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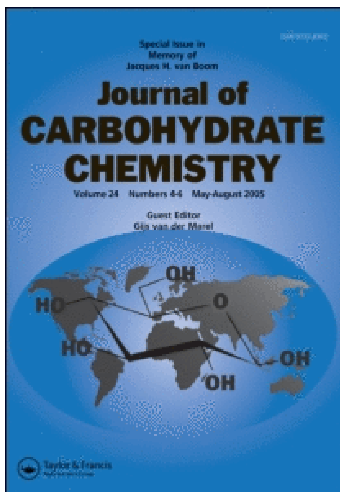

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New Surfactants with Sugar-Based Polar Heads Derived from Bile Acids: The *N*-Ursocholyl-d-glucosamine and *N*-Dehydrocholyl-d-glucosamine

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J. CARBOHYDRATE CHEMISTRY, 14(6), 805-817 (1995)

NEW SURFACTANTS WITH SUGAR-BASED POLAR HEADS DERIVED FROM BILE ACIDS : THE *N*-URSOCHOLYL-D-GLUCOSAMINE AND *N*-DEHYDROCHOLYL-D-GLUCOSAMINE

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ABSTRACT

A simple route to new surfactants with a sugar-based polar head group coupled to a steroidal hydrophobic group is described. These compounds, *N*-ursocholyl-D-glucosamine and *N*-dehydrocholyl-D-glucosamine, were obtained in a three stage process by acylation of D-glucosamine with *N*-acylthiazolidine-2-thiones derived from ursocholic and dehydrocholic acids.

INTRODUCTION

Proteins have to be extracted in a relatively pure state from their biological environment before their structures can be determined. Membrane proteins have an additional handicap in that they have to be solubilized by substances with surfactant properties, without being denatured. A satisfactory surfactant must both disrupt, or partially remove the membrane and then solubilize the protein. Membrane proteins only

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crystallize in a stable form in the presence of a high concentration of surfactant which must not also, in this step, denature the protein.

Most of the surfactants employed in this application are derived from sugars. The most commonly employed is the natural surfactant digitonin extracted from digitalis,[1] although various synthetic surfactants have been used, including alkyl glucosides, alkyl maltosides,[2] HECAMEG[3] and the *N*-alkylamino-1-deoxy-1-lactitols prepared in our laboratory.[4] Moreover, these surfactants can be used to solubilizate substrates of lypoxygenase.[5] All the surfactants with sugar heads prepared to date possess a long-chain alkyl hydrophobic part.

The valuable properties of digitonin for extraction of membrane proteins[1] prompted us to prepare new surfactants with a sugar polar head group and steroidal hydrophobic moiety. The steroidal moiety was chosen in view of the widespread nature of the interactions between cholesterol and lipid membranes.[6] Furthermore, bile salts are known to solubilize membrane proteins.[7]

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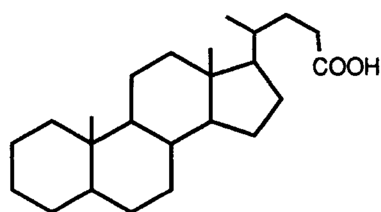
RESULTS AND DISCUSSION

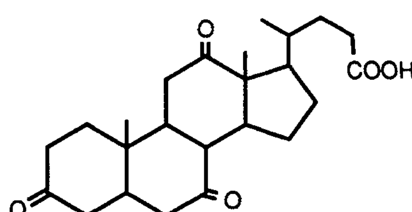
Synthesis of *N*-acyl-D-glucosamines derived from bile acids

The desired surfactants were obtained by joining functional groups to commercial bile acids in a three stage process using *N*-mercaptothiazoline to activate these acids as acylating agents. This process is known to selectively acylate amino alcohols, including D-glucosamine, on the amino group.[8]

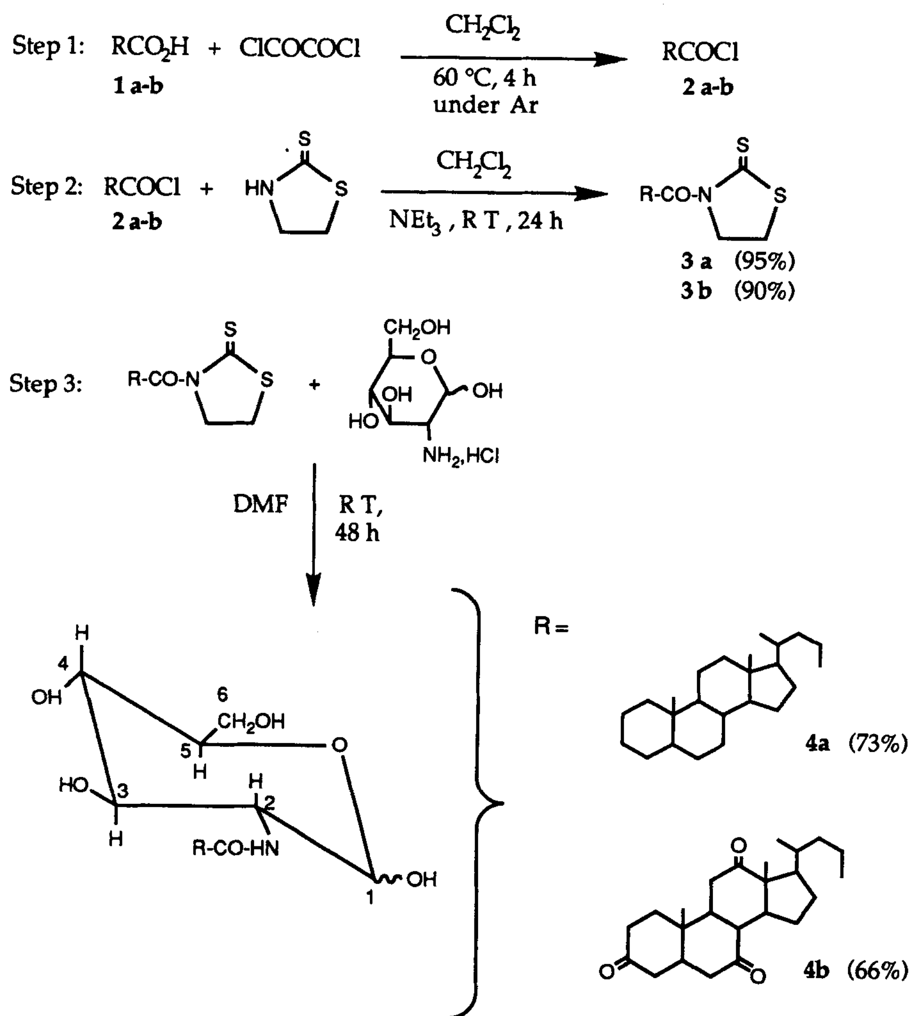
Ursocholic and dehydrocholic acids, which bear no functional groups other than the carboxylic acid residue, were activated in this way to acylate D-glucosamine as the polar head group in the target surfactants according to the synthetic Scheme:



Ursocholic acid **1a**



Dehydrocholic acid **1b**



Scheme

The acyl chlorides **2a** and **2b**, which are easily hydrolyzed, were characterized by ^1H and ^{13}C NMR spectroscopy and used in the following stage without purification. The corresponding derivatives of *N*-mercaptothiazoline **3a** and **3b** were recrystallized. The amido-sugars **4a** and **4b** were purified by column chromatography (cf. Experimental part) to give final yields of 73% and 66% respectively. They were characterized by IR, ^1H and ^{13}C spectroscopy, mass spectrometry (DCI/NH_3 or FAB) and microanalysis.

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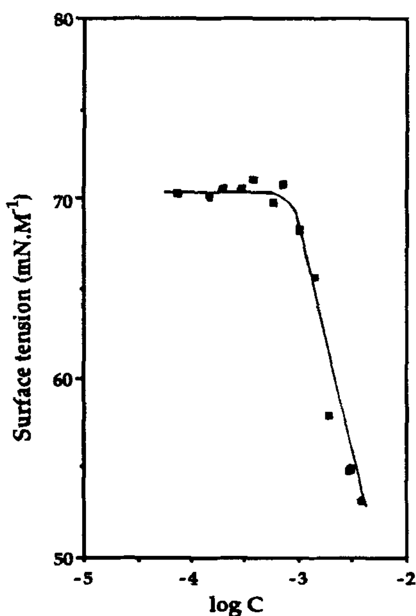

Figure 1a

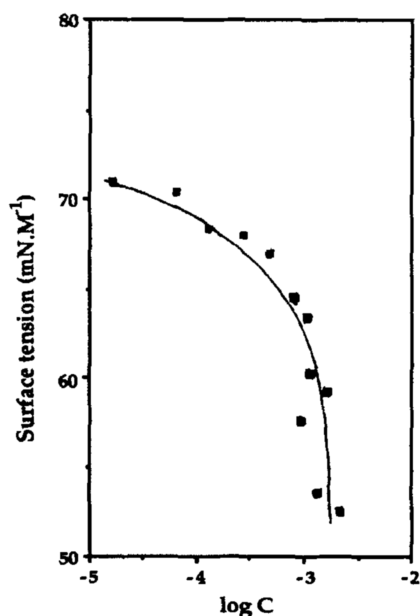

Figure 1b

Figure 1. Surface tension of aqueous solutions of compounds **4a** (Fig. 1a) and **4b** (Fig. 1b) versus log C ($mol.l^{-1}$) at 25 °C.

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The absolute configuration of C-2, as expected and shown from ^1H NMR studies, is preserved in the final structure of **4a** and **4b**. The α/β anomeric configuration ratio has also been resolved by ^1H NMR spectroscopy. The two forms α and β coexist in respective proportion of 68%/32% for compound **4a**, and 80%/20% for compound **4b** (cf experimental part).

Surface active properties of *N*-acyl-D-glucosamines 4a and 4b

The surfactant properties of compounds **4a** and **4b** were determined by measurement of their surface tensions. It can be seen from Figure 1 that both compounds have surfactant properties. The surface tension of water was reduced by 20 $mN.m^{-1}$ at their limit of solubility at 25 °C. This indicates that like bile salts they are localized at the water/air interface. In common with bile acids they also have no critical micellar concentration,[9] and molecular aggregation takes place over their entire range of concentrations in water.

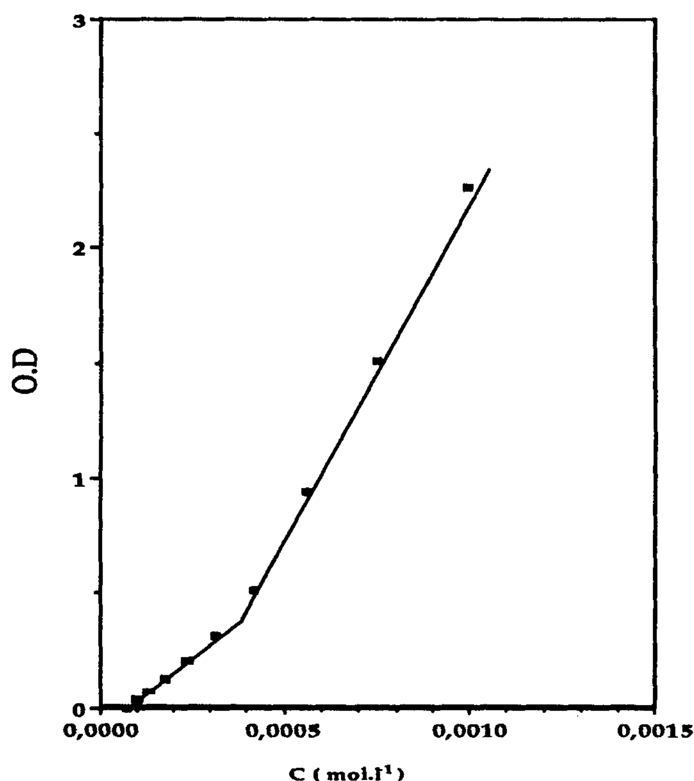

Figure 2. Absorbance of aqueous solutions of **4b** (λ = 216 nm; 25 °C).

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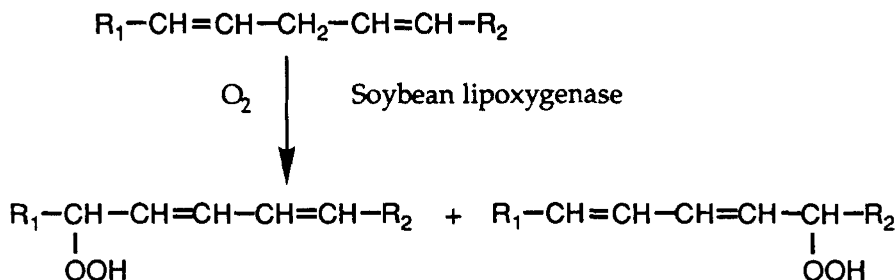
A complementary study by UV spectroscopy of compound **4b** (which absorbs in the UV region) found evidence for a critical concentration for molecular aggregation of $3.7.10^{-4}$ M at 25 °C (cf. Fig.2). Work is now in progress to determine the size of the objects by XR and neutron scattering. *N*-ursocholyl-D-glucosamine (**4a**) and *N*-dehydrocholyl-D-glucosamine (**4b**) would therefore appear to be of interest as surfactants for the extraction of membrane proteins and solubilization of substrates of enzymes as lipoxygenases.

Application : Solubilization of linoleic acid, a substrate for soybean lipoxygenase.

A large number of studies have been devoted to the lipoxygenases as they are widespread in both animals and plants,[10] in part because these enzymes have applications in the food and perfume industries.[11] Soybean

lipoxygenase is a dioxygenase which catalyses the incorporation of oxygen into polyunsaturated fatty acids bearing a *cis, cis* pentadienyl moiety to form a conjugated diene hydroperoxide[12] as follows:



The substrates of soybean lipoxygenase are essential fatty acids of mainly plant origin.[13] Linoleic acid is a substrate which is not readily solubilized or dispersed in aqueous media and the activity of the enzyme towards this substrate is not readily determined without addition of a surfactant such as Tween 20.[14] Unfortunately, this surfactant, which bears a polyoxyethylene head, rapidly denatures the enzyme (4% activity after 60 h incubation). We thus examined the ability of the new surfactants **4a** and **4b** to solubilize linoleic acid for use as a substrate for soybean lipoxygenase. We compared the behavior of **4a** and **4b** compounds with that of Tween 20 using the following procedure.

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Linoleic acid was dissolved in aqueous borate buffer containing equimolar quantities of surfactant and fatty acid. The activity of the enzyme was determined by following the formation of conjugated dienes which absorb at 234 nm (molar extinction coefficient = $2{,}5.10^4$ $M^{-1}.cm^{-1}$).[15] All experiments with lipoxygenase were performed at least three times and the values were the average of the different results. Table 1 list the results obtained.

It can be seen that the derivative **4a** had similar activity to that of Tween 20. On the other hand, higher activity was observed with the derivative **4b** than with Tween 20.

We then examined the stability of soybean lipoxygenase in the presence of the two surfactants. The enzyme was incubated in the presence of the two surfactants for at least three times, and the enzyme activity was then determined. The results are listed in Table 2 after incubation at 4 °C for 60 h.

There was a marked loss of activity in the presence of Tween 20, whereas there was little loss of activity in the presence of **4a** and **4b**

Table 1. Specific activity (SA) of soybean lipoxygenase (LG) in the presence of Tween 20, **4a** and **4b** compounds for dispersal of substrate.

Surfactant	Tween 20	**4a** compound	**4b** compound
SA of soybean LG (IU/mg protein)	14000	14600	17000

Table 2. Specific activity (SA) and relative specific activity (%) of soybean lipoxygenase (LG) in the presence of Tween 20, **4a** and **4b** compounds after incubation at 4 °C for 60 h.

Surfactant	Tween 20	**4a** compound	**4b** compound
SA of soybean LG (IU/mg protein)	533	12600	10850
Relative specific activity (%)	4	90	78

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derivatives. For compound **4a**, 90% of the initial activity remained after 60 h incubation, and only 22% activity was lost in the presence of compound **4b**.

The compounds **4a** and **4b** both solubilize linoleic acid without denaturing the enzyme soybean lipoxygenase. They thus represent a good alternative to Tween 20 for this type of formulation in industrial applications.[15]

CONCLUSION

We describe the synthesis of two new amphiphilic compounds based on bile acids with a sugar polar head. These compounds solubilize linoleic acid without denaturing the enzyme soybean lipoxygenase. These new surfactants potentially may have lots of applications in membranology, enzymology and extraction of proteins from biological systems. Work is in progress on such applications, and compounds based on other bile acids (as cholic, deoxycholic, lithocholic acids ...) and amino sugars are being prepared.

EXPERIMENTAL

General methods. Ursocholic acid (Sigma), dehydrocholic acid (Fluka, > 99%), oxalyl chloride (Aldrich, 2 M in CH_2Cl_2), mercaptothiazoline

(Aldrich, 98%), D-glucosamine hydrochloride (Fluka, puriss), soybean lipoxygenase (Serva, pH optimum 9), linoleic acid (Sigma, > 99%) were employed without further purification. All solvents were from Prolabo and were dried over 4 Å sieves. Elemental analyses were carried out at the microanalysis center at the Ecole de Chimie de Toulouse (France). In view of the highly hygroscopic nature of the compounds **4a** and **4b**, residual water content was determined with a Karl Fischer apparatus. Merck 60 silica gel (40-63 μm) was used for preparative column chromatography. The ^{13}C NMR and ^{1}H NMR spectra were recorded on a Brucker AC200 spectrometer (200 MHz for ^{1}H and 50.32 MHz for ^{13}C) or on a Brucker ARX400 spectrometer (400.13 MHz for ^{1}H and 100.62 MHz for ^{13}C). The UV spectra were recorded on a Hewlett Packard 8452A diode-array spectrophotometer, and the mass spectra were recorded on a Nermag R10-10 spectrometer by either DCI/NH_3 or FAB. Surface tension were measured on a Prolabo No.3 tensimat apparatus by the stirrup detachment method.

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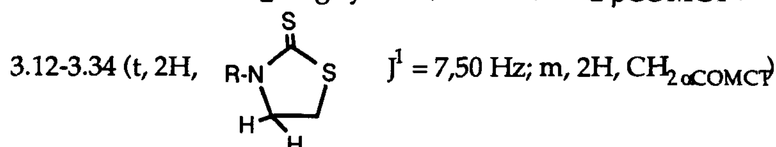
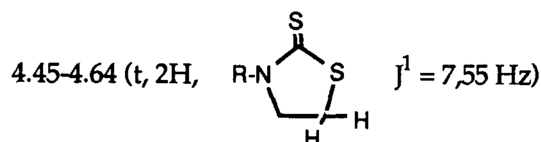
Synthesis of ursocholic acyl chloride 2a. Ursocholic acid **1a** (1.29 g, 3.57 mmol) was dissolved in 100 mL of anhydrous CH_2Cl_2 under argon at room temperature over 10 min. Oxalyl chloride (28.5 mmol) was then added under argon, and the mixture was refluxed for 4 h. The solvent was evaporated along with excess oxalyl chloride to give 1.35 g of crude product (white solid). ^{1}H NMR ($CDCl_3$, δppm) 0.63 (s, 3H, CH_3); 0.90-0.94 (m, 6H, 2CH_3); 1.27 (m, CH and CH_2 ring system); 1.68 (m, CH_2 β_{COCl}); 2.77-3.03 (m, 2H, $C\underline{H_2}$-COCl). ^{13}C NMR ($CDCl_3$, δppm) 12.10; 18.56; 24.31 (CH_3); 35.03; 35.93; 40.56; 43.76; 55.87; 56.65 (CH); 20.86; 21.39; 24.24; 26.59; 27.08; 27.29; 27.54; 28.24; 31.14; 35.4; 37.63; 40.31; 42.87; 44.43 (Cq and CH_2 inc. CH_2 αCOCl and CH_2 βCOCl); 174.28 ($\underline{C}$OCl). The hydrolysable product was stored under argon and used in the following stage without purification.

Synthesis of dehydrocholic acyl chloride 2b. The same method was used as for **2a** but with 1 g (2.48 mmol) of dehydrocholic acid **1b** and 20 mmol of oxalyl chloride to give 1.04 g of crude product (white solid).
^{1}H NMR ($CDCl_3$, δppm) 0.83 (s, 3H, CH_3); 1.06 (s, 3H, CH_3); 1.37 (s, 3H, CH_3); 2.18 (m, CH et CH_2 ring system); 2.81 (m, CH_2 βCOCl); 3.36 (m, 2H, $C\underline{H_2}$-COCl). ^{13}C NMR ($CDCl_3$, δppm) 11.84; 18.56; 21.94 (CH_3); 35.13; 45.52; 45.59; 46.85; 48.99; 51.79 (CH); 25.12; 27.66; 29.72; 30.61; 35.30; 36.05; 36.50; 38.64; 42.81; 44.58; 45.00 (Cq and CH_2 inc. CH_2 αCOCl and CH_2 βCOCl); 174.28 ($\underline{C}$OCl); 208.81; 209.01; 211.86 (CO ring system).

Synthesis of 3-ursocholyl-2-mercaptothiazoline 3a. Triethylamine (1 mL, 7.17 mmol) was added to a solution of 2-mercaptothiazoline (0.39 g, 3.27 mmol) in 100 mL of CH_2Cl_2. The ursocholic acid chloride **2a**, dissolved in 20 mL of CH_2Cl_2, was added dropwise to the mixture cooled in ice. The mixture was then stirred for 24 h at room temperature. The organic phase was washed with 0.5 N HCl followed by 5% $NaHCO_3$, dried over $MgSO_4$ and then concentrated to give 1.5 g of crude product (yellow solid). This compound was recrystallized from methanol to give 1.4 g of a yellow solid (mp 130 °C): yield=95%; ^1H NMR ($CDCl_3$, δppm) 0.63 (s, 3H, CH_3); 0.89-0.94 (m, 6H, $2CH_3$); 1.26 (m, CH and CH_2 ring system); 1.67 (m, CH_2 βCOMCT);



3.12-3.34 (t, 2H, [structure] J^1 = 7,50 Hz; m, 2H, CH_2 αCOMCT)

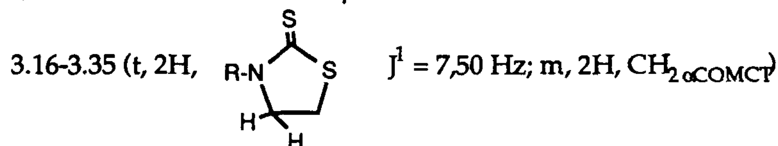


4.45-4.64 (t, 2H, [structure] J^1 = 7,55 Hz)

^{13}C NMR ($CDCl_3$, δppm) 12.12; 18.33; 18.58 (CH_3); 35.48; 35.94; 40.55; 43.79; 56.19; 56.64 (CH); 20.88; 21.39; 24.29; 26.6; 27.08; 27.30; 27.57; 28.30; 30.87; 35.41; 35.68; 37.64; 40.32; 42.84 (Cq and CH_2 inc. CH_2 αCOR and CH_2 βCOR); 56.14 (CH_2 αN); 60.19 (CH_2 αS); 174.54 (CO); 201.50 (CS).

Anal. Calcd for $C_{27}H_{43}ONS_2$: C, 70.23; H, 9.38; N, 3.03; Found : C, 70.69; H, 9.60; N, 2.84.

Synthesis of 3-dehydrocholyl-2-mercaptothiazoline 3b. The same method was used as for **3a** but with 0.6 mL (4 mmol) of triethylamine and 0.27 g (2.26 mmol) of 2-mercaptothiazoline giving 1.2 g of crude product (yellow solid). This compound was recrystallized from methanol to give 1.08 g of a yellow solid (mp 235 °C): yield=90%; ^1H NMR ($CDCl_3$, δppm) 0.83 (s, 3H, CH_3); 1.06 (s, 3H, CH_3); 1.36 (s, 3H, CH_3); 2.18 (m, CH and CH_2 ring system); 2.66-3.02 (m, CH_2 βCOMCT);



3.16-3.35 (t, 2H, [structure] J^1 = 7,50 Hz; m, 2H, CH_2 αCOMCT)

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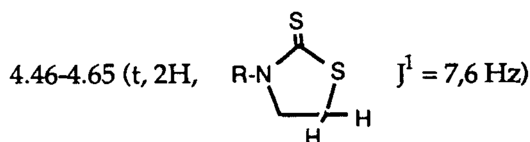
4.46-4.65 (t, 2H,  J^1 = 7,6 Hz)

^{13}C NMR ($CDCl_3$, δppm) 11.92; 18.95; 21.95 (CH_3); 37.76; 45.58; 45.97; 46.89; 49.03; 51.75 (CH); 25.19; 27.70; 28.28; 30.40; 35.32; 36.05; 36.15; 36.53; 38.67; 42.83; 45.02 (Cq and CH_2 inc. CH_2 αCOR and CH_2 βCOR); 56.11 (CH_2 αN); 56.95 (CH_2 αS); 175.21 (CO); 208.76; 209.05; 211.93 (CO ring system).

Anal. Calcd for $C_{27}H_{37}O_4NS_2$: C, 64.38; H, 7.40; N, 2.78; Found : C, 64.29; H, 7.50; N, 2.49.

Synthesis of *N*-ursocholyl-D-glucosamine 4a. 3-ursocholyl-2-mercaptothiazoline (1.4 g, 3.03 mmol) and D-glucosamine;HCl (0.59 g, 2.73 mmol) were stirred in 100 mL of DMF containing triethylamine (1 mL, 7.17 mmol) for 48 h at room temperature. The solvent was evaporated under reduced pressure. The residue was taken up in acetonitrile, filtered and the filtrate freeze-dried to give 1.09 g of crude product, yield 77% based on D-glucosamine. The product was purified by chromatography on a silica gel column ($CHCl_3$/MeOH; 2/1; v/v) to give 1.03 g of *N*-ursocholyl-D-glucosamine **4a** as a white solid: yield 73%; mp 192 °C. IR 3100-3500 cm^{-1} √OH, 1630 cm^{-1} √CONH. DCI/NH_3, [MH^+] 522; [MH^+ - H_2O] 504. ^{13}C NMR at 100.62 MHz (DMSO, δppm) 11.77; 18.25; 23.92 (3 CH_3); 34.95; 35.29; 42.99; 54.10; 55.62; 55.97; 57.02; 70.34; 71.05; 71.92 (10 CH, ring system, C-2, C-3, C-4 and C-5); 20.34; 20.71; 23.76; 26.06; 26.39; 26.60; 26.95; 27.63; 31.39; 32.16; 34.81; 37.03; 39.59; 42.16; 61.03 (2 Cq and 13 CH_2, ring system, C-6); 90.48; 95.49 (C-1 for α and β configurations respectively); 172.66; 173.12 (R_1-<u>C</u>O-NH-R_2 for α and β configurations respectively). ^1H NMR at 400.13 MHz (DMSO, δppm) 0.61 (s, 3H, CH_3); 0.87 (d, 3H, $J_{CH3,H}$ = 6,5 Hz, CH_3); 0.89 (s, 3H, CH_3); 1.0-2.2 (m, CH and CH_2 ring system inc. CH_2 α and β <u>R1</u>CONHR2); 3.0-3.7 (m, C<u>H</u>OH and C<u>H</u>$_2$OH); 4.35-5.0 (m, CHO<u>H</u> and CH_2O<u>H</u> exchangeable with D_2O); 6.37, 6.49 (2d, α and β configurations of OH-1 exchangeable with D_2O); 7.49, 7.65 (2d, N<u>H</u> for α and β configurations exchangeable with D_2O)

Anal. Calcd for $C_{30}H_{51}O_6N, 2H_2O$: C, 64.60; H, 9.94; N, 2.51; Found : C, 64.68; H, 10.09; N, 2.51.

<u>Identification of the anomeric protons corresponding to the α and β forms of 4a.</u>

For the α configuration : After adding D_2O : 3.58 (dd, J_{2-3} = 10.8 Hz and J_{2-1} = 3.2 Hz, H-2); 4.9 (d, J_{1-2} = 3.2 Hz, H-1); before adding D_2O : 6.37 (d, JOH-

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1,H-1 = 4.0 Hz, OH-1); 7.49 (d, $J_{NH,H-2}$ = 8.0 Hz, NH); 4.90 (dd, J_{1-2} = 3.2 Hz, $J_{H-1,OH-1}$ = 4.0 Hz, H-1); 3.58 (m, $J_{H-2,H-1}$ = 3.2 Hz, $J_{H-2,H-3}$ = 10.8 Hz, $J_{H-2,NH}$ = 8.0 Hz, H-2).

For the β configuration : After adding D_2O : 3.34 (dd, J_{2-3} = 10.2 Hz and J_{2-1} = 8.0 Hz, H-2); 4.4 (d, J_{1-2} = 8.0 Hz, H-1); before adding D_2O : 6.45 (d, $J_{OH-1,H-1}$ = 6.4 Hz, OH-1); 7.64 (d, $J_{NH,H-2}$ = 8.2 Hz, NH); 4.40 (dd, J_{1-2} = 8.0 Hz, $J_{H-1,OH-1}$ = 6.4 Hz, H-1) The H-2 signal is hidden by the HOD line of the solvent.

Synthesis of *N*-dehydrocholyl-D-glucosamine 4b. The same method was used as for **4a** but with 0.41 g (1,90 mmol) of D-glucosamine:HCl and 0.6 mL (4 mmol) of triethylamine giving 0.86 g of crude product, yield 80% based on D-glucosamine and 0.71 g of purified product : white solid; yield 66%; mp 230 °C. IR 3200-3500 cm^{-1} √OH, 1635 cm^{-1} √CONH, 1700-1715 cm^{-1} √CO ring system. FAB, [MH^+] 564; [MH^+ - H_2O] 546. ^{13}C NMR at 100.62 MHz (DMSO, δppm) 11.35; 18.80; 21.03 (3 CH_3); 35.08; 43.92; 45.33; 45.89; 47.88; 51.13; 54.14; 70.29; 71.03; 71.91 (10 CH, ring system, C-2, C-3, C-4 and C-5); 24.52; 27.20; 31.10; 32.48; 34.47; 35.52; 36.03; 38.27; 42.41; 44.44; 56.13; 61.02 (2 Cq and 10 CH_2, ring system, C-6); 90.46; 95.50 (C-1 for α and β configurations respectively); 172.57; 172.90 (R_1-<u>C</u>O-NH-R_2 for α and β configurations respectively).
^1H NMR at 400.13 MHz (DMSO, δppm) 0.77 (d, 3H,$J_{CH3,H}$ = 6.2 Hz, CH_3); 1.00 (s, 3H, CH_3); 1.33 (s, 3H, CH_3); 1.20-2.5 (m, CH and CH_2 ring system inc. CH_2 α and β <u>R1</u>CONHR2); 2.75-3.6 (m, C<u>H</u>OH and C<u>H2</u>OH); 4.4-5.0 (m, CHO<u>H</u> and CH_2O<u>H</u> exchangeable with D_2O); 6.38, 6.46 (2d, α and β configurations of OH-1 exchangeable with D_2O); 7.53, 7.67 (2d, N<u>H</u> for α and β configurations exchangeable with D_2O).

Anal. Calcd for $C_{30}H_{45}O_9N$, 1,7 H_2O : C, 60.63; H, 8.20; N, 2.35; Found : C, 60.64; H, 8.06; N, 2.31.

<u>Identification of the anomeric protons corresponding to the α and β forms of **4b**.</u>

For the α configuration : After adding D_2O : 3.56 (dd, J_{2-3} = 10.8 Hz and J_{2-1} = 3.2 Hz, H-2); 4.9 (d, J_{1-2} = 3.2 Hz, H-1); before adding D_2O : 6.38 (d, $J_{OH-1,H-1}$ = 4.2 Hz, OH-1); 7.53 (d, $J_{NH,H-2}$ = 7.9 Hz, NH); 4.90 (dd, J_{1-2} = 3.2 Hz, $J_{H-1,OH-1}$ = 3.8 Hz, H-1); 3.56 (m, $J_{H-2,H-1}$ = 3.1 Hz, $J_{H-2,H-3}$ = 10.8 Hz, $J_{H-2,NH}$ = 7.8 Hz, H-2).

For the β configuration : After adding D_2O : 3.36 (dd, J_{2-3} = 10.3 Hz and J_{2-1} = 8.0 Hz, H-2); 4.41 (d, J_{1-2} = 8.0 Hz, H-1); before adding D_2O : 6.44 (d, $J_{OH-1,H-1}$ = 8.0 Hz, OH-1); 7.67 (d, $J_{NH,H-2}$ = 8.3 Hz, NH); 4.41 (dd, J_{1-2} = 8.0 Hz,

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$J_{H\text{-}1,OH\text{-}1}$ = 8.0 Hz, H-1) The H-2 signal is hidden by the HOD line of the solvent.

Assay of enzyme activity. The activity of the enzyme was determined by measuring the rate of increase in absorbance at 234 nm on a Hewlett-Packard HP 8452A diode array spectrophotometer. The reaction mixture contained linoleic acid (0.80 μmol) dispersed with the different surfactants in equimolar proportions (0.8 μmol) in a total volume of 2,08 mL of borate buffer (pH 9) and 5 μL of enzyme solution (1 mg/mL). The kinetics of the reaction were followed directly in the spectrophotometer cuvette at 25 °C. The results were compared to those of an identical mixture containing Tween 20 as surfactant. A molar extinction coefficient of 25,000 $M^{-1}.cm^{-1}$ was employed to convert the absorbance at 234 nm into moles per liter of hydroperoxides formed. The specific activity was expressed in International Units per mg protein.

Stability of lipoxygenase. An identical solution to that used above but without substrate was incubated at 4 °C for 60 h. The activity of the enzyme was then determined at room temperature. The control solution contained Tween 20 as surfactant.

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