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Research paper

# Broad-spectrum antibacterial amphiphilic aminoglycosides: A new focus on the structure of the lipophilic groups extends the series of active dialkyl neamines

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ABSTRACT

Amphiphilic aminoglycosides (AAGs) constitute a new class of antibacterial compounds targeting the bacterial membranes. We have identified the 3′,6-dinonyl neamine **9** as a broad spectrum antibacterial AAG. Here, we report on the synthesis, antibacterial activity and eukaryotic cytotoxicity of new 3′,6-dialkyl neamines designed in order to finely delineate the structure-activity relationships relating their activity to a lipophilicity window. New broad-spectrum antibacterial derivatives were obtained carrying two identical linear or branched alkyl groups or two different linear alkyl groups. Two fluorescent antibacterial 3′,6-heterodialkyl neamines carrying a pyrenylbutyl fluorophore were also identified as potential tools for mechanistic study. Homodialkyl and heterodialkyl neamines appeared to be more active on Gram-negative bacteria than dinaphthylalkyl neamines. However, branched dialkyl neamines or heterodialkyl derivatives were found to be more cytotoxic on mammalian cells than **9.** The exposure of *P. aeruginosa* over one month to half-MIC of one of the most active derivatives **9** demonstrated the high difficulty of resistance emergence to AAGs.



## 1. Introduction

Antibacterial amphiphilic aminoglycosides (antibacterial AAGs) constitute a new class of antibiotic drugs candidates obtained by chemical modification of the polycationic antibiotic drugs aminoglycosides (AGs) such as neomycin B **1** (Fig. 1), kanamycins, tobramycin… that target the A site of bacterial 16 S rRNA to cause protein synthesis alteration [1–6]. AGs are highly potent broad-spectrum antibiotics and decades of widespread clinical use strongly reduced their clinical efficacy through the selection of resistant bacteria [7,8]. Originally, AAGs have been developed in the search for new AGs which are less susceptible to resistance. In this approach, Hanessian, Westhof, and coworkers have reported the first example of *in vivo* antibacterial lipophilic ether-modified AG paromomycin targeting rRNA with a novel mode of binding [9,10].

More lipophilic AAGs resulting from the introduction of one to three lipophilic groups on AG cores were shown to target bacterial membranes [1–6,11–24]. The corresponding strong increase in the AG lipophilicity results in a bacterial target shift from rRNA to membranes and significantly improves activity against bacterial strains resistant to the parent AG drugs and to antibiotic drugs of other classes. AAGs are active against resistant bacteria expressing different modes of resistance e.g. (i) reduction in the intracellular concentration of the antibiotics by efflux pump proteins, (ii) deactivation by AG-modifying enzymes, AG nucleotidyltransferases (ANTs), AG phosphotransferases (APHs), and AG acetyltransferases (AACs), and (iii) structural modification of the 16 S ribosomal RNA binding site that lead to reduced target affinity [7,8].

AAGs have appeared to be active mainly against susceptible and resistant Gram-positive bacteria to most antibiotic drugs or, more rarely, against susceptible and resistant Gram-positive and Gram-negative bacteria. The most active AAGs against Gram-negative bacteria are polyalkyl derivatives of neamine **2** (Fig. 1) [18–20,22], neosamine [20] and nebramine [16]. Antibacterial AAGs can also present immunomodulatory effects like tobramycin

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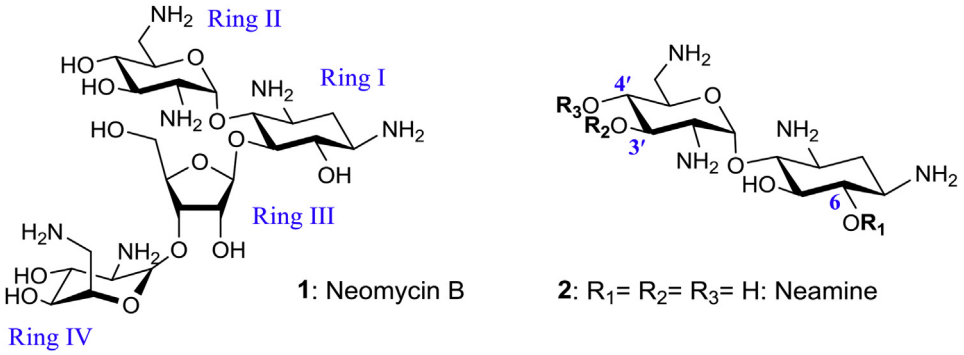


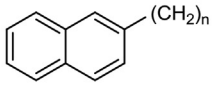

**Previous Neamine derivatives**

**3′,6-homodialkyl derivatives**

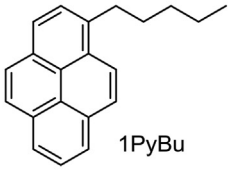
**3**: $R_1$= $R_2$= 2-naphthylmethyl (2NM), $R_3$= H
**4**: $R_1$= $R_2$= 2-naphthylpropyl (2NP), $R_3$= H
**5**: $R_1$= $R_2$= 2-naphthylbutyl (2NB), $R_3$= H
**6**: $R_1$= $R_2$= 2-naphthylhexyl (2NH), $R_3$= H
**7**: $R_1$= $R_2$= butyl (Bu), $R_3$= H
**8**: $R_1$= $R_2$= hexyl (Hx), $R_3$= H
**9**: $R_1$= $R_2$= nonyl (Nn), $R_3$= H
**10**: $R_1$= $R_2$= octadecyl (OcD), $R_3$= H

**3′,4′-homodialkyl derivative**

**11**: $R_1$= H, $R_2$= $R_3$= 2-naphthylpropyl (2NP)
**12**: $R_1$= H, $R_2$= $R_3$= nonyl (Nn)




2-naphthylalkyl groups
n = 1, 3, 4, 6



1PyBu

**New Neamine derivatives**

**3′,6-homodialkyl derivatives**

**13**: $R_1$= $R_2$= heptyl (Hp), $R_3$= H
**14**: $R_1$= $R_2$= octyl (Oc), $R_3$= H
**15**: $R_1$= $R_2$= decyl (De), $R_3$= H
**16**: $R_1$= $R_2$= undecyl (Ud), $R_3$= H
**17**: $R_1$= $R_2$= (3,7-dimethyl)octyl (DiMoc), $R_3$= H

**3′,4′-homodialkyl derivative**

**18**: $R_1$= H, $R_2$= $R_3$= (3,7-dimethyl)octyl (DiMoc)

**3′,6-heterodialkyl derivatives**

**19**: $R_1$= undecyl (Ud), $R_2$= heptyl (Hp), $R_3$= H
**20**: $R_1$= heptyl (Hp), $R_2$= undecyl (Ud), $R_3$= H
**21**: $R_1$= (1-pyrenyl)butyl (1PyBu), $R_2$= heptyl (Hp), $R_3$= H
**22**: $R_1$= heptyl (Hp), $R_2$= (1-pyrenyl)butyl (1PyBu), $R_3$= H

**Monoalkyl derivatives**

**23**: $R_1$= $R_3$= H, $R_2$= heptyl (Hp)
**24**: $R_1$= heptyl (Hp), $R_2$= $R_3$= H
**25**: $R_1$= $R_3$= H, $R_2$= undecyl (Ud)
**26**: $R_1$= undecyl (Ud), $R_2$= $R_3$= H

**Fig. 1.** Structure of the natural antibiotic aminoglycoside neomycin B **1**, of neamine **2** and of some amphiphilic neamines previously prepared **3**–**12** [18–20] and of new neamine derivatives **13**–**26** described here.

derivatives [25]. Furthermore, efficient antifungal AAGs exhibiting or lacking antibacterial activity have been also identified [26–29].

Interestingly, more recently, amphiphilic AG conjugates showed other antibacterial and antiviral properties. Amphiphilic tobramycin conjugates to lysine appeared to be able to sensitize multidrug resistant Gram-negative bacteria to antibiotic drugs [30,31], and conjugates to efflux pump inhibitors [32] or to a fluoroquinolone [33] were shown to overcome resistance in *P. aeruginosa* by enhancing bacterial outer membrane penetration and reducing efflux.

Amphiphilic neamine conjugates bearing a metal binding motif active against MDR *E. aerogenes* Gram-negative bacteria [34] have also been obtained using the chemistry previously developed in the search for antiviral AGs and AAGs [35,36]. AAGs also display *in vitro* antiviral activity like neamine- and neosamine-peptide nucleic acid (PNA) conjugates, the neamine and neosamine cores allowing cell penetration of PNA targeting viral RNA [37–39].

In the synthesis of AAGs, several strategies have been developed including partial conversion of (i) the AG amine groups into alkyl- or aryl-amide(s), reducing the number of positive charges carried at physiological pH, or (ii) the hydroxyl groups in carbamate, ether and thioether groups, respectively [1,2]. We have previously synthesized different mono-, di- and tri-*O*-alkyl derivatives of the small aminoglycoside neamine **2** prepared from the natural antibiotic drug neomycin B **1** and identified three series of antibacterial agents acting on susceptible and resistant Gram-positive and Gram-negative bacteria to AGs and other antibiotic drugs, the series of 3′,4′,6-trialkyl derivatives and the 3′,4′- and 3′,6-dialkyl derivative series (Fig. 1) [18–20].

The 3′,6-dialkyl derivatives appeared to be the most promising in regard to their broad-spectrum antibacterial activity and their lowest eukaryotic cytotoxicity [19]. In these series, we have identified three broad-spectrum antibacterial neamines, two 2-naphthylalkyl derivatives, the 3′,6-di-(2-naphthyl)propyl (3′,6-di2NP, **4**), and 3′,6-(2-naphthyl)butyl (3′,6-di2NB, **5**) neamines and, only, one alkyl derivative, the 3′,6-dinonyl neamine **9** (3′,6-diNn), the latter appearing to be the most active derivative against Gram-negative bacteria (Fig. 1) [19]. The 3′,6-dinaphthylmethyl (**3**), -dinaphthylhexyl (**6**), -dibutyl (**7**), -dihexyl (**8**) and -dioctadecyl (**10**) neamines have been found to be inactive or weakly active against Gram-negative bacteria. 3′,6-DiNn neamine **9** has appeared to inhibit *P. aeruginosa* biofilm growth and also shown antibacterial activity on *P. aeruginosa* strains resistant to the polycationic lipopeptide colistin targeting lipopolysaccharides (LPS) [22], colistin being one of the drug of last resort to combat multidrug-resistant (MDR) *P. aeruginosa* infections [40].

A window of lipophilicity has been delineated to fit to the optimal antibacterial activities of both 3′,6-dialkyl and 3′,6-di-2-naphthylalkyl neamines [19]. Such a window has been also observed in the 3′,4′,6-trialkyl series [19]. In regard to the unique

antibacterial properties of 3′,6-diNn neamine **9**, here, we report on the synthesis, antibacterial activity and eukaryotic cytotoxicity of new 3′,6-dialkyl neamines carrying linear alkyl chains, the 3′,6-diheptyl (diHp, **13**), -dioctyl (diOc, **14**), -didecyl (diDe, **15**) and -diundecyl (diUd, **16**) (Fig. 1). The studied dialkyl neamines were designed in order to finely tune the previously delineated SAR relating their activity expressed as 1/MIC (1/Minimum Inhibitory Concentration) values to clogP values that corresponds to a previously delineated activity window against MRSA and susceptible *P. aeruginosa* located between clogP = −12.7 and −9.0 [19]. The present study allows to split this window into two windows differentiating the alkyl derivatives and the previously synthesized naphthylalkyl derivatives and to identify new broad-spectrum antibacterial 3′,6-dialkylneamine, the dioctyl (**14**) and didecyl (**15**) derivatives. We also demonstrate here that dialkyl neamines carrying branched alkyl chains can have antibacterial activity through the synthesis and study of the 3′,6-dialkyl neamine derivative and its 3′,4′-isomer carrying two branched dimethyloctyl (DiMOc) chains, respectively **17** and **18** (Fig. 1).

In order to investigate the importance of the respective length and position of both linear alkyl groups grafted on the neamine core at positions 3′ and 6, we also report on the synthesis and antibacterial properties of two 3′,6-dialkyl neamines carrying two linear alkyl chains of different lengths, the 3′-heptyl-6-undecyl (3′-Hp-6-Ud, **19**) and 3′-undecyl-6-heptyl (3′-Ud-6-Hp, **20**) which are isomers having lipophilicities close to the lipophilicity of the antibacterial 3′,6-diNn neamine **9** (Fig. 1). Both isomers were found to be broad-spectrum antibacterial agents but more cytotoxic on eukaryotic cells than **9**. From the delineated SAR, two fluorescent antibacterial 3′,6-dialkyl neamine isomers **21** and **22**, carrying a heptyl and a pyrenylbutyl groups were identified, their activity making them useful for further mechanistic study. The antibacterial properties of these 3′,6-heterodialkyl neamines were compared to those of the corresponding 3′- or 6-monoalkyl derivatives **23**–**26**.

Antibacterial AAGs such as dialkyl neamines may use differences in the membrane composition to interact selectively with bacterial cells [3,41,42]. Our previous works have characterized the mechanism of action against *P. aeruginosa* [18,20–24]. By using membrane model systems, we demonstrated the interaction of 3′,6-diNn neamine **9** with outer membrane lipopolysaccharides [22] and inner membrane anionic phospholipids mostly cardiolipin leading to membrane permeabilization and depolarization [23]. By targeting cardiolipin-bacterial microdomains mainly located at the cell poles, 3′,6-diNn neamine leads to disassembly these microdomains into cardiolipin clusters and relocation of cardiolipin domains as confirmed by using membrane model systems mimicking inner membrane of *P. aeruginosa* [24]. Importantly, targeting cardiolipin-lipid domains results in bacterial morphological changes. These changes are characterized by a severe length decrease probably as a result from the decrease of the inner membrane fluidity/hydration induced by **9** which could impair the dynamics of cell-shape determining proteins like MreB [24]. Targeting cardiolipin could also explain the observed inhibition of the redox chain accompanied with an intracellular ATP concentration and pH decrease, and inhibition of cell growth induced by the amphiphilic neamine derivative [24]. Thus, several key membrane components present in multiple copies in the bacterial membranes are the targets of antibacterial amphiphilic neamines. Biochemical modifications of such multiple targets should have a high cost for the bacteria resulting in a slow development of resistances, in a rapid reversibility of the emerging resistances and in a high sensitivity to conventional antibiotic drugs of other classes [43].

In order to demonstrate the expected difficulty of resistance emergence to AAGs, we report here on the MIC changes against susceptible *P. aeruginosa* upon exposure over one month to half-MIC of one of the most active derivative, 3′,6-diNn neamine **9**, in comparison to the antibiotic drug ciprofloxacin.

## 2. Synthesis

The chemistry was developed from tetra-*N*-trityl neamine **27** [35] (Scheme 1) that was mono- and di-alkylated under phase transfer conditions for obtaining concomitantly tetratritylated 3′,6-dialkyl neamines and tetratritylated 3′- and 6-monoalkyl neamines intermediates.

Tetra-*N*-trityl neamine **27** was alkylated at 50 °C with the corresponding 1-bromoalkane, concentrated aqueous sodium hydroxide (30%), toluene and TBAI as a phase transfer agent. In the presence of TBAI, no tri- and tetra-alkylations were observed whereas using at room temperature TBAF as a phase transfer agent the 3′,4′,6-alkylated derivatives are formed [44].

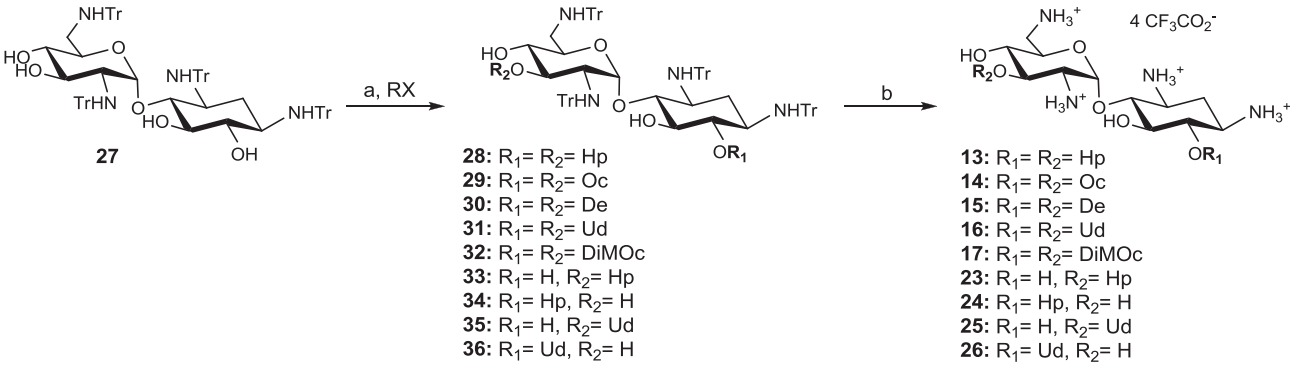
After purification, the tritylated 3′,6-alkylated neamines were deprotected in the presence of TFA and anisole to afford the potential antibacterial amphiphilic neamines.

The previously determined SAR window frame relating the antibacterial activity (1/MIC) and the calculated lipophilicity (clogP, Table 1) [45] of 3′,6-dialkyl neamine derivatives suggests that the clogP of antibacterial amphiphilic neamines should have a lipophilicity value higher than 3′,6-dihexyl (3′,6-diHx) neamine **8** (clogP = −14.2) and lower than 3′,6-di-2-naphthylhexyl (3′,6-di2NH) neamine **6** (clogP = −9.0) that are inactive against most of the selected bacteria [19]. In these studies, 3′,6-di-2-naphthylmethyl **3** (3′,6-di2NM) neamine (clogP = −12.7) showed unique properties being mainly active against susceptible and resistant Gram-positive bacteria. The clogP values of the tetraprotonated amphiphilic neamines calculated using the MarvinSketch software 5.11.4 have been used to define the window frame [45]. In this study, in order to define more accurately the window frame of SAR and to identify new strongly antibacterial amphiphiphilic neamines, 3′,6-diHp, (**13**), -diOc (**14**), -diDe (**15**), -diUd, (**16**) neamines carrying the same linear alkyl chains and the 3′,6-di(3,7-dimethyl)octyl (diDiMOc, **17**) neamine derivative carrying the same branched alkyl chains were prepared (Scheme 1, Table 1). During this synthesis, the formed tetratritylated 3′- and 6-monoheptyl (**33** and **34**) and 3′- and 6-monoundecyl neamines (**35** and **36**) were isolated for a potential second alkylation and/or for deprotection (Scheme 1).

After deprotection, 3′- and 6-monoheptyl (**23** and **24**, respectively) and 3′- and 6-monoundecyl neamines (**25** and **26**, respectively) were isolated and evaluated for comparison of their potential antibacterial activity to those of their parent 3′,6-dialkyl derivatives.

The second alkylation of the isolated tetratrityl 3′-monoHp (**33**) and 3′-monoUd (**35**) derivatives (Scheme 2) was performed under the same conditions in order to introduce a second undecyl and a heptyl group, respectively, and to obtain two isomeric 3′,6-dialkyl neamines having same clogP = −11.9 than the antibacterial 3′,6-diNn neamine **9** previously identified. After deprotection, the isomeric 3′-heptyl-6-undecyl (**19**) and 3′-undecyl-6-heptyl (**20**) neamines were isolated for antibacterial evaluation and comparison with **9**.

We also synthesized fluorescent amphiphilic neamines having clogP = −10.7, value corresponding to a potential antibacterial activity (Scheme 2). The pyrenyl ring was chosen as fluorophore and the lipophilic 4-(1-pyrenyl)butyl group (1PyBu) was selected to be introduced at the 3′- or the 6-position of the neamine core after introduction of one heptyl group at the 6- or 3′-position in order to maintain a lipophilicity close to the one of 3′,6-dinonyl neamine **9** This procedure could allow the obtention of fluorescent antibacterial amphiphilic neamines useful for mechanistic




**Scheme 1.** Synthesis of the studied 3′,6-dialkyl neamines and of their related 3′-mono- and 6-mono-alkyl compounds[a]
[a]Reagents and conditions: (a) 1-bromoalkane (RX), toluene, TBAI, aqueous NaOH (30%), 50 °C, 24 h; (b) TFA/$CH_2Cl_2$, anisole, rt, 2 h.

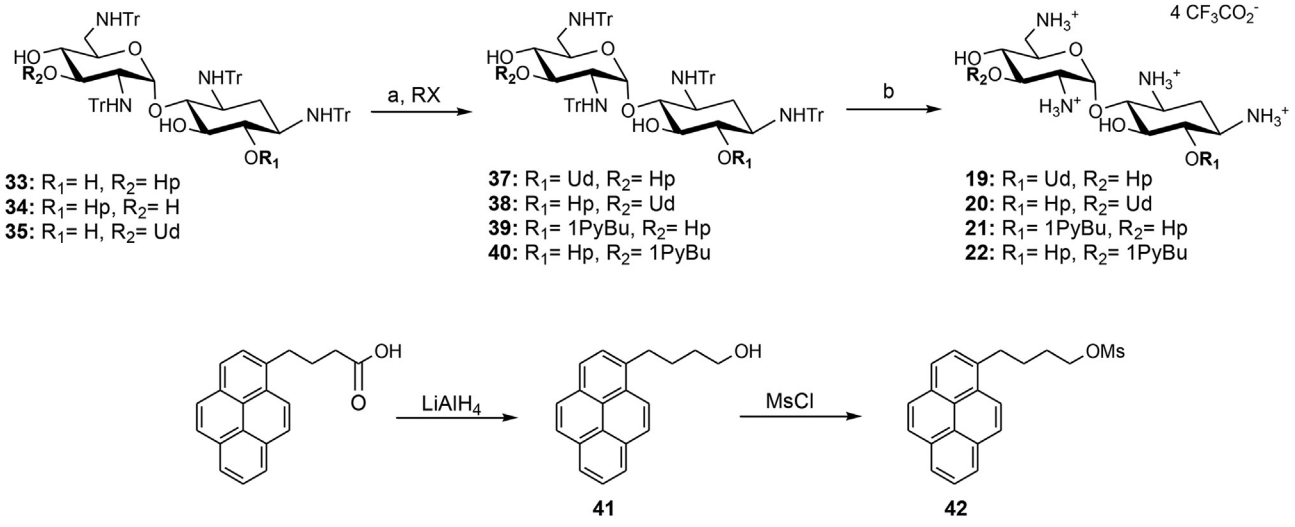
**Table 1**
ClogP values calculated using the MarvinSketch software 5.11.4 for the tetraprotonated amphiphilic neamine derivatives used in this study and for some antibacterial agents [45].

| Reference antibacterials | clogP | Dialkyl neamines | clogP |
|---|---|---|---|
| Neomycin B **1** | −29.9 | Dihexyl (diHx) **8** [19] | −14.2 |
| Colistin | −29.7 | Diheptyl (diHp) **13** | −13.5 |
| Neamine **2** | −19.4 | Dioctyl (diOc) **14** | −12.7 |
| Monoalkyl neamines | clogP | Dinonyl (diNn) **9** [19] and **12** [20], heptyl-undecyl **19** and **20** | −11.9 |
| Heptyl (MonoHp) **23** and **24** | −16.4 | Di(3,7-dimethyl)octyl (diDiMOc) **17** and **18** | −11.3 |
| Undecyl (MonoUd) **25** and **26** | −14.8 | Didecyl (diDe) **15** | −11.1 |
| | | Diundecyl (diUd) **16** | −10.3 |
| | | Pyrenylbutyl-heptyl (1PyBu-Hp) **21** and **22** | −10.7 |
| Dinaphthylalkyl neamines | clogP | | |
| Di(2-naphthyl)methyl (di2NM) **3** [18] | −12.7 | Di(2-naphthyl)butyl (di2NB) **5** [19] | −10.6 |
| Di(2-naphthyl)propyl (di2NP) **4** [19] and **11** [20] | −11.4 | Di(2-naphthyl)hexyl (di2NH) **6** [19] | −9.0 |

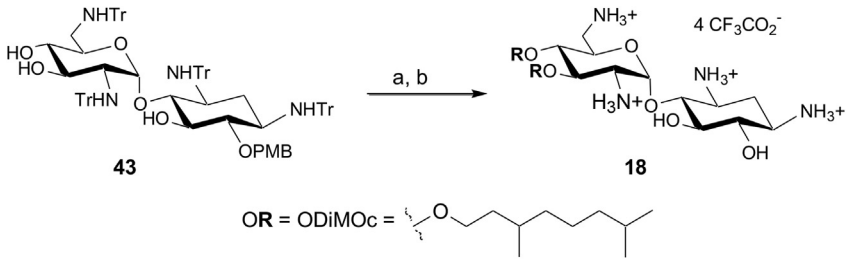
studies. 4-(1-pyrenyl)butyl methanesulfonate **42** was prepared in two steps from commercially available 4-(1-pyrenyl)butanoic acid: (i) reduction with $LiAlH_4$ to lead to the corresponding alcohol (**41**) and (ii) mesylation of the alcohol with methanesulfonyl chloride (Scheme 2).

The isolated tetratrityl 3′-monoheptyl (**33**) and 6-monoheptyl (**34**) neamines were alkylated with pyrenylbutyl sulfonate **42** under phase transfer conditions in the presence of TBAI. The tetratritylated 3′,6-dialkyl neamines formed were purified and deprotected to lead to 3′-heptyl-6-(4-(1-pyrenyl)butyl) (**21**) and 3′-(4-(1-pyrenyl)butyl)-6-heptyl (**22**) isomeric neamines.

The 3′,4′-di-2-naphthylpropyl (2NP) (**11**) and 3′,4′-dinonyl (Nn)




**Scheme 2.** Synthetic pathway used for the preparation of 3′,6-dialkyl neamines derivatives bearing two different alkyl chains[a]
[a]Reagents and conditions: (a) RX = 1-bromoalkane or 4-(1-pyrenyl)butyl mesylate **42** (synthesized from the corresponding acid via the alcohol **41**), toluene, TBAI, aqueous NaOH (30%), 50 °C, 24 h (b) TFA/$CH_2Cl_2$, anisole, rt, 2 h.




**Scheme 3.** Synthesis of 3′,4′-di-*O*,*O*′-(3,7-dimethyl)octyl neamine **18** (3′,4′-diDiMOc) from the *N*-tetratritylated *p*-methoxybenzyl neamine derivative **43** [44][a]
[a]Reagents and conditions: (a) NaH, DMF, 1-bromo-3,7-dimethyloctane, rt, 31%. (b) TFA/$CH_2Cl_2$, anisole, rt, 55%.

(**12**) neamine derivatives have shown interesting antibacterial activity against susceptible and resistant bacteria with MIC mostly slightly higher than those of their 3′,6-isomers **4** and **9**, respectively. Thus, we synthesized the 3′,4′-isomer **18** of compound **17** (3′,6-diDiMOc neamine) from the *N*-tetratrityl neamine derivative **43** [44] in which the 6-hydroxyl group is selectively protected by the *p*—methoxybenzyl group (Scheme 3). It was obtained in 17% yield in two steps: (i) alkylation of **43** in DMF with NaH and 1-bromo-3,7-dimethyloctane and (ii) deprotection with TFA/anisole.

## 3. Antibacterial activity, results and discussion

The synthesized amphiphilic neamines were evaluated against a large panel of susceptible and resistant Gram-positive and Gram-

**Table 2**
Minimum Inhibitory Concentrations (MIC) of the neamine derivatives synthesized and some representative AG against susceptible and resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa* strains. For AAGs, blue cells: MIC< 4 μg/mL, yellow cells: MIC = 4 to 8 μg/mL, non colored cells: MIC > 8 μg/mL.

| AGs | MIC μg/mL | | | | | | |
|---|---|---|---|---|---|---|---|
| | *S. aureus* | | | *P. aeruginosa* | | | |
| | ATCC 25923 | SA-1 Pump NorA | ATCC 33592 HA-MRSA | ATCC 27853 | Psa. FO3[a] | PA22[b] | PA406[c] |
| Amikacin | 4 | 2 | 8-16 | 2-4 | 8 | 8 | 1 |
| Gentamicin | 0.25-0.5 | 0.5 | 1 | 1 | >128 | 4 | < 0.25 |
| Tobramycin | 0.25-0.5 | 0.5 | 64 | < 0.25 | 128 | 0.5 | < 0.25 |
| Neomycin B **1** | 1-2 | 0.5-1 | >128 | 64 | 128 | 32-64 | 2-4 |
| Neamine **2** | 16-32 | 8 | >128 | >128 | >128 | >128 | 64 |
| Dialkyl neamines incorporating same alkyl groups | | | | | | | |
| 3′,6-di2NP **4**[19] | 2 | 2 | 2 | 8-16 | 16 | 16 | 2-4 |
| 3′,6-di2NB **5**[19] | 2 | 2-4 | 2 | 4 | 8 | 8 | 8 |
| 3′,6-diHp **13** | 8 | 8 | 8 | 32 | 8 | 16 | 4 |
| 3′,6-diOc **14** | 1 | 1 | 2 | 2 | 8 | 8 | 2 |
| 3′,6-diNn **9**[19] | 1 | 1 | 2-4 | 2-4 | 4-8 | 4 | 2-4 |
| 3′,4′-diNn **12**[20] | 4 | 4 | 4 | 8-16 | 8-16 | 8-16 | 8-16 |
| 3′,6-diDe **15** | 1 | 1 | 1-2 | 4 | 4 | 4 | 8 |
| 3′,6-diUd **16** | 8 | 8 | 64 | 16 | 16 | 16 | 16 |
| 3′,6-diDiMOc **17** | 1 | 1 | 1 | 4 | 8 | 4-8 | 2-4 |
| 3′,4′-diDiMOc **18** | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| Dialkyl neamines incorporating two different alkyl groups | | | | | | | |
| 3′-Hp-6-Ud **19** | 1 | 1 | 1 | 2 | 4 | 4 | 2 |
| 3′-Ud-6-Hp **20** | 1 | 32 | 1 | 2 | 4 | 4 | 2 |
| 3′-Hp-6-1PyBu **21** | 2 | 2 | 1 | 4 | 4 | 16 | 4 |
| 3′-1PyBu-6-Hp **22** | 1 | 4 | 2 | 8 | 4 | 16 | 8 |
| Monoalkyl neamines | | | | | | | |
| 3′-monoHp **23** | >128 | >128 | >128 | >128 | >128 | >128 | >128 |
| 6-monoHp **24** | >128 | >128 | >128 | >128 | >128 | >128 | >128 |
| 3′-monoUd **25** | 16 | 16 | 16 | >128 | >128 | >128 | 4-8 |
| 6-monoUd **26** | 32 | 8 | 8 | >128 | >128 | >128 | 16 |

a: Psa.F03 AAC6′-IIA; b: surexp MexXY; c: PAO509.5 ΔtriABC.

**Table 3**
Minimum Inhibitory Concentrations (MIC) of the neamine derivatives synthesized and some representative AG against selected bacterial susceptible and resistant *Acinetobacter lwoffii*, *Escherichia coli* and *Klebsiella pneumonia* strains. For AAGs, blue cells: MIC< 4 μg/mL, yellow cells: MIC = 4 to 8 μg/mL, non colored cells: MIC > 8 μg/mL.

| AGs | MIC μg/mL | | | | | |
|---|---|---|---|---|---|---|
| | *A. lwoffii* | | *E. coli* | | | *K. pneumonia* |
| | ATCC 17925 | AI.88-483[a] | ATCC 25922 | PAZ505H8101[b] | L8058.1[c] | ATCC 700603 |
| Amikacin | < 0.25 | 128 | 4 | 64 | 8 | 1 |
| Gentamicin | < 0.25 | 2 | 0.5 | 1 | 64 | 4-8 |
| Tobramycin | < 0.25 | 0.5-1 | 1 | 32 | 64 | 4-8 |
| Neomycin B **1** | < 0.25 | 64-128 | 2 | 4 | 1 | 4-8 |
| Neamine **2** | 0.5 | >128 | 32 | >128 | 32 | 32 |
| Dialkyl neamines incorporating same alkyl groups | | | | | | |
| 3′,6-di2NP **4**[19] | 4 | 32-64 | 8 | 4-8 | 16 | 32 |
| 3′,6-di2NB **5**[19] | 2 | >128 | 8 | 4 | 8 | 32 |
| 3′,6-diHp **13** | 32 | >128 | 32 | 16 | 32 | 64 |
| 3′,6-diOc **14** | 2-4 | 128 | 4 | 2-4 | 4 | 8 |
| 3′,6-diNn **9**[19] | 2 | 16 | 2-4 | 2-4 | 2-4 | 4 |
| 3′,4′-diNn **12**[20] | 4-8 | 32 | 4-8 | 4-8 | 8 | 16-32 |
| 3′,6-diDe **15** | 2 | 64 | 2 | 8 | 128 | 16 |
| 3′,6-diUd **16** | 32 | 32 | 4 | 16 | 16 | 32 |
| 3′,6-diDiMOc **17** | 1-2 | 4-8 | 2-4 | 1-2 | 2-4 | 2 |
| 3′,4′-diDiMOc **18** | 4 | 8 | 8 | 4 | 4 | 8 |
| Dialkyl neamines incorporating two different alkyl groups | | | | | | |
| 3′-Hp-6-Ud **19** | 2 | >128 | 16 | 4 | 4 | 8 |
| 3′-Ud-6-Hp **20** | 2 | >128 | 2 | 2 | 4 | 4 |
| 3′-Hp-6-1PyBu **21** | 4 | >64 | 4 | 4 | 16 | 8 |
| 3′-1PyBu-6-Hp **22** | 8 | >128 | 4 | 4 | 8 | 4 |
| Monoalkyl neamines | | | | | | |
| 3′-monoHp **23** | 64 | >128 | >128 | >128 | >128 | >128 |
| 6-monoHp **24** | 64 | >128 | >128 | >128 | >128 | >128 |
| 3′-monoUd **25** | 32 | >128 | 64 | 32 | 32 | >128 |
| 6-monoUd **26** | 64 | >128 | >128 | >128 | >128 | >128 |

a: APH3′-VIA; b: AAC6′-IB; c: ANT2′′-IA.

negative bacteria. In the former class of bacteria, the minimum inhibitory concentrations (MIC) were measured against susceptible *S. aureus* ATCC 25923 and two resistant strains, the SA-1 strain overexpressing resistance pump (NorA) and the Methicillin-resistant strains ATCC 33592 HA-MRSA (Table 2) [46]. In the Gram-negative class of bacteria, the effects were evaluated against susceptible and resistant strains of *P. aeruginosa* (Table 2), *A. lwoffii* and *E. coli* overexpressing aminoglycoside-modifying enzymes or efflux pumps and against the susceptible ATCC 700603 K. *pneumonia* strain (Table 3).

### 3.1. Antibacterial activity of the 3′,6-homodialkyl neamines diHp **13** to diUd **16** made of linear alkyl chains

In order to finely tune the antibacterial activity-lipophilicity relationship, we evaluated the synthesized 3′,6-dialkyl neamines carrying two linear alkyl chains heptyl (7 carbon atoms in both alkyl chains, Hp/C7, **13**), octyl (Oc/C8, **14**), decyl (De/C10, **15**) and undecyl (Ud/C11, **16**). The clogP of these derivatives (Table 1) have been selected to match with the −12.7 to −9.0 clogP window corresponding to derivatives having broad-spectrum activity. This window has been delineated from Fig. 2A on HA-MRSA and Fig. 3A on susceptible *P. aeruginosa* ATCC 27853, Figures in which the values of (1/MIC) are plotted versus clogP values [19].

Against *S. aureus* strains, the diOc (**14**), diNn (**9**) and diDe (**15**) neamines showed MIC values from 1 to 2–4 μg/mL (Table 2) with clogP values ranging from −12.7 to −11.1 (Table 1). The antibacterial activity decreases for the diHp (**13**) and diUd (**16**) neamines with MIC against MRSA of 8 and 64 μg/mL, respectively. The clog P values of the diHp (**13**) and diUd (**16**) neamines are −13.5 and −10.3, respectively, and correspond to the limits of the clogP windows (Fig. 2 A) [19].

Against *P. aeruginosa* strains (Table 2), the diOc (**14**), diNn (**9**) and diDe (**15**) neamines also showed the lower MICs (from 2 to 8 μg/mL). The antibacterial effect decreased for the diHp (**13**) and diUd (**16**) derivatives (MICs from 4 to 32 μg/mL) with a better effect of the diHp derivative **13** against PA406. The diNn derivative **9** appeared to be the most active derivative in the series.

Against susceptible *A. lwoffii* and *K. pneumonia* (Table 3), the most active derivatives are again the diOc (**14**), diNn (**9**) and diDe (**15**) neamines with MIC = 2–4 μg/mL and 4–16 μg/mL, respectively to the strains. Once again, the diNn derivative appeared to be the most active derivative in the series especially against resistant *A. lwoffii* overexpressing aminoglycoside-modifying enzymes (MIC = 16 μg/mL), the diOc (**14**) and diDe (**15**) derivatives showing high MIC values (128 and 64 μg/mL, respectively).

Against the susceptible *E. coli* strain (Table 3), the four diOc (**14**), diNn (**9**), diDe (**15**) and diUd (**16**) neamines showed similar good antibacterial activity (MIC = 2–4 μg/mL). The most active compounds are the diNn (**9**) and diDe (**15**) with close MIC from 2 to 4 μg/mL and, against resistant *E. coli* strains, the activity decreased mainly for the diDe (**15**) and diUd (**16**) derivatives (MIC from 8 to 128 μg/mL) whereas the activity of the diOc (**14**) and diNn (**9**) remained unchanged (MIC = 2–4 μg/mL).

The difference in the antibacterial activity of the 3′,6-dialkyl neamines appears in the graphs of (1/MIC) versus clogP plotted for HA-MRSA (Fig. 2) and susceptible *P. aeruginosa* ATCC 27853 (Fig. 3).

In Fig. 2B, the previously obtained graph plotted for MRSA (Fig. 2A) [19] is decomposed into two graphs, one for the neamine

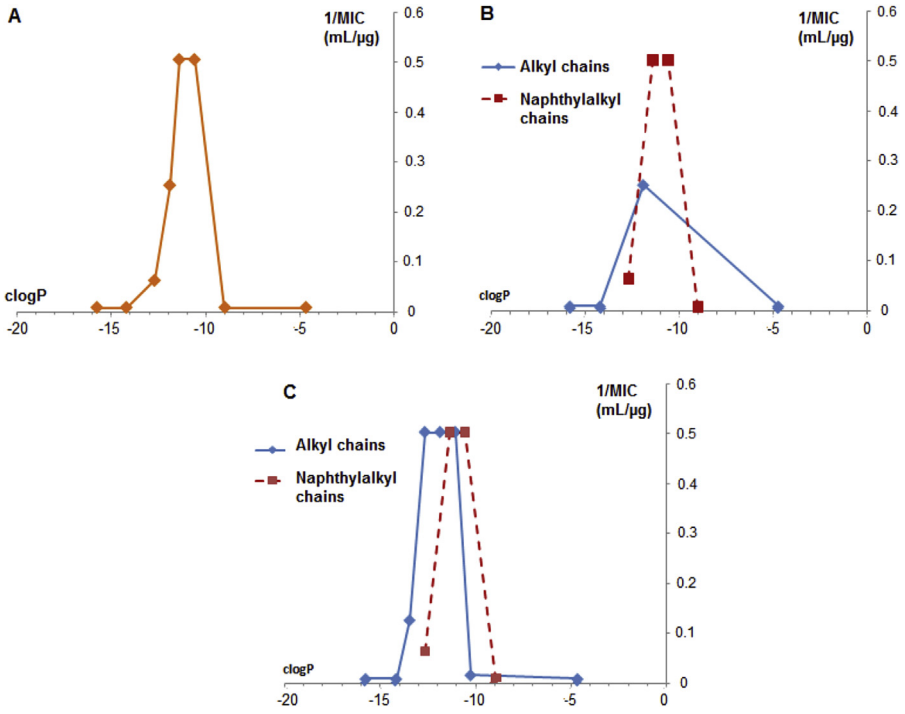



**Fig. 2.** Values of 1/(MIC (µg/mL)) as a function of clogP values for 3′,6-dialkyl neamines against ATCC 33592 HA-MRSA. A: Graph resulting from the previous study [19]; B: Previous graph decomposed into two graphs, one for neamines carrying linear alkyl chains (**7** (diBu), **8** (diHx), **9** (diNn) and **10** (diOcD): blue diamonds) and, the other one, for neamines carrying 2-naphthylalkyl groups (**3** (di2NM), **4** (di2NP), **5** (di2NB) and **6** (di2NH): red squares and red broken line); C: Graphs obtained with 3′,6-dialkyl neamines carrying linear alkyl chains including the new synthesized derivatives **13**–**16** (blue diamonds) in comparison to the naphthylalkyl derivatives **3**–**6** (red squares and red broken line).

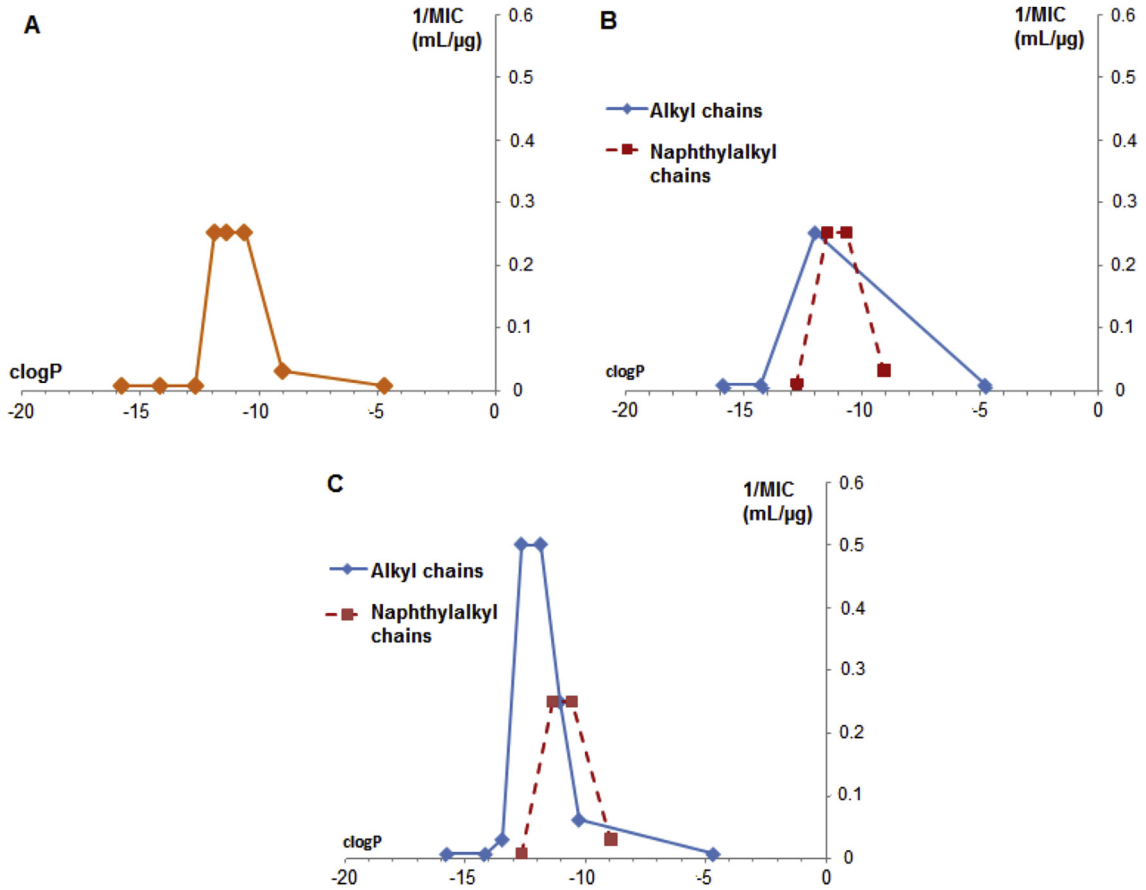



**Fig. 3.** Values of 1/(MIC (µg/mL)) as a function of clogP values for 3′,6-dialkyl neamines against susceptible *P. aeruginosa* ATCC 27853. A: Graph resulting from the previous study [19]; B: Previous graph decomposed into two graphs, one for neamines carrying linear alkyl chains (**7** (diBu), **8** (diHx), **9** (diNn) and **10** (diOcD): blue diamonds) and the other one for neamines carrying 2-naphthylalkyl groups (**3** (di2NM), **4** (di2NP), **5** (di2NB) and **6** (di2NH): red squares and red broken line); C: Graphs obtained with 3′,6-dialkyl neamines carrying linear alkyl chains including the new synthesized derivatives **13**–**16** (blue diamonds) in comparison to the naphthylalkyl derivatives **3**–**6** (red squares and red broken line).

derivatives carrying linear alkyl chains **7** (diBu), **8** (diHx), **9** (diNn) and **10** (diOcD) and the other for derivatives carrying 2-naphthylalkyl groups **3** (di2NM), **4** (di2NP), **5** (di2NB) and **6** (di2NH). Fig. 2B shows clearly the previous lack of data for linear alkyl chains. In Fig. 2C, the graph incorporating the data obtained with the new synthesized derivatives carrying diHp (diC7) to diUd (diC11) alkyl chains (neamines **13**, **14**, **9**, **15**, **16**) is compared to the graph obtained with the previously synthesized 2-naphthylalkyl derivatives. Clearly, two different windows of clogP values can be delineated for both series, one for the dialkyl derivatives from −13.5 to −10.5 and the other for the naphthylalkyl derivatives from −13.0 to −9.5. Fig. 2C also shows clearly that both dialkyl and dinaphthylalkyl derivatives can have similar good antibacterial activity.

In Fig. 3, the same window-splitting can be observed for the susceptible *P. aeruginosa* strain with delineation of two new windows of clogP values, one for the dialkyl derivatives from −13.5 to −10.5 and second for the naphthylalkyl derivatives from −13.0 to −9.5 (Fig. 3C). Fig. 3C also shows the higher antibacterial activity of the dialkyl neamines in comparison to the naphthylalkyl derivatives on susceptible *P. aeruginosa* that is also observed in Table 2 against *E. coli* and *K. pneumonia* strains.

### 3.2. Antibacterial activity of the 3′,6-diDiMOc (*17*) and 3′,4′-diDiMOc (*18*) neamines made of branched alkyl chains

The 3′,6-diDiMOc neamine **17** having a −11.3 clogP value (Table 1) included in the delineated lipophilicity window showed antibacterial activity against susceptible and resistant *S. aureus* strains (MIC = 1 μg/mL, Table 2) similar to those of the diOc (**14**), diNn (**9**) and diDe (**15**) neamines (MIC = 1 to 2–4 μg/mL). **17** also revealed activity against susceptible and resistant Gram-negative bacteria similar to those measured with the previously identified best antibacterial diNn neamine **9** (MIC = 1–8 and 2–16 μg/mL, respectively, Table 3) with a potential interest for resistant *A. lwoffii* (MIC = 8 μg/mL as compared to 16 μg/mL for diNn neamine).

Its 3′,4′-diDiMOc isomer **18** showed a slightly weaker antibacterial activity against susceptible and resistant Gram-negative and Gram-positive bacteria with MICs values ranging from 4 to 8 μg/mL, (Table 2). This activity appeared to be close to the activity of the previously studied 3′,4′-diNn neamine **12** (Table 2) [20].

### 3.3. Antibacterial activity of the 3′,6-heteroalkyl neamines, mixed n-heptyl/n-dodecyl derivatives (*19* and *20*) and fluorescent mixed n-heptyl/(1-pyrenyl)butyl (*21* and *22*)

In order to evaluate the importance of the hydrophobic chain length in the antibacterial activity maintaining the lipophilicity in the delineated windows of clogP values, 3′-Hp-6-Ud (**19**) and 3′-Ud-6-Hp (**20**) neamines carrying mixed linear C7 and C11 alkyl groups and two fluorescent derivatives **21** and **22** incorporating Hp (C7) and (1-pyrenyl)butyl (1PyBu) groups (clogP = −10.7) were synthesized. Their structures were designed to lead, for the dialkyl isomers, to clogP = −11.9 corresponding to the calculated value for the diNn derivative **9** and to clogP = −10.7 for the pyrenyl derivatives (Table 1). As expected, MIC values of both Hp/Ud neamines **19** and **20** appeared to be mainly from 1 to 4 μg/mL except for compound **19** against *E. coli* (MIC 16 μg/mL) and compound **20** against *S. aureus* NorA (32 μg/mL). They are inactive (MIC> 128 μg/mL) against resistant *A. lwoffii* (Tables 2 and 3).

Both fluorescent pyrenyl derivatives **21** and **22** designed and synthesized for mechanistic study showed similar antibacterial activity (Tables 2 and 3) slightly lower as compared to those of both mixed Hp/Ud derivatives **19** and **20** against all selected bacteria strains. Again, they are inactive on resistant *A. lwoffii* and weakly active on *P. aeruginosa* PA22.

The corresponding 3′-mono- and 6-mono-heptyl neamines (**23** and **24**, respectively) were found inactive or very weakly active (MIC> 64 μg/mL) (Tables 2 and 3). The 3′-mono- and 6-mono-C11 neamines (**25** and **26** respectively) also appeared to be mainly inactive (MIC = 8 to >128 μg/mL) (Tables 2 and 3). However, some MIC values were found to be relatively low (4–32 μg/mL against *S. aureus* strains and against PA406) but they are clearly higher than those observed with the derivatives **19**–**22** carrying two different alkyl chains.

## 4. Cytotoxicity

Using the MTT assay, the viability of murine J774 macrophages were evaluated in the presence of 10 and 30 μM of the antibacterial 3′,6-dialkyl neamines (**13**–**26**) described here and are compared in Table 4 to those of antibacterial neamines (**3**, **4**, **9**, **11** and **12**) previously described.

At 10 μM, the viability values measured in the presence of the 3′,6-homodialkyl neamines **13**, **14**, **9**, **15**, **16** carrying from Hp (C7) to

**Table 4**
Viability (%) of murine J774 macrophages determined using the MTT assay in the presence of 10 and 30 μM of the prepared neamine derivatives, the numbers of independent experiences are mentioned after the viability values in brackets.

| Reference AGs | Viability % 10 μM | 30 μM | Dialkyl neamines incorporating same alkyl groups | Viability % 10 μM | 30 μM |
|---|---|---|---|---|---|
| Amikacin | 81.2 (3) | 92.0 (2) | 3′,6-di2NM **3**[18] | 86.0 (7) | 75.7 (2) |
| Gentamicin | 88.4 (11) | 82.9 (4) | 3′,6-di2NP **4**[19] | 91.1 (13) | 89.5 (2) |
| Tobramycin | 99.7 (4) | 83.2 (2) | | | |
| Neomycin B **1** | 87.3 (10) | 69.8 (2) | 3′,6-diHp **13** | 96.3 (3) | 79.9 (3) |
| Neamine **2** | 94.8 (9) | 84.4 (2) | 3′,6-diOc **14** | 91.3 (4) | 65.1 (3) |
| Dialkyl neamines incorporating different alkyl groups | | | 3′,6-diNn **9**[19] | 86.7 (9) | 67.4 (3) |
| 3′-Hp-6-Ud **19** | 82.9 (3) | 38.8 (2) | 3′,6-diDe **15** | 85.5 (4) | 14.5 (3) |
| 3′-Ud-6-Hp **20** | 88.4 (3) | 39.1 (2) | 3′,6-diUd **16** | 81.7 (4) | 14.1 (3) |
| 3′-Hp-6-1PyBu **21** | 77.7 (3) | 23.8 (2) | 3′,6-diDiMOc **17** | 88.4 (8) | 58.7 (9) |
| 3′-1PyBu-6-Hp **22** | 55.9 (3) | 45.3 (2) | | | |
| Monoalkyl neamines | | | 3′,4′-diDiMOc **18** | 62.6 (2) | 75.4 (2) |
| 3′-monoHp **23** | 77.5 (4) | 70.2 (3) | 3′,4′-di2NP **11**[20] | 87.7 (6) | 66.9 (2) |
| 6-monoHp **24** | 84.3 (3) | 71.3 (2) | 3′,4′-diNn **12**[20] | 82.7 (4) | 48.6 (2) |
| 3′-monoUd **25** | 81.8 (4) | 77.1 (3) | | | |
| 6-monoUd **26** | 74.7 (3) | 83.4 (2) | | | |

Ud (C11) linear alkyl chains or the branched DiMOc chain (**17**) decreased slightly with the linear chain length and remains close to 80–90%. At 30 μM, the corresponding viability values decreased more drastically with the increase of the chain length and thus clogP, the values are near to 80% for the diHp (diC7, **13**), to 70% for the diNn (diC9, **9**) and to 65% for the diOc (diC8, **14**) neamines and much smaller (15% viability) for the diDe (C10, **15**) and diUd (diC11, **16**) neamines. The 3′,6-diDiMOc derivative **17** showed a viability of 59%, smaller than the 67% value measured in the presence of its 3′,6-diNn analogue **9** of close lipophilicity (clogP = −11.3 and −11.9, respectively).

A strong decrease of the viability at 30 μM in comparison to 10 μM was also observed for the 3′,6-dialkyl neamines **19** and **20** carrying mixed heptyl/undecyl groups (C7/C11) (around 85% at 10 μM and 39% at 30 μM, respectively). The fluorescent heptyl/pyrenylbutyl neamines **21** and **22** showed as expected reduced viability values (78 and 56% at 10 μM and 24 and 45% at 30 μM, respectively). However, the 3′-Hp-6-PyBu neamine **19** appeared to be strongly less cytotoxic at 10 μM than its 3′-PyBu-6-Hp isomer **20.** At 10 μM, the antibacterial 3′,4′-diDiMOc derivative **18** showed a reduced viability in comparison to its 3′,6-isomer **17** (63 and 88%, respectively). All the monoalkyl neamines **23**–**26** showed similar effects with viability roughly close to 70–80% both at 10 and 30 μM.

## 5. MIC changes against *P. aeruginosa* UPON long exposure to AAGs

We have previously studied the MIC changes against susceptible *P. aeruginosa* ATCC 27853 upon 12 days exposure to half-MIC of 3′,6-di2NP neamine (**4**) and its 3′,4′-isomer (**11**) in comparison to ciprofloxacin [20]. Exposure of *P. aeruginosa* to subinhibitory concentrations of ciprofloxacin, or di2NP neamines caused a decrease in susceptibility that has appeared later for the di2NP derivatives. We repeated this type of experiment with the 3′,6-diNn neamine derivative **9**, one of the most active neamine identified against susceptible and resistant *P. aeruginosa* strains. MIC of **9** against susceptible *P. aeruginosa* ATCC 27853, after exposure to half-MIC at different times over more than one month, appeared to be slightly increased from 1 to 4 μg/mL at day 15, 30 and 38 as compared to MIC values measured with ciprofloxacin which increased faster from 0.5 to 16 μg/mL at days 6, 21 and 24 (Fig. 4).

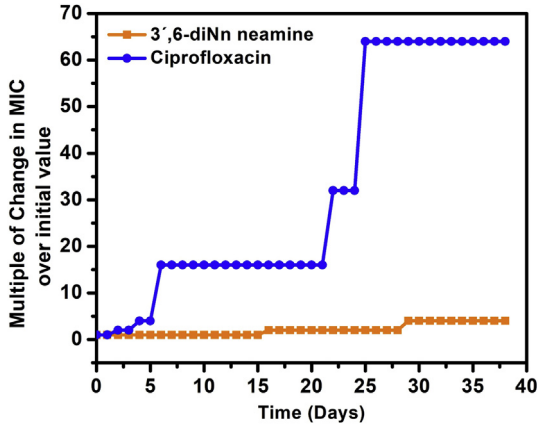



**Fig. 4.** Evolution of the MIC against *P. aeruginosa* of 3′,6-diNn neamine **9** (orange line) in comparison to the evolution of the MIC of ciprofloxacin (blue line) after exposure to half-MIC concentrations for the indicated times. The concentration of the antibiotic was re-adjusted each day to remain equivalent to half the MIC. Results are expressed in changes in MICs over initial value. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

## 6. Discussion and conclusion

Previously, we have identified three broad-spectrum antibacterial amphiphilic neamines carrying two lipophilic groups grafted at positions 3′ and 6 of the neamine core, two 3′,6-dinaphtylalkyl neamines **3**, **4** and **5** and one 3′,6-dialkyl neamine, the dinonyl derivative **9**. In the search for new antibacterial neamines, new 3′,6-dialkyl neamines **13**–**17** carrying two identical linear alkyl chains made of 7–11 carbon atoms (C7 to C11) were synthesized and evaluated in comparison to the previously active 3′,6-diNn derivative **9**. The best antibacterial activity against *S. aureus* strains was observed with the octyl, nonyl and decyl chains (compounds **14**, **9** and **15**, respectively; 3 strains among which MRSA). These derivatives also showed the best activity against the majority of susceptible and resistant Gram-negative bacteria. In comparison, the antibacterial activity of the diheptyl (diC7, **13**) and diundecyl (diC11, **16**) derivatives decreases against most bacteria strains and more strongly against Gram-negative bacteria.

These results well confirm the key role of the lipophilicity in the antibacterial activity and lead to differentiate the lipophilicity/activity window corresponding to linear 3′,6-dialkyl neamine (−13.5 to −10.5 clogP values) from the window corresponding to naphthylalkyl derivatives (−13.0 to −9.5 clogP values) (Fig. 2 against MRSA and Fig. 3 against susceptible *P. aeruginosa*). From this study, we may conclude that the 3′,6-dialkyl neamines are more active against most of Gram-negative bacteria than 3′,6-naphthylalkyl neamines (Fig. 3). The presence on the neamine core of linear alkyl chains more flexible than arylalkyl groups is probably more favorable to insertion of the antibacterial derivatives into Gram-negative bacteria membranes. Into the bacterial membranes, the linear alkyl chains can pack to the linear saturated alkyl chains carried by the targeted anionic lipids, lipopolysaccharides and cardiolipin.

The delineated values of clogP corresponding to good antibacterial activities can be used for the design of new antibacterial amphiphilic neamines but have no biological significance since we have shown previously that (i) the antibacterial activity is correlated directly to the lipophilicity of the grafted chains [19] and (ii) smaller amphiphilic neosamines having weaker clogP exibit similar antibacterial effects [20]. As demonstrated with di2NM (**3**) [18,21] and diNn (**9**) neamines [22–24] and neosamines derivatives [20], the antibacterial activity of the amphiphilic aminoglycosides results from outer effects on bacterial membranes and on different targets and, thus, the delineated clogP values cannot be easily related to these effects also depending on the size and shape of the active compounds. The number of positive charges carried by the amphiphilic neamines is also a critical parameter in the antibacterial activity and several protonated forms are present at physiological pH.

In order to evaluate the importance in the antibacterial effect of the alkyl chain linearity, the 3′,6-dialkyl neamine **17** carrying two branched dimethyloctyl (DiMOc) chains and having a lipophilicity close to the one of the most active diNn derivative **9** (clogP = −11.3 and −11.9, respectively) was synthesized and evaluated. **17** showed a broad-spectrum antibacterial activity similar to the one of diNn derivative **9** and, thus, branched chains can be used for obtaining antibacterial AAGs. Its 3′,4′-isomer (**18**) appeared to be mainly less active as well as the 3′,4′-diNn derivative **12** in comparison to their respective 3′,6-isomers. The difference of the molecular shapes and volumes of the 3′,4′- and 3′,6-dialkyl isomers involved in the membrane effects explains probably this result.

In order to investigate the importance of the respective length and position of both chains in the antibacterial activity, we synthesized 3′,6-heterodialkyl isomeric neamines carrying heptyl and

undecyl chains selected for maintaining a lipophilicity (clogP) close to the lipophilicity of the antibacterial diNn derivative **9** (clogP = −11.9). Both Hp/Ud (C7/C11) isomers **19** and **20** prepared revealed broad-spectrum antibacterial activity similar to the one of the best antibacterial 3′,6-homodialkyl neamines **9**, **14** and **15**, excepted for their lack of activity against the resistant *A. lwoffii* strain. A critical nonyl chain length at both positions is necessary for a significant antibacterial activity against this strain.

Both designed antibacterial fluorescent 3′,6-heteroalkyl neamines carrying a pyrenyl fluorophore and a heptyl chain **21** and **22** matching with the delineated lipophilicity window of activity were also found to be broad-spectrum antibacterial agents and will be used for mechanistic study.

Regarding the viability of murine J774 macrophages in the presence of the antibacterial 3′,6-homodialkyl derivatives, the cell viability decreased slightly at 10 μM from the diHp (**13**) to the diUd (**16**) derivatives increasing the linear chain lengths and for the 3′,6-diDiMOc derivative **17** (viability values from 96 to 82%). At 30 μM, the viability decreased strongly, especially for the most lipophilic diDe (**15**) and diUd (**16**) neamines and for the 3′,6-diDiMOc derivative. A similar strong decrease in mammalian cell viability at 30 μM was observed for the less lipophilic 3′,6-heterodialkyl neamines **19** and **20** carrying Hp/Ud chains and the fluorescent pyrenyl neamines **21** and **22**.

In conclusion, the fine tuning of the previously delineated window of lipophilicity corresponding to broad-spectrum activity of 3′,6-dialkyl neamines performed through a focus on the structure of their lipophilic groups led to the identification of new antibacterial derivatives carrying two linear octyl and undecyl chains or two branched dimethyloctyl chains.

This study led to the delineation of new structure-activity and structure-eukaryotic viability relationships showing that (i) 3′,6-homodialkyl neamines are more active against Gram-negative bacteria than 3′,6-homodinaphthylalkyl neamines but more cytotoxic on mammalian cells, (ii) the grafting of branched alkyl chains of similar lipophilicities preserves or increases the antibacterial activity (examples in the 3′,6- and the 3′,4′-dialkyl series) and, also, increases the eukaryotic cytotoxicity and (iii) 3′,6-heterodialkyl neamines can have good antibacterial activity and the dissymmetry of their chains increases their cytotoxicity.

Recently, Fridman and coworkers reported that an increased degree of unsaturation in the lipophilic chain of antifungal cationic amphiphiles derived from the aminoglycoside tobramycin significantly reduced immediate toxicity to mammalian cells in comparison to saturated derivatives [29]. A similar conclusion can be made here from the decreased eukaryotic cytotoxicity of the dinaphthylalkyl neamines in comparison to the one of the corresponding dialkyl neamines having close lipophilicity. The incorporation of small aromatic rings in the amphiphilic aminoglycoside chains decreases their cytotoxicity and reduced their antibacterial effects against Gram-negative bacteria but interestingly not against Gram-positive bacteria.

The previously identified 3′,6-dinonyl neamine **9** appeared to be the most active amphiphilic neamine against *P. aeruginosa* strains and the less cytotoxic derivative on mammalian cells. The exposure of susceptible *P. aeruginosa* to half-MIC of **9** at different times over one month demonstrated the expected high difficulty of resistance emergence to amphiphilic aminoglycosides with a highly weaker and slower increase of MIC in comparison to ciprofloxacin.

## Acknowledgments

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## Abbreviations Used

| | |
|---|---|
| AAC | aminoglycoside *N*-acetyltransferase |
| AAG | amphiphilic aminoglycoside |
| AG | aminoglycoside |
| ANT | aminoglycoside *O*-nucleotidyltransferase |
| APH | aminoglycoside *O*-phosphoryltransferase |
| CA | cationic amphiphiles |
| cfu | colony forming unit |
| De | decyl |
| DiMOc | 3,7-(dimethyl)octyl |
| Hx | Hexyl |
| Hp | heptyl |
| MRSA | methicillin resistant *S. aureus* |
| MIC | minimum inhibitory concentration |
| 2NB | 2-naphthylbutyl |
| 2NH | 2-naphthylhexyl |
| 2NM | 2-naphthylmethyl |
| Nn | nonyl |
| 2NP | 2-naphthylpropyl |
| Oc | octyl |
| OM | outer membrane |
| Ud | undecyl |

## Experimental section

### *Calculation of clogP Values [45].*

The lipophilicity character of the neamine derivatives prepared was estimated through the calculation of clogP values (octanol/water partition coefficients) using the MarvinSketch software [Marvin 5.11.4, 2012, ChemAxon (http://www.chemaxon.com)]. The clogP plug-in in this software calculates the octanol/water partition coefficient, which is used in QSAR analysis and rational drug design as a measure of molecular hydrophobicity. The calculation method used here is based on a modification of the method published by Viswanadhan and Ghose et al. (VG method) [45].

### *Synthesis. General procedures*

#### *Procedure I: General procedure used for the 3′-mono-, 6-mono- and 3′,6-di-O-alkylation of the tetra-N-tritylated neamine **27** alkylation under phase transfer conditions: [44]*

To a solution of compound **27** [35] (1.0 eq, 3.0 g) in toluene (150 mL) were added *N*-tetrabutyl ammonium iodide TBAI (1.5 eq), the halide (1.6–3.6 eq) and an aqueous solution of NaOH (30%, w/w, 100 mL). The mixture was vigorously stirred at 50 °C during 5 h. Another portion of halide (0.6 eq) was added and the reaction was heated again at 50 °C for 24 h. The mixture was cooled to room temperature and the organic phase was washed with water (3 × 50 mL) and brine (1 × 50 mL) before drying over $MgSO_4$. The solution was filtered off and the solvent evaporated to dryness under reduced pressure. The solid was chromatographed on silica gel eluting with mixtures toluene/ethyl acetate/$Et_3N$ (0.25%) from

100:0 to 80:20 to give the mono and dialkyl products.

*Procedure II: General procedure used for the deprotection of the alkylated N-tetratritylated neamine derivatives*

The tritylated compound was dissolved in DCM/TFA (4/1, v/v) and anisole was added (0.1 mL/mL of solvent mixture). The mixture was stirred at room temperature during 2 h and methanol was added (10 mL). The solvents and TFA were co-evaporated under reduced pressure. A second evaporation after addition of methanol (10 mL) led to the crude product which was dissolved in a mixture of $H_2O$/DCM (1/1). The aqueous phase was washed twice with DCM before evaporation under reduced pressure. The residue was purified by C18 reversed phase chromatography (gradient $H_2O$/MeOH) to give the desired product as the pure tetratrifluoroacetate.

*Purification*

The aminosugar purity of the evaluated compounds was ≥95%. Before the final deprotection step under acidic conditions, careful purifications of the tritylated derivatives allowing minor isomers or impurities removal were performed by chromatography on silica gel. The purity of the deprotected neamine derivatives was controlled by $^1$H NMR spectrometry and TLC on silica gel (eluent, EtOH/$H_2O$/($NH_3$, $H_2O$; 20%) 80:10:10; TLC visualization: ninhydrin spray (0.3 g, 3 mL AcOH, 100 mL of EtOH).

*Characterization of the synthesized amphiphilic neamines evaluated for their antibacterial activity*

The regioselective alkylation of the neamine core was confirmed by 2D NMR COSY/HMQC and mass spectrometry experiments performed with the new amphiphilic neamines described.

2D NMR COSY/HMQC experiments revealed for all new compounds, like for the previously synthesized derivatives, strong increase of the chemical shifts of the carbon atoms of the neamine core carrying an oxyalkyl group and decrease of the chemical shifts of the protons attached to these carbon atoms in comparison to the signals observed for the same carbon atoms of the core carrying a hydroxyl group and the corresponding protons, respectively. Such effects allowed to confirm the structure, for example, in 3′,6-dialkyl neamines, C3′ and C6 are strongly deshielded and the corresponding H3′ and H6 are shielded in comparison to neamine.

HRMS showed a cleavage of the neamine core to lead to a deoxystreptamine fragment corresponding to ring I of the neamine derivatives (Fig. 1) useful to confirm the regioselectivity of alkylation. Indeed, the *m/z* values detected for these fragments can reveal alkylation of ring I, for example, 3′-heptyl-6-undecyl neamine **19** and 3′-undecyl-6-heptyl neamine **20** separately led to a fragment at *m/z* = 317.2799 (z = +1, $C_{17}H_{37}N_2O_3$, *m/z* calc. 317.2799) and a fragment *m/z* = 261.2172 (z = +1, $C_{13}H_{29}N_2O_3$, *m/z* calc. 261.2173) corresponding to the presence of one undecyl group and one heptyl group on ring I, respectively.

*3′,6-Di-O-heptyl neamine **13**, 3′-mono-O-heptyl neamine **23** and 6-mono-O-heptyl neamine **24***

Compounds **13, 23** and **24** were synthesized following procedure I from **11** (1.0 eq, 3.9 mmol, 5.0 g) and 1-bromoheptane (3.5 eq, 14.3 mmol, 2.2 mL). The protected derivatives **28, 33** and **34** were obtained in 35%, 11% and 11% yield respectively (white solid).

**28**: HRMS (ESI$^+$) *m/z*: $[M+H^+]_{calcd}$: 1487.8498, $[M+H^+]_{found}$: 1487.8498; **33**: HRMS (ESI$^+$) *m/z*: $[M+H^+]_{calcd}$: 1389.7403, $[M+H^+]_{found}$: 1389.7405; **34**: HRMS (ESI$^+$) *m/z*: $[M+H^+]_{calcd}$: 1389.7403, $[M+H^+]_{found}$: 1389.7417. The deprotection was performed following procedure II to give derivative **13** (99%), **23** (95%) and **24** (92%) as white solid.

**13**: $^1$H NMR (500 MHz, MeOD) δ = 5.99 (d, *J* = 3.7 Hz, 1H), 4.04 (m, 2H), 4.00–3.85 (m, 3 H), 3.77–3.62 (m, 3H), 3.43 (m, 3H), 3.34 (m, 1H), 3.24 (m, 1H), 3.13 (m, 1H), 2.46 (m, 1H), 2.01 (d, *J* = 12.5 Hz, 1H), 1.66 (m, 4H), 1.31 (m, 16H), 0.89 ppm (m, 6H) ppm. $^{13}$C NMR (125 MHz, MeOD) δ = 95.3, 80.9, 77.2, 76.3, 76.0, 73.7, 73.3, 72.0, 70.4, 53.1, 49.3, 48.7, 40.3, 31.6, 29.7, 29.6, 28.9, 28.4, 25.6, 25.5, 22.3, 13.0 ppm. HRMS (ESI$^+$) *m/z*: $[M+H^+]_{calcd}$: 519.4116, $[M+H^+]_{found}$: 519.4116.

**23**: $^1$H NMR (400 MHz, MeOD) δ = 5.99 (d, *J* = 3.4 Hz, 1H), 4.10–3.93 (m, 3H), 3.86 (dd, *J* = 10.3, 8.8 Hz, 1H), 3.69 (m, 1H), 3.62 (t, *J* = 8.9 Hz, 1H), 3.51–3.38 (m, 4H), 3.19 (br.t, *J* = 9.0 Hz, 1H), 3.09 (dd, *J* = 13.2, 8.6 Hz, 1H), 2.45 (d, *J* = 12.2 Hz, 1H), 2.00 (m, 1H), 1.66 (m, 2H), 1.32 (m, 10H), 0.90 (m, 3H) ppm. $^{13}$C NMR (100 MHz, MeOD) δ = 96.9, 78.8, 77.7, 77.4, 74.9, 74.5, 73.7, 71.6, 54.8, 51.6, 50.4, 42.0, 33.1, 31.3, 30.5, 30.1, 27.1, 23.8, 14.5 ppm. HRMS (ESI$^+$) *m/z*: $[M+H^+]_{calcd}$: 421.3020, $[M+H^+]_{found}$: 421.3020.

**24**: $^1$H NMR (400 MHz, MeOD) δ = 5.94 (d, *J* = 4 Hz, 1H), 4.02 (m, 2H), 3.94 (m, 2H), 3.62–3.75 (m, 2H), 3.47–3.38 (m, 2H), 3.30–3.18 (m, 3H), 3.15–3.07 (m, 1H), 2.46 (m, 1H), 2.03 (m, 1H), 1.68 (m, 2H), 1.31 (m, 10H), 0.90 (s, 3H) ppm. $^{13}$C NMR (100 MHz, MeOD) δ = 97.7, 82.7, 79.7, 77.9, 75.4, 73.6, 71.5, 70.1, 55.8, 51.0, 50.5, 42.3, 33.3, 31.4, 30.7, 30.2, 27.2, 24.0, 14.7 ppm. HRMS (ESI$^+$) *m/z*: $[M+H^+]_{calcd}$: 421.3020, $[M+H^+]_{found}$: 421.3021.

*3′,6-Di-O-octyl neamine **14***

Compound **14** was synthesized following procedure I from **27** (1.0 eq, 1.1 mmol, 1.4 g) and 1-bromooctane (5 eq, 5.4 mmol, 93 μL). The protected derivative **29** was obtained in 21% yield (white solid). HRMS (ESI$^+$): $[M+H^+]_{calcd}$: 1515.8811, $[M+H^+]_{found}$: 1515.8816. The deprotection was performed following procedure II to give derivative **14** as a white solid (84%). $^1$H NMR (400 MHz, MeOD) δ = 5.98 (d, *J* = 3.5 Hz, 1H), 4.09–3.82 (m, 5H), 3.77–3.60 (m, 3H), 3.47–3.33 (m, 3H), 3.24 (m, 3H), 3.11 (dd, *J* = 13.4, 8.7 Hz, 1H), 2.45 (m, 1H), 2.00 (dd, *J* = 12.4, 0.2 Hz, 1H), 1.65 (m, 4H), 1.30 (m, 20H), 0.89 (m, 6H) ppm. $^{13}$C NMR (100 MHz, $D_2O$) δ = 95.3, 80.4, 78.1, 75.9, 75.4, 74.2, 73.7, 71.3, 69.9, 52.0, 49.1, 48.5, 39.6, 31.3, 29.4, 29.3, 28.8, 28.6, 28.3, 25.4, 25.1, 22.2, 13.6 ppm. HRMS (ESI$^+$) *m/z*: $[M+H^+]_{calcd}$: 547.4429, $[M+H^+]_{found}$: 547.4429.

*3′,6-Di-O-decyl neamine **15***

Compound **15** was synthesized following procedure I from **27** (1.0 eq, 2.3 mmol, 3.0 g) and 1-bromodecane (3.6 eq, 8.4 mmol, 1.7 mL). The protected derivative **30** was obtained in 20% yield (white solid), HRMS (ESI$^+$) *m/z*: $[M+H^+]_{calcd}$: 1571.9437, $[M+H^+]_{found}$: 1571.9436. The deprotection of **30** was performed following procedure II to give derivative **15** as a white solid (65%). $^1$H NMR (400 MHz, MeOD) δ = 5.96 (d, *J* = 3.4 Hz, 1H), 4.09–3.82 (m, 5H), 3.76–3.62 (m, 3H), 3.42 (m, 3H), 3.28 (m, 3H), 3.13 (dd, *J* = 12.0, 8.8 Hz, 1H), 2.45 (m, 1H), 2.02 (m, 1H), 1.68 (m, 4H), 1.31 (m, 28H), 0.90 (m, 6H) ppm. $^{13}$C NMR (100 MHz, MeOD) δ = 96.9, 82.3, 79.0, 77.7, 77.5, 75.1, 74.8, 73.4, 71.9, 54.5, 50.7, 50.2, 41.8, 33.1, 31.2, 31.1, 30.8, 30.7 30.5, 27.1, 27.0, 23.8, 14.4 ppm. HRMS (ESI$^+$) *m/z*: $[M+H^+]_{calcd}$: 603.5055, $[M+H^+]_{found}$: 603.5057.

*3′,6-Di-O-undecyl neamine **16**, 3′-mono-O-undecyl neamine **25** and 6-mono-O-undecyl neamine **26***

Compounds **16, 25** and **26** were synthesized following procedure I from **27** (1.0 eq, 2.3 mmol, 3.0 g) and 1-bromoundecane (3.6 eq, 8.4 mmol, 1.9 mL). The protected derivatives **31, 35** and **36** were obtained in 30%, 15% and 3% yield respectively (white solid). **31**: HRMS (ESI$^+$) *m/z*: $[M+H^+]_{calcd}$: 1599.9750, $[M+H^+]_{found}$: 1599.9736; **35**: HRMS (ESI$^+$) *m/z*: $[M+H^+]_{calcd}$: 1445.8029, $[M+H^+]_{found}$: 1445.8034; **36**: HRMS (ESI$^+$) *m/z*: $[M+H^+]_{calcd}$:

1445.8029, $[M+H^+]_{found}$: 1445.8027.

The deprotection was performed following procedure II to give derivative **16** (98%), **25** (54%) and **26** (94%) as white solid.

**16**: $^1$H NMR (400 MHz, MeOD) δ = 5.90 (d, *J* = 3.4 Hz, 1H), 4.01 (td, *J* = 12.0, 2.9 Hz, 1 H), 3.90 (m, 3H), 3.79 (dd, *J* = 10.0, 8.6 Hz, 1H), 3.72–3.58 (m, 3H), 3.43–3.32 (m, 3H), 3.24 (m, 3H), 3.07 (dd, *J* = 13.2, 8.8 Hz, 1H), 2.38 (br. s., 1H), 1.90 (m, 1H), 1.61 (m, 4H), 1.26 (m, 34H), 0.85 (m, 6H) ppm. $^{13}$C NMR (100 MHz, MeOD) δ = 96.8, 82.4, 79.3, 77.9, 77.7, 75.0, 74.7, 73.4, 71.7, 54.6, 50.8, 50.2, 41.8, 33.1, 31.2, 31.1, 30.8, 30.7, 30.5, 30.4, 27.1, 27.0, 23.8, 14.5 ppm. HRMS (ESI$^+$) *m/z*: $[M+H^+]_{calcd}$: 631.5368, $[M+H^+]_{found}$: 631.5369.

**25**: $^1$H NMR (400 MHz, MeOD) δ = 5.99 (d, *J* = 3.8 Hz, 1H), 4.01 (m, 3H), 3.86 (dd, *J* = 10.5, 8.6 Hz, 1H), 3.70 (m, 1H), 3.61 (t, *J* = 9.0 Hz, 1H), 3.42 (m, 4H), 3.19 (m, 1H), 2.44 (d, *J* = 12.3 Hz, 1H), 1.99 (d, *J* = 12.6 Hz, 1H), 1.65 (m, 2H), 1.29 (m, 18H), 0.90 (m, 3H) ppm. $^{13}$C NMR (100 MHz, MeOD) δ = 96.6, 78.5, 77.4, 77.3, 74.7, 74.3, 73.5, 71.4, 54.5, 51.3, 50.0, 41.8, 33.0, 31.1, 30.7, 30.6, 30.4, 26.9, 23.6, 14.3 ppm. HRMS (ESI$^+$) *m/z*: $[M+H^+]_{calcd}$: 477.3647, $[M+H^+]_{found}$: 477.3649.

**26**: $^1$H NMR (400 MHz, MeOD) δ = 5.90 (m, 1H), 4.03 (m, 1H), 3.92 (m, 3H), 3.75–3.61 (m, 2H), 3.42 (m, 1H), 3.27–3.15 (m, 3H), 3.11 (m, 1H), 2.42 (m, 1H), 1.92 (m, 1H), 1.67 (m, 2H), 1.30 (m, 18H), 0.90 (m, 3H) ppm. $^{13}$C NMR (125 MHz, MeOD) δ = 97.5, 82.6, 80.0, 77.7, 75.2, 73.4, 71.2, 70.1, 55.9, 50.9, 50.3, 42.1, 33.3, 31.2, 30.8, 30.5, 29.8, 27.1, 23.8, 14.6 ppm. HRMS (ESI$^+$) *m/z*: $[M+H^+]_{calcd}$: 477.3647, $[M+H^+]_{found}$: 477.3646.

### *3′,6-Di-O-[3,7-(dimethyl)octyl] neamine* ***17***

Compound **17** was synthesized following procedure I from **27** (1 eq, 2.3 mmol, 3.0 g) and 1-bromo-3,7-dimethyloctane (3.6 eq, 8.4 mmol, 1.7 mL). The protected derivative **32** was obtained in 5% yield (white solid), HRMS (ESI$^+$) *m/z*: $[M+H^+]_{calcd}$: 1571.9437, $[M+H^+]_{found}$: 1571.9429. The deprotection was performed following procedure II to give derivative **17** as a white solid (quantitative yield). $^1$H NMR (400 MHz, MeOD) δ = 5.98 (d, *J* = 3.0 Hz, 1H), 4.09–3.94 (m, 4H), 3.87 (dd, *J* = 10.4, 8.6 Hz, 1H), 3.77–3.65 (m, 3H), 3.46–3.33 (m, 4H), 3.24 (m, 3H), 3.13 (dd, *J* = 12.0, 8.6 Hz, 1H), 2.46 (m, 1H), 2.00 (m, 1H), 1.70 (m, 2H), 1.53 (m, 6H), 1.31 (m, 6H), 1.16 (m, 6H), 0.89 (m, 18H) ppm. $^{13}$C NMR (125 MHz, MeOD) δ = 96.8, 82.5, 78.8, 77.9, 77.8, 73.8, 73.7, 73.6, 71.8, 54.7, 50.8, 50.3, 41.9, 40.6, 38.9, 38.7, 38.3, 38.1, 31.4, 31.2, 30.0, 29.3, 26.0, 25.9, 23.2, 23.1, 20.1, 20.1 ppm. HRMS (ESI$^+$) *m/z*: $[M+H^+]_{calcd}$: 603.5055, $[M+H^+]_{found}$: 603.5054.

### *3′,4′-Di-O-[3,7-(dimethyl)octyl] neamine* ***18***

To a solution of compound **43** [44] (1.0 eq, 0.46 mmol, 650 mg) in anhydrous DMF (15 mL) were added NaH (60%, 2.5 eq, 1.90 mmol, 45 mg) and the solution was stirred at room temperature under argon atmosphere during 30 min. The 1-bromo-3,7-dimethyloctane (2.5 eq, 1.15 mmol, 0.25 mL) was then added dropwise and the mixture was stirred overnight at 50 °C then quenched with MeOH and the solvents were evaporated under reduced pressure. The crude product was dissolved in DCM (20 mL) and the solution was washed with an aqueous solution of $KH_2PO_4$ 3% (3 × 20 mL). The organic layer was dried over $MgSO_4$ and evaporated under reduced pressure. The crude product was purified by flash chromatography (silica gel, elution: toluene/ethyl acetate 100:0 to 98:2 with 0.25% $Et_3N$) to give the tritylated intermediate in 31% yield (white solid), HRMS (ESI$^+$) *m/z*: $[M+H^+]_{calcd}$: 1692.0012, $[M+H^+]_{found}$: 1692.0015. The tritylated intermediate (1.0 eq, 0.14 mmol, 240 mg) was dissolved in DCM/TFA (4/1: w/w) (4 mL) and anisole (0.1 mL/mL of solvent) was added. The mixture was stirred at room temperature during 2 h and MeOH (10 mL) was added to quench the TFA then the solvents were evaporated under reduced pressure. The crude product was dissolved in cyclohexane (10 mL) and the white precipitate was filtered off. The residue was purified by flash chromatography (silica gel, elution: EtOH/$H_2O$/$NH_4OH$ 8/1/1) to give **44** as a pure TFA salt in 55% yield (white solid). $^1$H NMR (400 MHz, MeOD) δ = 5.93 (m, 1H), 4.08 (m, 2H), 3.96–3.72 (m, 4H), 3.70–3.53 (m, 2H), 3.49–3.36 (m, 3H), 3.27–3.21 (m, 2H), 3.18–3.10 (m, 2H), 2.42–2.37 (m, 1H), 2.10–1.99 (m, 1H), 1.78–1.61 (m, 2H), 1.59–1.48 (m, 5H), 1.42–1.25 (m, 8H), 1.21–1.12 (m, 5H), 0.95–0.85 (m, 18H) ppm. $^{13}$C NMR (125 MHz, MeOD) δ = 96.6, 81.2, 78.6, 77.5, 77.3, 74.3, 73.1, 72.8, 71.0, 51.5, 50.1, 49.8, 41.6, 40.5, 38.8, 38.7, 38.5, 38.2, 31.5, 31.4, 31.2, 30.9, 29.7, 25.9, 25.8, 23.1, 23.0, 20.3, 20.0. HRMS (ESI$^+$) *m/z*: $[M+H^+]_{calcd}$: 603.5055, $[M+H^+]_{found}$: 603.5051.

### *3′-O-heptyl-6-O-undecyl neamine* ***19***

Compound **19** was synthesized following procedure I from **33** (1 eq, 0.22 mmol, 300 mg) and 1-bromoundecane (3.5 eq, 0.76 mmol, 170 μL). The protected derivative **37** was obtained in 19% yield (white solid), HRMS (ESI$^+$) *m/z*: $[M+H^+]_{calcd}$: 1543.9111, $[M+H^+]_{found}$: 1543.9119. The deprotection was realized following procedure II to give derivative **19** as a white solid (quantitative yield). $^1$H NMR (400 MHz, MeOD) δ = 5.96 (d, *J* = 8 Hz, 1H), 4.08–4.00 (m, 2H), 4.01–3.83 (m, 3H), 3.76–3.61 (m, 3H), 3.43 (m, 3H), 3.28–3.18 (m, 3H), 3.12 (dd, *J* = 12.0, 8.0 Hz, 1H), 2.45 (m, 1H), 2.03 (m, 1H), 1.66 (m, 4H), 1.32 (m, 24H), 0.90 (m, 6H) ppm. $^{13}$C NMR (100 MHz, MeOD) δ = 96.9, 82.4, 78.8, 77.7, 77.4, 75.1, 74.8, 73.3, 71.9, 54.6, 50.7, 49.8, 41.8, 33.0, 31.2, 31.0, 30.8, 30.5, 30.3, 27.0, 23.7, 14.4 ppm. HRMS (ESI$^+$) *m/z*: $[M+H^+]_{calcd}$: 575.4742, $[M+H^+]_{found}$: 575.4748.

### *3′-O-undecyl-6-O-heptyl neamine* ***20***

Compound **20** was synthesized following procedure I for alkylation from **35** (1 eq, 0.07 mmol, 100 mg) and 1-bromoheptane (2.5 eq, 0.17 mmol, 27 μL). The protected derivative **38** was obtained in a quantitative yield (white solid), HRMS (ESI$^+$) *m/z*: $[M+H^+]_{calcd}$: 1543.9124, $[M+H^+]_{found}$: 1543.9116. The deprotection was performed following procedure II to give derivative **20** as a white solid (86%). $^1$H NMR (400 MHz, MeOD) δ = 5.97 (d, *J* = 3.7 Hz, 1H), 4.05 (t, *J* = 8 Hz, 2H), 4.00–3.83 (m, 3H), 3.69 (m, 3H), 3.43 (m, 3H), 3.29–3.19 (m, 3H), 3.12 (dd, *J* = 12,8 Hz, 1H), 2.46 (m, 1H), 2.03 (m, 1H), 1.68 (m, 4H), 1.31 (m, 24H), 0.90 (m, 6H) ppm. $^{13}$C NMR (100 MHz, MeOD) δ = 97.2, 82.7, 79.1, 78.0, 77.7, 75.6, 75.1, 73.7, 72.2, 54.9, 51.0, 50.5, 42.0, 33.4, 33.3, 31.5, 31.4, 31.1, 31.0, 30.8, 30.7, 30.2, 27.4, 27.2, 24.1, 24.0, 14.9 ppm. HRMS (ESI$^+$) *m/z*: $[M+H^+]_{calcd}$: 575.4742, $[M+H^+]_{found}$: 575.4741.

### *3′-O-heptyl-6-**O**-[4-(1-pyrenyl)butyl] neamine* ***21***

Compound **21** was synthesized following procedure I from **33** (1.0 eq, 0.59 mmol, 810 mg) and 4-(1-pyrenyl)butyl methanesulfonate (3.0 eq, 1.80 mmol, 620 mg). The protected derivative **39** was obtained in 14% yield (white solid), HRMS (ESI$^+$) *m/z*: $[M+H^+]_{calcd}$: 1645.8655 $[M+H^+]_{found}$: 1645.8673. The deprotection was performed following procedure II to give derivative **21** as a white solid (95%). $^1$H NMR (500 MHz, MeOD) δ = 8.33 (d, *J* = 10 Hz, 1 H), 8.19 (d, *J* = 7.5 Hz, 2H), 8.13 (m, 2H), 8.06–7.97 (m, 3H), 7.90 (d, *J* = 9.2 Hz, 1H), 5.93 (m, 1H), 4.11–3.97 (m, 3H), 3.97–3.83 (m, 2H), 3.79 (m, 1H), 3.76–3.60 (m, 2H), 3.46–3.36 (m, 4H), 3.26–3.16 (m, 2H), 3.15–3.05 (m, 1H), 2.42 (m, 1H), 2.06–1.74 (m, 5H), 1.67 (m, 2H), 1.31 (m, 10H), 0.90 (s, 3H) ppm. $^{13}$C NMR (100 MHz, MeOD) δ = 138.2, 133.0, 132.9, 132.4, 131.3, 130.0, 128.7, 128.4, 127.9, 127.0, 126.4, 126.3, 126.1, 126.0, 125.9, 124.5, 97.0, 82.5, 77.7, 75.2, 74.6,

73.6, 71.9, 54.7, 50.8, 50.2, 41.9, 34.3, 33.0, 31.4, 31.1, 30.5, 29.7, 27.1, 23.9, 14.5 ppm. HRMS ($ESI^+$) *m/z*: $[M+H^+]_{calcd}$: 677.4273, $[M+H^+]_{found}$: 677.4274.

*3′-O-[4-(1-pyrenyl)butyl]-6-O-heptyl neamine* ***22***

Compound **22** was synthesized following procedure I from **34** (1.0 eq, 0.67 mmol, 930 mg) and 4-(1-pyrenyl)butyl methanesulfonate (3 eq, 2.00 mmol, 700 mg). The protected derivative **40** was obtained in 21% yield (white solid), HRMS ($ESI^+$) *m/z*: $[M+H^+]_{calcd}$: 1645.8655, $[M+H^+]_{found}$: 1645.8615.

The deprotection was performed following procedure II to give derivative **22** as a white solid (90%). $^1H$ NMR (500 MHz, MeOD) δ = 8.34 (d, *J* = 9.2 Hz, 1H), 8.18 (dd, *J* = 7.8, 1.4 Hz, 2H), 8.14 (dd, *J* = 7.8, 1.4 Hz, 2H), 8.02 (m, 3H), 7.91 (d, *J* = 7.9 Hz, 1H), 5.93 (m, 1H), 4.10–3.97 (m, 3H), 3.96–3.82 (m, 2H), 3.79 (m, 1H), 3.71 (m, 1H), 3.65 (m, 1H), 3.45–3.34 (m, 4H), 3.27–3.19 (m, 2H), 3.08 (dd, *J* = 15,0 10,0 Hz, 1H), 2.43 (m, 1H), 2.06–1.78 (m, 5H), 1.67 (m, 2H), 1.32 (m, 10H), 0.90 (m, 3H) ppm. $^{13}C$ NMR (125 MHz, MeOD) δ = 138.2, 133.0, 132.5, 131.3, 130.1, 128.7, 128.7, 128.4, 127.8, 127.2, 126.4, 126.3, 126.1, 126.0, 125.9, 124.4, 97.0, 82.5, 79.2, 77.8, 77.7, 75.3, 74.7, 73.5, 71.9, 54.7, 50.8, 50.2, 41.9, 34.3, 33.1, 31.3, 31.2, 30.5, 29.6, 27.0, 23.8, 14.5 ppm. HRMS ($ESI^+$) *m/z*: $[M+H^+]_{calcd}$: 677.4273, $[M+H^+]_{found}$: 677.4276.

*4-(1-pyrenyl)butanol* ***41***

To a solution of 4-(1-pyrenyl)butanoic acid (1.0 eq, 8.7 mmol, 2.5 g) in anhydrous THF (25 mL) at 0 °C was added dropwise a solution of lithium aluminate hydride $LiAlH_4$ 2 M in THF (2.0 eq, 17.3 mmol, 8.7 mL) and the mixture was stirring at 0 °C for 2 h. The reaction was quenched with ethyl acetate and the organic phase was washed with $H_2O$ (3 × 50 mL), dried over $MgSO_4$ and concentrated under vacuum. The crude product was purified by flash chromatography (silica gel, elution: cyclohexane/ethyl acetate 70/30) to give yellow oil. (1.79 g, 75%). $^1H$ NMR (400 MHz, $CDCl_3$): δ = 8.29 (d, *J* = 9.3 Hz, 1 H), 8.20–8.15 (m, 2 H), 8.12 (dd, *J* = 8.6, 2.7 Hz, 2 H), 8.04–7.98 (m, 3 H), 7.88 (d, *J* = 7.8 Hz, 1 H), 3.72 (t, *J* = 6.6 Hz, 2 H), 3.39 (t, *J* = 7.7 Hz, 2 H), 2.00–1.92 (m, 2 H), 1.80–1.72 (m, 2 H) ppm. $^{13}C$ NMR (100 MHz, $CDCl_3$) δ = 136.6, 131.4, 130.9, 129.8, 128.6, 127.5, 127.2, 127.2, 126.6, 125.8, 125.1, 125.0, 124.8, 124.8, 124.7, 123.4, 62.8, 33.2, 32.7, 27.9 ppm. HRMS ($ESI^+$) *m/z*: $[M+H^+]_{calcd}$: 275.1430, $[M+H^+]_{found}$: 275.1429.

*4-(1-pyrenyl)butyl methanesulfonate* ***42***

To a solution of 4-(1-pyrenyl)butanol **41** (1.0 eq, 6.5 mmol, 1.79 g) and $Et_3N$ (5.5 eq, 35.7 mmol, 5.0 mL) in anhydrous dichloromethane (40 mL) under argon, methanesulfonyl chloride (1.5 eq, 9.8 mmol, 0.75 mL) was added dropwise to the solution and the mixture was stirred at room temperature for 2 h. The organic phase was then washed with water (2 × 100 mL), dried over $MgSO_4$, filtered off and concentrated under vacuum to give **41** as a brown oil (2.1 g, 91%). $^1H$ NMR (400 MHz, $CDCl_3$) δ = 8.26 (d, *J* = 9.1 Hz, 1H), 8.20–8.16 (m, 2H), 8.15–8.11 (m, 2H), 8.05–7.98 (m, 3H), 7.87 (d, *J* = 7.8 Hz, 1H), 4.28 (t, *J* = 6.2 Hz, 2H), 3.41 (t, *J* = 7.5 Hz, 2H), 2.96 (s, 3H), 2.05–1.86 (m, 4H) ppm. $^{13}C$ NMR (100 MHz, $CDCl_3$) δ = 135.4, 131.1, 130.5, 129.7, 127.1, 127.1, 126.9, 126.4, 125.6, 124.8, 124.7, 124.5, 122.8, 69.4, 37.0, 32.4, 28.7, 27.6 ppm. HRMS ($ESI^+$) *m/z*: $[M+H^+]_{calcd}$: 353.1206, $[M+H^+]_{found}$: 353.1205.

*Biological and biochemical assays*

*MIC Determination.* All strains were grown overnight at 37 °C on trypticase soy agar (TSA) petri dishes (BD Diagnostics, BD, Franklin Lakes, NJ). MICs were determined by microdilution using a fresh culture in cation-adjusted Mueller–Hinton broth (CA-MHB) and a starting inoculum of $10^6$ cells, according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI) [46]. Assessment of Eukaryotic Cell Viability. Cell viability was assessed by evaluating their metabolic activity using the MTT assay (reduction in mitochondria of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium in purple formazan crystal) [47]. Briefly, cells exposed for 24 h to 10 or 30 μM of compounds were incubated for 1 h with 0.2 mg/mL MTT. The reaction was stopped by addition of dimethyl sulfoxide (DMSO). The OD was measured at 570 nm.

*Resistance selection method*

A serial passage method was used in which *P. aeruginosa* ATCC 27853 were grown in the continuous presence of a drug concentration corresponding to half of the MIC. The bacteria were examined daily for a change in MIC, followed by a corresponding increase of the drug concentration for up to 38 days. For this purpose, an initial inoculum of $2.5x10^6$ cfu/mL of each of the original strain was exposed in broth to a range of antibiotic concentrations from 0.1 to 5–10-fold their original MIC (using arithmetic increases). After 24 h at 37 °C, the tubes were examined to determine the minimal drug concentration preventing bacterial growth (this value was equal to the MIC determined on an agar plate for the corresponding strain). Bacteria growing at a drug concentration of half this value were then readjusted at a density of $2.5x10^6$ cfu/mL and again exposed for 24 h to drug concentrations from 0.1 to 5–10-fold the MIC. This process was repeated each day, looking for growth at drug concentrations larger than the original MIC. If this was observed, the new minimal drug concentration preventing bacterial growth was determined based on visual inspection of the cultures (and samples taken for confirmation of the MIC by agar dilution). Bacteria growing at a concentration corresponding to half of this new value were then used for continuation of the experiment, for a total of 38 days.

## Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ejmech.2018.08.022.

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# Supporting information

# Broad-Spectrum Antibacterial Amphiphilic Aminoglycosides: A New Focus on the Structure of the Lipophilic Groups Extends the Series of Active Dialkyl Neamines

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## Purity determination of the evaluated derivatives prepared

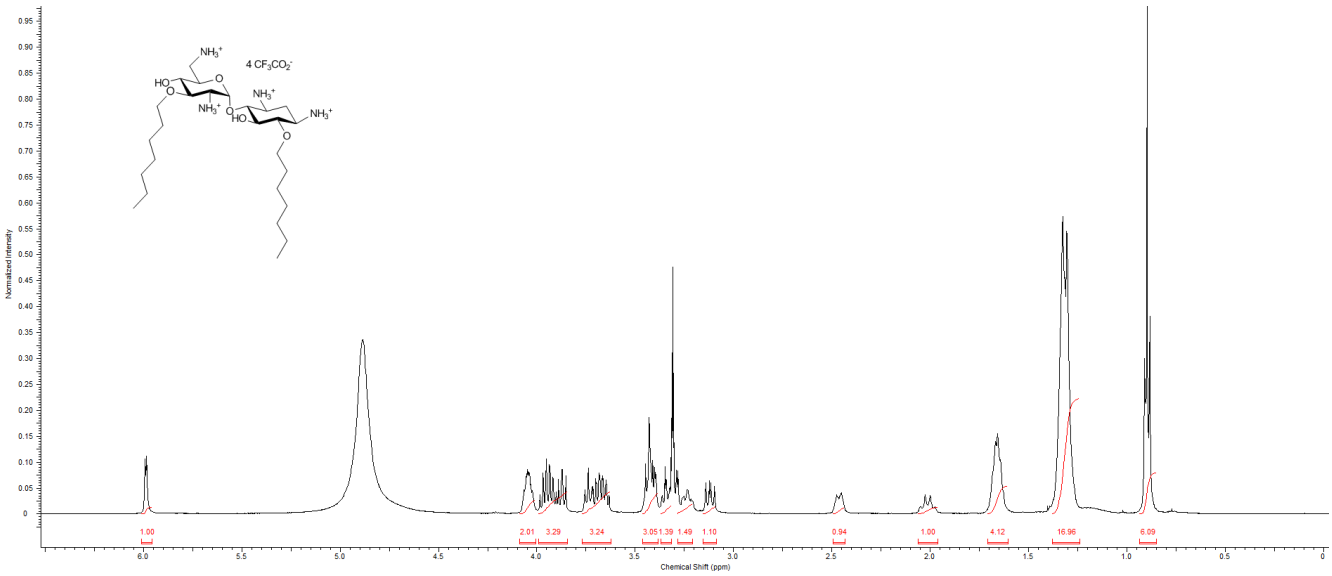
Before the final deprotection step under acidic conditions, careful purifications of the tritylated derivatives allowing removal of minor isomers and impurities were performed by chromatography on silica gel. The purity of the compounds was not determined by elemental analysis since (i) the main difficulty in the purification was the removal of isomers (for example, the 3′,4′-isomers of the 3′,6-neamine derivatives) and (ii) the final compounds are very hygroscopic.

TLC and $^{1}H$ NMR spectrometry allowed to detect the presence of isomers (eluent: EtOH/$H_2O$/($NH_4OH$; 20%) 80:10:10 and TLC visualization: ninhydrin spray (0.3 g, 3 mL AcOH, 100 mL EtOH).

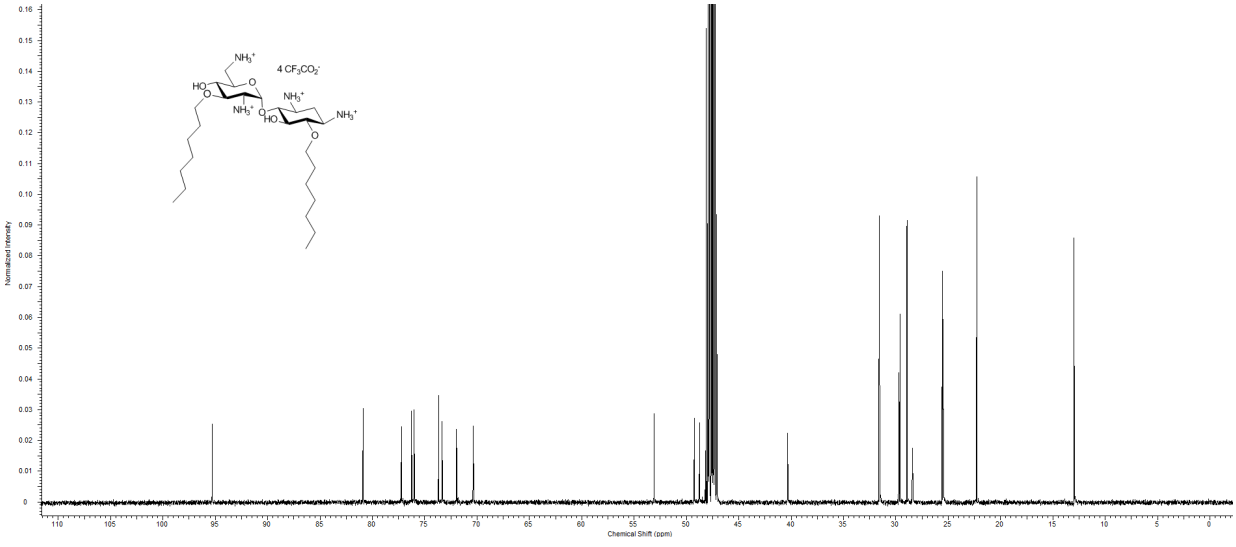
# $^{1}$H and $^{13}$C NMR spectra of compounds

Compound **13**
**3′,6-Di-*O*-heptylneamine**

$^{1}$H NMR $CD_3OD$




$^{13}$C NMR $CD_3OD$



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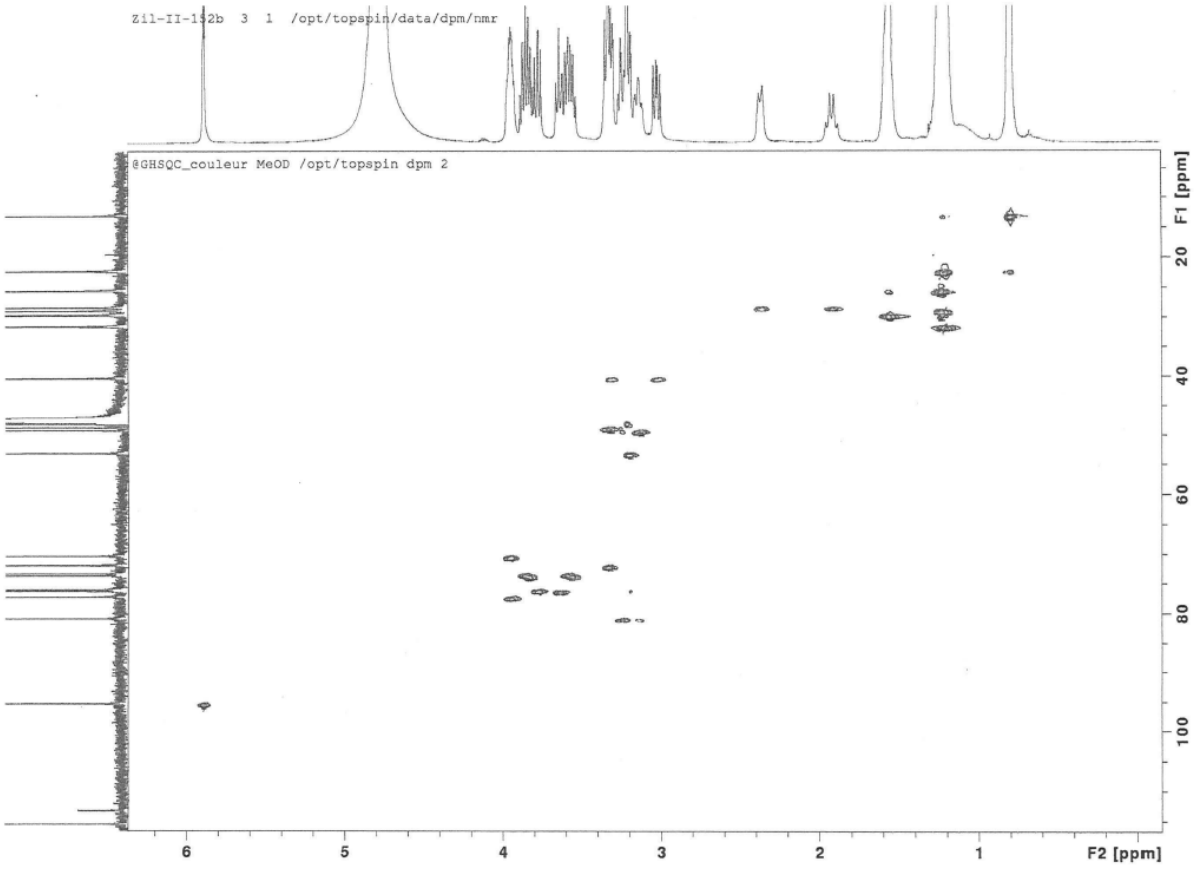
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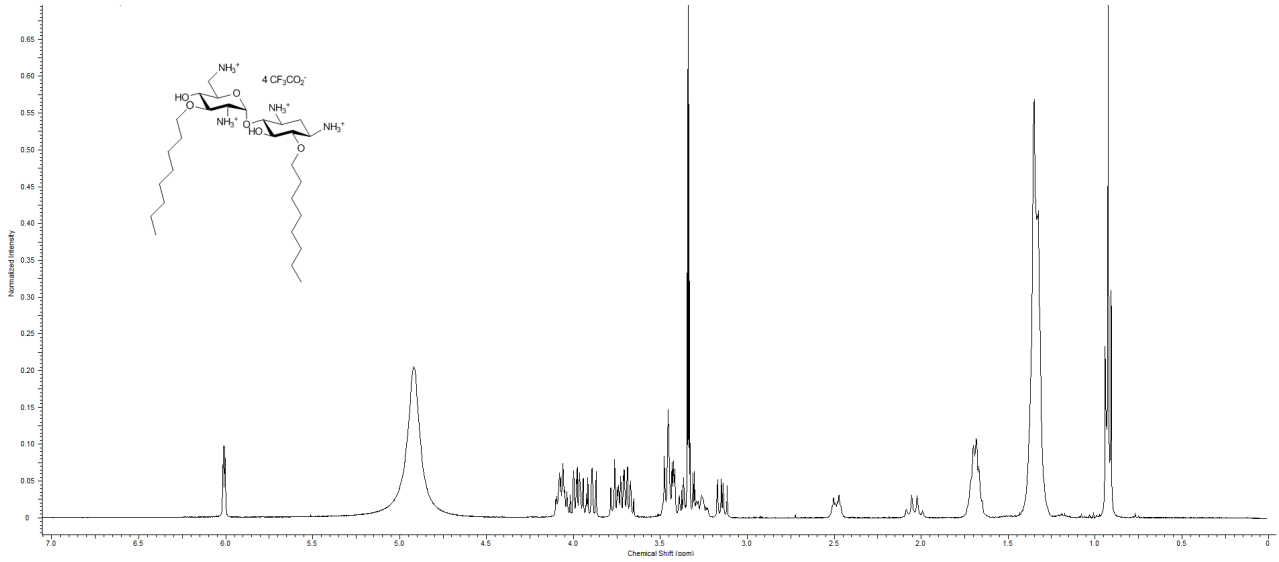
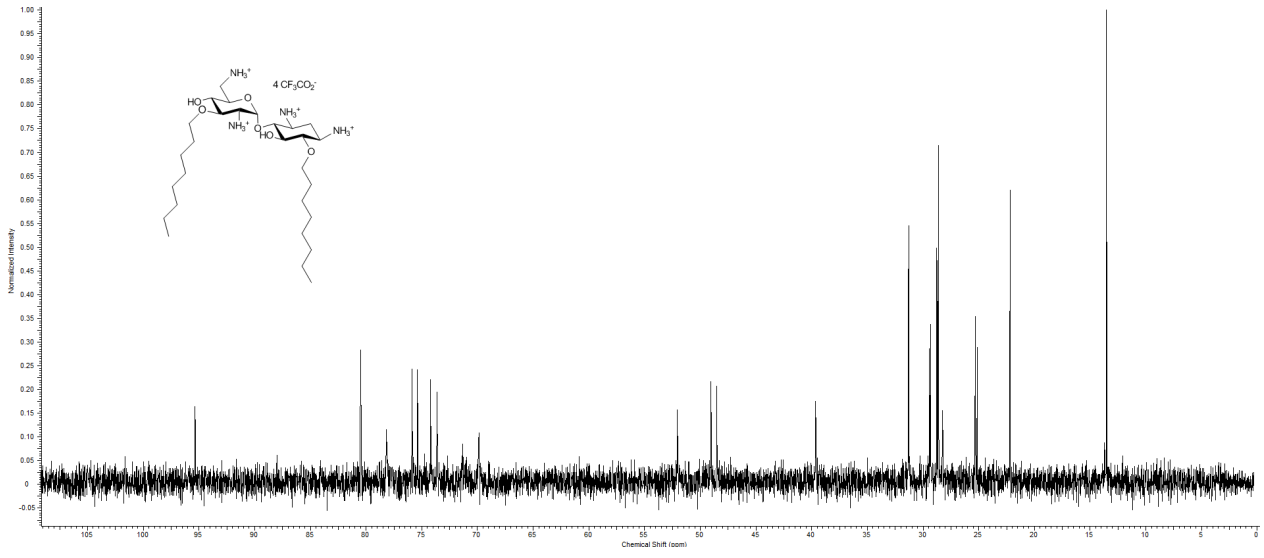
F1 [ppm]
20
40
60
80
100
6
5
4
3
2
1
F2 [ppm]

Compound **14**
**3′,6-Di-*O*-octylneamine**

$^{1}$H NMR $CD_3OD$

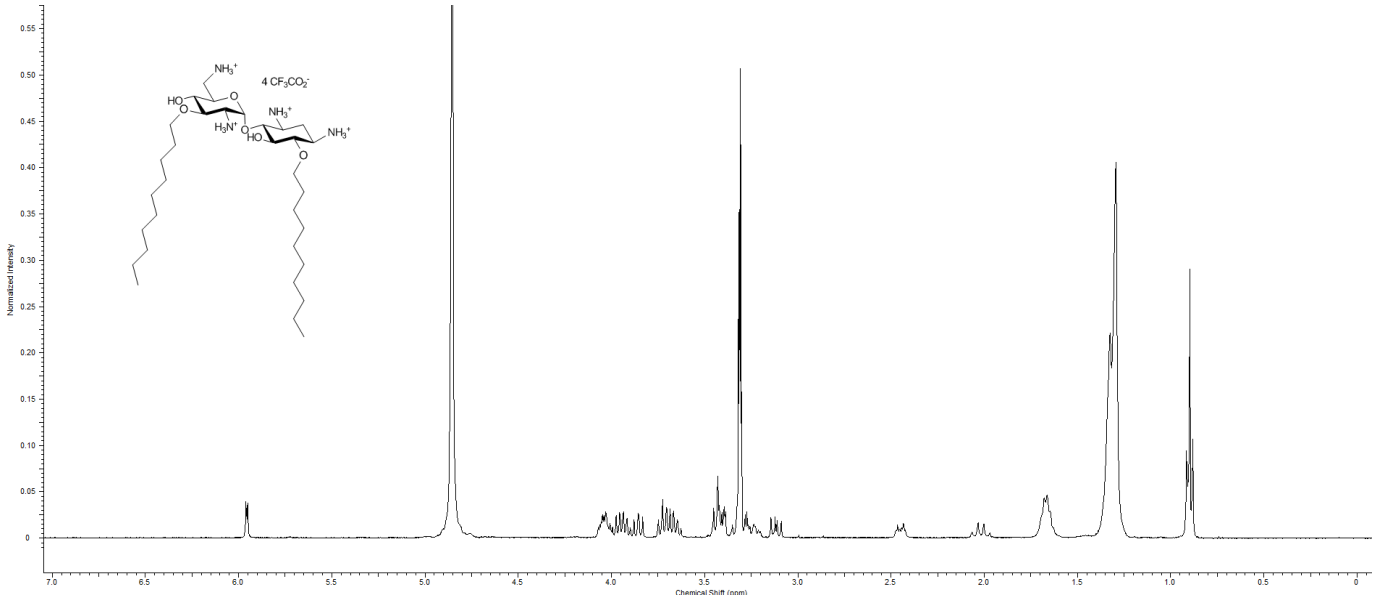



$^{13}$C NMR $D_2O$

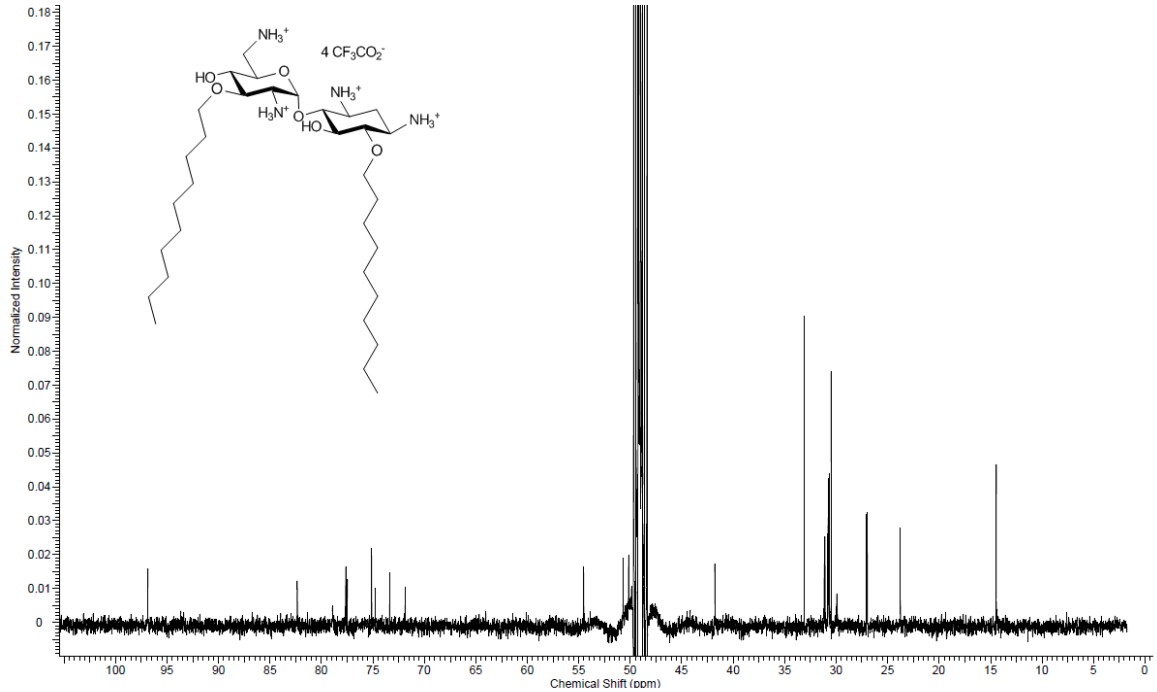


Compound **15**
**3′,6-Di-*O*-decylneamine**

$^{1}$H NMR $CD_3OD$



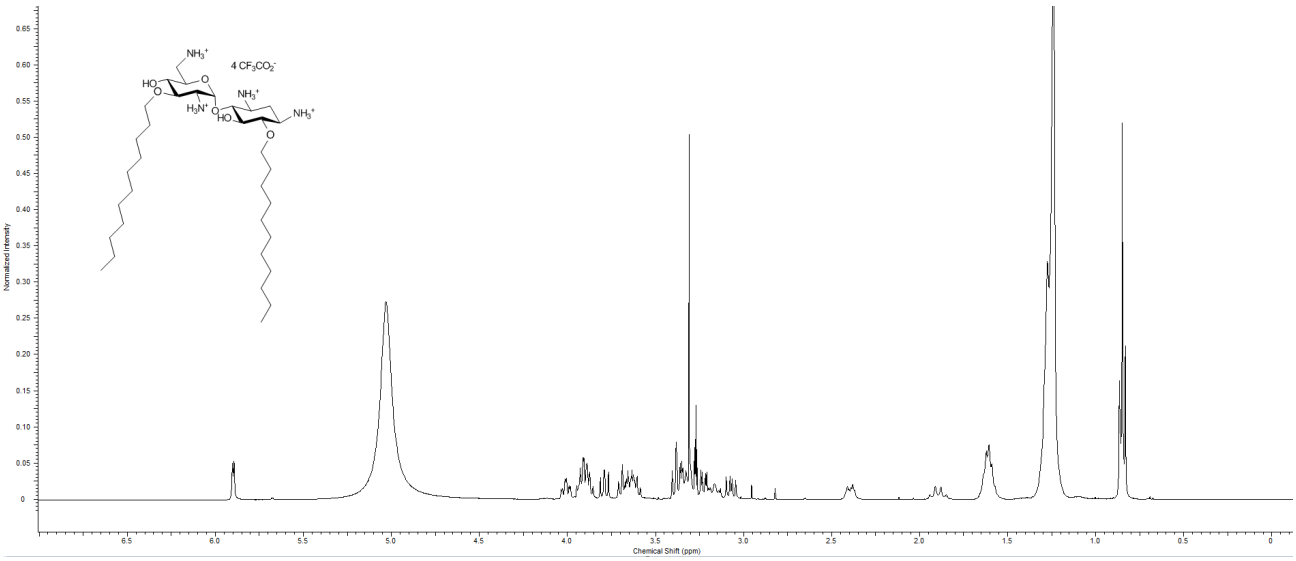

$^{13}$C NMR $CD_3OD$



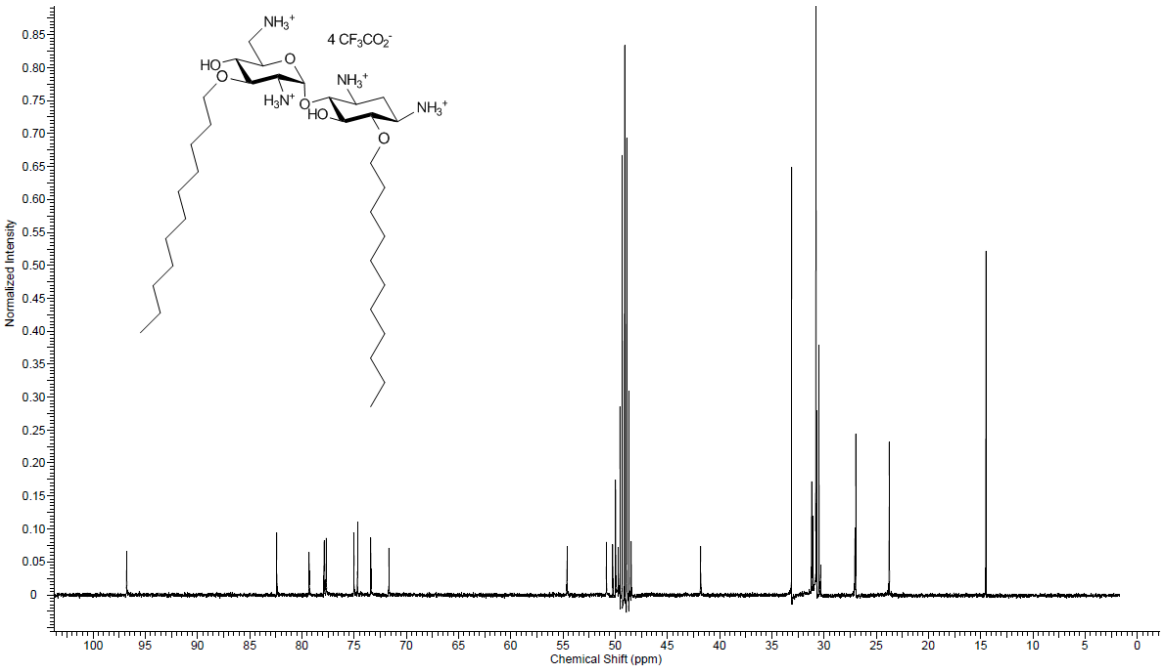
Compound **16**

**3′,6-Di-*O*-undecylneamine**

$^{1}$H NMR $CD_3OD$



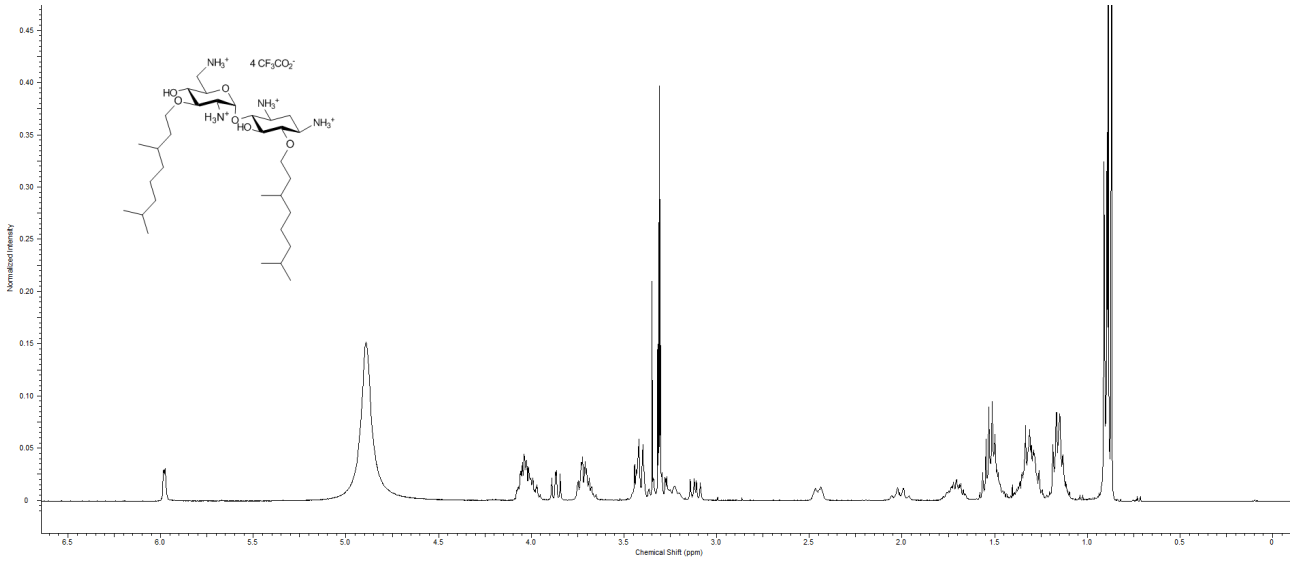

$^{13}$C NMR $CD_3OD$



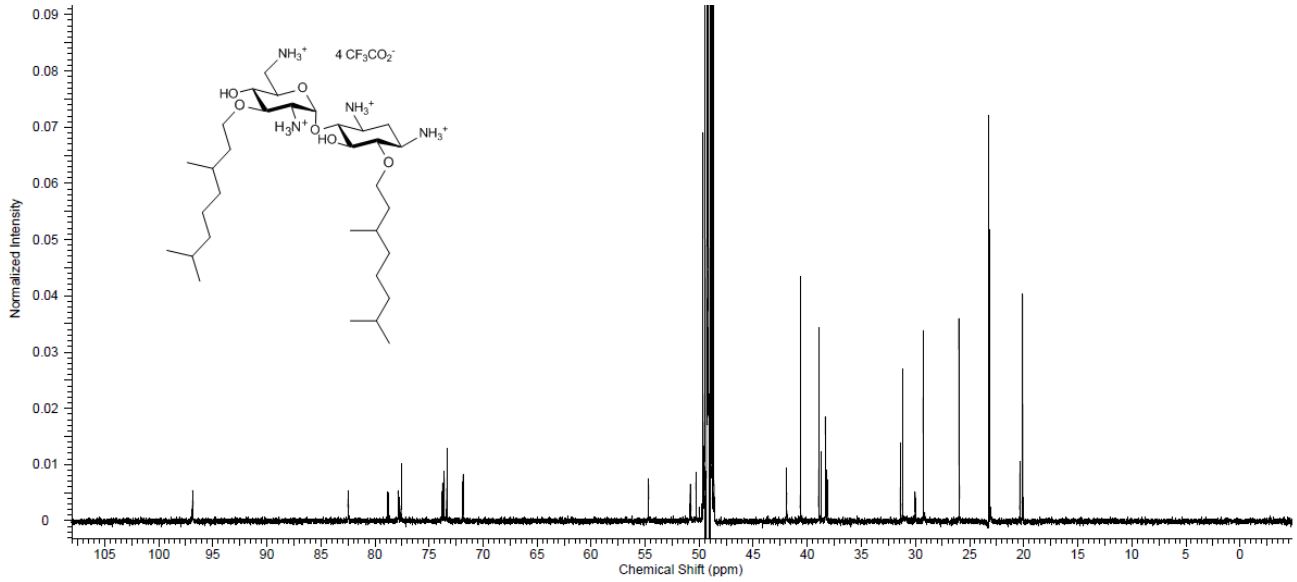
Compound **17**

**3′,6-Di-*O*-[3,7-(dimethyl)octyl]neamine**

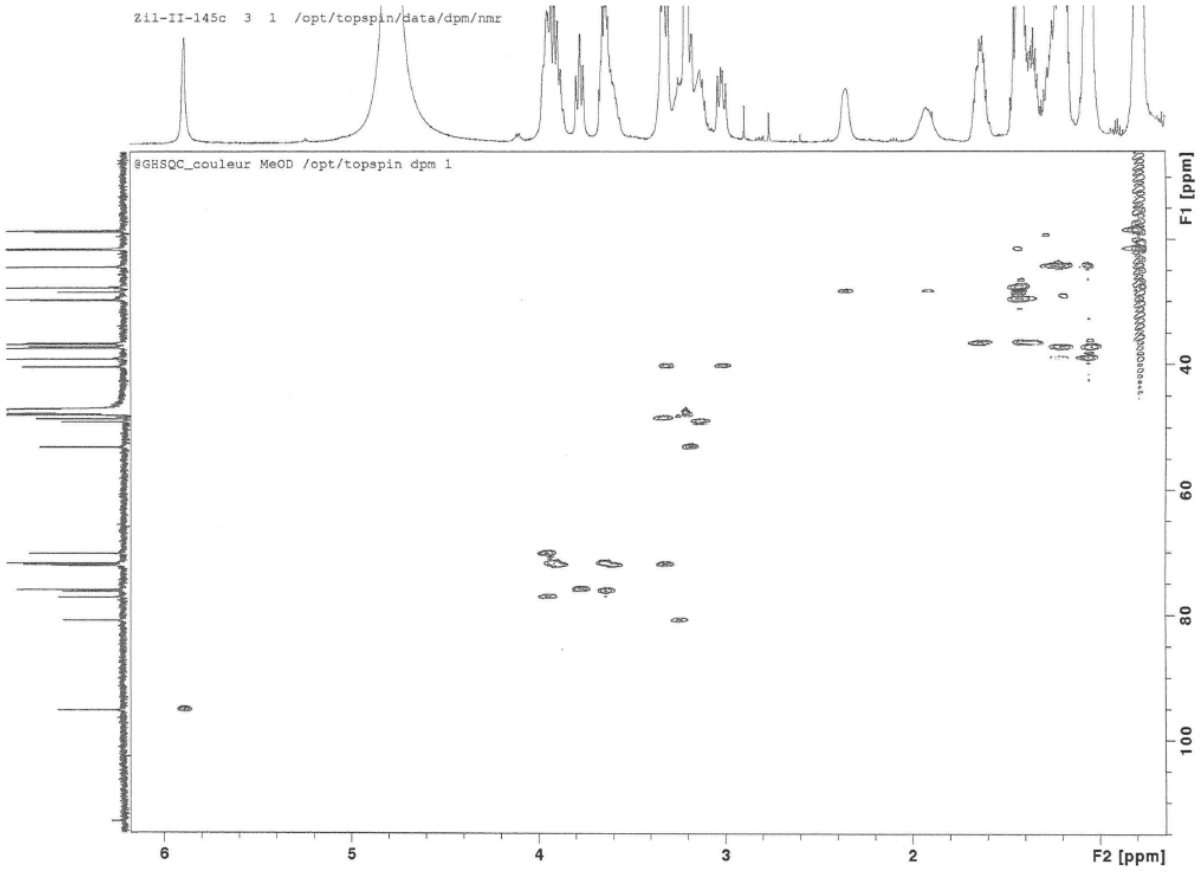
$^{1}$H NMR $CD_3OD$




$^{13}$C NMR $CD_3OD$



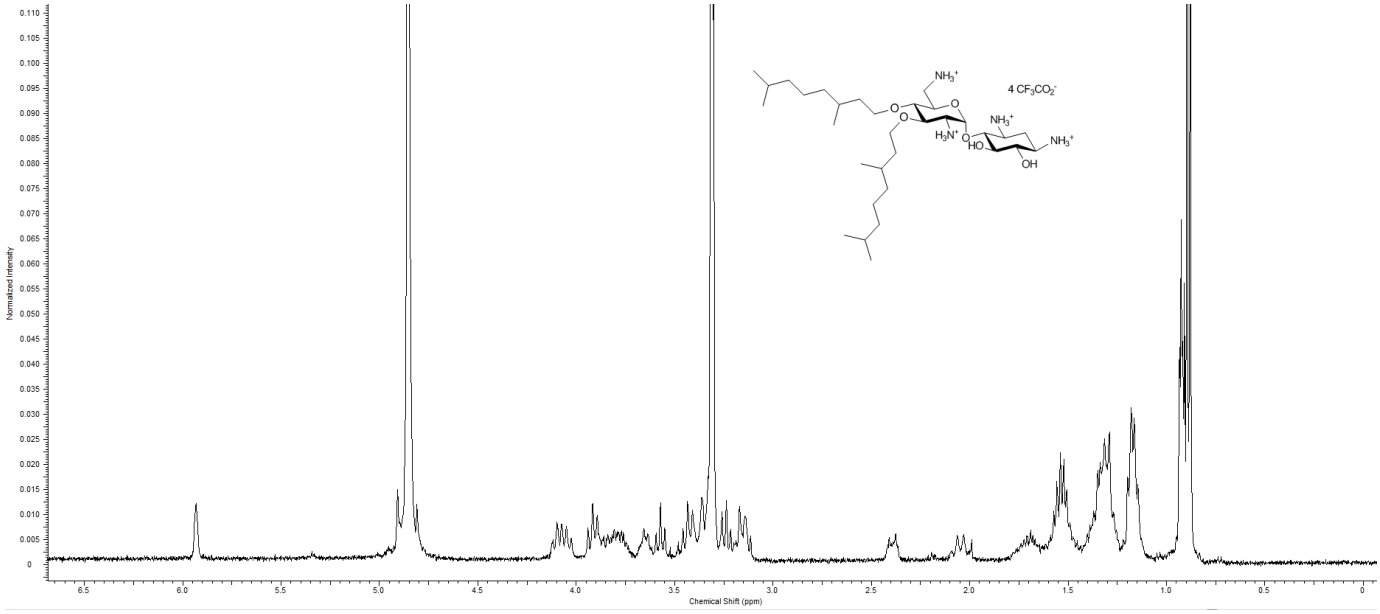
HMQC


Zil-II-145c 3 1 /opt/topspin/data/dpm/nmr
$GHSQC_couleur MeOD /opt/topspin dpm 1
F1 [ppm]
40
60
80
100
6
5
4
3
2
F2 [ppm]

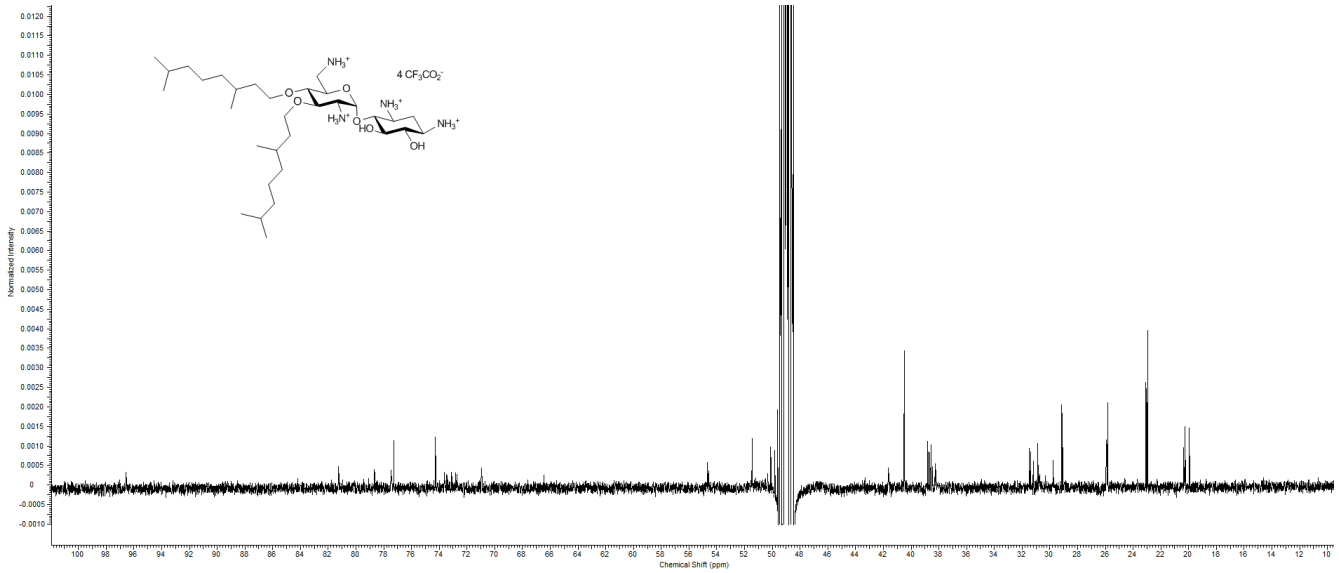
Compound **18**

**3′,4′-Di-*O*-[3,7-(dimethyl)octyl]neamine**

$^{1}$H NMR $CD_3OD$



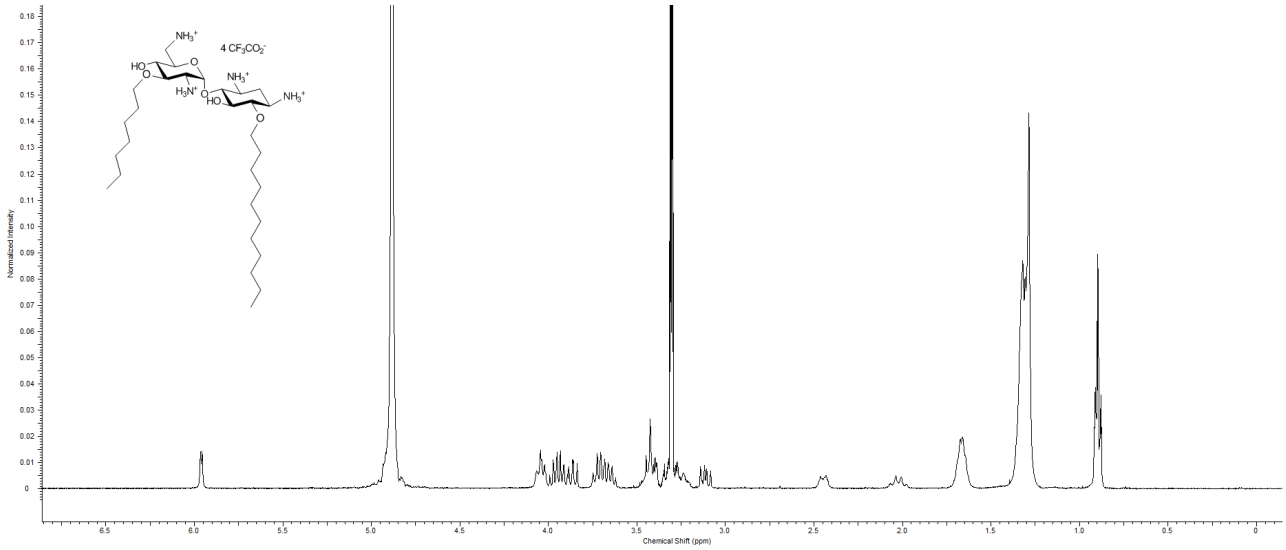

$^{13}$C NMR $CD_3OD$



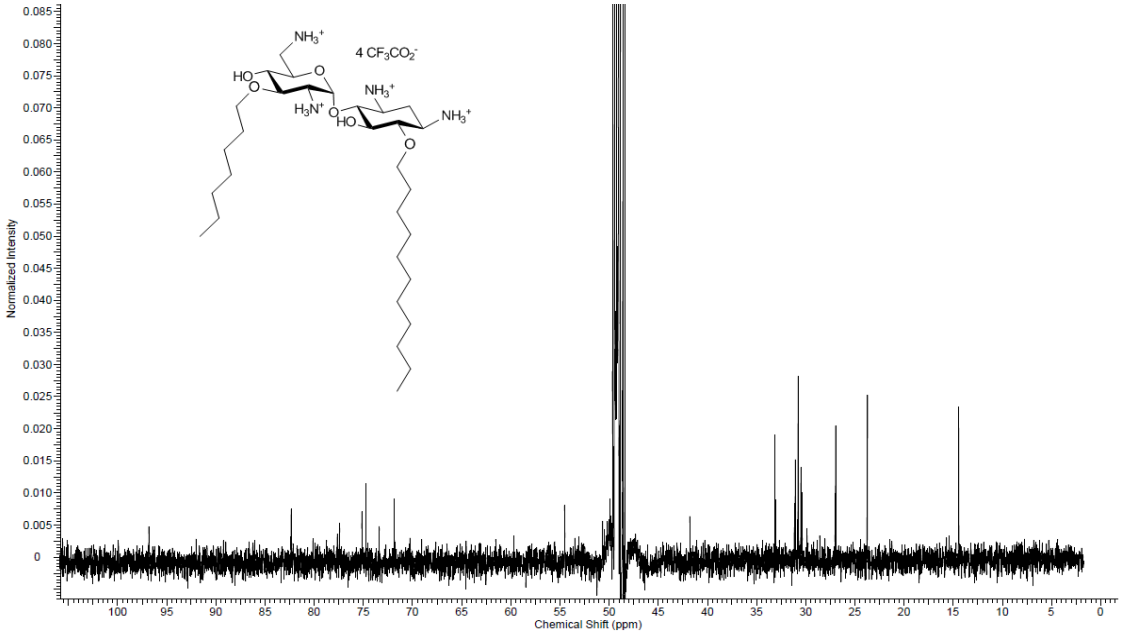
Compound **19**

**3′-*O*-heptyl-6-*O*-undecylneamine**

$^{1}$H NMR $CD_3OD$



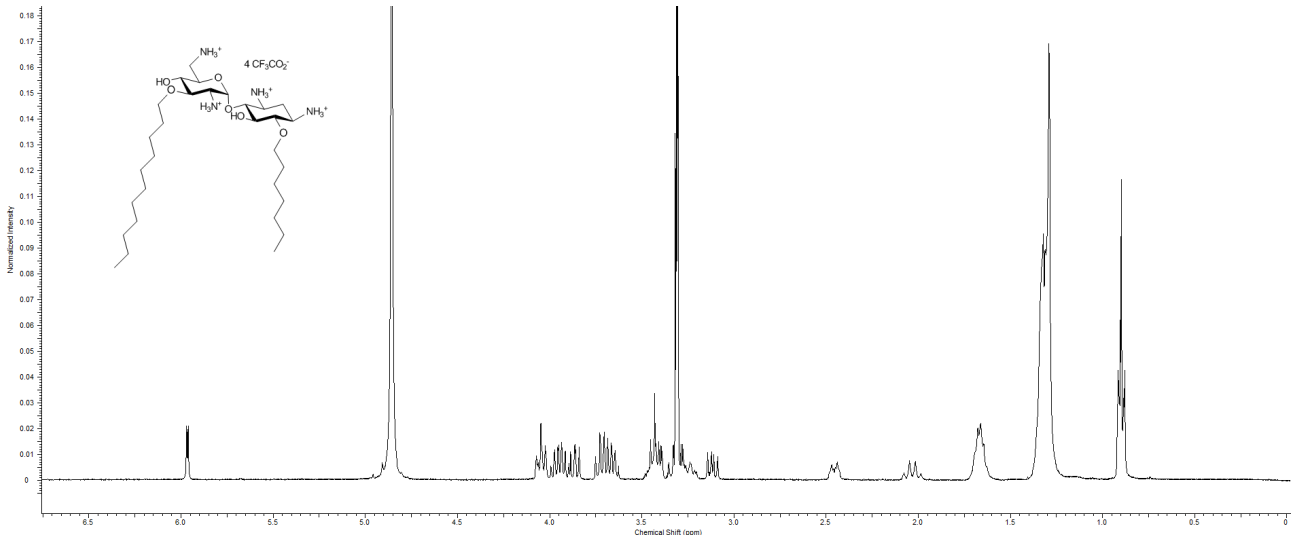

$^{13}$C NMR $CD_3OD$



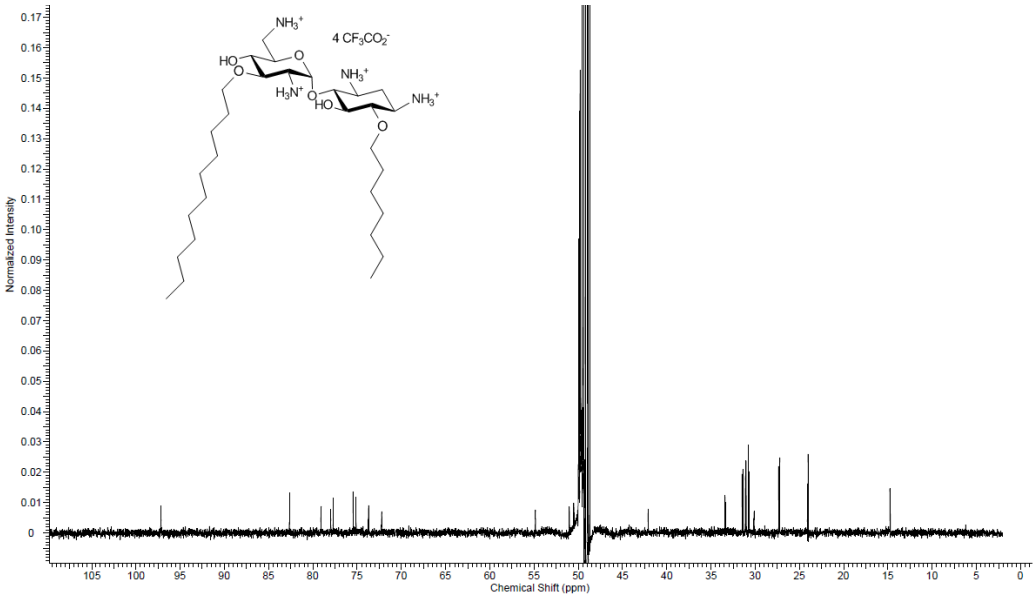
Compound **20**

**3′-*O*-undecyl-6-*O*-heptylneamine**

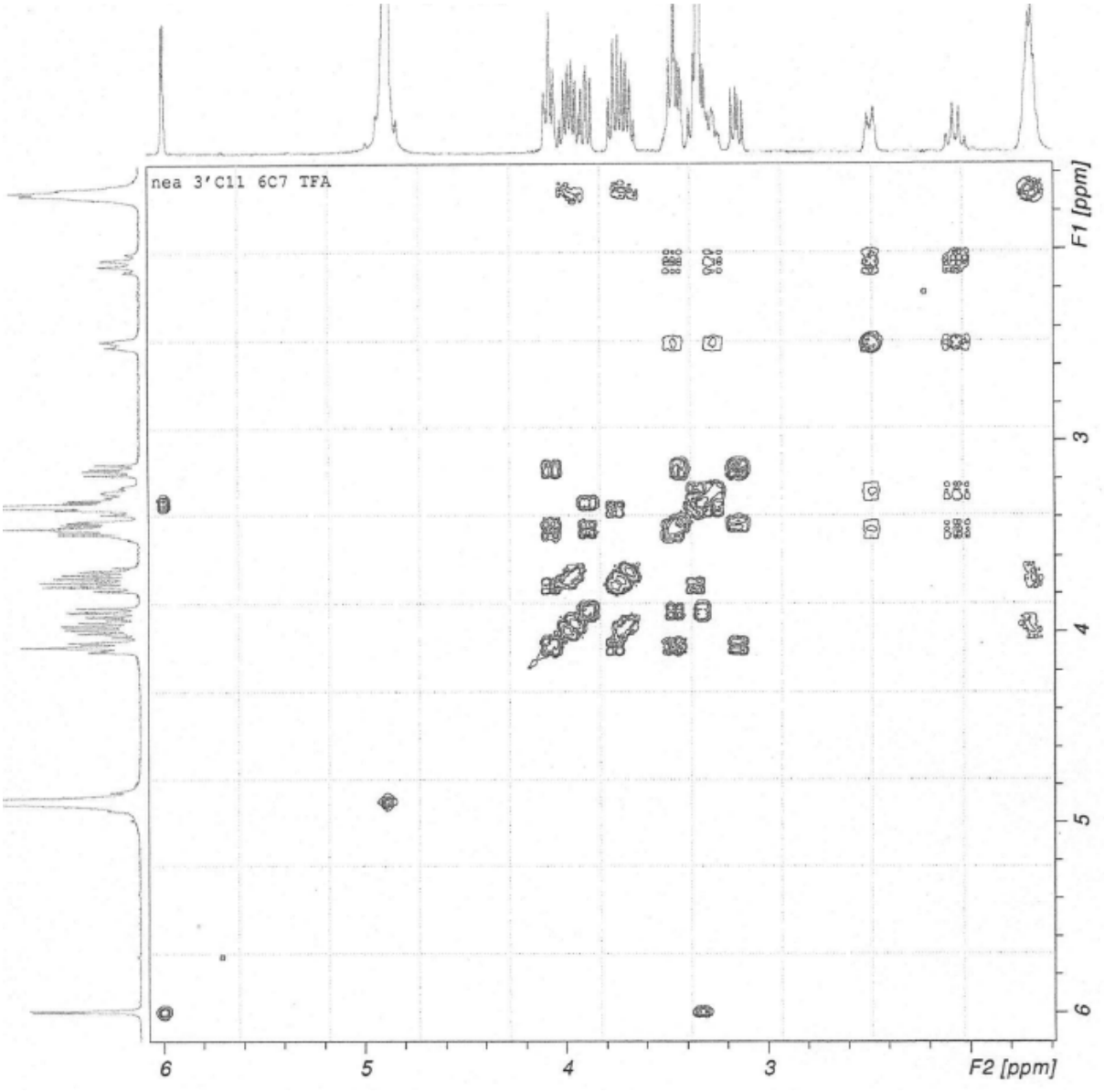
$^{1}$H NMR $CD_3OD$



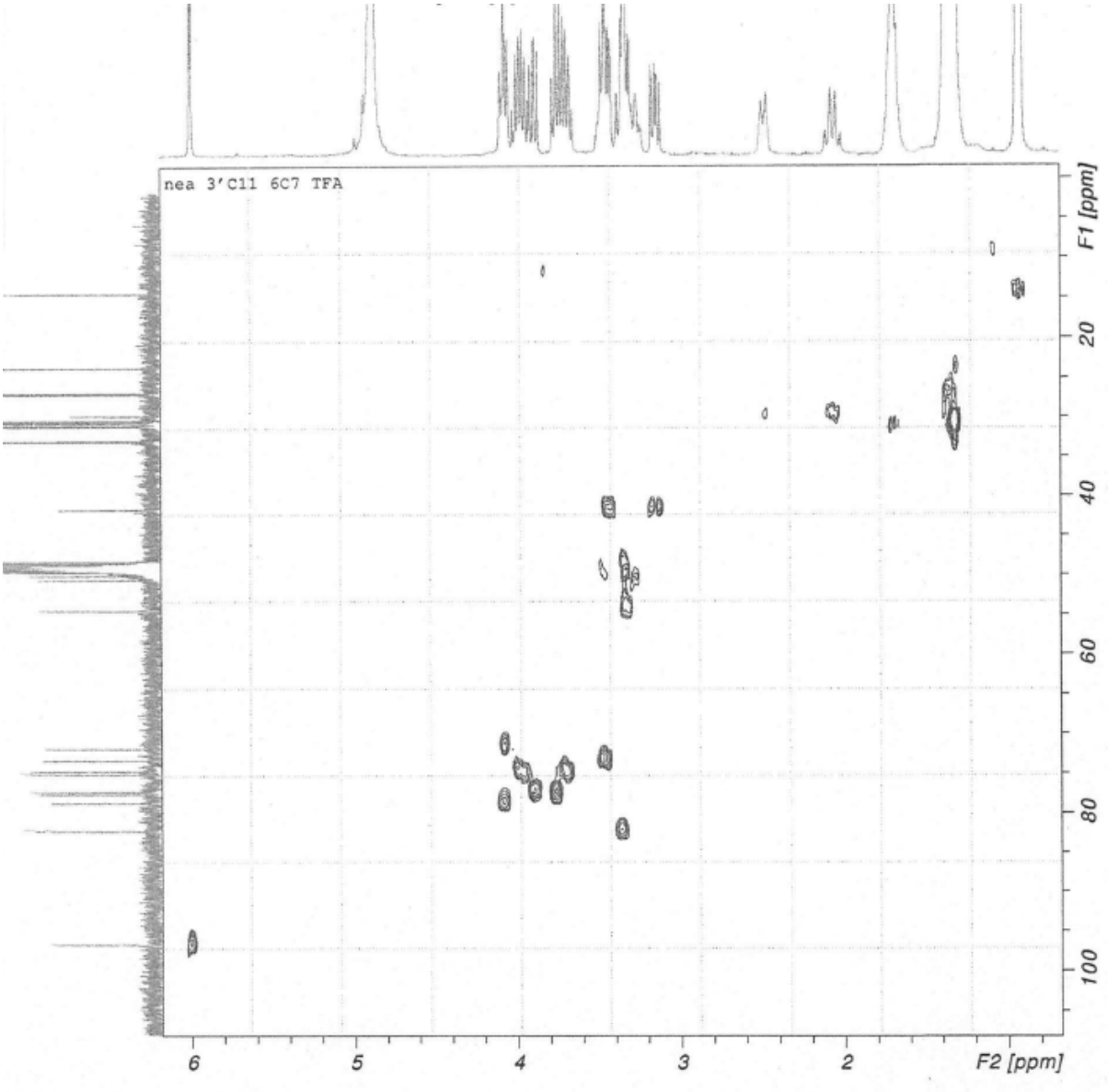

$^{13}$C NMR $CD_3OD$



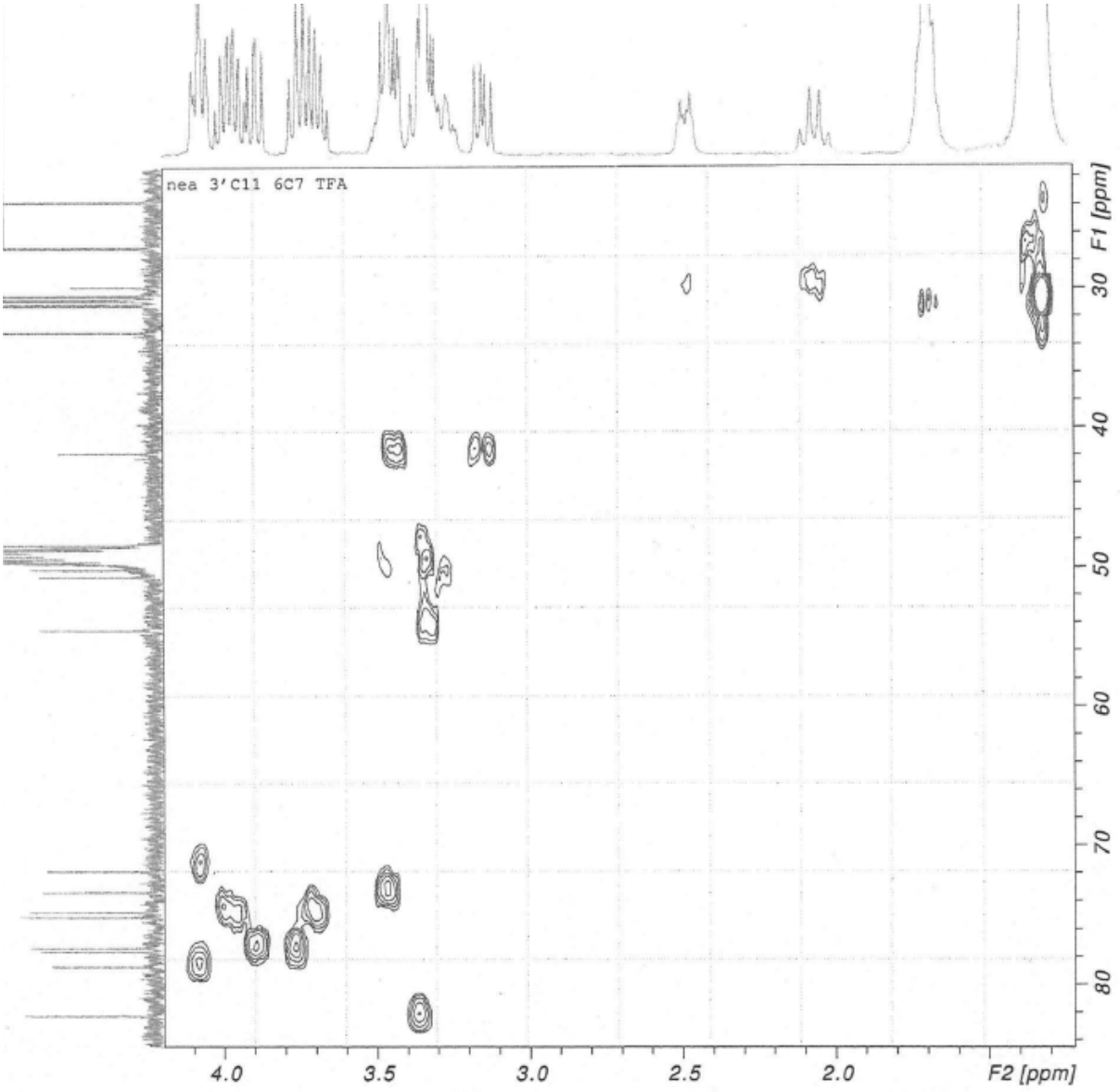
COSY



HMQC 1



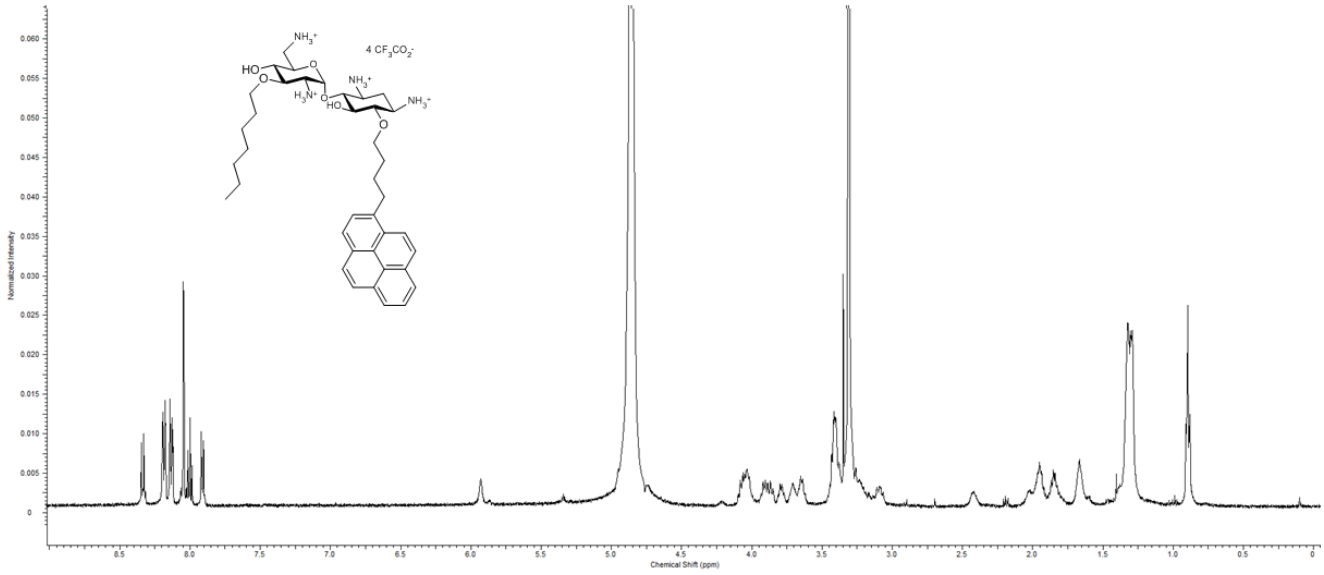
HMQC 2



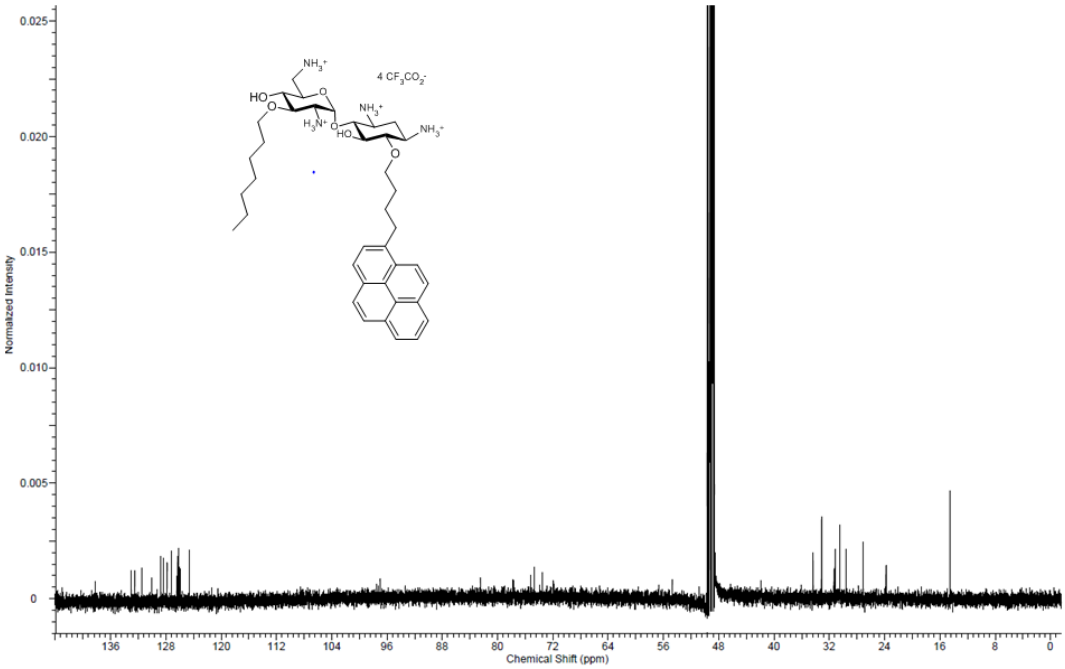
Compound **21**

**3′-*O*-heptyl-6-*O*-[4-(1-pyrenyl)butyl]neamine**

$^{1}$H NMR $CD_3OD$



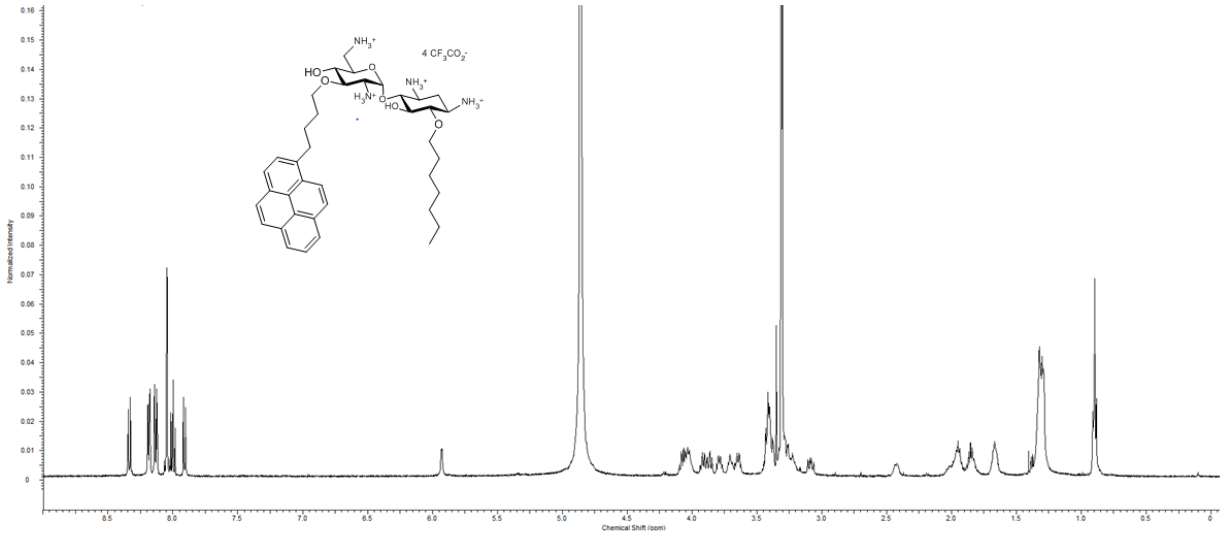

$^{13}$C NMR $CD_3OD$



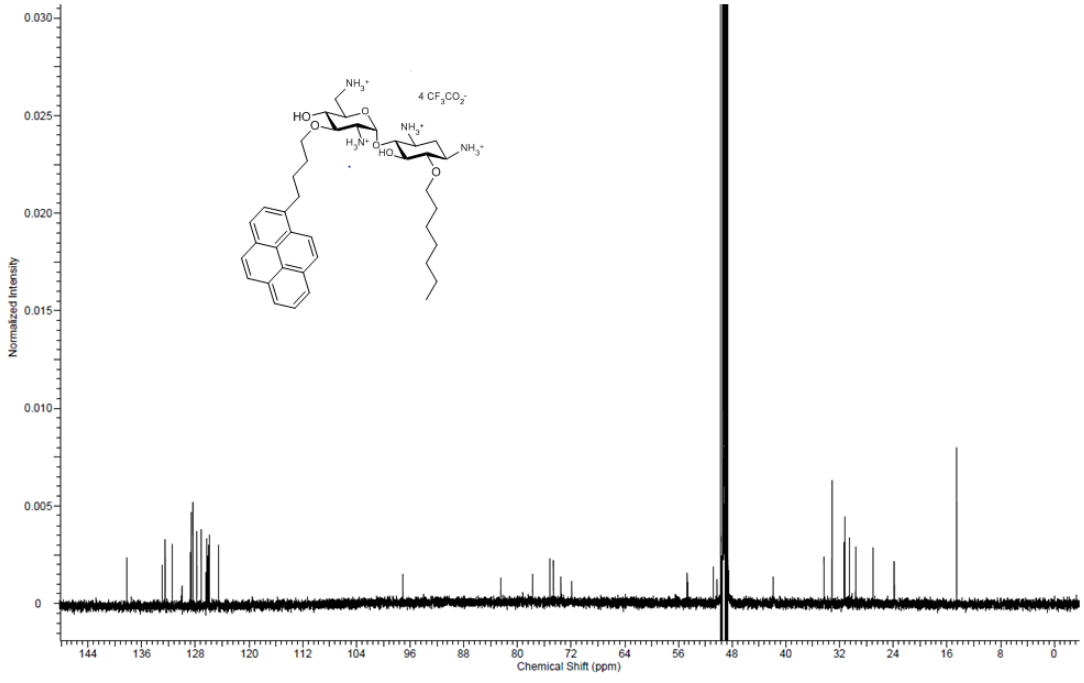
Compound **22**

**3′-*O*-[4-(1-pyrenyl)butyl]-6-*O*-heptylneamine**

$^{1}$H NMR $CD_3OD$



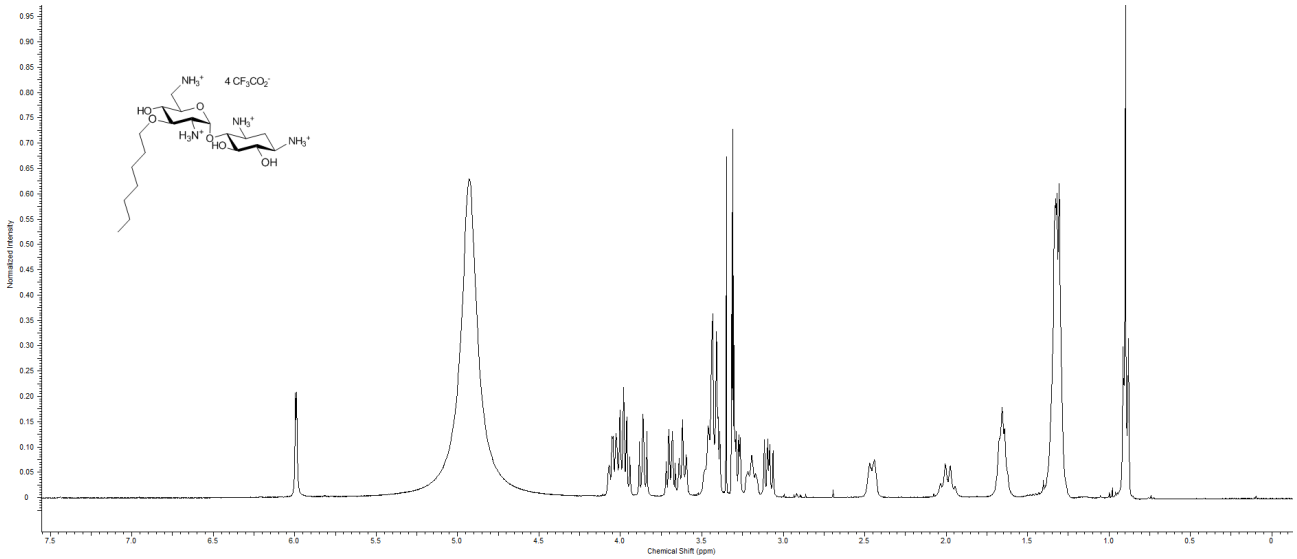

$^{13}$C NMR $CD_3OD$



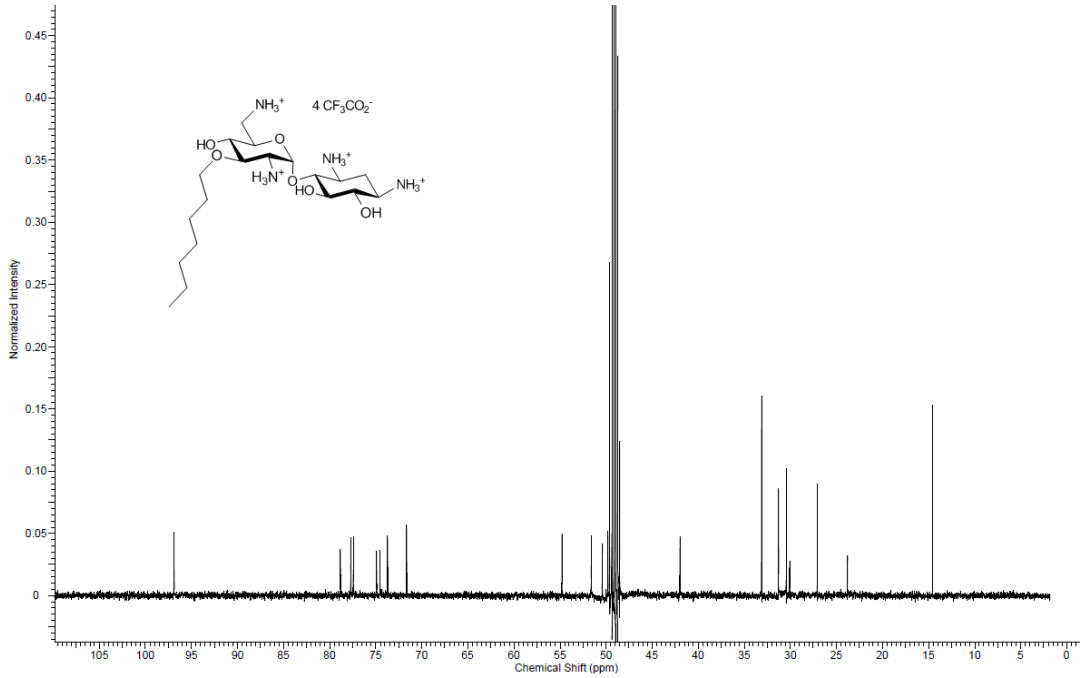
Compound **23**

**3´-mono-*O*-heptylneamine**

$^{1}$H NMR $CD_3OD$

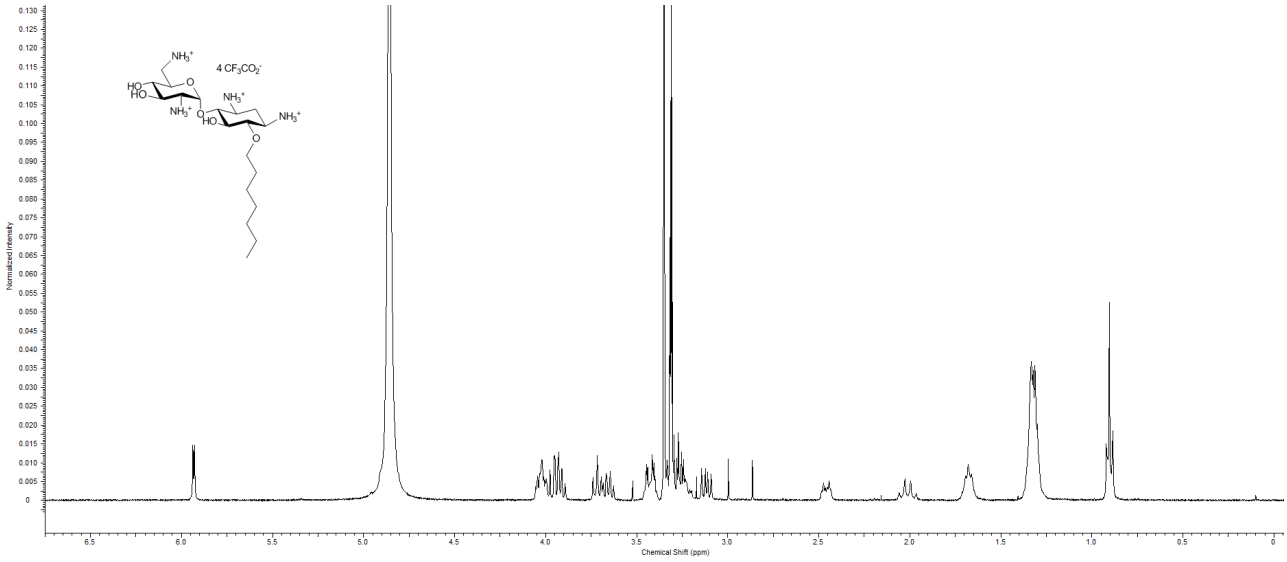



$^{13}$C NMR $CD_3OD$

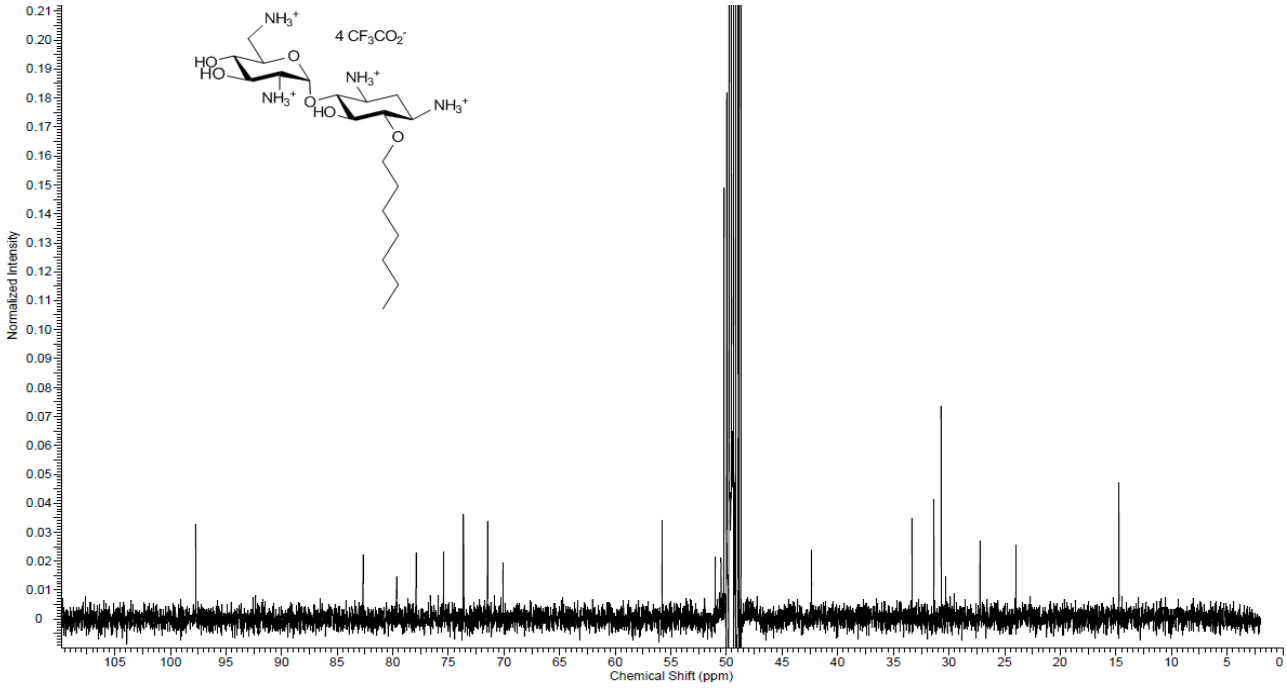


Compound **24**
**6-mono-*O*-heptylneamine**

$^{1}$H NMR $CD_3OD$



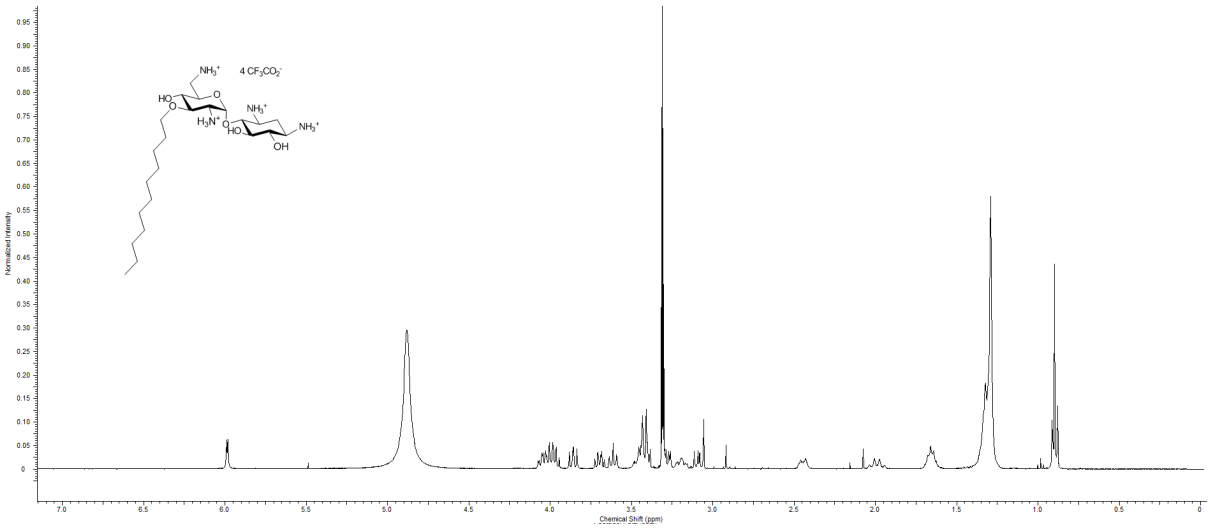

$^{13}$C NMR $CD_3OD$



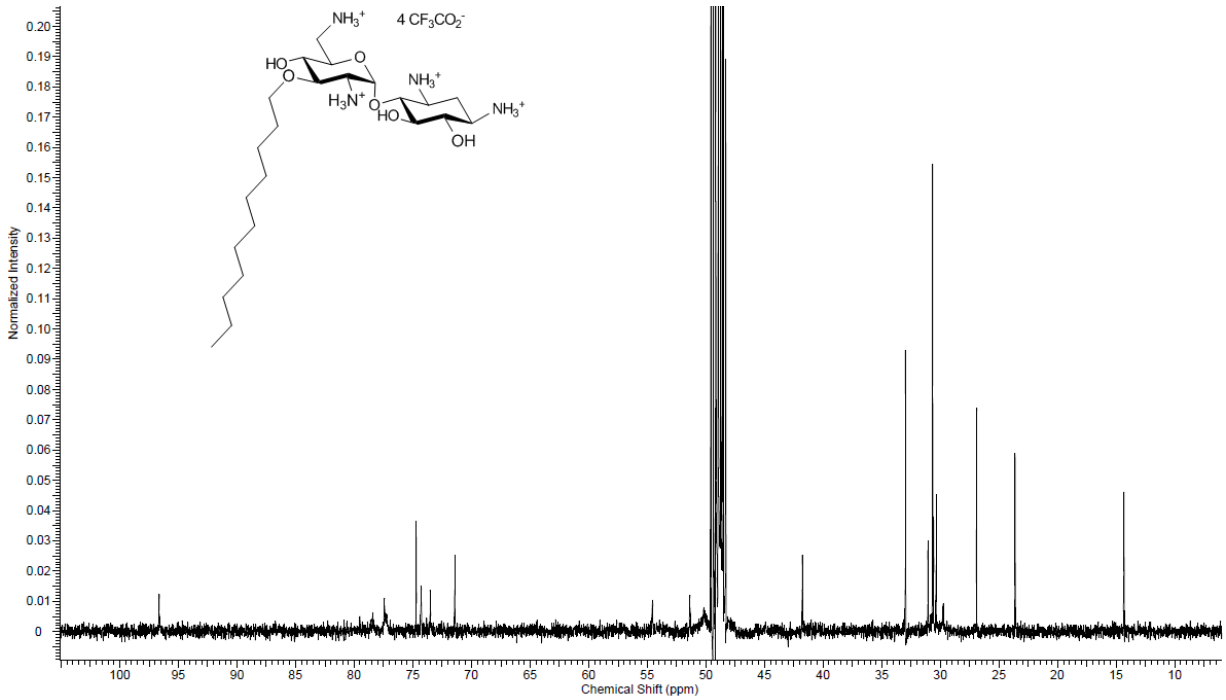
Compound **25**

**3´-mono-*O*-undecylneamine**

$^{1}$H NMR $CD_3OD$

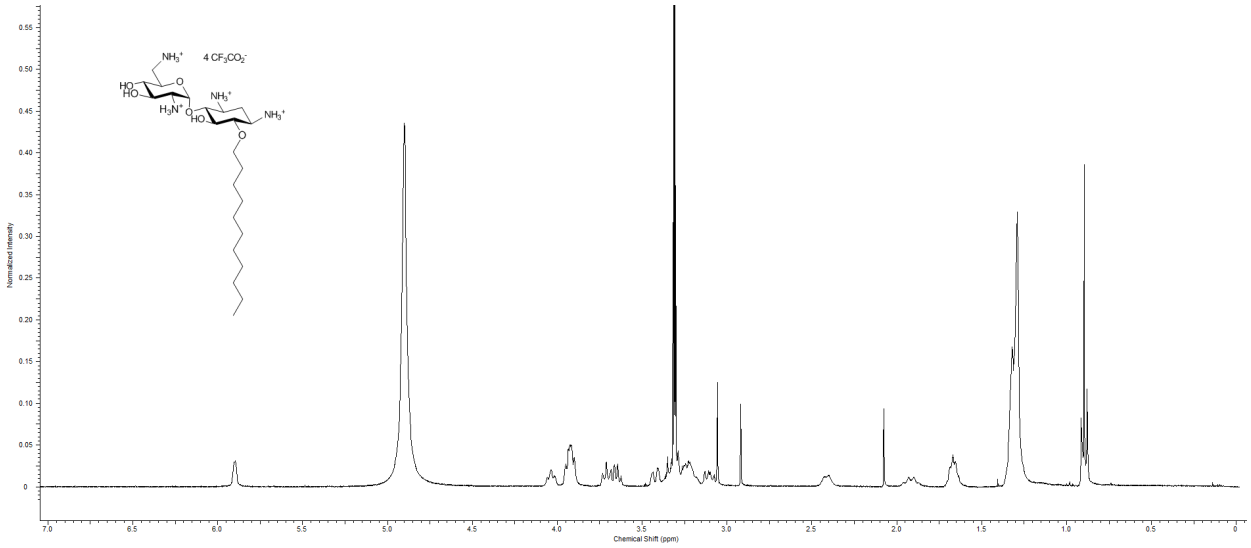



$^{13}$C NMR $CD_3OD$

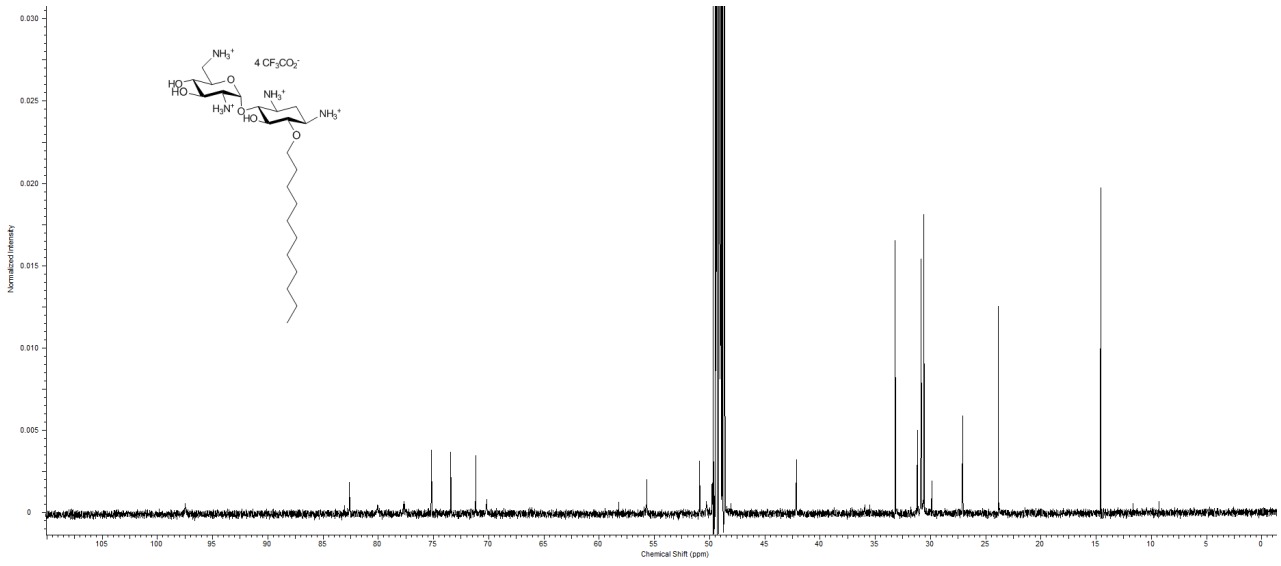


Compound **26**
**6-mono-*O*-undecylneamine**

$^{1}H$ NMR $CD_3OD$

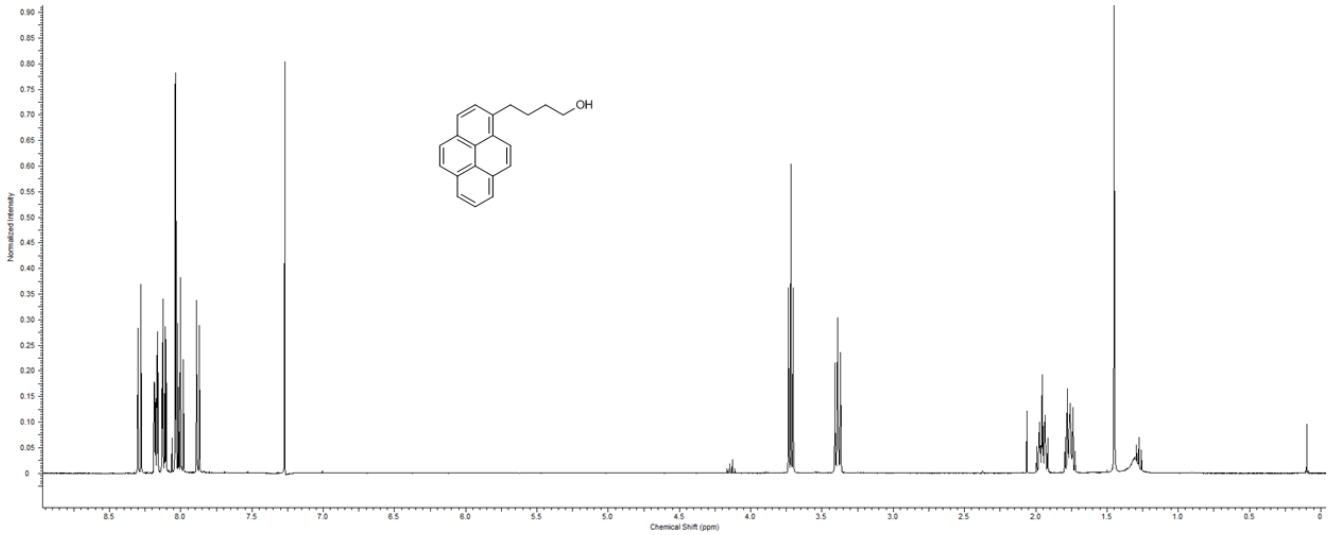



$^{13}C$ NMR $CD_3OD$

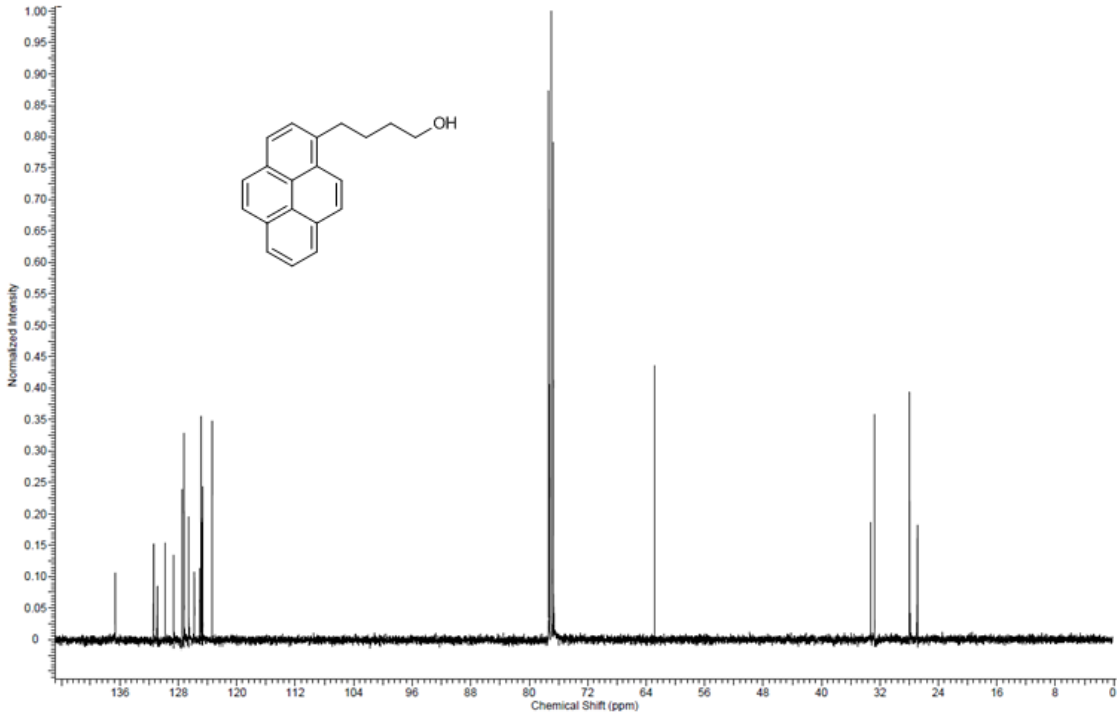


Compound **41**
**4-(1-pyrenyl)butanol**

$^{1}H$ NMR $CDCl_3$



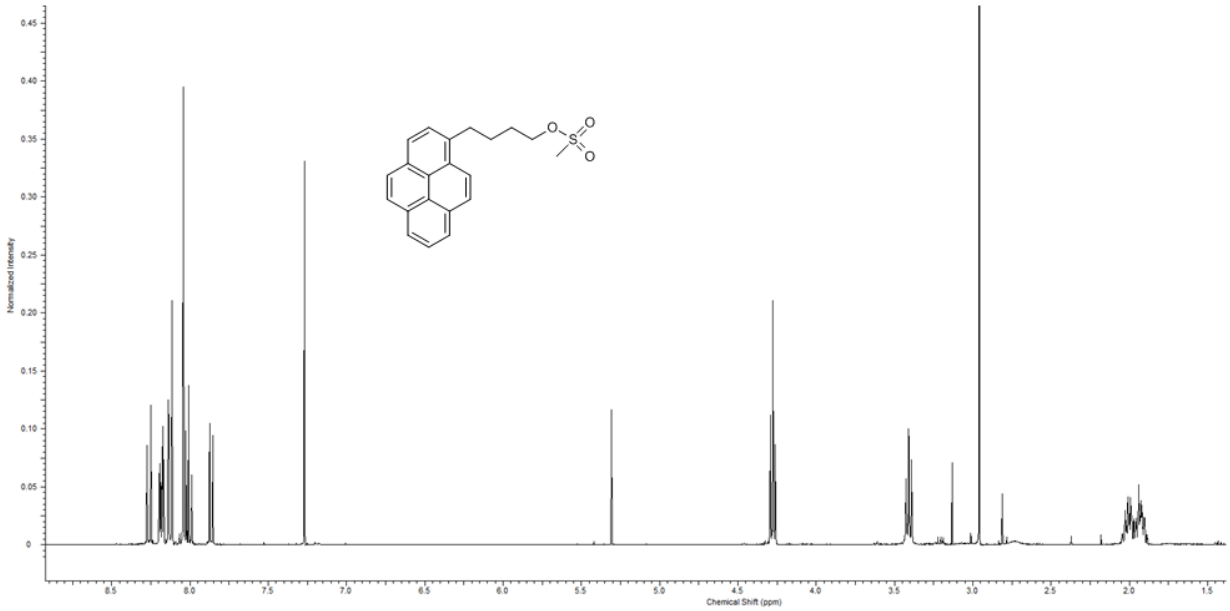

$^{13}C$ NMR $CDCl_3$



Compound **42**

**4-(1-pyrenyl)butyl methanesulfonate**

$^{1}H$ NMR $CDCl_3$




$^{13}C$ NMR $CDCl_3$