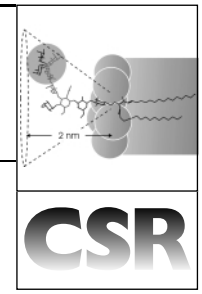
Chemistry of glycosphingolipids—carbohydrate molecules of biological significance



Yashwant D. Vankar[a] and Richard R. Schmidt*[b]

[a] *Department of Chemistry, Indian Institute of Technology Kanpur, Kanpur-208 016, India*
[b] *Fachbereich Chemie, Universität Konstanz, M 725, 78457 Konstanz, Germany*

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The chemistry for the synthesis of glycosphingolipids has been greatly advanced in the last two decades, and now almost any compound of any structural complexity can be prepared. The methodology is based on the development of efficient glycoside bond formation strategies in order to obtain the oligosaccharide moiety, on the synthesis of useful sphingosine intermediates, and finally on their successful ligation in order to provide neutral as well as acidic glycosphingolipids (*i.e.* gangliosides). Also enzymatic approaches towards this goal have been investigated. Recent conformational and biological studies exhibit a rapidly growing understanding of the physical properties and the importance of glycosphingolipids which is essentially based on the great synthetic achievements discussed in this review.

1 Introduction

Glycosphingolipids (GSLs) are characteristic membrane components of eukaryotic cells where they are found in the carbohydrate-rich glycocalix, which consists of glycoproteins and glycosaminoglycans in addition to GSLs.[1] Each GSL carries a hydrophobic ceramide (Cer) moiety and a hydrophilic extracellular oligosaccharide chain which protrudes from the membrane surface. Because of their great structural variations they are generally classified as follows:

(i) Cerebrosides (containing one sugar residue)
(ii) Sulfatides (containing one sugar residue with a sulfate group)
(iii) Neutral Glycosphingolipids (containing one or more—perhaps up to 30—uncharged sugar residues)
(iv) Gangliosides (containing one or more neuraminic acid residues)

Representative examples of each of the above kind are shown in Fig. 1. The major types of sugars that are found in GSLs in animals, including humans, are glucose (Glc), galactose (Gal), fucose (Fuc), *N*-acetylgalactosamine (GalNAc), and *N*-acetylglucosamine (GlcNAc). Other sugars have also been identified albeit in rare instances. Typically, gangliosides contain one or more sialic acids in the form: *N*-acetylneuraminic acid (Neu5Ac) or *N*-glycolylneuraminic acid (Neu5Gc) or deriva-

Yashwant D. Vankar was born in Varansi, India and obtained his M.Sc. degree from Banaras Hindu University (1971). He then obtained his Ph.D. degree (1976) under the guidance of Professor B. D. Tilak at the National Chemical Laboratory, Pune. After post-doctoral experiences at different laboratories (Professor D. I. Davies, King's College, London, 1976–1977, Professor G. A. Olah, University of Southern California, Los Angeles, 1977–1979 and Professor E. Wenkert, Rice University, Houston, 1979–1980), he joined the faculty of the Indian Institute of Technology, Kanpur in 1981 where he has been a full professor since 1991. He has also been an Alexander-von-Humboldt fellow in the research group of Professor Dr R. R. Schmidt, University of Konstanz, Konstanz (1990–1991, 1995, 1999). Professor Vankar's research interests revolve around developing synthetic methods of contemporary interest in organic synthesis addressing selectivities, particularly dealing with nitroaliphatics and functionalised epoxides. More recently his group has also initiated work in the area of carbohydrate chemistry, in particular, pertaining to O- and C-glycosylations and amino sugars.

Yashwant D. Vankar

Richard R. Schmidt received his Ph.D. at the University of Stuttgart in 1962, where he worked under the guidance of Professor Rudolf Gompper on push–pull stabilized quinone methides. From 1965 to 1966 he spent a postdoctoral year with Professor Frank M. Huennekens at the Scripps Research Foundation in La Jolla/USA on coenzyme B_{12} metabolism. He returned to Stuttgart and became Dozent and Associate Professor. Since 1975 he has been a full professor at the University of Konstanz, he has stayed there ever since and denied calls to other universities. His main research interest is devoted to carbanion chemistry, stereochemistry, cycloaddition reactions, chemistry of heterocycles and natural products. In recent years his work has been mainly dedicated to carbohydrate and particularly glycoconjugate chemistry and their biological relevance.

Richard R. Schmidt

tives thereof which are attached to a neutral sugar(s) by an α-ketosidic linkage.

The ceramide part is made up of a sphingosine moiety which is a long chain amino alcohol with 18–20 carbon atoms, and a long chain fatty acid.[2] The sphingosine most frequently found in a glycosphingolipid is C_{18}-sphingosine, although large amounts of C_{20}-sphingosine have been detected in brain ganglioside along with C_{18}-sphingosine. Glycosphingolipids may be structurally related to one another through the stepwise addition or removal of single monosaccharides. Such glycolipids can therefore be arranged in series. Sialic acid containing glycolipids, *i.e*. gangliosides occur within all these carbohydrate series (Fig. 2). The main families of GSLs start with lactose as the first building block, further extension by *N*-acetylglucosamine, galactose or *N*-acetylgalactosamine leads to the formation of oligosaccharides of the *lacto*-, *globo*- or the *ganglio*-series, respectively.

Some related glycolipids lack glucose as the first sugar. Instead, they commence their oligosaccharide chain with galactose attached to the ceramide. Such glycolipids occur, substituted by sialic acid or sulfate, or they are extended by neutral sugars in much the same way as other compounds of the *ganglio*- or the *globo*-series.

Glycosphingolipids serve[2] a variety of functions through interaction with many biofactors by inhibiting or interfering with the physiological effects of these factors or cells.[3] These effects have been interpreted as being receptors, at which GSLs are involved with different biofactors. At the cell surface, GSLs can interact with toxins, viruses and bacteria.[4] These pathogens benefit from the close spatial neighborhood of specific carbohydrate recognition sites on the cell surface and the plasma membrane. Cell adhesion phenomena of this type result from a binding of the carbohydrate moiety of the membrane-bound GSLs to lectins (proteins) on the surface of neighboring

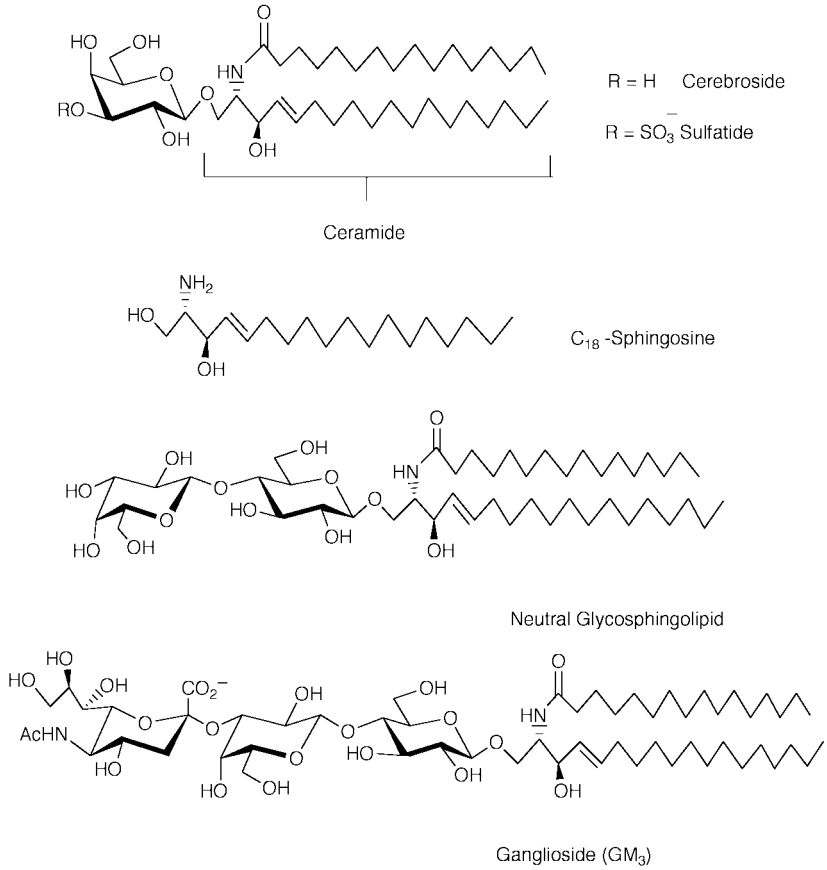


Fig. 1

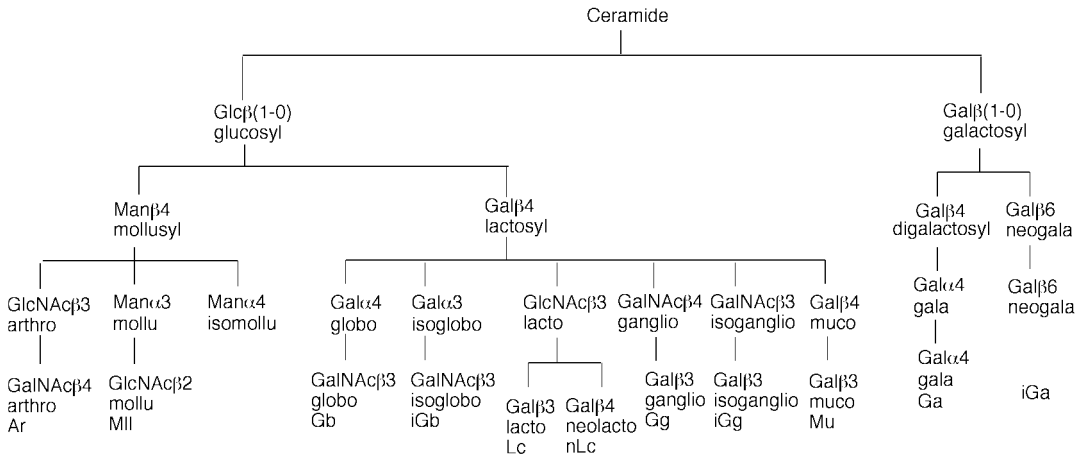


Fig. 2

cells. Interactions of GSLs with receptors and enzymes which are located in the same membrane have been described to be of some physiological significance. Ganglioside G_{M1}, for example, can activate the nerve growth factor,[4] and ganglioside G_{M3} inhibits tyrosine phosphorylation of the epidermal growth factor receptor.[5] Interestingly, lipophilic intermediates of GSL metabolism such as sphingosine, ceramide, and their phosphorylated derivatives have been recently identified as novel signal substances.[5]

Glycosphingolipids are also essential for the function of human skin where they contribute to the formation of the water permeability barrier.[6] However, the structures of the ceramides involved here differ in a characteristic manner from the membrane anchors normally found in the GSLs of vertebrates. Unusually long fatty acid residues up to 34 carbon atoms and sphingoid bases that are hydroxylated at various positions are found. It has also become clear that many GSLs are blood group antigens and their studies not only offer chemical structures of blood group antigens, but also delineate their immunological significance.[7]

A number of glycosphingolipids play a role as tumor associated antigens and in immunotherapy of individual cancer forms.[7] Most neurons, especially of the central nervous system, contain a variety of gangliosides which are present in relatively high overall concentration. Immunochemical and biochemical studies demonstrate that changes in GSLs of neuronal membranes are related to degenerative changes, for instance characteristics of Alzheimer's disease.[8] Further, GSLs have been found to display neuroprotective and neuroregenerative effects in diseases like Parkinson's disease or ischemic stroke.[8]

2 Isolation

One of the most commonly used methods for the isolation of gangliosides is based on the special ability of these compounds to partition from the organic solvent in which they are extracted into an aqueous phase. However, this partition procedure cannot be used to quantitatively isolate all species of gangliosides from the aqueous phase because the less polar gangliosides could not be totally extracted from the organic solvent phase. Moreover, neutral GSLs with more than four oligosaccharide residues tend to partition into the aqueous phase, thereby creating more problems in purification of the gangliosides during later steps. To overcome these disadvantages, anion exchangers such as DEAE–Sephadex, DEAE–Sepharose, Sepherosil–DEAE–dextran, DEAE–Sephacel, DEAE–CPG, and DEAE–silica have been used. However, for both neutral GSLs and gangliosides the use of DEAE–silica gel is preferred for a number of reasons. Preparative thin layer and column chromatography using varying proportions of $CHCl_3$–MeOH and water are also used in isolating GSLs. In view of the fact that the above mentioned methods of isolation of GSLs take a long time, recently Schwarz *et al*.[9] have reported a rapid isolation of gangliosides by cloud-point extraction using a non-ionic detergent *viz*. hexaethylene glycol mono-tetradecyl ether.

3 Chemical synthesis

Careful examination of the various GSLs (neutral as well as acidic) suggests clearly that synthesis involves a linkage between three fragments *viz*. the oligosaccharide part, the sphingosine moiety, and the fatty acid part. A literature survey reveals that most of the syntheses begin by procuring the properly protected oligosaccharide part followed by attachment of the sphingosine moiety (or its precursor *e.g*. an azidosphingosine), *N*-acylation with the fatty acid and finally deprotection of the protecting groups. Therefore in this review we present, in the beginning, some commonly used and efficient methods of the synthesis of (i) oligosaccharides and (ii) azidosphingosines followed by a few examples of the synthesis of (iii) neutral glycosphingolipids and (iv) gangliosides (acidic GSLs).

3.1 Oligosaccharide synthesis—general strategies

One of the most important aspects of oligosaccharide synthesis is stereocontrol (α or β) in the glycoside bond formation (glycosylation). This aspect necessitates a two step procedure with the following requirements.[10] The first step is the activation of the anomeric center generating the glycosyl donor which requires convenient formation of a stereochemically uniform glycosyl donor having either α- or β-configuration at will, and possessing thermal stability at least up to room temperature. This should eventually lead to a possibility of chromatographic purification. The second step is glycosyl transfer to appropriate acceptors providing a glycosidic bond. Clearly, this step imposes several restrictions *viz*. (i) use of a simple catalyst, (ii) irreversibility of the reaction, (iii) stability of other glycosidic linkages within the system, (iv) high chemical yield, and most importantly (v) high α- or β-selectivity (anomeric control) at the anomeric centre.

The two principles that have been successfully employed for glycosylation reactions include (i) anomeric exchange reactions containing the Koenigs–Knorr procedure and its many variants, for instance, fluoride, sulfur and sulfoxide activation and (ii) anomeric oxygen retaining reactions containing the anomeric *O*-alkylation and trichloroacetimidate, phosphate, phosphite, sulfate and sulfite activation (Fig. 3). A brief description of each of the important glycosylation procedures commonly employed in GSL syntheses is given below.

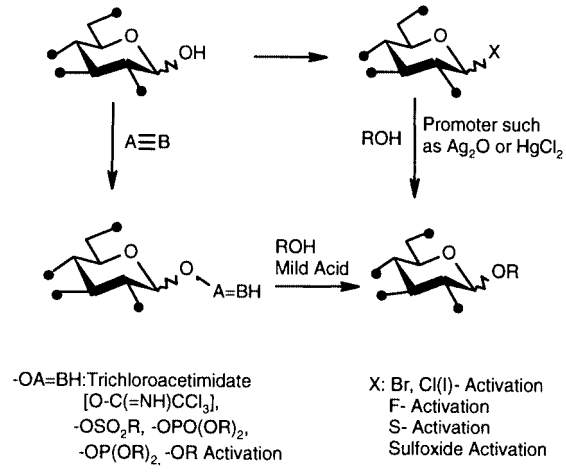


Fig. 3

3.1.1 Koenigs–Knorr procedure. In the classical Koenigs–Knorr procedure,[11] the exchange of the anomeric hydroxy group for chlorine or bromine is carried out with typical halogenating agents while all other hydroxy groups are *O*-protected. The halogen atom usually occupies the *axial* position due to the "thermodynamic" anomeric effect. Towards forming a glycoside bond, these glycosyl halides are reacted, *in situ*, with acceptors in the presence of promoters such as silver and mercury salts which readily permit C–O bond formation at the anomeric center. For the most common promoter systems the following order of reactivity has been generally confirmed:[10] $AgOTf/Ag_2CO_3 > AgClO_4 > Hg(CN)_2/HgBr_2 > Hg(CN)_2$. The formation of acid (HX, Fig. 4) can be circumvented by the addition of Ag_2CO_3 and generated water is usually removed with drierite or molecular sieves.

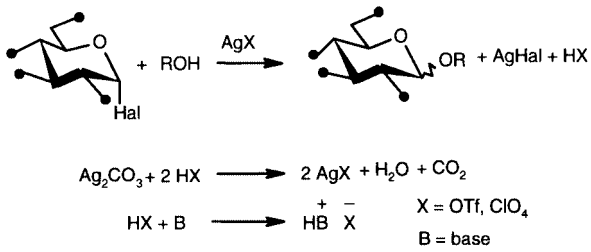


Fig. 4

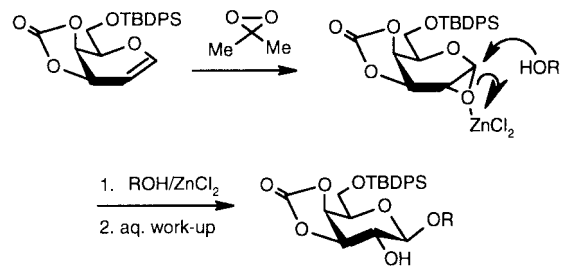


Fig. 6

Depending on the nature of the protecting groups, halogens and the *gluco*- or *manno*-configurations of the glycosyl donors, often either α- or β-selectivity is observed. Many applications have been found for the use of Koenigs–Knorr glycosylation methodology in GSLs synthesis,[11] including the attachment of a sialyl group to an oligosaccharide part.

3.1.2 Fluoride as a leaving group. Because of the stronger C–F bond, the use of glycosyl fluoride as a glycosyl donor has recently become popular.[12] Sialyl fluorides have also been utilised to form α-anomers (Fig. 5), however, the use of special fluorophilic promoters such as $SnCl_2$–$AgClO_4$, $Sn(OTf)_2$, $CpZr(OTf)_2$, $CpHF(OTf)_2$ in equimolar amounts is a drawback with this method.

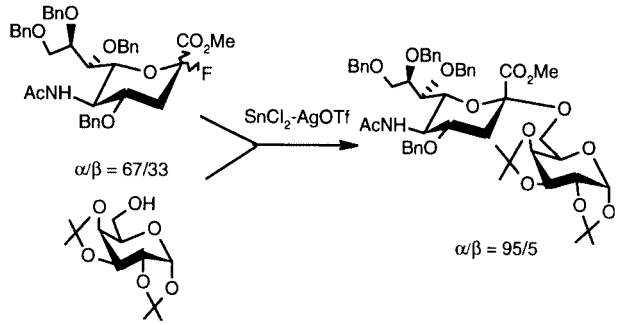


Fig. 5

3.1.3 1,2-Anhydro (or 1,2-epoxide) sugars as glycosyl donors. Ready reactivity of 1,2-anhydro (or 1,2-epoxy) sugars towards nucleophilic ring opening has made such structural features useful substrates for *O*-glycosylations and this strategy (also known as the glycal assembly approach) has been employed by Danishefsky[13] in many complex oligosaccharide syntheses. This has been possible because of the easy accessibility of the 1,2-epoxides (Fig. 6) from the corresponding glycals by reacting them with dimethyldioxirane. Opening of the epoxide ring catalyzed by Lewis acids such as $ZnCl_2$ in the presence of a glycosyl acceptor provides 1,2-*trans* linked *O*-glycosides.

3.1.4 Sulfur as a leaving group. Thioglycosides[14] have attracted considerable attention because they are stable under most reaction conditions frequently used for the construction of *O*-glycosides. Further, a number of thiophiles such as NIS–TfOH, MeOTf, DMTST, IDCP, PhSeOTf are available which could permit a choice of them depending on the suitability. Also, the thioglycosides could be converted to other glycosyl donors commonly used in oligosaccharide synthesis *viz*. sulfoxides,[15] fluorides,[12] and halides.[10,11] However, this method also, like that of fluorides, suffers from the disadvantage of using at least equimolar amounts of the promoters. Nevertheless, thioglycosides have been extensively used in complex oligosaccharide synthesis, including sialylation (Fig. 7).

In this category of leaving groups, recently glycosyl sulfoxides[15] have been used as glycosyl donors. They are obtained from the corresponding thioglycosides in a further step, *i.e.* by MCPBA oxidation, and their activation is performed by strong acids or acid anhydrides such as TMSOTf, triflic acid or triflic anhydride.

3.1.5 The trichloroacetimidate method. Glycosylation with anomeric trichloroacetimidate donors[10] has proven to be a highly attractive and powerful method and is currently one of the most frequently applied strategies for the preparation of *O*-glycosides. The possibility of obtaining both the anomers *viz*. α or β depending on the nature of the base (*e.g.* NaH, DBU, Cs_2CO_3 for α-anomers and K_2CO_3 for β-anomers of glucose and galactose) used is an added advantage. Activation is performed in the presence of catalytic amounts of $BF_3{\cdot}Et_2O$, TMSOTf, AgOTf or $ZnBr_2$. Depending on the nature of the protecting group, solvent and the catalyst, α- or β-glycosides are formed (Fig. 8).

3.1.6 The phosphite method. For sugars requiring relatively low activation to generate glycosyl donor properties [ketoses, 3-deoxy-2-glyculosonates: 3-deoxy-2-octulosonic acid (KDO), Neu5Ac], the phosphite method[10] which has recently been introduced by Martin and Schmidt[16] has found application in oligosaccharide synthesis especially in procuring α-glycosides using sialyl phosphite donors in acetonitrile as a solvent (Fig. 9).

3.1.7 Some additional aspects. Apart from the above mentioned methods of *O*-glycosylations there have been a few more methods[10] such as in pent-4-enyl glycosides and but-3-en-2-yl glycosides synthesis which are also useful.

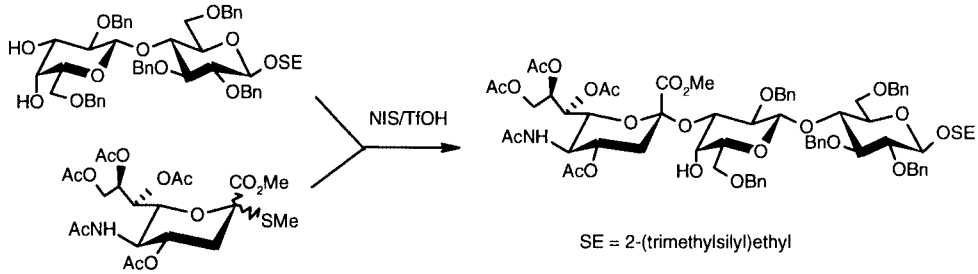


Fig. 7

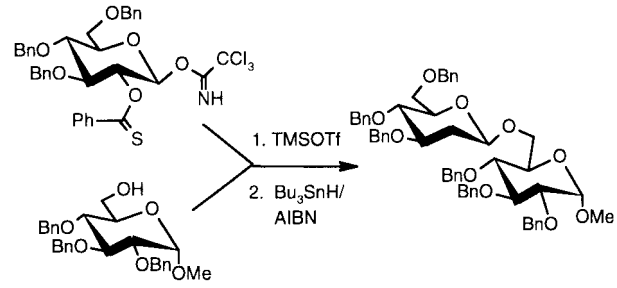


Fig. 8

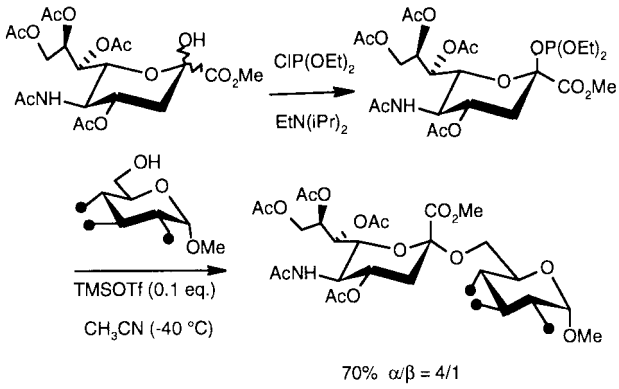


Fig. 9

Protecting groups play an important role in terms of stereoselectivity of glycoside bond formation (α or β) and also on the yield of such reactions. In this regard pivaloyl group has been found to be a very useful protecting group especially at C-2 to avoid orthoester formation and yet give β-glycosidation.

Having procured the desired oligosaccharide unit, the attachment of the ceramide part needs to be addressed. Towards this end, basically two strategies have been adopted *viz*. (i) direct attachment of the preformed ceramide unit to the oligosaccharide or (ii) attachment of a sphingosine moiety (or its precursor) followed by its conversion to the ceramide part by *N*-acetylation with an appropriate fatty acid.

However, it has generally been observed that coupling of the ceramide unit to the oligosaccharide part is rather low yielding. Therefore, the second strategy of the attachment of a sphingosine (or its precursor) is generally preferred. In this regard, azidosphingosine[17–19] has been found to be an excellent precursor of sphingosine thus leading to the "azidosphingosine glycosylation procedure".[18] This is mainly because an azide group is non-nucleophilic and hence does not compete with a hydroxy group towards glycosylation. Since reduction of an azide is relatively easy, usually azidosphingosines are glycosylated with appropriate sugar residues followed by reduction of the azide to an amino group and subsequent *N*-acylation in order to generate the ceramide moiety.[18] A large number of syntheses of azidosphingosine(s) have, therefore, been reported in the literature.[19] A few important syntheses of azidosphingosine(s) (or their derivatives) are presented here.

3.2 Sphingosine synthesis

Syntheses of sphingosines (C_{18} or C_{20}) in chiral form *via* azidosphingosines have been reported[19] by mainly two approaches, *viz*. (i) the Chiral Pool Approach and (ii) the Asymmetric Induction Approach.

3.2.1 Chiral Pool Approach. In this approach naturally occurring chiral substrates such as D-galactose, D-glucose and D-xylose have been employed. Naturally occurring sphingosine (**3**, Fig. 10) has the D-*erythro* configuration and carbohydrates mentioned above possess the D-*threo*-diol structure and therefore at some stage nitrogen is introduced with inversion of configuration to obtain the natural sphingosine geometry. Likewise, D-tartaric acid, an unnatural substance, possessing the D-*threo*-diol structure has also been employed.[19]

The most effective synthesis[17] to date, requiring only seven steps and starting from D-galactose proceeded *via* 2,4-*O*-benzylidene-D-threose **1** (Fig. 10). This threose derivative was elaborated to the valuable azidosphingosine **2** *via* a *trans*-selective Wittig reaction, stereoselective azide introduction on a triflate, followed by hydrolysis. Reduction of **2** with H_2S in pyridine led to the formation of the desired D-*erythro*-sphingosine **3** in 95% yield.

Another relatively short synthesis of **3** begins[20] with D-xylose involving an S_N2'-type reaction for the attachment of the long chain and introduction of the *E*-configured double bond as one of the key steps. Thus, D-xylose was converted into 3,5-dibenzyl-D-xylose **4** (Fig. 11) *via* 1,2-*O*-isopropylidene-D-xylose. The Wittig reaction of **4** with methylidenetriphenylphosphorane followed by mesylation gave the dimesylate **5** which was reacted with the Grignard reagent $C_{12}H_{25}MgBr$ in the presence of CuCN to effect C–C bond formation, in a highly stereoselective manner. The monomesylate **6** so obtained was converted into D-*erythro*-sphingosine **3** in three steps, as shown in Fig. 11, which involved condensation with NaN_3, reduction of the azido group to an amino functionality and then deprotection of the benzyl groups under Birch reduction conditions.

3.2.2 Asymmetric Induction Approach. Chiral auxiliaries such as that of Evans' oxazolidinone and Enders' SAMP hydrazone have been used for the synthesis of sphingosines.[19] Thus, Nicolaou *et al*.[21] have reported the use of the Evans auxiliary by condensing the oxazolidinone unit with bromo-

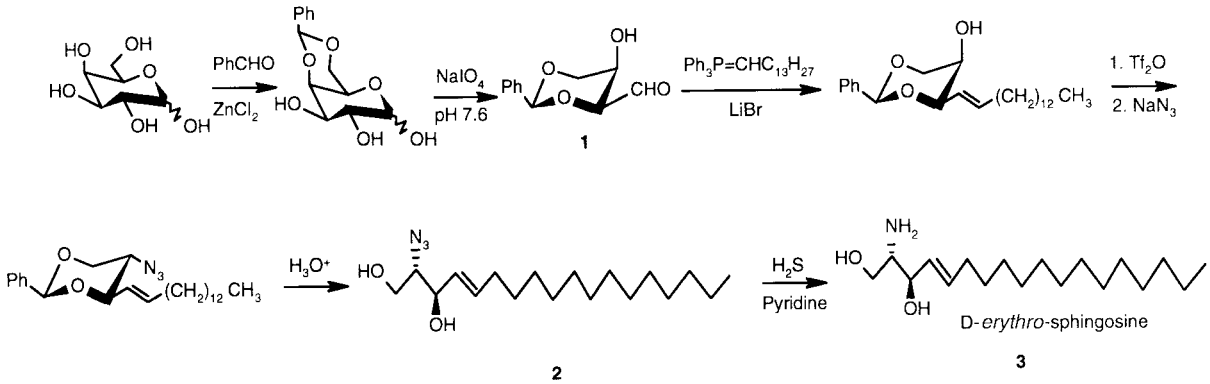


Fig. 10

acetic acid to obtain **7** (Fig. 12) which was converted into its boron enolate and condensed with the α,β-unsaturated aldehyde **8** to afford **9** in 72% yield. Compound **9** was obtained along with a small amount (5%) of the corresponding L-*threo* isomer. The major D-*threo* isomer *viz*. **9** was, however, separated and subsequently transformed to the corresponding azido derivative **10** in which the hydroxy group was silylated with *tert*-butyldimethylsilyl triflate in the presence of 2,6-lutidine. Reduction of this oxazolidinone derivative **10** with $LiBH_4$ followed by fluoride induced desilylation afforded the requisite diol **11** in which the azide moiety was reduced, by reaction with propanedithiol in the presence of Et_3N, to complete the synthesis of the desired D-*erythro*-sphingosine **3**.

Although there are only a few reports[19] based on chemoenzymatic approaches for the synthesis of sphingosine, a recent one by Hudlicky *et al*.[22] leading to azidosphingosine appears to be useful. In this approach, chlorobenzene is oxidized with a microorganism, *Pseudomonas putida* 39 D, to an optically pure *cis*-diol **12** which is transformed into D-*erythro*-sphingosine **3** through a series of reactions as shown in Fig. 13. Thus, the diene diol **12** was protected as an acetonide and converted to the epoxide **14** in two steps *via* a bromohydrin **13** as shown in Fig. 13. This bromohydrin was subsequently transformed into an azido alcohol **16** *via* yet another bromohydrin **15**. Thus, treatment of **13** with NaOH in the presence of a phase transfer catalyst (Bu_4NHSO_4) afforded the epoxide **14** which was stereo- and regioselectively opened by LiBr to give **15** in 94% yield. The azido alcohol **16** was obtained in 75% yield by subsequent nucleophilic substitution on the bromohydrin **15** with NaN_3 in DMSO. Treatment of **16** with excess ozone at −78 °C, followed by excess $NaBH_4$ gave the azido-D-allose **17**. Acetonide deprotection under aqueous acidic conditions followed by $NaIO_4$ mediated cleavage afforded azido-L-erythrose **18** *via* loss of the glyoxalate residue. Direct Wittig olefination with *n*-tetradecyltriphenylphosphonium bromide in the presence of *n*-BuLi gave a mixture of *cis*- and *trans*-azidosphingosines in 14 and 3.8% yield, respectively. However, the *cis*-azidosphingosine could be readily photoisomerized to *trans*-azidosphingosine **2** by means of a Hanovia 400 W lamp, Pyrex filter, and diphenyl disulfide in a 4 : 1 mixture of hexanes and dioxane. Reduction of **2** with H_2S in pyridine afforded D-*erythro*-sphingosine **3**.

3.3 Synthesis of neutral glycosphingolipids

Because of their being tumor associated antigens, neutral glycosphingolipids *viz*. H-antigen, Lewis A (Le^a), Lewis B (Le^b), Lewis X (Le^x) and Lewis Y (Le^y) have become important targets for synthesis since these compounds cannot be easily isolated in sufficient quantities in pure forms for further biological studies. Synthesis of these antigens, especially Le^x and Le^y family ones, is not simple since they exist in monomeric, dimeric, and even trimeric forms (Fig. 14). These syntheses basically comprise two parts: (a) synthesis of the oligosaccharide part and (b) attachment of the ceramide part (or attachment of the sphingosine unit followed by its conversion to the ceramide part). As has been mentioned above, direct attachment of the ceramide part is not high yielding and therefore attachment of a sphingosine unit (mainly azidosphingosine[17–19]) first and then its conversion to ceramide is now generally adopted. In the following discussion, a few approaches towards the synthesis of some important neutral glycosphingolipids are described.

A prominent tumor associated antigen is the Le^x determinant Galβ(1–4)[Fucα(1–3)]GlcNAc and, as mentioned above, it has been found to exist as a monomer or dimer (Fig. 14). Several syntheses of Le^x antigens have employed different strategies, different building blocks, and/or different protecting groups. A strategy[23] based on azidoglucose, for which convenient protecting group patterns could be developed, was adopted. Thus, *tert*-butyldimethylsilyl 2-azido-4,6-*O*-benzylideneglucopyranoside **19** (Fig. 15), derived from glucose, was used for synthesis of the desired Le^x trisaccharide building block **23** through (i) 3-*O*-fucosylation of **19** with the trichloroacetimidate **20**, (ii) ensuing reductive benzylidene ring cleavage, in the disaccharide **21**, with $NaCNBH_3$–HCl and (iii) 4-*O*-galactosylation with yet another trichloroacetimidate derivative **22** of galactose in the presence of TMSOTf. Conversion of **23** into a trisaccharide donor **24** (α,β-trichloroacetimidates) and into a trisaccharide acceptor **25** was readily performed as shown in Fig. 15. Reaction of these two trisaccharides **24** and **25** in acetonitrile in the presence of TMSOTf at low temperature (nitrile effect)[10,23] afforded exclusively a β-connected hexasaccharide **26** which was transformed into hexasaccharide donor **27α,β** after deprotection of the 1-*O*-silyl bond with TBAF followed by treatment with CCl_3CN in the presence of DBU. The desired octasaccharide **29** was obtained from **27α,β** and a lactose derivative **28**, having a 2a-*O*-pivaloyl protection, *via* glycosylation in the presence of TMSOTf as a catalyst and acetonitrile as a solvent, azide reduction with H_2S in pyridine–H_2O and ensuing acetylation with acetic anhydride in pyridine. Hydrogenolytic *O*-debenzylation and concomitant *O*-debenzylidenation with Pd/C as catalyst in the solvent system acetic acid–acetone–methanol and then *O*-acetylation with acetic anhydride in pyridine afforded *O*-acyl-protected octasaccharide **30**. Regioselective 1a-*O*-deacetylation of **30** with N_2H_4·HOAc gave compound **31** in 90% yield which was treated with CCl_3CN in the presence of DBU as a base to afford exclusively[9] α-trichloroacetimidate **32** in 87% yield. This compound was then ready for connecting with azidosphingosine.[16,17] Thus, reaction of **32** with 3-*O*-benzoylazidosphingosine **33** in the presence of a catalytic amount of TMSOTf gave glycoside **34** in 55% yield. Reduction of the azide moiety with H_2S in pyridine–water followed by reaction with hexadecanoic acid in the presence of *N*-[3-(dimethylamino)propyl]-*N*′-ethylcarbodiimide hydrochloride (Merck), a water-soluble-carbodiimide (WSC) and deacetylation with NaOMe in MeOH led to the target dimeric neutral glycosphingolipid antigen **35** (Le^x dimer).

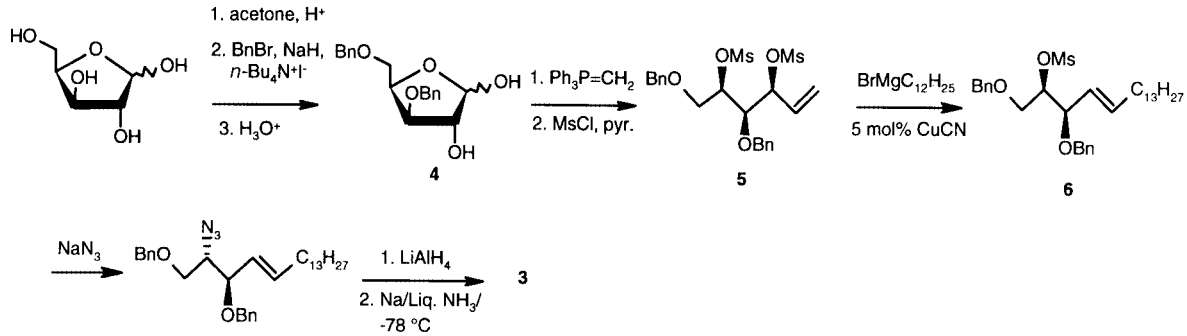


Fig. 11

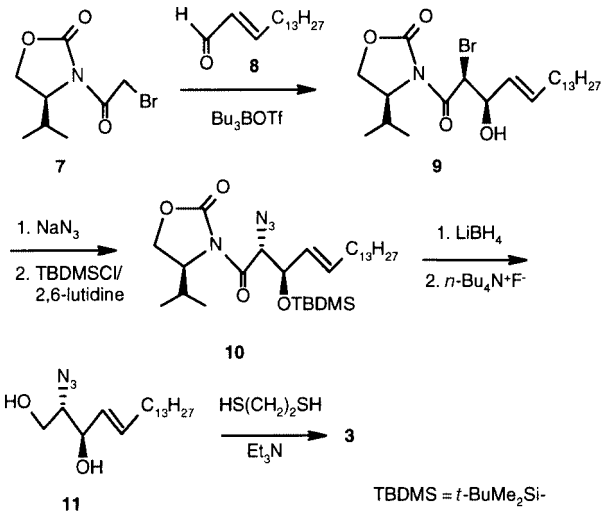


Fig. 12

The above described strategy for the synthesis of Le^x dimer involves two main methodologies *viz.* (i) application of trichloroacetimidate activation for *O*-glycosylation (*cf.* section 3.1.5) and (ii) use of azidosphingosine for ceramide attachment. The trimeric Le^x could also be synthesized by attaching the third Le^x determinant in an analogous manner. This method was also successfully applied to the synthesis of sialyl dimer Le^x.[30]

Another important tumor associated antigen isolated by Hakomori *et al.*[24] from human breast cancer cell line MCF-7 belongs to the "globo-series" of glycosphingolipids. This globo 'H' antigen **52** has been synthesized by Lassaletta and Schmidt[25] and also by Danishefsky *et al.*,[26] thus, providing proof of its structure (Fig. 16), in addition to making it available for further biological studies.

In the first approach,[25] the hexasaccharide unit **50** of the globo 'H' antigen was constructed from a disaccharide **49** and a tetrasaccharide building block **48** was obtained starting from galactose derived donor **36** and acceptor **37** which reacted in the presence of TMSOTf in dichloromethane at room temperature to provide the desired β(1→3)-connected disaccharide **38** in 91% yield. Removal of the *O*-acetyl groups using NaOMe in MeOH followed by benzoylation with one equivalent of benzoyl cyanide in the presence of triethylamine gave regioselectively 3b-*O*-benzoyl derivative **39**. Fucosylation of **39** with glycosyl donor **40** in ether as solvent resulted in the required α-(1→2)-linkage and gave trisaccharide **41** in 89% yield. Ensuing removal of the 1-*O*-TBS group with tetrabutylammonium fluoride (TBAF) in THF led to 1-*O*-unprotected derivaitive **42** which reacted with CCl_3CN in the presence of DBU as a base to form the trichloroacetimidate **43** (91%, α:β = 1:6). Reaction of this mixture with acceptor **44** in CH_3CN at −40 °C in the presence of TMSOTf as catalyst led to the desired β(1→3)-linkage and furnished tetrasaccharide **45** in 79% yield, thus displaying the usefulness of the nitrile effect.[10,23] Desilylation of **45** with TBAF in THF followed by reaction with allyl bromide in the presence of NaH as a base gave *via* anomeric *O*-allylation **46α,β** (80%, α:β = 3:1). 2a-*O*-Benzylation of **46** followed by 1a-*O*-deallylation using bis(methyldiphenylphosphine)cyclooctadieneiridium hexafluorophosphate/hydrogen catalyst system and then with *N*-bromosuccinimide in the presence of water afforded 1-*O*-unprotected **47** in 91% yield. Compound **47** gave the trichloroacetimidate **48** (94%, α:β = 3:1), when reacted with CCl_3CN–DBU which in turn was subsequently used for glycosylating **49**, a lactose derivative, in the presence of TMSOTf as catalyst in ether to form the desired hexasaccharide **50**, with α(1→4) linkage in 58% yield. Conversion of **50** into the peracetylated hexasaccharide **51** was carried out as described above for the conversion of **29** into **31** except that the reduction of the azido to the amino group was carried out using $Pd(OH)_2$ on carbon. This hexasaccharide could be readily transformed into globo H antigen **52** using the azidosphingosine (glycosylation) procedure.[17,18]

The synthesis reported by Danishefsky *et al.*[26] also employs the connection of an azidosphingosine to a hexasaccharide moiety similar to **51** (Fig. 16) but their approach to glycosylations is based on the 'Glycal Assembly' method (*cf.* section 3.1.3). Reduction of the azido group to the amino functionality followed by *N*-acetylation with palmitic anhydride and subsequent removal of the protecting groups led to the completion of the synthesis of the globo 'H' antigen **52**.

3.4 Synthesis of gangliosides

As mentioned in the beginning (*cf.* Section 1), gangliosides are sialic acid (neuraminic acid) containing glycosphingolipids, present in the outer membranes of living cells. The sialyl Lewis X (sLe^x) epitope Neu5Acα(2–3)Galβ(1–4)[Fucα(1–3)]GlcNAc has become a prominent target for isolation, synthesis, and biological studies because of its role in cell-adhesion and its implication in inflammation through binding to selectins, a

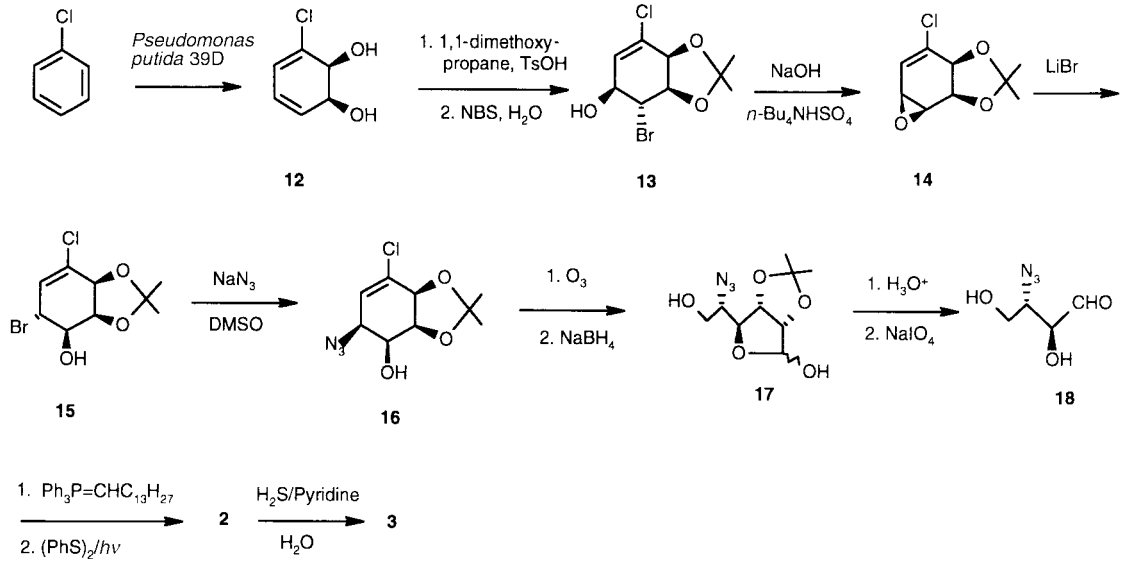
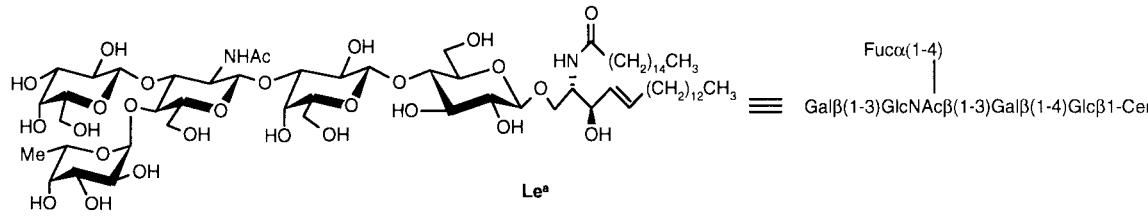


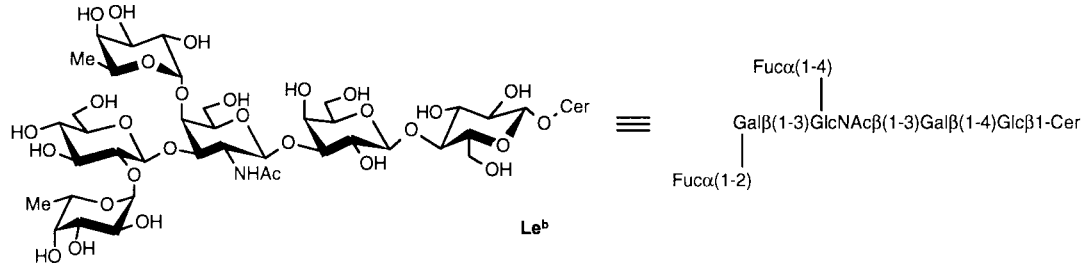
Fig. 13

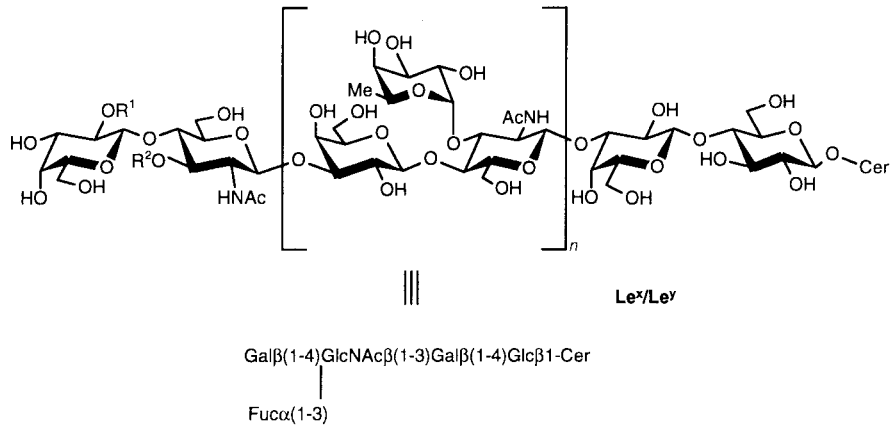
group of cell surface lectins (proteins). The sialyl dimer Lewis X form (dimer 'sLe^x') (Fig. 18) exhibits high potency among the natural epitopes of E-selectin, in addition to its being a tumor associated antigen (a member of the lactoneo series antigens).

In the synthesis of gangliosides, sialylation is one of the most important steps; when halogenoses of *O,N*-acylated neuraminic acid esters are used as donors (L = Hal in Fig. 17) which are activated by silver or mercury salts as promoter (= P) only modest yields of the desired α-products are obtained, especially when secondary hydroxy groups are used in the acceptor. Thioglycosides of neuraminic acid could also be used as sialyl donors but the use of at least equimolar amounts of thiophiles (NIS, DMTST, MeSBr, or AgOTf) as promoters constitutes a major drawback; this is even more so because often up to two to four fold excess of the promoter is required for reaction. In this regard, the phosphite method for *O*-glycosylation is remarkably effective in α-sialylation since the leaving groups, *viz.* $HOPO_2R$, being weakly basic do not consume the promoter (TMSOTf or TfOH) which acts as a true catalyst and α-sialylation is predominant in acetonitrile as solvent.

The first total synthesis of sialyl dimeric Le^x ganglioside **68** has been reported by Hasegawa *et al.*[27] utilizing thioglycoside based glycosyl donors and a pentasaccharide **55**.[28] For this purpose, three thioglycosides *viz.* **54**, **58** and **61** (Fig. 18) were chosen as the building blocks. Compound **54** was obtained sequentially by 4-methoxybenzylation of phenyl 4,6-*O*-benzylidene-2-deoxy-2-phthalimido-1-thio-β-D-glucopyranoside **53**, reductive ring opening of benzylidene acetal with $NaCNBH_3$–HCl and subsequent 4-*O*-acetylation. Regioselective glycosylation of the pentasaccharide **55** with **54** in the presence of *N*-iodosuccinimide (NIS)–triflic acid (TfOH) and molecular sieves 4 Å (MS-4 Å) gave the hexasaccharide **56** in 62% yield which was converted into **57** by successive treatments with hydrazine monohydrate and acetic anhydride in MeOH. The glycosylation of **57** with sialyl-α(2→3)Gal donor[27] **58** in







R^1	R^2	*n*	
Fucα	H	0	H-Antigen
H	Fucα	0	Lewis X (Le^x)
Fucα	Fucα	0	Lewis Y (Le^y)
H	Fucα	1(2)	Dimer (trimer) Le^x
Fucα	Fucα	1(2)	Dimer (trimer) Le^y

Fig. 14

CH_2Cl_2 at −15 °C in the presence of dimethyl(methylthio)sulfonium triflate (DMTST) and MS-4 Å, gave the desired octasaccharide **59** in 55% yield which was characterized as its acetate **60**. Deprotection of the *p*-methoxybenzyl group in **60** using ceric ammonium nitrate followed by coupling with methyl 2,3,4-tri-*O*-benzyl-1-thio-β-L-fucopyranoside **61** in the presence of NIS–TfOH as the glycosyl promoter and powdered MS-4 Å at −15 °C gave the sialyl dimeric Le^x derivative **62**.

Benzyl group deprotection, with subsequent *O*-acetylation followed by treatment with CF_3COOH resulted in the hemiace-

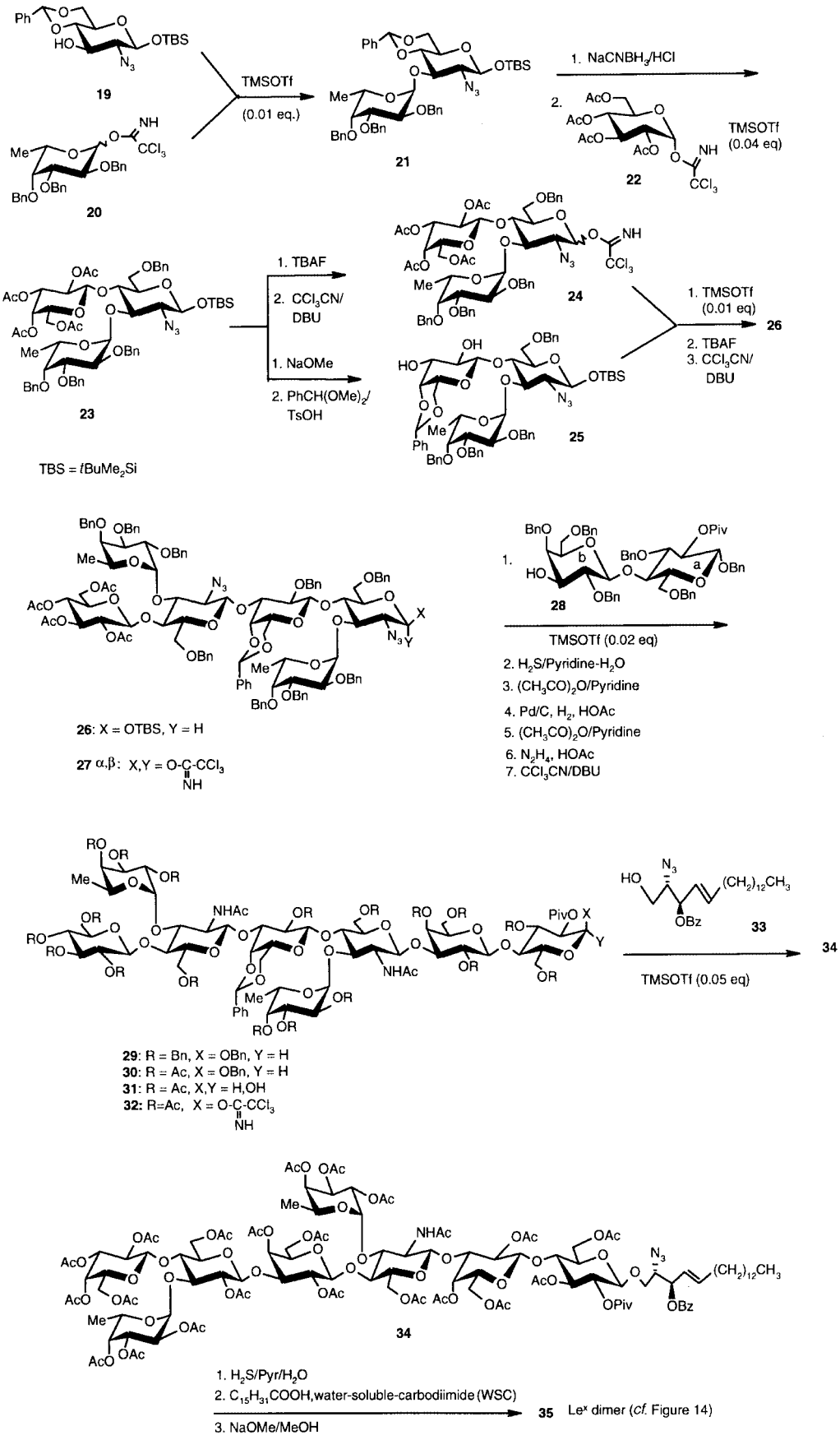


Fig. 15

tal derivative **63** which reacted with CCl_3CN in the presence of DBU to give the α-trichloroacetimidate **64**. At this stage, glycosylation of an azidosphingosine derivative **65** with **64** in the presence of $BF_3{\cdot}Et_2O$ at 0 °C afforded the β-glycoside **66** in 33% yield. Selective reduction of the azide group in **66** with Ph_3P in benzene followed by condensation with octadecanoic acid using 2-chloro-1,3-dimethylimidazolium chloride (DMC) gave the fully protected sialyl dimeric Le^x ganglioside **67** in 57% yield. Sequential deprotections using TBAF, NaOMe–MeOH and saponification led to the completion of the synthesis of sialyl dimeric Le^x ganglioside **68**.

The approach by Ogawa *et al.*[29] also involves the use of a pentasaccharide **69** (Fig. 19) similar to that used by Hasegawa *et al*. (**55**, Fig. 18), however, the sialylation involved the use of the phosphite method.[16] Their synthesis began with a trisaccharide **71** which was glycosylated with a sialic acid donor **72**, a phosphite derivative, in the presence of TMSOTf as catalyst to obtain the desired α-linked tetrasaccharide **73** in 40% yield. It may be noted that sialylation of **71** with the corresponding thio sialyl donor **74** gave a mixture of α- and β-isomers. The α-isomer **73** was acetylated, and then converted into the trichloroacetimidate **75** after deallylation using (i) $[Ir(COD)(PMePh_2)]PF_6$ in THF (*cf*. Fig. 16), and (ii) I_2 in aq. THF followed by treatment with CCl_3CN in the presence of DBU. Glycosylation between **75** and **69** using $BF_3{\cdot}Et_2O$ as a catalyst in CH_3CN at −40 °C gave the nonasaccharide **76** in 52% yield, in a highly regio- and stereocontrolled manner, which was characterized as its acetate **77**. Hydrogenolysis of **77** with Perlman's catalyst $[Pd(OH)_2/C]$, subsequent acetylation to obtain **78** followed by chemoselective cleavage of the anomeric acetate with hydrazinium acetate in DMF led to the hemiacetal **79**. Treatment of this hemiacetal **79** with CCl_3CN in the presence of DBU afforded the α-trichloroacetimidate **80** in 87% yield which was coupled with **81**, a ceramide derivative, in the presence of $BF_3{\cdot}Et_2O$ at −15 °C to form **82** in 39% yield. Conversion of **82** into the natural sialyl Le^x dimer **68** was carried out using four steps *viz*. (i) refluxing of **82** with excess LiI in pyridine to form the Li salt of **83**, (ii) subsequent treatment with NH_2NHMe in refluxing

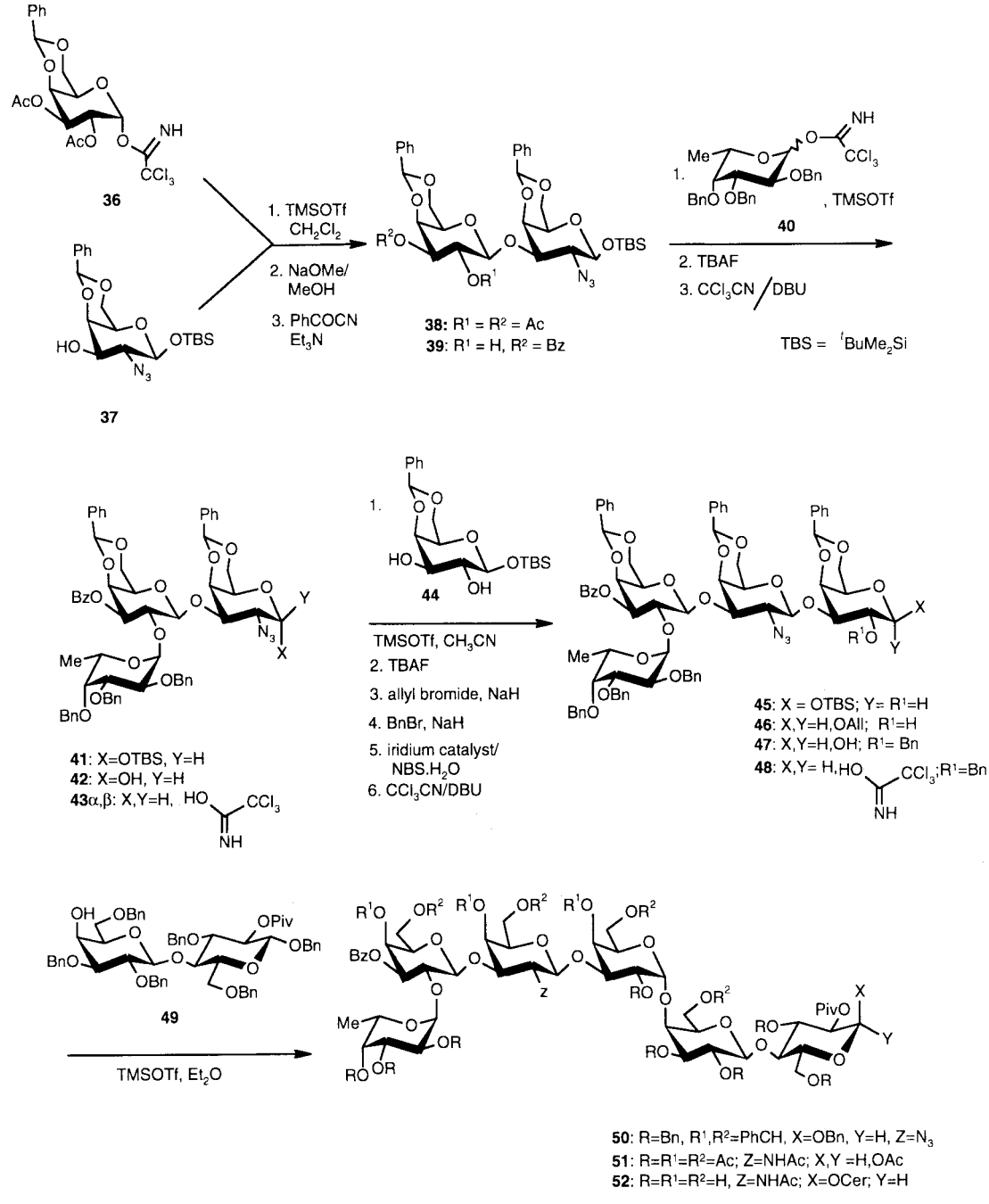


Fig. 16

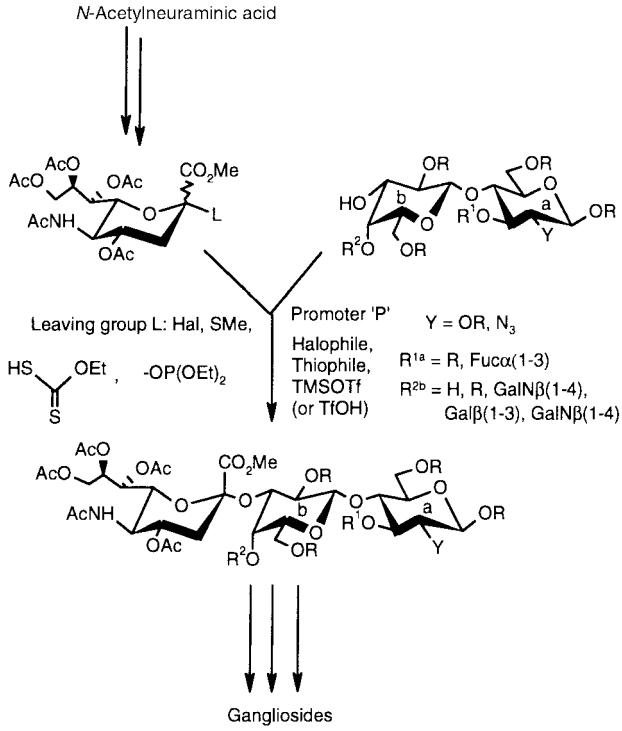


Fig. 17

EtOH, (iii) Ac_2O in MeOH, (iv) aq. NaOH in 1:1 MeOH–THF.

The strategy developed[23] for the synthesis of Le^x dimer **35** (*cf*. Fig. 15) has also been successfully applied to the synthesis of sialyl Le^x dimer **68** by Hummel and Schmidt.[30]

4 Approaches to enzymatic synthesis

In this kind of approach successful syntheses of a few gangliosides such as G_{M3}, G_{M4} and a few analogs have been reported by using chemoenzymatic methods. Typically one or two steps involved the use of an enzyme, generally depending on its availability. Among these, attachment of a sialyl group using sialyltransferase[31,32] and ceramide attachment using ceramide glycanase[33] have been investigated. Thus, Zehavi and Tuchinsky[31] have reported the synthesis of G_{M3} **90** using a water soluble polymer support **87** (Fig. 20) and a photosensitive protection for the amino functionality of the sphingosine unit.

Light-sensitive 2-nitrobenzyl derivative **84** of lactosyl-sphingosine was saponified to **85**. An amino-functionalized polyacrylamide **87**, possessing a four atom spacer, was procured from the water soluble polymer polyacrylamide–poly(*N*-acryloxysuccinimide) (PAN) upon reaction with *N*-(benzyloxycarbonyl)ethane-1,2-diamine followed by hydrogenolysis.

Coupling of polymer **87** with **85** using 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide (EDCD) in water led to a lactosyl-β-(1–1)-sph polymer **86**. Sialylation of **86** was achieved by using Galβ-1,4-GlcNAcα-2,3-(N)-sialyltransferase including ^{14}C labeled CMP-NeuNAc as an *N*-acetylneuraminyl donor and calf intestinal alkaline phosphatase was used in the reaction mixture to destroy the nucleotide phosphate inhibitor (CMP) that is released during the glycosyl transferase reaction, to give immobilized *lyso* G_{M3}.

Photolysis (> 350 nm) of the 2-nitrobenzylurethane group in **88** gave **89** whose subsequent acylation with stearoyl chloride led to G_{M3} **90**.

From this synthetic endeavour it is clear that the use of enzyme circumvents the need for elaborate protecting group chemistry. The photolysis of the 2-nitrobenzylurethane unit is also an important step here as the conditions are mild which could be even more useful in more complex GSLs synthesis.

Likewise, Liu and Danishefsky[32] have made use of their 1,2-anhydrosugar chemistry (Glycal Assembly) in combination with employing α-(2,3)-sialyltransferase as shown in Fig. 21. Once again, sialylation in a regio- and stereoselective manner with minimum protection–deprotection sequences highlights the possibilities of enzymes in GSLs synthesis.

Because of the lower number of steps involved in enzymatic reactions coupled with high regio- and stereoselectivities it is likely that more use of enzymes will be seen in future for GSLs synthesis.

5 Conformational studies

The three-dimensional structures of biomolecules and particularly biopolymers are known to play important roles in a variety of biological functions. The cellular environments in which glycosphingolipids are found are the amphiphilic membranes. This must be taken into account when experimental data for conformational analyses are acquired. Therefore, in addition to the structure and mobility of the oligosaccharide chain, the relative orientation of the carbohydrate moiety and membrane is relevant. Another quality of glycosphingolipids which was discovered only recently is the demixing of membrane components and the formation of higher order structures.[34] Since in many cases the crystal structures of carbohydrates are not available, the best method with which to study solution structure of GSLs with accuracy is high-resolution NMR spectroscopy. It also helps in obtaining molecular parameters like interatomic distances or torsion angles of oligosaccharides in solution. Carbohydrate mobility can be quantified on different timescales with relaxation parameters (10^{-9} to 10^{-6} s) or with coupling constant and chemical shift data (10^{-3} s). The spectroscopic studies must be performed in reproducible model surroundings. A simple model membrane is available with fully deuterated SDS micelles where gangliosides can be studied with high resolution NMR methods. A more biomimetic environment is disc shaped micelles, so-called bicelles.[35]

Three rotatable bonds between the anomeric carbon of the reducing sugar and the C-2 of the ceramide aglycon form a flexible joint between the carbohydrate moiety and the membrane. NMR studies clearly define a close to perpendicular orientation of the lactose and the membrane. A realistic description of a carbohydrate chain is a rotation cone,[36] as shown in Fig. 22.

Linear gangliosides like globosides are characterized by overall flexibility. Well-defined solution conformations are found for branched carbohydrate structures where steric interactions between vicinal pyranoses strongly reduce the number of accessible conformations. Cooperative stabilization of branched oligosaccharides was detected for the Lewis antigens where additional hydrophobic contacts between parallel oriented pyranose ring planes are found.[36] The central trisaccharide core of G_{M1} is found in a rigid conformation and a hydrogen bond can be formulated between the carboxylic acid group and the amide proton (Fig. 23).[37] It has also been reported[37] that overall structural features of G_{A1} (an asialoganglioside G_{M1}) are very similar to those of G_{M1}. In G_{A1} there is a stable intramolecular hydrogen bond between the third hydroxy group in ring I and the ring oxygen atom in ring II (Fig. 23). There is a weak hydrogen bond between the second hydroxy group in ring IV and the acetamido group in ring III; the β-D-GalNAc unit is surrounded by ring II and ring IV in a compact manner: this amido proton cannot interact with the solvent easily and that is why this proton has been observed to exchange slowly.

These electrostatic interactions and through space interactions between the sialic acid have been found to cause the

chemical shift dispersions in G_{M1}.There are more chemical shift dispersions in G_{M1} than in G_{A1}. However, this study shows that tertiary foldings of G_{A1} are very similar to those in G_{M1}. It is suggested[38] that the differences in the biological specificities between G_{M1} and G_{A1} are caused not by their tertiary foldings, but mainly by electrostatic properties due to the presence or absence of sialic acid. Since it is evident that G_{A1} is more hydrophobic than G_{M1}, a receptor with a hydrophobic binding site can recognize the G_{A1} better than G_{M1}.

6 Some recent biological studies

Recent biological studies indicate that some of the GSL antigens have been identified as adhesion molecules recognized by carbohydrate-binding proteins or by complementary carbohydrates on target cells. Such adhesion, coupled with signaling, is believed to initiate the metastatic process.

The P histo-blood group-related glycosphingolipid, sialyl galactosyl globoside (SGG), has been implicated as a preferred

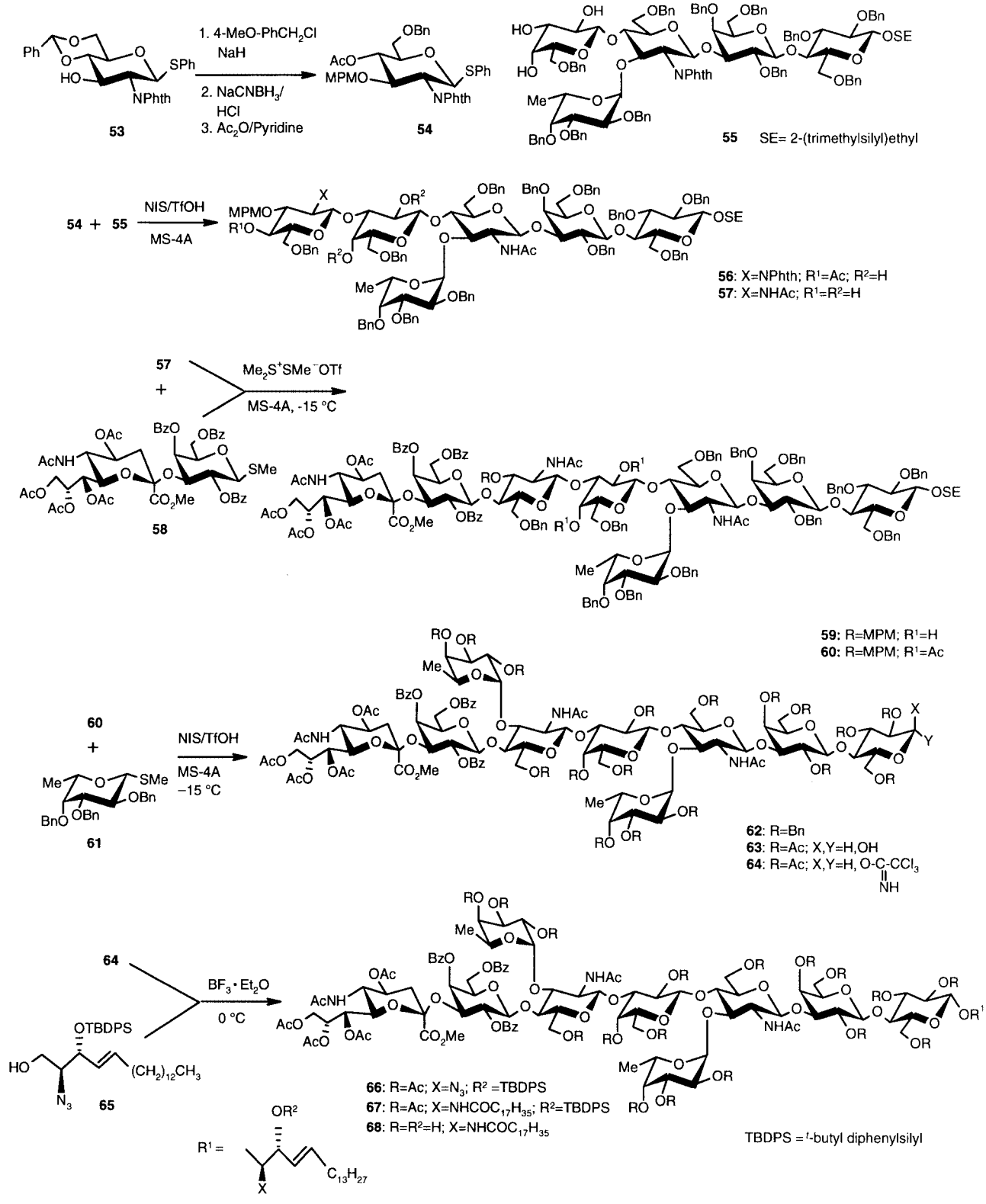


Fig. 18

binding receptor for uropathogenic *Escherichia coli*. From normal human kidney, SGG has been isolated and the significance of this structure as a unique receptor in human kidney for uropathogenic *E. coli* and its role in the pathogenesis of urinary tract infections have been studied.[39]

Likewise, G_{M1} has been expected[40] to have antineurotoxic, neuroprotective and neurorestorative effects on various central neurotransmitter systems. Further, these studies may point to novel approaches for treating neuroinjury and a variety of degenerative conditions, including aging.

Gangliosides have been found[41] to induce an α-helical structure in β-amyloid peptide, a 39–43 residue peptide, and thereby diminish fibrillogenesis. It is also observed that the sialic acid moiety of gangliosides is necessary for the induction of α-helical structure.

Ganglioside G_{M2} is particularly important because (i) it is expressed on the cell surface of a number of human cancers, including melanoma, sarcoma, and renal cancer; (ii) G_{M2}-reactive antibodies are cytotoxic *in vitro* against G_{M2}^{+} human cancer cells; (iii) G_{M2} is immunogenic in humans due to the presence of naturally occurring low-titer IgM serum antibodies against G_{M2}, due to the relative ease of isolating G_{M2} monoclonal antibodies from humans, and to the induction of G_{M2} antibodies in melanoma patients following immunization

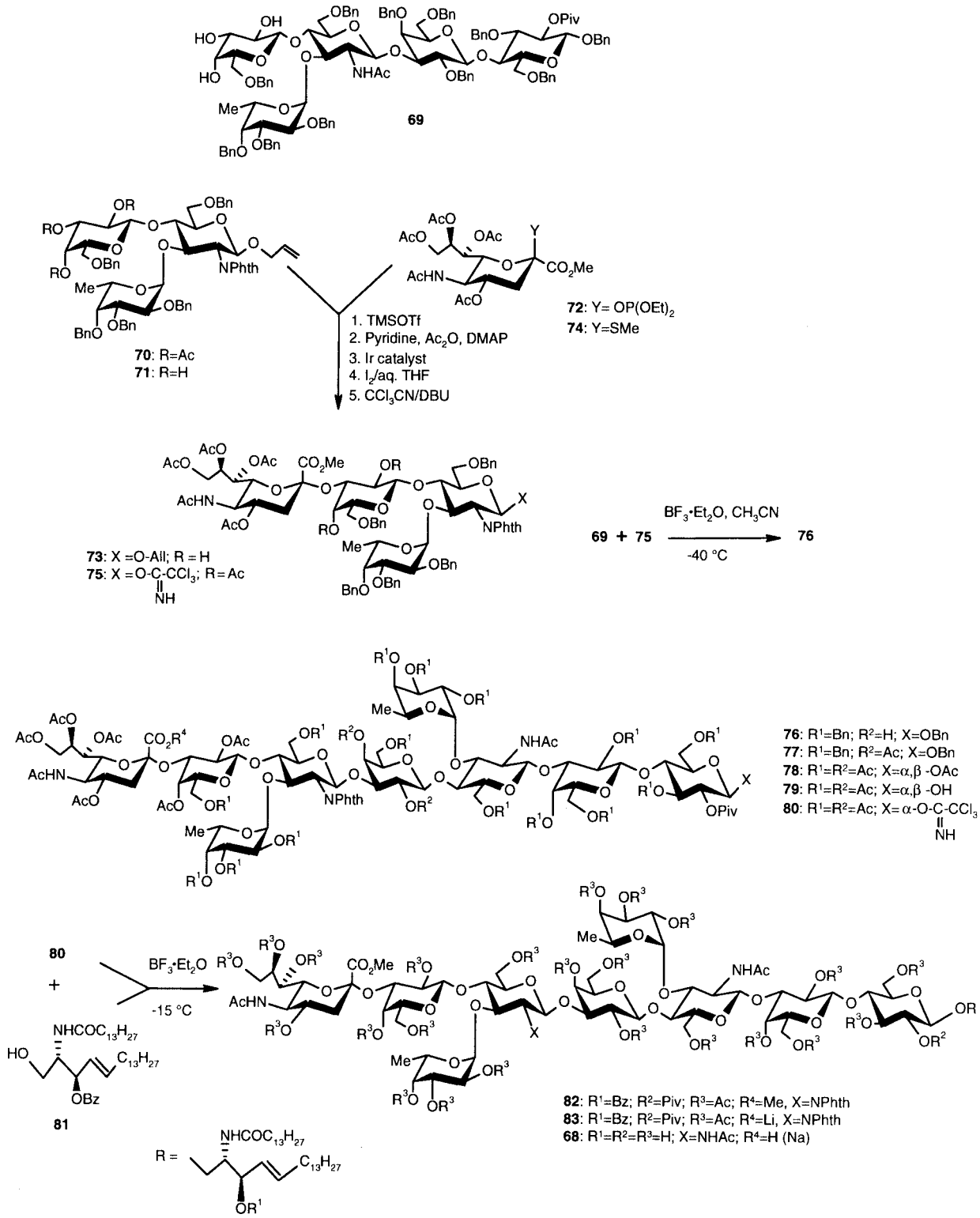


Fig. 19

with G_{M2}-containing vaccines; (iv) the presence of G_{M2} antibodies in melanoma patients appears to be associated with an improved survival rate and a longer disease-free interval; and (v) no deleterious side effects associated with an immune response to G_{M2} have been observed.

Various approaches for inducing immune response against G_{M2}^{+} cancer cells in melanoma patients have been pursued. A recent study[42] suggests that ceramide containing a fatty acid longer than ten carbon atoms is required for full and specific immune recognition of G_{M2}. Based on this observation, G_{M2}–KLH (keyhole hemocynin) conjugate vaccines have been prepared by using synthetic G_{M2} containing stearic acid and a clinical trial study is being carried out.

7 Summary and outlook

The great progress in oligosaccharide synthesis methodology—particularly the improvements in the Koenigs–Knorr procedure, the use of *O*-glycosyl trichloroacetimidates, thioglycosides, and 1,2-anhydrosugars as glycosyl donors—within the last twenty years has resulted also in a breakthrough in GSL synthesis. The use of now chemically readily available azidosphingosine as

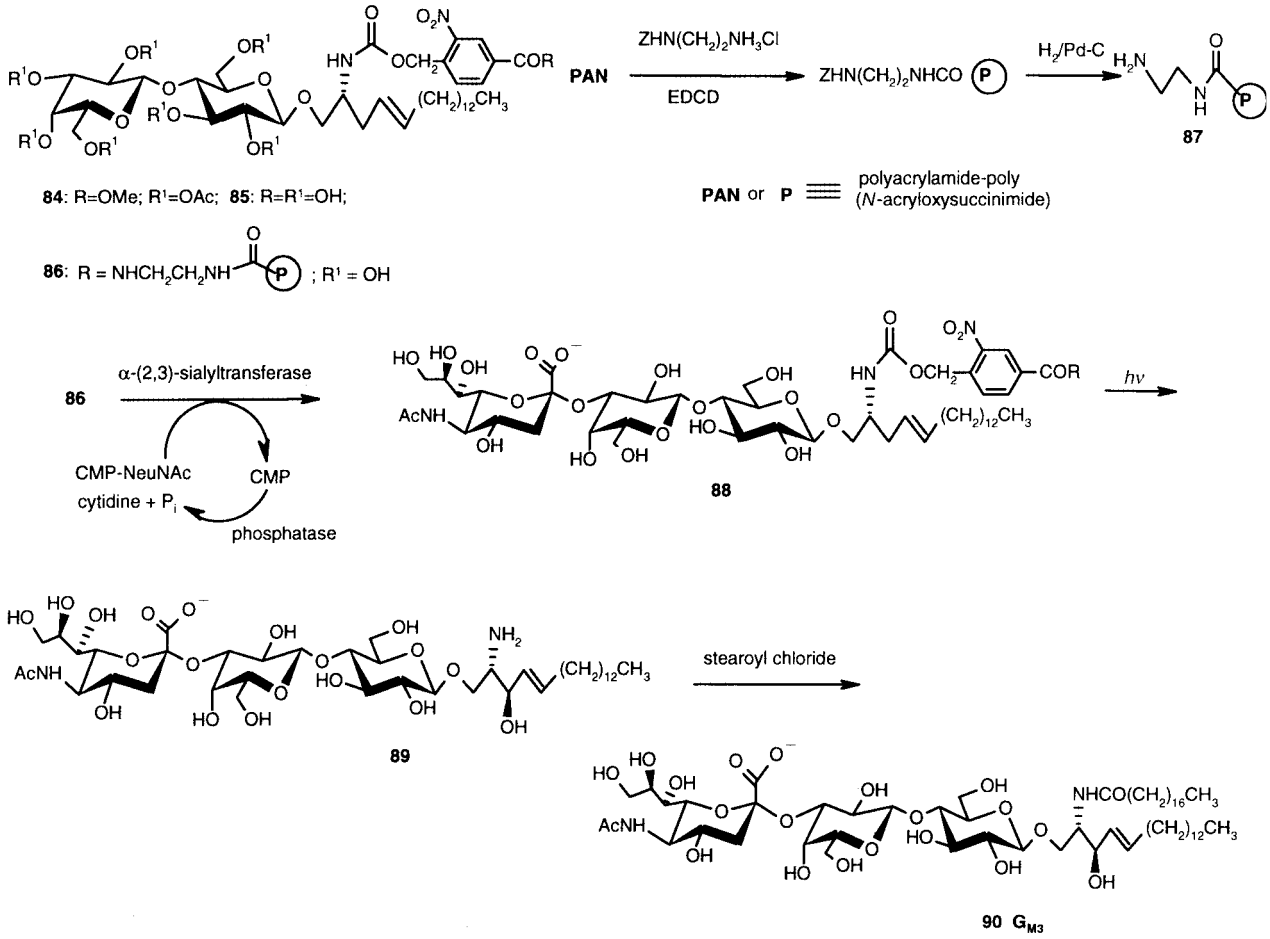


Fig. 20

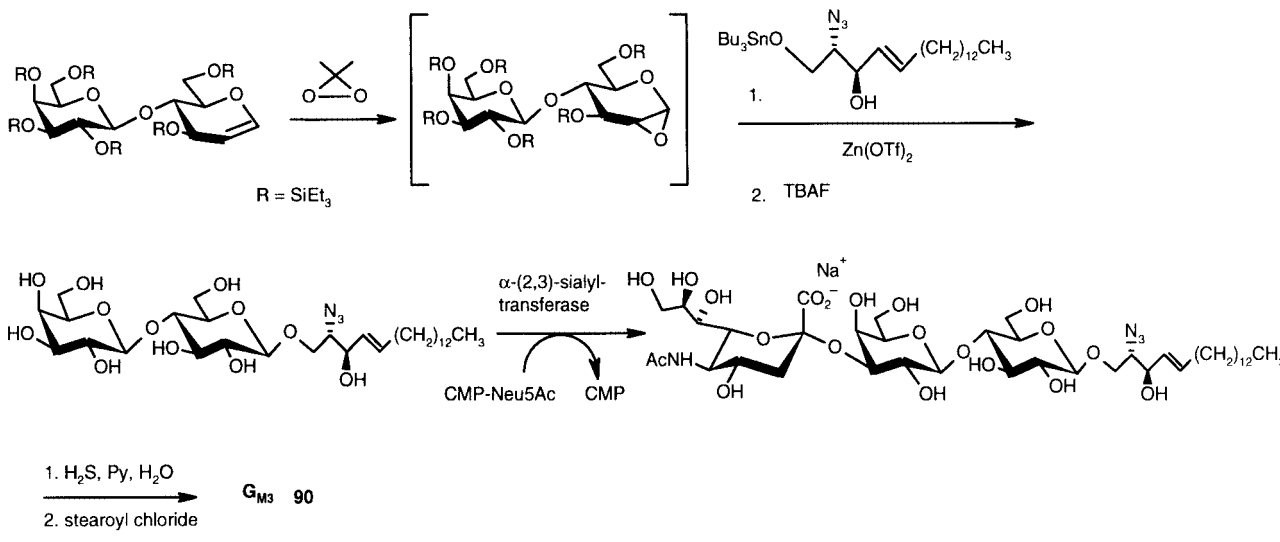


Fig. 21

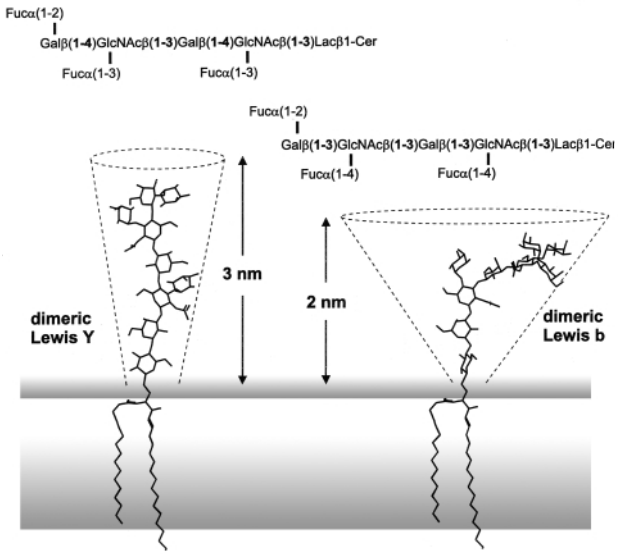


Fig. 22

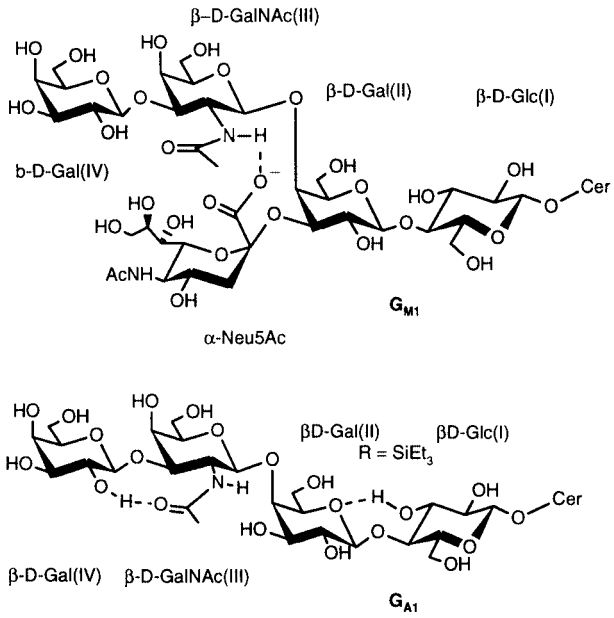


Fig. 23

oligosaccharide acceptor from corresponding glycosyl donors ("azidosphingosine glycosylation procedure") permitted a reliably high yielding linkage between the two moieties at a rather late stage of the synthetic strategy. Thus, various neutral GSLs could be obtained in good to excellent overall yields. For the synthesis of gangliosides (Neu5Ac containing GSLs), phosphite and sulfide leaving groups at the Neu5Ac residue and the use of nitriles as solvents ("nitrile effect") at low temperatures greatly improved the yields and the desired α-selectivity in the sialylation step. Thus, even access to sialyl dimer Lewis^x or various gangliosides of the ganglio series carrying even more than one Neu5Ac residue was permitted. The relatively mature stage of chemical synthesis of GSLs will be more and more complemented also by chemoenzymatic approaches on condition that more stable glycosyltransferases become (commercially) available.

The availability of GSLs and particularly structurally modified derivatives thereof by chemical synthesis has initiated great interest for biological studies. This outstanding development of chemical synthesis methodology nicely coincides with the focus on the molecular basis, for instance, of cell–cell adhesion, membrane signal transduction, membrane caveoloe formation, and receptor/epitope recognition. Therefore, the interaction of chemistry and biology will offer new dimensions to this research field.

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