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Synthesis, purification, and cell-toxicity of a choline betainate

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ABSTRACT

In this work, choline chloride and betaine hydrochloride were condensed into a - to our knowledge - unreported choline betainate (*N,N,N*-trimethyl-2-oxo-2-(2-(trimethylammonio)ethoxy)ethanaminium chloride) using 1,1′-carbonyldiimidazole (CDI) activation of betaine hydrochloride in dimethylsulfoxide. The product and reaction intermediates were isolated, purified by preparative HPLC and analyzed in detail by infrared and nuclear magnetic resonance spectroscopy. The final product has a high cytotoxicity for L929 mouse fibroblasts, and low antibacterial activity against *P. Aeruginosa* and *S. Aureus* at concentrations of up to 20 mg/ml. It could potentially further be investigated for similar uses as suxamethonium chloride, a muscle relaxant drug.

1. Introduction

Choline and betaine are essential molecules of life. Betaine serves as a methyl donor in methionine biosynthesis [1]. Betaine and choline can be synthesized in the human body, but both are mostly acquired through certain dietary sources like eggs, meat, vegetables, and dairy products [2]. Consequently, choline plays a vital role in the synthesis of acetylcholine, phosphatitylcholines, and therefore for a normal function of the central nervous system [3], metabolism of methyl groups, and for healthy cell membranes [1]. In chemical reactions, choline and betaine have often been used as part of deep eutectic solvents [4], or in ionic liquids [5]. Quaternary ammonium based deep eutectic solvents increased the stability [4], and solubility of compounds [5], could be used for CO_2 capture [6], or have been used as green extraction solvents [7]. Extensive research proved that choline and betaine are non-toxic [7–11] and environmentally friendly [12,13]. Cationization of polymers with either choline or betaine enhanced delivery of DNA into cells [14], inhibited acetylcholine esterase, [15] or increased the biocompatibility and antimicrobial properties [16]. One commercially available choline derivative bearing two cationic charges is suxamethonium chloride also called succinylcholine, used as a drug in anaesthesia [17]. As already known, quaternary ammonium compounds act as excellent nonselective antibacterial agents, due to their cationic moiety [18]. For that particular case, choline was used as disinfectant [19] and antimicrobial drug delivery agent [20], mostly for topical applications, in combination with other compounds [21]. Further investigations of compounds comprising choline and betaine that also imitate suxamethonium chloride are therefore of interest.

In this work choline chloride and betaine hydrochloride were condensed through ester bonds using carbonyldiimidazole (CDI) as activating agents. The products and intermediates were characterized with attenuated total reflectance infrared spectroscopy (ATR-IR) and nuclear magnetic resonance (NMR) before and after preparative high performance liquid chromatography (HPLC). Finally, the antimicrobial activity against *Pseudomonas Aeruginosa* and *Staphylococcus Aureus,* and the viability of L929 fibroblasts in contact with the substance was assessed.

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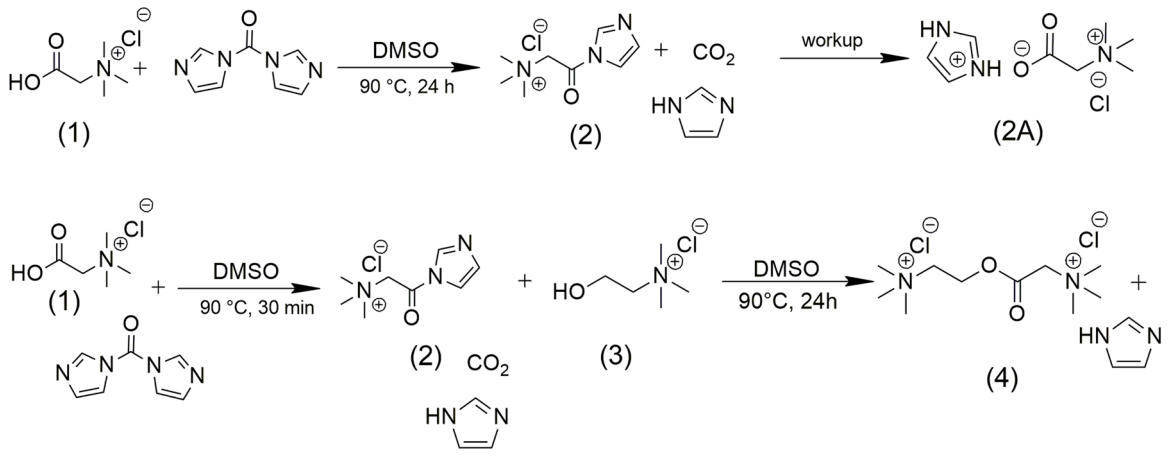


Fig. 1. Formation of compounds (2), (2A) after workup, and (4), form betaine hydrochloride (1) and choline chloride (3) through CDI coupling.

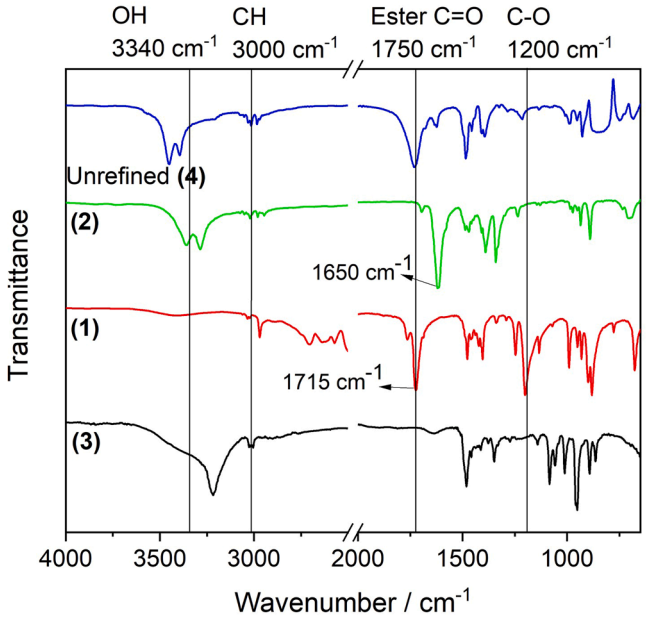


Fig. 2. ATR-IR spectra of unrefined (4) and compounds (2/2A), (1), and (3).

2. Experimental section

2.1. Materials

Betaine hydrochloride (BET HCl); betaine (BET); dimethylsulfoxide (DMSO) anhydrous; 1,1′-carbonyldiimidazole (CDI); sodium pyruvate (P2256); L-glutamine (G7513); penicillin/streptomycin (1 %, P0781); and methanol, suitable for HPLC, ≥ 99.9 % were purchased from Sigma-Aldrich/Merck (Germany). Choline chloride (ChCl) was purchased from Acros (Geel, Belgium). Acetone was purchased from Carlo Erba (Val-de-Reuil, France).

Dialysis tubes (regenerated cellulose membrane, MWCO 14 kDa) were purchased from Carl Roth (Karlsruhe, Germany). Minimum Essential Medium (MEM; 51,200–046) and Foetal bovine serum (100,500–064) were from Gibco™ (Amarillo, TX, USA). Trypsin was from Invitrogen (Waltham, MA, USA). Etoposide (ET) was from Santa Cruz Biotechnology (Dallas, TX, USA). 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide (MTT) was from Abcam (Cambridge, UK). Phosphate buffered saline (PBS) was from PAA Laboratories (Toronto, Canada). Ultra-pure water from a Millipore (MA, USA) water purification system (resistivity ≥ 18.2 MΩ cm, pH 6.8) was used for the preparation of all aqueous solutions throughout the whole work.

2.2. Synthesis of (2-(1H-imidazol-1-yl)-N,N,N-trimethyl-2-oxoethanaminium chloride) (2) and formation of imidazolium salt (2A) during workup

In a 100 mL round beaker, previously dried BET HCl (compound **(1)**, Fig. 1 (24 h, 40 °C, 100 mbar), 1 g, 6.5 mmol) was added to DMSO (40 mL, 563.16 mmol, 90 °C, 200 rpm) and left to wet for 30 min. Compound **(1)** is insoluble in DMSO. With the addition of CDI (1 g, 6.2 mmol), activation of BET HCl was conducted for 24 h at 90 °C. After 10 min of mixing, the reaction solution became clear and remained constant thereafter, indicating the formation of compound **(2,** Fig. 1**)**. The reaction solution was precipitated in 100 mL of acetone, filtered through filter paper previously soaked in acetone on a funnel, and washed with 3 × 50 mL of acetone after each filtration. Formed imidazole and CDI are soluble in acetone, whereas **(1),** likely **(2)** and **(2A)** are not. The sample was dried in a vacuum overnight (40 °C, 100 mbar). Yield: 1.3 g.

2.3. Synthesis of N,N,N-trimethyl-2-oxo-2-(2-(trimethylammonio) ethoxy)ethanaminium chloride (4)

In a 100 mL round beaker, previously dried (24 h, 40 °C, 100 mbar) betaine HCl (compound **(1)**, 1 g, 6.5 mmol) was added to DMSO (40 mL, 563.16 mmol, 90 °C, 200 rpm) and left to wet for 30 min. With the addition of CDI (1 g, 6.2 mmol), formation of **(2)** was conducted for 30 min at 90 °C (Fig. 1). Previously dried choline chloride **(3)** (1 g, 7.1 mmol)) was added to the solution and dissolved within minutes. The solution was left to react for 24 h at 90 °C, precipitated into 100 mL acetone, filtered through filter paper previously soaked with acetone on a funnel, washed with 3 × 50 mL acetone after each filtration, and the crude product dried in vacuum overnight (40 °C, 100 mbar). Yield: 1.2 g. CDI, and formed imidazole are soluble in acetone, whereas compounds **(1), (2), (3)** and **(4)** are not, requiring further preparative purification (see Section 5.2). The products are analysed by ATR-IR and NMR.

2.4. Attenuated total reflectance infrared spectroscopy (ATR-IR)

Attenuated total reflectance infrared spectroscopy (ATR-IR) spectra of all samples were measured using a PerkinElmer FTIR System Spectrum GX Series-73,565 at a scan range of 4000–650 cm^{-1} with 32 scans and 4 cm^{-1} resolution.

2.5. Nuclear magnetic resonance (NMR)

NMR measurements were carried out on Bruker BioSpin DD2 300 MHz NMR spectrometer. NMR samples were prepared by dissolving 0.1

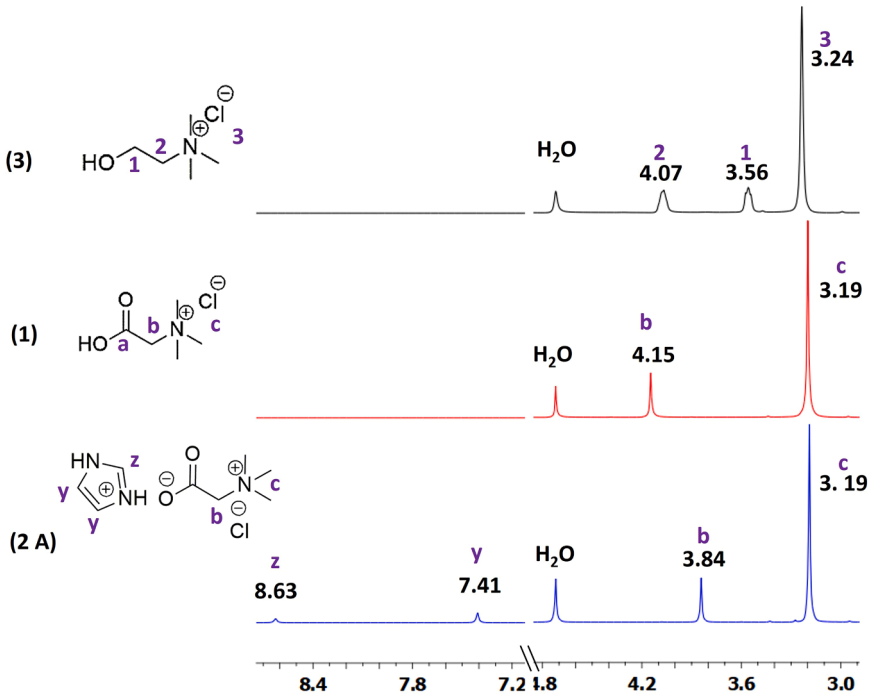


Fig. 3. ^{1}H NMR spectra of compounds (3), (1) and (2/2A) in D_2O.

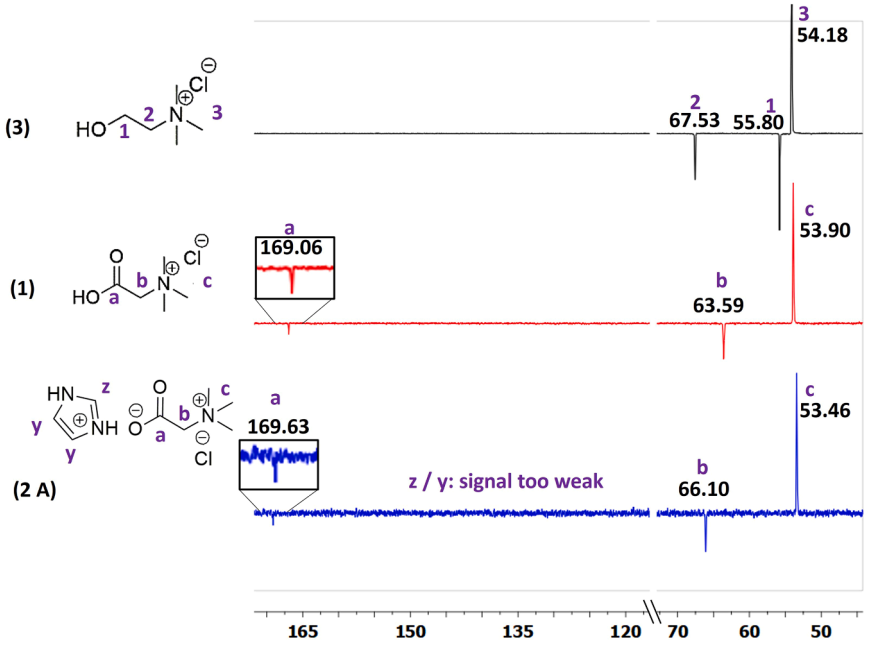


Fig. 4. ^{13}C NMR spectra of compound (3), (1) and (2/2A) in D_2O.

g of the sample in 0.6 mL D_2O or CD_3OD. Identification was performed using the characteristic NMR resonances, assigned based on their chemical shifts. 1D ^{1}H NMR spectra were recorded using zg30 pulse sequence. A relaxation delay of 1.0 s was used, and 65,536 points and 16 scans recorded. ^{13}C NMR data were acquired using jmod pulse sequence. Spectral width was set to 18,028.8 Hz. A relaxation delay of 2.0 s was used, and 65,536 points and 256 scans recorded.

2.6. High performance liquid chromatography (HPLC)

After precipitation and purification with acetone, compound **(4)** was further purified using preparative HPLC. The separation was performed on PurifFlash 5.250 (Interchim, Montluçon, France) preparative chromatograph equipped with PDA and ELSD detectors. The SiO_2 normal phase (US10Si-250/212, Interchim, Montluçon, France) chromatographic column with an inner diameter of 21.2 mm and length of 250 mm (particle size 10 mm) was used for the separation of compounds, where 80% aqueous MeOH solution was used as mobile phase (flow rate = 16 ml/min). The presence of analytes was detected using an ELSD and UV/Vis (l = 220 nm) detector. Additionally, the spectrum of eluted compounds was monitored in the wavelength range between 200 nm and 800 nm. Overlapping of the peaks were overcome with

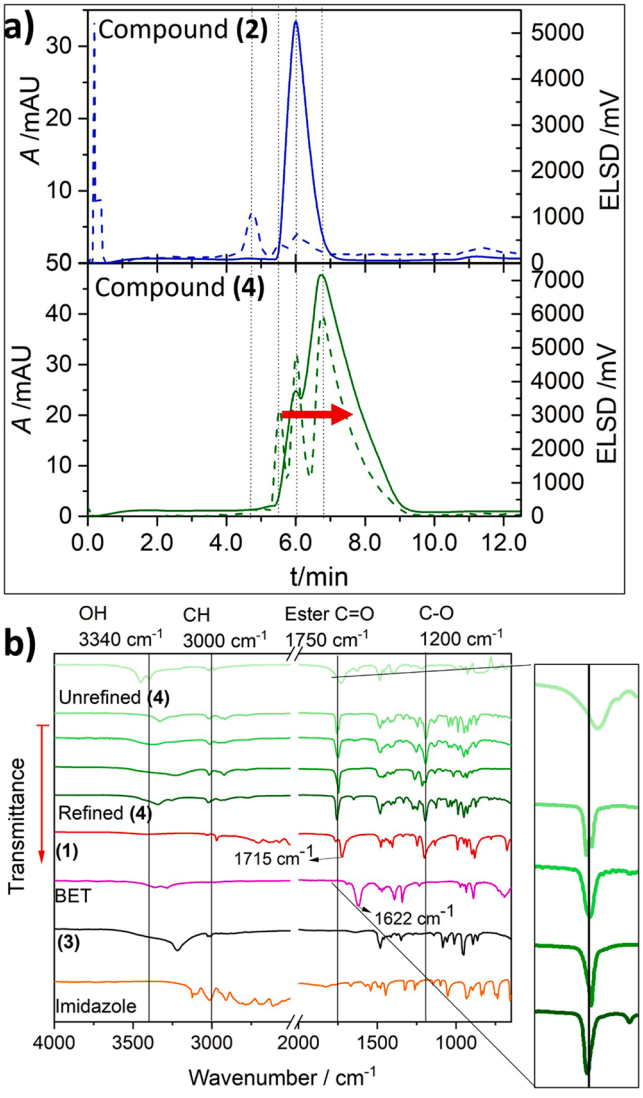


Fig. 5. a) Chromatograms, of the acetone precipitates of the reaction products. Blue curve compound (2/2A), green curve compound (4) (solid line: ELSD detector, dashed line UV/Vis detector at 220 nm). b) Arrow shown in the chromatogram depicts the order of the fractions identified by ATR-IR after removal of mobile phase through evaporation and freeze drying compared to the starting materials betaine hydrochloride, betaine chloride, choline chloride and imidazole.

repurification of fraction in question.

After repurification, fractions of **(4)** were collected, and MeOH was evaporated using a rotary evaporator (Büchi, Flawil, Switzerland). Finally, a dry product was obtained using a freeze-dryer (Mini Lyotrap, LTE Scientific, UK). Separately isolated compound **(2/2A)** (chapter 2.2.) was run over the HPLC column under the same conditions.

3. Cytotoxicity assessment with MTT assay

Biological reactivity and potential cytotoxic activity of compound **(4)** obtained from the preparative HPLC was evaluated *in vitro* in mouse fibroblasts (NCTC clone 929: CCL 1; L929; American Type Culture Collection, ATCC, Manassas, VA, USA)) and compared to compounds **(1)** and **(3)**) also evaluated in a previous study [22]. The L929 cells were seeded onto 96-well microplates (Nunc, Naperville, IL, USA) at a density of 10 000 cells/well, which corresponds to 50 000 cells/mL, and were incubated for 24 ± 1 h at 37 ± 1 °C and 5 ± 1 % CO_2 in humidified atmosphere to attach. The cytotoxicity was evaluated after 24 ± 1 h of exposure with the 3 (4,5 dimethylthiazol-2-yl)−2,5 diphenyltetrazolium bromide (MTT) assay in accordance with the International Standard ISO 10,993–5:2009, Biological evaluation of medical devices—Part 5: Tests for *in vitro* cytotoxicity, as previously described [23]. The biological reactivity was visually evaluated following exposure to the samples for 24 ± 1 and 48 ± 1 h, by light microscopy, and graded on a scale of 0 to 4 as described in ISO 10,993–5:2009. Compound **(4)** was dissolved in complete growth medium (MEM supplemented with 10 % FBS, 4 mM L-glutamine, 0.11 mg/mL sodium-pyruvate, 100 IU/mL penicillin, and 100 μg/mL streptomycin) at the concentration 20 mg/mL. Graded concentrations in the range of 0.001 to 20 mg/mL were tested, and half maximal inhibitory concentration (IC_{50}) values determined. In each experiment, a negative control (complete growth medium) and a positive control (5% DMSO) were included. Three independent experiments were performed in five replicates. The program GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA) was used for statistical analysis and calculation of IC_{50} values. ANOVA (one-way analysis of variance) and Dunnet's multiple comparison test were used to determine statistically significant differences in cell viability between the control and the samples. The IC_{50} represents the concentration of a compound required for a 50 % inhibition of growth *in vitro*. The IC50 values were determined using nonlinear regression (log(agonist) vs. normalized response—variable slope) using GraphPad Prism 9.

4. *In vitro* evaluation of antibacterial activity

For antimicrobial tests, the samples were dissolved in sterilized distilled water. From these solutions, the materials were subsequently diluted from base concentration of 400 mg/mL with two-fold dilution until 0.39 mg/mL. The final concentration in the test tube ranged from 20 mg/mL to 0.02 mg/mL. Two samples for each concentration were prepared. Antimicrobial activity was tested against *S. Aureus* (DSM 799) and *P. Aeruginosa* (DSM 1128). In the solutions, bacteria were added, and incubated for 24 h at 37 °C, where the growth of the bacteria was observed, and minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) were determined.

5. Results

5.1. ATR-IR and NMR spectroscopy of compound (2)

The ATR-IR spectra of the crude product of compound **(2)** (Fig. 2), shows a peak at 1650 cm^{-1} attributed to either the C=O stretching vibration of the *N*-acyl imidazole amide or the imidazolium betainate carboxylate salt, indicating a mixture of both compounds. The protonated betaine hydrochloride **(1)** has a C = O stretching vibration at 1715 cm^{-1} (Fig. 2). A decrease of the intensity of the O—H vibrations (3340 cm^{-1}) of **(3)**, indicates the formation of the ester bonds visible already in the raw product of **(4)**.

The NMR spectra of **(2/2A)** was compared with the base materials **(1)** and **(3)**. The ^{1}H NMR spectra of **(2A)** shows only two peaks of the aromatic imidazole (Fig. 3) confirming the presence of only the imidazolium salt of betaine and not compound **(2)**. The ^{13}C NMR (Fig. 4) spectra does not show the presence of two different imidazole carbon atoms together with the trimethyl and carbonyl groups of either **(1)** or **(2)**. This can be addressed to the fact that the imidazolium salt is not formed in stochiometric amounts and the signal to noise ratio therefore is too low for these carbon signals to be detected.

For comparison measurements in CD_3OD were performed. The NMR spectra of **(2A)** in CD_3OD, was compared to compound **(1), (3)**, and the inner salt of betaine (further referred to as compound **(5)**, Figs. S1 and S2). Peaks at 8.63 and 7.41 ppm (Fig. S1) were attributed to protons of the imidazole ring, whereas in ^{13}C NMR (Fig. S2) only one carbon could be attributed to the imidazole ring at 120.62 ppm for sensitivity reasons.

(2A): ^{1}H NMR (300 MHz, D_2O, δ in ppm TMS) δ 8.63 (s), 7.41 (s), 3.84 (s), 3.19 (s). ^{13}C NMR (75.53 MHz, D_2O, δ in ppm TMS) δ 169.63,

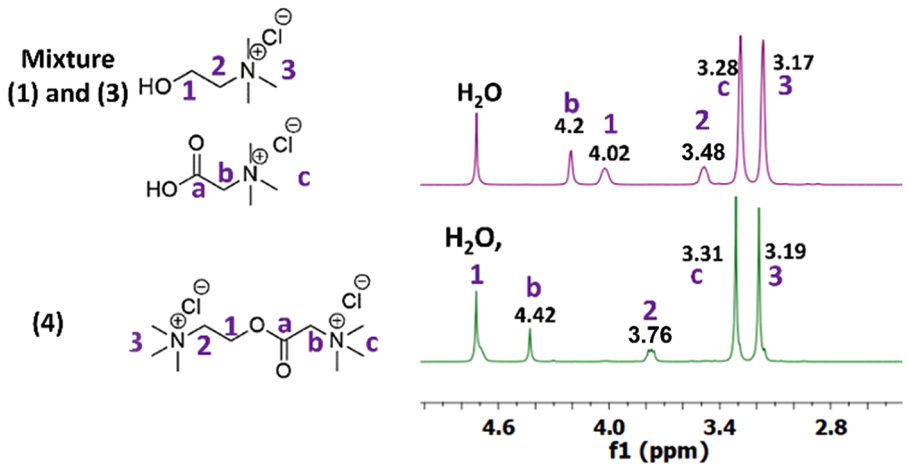


Fig. 6. ^{1}H NMR spectra of a mixture of (1) and (3) and compound (4) in D_2O.

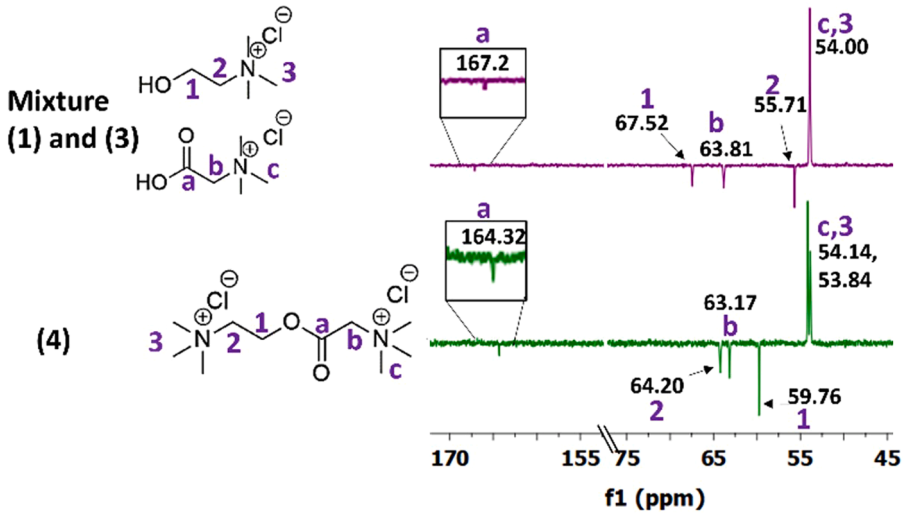


Fig. 7. ^{13}C NMR spectra of a mixture of (1) and (3), compound (4) in D_2O.

66.10, 53.46.

5.2. Preparative HPLC, ATR-IR and NMR of compound (4)

The crude product of compound **(4)** was further purified by preparative HPLC. Fig. 5a shows the chromatograms of the acetone precipitate of compound **(2/2A)** (blue curve, minor peak 4.8 min, main peak at 6.0 min, dashed line UV, solid line ELSD detector) which mainly contains **(2A)** and **(1)**. An additional peak (6.8 min) is observed in the crude product of compound **(4)** (green curve, dashed line UV, solid line ELSD detector), which likely also contains **(3).** The fractions of the 6.8 min peak were collected, methanol evaporated, and water removed by freeze-drying. The samples were characterized using ATR-IR spectroscopy (Fig. 5b). A splitting of the peak at 1750 cm^{-1} was observed, corresponding to the C = O stretching vibration of either betaine hydrochloride **(1)**, or the ester of **(4)**. At longer elution time at 7 min, only one carbonyl peak remained. Due to similar elution times of both compounds, a second chromatography run was performed. Neither free nor bound imidazole, nor betaine chloride were present in the refined product of **(4)** as confirmed by comparative ATR-IR (Fig. 5b) and NMR measurements (Figs. 6 and 7).

NMR

The spectra of the HPLC fractionated compound **(4)** shows the presence of several peaks different to a physical mixture of the reactants (Figs. 6 and 7). The protons of the ammonium groups can be attributed to peaks from 3.15 – 3.39 ppm (Fig. 6) and do not experience a large chemical shift. The downfield chemical shifts of the methylene groups of betaine (from 4.20 to 4.42 ppm and 63.81 ppm to 64.20 ppm), and of choline (from 3.48 to 4.71 ppm and 55.75 to 63.17 ppm) are due to the electron withdrawing character in the newly formed ester compound (Figs. 6 and 7).

Also, shifts in the carbonyl carbon signals are observed for the ester bonds from 167.2 ppm to 164.3 ppm (Fig. 7). The carbon signals (54 ppm) of the methyl groups do not show large differences in chemical shifts.

A lack of observed DMSO peaks [24] in the ^{1}H and ^{13}C NMR spectra confirmed a sufficient removal of the solvent.

Mixture of (1) and (3): ^{1}H NMR (300 MHz, D_2O, δ in ppm TMS) δ 4.2 (s), 4.02 (m), 3.48 (t), 3.28 (s), 3.17 (s). ^{13}C NMR (75.53 MHz, D_2O, δ in ppm TMS) δ 167.21, 67.52, 63.81, 55.71, 54.00. **(4)**: ^{1}H NMR (300 MHz, D_2O, δ in ppm TMS) δ 4.71 (m), 4.42 (s), 3.48 (t), 3.31 (s), 3.19 (s). ^{13}C NMR (75.53 MHz, D_2O, δ in ppm TMS) δ 164.32, 64.20, 63.17, 59.76, 54.14, 53.84.

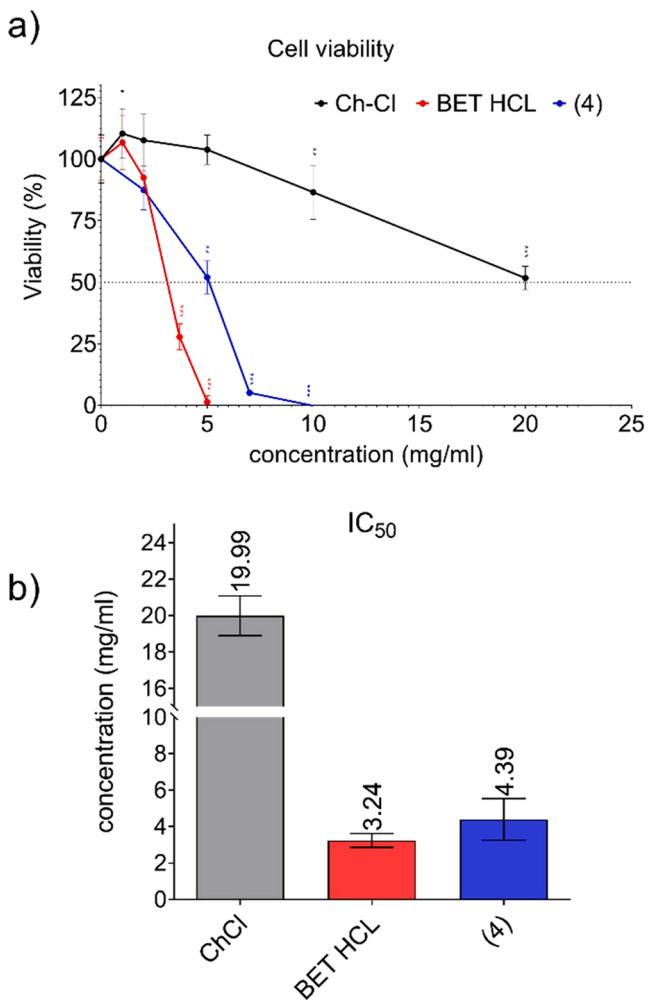


Fig. 8. Cytotoxicity (a) and LD50 (b) compound (4) after 24 h of exposure in the L929 mouse fibroblast cell line in comparison to samples ChCl (3) and BET HCl (1) (determined in a previous study ref. [22]). The negative control (cell growth medium) and the positive control (5 % DMSO) performed as anticipated (data not shown). The asterisks denote statistically significant difference to the negative control (ANOVA and Dunnet's multiple comparison test; $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$).

5.3. Biological activity of N,N,N-trimethyl-2-oxo-2-(2-(trimethylammonio)ethoxy)ethanaminium chloride, (compound (4))

5.3.1. Cytotoxicity and biological reactivity

The cytotoxicity of compound **(4)** was evaluated according to ISO 10,993–5 using the MTT assay in the L929 mouse fibroblast cell line (Fig. 8). The cytotoxicity of compound **(4)** (IC_{50} value: 4.39 ± 1.14 mg/ml), was similar to BET HCl **(1)** (IC_{50} value: 3.24 ± 0.37 mg/ml), but higher than for ChCl **(3)** from a previous study [22] (IC_{50} value: 19.99 ± 1.09 mg/ml). The biological reactivity (Fig. 9) evaluated under the microscope after 24 and 48 h of exposure reflected the cytotoxicity results. Compound **(4)** showed no reactivity (Grade 0) after 24 h of exposure at concentrations up to 2 mg/ml. After 48 h a slight reactivity was observed in cells exposed to 2 mg/ml of compound **(4)**. At higher concentrations (10 and 20 mg/ml), a severe reactivity (Grade 4) was observed at both test time points. The biological reactivity of compound **(4)** was similar to the one observed for BET HCl **(1)** but significantly higher than that of sample ChCl **(3)** [22].

According to Debbasch et al. [25], quaternary ammonium compounds have the tendency to cause oxidative stress and apoptosis at low concentration. However, further investigations need to be conducted to elucidate the mechanism leading to these lower IC_{50} values.

Previously reported literature claims that MIC increases with longer alkyl chains between two cationic moieties [26] whereas longer alkyl chains on the nitrogen increase efficacy [27]. Both factors need to be studied further with different human cell lines and bacteria.

5.3.2. Antimicrobial activity

No significant antimicrobial activity was observed for ChCl **(3)**and **(4)**, whereas BET HCl **(1)** showed slight antimicrobial properties for the two strains tested (Table 1, MIC BET HCl: *S. Aureus* 5 mg/mL, *P. Aeruginosa* 2.5 mg/mL) which might be caused by the acidic nature of BET HCl **(1)**. To obtain more detailed information regarding the concentration which can be used to achieve antimicrobial properties, MBC was determined, which for BET HCl **(1)** against *S. Aureus* is at 20 mg/mL, and against *P. Aeruginosa* is at 10 mg/mL.

According to previous literature, usually Gram-positive bacteria are more susceptible towards cationic moieties leading to lower concentrations necessary for bacteria inhibition [28], whereas for BET HCl **(1)**, that is obviously not the case. ChCl **(3)** does not show any antimicrobial activity against both bacteria, while compound **(4)** showed slight antimicrobial activity at 20 mg/mL against *P. Aeruginosa*.

6. Conclusion

The synthesis of *N,N,N*-trimethyl-2-oxo-2-(2-(trimethylammonio) ethoxy)ethanaminium chloride (compound **(4)**) was possible using carbonyl diimidazole (CDI) as a coupling agent. The imidazolium salt of betaine, (compound **2A**) was formed upon workup, and it is proposed that the activated form of betaine (compound **2**) is very short lived and unstable. Preparative HPLC was used to separate the crude reaction product and yielded a condensed choline betainate ester as confirmed by IR and NMR measurements. Although containing quaternary ammonium ions, the ester showed no significant antimicrobial activity against *S. Aureus* or *P. Aeruginosa,* but certain cytotoxic properties against mouse fibroblasts. The synthesized compound could be further investigated for properties similar to Suxamethonium chloride in follow up studies, including a detailed study on the mechanism of toxicity and the stability of the ester bond in aqueous environments at different pH values. More work could be performed by extending the alkyl chains and spacers, and by modifying the scaffold carrying multiple positive charges.

CRediT authorship contribution statement

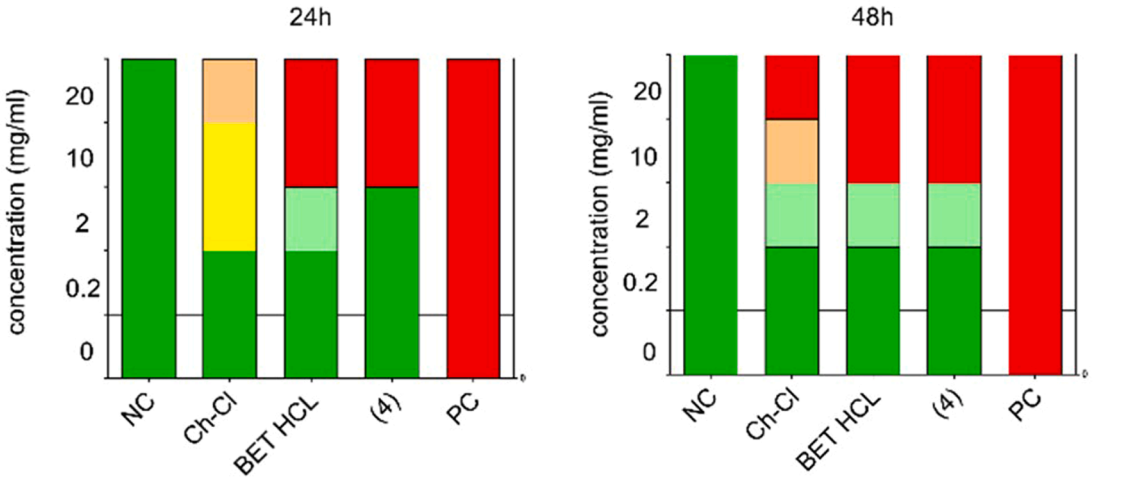
Lucija Jurko: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Gregor Hostnik:** Writing – review & editing, Writing – original draft, Investigation, Data curation. **Tobias Steindorfer:** Investigation, Data curation. **Alja Štern:** Writing – review & editing, Writing – original draft, Investigation, Data curation. **Perica Bošković:** Writing – review & editing, Investigation, Conceptualization. **Matej Bračič:** Data curation, Writing – review & editing. **Bojana Žegura:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Rupert Kargl:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.



Grade Reactivity Conditions in accordance with ISO 10993-5		
Grade 0	No reactivity	Discrete intracytoplasmatic granules, no cell lysis, no reduction of cell growth.
Grade 1	Slight reactivity	Not more than 20 % of the cells are round, loosely attached and without intracytoplasmatic granules, or show changes in morphology; occasional lysed cells are present; only slight growth inhibition observable.
Grade 2	Mild reactivity	Not more than 50 % of the cells are round, devoid of intracytoplasmatic granules, no extensive cell lysis; not more than 50 % growth inhibition observable.
Grade 3	Moderate reactivity	Not more than 70 % of the cell layers contain rounded cells or are lysed; cell layers not completly destroyed, but more than 50 % growth inhibition observable.
Grade 4	Severe reactivity	Nearly complete or complete destruction of the cell layers.

Fig. 9. Biological reactivity of compound (4) after 24 and 48 h of exposure in the L929 mouse fibroblast cell line in comparison to samples ChCl (3) and BET HCl (1) (determined in a previous study ref. [22]). Morphological changes were evaluated and graded according to ISO 10,993–5. NC is the negative control (cell growth medium), PC is the positive control (5 % DMSO). The controls were tested at only one concentration, and a uniform bar is shown for visualization.

Table 1
Antimicrobial activity of BET HCl, ChCl and compound (4).

	S. Aureus		*P. Aeruginosa*	
	MIC / mg mL^{-1}	MBC / mg mL^{-1}	MIC / mg mL^{-1}	MBC / mg mL^{-1}
BET HCl	5	20	2.5	10
ChCl	>20	/	>20	/
COMPOUND (4)	>20	/	20	>20

Acknowledgements

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.molstruc.2024.138581.

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Further reading

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