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Broadening Deoxysugar Glycodiversity: Natural and Engineered Transaldolases Unlock a Complementary Substrate Space

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Abstract: The majority of prokaryotic drugs are produced in glycosylated form, with the deoxygenation level in the sugar moiety having a profound influence on the drug's bioprofile. Chemical deoxygenation is challenging due to the need for tedious protective group manipulations. For a direct biocatalytic de novo generation of deoxysugars by carboligation, with regiocontrol over deoxygenation sites determined by the choice of enzyme and aldol components, we have investigated the substrate scope of the F178Y mutant of transaldolase B, TalB^{F178Y}, and fructose 6-phosphate aldolase, FSA, from *E. coli* against a panel of variously deoxygenated aldehydes and ketones as aldol acceptors and donors, respectively. Independent of substrate structure, both enzymes catalyze a stereospecific carboligation resulting in the D-*threo* configuration. In combination, these enzymes have allowed the preparation of a total of 22 out of 24 deoxygenated ketose-type products, many of which are inaccessible by available enzymes, from a [3×8] substrate matrix. Although aliphatic and hydroxylated aliphatic aldehydes were good substrates, D-lactaldehyde was found to be an inhibitor possibly as a consequence of inactive substrate binding to the catalytic Lys residue. A 1-hydroxy-2-alkanone moiety was identified as a common requirement for the donor substrate, whereas propanone and butanone were inactive. For reactions involving dihydroxypropanone, TalB^{F178Y} proved to be the superior catalyst, whereas for reactions involving 1-hydroxybutanone, FSA is the only choice; for conversions using hydroxypropanone, both TalB^{F178Y} and FSA are suitable. Structure-guided mutagenesis of Ser176 to Ala in the distant binding pocket of TalB^{F178Y}, in analogy with the FSA active site, further improved the acceptance of hydroxypropanone. Together, these catalysts are valuable new entries to an expanding toolbox of biocatalytic carboligation and complement each other well in their addressable constitutional space for the stereospecific preparation of deoxysugars.

Keywords: biocatalysis · carbohydrates · enzymes · protein engineering · stereoselectivity

Introduction

The majority of prokaryotic small-molecule natural products interesting for pharmaceutical applications, such as those in clinical use for the treatment of bacterial or fungal infections, cancer, and other human diseases, are produced in glycosylated form. Structural changes in the sugar attachment can have a profound influence on the drugs' bioactivity, pharmacokinetic properties, and target specificity at the tissue, cellular, or molecular level.[1] Although natural products by themselves constitute a library of core structures encompassing a large and privileged structural diversity, glycoconjugation greatly further enhances the accessible chemical diversity by employing more than 100 different sugars.[2,3] Most of these sugars are deoxygenated and unknown in eukaryotes and the role that they play in many physiological processes is attributed in part to the enhanced hydrophobicity that they display with respect to the oxygenated analogues. A variety of non-glycosylated therapeutics could be improved by glycoconjugation, including mitomycin,[4] morphine,[5] ifosfamide mustard,[6] podophyllotoxin,[7] colchicine,[8] rapamycin,[9] or taxol.[10] Natural glycodiversity has recently inspired the development of both in vitro and in vivo strategies for manipulating the sugar biosynthetic machinery with a view to generating novel "glycol-randomized" natural product analogues[2,11] in the search for improved pharmaceutical or agricultural drug lead compounds.[12] The biosynthesis of most carbohydrate moieties found in natural products proceeds via nucleotide-activated hexose intermediates by different multistep combinations involving oxidation, reduction, deoxygenation, epimerization, isomerization, group transfer, and rearrangement reactions before the glycoconjugation step.[2] In particular, the enzymatic creation of deoxygenation sites is a most challenging step.[13] Chemical methods to prepare deoxysugars by total synthesis[14] or by regiospecific deoxygenation of natural

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monosaccharides[15] usually require tedious multistep conversions due to the need for protective group manipulations.

A more direct biocatalytic generation of deoxysugars is possible by de novo construction using carboligation enzymes with immediate control over sites of deoxygenation by the choice of suitable enzyme and aldol components.[16] In particular, dihydroxyacetone phosphate-dependent aldolases have been instrumental in the generation of a plethora of natural and non-natural sugars[17] and isomerases have been used to convert ketoses into aldoses, including various deoxysugars.[18] Despite these numerous examples, limitations to this approach are the often stringent selectivity of aldolases for their nucleophilic substrate, which limits the structural diversity accessible to each individual catalyst, and the typical selectivity for phosphorylated substrates, which introduces an unsought economic burden. Thus, we set out a program to investigate novel enzymes from the transaldolase family as potential catalysts for asymmetric synthesis with a particular focus on deoxysugar preparation.

Transaldolase is a ubiquitous enzyme in all domains of life, which, via a Schiff base intermediate, catalyzes the reversible transfer of dihydroxyacetone (DHA) among ketose donors and aldose acceptors in the pentose phosphate pathway. As a first result of our joint program focusing on the directed evolution of transaldolase B from *E. coli* (TalB)[19] towards novel specificities, very recently some of us reported that replacement of a single amino acid residue, phenylalanine 178, by tyrosine in the active site confers true aldolase, instead of transaldolase, activity to the mutant enzyme ($TalB^{F178Y}$; Scheme 1).[20]

Herein we report on the donor and acceptor substrate space of this engineered $TalB^{F178Y}$ mutein in comparison with the related wild-type fructose 6-phosphate aldolase (FSA) from *E. coli*, another member of the transaldolase class.[21] The latter was recently studied for application in the synthesis of iminocyclitols using azido or Cbz-protected amino aldehydes.[22] Despite the poor similarity between their primary protein sequence (18.1% identity, 35.1% similarity), $TalB^{F178Y}$ and FSA show a highly similar 3D structure at the level of the monomer fold ($(\beta/\alpha)_8$ barrel) as well as in the construction of their active sites; all 11 functionally equivalent amino acid residues in the active site show a similar spatial orientation (Figure 1) even if they are not conserved in sequence.[23,24] However, in the course of this study we found that engineered $TalB^{F178Y}$ and wild-type FSA have a rather different, but highly complementary capacity, for completely stereoselective applications in deoxysugar synthesis. We demonstrate their rather general utility as tools for asymmetric synthesis by the preparation of a large set of stereochemically homogeneous deoxysugars from simple precursors and discuss their individual strengths and limitations with respect to differences in protein structure.

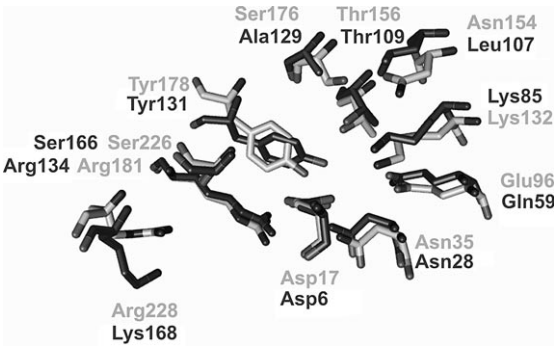
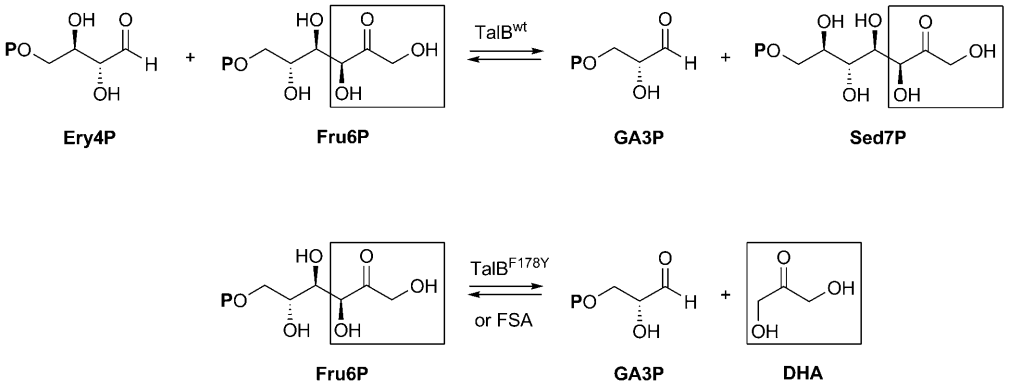


Figure 1. Comparison of the X-ray structures of the active site of FSA (dark gray; pdb 1L6w)[23] and $TalB^{F178Y}$ (light gray; pdb 3cwn).[20] The figure was prepared by using PyMOL.[25]

Results and Discussion

To evaluate the utility of $TalB^{F178Y}$ as a potential catalyst for the preparation of deoxysugars, variably deoxygenated donors (Figure 2) and acceptors (Figure 3) were selected for small-scale preparative reactions under standard conditions to gain an insight into the tolerance of structural modifications and the consequences for the stereoselectivity of car-



Scheme 1. Exemplary metabolic reaction of wild-type transaldolase ($TalB^{wt}$) and the evolved catalytic activity of $TalB^{F178Y}$ for reversible D-*threo*-aldol formation shared by wild-type fructose 6-phosphate aldolase (FSA).

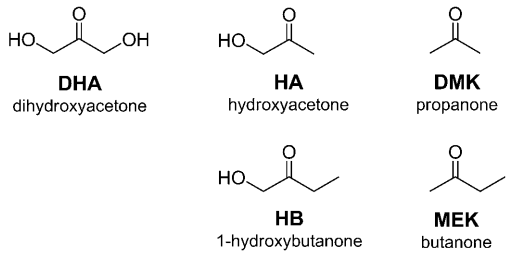


Figure 2. Aldol donors considered in this study.

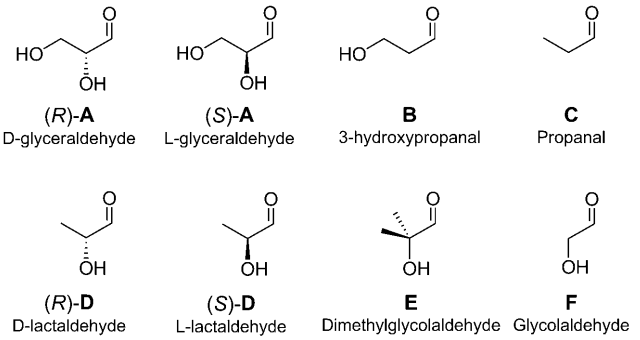
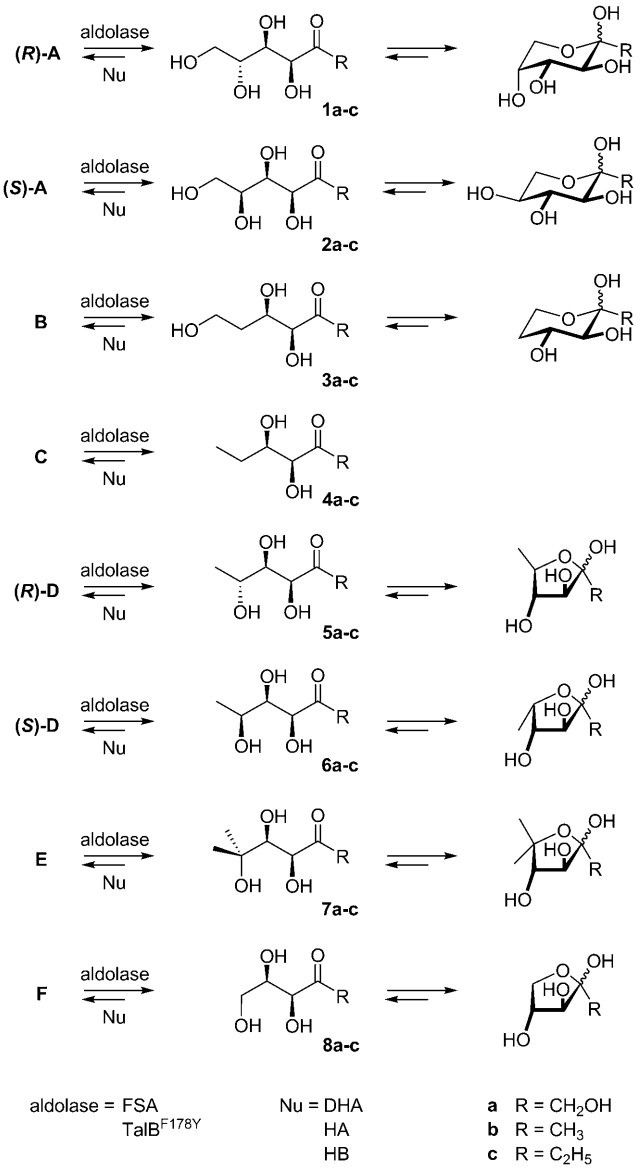


Figure 3. Systematic structural variation of aldol acceptors showing the stereoconfiguration and degree of functionalization.

boligations. Sufficiently high substrate concentrations (100 mM acceptor aldehyde, 150 mM donor) were chosen to compensate for potentially low K_m values and reactions were performed under standard conditions in 50 mM Gly-Gly buffer at pH 8.5 and 25 °C. The outcomes were then analyzed in comparison with the corresponding reactions with FSA as a reference enzyme. The progress of the reaction was monitored by TLC/HPLC and by in situ NMR (^{1}H, ^{13}C) experiments in D_2O for a period of 24–48 h. The latter analysis served to provide an unambiguous proof of product constitution as well as relative configuration. The NMR data also clearly reflect the relative kinetic differentiation among substrate classes in the direction of synthesis because the reverse aldol cleavage reaction becomes disfavored owing to the cyclization of primary products to form more stable furanose or pyranose isomers, which thereby withdraws products from the aldol equilibrium (except for substrate **C**).[16,17] Finally, reaction mixtures were lyophilized and products purified by silica gel chromatography for individual characterization.

To test the suitability of different donor substrates, non-phosphorylated D-glyceraldehyde ((*R*)-**A**) was the most plausible acceptor because it bears an absolute configuration identical to the natural substrate D-glyceraldehyde 3-phosphate (GA3P). Although the original screening of the TalB mutant libraries had been performed for their ability to synthesize fructose 6-phosphate (Fru6P) from DHA and GA3P,[20] the latter was dismissed as a potential reference component for judging synthetic applications because of its inherent instability towards decomposition.[26] By using DHA as a donor, TalB^{F178Y} indeed produced a single product, as indicated by a new spot on TLC that was subsequently identified as D-fructose **1a** by its typical sets of NMR signals (Scheme 2). Spectral correlation also ascertained a stereoselective 3*S*,4*R* aldol addition as NMR analysis showed no indication of the formation of other diastereomers (> 95 % *de*; see the Supporting Information). The stereoselectivity of the addition was further confirmed by an enzyme-coupled photometric assay for the formation of D-fructose.[27] The relative kinetic parameters were determined by using DHA as donor and DL-GA or the enantiopure D-GA as acceptor (Table 1). For the donor substrate DHA, TalB^{F178Y} and FSA showed similar kinetic properties and cat-



Scheme 2. Structural set of deoxysugars accessible by TalB^{F178Y}/FSA catalysis using substrate combinations from Figures 3 and 4. **a**: R = CH_2OH; **b**: R = CH_3; **c**: R = C_2H_5.

Table 1. Comparison of the steady-state kinetics for FSA and TalB^{F178Y} using donor substrate DHA and acceptor substrates glyceraldehyde 3-phosphate and glyceraldehyde to form D-fructose 6-phosphate and D-fructose, respectively.[a]

Kinetic parameters[b]	DHA + DL-GA3P⇌Fru6P		DHA + DL-GA⇌D-Fru[c]	
	FSA	TalB^{F178Y}	TalB^{F178Y}(DL-GA)	TalB^{F178Y}(D-GA)
V_{max} [U mg^{-1}]	31	20	0.87	0.86
K_m [mM]	1.9	2.4	>120	59
k_{cat}[s^{-1}][d]	13	12	0.54	0.53
k_{cat}/K_m [M^{-1} s^{-1}]	6.6×10^3	5.0×10^3	<4.3	9.0

[a] Data are average values of two independent measurements. [b] The enzyme-coupled assays employed only detect the specific formation of D-fructose 6-phosphate and D-fructose, respectively. [c] Ref. [27]. [d] k_{cat} was calculated as turnover number per active site, that is, monomeric subunit.

alytic efficiencies.[20] Although the TalB mutant showed good reactivity with both sources of glyceraldehyde (DL- and D-GA), no activity was observed by using FSA under the conditions of the spectrophotometric assay (<0.05 U mg^{-1}). This is in agreement with previous reports that D-GA is a very weak substrate for FSA, especially in combination with DHA as donor.[21b,28a] For TalB^{F178Y}, substrate inhibition was only observed at rather high D-GA concentrations (>100 mM). In comparison, by using the phosphorylated acceptor DL-GA3P, both FSA and TalB^{F178Y} showed similar V_{max} and K_m values of 31 and 20 U mg^{-1} and 1.9 and 2.4 mM, respectively (Table 1). This resulted in similar catalytic efficiencies (k_{cat}/K_m) of 6600 and 5000 M^{-1} s^{-1}, respectively. At concentrations >4 mM, strong substrate inhibition was observed with FSA, but not with TalB^{F178Y} within the range of DL-GA3P concentrations studied. Although the data demonstrates that the phosphorylated substrate is clearly preferred by TalB^{F178Y} because the catalytic efficiency for DL-GA3P is three orders of magnitude larger than for DL-GA, it is of practical significance that TalB^{F178Y} shows considerable activity at a V_{max} of around 0.9 U mg^{-1}, even with the non-phosphorylated substrate.

Despite a recent report stating hydroxyacetone (HA) to be unacceptable to TalB^{F178Y} on account of a negative HPLC analysis,[20] we envisaged chances to bring about conversion under appropriate reaction conditions as the molecular size is largely comparable to that of DHA as the perceived "natural" substrate. Remarkably, with (*R*)-**A** as an acceptor, the mutant displayed moderate tolerance for the non-natural deoxygenated HA substrate reaching >50% conversion (TLC analysis) within the time window of the analysis. According to the NMR analysis, the reaction yielded the 1-deoxy analogue **1b** exclusively, which points to a unique binding orientation of HA and regiospecific deprotonation towards a hydroxyenamine as the activated nucleophile. No reaction took place in the presence of non-hydroxylated propanone, however, which indicates that the hydroxymethyl unit is an indispensable structural precondition for the aldol donor component. By using 1-hydroxybutanone (HB) as a potential chain-extended nucleophile, the protein precipitated at donor concentrations as low as 50 mM with no detectable product formation. Thus, by using (*R*)-**A** as the acceptor, TalB^{F178Y} shows a clear donor preference for the "natural" substrate DHA followed by HA, whereas HB leads to rapid inactivation. Identical reactions, but run with FSA instead for comparison, revealed a distinctly different reactivity pattern.[21b] The FSA-catalyzed reaction with DHA showed only a very low conversion to **1a**, which corroborates earlier findings that DHA is a poor substrate for this enzyme with (*R*)-**A** as an acceptor[21b,22a] but stands in clear contrast to the reported equal efficiency of all three donors DHA, HA, and HB with GA3P as the acceptor.[22b] On the other hand, the FSA-catalyzed reaction of (*R*)-**A** with HA to yield **1b** was more rapid than that using TalB^{F178Y}, and notably, with HB it gave a modest conversion to **1c** as a single product whereas TalB^{F178Y} gave none. Neither catalyst is able to accept propanone as a nucleophile. By using wild-type transaldolase, as expected no product formation could be detected from any of these non-natural substrate combinations.

To elucidate a possible stereochemical substrate discrimination, the relative reaction rates were determined with (*S*)-**A** (L-glyceraldehyde) under otherwise identical conditions. TalB^{F178Y}-catalyzed addition of DHA led to a rapid conversion with L-sorbose **2a** identified as the only product by TLC and NMR analysis. Initially, the conversion of (*S*)-**A** seemed to proceed more rapidly than that of the enantiomer (*R*)-**A** according to TLC analysis. However, a competitive experiment using *rac*-**A** (DL-glyceraldehyde) under kinetically controlled conditions with in situ ^{1}H NMR monitoring identified a 5:2 ratio of **1a**/**2a**, which clearly demonstrates a preference for (*R*)-**A** but indicates that TLC cannot be used as the only method of product analysis because of potential errors arising from unequal staining intensity. An unexpected but true preference for (*S*)-**A** was exhibited with HA as the donor although the conversion to the corresponding 1-deoxy-L-sorbose **2b** was less rapid than for DHA. Complementary FSA-catalyzed reactions with acceptor (*S*)-**A** showed the same overall trend of donor qualities as with (*R*)-**A**. Again, DHA proved a weak donor for FSA leading to slow conversion to **2a**, whereas HA and HB proved to be much superior donors with completely regio- and stereoselective conversions to **2b** and the higher homologue **2c**, respectively. FSA also clearly displayed a preference for the 2*S*-configured acceptor.

By using the 2-deoxy analogue of glyceraldehyde **B**, eliminating configurational predilection, TalB^{F178Y} demonstrated excellent conversion of DHA to afford 5-deoxy-D-fructose **3a** as a single product in an isolated yield of 83%. As the acceptor lacks a chiral reference center, the absolute configuration was verified by optical rotation ($[\alpha]_D^{25}=-60.7$ ($c=1.04$, H_2O); lit.:[18a] $[\alpha]_D^{25}=-64.6$ ($c=4.3$, H_2O)). Changing the donor to HA also showed good conversion within 24 h (ca. 66% by NMR spectroscopy) to furnish 1,5-dideoxyhexulose **3b** as the sole product. For comparison, a remarkably

good reaction was also observed in the FSA-catalyzed addition of DHA to **B**, whereas reactions using HA and HB donors again proceeded smoothly with near quantitative conversion to provide deoxysugars **3b** and **3c**, respectively.

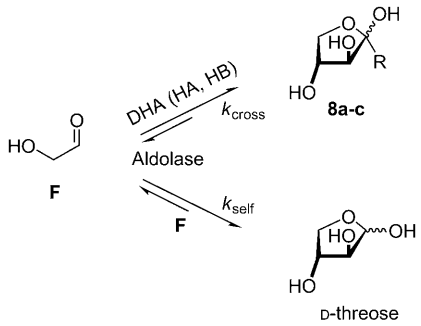
In the presence of DHA, propanal (**C**; "2,3-dideoxyglyceraldehyde") was converted by TalB^{F178Y} to a moderate extent furnishing the corresponding open-chain "hexulose" **4a**. Even in combination with HA this substrate was well tolerated, producing the dihydroxyketone **4b**. By using **C**, FSA again catalyzed the addition of DHA fairly well and converted HA and HB rapidly to provide **4b** and **4c**, respectively.

Enantiomers of lactaldehyde ("3-deoxyglyceraldehyde") (*R*)-**D** and (*S*)-**D** were expected to provide more insight into the capacity of the enzyme catalysts for kinetic enantiomer differentiation. By using (*R*)-**D**, TalB^{F178Y} catalyzed a rather slow conversion with DHA to yield 6-deoxyhexulose **5a** as a single product. Furthermore, no reaction was observed on changing the donor to HA. This behavior was rather perplexing considering the structural relationship with acceptor (*R*)-**A**, which has an identical configuration at C-2, and with acceptor **C**, which has an identical degree of deoxygenation at C-3, both of which were better substrates than (*R*)-**D**. Interestingly, FSA showed no activity at all in the reactions of (*R*)-**D** with each of the three donors. Indeed, (*R*)-**D** was identified as a strong inhibitor of FSA activity when the latter was assayed in the cleavage of Fru6P (see the Supporting Information).

In contrast, the outcome with the enantiomeric (*S*)-**D** was quite different as TalB^{F178Y} catalyzed a rapid conversion with DHA to the expected hexulose **6a** and showed a moderate rate with HA to afford **6b**. FSA acted as anticipated, yielding a less productive reaction with DHA and speedy conversions to products **6b** and **6c** with HA and HB, respectively. Thus, in comparison with the reactions with fully hydroxylated **A**, with 3-deoxygenated acceptors **D** not only were the donor preferences of TalB^{F178Y} and FSA more pronounced, but also the preference for the *S* enantiomer.

The TalB^{F178Y}-catalyzed reaction with DHA and compound **E**, the symmetrical analogue of **D** lacking an element of chirality, proceeded well to furnish a single product **7a**, whereas with HA the sugar **7b** was generated at only a modest rate. Similarly, FSA catalysis was moderately productive with HA and HB, providing deoxy- and dideoxysugars **7b** and **7c**, respectively, whereas with DHA only a fair reaction occurred. Thus, with both enzymes acceptor **E** behaved similarly to the *S* enantiomer of **D**, although it was slightly attenuated. Apparently, steric hindrance directly adjacent to the reactive carbonyl group of the acceptor component seems to be tolerated rather well by both catalysts.

It was speculated that the structurally most simple acceptor, 2-hydroxyaldehyde **F**, offers a borderline case with the potential of acting both as a good electrophile as well as a potential donor.[28a] By using TalB^{F178Y} in the presence of DHA, **F** was converted into D-xylulose **8a** as the major product. However, TLC analysis as well as distinct signals in the NMR experiments indicated the presence of a second



Scheme 3. Reactions of acceptor **F**, also acting as a competing nucleophile. **a**: R = CH_2OH; **b**: R = CH_3; **c**: R = C_2H_5.

component that consequently was established to be D-threose stemming from the homoaldolization of **F** (Scheme 3). This novel side-reaction has recently been also observed by other authors for FSA[28] and its occurrence in the TalB^{F178Y}-catalyzed reaction reflects the similar catalytic environment of these two catalysts. However, the self-aldolization was only a minor activity (< 10% relative to cross-aldol product **8a**) in comparison with that observed with FSA for which D-threose formation was by far the predominant pathway. Considering the lower reactivity of TalB^{F178Y} with HA compared with DHA, a more pronounced competition was to be expected for the donor HA towards **F** (cross- and self-aldolization) and indeed observed, although modest conversion into the cross-aldol product **8b** was also attained. In line with the observations of others,[28] FSA-catalyzed reactions of acceptor **F** with DHA showed the presence of large amounts of D-threose and practically no conversion to the cross-aldol product **8a**. In contrast, from the FSA-catalyzed reactions with HA and HB, the major products were the cross-aldol products **8b** and **8c**, respectively, which were accompanied by only modest amounts of D-threose.

From the entire set of reactions discussed above, each component of the [3×8] product library (Scheme 2, Table 2) could be isolated generally in good-to-high yields after a sufficiently extended reaction time. This outcome is facilitated by the fact that the enzymatic carboligation equilibrium generally favors product formation due to the ensuing cyclization of primary structures to more stable cyclic furanose or pyranose forms (Scheme 2), which withdraw the products from the retro-aldol equilibrium.[16] For components involving DHA, TalB^{F178Y} proved to be clearly superior, whereas for components involving HB, FSA is the only choice. For conversions based on the component HA, both TalB^{F178Y} and FSA are suitable with a subtle preference for each depending on particular combinations (Figure 4). Thus, the two catalysts complement each other well to address a large constitutional space of regiospecifically deoxygenated sugars that in part (specifically, all compounds in series **b** and **c**) were not accessible with available enzymes before.[16,17] The only exceptions (**5b**,**c**) observed are caused by the low reactivity of (*R*)-**D** (D-lactaldehyde) with both biocatalysts. De-

Table 2. Classification of substrate quality.

Electrophile	Dihydroxyacetone (DHA)				Hydroxyacetone (HA)			Hydroxybutanone (HB)		
	TalB^{wt}	TalB^{F178Y}	FSA	Product	TalB^{F178Y}	FSA	Product	TalB^{F178Y}	FSA	Product
D-glyceraldehyde (**(*R*)-A**)	–	+++	+	**1a**	++	+++	**1b**	–	+++	**1c**
L-glyceraldehyde (**(*S*)-A**)	–	+++	+	**2a**	+++	+++	**2b**	–	+++	**2c**
3-hydroxypropanal (**B**)	–	+++	++	**3a**	++	+++	**3b**	–	+++	**3c**
propanal (**C**)	–	++	++	**4a**	++	+++	**4b**	–	++	**4c**
D-lactaldehyde (**(*R*)-D**)	–	++	–	**5a**	–	–	**5b**	–	–	**5c**
L-lactaldehyde (**(*S*)-D**)	–	+++	+	**6a**	++	+++	**6b**	–	+++	**6c**
2-methyl-2-hydroxypropanal (**E**)	–	++	+	**7a**	+	++	**7b**	–	++	**7c**
glycolaldehyde (**F**) [b]	–	++	+	**8a**	+	+++	**8b**	–	++	**8c**

[a] Substrates are indicated as good (+++; conversion >75%), moderate (++; 25–75%), fair (+; <25%), or poor (–; no product detectable by TLC). Data are based on TLC analysis after 1 h of reaction time (100 mM acceptor, 150 mM donor, 20 mg FSA or 10 mg TalB^{F178Y}; glycyl-glycine buffer 50 mM at pH 8.5). [b] All reactions involving **F** are accompanied by D-threose formation from self-aldolization.

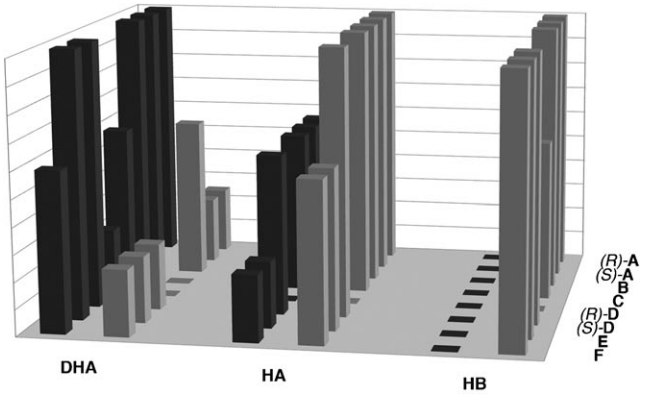


Figure 4. Three-dimensional illustration of the relative rates of product formation (*y* axis) upon catalysis by TalB^{F178Y} (dark bars) or FSA (light bars) for the [3×8] substrate array of structurally related aldol donors (*x* axis) and acceptors (*z* axis).

spite the deliberate restriction of this study to small aldehydes, it is apparent that for both catalysts the scope of acceptor substrates is rather wide, in common with other aldolases, and even tolerates steric bulk directly adjacent to the reactive carbonyl group. It ought to be mentioned that the preparation of **3a** is the first efficient one-step preparation of 5-deoxy-D-fructose, which is discussed as a potential artificial sweetener,[18a,29] from inexpensive starting materials.

Both catalysts deliver aldol adducts having a common D-*threo* configuration, identical to that of fructose 1,6-bisphosphate aldolase (FruA).[30] According to the NMR analysis of the reaction monitored in situ and of the crude product mixtures, all conversions proceeded with excellent stereoselectivity. Products **1a–c**, **2a–c**, and **6a–c** from enantiomerically pure 2-hydroxyaldehydes (*R*)-**A**, (*S*)-**A**, and (*S*)-**D** allow an immediate determination of the diastereoselectivity due to the internal reference of the specific absolute configuration introduced by the aldehyde chiral center; the formation of a single stereoisomer indicates complete selectivity in the asymmetric carboligation step. Similarly, from the 2-hydroxyaldehydes **E** and **F**, single products **7a–c** and **8b**,**c** were obtained and the 3-hydroxyaldehyde **B** yielded single diastereomers **3a–c**. This correlates with numerous observations using FruA and other DHAP-dependent aldolases, for which hydroxylation at the 2- or 3-position facilitates the correct binding of the aldehyde electrophile in the active site.[16,31]

It became evident from X-ray studies of DHAP aldolases that the absolute stereospecificity of the configuration at the nucleophilic carbon usually is fully controlled by the enzyme as stereospecific deprotonation is assisted by hydrogen bonding of the hydroxy group to give a *cis* or *trans* enediolate (or hydroxyenamine equivalent) with spatial protection of one prochiral hemisphere of the "carbanion" equivalent from the protein backbone. Diastereoselectivity results from two-fold hydrogen bonding of the aldehyde carbonyl by active-site residues to activate and correctly position the electrophilic center for stereoselective attack by the enolate (or enamine) nucleophile with retention of its configuration.[31] The 5:2 kinetic preference for *R*-configured aldehyde **A** is in line with the configuration of the natural D-GA3P substrate. However, the inverse preference for (*S*)-**D** seems to be peculiar. The latter may result from an overriding inhibition by the *R* antipode, as observed in the inability to form adducts **5b**,**c**, both for TalB^{F178Y} or FSA catalysts. Contrary to the situation with **A**, the nonpolar end of the acceptor may lead to a nonproductive binding mode, possibly by covalent binding to the catalytic lysine 132 of TalB through carbinolamine (or imine) formation, which would competitively interfere with the regular mode of catalysis. Support for the latter option may be seen in the apparent formation of an aldehyde-derived carbinolamine ligand during crystallization for X-ray structure determination of FSA.[23]

Most remarkable, however, are the TalB^{F178Y}- and FSA-catalyzed conversions involving **C** in the generation of single diastereomers **4a–c** for three reasons: 1) Such substrate combinations are challenging for aldolases, particularly for transaldolase descendants designed by nature for the conversion of highly polar phosphorylated and polyhydroxylated substrates because more generic structures with rather low oxygenation levels possess a decreased aldol acceptor electrophilicity and lack opportunities for hydrogen bonding other than to aldehyde carbonyl; 2) these are the first examples of non-carbohydrate products with this low-level substitution pattern produced by an aldolase;[16,17,32] 3) aliphatic α,β-dihydroxy ketones of the type **4b**,**c** with a strictly de-

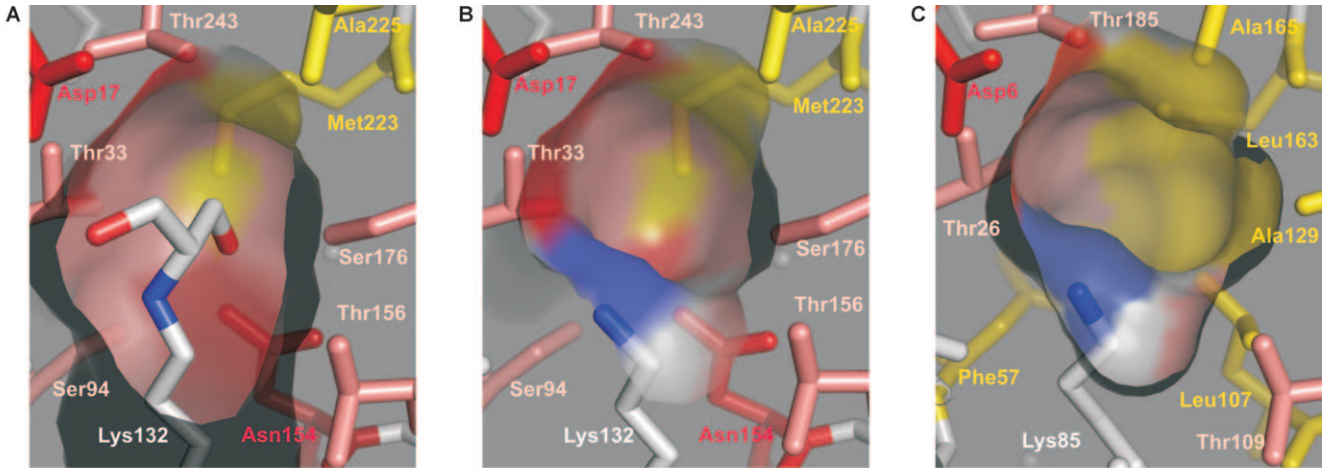


Figure 5. Comparison of the X-ray structures of the active site of wild-type TalB (**B**, pdb 1onr)[24a] and FSA (**C**, pdb 1L6w).[23] For both enzymes the binding pocket corresponding to the C1 carbon of the Schiff base intermediate is shown. Except for the Schiff base forming lysine, the residues and active site surfaces are stained according to their polarity profile (polar or charged residues in red, nonpolar residues in yellow). For comparison, the reduced Schiff base intermediate with Lys132 is shown for $TalB^{wt}$ (**A**, pdb 1ucw).[24b] The figure was prepared by using PyMOL.[25]

fined absolute and relative *syn* configuration are currently not accessible with similar efficiency by enantioselective chemical catalysis.[33] For known FruA enzymes, for comparison, diastereoselectivity with propanal amounts to only 96% *de* with the FruA from rabbit muscle or *Staphylococcus carnosus*.[30]

With increasing deoxygenation increments in the substrate components, selectivity for donor type and acceptor enantiomeric configuration becomes more pronounced when compared with the fully hydroxylated combination, which seems to point to a more compact transition state and thus higher energetic discrimination of the respective reaction pathways of deoxygenated compounds. This effect is potentially caused by the reduced hydrogen-bonding interactions available to substrates with a lower number of hydroxy functions, both intramolecularly as well as to protein residues (Figure 5).

Indeed, a comparison of the residues that surround the C1 carbon of the glyceryl moiety in the Schiff base intermediate within a distance of 5 Å in the two enzymes, FSA and wild-type TalB,[23,24] reveals that some of the polar residues in the active site of TalB are replaced by nonpolar residues in FSA (Table 3). This includes the residues Asn154 and Ser176 in TalB, which form a hydrogen bond to the hydroxy group at C1. These residues are replaced by the aliphatic side-chains Leu107 and Ala129 in FSA. The consequences become even more apparent with a glance at the structures (Figure 5). The surface of the binding pocket accommodating the C1 moiety in TalB is mainly stained in red, which corresponds to polar and charged residues forming this cleft. In contrast, the binding site in FSA is predominantly stained in yellow and indicates a hydrophobic surface formed from nonpolar residues. These differences in polarity and the absence of hydrogen-bond donors might explain why FSA 1) prefers HA with a nonpolar methyl group at C1 over DHA as donor substrate and 2) how this preference may contribute to the regiospecificity of the aldol reaction with HA (and HB) to produce 1-deoxysugars exclusively. On the other hand, DHA can form more interactions with the active site of TalB than HA. Therefore, the binding of DHA is preferred over HA in the case of TalB (or its mutant).

Table 3. Residues located within 5 Å distance of the glyceryl C1 carbon in the reduced Schiff base intermediate of TalB (pdb 1ucw)[24] and the corresponding residues in FSA (pdb 1L6w).[23] Conserved residues are shown in italics.

TalB	*Thr33*	Ser94	Asn154	*Thr156*	Ser176	Met223	*Ala225*	*Thr243*
FSA	*Thr26*	Phe57	Leu107	*Thr109*	Ala129	Leu163	*Ala165*	*Thr185*

As a control experiment to verify this interpretation of nucleophilic substrate recognition sites, the polar residue Ser176 of $TalB^{F178Y}$, involved in the binding of the terminal CH_2OH function of the donor, was replaced by a nonpolar Ala residue in a step towards mimicking the more hydrophobic binding environment of FSA (Table 3). The relative donor preference was evaluated in a competition experiment by using equal concentrations of both DHA and HA nucleophiles together with 3-hydroxypropionaldehyde (**B**), selected for its lack of configurational complications and for the pyranoid cyclization mode that stabilizes the derived products. Indeed, the double mutant $TalB^{F178Y,S176A}$ showed practically identical conversion of HA to 1,5-dideoxy-D-*threo*-hexulose **3b** in comparison with the DHA-derived 5-deoxy-D-*threo*-hexulose **3a**, according to an NMR analysis (ratio **3a**/**3b** = 1:1). Under identical kinetically controlled conditions, the single mutant $TalB^{F178Y}$ almost exclusively produced **3a**, whereas FSA catalysis exclusively gave **3b** (see the Supporting Information). This finding complements well an inverse mutagenesis experiment with FSA in which FSA^{A129S} shows improved activity with the more polar DHA donor.[28b]

In comparison with the productive binding of HA by both of these TalB muteins, their behavior towards the higher homologous HB remains puzzling. Although TalB^{F178Y} led to rapid precipitation upon exposure to HB concentrations even as low as 10 mM, the double mutant TalB^{F178Y,S176A} seemed to be more stable but still precipitated at concentrations >50 mM HB. Under no reaction conditions was product formation (**3c**) detectable. The limitations for the use of HB as a donor substrate seem to be rather particular to the structure of the compound. The bulk of the hydrophobic moiety alone cannot be the imposing factor because propanal (**C**) is a fairly good substrate that is tolerated at much higher concentrations. As a first hypothesis, the extra methylene group in HB may not be adaptable in the active site without causing major reorientation of active-site residues thereby causing a destabilization of the overall fold and/or subunit aggregation state. Replacement of Ser176 by Ala seems to ameliorate the effect but without allowing complete adaptation. Possibly, the larger size of Met223 relative to Leu163 in FSA is another crucial factor to be considered for further mutagenesis experiments.

The fact that propanone (acetone) is inappropriate for both TalB^{F178Y} and FSA as a nucleophile even at high substrate concentration indicates that the hydroxyacetyl portion in the donor is an absolute structural requirement in either or both Schiff base formation and nucleophile generation. Homoaldol formation from glycolaldehyde, observed for both catalysts, albeit at different relative rates, is proof that this aldehyde can bind and act similarly as a donor, in structural analogy to the hydroxymethyl ketone donors. The very poor donor efficiency of TalB^{F178Y} towards glycolaldehyde relative to the distinct potency of FSA, which results in an incompletely filled void in the donor pocket upon binding of an aldehyde instead of a ketone group, may be interpreted as a higher adaptive flexibility of the FSA active site. Indeed, the efficiency of glycolaldehyde binding seems to correlate with the more pronounced inhibition (nonproductive Schiff base formation) observed with D-lactaldehyde, as discussed above.

On the other hand, D-GA and other non-phosphorylated aldol acceptors containing varying degrees and location of deoxygenation sites have been demonstrated to be good substrates, despite the fact that the metabolic function of the enzymes targets fully oxygenated and phosphorylated sugar substrates. Thus, an efficient one-step preparation of 5-deoxyfructose, which is of interest as a potential artificial sweetener,[29] was achieved starting from inexpensive starting materials. Although this study was restricted to small C_2–C_3 aldehydes for the sake of limiting the substrate library to a manageable size, we expect that larger structures will behave similarly, as is well documented for DHAP aldolases and related enzymes.[16,17] Significantly, the two catalysts even seem to tolerate α-branching in the acceptor component rather well, causing steric hindrance directly adjacent to the reactive carbonyl group. This and other aspects need to be further investigated.

Conclusion

We have studied two enzymes, TalB^{F178Y} and FSA, as new entries to an expanding toolbox of biocatalytic carboligation and demonstrated that these catalysts are useful and reliable for preparative applications similar to the "classic" fructose 1,6-bisphosphate aldolase (FruA) but without the need for a costly phosphorylated nucleophile. The catalysts show an interesting complementary tolerance for the aldol donor component with TalB^{F178Y} preferring the fully hydroxylated DHA nucleophile and FSA the more deoxygenated nucleophile, and even the elongated aliphatic HB that is unacceptable toTalB^{F178Y}. Although FSA shows a somewhat broader substrate tolerance, utilizing HB as efficiently as HA, its reactions involving DHA are sluggish and less productive; this catalyst prefers a higher degree of deoxygenation in its donor as well as acceptor substrates. TalB^{F178Y} is able to use glycolaldehyde as an aldol donor but, in comparison with FSA, only as a minor side-reaction to the use of this compound as an aldol electrophile. Factors governing the discriminate substrate selectivity have been elucidated by site-specific mutagenesis of the polar Ser176 residue in TalB^{F178Y} to approach an FSA-like active-site composition.

In combination, these enzymes have allowed the preparation of a total of 22 out of 24 structures, many of which are unique for enzymatic carboligation, from a [3×8] substrate matrix of deoxygenated ketose-type products with a specific D-*threo* or *syn*-3*S*,4*R* configuration (4*S*,5*R* in case of the HB nucleophile) by using variously modified aldehydes. The only two omissions in the matrix concern the special case of D-lactaldehyde, which seems to pose a specific problem of inactive substrate binding. Further studies to adapt the substrate tolerance of the enzymes for alternative donors and acceptors, and towards further preparative applications are in progress. The results will be communicated in due course.

Experimental Section

Enzyme sources: Fructose 6-phosphate aldolase (FSA; 3.2 U mg^{-1} protein) and transaldolase B mutant F178Y (TalB^{F178Y}; 0.32 U mg^{-1} protein) were prepared as lyophilized powders according to published procedures.[20,21a] For both biocatalysts, activity was assayed as the cleavage rate of Fru6P with the formation of GA3P being monitored by coupled enzymatic consumption of NADH;[21a] the protein was quantified by the Bradford assay.[34] One unit of FSA or TalB^{F178Y} is defined as the amount of protein that will cleave 1 μmol of Fru6P to afford D-GA3P and DHA per minute at 25 °C and pH 8.5 (glycyl-glycine buffer, 50 mM).

Determination of kinetic constants: The K_m value for DL-GA3P was determined within a concentration range of 0.14–11.2 mM (FSA) and 0.28–11.2 mM (TalB^{F178Y}) at 30 °C in 50 mM glycyl-glycine buffer (pH 8.5) containing 1 mM dithiothreitol (DTT). The formation of Fru6P was detected as described previously[20,21a] using the coupling enzymes phosphoglucose isomerase and glucose 6-phosphate dehydrogenase (activities were monitored at 340 nm for 10 min). The concentration of DHA was kept constant at 300 mM (FSA) or at 150 mM (TalB^{F178Y}). The concentration of DHA was saturating as the K_m value is 62 mM for FSA and 30 mM for TalB^{F178Y}.[20] The specific activity was plotted against the concentration of DL-GA3P. As a result of the strong substrate inhibition observed in some reactions the kinetic constants were calculated from Hanes plots[35] by

using SigmaPlot 9.0. The formation of D-fructose from DHA (150 mM) and DL-GA or D-GA was followed by analyzing the formation of NADPH (0.5 mM $NADP^+$) in 50 mM glycyl-glycine buffer (pH 8.5) containing 1 mM DTT by using the coupling enzymes fructokinase (0.5 U) from *Zymomonas mobilis*,[36] phosphoglucose isomerase (0.5 U), and glucose 6-phosphate dehydrogenase (0.5 U). ATP (1.2 mM) and $MgCl_2$ (10 mM) were added.[27] The K_m value for DL-GA (or D-GA) was determined by using aldehyde concentrations ranging from 2 to 140 mM for $TalB^{F178Y}$. The concentration of DHA was kept constant at 150 mM.

Site-directed mutagenesis: Site-directed mutagenesis for the replacement of Ser176 with Ala was carried out by using a modified version of the QuikChange protocol. In this case the plasmid pJF119*talBF178Y*[20] was used as template and the PfuUltra DNA Polymerase (Stratagene) for amplification. The following primers were used: Forward 5′-ggcgtgttcctgatcgcgccgtacgttggc-3′, reverse 5′-gccaacgtacggcgcgatcaggaacacgcc-3' (mutated codon is underlined). The correct sequence was confirmed by custom sequencing (GATC, Konstanz, Germany).

General procedures for substrate evaluation

In situ NMR experiments: All component solutions were prepared in D_2O. Solutions of donor and aldehyde acceptor components at final concentrations of 150 and 100 mM, respectively, in a total volume of 1 mL were added to a solution of the aldolase (20 mg FSA or 10 mg $TalB^{F178Y}$ as lyophilized powder) in glycyl-glycine buffer (50 mM, pH 8.5). From this mixture, a sample of 700 μL was withdrawn and carefully transferred to an NMR tube. The progress of the reaction progress was monitored by recording ^1H NMR spectra (500 MHz) after 0, 1, 12, and 24 h of reaction time (48 h for slow reactions). The NMR experiments were analyzed for proof of stereochemical preference as well as effective conversion.

To assess the donor preferences of FSA and mutants $TalB^{F178Y}$ and $TalB^{F178Y,S176A}$, competition experiments (in D_2O) were performed as follows. Equal stoichiometric quantities of DHA, HA, and acceptor aldehyde (each at 100 mM final concentration) in a total volume of 1 mL were added to the aldolase solution (700 μL; 20 mg FSA; 10 mg $TalB^{F178Y}$; 10 mg $TalB^{F178Y,S176A}$) in glycyl-glycine buffer (50 mM, pH 8.5). The reactions were followed by recording ^1H NMR spectra (500 MHz) after 0, 1, 6, 12, and 24 h.

Preparative synthesis: Lyophilized fructose 6-phosphate aldolase powder (200 mg; 100 mg of $TalB^{F178Y}$) was added to a solution (10 mL total reaction volume) containing the respective donor (DHA, HA, HB; 150 mM) and acceptor (**A**–**F**; 100 mM) components in glycyl-glycine buffer (50 mM, pH 8.5) and the resultant mixture was stirred at room temperature. The addition of DTT (1 mM) recommended for kinetic assays[20,21a] was omitted in the preparative reactions as the enzymes were found to be stable in its absence. The reaction was monitored at regular intervals by TLC (chloroform/methanol; 5:1 or 2:1) and worked up after 24–48 h depending on the rate of consumption of the aldehyde. The relative rates of conversion were determined on the basis of densitometric TLC monitoring, by signal integration of in situ NMR experiments, as well as by gravimetric quantification of the isolated product. Conversion is denoted as +++ (>75%), ++ (25–75%), + (<25%), or – (no detectable product formation). For product characterization, the individual reaction mixtures were worked up by lyophilization of the crude reaction mixture followed by silica gel column chromatography of the residue using chloroform/methanol as the eluent (20:1–5:1) to provide the pure product. The products were characterized by recording their mass and NMR spectra. The ^1H and ^{13}C NMR spectra were recorded and whenever required further information was obtained by ^{13}C DEPT, ^1H–^1H COSY, and HSQC experiments.

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