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Biosynthesis of conjugatable saccharidic moieties of GM_2 and GM_3 gangliosides by engineered *E. coli*†

Sébastien Fort,* Lemonia Birikaki, Marie-Pierre Dubois, Tatiana Antoine, Eric Samain and Hugues Driguez

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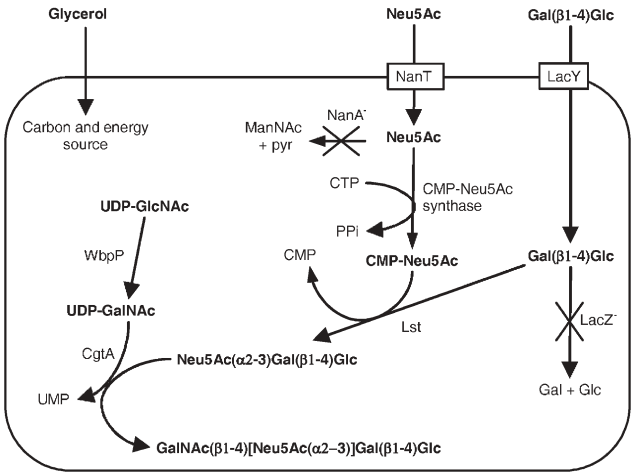
Oligosaccharidic moieties of GM_2 and GM_3 gangliosides bearing an allyl or a propargyl aglycon, are efficiently biosynthesized on the gram scale by growing metabolically engineered *Escherichia coli* cells in the presence of the corresponding lactoside acceptors and sialic acid.

Recent advances in glycobiology have revealed the significant role of glycans present on the cell surface as key elements in recognition processes occurring during fertilization, embryogenesis, metastasis, inflammations and host–pathogen adhesion. Further research on the biological functions of glycoconjugates and the development of carbohydrate-based therapeutics will undoubtedly be closely related to the availability of complex carbohydrate structures.[1] Thus, there exists a real need to access large quantities of oligosaccharides and glycoconjugates. Bacterial metabolic pathway engineering has recently emerged as a powerful method for the large scale synthesis of oligosaccharides.[2] Glycosylation reactions are performed by whole cells overexpressing the genes encoding the appropriate glycosyltransferases and sugar-nucleotide biosynthesis. We have recently reported an efficient sialyllactose synthesis[3] (2.6 g L^{-1}) by high density culture of a metabolically engineered *E. coli* strain that overexpresses the *Neisseria meningitidis* genes for α-2,3-sialyltransferase and CMP-NeuAc synthase (Scheme 1). Sialic acid containing oligosaccharides such as gangliosides constitute attractive targets for pharmaceutical development. Present in all vertebrate cells, gangliosides are expressed in human tumors of neuroectodermal origin (melanoma, glioma, neuroblastoma) and GM_2/GM_3 among others have been identified as potential targets for vaccine based therapy of cancer.[4] Exogenous lactose and NeuAc were actively internalized by the *E. coli* β-galactoside and NeuAc permeases. They accumulated in the cytoplasm without being degraded, since a strain devoid of β-galactosidase and NeuAc aldolase activities was used. NeuAc was converted into CMP-NeuAc and transferred onto lactose to form sialyllactose. The saccharidic portion of GM_2 was further produced at 1.25 g L^{-1} by a strain overexpressing the additional genes for a β-1,4-GalNAc transferase and an UDP-GlcNAc C4 epimerase necessary to provide UDP-GalNAc in the cells.[5]

Potential biomedical applications of gangliosides and other oligosaccharidic antigens for diagnosis and anticancer vaccine development, encouraged us to synthesize conjugatable forms of these molecules. We now report for the first time the biosynthesis of GM_3 and GM_2 carbohydrate moieties bearing allyl and propargyl aglycons.



Scheme 1 Metabolically engineered pathway of GM_3 and GM_2 saccharidic portion biosynthesis in *Escherichia coli* K12. Lactose and Neu5Ac, which are internalized by the specific permeases LacY and NanT, cannot be degraded because of β-galactosidase (LacZ) and aldolase (NanA) inactivation. Neu5Ac is converted into a nucleotide-activated form (CMP-Neu5Ac) by CMP-Neu5Ac synthase and then transferred onto lactose by α-2,3-sialyltransferase (encoded by Lst), to form sialyllactose. Use of the endogenous pool of UDP-GalNAc produced by the recombinant UDP-GlcNAc C4 epimerase (WbpP), allows β-1,4-GalNAc transferase (CgtA) to catalyze the glycosylation of sialyllactose to form the GM_2 saccharidic moiety. CTP, cytidine triphosphate; PPi, inorganic pyrophosphate.

Allyl and propargyl β-lactosides[6] **1** and **2** (Fig. 1) were chosen for the range of specific water-compatible reactions possible on their aglycon. The alkene function can alternatively be transformed into an aldehyde or an amino group through ozonolysis or photochemical addition of cysteamine. The former is a classical way to couple a carbohydrate moiety to the lysine amino acids of proteins through reductive amination[7] while the latter gives access to a versatile amino group.[8] The chemistry of terminal alkynes has recently emerged as a powerful tool for the conjugation of bioactive species in mild conditions through Huisgen 1,3-dipolar addition. The chemoselectivity of azido addition onto alkynes in aqueous conditions offers great perspectives and this bioconjugation technique, promoted by Sharpless *et al*. as "click chemistry", was successfully applied to peptide, DNA and carbohydrate fields.[9]

Strain TA01[5] was cultivated at high cell density on glycerol in a 2-litre reactor as previously described. The culture was supplied

† Electronic supplementary information (ESI) available: experimental details and NMR spectra. See http://www.rsc.org/suppdata/cc/b5/b500686d/

*Sebastien.Fort@cermav.cnrs.fr

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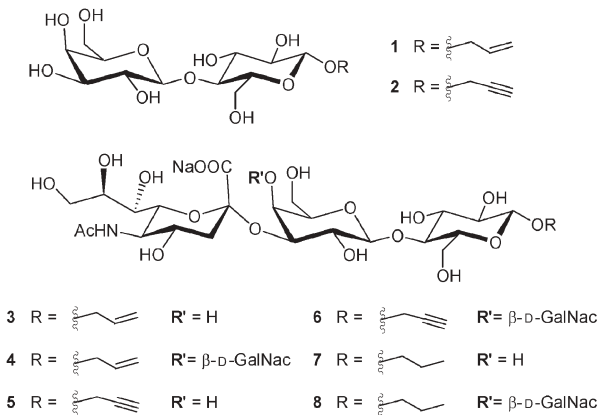
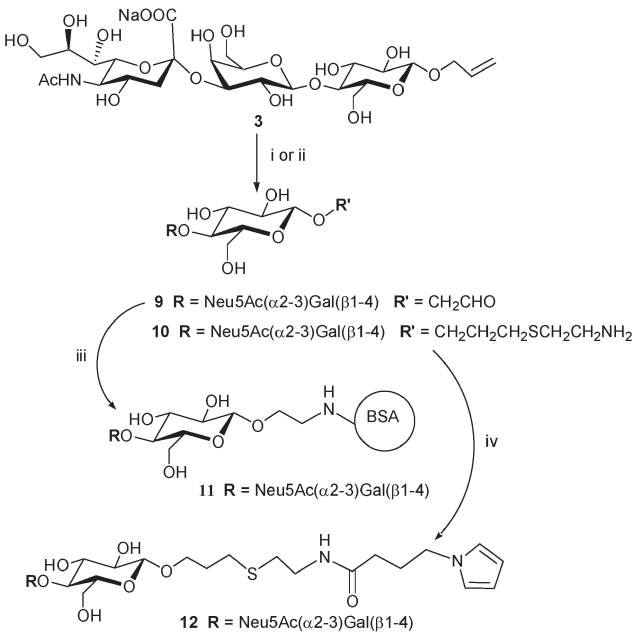


Fig. 1

with lactoside **1** (1.7 g) and sialic acid (1 equiv.) at the beginning of the fed-batch phase. TLC analysis of the fermentation time course showed that **1** accumulated transiently into the cell and was converted into the more polar sialyllactoside **3**, which accumulated first in the intracellular fraction, before being partly released in the supernatant. Both fractions were purified as previously described, by adsorption on activated charcoal followed by ion exchange chromatography, affording 840 mg of the expected trisaccharide **3**. The structure of this sialyllactoside was confirmed by mass spectrometry (*m*/*z*: 696 $[M + H]^+$; 718 $[M + Na]^+$) as well as by ^1H and ^{13}C NMR spectroscopy, which also demonstrated the good purity of the product. As allyl β-lactoside proved to be a good acceptor for the synthesis of **3**, the corresponding GM_2 saccharidic portion GalNAcβ1,4(NeuAcα2,3)Galβ1,4Glc was prepared. Cultivation of strain TA05[5] on glycerol supplemented with **1** (1.5 g) and NeuAc, afforded the allyl tetrasaccharide **4** (1.25 g), which was characterized by ^1H, ^{13}C NMR and mass spectrometry after purification as described earlier.

Propargyl β-lactoside **2**, which was prepared on 50 g scale in the laboratory, was also efficiently internalized and glycosylated within the living factory process. Saccharidic portions of GM_3 and GM_2 bearing the terminal alkyne function at the reducing end were efficiently prepared following the previous procedure. The tri- and tetrasaccharides **5** (670 mg) and **6** (900 mg) were isolated following the same procedure from 1-litre culture (1.7 g and 3 g of **2** respectively) and were characterized by MS and FT-IR spectroscopy. The characteristic C≡C vibrational band at 2114 cm^{-1} indicated the presence of the alkynyl group. NMR spectra of **5** and **6** confirmed the oligosaccharidic structures, although the proton and carbon signals of the triple bond were sometimes missing on spectra recorded in D_2O. This phenomenon, which was dependent on the NMR sample preparation, could be suppressed in DMSO-d_6. However, the resolutions of the spectra were dramatically decreased. For unambiguous characterization, NMR experiments were finally recorded in D_2O after reduction of the alkyne group by hydrogenolysis over $Pd(OH)_2$.

Other β-lactosides with nitrogen-containing aglycons were also tested as potential acceptors in the fermentation process. *N*-Allyl acetamide β-lactoside failed to be internalized into the cell whereas an azido ethyl lactoside gave poor results. The polarity of the aglycon seems to have a significant influence on the ability to be internalized, showing that the choice of suitable acceptors is not



Scheme 2 Preparation of GM_3 conjugates. i) O_3, MeOH, −50 °C, 1 h then Me_2S; ii) cysteamine hydrochloride (10 equiv.), H_2O, UV (254 nm), RT; iii) $NaBH_3CN$, BSA, H_2O, 37 °C; iv) pentafluorophenyl 4-(pyrrol-1-yl)butanoate, Et_3N, DMF, RT, 94% (2 steps).

trivial and acceptors have to be screened to extend our methodology.

To demonstrate the field of application offered by this approach, two GM_3 glycoconjugates were prepared. The protein conjugate **11** (Scheme 2) was obtained by ozonolysis of **3**, followed by reductive amination of aldehyde **9** with BSA as previously described by Hall.[7] After purification by filtration on a P6 biogel cartridge, the GM_3 content (26 mol of trisaccharide per mol of protein) was determined by a phenol–sulfuric acid assay.[10]

The pyrrole derivative **12** was further synthesized to develop a microarray for the study of carbohydrate–protein interactions. Pyrrole conjugates can be electropolymerized into thin electroconducting films that have proved to be remarkable biosensors.[11] Compound **12** was prepared through radical addition of a cysteamine linker onto the allyl group of **3**. The reaction was carried out in water by UV irradiation (254 nm) at room temperature for 48 hours with an excess of cysteamine hydrochloride (10 equiv.).[12] The resulting amine **10** was freeze dried and directly used in the next reaction without prior treatment, although it can be purified by Dowex® 50W × 4 cation exchange resin. The pyrrole motif was finally introduced by coupling **10** with an excess of a pentafluorophenyl activated carboxylic pyrrole[13] (see ESI†) in anhydrous dimethylformamide in the presence of triethylamine. After purification by flash chromatography on silica gel, **12** was isolated in 94% yield after the two steps.

In conclusion we have demonstrated that β-lactosides bearing latent chemical functions such as an allyl and a propargyl group, are efficiently internalized by the lactose permease into metabolically engineered *E. coli* cells. These lactosyl acceptors can be glycosylated by appropriate glycosyltransferases giving access, on the gram scale, to conjugatable forms of GM_2 and GM_3 saccharidic portions. Synthesis of GM_2 glycoclusters based on click chemistry, is currently underway and will be reported

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elsewhere in due course. A more detailed study of the range of modified lactosyl acceptors is also going on.

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Sébastien Fort,* Lemonia Birikaki, Marie-Pierre Dubois, Tatiana Antoine, Eric Samain and Hugues Driguez
Centre de Recherches sur les Macromolécules Végétales, CERMAV-FR-CNRS 2607, affiliated to Université Joseph Fourier Grenoble, BP 53, 38041 Grenoble Cedex 9, France.
E-mail: Sebastien.Fort@cermav.cnrs.fr; Fax: +33 (0)4 76 54 72 05; Tel: +33 (0)4 76 03 76 03

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