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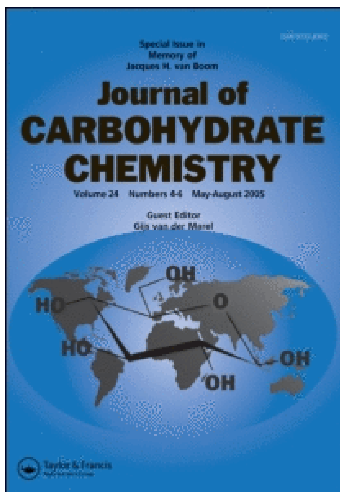

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An Efficient Acylation of Free Glycosylamines for the Synthesis of *N*-Glycosyl Amino Acids and *N*-Glycosidic Surfactants for Membrane Studies

Daniel Plusquellec[a]; Catherine Brenner-Hénaff[b]; Pascale Léon-Ruaud[a]; Stéphane Duquenoy[a]; Martine Lefeuvre[a]; Henri Wróblewski[b]
[a] ENSCR, Département de Chimie Organique, Laboratoire de Synthèses et Activations de Biomolécules, associé, Rennes, France [b] Département "Membranes et Osmorégulations", Laboratoire d'Immunochimie des Membranes Bactériennes, associé, Université de Rennes I, Rennes cedex, France

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AN EFFICIENT ACYLATION OF FREE GLYCOSYLAMINES FOR THE SYNTHESIS OF *N*-GLYCOSYL AMINO ACIDS AND *N*-GLYCOSIDIC SURFACTANTS FOR MEMBRANE STUDIES.

Daniel Plusquellec,[1*] Catherine Brenner-Hénaff,[2] Pascale Léon-Ruaud,[1] Stéphane Duquenoy,[1] Martine Lefeuvre[1] and Henri Wróblewski [2]

[1] ENSCR, Département de Chimie Organique, Laboratoire de Synthèses et Activations de Biomolécules, associé au CNRS URA D1467, Avenue du Général Leclerc, 35700 Rennes, France.

[2] Université de Rennes I, Département "Membranes et Osmorégulations", Laboratoire d'Immunochimie des Membranes Bactériennes, associé au CNRS URA D0256, Campus de Beaulieu, 35042 Rennes cedex, France.

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ABSTRACT

Treatment of free glycosylamines with 3-acyl-5-methyl-1,3,4-thiadiazole-2 (3*H*)-thiones **6** or with acids and 5-methyl-2-thioxo-1,3,4-thiadiazole-3(2*H*)-carbothioic acid *S*-(5-methyl-1,3,4-thiadiazol-2-yl) ester **7** in hydroorganic media afforded *N*-acylglycosylamines in high yields and without any competitive deglycosylation. This reaction found applications in the synthesis of *N*-glycopeptide building blocks and of glycosidic non ionic surfactants. Results concerning surface activities of two *N*-acylglycosylamines are reported. The new non ionic *N*-octanoyl-β-D-glucosylamine surfactant exhibited efficacy and selectivity in the extraction of membrane proteins, enhanced the activity of a membrane succinate dehydrogenase and proved thus useful for membrane studies.

INTRODUCTION

The selective synthesis of *N*-acylglycosylamines under mild conditions in the presence of unprotected hydroxyl groups is of current interest due to the presence of such linkages in a number of glycoproteins[1] or in synthetic immunomodulators.[2] Methods presently used for the preparation of *N*-glycopeptides models include condensation of *N*-protected aspartic acid α-monoesters with protected β-D-glycosylamines using various

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coupling reagents to give a protected asparagine-carbohydrate conjugate.[1] The amine has been previously lengthily prepared from the fully acylated glycosyl chloride *via* the azide. To avoid this tedious azide route, methods based on the use of unprotected glycosylamines were developed more recently.[3] Due to the high lability of unprotected 1-aminosugars and their tendancy to dimerise and to deglycosylate,[4] realization of the glycosylamide linkage with a high yielding coupling requires mild conditions.

In the course of our ongoing program devoted to the synthesis [5] and uses [6] of non ionic glycosidic surfactants for the selective extraction of membrane proteins with retention of their native properties, we were interested in designing glycosylamides derived from fatty acids and various glycosylamines.[7] We describe herein a simple and efficient synthetic method that leads to large scale syntheses of such compounds. This method proved furthermore to be very effective in the preparation of *N*-glycopeptide building blocks.

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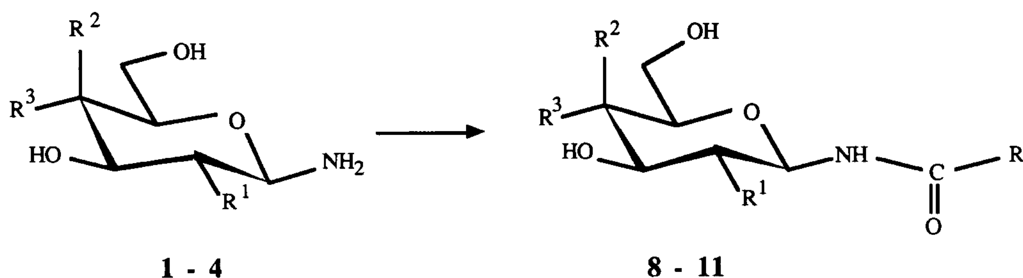
RESULTS AND DISCUSSION

Synthesis of N-acylglycosylamines and N-glycosylamino acids

Glycopyranosylamines **1-4** (Fig. 1) were prepared essentially as previously described by treating the corresponding reducing sugars either with methanolic ammonia [8] or aqueous ammonium hydrogenocarbonate [9] and the major products crystallized as the β-anomers owing to the weak anomeric effect of the nitrogen atom.[10,11] Glycosylamines, especially those that do not contain an acetamido group at the C2 of the pyranose ring, undergo spontaneous hydrolysis in neutral or slightly acidic aqueous solutions.[4,12] We have therefore selected β-D-glucopyranosylamine **1** as substrate for the demonstration of the methodology.

When a solution of compound **1** in anhydrous DMF was treated with lauroyl chloride in the presence of pyridine, deglycosylation occurred readily and the primary dodecanoylamide was isolated quantitatively. Surprisingly the *N*-lauroylthiazolidine-2-thione reacted slowly with amine **1** at room temperature in pyridine or dimethylformamide, but reaction was achieved within 4-5 hours at 60-70 °C. Nevertheless, the expected *N*-lauroyl-β-D-glucopyranosylamine **8c** was obtained in low yield (*ca.* 10 %) associated with the primary dodecanoylamide and a mixture of anomeric diglucosylamines isolated as their *O*-acetylated derivatives. These results have to be compared with previous works, *e.g.* acetolysis of free glycosylamines which causes drastic alterations in the substrates.[13]

To overcome these problems, we therefore used reagent **6a** derived from 2-mercapto-5-methyl-1,3,4-thiadiazole.[5a] Effectively, the reaction of **6a** with glucosylamine

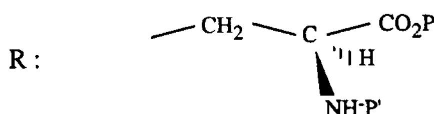


1: R^1=R^3=OH; R^2=H (β-D-Glucopyranosylamine)→ **8a-e**
2: R^1=R^2=OH; R^3=H (β-D-Galactopyranosylamine) → **9a,c**
3: R^1=OH; R^2=H; R^3=O-β-D-galactopyranosyl (β-D-Lactosylamine)→ **10c**
4: R^1=NH-Ac; R^2=H; R^3=OH (2-acetamido-2-deoxy-β-D-glucopyranosylamine)→ **11f**

R: - $(CH_2)_m$ - CH_3

	a	b	c	d
m	6	8	10	12

R :



e: P = Bn; P' = BOC
f: P = CH_3; P' = Ac

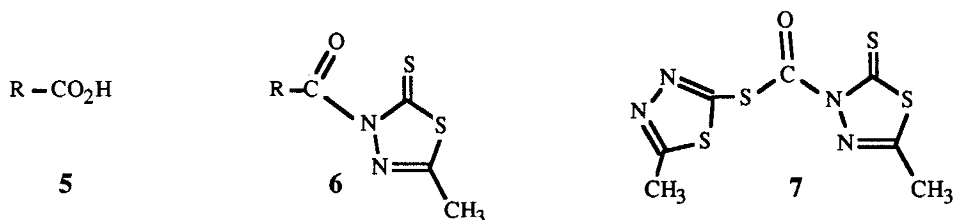


Fig. 1. Reaction scheme for the synthesis of *N*-acyl-β-D-glycosylamines

1, in DMF solution at room temperature for 30 min, afforded the expected *N*-octanoyl-β-D-glucosylamine **8a** in a 70 % yield (Table 1, entry 1) without any deglycosylation product or amido ester.

We expected to improve the procedure for synthesizing *N*-glycosylamides in water-organic solvent mixtures if the pH of the reaction medium could be maintained slightly

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Table 1. Conversion of glycosylamines **1-4** to the corresponding *N*-acylglycosylamines

Entry	Glycosylamine	Reagent(s)	Procedure [a]	*N*-Acylglycosylamine (%)
1	**1**	**6a**	A	**8a** (70)
2	**1**	**6a**	B	**8a** (75)
3	**1**	**5a,7**	C	**8a** (86)
4	**1**	**5b,7**	C	**8b** (78)
5	**1**	**5c,7**	C	**8c** (90)
6	**1**	**5d,7**	C	**8d** (88)
7	**1**	**5e,7**	C	**8e** (72)
8	**2**	**6a**	A	**9a** (49)
9	**2**	**5c,7**	C	**9c** (84)
10	**3**	**5c,7**	C	**10c** (85)
11	**4**	**5f,7**	C	**11f** (72)

a. Procedure A: reagent **6**, DMF, RT for 30 min. Procedure B: reagent **6**, acetone/water (1:2, v/v), RT for 1 h. Procedure C: acid **5** and reagent **7**, Et_3N, acetone/water (9:2, v/v), RT for 2 h.

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basic. Indeed, when a solution of **6a** in acetone was added, in one portion, to a freshly prepared aqueous solution of β-D-glucosylamine **1** in water, acylation occurred selectively within 1 hour at room temperature. Acetone was removed under reduced pressure and the residue was next subjected to our usual procedure. Compound **8a** was thus isolated in a 75 % yield (Table 1, entry 2).

To avoid the preparation of the intermediate **6a** and in view of the synthesis of models of β-*N*-glycopeptides by coupling aspartic acid derivatives with glycosylamines, we then used the coupling reagent **7**.[14] Thus, a solution of octanoic acid **5a**, thiocarbamate **7** and triethylamine in acetone was stirred for 1 hour at room temperature. An aqueous solution of β-D-glucosylamine **1** was then added along with a catalytic amount of DMAP. Decolourization of the yellow solution occurred within 2 hours at the same temperature and *N*-octanoyl-β-D-glucosylamine **8a** was thus obtained in a 85 % yield.

These encouraging results prompted us to employ this coupling procedure to a series of fatty acids and to glycosylamines **1-3**. Our results are summarized in Table 1. In all cases, the acid, the coupling reagent, the organic base and the amine were used in stoichiometric ratios. Obviously, this method allows convenient access to the synthesis of a variety of *N*-acylglycosylamines derived from monosaccharides **1-2** and from lactosylamine **3** in very high yields.

In studies on the coupling of glycosylamines with the 4-carboxyl of aspartic acid derivatives, a high yield coupling reaction without any risk of anomerization of the glycosidic bond nor racemization was required. Treatment of the side chain of aspartate derivatives **5e** and **5f** with reagent **7** in acetone solution containing NEt_3 and then with an aqueous solution respectively of amine **1** or **4**, afforded the corresponding β-*N*-glycosylaminoacids **8e** and **11f** (Table 1, entries 7 and 11) in high yields of pure products. None of the α-anomers were detected by TLC or NMR even for derivative **8e** lacking an acetamido group at C_2. In the ^1H NMR spectra of compounds **8e** ($DMSO+D_2O$) and **11f** (D_2O) the H_1 protons appeared as doublets respectively at 4.75 ppm ($J_{1,2}$ = 8.9 Hz) and 4.8 ppm ($J_{1,2}$ = 9.2 Hz) indicating a β configuration of the anomeric linkage. Compound **11f** was easily purified by preparative column chromatography and **8e** was obtained without chromatography by recrystallization from ethyl acetate. These results suggest a widespread applicability of the coupling method described herein.

Surface active properties and critical micellar concentrations (CMC's) of N-acylglycosylamines

Compounds **8a** and **9a** proved to be soluble in water at concentrations up to 30 g/L for **8a** and 25 g/L for **9a**. Neutral and slightly basic (pH 8.5) aqueous solutions were stable at room temperature for several weeks. The surface tension of pure water (72 mN/m at 25 °C)[15] and that of Na-phosphate buffers were reduced by surfactants **8a** and **9a** to about 30 mN/m at concentrations above the CMC. Plotting γ (mN/m) *vs* log C indicated a CMC of about 70 mM for **8a** and 45 mM for **9a** in pure water (Fig. 2). The previous results were corroborated spectrofluorometrically using ANS and DPH as fluorescent probes.[16] A CMC of 80 mM in 0.1 M phosphate buffer at pH 7.5 was found for compound **8a**. These results reflect the hydrophobic head group effect on the CMC's of glycosidic surfactants showing the same hydrocarbon chain. Elucidation of this phenomenon requires further investigation but we believe that the lower CMC of **9a** might result from a lesser extent of hydration of a galactosyl moiety as compared to that of a glucosyl one. These high CMC values make these surfactants especially suitable for biological applications when removal of the detergent by dialysis is necessary.

Membrane protein extraction with detergent 8a and effect on membrane succinate dehydrogenase

Previous studies [6,17] have shown that glycosidic amphiphiles are of general interest in membranology for the solubilization and the purification of membrane proteins in the perspective of their structural and functional characterization. Moreover, these surfactants

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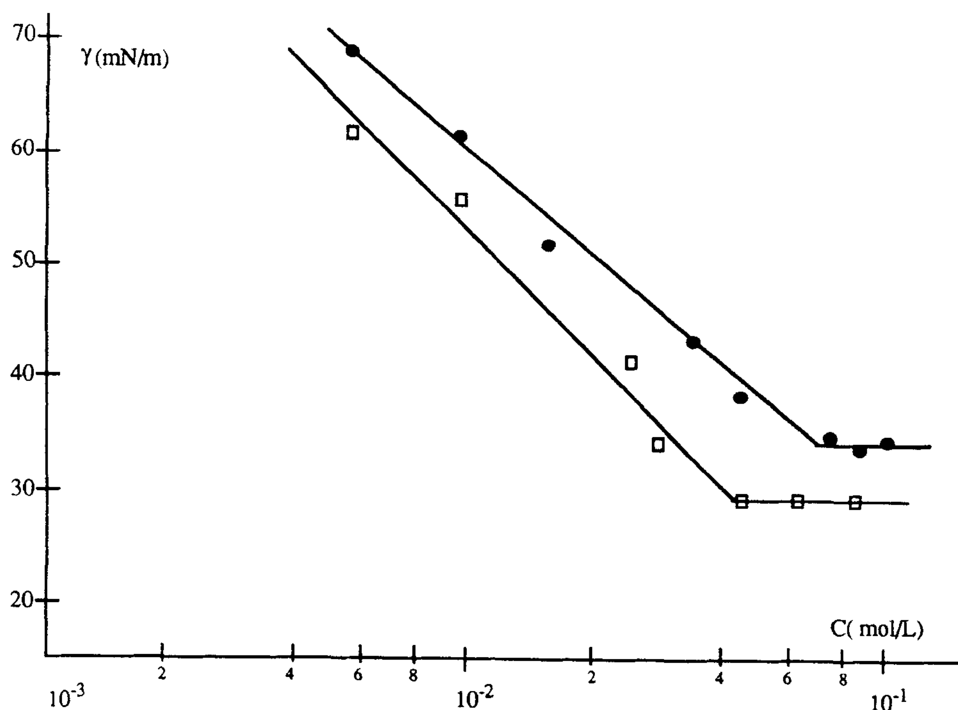

Fig. 2. Plot of the surface tension of pure water *versus* the concentration of compounds **8a** (•) and **9a** (□) in water at 25 °C.

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generally allow proteins to be obtained without denaturation.[17] Membrane proteins of *Acholeplasma laidlawii* A were extracted using *N*-octanoyl-β-D-glucosylamine **8a** (trivial name NOGA). Figure 3 shows that, as expected, membrane protein solubilization strongly depended on NOGA concentration with, however, two distinct levels of efficacy. Extraction started well below the CMC and a plateau of 24 % extracted protein was reached for concentrations *ca.* 50 mM NOGA, corresponding to a ratio of 24 μg membrane protein/μmode surfactant. The efficacy was shifted up by using NOGA above its CMC. The maximum level of extraction, *i.e.* 42 % solubilized protein, was reached for a surfactant concentration of 80 mM, corresponding to a ratio of 26 μg protein/μmole of NOGA. This phenomenon was confirmed, in the present case, by SDS-PAGE analysis (see Fig.3) and was also observed, though to a lesser extent, in the case of a *Spiroplasma* membrane.[26]

SDS-PAGE analysis (Fig. 4) revealed that 15 polypeptides of the 38 polypeptides detected by the technique, were selectively extracted. It is noteworthy that, except a

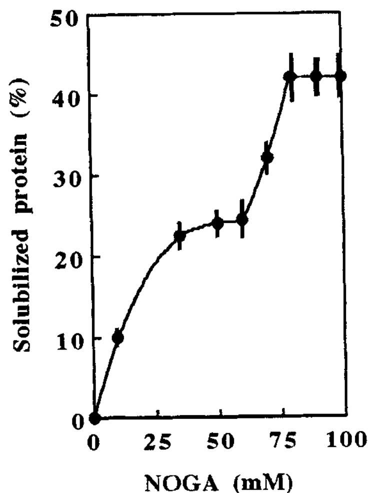

Fig. 3. Extraction of *A. laidlawii* membrane proteins with NOGA. Membrane proteins were extracted with different concentrations of NOGA as described under Materials and Methods. Protein concentrations were determined in the soluble fractions and expressed as the percentage of total protein concentration in the starting membrane material (5 mg protein/mL).

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polypeptide of 15 kDa, all the other components which were extracted displayed molecular masses above 27 kDa. The highest degree of selectivity was observed with 50 mM NOGA (Fig. 3, lane 5), corresponding to a ratio of 24 µg membrane protein/µmole surfactant. Indeed, in these conditions, only 10 polypeptides were extracted, with the 40, 45.5 and 48 kDa components being the major ones.

The effect of NOGA on the activity of *Pasteurella multocida* succinate dehydrogenase, a bacterial integral plasma membrane enzyme, is summarized in Table 2. Below the CMC, NOGA slightly increased the K_m of the enzyme but had no effect on the V_{max}. However, when used above the CMC, the K_m was decreased more than twofold whereas the maximum velocity was increased by a factor of 1.5, suggesting an activation of the enzyme. In the case of enzymes bound to the inner face of closed vesicles an apparent activation may result from permeabilization or solubilization of the membranes by the surfactant.[25] However, since we have used reverted membrane vesicles obtained by the French press method and exposing succinate dehydrogenase to the outer medium, this explanation does not hold in the present study. We instead believe that the enhancement of enzymatic activity reflects a true activation of the enzyme by high NOGA concentrations. Elucidation of this mechanism requires further investigation.

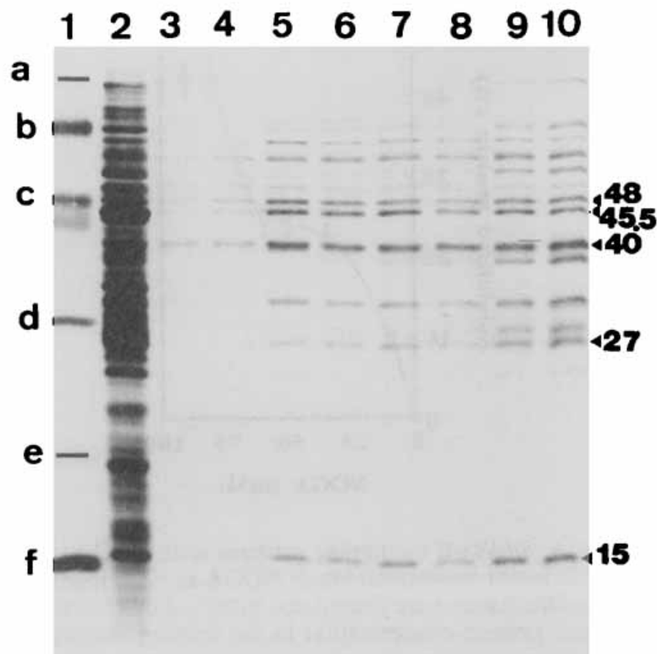

Fig. 4. SDS-PAGE analysis of proteins extracted with NOGA from the membrane of *A. laidlawii*. Lanes: (1) Molecular mass marker proteins: (a) phosphorylase b, 94kDa; (b) serum albumin, 67 kDa; (c) ovalbumin, 43 kDa, (d) carbonic anhydrase, 30 kDa; (e) trypsin inhibitor, 20.1 kDa; and (f) α-lactalbumin, 14.4 kDa. (2) 10 μg of *A. laidlawii* membrane protein extracted with 1 % SDS (this sample is the reference corresponding to 100 % of extracted protein). (3) 1.0 μg of protein extracted with 10 mM NOGA. (4) 2.2 μg of protein extracted with 35 mM NOGA. (5) 2.4 μg of protein extracted with 50 mM NOGA. (6) 2.4 μg of protein extracted with 60 mM NOGA. (7) 3.2 μg protein extracted with 70 mM NOGA. (8) 4.2 μg protein extracted with 80 mM NOGA. (9) 4.2 μg protein extracted with 90 mM NOGA. (10) 4.2 μg protein extracted with 100 mM NOGA. Proteins bands were silver stained. The polypeptides mentioned in the text are pointed out with arrow heads and their masses are given in kilodaltons.

Table 2 . Effect of NOGA on membrane succinate dehydrogenase

NOGA conc.		$K_m \pm SD$	V_{max}
%	mM	(μM)	(μmol/min/mg protein)
0	0	0.40 ± 0.03	53.41
0.3	10	0.42 ± 0.01	54.35
3.1	100	0.15 ± 0.01	82.00

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Altogether, these data show that NOGA **8a** is a mild surfactant displaying selectivity toward membrane protein extraction and capable of enhancing membrane enzyme activity. Interestingly, in the case of the *A. laidlawii* plasma membrane, the highest degree of selectivity was observed when using NOGA at concentration close to 50 mM *i.e.* below the CMC of the surfactant.

CONCLUSION

Employing the methodology described herein, high yielding acylations of unprotected glycosylamines were performed without deglycosylation nor epimerization. *N*-octanoyl-β-D-glucosylamine proved *inter alia* to be easily purified at low cost for membrane studies and exhibited a good solubilizing power towards membrane proteins with a high selectivity for three polypeptides out of the 38 proteins detected in the membrane of *A. laidlawii*, a bacterial model system which has been widely used for membrane research. Its mildness towards proteins was on the other hand noteworthy. The suitability of this surfactant for the selective extraction of integral antigens has recently been reported.[25]

As an extension to these studies, our methodology proved to be useful in the synthesis of *N*-glycopeptide building blocks. Our current focus is to test the limits of this reaction regarding the size of the glycosyl and the peptides moieties.

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EXPERIMENTAL

General procedures. Melting points were determined with a Reichert melting point apparatus and are uncorrected. Optical rotations were measured using a Polartronic D polarimeter. ^{1}H and ^{13}C NMR spectra were obtained with a JEOL FX 90 and 22.5 MHz Fourier transform spectrometer; chemical shifts were expressed in δ values (ppm), using tetramethylsilane as an internal standard. Infrared spectra were recorded on a Pye-Unicam SP200 spectrophotometer. TLC was performed on Merck 60 F 254 silica gel unactivated plates with a mixture of chloroform, methanol, acetic acid and water (37: 8: 4: 1, v/v) as the solvent system. Compounds were visualized by spraying the plates with 5 % sulfuric acid in ethanol and by charring them on a hot plate. Preparative column chromatography was performed with Merck 60 H (5-40 μm) silica gel with the solvent systems specified. Elemental analyses were made by the "Service Central d'Analyse du CNRS", Vernaison (France) or by the "Service de Microanalyses de l'ENSCR", Rennes, (France).

2-Mercapto-5-methyl-1,3,4-thiadiazole was purchased from Janssen Chimica (Belgium) and *N-t*-BOC-L- aspartic acid α-benzyl ester **5e** from Sigma Chimie (France). Acylating reagent **6a** was prepared from chloride and 2-methyl-5-mercapto-1,3,4-thiadiazole in dichloromethane in the presence of triethylamine according to our previous work.[5a] β-D-glucosylamine **1**, β-D-galactosylamine **2** and β-D-lactosylamine were obtained from the corresponding reducing sugars and ammonia.[8] 2-Acetamido-2-deoxy-β-D-glucopyranosylamine **4** was prepared according to procedures previously described by Kallin *et al.* and Likhosherstov *et al.*.[9] Aspartic acid derivative **5f** was synthesized following classical routes according to previous works.[19] All reagents were of commercial quality and were purchased from Janssen Chimica or Aldrich Chemie.

General procedures for the synthesis of *N*-acyl-β-glycopyranosylamines (8-11). Method A. β-D-glucopyranosylamine **1** (1.34 g, 7.5 mmol) was dissolved in 60 mL of anhydrous dimethylformamide by heating the mixture at 70 °C for 5 min. The solution was allowed to cool at room temperature and the acylating reagent **6a** (5 mmol) was added in one portion. The solution was stirred at the same temperature until decolourization of the yellow solution had occured (30 min). The solvent was removed *in vacuo* at 35-40 °C and the solid residue was then diluted with a potassium phosphate buffer (pH 7.0, 40 mL) and 1-butanol (40 mL). The aqueous phase was extracted twice with 1-butanol (2 x 20 mL) and the combined organic layers were washed with water (3 x 10 mL) and concentrated. The residue was subjected to column chromatography eluting with a chloroform/methanol step gradient mixture that varied from 1 % to 20 % methanol as eluent to give *N*-octanoyl-β-D-glucosylamine (65 %) as a white powder.

Method B. To a solution of β-D-glucopyranosylamine **1** (8.05 g, 45 mmol) in 25 mL of water was added a solution of amide **6a** (7.75 g, 30 mmol) in 60 mL of acetone. After 60 min at room temperature the organic solvent was removed and the aqueous layer was extracted three times with 1-butanol (3 x 20 mL). The solvent was removed under vacuum and column chromatography was used to separate *N*-octanoyl-β-D-glucosylamine **8a** from 2-mercapto-5-methyl-1,3,4-thiadiazole *(vide supra)*. Fractions containing the low Rf material were combined and evaporated to obtain 6.6 g (73 %) of **8a**.

Method C. 5-methyl-2-thioxo-1,3,4-thiadiazole-3(*2H*)-carbothioic acid *S*-(5-methyl-1,3,4-thiadiazol-2-yl) ester 7. To a stirred solution of phosgene (7.77 mL of a 1.93 M solution in toluene, 15 mmol) in dry dichloromethane (20 mL), cooled to -15 °C, was added a solution containing 2.96 g (30 mmol) of 2-mercapto-5-methyl-1,3,4-thiadiazole and pyridine (4.84 mL, 60 mmol) in dichloromethane (50 mL). The mixture was stirred at -10 °C for 1 h and at 0 °C for an additional 1h while the course of the reaction was monitored by TLC. The solution was successively washed with 0.5 N

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HCl (2 x 20 mL) and water (2 x 40 mL), dried ($MgSO_4$) and concentrated to a solid residue which was recrystallized from chloroform. Compound **7** (4.85 g, 85 %) was obtained as yellow needles: mp 172-176 °C; IR (HCB) 1760 and 1735 cm^{-1} (carbonyl); ^1H NMR ($CDCl_3$) δ 2.56 (s, 3H, CH_3) and 2.84 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ 15.85 (CH_3), 16.48 (CH_3), 157.25, 157.65 and 162.28 (3 C=N), 169.12 (C=O) and 185.04 (C=S).

Anal. Calcd for $C_7H_6ON_4S_4$: C, 28.94; H, 2.08. Found: C, 28.81; H, 2.28.

***N*-Acylglycosylamines.** Reagent **7** (8.7 g, 30 mmol) was added at room temperature to a solution containing the appropriate carboxylic acid **5** (30 mmol) and triethylamine (4.15 mL, 30 mmol) in acetone (90 mL) and the mixture was stirred at room temperature for 1 h. To this solution was added the β-D-glucosylamine **1** (30 mmol) and DMAP (0.36 g, 3 mmol) in water (20 mL). The mixture was stirred at the same temperature for 2.5 h. Acetic acid was added and the organic solvent was removed under reduced pressure. To the residue were added 1-butanol (40 mL) and a phosphate buffer at pH 7 (20 mL). The aqueous layer was further extracted twice with a mixture of 1-butanol, ethyl acetate (1:1, v/v) (2 x 20 mL) and the combined extracts were washed with water and concentrated. The residue was either chromatographed or recrystallized twice to give the expected *N*-acylglycosylamines in high yields.

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***N*-Octanoyl-β-D-glucopyranosylamine (8a).** Yield 86 % (method C); white powder when crystallized from ethyl acetate and methanol; mp 185-186 °C; $[\alpha]_D^{20}$ + 15.0 (*c* = 0.01 g/mL in dimethylformamide); IR (hexachlorobutadiene) 3440 (broad, OH), 3320 (NH) and 1630 cm^{-1} (amide I); ^1H NMR (DMSO-d_6) δ 0.88 (3H, t), 1.27 (8H, m), 1.50 (2H, m), 2.11 (2H, t), 3.10-3.70 (6H, m), 4.50 (1H,t), 4.6-5.0 (3H, m), 4.72 (1H, d, after isotopic exchange with D_2O, $J_{1,2}$ = 8.5 Hz), 8.27 (1H, d); ^{13}C NMR (DMSO-d_6) δ 14.55 (CH_3), 22.76, 25.63, 29.15, 29.34, 31.86 and 36.20 (CH_2), 61.58 (C_6), 70.43 (C_4), 72.74 (C_2), 77.72 (C_5), 78.64 (C_3), 80.02 (C_1), 175.02 (C=O).

Anal. Calcd for $C_{14}H_{27}NO_6$: C, 55.06; H, 8.91. Found: C, 54.80; H, 8.96.

***N*-Decanoyl-β-D-glucopyranosylamine (8b).** Yield 78 % (method C); mp 186-188 °C from EtOH; lit.[20]; $[\alpha]_D^{20}$ + 14.3° (*c* = 0.01 g/mL in dimethylformamide); IR and NMR data were identical with those of compound **8a**.

Anal. Calcd for $C_{16}H_{31}NO_6$: C, 57.63; H, 9.37. Found: C, 58.00; H, 9.15.

***N*-Dodecanoyl-β-D-glucopyranosylamine (8c).** Yield 90 % (method C); mp 185-187 °C from EtOH; lit.[20]; $[\alpha]_D^{20}$ + 13.9° (*c* = 0.01 g/mL in dimethylformamide); IR and NMR were identical with those of compound **8a**.

***N*-Tetradecanoyl-β-D-glucopyranosylamine (8d).** Yield 88 % (method C); mp 183-186 °C from EtOH; lit. [20]; $[\alpha]D^{20}$ + 9.1 (*c* = 0.01 g/mL in dimethylformamide; IR and NMR were identical with those of compound **8a**.

Anal. Calcd for $C_{20}H_{39}NO_6$: C, 61.66; H, 10.99. Found: C, 61.52; H, 10.10.

***N*-[1-benzyl *N*-(*tert*-butoxycarbonyl)-L-aspart-4-oyl]-β-D-glucopyranosylamine (8e).** Yield 72 % (method C); mp 162-164 °C from ethyl acetate; $[\alpha]_D^{20}$ -8 (*c* = 0.01 g/mL in methanol); IR (hexachlorobutadiene) 3400 (OH), 3300 (NH), 1730 and 1690 (ester and carbamate), 1635 cm^{-1} (amide); ^{13}C NMR (DMSO-d_6)δ 28.15 (CH_3), (CH_2), 50.04 (CH), 61.03 (C-6), 66.02 (CH_2-C_6H_5), 70.04 (C-4), 72.55 (C-2), 77.42 (C-5), 78.53 [C-3 and *C* $(CH_3)_3$], 79.70 (C-1), 127.67, 127.97, 128.37 and 135.96 (arom), 155.22 (carbamate CO), 169.44 and 171.79 (ester and amide CO).

Anal. Calcd for $C_{22}H_{32}O_{10}N_2$: C, 54.53; H, 6.66; N, 5.78. Found: C, 54.26; H, 6.88; N, 5.54.

***N*-Octanoyl-β-D-galactopyranosylamine (9a).** Compounds **9a** was synthesized using method A previously described and was purified by column chromatography eluting with chloroform and then with chloroform-methanol mixtures that varied from 10 % to 17 % methanol as eluents. Yield 49 %; mp 175-178 °C from ethyl acetate and methanol; $[\alpha]_D^{20}$ + 27.9° (*c* = 0.01 g/mL in dimethylformamide); IR (hexachlorobutadiene) 3440 (broad, OH), 3320 (NH) and 1630 cm^{-1} (amide I); ^1H NMR (DMSO-d_6) δ 0.88 (3H, t), 1.26 (8H, m), 1.50 (2H, m), 2.11 (2H, t), 3.10-3.70 (6H, m), 4.40-4.79 (4H,m), 4.68 (1H, d, after isotopic exchange with D_2O, $J_{1,2}$ = 9.1 Hz), 8.27 (1H, d); ^{13}C NMR (DMSO-d_6) δ 13.93 (CH_3), 22.08, 24.95, 28.53, 28.75, 31.21 and 35.49 (CH_2), 60.52 (C_6), 68.29 (C_4), 69.73 (C_2), 74.28 (C_3), 76.58 (C_5), 80.00 (C_1), 172.74 (C=O).

Anal. Calcd for $C_{14}H_{27}NO_6$: C, 55.06; H, 8.91. Found: C, 55.41; H, 8.73.

***N*-Dodecanoyl-β-D-galactopyranosylamine (9c).** Compound **9c** was prepared as previously described in method C and was then chromatographed on a column of silica gel using 85:15 chloroform-methanol as the eluent. Yield 84 %; mp 187-189 °C from ethanol; $[\alpha]_D^{20}$ + 25.5 (*c* = 0.01 g/mL in dimethylformamide); IR and NMR were identical with those of compound **9a**.

Anal. Calcd for $C_{18}H_{35}NO_6$: C, 59.83; H, 9.76. Found: C, 59.98; H, 9.86.

***N*-Dodecanoyl-β-D-lactosylamine (10c).** Compound **7c** was prepared from 2.57 g (7.5 mmol) of β-D-lactosylamine **3** and 1.00 g (5 mmol) of lauric acid and 1.45 g (5 mmol) of reagent **7** in a water:acetone mixture as described in method C. After the reaction had occurred to completion, the mixture was worked up and the residue was recrystallized twice from ethanol/water (90:10, v/v) to give 2.38 g (85 %) of **10c** which crystallized as a dihydrate. mp 246-248 °C from ethanol and water; $[\alpha]_D^{20}$ + 13.8° (*c* = 0.01 g/mL in dimethylformamide); IR (hexachlorobutadiene) 3380 (broad, OH), 3280 (NH) and 1660 (amide I) cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.88 (3H, t), 1.26 (16H, m), 1.50 (2H, m), 2.11 (2H, t), 3.10-3.70 (12H, m), 4.25 (1H,m, $H_{1'}$), 4.52-5.15 (7H, m),

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4.55-5.18 (7H, m), 4.76 (1H, d, after isotopic exchange with D_2O, H_1, $J_{1,2}$ = 8.5 Hz); ^{13}C NMR (DMSO-d_6) δ 13.85 (CH_3), 22.08, 24.93, 28.72, 29.02, 31.32 and 35.49 (CH_2), 60.62 (C_6 and $C_{6'}$), 68.29 ($C_{4'}$), 70.70 ($C_{2'}$), 72.14 (C_2), 73.30 ($C_{3'}$), 75.63 (C_5 and $C_{5'}$), 76.39 (C_3), 80.65 (C_1) 103.78 ($C_{1'}$), 172.85 (C =O).

Anal. Calcd for $C_{24}H_{45}NO_{11}.2H_2O$: C, 51.43; H, 8.81. Found: C, 51.43; H, 8.70.

2-Acetamido *N*-[1-methyl *N*-(acetamido)-L-aspart-4-oyl]-2-deoxy-β-D-glucopyranosylamine (11f). Compound **11f** was obtained as previously described in method C from 1.1 g (5 mmol) of glycosylamine **4**, 0.94 g (5 mmol) of aspartate derivative **5f**, 1.45 g (5 mmol) of reagent **7**, 0.7 mL (5 mmol) of triethylamine and 10 mg of DMAP in a water acetone mixture (20 mL, 1:4, v/v). The crude product was purified by open column chromatography affording 1.4 g (72 %) of compound **11f** whose analytical data were identical with those of an authentical sample.[19]

Determination of surface tensions and critical micellar concentrations (CMC). The water soluble *N*-octanoyl-β-D-glycosylamines **8a** and **9a** were tested for physical properties (surface tension and CMC). For surface tension measurements, compounds **8a** and **9a** were dissolved in a 0.1 M sodium phosphate buffer (pH 7.4) or in twice distilled water at 25 °C and surface tensions were measured as a function of concentration using a Krüss K12 tensiometer with a Du Noüy ring at the same temperature. Each measurement was performed two or three times. The CMC of the surfactant **8a** was also determined spectrofluorometrically with the techniques of de Vendittis *et al.* and Chattopadhyay and London using the fluorescent probes ANS (λ_{exc} = 370 nm, λ_{em} = 490 nm) and DPH (λ_{exc} = 358 nm, λ_{em} = 430 nm), respectively.[16] Measurements were performed with a Kontron SFM 25 spectrofluorimeter equipped with a thermostated cell holder, using 1-cm path length quartz cuvettes. The samples were held in the dark to avoid photoisomerization of DPH.

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Membranes preparation and protein extraction with surfactants. Membranes of *Acholeplasma laidlawii* A (strain PG8) and of *Pasteurella multocida* (strain 9222) were prepared as described previously.[6] Membrane protein extraction was performed by mixing one volume of membrane suspension (10 mg of protein/mL) with one volume of surfactant in 0.1 M Na phosphate buffer (pH 7.5). Extractions with SDS were performed for 10 min at room temperature. Extractions with neutral surfactants were performed for 1 h at 4 °C. The mixtures were centrifuged at 260.000 g for 15 min (Beckman TL-100 ultracentrifuge, TLA 100.1 rotor) and the supernatants were recovered and stored at -30 °C.

SDS-polyacrylamide gel electophoresis (SDS-PAGE). Solubilized membrane proteins were separated in 120 x 120 x 1-mm polyacrylamide gels in the

presence of 0.1 % SDS.[21] Compositions of the stacking gel and of the separating gel were T = 4.8 %, C = 2.6 % and T = 12.6 %, C = 2.6 %, respectively.[22] Polypeptides were silver stained according to the method of Tunon and Johansson.[23]

Protein and NOGA determinations. Protein concentration was determined with the bicinchoninic acid method, [24] using serum albumin as standard.

Assay of succinate dehydrogenase. Succinate dehydrogenase of the plasma membrane of *P. multocida* was assayed as described in Osborn *et al.* [18] in 50 mM sodium phosphate buffer (pH 7.4) containing 10 mM KCN, sodium succinate (concentration range: 0.25 to 25 mM), 30 μg of thiazolyl blue and 10μg of phenazine methosulfate per milliliter.

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