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SHORT COMMUNICATION

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 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**.

This paper is dedicated to the memory of Prof. Maurizio Botta (1950–2019) who coordinated the first steps of this work, contributing to conceiving the presented idea [*« A teacher affectseternity; he can never tell where his influence stops.» Henry B. Adams*].

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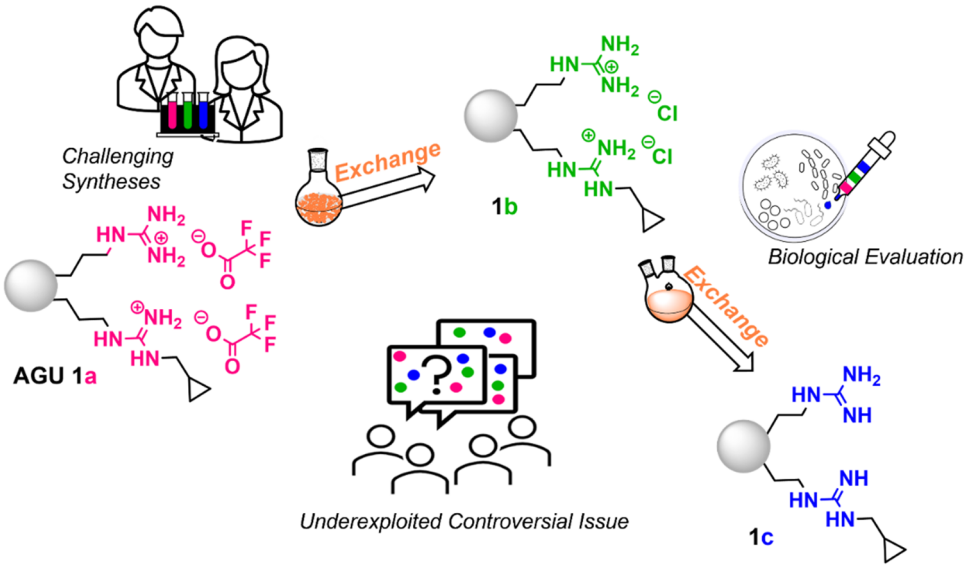
Graphical abstract



The controversial issue of organic compounds counterions impact in biological assays: challenging synthesis of AlkylGuanidino Urea **1** as TF-Acetate **(a)** and HCl **(b)** salts and free base **(c)** and antibacterial susceptibility evaluation.

Keywords Antibacterials · Guanidine · Counterion · Trifluoroacetate salt · Hydrochloride salt · Free base guanidine

Abbreviations

ADME	Absorption, distribution, metabolism, and excretion
AGU	Alkylguanidino urea
AMP	Antimicrobial peptide
AMR	Antimicrobial resistance
ATCC	American type culture collection
Boc	*tert*-Butoxycarbonyl
CCUG	Culture collection of University of Gothenburg
CFU	Colony-forming unit
CLSI	Clinical laboratory standards institute
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DMSO	Dimethyl sulfoxide
EUCAST	European committee on antimicrobial susceptibility testing
MBC	Minimal bactericidal concentration
MIC	Minimal inhibitory concentration
MW	Microwave
PHMG	Polyhexamethylene guanidine
PK	Pharmacokinetics
TEA	Triethylamine
TFA	Trifluoroacetic acid
TF-Acetate	Trifluoroacetate

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 trifluoroacetylating 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

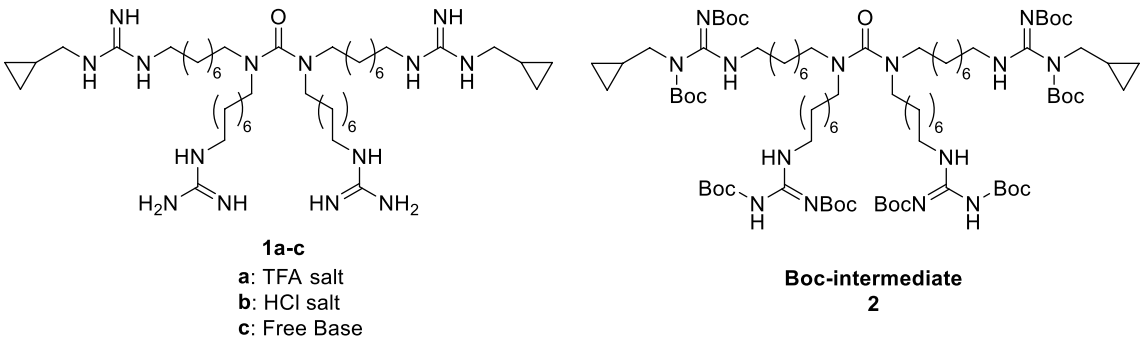


Fig. 1 Structure of AGU **1** as TF-Acetate (**a**) and HCl (**b**) salts or free base (**c**) and the Boc-intermediate **2**

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.

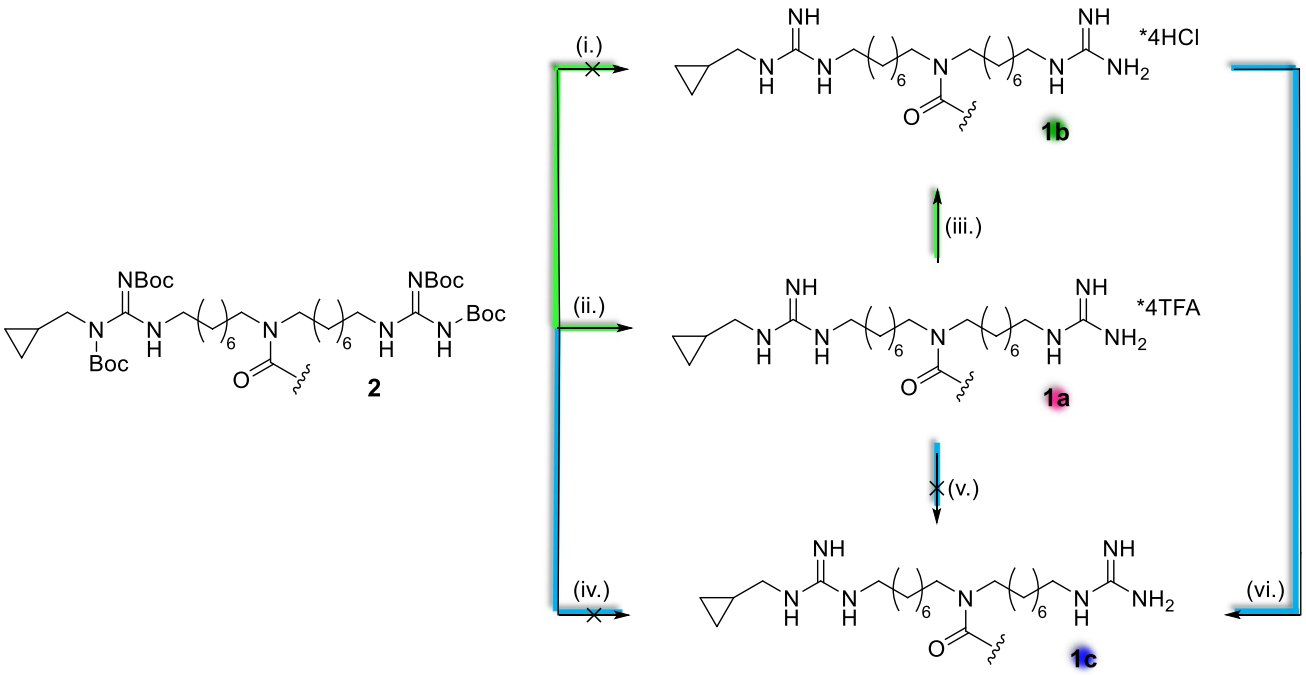
Different synthetic strategies were explored and some attempts are reported in Table 1.

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 ^{19}F NMR since its fluorine atoms show a characteristic singlet signal at 77 ppm in CD_3OD [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



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 CH_2Cl_2, sealed flask, r.t., 5 h [10]*; iii.* Amberlite IRA400 chloride form, CH_3OH, r.t., 72 h; *vi.* NaOEt in situ dry EtOH, N_2, 0 °C-r.t., 30 min. Unsuccessful reactions are reported in Tables 1, S1, and S2

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_3OH 5 µM, sealed flask, r.t., 24 h	Compound not isolated
iii	3	HCl 5 N, CH_3OH 5 µM, r.t., 16 h	TF-Acetate still present
	4	1. TEA (up to 40 eq.), CH_2Cl_2, 20 µM, r.t., 16 h; 2. HCl 2 N, CH_3OH 10 µM, r.t., 48 h	TF-Acetate still present
	5	1. DBU (up to 40 eq.), CH_3OH 20 µM, r.t., 16 h; 2. HCl 2 N, CH_3OH 10 µM, r.t., 48 h	Compound degradation
	6	1. NaOH 2.5 N, CH_3OH 10 µM, r.t., 48 h; 2. HCl 2 N, CH_3OH 10 µM, r.t., 48 h	TF-Acetate still present
	7	Amberlite IRA 400 chloride form, CH_3OH 6 µM, r.t., 72 h	Reaction occurred and product isolation

Reagents, reaction conditions, and main results are reported for each reaction

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 ^{19}F 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 aqueous solution. Although basic molecules are generally available on the market in the form of acid salts [45], several AMPs and guanidino compounds were prepared and tested as free base derivatives. Indeed, bacterial culture media are characterized by pH values above 6.0 [19] and free base guanidines will fast pick up protons from the environment to increase their chemical stability.

On that basis, we focused our chemical efforts toward the preparation of **1c**. 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 rate 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 counteranion, 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 2 reports the antibacterial activity of the same AGU compound (**1**), in its salt (**a-b**) and free base (**c**) forms, in both µg/mL and µM, the latter unit limiting the influence of variations of molecular weights. The Clinical & Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) do not provide guidelines on the unit system for reporting MICs. Indeed, both weight/volume (µg/mL) and molarity (mol/L) unit systems can be used. In general, in clinical practice, a

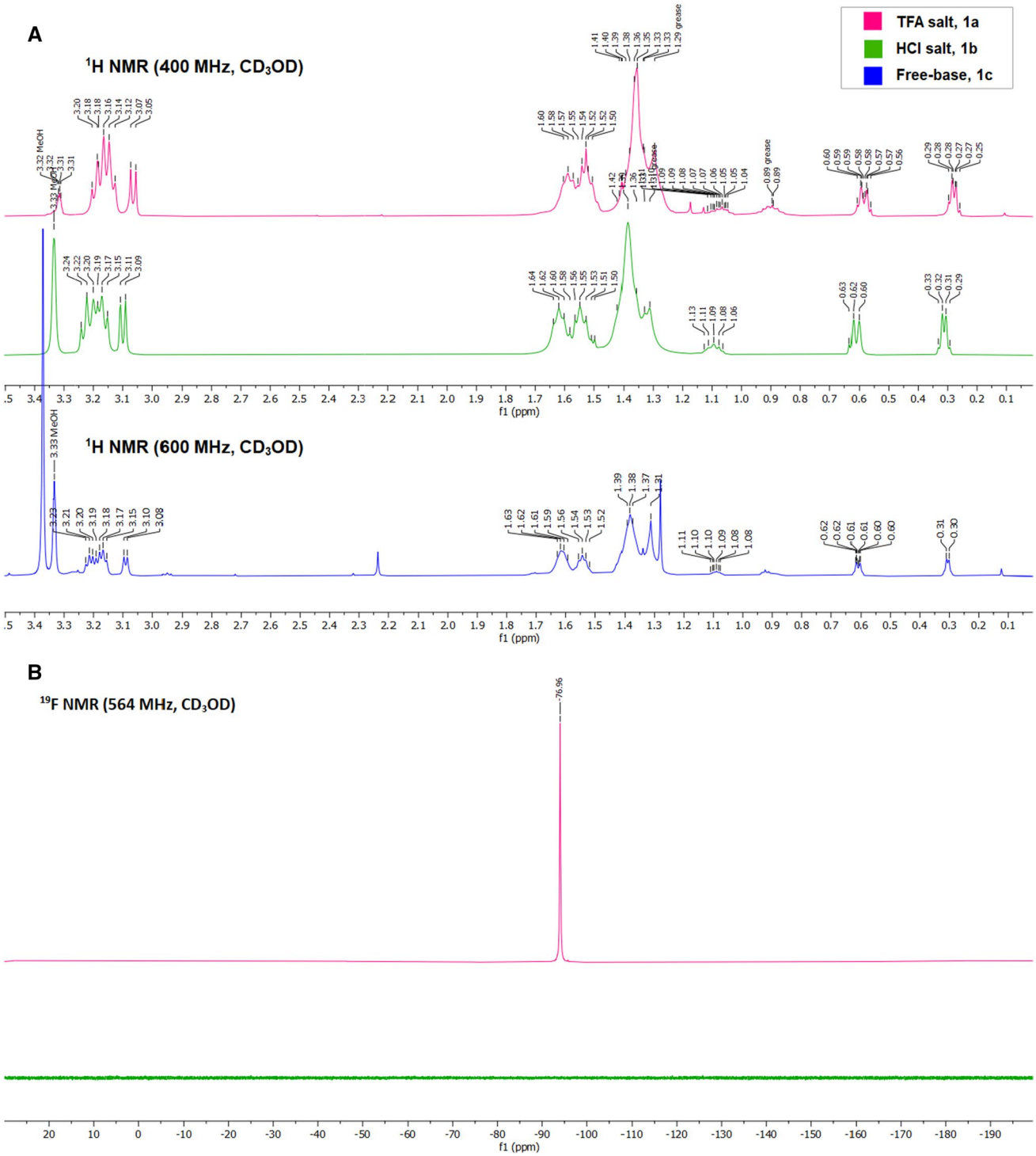


Fig. 2 Comparison of ^{1}H and ^{19}F NMR spectra of AGUs **1a–c**. **A** ^{1}H NMR spectra of **1a** (purple) and **1b** (green) were recorded at Bruker 400 MHz. ^{1}H NMR spectrum of **1c** (blue) was recorded at Bruker 600 MHz. **B** ^{19}F NMR spectra of **1a** (purple) and **1b** (green) were recorded at Bruker 600 MHz. All the experiments were performed in CD_3OD. Spectra were visualized and analyzed through MestreNova 14.2 [44]

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.

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)[a]		
	COL	VAN	DAP	1a	1b	1c	1a	1b	1c
B. subtilis ATCC 6633	–[b]	0.5	1	2[c]	1	8	1.54	1.18	9.46
E. faecalis ATCC 19,433	–	1	1	2[c]	2	8	1.54	2.02	9.46
S. aureus ATCC 25,923	–	0.5	0.125	2[c]	2	8	1.54	2.02	9.46
S. epidermidis ATCC 14,990	–	0.5	0.125	1[c]	1	4	0.77	1.01	4.73
S. pyogenes ATCC 12,344	–	0.5	0.125	1[c]	0.5	4	0.77	0.50	4.73
E. coli CCUG^T	0.5	–	–	2[c]	2	16	1.54	2.02	18.92
K. pneumoniae ATCC 13,833	0.5	–	–	2[c]	2	16	1.54	2.02	18.92
A. baumannii ATCC 17,978	1	–	–	8[c]	8	32	6.15	8.07	37.85
P. aeruginosa ATCC 27,853	0.5	–	–	8[c]	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	1	16	49.18	1.01	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	–	–	1[c]	2	128	0.77	2.02	151.42
K. pneumoniae 081R	64	–	–	2[c]	2	128	1.54	2.02	151.42
A. baumannii AC-54/97	0.25	–	–	2[c]	4	32	1.54	4.04	37.86
A. baumannii N50	32	–	–	16	4	64	12.29	4.04	75.71
B. cepacia SI-R2	0.5	–	–	16[c]	2	32	12.29	2.02	37.86
S. maltophilia 634/08	0.25	–	–	16[c]	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

[a]MICs conversion in molarity (μM) was calculated through the formula: $\frac{MIC(\mu g/mL)}{m.w.} \times 1000$, considering 1301.42, 991.16, and 845.33 g/mol as the molecular weight for **1a**, **1b**, and **1c,** respectively

[b]-: not determined

[c]Data from references 7–10

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 no 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

Table 3 MBCs of **1a–c** against representative drug-resistant bacterial species

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: $\frac{MIC(\mu g/mL)}{m.w.} \times 1000$, 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: CH_2Cl_2 was dried over calcium hydride and alcohols over I_2/Mg. TLCs were visualized under UV light and stained with ninhydrin or basic permanganate stains. ^{1}H NMR spectra were recorded on Bruker Avance 400 or 600 spectrometers at 400 or 600 MHz, while ^{13}C NMR spectra on Bruker Avance 400 at 100 MHz. ^{1}H NMR spectra were reported in parts per million (δ scale) and internally referenced to CD_3OD 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 CD_3OD 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). ^{19}F 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 CH_3OH/H_2O. 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].

1,3-Bis(8-carbamimidamidooctyl)-1,3-bis({8-[*N*′-(cyclopropylmethyl)carbamimidamido]octyl})urea Hydrochloride Salt (1b) To a solution of **1a** (45.7 mg, 0.035 mmol) in CH_3OH (5.8 mL), Amberlite IRA-400 Chloride form (137.0 mg) was added and the reaction mixture was stirred through a rocker shaker at room temperature for 24 h. Then, the mixture was diluted with CH_3OH and filtered through a 0.45 μm PTFE filter. The filtrate was evaporated *under vacuum* to furnish the desired product as a pale yellow oil. Yield: 90%.^{1}H NMR (CD_3OD, 400 MHz) δ (ppm): 3.24–3.16 (m, 16H), 3.10 (d, J = 7.0 Hz, 4H), 1.64–1.55 (m, 8H), 1.53–1.50 (m, 8H), 1.43–1.29 (m, 32H), 1.09 (p, J = 5.6 Hz, 2H), 0.62 (d, J = 7.6 Hz, 4H), 0.31 (d, J = 5.0 Hz, 4H). ^{13}C NMR (CD_3OD, 100 MHz) δ (ppm): 3.4, 9.5, 26.2, 26.5, 27.5, 28.4, 28.4, 28.8, 28.9, 29.2, 41.0, 41.1, 45.8, 46.9, 47.1, 47.3, 47.5, 47.7, 47.9, 48.1, 155.6, 165.4. LCMS(ES+) m/z = 845.8 $[M+H]^+$, 423.1 $[M+2H]^{2+}$, 282.5 $[M+3H]^{3+}$, 212.1 $[M+4H]^{4+}$.

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 CH_3OH (7.2 mL) was added dropwise at 0 °C in an ice bath, under N_2. 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 CH_2Cl_2 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 several times through 0.45 µm PTFE filters. Aliquots of the sample were analyzed with the silver nitrate assay to ensure the absence of chlorine atoms. After evaporation, the yellowish oil was treated with CH_2Cl_2 (5 × 10.0 mL) and filtered again with a 0.45 µm PTFE filter. After evaporation, a colorless oil was obtained. An aliquot was subjected to the silver nitrate assay. In brief, a 0.1 M solution of silver nitrate in deion. H_2O was freshly prepared and used to assess the complete conversion of **1b** to **1c** and the removal of residual NaCl. In presence of chloride ions, silver chloride is formed as a white precipitate. No evidence of precipitation was observed. Yield: 80%. ^{1}H NMR (CD_3OD, 600 MHz) δ (ppm): 3.24–3.15 (m, 16H), 3.09 (d, J = 7.0 Hz, 4H), 1.63–1.59 (m, 8H), 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). ^{13}C 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 5×10^4 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.

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