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Synthesis and Evaluation of the Antimicrobial Activity of Dodecyl trimethyl ammonium and N-(N, N-dimethylamino) Propyl Glucosylamine

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ABSTRACT

In this study Dodecyl trimethyl ammonium sulphate (DTA) was obtained through quaternization reaction between dodecylamine and dimethyl sulfate (yield: 51.5%) while Dimethylamino propyl glucosylamine (DAPGA) was successively synthesized from D-glucose and 3-Dimethylamino propylamine (yield: 93.6%). Both compounds were analyzed through NMR spectroscopy. Evaluation of their biological activity against *Salmonella* Typhimurium, *Staphylococcus aureus and Fusarium oxysporum cubens* indicated that at a concentration greater than 0.001 10^{-4} mole mL^{-1}, DTA significantly inhibited growth of *Salmonella* Typhimurium and *Staphylococcus aureus* while DAPGA was not effective. Furthermore, these two compounds showed a pronounced antifungal activity against a toxicogen fungal, *Fusarium oxysporum cubens*.

Key words: Dodecyl trimethyl ammonium, Dimethylamino propyl glucosylamine, *Salm.* Typhimurium, *Staph. aureus, F. oxysporum cubens,* biocides.

INTRODUCTION

Development of pathogen bacteria and mycotoxicogen fungi in food constitute a danger for humanity. These harmful microorganisms can be major source of food toxi-infection leading to different human diseases. For instance *Salmonella* and *Listeria* species, very resistant mircroorganisms at different conditions used for food preservation, can lead to salmonellose and listeriose, two known human fatal diseases [1-6]. Furthermore, fungi such us *Aspergillus*, *Penicillium* and *Fusarium* species essentially found on cereal are responsible of teratogenic and carcinogenic toxins which seriously affect human life[7-14]. Due to the diversity and ravages of these harmful microorganisms, human prefer to use antimicrobials to fight against them. Nevertheless, even if the importance of these chemicals is no longer demonstrable, some of them crop up serious eco-toxicological problems and therefore were interdicted or limited from the markets[15, 16]. These

decisions come up very rapidly and left questions on the means to be used for replacing them[17]. Furthermore, future effectiveness of antimicrobial therapy is somewhat in doubt since various microorganisms are becoming more resistant to the existing antimicrobial agents[1-6]. Therefore, it is the responsibility of scientists to work hard and to find out environment friendly active compounds which can be used in this struggling. Previous reports indicated that amino sugars and ammonium compounds could be one of the good candidates to reach the goal[18-24]. This study is our contribution in this scientists' task and was enabled to synthesize two molecules with promised results.

EXPERIMENTAL

Chemicals such us glucose (Sigma Aldrich), dodecylamine 98% (Sigma Aldrich), 3-dimethylamino propylamine (Sigma Aldrich), dimethyl sulphate 99% (Aldrich), Sodium sulphate 99% (Aldrich), chloroform (Prolabo), ethanol (Schorlau) and methanol (Labosi) were used with out any further purification. Culture media Mueller Hinton agar (Biomérieux), Nutrient broth (Difco) and Potato dextrose Agar (Himedia RM 007) were used to assess biological activity. Azithromycin (Alice Pharma PVT, LTD, India) was used as positive reference.

General methods

^{1}H NMR spectra of DTA and DAPGA were recorded at 300 MHZ on a Bruker Avance 300 spectrometer and chemical shifts are given in ppm. The melting point of DTA was determined by an Electrothermal 9100 Digital Melting Point Apparatus IA 9100.

Synthesis of DTA

1-Dodecylamine (0.0539 mole) was dissolved in 20 mL of chloroform. The mixture was refluxed at 50°C and under stirring a small quantity of sodium sulphate was added. After 30 min of reaction, 0.2158 mole of dimethyl sulphate was carefully added to the mixture and reflux was maintained for 6 hours. Solvent was then removed under reduced pressure and residue was co-evaporated four times in methanol to recuperate a white solid which was finally recristallized in ethanol to obtain 9 grams of DTA (Figure 1).

Synthesis of DAPGA

Reported method of Petit (1999)[25] was adapted to synthesize DAPGA (Fig. 2). Ten grams of glucose (0.0555 mole) and 5.7 g (0.0558 mole) of 3-dimethylamino propylamine were dissolved in 66 mL of methanol. The reaction mixture was refluxed at 40°C under stirring for 4 hours. The solvent was removed under reduced pressure using a rotary evaporator and a viscous yellowish liquid of 9.3 g was obtained.

Microorganisms

The fungal strain, *Fusarium oxysporum cubens,* was isolated and characterized in the laboratory of University of Rwanda, College of Science and Technology. Two bacteria *Salmonella* Typhimurium (Institut Pasteur 5858) and *Staphylococcus aureus* (Institut Pateur 25923) were obtained from the Laboratory of US_2B, Université Bordeaux 1, France.

Antifungal activity assessment

Potato dextrose agar (PDA) medium was used to isolate, identify and revivify *F. oxysporum cubens*. The radial growth method[17] was used to assess the antifungal activity of DTA and DAPGA against *F. oxysporum cubens*. Concentrations of 0.4×10^{-4} and 1.5×10^{-4} mole mL^{-1} for both compounds were evaluated. Distilled water and methanol were used to dissolve DTA and DAPGA, respectively. Each essay was done in triplicate and control experiments which contain either distilled water or methanol were done in parallel. The percentage of inhibition from the two products was calculated after 8 days of incubation using the formula previously reported[26]. In addition, the diameter of fungi was daily measured during 8 days of incubation to evaluate the effect of the two products on the kinetic growth of fungi.

Antibacterial activity assessment on agar media

Mueller-Hinton culture medium was used to assess biological activity of DTA and DAPGA against *Salm.* Typhimurium and *Staph. aureus*. Disc diffusion method was used to conduct the test[17, 27].

Assessment of antibacterial efficacy of DTA and DAPGA in liquid medium

Nine milliliters of liquid medium, nutrient broth, in test tubes were separately contaminated by 1 mL of 18h revivified bacteria strains, *Salm.*

Typhimurium and *Staph. aureus,* separately. Then a given quantity of DTA or DAPGA was added prior to have a final concentration of $0.08.10^{-4}$ mole mL^{-1} of these products. The obtained tubes were then incubated at 37°C for 24 hours. Control tubes without drugs were concurrently incubated. After 24 hours of incubation, 1 mL of this microbial suspension was previously 10^6 times diluted and 100 mL were deposited on the Mueller-Hinton agar medium and gently spread onto the surface of this prior to incubation at 37°C for 24h. Forming colony unities were then counted and number recorded. The experiment was conducted in triplicate.

Statistical analysis

Results from antibacterial assays were statistically analyzed using one-way analysis of variance (ANOVA). The significant difference between activities of DTA and DAPGA or between these drugs and control experiments was obtained when the probability (p) found was higher than the significance threshold of 0.05.

RESULTS AND DISCUSSION

Chemical Synthesis

In this study, both DTA and DAPGA were successively synthesized and characterized by ^1H NMR spectroscopy.

DTA

Yield: 51.5%, melting point: 75.1°C, ^1H NMR (300 MHz, D_2O, Figure 3): Σ_H 3.4 (2H, t, $-NCH_2-$), 3.19 (9H, s, $-N^+(CH_3)_3$), 1.7-1.9 (2H, br, $-CH_2CH_3$), 1.4-1.5 (18H, $-CH_2-$), 0.9-1 (3H, t, $-CH_3$). The total synthesis of DTA was indicated by a signal in the form of singulet at 3.19 ppm representing $^+N(CH_3)$ protons (Figure 3).

Our result was confirmed by previous reports[21, 24, 26, 28] in which quaternary ammonium protons were characterized by signals found at 3.1, 3.33 and 3.67 ppm respectively, depending on the type of chemical groups surrounding them.

DAPGA

Yield: 93.6%, ^1H NMR (300 MHz, D_2O): d_H 3.9-4.0 (1H, d, H-1), 3.8-3.9 (1H, dd, H-6a), 3.6-3.7 (1H, dd, H-6b), 3.4-3.5 (1H, t, H-3), 3.3-3.4 (2H, complex, H-4 and H-5), 3.1-3.2 (1H, t, H-2), 2.78-2.86 (2H, mm, $-NHCH_2-$), 2.35 (2H, t, $-CH_2N=$), 2.15 (6H, s, $-N(CH_3)_2$), 1.54-1.65 (2H, m, $-CH_2-$). The synthesis of DAPGA was confirmed by H-1 signal in the form of doublet found at lower chemical shift of 3.9-4.0 ppm compared to H-1a and H-1b of glucose which were reported to be around 5.23 and 4.65 ppm[29], respectively, and this indicated the replacement of OH-1 group by -NH- group in glucosylamine. The same observation was reported in various previous works[17, 27, 29, 30].

Antifungal activity of DTA and DAPGA on *F. oxysporum* cubens

Some fungi are harmful either to animals or plants by intoxicating them. Among these fungi we chose to work with one toxicogen fungus called

Table 1: Inhibition of DTA, methanol and DAPGA on the radial growth of *F. oxysporum cubens* at the 8th day of incubation

Biocides	Concentration (mol ml^{-1})10^{-4}	Percentage of inhibition ±SEM
DTA	0.4	100.0 ± 0.0
	1.5	100.0 ± 0.0
DAPGA	0.4	81.5 ± 1.2
	1.5	100.0 ± 0.0
Distilled water	1 mL	0.0 ± 0.0
Methanol	1 mL	11.5 ± 1.4

Table 2: Inhibition diameter values of DTA on *Staph. aureus* and *Salm t*yphimurium Inhibition diameter ± SEM (in mm)

Bacteria	Concentrations (mol mL^{-1})10^{-4}				
	0.08	0.04	0.02	0.01	0.001
Staph. aureus	24.3 ± 0.3	20.3 ± 0.5	18.3 ± 0.7	9.2 ± 0.8	0.0 ± 0.0
Salm. Typhimurium	15.3 ± 0.9	9.7 ± 0.3	8.0 ± 0.0	2.1 ± 0.5	0.0 ± 0.0

Table 3: Antibacterial activity of DTA and DAPGA in water

Drugs	Antibacterial activity (UFC ± SD)	
	***Salm* typhimurium**	***Staph aureus***
Control experiment	374 ± 13	299 ± 23
DTA	0 ± 0	0 ± 0
DAPGA	362 ± 17	277 ± 20

Table 4: Inhibition diameter values of DAPGA, DoGA and 3-DMAP on *Staph aureus* and *Salm* typhimurium

Biocides	Inhibition diameter ± SEM (in mm)		
	Concentration (mole mL^{-1}) × 10^{-4}	***Staph aureus***	***Salm* typhimurium**
DoGA	0.5	16.0 ± 0.5	10.6 ± 0.5
DAPGA	0.5	0.0 ± 0.0	0.0 ± 0.0
	2.0	6.0 ± 0.3	8.0 ± 0.6

Fusarium oxysporum cubens. *Fusarium* species are known, not only to be implicated in the annually worldwide harvest lost[12], but also to produce a variety of toxins such as fumonisins, zearalenone and trichothecenes in food mainly cereal, and thus exhibiting chronic toxicity[7,8,10,14,31]. In this study, both two products DTA and DAPGA were effective against the growth of *Fusarium oxysporum cubens* (Figure 5 and Table 1). At all concentrations used, 0.4 x 10^{-4} and 1.5 x 10^{-4} mole mL^{-1}, DTA completely inhibited the growth of *Fusarium oxysporum cubens* while DAPGA exhibited a significant antifungal activity (percentage of inhibition of 81.5 %) when a concentration of 0.4 x 10^{-4} mole mL^{-1} was tested and completely inhibited the growth of fungal at a concentration of 1.5 x 10^{-4} mole mL^{-1}. Methanol and water used as negative control exhibited non significant antifungal activity.

Furthermore, the antifungal activity of these two compounds was observed during a week to visualize well what was happened during incubation of fungus. At concentration of 0.4 x 10^{-4} mole mL^{-1}, DTA completely inhibited the growth of

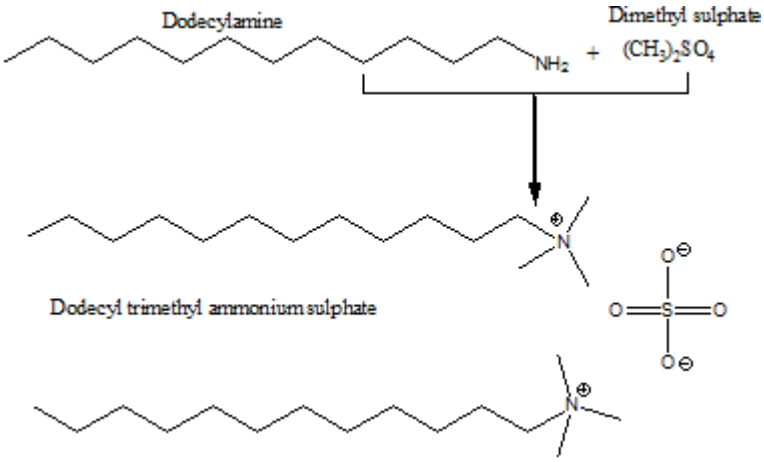


Fig. 1: Synthesis scheme of DTA

fungi while DAPGA significantly affected the kinetic growth of fungus by strongly changing the shape of its kinetic growth. Methanol used as negative control did not show significant effect on the kinetic growth of fungal but slightly delayed it. All these observations were done in comparison with the effect of distilled water used as control (Figure 6).

At all concentrations used DAPGA did not affect the growth of both bacteria while DTA showed its efficacy in inhibiting these microbes. At the concentration of 0.08 x 10^{-4} mole mL^{-1}, DTA exhibited higher activity against both bacteria. It inhibited the growth of *Staph. aureus* and *Salm.* Typhimurium with inhibition zones of 24 and 15 mm respectively. These inhibition zones are too sufficient to consider DTA as effective against both bacteria [32]. In this study, at a concentration previously quoted, DTA led to an inhibition diameter which was closed to 16 mm (viz 15.3 ± 0.9) for *Salm.* Typhimurium and thus can be used as the minimal effective concentration against that bacterium. Furthermore, a concentration of around 0.02 mole mL^{-1} can be taken as minimal effective one for *Staph. aureus*. As shown in the table 2, DTA with a concentration greater than 0.001.10^{-4} mole mL^{-1} had higher antibacterial activity on *Staph. aureus*, Gram positive bacterium, than the antibacterial activity it had on a Gram negative bacterium, *Salm.* Typhimurium. This may partially due to the chemical composition of their cell-wall structures which are different and thus conduct to their different permeability towards drugs[33, 34]. In addition, at a concentration of 0.08 mole mL^{-1}, Azithromycin, an antibiotic used as positive control, exhibited comparable antibacterial activity with DTA. DTA indicated inhibition zones of 24.3 ± 0.3 and 15.3 ± 0.9 for *Staph. aureus* and *Salm.* Typhimurium (Table 2), respectively, while for Azithromycin the inhibition zones were found to be 26.7 ± 1.2 and 20.3 ± 1.8 mm respectively for *Staph. aureus* and *Salm.* Typhimurium. Comparison of these results indicated that efficiency of DTA is closely comparable to that from know antibiotic, Azithromycin. At concentrations lower than or equal to 0.08 x 10^{-4} mole mL^{-1}, DAPGA did not show any significant antibacterial activity.

To verify the reason of difference between biological activities of DTA and DAPGA, the efficacy of these two drugs were also assessed in liquid media. DTA completely inhibited the growth of both bacteria while DAPGA didn't show any significant inhibition compared to control experiments (Table 3).

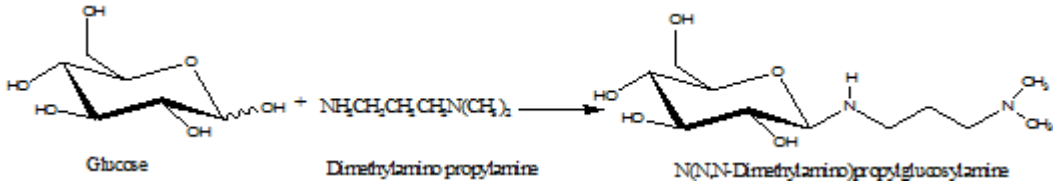


Fig. 2: Synthesis scheme of DAPGA

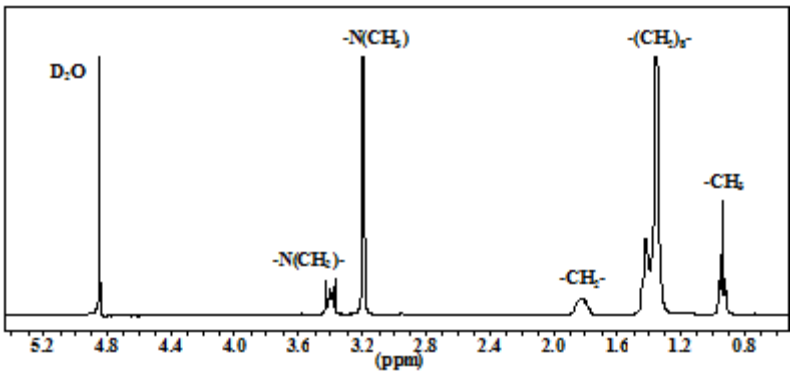


Fig. 3: ^{1}H NMR spectrum of DTA

Having these observations, decomposition of DAPGA in D_2O was followed using ^{1}H-NMR. Both disappearance of –NH(CH_2)- and decreasing intensity of H-1 signals were served as references (Figure 8). We chose to work with D_2O as solvent, because of its quite same properties, in hydrolysis process, with H_2O used to prepare liquid medium in which both compounds was tested. After 24 h in D_2O, the initial DAPGA decreased of 50% while at 48 h, only 37.5% of DAPGA was remained in the medium without any decomposition (Figure 8).

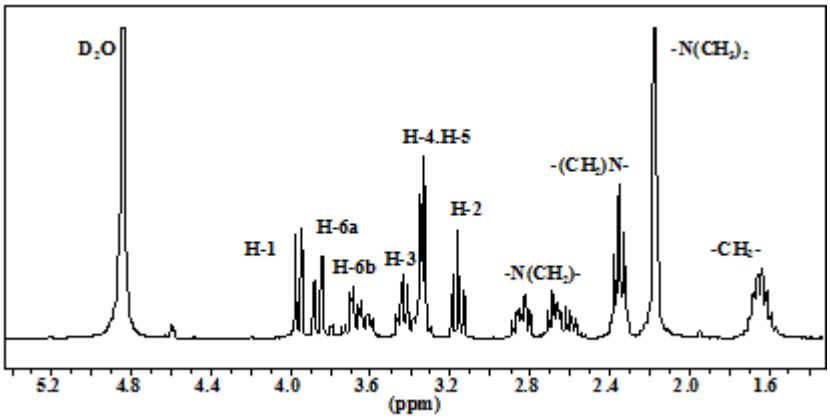


Fig. 4: ^{1}H NMR spectrum of DAPGA

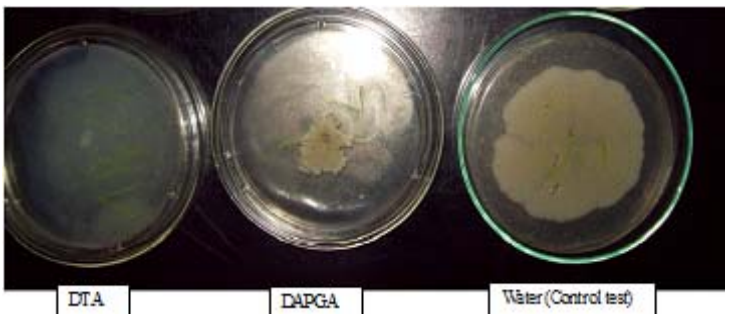


Fig. 5: Illustration of antifungal activity of DTA and DAPGA on *Fusarium oxysporum cubens* at a concentration of 0.4 x 10^{-4} mole.mL^{-1}

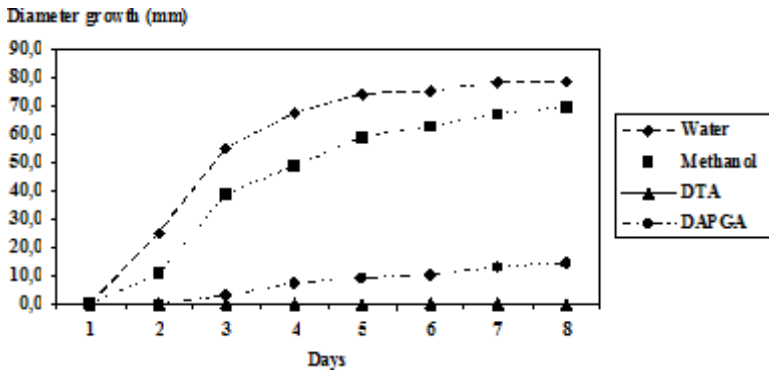
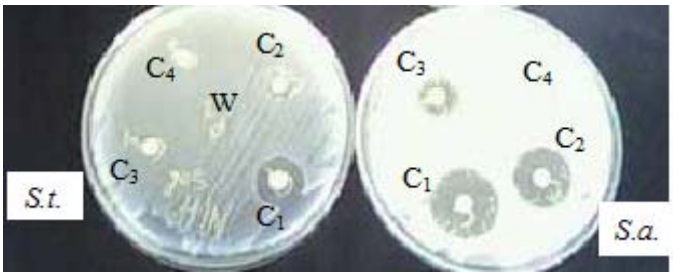


Fig. 6: The effect of synthesized products on the kinetic growth of *F. oxysporum cubens* at the concentration of 0.4 x 10^{-4} mole mL^{-1}

This remarked rapid decomposition of DAPGA could be the reason of its poor antibacterial activity. As previously reported[17], the lack of activity of glucosylamines against bacteria, may be partially explained by their rapid hydrolysis during the first 24 hours. To verify this assumption, the antibacterial activity of DAPGA was compared to that of Dodecyl glucosylamine (DoGA), a molecule which was previously synthesized and found to be not highly hydrolyzed[17]. At a concentration of 0.5 x 10^{-4} mole mL^{-1}, both *Salm.* Typhimurium and *Staph. aureus* were exactly sensible to DoGA but not to DAPGA (Table 4).

DAPGA started to exhibit antibacterial activity at higher concentration of 2.0 x 10^{-4} mole mL^{-1} compared to DoGA and this observed activity was even not significant.



Note: S.t.: *Salm. Typhimurium*, S.a: *Staph. aureus*, C_1, C_2, C_3 and C_4: concentrations of drugs which are equal to: 0.04, 0.02, 0.01 and 0.001 (x 10^{-4}) mole mL^{-1} respectively, w: water

Fig. 7: Illustration of antibacterial activity of DTA on *Salm typhimurium* and *Staph aureus*

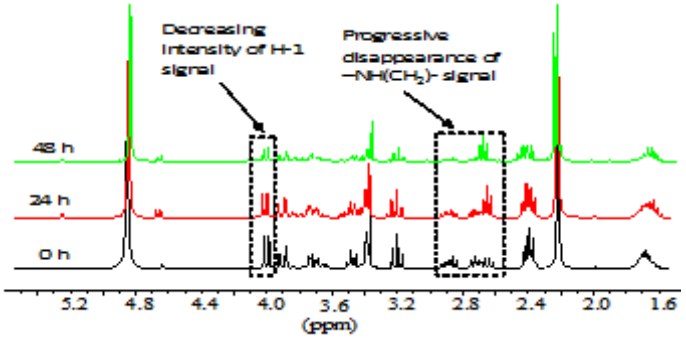


Fig. 8: Decomposition of DAPGA in D_2O for 48 h

In conclusion, results revealed that at all concentrations used, DTA and DAPGA were effectiveness to inhibit the growth of *F. oxysporum cubens*. Furthermore, Dodecyl trimethyl ammonium (DTA) showed significant antibacterial activity against the growth of *Salm.* Typhimurium and *Staph. aureus*. The lack of significant antibacterial of N-(N,N-Dimethylamino) propyl glycosylamine against *Staph. aureus* and *Salm.* Typhimurium could be due to its remarked decomposition in D_2O and research on its possible stabilization should be of interest. Through our findings, the reported antibacterial efficacy of ammonium compounds[21, 24, 28] was confirmed: satifying antimicrobial activity was obtained from small biodegradable molecule, dodecyl trimethyl ammonium sulphate. It is however to note that the toxicity assessment of this compound should be carefully realized before any application of this compound in various domains.

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