J|A|C|S
ARTICLES
Published on Web 11/07/2007

D-Fructose-6-Phosphate Aldolase-Catalyzed One-Pot Synthesis of Iminocyclitols

Masakazu Sugiyama, Zhangyong Hong, Pi-Hui Liang, Stephen M. Dean, Lisa J. Whalen, William A. Greenberg,* and Chi-Huey Wong*

Contribution from the Department of Chemistry and the Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037

Received May 30, 2007; E-mail: wong@scripps.edu

Abstract: A one-pot chemoenzymatic method for the synthesis of a variety of new iminocyclitols from readily available, non-phosphorylated donor substrates has been developed. The method utilizes the recently discovered fructose-6-phosphate aldolase (FSA), which is functionally distinct from known aldolases in its tolerance of different donor substrates as well as acceptor substrates. Kinetic studies were performed with dihydroxyacetone (DHA), the presumed endogenous substrate for FSA, as well as hydroxy acetone (HA) and 1-hydroxy-2-butanone (HB) as donor substrates, in each case using glyceraldehyde-3-phosphate as acceptor substrate. Remarkably, FSA used the three donor substrates with equal efficiency, with k_{cat}/K_M-values of 33, 75, and 20 M^{-1} s^{-1}, respectively. This level of donor substrate tolerance is unprecedented for an aldolase. Furthermore, DHA, HA, and HB were accepted as donors in FSA-catalyzed aldol reactions with a variety of azido- and Cbz-amino aldehyde acceptors. The broad substrate tolerance of FSA and the ability to circumvent the need for phosphorylated substrates allowed for one-pot synthesis of a number of known and novel iminocyclitols in good yields, and in a very concise fashion. New iminocyclitols were assayed as inhibitors against a panel of glycosidases. Compounds **15** and **16** were specific α-mannosidase inhibitors, and **24** and **26** were potent and selective inhibitors of *β-N*-acetylglucosaminidases in the submicromolar range. Facile access to these compounds makes them attractive core structures for further inhibitor optimization.

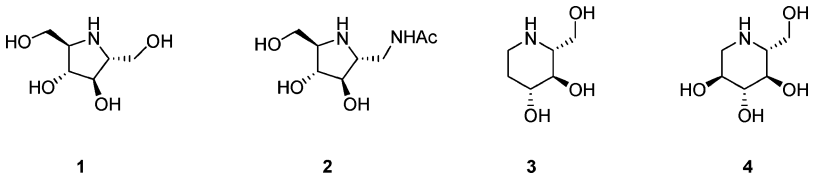
Introduction

The field of biocatalysis involves the application of enzymes as tools for organic synthesis.[1] The catalytic power and specificity of enzymes allows them to effect difficult transformations under mild conditions and with exceptional stereoselectivities. Many classes of enzymes have been exploited as synthetic tools, and one of the most prominent is the aldolases, which catalyze reversible formation of C−C bonds with high enantioselectivities.[2] A common limitation of enzymes as synthetic catalysts, and of aldolases in particular, is due to their substrate specificity. Substrates frequently need to be phosphorylated, and small changes in substrate structure typically lead to significant reduction in activity. Lack of generality and inability to use readily available substrates has limited the practicality of aldolases as catalysts for organic synthesis. Approaches to broadening the synthetic utility of aldolases include directed evolution, substrate engineering, and discovery of novel enzymes in nature.[3]

The polyhydroxylated pyrrolidines and piperidines, commonly called iminocyclitols, are potent glycosidase and glycosyltransferase inhibitors, due to their mimicry of the transition state of the enzymatic reactions.[4] Glycosidases and glycosyltransferases play key roles in metabolism, lysosomal catabolism, and glycoprotein processing. Glycoproteins and glycoconjugates on the cell surface regulate many significant biological events such as viral infection, cell−cell recognition, and inflammation. As a result, iminocyclitols have been attractive as drug candidates for a number of diseases such as cancer, viral infection, lysosomal storage disorders, and diabetes.[5] Several examples of important iminocyclitols are illustrated in Figure 1. The elegant work of Schramm and co-workers has led to discovery of iminocyclitol nucleoside derivatives that are exceptionally potent inhibitors of purine nucleotide phosphorylase and MTAN nucleosidase, illustrating the value of iminocyclitols in the design of inhibitors of glycoprocessing enzymes.[6] However, structure−

(1) (a) Powell, K. A.; Ramer, S. W.; del Cardayré, S. B.; Stemmer, W. P. C.; Tobin, M. B.; Longchamp, P. F.; Huisman, G. W. *Angew. Chem., Int. Ed.* **2001**, *40*, 3948−3959. (b) Panke, S.; Held, M.; Wubbolts, M. *Curr. Opin. Biotechnol.* **2004**, *15*, 272−279. (c) Tao, J.; Zhao, L.; Ran, N. *Org. Process Res. Dev.* **2007**, *11*, 259−267.
(2) (a) Machajewski, T. D.; Wong, C.-H. *Angew. Chem., Int. Ed.* **2000**, *39*, 1352−1375. (b) Franke, D.; Hsu, C.-C.; Wong, C.-H. *Methods Enzymol.* **2004**, 224−238.
(3) Dean, S. M.; Greenberg, W. A.; Wong, C.-H. *Adv. Synth. Catal.* **2007**, *349*, 1308−1320.
(4) (a) Winchester, B.; Fleet, G. W. J. *Glycobiology* **1992**, *2*, 199−210. (b) Butters, T. D.; Dwek, R. A.; Platt, F. M. *Chem. Rev.* **2000**, *100*, 4683−4696. (c) Wang, R.-W.; Qing, F.-L. *Org. Lett.* **2005**, *7*, 2189−2192. (d) Sears, P.; Wong, C.-H. *Angew. Chem., Int. Ed.* **1999**, *38*, 2300−2324. (e) Ouchi, H.; Mihara, Y.; Takahata, H. *J. Org. Chem.* **2005**, *70*, 5207−5214.
(5) (a) Asano, N. *Glycobiology* **2003**, *13*, 93R−140R. (b) Dwek, R. A.; Butters, T. D.; Platt, F. M.; Zitzmann, N. *Nat. Rev. Drug Discovery* **2002**, *1*, 65−75. (c) Jacob, G. S. *Curr. Opin. Struct. Biol.* **1995**, *5*, 605 −611.
(6) (a) Schramm, V. L. *Acc. Chem. Res.* **2003**, *36*, 588−596. (b) Singh, V.; Evans, G. B.; Lenz, D. H. Mason, J. M.; Clinch, K.; Mee, S.; Painter, G. F.; Tyler, P. C.; Furneaux, R. H.; Lee, J. E.; Howell, P. L.; Schramm, V. L. *J. Biol. Chem.* **2005**, *280*, 18265−18273.

10.1021/ja073911i CCC: $37.00



1 2 3 4

Figure 1. Iminocyclitols. **1**, DMDP; **2**, β-hexosaminidase inhibitor; **3**, fagomine; **4**, DNJ.

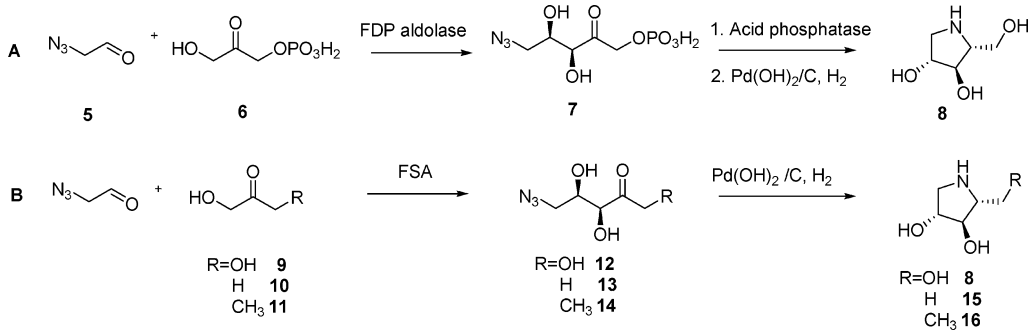


Figure 2. Aldolase-catalyzed synthesis of iminocyclitols. (A) Original synthesis with D-fructose-1,6-diphosphate(FDP) aldolase; (B) synthesis with D-fructose-6-phosphate aldolase (FSA).

activity relationships for iminocyclitol glycosidase inhibitors can be difficult to elucidate, making rational inhibitor design a frustrating exercise.[7] This is well illustrated by the recent observation by Fleet and co-workers that the L-enantiomers of rationally designed D-iminocyclitols can be much more potent inhibitors of the D-sugar processing enzymes.[8] Considering this situation, there is a need for facile methods of accessing a broad range of iminocyclitol structures in sufficient quantities to allow for synthesis of analogues, so that structurally diverse libraries can be screened for inhibitory activity.

Among the reported methods of preparing iminocyclitols,[9] chemoenzymatic synthesis using aldolases provides rapid access to multiple scaffolds in a highly selective manner.[10a−c] The dihydroxyacetone phosphate (DHAP)-dependent aldolases catalyze the addition of DHAP to acceptor aldehydes, and are capable of accepting aldehydes containing azide or *N*-Cbz-amine substitutions, thus providing the corresponding azido- or *N*-Cbz-amino polyhydroxy ketones. After removal of the phosphate group with phosphatase, reduction of the azide or deprotection of *N*-Cbz group followed by intramolecular reductive amination affords a series of iminocyclitols (Figure 2A).[10] However, DHAP-dependent aldolases possess the drawback of strict donor substrate specificity toward DHAP, and nonphosphorylated dihydroxyacetone (DHA) cannot be used. The high cost and instability of DHAP, as well as the requirement of a phosphatase to remove the phosphate ester, reduce the practicality of the process. Significant effort has been expended toward a more practical access to DHAP,[11] but it remains difficult. A more desirable solution would be the elimination of the requirement for DHAP, and facilitation of the use of readily available, inexpensive DHA in its place. One approach to achieving this end is directed evolution of the enzyme. Several examples of the application of directed evolution to change the substrate specificity or synthetic utility of an aldolase have been published recently.[12] Another approach to improving synthetic utility of an aldolase is substrate or reaction engineering, as in our recent application of borate to allow RhaD aldolase to accept DHA.[13] A third route is the discovery of novel enzymes from nature that catalyze the desired reaction.[14] In parallel with these biocatalytic efforts, many organocatalytic asymmetric aldol[15] methods have been reported, including several that utilize DHA or hydroxy acetone as donors.[15b−f] Not limited by the strict substrate dependence of enzymes, a great deal of research in organocatalytic aldol chemistry is focused on improving catalytic efficiency and stereoselectivity to enzymelike levels.

(7) Gloster, T. M.; Meloncelli, P.; Stick, R. V.; Zechel, D.; Vasella, A.; Davies, G. J. *J. Am. Chem. Soc.* **2007**, *129*, 2345−2354.

(8) (a) Yu, C.-Y.; Asano, N.; Ikeda, K.; Wang, M.-X.; Butters, T. D.; Wormald, M. R.; Dwek, R. A.; Winters, A. L.; Nash, R. J.; Fleet, G. W. J. *Chem. Commun.* **2004**, 1936−1937. (b) Asano, N.; Ikeda K.; Yu, L.; Kato, A.; Takebayashi, K.; Adachi, I.; Kato, I.; Ouchi, H.; Takahata, H.; Fleet, G. W. J. *Tetrahedron: Asymmetry* **2005**, *16*, 223−229.

(9) (a) Ayad, T.; Genisson, Y.; Baltas, M. *Curr. Org. Chem.* **2004**, *8*, 1211−1233. (b) Moriarty, R. M.; Mitan, C. I.; Branza-Nichita, N.; Phares, K. R.; Parrish, D. *Org. Lett.* **2006**, *8*, 3465−3467.

(10) (a) Samland, A. K.; Sprenger, G. A. *Appl. Microbiol. Biotechnol*. **2006**, *71*, 253−264. (b) Whalen, L. J.; Wong, C.-H. *Aldrichimica Acta* **2006**, *39*, 63−71. (c) Gijsen, H. J. M.; Qiao, L.; Fitz, W.; Wong C.-H. *Chem. Rev.* **1996**, *96*, 443−474. (d) Kajimoto, T.; Liu, K. K.-C.; Pederson, R. L.; Zhong, Z.; Ichikawa, Y.; Porco, J. A.; Wong, C.-H. *J. Am. Chem. Soc.* **1991**, *113*, 6187−6196. (e) Von der Osten, C. H.; Sinskey, A. J.; Barbas, C. F., III; Pederson, R. L.; Wang, Y.-F.; Wong, C.-H. *J. Am. Chem. Soc.* **1989**, *111*, 3924−3927. (f) Liu, K. K.-C.; Kajimoto, T.; Chen, L.; Zhong, Z.; Ichikawa, Y.; Wong, C.-H. *J. Org. Chem*. **1991**, *56*, 6280−6289.

(11) For chemical synthesis of DHAP, see: (a) Gefflaut, T.; Lemaire, M.; Valentin, M.-L.; Bolte, J. *J. Org. Chem.* **1997**, *62*, 5920−5922. (b) Jung S.-H.; Jeong, J.-H.; Miller, P.; Wong, C.-H. *J. Org. Chem*. **1994**, *59*, 7182−7184. For recent enzymatic syntheses of DHAP, see: (c) Sánchez-Moreno, I.; García-García, J. F.; Bastida, A.; García-Junceda, E. *Chem. Commun.* **2004**, 1634−1635. (d) Schoevaart, R.; Van Rantwigk, F.; Sheldon, R. A. *Chem. Commun*. **1999**, 2465−2466. (e) Fessner, W.-D.; Sinerius, G. *Angew. Chem., Int. Ed. Engl*. **1994**, *33*, 209−212.

(12) (a) Fong, S.; Machajewski, T. D.; Mak, C. C.; Wong, C.-H. *Chem. Biol.* **2000**, *7*, 873−883. (b) Hsu, C.-C.; Hong, Z.; Wada, M.; Franke, D.; Wong, C.-H. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 9122−9126. (c) Woodhall, T.; Williams, G.; Berry, A.; Nelson, A. *Angew. Chem., Int. Ed.* **2005**, *44*, 2109−2112. (d) Williams, G. J.; Woodhall, T.; Farnsworth, L. M.; Nelson, A.; Berry, A. *J. Am. Chem. Soc.* **2006**, *128*, 16238−16247.

(13) Sugiyama, M.; Hong, Z.; Whalen, L. J.; Greenberg, W. A.; Wong, C.-H. *Adv. Synth. Catal*. **2006**, *348*, 2555−2559.

(14) (a) Samland, A.; Sprenger, G. A. *Appl. Microbiol. Biotechnol.* **2006**, *71*, 253−264. (b) Schurmann, M.; Sprenger, G. A. *J. Biol. Chem*. **2001**, *276*, 11055−11061. (c) Schurmann, M.; Schurmann, M.; Sprenger, G. A. *J. Mol. Catal. B: Enzym.* **2002**, *19*, 247−252.

(15) (a) Dalko, P. I.; Moisan, L. *Angew. Chem., Int. Ed.* **2004**, *43*, 5138−5175. (b) Notz, W.; List, B. *J. Am. Chem. Soc.* **2000**, *122*, 7386−7387. (c) Sakthivel, K.; Notz, W.; Bui, T.; Barbas, C. F. *J. Am. Chem. Soc.* **2001**, *123*, 5260−5267. (d) Cordova, A.; Notz, W.; Barbas, C. F. *Chem. Commun.* **2002**, 3024−3025. (e) Fernandez-Lopez, R.; Kofoed, J.; Machuqueiro, M.; Darbre, T. *Eur. J. Org. Chem.* **2005**, 5268−5276. (f) Ramasastry, S. S. V.; Zhang, H.; Tanaka, F.; Barbas, C. F. *J. Am. Chem. Soc.* **2007**, *129*, 288−289.

Table 1. Kinetic Values[a] for FSA with Three Donor Substrates, Using Glyceraldehyde-3-phosphate as Acceptor Substrate

donor	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}s^{-1}$)
dihydroxyacetone	40 ± 18	1.31 ± 0.20	33
hydroxy acetone	11 ± 6	0.82 ± 0.15	75
1-hydroxybutan-2-one	32 ± 17	0.63 ± 0.10	20

[a] Values were obtained as described in the Experimental Section.

Here we report the application of fructose-6-phosphate aldolase (FSA) from *Escherichia coli*[14b] for practical one-pot synthesis of a broad range of iminocyclitols, in a procedure that avoids the need for a phosphorylated substrate. Previous reports from Sprenger and co-workers showed that FSA catalyzes the reversible aldol reaction from D-fructose-6-phosphate to D-glyceraldehyde-3-phosphate and the non-phosphorylated DHA and also that it uses hydroxy acetone in the place of DHA.[14c] The lack of strict donor substrate preference exhibited by FSA is unusual for an aldolase. The sequence of FSA is also unusual and is, in fact, more homologous to transaldolases, although transaldolase activity was not observed for this enzyme.[14b] On the basis of these findings, we hypothesized that this unique aldolase could be used to synthesize iminocyclitols and related compounds by using DHA (or other donors) instead of DHAP as the donor substrate. During the course of our efforts, Clapés and co-workers reported the first application of FSA for the synthesis of iminocyclitols, using a strategy similar to the one outlined here to synthesize **3** and a series of *N*-alkyl derivatives.[16] In the present work, FSA was used to synthesize iminocyclitols from DHA, HA, or HB, and a range of acceptor substrates (Figure 2B). Finally, eliminating the need for DHAP has enabled the development of a practical one-pot synthesis of iminocyclitols by performing the aldol and reductive amination reactions in a single vessel. The new methods allowed for extremely facile access to a broad range of iminocyclitols, which were assayed against a panel of glycosidases.

Results and Discussion

To determine the catalytic efficiency of FSA with a variety of donor substrates, kinetics experiments were performed with dihydroxyacetone, hydroxy acetone, or 1-hydroxy-2-butanone as donor, and D-glyceraldehyde-3-phosphate as acceptor. We observed that DHAP was not a viable donor substrate for FSA, which concurs with the original observations of Sprenger and co-workers.[14b] Kinetic values were determined in the synthetic direction by following the consumption of the acceptor D-glyceraldehyde-3-phosphate (G-3-P) over the course of the reaction by withdrawing aliquots at various time points and measuring remaining G-3-P concentration by an enzyme-coupled assay utilizing triosephosphate isomerase and α-glycerophosphate dehydrogenase. Consumption of NADH, as measured by absorbance at 340 nm, was equated with remaining G-3-P concentration. Remarkably, the unnatural substrates HA and HB were not merely tolerated, but were equally efficient substrates when compared to the presumed endogenous substrate DHA, as shown in Table 1. In fact, k_{cat}/K_M for HA was slightly higher than for DHA, although all three substrates had catalytic efficiencies within the same order of magnitude of each other. This level of plasticity with respect to donor substrate structure is unprecedented for an aldolase and suggests that FSA could be a biocatalyst with an exceptionally broad substrate scope and utility. This possibility was explored by preparing a range of iminocyclitol products using FSA.

Initial preparative experiments were aimed at the synthesis of 1,4-dideoxy-1,4-imino-D-arabitol, **8**, a naturally occurring five-membered iminocyclitol which inhibits α-glucosidases.[17] FSA accepted azidoacetaldehyde and DHA to give a single diastereomer of the aldol product **12** (Figure 2B). The aldol product was subjected to reductive amination to afford **8**. The NMR spectra of the product matched that of authentic **8**, and no peaks from other diastereomers were observed, confirming that FSA possesses the same (*3S,4R*) stereoselectivity as that of D-fructose-diphosphate (FDP) aldolase.

The broad donor substrate specificity of FSA was exploited to synthesize a large series of iminocyclitols (Table 2, 3), including many that were not previously accessible by aldolase-catalyzed methods, due to substrate tolerance restrictions. When using azidoacetaldehyde as acceptor, FSA was able to efficiently use HA and HB as donor substrates, to give methyl derivatives **13** or ethyl-substituted polyhydroxy ketones **14**, respectively (Figure 2). The reductive amination of azidoketoses was diastereoselective (consistent with our previous reports[10]) with hydrogen attacking the imine intermediate from the face opposite to the axial OH-group being favorable.

A series of 2-azido aldehydes were used as acceptor aldehydes to prepare five-membered iminocyclitols (Table 2), and *N*-Cbz 3-amino aldehydes were used to prepare six-membered iminocyclitols, as first described by Clapés and co-workers[16] (Table 3). Using HA and HB as donor substrates, azidoaldehydes **5**, **17**, and **22** were all accepted by FSA. Compounds **19** and **21** are deoxy- or ethyl derivatives of 2,5-dideoxy-2,5-imino-D-mannitol (DMDP), **1**,[18] a naturally occurring *β*-glycosidase inhibitor, and compounds **24** and **26** are derivatives of compound **2**, a potent inhibitor of *N*-acetyl-*β*-hexosaminidase[19] that may be useful in the treatment of osteoarthritis. Interestingly, FSA was highly enantioselective for 2-azido-3-substituted aldehydes **17** and **22**, with (*R*)-aldehydes serving as better substrates than (*S*)-enantiomers. Reactions with the *N*-Cbz amino aldehydes were performed in DMF/water (1:4) cosolvent system because of the low aqueous solubility of the substrate. The cosolvent did not adversely affect enzyme activity, suggesting that organic cosolvents are well-tolerated by FSA and may be used for other substrates that have poor aqueous solubility. DHA and **27** were condensed by FSA to yield one diastereomer **28**, followed by reductive amination to give the naturally occurring glucosidase inhibitor fagomine **3**. The specific rotation obtained for this sample of **3** was +18.1°, whereas the reported value for **3** isolated from natural sources is +19.5°,[20] suggesting the enzymatically produced iminocyclitol has an enantiomeric excess of 93%. A detailed analysis of the enantioselectivity and

(16) Castillo, J. A.; Calveras, J.; Casas, J.; Mitjans, M.; Vinaedell, M. P.; Parella, T.; Inoue, T.; Sprenger, G. A.; Joglar, J.; Clapes, P. *Org. Lett.* **2006**, *8*, 6067−6070.

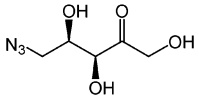
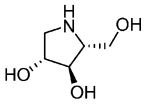
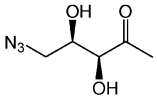
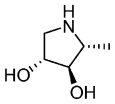
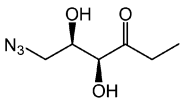
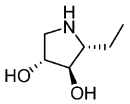
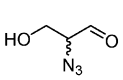
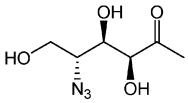
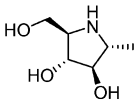
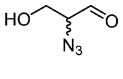
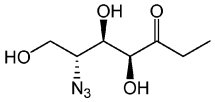
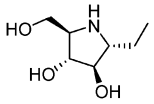
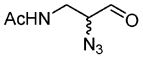
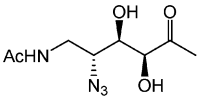
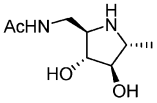
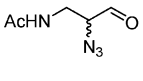
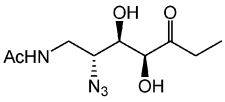
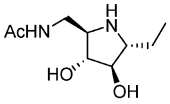
(17) (a) Fleet, G. W. J.; Nicholas, S. J.; Smith, P. W.; Evans, S. V.; Fellows, L. E.; Nash, R. J. *Tetrahedron Lett.* **1985**, *26*, 3127−3130. (b) Fleet, G. W. J.; Smith, P. W. *Tetrahedron* **1986**, *42*, 5685−5692.

(18) (a) Pederson, R. L.; Wong, C.-H. *Heterocycles* **1989**, *28*, 477−480. (b) Ziegler, T.; Straub, A.; Effenberger, F. *Angew. Chem., Int. Ed. Engl.* **1988**, *27*, 716−717.

(19) (a) Liu, J.; Numa, M. M. D.; Liu, H.; Huang, S.-J.; Sears, P.; Shikhman, A. R.; Wong, C.-H. *J. Org. Chem.* **2004**, *69*, 6273−6283. (b) Liu, J.; Shikhman, A. R.; Lotz, M. K.; Wong, C.-H. *Chem. Biol.* **2001**, *8*, 701−711. (c) Liang, P.-H.; Cheng, W.-C.; Lee, Y.-L.; Yu, H.-P.; Wu, Y.-T.; Lin, Y.-L.; Wong, C.-H. *ChemBioChem* **2006**, *7*, 165−173.

(20) Kato, A.; Asano, N.; Kizu, H.; Matsui, K.; Watson, A. A.; Nash, R. J. *J. Nat. Prod.* **1997**, *60*, 312−314.

Table 2. Iminocyclitol Synthesis from Azidoaldehydes

Entry	Acceptor	Donor	Aldol product	Iminocyclitol	Yield (%) 2-step	Yield (%) 1-pot
1	 **5**	DHA	 **12**	 **8**	57	73
2	 **5**	HA	 **13**	 **15**	64	72
3	 **5**	HB	 **14**	 **16**	62	83
4	 **17**	HA	 **18**	 **19**	27	32
5	 **17**	HB	 **20**	 **21**	27	33
6	 **22**	HA	 **23**	 **24**	39	57
7	 **22**	HB	 **25**	 **26**	31	53

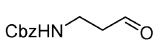
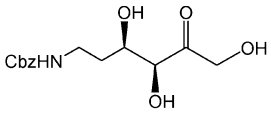
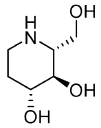
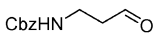
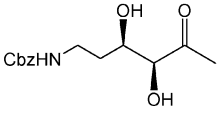
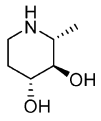
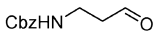
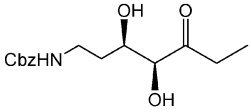
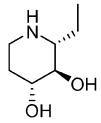
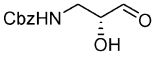
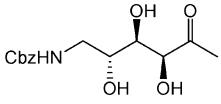
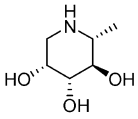
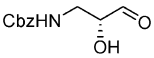
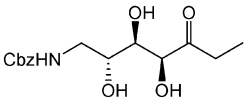
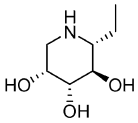
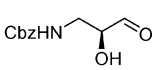
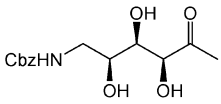
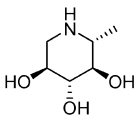
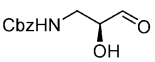
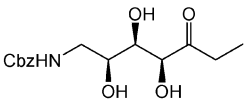
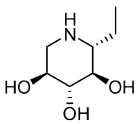
diastereoselectivity of FSA-catalyzed reactions is in progress, although it requires samples of the enantiomers, which are not readily accessible except by multistep synthesis.

Deoxyfagomine **30** and the ethyl fagomine analogue **32** were also synthesized. In order to synthesize 1-deoxynojirimycin (DNJ) derivatives or 1-deoxymannojirimycin (mannoDNJ) derivatives, both pure enantiomers of 3-[(benzyloxycarbonyl)-amino]-2-hydroxypropionaldehyde **33** and **38** were prepared (see Supporting Information). DNJ and its derivatives constitute an important class of iminocyclitols, including *N*-butyl-DNJ (Miglustat), which is a potent glucosylceramide synthase inhibitor. It is used clinically for the treatment of the glycolipid storage disorder, type I Gaucher disease.

As noted, FSA possesses unusually wide donor substrate specificity, which is useful because strict donor substrate specificities of aldolases have often limited their applications. While the physiological role of FSA is still not entirely clear, HA and HB are equally good substrates from a kinetic standpoint compared to DHA and appear to be more versatile donor substrates for FSA than DHA from a synthetic standpoint, because they reacted efficiently with all the azidoaldehydes or *N*-Cbz amino aldehydes tested in this study. In contrast, DHA was compatible only with 2-azidoaldehyde **5** and 3-[(benzyloxycarbonyl)amino]propionaldehyde **27**, not with azido- or Cbz-amino aldehydes containing branching at the 2-position. The observation that the acceptor substrate tolerance of the aldolase is dependent on the nature of the donor substrate is intriguing. FSA has previously been noted to have significant sequence homology (~50%) to transaldolase B,[14b] which catalyzes the reversible transfer of a DHA moiety from fructose-6-phosphate to erythrose-4-phosphate, to form sedoheptulose-7-phosphate and release glyceraldehyde-3-phosphate. The unusual substrate tolerance of FSA may be related to an evolutionary origin as a transaldolase, an enzyme class that must accommodate four separate substrates in its active site over the course of its catalytic cycle.

Further optimization of FSA-catalyzed synthesis of iminocyclitols was achieved by developing methods for performing all

Table 3. Iminocyclitol Synthesis from Cbz-Protected Aminoaldehydes

Entry	Acceptor	Donor	Aldol product	Iminocyclitol	Yield (%) 2-step	Yield (%) 1-pot
8	 **27**	DHA	 **28**	 **3**	65	55
9	 **27**	HA	 **29**	 **30**	78	64
10	 **27**	HB	 **31**	 **32**	78	82
11	 **33**	HA	 **34**	 **35**	40	48
12	 **33**	HB	 **36**	 **37**	51	20
13	 **38**	HA	 **39**	 **40**	60	53
14	 **38**	HB	 **41**	 **42**	47	57

operations in a single pot (Figure 3). FSA-catalyzed aldolase reactions were performed in sodium borate buffer (pH 7.6), followed by the reductive amination in the same vessel, in the presence of excess equivalents of diethylamine. The addition of diethylamine protected the secondary amine of the resulting iminocyclitols from further intermolecular reductive amination by any residual aldehyde or ketone. Thus, the iminocyclitol synthesis could now be completed in a one-pot fashion, without isolation of intermediates. All iminocyclitols listed in Tables 2 and 3 were synthesized by the one-pot procedure, as well as the two-step procedure. Analysis of crude product mixtures by NMR showed small quantities of diastereomeric products, typically in the range of 3−5%. This observation is in good agreement with the 93:7 diastereoselectivity observed by Clapés and co-workers for preparation of compound **3**.[16] The major source of yield loss was the difficulty in purification of these polar molecules. Compounds **15**, **21**, **24**, **26**, **32**, **37**, and **42** are previously unreported iminocyclitols, and preliminary inhibitory activities toward a series of glycosidases were determined (Table 4).

Compounds **15** and **16** were found to be slightly more potent inhibitors of α-mannosidase from jack beans than the known inhibitor **8**, with 26% and 14% inhibition at 1 μM, respectively. While not exceptionally potent, **16** is quite specific for α-mannosidase, whereas **8** inhibits both α- (K_i 860 nM) and β-glucosidases in addition to α-mannosidase. Further structural elaboration of **16**, which can be readily accessed by this synthetic method, should lead to more potent and specific mannosidase inhibitors. Such compounds may be useful for preventing aberrant glycosylation in cancer. Compounds **24** and **26** are potent and specific inhibitors of β-*N*-acetylglucosaminidases from jack beans and human placenta. Individual K_i values were determined to be in the submicromolar range in each case (Table 4). Compound **24** is a 320 nM inhibitor of the plant enzyme,

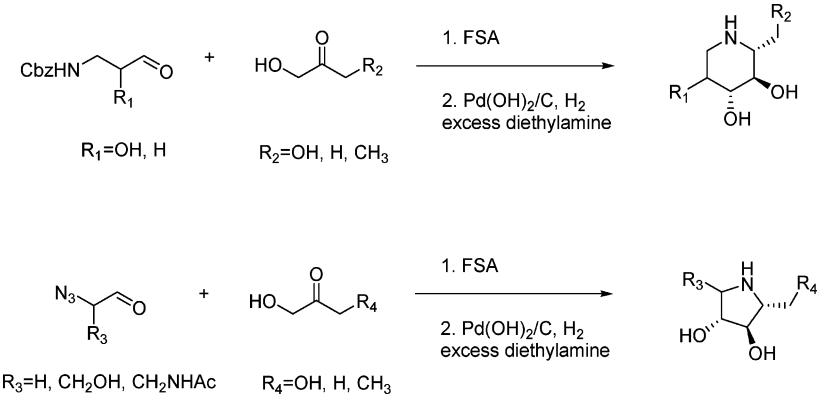


Figure 3. One -pot synthesis of iminocyclitols.

Table 4. Glycosidase Inhibition Activities[a] of Synthesized Iminicyclitols

enzyme[b]	**3**	**8**	**15**	**16**	**19**	**21**	**24**[c]	**26**[c]	**35**	**37**
α-Glu	3	100(58)	54 (4)	4	39	3	0	0	6	0
β-Glu	0	84(4)	55 (4)	69 (0)	50	40	0	6	11	0
α-Man	14	81(9)	95 (26)	95 (14)	25	11	11	8	85 (2)	42
β-Man	25	38	10	20	47	49	0	0	6	0
β-Gal	34	47	0	4	13	19	0	0	4	6
β-*N*-GlcNAc[1]	6	0	0	0	29	3	100 (61) **0.32**	100 (37) **0.76**	9	21
β-*N*-GlcNAc[2]	0	0	0	0	0	0	100 (59) **0.51**	100 (68) **0.29**	11	22

[a] Values represent percent inhibition at 200 μM compound. For the most potent inhibitors, percent inhibition was also determined at 1 μM, shown in parentheses. [b] α-Glu, α-glucosidase from jack beans; *β*-Glu, *β*-glucosidase from almonds; α-Man; α-mannosidase from jack beans; *β*-Man, *β*-mannosidase from snail; *β*-Gal, *β*-galactosidase from *Aspergillus oryzae*; *β*-*N*-GlcNAc,[1] *β*-*N*-acetylglucosaminidase from jack beans; *β*-*N*-GlcNAc[2], *β*-*N*-acetylglucosaminidase from human placenta. [c] For compounds **24** and **26**, K_i values against the two *β*-*N*-acetylglucosaminidases are shown in bold (μM).

whereas **26** is a 290 nM inhibitor of the human enzyme. These structures may serve as readily accessible cores for optimization, leading to discovery of new treatments for osteoarthritis.[19c] Inhibition activities of new compounds toward α-galactosidase from green coffee beans and α-fucosidase from bovine kidney were also determined, but no significant inhibition was observed, except compound **35** showed 48% inhibition against α-fucosidase at 200 μM (data not shown).

Conclusions

In conclusion, a newly discovered aldolase, FSA, has been investigated as a catalyst for organic synthesis. Kinetic measurements showed that FSA did not merely tolerate structural variation in the donor substrate but also accepted hydroxy acetone and 1-hydroxy-2-butanone equally well compared to dihydroxyacetone, the presumed endogenous substrate. This level of donor substrate tolerance is unprecedented for an aldolase and makes FSA a practical catalyst for synthesis of a wider range of compounds than is typically possible with an aldolase. To illustrate the power of FSA as a biocatalyst, a one-pot chemoenzymatic method for the synthesis of a variety of iminocyclitols from readily available, non-phosphorylated donor substrates has been developed. In addition to dihydroxyacetone, the unnatural donors hydroxy acetone and 1-hydroxybutan-2-one were also accepted in the FSA-catalyzed aldol reactions with both azido- and Cbz-amino aldehyde acceptors. The tolerance observed for both donor and acceptor suggests that FSA has a significant amount of plasticity in its binding site, which may be related to its high homology to transaldolases. Directed evolution may allow for even greater substrate tolerance and synthetic utility. The broad substrate tolerance of FSA allowed for the synthesis of a number of known and novel iminocyclitols in relatively high yields, and in an extremely concise fashion. Glycosidase inhibition studies of the new compounds revealed that **24** and **26** are potent and specific inhibitors of *β*-*N*-acetylglucosaminidases. This new synthetic method renders these compounds accessible and will enable optimization studies to develop more active inhibitors as potential treatments for osteoarthritis and other disorders related to glycoprocessing enzymes. Efforts are underway on the expansion of the substrate tolerance of FSA, with the goal of developing practical biocatalysts with very broad utility.

Experimental Section

General. Solvents, starting materials, and reagents were used as purchased without further purification. UV kinetic assays were performed on a Beckman DU-650 spectrophotometer. Proton NMR spectra (^{1}H NMR) were recorded at 500 MHz using a Bruker 5 mm DCH CryoProbe. Chemical shifts are expressed in parts per million (δ) and are referenced to residual protium in the NMR solvent: CD_2HOD, δ 3.31; DOH, δ 4.80. Carbon NMR (^{13}C NMR) spectra were recorded at 125 MHz using the same probe. Chemical shifts (δ ppm) are referenced to the carbon signal for the solvent: CD_3OD, δ 49.05. Optical rotation measurements were taken on a Perkin-Elmer 241 polarimeter with an aperture of 2 mm using a microcell with 1 cm path length. Aldehyde dehydrogenase, glycerophosphate dehydrogenase, and triosephosphate isomerase were purchased from Sigma. For glycosidase inhibitor assays, samples in 96-well plates were analyzed on a Packard Fusion Universal Microplate Analyzer spectrophotometer.

Overexpression of His-Tagged FSA in *E. coli*. The *mipB*[14b] gene encoding FSA was amplified from chromosomal DNA of *E. coli* W3110 by PCR with following two primers; MipB5Nde, 5′-gatgcatatggaactgtatctggatac-3′, and MipB3Xho, 5′-ggcctcgagaatcgacgttctgccaaacg-3′.

The amplified 660 bp fragment was cloned into the *Nde*I and *Xho*I site of pET20b(+) vector (Novagene) to give pETFSA. *E. coli* BL21 (DE3) was transformed with pETFSA, and transformants were cultivated in LB medium containing 50 μg/mL carbenicillin, 10 μM IPTG at 37 °C for 16 h. The recombinant FSA was expressed in the soluble fraction and appeared as a major band on SDS-PAGE. The cells were harvested by centrifuge, washed with saline, and used as whole cell catalysts. His-tagged FSA was also purified by Ni^{2+} affinity column chromatography. Cells were suspended in binding buffer (50 mM glycylglycine buffer (pH 8.5), 300 mM NaCl, and 10 mM imidazole) and disrupted by sonication. After removing debris by centrifuge, the soluble fraction was applied onto a Ni^{2+} affinity column; the column was washed with the binding buffer, and His-tagged FSA was eluted with elution buffer (binding buffer containing 250 mM imidazole). The eluant was collected and dialyzed against 20 mM HEPES/NaOH buffer (pH 7.5).

Kinetic Assays. A solution of D/L-glyceryaldehyde-3-phosphate was obtained by deprotection of the commercially available D/L-glyceraldehyde-3-phosphate diethylacetal following the manufacturer's protocol.

Donor substrate (dihydroxyacetone, hydroxy acetone, or 1-hydroxybutan-2-one) was added in amounts varying from 5 to 120 mM to a solution containing 3 mM D-glyceraldehyde-3-phosphate and 0.03 mg/mL purified FSA in 50 mM glycylglycine buffer (pH 8.5) plus 1 mM 2-mercaptoethanol at 25 °C. Aliquots of 25 μL of these reactions were removed every 2 min and quenched by addition to a solution containing 0.23 mM NADH and 8 units/mL α-glycerophosphate dehydrogenase-triosephosphate isomerase in 50 mM Tris buffer (pH 7.5). Previous work has shown that FSA is inhibited by Tris buffer, alleviating the need for a separate quenching step.[14b] Absorbance at 340 nm was measured, and consumption of NADH was equated to D-glyceraldehyde-3-phosphate consumed. By measuring the consumption of d-glyceraldehyde-3-phosphate over the course of the reaction, the velocity of the reaction at varying concentrations of donor was determined. The kinetic parameters (K_M, k_{cat}) were obtained by nonlinear least-squares curve-fitting using the GRAFIT program (Erithacus Software).

General Procedure for FSA-Catalyzed One-Pot Synthesis of Iminocyclitols. To a solution of acceptor aldehyde (0.5 mmol) and donor (DHA **9**, hydroxy acetone **10**, or 1-hydroxybutan-2-one **11**, 1.0 mmol) in 50 mM sodium borate buffer (pH 7.6, 10 mL) were added toluene (100 μL) and *E. coli* BL21 (DE3) cells harboring pETFSA (0.3 g, wet weight). For the racemic acceptor aldehydes (entries 4−7), acceptor aldehyde (1.0 mmol) and donor (**10** or **11**, 0.5 mmol) were used. For the Cbz-protected amino aldehydes **27**, **33**, and **38** (entries 8−14), aldol condensations were performed in water/DMF (4:1) cosolvent system instead of water. The reaction mixture was shaken at room temperature for 6 h, and cells were removed by centrifuge. To this mixture was added MeOH (10 mL), diethylamine (0.73 g, 10.0 mmol), and $Pd(OH)_2/C$ (10%, 50 mg), and the mixture was subjected to hydrogenation (400 psi H_2) overnight at room temperature. After filtration over Celite and concentration, iminocyclitols were purified by flash chromatography (CH_2Cl_2/MeOH/water/NH_4OH).

General Procedure for Two-Step Synthesis of Iminocyclitols. The FSA-catalyzed aldol reactions were carried out in HEPES/NaOH buffer (100 mM, pH 7.5, 10 mL). After the aldol condensation and removal of the cells, the enzymatic reaction solution was lyophilized, and the residue was purified by flash chromatography (silica, CH_2Cl_2/MeOH) to afford the azidoketones. The purified azidoketone was then dissolved in 1.5 mL of MeOH and water (1:1), and $Pd(OH)_2/C$ (10%, 50 mg/mmol substrate) was added. The reaction was hydrogenated (50 psi H_2) at room temperature for 12 h. Filtration over Celite and flash chromatography (silica, CH_2Cl_2/MeOH/water/NH_4OH) provided iminocyclitols.

Enzymatic Inhibition Assays. Inhibitory activities of synthesized iminocyclitols were evaluated toward α-glucosidase (from jack beans), β-glucosidase (from almonds), α-mannosidase (from jack beans), β-mannosidase (from snail), α-galactosidase (from green coffee beans), β-galactosidase (from *Aspergillus oryzae*), α-fucosidase (from bovine kidney), β-*N*-acetylglucosaminidase (from jack beans and human placenta). All enzymes and corresponding *p*-nitrophenyl glycosides were purchased from Sigma. Reactions were performed in 100 μL of reaction mixture containing 50 mM HEPES/NaOH buffer (pH 6.8), 1 mM *p*-nitrophenyl glycoside substrates, appropriate amounts (typically 0.5 to 1.0 U/mL) of glycosidases, and 200 μM (or 1 μM) iminocyclitols at final concentrations, respectively. Increase of absorbance at 405 nm was determined by a plate reader at 25 °C for 10 min. All assays were performed in duplicate, and percent inhibition was calculated based on the activities in the presence or absence of iminocyclitols. For the compounds showing significant inhibitory activities at 200 μM, assays were also performed at 1 μM. The K_i values of compounds **8**, **24**, and **26** were determined from a double reciprocal plot ($1/v$ vs 1/[S]) to give apparent K_m values in the presence of inhibitors. The secondary plot was generated by plotting the apparent K_m values as a function of inhibitor concentrations. K_i was calculated from the negative value of the *x*-intercept of this plot.

Acknowledgment. We are grateful for support from the NIH (GM044154). L.J.W. thanks the Skaggs Institute for Chemical Biology for a postdoctoral fellowship. M.S. was supported by Ajinomoto Co. Inc.

Supporting Information Available: Synthesis of aldehyde acceptors, characterizations of all new compounds, and scanned NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

JA073911I