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LIPASE-CATALYZED CHEMOSELECTIVE N-ACYLATION OF AMINO-SUGAR DERIVATIVES IN HYDROPHOBIC SOLVENT : ACID-AMINE ION-PAIR EFFECTS.

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Abstract : Enzymatic N-acylation of N-methyl-glucamine (1-deoxy-1-methylamino-D-glucitol) in hexane using lipase from *Rhizomucor miehei* (Lipozyme®) is described. N-methyl-glucamine was solubilized by oleic acid addition which resulted in the formation of an ion-pair between acid and amine function. This ion-pair, identified by Infra-Red spectroscopy, is essential for amide or ester synthesis. Its stability in hexane was also found to be the limiting factor of reaction yield which never exceeded 50 % of acid conversion. The chemoselectivity of the reaction between oleic acid and N-methyl-glucamine towards amide or ester synthesis was under the control of acid/amine ratio. This is the first report showing the key role of substrate ionic state when operating enzyme catalysis in non-conventional media. © 1997 Elsevier Science Ltd.

INTRODUCTION

Most surfactants presently used in cosmetic products or detergent compositions are based on chemical derivatives. Because of increased concern over environmental issues, it seems advantageous to develop surfactants from raw materials of agricultural origin such as carbohydrates[1]. These natural compounds represent a source of renewable raw materials which are synthetically versatile, inexpensive, optically pure and environmentally safe. From this point of view, sugar amide surfactants and derivatives constitute an extremely important class of industrial chemicals. Such non-ionic amide surfactants are biodegradable and more stable in alkaline media[2] than ester-based surfactants.

One traditional method for preparing sugar fatty amide surfactants includes the Schotten-Baumann reaction between an amine and a fatty acid chloride in aqueous alkaline medium. But with this method, it is necessary at the end of the reaction to remove the chloride salt coproduced with the amide in order to preserve the good detergent live nature of the compound. In view of the numerous difficulties encountered in the production of sugar amide surfactants by chemical synthesis, alternative enzymatic methods have been suggested. In fact, the enzymes which are highly regioselective and enantioselective may be employed for the selective acylation of sugars and amino-sugar molecules. However, an obstacle to the aforementioned

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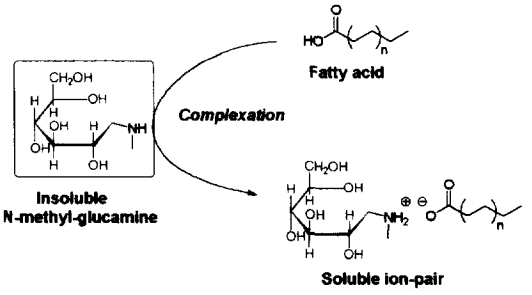
enzymatic approach, arises from the very low solubility of unprotected sugars in hydrophobic solvents. The use of hydrophilic solvents, such as pyridine and DMF, increases the toxicity of the reaction medium and generally reduces enzymatic activity, leading to long reaction times[3]. Another way to produce an acylation of sugar, is the modification of the hydrophilic molecule to increase its miscibility in hydrophobic solvents such as hexane. For this, it is possible to use a solubilizing agent (organo boronic acid, isopropylidene) to form a complex with sugar or derivatives, but this process requires an additional step and increases the toxicity of the medium[4].

In this paper, a non-toxic process for the lipase-catalyzed synthesis of sugar fatty amide derivatives such as the oleoyl-N-methyl-glucamide **3**, from N-methyl-glucamine (1-deoxy-1-methylamino-D-glucitol) **1** and oleic acid **2** without any additives to solubilize the N-methyl-glucamine, is described. Among the amino-sugar derivatives, N-methyl-glucamine was chosen because it is easily obtained by reductive amination of glucose in the presence of methylamine. Investigations were more particularly focused on the effect of ion-pair (acid-amine) on reaction yield, solubility and chemoselectivity.

RESULTS AND DISCUSSION

N-methyl-glucamine solubilization.

N-Methyl-glucamine **1** is not soluble in hydrophobic solvents such as hexane, but in the presence of oleic acid **2**, N-methyl-glucamine was solubilized by ion-pair formation. As shown in Table 1, the N-methyl-glucamine solubilization increased with acid / amine ratio. With an acid / amine ratio of 6, 100 % of N-methyl-glucamine was solubilized. The composition of the medium, especially the carbonyl species, was analyzed by Infra-Red spectroscopy (Figure 1). When only oleic acid is solubilized in hexane, only one carbonyl band is observed at 1715 cm^{-1} corresponding to the acid form. When N-methyl-glucamine is added to the medium, the carbonyl acid band diminishes and a band at 1562 cm^{-1} is detected coming from the carboxylate ion. This additional band demonstrates that there is an ion-pair formed when N-methyl-glucamine is present (Scheme 1). Fatty acid acts as a phase-transfer catalyst for N-methyl-glucamine. In hexane (solvent of low dielectric constant) the ion-pair is very stable because the ions cannot be solvated.



Scheme 1

Table 1 : N-methyl-glucamine solubilisation in hexane at 55°C, versus acid / amine ratio.

Acid/Amine (mM) / (mM)	Soluble N-methyl-glucamine (%)
60/60	50
60/50	60
60/40	75
60/30	85
60/20	90
60/10	100

The concentration of the complex formation was determined by HPLC analysis.

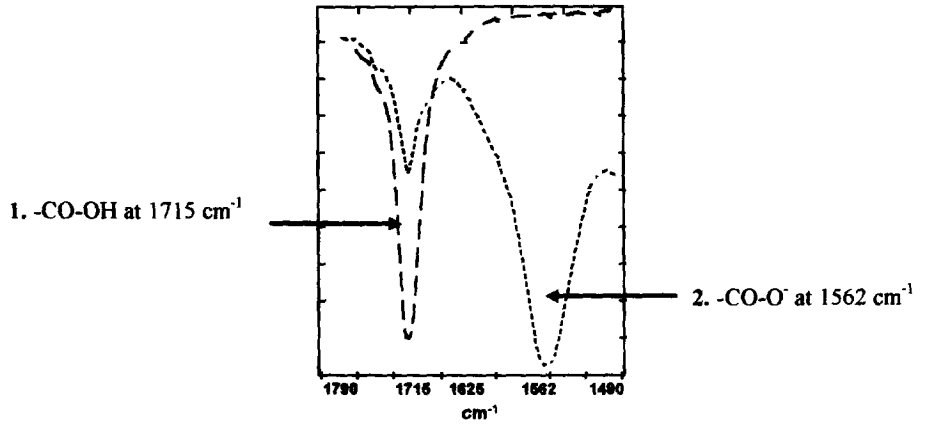
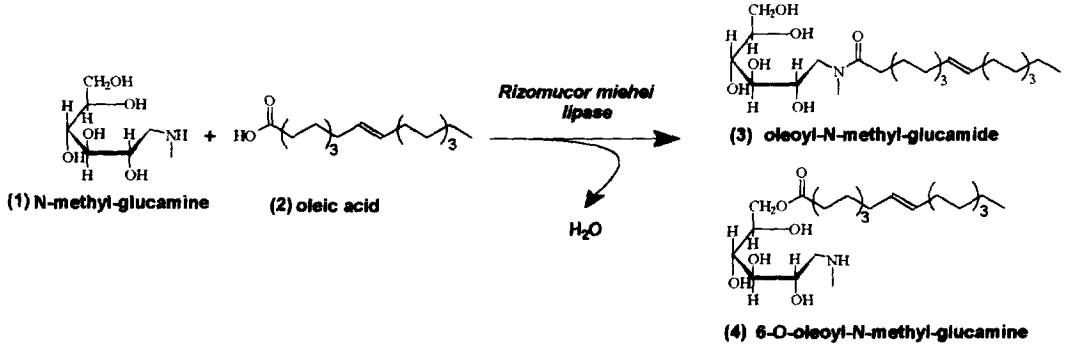


Figure 1 : IR analysis of the various carbonyl groups present at the start of the reaction.

1. Oleic acid in hexane (60 mM)
2. Oleic acid (60 mM) and N-methyl-glucamine (60 mM) in hexane

Influence of ion-pair Acid-N-methyl-glucamine on acylation of N-methyl-glucamine in hexane by lipase from Rhizomucor miehei (Lipozyme®) :

N-Methyl-glucamine contains the amine and five hydroxyl functions that can react with oleic acid, to produce either the amide compound (**3**) or the N-methyl-glucamine mono-ester (**4**)(Scheme 2). The reaction was first carried out in batch, at 55°C with a molar acid / amine ratio of 1. The results obtained are presented in Figure 2. In these conditions, after 80 hours at 55°C, 50 % oleic acid was converted. The decrease of oleic acid concentration was concomitant with the synthesis of two products **3a** and **4a**. These derivatives were easily distinguished by HPLC analysis (Figure 3). In UV (210 nm), product **3a** absorbed much more than product **4a**, suggesting that it contained an amide bond.



Scheme 2

The isolation, purification and characterization of products **3a** and **4a** further confirmed that product **3a** corresponds to oleoyl-N-methyl-glucamide and product **4a** to 6-O-oleoyl-N-methyl-glucamine. Indeed, the band at 1620 cm^{-1} on the Infra-Red spectrum and the peak at 175 ppm on ^{13}C NMR spectrum confirm the presence of the amide bond in compound **3a**. For compound **4a**, the signals corresponding to ester bond were also recorded at 1735 cm^{-1} and 178 ppm on Infra-Red and ^{13}C NMR spectrum respectively.

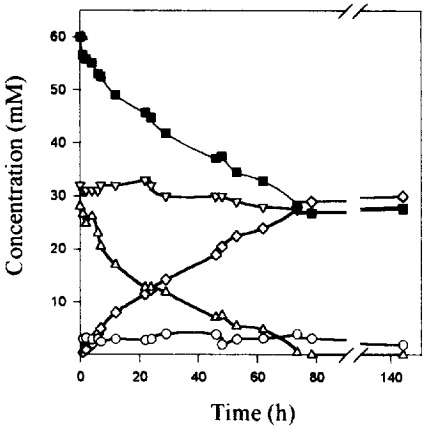


Figure 2 : Condensation of N-methyl-glucamine (60 mM) with oleic acid (60 mM) (Reaction carried out in hexane at 55°C with 10 g/l of Lipozyme®)
◆ oleoyl-N-methyl-glucamide, O O-oleoyl-N-methyl-glucamine, ▼ Soluble N-methyl-glucamine, ▲ Insoluble N-methyl-glucamine, ■ oleic acid.

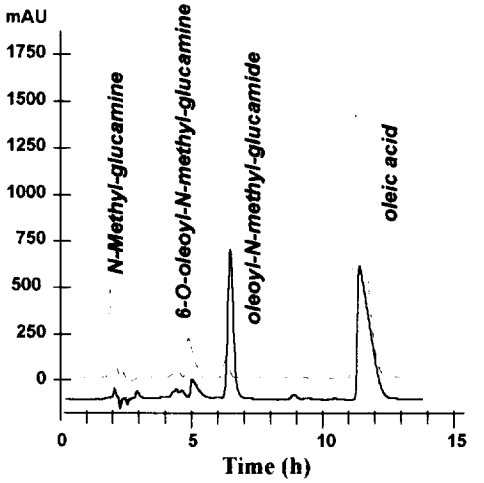


Figure 3 : HPLC Analysis of the N-methyl-glucamine acylation by oleic acid.
Column C18 (250 x 4 mm), methanol water TFA (90 10 0.3, v v v), 1 ml min., 40°C.
Detection : UV at 210 nm (—) and Refractometer(- -)

The acid conversion did not exceed 50 %. It was first assumed that this was due to the ion-pair formed between fatty acid and N-methyl-glucamine. Infra-Red analysis of carbonyl frequencies of the different compounds at the end of the reaction in hexane confirmed this hypothesis (Figure 4). In fact, this complex is very stable in hexane. As a result, at the end of the reaction, 50 % of oleic acid is not available to react with the

enzyme. The ion-pair formation appears to be the limiting factor of the reaction. Moreover, at the end of the reaction, the amide compound is present (carbonyl amide 1625 cm^{-1}) but there is no free acid (unionised) in the medium. All the acid present in the medium was transformed into the carboxylate salt of N-methyl-glucamine (1562 cm^{-1}). In the absence of available free acid, the reaction stopped. By addition of acid to the medium, it was possible to reactivate the synthesis.

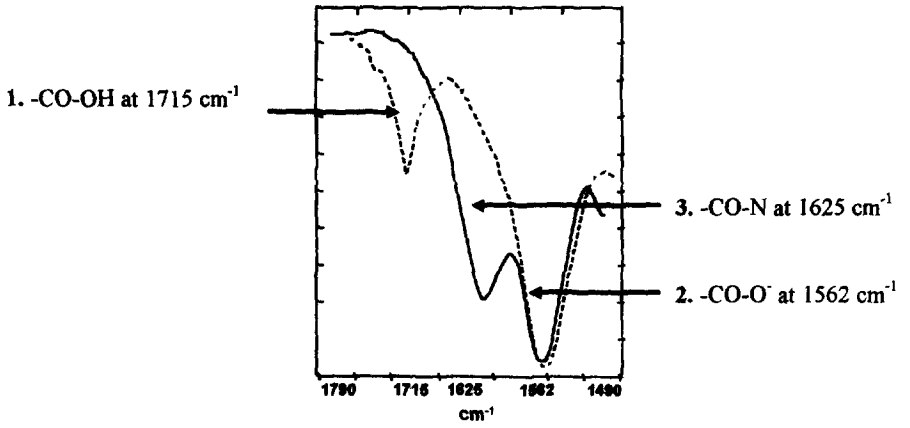


Figure 4 : IR analysis of the various carbonyl groups present at the end of the reaction.

2. Oleic acid (60 mM) and N-methyl-glucamine (60 mM) in hexane (initial condition)
3. Final reaction conditions (30 mM of amide, 30 mM of ion-pair)

Influence of the molar ratio Acid / N-methyl-glucamine

The effect of the molar oleic acid / N-methyl-glucamine ratio on the amide yield as well as on chemoselectivity, was then investigated. The results are reported in Table 2. By increasing the acid / amine ratio, it was possible to improve the amine transformation rate, and the ester synthesis at the expense of amide synthesis. For a ratio of 8, 100 % of N-methyl-glucamine transformation was obtained, exclusively yielding monoester **4a**. On the contrary, amidification was exclusive when the ratio was lower than 1. An excess of acid promotes esterification while an increase in amine orientates the reaction towards amidification. A similar effect was observed when non reactive amine such as triethylamine was added. For an acid / N-methyl-glucamine ratio equal to 60 mM / 40 mM (1.5/1) (giving 50 % of acid conversion with 37 % of N-acylation), the addition of triethylamine (60 mM) in the medium improves amidification (65 %), but the conversion rate decreases (35 % of acid conversion). In all cases, acid conversion never exceeds 50 %. It seems that both acidic-basic conditions and particularly the ion-pair formed between acid and amine may explain these results. To obtain a reaction, it is necessary to solubilize the N-methyl-glucamine and to form the ion-pair. With methyl oleate, no solubilization of N-methyl-glucamine was observed, and enzymatic acylation was produced with very low yields. This result indicates that N-methyl-glucamine must be solubilized with fatty acid to be reactive. As already noted, this ion-pair is stable in hexane and probably blocks the amine function which cannot react with the acyl-enzyme. Consequently, the ester is preferentially obtained when acid is in excess. But with the increase of amine in the

medium (N-methyl-glucamine or triethylamine), amidification occurs preferentially showing that amine groups are more reactive. In the aqueous phase of the catalytic site, it may be possible that in the presence of an excess of base the amine is de-protonated and consequently will react to preferentially form the amide. The catalytic groups of the active site may be influenced by the acidic-basic conditions of the medium.

Table 2 : Chemoselective acylation of N-methyl-glucamine versus molar ratio acid / amine.

Molar ratio acid/amine	Acid conversion (%)	Amine conversion (%)	Amidification (%)
8/1	10	100	0
4/1	25	100	10
2/1	33	85	20
1.5/1	50	75	30
1.2/1	50	60	70
1/1	50	50	90
1/2	40	20	100

(Reaction realized at 55°C, in 10 ml of hexane, with oleic acid (0.6 mmole), N-methyl-glucamine (0.07 to 1.2 mmole) and 100 mg of Lipozyme)

Influence of the fatty acid nature

Using the standard reaction procedure, other fatty acids were tested. Results reported in Table 3, show that all the fatty acids had a lower reactivity than oleic acid. Among the saturated fatty acid, lauric acid was the more reactive. Capric acid reacted poorly as a substrate because of its very low solubility in the medium.

Table 3 : Acylation of N-methyl-glucamine with various fatty acids, catalyzed by *Rhizomucor miehei* Lipase in hexane.

Acylating agent	Conversion after 130 h (%)	Compounds obtained
oleic acid	50	3a
stearic acid	40	3b
palmitic acid	44	3c
lauric acid	46	3d
capric acid	10	3e

The degree of conversion was determined by HPLC on the base on the disappearance of the substrates. No acylation was observed without lipase.

CONCLUSION

A method for a chemoselective acylation of N-methyl-glucamine with fatty acid, without any protecting groups, using *Rhizomucor miehei* immobilized lipase (Lipozyme®) in hexane, has here been reported. This original process, describes amide bond synthesis from a secondary amine and fatty acid. It has been shown that N-methyl-glucamine is solubilized by complexation with fatty acid which resulted in the formation of an ion-pair between acid and amine function. The identification of the ion-pair is possible by Infra-Red analysis. With oleic acid, the reaction, because of ion-pairs, was limited to 50 % of acid conversion. This is the first report showing the key role of substrate ionic state when operating enzyme catalysis in non-conventional media. The acidic-basic conditions of the reaction but more generally molar acid / amine ratio, were identified as parameters controlling chemoselectivity and the yield of the acylation reaction. Finally, this synthesis can be extended to various sources of fatty acids.

EXPERIMENTAL PROCEDURES

Biological and Chemical material

Lipozyme® (lipase from *Rhizomucor miehei* immobilized on an anionic macroporous resin, Duolite 568N), was a gift from Novo Industries (Denmark). The solvents used for synthesis were all pure, purchased from Fluka. The N-methyl-glucamine (1-deoxy-1-methylamino-D-glucitol) and triethylamine (Sigma Chemical Co.), were more than 99 % pure. All the acyl donors were more than 99 % pure, and were supplied by Sigma Chemical Co, USA.

General procedure for the enzymatic reaction

N-methyl-glucamine, acyl agent and *Rhizomucor miehei* Lipase (Lipozyme®) were mixed in Hexane. The reactions were carried out in 25 ml flasks mechanically stirred. The standard conditions were : 0.6 mmole of oleic acid were mixed with 0.6 mmole of N-methyl-glucamine and 100 mg of Lipozyme in 10 ml of Hexane. The reaction was realized at 55°C under atmospheric pressure for 120 hours. These standard conditions were used except when otherwise stated in the text.

HPLC Analysis

Analysis were performed with an HPLC system from Hewlett Packard (processor, pump, UV detector and injector model 1050, differential refractometer (RI) model 1047A), equipped with an Ultrasep C18 (250 x 4 mm, 6 µ) reverse phase column from ICS, France. 25 µl of the suitably diluted reaction mixture were injected. For reactions with long chain fatty acids (more than 12 carbon atoms), a mixture of methanol/water/TFA, 90/10/0.3 (v/v/v) was used as eluent at 40°C and a flow rate of 1 ml/min. For reactions with short chain fatty acids (fewer than 12 carbon atoms), a mixture of methanol/water/TFA, 80/20/0.3 (v/v/v) was used as eluent at 40°C at flow rate of 1 ml/min. Products were detected using a UV detector at 210 nm and a differential refractometer. The samples were quantified by means of pure reagent calibration curves.

Purification of reaction products

At the end of the reaction, the biocatalyst was removed by filtration of the mixture and the solvent evaporated under reduced pressure. The remaining oil was separated into amide (N-acyl) and monoesters of N-methyl-glucamine (O-acyl), by chromatography using silica gel (60 H, Merck) column (30 cm x 20 mm). The concentrated oil sample was diluted in a minimum volume of chloroform/methanol (9/1, v/v) and was deposited at the top of the column previously equilibrated with chloroform/methanol (9/1, v/v). The column was eluted with chloroform/methanol mixtures from 9/1 to 7/3 (v/v) for elution. All the fractions obtained were verified by HPLC analysis before structural analysis. The solvent of fractions was evaporated in a rotary evaporator. The oily residu was rapidly triturated in ethyl ether. A waxy, white solid was obtained.

Structural analysis

Carbon 13 Nuclear Magnetic Resonance (^{13}C NMR) spectra were recorded using an AC 250 MHz spectrometer from Brüker, with an internal reference of Tetramethylsilane. Mass spectra were obtained by chemical ionisation (DCI/NH_3), using NERMAG R10-10 spectrometer. Infra-Red (IR) spectra were recorded using Perkin Elmer IRFT 1760-x spectrometer for KBr pellets. For the Infra-Red analysis of the reaction medium, 500 µl of the reaction mixture at 55°C (60 mM) were injected into the analytic cell. A hexane solution was used as reference.

1-deoxy-1-[methyl(1-oxo-9-octadecenyl)amino]-D-glucitol, (oleoyl-N-methyl-glucamide)(3a):

IR : ν (OH) = 3500 cm^{-1}, ν (CH) = 2800-2900 cm^{-1} and ν (CO-N) = 1620 cm^{-1}.

^{13}C NMR/$CDCl_3$ (δ in ppm) : 175 (CO-N), 130 (2 CH=CH), 22.7-35.8 (14 CH_2), 14 (CH_3), 70-73 (4 CH-OH), 63.7 (CH_2OH), 51(CH_2N), 37.5 (CH_3N). Anal. Calcd for $C_{25}H_{49}NO_6$: C, 65.42 ; H, 10.66. Found : C, 65.53 ; H, 10.74. Mass (DCI/NH_3) : 461 ($M + H^+$), 196 (CH_2OH-$(CHOH)_4$-CH_2-NH-CH_3+ H^+).

1-deoxy-1-methylamino-6-(1-oxo-9-octadecenyl)-D-glucitol, (6-O-oleoyl-N-methyl-glucamine)(4a):

IR : ν (OH) = 3500 cm^{-1}, ν (CH) = 2800-2900 cm^{-1} and ν (CO-O) = 1735 cm^{-1}.
^{13}C NMR/$CDCl_3$ (δ in ppm) : 178 (CO-O), 130 (2 CH=CH), 22.7-35.8 (14 CH_2), 14 (CH_3), 70-73 (4 CH-OH), 65.7 (CH_2O), 49(CH_2NH), 37.5 (CH_3N). Anal. Calcd for $C_{25}H_{49}NO_6$: C, 65.42 ; H, 10.66. Found : C, 65.45 ; H, 10.84. Mass (DCI/NH_3) : 461 ($M + H^+$), 196 (CH_2OH-$(CHOH)_4$-CH_2-NH-CH_3+ H^+)

1-deoxy-1-[methyl(1-oxooctadecyl)amino]-D-glucitol, (stearoyl-N-methyl-glucamide)(3b).

IR : ν (OH) = 3500 cm^{-1}, ν (CH) = 2800-2900 cm^{-1} and ν (CO-N) = 1620 cm^{-1}.
^{13}C NMR/$CDCl_3$ (δ in ppm) : 175 (CO-N), 22.7-35.8 (16 CH_2), 14 (CH_3), 70-73 (4 CH-OH), 63.7 (CH_2OH), 51(CH_2N), 37.5 (CH_3N). Anal. Calcd for $C_{25}H_{51}NO_6$: C, 64.93 ; H, 11.03. Found : C, 64.97 ; H, 11.12.
Mass (DCI/NH_3) : 463 ($M + H^+$), 196 (CH_2OH-$(CHOH)_4$-CH_2-NH-CH_3+ H^+).

1-deoxy-1-[methyl(1-oxohexadecyl)amino]-D-glucitol, (palmitoyl-N-methyl-glucamide)(3c).

IR : ν (OH) = 3500 cm^{-1}, ν (CH) = 2800-2900 cm^{-1} and ν (CO-N) = 1620 cm^{-1}.
^{13}C NMR/$CDCl_3$ (δ in ppm) : 175 (CO-N), 22.7-35.8 (14 CH_2), 14 (CH_3), 70-73 (4 CH-OH), 63.7 (CH_2OH), 51(CH_2N), 37.5 (CH_3N). Anal. Calcd for $C_{23}H_{47}NO_6$: C, 63.59 ; H, 10.82. Found : C, 63.35 ; H, 10.78.
Mass (DCI/NH_3) : 435 ($M + H^+$), 196 (CH_2OH-$(CHOH)_4$-CH_2-NH-CH_3+ H^+).

1-deoxy-1-[methyl(1-oxododecyl)amino]-D-glucitol, (lauroyl-N-methyl-glucamide)(3d):

IR : ν (OH) = 3500 cm^{-1}, ν (CH) = 2800-2900 cm^{-1} and (CO-N) = 1620 cm^{-1}.
^{13}C NMR/D_2O (δ in ppm) : 177.8 (CO-N), 25.2-36.5 (10 CH_2), 16.3 (CH_3), 72.2-74.5 (4 CH-OH), 65.4 (CH_2OH), 54.3 (CH_2N), 39.6 (CH_3N). Anal. Calcd for $C_{19}H_{39}NO_6$: C, 60.39 ; H, 10.33. Found : C, 61.02 ; H, 10.12. Mass (DCI/NH_3) : 379 ($M + H^+$), 196 (CH_2OH-$(CHOH)_4$-CH_2-NH-CH_3+ H^+).

1-deoxy-1-[methyl(1-oxodecyl)amino]-D-glucitol, (decanoyl-N-methyl-glucamide)(3e):

IR : ν (OH) = 3500 cm^{-1}, ν (CH) = 2800-2900 cm^{-1} and (CO-N) = 1620 cm^{-1}.
^{13}C NMR/$CDCl_3$ (δ in ppm) : 175.4 (CO-N), 22.7-34.2 (8 CH_2), 14.3 (CH_3), 70.2-72.5 (4 CH-OH), 63.4 (CH_2OH), 51.3 (CH_2N), 37.4 (CH_3N). Anal. Calcd for $C_{17}H_{35}NO_6$: C, 58.37 ; H, 10.01. Found : C, 58.43 ; H, 10.20. Mass (DCI/NH_3): 350 ($M + H^+$), 196 (CH_2OH-$(CHOH)_4$-CH_2-NH-CH_3 + H^+).

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