N, N, N-triethyl ammonium L-Phenylalanine esters as potential antimicrobial agents.

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Quaternary ammonium compounds are an important class of molecules with wide variety of applications, mainly in the antimicrobial field. Quaternary ammonium compounds have the ability to bind with the anionic moiety of phospholipids of the bacterial membrane, disrupting the cell membrane of the microorganisms. However, classical quaternary ammonium salts can have adverse effects on the environment. Therefore, the use of amino acid analogues are preferred as raw materials for their synthesis. In this study, quaternary ammonium salts, namely *N, N, N*-triethyl ammonium L-Phenylalanine esters were synthesised by tethering the amino end with ethyl groups. The ease of quaternisation with increasing ester chain length of the amino acid was studied. The yields of the quaternary ammonium compounds were found to decrease with increasing alkyl chain of the phenylalanine esters (35% - 6%). The antibacterial activity of the quaternary ammonium compounds were compared to that of the unquaternised phenylalanine ester using the Kirby-Bauer disk diffusion method against gram positive (B.*cereus* ATTC 11778, S.*aureus* ATCC 29213) and gram negative bacteria (S. *typhimurium* ATCC 14028, P.*aeroginosa* ATTC 27853). The quaternised ammonium esters were found to be better potential antibacterial agents as compared to the unquaternised esters.

Keywords: Quaternary ammonium compounds, L-Phenylalanine esters, gram-positive bacteria, gram negative bacteria.

Introduction

In spite of a relatively wide choice of disinfection agents nowadays the microorganisms represent hitherto a serious sanitary and economic problem. The phenomenon of intrinsic resistance of some species as well as the ability of microorganisms to adapt for high concentrations of usual disinfectants stimulates the search for new antimicrobial compounds with higher effectiveness [1]. Quaternary ammonium compounds (QUATs) belong to the group of reliable antimicrobial compounds and has been known to have a wide variety of applications such as fungicides and biocides [2]. Nowadays the antimicrobial mode of action

of the ammonium salts has been elucidated. These compounds cause a generalized damage of the cytoplasmic membrane so that the positively charged "head" of the molecule interacts with the negatively charged membrane components followed by penetration of nonpolar tenside constituent of its hydrophobic part. The crucial first step at the membrane destruction is in this case the decrease of its electrical potential by Coulomb interactions [3-5]. There are several different types of QUATS used as biocides, with the benzalkonium being the most common [6]. Although a number of reports have indicated that benzalkoniums are effective as biocides, recent data indicates that various strains of benzalkonium-resistant bacteria have come into being [7, 8]. Therefore, there is a need to develop more QUATS with antimicrobial activities. Quaternary ammonium compounds, in general, have toxic effects toward mammalian cells [9]. In humans and animals they are considered too toxic for systemic applications, but acceptable for topical applications. The sustained toxicity and environmental impact of these antibacterial compounds are related to their chemical stability. To overcome these limitations, QUATS derived from natural precursors such as amino acids have been developed [10, 11]. In 2010, Lucac *et al*. reported the antimicrobial activities of a series of quaternary ammonium compounds from phenylalanine [12]. The compounds were found to possess high antibacterial properties with MIC value of up to 13.5 μM for *S.aureus*. The activity was also found to be influenced by the alkyl chain length. In this work, we report the synthesis and preliminary antibacterial screening of quaternary ammonium compounds, namely *N,N,N*-triethyl ammonium L-Phenylalanine esters. They were prepared by esterification of phenylalanine followed by tethering the amino group of the phenylalanine moiety with ethyl groups. Esters of increasing chain length were quaternised since antibacterial activity is also affected by chain length.

Materials and methods

L-Phenylalanine was obtained from HiMedia Laboratories (India). Bromoethane was obtained from Sigma Aldrich (St Louis, USA). Mueller Hinton agar and Mueller Hinton broth were obtained from Oxoid Ltd (United Kingdom). Cetyl trimethyl ammonium bromide (CTAB) was obtained from BDH Laboratory Supplies, England. The different bacterial strains were obtained from Microbiologics® (St Cloud, MN, USA) and Oxoid Ltd (United Kingdom). ^{1}H NMR and ^{13}C NMR spectra were recorded at 250 MHz and 62.9 MHz on a Bruker electro spin NMR spectrometer using $CDCl_3$, D_2O and DMSO-d_6 as solvents.

***N, N, N*-triethyl ammonium L-Phenylalanine methyl ester**

L-Phenylalanine methyl ester was synthesized according to a modified procedure [13]. L-Phenylalanine (1.00 g, 6 mmol) was stirred with the corresponding alcohol (15 mL) at 0°C. Thionyl chloride (1 mL, 13.8 mmol) was added dropwise to the reaction mixture which was stirred at room temperature for 48 hours. Excess alcohol was evaporated in *vacuo* and the residue washed with diethyl ether to yield the ester as a white solid.

L-Phenylalanine methyl ester (0.5 g, 3 mmol) and K_2CO_3 (1.2 g) was stirred in acetonitrile. Bromoethane (4mL) was then added and heated to 90°C in a sealed tube for 18 hours. The reaction mixture was filtered to remove excess K_2CO_3. The solvent was then removed in vacuo to yield the desired product as white solid.

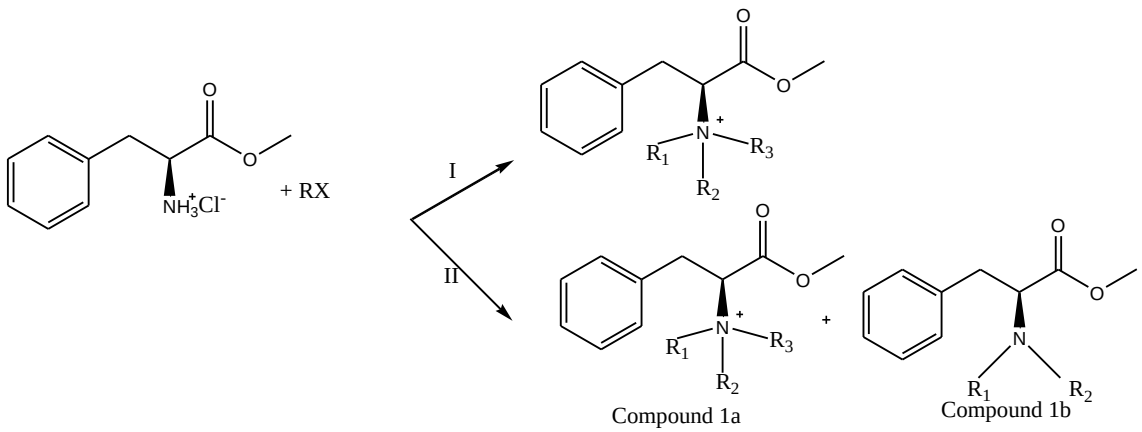
Antimicrobial evaluation.

A growth inhibition assay was performed against the gram positive bacteria *Staphylococcus aureus* (ATCC 29213) and *Bacillus cereus* (ATCC 11778) and two gram negative bacteria *Pseudomonas aeroginosa* (ATCC 27853) and *Salmonella Typhimurium* (ATCC 14028) using the Kirby-Bauer disc diffusion method [14]. Solutions of the compounds were prepared in distilled water with concentration of 100 mg/mL. 10 µL of these sample solutions were pipetted onto the discs and allowed to incubate at 37°C for 24 hours. The activities of the compounds were determined by measuring the diameter of the zone of inhibition in mm. CTAB which is commonly use as an antiseptic was used as a positive control using the same concentration of the tested compounds.

Results and Discussions

The synthesis of *N,N,N* triethyl phenylalanine methyl ester consists of esterifying the amino acid followed by quaternising the amino group. Methods for quaternising primary and secondary amines involve alkylating agents and a strong inorganic base to bind the acid which is generated and often involves prolonged reaction times and heat which are conducive to undesirable side reactions [15]. Francis *et al*. (1976) reported the efficient quaternisation of primary, secondary and tertiary amine using methyl iodide and $KHCO_3$ or $NaHCO_3$ as base in methanol at room temperature [15]. The method is mild, reasonably selective and of general applicability. When this method was used, quaternisation was successful with MeI, giving rise to the *N,N,N*-trimethyl phenylalanine ester derivative. But, when quaternisation was attempted with ethyl iodide, only the dialkylated product was formed rather than the *N,*

N, N-triethyl derivative. The formation of the dialkylated derivative was also observed by heating the reaction to 90°C in a sealed tube for 18 hours. NaOH was then used as a stronger base compared to $NaHCO_3$. Hurley *et al.* carried out quaternisation reaction with MeI in the presence of NaOH at 90°C for 6 hours [16]. The same reaction condition was used to quaternise phenylalanine methyl ester using ethyl iodide. In this case, the amino group was quaternised with the ethyl groups but, the ester moiety was found to have undergone hydrolysis due to the presence of NaOH. TEA was then used as base. In this case, the formation of the quaternised base may render the isolation of the desired quaternary ammonium compound difficult. In order to overcome this issue, the phenylalanine ester hydrochloride was first made to react with TEA, to form the free amine which was then made to react with EtI. No reaction took place due to the absence of base in the reaction medium during quaternisation. Quaternisation was then attempted using K_2CO_3 as base with EtBr in acetonitrile at 90°C in a sealed tube to give a mixture of the desired product (compound 1a) together with the dialkylated product(compound 1b) as the major product.

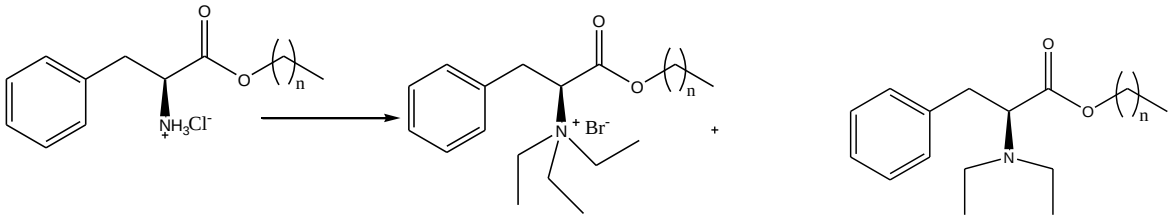


I: R= CH_3, R_1=R_2=R_3=CH_3

II: R= C_2H_5, R_1=R_2=R_3= C_2H_5

Scheme 1: Synthesis attempts of *N,N,N*-triethyl phenylalanine methyl ester

The effect of the alkyl ester chain length on the quaternisation of the L-Phenylalanine esters was studied. Different phenylalanine esters with alkyl chain length varying from C_1 to C_4 were quaternised with ethyl groups. An increase of the alkyl ester chain length from methyl to propyl ester causes a decrease in the yield of the quaternary ammonium compound and an increase in the diethyl derivative. In the case of the butyl ester, the diethyl derivative was formed as the only product.



I **II**

Compound number	n	Yield %	
		I	II
1a, 1b	0	35	63
1c, 1d	1	28	70
1e,1f	2	6	76
2a	3	0	78

Scheme 2: Effect of alkyl ester chain length on quaternisation of phenylalanine esters

An increase in the alkyl chain length of the esters render quaternisation more difficult, possibly due to steric hindrance. The bulky ethyl groups renders quaternisation unfavourable with phenylalanine esters of chain length > 3.

Quaternisation of phenylalanine butyl ester gave rise to the diethyl derivative (Compound 2a). Quaternisation was then attempted by reacting the diethyl derivative of phenylalanine butyl ester with excess ethyl bromide and no product was obtained. However, when the diethyl derivative was reacted with MeI, the desired quaternary ammonium compound 2b was formed.

Antibacterial activity

The antibacterial activity of *N,N,N* triethyl quaternary ammonium phenylalanine methyl ester was compared to the unquaternised phenylalanine methyl ester, to study the effect of the quaternised nitrogen moiety.

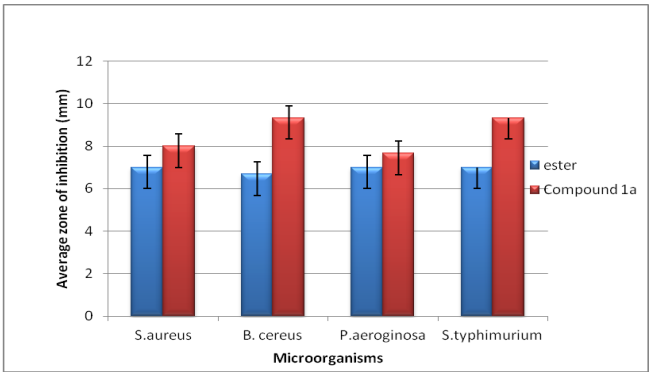


Figure 1: Activity of compound 1a compared to phenylalanine methyl ester

From Figure 1, it can be seen that the activity of compound 1a is greater than that of the unquaternised phenylalanine methyl ester. The higher activity of compound 1a might be attributed to a higher affinity of the compound for the bacterial membrane due to the extra ethyl groups which provide more hydrophobic interactions (Figure 2).

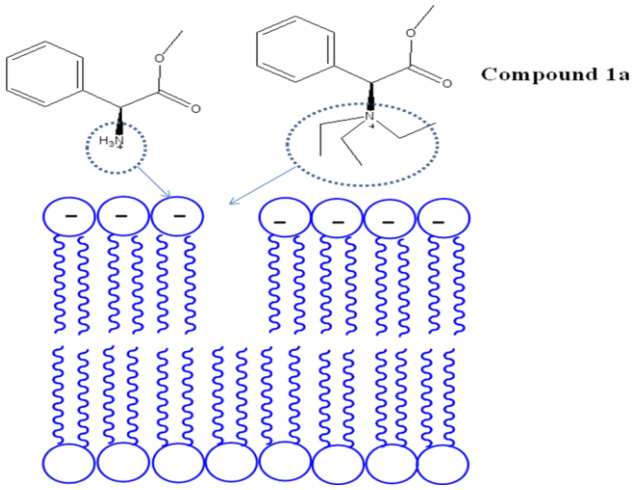


Figure 2: Interaction between phenylalanine methyl ester and compound 1a with bacterial membrane

Conclusion

The study shows that the synthesised N, N, N-triethyl ammonium phenylalanine esters have potential antibacterial actitvities against both gram positive and gram negative bacteria. They display better activity compared to the phenylalanine ester hydrochloride. This shows that the quaternary ammonium moiety have better interactions with the bacterial cell membrane.

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