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Preparation of photoreactive 12-[(4-azidosalicyl)amino] dodecanoic acid acylated derivatives of gangliosides radioioiodinated to high specific radioactivity.

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SUMMARY

We describe the preparation of ^{125}I labeled 12[(4-azidosalicyl)amino]dodecanoic acid (ASD) acylated derivatives of G_{M3}, G_{D3}, G_{M1} and SPG gangliosides. Gangliosides isolated from natural sources were deacylated, reacylated with ASD and radioiodinated with ^{125}I and chloramine T as an oxidant. During purification by HPLC each radioiodinated ganglioside derivative emerged from the column as two peaks differing in the substitution of 4-azidosalicylic acid residue. Radioiodinated gangliosides-ASD were of over 95% purity and about 2200 Ci/mmole specific activity. They were used for photolabeling of erythrocyte membrane proteins.

Key words: gangliosides, photolabeling, 12[(4-azidosalicyl)amino]dodecanoate, radioiodination, HPLC

INTRODUCTION

Gangliosides are sialic acid containing glycosphingolipids ubiquitously present in animal cell plasma membranes (1). Photoreactive ganglioside derivatives of high specific radioactivity and purity should expedite studies on ganglioside uptake from cell media, in metabolism, and other functions (2).

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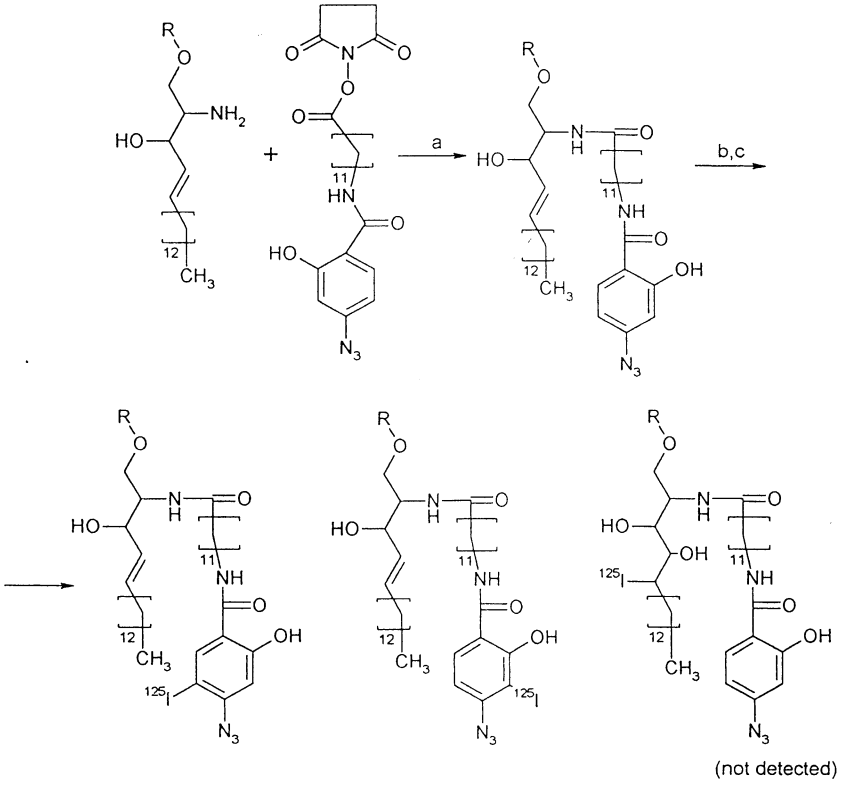
We describe the preparation of 12[(4-azidosalicyl) amino]dodecanoic acid (ASD) acylated ganglioside derivatives (gangliosides-ASD) radioiodinated to about 2200Ci/ mmole specific activity.

RESULTS AND DISCUSSION



a) DMF, DIPEA; b) $Na^{125}I$, chloramine T; c) HPLC
R= G_{M3}: Sialα2-3Galβ1-4Glc-; G_{D3}: Sialα2-8Sialα2-3Galβ1-4Glc-; SPG: Sialα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc-; G_{M1}: Galβ1-3GalNAcβ1-4[Sialα2-3]Galβ1-4Glc-

Scheme 1. Preparation of radioiodinated gangliosides-ASD.

The preparation of ^{125}I labeled gangliosides-ASD was carried out by the route outlined in Scheme 1. After radioiodination, gangliosides-ASD were separated by HPLC into two peaks containing over 80% of the radioactivity injected into the column. Iodination of gangliosides-ASD should result in formation of at least four reaction

products. Iodine can be added to the double bond of sphingosine with the formation of iodohydrin, a reaction exploited for radioiodination of unmodified G_{M1} ganglioside (3). 4-Azidosalicylic acid (ASA) residue of ASD should give three products: monoiodosubstituted at 5' and 3' positions, respectively, and diiodosubstituted at both.

Table 1

HPLC purification of radioiodinated gangliosides-ASD.

Ganglioside - ASD	Solvent (MeCN:buffer)	Retention time (min)		
		Unmodified	Peak I	Peak II
G_{M3}-ASD	65:40	20.67	20.67[a]	29.67
G_{D3}-ASD	53:47	22.17	26.17	36.50
SPG-ASD	60:40	23.33	23.33[a]	33.42
G_{M1}-ASD	60:40	19.58	19.58[a]	28.58
G_{M1}-ASD[b] reduced	60:40	35.40	35.40[a]	48.35

Radioiodination mixtures were purified by HPLC on a 250-4 Superspher 100 RP-18 end capped column. [a]Radioactive peak I is eluted together with the unmodified ganglioside-ASD. [b]Reduction of the double bond of sphingosine strongly affects the retention time of G_{M1}-ASD.

To discriminate between formation of iodohydrin and substitution of ASA, we took for radioiodination the G_{M1}-ASD ganglioside which contained C_{18} sphinganine obtained through reduction of the double bond of sphingosine with H_2 (4), a modification precluding formation of iodohydrin. HPLC analysis of radioiodination products with this G_{M1}-ASD also revealed the presence of two radioactive peaks, in keeping with the assumption that gangliosides-ASD have the ASA residue substituted with ^{125}I at different positions. In all radioiodination experiments the ratio of radioactivity recovered in the two peaks ranged from 1:1.5 to 1:2.5 in favor of the slower emerging material. Routinely the slower eluted peaks contained 21%-26% of the ^{125}I used for iodinations. Radioiodinated gangliosides-ASD emerging in these peaks were used for experiments described in this study. Their specific radioactivity, calculated on the basis of HPLC detector response, ranged between 2200-2400 Ci/mmole, an overestimation reflecting

inaccuracy of our assays. Purity of ganglioside-ASD preparations, estimated after TLC by the Phosphor-Imager, exceeded 98% while, when checked by HPLC, it was over 95%. Treatment with ceramide glycanase (5) and neuraminidases (6) strongly affected TLC mobility of radioiodinated gangliosides-ASD in keeping with their ganglioside character.

Radioiodinated gangliosides-ASD were taken up by erythrocytes in a manner dependent upon the structure of their oligosaccharide moieties. Incubation of erythrocytes prepared in phosphate buffered saline (PBS) as a 2% suspension ($2x10^8$ cells in 1 ml) with different radioiodinated gangliosides-ASD at a 2 nM concentration for 2h at 37 °C, resulted in the incorporation per 10^6 cells of 5.5 fmoles G_{D3}-ASD and of 2.8 - 3.2 fmoles of the remaining gangliosides-ASD. Preferential incorporation of G_{D3}-ASD seems to be a specific property of erythrocytes, as it was not observed in similar experiments with HL-60 cells. Radioiodinated gangliosides-ASD incorporated into erythrocytes can be removed at 37 °C with 1% bovine serum albumin (BSA) in a back

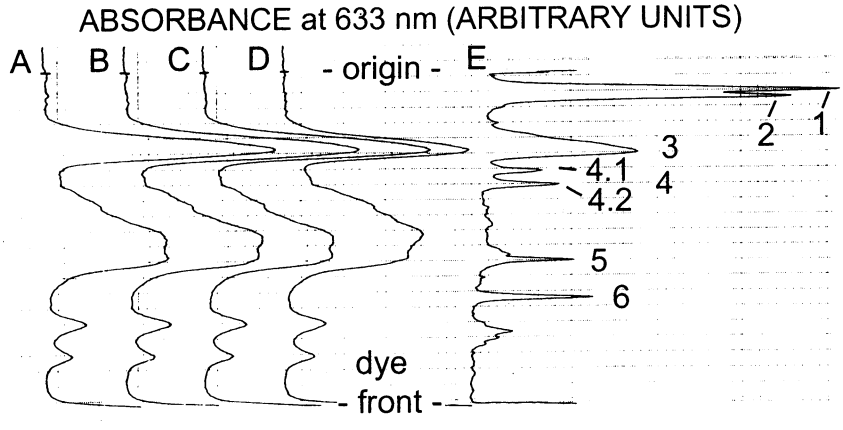


Figure1. Pattern of photolabeling of erythrocyte membrane proteins with radioiodinated gangliosides-ASD. Solubilized membrane samples containing 120,000 dpm of radioactivity were subjected to polyacrylamide gel electrophoresis under denaturing conditions (7, 8) (Methods). A, B, C, and D are densitometric tracings of autoradiograms of the separated membrane proteins photolabeled with radioiodinated SPG-, G_{M1}-, G_{D3}-, and G_{M3}-ASD, respectively. E shows the densitometric tracing of Coomassie brilliant blue stained protein bands numbered after Fairbanks et al. (9).

exchange process, but they are completely resistant to treatment with 0.25% trypsin; this indicates that they are not adsorbed on, but intercalate erythrocyte membranes.

Upon photolysis, the radioiodinated gangliosides-ASD incorporated into erythrocytes crosslink to a number of membrane proteins (Fig 1). Photolabeling patterns were similar for different gangliosides-ASD and did not depend either upon the length of incubation time or BSA treatment preceding photolysis.

The preparation of radioiodinated gangliosides-ASD outlined is simple and compounds obtained are of high purity and specific radioactivity, the highest reported thus far for a ganglioside derivative. When stored diluted in ethanol for 1 month at -20°C they had similar TLC mobility and purity as the freshly prepared material, thus, by this criterion, radiation damage was not substantial.

EXPERIMENTAL

HPLC. The Shimadzu apparatus consisting of two LC-10 AD pumps, SCL-10A controller and SPD-10A detector was used. Characterization of N-hydroxysuccinimidyl ester of ASD was performed with the Hewlett-Packard HP 1100 system consisting of a 1100 binary pump, HP 1100 diode array detector and HP 1100 mass spectrometric detector equipped with an atmospheric pressure chemical ionisation interface.

TLC. Gangliosides-ASD were analyzed in a solvent system of chloroform:methanol:0.2% aqueous $CaCl_2$ (60:35:8).

12-[(4-azidosalicyl)Amino]dodecanoic acid (ASD). The acid was synthesized as described (10) with minor modifications. Its purity, as estimated by HPLC on a 250-4 Superspher 100RP 18 (4μm) column (Merck) eluted with acetonitrile:water:glacial acetic acid (74:25:1), was about 99%. MS (EI) analysis with the parent ion at m/z 376(M^+) and 51.6% relative intensity, HRMS analysis with m/z at 376.209 (calcd. for $C_{19}H_{28}O_4N_4$, 376.211) and elemental analysis (calcd.: C, 60.64; H, 7.45; N, 14.89; found: C, 60.54; H, 7.61; N, 14.81) confirmed its identity.

12-[(4-azidosalicyl)Amino]dodecanoylsuccinimide (NHS-ASD). The ester was prepared as described (11,12) and used without purification. HRMS analysis of the reaction mixture detected the parent ion at m/z 473.224 (M^+) (calcd. for $C_{23}H_{31}O_6N_5$, 473.227). When

analyzed by HPLC with a diode array and MS detectors the reaction product representing about 70% of the material (total ion current) was identified as NHS-ASD because its peak, emerging from the column after ASD, contained the parent ion at m/z 474 ($M+H^+$), and was characterized by the UV spectrum, with maxima at 269 and 309 nm, which was similar to that for ASD.

Preparation of lysogangliosides and their N-acylation with ASD. This step was performed as described (12, 13). HPLC purification of lysogangliosides and gangliosides-ASD will be reported elsewhere. Glc analysis of long chain bases of the lysogangliosides revealed only the presence of C_{18} sphingosine. Their HPLC purity was between 95.3 % for G_{D3}-ASD and 97.5% for G_{M1}-ASD. The UV spectra of gangliosides-ASD dissolved in acetonitrile:water (4:1) had peaks at around 270 nm and 309 nm, which disappeared during irradiation with a UV lamp (14). Molar extinction coefficients at 270 nm calculated on the basis of sialic acid content ranged from 2.1 $x10^4 M^{-1}$ cm^{-1} for G_{M3}-ASD to 2.3 $x10^4 M^{-1}$ cm^{-1} for G_{D3}-ASD.

Radioiodination of gangliosides-ASD. To a small glass tube were added: ganglioside-ASD, 3 nmoles in 5 µl of water; Na-phosphate buffer, pH 7.4, 1 µmole, 5 µl; $Na^{125}I$, 0.5 mCi, 0.23 nmole; and methanol, 25 µl. The tube was sonicated briefly in an ultrasonic cleaning bath, then iodination was initiated with 17 nmoles of chloramine T added in 10 µl of chloroform:methanol (1:1) and terminated after 7 min by the addition of 100 nmoles of β-mercaptoethanol in 10 µl of methanol. The iodination mixture was diluted with 0.5 ml of methanol:1% aqueous NaCl (1:3) and applied to a Sep-Pak C_{18} light cartridge. The radioiodination tube was rinsed 3 times, each time by successive addition of 100 µl of methanol followed by vortexing and addition of 300 µl of 1% NaCl in water. The rinsing solutions were transferred to the cartridge which was washed successively with 5 ml of methanol:1% NaCl (1:3), 3 ml of methanol:water (1:3) and 1 ml of methanol:water (1:2). The washing solutions were discarded to radioactive waste. The radioiodinated gangliosides-ASD were eluted from the cartridge with 2 ml of methanol.

HPLC purification of radioiodinated gangliosides-ASD. The radioiodinated material was reduced in volume under N_2 to about 40 µl, a similar volume of acetonitrile was added and the tube was vortexed and sonicated in an ultrasonic bath. The liquid collected

in the tip of the tube after centrifugation was injected into a Rheodyne 200 µl sample loop. The tube was rinsed twice with 30 µl aliquots of acetonitrile:5mM Na-phosphate buffer (1:1), the rinses also injected into the loop (total volume not exceeding 150 µl) and the sample purified on a 250-4 Superspher 100 RP-18 end capped column which was eluted at 0.5 ml/min with acetonitrile:5mM Na-phosphate buffer, pH 7.0. The elution was monitored at 270 nm, 0.5 ml fractions were collected and their radioactivity determined in the γ counter (LKB/Wallack 1272 Clinigamma). The fractions containing radioiodinated gangliosides-ASD were pooled, diluted with 1% NaCl in water to lower acetonitrile concentration in the mixture below 20%, and applied to a Sep-Pak C_{18} light cartridge. The cartridge was washed with 5 ml of water and the radioiodinated gangliosides-ASD were eluted with 2 ml of methanol. The samples were reduced in volume under N_2 to about 200 µl, diluted with ethanol containing 1% of 0.1M HEPES buffer, pH 7.4, to about 20 µCi/ml and stored at -20 °C until used.

Characterization of radioiodinated gangliosides-ASD. Purity of the gangliosides-ASD was estimated by TLC and Phosphor-Imager with Quant 3 program of Molecular Dynamics, as well as by HPLC. Aliquots of the material ($2x10^6$cpm) were analyzed under the same conditions as used for purification of radioiodinated gangliosides-ASD. To prove the ganglioside character of the radioiodinated material, aliquots were treated with neuraminidases (6) and ceramide glycanase (5). After overnight incubation at 37°C the samples were freed of salts on Sep-Pak C_{18}light cartridges and analyzed by TLC. The distribution of radioactivity was detected by autoradiography.

Incorporation of radioiodinated gangliosides-X-ASA into erythrocytes. These experiments as well as determination of the susceptibility of erythrocyte bound radioiodinated gangliosides-ASD to treatment with 1% BSA and 0.25% trypsin were performed as previously described (8, 13).

Photolabeling of erythrocyte membrane proteins. The erythrocytes incubated with radioiodinated gangliosides-ASD for 2 h were washed with PBS or, in some experiments, treated with 1% BSA at 37°C for 15 min and photolabeled as previously described (8,13). Erythrocyte membranes were prepared (15), solubilized and subjected to sodium dodecyl sulfate/polyacrylamide gel electrophoresis in 10% gels. The gels were stained with

Coomassie brilliant blue, dried and exposed to X-ray films. Protein content and distribution of radioactivity in the separated bands were estimated by densitometric scanning.

Other procedures. Long chain bases in lysogangliosides were analyzed as trimethylsilyl derivatives (16). Sialic acid content was estimated colorimetrically (17). Protein content was determined with bicinchoninic acid according to the protocol of Pierce Chemical Co. Mass spectrometric analyses were performed with the AMD 604 apparatus.

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