Oxidative release of natural glycans for functional glycomics

Xuezheng Song1, Hong Ju1, Yi Lasanajak1, Matthew R Kudelka1, David F Smith1 & Richard D Cummings2

Glycans have essential roles in biology and the etiology of many diseases. A major hurdle in studying glycans through functional glycomics is the lack of methods to release glycans from diverse types of biological samples. Here we describe an oxidative strategy using household bleach to release all types of free reducing N-glycans and O-glycan-acids from glycoproteins, and glycan nitriles from glycosphingolipids. Released glycans are directly useful in glycomic analyses and can be derivatized fluorescently for functional glycomics. This chemical method overcomes the limitations in glycan generation and promotes archiving and characterization of human and animal glycomes and their functions.

Glycomics has received considerable interest in recent years1,2, but it lags behind genomics and proteomics because of analytical and preparative difficulties. Glycomics focuses on analyses of glycan structures3, whereas functional glycomics studies the recognition of glycans by glycan-binding proteins (lectins) either free or on cells, bacteria and viruses4,5, as well as glycan binding by antibodies. Detailed glycomics analyses require sufficient amounts of glycans for studies by NMR, crystallography and other methods, including HPLC6–8, mass spectrometry (MS)9–15 and matrix-assisted laser desorption/ionization (MALDI)–time-of-flight (TOF) MS analysis showed intact N-, O- and GSL-glycans, which can be tagged specifically for chromatographic separation and structural elucidation (Fig. 1).

RESULTS
NaClO releases free reducing N-glycans from glycoproteins
We previously reported that N-bromosuccinimide (NBS), a mild oxidant, oxidatively decarboxylates small N-glycopeptides with short peptide chains without affecting glycan structures36. This method, however, requires prior proteolysis of intact glycoproteins for effectiveness. In exploring other oxidative release or degradation approaches, we discovered that NaClO, which is known to degrade proteins37,38, effectively and selectively degrades the aglycon portion of native glycoconjugates to release intact glycans. Brief treatment of glycoproteins with NaClO releases free N-glycans, which can be specifically derivatized throughout the reducing end (Fig. 2a). NaClO degrades glycoproteins in minutes (Online Methods), and released glycans are easily visualized by their high mobility compared to untreated glycoproteins (for example, ovalbumin) in thin-layer chromatography (TLC) (Supplementary Fig. 1a). Analysis of treated glycoproteins by SDS-PAGE and Coomassie staining showed complete loss of intact glycoprotein material after NaClO treatment. Matrix-assisted laser desorption/ionization (MALDI)–time-of-flight (TOF) MS analysis showed that N-glycans are produced as glycosylamines, which spontaneously convert to free reducing glycans in aqueous conditions at room temperature (Supplementary Fig. 2). We found that ORNG is applicable to all types of common glycoproteins (Fig. 2b), including ovalbumin, bovine IgG and horseradish peroxidase (HRP), the last of which is resistant to PNGase F digestion owing to core α3-fucose modification of N-glycans39. The typically labile sialic acid residues are well preserved during ORNG, as demonstrated with bovine fetuin (Fig. 2c).

As noted, N-glycans derived by ORNG react with amine-based tags, such as the bifunctional fluorescent linker 2-amino-N-(2-aminoethyl)benzamide (AEAB) or other fluorescent tags, to facilitate chromatographic separation and purification. AEAB conjugates

1Department of Biochemistry, Emory University School of Medicine, Atlanta, Georgia, USA. 2Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts, USA. Correspondence should be addressed to X.S. (xsong2@emory.edu) or R.D.C. (rcummin1@bidmc.harvard.edu).

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of glycans released from bovine fetuin by ORNG showed the same glycans as those released by PNGase F (Fig. 2d). MALDI-TOF-MS profiles of permethylated glycans from human plasma released by ORNG and PNGase F were very similar (Fig. 2e). Several fucosylated glycans, presumably from serum IgG, were more abundant in the ORNG-released glycans, probably because some N-glycans may be resistant to PNGase F digestion without predigestion of glycoproteins with trypsin. We also obtained an N-glycan profile of human saliva after brief NaClO treatment (Supplementary Fig. 3). We observed that saliva-derived N-glycans had masses predictive of high fucosylation, which is consistent with previous reports. These results demonstrate that ORNG has great potential in high-throughput analyses of N-glycosylation of large-scale biological samples with minimal sample preparation and no prior proteolysis.

To determine the large-scale utility of ORNG, we analyzed store-bought chicken eggs, a readily available and inexpensive source of animal glycoproteins. We separated egg whites from yolks and treated several hundred grams of these materials directly with NaClO (Online Methods). The N-glycan profile of egg white by MALDI-TOF-MS analysis (Fig. 2f) was very different from that of yolk. Whereas egg white showed a profile including high-mannose and hybrid type N-glycans similar to that of ovalbumin, as expected (ovalbumin constitutes ~80% of egg white), the egg yolk profile indicated an abundance of high-mannose and complex type N-glycans. Several hundred grams of porcine tissues were also treated with NaClO to easily obtain gram-scale amounts of recoverable N-glycans from wet animal tissue were 0.5–1%, which approximates the predicted carbohydrate content for egg yolk of 0.7–1% (ref. 43).

As to the potential mechanism of NaClO release of N-glycans, we propose that the glycan-peptide amide bond is chlorinated to form an N-chloroamide (Supplementary Fig. 1b). A pericyclic reaction involving an asparagine proton gives a glycan-isocyanate intermediate, which is hydrolyzed to glycosylamine and then to free reducing glycan. This mechanism is consistent with the occurrence of glycosylamines as the major products after reaction with NaClO. We obtained clear spectra, and anomic region chemical shifts of six N-glycans isolated from egg yolk were tentatively assigned (Supplementary Fig. 6a) using CASPER (Supplementary Fig. 7).

We applied ORNG to the preparative release of N-glycans from various other sources, including ovalbumin, bovine IgG and FBS. Released glycans were AEAB tagged and separated by multidimensional HPLC. These separations yielded individual glycans of >95% purity (Supplementary Fig. 4), which are salt-free and useful for MS characterization and microarray printing. A library of 67 complex N-glycans with substantial quantities (micrograms to milligrams) was readily obtained from gram quantities of these glycoproteins (Supplementary Tables 1 and 2). MS and MS/MS analysis of these glycans yielded substantial structural information (Supplementary Fig. 5). To confirm the robustness of ORNG, we selected several purified N-glycans, including high-mannose, hybrid and complex types and neutral and acidic glycans for characterization by 1H NMR spectroscopy (Supplementary Fig. 6a). We obtained clear spectra, and anomic region chemical shifts of six N-glycans isolated from egg yolk were tentatively assigned (Supplementary Fig. 6b) using CASPER (Supplementary Fig. 7).

We printed an N-glycan microarray from this library and analyzed binding of immobilized glycans to several plant lectins (Fig. 3 and Supplementary Table 3). The interactions showed consistent and predictable results, demonstrating that glycans prepared by ORNG retain their conformation and ability to be recognized. The binding profiles of lectins toward glycans, which provide information about specific glycan determinants, aid in the structural characterization (Supplementary Fig. 5). Thus, ORNG permits the exploitation of ‘shotgun glycomics’ as an effective method to identify potential ligands for glycan-binding proteins and anti-glycan antibodies and to sequence the glycans within a given metaglycome.

Our results indicate that glycans are degraded much more slowly than peptide backbones. To test this directly, we treated a free reducing glycan lacto-N-neotetraose with 1% NaClO. We observed only a slight degradation at the reducing end after a long treatment (15 min) (Supplementary Fig. 8). Coupled with our results showing similarity between N-glycans produced by PNGase F and those by ORNG, these results indicate that under controlled conditions, glycans are stable to NaClO treatment.
NaClO treatment of glycoproteins releases O-glycan acids

We also found, unexpectedly, that ORNG is useful for the preparation of O-glycan derivatives (Fig. 4a). As might be predicted given the above data, O-glycosidic linkages are more stable than N-glycoside upon NaClO treatment (Online Methods), but the protein is quickly degraded (Supplementary Fig. 9a). MALDI-TOF-MS analysis of the NaClO treatment product from porcine stomach mucin, a glycoprotein rich in O-glycans linked to serine or threonine residues, showed recovered glycans linked at the reducing end to glycolic acid or lactic acid (O-glycan-acids) (Fig. 4b). The acidic aglycons, glycolic acid and lactic acid, presumably arise from the serine and threonine residues, respectively, to which the glycans are attached. Substantially larger amounts of NaClO are needed to degrade peptide chains sufficiently to release O-glycans compared to that for N-glycans. MALDI-TOF-MS analysis of O-glycan-acids released from porcine stomach mucin and fetuin after permethylation showed two sets of signals clearly matching these O-glycan-acid products, along with
some permethylated free reducing glycans (Fig. 4c). The availability of O-glycans that retain their linkage to the glycoprotein yields an unexpected advantage and will facilitate study of glycan recognition, especially where O-glycan linkage and anomerity is necessary. For glycomic analyses of O-glycan-acids, permethylation under typical highly basic conditions is accompanied by release of O-glycans from incompletely degraded O-glycopeptides through β-elimination, as reported previously for pronase-digested glycoproteins. Unlike prevailing β-elimination methods, 9-O-acetylated sialic acid in bovine submaxillary mucin (BSM) was retained after ORNG and was stable to methylation of carboxylic acids (Supplementary Fig. 9b). To facilitate the separation and purification of the products, the glycolic acid or lactose aglycon can be easily derivatized by activation with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC/NHS) and a fluorescent linker with an amino group, such as mono-9-fluorenyl-methoxycarbonyl (mono-Fmoc) ethylenediamine, which can be analyzed by MALDI-TOF-MS and separated by HPLC (Fig. 4d,e) to prepare O-glycan libraries for microarray preparation. This derivatization does not substantially affect sialic acids as reported previously.

We used ORNG and multidimensional chromatography to prepare an O-glycan library from porcine stomach mucin (Online Methods). Such a library is impractical with common methods of O-glycan release, which typically require NaOH–NaBH₄ treatment and results in reduction of O-glycans. A library of 65 major O-glycan-acids was obtained (Supplementary Table 4) and analyzed by MS and MS/MS (Supplementary Fig. 10). Many sulfated glycans were identified in the library, confirming the compatibility of ORNG with O-sulfation of glycans. These glycans were deprotected by piperidine to expose the amino group, printed onto microarray slides and analyzed by plant lectins. Distinct and specific lectin binding (Supplementary Fig. 11) was observed, validating the ORNG approach with O-glycans.

Mouse gastrointestinal tract glycan analysis
To demonstrate the utility of ORNG in comparative tissue glycomic analysis, we prepared O-glycans from the gastrointestinal tract tissues of a C57BL/6 wild-type mouse (6 months old), including stomach, small intestine and colorectum (Online Methods). Glycans were released by NaClO, purified by C₁₈ and carbon (Hypercarb) solid-phase extraction (SPE) cartridges and permethylated for MS analysis (Supplementary Fig. 12 and Supplementary Table 5). As expected, we found contamination of heose oligomers (m/z 681, 885 and so on), presumably from food digest, in all tissues in the eluate from the Hypercarb cartridges. Only low-abundance N-glycans were observed as high-mannose structures for all three tissues. Notably, the three tissues showed different O-glycan profiles in elutions from both the C₁₈ and Hypercarb cartridges. After NaClO treatment (Online Methods), the mucin glycoproteins were not fully degraded to yield O-glycan acids, owing to the limited amount of NaClO used. Instead, partially degraded glycopeptides were obtained.
and retained on both C18 and Hypercarb SPE cartridges. These O-glycopeptides, upon treatment with DMSO, NaOH and iodomethane, release and permethylate O-glycans efficiently. Mouse stomach showed abundant fucosylated O-glycans, similar to that reported for porcine stomach mucin with nearly no sialylated O-glycans. Small intestine showed much less complex profiles, with more monosialylated glycans. Colon shows even more sialylation, including many disialylated glycans that were not observed in stomach and small intestine. These results demonstrate that mucin O-glycan expression and sialylation is highly regulated in different regions of the murine gastrointestinal tract (Supplementary Table 5).

**NaClO treatment of GSLs releases glycan nitriles**

We also found, unexpectedly, that ORNG can degrade GSLs, a challenging class of glycoconjugates (Fig. 5a). We treated unmodified porcine brain gangliosides containing the common ceramide lipid moiety with NaClO in aqueous conditions (Online Methods) and analyzed the products by MS. We observed loss of the lipid moiety and a 39-Da increase in molecular mass in the major products over the corresponding free reducing glycans (Fig. 5b), suggesting formation of cyanomethyl glycosides. Notably, porcine brain tissue can be directly treated in aqueous conditions without organic solvent extraction. Consistently, the permethylated product gave a MS profile matching cyanomethyl glycosides (Fig. 5c). Similarly, we also found that NBS treatment of gangliosides at 65 °C yielded glycan nitriles (Supplementary Fig. 13). However, NBS cannot release glycan nitriles directly from brain tissue. After reduction of the nitriles with nickel chloride (NiCl2) and sodium borohydride (NaBH4), the resulting product showed a 4-Da mass shift, matching the expected aminoethyl glycosides (Fig. 5d). When this product was permethylated, an expected profile of tertiary ammonium cations was observed (Fig. 5d), confirming that NaClO treatment of GSLs in aqueous conditions oxidatively delipidated the glycan moieties, converting them to cyanomethyl glycosides.

We propose a potential mechanism for this reaction, which has not to our knowledge been previously reported, of NaClO and GSLs (Supplementary Fig. 14). Two consecutive oxidative elimination steps through pericyclic intermediates convert the amide of the lipid moiety to a nitrile, which is stable toward further oxidation. Nitriles are normally stable but can be specifically activated under certain reductive conditions for further modification and conjugation. We applied the mild, specific NiCl2–NaBH4 reduction to the glycan nitriles. The resulting alkyamine was easily protected with an Fmoc group as a reversible fluorescent tag, which facilitates detection during chromatographic separation. This strategy permits an easy functional labeling of gangliosides, which we validated by MALDI-TOF–MS (Fig. 5e) and a clean HPLC profile (Fig. 5f). No nitriles were observed in the products. A C18 SPE column is useful to purify the glycan–Fmoc products on the basis of Fmoc hydrophobicity. Fmoc groups can be removed by piperidine treatment for further modification, including microarray immobilization or linkage to amine-reactive aglycon moieties.

We also developed a method to directly utilize the nitrile functionality for fluorescent tagging. Nitriles have been used in palladium on carbon (Pd/C)–catalyzed N-alkylation of amines but often applied in excess. Glycan nitriles generated from N-glycans by pronase and NBS can be efficiently tagged with 2-aminobenzamide (2-AB). When we treated nitriles directly with 2-AB along with Pd/C catalyst and ammonium formate, we observed efficient tagging of the glycan nitriles, as demonstrated by MALDI-TOF–MS and HPLC profiles (Supplementary Fig. 15).
No nitriles were found after the conjugation, and only trace amounts of glycan amines (from direct reduction of nitriles) were observed on mass spectra.

**DISCUSSION**

Explorations of structures and functions of highly complex human and animal glycomes have been hampered by the lack of methods to obtain sufficient quantities of natural material and high-throughput sequencing. We report a method to release glycans with inexpensive and mild reagents that substantially improves the simplicity and accessibility of the study of glycoconjugates.

We compared the ORNG method with several traditional glycomics approaches (Supplementary Table 6). Bleach is commonly used for sterilization owing to its strong oxidative power toward proteins in microorganisms. However, there has been no prior systematic study on NaClO in the preparation and release of glycans from glycoproteins and GSLs. NaClO can be used to solubilize the cell walls of yeast to facilitate extraction of glucan polysaccharides and has been shown to degrade some glycosaminoglycans. Although NaClO is an oxidative reagent, under controlled conditions it can only degrade glycans and modifications (such as O-acetylation and O-sulfation) very slowly owing to the high oxidation state of carbohydrates. Because it attacks peptide bonds, ORNG should be applicable to plant and other nonmammalian glycans that might not be cleavable by traditional methods. The three major classes of glycans released by ORNG can be tagged using fluorescent molecules that are commercially available or can be easily prepared. Therefore, ORNG can be used for rapid and large-scale production of glycans from animal glycoconjugates in forms that can be directly purified, derivatized and functionally explored, as in glycan microarrays.

Owing to the chemical nature of NaClO, glycans with functional groups such as primary amines (for example, heparin or heparan sulfate) or sulphydryl or carbon double bonds might be partly degraded by this approach.

The sensitivity of glycoconjugates to oxidative degradation may have biological implications beyond the technical observations described here. Because hypohalous acids are known to be important natural oxidants with bioactivities, the generation of N-, O- and GSL-glycans from natural glycoconjugates raises the possibility of glycans as secondary metabolites, a process that might occur naturally. Studies have shown that free glycans occur in body fluids, such as N-glycans with a cleaved chitobiosyl core and have been observed on prolonged NaClO treatment. Although we focused here on in vitro analysis and preparation of these glycans, our findings warrant further investigation of in vivo glycans that may be generated through a similar oxidative degradation pathway.

**METHODS**

Methods and any associated references are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

X.S., D.F.S. and R.D.C. conceived the method; X.S., H.J., M.R.K. and Y.L. performed experiments; X.S., D.F.S. and R.D.C. analysed the data and wrote the paper.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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**ONLINE METHODS**

**Materials.** All chemicals and HPLC solvents were purchased from Sigma-Aldrich, Acros, Oakwood chemicals or Fisher Scientific. Any commercial source of NaClO could potentially be used for this method but should be compared to NaClO from a chemical supplier for validation. Milli-Q water was used to prepare all aqueous solutions. NaClO solutions were from Clorox (6.15% NaClO), Pure Bright (6% NaClO), Up and Up (8.25% NaClO) or Sigma-Aldrich (5% chlorine) and prepared freshly by addition of water. Bleach stored for more than 6 months at room temperature as 6% NaClO has been used successfully. Pd/C (10% palladium on carbon) was from Sigma-Aldrich. C18 Sep-Pak cartridges were from Waters; HyperCarb cartridges were from Thermo Scientific. Mouse gastrointestinal tract tissues were obtained under approved IACUC protocol 2002319 (Emory University). Normal human plasma was obtained under approved IRB protocol IRB00000408 (Emory University).

**Mass spectrometry (MS).** A Bruker Daltonics Ultraflex-II MALDI-TOF/TOF system and an anchoring target plate were used for MS analysis. Reflective positive mode was used for glycans before and after permethylation. 2,5-dihydroxybenzoic acid (DHB) (5 mg/mL in 50% acetonitrile with 0.1% trifluoroacetic acid) was used as matrix.

**High-performance liquid chromatography analyses.** A Shimadzu HPLC CBM-20A system with UV detector SPD-20A and fluorescence detector RF-10Axl was used for HPLC analysis. UV absorption at 330 nm excitation and 420 nm emission was also used. Agilent amino columns were used for normal phase HPLC. Both C18 and PGC columns were used for reverse-phase HPLC separation. The mobile phases were acetonitrile, water, and aqueous ammonium acetate buffer at pH 4.5. A linear gradient from 20 mM ammonium acetate in 80% acetonitrile to 200 mM ammonium acetate at pH 4.5 was used. Normal human plasma samples, a 50 mg/mL protein concentration was assumed. Human plasma (10 µL) was mixed with 50 µL saturated Borax solution and 10× 1% NaClO were ground together for 2 min at room temperature and quenched with 0.2× 1% formic acid. The mixture was centrifuged to remove insoluble material, and the supernatant was passed through a Sep-Pak C18 cartridge (500 mg) and washed with 5 × 3 mL water. Glycans were eluted with 3 mL 50% acetonitrile and 0.1% TFA. The elution was dried by SpeedVac, permethylated and analyzed by MALDI. The flow-through fraction and first 3-mL wash fraction were collected and passed through a HyperCarb SPE cartridge (50 mg). The carbon cartridge was further washed with 5 × 1 mL water. Glycans were eluted with 1 mL 50% acetonitrile and 0.1% TFA. The elution was dried by SpeedVac, permethylated and analyzed by MALDI.

**Thin-layer chromatography analyses.** Glycoproteins or glycans in aqueous solutions (2 µL) were spotted on silica gel TLC plates, dried, and developed with two different solvent systems. After air drying, the plates were sprayed with 0.1% orcinol in 5% sulfuric acid. The plates were heated on a hot plate until a clear pattern appeared.

**Permethylation and MS analysis.** Permethylation of glycan samples was carried out according to reported procedures to increase the sensitivity of MS analysis. Briefly, a lyophilized sample was treated with a DMSO-NaOH slurry (100–200 µL) and methyl iodide (25–50 µL) for 10–30 min. The supernatant was then partitioned between water (500 µL) and chloroform (500 µL). The organic layer was washed with 500 µL water, dried, and redissolved in 50% methanol for MS analysis.

**NaClO release of N-glycans for analysis. Method 1.** Glycoprotein (50 µL, 10 mg/mL) was mixed with 50 µL saturated sodium borate (Borax) solution. 100 µL 1% NaClO was added and the mixture was shaken for 1 min. Formic acid (10 µL) was added to quench the reaction. After briefly cooling on ice (2 min), the mixture was centrifuged at 10,000 × g for 2 min, and the supernatant was transferred into a suspension of 5 mg 10% Pd/C in 200 µL water in a centrifuge filter with a 0.2-µm nylon membrane. When Pd/C was used instead of normal activated carbon for absorption of glycans, multisialylated glycans were stabilized. After shaking for 5 min at room temperature, the mixture was filtered by centrifugation and the filtrate was discarded. The Pd/C powder was washed with 3 × 250 µL 1% formic acid. To the Pd/C powder, 100 µL 0.1% formic acid was added and the mixture shaken at 37 °C for 1 h and centrifuged to remove the filtrate. The Pd/C powder was washed with 250 µL 0.1% trifluoroacetic acid (TFA). Glycans were eluted with 50 µL acetonitrile and 0.1% TFA and analyzed by MALDI directly. The eluate was dried and permethylated for MALDI analysis. For human plasma samples, a 50 mg/mL protein concentration was assumed. Human plasma (10 µL) was mixed with 40 µL water and processed as described above.

**Method 2.** Mouse tissue was treated with pestle and mortar. For x mg of tissue, 4× µL water and 5× µL saturated Borax solution and 10× 1% NaClO were ground together for 2 min at room temperature and quenched with 0.2× 1% formic acid. The mixture was centrifuged to remove insoluble material, and the supernatant was passed through a Sep-Pak C18 cartridge (500 mg) and washed with 5 × 3 mL water. Glycans were eluted with 3 mL 50% acetonitrile and 0.1% TFA. The elution was dried by SpeedVac, permethylated and analyzed by MALDI. The flow-through fraction and first 3-mL wash fraction were collected and passed through a HyperCarb SPE cartridge (50 mg). The carbon cartridge was further washed with 5 × 1 mL water. Glycans were eluted with 1 mL 50% acetonitrile and 0.1% TFA. The elution was dried by SpeedVac, permethylated and analyzed by MALDI.

**Preparative NaClO treatment for the production of N-glycans.** Glycoproteins (1–10 g) were dissolved in water to 20 mg/mL. To this solution, 0.2 volume of 6% NaClO was added under stirring. After 15 min at room temperature, 0.01 volume of formic acid was added to the reaction mixture slowly and stirred for another 5 min and centrifuged to remove insoluble material. The supernatant was dried on a rotary evaporator and the residue was suspended in water and centrifuged to remove insoluble material. The supernatant was desalted over a Sephadex G-25 column (1.6 × 60 cm), and the desalted solution was passed through a C18 Sep-Pak column (2–10 g resin). The flow-through solution was dried and ready for AEAB conjugation as described previously.

For egg yolk, egg white, and other animal tissues, tissues were homogenized with ice-cold water using a Waring blender so that the final protein concentration was ~20 mg/mL on the basis of average protein content estimation. For example, 18 egg yolks (345 g) were mixed with 2,400 mL water in a mechanical stirrer. 6% NaClO (550 mL) was added and the mixture was stirred. NaClO was quickly consumed along with a quick drop in pH from 12 to 9 within 5 min. The mixture was stirred for 15 min at room temperature. Octanol (3 mL) and formic acid (30 mL) were added slowly and the mixture was stirred for 5 min. The mixture was centrifuged at 9,500 × g for 30 min. The supernatant was collected and dried on rotary evaporator. The residue was resuspended in 200 mL water, filtered and dialyzed in molecular-weight-cutoff (MWCO) 1 kD tubes for 4 h against running water. The dialysate was brought to a volume of 1,100 mL by addition of water, and pH was adjusted to 9 by addition of 50% sodium hydroxide solution.
To this mixture, 46 mL 6% NaClO was added slowly over 10 min, and the solution stirred for another 2 min. Formic acid (10 mL) was added, and the mixture was dried on rotary evaporator. The residue was dissolved in 100 mL water and filtered through a 0.45-μm membrane. The filtrate was desalted with a Sephadex G25 column (5 × 100 cm). Fractions positive with phenol-sulfuric acid assay were collected and lyophilized to give 4.7 g crude glycans. For solid animal tissue or organs, a 20% protein concentration was used for calculation.

**NaClO treatment for the production of O-glycans.** Porcine stomach mucin (10 g dry weight) was dissolved and suspended in 500 mL water. To this, 250 mL of 6% NaClO was added under stirring. After 30 min at room temperature, formic acid (7.5 mL) was added slowly to the reaction mixture. The mixture was stirred for another 5 min and centrifuged to remove insoluble material. The supernatant was dried on a rotary evaporator, and the residue was dissolved in water and filtered through a 0.45-μm membrane. The filtrate was brought to a volume of 500 mL by addition of water and adjusted to pH 7.6 by addition of NaOH. To this mixture, 16.6 mL 6% NaClO was added and the mixture was stirred for 24 h at room temperature. Formic acid (2 mL) was added, and the mixture was dried on rotary evaporator. The residue was dissolved in 100 mL water and desalted with a Sephadex G25 column (5 × 100 cm). Fractions positive with phenol–sulfuric acid assay were collected and lyophilized to give 4.3 g crude glycans.

**NaClO treatment for the production of GSL-glycans from porcine brain.** Porcine brain (220 g wet weight) obtained from a local farmer’s market was diced into small cubes and blended with 440 mL cold water to a homogeneous mixture. To this suspension, 1,320 mL 6% NaClO was added under vigorous stirring. After 30 min, octanol (10 mL) and formic acid (30 mL) were added. The mixture was stirred briefly and stored at 4 °C over night. The mixture was centrifuged to remove the upper (fatty) layer. The residual aqueous material was dried in a rotary evaporator. The residue was dissolved in 100 mL water and desalted with a Sephadex G25 column (5 × 100 cm). Fractions positive in phenol–sulfuric acid assay were collected and lyophilized to give 2.5 g crude GSL-derived glycans.

**Fluorescent tagging of O-glycans using Mono-Fmoc ethylenediamine.** O-glycan-glycolic/lactic acids were dissolved in 0.5 M MES buffer (pH 5.5) to 25 mg/mL. Equal volumes of freshly prepared N-hydroxysuccinimide (NHS) (100 mg/mL in DMSO) and EDC (100 mg/mL in DMSO) solutions were added. The mixtures were stirred at room temperature for 15 min. An equal volume of mono-Fmoc ethylenediamine (50 mg/mL in DMSO) was added, followed by sodium bicarbonate (100 mg/mL of total volume). The mixture was stirred for 1 h and centrifuged. The supernatant was precipitated in 10 volumes of acetonitrile at ~20 °C for 1 h. After centrifugation, the pellet was collected and redissolved in water for HPLC purification.

**Fluorescent tagging of glycan-nitriles with 2-aminobenzenesulfonic acid (2-AB) by Pd/C-mediated N-alkylation.** In a typical procedure, desalted glycan nitriles released from GSLs were mixed with 2-AB (25 mM) and ammonium formate (0.5–1 M) in 9:1 water/methanol. Then palladium (10% on carbon (Pd/C)) was added (1–2 mg/mL). The mixture was mixed by rotation at 50 r.p.m. at room temperature for 4 h, and more decolorizing carbon was added to absorb the glycans. The mixture was filtered and washed, and glycan eluted from carbon by 50% acetonitrile with 0.1% TFA.

**Microarray printing, binding assay and scanning.** Non-contact printing on NHS-activated slides was used. N-glycans were printed at 100 μM in 100 mM sodium phosphate (pH 8.5) in replicates of four. Biotinylated lectins (Vector Labs) except Con A were assayed at 10 μg/mL (Con A was assayed at 1 μg/mL). Alexa 488–streptavidin (Invitrogen, 5 μg/mL) was used for the detection of binding using a fluorescent scanner (Molecular Diagnostics). For O-glycans, before printing, the Fmoc-tagged O-glycans were incubated with a mixture of water, DMF and piperidine (40:40:20, v/v/v) for 10 min. The mixture was then dried in a SpeedVac and reconstituted in 100 mM sodium phosphate (pH 8.5) to 50 μM for microarray printing.

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