The impact of counterions in biological activity: case study of antibacterial alkylguanidino ureas

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Abstract

Trifluoroacetic acid (TFA), due to its strong acidity and low boiling point, is extensively used in protecting groups-based synthetic strategies. Indeed, synthetic compounds bearing basic functions, such as amines or guanidines (commonly found in peptido or peptidomimetic derivatives), developed in the frame of drug discovery programmes, are often isolated as trifluoroacetate (TF-Acetate) salts and their biological activity is assessed as such in vitro, ex vivo, or in vivo experiments. However, the presence of residual amounts of TFA was reported to potentially affect the accuracy and reproducibility of a broad range of cellular assays (e.g., antimicrobial susceptibility testing, and cytotoxicity assays) limiting the further development of these derivatives. Furthermore, the impact of the counterion on biological activity, including TF-Acetate, is still controversial. Herein, we present a focused case study aiming to evaluate the activity of an antibacterial AlkylGuanidino Urea (AGU) compound obtained as TF-Acetate (1a) and hydrochloride (1b) salt forms to highlight the role of counterions in affecting the biological activity. We also prepared and tested the corresponding free base (1c). The exchange of the counterions applied to polyguanidino compounds represents an unexplored and challenging field, which required significant efforts for the successful optimization of reliable methods of preparation, also reported in this work. In the end, the biological evaluation revealed a quite similar biological profile for the salt derivatives 1a and 1b and a lower potency was found for the free base 1c.
Introduction

Nowadays, a large number of hospital-acquired infections are caused by antibiotic-resistant opportunistic pathogens. Furthermore, several clinically relevant organisms are rapidly evolving towards multidrug and even pan-drug-resistant phenotypes, whose global spread represents a significant Public Health issue. Whereas tackling the problem of antimicrobial resistance (AMR) relies on the implementation of several approaches (e.g., antibiotic stewardship), the discovery or development of new antibiotics, especially those active on carbapenem-resistant Enterobacteriaceae, Pseudomonas spp., and Acinetobacter spp., remains a critical priority [1].

The current COVID-19 pandemic increased awareness of the fragility of health systems worldwide [2, 3] and further highlighted the urgent need for novel pharmacologic treatments to tackle drug-resistant pathogens. Indeed, although the actual impact of the SARS-CoV-2 pandemic on AMR is still debated [4], the incidence of bacterial coinfections was recently reported to be as high as 30% [5]. Furthermore, as antibiotic prophylaxis is also part of the COVID-19 management [6], the related overuse of such valuable therapeutics [4] could have worsened this already dramatic scenario.

In the last years, we have been involved in the development of AlkylGuanidino Ureas (AGUs) endowed with remarkable antibacterial properties [7–9]. Among them,
compound 1a (Fig. 1) showed a broad-spectrum bactericidal activity, including against antibiotic-resistant Gram-negative species (Minimal Inhibitory Concentration (MIC) values as low as 1 µg/mL); in-depth biological characterization and in vitro preliminary Absorption, Distribution, Metabolism, and Excretion (ADME) profile were also recently reported [9, 10].

However, compound 1a and the whole AGUs library were obtained and tested as TF-Acetate salts since the final reaction step involved the treatment of the intermediates like 2 (Fig. 1) with TFA. Briefly, all the reported synthetic routes were based on the tert-butoxycarbonyl (Boc)-protecting group strategy to soften the high polarity of free guanidino functions and to enhance their synthetic accessibility and handling. Thus, after the Boc-cleavage in TFA solution, the final compounds are endowed with the guanidino moieties positively charged and TF-Acetate counterions.

Although TFA is widely employed in protecting groups-based strategies and peptide synthesis, especially in solid-supported techniques [11] in relation to its strong acidity ($pK_a = 0.52$) and low boiling point, the presence of residual amounts (up to $10^{-7}$ M) of TFA or TF-Acetate counteranion in the final samples subjected to biological assays represents a thoroughly debated issue for the scientific community [12]. In fact, it was proved to affect the accuracy and reproducibility of cellular assays by inhibiting cell proliferation [12] or favoring the viability of several cell types unspecifically [12–14], limiting further in vivo experiments [15] and potential medical uses. This is especially true for synthetic antimicrobial peptides (AMPs) or compounds bearing positively charged residues or moieties and strong bases. The TF-Acetate content should thus be appropriately considered, even if studies on the potential effects of counterions on cell-based assays are sparse [16] and a straightforward correlation between counterion type and in vitro biological activity still has to be established [17]. In general, no significant differences emerged when testing a compound as a free base or as salts from different organic or inorganic acids [18, 19]. However, especially in the case of antimicrobial compounds, a modulation of their activity was noticed [20–22], resulting in MIC values differing by at 2 log2 dilutions (fourfold). This could be in part explained by the different molecular weights of the test compounds, since MIC values are commonly reported in µg/mL (or mg/L), rather than being a direct influence on antimicrobial action [20], but it is unclear whether the presence of counterions could further impact on the antibacterial activity. Furthermore, contaminant TFA or TF-Acetate counterion is highly detrimental to biological material also in preclinical and in vivo Pharmacokinetics (PK) studies [17]. In fact, in vivo TFA is reported to trifluoroacytating proteins, causing hepatitis [23], and breaking down the intermolecular structure of water, while TF-Acetate anions can interfere with or disrupt membrane function, enzymatic catalysis, secondary structures of proteins, and protein stability [24] and lead to immune response-inductions [25–27]. Also, compounds as TF-Acetate salts were reported to exert a low pharmacological efficacy compared to the same compounds with other counterions [28]. Hence, each case should be considered individually and more than one counterion should be investigated to achieve the optimal biological profile [17].

Hence, starting from a TF-Acetate AGU (1a), we synthesized the corresponding compound with a different counterion as a hydrochloride salt (1b) or as a free base (1c) (Fig. 1), in order to compare their antibacterial activity and to gain insights on the influence of counterions in such preparations.

Results and discussion

Although several protocols avoiding the use of TFA were reported [29–32], such synthetic approaches were barely used for compounds with several guanidino functions on an aliphatic skeleton. As an example, preparation of the antimicrobial polyhexamethylene guanidine (PHMG) polymer involved the use of non-protected guanylating agents, yielding the guanidinium hydrochloride without additional

![Fig. 1](image-url)
reactions involving strong acids [33]. It would not be unlikely that the presence of several guanidines and aliphatic chains could hamper the reaction completion or promote the degradation of the compound.

Surprisingly, the use of HCl on the Boc-intermediate 2 (Scheme 1) was found unsuccessful to yield the desired product, although HCl-based protocols are generally widely used in Boc cleavage due to their faster kinetics compared to that using TFA. It is common knowledge that, when the milder TFA is employed, higher reaction concentrations and amounts of acid are required to achieve reasonable reaction times [34]. However, the synthesis of 1b was very challenging and passed across TF-Acetate derivative 1a (ii, Scheme 1), as shown in Scheme 1.

Different synthetic strategies were explored and some attempts are reported in Table 1.

The first strategies to obtain 1b were based on the Boc-cleavage of intermediate 2 through the direct employment of hydrochloric acid in 1,4-dioxane saturated solution [35] (Entry 1, Table 1) or in situ-generated from acetyl chloride in methanol [36] (Entry 2). However, chromatographic purification in alumina or reverse-phase silica failed to provide an acceptable purity of the sample for biological evaluation (> 95%), as determined by HPLC–MS analysis. Isolation of such compound, using semi-preparative HPLC, could not be investigated further due to the low intensity of UV absorbance and an unsatisfactory resolution of compounds 1a and 1b when neither acids nor bases were added to the mobile phase in analytical HPLC.

Then, a different strategy starting from the synthesis of the TF-Acetate salt 1a [10, 37] (ii, Table 1) and the counterion exchange with the chloride anion was implemented. To confirm the completion of the anion exchange, we resorted to the detection of the presence/disappearance of the TF-Acetate in the samples by 19F NMR since its fluorine atoms show a characteristic singlet signal at 77 ppm in CD3OD [17, 38]. In fact, in our case, the traditionally employed IR spectroscopy technique is not effective in monitoring the anion exchange since the absorption bands of urea and guanidines belonging to AGUs overlap to that of TF-Acetate, complicating the interpretation of the resulting spectra [15].

We also excluded RP-HPLC technique since it shows limited efficacy in anion exchange [15, 17] and not possible in our case. In fact, the optimized analytical protocol set up for the characterization of AGU compounds relied on the use of ammonium acetate as a mobile phase additive, while the use of acids resulted in very broad and low-resolved signals in the UV-chromatogram. Thus, several in-batch synthetic approaches were tried (Entries 3–7, Table 1).

Initially, 1a was treated with HCl 5 N in methanol [39] (Entry 3) but no conversion was observed. Then we tried a protonation/reprotonation approach consisting of the in situ flask, r.t., 5 h [10]; iii. Amberlite IRA400 chloride form, CH3OH, r.t., 72 h; vi. NaOEt in situ dry EtOH, N2, 0 °C-r.t., 30 min. Unsuccessful reactions are reported in Tables 1, S1, and S2.

Scheme 1 Synthetic routes explored to obtain 1b and 1c. Successful (iii, vi) and failed (i, iv, v) reaction steps. In green and blue are presented the approaches for 1b and 1c, respectively. Reagents and reaction conditions: ii. freshly dist. TFA (20% v/v), dry CH2Cl2, sealed
generation of the free base guanidino intermediate 1c, followed by the addition of concentrated HCl (37% w/w). Thus, we stirred 1a with different organic bases, such as triethylamine (TEA) (Entry 4) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (Entry 5). On one hand, the low basicity of TEA ($pK_b = 3.2$) was not sufficient to deprotonate guanidinium moieties, as confirmed by $^{19}$F NMR spectra, while treatment with DBU, which possesses a lower $pK_b$ (1.1), caused the compound degradation. In Entry 6, an attempt with a caustic alkali in an aqueous medium [40] is reported but, also in this case, the isolation of 1b failed.

Hence, in the end, we resorted to a resin-based procedure (iii., Table 1), which resulted to be successful in yielding the pure chloride salt 1b. Briefly, we converted TF-Acetate 1a into 1b through a long-period stirring in presence of Amberlite IRA 400 chloride form resin [41–43]. The completion of conversion was checked by $^{19}$F NMR spectroscopy. $^1$H and $^{13}$C NMR spectra of 1a and 1b are reported in Fig. 2 and in the Supplementary Information.

The expected small changes in chemical shifts were observed (A, Fig. 2), confirming the absence of fluorine atom after anion exchange (B).

The stability issue of the free base compounds remains unsolved, due to their high hygroscopicity and tendency to capture CO$_2$ after air exposure to form carbonate salts in unsolved, due to their high hygroscopicity and tendency to absorb moisture. However, as expected, great synthetic difficulties were encountered and several approaches were tried by exploring both in batch, microwave (MW), and salt-exchange procedures, starting from salts 1a and 1b or Boc-intermediate 2, as shown in Scheme 1. Attempts involving 2 were unsuccessful, probably due to the high complexity of polyguanidines cleavage and are described in Table S1 in the Supplementary Information. To the best of our knowledge, the acid and base-free cleavage of highly guanylated substrates is sparsely reported in literature and during these experiments, degradation phenomena or by-products formation often occurred. Further experiments were performed aimed at obtaining 1c from the TF-Acetate 1a and the reaction conditions are reported in Table S2 in the Supplementary Information. Unfortunately, no product was obtained since the TF-Acetate counterion strongly interacts with the positively charged guanidines, resulting in a challenging displacement [15] that requires first a replacement with a stronger acid-derived counterion, such as the chloride ion from hydrochloric acid ($pK_a = -5.9$) [46].

Hence, we treated the hydrochloride derivative 1b with sodium ethoxide in absolute ethanol [47], based on the poor solubility of the resulting sodium chloride in the reaction solvent [48] and the possibility to be easily filtered off. To assess the total absence of hydrochloride counterion, a precipitation assay-based analysis with silver nitrate was performed. The pictures of an ethanolic solution of 1b and 1c after filtration are reported in Figure S1 in Supplementary Information.

Finally, derivatives 1b and 1c were tested against a panel of representative bacterial species and data are shown in Table 2.

Table 1: Attempts to synthesize compound 1b

| Entry | Reagents and Conditions | Results |
|-------|-------------------------|---------|
| i 1   | HCl 4 N in dry 1,4-dioxane, 2.3 µM, 0 °C, 8 h | Compound degradation |
| 2     | Freshly dist. acetyl chloride (20% v/v), dry CH$_3$OH 5 µM, sealed flask, r.t., 24 h | Compound not isolated |
| iii 3 | HCl 5 N, CH$_3$OH 5 µM, r.t., 16 h | TF-Acetate still present |
| 4     | 1. TEA (up to 40 eq.), CH$_2$Cl$_2$ 20 µM, r.t., 16 h; 2. HCl 2 N, CH$_3$OH 10 µM, r.t., 48 h | Compound degradation |
| 5     | 1. DBU (up to 40 eq.), CH$_3$OH 20 µM, r.t., 16 h; 2. HCl 2 N, CH$_3$OH 10 µM, r.t., 48 h | Compound degradation |
| 6     | 1. NaOH 2.5 N, CH$_3$OH 10 µM, r.t., 48 h; 2. HCl 2 N, CH$_3$OH 10 µM, r.t., 48 h | TF-Acetate still present |
| 7     | Amberlite IRA 400 chloride form, CH$_3$OH 6 µM, r.t., 72 h | Reaction occurred and product isolation |

Reagents, reaction conditions, and main results are reported for each reaction.
relevant parameter to understand the potency of an antibiotic is the ratio between MIC value and blood concentration which is conventionally expressed as weight/volume. Also, microbiologists usually resort to the latter system although molar concentrations can also be used when the tested compounds are pure and fully characterized antimicrobials.

Fig. 2  Comparison of $^1$H and $^{19}$F NMR spectra of AGUs 1a–c. A $^1$H NMR spectra of 1a (purple) and 1b (green) were recorded at Bruker 400 MHz. $^1$H NMR spectrum of 1c (blue) was recorded at Bruker 600 MHz. B $^{19}$F NMR spectra of 1a (purple) and 1b (green) were recorded at Bruker 600 MHz. All the experiments were performed in CD$_3$OD. Spectra were visualized and analyzed through MestreNova 14.2 [44].
When the MIC values were converted from μg/mL to μM, no significant differences were observed between the hydrochloride AGU 1b and the free base 1c, whose molecular weight was approximately 24 and 35% lower than that of the corresponding TF-Acetate 1a, resulting in a negligible difference. However, despite compounds 1a and 1b showing similar antibacterial activities, the free base AGU (1c) showed a low potency on Gram-negative species, both considering data in weight/volume or molar systems, although a good profile is still retained on Gram-positive strains. This indicates that the counterions may affect the intrinsic antibacterial activity of AGU derivatives. The most significant drop in activity was observed on Gram-negative pathogens, particularly towards P. aeruginosa, pointing out that the presence of the counterion may somehow contribute to enhancing the antibacterial activity of these compounds (e.g. by altering the physicochemical conditions of the medium or modifying the permeability of biological membranes).

Compounds 1b and 1c were also tested on resistant clinical isolates and the panel was also extended for the reference compound 1a. HCl salt form 1b exhibited an enhanced antibacterial profile on C. meningosepticum, A. hydrophila, B. cepacia, and S. maltophilia. However, a detrimental decrease of the antibacterial properties was observed for 1c. Among the different bacterial resistance mechanisms, the changes in the lipid composition and distribution in the membranes are very common and, along with the counterion effect, may have led to activity fluctuations depending on the strain and the counterion type or presence.

Minimal bactericidal concentrations (MBC) values were also measured on a panel of multi-drug resistant bacterial strains, as shown in Table 3. Data outlined a good bactericidal profile, as the obtained MBC values are almost identical to those of MIC, as already observed for different AGUs derivatives [10]. Moreover, the counterion does not seem to affect the bactericidal behavior of compounds 1a-c proving that the salt form does not have an impact on the effect of the compounds on the bacterial cultures.

**Conclusion**

We prepared the hydrochloride 1b and the free base 1c forms of a previously characterized AGU compound 1a, showing potent and broad-spectrum antibacterial activity, including on antibiotic-resistant clinical isolates. Compounds 1b and 1c were obtained using anion-exchange and in batch protocols, respectively. Several synthetic strategies, which were

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### Table 2 Antibacterial activity of compounds 1a–c against representative Gram-positive and Gram-negative bacterial species, including multi-drug resistant clinical isolates

| Bacterial strain | MIC (µg/mL) | MIC (µM) |
|------------------|-------------|----------|
|                  COL | VAN | DAP | 1a | 1b | 1c | 1a | 1b | 1c |
| B. subtilis ATCC 6633 | 0.5 | 1 | 2c | 1 | 8 | 1.54 | 1.18 | 9.46 |
| E. faecalis ATCC 19,433 | – | 1 | 1 | 2c | 2 | 8 | 1.54 | 2.02 | 9.46 |
| S. aureus ATCC 25,923 | – | 0.5 | 0.125 | 2c | 2 | 8 | 1.54 | 2.02 | 9.46 |
| S. epidermidis ATCC 14,990 | – | 0.5 | 0.125 | 1c | 1 | 4 | 0.77 | 1.01 | 4.73 |
| S. pyogenes ATCC 12,344 | – | 0.5 | 0.125 | 1c | 0.5 | 4 | 0.77 | 0.50 | 4.73 |
| E. coli CCUG7 | 0.5 | – | – | 2c | 2 | 16 | 1.54 | 2.02 | 18.92 |
| K. pneumoniae ATCC 13,833 | 0.5 | – | – | 2c | 2 | 16 | 1.54 | 2.02 | 18.92 |
| A. baumannii ATCC 17,978 | 1 | – | – | 8c | 8 | 32 | 6.15 | 8.07 | 37.85 |
| P. aeruginosa ATCC 27,853 | 0.5 | – | – | 8c | 8 | 64 | 6.15 | 8.07 | 75.71 |
| C. meningosepticum CCUG 4310 | > 256 | – | – | 64 | 16 | 128 | 49.18 | 16.14 | 151.42 |
| A. hydrophila ATCC 7966 | 0.25 | – | – | 64 | 16 | 16 | 49.18 | 10.18 | 18.93 |
| A. faecalis FL 424/98 | 0.5 | – | – | 4 | 4 | 128 | 3.07 | 4.04 | 151.42 |
| E. cloacae VA-417/02 | 0.25 | – | – | 1c | 2 | 128 | 0.77 | 2.02 | 151.42 |
| K. pneumoniae 081R | 64 | – | – | 2c | 2 | 128 | 1.54 | 2.02 | 151.42 |
| A. baumannii AC-54/97 | 0.25 | – | – | 2c | 4 | 32 | 1.54 | 4.04 | 37.86 |
| A. baumannii N50 | 32 | – | – | 16 | 4 | 64 | 12.29 | 4.04 | 75.71 |
| B. cepacia S1-R2 | 0.5 | – | – | 16c | 2 | 32 | 12.29 | 2.02 | 37.86 |
| S. maltophilia 634/08 | 0.25 | – | – | 16c | 2 | 32 | 12.29 | 2.02 | 37.86 |

MIC values, expressed in both µg/mL and µM, are the average values from experiments performed at least in triplicate. Colistin (COL), Vancomycin (VAN), and Daptomycin (DAP) were used as control antibiotics.

b: not determined
c: Data from references 7–10

MICs conversion in molarity (µM) was calculated through the formula: 

\[
\text{MIC (µM)} = \text{MIC (µg/mL)} \times 1000
\]

considering

1301.42, 991.16, and 845.33 g/mol as the molecular weight for 1a, 1b, and 1c, respectively.
reported in literature to be successful for monoguanidino compounds, were attempted. However, these approaches were found unable to produce the desired compounds, being less efficient when applied to the preparation of polyguanidino compounds.

Quite strikingly, the antibacterial activity, determined using a panel of relevant bacterial species, of TF-Acetate (1a) and HCl (1b) salts were comparable, besides an improvement of activity highlighted by 1b especially towards resistant clinical isolates. However, the free base 1c retained the activity on a few Gram-positive bacterial species, exhibiting a somewhat decreased potency on Gram-negative strains and clinical isolates with resistant phenotypes. While it remains unclear whether this could be more relevant when testing polycationic substances, these data highlight the need to carefully consider the potential impact of counterions when testing antimicrobial compounds, as their presence may modulate the intrinsic activity of such compounds.

### Material and methods

#### Chemistry

All commercially available chemicals and solvents were used as purchased. Anhydrous reactions were performed into flame-dried glassware after three cycles of vacuum/dry nitrogen and were run under a positive pressure of dry nitrogen. Anhydrous solvents were prepared prior to use: CH₂Cl₂ was dried over calcium hydride and alcohols over I₂/Mg. TLCs were visualized under UV light and stained with ninhydrin or basic permanganate stains. ¹H NMR spectra were recorded on Bruker Avance 400 or 600 spectrometers at 400 or 600 MHz, while ¹³C NMR spectra on Bruker Avance 400 at 100 MHz. ¹H NMR spectra were reported in parts per million (δ scale) and internally referenced to CD₂OD signal at δ 3.31 ppm. Chemical shifts for carbon are reported in parts per million (δ scale) and referenced to the carbon resonances of CD₃OD at δ 49.00 ppm. Data are shown as following: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, qi = quintet, m = multiplet and/or multiplet resonances, br = broad signal), integration and coupling constants (J) in Hertz (Hz). ¹⁹F NMR spectra were recorded on a Bruker 600 spectrometer at 564 MHz. Mass spectra (LCMS) were acquired using an Agilent 1100 LC-MSD VL system (G1946C) by direct injection with a 0.4 mL/min flow rate using a binary solvent system of 95/5 CH₃OH/H₂O. UV detection was monitored at 221 or 254 nm. Mass spectra were acquired in positive or negative mode scanning over the mass range 100–1500 m/z, using a variable fragmentor voltage of 0–70 V.

The purity of final products was assessed by HPLC–UV-MS analysis as reported [10].

| Bacterial strain        | MBC (µg/mL) | MBC (µM) |
|-------------------------|-------------|----------|
|                         | 1a  | 1b  | 1c  | 1a  | 1b  | 1c  |
| *C. meningosepticum* CCUG 4310 | 64  | 32  | 128 | 49.18 | 32.28 | 151.42 |
| *A. hydrophila* ATCC 7966 | 64  | 2   | 16  | 49.18 | 2.02  | 18.93  |
| *A. faecalis* FL 424/98  | 4   | 4   | 128 | 3.07  | 4.04  | 151.42 |
| *E. cloacae* VA-417/02   | 1   | 2   | 128 | 0.77  | 2.02  | 151.42 |
| *K. pneumoniae* 081R     | 2   | 2   | 128 | 1.54  | 2.02  | 151.42 |
| *A. baumannii* AC-54/97  | 2   | 4   | 64  | 1.54  | 4.04  | 75.71  |
| *A. baumannii* N50       | 16  | 4   | 64  | 12.29 | 4.04  | 75.71  |
| *B. cepacia* SI-R2       | 16  | 2   | 32  | 12.29 | 2.02  | 37.86  |
| *S. maltophilia* 634/08  | 32  | 2   | 32  | 24.58 | 2.02  | 37.86  |

MBC values, expressed in both µg/mL and µM, are the average values from experiments performed at least in triplicate. the unit system conversion was calculated through the formula: \( \frac{\text{MIC (µg/mL)}}{m \times \text{w}} \times 1000 \), considering 1301.4, 991.16, and 845.33 g/mol as the molecular weight for 1a, 1b, and 1c, respectively.
1,3-Bis(8-carbamimidamidooctyl)-1,3-bis(8-[N'-((cyclopropylmethyl)carbamimidamido)octyl])urea Free Base (1c). To Boc-protected 2 (28.0 mg, 0.017 mmol), a solution of freshly distilled acetyl chloride (800 µL) in dry CH₂OH (7.2 mL) was added dropwise at 0 °C in an ice bath, under N₂. The reaction mixture was stirred at rt for 30 min. The completion of the reaction was assessed by LCMS analysis. Then, toluene and hexane were added and the mixture was evaporated. The crude was washed with hexane and CH₂Cl₂ several times. Then, it was treated with a freshly prepared ethanolic solution of sodium ethoxide: sodium ethoxide was prepared in situ by adding sodium (3.2 mg, 0.14 mmol) to dry absolute ethanol (4.25 mL) at 0 °C. The reaction mixture was stirred at room temperature for 1 h till precipitation of a white powder (NaCl). Then, the mixture was diluted with dry ethanol (5 × 10.0 mL) and filtered again with a 0.45 µm PTFE filter. After evaporation of residual NaCl. In presence of chloride ions, silver nitrate is formed as a white precipitate. No evidence of precipitation was observed. Yield: 80%. ¹H NMR (CD3OD, 600 MHz) δ (ppm): 3.24–3.15 (m, 16H), 3.09 (d, J = 7.0 Hz, 4H), 1.63–1.59 (m, 16H), 1.56–1.52 (m, 8H), 1.39–1.37 (m, 24H), 1.31 (m, 8H), 1.11–1.08 (m, 1H), 0.62–0.60 (m, 4H), 0.31 (d, J = 6.0 Hz, 4H). ¹³C NMR and LCMS(ES +) spectra are consistent with those of 1b.

In vitro antibacterial activity testing

Bacterial strains, including representatives of both Gram-positive and Gram-negative bacterial species, were obtained from the ATCC or CCUG culture collections (except clinical isolates, which were already present in our collection). Compounds were resuspended in dimethylsulfoxide (DMSO) at a final concentration of 50 or 100 mg/mL and subsequently diluted in the culture medium. MIC and MBC values of the compounds were determined using the micro-dilution broth method using Mueller–Hinton broth as recommended by CLSI. Bacterial inoculum was 5 × 10⁴ CFU/well. MICs and MBCs were recorded after 18 h of incubation at 35 °C.

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Author contributions ID, CA, and JDD conceived the presented idea and designed the compounds. ID, CA, and CP synthesized and characterized the compounds. FS and JDD performed the biological assays. ID, CA, and JDD wrote the first draft of the manuscript. ID, CA, ED, LB, and JDD analyzed data and results. All authors have read and approved the final version of the manuscript.

Data availability The datasets generated and analyzed in the current study are available from the corresponding authors on reasonable request.

Declarations

Conflict of interest The authors declare no competing interest.

References

1. Genscript Impact of TFA—A Review, https://www.genscript.com/review-impact-of-tfa-in-peptide.html. Accessed 18 Jun 2022.
2. Food and Drug Administration of the United Nations. Prominent global leaders in science, industry and government meet to step up fight against antimicrobial resistance. https://www.fao.org/news/story/en/item/13710147/code. Accessed 18 Jun 2022.
3. Lul A, Erondo NA, Heymann DL et al (2021) Fragmented health systems in COVID-19: rectifying the misalignment between global health security and universal health coverage. Lancet 397:61–67. https://doi.org/10.1016/S0140-6736(20)32228-5
4. Subramanyan SH, Czyż DM, Acharya KP, Humphreys H (2021) The potential impact of the COVID-19 pandemic on antimicrobial resistance and antibiotic stewardship. VirusDisease. https://doi.org/10.1007/s13337-021-00695-2
5. Nasir N, Rehman F, Omair SF (2021) Risk factors for bacterial infections in patients with moderate to severe COVID-19: a case-control study. J Med Virol 93:4564–4569. https://doi.org/10.1002/jmv.27000
6. Rawson TM, Moore LSP, Zhu N et al (2020) Bacterial and fungal coinfection in individuals with coronavirus: a rapid review to support COVID-19 antimicrobial prescribing. Clin Infect Dis 71:2459–2468. https://doi.org/10.1093/cid/ciaa530
7. Botta M, Maccari G, Sanfilippo S, et al (2015) Linear guanidine derivatives, methods of preparation and uses thereof. Int. Patent Appl. WO2016/055644 A1
8. Zamperini C, Maccari G, Deodato D et al (2017) Identification, synthesis and biological activity of alkyl- guanidine oligomers as potent antibacterial agents. Sci Rep 7:1–11. https://doi.org/10.1038/s41598-017-08749-6
9. Pasero C, D’Agostino I, De Luca F et al (2018) Alkyl-guanidine compounds as potent broad-spectrum antibacterial agents: chemical library extension and biological characterization. J Med Chem. https://doi.org/10.1021/jacsmedchem.8b00619
10. D’Agostino I, Ardino C, Poli G et al (2022) Antibacterial alkyl-guanidino ureas: molecular simplification approach, searching for membrane-based MoA. Eur J Med Chem. https://doi.org/10.1016/j.ejmech.2022.114158
11. López SE, Salazar J (2013) Trifluoroacetic acid: uses and recent applications in organic synthesis. J Fluor Chem 156:73–100. https://doi.org/10.1016/j.jfluchem.2013.09.004
12. Cornish J, Callon KE, Lin CQX-X et al (1999) Trifluoroacetic acid, a contaminant in purified proteins, inhibits proliferation of...
13. Ma TG, Ling YH, McClure GD, Tseng MT (1990) Effects of trifluoroacetic acid, a halothane metabolite, on C6 glioma cells. J Toxicol Environ Health 31:147–158. https://doi.org/10.1080/15287399009351444

14. Tipps ME, Iyer SV, John Mihic S (2012) Trifluoroacetate is an allosteric modulator with selective actions at the glycine receptor. Neurpharmacology 63:368–373. https://doi.org/10.1016/j.neuroph.2012.04.011

15. Roux S, Zéki E, Rousseau B et al (2008) Elimination and exchange of trifluoroacetate counter-ion from cationic peptides: a critical evaluation of different approaches. J Pept Sci 14:354–359. https://doi.org/10.1002/jpsc.951

16. Sikora K, Jaśkiewicz M, Neubauer D et al (2018) Counter-ion effect on antistaphylococcal activity and cytotoxicity of selected antimicrobial peptides. Amino Acids 50:609–619. https://doi.org/10.1007/s00726-017-2536-9

17. Sikora K, Jaśkiewicz M, Neubauer D et al (2020) The role of counter-ions in peptides—an overview. Pharmaceuticals 13:1–26. https://doi.org/10.3390/ph13010013

18. Gausser H, Morency H, Lavoie MC, Subirade M (2002) Replacement of trifluoroacetic acid with HCl in the hydrophobic purification steps of pediocin PA-1: a structural effect. Appl Environ Microbiol 68:4803–4808. https://doi.org/10.1128/AEM.68.10.4803–4808.2002

19. Rizzello CG, Losito I, Gobbetti M et al (2005) Antibacterial activity of peptides from the water-soluble extracts of Italian cheese varieties. J Dairy Sci 88:2348–2360. https://doi.org/10.3168/jds.S0022-0302(05)72913-1

20. Greber KE, Dawgul M, Kamysz W, Sawicki W (2017) Cationic net charge and counter ion type as antimicrobial activity determinant factors of short lipopeptides. Front Microbiol 8:123. https://doi.org/10.3389/fmicb.2017.00123

21. Zhang C, Jiang Y, Hu H et al (2017) “Organic counterion” modified quaternary ammonium salt: Impact on antibacterial activity & application properties. J Mol Liq 241:638–645. https://doi.org/10.1016/j.molliq.2017.06.062

22. Dutta S, Shome A, Kar T, Das PK (2011) Counterion-induced modulation in the antimicrobial activity and biocompatibility of amphiphilic hydrogelators: Influence of in-situ-synthesized ag-nanoparticle on the bacterialic property. Langmuir 27:5000–5008. https://doi.org/10.1021/la104903z

23. Gut J, Christen U, Frey N et al (1995) Molecular mimicry in halothane hepatitis: Biochemical and structural characterization of lipoylated autoantigens. Toxicology 97(1–3):199–222. https://doi.org/10.1016/0300-5978(95)00011-0

24. Collins KD, Washabaugh MW (1985) The Hofmeister effect and the behaviour of water at interfaces. Q Rev Biophys 18:323–422. https://doi.org/10.1017/S0033583585000019

25. You Q, Cheng L, Reilly TP et al (2006) Role of neutrophils in a mouse model of halothane-induced liver injury. Hepatology 44:1421–1431. https://doi.org/10.1002/hep.21425

26. Trudell JR, Ardiez CM, Anderson WR (1991) Antibodies raised against trifluoroacetyl-protein adducts bind to N-trifluoroacetyl-phosphatidylethanolamine in hexagonal phase phospholipid micelles. J Pharmacol Exp Ther 257(2):657–662

27. You Q, Cheng L, Ju C (2010) Generation of T cell responses targeting the reactive metabolite of halothane in mice. Toxicol Lett 194:79–85. https://doi.org/10.1016/j.toxlet.2010.02.009

28. Boulterne AI, Polak PE, Braun D et al (2014) Effects of peptide fraction and counter ion on the development of clinical signs in experimental autoimmune encephalomyelitis. J Neurochem 129:696–703. https://doi.org/10.1111/jnc.12664

29. George N, Ofori S, Parkin S, Awuah SG (2020) Mild deprotection of the N-tert-butoxy carbonyl (N-Boc) group using oxalyl chloride. RSC Adv 10:24017–24026. https://doi.org/10.1039/D0RA04110F

30. Jacquemard U, Bénéteau V, Lefoix M et al (2004) Mild and selective deprotection of carbamates with Bu4NF. Tetrahedron 60:10039–10047. https://doi.org/10.1016/j.tet.2004.07.071

31. Tom NJ, Simon WM, Frost HN, Ewing M (2004) Deprotection of a primary Boc group under basic conditions. Tetrahedron Lett 45:905–906. https://doi.org/10.1016/j.tetlet.2003.11.108

32. Wuts PGMM, Greene TW (2006) Greene’s protective groups in organic synthesis, 4th edn. Wiley-Interscience, Hoboken, N.J. https://doi.org/10.1002/0470047005

33. Zhang Y, Jiang J, Chen Y (1999) Synthesis and antimicrobial activity of polymeric guanidine and biguanidine salts. Polymer (Guildf) 40:6189–6198. https://doi.org/10.1016/S0032-3861(98)00828-3

34. Ashworth IW, Cox BG, Meyrick B (2010) Kinetics and mechanism of N-Boc cleavage: evidence of a second-order dependence upon acid concentration. J Org Chem 75:8117–8125. https://doi.org/10.1021/jo101767h

35. Han G, Tamaki M, Hruby VJ (2001) Fast, efficient and selective deprotection of the tert-butoxycarbonyl (Boc) group using HCl/ dioxane (4:1). J Pept Res 58:338–341. https://doi.org/10.1034/j.1399-3011.2001.00935.x

36. Nudelman A, Bechor Y, Falb E et al (2006) Acetyl chloride-methanol as a convenient reagent for: A) quantitative formation of amine hydrochlorides B) carbamate ester formation C) mild removal of N-t-boc-protective group. Synth Commun 28:471–474. https://doi.org/10.1080/00339791980805101

37. Balestri LJI, D’Asgostino I, Rango E et al (2022) Focused library of phenyl-fused macrocyclic amidinoureas as antifungal agents. Mol Divers 1:1–11. https://doi.org/10.1007/S11030-022-10388-7

38. Lloyd L, Boguszewski P (2010) Freebasing of peptide salts and the removal of acidic ion-pairing reagents from fractions after hplc purification, agilent: Application Note SI-02449, https://www.agilent.com/cs/library/applications/SI-02449.pdf. Accessed 20 Jun 2022

39. Spivak A, Khalitova R, Nedopekina D (2018) Synthesis and evaluation of anticancer activities of novel C-28 guanidine-functionalized triterpene acid derivatives. Molecules 23:3000. https://doi.org/10.3390/MOLECULES23113000

40. Capua M, Perrone S, Perna FM et al (2016) An expeditious and greener synthesis of 2-aminoimidazoles in deep eutectic solvents. Molecules. https://doi.org/10.3390/MOLECULES21070924

41. Nagle PS, Rodriguez F, Kahvedzic A et al (2009) Asymmetrical diaromatic guanidinium/2-aminoimidazolium derivatives: synthesis and DNA affinity. J Med Chem 52:7113–7121. https://doi.org/10.1021/JM901017T

42. Bachand B, DiMaio J, Siddiqui MA (1999) Synthesis and structure-activity relationship of potent bicyclic lactam thrombin inhibitors. Bioorg Med Chem Lett 9:913–918. https://doi.org/10.1016/S0960-894X(99)00130-4

43. Diez-Cecilia E, Kelly B, Perez C et al (2014) Guanidinium-based derivatives: searching for new kinase inhibitors. Eur J Med Chem 69:336–345. https://doi.org/10.1016/j.ejmech.2014.05.025

44. Willcott MR (2009) MesoRe Nova. J Am Chem Soc 131:13180–13180. https://doi.org/10.1021/ja906700T

45. Thurston JT (1941) Preparation of free guanidine and biguanide bases, Official Gazette of the United States Patent Office. Appl. 383277

46. Trummal A, Lipping L, Kaljurand I et al (2016) Acidity of strong acids in water and dimethyl sulfoxide. J Phys Chem A 120:3663–3669. https://doi.org/10.1021/ACS.JPCA.6B02253
47. Sweis R, Edmondson S, Kaelin D (2008) Substituted aminopyrimidines as cholecystokinin-i receptor modulators background of the invention. Int. Patent Appl. WO 2008/091631 A1
48. Pinho SP, Macedo EA (2004) Solubility of NaCl, NaBr, and KCl in water, methanol, ethanol, and their mixed solvents. J Chem Eng Data 50:29–32. https://doi.org/10.1021/JE049922Y

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