Practical synthesis of carbohydrates based on aldolases and glycosyl transferases

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Abstract: With various recombinant DNA and protein engineering techniques now available, enzyme-based technologies are emerging as practical methods for large-scale synthesis of chiral intermediates and bioactive molecules, especially carbohydrates, oligosaccharides, their conjugates and related substances. This paper describes recent developments in the synthesis of novel monosaccharides and aza sugars based on aldolases, and the synthesis of oligosaccharides and analogs based on glycosyltransferases coupled with in situ regeneration of sugar nucleotides.

As many enzymes are available for the stereocontrolled synthesis of chiral synthons (1), attention has been extended to the development of more effective and stable enzymes for the synthesis of molecules with increasing complexity (2). One class of such complex molecules are carbohydrates and their conjugates, especially those that exist on cell surfaces (3). These molecules are involved in many types of recognition phenomena (3-6); however, most of their precise functions have not been clearly identified at the molecular level. Part of the reason is that these molecules have been difficult to isolate, characterize and synthesize. Enzyme-based technology seems to be well suited for the synthesis of glycoconjugates and related substances for the study of their functions as these molecules are multifunctional and highly soluble in polar solvents, and many enzymes are available for the transformation of these molecules (7). The following describes some new technologies developed for the synthesis of sugar- and peptide-related substances based on recombinant or engineered enzymes.

ALDOLASES

Catalytic asymmetric aldol condensation is one of the most effective methods for carbon-carbon bond forming reactions. Enzyme-catalyzed aldol condensation holds great potential in this regard (7,8). More than 20 aldolases are known and several of them have been explored for synthesis. The aldolases that have been cloned and overexpressed include fructose-1,6-diphosphate aldolase (9,10), fuculose-1-phosphate aldolase (11,12), rhamnulose-1-phosphate aldolase.
aldolase (12), DAHP synthase (13), and 2-deoxyribose phosphate aldolase (14). In general, the type II Zn\(^{++}\)-containing aldolases are more stable than the Type I Schiff base forming enzymes. A particular important application of aldolases is the synthesis of aza sugars - a class of molecules useful as glycosidase inhibitors (8,15,16). All the aldolases explored so far possess two common features: first, they are highly specific for the donor substrate but flexible for the acceptor component; second, the stereoselectivity in aldol condensations is controlled by the enzyme not by the substrate, with some exceptions observed in the sialic acid aldolase reactions (17). These two common features led to the development of a general strategy for the synthesis of aza sugars (8,15,16,18,19). As indicated in Figure 1, the combined enzymatic aldol condensation and Pd-mediated reductive amination is one of the most effective and practical ways available now for the synthesis of nitrogen-containing sugars. The phosphate group contained in the enzyme product facilitates product recovery. It can also serve as an activating group during the reductive amination step to give 1,α-dideoxy aza sugars. Both thermodynamic and kinetic approaches can be employed to prepare a single diastereomeric product starting with a racemic aldehyde. Although aldolases are generally very specific for the donor component, no site-directed mutagenesis has been employed to alter the active site of aldolases to change their donor specificity or stereospecificity. The X-ray structure study of fructose-1,6-diphosphate aldolase suggests that the C-terminal peptides of the enzyme mediate the entry of substrates to the active site (20). Alteration of the active site or the C-terminal residues thus may lead to new aldolase activities. Figures 2-4 are representative syntheses of sugars and related compounds based on aldolases.

![Figure 2. Representative aldolase-catalyzed synthesis of aza sugars.](image-url)

**LARGE-SCALE SYNTHESIS OF OLIGOSACCHARIDES USING GLYCOSYLTRANSFERASES WITH IN SITU REGENERATION OF SUGAR NUCLEOTIDES**

Glycosidases (21,22,23) and glycosyltransferases (6,24-25) have been used in oligosaccharide synthesis. Although these two types of enzymes are complementary with regard to their synthetic utilities, the sugar nucleotide-dependent glycosyltransferases seem to be more suitable for the synthesis of complex oligosaccharides as the enzymatic reactions are stereo- and regioselective for various complex acceptor structures. The major problems are that the membrane-bound glycosyltransferases are not very stable and readily available, and that sugar nucleotides are too expensive to be used as stoichiometric reagents. Furthermore, the reactions often exhibit product inhibition caused by the released nucleoside phosphates. A simple solution to these problems is to regenerate the sugar nucleotide from the released nucleoside phosphate. It has been demonstrated that UDP-glucose and UDP-galactose can be regenerated in situ in a 50-g synthesis of the disaccharide N-acetyl-lactosamine (24). Recently, new procedures for the regeneration of
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CMP-sialic acid (26), GDP-fucose (27,28), GDP-mannose (27,28), and UDP-glucuronic acid (29) have been developed. It has also been demonstrated that two glycosyltransferases can be used in a one-pot reaction, coupled with the regeneration of more than two sugar nucleotides, for the formation of two glycosidic bonds starting with three unactivated monosaccharides (30). No product inhibition was observed in the reactions. The enzymes can be immobilized on a polyacrylamide support and recovered for reuse. It would be interesting to determine whether this multiple enzyme system can be applied to the one-pot synthesis of oligosaccharides containing more than four monosaccharide units. There are eight sugar nucleotides commonly used in mammalian systems for glycosyltransferase reactions, and they can now be regenerated for enzymatic oligosaccharide synthesis. The remaining issues will be to make the glycosyltransferases more stable and readily available. Although several cloning

\[ \text{D-DNJ} \]
\[ \text{L-DNJ} \]

Figure 3. Enantiocomplementary nature of FDP aldolase and Rham-1-P aldolase.

\[ \text{D-KDN (R=OH)} \]
\[ \text{L-KDN (R=OH)} \]

Figure 4. Sialic acid aldolase-catalyzed addition reaction with normal (\(si\) face attack) and unusual (\(re\) face attack) stereoselectivity. Synthesis of enantiomeric high-carbon sugars.
Figure 5. Regeneration of sugar nucleotides for glycosyltransferase-catalyzed oligosaccharide synthesis.

E1: Gal transferase
E2: Glc transferase
E3: Sia transferase
E4: GlcU transferase
E5: GalNAc transferase
E6: GlcNAc transferase
E7: Fuc transferase
E8: Man transferase
E9: Pyruvate kinase
E10: UDP-Glc pyrophosphorylase
E11: UDP-Gal 4-epimerase
E12: Inorganic pyrophosphorylase
E13: Gal-1-P uridylytransferase
E14: Galactokinase
E15: Nucleoside monophosphate kinase
E16: CMP-NeuAc synthetase
E17: UDP-Glc dehydrogenase
E18: UDP-GalNac 4-epimerase
E19: UDP-GalNac pyrophosphorylase
E20: UDP-GlcNac pyrophosphorylase
E21: GDP-Fuc pyrophosphorylase
E22: GDP-Man pyrophosphorylase
E23: GDP-Fuc synthesizing enzyme
E24: \textit{Thermoanaerobium brockii} alcohol dehydrogenase
systems have been developed (31,32), there is still a need for the development of more practical expression systems than that based on CHO cells. Since the soluble portion of glycosyltransferases (the so-called catalytic domain) (31) is stable and fully active, one possibility is to clone and express the catalytic domain of glycosyltransferases in microorganisms. The active domain of human β1,4-galactosyltransferase, for example, has been cloned and expressed in E. coli. (33) An α2,3-sialyltransferase has been overexpressed in a baculovirus system (28). Similar approaches could be applied to other glycosyltransferases. Another issue of interest is to alter the regioselectivity of glycosyltransferases using site-directed mutagenesis. With regard to substrate specificity, glycosyltransferases exhibit relaxed donor and acceptor specificities under certain conditions as indicated in the recent studies on β1,4-galactosyltransferase (34,35) and α1,3fucosyltransferase (36). This relaxed substrate specificity allows the use of glycosyltransferases in the synthesis of certain unnatural oligosaccharides. Glycosyltransferases usually have high $K_m$ values for unnatural substrates while the $V_{max}$ values are still significant. This low affinity for unnatural substrates may partly contribute to the origin of specificity of glycosyltransferases in vivo. Given the increasing interests in oligosaccharides and their conjugates, it is expected that practical oligosaccharide synthesis will remain a very active subject for study in the next several years. The regeneration systems now available should be useful for the large-scale synthesis of many oligosaccharides, provided that glycosyltransferases become readily available. Sialyl Le$^a$, a ligand of endothelial leukocyte adhesion molecule (ELAM-1) (37), its terminal glycal, and 5-thioglucose- or deoxynojirimycin-containing oligosaccharides, for example, have been prepared enzymatically coupled with in situ regeneration of sugar nucleotides (28) (Figure 6). Since sialyl Le$^a$ glycal is conformationally similar (based on our 2-D NMR analysis) to sialyl Le$^a$, it may act as an antagonist of ELAM-1. Work is in progress to employ our developed engineered serine proteases (e.g. thiosubtilisin, methylchymotrypsin) (27) as peptide ligases to join glycosylpeptide fragments in aqueous solution in a kinetically controlled mode. The glycosylpeptides will then be used as substrates for glycosyltransferases to form oligosaccharyl polypeptides.

CONCLUSION

The practicality of enzyme-based synthetic technology can be improved via protein engineering to alter the performance of enzymes. As more enzymes become available via advanced recombinant techniques, enzyme-catalyzed synthesis of complex biomolecules such as glycoconjugates and their intermediates and related substances will become a very effective and practical approach for use to study the structure-function relationship of glycoconjugates and to develop new compounds for therapeutic evaluation.
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