thermodynamic diastereoselectivity in a limited number of cases when racemic mixtures of aldehydes reacted with DHAP in RAMA-catalyzed reactions: reaction of **2** or **19** with DHAP showed kinetic diastereoselectivity and reaction of **100** with DHAP showed thermodynamic diastereoselectivity, i.e., a discrimination between the antipodes in a racemic

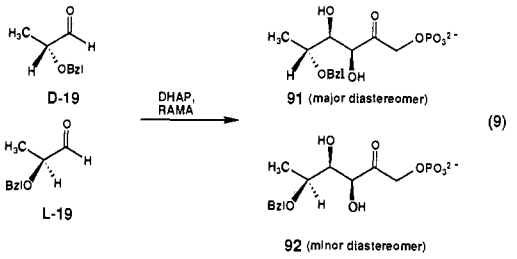
(67) Mendicino, J. F. *J. Am. Chem. Soc.* **1960**, *82*, 4975.

Table IV. Kinetic Diastereoselectivity of RAMA with Aldehydes (RCHO)

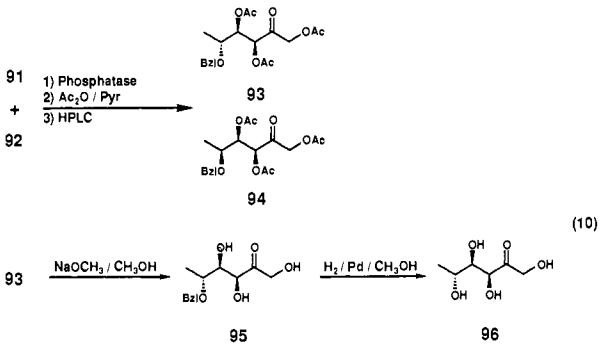
R		% conv	stereoselectivity
$P_iOCH_2CH(OH)$	**2**	40	20:1
$Ph(CH_3)CH$	**15**	60	1.3:1
$Ph(CH_3O)CH$	**16**	60	1.6:1
$CH_3CH(OBzl)$	**19**	45	3.0:1

mixture of aldehyde caused by a difference in the relative stability of the diastereomeric products that are formed by use of RAMA.

We examined four aldehydes, **2**, **15**, **16**, and **19**, for evidence of kinetic diastereoselectivity (Table IV). Aldehydes **15** and **16** did not show useful diastereofacial selectivity at 60% conversion of DHAP in the presence of a 2-fold excess of aldehyde. The reaction of 2.5 equiv of a racemic mixture of **19** with 1 equiv of DHAP, however, gave two diastereomeric products **91** and **92** in a ratio of 3:1 after 45% of the DHAP was consumed; the major diastereomer was **91** (eq 9). We determined the stereochemistry



of the adducts by two independent methods: (i) by synthesizing an enantiomerically enriched mixture of aldehyde L-**19** from L-lactic acid, subjecting it to excess DHAP in the presence of RAMA, and assigning the stereochemistry of the adducts by spectroscopic methods; (ii) by dephosphorylating and peracetylating **91** and **92** to give **93** and **94** which could be separated by HPLC. Deprotection of **93** ($NaOCH_3$–CH_3OH followed by H_2–Pd) gave 6-deoxy-D-fructose (**96**); L-sorbose (**97**) was not detected. The spectral properties of **96** were those reported in the literature (eq 10).



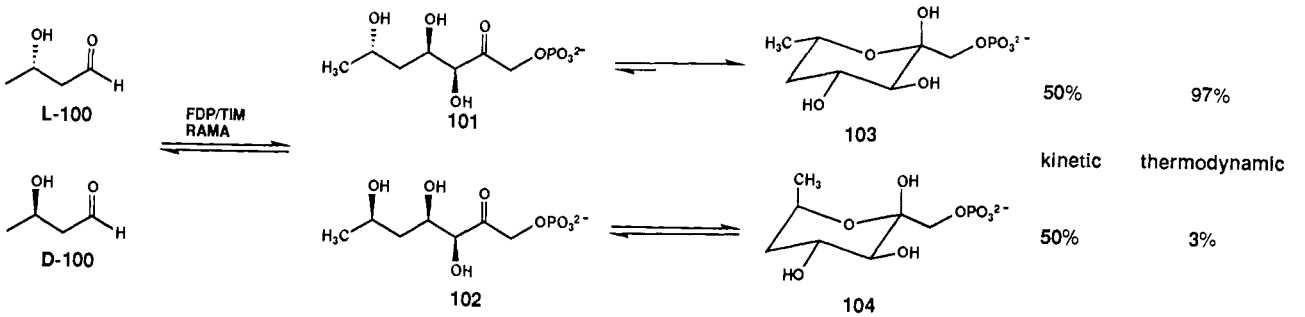
We also confirmed[68] that RAMA selects D-G-3-P (D-**2**) in preference to L-G-3-P (L-**2**). Our experiment monitored the consumption of DHAP by ^{31}P NMR spectroscopy and used enzymatic assays specific for D-**2** and L-**2** to measure the relative reactivities of these two enantiomers. When a mixture containing 2 equiv of racemic G-3-P and 1 equiv of DHAP reacted in the presence of RAMA, the ratio of the consumption of D-**2** to L-**2** was 98:2 after 98% of the DHAP had reacted. In separate experiments, we measured the relative reactivity of D-G-3-P and L-G-3-P and found that D-G-3-P reacted 7 times faster than L-G-3-P.

We also examined several aldehydes for evidence of thermodynamic diastereoselectivity[69,70] in RAMA-catalyzed aldol re-

(68) Tung, T.-C.; Ling, K.-H.; Byrne, W. L.; Lardy, H. A. *Biochem. Biophys. Acta* **1954**, *14*, 488.

(69) An elegant example illustrates the use of proteases to resolve racemic mixtures of esters with in situ racemization: Fülling, G.; Sih, C. J. *J. Am. Chem. Soc.* **1987**, *104*, 2845.

Scheme II. An Example of Thermodynamic Selectivity in a RAMA-Catalyzed Reaction[a]



[a] The formation of diastereomer **103** having the 5-methyl group in the equatorial position is favored compared with the formation of diastereomer **104** having the 5-methyl group in the axial position after cyclization of **101** and **102**. The ratio at thermodynamic equilibrium is 97:3 (**103** to **104**).

actions. With racemic mixtures of the α-substituted aldehydes **15**, **16**, **18**, and **19**, we observed a 1:1 mixture of the acyclic diastereomeric aldol adducts after reaction times of 1 week; there was no thermodynamic preference for either diastereomer. When a racemic mixture of aldehydes L-**100** and D-**100** (generated in situ from D,L-pent-4-en-2-ol) reacted with DHAP in the presence of RAMA, however, the ratio of the two diastereomeric products **103** and **104** was 97:3 after 5 days (Scheme II). In this case, the initial diastereomeric adducts (**101** and **102**) cyclize to pyranose rings in which the methyl group can be in either the equatorial (**103**) or axial (**104**) position; the energy difference between these forms causes the observed thermodynamic stereoselectivity. We confirmed that this ratio is based on relative thermodynamic stability by isolating the minor diastereomer **104** and reacting it again with a solution of DHAP, racemic **100**, and RAMA; the same 97:3 ratio of products was observed for **103** and **104**.

Experimental Section

Materials and Methods. Chemicals were purchased from Aldrich and were reagent grade. Enzymes and biochemicals were obtained from Sigma. Enzyme immobilization or enclosure in membranes were carried out as described.[35,36] Optical rotations were measured with a Perkin-Elmer 241 polarimeter. NMR spectra were recorded by using tetramethylsilane (TMS), sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS), chloroform ($CHCl_3$), or phosphate (H_3PO_4) as internal standard. Elemental analyses have been prepared by Galbraith Laboratories. Mass spectra were obtained with a Kratos MS 50.

Enzymatic Assays. Kinetic Measurements. To 1 mL of 0.2 M triethanolamine (TEA) buffer (pH 7) containing DHAP or G-3-P (50 mM) and the potential substrate (50 mM) was added 20 μL of a solution containing aldolase ($\sim$500 U/mL of the TEA buffer). At time intervals of 1 min during the course of 5–10 min, 0.1 mL of the assay solution was withdrawn, quenched with 30 μL of 7% perchloric acid solution, neutralized with 20 μL of 1 N NaOH, and diluted with 0.5 mL of 0.2 M pH 7 TEA buffer. This solution (0.05-mL aliquot) was subsequently assayed for either DHAP[71,72] or G-3-P.[28] A control reaction containing the natural substrates was run and assayed at the same time for reference. The rate of reaction for aldehyde analogues (defined as V_{sub}, in units of micromole of product produced per minute) was calculated by plotting time versus consumption of DHAP. The relative rate (V_{rel}) for analogues of G-3-P is defined as the ratio $V_{sub}/V_{G\text{-}3\text{-}P}$. The relative rate for analogues of DHAP is V_{sub}/V_{DHAP} where V_{sub} is a measure of the consumption of the analogues of DHAP.

Assay of Preparative-Scale Experiments. Large-scale reactions were monitored as follows: 100-μL aliquots of the reaction mixture were withdrawn, and the protein was denatured by addition of 100 μL of 7% perchloric acid solution. After neutralization with 1 N NaOH and centrifugation in an Eppendorf centrifuge, an aliquot (100 μL) of the supernatant was dissolved in 2.9 mL of 0.04 M TEA buffer (pH 7.6, 40 mM EDTA) and transferred to a 3-mL plastic cuvette. To this cuvette was added 50 μL of a 5 mM solution of NADH in the above buffer, 50 μL of a solution of glycerol-3-phosphate dehydrogenase (40 U/mL) as a suspension in ammonium sulfate, and 50 μL of a suspension of aldolase in ammonium sulfate (13.5 U/mL).[29] The oxidation of NADH to NAD was monitored at 340 nm to give the value of ΔA_1. After 20 min, 50 μL of a suspension of triosephosphate isomerase (TIM) in ammonium sulfate (250 U/mL) was added and the disappearance of NADH was again recorded to give the value of ΔA_2. The percent of DHAP consumed is $(\Delta A_1 - \Delta A_2)/(\Delta A_1 + \Delta A_2) \times 100$. The error in this measurement is $\pm$10%.

5,6-Dideoxy-D-2-*threo*-hexulose 1-Phosphate (82). A 0.2 M solution of FDP (**3**; 3.75 L) was prepared by stirring the dicalcium salt of FDP with ion-exchange resin (Dowex 50W-X8, H^+ form; resin was added until the solution became homogenous) for 24 h and then removing the resin by filtration. The pH was adjusted to 7, and after degassing with nitrogen, 750 U of aldolase and 1000 U of TIM (both immobilized on PAN gel[35]) were added. After the resultant mixture was stirred for 1 h, 162 mL (2.25 mol) of freshly distilled propionaldehyde (**6**) was added dropwise during 20 h. After 24 h, the same amount of immobilized enzymes was added, and after an additional 8 h, this procedure was repeated. Stirring was continued for 48 h, and the gel then was removed by centrifugation. After the supernatant was cooled to 4 °C, 421 g of barium acetate (1.7 mol) was added in portions with stirring and the pH was adjusted to 7.5. Acetone (3.5 L) was added, and the resulting suspension was stored at 4 °C for 3 days. The precipitate formed was isolated by centrifugation, successively washed with acetone and ether, and then dried in vacuo to yield 515 g of a yellowish solid. Enzymatic analysis indicated that 1.1 mol of product **82** (73% yield) was present. The compound was not further characterized and was used directly in the following step.

5,6-Dideoxy-D-2-*threo*-hexulose (83). A half liter of a 0.2 M solution of aldol adduct **82** ($\sim$100 mmol) was prepared by stirring the barium salt with ion-exchange resin (Dowex 50W-X8, H^+ form) overnight. After filtration, the pH was adjusted to 5.0, and after purging with nitrogen, 600 U of acid phosphatase was added. After the resultant mixture was stirred for 6 days at room temperature, the enzyme was denatured by heating to 75 °C. The solution was concentrated to 50 mL and then continuously extracted with ethyl acetate for 24 h. The aqueous layer was evaporated at reduced pressure nearly to dryness and then extracted five times with 100-mL portions of boiling acetone. The combined organic layers were dried over $MgSO_4$ and concentrated in vacuo. The remaining residue was chromatographed (silica gel, ethyl acetate–hexane 1:1) to yield 10 g (68%) of **83** as a yellow oil: $[\alpha]_D = -14.6°$ (c = 1, CH_3OH) [lit.[26] $[\alpha]_D = -13°$ (c = 1.2, CH_3OH)]; ^1H NMR (CD_3OD, 300 MHz) δ 4.53 (d, J = 19.2 Hz, 1 H, $CH_{2a}OH$), 4.42 (d, J = 19.3 Hz, 1 H, $CH_{2b}OH$), 4.14 (d, J = 2.2 Hz, CH(OH)CO), 3.78 (ddd, J_1 = 2.2 Hz, J_2 = 6.3 Hz, J_3 = 7.8 Hz, 1 H, CH(OH)C), 1.58 (m, 2 H, CH_2), 0.95 (t, J = 7.5 Hz, 3 H, CH_3); ^{13}C NMR (CD_3OD, 75 MHz) δ 214.0 (CO), 78.7 (CH(OH)CO), 74.8 (CH(OH)CH_2), 67.7 (CH_2OH), 27.0 (CH_2), 10.5 (CH_3); IR (neat) 1725 (C=O) cm^{-1}.

5-*O*-Methyl-D-fructose 1-Phosphate (84). An aqueous solution of 2-*O*-methyl-D-glyceraldehyde[20] (**25**; 9 mmol in 15.7 mL of water) was mixed with 10 mL of a solution containing 9 mmol of DHAP prepared enzymatically by using glycerol kinase,[37] and the solution was adjusted to pH 7 with 1 N KOH. Aldolase immobilized in PAN gel[35] (58 U) was added; the total volume was ca. 60 mL. After the mixture was stirred at room temperature for 5.5 h, the gel was separated by centrifugation and the supernatant was filtered through a layer of Celite. After concentration of the filtrate to a volume of 20 mL, the pH of the mixture was adjusted to 8.2, a solution of barium acetate (2.76 g, 10.8 mmol) in 5 mL of water was added, and the solution was readjusted to pH 8.2. Ethanol (250 mL) was added, and the resulting suspension was kept at 4 °C overnight. The precipitate formed was separated by centrifugation,

(70) A chemical example using a similar strategy has recently been reported: Reider, P. J.; Davis, P.; Hughes, D. L.; Grabowski, E. J. J. *J. Org. Chem.* **1987**, *52*, 955.

(71) Bergmeyer, H. U. In *Methods of Enzymatic Analysis*, 3rd ed.; Bergmeyer, H. U., Bergmeyer, J., Grassl, M., Eds.; Academic: New York, 1984; Vol. VI, p 342.

(72) Wood, W. A. *Methods Enzymol.* **1966**, *9*, 210.

washed with ethanol, and dried in vacuo to give 3.08 g of a white powder. This material was not characterized further and was used directly in the following step.

5-*O*-Methyl-D-fructose (85). The barium salt of **84** (2.5 g) was stirred with ion-exchange resin (Dowex 50W-X8, H^+ form) overnight. The resin was removed by filtration and washed with water, and the combined filtrates were concentrated to a volume of 20 mL. This solution was mixed with 25 mL of 2 N sulfuric acid, and the total volume of the mixture was diluted to 50 mL with water. The solution was heated to 87 °C, and the reaction was monitored by analyzing the release of inorganic phosphate.[30] After 3 h, the reaction was complete and the mixture was passed through a column of Dowex 1-X8 (HCO_3^- form). The eluant was concentrated in vacuo to give **85** as a pale yellow foam (796 mg, 56% yield based on **25**): $[\alpha]_D = -150°$ ($c = 1$, H_2O) [lit.[66] $[\alpha]_D = -156°$ ($c = 1$, H_2O)]; ^{13}C NMR (D_2O, 75 MHz) δ 98.5 (C-2), 79.6 (C-5), 70.1 (C-4), 68.8 (C-3), 64.7 (C-1), 60 (C-6), 57.9 (OCH_3).

5-*O*-Benzyl-D-xylulose (86). A solution of (benzyloxy)acetaldehyde (**37**; 2 g, 13.2 mmol), FDP (sodium salt, 3 g, 7 mmol), distilled water (60 mL), and DMSO (4 mL) was adjusted to pH 6.8 with 1 N NaOH. This solution was purged with nitrogen, and 60 U of aldolase and 40 U of TIM coimmobilized on PAN gel[35] were added. After the reaction mixture was stirred at room temperature for 10 h, an additional 10 mg (130 U) of lyophilized aldolase was added. After the solution was stirred under nitrogen for 48 h, enzymatic assay (see description of assays above) indicated approximately a 92% conversion to products. Addition of 1 N HCl adjusted the solution to pH 5.6, and 100 mg (70 U) of acid phosphatase was added. After 24 h, TLC analysis (silica gel, 1-butanol–acetone–water 5:3:2) indicated that the reaction was complete. Following continuous extraction with ethyl acetate for 24 h, the organic layer was dried over $MgSO_4$ and concentrated in vacuo. Purification of the residual oil (silica gel, ethyl acetate–hexane 1:1) gave 2.6 g of **86** (82% yield for the phosphate cleavage; 75% yield based on **37**): $[\alpha]_D = -2.2°$ ($c = 0.98$, $CHCl_3$); ^1H NMR ($CDCl_3$–D_2O 9:1, 500 MHz) δ 7.5–7.1 (m, 5 H), 4.55 (d, $J = 19.6$ Hz, 1 H), 4.54 (s, 2 H), 4.41 (d, $J = 19.6$ Hz, 1 H), 4.33 (d, $J = 2.4$ Hz, 1 H), 4.15–4.11 (m, 1 H), 3.67–3.6 (m, 2 H); ^{13}C NMR ($CDCl_3$–D_2O 9:1, 125 MHz) δ 187.3, 137.4, 128.4, 127.8, 75.9, 73.4, 70.7, 70.6, 66.5; IR ($CHCl_3$) 3700, 3510, 3025, 2405, 1725, 1520, 1420, 1200 cm^{-1}. Anal. Calcd for $C_{12}H_{16}O_5$. C, 59.99; H, 6.71. Found. C, 59.70; H, 6.85.

5-*O*-Tetrahydropyranyl-D-xylulose (87). (Tetrahydropyranyloxy)-acetaldehyde (**38**; 1.6 g, 11.1 mmol) and FDP (**3**) (trisodium salt, 1.11 g, 2.2 mmol) were treated with aldolase and TIM by following a similar procedure used for the **37**. When enzymatic assay indicated approximately a 91% conversion of reactants to products, the reaction mixture was treated with acid phosphatase and worked up as described for compound **86** to provide 750 mg of **87** as a 1:1 mixture of stereoisomers (80% yield for the phosphate cleavage; 73% yield based on FDP): ^{13}C NMR ($CDCl_3$, 125 MHz) δ 187.5, 187.2, 100.3, 100.0, 75.93, 75.9, 70.84, 70.79, 69.65, 68.36, 66.86, 66.61, 63.39, 63.34, 30.46, 30.39, 24.98, 19.86, 19.8. Anal. Calcd for $C_{10}H_{18}O_6$: C, 51.3; H, 7.75. Found: C, 51.27; H, 7.91.

D-Xylulose (88) by Hydrolysis of 87. Ion-exchange resin (Dowex 50W-X8, H^+ form) was added to a solution of **87** (100 mg, 0.42 mmol) in 5 mL of water, and the mixture was stirred at room temperature for 3 h. The resin was removed by filtration and washed with water, and the combined filtrates were extracted three times with dichloromethane. Evaporation in vacuo of the aqueous solution yielded 63 mg (95%) of **88**: $[\alpha]_D = -31.8°$ ($c = 2$, H_2O) [lit.[25] $-33°$ ($c = 2.5$, H_2O)]; ^{13}C NMR (D_2O, 125 MHz) δ 195.0, 107.2, 104.4, 98.6, 82.2, 77.8, 77.4, 76.8, 76.5, 73.5, 73.3, 71.8, 67.5, 64.6, 64.0, 63.3. The spectral data were indistinguishable from those of a sample purchased from Sigma.

D-Xylulose (89) by Hydrogenolysis of 86. Benzyl ether **86** (150 mg, 0.62 mmol) was dissolved in ethanol; 20 mg of 10% Pd/C catalyst was added, and this suspension was stirred at room temperature under hydrogen for 12 h. A mixture of Celite and Florisil was then added, and the mixture was filtered through a layer of Celite and chromatographed on P-2 Biogel (eluant, H_2O). Concentration of the filtrate in vacuo gave **88** (82 mg, 87%). The product was indistinguishable in its spectroscopic properties from the sample obtained through hydrolysis of **87**.

(*S*,*S*,*R*)-5-(Benzyloxy)-1,3,4-trihydroxy-2-hexanone 1-Phosphate (91) and (*S*,*S*,*S*)-5-(Benzyloxy)-1,3,4-trihydroxy-2-hexanone 1-Phosphate (92). A solution of D-**19** and L-**19** (65 mg, 0.40 mmol), DMSO (0.20 mL), and DHAP (0.24 mmol) in 4 mL of 200 mM triethanolamine (TEA) and 200 mM EDTA (pH 6.7) was added to a 10-mm NMR tube. A ^{31}P NMR spectrum was recorded and aldolase was added (50 U, 100 μL of a solution containing 500 U/mL in a solution of 0.2 M TEA buffer). ^{31}P NMR spectra, recorded at intervals of 5 min, showed four peaks in addition to the peak at 1.82 ppm corresponding to the reference peak of inorganic phosphate: two peaks corresponding to the diastereomeric products **91** and **92** of the aldol reaction (3.45 and 3.48 ppm) and two peaks corresponding to DHAP (4.40 ppm, hydrate; 3.58 ppm, ketone). The calculated percent conversion was the ratio of the sum of the areas of the peaks corresponding to the products divided by the sum of the areas of all four peaks. The calculated diastereoselectivity was the ratio of the areas of the peaks corresponding to the products. After 15 min the calculated diastereoselectivity of **91** to **92** was 3:1, and ~45% of the DHAP had been consumed. A similar assay procedure determined the diastereomeric ratio of **15** to **16**. The reaction was quenched with 2 mL of 1 N HCl and left at room temperature for 2 h. The solution was filtered, adjusted to pH 5.3 with 1 N NaOH, and diluted with water to a volume of 35 mL. The solution was extracted three times with 20-mL portions of methylene chloride, and the aqueous layer was transferred to a 50-mL graduated cylinder. Acid phosphatase (~100 U) was added to the stirred reaction mixture. After 12 h, analysis by ^{31}P NMR spectroscopy indicated that the cleavage of phosphate was complete. The solution was concentrated in vacuo to a volume of 2 mL and extracted three times with 25-mL portions of ethyl acetate. The combined organic layers were dried over $MgSO_4$ and concentrated in vacuo. Chromatography (silica gel; eluant, hexane–ethyl acetate 1:1–3:1) yielded 9.6 mg (20%) of a mixture of diastereomers of dephosphorylated **91** and **92**. When the conversion of DHAP reached 85% in a similar reaction, the calculated diastereoselectivity of **91** to **92** was 1.2:1. The stereochemistry of the major isolated product was proven by its conversion to 6-deoxy-D-fructose (**97**) as described below.

5-*O*-Benzyl-6-deoxy-D-fructose Triacetate (93) and 5-*O*-Benzyl-6-deoxy-L-sorbose Triacetate (94). A solution of racemic **19** (1.0 g, 6 mmol), FDP (trisodium salt, 1.6 g, 3.1 mmol) DMSO (2 mL), and aldolase in 40 mL of water was adjusted to pH 7 with 1 N NaOH. After the reaction solution was stirred for 24 h, analysis by enzymatic assay indicated ~40% consumption of FDP. The solution was filtered, the reaction flask was rinsed with ~40 mL of water, and the combined aqueous washes were extracted twice with 50-mL portions of methylene chloride. The combined organic phases were back-extracted with two 20-mL portions of water. The combined aqueous layers were concentrated in vacuo to 80 mL, and the solution was adjusted to pH 5.0 with 1 N HCl. The solution was then treated with acid phosphatase as described above. After ~24 h, the mixture was concentrated in vacuo to a volume of 3 mL and extracted three times with 100-mL portions of ethyl acetate. The organic phases were concentrated in vacuo and chromatographed (silica gel; eluant, hexane–ethyl acetate 1:1–1:3) to yield 338 mg (55% yield) of a mixture of **91** and **92**. A portion of this mixture (140 mg) was acetylated by standard procedures (acetic anhydride, DMAP, pyridine) and purified (silica gel; eluant, hexane–ethyl acetate 3:1–1:1) to provide 180 mg (86%) of a mixture of 5-*O*-benzyl-6-deoxy-D-fructose triacetate (**93**) and 5-*O*-benzyl-6-deoxy-L-sorbose triacetate (**94**). A portion of this mixture was purified by HPLC (Waters μ-Porasil column, 3.9 mm × 30 cm; hexane–ethyl acetate 3:1; flow, 0.6 mL/min). Compound **93**: ^1H NMR ($CDCl_3$, 500 MHz) δ 7.35–7.24 (m, 5 H), 5.63 (d, $J = 2.3$ Hz, 1 H), 5.28 (dd, $J = 8.3$, 2.2 Hz, 1 H), 4.82 (d, $J = 17.1$ Hz, 1 H), 4.75 (d, $J = 17.1$ Hz, 1 H), 4.59 (d, $J = 11.5$ Hz, 1 H), 4.35 (d, $J = 11.4$ Hz, 1 H), 3.70 (dq, $J = 8.3$, 6.1 Hz, 1 H), 2.08 (s, 3 H), 2.05 (s, 3 H), 2.03 (s, 3 H), 1.21 (d, $J = 6.1$ Hz, 3 H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 198.40, 170.06, 169.78, 137.42, 128.51, 128.20, 127.98, 74.41, 73.12, 71.52, 70.81, 66.49, 20.61, 20.34, 15.70. Compound **94**: ^1H NMR ($CDCl_3$, 500 MHz) δ 7.34–7.24 (m, 5 H), 5.39 (d, $J = 4.6$ Hz, 1 H), 5.31 (t, $J = 4.7$ Hz, 1 H), 4.80 (d, $J = 17.0$ Hz, 1 H), 4.76 (d, $J = 17.0$ Hz, 1 H), 4.54 (d, $J = 11.7$ Hz, 1 H), 4.39 (d, $J = 11.5$ Hz, 1 H), 3.80 (dq, $J = 4.8$, 6.4 Hz, 1 H), 2.15 (s, 3 H), 2.10 (s, 3 H), 2.08 (s, 3 H), 1.19 (d, $J = 6.4$ Hz, 3 H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 197.55, 169.94, 169.70, 169.64, 137.80, 128.35, 127.82, 127.72, 74.19, 73.51, 72.70, 71.22, 66.75, 20.68, 20.39, 20.35, 15.48.

5-*O*-Benzyl-6-deoxy-D-fructose (95). Compound **93** (4.5 mg, 0.012 mmol) was dissolved in 2 mL of methanol, and sodium methoxide was added until the pH of the solution was ~8. After 2 h, ion-exchange resin (Dowex 50W-X8, H^+ form) was added to neutralize the solution. The reaction mixture was filtered through glass wool and concentrated in vacuo, and the residue was purified (silica gel; eluant, ethyl acetate–hexane 3:1–5:1) to yield 1.4 mg (47%) of **95**: ^1H NMR ($CDCl_3$, 500 MHz) δ 7.37 (m, 5 H), 4.67 (d, $J = 11.3$ Hz, 1 H), 4.57 (s, 1 H), 4.55 (d, $J = 19.6$ Hz, 1 H), 4.44 (d, $J = 20$ Hz, 1 H), 4.42 (d, $J = 11.2$ Hz, 1 H), 3.82–3.76 (m, 2 H), 1.32 (d, $J = 6.0$ Hz, 3 H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 137.44, 128.67, 128.19, 127.95, 75.60, 74.57, 71.65, 66.91, 16.22.

6-Deoxy-D-fructose (96). Compound **95** (0.7 mg, 0.003 mmol) and ~5 mg of 10% Pd/C catalyst were added to 1 mL of methanol. The reaction mixture was then stirred at room temperature under hydrogen (1 atm). After 6 h, the reaction mixture was filtered and the filtrate was concentrated in vacuo to give **96**. The spectral properties of this sample agreed with those reported in the literature. Major ring form: ^1H NMR (D_2O, 500 MHz) δ 4.06 (d, $J = 8.4$ Hz, 1 H), 3.88 (t, $J = 8.2$ Hz, 1 H),

3.79 (dq, J = 8.0, 6.2 Hz, 1 H), 3.56 (d, J = 12.1 Hz, 1 H), 3.49 (d, J = 12.0 Hz, 1 H), 1.32 (d, J = 6.2 Hz, 3 H); ^{13}C NMR (D_2O, DSS, 125 MHz)[73] δ 103.74, 82.09, 79.04, 77.88, 65.56, 21.60. Only one resonance of the minor stereoisomer could be assigned in the ^{1}H NMR spectrum; 1.30 (d, J = 6.2 Hz).

(*S*,*R*,*S*)-5-Phenyl-1,3,4-trihydroxy-2-hexanone (98). To a solution of 2-phenylpropanal (**15**; 370 mg, 2.7 mmol), FDP (sodium salt, 970 mg, 2.4 mmol), water (30 mL), and DMSO (6 mL) was added 1 N NaOH to adjust the solution to pH 7.0. The solution was purged with nitrogen, and 100 U of aldolase and 40 U of TIM coimmobilized on PAN gel[35] were added. After the reaction mixture was stirred at room temperature for 24 h, 100 U of lyophilized aldolase and 250 U of TIM were added. After the reaction was stirred for an additional 36 h, the above procedure was repeated. After an additional 24 h, analysis by enzymatic assay indicated that 60% of the DHAP originally present had been consumed. The reaction mixture was extracted three times with 30-mL portions of CH_2Cl_2, and the aqueous layer was transferred to a 100-mL Erlenmeyer flask. The solution was adjusted to pH 5.6 with 1 N HCl, and 200 mg (140 U) of acid phosphatase was added. After being stirred for 48 h, the reaction mixture was continuously extracted with ethyl acetate for 24 h. The organic layer was dried over $MgSO_4$ and concentrated in vacuo. Purification on silica gel (ethyl acetate–hexane 1:1) yielded 210 mg (30%) of a mixture of diastereomers of **98** (1.3:1). Separation using HPLC on silica (Waters Porasil column; ethyl acetate–hexane 3:1) yielded the major diastereomer as a homogenous compound: ^{1}H NMR (500 MHz, $CDCl_3$ and D_2O (100 μL)) δ 7.5–7.2 (m, 5 H), 4.49 (d, J = 19.2 Hz, 1 H), 4.30 (d, J = 19.2 Hz, 1 H), 3.94 (d, J = 9.8 Hz, 1 H), 3.90 (br s, 1 H), 3.2–3.0 (m, 1 H), 1.42 (d, J = 6.9 Hz, 3 H); IR ($CHCl_3$) 1726 cm^{-1}; MS, m/e 224 (M^+).

4-(*N*-Acetylpyrrolidin-2-yl)-1,3,4-trihydroxybutan-2-one 1-Phosphate (99). A 100-mL graduated cylinder equipped with a magnetic stirring bar was charged with 5.3 g (13 mmol) of the sodium salt of FDP in 70 mL of H_2O, and the solution was adjusted to pH 6.7 with 1 N NaOH. Following the addition of 3.0 g (20 mmol) of **60**,[46] the solution was degassed for 30 min with nitrogen and 2-mercaptoethanol (15 μL) was added. A solution of aldolase (500 U) and TIM (ca. 500 U) in 2 mL of the reaction mixture was placed in a dialysis bag (SpectraPor 2, MWCO 12-14,000, 12-mm width) which was then placed in the reaction mixture. After the reaction mixture was stirred for 5 days at room temperature, analysis by enzymatic assay showed that ~35% of the FDP originally present had been consumed. The reaction mixture was concentrated in vacuo and chromatographed [AG-1X ion-exchange resin, formate form; eluant, H_2O (150 mL), 1 N HCOOH (100 mL), 2 N HCOOH (100 mL), 4 N HCOOH (400 mL)] to yield 0.96 g (3 mmol, 11%) of **99** as a mixture of two isomers (due to restricted rotation about the amide bond): ^{1}H NMR (D_2O, 500 MHz, major peaks) δ 4.21 (dd), 3.82 (d), 3.75 (d), 3.7–3.6 (m), 3.5–3.3 (m), 2.0–1.6 (m containing 1.95 (s), 1.91 (s)); MS (FAB), m/e 254 ($[M + Na]^+$).

5,7-Dideoxy-L-*gluco*-2-heptulopyranose 1-Phosphate (103). A solution of D,L-pent-4-en-2-ol (250 mg, 2.9 mmol) in dry methanol (20 mL) was cooled to −78 °C and treated with ozone until a pale blue color persisted. Dimethyl sulfide (1.0 mL, 13.6 mmol) was added, and the mixture was stirred (ca. 2 h) at room temperature until a negative iodine–starch test indicated the absence of peroxides. Evaporation of the solution in vacuo at 0 °C provided **100**, which was taken up in water (15 mL) to give a clear, colorless solution. FDP (sodium salt, 125 mg, 0.25 mmol) was added, and the solution was carefully adjusted to pH 6.8 with 1 N NaOH. After addition of soluble aldolase (20 U) and TIM (50 U), the mixture was allowed to stand at room temperature. The disappearance of the aldehyde and appearance of the products were determined by analyzing the 500-MHz ^{1}H NMR spectra of lyophilized aliquots (0.2 mL). After 6 h, all of the FDP had been consumed and two sets of signals in a ratio of 1:1 indicated that two diastereomers had formed. The signals assigned to **104** diminished after 5 days to give a final ratio of 97:3 of **103** to **104**. Compound **103**: ^{1}H NMR (500 MHz, D_2O) δ 4.14 (m, 1 H), 3.91 (ddd, 1 H, J = 11.7, 9.6, 2.1 Hz), 3.84 (dd, 1 H, J = 11.1, 7.4 Hz), 3.74 (dd, 1 H, J = 11.1, 5.5 Hz), 3.48 (d, 1 H, J = 9.6 Hz), 2.04 (ddd, 1 H, J = 12.8, 11.7, 5.0 Hz), 1.39 (quart, 1 H, J = 12.8, 11.7 Hz), 1.19 (d, 3 H, J = 6.3 Hz). Compound **104**: ^{1}H NMR (500 MHz, D_2O) δ 4.31 (m, 1 H), 4.08 (quart, J = 3.2, 2.5 Hz, 1 H), 3.93 (dd, J = 11.3, 9.8 Hz, 1 H), 3.75 (d, J = 3.2 Hz, 1 H), 3.48 (dd, J = 11.3, 6.6 Hz, 1 H), 1.75 (m, 1 H), 1.69 (m, 1 H), 1.19 (d, J = 6.3 Hz, 3 H); MS (FAB), m/e 267 ($[M (d_4) + H - OD]^+$).

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Registry No. 1, 57-04-5; **2**, 591-57-1; **3**, 488-69-7; **4**, 50-00-0; **5**, 75-07-0; **6**, 123-38-6; **7**, 123-72-8; **8**, 110-62-3; **9**, 78-84-2; **10**, 590-86-3; **11**, 122-78-1; **12**, 107-02-8; **13**, 4170-30-3; **14**, 630-19-3; **15**, 34713-70-7; **16**, 19190-53-5; **17**, 141-46-8; **18**, 3913-65-3; (±)-**19**, 41954-96-5; (D)-**19**, 81445-45-6; (L)-**19**, 81445-44-5; **20**, 117706-94-2; **21**, 117706-95-3; **22**, 40364-80-5; **23**, 56-82-6; **24**, 105121-51-5; **25**, 114790-43-1; **26**, 114790-45-3; **27**, 583-50-6; **28**, 585-18-2; **29**, 50-69-1; **30**, 4300-28-1; **31**, 10323-20-3; **32**, 13137-52-5; **33**, 154-17-6; **34**, 3573-50-0; **35**, 50-99-7; **36**, 56-73-5; **37**, 60656-87-3; **38**, 699-13-8; **39**, 105444-34-6; **40**, 64821-54-1; **41**, 15186-48-8; **42**, 3268-49-3; **43**, 107-22-2; **44**, 111-30-8; **45**, 78-98-8; **46**, 2706-75-4; **47**, 107-20-0; **48**, 17157-48-1; **49**, 6139-84-0; **50**, 75-87-6; **51**, 65032-54-4; **52**, 58657-26-4; **53**, 73707-26-3; **54**, 58503-60-9; **55**, 64790-08-5; **56**, 89711-08-0; **57**, 67561-03-9; **58**, 73323-67-8; **59**, 872-53-7; **60**, 73323-64-5; **61**, 117625-90-8; **62**, 117706-96-4; **63**, 100-52-7; **64**, 1003-29-8; **65**, 98-01-1; **66**, 500-22-1; **67**, 1121-60-4; **68**, 117625-91-9; **69**, 51827-90-8; **70**, 117625-92-0; **71**, 96-26-4; **72**, 117625-93-1; **73**, 117625-94-2; **74**, 117625-95-3; **75**, 117625-96-4; **76**, 23743-97-7; **77**, 24472-75-1; **78**, 19794-30-0; **82**·Ba, 117625-97-5; **83**, 117625-98-6; **84**·Ba, 117625-99-7; **85**, 58794-68-6; **86**, 117626-00-3; **87** (isomer 1), 117626-01-4; **87** (isomer 2), 117652-05-8; **88**, 551-84-8; **91**, 117626-02-5; **92**, 117626-03-6; **92** (dephosphorylated), 117626-11-6; **93**, 117626-04-7; **94**, 117626-05-8; **95**, 117626-06-9; **96**, 4429-06-5; **98** (isomer 1), 117626-07-0; **98** (isomer 2), 117626-12-7; **99**, 117626-08-1; (D)-**100**, 117706-97-5; (L)-**100**, 117706-98-6; **103**, 117626-09-2; **104**, 117626-10-5; RAMA, 9024-52-6; TIM, 9023-78-3; (DL)-pent-4-en-2-ol, 111957-98-3.

(73) The ^{13}C spectrum of **97** was obtained from a sample that also contained 6-deoxy-L-sorbose.