We have devised a facile route to monodisperse PAHs of 3.0 (**6**) and 4.5 nm (**7**) length,[14] which, regardless of their considerable size, can be handled and characterized (Table 3) like any low molecular weight compound. To our knowledge PAH **7** is the most extended, fully characterized aromatic compound known today.

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[4] For an alternative approach to double-stranded, unsaturated polymers, see U. Scherf, K. Müllen, *Synthesis* **1992**, 23.
[5] Compound **4** was prepared according to the method of C. F. Allen, *Chem. Rev.* **1962**, *62*, 653. See also B. Schlicke, A.-D. Schlüter, *Synlett* **1996**, 425.
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[7] The LDI-TOF spectra of **6**, **7**, and other related compounds show, besides the molecular ion peak (low intensity), patterns of signals with a Gaussian type distribution at somewhat lower masses (relatively high intensity). The peaks of these patterns are separated by a constant $m/e = 28$ Da. The reason for this finding is not yet understood. A possible systematic distribution of molecular weights caused by an inadvertent employment of reagents with an even distribution of methylene groups in the alkyl chains is unlikely. The alkyl chains are introduced into the whole sequence as bromides and alcohols (see Refs. [3d, 5]), the purity of which was greater than 98% (checked by (GC)).
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[12] The reason for the weak fluorescence of **7** is presently under investigation.
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[14] The lengths were calculated with the program Spartan Silicon Graphics, Version 4.1.1., on the assumption that conformations are planar. Hydrogen atoms were disregarded.

Efficient Synthesis of Ganglioside GM2 for Use in Cancer Vaccines**

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Sheila R. Fortunato, Stefan Reinhardt, Lloyd J. Old,
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A number of carbohydrate antigens that are expressed on human cancer cells on glycolipids and glycoproteins are considered attractive targets for immunotherapy with monoclonal antibodies and vaccines.[1] Examples include the gangliosides GM2, GD2, GD3, *O*-acetyl-GD3, and fucosyl-GM1, the neutral glycosphingolipid globo-H, the T, Tn, and sTn epitopes expressed on glycoproteins, and the Le^Y epitope expressed on both glycolipids and glycoproteins. Of these potential targets, ganglioside GM2 is of particular interest, because a) GM2 is expressed on the cell surface of a number of human cancers, including melanoma, sarcoma, and renal cancer;[2] b) GM2-reactive antibodies (mouse and human polyclonal and monoclonal antibodies) are cytotoxic in vitro against $GM2^+$ human cancer cells;[3,4] c) GM2 is immunogenic in humans due to the presence of naturally occurring low-titer IgM serum antibodies against GM2,[4] to the relative ease of isolating GM2 monoclonal antibodies from humans,[5] and to the induction of GM2 antibodies in melanoma patients following immunization with GM2-containing vaccines;[3,4,6–8] d) the presence of GM2 antibodies in melanoma patients appears to be associated with an improved survival rate and a longer disease-free interval;[4] and e) no deleterious side effects associated with an immune response to GM2 have been observed.[4]

Various approaches for inducing an immune response against $GM2^+$ cancer cells in melanoma patients have been pursued, including immunization with GM2-expressing tumor cells,[7] with purified GM2 mixed with various immunological adjuvants,[3,4] and more recently, with vaccines consisting of purified GM2 chemically conjugated to an immunogenic carrier protein.[6] Antibodies induced by whole cell vaccines or GM2 adherent to bacillus Calmette Guerin (BCG) were generally of the IgM isotype (low-titer, short-lived, and nonboostable), consistent with a T-cell-independent immune response.[3,4] In more recent studies vaccination with GM2 chemically conjugated to keyhole hemocyanin (KLH) and mixed with QS21, an adjuvant of the saponin family, resulted in more strongly cytotoxic IgM antibodies and the frequent induction of IgG antibodies against GM2 in ELISA (enzyme-linked immunosorbent assay).[6,8] However, the majority of the IgG antibodies against GM2 failed to react with GM2-expressing human cancer cells.[8] This could be due, among other explanations, to the induction of IgG antibodies against GM2 epitopes that are hidden or buried on the cell surface, possibly as a consequence of the particular vaccine formulation.

The GM2 used in these studies was derived from mammalian tissues (e.g., bovine brain, cat Tay–Sachs brain, or human melanoma). However, extraction and purification of GM2 from tissue bears the risk of biological contamination. In addition, mammalian tissue-derived GM2 comprises a mixture of different molecular species, mostly varying in the ceramide moiety. For systematic clinical vaccine development, a consistent source of a single, well-defined, synthetic GM2 species would be desirable, particularly for vaccines to be used in large groups of patients. We have therefore developed a practical method for the chemical synthesis of ample quantities of GM2 oligosaccharide building blocks and GM2 ganglioside for use in GM2 cancer vaccines.

The strategies pursued in previous GM2 syntheses[9–11] follow the GA2 or the GM3 route,[12] which is also employed here (Scheme 1). The most important synthetic problems in this endeavor are a) convenient generation of a suitable lactose building block, b) α-selective attachment of a sialyl donor to the 3b-hydroxyl group of this lactose constituent, c) high-yield β-selective attachment of an N-protected galactosamine residue to the low-reactive 4b-hydroxyl group of the GM3 trisaccharide intermediate, and d) convenient transformation of the N-protected galactosamine residue into the *N*-acetyl-galactosamine constituent. Problems (a)–(d) can be solved by means of build-

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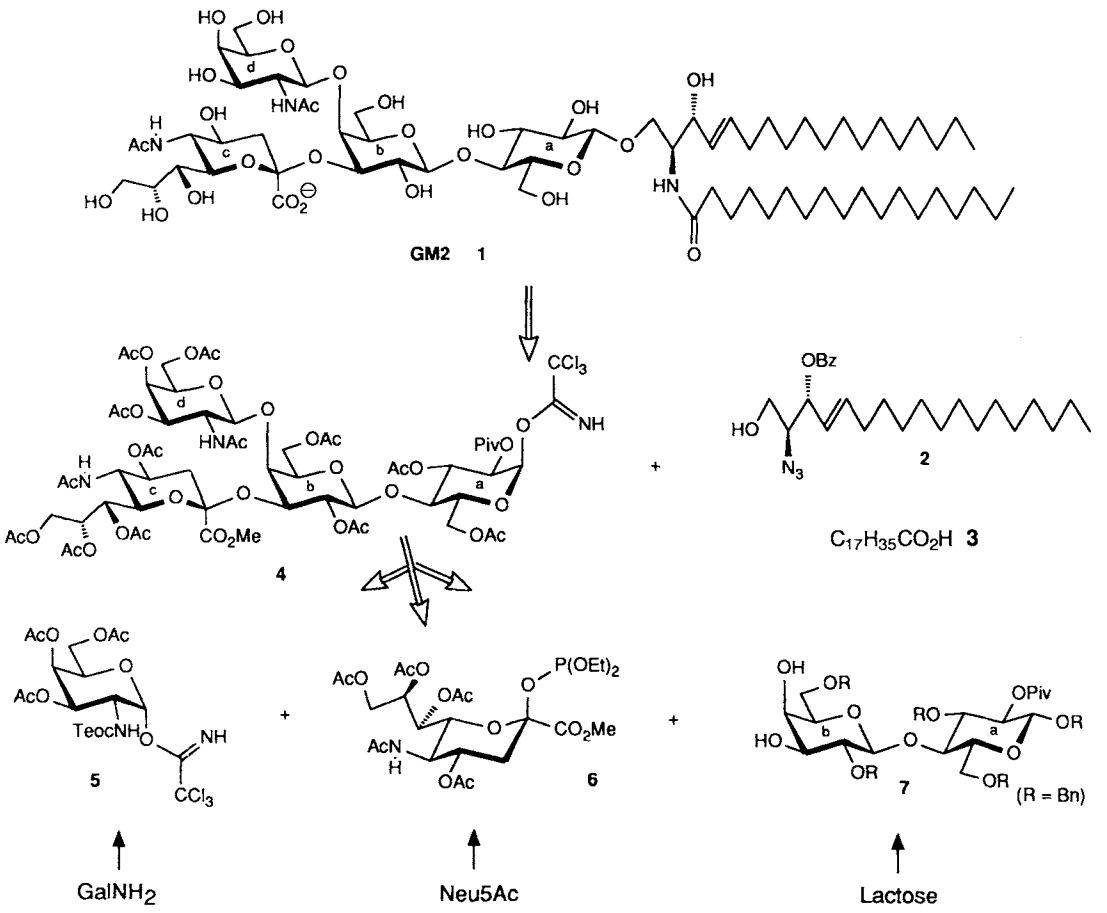
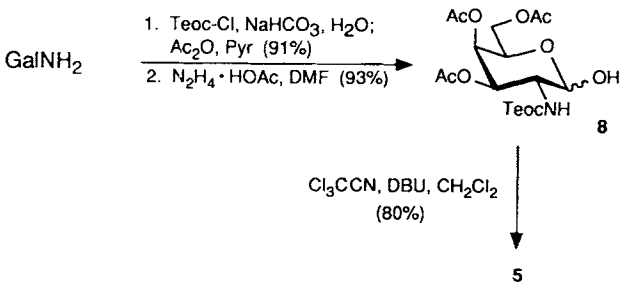


Scheme 1. Retrosynthesis of GM2 (**1**).

ing blocks **2**,[13] **3**, and **5**–**7**, which constitute tetrasaccharide intermediate **4**.

After investigating various galactosamine donors, we selected *N*-trichloroethoxycarbonyl(Teoc)-protected trichloroacetimidate **5**,[14] which was obtained from galactosamine in three convenient steps via the 1-O unprotected intermediate **8** in high yield (Scheme 2). This procedure avoided the tedious azidogalactose production[11,12] and the difficult removal of *N*-phthaloyl protecting groups in the presence of Neu5Ac residues, required in previous GM2 syntheses.[9,10] Simple treatment of *N*-Teoc-containing compounds with Zn/acetic anhydride leads to direct replacement of *N*-Teoc by an acetyl group.[15] The known diethyl phosphite derivative **6**[16] was employed as Neu5Ac donor.

The 3b,4b-O-unprotected 2a-*O*-pivaloyllactose residue **7**[17] facilitates the desired consecutive regioselective attack at the 3b- and then at the 4b-hydroxyl group, because of the very different



Scheme 2. Synthesis of the trichloroacetimidate **5** (Pyr = pyridine).

reactivities of these groups. The number of steps required for the reported synthesis of **7**[17] was greatly reduced by employing 1a,2a-*O*-silyl-group migration[18] for the regioselective introduction of the 2a-*O*-pivaloyl group. Isopropylidenation of lactose[19] and then silylation with thexyldimethylsilyl chloride (TDS-Cl) in the presence of base led to 1-*O*-silylated 3b,4b-*O*-isopropylidenelactose **9** (Scheme 3), which can be conveniently obtained also via the per-O-acetyl derivative of 3b,4b-*O*-isopropylidenelactose.[20] Benzylation of **9** with benzyl bromide and NaH as base in DMF leads to reversible silyl-group migration between the 1a and 2a oxygen atoms. Owing to the high nucleophilicity of the oxide oxygen atom of the β anomer, **10** is directly obtained from these intermediates in good yield by an irreversible anomeric *O*-alkylation process[18,21]. Removal of the 2a-*O*-silyl group with tetra-*n*-butylammonium fluoride (TBAF) in THF, pivaloylation, and acid-catalyzed cleavage of the isopropylidene group furnished target molecule **7** in high yield.

Reaction of sialyl donors with 2,3,4-*O*-unprotected galactosyl residues provides generally good α-glycosylation results. This is usually not found for 3,4-*O*-unprotected galactose derivatives.[16,22] Investigations with donor **6** and acceptor **7** and various catalysts showed that tin(II) triflate in acetonitrile at $-40\,^{\circ}C$ leads to high α selectivity ($\alpha:\beta = 9:1$) and good yields of GM3 intermediate **11** (Scheme 4); at room temperature sialoside yields of up to 80% were obtained with a slightly higher content of the β anomer ($\alpha:\beta = 4:1$); the α product **11** can be separated by flash chromatography in high purity. Subsequent glycosylation with donor **5** gave the desired tetrasaccharide in almost quantitative yield. Replacement of the Teoc group

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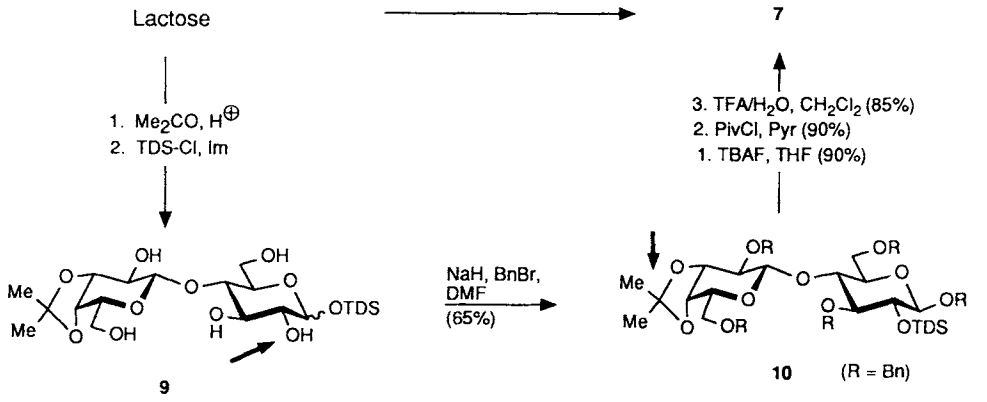
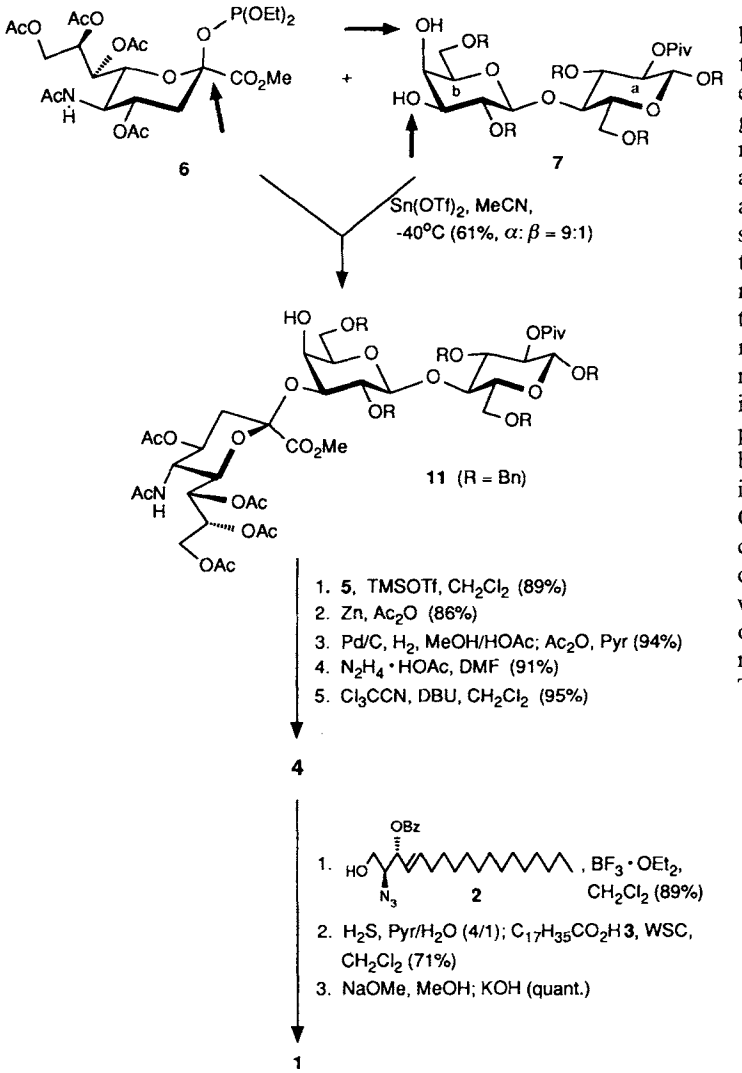


Scheme 3. Synthesis of the pivaloyllactose **7**. Im = imidazole, Piv = pivaloyl, TFA = trifluoroacetic acid. For synthesis of **7** from lactose, see also ref. [17].



Scheme 4. Synthesis of GM2 (**1**) from the building blocks **6**, **7**, **5**, and **2**. Tf = F_3CSO_2, TMS = trimethylsilyl, WSC = water-soluble carbodiimide [*N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride].

by an acetyl group with Zn/Ac_2O proceeded smoothly. Hydrogenolysis with Pd/C in MeOH/HOAc, followed by treatment with Ac_2O in pyridine led to replacement of all *O*-benzyl groups by *O*-acetyl groups. All subsequent procedures followed our previously introduced standard methods:[12] regioselective removal of the anomeric *O*-acetyl group with $N_2H_4 \cdot$HOAc and then treatment with CCl_3CN in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) as base afforded tetrasaccharide donor **4**. Application of the "azidosphingosine glycosylation procedure"[13] to azidosphingosine derivative **2**,[13] transformation of the azido into the amino group, attachment of the stearoyl residue (or other acyl group; see below), and then removal of all protective groups under basic conditions furnished target molecule **1** that was identical to previously synthesized material.[8, 9]

The availability of a GM2 oligosaccharide building block in large quantities by chemical synthesis facilitates the construction of GM2 gangliosides with different defined ceramide moieties and novel GM2 neoglycoconjugates for use as immunogens in GM2 vaccines. For instance, we have synthesized by this methodology GM2 having fatty acids of various lengths (capric acid, stearic acid, and lignoceric acid) in the ceramide moiety, and we have linked GM2 oligosaccharide to a short lipophilic spacer molecule (azidohexanol) to facilitate further conjugation to carrier molecules. To identify potential serological differences resulting from the composition of the lipid moiety, we tested these compounds and GM2 derived from bovine brain for their reactivity with a number of serological reagents (including mouse and human monoclonal antibodies against GM2, rabbit immune serum with GM2 reactivity, and sera from melanoma patients who had been immunized with vaccines containing bovine-brain GM2) by ELISA, dot blot immune stains, and immune thin-layer chromatography. We found that synthetic GM2 with stearic acid or lignoceric acid and sphingosine in the ceramide moiety was serologically indistinguishable from GM2 derived from bovine brain (Figures 1 and 2). However, GM2 with a ceramide containing a shorter fatty acid (e.g., capric acid) or GM2 oligosaccharide linked to azidohexanol showed dramatically reduced reactivity towards GM2-reactive antibodies. This suggests that ceramide containing a fatty acid longer than

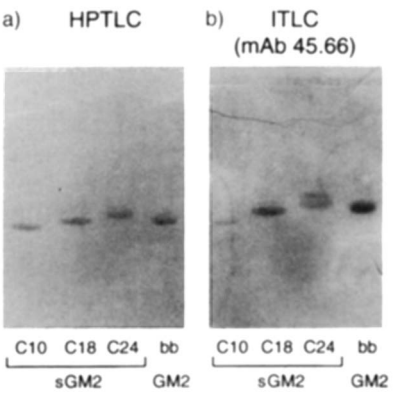


Figure 1. Reaction of a human monoclonal GM2 antibody (mAb 45.66) from a patient with malignant melanoma with synthetic GM2 containing capric acid (C10sGM2), stearic acid (C18sGM2), lignoceric acid (C24sGM2), or GM2 derived from bovine brain (bbGM2). The amount of ganglioside spotted per lane was 1 mg. The TLC was developed in chloroform/methanol/$CaCl_2$ (0.2%) in H_2O (55/45/10 v/v/v) and treated with hybridoma supernatant 45.66 diluted 1:10 with phosphate-buffered saline. Specific reactivity was made visible by using an anti-human IgM antibody conjugated to horseradish peroxidase and diaminobenzidine (b). Gangliosides were stained with orcinol/H_2SO_4 after immunostaining (a).

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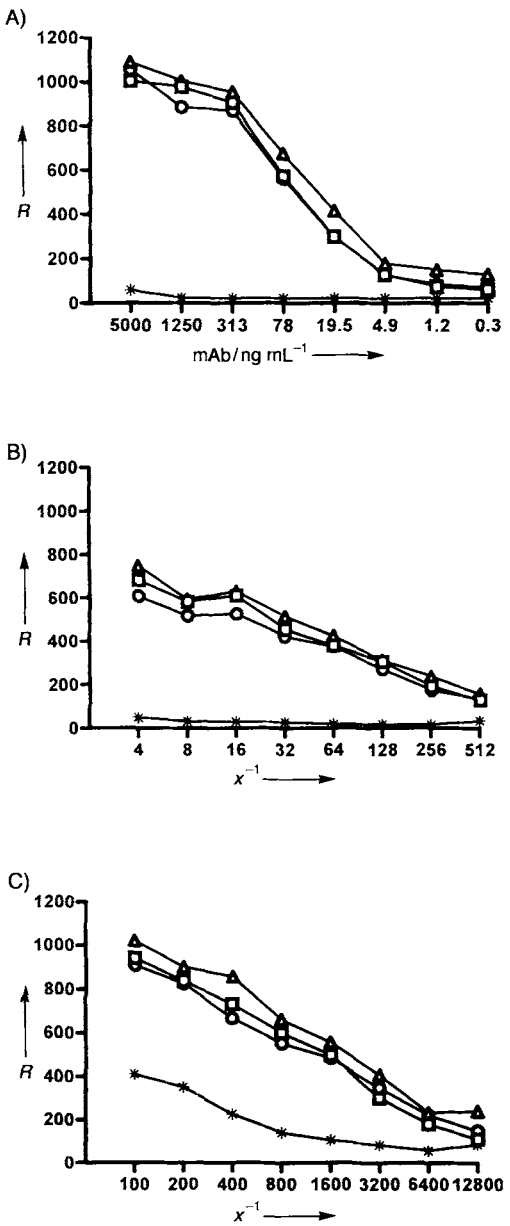

Figure 2. ELISA reactivity of GM2 antibodies with synthetic GM2 containing C10:0 (•), C18:0 (○), or C24:0 (△) fatty acid or with bovine brain-derived GM2 (□). A) Mouse monoclonal antibody 10.11 (purified IgM). B) Human monoclonal antibody 45.66 (tissue culture supernatant, IgM). C) Serum of a melanoma patient immunized with bovine brain-derived GM2-KLH/QS21 vaccine (IgG). No reactivity was observed with control antibodies or other gangliosides. *Method*: 200 pmol GM2 were incubated with varying amounts of GM2 antibody, and reactivity was quantitated by using Fitc-conjugated species, isotype specific secondary antibodies, and a microplate fluorospectrometer.

ten carbon atoms is required for full and specific immune recognition of GM2. Based on this observation, we have now prepared GM2–KLH conjugate vaccines by using synthetic GM2 containing stearic acid. Clinical trials with these vaccines will be initiated in the near future in patients with advanced malignant melanoma. By utilizing fully synthetic GM2, which is structurally well defined and free of biological contaminants, we hope to come a step closer towards our goal of developing a safe and efficient vaccine able to induce consistently a high-titered and long-lived cytotoxic antibody response in cancer patients.

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