Lipases in the preparation of beta-blockers

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Lipases in the preparation of β-blockers

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β-Adrenergic blocking agents are pharmaceutical products used for the treatment of hypertension and angina pectoris. These β-blockers can be prepared in optically active form by conventional crystallization procedures, by asymmetric chiral synthesis and by using stereoselective enzymes.

Lipases (triacylglycerol acylhydrolase EC 3.1.1.3) occur widely in nature. They belong to the class of serine hydrolases, and therefore do not require cofactors. Their biological function is to catalyse the

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hydrolysis of triacylglycerols to yield free fatty acids, di- and mono-acylglycerols and glycerol. The reaction is reversible: lipases can also catalyse the formation of acyl-glycerols from free fatty acids and glycerol[1].

Lipases are active at oil–water interfaces of a heterogeneous reaction system, and are thus differentiated from esterases which act on water-soluble substrates in a homogeneous milieu. The preference of lipases for substrates at interfaces seems to be conferred by the presence of interfacial binding sites on the enzyme and not by unique properties of the catalytic site. Since lipases function at an oil–water interface their characterization is rather complicated – one of the reasons why lipases have been studied less extensively than other industrial enzymes such as amylases and proteases.

Applications of lipases

During the last decades, lipolytic enzymes have been used in the dairy and food industry (cheese ripening, production of flavors and cocoa butter substitutes), in detergents, for tanning, sewage treatment and cosmetics[2–4]. For drugs (and agrochemicals) there is an increasing trend towards the use of optically pure stereoisomers, which are more target-specific and show fewer side-effects than racemic mixtures of isomers. Consequently, approval procedures from the FDA and equivalent regulatory bodies are likely to tighten in the near future. There are therefore important stimuli for companies to market optically pure isomers. This leads to an increasing demand for efficient processes for the industrial-scale synthesis of optically active compounds, which in turn

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Fig. 1

Typical structure of β-blockers.

stimulates increased research and development on the production of specialty chemicals via, for instance, lipases or esterases[5]. Development, in some cases, is well advanced: at Chemie-Linz in Austria, a process for the resolution of racemic α-halopropionic acids by lipases[6–8] is operating on pilot-plant scale; and at Andeno–DSM[9] the resolution of racemic alkanoic acid oxyranyl methyl esters (glycidyl esters) is performed on a commercial scale.

In this paper we describe the potential application of lipases in the chemoenzymatic synthesis of chiral intermediates for optically active β-adrenergic blocking agents (β-blockers) and compare it with competing technologies.

β-Blockers

β-Blockers are effective in humans for the treatment of hypertension and angina pectoris. In the pharmaceutical industry some fifty different compounds having β-blocking activity have been brought to some stage of commercial development. About two dozen of these are approved for use and accounted worldwide for $2.2 billion in revenues in 1985 (Ref. 10).

The aryloxypropanolamine struc-

ture containing one chiral center (Fig. 1) is characteristic of almost all of the FDA-approved β-blockers. In general, β-blocking activity resides in the (S)-enantiomer. For instance, (S)-propranolol (Ar, 1-naphthyl; R, isopropyl) is 100 times more active than the (R)-isomer. However, with the exceptions of (S)-timolol (Merck, Sharp & Dohme), (S)-penbutolol (Hoechst) and (S)-levobunolol (Warner-Lambert) (Fig. 2)[11–13], all of the FDA-approved β-blockers are marketed as racemates.

For some compounds benefits have been claimed for the (R)-enantiomer (e.g. anti-glaucoma activity). Of the ophthalmic β-blockers, both enantiomers are reported to be equally effective in the treatment of glaucoma. Nevertheless, in this case, reduction of the unwanted side-effect (cardiovascular β-blocking) can only be accomplished by removing the (S)-isomer.

Preparation of optically active β-blockers

Until the early 1980s, only a few methods, all non-enzymatic, had been described for the preparation of optically active β-blockers. They could be prepared from D-mannitol via (R)-2,3-O-8-isopropylidene glyceraldehyde[14,15], from racemic β-blockers by optical resolution[16,17], or from racemic 3-tert-butylamino-1,2-propanediol by optical resolution[18,19] (Fig. 3). Subsequently, the list of publications and patents concerning enzyme-catalysed processes to chiral intermediates of β-blockers has been growing.

Iriuchijima and colleagues at Sagami hydrolysed (±)-1,2-diacetoxy-3-chloropropane enantioselec-

tively with lipoprotein lipase to produce the (S)-enantiomer in 90% enantiomeric excess[20] (Fig. 4a). Under alkaline conditions, the (S)-enantiomer could be converted with various phenols into the corresponding (S)-3-aryloxy-1,2-propanediols from which several β-blockers [e.g. (S)-propranolol] were synthesized chemically.
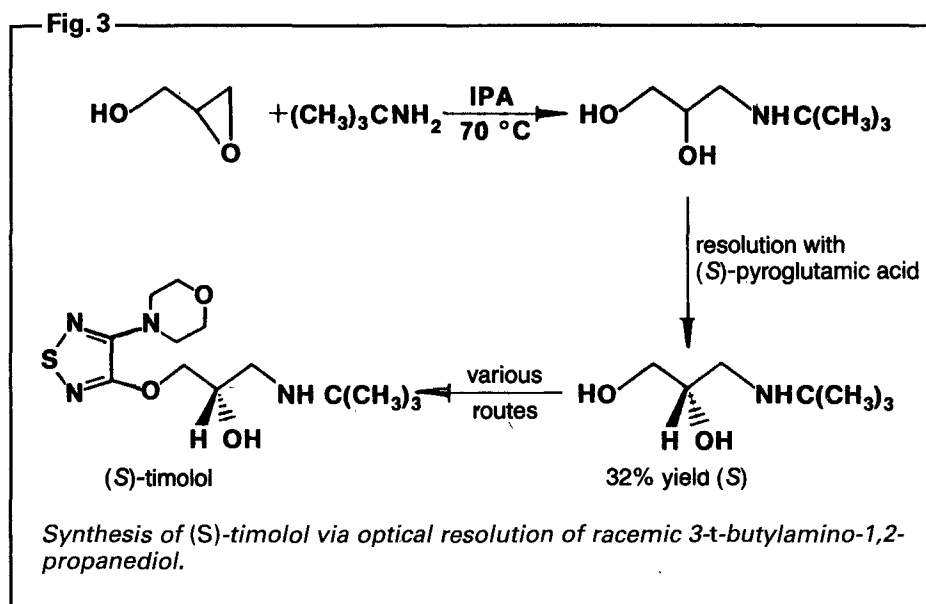
Iriuchijima's group also investigated the asymmetric hydrolysis of (±) 1-acetoxy-2,3-dichloropropane (Fig. 4b), which is easily prepared from 1-chloro-2-propene (Ref. 21). The recovered ester, (S)-1-acetoxy-2,3-dichloropropane was converted, under basic conditions, into (R)-epichlorohydrin, which was converted in situ into (S)-2,3-dichloropropylphenylcarbamate (a compound with herbicidal activity) (S)-propranolol and (S)-pindolol.

Optically active epichlorohydrin is a potentially attractive intermediate for a number of industrially relevant chiral chemicals (e.g. β-blockers, L-carnitine, insect pheromones) and the preparation of its separate isomers has been the subject of much research lately. For instance, chiral epichlorohydrin has also been prepared by:

- stereoselective hydrolysis of 2-acyloxy-3-chloropropyl-p-toluenesulphonate by lipase from *Pseudomonas aeruginosa*[22];
- esterification of 2,3-dichloro-1-propanol by lipase from *Candida cylindracea* and tributyrin[23];
- removal of (R)-(+)-2,3-dichloro-1-propanol from a racemic mixture by a strain of *Pseudomonas* to yield the (S)-isomer[24];
- stereospecific epoxidation of 3-chloro-1-propene by a *Mycobacterium* sp.[25];
- chemical means (see for example Refs 26 and 27).

Other chiral intermediates of β-blockers have also been produced enzymatically. Ohno and colleagues from Sumitomo used lipase from a *Pseudomonas* species to catalyse asymmetric hydrolysis of (R,S)-1-acetoxy-2-aryloxypropionitrile[28] (Fig. 4c). (S)-1-acetoxy-2-α-naphthyloxypropionitrile, obtained by lipolytic resolution, was converted in two steps into (S)-propranolol. The same approach was

Fig. 2

(S)-timolol   (S)-penbutolol   (S)-levobunolol

Structures of (S)-timolol, (S)-penbutolol and (S)-levobunolol.

Fig. 3

Synthesis of (S)-*timolol via optical resolution of racemic 3-t-butylamino-1,2-propanediol.*
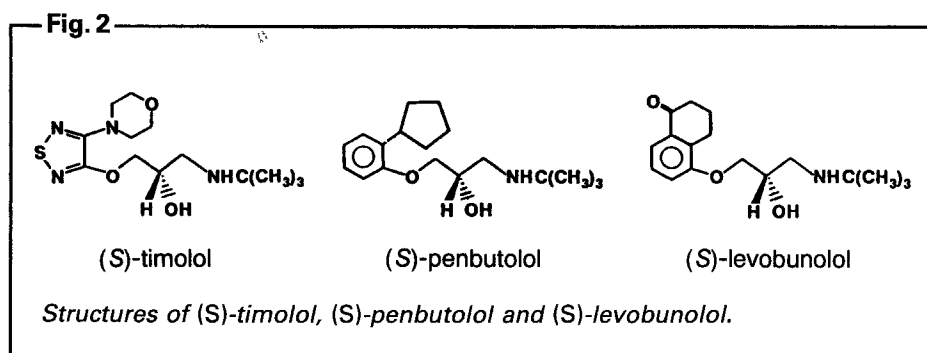
followed by Ohta *et al.*[29] who used growing cells of a *Bacillus* strain.

Another route has been developed at Kanegafuchi. Watanabe *et al.*[30] synthesized 2-oxazolidinone esters, from glycidol or allylalcohol. The racemic mixtures were subsequently selectively hydrolysed by lipoprotein lipase. Thus, *(R,S)*-5-acyloxymethyl-3-alkyl-2-oxazolidinones were converted into the corresponding *(R)*-5-hydroxymethyl-3-alkyl-2-oxazolidinones together with the *(S)*-enantiomer of the original 5-acyloxy-

methyl derivative (Fig. 4d). Treatment of this *(S)*-derivative with sodium hydroxide yielded the desired *(S)*-oxazolidinone; the unwanted *(R)*-by-product could be inverted, giving *(S)*-hydroxymethyl-3-alkyl-2-oxazolidinone in high overall yield[31].

Recently an elegant method for lipase-mediated enantioselective hydrolysis of esters of epoxy alcohols was reported by Ladner and Whitesides[32]: optically active *(R)*-glycidyl esters were isolated at laboratory

scale using a commercially available lipase preparation from porcine pancreas.

At Andeno–DSM the lipase-catalysed resolution of racemic glycidyl butyrate (R in Fig. 4e, C_3H_7) has been improved considerably and is now being exploited on a commercial scale to give *(R)*-glycidyl butyrate and *(R)*-glycidol (Fig. 4e) with high enantiomeric excesses. From these chiral products both *(R)*- and *(S)*-glycidyl tosylate of high enantiomeric purity are prepared.

The high selectivity for *O*-nucleophilic attack at C-1, makes these tosylates highly attractive intermediates for a number of industrially important chemicals[33,34] (Fig. 5). In addition, these chiral glycidyl tosylates exhibit an excellent thermal stability, both chemical and optical. Both *(R)*- and *(S)*-glycidyl tosylate are being marketed*.

Fig. 4

Potential applications of lipases in the preparation of chiral intermediates to β-blockers.
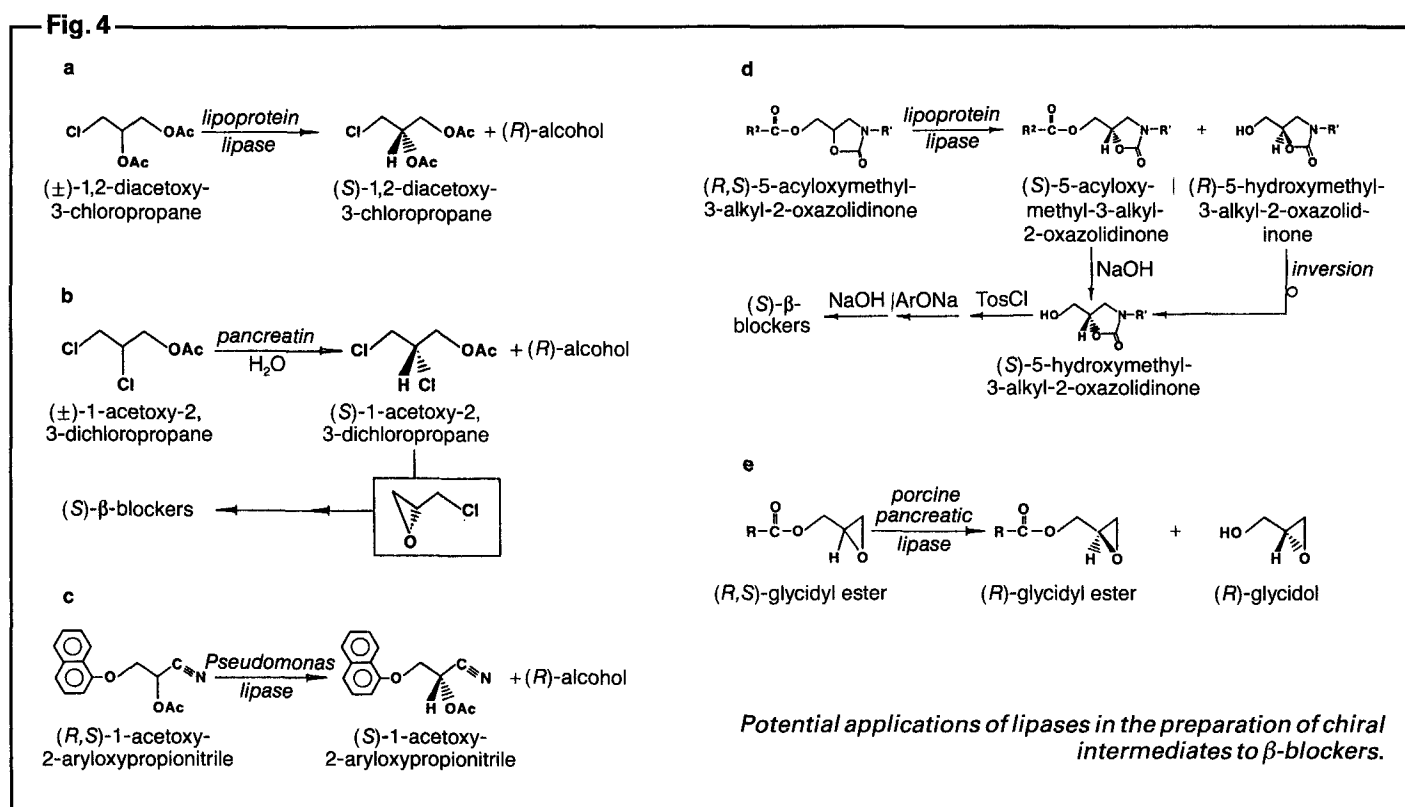
Competing technologies
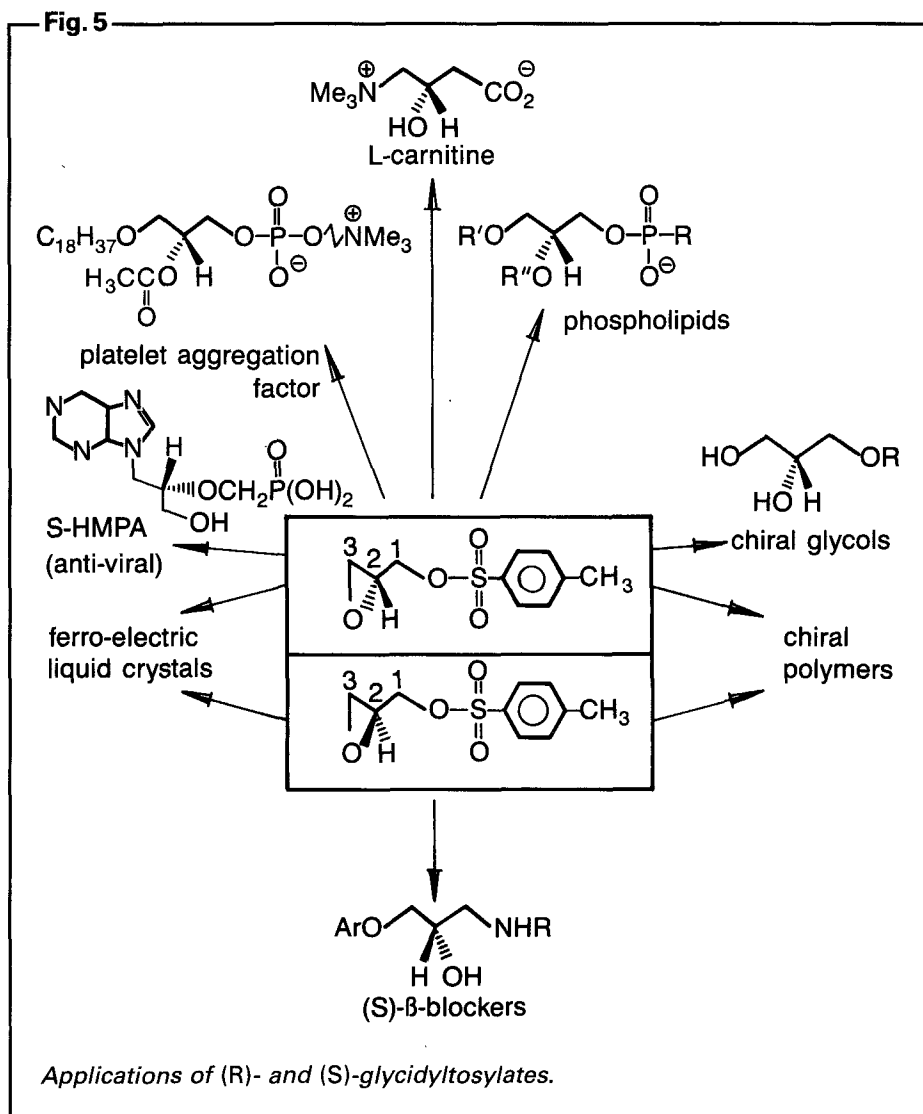
Alternative methods for the preparation of chiral intermediates for β-blockers have been reported. For instance, microbial epoxidation of aryl allyl ethers yielding (+)-aryl glycidyl ethers has been reported by scientists from Ibis[35,36]. So far, the reaction has only been performed at low substrate concentrations and the microorganisms appear not to have a broad substrate specificity. In a more recent patent application from Ibis[37] it is disclosed that (R)-1,3-dioxolane-4-methanol [(R)-solketal] can be prepared by treating racemic solketal with microbial cells that stereoselectively metabolize the (S)-isomer. Nevertheless, the large number of chemical steps needed to convert (R)-solketal into β-blockers seems to prohibit its use in this way.

An elegant chemical approach to the production of chiral glycidols by asymmetric epoxidation of allyl alcohol was developed by Sharpless and co-workers[38]. In the original paper, poor yields were obtained because of low extraction yields but improvements have been reported[34].

When compared with these technologies or with the production of chiral epichlorohydrin[22–27] the production of chiral epoxides by a lipase-catalysed resolution of glycidyl esters is very attractive. It is simple, easy to scale up and the reactions can be performed at very high substrate concentrations. In general, for a lipase-catalysed hydrolytic reaction, separation of the emulsified layers can be a serious problem on an industrial scale. Thus selection of a suitable solvent for extraction is essential. In optimizing our process for the lipase-mediated resolution of racemic glycidyl esters we have managed to solve these problems effectively without recourse to immobilizing the lipase or using a membrane reactor.

Future developments

This review has shown that many chiral intermediates to industrially relevant compounds [e.g. (S)-β-blockers, (R)- and (S)-epichlorohydrin] are easily produced using lipolytic enzymes. In our opinion, more attention should be directed towards two ends in particular:

Fig. 5

L-carnitine

platelet aggregation factor

phospholipids

S-HMPA (anti-viral)

chiral glycols

ferro-electric liquid crystals

chiral polymers

ArO — NHR
H OH
(S)-β-blockers

Applications of (R)- *and* (S)-*glycidyltosylates.*

(1) screening for novel lipases with industrially relevant properties (e.g. thermostability, stereoselectivity); and (2) regulating the synthesis of exocellular microbial lipase in order to produce the biocatalyst.

Screening

Some general criteria for goal-orientated screening programmes have been put forward by Cheetham[39]: one can consider using such methods as recombinant-DNA techniques, mutagenesis, medium engineering, and isolation of microorganisms from extreme environments[39,40].

Laborious random screening programmes can also be successful; such programmes performed by Japanese scientists from Sumitomo, Sagami and Kanegafuchi led to systems for the resolution of racemates[20,21,28,30,31]. It has been estimated that less than 1% of the world's microorganisms have been properly examined, so it seems that much scope remains in this area.

Lipase synthesis

In general, the activity and amount of lipase produced by microorganisms is strongly dependent on environmental factors such as medium composition and culture conditions. Moreover, culture conditions may also influence the ratio of extra- to intra-cellular lipase production and their specific properties. With respect to medium composition, lipase production is often stimulated by lipids such as lard, olive oil, butter and fatty acids. Effects of polysaccharides, inorganic substances and nitrogen and carbon sources have also been reported.

For fungi, the use of submerged cultures may enhance lipase production in some species, whereas semi-solid cultivation is favorable to others (e.g. *Aspergillus* sp.[41]). For instance, *Rhizopus delamar* in semi-solid cultures mainly produced amylase and protease activity, whereas in submerged cultures there was a substantial production of

multiform (A, B and C) lipases without the production of proteases[42]. Whether the organism produced B or C lipases could be controlled using selected nitrogen sources and phospholipids[42]. Large-scale production of naturally occurring lipases in genetically engineered organisms is also becoming a reality.

Solvent studies

Investigations on model systems for lipase-catalysed reactions should be emphasized in order to obtain a better understanding of the factors that influence the stereo- and regioselectivity of these reactions. Initial results indicate that addition of organic co-solvents markedly influences the regio- and stereoselectivity, stability and activity of some lipases. For instance, a 24-fold increase in enzyme activity was noted when acetone was replaced by more apolar co-solvents (e.g. hexane, di-*n*-butylether) during the regioselective hydrolysis of a per-*O*-acetylated carbohydrate by lipase from *Candida cylindracea*[43]. Solvents such as carbon tetrachloride[6–8] and isooctane[44] also affected the stereoselectivity, enzyme stability and activity of lipase from *Candida cylindracea*. Lipases can retain their esterification and transesterification activity in nearly anhydrous organic solvents, even at higher temperatures[45]. By covalent attachment to an amphipathic polyethylene glycol polymer, lipases can even be made soluble in organic solvents with (partial) retention of activity[46].

Protein engineering

Properties of lipases that are potential targets of modification in the future include, for example, stereoselectivity, substrate specificity and affinity, pH and temperature optima, and resistance to inactivation by non-aqueous solvents, heat, high salt concentrations, proteolysis or oxidizing substances. Current knowledge of structure–function relationships of lipases is limited: although more than 300 protein structures have been determined, only one of these is of a lipase (from *Geotrichum candidum*, determined at 2.5 Å resolution)[47]. Thus it will take considerable time to accumulate sufficient knowledge on structure–function relationships in lipases to be able to design a lipolytic enzyme with more desirable properties.

Effective future work on the improvement of lipase-based systems for producing pharmaceuticals will demand a coordinated multidisciplinary approach incorporating genetics, recombinant DNA technology, microbial physiology, biochemical engineering, enzymology, protein chemistry and crystallography.

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Enzymatic synthesis of oligosaccharides

Kurt G. I. Nilsson

As the importance of the oligosaccharide moieties of glycoproteins and glycolipids is being increasingly recognized, efforts to synthesize them are expanding. The number of functional groups of carbohydrate monomers and the variety of configurations that oligomers can adopt is greater than with nucleotides/nucleic acids or amino acids/peptides. By reversing the hydrolytic action of glycosidases and by using highly regiospecific glycosyltransferases, enzymatic oligosaccharide synthesis can be performed.

Why is it necessary to synthesize oligosaccharides? The fundamental answer is that we want to explore and exploit for human ends the biological activity of these polymers following synthetic and biosynthetic work on oligopeptides and oligonucleotides. The complex oligosaccharide chains (glycans) of glycoproteins and glycolipids mediate or modulate a variety of biological processes[1–9]. For example, the glycoconjugate oligosaccharides serve as cell surface receptors (e.g. for influenza and other viruses, bacteria, bacterial toxins, blood-group and tumor-specific antibodies, circulating lymphocytes and for a variety of lectins); they are important for intracellular migration and secretion of glycoproteins and for clearance of glycoproteins from circulation by hepatocytes; they are involved in cell adhesion; they serve as modulators of cell growth; and they change during cellular differentiation. Moreover, there are numerous reports on alterations of glycans after malignant transformation[2,4,6–11]. Antibodies against cancer-associated carbohydrate anti-

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gens are being used in diagnostic kits (e.g. pancreas, colon cancer)[6] or in immunotherapy (e.g. melanoma patients)[2]. The importance of the carbohydrate portion of serum glycoproteins for their half-lifes in the circulation and their immunogenicity has been recognized[12] and *in-vitro* glycosylation of recombinant proteins has been attempted[13].

Knowledge of the various glycoprotein and glycolipid glycan structures has increased dramatically in the last decade because of the development of permethylation[14] and NMR analysis. It is well known that the combination of different amino acids gives a huge variety of peptides and proteins. But the number of possible combinations of a given number of carbohydrates monomers is much higher because there are many possible linkage sites on each and at each site there is the possibility of different anomeric configuration (α- or β-glycosidic linkages). However, the glycan structures of glycoconjugates are not randomly constructed. On the contrary, they may be divided into families in which structures are similar and contain common oligosaccharide sequences (Table 1). The most common carbohydrate chains

in glycoproteins are high-mannose- and complex-type, asparagine-linked (*N*-glycosidic) or serine/threonine-linked (*O*-glycosidic) oligosaccharides. Similarly, glycolipids can be divided into five main structural series. Nevertheless, the diversity of the glycoconjugate oligosaccharides evident from Table 1 allows for biological specificity. Indeed, it has been proposed that 'the specificity of many natural polymers is written in terms of sugar residues and not of amino acids or nucleotides' (Ref. 15). The synthesis of such complexes represents a stiff challenge.

Importantly, however, short fragments of glycan structures (Table 1) are sufficient for biological specificity[1,2,6,11]. These have been used in affinity chromatography, for preparing neoglycoconjugates, for incorporation into liposomes, for immunization, and for characterization of antibodies, glycosidases, glycosyltransferases and lectins. They have also been used in the development of diagnostic kits or targeting of drugs[7–9,16–19]. For example, a sensitive and specific assay for identifica-