Acta Chemica Scandinavica, 1996: 50: 211–218
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ACTA
CHEMICA SCANDINAVICA
ISSN 0904–213X

Chemoenzymatic Synthesis: Application to the Study of Carbohydrate Recognition†

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Wong, C.-H., 1996. Chemoenzymatic Synthesis: Application to the Study of Carbohydrate Recognition. – Acta Chem. Scand. 50: 211–218 © Acta Chemica Scandinavica 1996.

Reported here are useful strategies recently developed for the large-scale synthesis of complex and polyfunctional molecules using native or engineered enzymes as catalysts. Several important issues in the field regarding the problems of substrate specificity, product inhibition, reaction reversibility, enzyme stability and catalytic efficiency are addressed in the representative synthesis of carbohydrates and carbohydrate mimetics designed for use to study carbohydrate-mediated cell adhesion.

Recent advances in recombinant DNA technology and structural biology have made possible the production of virtually any enzyme and its engineered variants in large quantities for the exploitation of their synthetic utility. As transition-metal-based asymmetric catalysis has become a useful approach to the synthesis of small molecules in organic solvents, enzyme-catalyzed asymmetric catalysis has become more appealing in the transformation of complex molecules with multi-functionalities in aqueous solution.

When using enzymes as catalysts in organic synthesis, several important issues regarding the problems of substrate specificity, product inhibition, reaction reversibility, enzyme stability and catalytic efficiency have to be addressed in order to evaluate the practicality of a given enzymatic process (Fig. 1). As illustrated in the following sections regarding the use of enzymes in the synthesis of carbohydrates and related structures, new strategies and methods have been developed to address these issues.

Carbohydrates on cell surfaces represent an effective class of biomolecules coding for a vast amount of information required in various biological recognition processes such as bacterial and viral infections, cell adhesion in inflammation and metastasis, differentiation, development, regulation and many other intercellular communication and signal transduction processes. The pace of development of carbohydrate-based pharmaceuticals has, however, been slower than that of other classes of biomolecules. Part of the reason is due to the difficult problem associated with the synthesis of carbohydrates on a large scale for therapeutic evaluation. The requirement of multiprotection and deprotection steps in conventional carbohydrate synthesis makes it difficult for large-scale processes. Enzymes are able to contribute to the resolu-

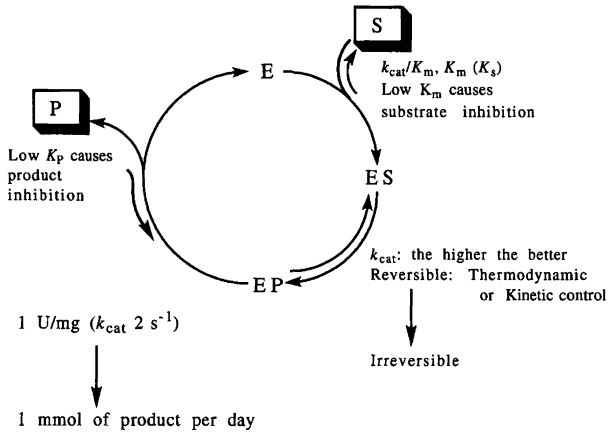


Fig. 1. The catalytic cycle of an enzymatic reaction indicating several important issues to be considered when used in synthesis. A low dissociation constant for the enzyme–substrate complex (ES) or the enzyme–product complex (EP) may lead to low catalytic efficiency or product inhibition. Useful K_m values are in the range 0.1–1 mM as the upper limit of k_{cat}/K_m in most enzymatic reactions is ca. 10^8 M^{-1} s^{-1}. The ES to EP step is determined by k_{cat}, the higher the better. When the reaction is reversible, one has to control the reaction with a kinetic or thermodynamic approach to optimize the process, or to make the reaction irreversible to increase selectivity. With regard to specific activity, an enzyme with 1 U mg^{-1} (i.e., $k_{cat} \approx 2$ s^{-1}) will produce about 1 mmol of product a day (per mg of enzyme), and enzymes with specific activity at this level or higher are synthetically useful.

† Contribution presented at the Nobel Symposium on *Catalytic Asymmetric Synthesis*, September 3–7, 1995 at Tammsvik, Bro, Sweden.

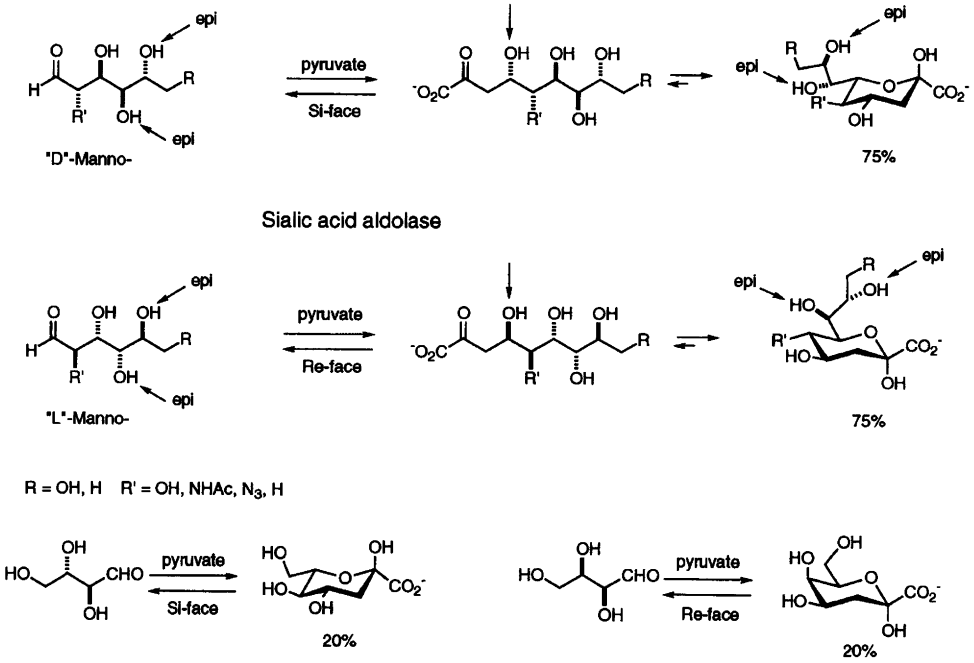


Fig. 2. Sialic acid aldolase-catalyzed reaction for the synthesis of sialic acids and analogs. Because of the reversibility of the reaction, an inversion of stereoselectivity (*Re*-face attack) occurs with enantiomeric substrates at equilibrium to form a more stable product.

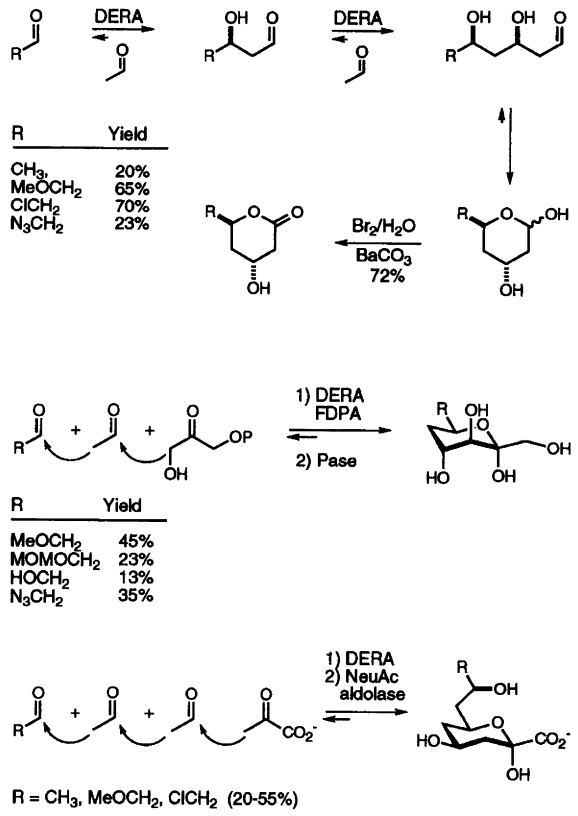


Fig. 3. One-pot sequential aldol reactions using three substrates—one enzyme or three substrates—two enzymes. DERA, 2-deoxyribose 5-phosphate aldolase; FDPA, fructose 1,6-diphosphate aldolase; NeuAc, sialic acid or neuraminic acid.

tion of this issue, and have been increasingly considered as a useful class of catalysts for organic synthesis.[1] Numerous novel monosaccharides can now be prepared based on aldolase-catalyzed reactions.[2] In addition, bioactive oligosaccharides and their conjugates are now accessible in large quantities based on glycosyltransferase reactions coupled with the regeneration of sugar nucleotides.[3]

Aldolase-catalyzed synthesis of novel monosaccharides and mimetics

Of the more than 20 aldolases known, approximately half have been exploited for synthesis.[2] Aldolases catalyze aldol addition reactions, and the most useful application of these enzymes is in carbohydrate synthesis. Owing to the high degree of flexibility in accepting the acceptor component in most aldolase-catalyzed addition reactions, many common and uncommon sugars can be synthesized. Figures 2–4 illustrate some recent applications of this class of enzymes. The four dihydroxyacetone phosphate-dependent aldolases have been cloned and overexpressed,[4,5] and are commercially available for synthesis. Combined with the Sharpless osmium-catalyzed asymmetric dihydroxylation for the preparation of optically pure hydroxy aldehydes, these enzymes are useful for the synthesis of enantiomeric ketoses.[6] Aldolases have also been used in the synthesis of pyrrolidines, piperidines[7] and deoxythiosugars.[5] The use of pyruvate-dependent aldolases in synthesis is illustrated by the

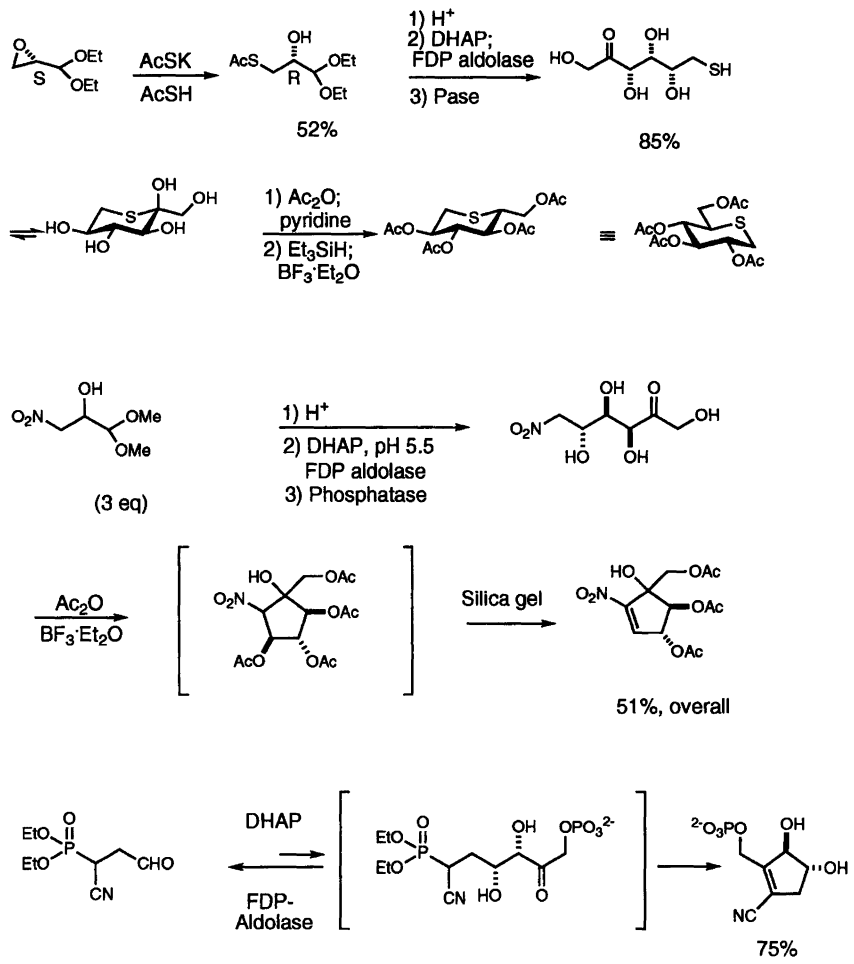


Fig. 4. Aldolase-mediated synthesis of thiosugars and cyclitols. DHAP, dihydroxyacetone phosphate.

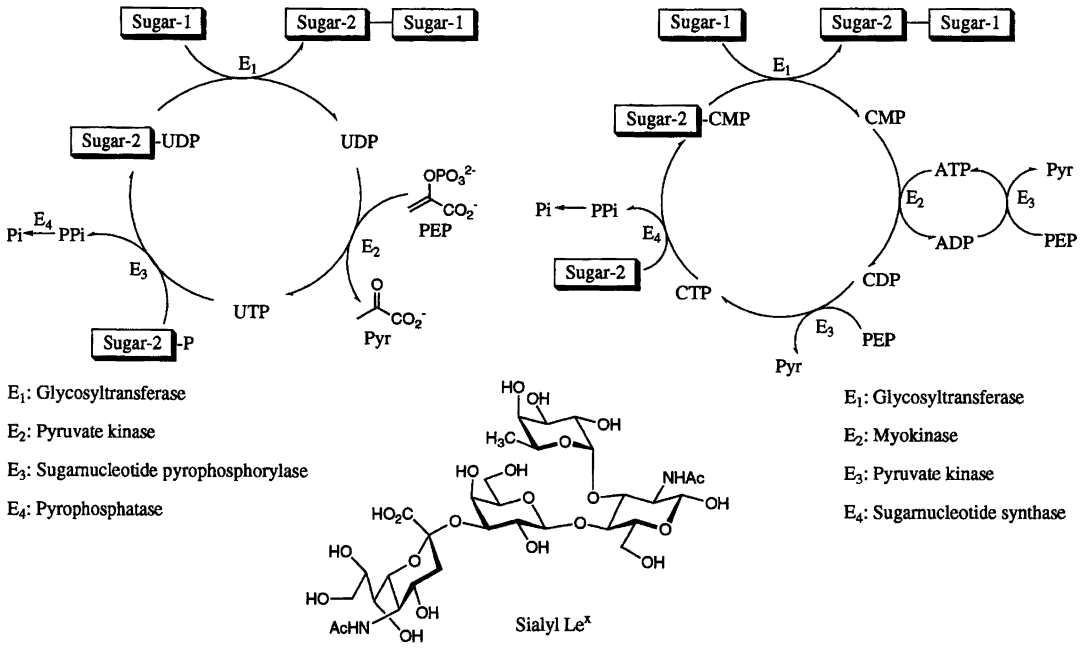


Fig. 5. Regeneration of sugar nucleotides in glycosyltransferase-catalyzed synthesis of oligosaccharides.

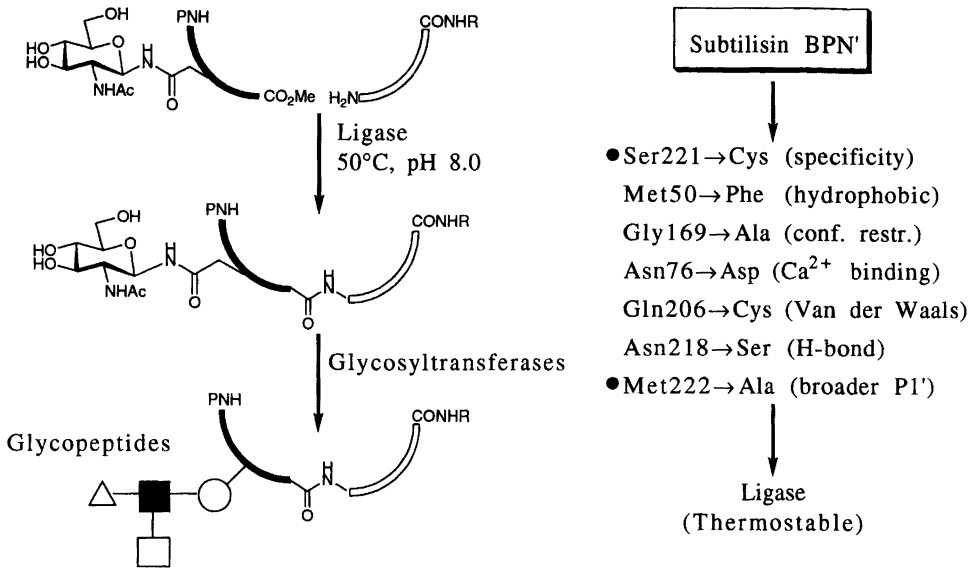
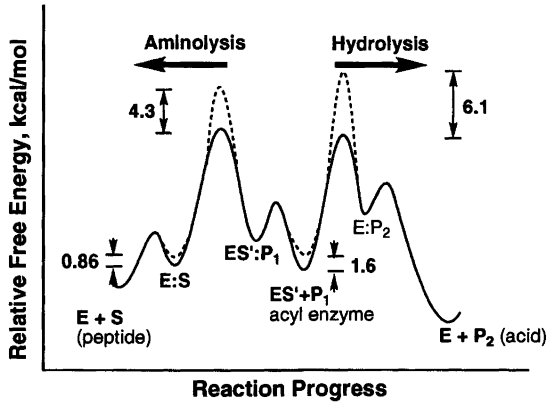


Fig. 6. Glycopeptide coupling using engineered subtilisins as peptide ligases in aqueous solution coupled with glycosyltransferase reactions. The serine protease was altered (as indicated) to change the specificity and stability. The engineered enzyme has a half-life of more than 50 h at 50°C in aqueous solution (pH 8.0) compared to 1 h for the wild-type enzyme, and the aminolysis to hydrolysis ratio is about 1000 times higher. The variant is, however, about 1000 times less active than the wild-type enzyme.

representative synthesis of high-carbon keto acids, catalyzed by sialic acid aldolase (Fig. 2).[8] In reaction with its natural substrate *N*-acetylmannosamine, the enzyme catalyzes the addition of pyruvate to the *Si*-face of the acceptor carbonyl group. When certain acceptors are used as substrates, a complete inversion of the facial selectivity (i.e., *Re*-face attack) is observed – suggesting a thermodynamically controlled process operating in the enzymatic reaction as the inverted stereoselectivity leads to the formation of a more stable product (Fig. 2). Further study of enzymatic aldol reactions leads to the development of new sequential aldol reactions with three substrates (e.g., with the use of 2-deoxyribose 5-phosphate aldolase and fructose 1,6-diphosphate aldolase or sialic acid aldolase) reacting in one pot (Fig. 3).[10] In the former case, the reaction stops when the product forms a stable cyclic hemiacetal. In the latter case, the first aldolase reaction provides a product which is a substrate for the second aldolase. With the increasing understanding of the specificity of various aldolases, these two sequential aldolase reaction processes are expected to find use in organic synthesis. When mercaptoaldehydes are used as substrates, thiosugars are produced.[5] When nitroaldehydes are used as substrates, the products undergo a non-enzymatic intromolecular nitroaldol reaction to give nitrocyclitols which may be a useful source of aminocyclitols.[11] When phosphonate-containing aldehydes are used as substrates the products spontaneously undergo a Horner–Wadsworth–Emmons olefination to give another type of cyclitol (Fig. 4).[12]



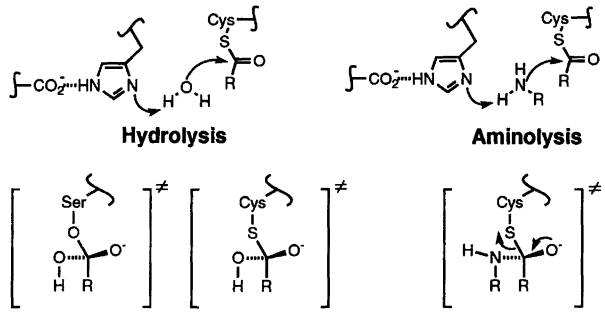


Fig. 7. An energy diagram indicating the mechanism of thiosubtilisin-catalyzed aminolysis in aqueous solution. The hydrolysis of the acyl–enzyme intermediate is energetically less favorable than aminolysis. The numbers indicated are in kcal mol^{-1}.

Synthesis of oligosaccharides and glycopeptides

Owing to the advances in recombinant DNA technology, glycosyltransferases are now available in large quantities for the synthesis of oligosaccharides. Coupled with the regeneration of sugar nucleotides (Fig. 5), these enzymes have been developed for the large-scale synthesis of oligosaccharides.[3] The cofactor regeneration scheme not only reduces the cost of sugar nucleotides, but also lessens the problem of product inhibition caused by the leaving nucleoside phosphate. This enzymatic strategy of oligosaccharide synthesis has been applied to the kilogram-scale synthesis of the oligosaccharide sialyl Lewis x[13] which is in clinical trials as a new anti-inflammatory agent for the treatment of heart attack or reperfusion injury. When a glycosyltransferase is not available, it may be replaced with a glycosidase for the formation of the corresponding glycosidic bond. An example is the synthesis of sialyl Lewis a,[14] in which the β-1,3-linked *N*-acetyllactosamine was prepared via regioselective acetylation of galactal with an engineered subtilisin in high concentrations of DMF followed by β-galactosidase reaction and azidonitration. The product is subject to further enzymatic glycosylation using sialyl and fucosyl transferases to yield the desired product. There are eight sugar nucleotides commonly found in mammalian systems as substrates for glycosyltransferases, and methods for the regeneration of each sugar nucleotide have been developed.[15] This sugar nucleotide regeneration strategy has been used in the synthesis of many oligosaccharides, including the recent synthesis of hyaluronic acid with molecular weight of ca. 500000.[16]

Glycosyltransferases have also been used in the solid-phase and solution-phase synthesis of glycopeptides.[18] In the latter case, glycopeptides were prepared non-enzymatically and coupled with another peptide fragment in aqueous solution via protease-catalyzed aminolysis. Several thermostable subtilisin variants have been developed for efficient aminolysis reactions via site-specific change of amino acids (Fig. 6).[19] A mechanistic investigation of a thermostable thiosubtilisin variant was conducted and an energy diagram was constructed to explain the high ratio of aminolysis to hydrolysis reaction (Fig. 7).[19]

In another effort directed toward the development of new methods for the synthesis of complex carbohydrates, a method for the introduction of a sulfate group to an oligosaccharide has been developed using a recombinant sulfotransferase coupled with regeneration of the sulfation cofactor 3′-phosphoadenosine 5′-phosphosulfate (PAPS) (Fig. 8).[21] This method should be generally useful for the enzymatic synthesis of oligosaccharide sulfates such as heparin.

Synthesis of carbohydrate mimetics

One of the major problems in the development of carbohydrate-based pharmaceuticals is, in addition to the difficulty in synthesis, that carbohydrates are unstable and orally inactive. This problem requires a new ap-

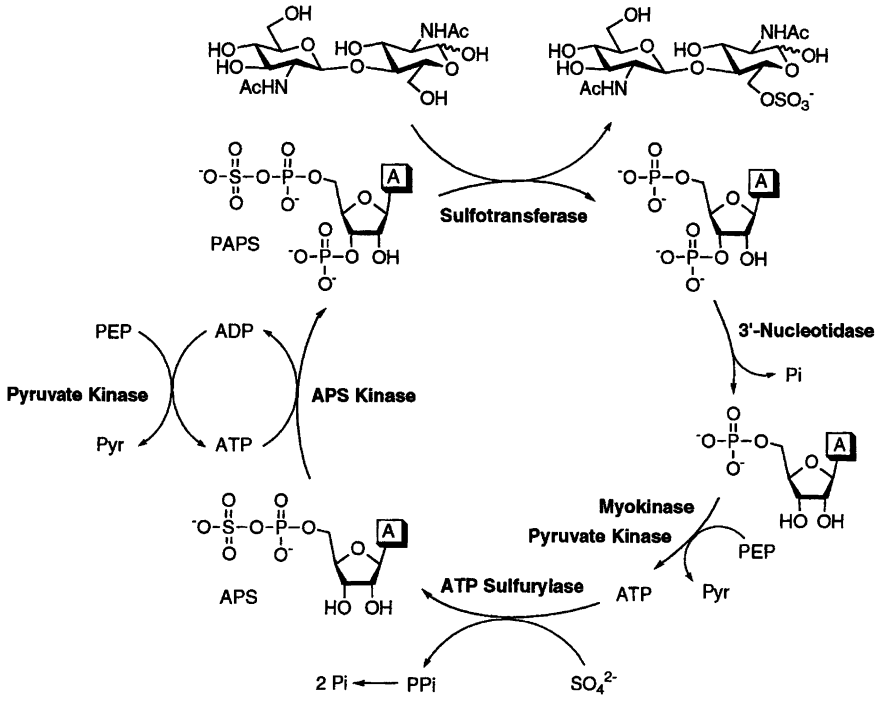


Fig. 8. Regeneration of PAPS in enzyme-catalyzed sulfation of oligosaccharides. A stands for adenine.

proach to the development of inhibitors of glycosidases and glycosyltransferases. In addition, many interesting oligosaccharide ligands potentially useful as inhibitors of carbohydrate binding proteins such as lectins, may only be useful as injectable drugs for acute symptoms, not as orally active forms for the treatment of chronic diseases. Development of carbohydrate mimetics is therefore of great interest. With regard to the inhibition of glycosidases and glycosyltransferases, various cyclic imitols have proved to be effective and aldolases have been shown to be useful for the synthesis of pyrrolidines and piperidines and their homoanalogs (Fig. 9).[2] Cyclic guanidino-sugars recently developed are also effective glycosidase inhibitors.[21] The starting material used in the synthesis of cyclic guanidino-sugars was prepared from a lipase-catalyzed hydrolysis of a *meso*-epoxy diol (Fig. 9).[22]

Both homopyrrolidines and piperidines can also be used as building blocks in the synthesis of sequence-specific inhibitors of glycosidases and glycosyltransferases.[23]

In an effort toward the development of oligosaccharide mimetics, several sialyl Lewis x mimetics have recently been designed and synthesized, and two such mimetics have inhibitory activities comparable to that of sialyl Lewis x (Fig. 10).[24,25] A key component used in the synthesis is (2*S*, 3*R*)-2-amino-3,4-dihydroxybutanoic acid (L-δ-hydroxythreonine), which can be easily prepared via a threonine aldolase-catalyzed addition reaction (Fig. 10).[26]

In summary, the aldolase reaction strategy described above provides a new approach to the synthesis of novel monosaccharide structures and mimetics, and the glycosyltransferase reaction has proved useful for the large-scale synthesis of oligosaccharides. One can also alter the specificity and stability of subtilisin to peptide ligases for the synthesis of glycopeptides. As various monosaccharide building blocks and glycosyltransferases become readily available, many carbohydrate recognition problems in biological systems can be studied, and the development of novel carbohydrates and mimetics for use to control the function of carbohydrates involved in certain diseases and metabolic disorders will follow.

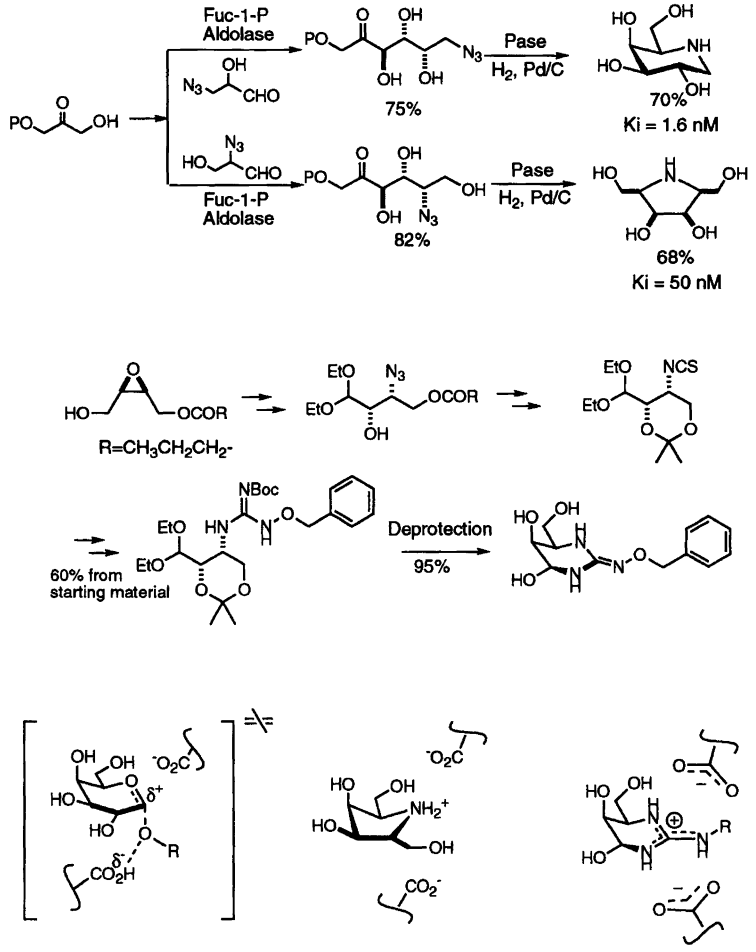


Fig. 9. Synthesis and proposed modes of action of inhibitors of α-galactosidase. The neutral form of the inhibitors is bound to the enzyme, followed by a proton transfer to form a tight complex.

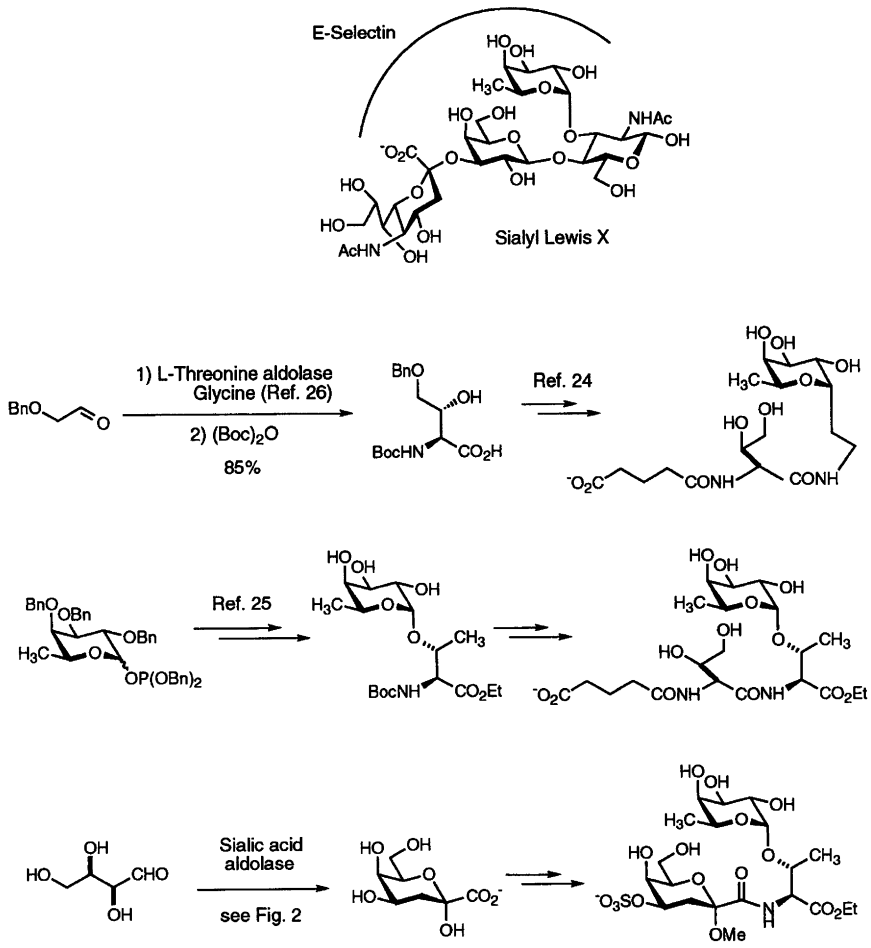


Fig. 10. Chemo-enzymatic synthesis of sialyl Lewisxmimetics. The E-selectin binding site was determined and used in the design of mimetics.

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Received September 5, 1995.