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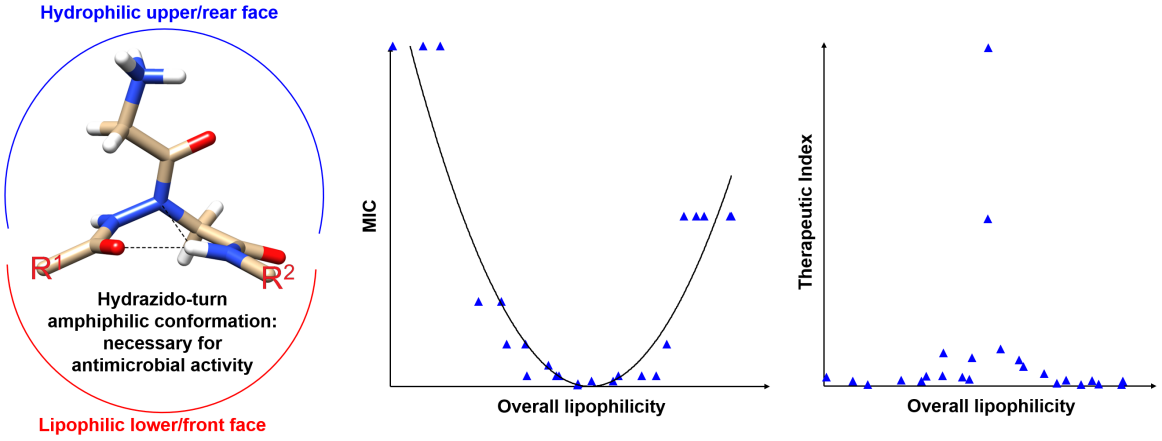

Simple Amphiphilic *α*-Hydrazido Acids: Rational Design, Synthesis, and In Vitro Bioactivity Profile of a Novel Class of Potential Antimicrobial Compounds

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ABSTRACT

Pursuing the search for a new class of structurally simple mimics of antimicrobial peptides, we optimized a short, cheap and high-yielding synthesis of mono-charged amphiphilic *α*-hydrazido acid

derivatives. The most active derivatives furnished MICs that are among the best values reported in literature for synthetic amphiphilic membranolytic compounds. They exhibited a broad-spectrum in vitro activity against a variety of Gram-positive and Gram-negative bacteria, including two multidrug-resistant strains. In spite of the minimal cationic charge, the best compounds demonstrated to be selective toward bacterial cell membranes over mammalian cell membranes. The relationship between either the antibacterial or the hemolytic activity and the overall lipophilicity furnished an easy way to individuate the best dimensional range for the hydrophobic portions. The importance of a non-disrupted amphiphilicity was also demonstrated. Considering the bioactivity profile and the ease of synthesis, these chemically and proteolitically stable hydrochlorides are suitable for development of a new class of wide-spectrum antibiotics.

KEYWORDS

Rational design
Cationic amphiphilic *α*-hydrazido acids
Wide-spectrum antibacterials
Overall lipophilicity
Non-disrupted amphiphilicity
Membranolytic compounds

ABBREVIATIONS

AMPs, antimicrobial peptides; HDPs, host defense peptides; CSAs, cationic steroid antimicrobials; PNA, peptide nucleic acid; *α*-AApeptides, *N*-acylated-*N*-aminoethyl *α*-peptides; DFT, density functional theory; IEF-PCM, integral equation formalism version of polarizable continuum model method; NBO, natural bond orbital; AIM, atoms in molecules; DIPEA, *N*,*N*-diisopropylethylamine; anh., anhydrous; DCM, dichloromethane; rt, room temperature; Boc, *tert*-butyloxycarbonyl protecting group; Gly, glycine; EDCI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; TFA, trifluoroacetic acid; LR-MRSA, linezolid- and methicillin-resistant *Staphylococcus aureus* AOUC-0915; GR-CREc, gentamicin- and colistin-resistant *Escherichia coli* 288328; HPLC, high performance liquid chromatography; RP-HPLC, reverse-phase HPLC; MIC, minimum inhibitory concentration; HC_{50}, 50% hemolytic concentration; TI, therapeutic index; *mcr*, mobilized colistin resistance; *aac(3)-IIa*, aminoglycoside *N*(3)-acetyltransferase; rRNA, ribosomal ribonucleic acid; LPS,

lipopolysaccharide; PBP, penicillin-binding protein; PBS, phosphate buffered saline; PI, propidium iodide; NPN, *N*-phenyl-1-naphthylamine THF, tetrahydrofuran; *i*PrOH, iso-propanol.

1. Introduction

The exponentially rising number of multidrug resistant bacteria, together with the slowing down of the discovery of new antibacterial compounds, is a well-known world public health priority. The non-judicial use or abuse of antibiotics, as well as their widespread use in livestock [1], are further favoring the spread of antibiotic resistance [2] and the emergence of bacterial strains even resistant to last-resort antibiotics [3].

In the search for new classes of antibiotics, the natural cationic antimicrobial peptides (AMPs), have been extensively taken into consideration in the last decades [4-6]. Their amphiphilic active secondary structure is essential to cause lysis of the bacterial cell membrane, even if occasionally they can also act on intracellular targets [7-10] and modulate the innate immune response [11]. It is usually claimed that bacteria can hardly develop resistance toward AMPs [8], but there are growing reports both on intrinsic and acquired resistance, mainly due to membrane modifications [11].

Even if AMPs have a high net positive charge and are moderately selective in vitro toward bacterial cells over mammalian cells, due to the different contents of anionic and uncharged/zwitterionic lipids in membranes [12], their in vivo low efficacy and high toxicity has hampered the clinical use. Only polymyxins have been systemically administered since the 1950s [13], but their use has been strongly limited by the high incidence of nephrotoxicity and neurotoxicity [14], and the vast majority of antimicrobial peptides that are currently in clinical development are for topical use only [11]. Other important drawbacks, such as high cost of manufacture, instability toward proteases, and low bioavailability, have further hindered the application of natural AMPs in clinical therapeutics [5,15].

In order to circumvent these problems, several groups have been working on synthetic mimics of AMPs (Figure 1). Apart from some rationally designed short α-helices [16,17] and α-peptidic dendrimers [18,19], most of the oligomeric structures with a number of residues between 5 and 20 units are based on the diverse non-natural stable helices (foldamers) formed in solution by β-peptides [20-25] (Figure 1), oligoureas [26,27], and α-peptoids [28-31]. However, foldamers rely mainly on expensive chiral monomers joined together by a multistep solid phase peptide synthesis followed by a demanding purification, which are not suitable for industrial applications. Stable amphipathic conformations also

characterize shorter oligomers, such as de novo designed small arylamide foldamers [34-36] (Figure 1). In all of these cases, the preorganization of monomers is at the basis of inherently stable secondary structures [21,24,35-37], which do not have to spend free energy in order to arrange into an amphiphilic folding. Examples of amphipathic antimicrobials based on an extremely rigid backbone can also be found in synthetically-produced cationic steroid antimicrobials (CSAs) [38-41], as well as in triaryl derivatives [42] where the facially amphiphilic topology is constitutionally guaranteed by the proper 1,3,5-substitution of aryl scaffolds (Figure 1).

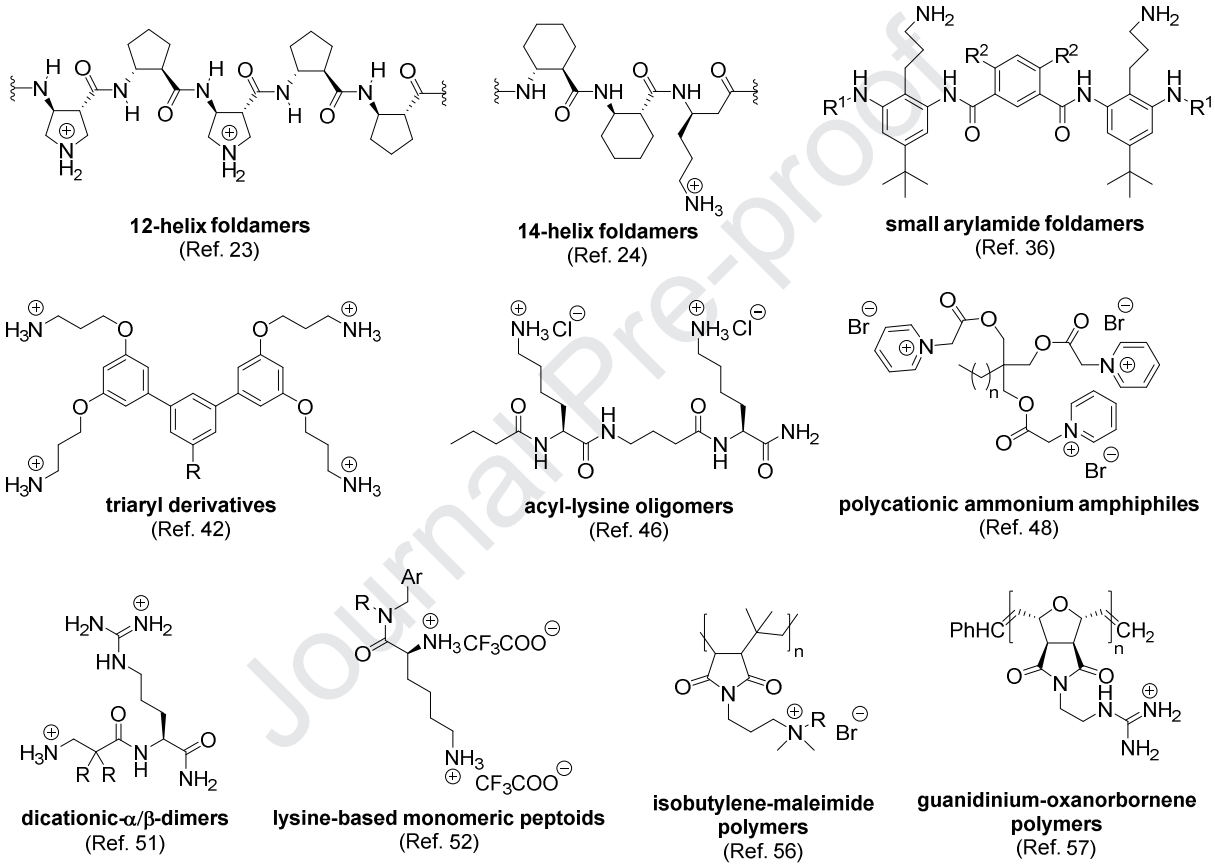


Figure 1. Representative synthetic mimics of antimicrobial peptides.

The opposite approach has also been used, thus mimicking AMPs like indolicidin, which is unstructured in aqueous environment, but shows a stable polyproline II-like structure upon interaction with liposomes and is a membrane permeabilizing peptide [43]. Synthetic oligomeric species not based on preorganized monomers, thus having much more flexible backbones, may penetrate the bacterial cell wall barrier in an easier way, compared to conformationally rigid antimicrobials, but they can still easily assume amphiphilic conformations and then have a potent disrupting action on membranes [44].

Members of these flexible oligomeric antimicrobials are *α*-AApeptides [45], acyl-lysine oligomers [46], a few dicationic oligo-*α*-peptide analogues with a minimalist design [47], and various polycationic ammonium amphiphiles [48-50] (Figure 1). Interesting examples of apparently unstructured antibacterials with a very small size have been recently reported in literature, such as dicationic *α/β*-dimers [51] and extremely simple, but still potent, lysine-based monomeric peptoids [52] (Figure 1).

Polycationic antimicrobial polymers have also been extensively investigated and reviewed on all aspects [53-55]. Remarkable examples of polymers having a good bioactivity profile are the isobutylene-maleimide [56] and the guanidinium-oxanorbornene [57] polymers (Figure 1).

Herein we report the synthesis and the in vitro antibacterial and hemolytic activities of small amphiphilic achiral *α*-hydrazido acids, which were demonstrated to be membranolytic. In view of a possible practical implementation, other important features (i.e. stability toward enzymatic and chemical degradation) were also evaluated. In their design, the hydrophobic portion is composed of both the *N*- and *C*-terminal lipophilic groups, whereas the single charge is furnished by the ammonium ion of the glycine side chain (Figure 2). The *α*-hydrazido acids are mimics of *β*-amino acids and have been chosen because oligomers of their conformationally constrained version are prone to form the peculiar intramolecular hydrogen bond pattern of the hydrazido-turn secondary structure [58]. Thus, this folding could also be assumed by the present non-preorganized acyclic *α*-hydrazido acids and used to segregate polar and charged molecular portions from the lipophilic moieties. However, the lack of conformational constrictions in their flexible backbone should facilitate the penetration through the bacterial cell wall barrier and give rise to a more potent activity, as demonstrated for amphiphilic compounds of different dimensions [46,59,60]. In this initial investigation we devoted our attention only to mono-charged compounds, which are likely to be less potent as antimicrobial and less selective toward mammalian cells, so that this choice should also ensure the possibility for easy future improvements, by simply using a multiple charge-bearing side chain.

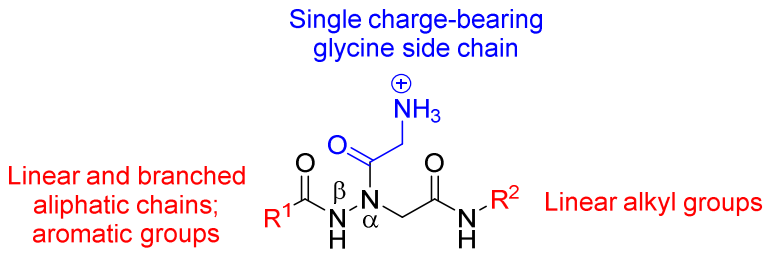


Figure 2. General structure of antibacterial *α*-hydrazido acids, with the common nomenclature for nitrogen atoms in these compounds.

2. Results and discussion

2.1 Design of the amphiphilic lead structure

The idea at the basis of the choice of this particular backbone, that is its innate tendency to form amphiphilic structures, was the subject of a high-level density functional theory (DFT) analysis. After a complete conformational search at a lower theory level on a model compound with $R^1 = R^2$ = Me, all the previously found energy minima were refined using the well-performing hybrid functional ωB97X-D3(0) [61], together with the very large 6-311++g(3df,3pd) basis set, describing the solvent bulk with the integral equation formalism version of polarizable continuum model method (IEF-PCM) [62]. The highly predominating conformer of the model compound was then used for constructing selected conformers of an actually used compound, namely **4Cb**, all having the aliphatic chains in the most stable all-antiperiplanar conformation (see Supplementary Data for all the other structures and additional computational details). The theoretical approach indicated that the preferred conformation in water for this monomeric compounds is the same hydrazido-turn assumed by their conformationally constrained oligomeric versions [58] (Figure 3).

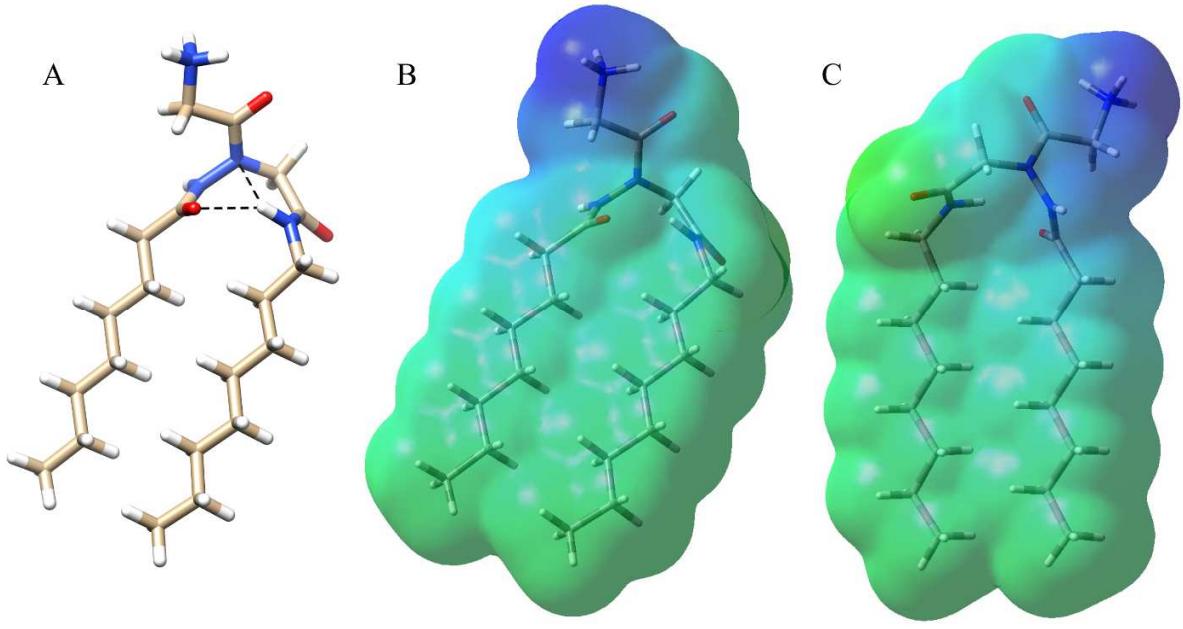


Figure 3. (A) Structure of most stable conformer of ammonium cation $C_7H_{15}CO$-HydrGlyH$^+$-NHC_8H_{17} (**4Cb**, R^1 = *n*-C_7H_{15}, R^2 = *n*-C_8H_{17}), computed at ωB97X-D3(0)/6-311++g(3df,3pd)/IEF-PCM level in water. (B) Front and (C) rear views of electrostatic potential surface.

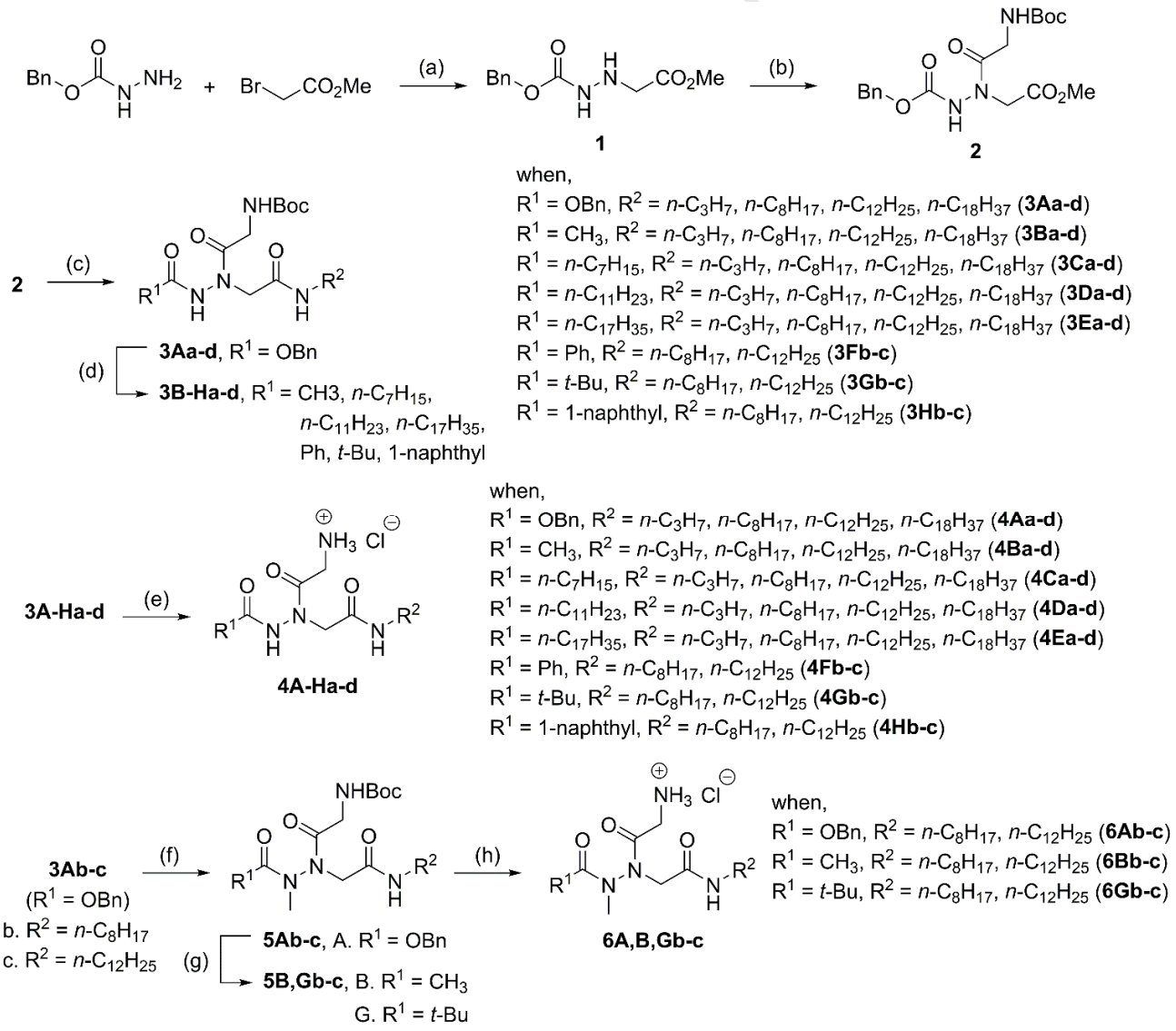
The Natural Bond Orbital (NBO) analysis [63,64] pointed out that this structure is mainly stabilized by the C=O□□□H-N hydrogen bond (2.08 Å), which is characterized by an important covalent contribution and forms an eight-membered pseudocycle, and, to a lesser extent, by the N^{α}□□□H-N H-bond (2.38 Å), which is instead essentially electrostatic in nature (Tables S3 and S5). The study exploiting the Atoms In Molecules (AIM) theory [65,66] confirmed the existence of a bond critical point associated with a quite strong C=O□□□H-N hydrogen bond, whereas no critical point was found in the case of N^{α}□□□H-N interaction, thus describing this latter hydrogen bond as purely electrostatic (Table S6 and Figure S20). It is easy to see from the electrostatic potential surfaces of **4Cb** as a cation (Figure 3) or considering the chloride counterion (Figure S24) that the only hydrogen atom belonging to hydrocarbon chains that does not have an almost neutral potential is the one experiencing hyperconjugation with the *N*-terminal C=O (light blue).

Apart from the obvious contribution of the charged glycine side chain, within this hydrophilic molecular portion the *C*-terminal carbonyl and the *N*-terminal NH functionalities must play an important role in forming hydrogen bonds with water and charged lipid heads while interacting with phospholipid bilayers. The ability of hydrazide NHs to form quite strong hydrogen bonds is also readily apparent considering that their experimental proton chemical shifts in diluted deuterochloroform solutions range from 7.81 to 8.03 ppm when R^1 = OBn, and reach very high values (8.74-9.87 ppm) when R^1 is a hydrocarbon chain, thus experimentally confirming their high positive charge (see Supplementary Data). Calculations of atomic charges of ammonium cation with NBO, AIM, Merz-Singh-Kollman [67,68], CHelpG [69], and Hu-Lu-Yang [70] schemes (Figures S19-S23), as well as the computed electrostatic potential and charges taking also into account the chloride anion (Figures S24-S25), plenty confirmed these findings about the segregation of polar/charged and apolar portions. It must be noted here that only inherently very stable structures can display amphipathic conformations in water, whereas the use of organic co-solvents or the presence of liposomes and vesicles greatly facilitates the formation of the amphiphilic secondary structure even for compounds which are substantially unstructured in water or aqueous buffers [37,71,72]. These observations strongly point toward an amphiphilic arrangement of our *α*-hydrazido acid hydrochlorides during the action on phospholipidic bilayers.

2.2 Synthesis

Benzyl carbazate was reacted with methyl bromoacetate in the presence of *N*,*N*-diisopropylethylamine, obtaining compound **1** in excellent yield (Scheme 1). Benzyl carbazate demonstrated to be a poor nucleophile, thus concentrated dichloromethane solutions had to be used in order to obtain reasonable reaction times. Moreover, DIPEA was necessary to avoid the competition in the nucleophilic substitution from less hindered amines, such as triethylamine, which invariably led to unsustainable decreases in yields, due to the substantial formation of the corresponding quaternary ammonium salts with methyl bromoacetate.

Scheme 1. General Synthetic Scheme for the Preparation of Amphiphilic α-Hydrazido Acid Derivatives[a]



[a] (a) *N*,*N*-diisopropylethylamine (DIPEA), anh. DCM (0.5 mL per mmol of benzyl carbazate), rt, 48 h, 96%; (b) Boc-Gly-OH, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI), anh. DCM (0.5 mL per mmol of **2**), -20 °C, 2 h, 86%; (c) R^2NH_2 (R = alkyl group), anh. MeOH, 7 h at rt for **3Aa**, 18, 24 or 36 h at reflux for **3Ab**, **3Ac**, and **3Ad**, 88-94%; (d) HCO_2H, Pd/C, anh. DCM, rt, 1 h, then pyridine, R^1COCl, anh. DCM, rt, 1 h, 65-95%; (e) Trifluoroacetic acid (TFA)/DCM 1:3, rt, 20 min, chromatographic purification of free amine, then 3 M HCl in anh. MeOH, 68-98%; (f) Lithium bis(trimethylsilyl)amide (LHMDS), anh. THF, Ar, 0 °C, 10 min, then methyl iodide, rt, 24 h, **5Ab**: 87% **5Ac**: 90%; (g) HCO_2H, Pd/C, anh. DCM, rt, 1 h, then pyridine, R^1COCl, anh. DCM, rt, 1 h for **5Bb-c**, 20 h for **5Gb-c**, 81-92%; (h) Trifluoroacetic acid (TFA)/DCM 1:3, rt, 20 min, chromatographic purification of free amine, then 3 M HCl in anh. MeOH, 75-98%.

Then compound **1** was reacted with Boc-Gly-OH, using EDCI as the coupling agent, obtaining the desired common precursor **2** in very good yield, but even in this case it was necessary to circumvent the scarce reactivity of **1** by using concentrated solutions and also optimizing all the other experimental conditions. After reaction of **2** with the suitable alkyl amines, the first four compounds ready to be deprotected, **3Aa-d** (R^1 = OBn), where successfully synthesized, and the change of R^1 group by means of the carboxybenzyl removal/free hydrazide acylation sequence furnished the remaining compounds **3B-Ha-d** in good to excellent overall yields. Hydrochlorides **4** were then obtained in pure form by Boc removal with trifluoroacetic acid, followed by an easy chromatographic purification of free amines on silica gel and re-salification with hydrochloric acid. It must be pointed out that the achievement of the best possible antibacterial efficacy was out of the scope of this initial evaluation, so a coarse grid approach was voluntarily chosen, and only alkyl chains differing one from another for a large number of methylenic units were chosen. Moreover, when R^1 groups different from linear alkyl chains were taken into account, not all the possible combinations among R^1 and R^2 groups were synthesized and tested. In fact, as correctly reported in Scheme 1, this was done when the *N*-terminal chain, R^1, was the parent carboxybenzyl protecting groups, and for all the alkyl chains R^1 = CH_3-$C_{17}H_{35}$. After this screening, the best performing *C*-terminal chains (R^2 = C_8H_{17}, $C_{12}H_{25}$) were chosen to synthesize selected compounds with R^1 = *t*-Bu, Ph, and 1-naphthyl.

In order to verify the hypothesis that the well-defined amphiphilic character of the preferred conformations should be determining in favoring the destabilizing action on phospholipid bilayers, the *N*-methylated versions of six selected compounds were also synthesized (Scheme 1). Starting from **3Ab** (R^1 = OBn, R^2 = *n*-C_8H_{17}) and **3Ac** (R^1 = OBn, R^2 = *n*-$C_{12}H_{25}$), deprotonation with lithium bis(trimethylsilyl)amide followed by reaction with iodomethane directly gave the corresponding *N*-methylated compounds **5Ab** and **5Ac** in very good yields. Compounds **5Bb** (R^1 = CH_3, R^2 = *n*-C_8H_{17}), **5Bc** (R^1 = CH_3, R^2 = *n*-$C_{12}H_{25}$), **5Gb** (R^1 = *t*-Bu, R^2 = *n*-C_8H_{17}), and **5Gc** (R^1 = *t*-Bu, R^2 = *n*-$C_{12}H_{25}$)

were then obtained in good overall yields following the same methodology reported above for the corresponding unmethylated compounds, but using a prolonged reaction time when the acylating reagent was pivaloyl chloride. Exploiting the sequence acidic Boc removal/purification of free amine/re-salification, hydrochlorides **6** were eventually obtained in pure form in yields ranging from 75% to 98%.

2.3 Antibacterial and hemolytic activities

The antibacterial activity was first tested against three Gram-positive (*Enterococcus faecalis* ATCC 29212, *Enterococcus faecium* 135562 (35C), and *Staphylococcus aureus* ATCC 29213) and two Gram-negative (*Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853) collection strains (Table 1). The minimum inhibitory concentrations (MICs) were evaluated using the broth microdilution method in 96-well microtiter plates, following the CLSI guidelines [73], while hemolytic activity was determined according to a reported procedure [28]. Compounds **4Ae** (R^1 = *n*-$C_{17}H_{35}$, R^2 = *n*-$C_{18}H_{37}$) and **4Hc** (R^1 = 1-naphthyl, R^2 = *n*-$C_{12}H_{25}$) were too insoluble to be tested. In addition, two among the most active compounds were subsequently tested against multidrug-resistant bacteria, namely the linezolid- and methicillin-resistant *Staphylococcus aureus* AOUC-0915 (LR-MRSA) and the gentamicin- and colistin-resistant *Escherichia coli* 288328 (GR-CREc). MICs and hemolysis results were reproducible between three independent experimental replicates. These results, as well as the reverse-phase HPLC-derived overall lipophilicity, are reported in Table 1.

From the results in Table 1, many general deductions can be drawn. First, the hydrochlorides of *α*-hydrazido acids are somewhat more active against Gram-positive bacteria, *E. faecium* being the most sensitive, with respect to Gram-negatives, especially *Pseudomonas aeruginosa*. This observation makes readily apparent that the thick peptidoglycan layer of Gram-positive bacteria cannot prevent the entry of these flexible molecules, even when a large and rigid 1-naphthyl group is present as R^1 (**4Hb**), because of the nano-sized pores in the membrane of Gram-positive bacteria [74]. Moreover, the lower sensitivity of Gram-negatives is common in literature for cationic amphipathic membranolytic compounds, and is usually claimed as mainly caused by the reduced active concentration that can reach and disrupt both the outer and inner membranes of Gram-negative bacteria. This in turn is due to inability to permeabilize their primary barrier for hydrophobic compounds, which is the electrostatic network of negatively charged lipopolysaccharide molecules bound to divalent cations in the outer leaflet [75]. However, the lower activity toward Gram-negative bacteria could also be due to their high

content of zwitterionic phosphatidylethanolamine, whereas Gram-positives are usually richer in negatively charged components [39], or to the substantial amounts of negative curvature-inducing lipids into their phospholipid bilayers [76].

Table 1. In Vitro Antibacterial and Hemolytic Activities of *α*-Hydrazido Acids

			MIC *vs* drug sensitive bacteria (μg mL^{-1})[a]								
Compd	**R^1**	**R^2**	***E. faecalis***	***E. faecium***	***S. aureus***	***E. coli***	***P. aeruginosa***	**HC$_{50}$[b] (μg mL^{-1})**	**TI *vs* *S. aureus***	**TI *vs* *E. coli***	**Retention time (min)[c]**
4Aa	OBn	C_3H_7	1024	512	512	512	512	775±19	1.5	1.5	3.4
4Ab	OBn	C_8H_{17}	128	64	64	64	128	189±7	3.0	3.0	14.3
4Ac	OBn	$C_{12}H_{25}$	16	8	8	32	128	86±5	11	2.7	21.2
4Ad	OBn	$C_{18}H_{37}$	512	256	512	512	512	243±13	0.5	0.5	30.9
4Ba	CH_3	C_3H_7	2048	2048	1024	2048	2048	2700±43	2.6	1.3	0.3
4Bb	CH_3	C_8H_{17}	256	128	128	256	256	223±9	1.7	0.9	9.3
4Bc	CH_3	$C_{12}H_{25}$	32	32	64	64	64	127±8	2.0	2.0	17.5
4Bd	CH_3	$C_{18}H_{37}$	32	32	256	512	512	228±18	0.9	0.5	28.0
4Ca	C_7H_{15}	C_3H_7	1024	1024	1024	1024	2048	433±10	0.4	0.4	5.2
4Cb	C_7H_{15}	C_8H_{17}	32	16	16	16	32	131±8	8.2	8.2	17.8
4Cc	C_7H_{15}	$C_{12}H_{25}$	32	16	64	256	512	367±24	5.7	1.4	24.0
4Cd	C_7H_{15}	$C_{18}H_{37}$	>256	>256	>256	>256	>256	267±18	<1.0	<1.0	33.1
4Da	$C_{11}H_{23}$	C_3H_7	64	64	64	64	64	167±16	2.6	2.6	16.6
4Db	$C_{11}H_{23}$	C_8H_{17}	16	8	64	128	128	486±21	7.6	3.8	23.5
4Dc	$C_{11}H_{23}$	$C_{12}H_{25}$	128	128	>128	>128	>128	438±12	<3.4	<3.4	29.1
4Dd	$C_{11}H_{23}$	$C_{18}H_{37}$	512	512	256	>512	512	364±11	1.4	<0.7	35.9
4Ea	$C_{17}H_{35}$	C_3H_7	32	16	64	512	512	234±4	3.7	0.5	26.5
4Eb	$C_{17}H_{35}$	C_8H_{17}	512	64	128	512	512	200±31	1.6	0.4	32.2
4Ec	$C_{17}H_{35}$	$C_{12}H_{25}$	512	256	256	512	512	124±2	0.5	0.2	35.8
4Ed	$C_{17}H_{35}$	$C_{18}H_{37}$	N.D.[d]	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	41.8
4Fb	Ph	C_8H_{17}	128	128	128	128	128	368±6	2.9	2.9	12.3
4Fc	Ph	$C_{12}H_{25}$	4	4	4	4	64	195±13	49	49	19.7
4Gb	*t*-Bu	C_8H_{17}	256	256	512	256	512	775±22	1.5	3.0	11.7
4Gc	*t*-Bu	$C_{12}H_{25}$	8	8	4	8	16	394±28	99	49	19.8
4Hb	Naph	C_8H_{17}	32	16	32	16	64	311±31	9.7	19	14.4
4Hc	Naph	$C_{12}H_{25}$	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	21.6

MSI-78	---	64[e]	8-16[e]	16-32[e]	---	120[f]	7.5-15	3.8-7.5	---
Tetracycline[g]	8-32	---	0.12-1	0.5-2	8-32	---	---	---	---
Gentamicin[g]	4-16	---	0.12-1	0.25-1	0.5-2	---	---	---	---
Methicillin[g]	>16	---	0.5-2	---	---	---	---	---	---

MIC *vs* multidrug-resistant bacteria (μg mL^{-1})

			LR-MRSA[h]	GR-CREc[i]	HC_{50} (μg mL^{-1})	TI *vs* LR-MRSA	TI *vs* GR-CREc	Retention time (min)
4Fc	Ph	$C_{12}H_{25}$	4	4	195±13	49	49	19.7
4Gc	*t*-Bu	$C_{12}H_{25}$	4	8	394±28	99	49	19.8
Gentamicin[j]			---	128	---	---	---	---
Methicillin[j]			>1024	---	---	---	---	---

[a] Conservative estimates of at least three independent trials. [b] HC_{50} is defined as the concentration of compound that kills 50% red blood cells. Standard errors obtained by nonlinear regressions of average data (three independent trials for each compound) are reported. [c] Adjusted retention time, computed as the instrumental retention time minus the hold-up time. [d] N.D. stands for "not determined". [e] Values taken from Ref. 78. [f] Value taken from Ref. 34. [g] Values taken from Ref. 73. [h] Linezolid- and methicillin-resistant *S. aureus* AOUC-0915. [i] Gentamicin- and colistin-resistant *E. coli* 288328. [j] Values determined in this work.

As expected from the wide dimensional range of substituents used, MICs vary to a very large extent. As an example, while compound **4Ba** (R^1 = CH_3, R^2 = C_3H_7) having the shortest chains is almost completely inactive (e.g. MICs = 1024 μg mL^{-1} *vs S. aureus* and 2048 μg mL^{-1} *vs E. coli*), likely due to a poor interaction with the apolar portions of phospholipid bilayer, compound **4Cb** (R^1 = *n*-C_7H_{15}, R^2 = *n*-C_8H_{17}) shows a good activity (MICs = 16 μg mL^{-1} *vs S. aureus* and *E. coli*), and the MIC increases again for too long chains, as for **4Cd** (R^1 = *n*-C_7H_{15}, R^2 = *n*-$C_{18}H_{37}$, MICs >256 μg mL^{-1} *vs S. aureus* and *E. coli*), **4Dd** (R^1 = *n*-$C_{11}H_{23}$, R^2 = *n*-$C_{18}H_{37}$, MICs = 256 μg mL^{-1} *vs S. aureus* and >512 μg mL^{-1} *vs E. coli*), and **4Ec** (R^1 = *n*-$C_{17}H_{35}$, R^2 = *n*-$C_{12}H_{25}$, MICs = 256 μg mL^{-1} *vs S. aureus* and 512 μg mL^{-1} *vs E. coli*). Even in this case, the necessity to avoid either too small or too large apolar fragments parallels the findings for amphiphilic antibacterials with different dimensions, such as polymers [77], foldamers [23], and small peptoids [52]. The best MICs (**4Fc**, R^1 = Ph, R^2 = *n*-$C_{12}H_{25}$, 4 μg mL^{-1} *vs S. aureus* and *E. coli*, and **4Gc**, R^1 = *t*-Bu, R^2 = *n*-$C_{12}H_{25}$, 4 μg mL^{-1} *vs S. aureus* and 8 μg mL^{-1} *vs E. coli*), are equal to or better than the values for the magainin derivative MSI-78 (pexiganan, MIC = 8-16 μg mL^{-1} *vs S. aureus* and 16-32 μg mL^{-1} *vs E. coli*) [78], which is also more hemolytic (HC_{50} = 120 μg mL^{-1}) [34]. Despite the lack of any thorough optimization, these extremely simple and mono-charged

compounds are competitive toward much more complex and expensive 12- and 14-helix foldamers (best MICs = 3.1-3.2 μg mL^{-1} *vs S. aureus*) [23,24], and either isobutylene-maleimide or guanidinium-oxanorbornene polymers (best MICs = 3-12 μg mL^{-1} *vs S. aureus*) (Figure 1) [56,57]. Taking into consideration small molecules, *α*-hydrazido acids outperform tricationic tripeptides based on 2,5,7-tri-*tert*-butyl tryptophan in terms of both antimicrobial activity and therapeutic window [32], whereas only the most effective among tetracationic triaryl derivatives (best MICs = 0.78 μg mL^{-1} *vs S. aureus*, 3.13 μg mL^{-1} *vs E. coli*; best therapeutic indices = 406 *vs S. aureus*, 172 *vs E. coli*) give better overall results [42] (Figure 1). Even some among the structurally very simple lysine-based monomeric peptoids show a slightly more powerful antimicrobial efficacy (best MICs = 2.2 μg mL^{-1} *vs S. aureus*, 2.9 μg mL^{-1} *vs E. coli*), but despite the double positive charge they are less selective toward erythrocytes and have worst therapeutic indices [52] (best TIs = 34 *vs S. aureus*, 23 *vs E. coli*) (Figure 1).

It is worth noting that these *α*-hydrazido acids show identical activities towards two multidrug resistant strains, the linezolid- and methicillin-resistant *S. aureus* AOUC-0915 (LR-MRSA, MICs = 4 μg mL^{-1} for both **4Fc** and **4Gc**) and the gentamicin- and colistin-resistant *E. coli* 288328 (GR-CREc, MICs = 4 μg mL^{-1} for **4Fc** and 8 μg mL^{-1} for **4Gc**, Table 1). This is not surprising for LR-MRSA, whose resistances to methicillin and linezolid rely, respectively, on the presence of PBP2a, a penicillin binding protein with extremely low affinity for all *β*-lactams, except for last-generation cephalosporins [79], and an rRNA methyltransferase that catalyzes post-transcriptional methylation to the C8 position of nucleotide A2503 in 23S rRNA, causing a decreased binding affinity for linezolid [80]. Thus, the phospholipidic bilayers of LR-MRSA membrane are not substantially changed in comparison to the drug sensitive counterpart, *S. aureus* ATCC 29213. On the other hand, the conservation of efficacy of these cationic *α*-hydrazido acids toward GR-CREc was not predictable. In fact, whereas the resistance to gentamicin of GR-CREc is due to the *aac(3)-IIa* gene, which causes the covalent modification of gentamicin and lead to a poor binding to the ribosome target [81], the resistance to colistin is due to the MCR-1 protein, which lead to addition of phosphoethanolamine to lipid A. Consequently, the binding between the less negatively charged lipopolysaccharide (LPS) and the positively charged colistin is much less effective [82], and the same reduced binding affinity was demonstrated to be at the basis of an intrinsic resistance to cationic AMPs [83]. However, in the present case the possible decrease in electrostatic interaction between the cationic *α*-hydrazido acids and the outer membrane external leaflet, that is the first mandatory step for the following insertion and damaging action on phospholipid bilayers, does not appear to be detrimental for the antimicrobial activity. This observation suggests that

these new amphiphilic organic compounds might also be active against other MDR strains with the same compositions of lipid bilayers.

As far as hemolytic activity is concerned, it is evident from data in Table 1 that the smallest and less active compounds show larger HC_{50} values (e.g. **4Ba**, $R^1 = CH_3$, $R^2 = C_3H_7$, has $HC_{50} = 2700$ μg mL^{-1}), while the most active species with medium-sized R^1 and R^2 chains are also more active against red blood cells. However, the two most powerful antibacterial compounds (**4Fc**, R^1 = Ph, R^2 = *n*-$C_{12}H_{25}$, HC_{50} = 195 μg mL^{-1}, and **4Gc**, R^1 = *t*Bu, R^2 = *n*-$C_{12}H_{25}$, HC_{50} = 394 μg mL^{-1}) have hemolytic concentrations that are much higher than their MICs, thus leading to substantially better therapeutic indices than all the other compounds. In addition, the percent hemolysis computed at a concentration equal to the MIC for either *S. aureus* or *E. coli* was also evaluated using the parameters obtained from the non-linear regression of experimental hemolysis data by the Hill equation [84,85] (Table S2). From these data, it is easy to appreciate that also some less selective compounds with poorer therapeutic indices actually show extremely low percent hemolysis at MICs. As an example, compound **4Ab** (R^1 = OBn, $R^2 = C_8H_{17}$) has low TIs toward both *S. aureus* and *E. coli* (3.0, Table 1), but the computed percent hemolysis at MICs (64 μg mL^{-1}) are almost null (9 10^{-3}%, Table S2). Interestingly, contrarily to antibacterial efficacy, the hemolytic activity does not decrease when large *N*- and *C*-terminal substituents are present (e.g. **4Ec**, $R^1 = C_{17}H_{35}$, $R^2 = C_{12}H_{25}$, HC_{50} = 124 μg mL^{-1}), and has a more erratic general behavior. Both of these characteristics, taken together with the wide dimensional range of substituents considered, are directly involved in the very large interval of therapeutic indices obtained for all bacteria (e.g. from 0.5 to 99 for *S. aureus*).

It is easy to find the first structure-activity relationship considering only compounds **4A-Ea-d**, for which all the possible twenty combinations of R^1 (OBn, methyl, heptyl, undecyl, or heptadecyl) and R^2 (propyl, octyl, dodecyl, or octadecyl) were synthesized. For the shortest R^1 tail (Me), there is a striking improvement in antibacterial efficacy toward *E. faecium* passing from R^2 = propyl (MIC = 2048 μg mL^{-1}) to the octyl (MIC = 128 μg mL^{-1}), and then the dodecyl (MIC = 32 μg mL^{-1}) and octadecyl (MIC = 32 μg mL^{-1}) chains (Figure 4A, see Figures S1-S5 for the other bacteria). On the contrary, for the longest R^1 (*n*-$C_{17}H_{35}$), the best-performing R^2 fragment is the propyl (MIC = 16 μg mL^{-1}), whereas a continuous increase in MICs is observed as the length of R^2 increases. For all the other R^1, there is a clear decrease of antibacterial potency at both sides of the R^2 that best matches the particular R^1. The same findings can be deduced considering how MICs vary according to different R^1 moieties, taking constant R^2 group (Figure 4B). Analyzing the complete series of graphs (Figures S1-S5), it is also worth noting that *enterococci* are more susceptible to compounds having *N*- and *C*-terminal chains

longer than those required to inhibit growth of *S. aureus*, which in turn is more sensitive to chains longer than those necessary against Gram-negative bacteria. For example, when R^1 = *n*-$C_{11}H_{23}$, the best activities toward *E. faecalis* and *E. faecium* are obtained for R^2 = *n*-C_8H_{17}, while in the case of *S. aureus* R^2 = *n*-C_3H_7 and *n*-C_8H_{17} show the same potency, and for *E. coli* and *P. aeruginosa* the shortest R^2 furnished the lowest MICs (Figures S1-S5).

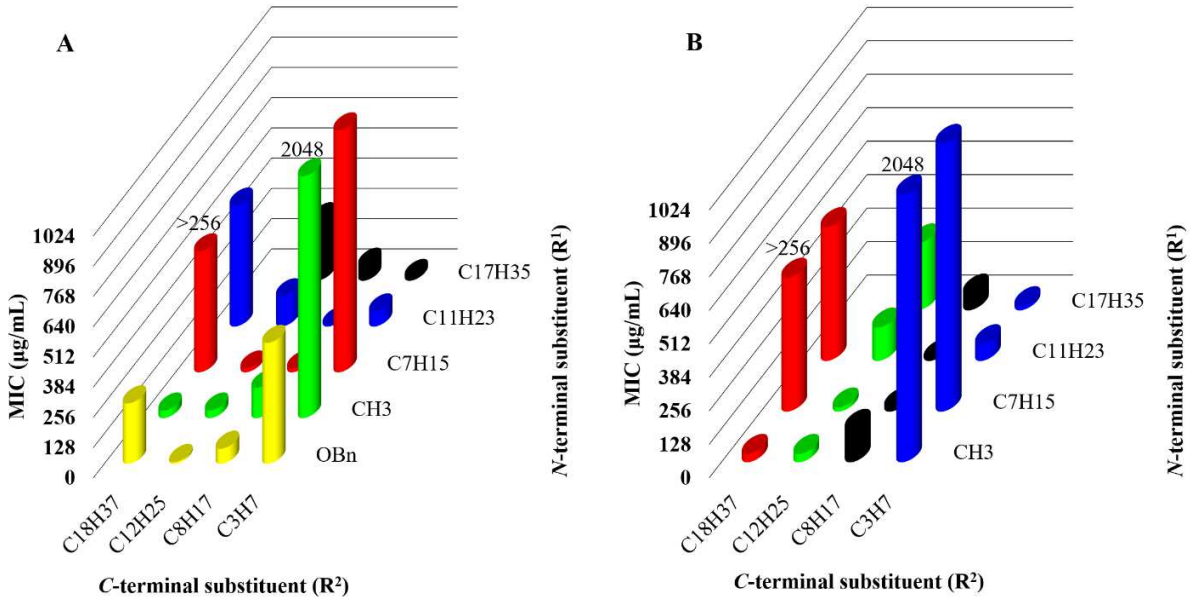


Figure 4. Variation of antimicrobial activity toward *E. faecium* with R^1 and R^2 substituents, for compounds **4A-Ea-d**. (A) bar graphs colored taking constant R^1 groups, highlighting the dependence of antimicrobial activity on R^2 length. (B) bar graphs colored taking constant R^2 groups, highlighting the dependence of antimicrobial activity on alkyl R^1 length (compounds **4Aa-d**, with R^1 = OBn , are not reported).

Figure 4 was useful for comparing homogenous series of compounds with the same type of substituents, but after the initial investigation we introduced *ad hoc* variations in R^1 groups, taking constant the two most active *C*-terminal R^2 groups, the octyl and dodecyl chains (Table 1). A possible way to develop a more general model for antibacterial activity, and also for hemolytic activity, is to use the reverse-phase HPLC-based (RP-HPLC) overall lipophilicity, in the form of either the retention time [32,42,52,86] or the percentage of less polar eluent at retention time [29,30]. This kind of structure-activity relationship highlights that, for all the bacteria tested, there is a parabolic trend of MICs toward retention time (Figures 5A and 5B for *S. aureus*, see Figure S6 for other bacteria).

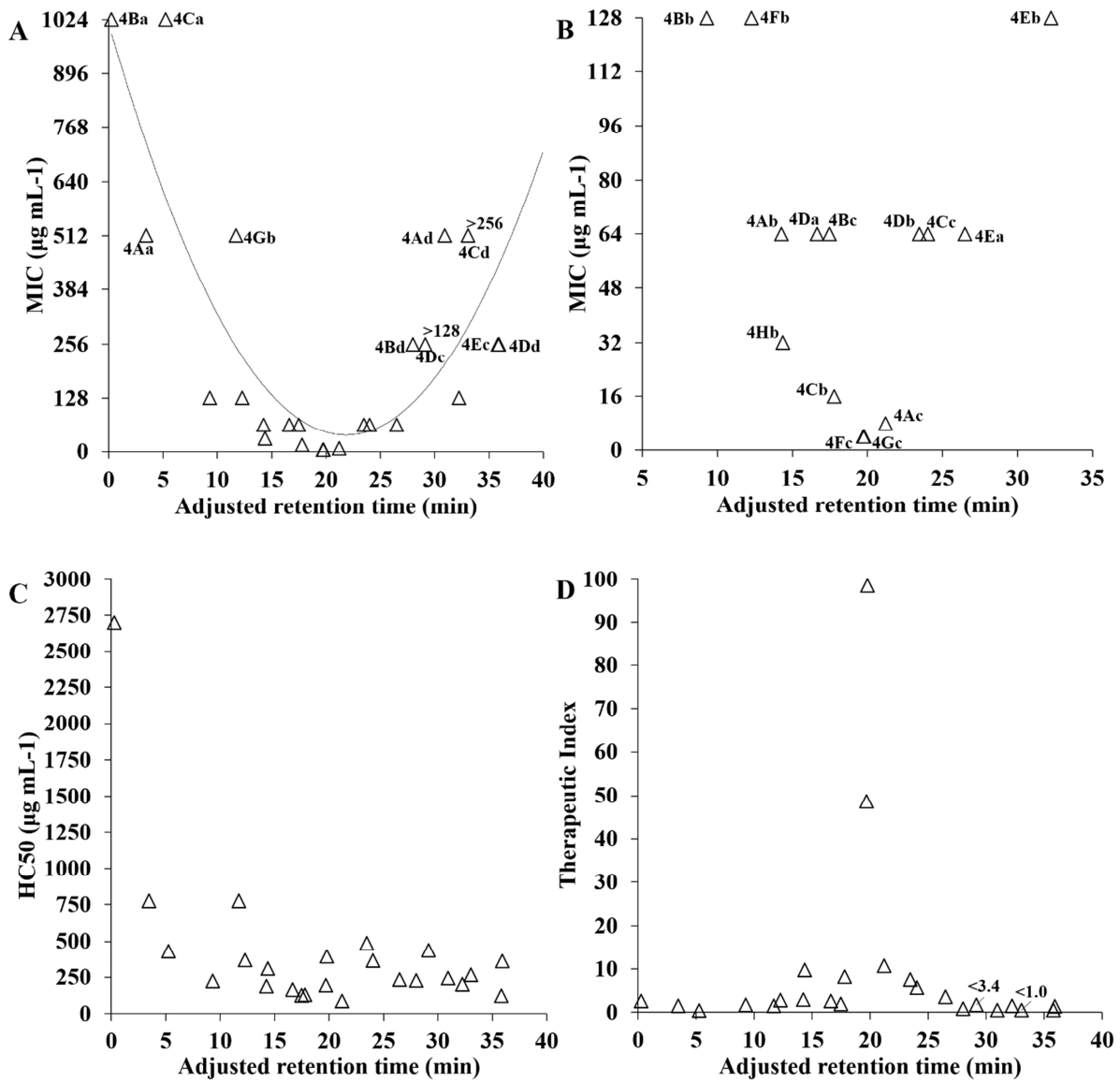


Figure 5. Variation of (A,B) antibacterial activity toward *S. aureus* (two different vertical scales), (C) hemolytic activity, and (D) therapeutic index toward *S. aureus* with adjusted retention time (instrumental retention time minus the hold-up time; Hewlett-Packard Lichrosorb RP 18 column, 5 µm, L × I.D. 200 × 4.6 mm, flow rate = 0.5 mL min^{-1}; elution gradient: from water:2-propanol = 70:30 to water:2-propanol = 10:90 in 40 minutes).

Of course, due to the fact that MICs determined with the adopted protocol were measured in powers of 2 [73], and that in some cases only minimum theoretical values corresponding to the highest concentration tested were available, coefficient of determination, r^2, indicating a very good parabolic interpolation could not be expected. However, r^2 of 0.88, 0.80, 0.68, 0.76, and 0.63 for *E. faecalis*, *E.*

faecium, *S. aureus*, *E. coli*, and *P. aeruginosa*, respectively, still confirm the visual impression that MIC varies with retention time with an approximately parabolic trend and, most important, that overall lipophilicity is by far the main parameter governing the antimicrobial activity for these compounds.

Albeit this finding is of obvious practical importance for directing the future synthesis of most active compounds in a more effective way, a simple interpretation of the observed raise of MICs after the optimal retention time ranges cannot be done. Of course, it is striking that the reason must relies on the differences between the ability of these compounds to simply bind to covalently linked monolayers of linear C18 hydrocarbon chains in a RP-HPLC column, and their capability to be attracted to the external bacterial surface, reach the fluid double layers of differently composed membranes, insert and then weaken or damage them by one or more of the known mechanisms. In fact, while the former clearly appears to be a monotonically raising additive function of van der Waals interactions, the latter is a much more complex phenomenon that encompasses many different molecular movements and interactions. Depending on the particular mechanism, different dispositions of amphiphilic compounds are required for either a regular pores formation, a generalized membrane weakening/permeabilization, or a less ordered membrane dissolution/destruction with a detergent-like effect [7-10]. Thus, it is not obvious that the ability of these compounds to kill bacteria must always increase as the interactions with the apolar stationary phase in a RP-HPLC strengthen. In addition, even if overall lipophilicity is evidently the main parameter for quantitatively describing the growth-inhibiting effect of these amphiphilic compounds, there must also be secondary variables, such as substituent-specific effects relying on shape and/or electronic features, which can hardly be described at the moment. In fact, for all the bacteria there are some cases in which compounds with very close retention times have different antibacterial activities, as well as cases of compounds with fairly different retention times that show the same MICs (Figure 5 and Figure S6).

The higher sensitivity of Gram-negative bacteria to shorter alkyl chains than those required for Gram-positives, previously highlighted analyzing complete homogeneous series in Figure 4 and Figures S1-S5, can be deduced here in a more comprehensive way taking into consideration the ranges of retention times in which compounds show good antimicrobial efficacy. In fact, retention time ranges where MICs drop below a given value (e.g. $\leq$64 μg mL^{-1}) in Figure 5 and Figure S6, become narrower and globally shift toward lesser values passing from *E. faecium* (14.3-32.2 minutes), to *E. faecalis* (14.4-28.0 minutes), to *S. aureus* (14.3-26.5 minutes), to *E. coli* (14.3-21.2 minutes), and eventually to *P. aeruginosa* (14.4-19.7 minutes). To the best of our knowledge, in all cases available in literature of RP-HPLC-derived overall lipophilicity measured for cationic antibacterial amphiphiles, such a clear

parabolic relationship has never been found, and only a few cases of increases in MICs for large lipophilic chains have been reported so far. However, this lack of previously reported similar behaviors could be due to the usually much smaller dimensional span of hydrophobic moieties investigated, thus generally leading to a monotonic increase in antibacterial activity as the overall lipophilicity increases [30,32,42,86]. Only in one case for lysine-based monomeric peptoids [52] (Figure 1), and in two cases for *N*-terminal alkylated oligopeptoids [29], a sharp decrease of antimicrobial efficacy with extended retention times was evidenced for structurally homogenous backbones bearing too large lipophilic portions.

In contrast to the approximately parabolic trend of minimum inhibitory concentrations, HC_{50} values show a sharp decrease as soon as the overall lipophilicity starts increasing and then, for the remaining range of retention times, they have a quite erratic behavior but without any overall reduction or increase (Figure 5C). This difference with respect MICs is also readily apparent directly analyzing only data for compounds belonging to the complete series with all the combinations of R^1 and R^2 used in the initial investigation (Table 1 and Figure S8). As an example, within the series of compounds **4Ca-d** (R^1 = *n*-C_7H_{15}), **4Da-d** (R^1 = *n*-$C_{11}H_{23}$), and **4Ea-c** (R^1 = *n*-$C_{17}H_{35}$), the variation of hemolytic concentration with R^2 groups is strikingly different from the homogenous trends of MICs toward any of the bacteria tested, as reported for *E. faecium* in Figure 4 (see also Figures S1-S5). The same applies to the dependence of HC_{50} on R^1 groups, taking into consideration compounds having the same R^2 chains (Figure S8), thus confirming a somewhat more unpredictable behavior of hemolytic activity. In addition, there are no other evident variables, such as shape or electronic features, identifying subgroups of clearly more (or less) hemolytic compounds.

Due to the peculiar and extremely different trends of MIC and HC_{50} with respect to retention time, the resulting pointed graphs of computed therapeutic indices (TI = HC_{50}/MIC) as a function of overall lipophilicity are not surprising (Figure 5A for *S. aureus*, Figure S7 for other bacteria), and exactly the same behavior has already been found for analogs of antimicrobial peptide gramicidin S [87]. For all bacteria, compounds having too short or too long retention times invariably show extremely low TIs, thus being completely unsafe. Only within the range of optimal overall lipophilicity, corresponding to the range of moderate to good antimicrobial activity (approximately from 14 to 27 minutes toward *S. aureus*), α-hydrazido acids can show a better in vitro selectivity, confirming the usefulness of this quantitative evaluation of overall lipophilicity by means of adjusted retention times. Obviously, due to the definition of TI itself, the wider or narrower ranges of best therapeutic indices toward other bacteria must parallel the wider intervals of overall lipophilicity furnishing good antimicrobial activity toward

enterococci, as well as the narrower intervals toward Gram-negative strains. In addition, based on the general order of sensitivity of bacteria to these compounds reported above (Table 1), TI values for many amphiphilic *α*-hydrazido acids decrease in the order *E. faecium* > *E. faecalis* > *S. aureus* > *E. coli* > *P. aeruginosa*. However, mainly due to the fluctuating behavior of hemolytic activity, even within the optimal range for each bacterium there are some compounds showing a fairly reduced therapeutic window (Figures 5A and S7, Tables 1 and S1). The two most powerful antibacterials, **4Fc** (R^1 = Ph, R^2 = *n*-$C_{12}H_{25}$, TIs = 49 for both *S. aureus* and *E. coli*) and **4Fc** (R^1 = *t*-Bu, R^2 = *n*-$C_{12}H_{25}$, TIs = 99 for *S. aureus* and 49 for *E. coli*), are by far the most selective compounds and have almost identical retention times, thus they appear as a sort of spike in most of the graphs. They also show the same good selectivity when MDR bacteria are taken into consideration (Table 1), and **4Fc** is the only one having a quite good therapeutic index for *Pseudomonas aeruginosa* (25), toward which all the other compounds show much poorer TIs (<5). The only remarkable exception to their superiority is *α*-hydrazido acid **4Db** (R^1 = *n*-$C_{11}H_{23}$, R^2 = *n*-C_8H_{17}) toward *E. faecium*, whose highest therapeutic index, 61 (Table S1), is caused by both its high activity toward that bacterium and its inherently low hemolytic potency.

2.4 Permeabilization of outer and inner membranes

To ascertain beyond doubt the permeabilizing and destabilizing action on bacterial membranes of *α*-hydrazido acid amphiphiles, we slightly modified previously reported procedures [49] and used compound **4Gc** (R^1 = *t*-Bu, R^2 = *n*-$C_{12}H_{25}$) at its MIC as a model system toward both the susceptible (ATCC 25922) and the MDR (GR-CREc, 288328) *E. coli* strains (Figure 6). *N*-phenyl-1-naphthylamine (NPN) was chosen as a fluorescent probe for the outer membrane permeabilization, due to its gain in fluorescence when passing from an aqueous solution to a hydrophobic environment (i.e. the membrane lipid bilayers), whereas propidium iodide (PI) was chosen as an indicator of inner membrane permeabilization, because its fluorescence is greatly enhanced when it binds to nucleic acids. The fluorescent probes showed a null (NPN) or extremely reduced (PI) uptake in absence of **4Gc**, while rapid increases in normalized fluorescence were observed in both cases by addition of the amphiphile to suspensions containing either the sensitive (Figure 6 A) or the MDR strain (Figure 6 B), thus plenty confirming the permeabilizing and damaging action of *α*-hydrazido acid hydrochlorides on bacterial membranes.

Even if the raise in normalized fluorescence for the susceptible ATCC collection strain seems to be extremely reduced for both probes in comparison to the gentamicin- and colistin-resistant GR-CREc

strain, this is mainly due to substantially different starting points in terms of absolute fluorescence intensity (see Figures S12-S15). The computed permeabilization rate constants must therefore be considered in order to effectively compare the behavior of compound **4Gc** toward the two strains. At least with these experimental conditions and for the time considered (10 minutes), the permeabilization of both membranes always demonstrated to follow an exponential raise to maximum ($r^2 \geq 0.99$), and the actual rate constants computed for outer membrane (Figures S12-S13) and inner membrane (Figures S14-S15) permeabilization are quite close for the two strains.

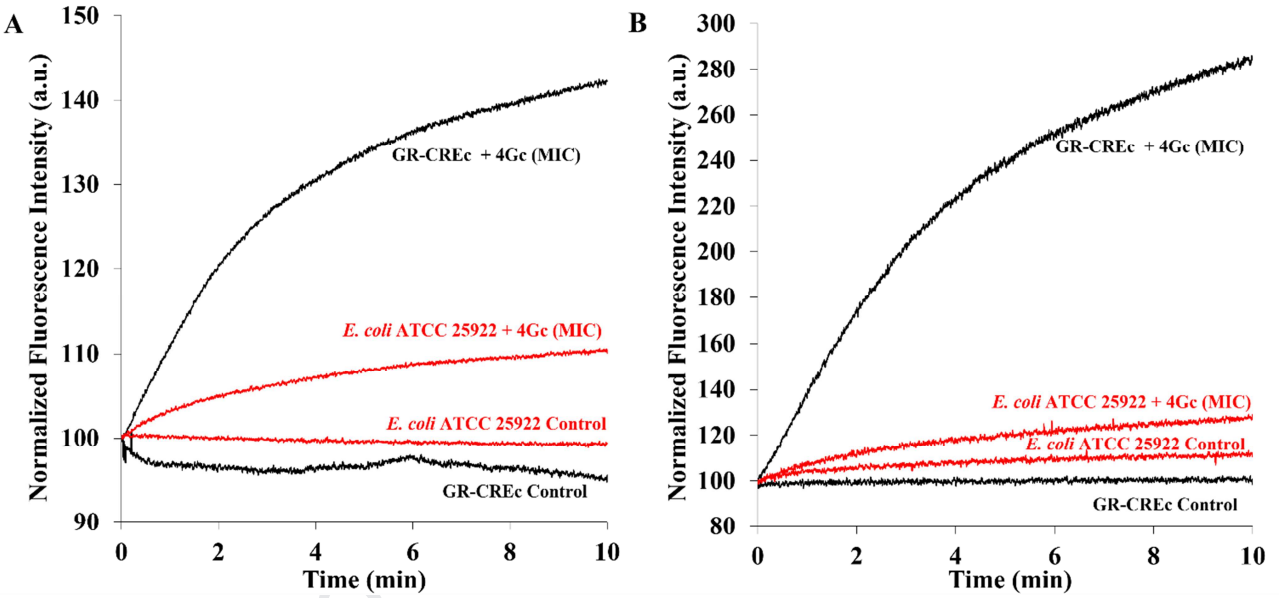


Figure 6. Variation of (A) NPN and (B) PI fluorescence with time, as a measure of outer and inner membrane permeabilization, respectively, caused by compound **4Gc** [red: *E. coli* ATCC 25922; black: GR-CREc (*E. coli* 288328)].

Interestingly, the outer membrane permeabilization rate constant measured by NPN uptake for GR-CREc (0.31 min^{-1}) resulted to be about 1.2 times greater than for the sensitive bacterium (0.25 min^{-1}), whereas for the inner membrane permeabilization the rate constant of PI uptake for GR-CREc (0.24 min^{-1}) was about 1.1 times greater than for *E. coli* ATCC 25922 (0.22 min^{-1}). Thus, not only the less negatively charged LPS of GR-CREc was unable to prevent the action of these amphiphiles more effectively than that of susceptible *E. coli*, but it resulted even more permeable to *α*-hydrazido acid hydrochlorides. This is in agreement with the identical MIC values of compounds **4Fc** and **4Gc** obtained against either susceptible or resistant *S. aureus* and *E. coli* strains (Table 1).

2.5 Stability toward enzymatic and chemical degradation

One of the main issues of AMPs, that is their sensitivity to proteolytic degradation [88], could not be excluded *a priori* for *α*-hydrazido acids, due to the presence of three different potentially hydrolysable carbonyl-nitrogen bonds, one of which linking the N^{α} atom to a glycine side chain. Thus, the minimum inhibitory concentrations toward *S. aureus* of compounds **4Cb** (R^1 = *n*-C_7H_{15}, R^2 = *n*-C_8H_{17}), **4Fc** (R^1 = Ph, R^2 = *n*-$C_{12}H_{25}$), and **4Gc** (R^1 = *t*-Bu, R^2 = *n*-$C_{12}H_{25}$), were evaluated after preincubation in fresh 50% blood plasma solution for 0, 3 and 6 hours at 37 °C [52].

Albeit MICs of both compounds **4Fc** and **4Gc** were subjected to a two-fold increase in presence of blood plasma, whereas for **4Cb** no variation was observed, it is very remarkable that all antimicrobial activities remained constant even after 6 hours preincubation (Figure 7), therefore demonstrating the resistance to proteolytic degradation for physiologically relevant time intervals.

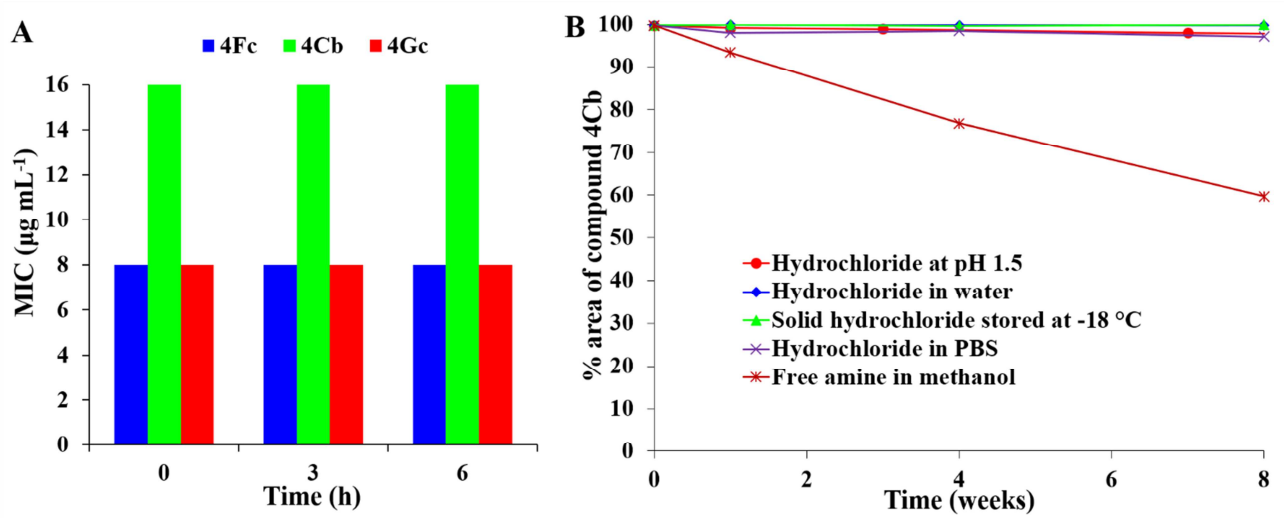


Figure 7. (A) MICs toward *S. aureus* of compounds **4Cb**, **4Fc**, and **4Gc**, after preincubation in 50% blood plasma solution for 0, 3 and 6 h at 37 °C. Conservative estimates of three trials for each compound. (B) Percent areas of samples of compound **4Cb**, stored in different conditions at room temperature, unless otherwise indicated (Hewlett-Packard Lichrosorb RP 18 column, 5 µm, L × I.D. 200 × 4.6 mm, flow rate = 0.5 mL/min; eluent water:2-propanol = 50:50).

The stability toward chemical degradation in different conditions was also important in view of a potential practical implementation, and some decomposition had already been noted for compounds stored for prolonged periods (>3 months) at -18 °C as free amines. Without re-salification after the chromatographic purification, the main side reaction in diluted conditions was demonstrated to be the transacylation, with transfer of *N*-terminal acyl group to the glycine amine functionality by means of an

intramolecular nucleophilic acyl substitution (see synthesis of compound **S1** in Supplementary Data). On the other hand, solid free amines intentionally stored at room temperature for more than one month also underwent intermolecular attacks and gave complex mixtures of products. Conversely, solid hydrochlorides demonstrated to be completely stable at -18 °C for at least 6 months. To better evaluate resistance to chemical degradation, samples of compound **4Cb** (R^1 = *n*-C_7H_{15}, R^2 = *n*-C_8H_{17}) as hydrochloride (1 μg mL^{-1}) in PBS (pH = 7.4), pure water, and an aqueous solution mimicking the lowest value for the stomach pH range (1.5), were stored at room temperature for 8 weeks, together with a methanolic solution of the free amine (Figure 7). Starting with a freshly prepared compound having a 99.7% purity, based on HPLC areas, the hydrochloride in water did not show any decomposition up to the maximum time, whereas only marginal changes were experienced by the samples in PBS and acidic solution (97.1% and 97.2% final purities, respectively). As expected, the methanolic solution of free amine was subjected to a pronounced degradation, with a loss of purity of more than 6% within the first week and about 40% after 8 weeks.

2.6 Importance of hydrazide NH and non-disrupted amphiphilicity

As initially reported, a series of six *N*-methylated compounds, whose parent unmethylated hydrochlorides showed a wide range of antimicrobial activities, was synthesized with the aim of verifying if the effective facial segregation of hydrophilic and lipophilic faces and the presence of highly positive hydrazide NH hydrogens were decisive in favoring a good antimicrobial activity.

Comparing the antibacterial activities of NMe hydrochlorides in Table 2 with the MICs of the corresponding unmethylated compounds in Table 1, it is easy to see that in only one case the *N*-methylated version is the most active (hydrochlorides **4Ac**, MIC = 16 μg mL^{-1} , and **6Ac**, MIC = 8 μg mL^{-1}, against *E. faecalis*), and in few other cases NMe derivatives have the same potency than their parent NH compounds.

Table 2. In Vitro Antibacterial Activities of *N*-Methylated *α*-Hydrazido Acids

			MIC *vs* drug sensitive bacteria (μg mL^{-1})[a]					
Compd	**R^1**	**R^2**	***E. faecalis***	***E. faecium***	***S. aureus***	***E. coli***	***P. aeruginosa***	**Retention time (min)[b]**
6Ab	OBn	C_8H_{17}	128	128	128	128	256	13.8
6Ac	OBn	$C_{12}H_{25}$	8	8	8	64	512	20.7

6Bb	CH_3	C_8H_{17}	1024	1024	2048	2048	2048	9.1
6Bc	CH_3	$C_{12}H_{25}$	32	32	64	128	256	17.1
6Gb	*t*-Bu	C_8H_{17}	512	512	512	256	512	11.7
6Gc	*t*-Bu	$C_{12}H_{25}$	16	16	16	64	512	18.9

[a] Conservative estimates of at least three independent trials. [b] Adjusted retention time, computed as the instrumental retention time minus the hold-up time.

However, in most cases there is a great increase (from two-fold to sixteen-fold) in minimum inhibitory concentrations, and the overall decrease in activity toward Gram-negative bacteria is more pronounced than that against Gram-positives. For all NMe compounds there is also a reduction up to 0.9 minutes in retention times, with respect to their NH counterparts. This decrease in the RP-HPLC measured overall hydrophobicity could be due to the contribution of a low energy conformation with an unconventional C=O□□□H-C hydrogen bond between the *C*-terminal hydrazide carbonyl and one of the slightly positive *N*-methyl hydrogens, as computationally demonstrated for the model *N*-methylated ammonium cation mAc-Hydr(Me)GlyH$^+$-NHMe (see Supplementary Data). This conformation has a worst directionality of the hydrophobic R^1 and R^2 groups, compared to the hydrazido-turn arrangement, thus probably leading to a poorer interaction with both the C18 hydrocarbon chains of the stationary phase in the HPLC column, and the lipid portions of membrane bilayers.

Despite the observed reductions in overall lipophilicities, it must be pointed out that retention times for the most active compounds in Table 2 (**6Ac**, **6Bc**, and **6Gc**) are well within the optimal ranges determined for unmethylated hydrochlorides **4**. Thus, overall hydrophobicity still remains the main parameter for describing the antimicrobial activity even in the case of *N*-methylated hydrochlorides, but other concepts must be invoked in order to explain the deleterious effect of such an apparently small structural change. To this end, the two most stable unmethylated and *N*-methylated hydrazido-turn conformers of either the simplified model compound (R^1 = R^2 = Me) or **4Cb** (R^1 = *n*-C_7H_{15}, R^2 = *n*-C_8H_{17}), and their corresponding *N*-methylated versions, were analyzed comparing the electrostatic potential surfaces and the atomic charges (see Supplementary data). All the charge calculations schemes confirmed that, despite the electronwithdrawing hydrazide nitrogen, the three hydrogens of the NMe group in the methylated species only have very small positive charges, therefore being essentially hydrophobic in nature, which was experimentally demonstrated for Boc-protected compounds **5** in deuterochloroform (see proton NMR spectra in Figures S59-S64). On the contrary, for the hydrazide NH of unmethylated compounds high positive charges were invariably obtained. Considering also the

chloride counterion for both **4Cb** and its NMe version allowed to better highlight the partial disruption of amphiphilicity in *N*-methylated compounds, which arises from the substantially lipophilic character of the methyl group on the N^{α} atom (Figure 8, see also Figures S29-S30). Even if it is the smallest alkyl group, the methyl on hydrazide nitrogen behaves like an "hydrophobic bulge", which very likely negatively affects the interaction with phospholipid heads and water molecules during the destabilizing and damaging action on membrane bilayers.

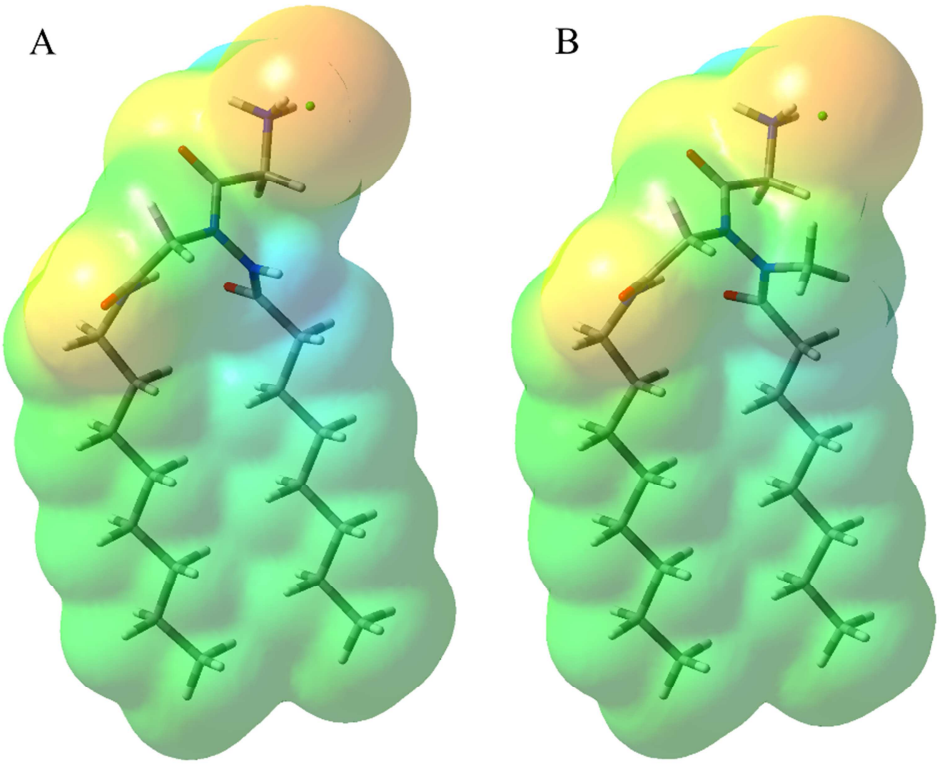


Figure 8. Rear views of electrostatic potential surfaces for the most stable structures of hydrochlorides (A) $C_7H_{15}CO$-HydrGly-NHC_8H_{17} □ HCl (**4Cb**, R^1 = *n*-C_7H_{15}, R^2 = *n*-C_8H_{17}), and (B) its *N*-methylated version $C_7H_{15}CO$-Hydr(Me)Gly-NHC_8H_{17} □ HCl, computed at ωB97X-D3(0)/6-311++g(3df,3pd)/IEF-PCM level in water.

These findings about the decrease of activity induced by a partly disrupted amphiphilic topology are similar and complementary to the results obtained for triaryl derivatives whit an additional pendant lipophilic group R [42] (Figure 1). In both cases, amphiphilicity seems to have a greater importance for the activity against Gram-negative bacteria. In *α*-hydrazido acids amphiphilicity is ruined by addition of a small lipophilic group in the hydrophilic face, with the concomitant loss of a hydrogen bond-donating functionality, whereas in the case of tetracationic triaryl derivatives the negative effect is

obtained by insertion of an amide linker in an otherwise completely hydrophobic region. However, for those triaryl derivatives with facially disrupted amphiphilicity, the almost complete loss of activity against Gram-negative bacteria was ascribed to a much less efficient insertion of the negative curvature-inducing hydrophobic portions, caused by the high content in phosphatidylethanolamine (PE) of Gram-negatives, which is itself a negative-inducing lipid. On the contrary, for the present *α*-hydrazido acids the lipophilic portions are unchanged with respect their unmethylated versions, whereas the *N*-methylation should reduce the overall ability of the polar and charged groups to act as a positive curvature-inducing components. The correct balance between components inducing both positive and negative curvature was demonstrated to be crucial to induce negative curvature in phospholipid bilayers [89], which in turn is necessary for the formation of pores in bacterial membranes [90]. Thus, complementary reasons could be at the basis of the more pronounced decrease in antibacterial efficacy toward Gram-negative bacteria for tetracationic triaryl derivatives and *α*-hydrazido acids.

3. Conclusions

Starting from the idea that an *α*-hydrazido acid skeleton with suitable derivatizations has an inherent propensity to the formation of the hydrazido-turn motif, simple mono-charged amphiphilic derivatives were first computationally analyzed. Then a short, cheap and high-yielding synthesis furnished a number of these novel mimics of antimicrobial peptides, which were submitted to biological assays. The membranolytic action of amphiphilic *α*-hydrazido acids was ascertained, and the most active compounds exhibited a broad-spectrum in vitro activity against a variety of Gram-positive and Gram-negative bacteria, which did not change when multidrug resistant strains were employed. Structure-activity relationships demonstrated that the overall lipophilicity is the main parameter governing the bacteriostatic activity and the selectivity with respect hemolysis, whereas the change of a hydrazide NH in favor of a methyl group caused both the loss of a good hydrogen bond donor and the partial disruption of amphiphilicity, thus reducing the antibacterial activity. Considering that the results reported here were obtained without performing any optimization in the search for the best bioactivity profile, we believe that these chemically and proteolytically stable *α*-hydrazido acids are promising lead compounds for the development of a new class of wide-spectrum antibiotics. Further studies are ongoing to evaluate the effect of di- and tricationic side chains, as well as different *N*- and *C*-terminal substituents, in order to find safer and more active compounds.

4. Experimental section

4.1 Procedures for biological assays

4.1.1 General important notes

All the hydrochlorides of *α*-hydrazido acids are surfactants, and their tendency to form foams very negatively affected the first preliminary evaluations of MICs and HC_{50}s. This was especially true for more concentrated solutions, and was ascertained to be related to the speed of withdrawal and, to a lesser extent, of the addition, causing evident dispersion and unreliability of results. These observations also applied to withdrawal and addition of 0.2 vol % Triton X-100. As an example, when both the withdrawal and the addition of nominal values of 150 μL of 0.2 vol % Triton X-100 solution with a Gilson P200 pipette were conducted employing about 0.5-1 seconds, the weights of solutions actually transferred into the vials for five trials were much less than expected and not constant (126.9±12.5 mg, mean±standard error), as determined with an analytical balance. On the contrary, employing about three seconds for both the withdrawal and the addition steps, the reliability and reproducibility of weights greatly improved (150.3±2.3 mg). To make much more reliable and reproducible the results, a time of at least three seconds was always used in definitive assays for all the withdrawal and addition steps. For the same reasons, the clear and homogeneous stock solutions were not vortexed immediately before the experiments. Another variable that is usually underestimated, that is the rigorous mixing in each well during the serial dilutions, was ascertained to be a secondary factor of error during the preliminary trials.

4.1.2 General procedure for the evaluation of minimum inhibitory concentrations

All tested compounds were dissolved in sterile water at the maximum possible concentration. Bacterial strains were grown for 6 hours in Brain Heart Infusion (BHI) broth and diluted in Mueller-Hinton II (MHII) broth (Oxoid spa, Milan, Italy) to give a final concentration of 1×10^6 cfu/mL. Serial dilutions of the tested compounds in MHII broth were prepared in 96-well microtiter plate (Cellstar, Greiner bio-one, Kremsmünster, Austria) (50 μL per well) and 50 μL of diluted bacterial suspension were added into each well. The wells with bacteria alone were used as positive growth control wells. Tetracycline was used as internal control, starting from a 1024 μg/mL stock solution made from a freshly prepared 10000 μg/mL solution. The plate was aerobically incubated at 37 °C for 24 hours. All

tests were performed in triplicate. The MICs were defined as the lowest concentrations of compounds inhibiting visible growth after 24 hours of incubation.

4.1.3 General procedure for the evaluation of hemolytic concentrations

Hemolysis experiments were performed with a slight modification of a reported procedure [28]. 4 mL of freshly drawn heparinized human blood were diluted with 25 mL of phosphate buffered saline (PBS) pH 7.4, centrifuged at 1000 g for 10 minutes and resuspended in 25 mL of PBS for three times. After washing, the pellet was resuspended in PBS to ~20 vol % and, in a 96-well microtiter plates, 100 μL of erythrocyte suspension were added to 100 μL of different concentrations of the tested compounds (1:2 serial dilutions in PBS) and incubated for 1 hour at 37 °C. The negative and positive controls were 100 μL of PBS and 100 μL of 0.2 vol % Triton X-100, respectively. After incubation, each well was supplemented with 150 μL of PBS and the plate centrifuged at 1.200 g for 15 minutes. The supernatant was diluted 1:60 (5 μl of supernatant in 295 μl of PBS), transferred in a new plate, and its absorbance at a wavelength of 350 nm (A_{350}) was measured using the Synergy HT microplate reader spectrophotometer (BioTek, Winooski, VT, USA). The percent hemolysis was determined as follows: $[(A - A_0)/(A_{total} - A_0)] \times 100$, where A is the absorbance of the test well, A_0 is the absorbance of the negative control, and A_{total} is the absorbance of the positive control. Evaluation of HC_{50} and b slope were carried out by nonlinear regression of the four-parameter logistic model of Hill [56], in all cases were 100% hemolysis was reached in the experiments conducted using stock solutions of the tested compounds at the maximum possible concentration. For compounds for which the 100% hemolysis could not be obtained, the three-parameter logistic model of Hill with the constrain of 100% hemolysis as the final value was used. The errors of the experiments were always less than 10%. The mean values of three replicates were reported for HC_{50} and b slope.

4.1.4 General procedures for the evaluation of outer and inner membrane permeabilization

Outer Membrane Permeabilization Assay. The Perkin Elmer LS 50 spectrometer was used, operating with the following parameters: 350 nm (slit width 10 nm) for excitation and 420 nm (slit width 10 nm) for emission, with a measure every 0.1 seconds for 10 minutes. All measurements were performed at 25 °C on freshly prepared samples, using quartz cuvettes with 10 mm path length. Stock solution of *N*-phenyl-1-naphthylamine (NPN, 0.5 mM) in acetone was prepared weekly and stored at 4 °C in a dark place. Midlog-phase *E. coli* cells (grown for 6 h, 10^8 cells/mL) were harvested (4000 rpm, 10 min at room temperature), washed, and resuspended in PBS buffer at pH 7.2. The bacterial

suspension was stored at 4 °C and used within 3 hours. Then, the cuvette was supplemented with 2.97 mL of bacterial suspension and, after 15 minutes thermostatation at 25 °C, 6 μL of a 0.5 mM solution of NPN in acetone (working concentration 1 μM) and 23.4 μL of a 1024 μg mL^{-1} solution of compound **4Gc** in sterile water (working concentration 8 μg mL^{-1}) were added. After a rapid mixing, the outer-membrane permeabilization was measured by the increase in fluorescence of NPN. Control experiments were performed with (i) 3 mL of bacterial suspension and 6 μL of 0.5 mM solution of NPN, (ii) 2.97 mL of PBS buffer at pH 7.2, 6 μL of a 0.5 mM solution of NPN and 23.4 μL of a 1024 μg mL^{-1} solution of compound **4Gc**, (iii) 3 mL of PBS buffer at pH 7.2 and 6 μL of 0.5 mM solution of NPN, and (iv) 3 mL of PBS buffer at pH 7.2 (see Supplementary Data).

Inner-Membrane Permeabilization Assay. The Perkin Elmer LS 50 spectrometer was used, operating with the following parameters: 535 nm (slit width 10 nm) for excitation and 617 nm (slit width 10 nm) for emission, with a measure every 0.1 seconds for 10 minutes. All measurements were performed at 25 °C on freshly prepared samples, using quartz cuvettes with 10 mm path length. Midlog-phase *E. coli* cells (grown for 6 h, 10^8 cells/mL) were harvested (4000 rpm, 10 min at room temperature), washed, and resuspended in PBS buffer at pH 7.2. The bacterial suspension was stored at 4 °C and used within 3 hours. Then, the cuvette was supplemented with 2.95 mL of bacterial suspension and, after 15 minutes thermostatation at 25 °C, 30 μL of a 1.5 mM solution of propidium iodide (PI, 1 mg mL^{-1} in water, ready to use solution stored at 4 °C in a dark place, working concentration 15 μM) and 23.4 μL of a 1024 μg mL^{-1} solution of compound **4Gc** in sterile water (working concentration 8 μg mL^{-1}) were added. After a rapid mixing, the inner-membrane permeabilization was measured by the increase in fluorescence of PI. Control experiments were performed with (i) 2.97 mL of bacterial suspension and 30 μL of 1.5 mM solution of propidium iodide, (ii) 2.95 mL of PBS buffer at pH 7.2, 30 μL of 1.5 mM solution of propidium iodide and 23.4 μL of a 1024 μg mL^{-1} solution of compound **4Gc**, (iii) 2.97 mL of PBS buffer at pH 7.2 and 30 μL of 1.5 mM solution of propidium iodide, and (iv) 3 mL of PBS buffer at pH 7.2 (see Supplementary Data).

4.1.5 General procedure for the evaluation of antibacterial activity in plasma

S. aureus ATCC 29213 was grown for 6 hours in brain heart infusion (BHI) broth and diluted in Mueller Hinton II broth (Oxoid spa, Milan, Italy) to give a final concentration of 1×10^6 cfu/mL. Fresh human blood cells were centrifuged at 3000 rpm for 5 minutes to separate the plasma from the red blood cells. Three aliquots for each tested compound were dissolved in water at a concentration of 512 μg/mL and diluted twofold in the plasma to reach the final concentration of 256 μg/mL. The aliquots

were preincubated at 37 °C for 0, 3, and 6 hours, and then used to perform MIC assays according to the broth microdilution method in 96-well microtiter plates. The stability of the compounds into the plasma was considered positive in absence of any change of their MIC values among the trials at different preincubation times.

*4.1.6 General procedure for the evaluation of chemical stability of compound **4Cb***

Four 1 mg/mL solutions of compound **4Cb** (free amine in methanol, hydrochloride dissolved in water, PBS, and water at pH 1.5 prepared using phosphoric acid) were stored at room temperature up to 8 weeks (temperature ranging from 20 to 25 °C). Suitable aliquots were taken and analyzed at the times reported in Figure 7. The fifth sample, that is the solid hydrochloride stored at -18 °C, was analyzed taking a small amount of compound (about 1 mg) and dissolving it in methanol. Percent areas of different samples of compound **4Cb** as a function of time were taken on a reverse-phase Hewlett-Packard Lichrosorb RP 18 column, 5 µm, L × I.D. 200 × 4.6 mm, with a flow rate 0.5 mL/min and water:2-propanol = 50:50, both containing 0.1% of trifluoroacetic acid, as the eluent. Elution was continued up to 1 hour. Areas of spikes at the hold-up time were not considered for integration.

4.2 Chemistry

4.2.1 General materials and methods

Melting points were obtained on an Electrothermal apparatus IA 9000 and are uncorrected. Melting points for hydrochlorides were not taken, due to decomposition at about 100 °C for all compounds, which was caused by loss of hydrogen chloride. ^{1}H and ^{13}C NMR spectra were determined at 25 °C on a Varian MR400 spectrometer, at 400 and 100 MHz for ^{1}H and ^{13}C, respectively, in $CDCl_3$ unless otherwise reported. Chemical shifts are reported in ppm relative to residual solvent signals (δ = 7.26 and 77.16 ppm for ^{1}H and ^{13}C NMR, respectively), and coupling constants (J) are given in Hz. Identity of hydrochlorides **4** and **6** was confirmed by elemental analyses performed in triplicates with a Thermo Scientific FLASH 2000 Elemental Analyzer. RP-HPLC analyses were performed with a Hewlett-Packard 1100 chromatograph equipped with a diode-array detector (λ = 210 nm) and a Lichrosorb RP18 5 µm column, L × I.D. 200 × 4.6 mm, using a constant flow rate of 0.5 mL/min. Water:2-propanol mixtures were used as eluents, both solvents containing 0.1% v/v of TFA. The hydrochlorides were dissolved in a water:2-propanol 70:30 mixture (0.5-1 mg/mL) and submitted to a gradient elution from water:2-propanol 70:30 to water:2-propanol 10:90 in 40 min (gradient +1.5% 2-propanol/min),

followed by additional 20 min with water:2-propanol 10:90. After return to the initial eluent composition, the column was re-conditioned for at least 20 min before the following analysis. Adjusted retention times, t'_R, are reported as the instrumental retention time, t_R, minus the hold-up time, t_M, which was determined to be 4.7 minutes for a flow rate of 0.5 mL/min. The purity of hydrochlorides **4** and **6** was ascertained by isocratic elutions, using water:2-propanol mixtures having amounts of 2-propanol 5 or 10% less than the percentages calculated at the adjusted retention times, t'_R, obtained in the gradient elutions. All the analysis were continued up to at least three times the values of the retention times obtained in isocratic elutions. With the exception of compound **4Ba**, which was used as crude product with 95% purity, all the freshly synthesized hydrochlorides **4** and **6** were always determined to have >95% purity by integration of HPLC areas, excluding spikes at the hold-up time. LCMS electrospray ionization mass spectra were obtained with a Finnigan Navigator LC/MS single-quadrupole mass spectrometer, cone voltage 25 V and capillary voltage 3.5 kV, injecting samples dissolved in methanol. Column chromatography was performed using Kieselgel 60 Merck (230-400 mesh ASTM). All the starting compounds were reagent grade and used without further purification. Ethyl acetate and cyclohexane used for chromatographic purifications were distilled at reduced pressure, using a rotary evaporator. Dichloromethane, methanol and dimethylformamide were distilled from calcium hydride, sodium, and phosphorus pentoxide, respectively, under an argon atmosphere. TLC analysis was performed with sheets of silica gel Fluka TLC-PET, using exposure to UV light and immersion in aqueous $KMnO_4$, followed by heating and by possible immersion in H_2SO_4 9 M. Retention factors (R_f) are reported, for a given eluent, as the ratio of the distance moved by the solute to that moved by the solvent. In the case of hydrochlorides **4** and **6**, the R_f factors are referred to free amines.

*4.2.2 Benzyl 2-(2-methoxy-2-oxoethyl)hydrazine-1-carboxylate, Cbz-Hydr-OMe, (**1**).* To a solution of benzyl carbazate (105 mmol, 17.45 g) and methyl bromoacetate (100 mmol, 9.76 mL) in anhydrous DCM (50 mL) at room temperature under inert atmosphere, DIPEA (105 mmol, 18.34 ml) was added. The reaction was stirred for 48 hours. After evaporation under vacuum, the residue was extracted using a mixture of cyclohexane/ethyl acetate 1:1 (400 mL) as the organic phase and water (100 mL). The organic phase was washed with HCl 1 M (2 × 10 mL) and water (10 mL), then the aqueous phases were sequentially extracted with additional 400 mL of cyclohexane/AcOEt 1:1. The second organic phase was washed with HCl 1 M (2 × 10 mL) and water (10 mL), then the reunited organic phases were dried over anhydrous sodium sulphate. After evaporation under vacuum, the crude product was

purified by flash chromatography on silica gel (cyclohexane/AcOEt), obtaining the pure product **1** as a colorless oil in a 96% yield (96 mmol, 22.87 g). R_f = 0.32 (cyclohexane/AcOEt = 1:1). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.69 (s, 2H), 3.72 (s, 3H), 5.13 (s, 2H), 6.93 (bs, 1NH), 7.31 (bs, 1NH), 7.34 (s, 5ArH) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 52.2, 52.6, 67.4, 128.4, 128.5, 128.7, 135.9, 156.9, 171.5 ppm. LCMS: *m/z* = 239.1 $[M+H]^+$, 261.1 $[M+Na]^+$.

*4.2.3 Benzyl 2-((tert-Butoxycarbonyl)glycyl)-2-(2-methoxy-2-oxoethyl)hydrazine-1-carboxylate, Cbz-HydrGly-OMe, (**2**).* To a solution of compound **1** (45 mmol, 10.72 g) in dry DCM (22.5 mL) under inert atmosphere, Boc-Gly-OH (58.5 mmol, 10.04 g) was added and the reaction mixture was thermostated at -20 °C. EDCI (67.5 mmol, 12.94 g) was added and the mixture was stirred vigorously at -20 °C for 1 hour, then the reaction mixture was diluted with AcOEt (400 mL) and water (50 mL). After separation, the organic phase was sequentially washed with HCl 1 M (3 × 10 mL) and saturated solution of Na_2CO_3 (3 × 10 mL). The aqueous phases were sequentially extracted with AcOEt (250 mL) and the organic phase was washed with HCl 1 M (3 × 5 mL) and water (50 mL). The combined organic phases were dried over anhydrous Na_2SO_4, the solvent was evaporated under reduced pressure and the residue was purified by silica gel chromatography (cyclohexane/AcOEt mixtures as eluents) to give the pure compound **2** as a colorless low-melting wax in 86% yield (38.7 mmol, 15.30 g). R_f = 0.50 (cyclohexane/AcOEt = 1:1). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.44 (s, 9H), 3.64 (bs, 1H), 3.73 (s, 3H), 4.09 (bs, 2H), 5.06 (bs, 1H), 5.17 (s, 2H), 5.25 (bs, 1NH), 7.30-7.51 (m, 5ArH+1NH) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 28.4, 42.0, 48.5, 52.6, 68.5, 80.0, 128.5, 128.8, 135.0, 154.8, 155.9, 169.4, 171.9 ppm. LCMS: *m/z* = 396.2 $[M+H]^+$, 418.2 $[M+Na]^+$.

4.2.4 General procedure for the synthesis of C-terminal derivatives

Note: the representative procedure is referred to 1 mmol of starting compound. To a solution of compound **2** (1 mmol) in dry MeOH (2 mL) under inert atmosphere, the suitable amine was added (3 eq. for propylamine and 1.5 eq. for octylamine, dodecylamine and octadecylamine). The reaction was stirred for 7 hours at room temperature for the synthesis of compound **3Aa**, and refluxed for 18, 24 and 36 hours for the syntheses of compounds **3Ab**, **3Ac**, and **3Ad**, respectively. Thus, the volatile species were removed under vacuum and the residue was submitted for three times to the dissolution in few milliliters of anhydrous dichloromethane, followed by in vacuo evaporation. The residue was purified by silica gel chromatography (cyclohexane/AcOEt mixtures as eluents) to give the corresponding pure compound.

*4.2.4.1 Benzyl 2-((tert-butoxycarbonyl)glycyl)-2-(2-oxo-2-(propylamino)ethyl)hydrazine-1-carboxylate, Cbz-HydrGly-NHC$_3$H$_7$, (**3Aa**).* Starting from **2** (2.00 mmol, 791 mg) and following the general procedure, compound **3Aa** was obtained in 94% yield (1.88 mmol, 794 mg) as a white amorphous solid. R_f = 0.55 (cyclohexane/AcOEt 2:8). M.p. = 49-50 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.89 (t, *J* = 7.4 Hz, 3H), 1.42 (s, 9H), 1.45-1.54 (m, 2H), 3.14-3.20 (m, 2H), 3.75-4.51 (m, 4H), 5.17 (s, 2H), 5.24 (bs, 1NH), 6.77 (bs, 1NH), 7.32-7.40 (m, 5ArH), 7.97 (bs, 1NH) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 11.4, 22.5, 28.4, 41.4, 41.9, 52.9, 68.4, 80.0, 128.4, 128.7, 135.1, 156.1, 156.2, 167.9, 172.3 ppm. LCMS: *m/z* = 423.2 $[M+H]^+$, 445.2 $[M+Na]^+$.

*4.2.4.2 Benzyl 2-((tert-butoxycarbonyl)glycyl)-2-(2-(octylamino)-2-oxoethyl)hydrazine-1-carboxylate, Cbz-HydrGly-NHC$_8$H$_{17}$, (**3Ab**).* Starting from **2** (5.00 mmol, 1.98 g) and following the general procedure, compound **3Ab** was obtained in 88% yield (4.41 mmol, 2.17 g) as a white waxy solid. R_f = 0.60 (cyclohexane/AcOEt 2:8). M.p. = 74-76 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.87 (t, *J* = 7.0 Hz, 3H), 1.26 (bs, 10H), 1.42 (s, 9H), 1.46 (bs, 2H), 3.12-3.26 (m, 2H), 3.75-4.47 (m, 4H), 5.17 (s, 2H), 5.25 (bs, 1NH), 6.77 (bs, 1NH), 7.32-7.38 (m, 5ArH), 8.02 (bs, 1NH) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.1, 22.7, 26.9, 28.4, 29.26, 29.29, 31.9, 39.7, 41.88, 41.90, 52.9, 68.4, 79.9, 128.4, 128.68, 128.72, 135.1, 156.0, 156.2, 167.7, 172.2 ppm. LCMS: *m/z* = 493.3 $[M+H]^+$, 515.3 $[M+Na]^+$.

*4.2.4.3 Benzyl 2-((tert-butoxycarbonyl)glycyl)-2-(2-(dodecylamino)-2-oxoethyl)hydrazine-1-carboxylate, Cbz-HydrGly-NHC$_{12}$H$_{25}$, (**3Ac**).* Starting from **2** (5.00 mmol, 1.98 g) and following the general procedure, compound **3Ac** was obtained in 88% yield (4.39 mmol, 2.41 g) as a white amorphous solid. R_f = 0.43 (cyclohexane/AcOEt = 2:8). M.p. = 76-78 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.87 (t, *J* = 7.0 Hz, 3H), 1.25 (bs, 18H), 1.42 (s, 9H), 1.45 (bs, 2H), 3.14-3.24 (m, 2H), 3.74-4.46 (m, 4H), 5.17 (s, 2H), 5.25 (bs, 1NH), 6.73 (bs, 1NH), 7.29-7.39 (m, 5ArH), 8.01 (bs, 1NH) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.1, 22.8, 27.0, 28.4, 29.3, 29.4, 29.5, 29.67, 29.73, 29.76, 32.0, 39.8, 41.9, 52.8, 68.5, 80.0, 128.4, 128.7, 128.8, 135.1, 156.04, 156.07, 167.7, 172.2 ppm. LCMS: *m/z* = 549.4 $[M+H]^+$, 571.4 $[M+Na]^+$.

*4.2.4.4 Benzyl 2-((tert-butoxycarbonyl)glycyl)-2-(2-(octadecylamino)-2-oxoethyl)hydrazine-1-carboxylate, Cbz-HydrGly-NHC$_{18}$H$_{37}$, (**3Ad**).* Starting from **2** (5.00 mmol, 1.98 g) and following the general procedure, the compound **3Ad** was obtained in 90% yield (4.50 mmol, 2.85 g) as a white waxy

solid. R_f = 0.63 (cyclohexane:AcOEt 2:8). M.p. = 84-86 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.88 (t, *J* = 7.0 Hz, 3H), 1.25 (bs, 30H), 1.43 (s, 9H), 1.47 (bs, 2H), 3.15-3.25 (m, 2H), 3.74-4.56 (m, 4H), 5.18 (s, 2H), 5.23 (bs, 1NH), 6.61 (bs, 1NH), 7.32-7.40 (m, 5ArH), 7.81 (bs, 1NH) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.1, 22.7, 27.0, 28.3, 29.2, 29.36, 29.40, 29.6, 29.70, 29.75, 32.0, 39.7, 41.9, 53.0, 68.3, 79.9, 128.3, 128.6, 128.7, 135.1, 156.1, 156.3, 167.8, 172.2 ppm. LCMS: *m/z* = 655.5 $[M+Na]^+$.

4.2.5 General procedure for the synthesis of N-terminal derivatives

Note: representative procedure referred to 1 mmol of starting compound. To a solution of compound **3Aa-d** (1 mmol) dissolved in dry DCM (2 mL) under inert atmosphere at room temperature, Pd/C (100 mg) and formic acid (76 µL, 2 mmol) were sequentially added and the mixture was stirred for 1 hour. The volatile species were removed under vacuum at room temperature, then DCM (20 mL) was added and the reaction mixture was filtered through Celite, washing with DCM (3 × 10 mL). The organic phase was washed with a saturated solution of Na_2CO_3 (5 mL), then the aqueous phase was newly extracted with DCM (25 mL) and, after separation, the second organic phase was washed with a saturated solution of Na_2CO_3 (5 mL). The combined organic phases were dried over anhydrous Na_2SO_4, the solvent was evaporated under vacuum at room temperature and the free hydrazide intermediate was directly submitted to the following acylation reaction. The free hydrazide was dissolved in dry DCM (5 mL) under inert atmosphere, then pyridine (121 µL) was added, followed by dropwise addition of the suitable acyl chloride (1.1 eq). The solution was stirred for 1 hour, then all the volatile species were removed in vacuo at room temperature and the residue was diluted with AcOEt (30 mL) and water (5 mL). After separation, the organic phase was washed with HCl 1 M (2 × 3 mL), saturated aqueous sodium carbonate (5 mL) and water (5 mL). The aqueous phases were sequentially extracted with additional 30 mL of ethyl acetate, then the second organic phase was washed with HCl 1 M (2 × 3 mL), saturated aqueous sodium carbonate (5 mL) and water (5 mL). The combined organic phases were dried over anhydrous sodium sulfate and evaporated under vacuum, then the crude product was purified by column chromatography on silica gel (cyclohexane/AcOEt mixtures as eluents), to give the pure compound.

*4.2.5.1 tert-Butyl (2-(2-acetyl-1-(2-oxo-2-(propylamino)ethyl)hydrazinyl-2-oxoethyl)carbamate, CH_3CO-HydrGly-NHC_3H_7, (**3Ba**).* Starting from **3Aa** (265 µmol, 117 mg) and following the general procedure, compound **3Ba** was obtained in 65% yield (172 µmol, 56.8 mg) as a white waxy solid. R_f =

0.23 (AcOEt:MeOH 95:5). M.p. = 138-140 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.92 (t, *J* = 7.4 Hz, 3H), 1.43 (s, 9H), 1.51-1.60 (m, 2H), 2.09 (s, 3H), 3.22 (q, *J* = 6.6 Hz, 2H), 4.00 (bs, 2H), 4.23 (bs, 2H), 5.28 (bs, 1NH), 7.63 (bs, 1NH), 9.32 (bs, 1NH) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 11.5, 20.8, 22.6, 28.5, 41.6, 41.8, 53.8, 80.2, 156.2, 168.3, 171.1, 171.7 ppm. LCMS: *m/z* = 331.2 $[M+H]^+$, 353.2 $[M+Na]^+$.

*4.2.5.2 tert-Butyl (2-(2-acetyl-1-(2-(octylamino)-2-oxoethyl)hydrazinyl)-2-oxoethyl)carbamate, CH_3CO-HydrGly-NHC_8H_{17}, (**3Bb**).* Starting from **3Ab** (1.10 mmol, 542 mg) and following the general procedure, compound **3Bb** was obtained in 95% yield (1.05 mmol, 419 mg) as a colorless pitchy compound. R_f = 0.22 (AcOEt). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.87 (t, *J* = 7.0 Hz, 3H), 1.24-1.32 (m, 10H), 1.43 (s, 9H), 1.48-1.55 (m, 2H), 2.09 (s, 3H), 3.24 (q, *J* = 6.6 Hz, 2H), 3.99 (bs, 2H), 4.23 (bs, 2H), 5.28 (bs, 1NH), 7.60 (bs, 1NH), 9.31 (bs, 1NH) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.2, 20.8, 22.8, 27.0, 28.5, 29.2, 29.3, 31.9, 40.0, 54.0, 80.2, 156.2, 168.3, 171.0, 171.6 ppm. LCMS: *m/z* = 401.4 $[M+H]^+$, 423.4 $[M+Na]^+$.

*4.2.5.3 tert-Butyl (2-(2-acetyl-1-(2-(dodecylamino)-2-oxoethyl)hydrazinyl)-2-oxoethyl)carbamate, CH_3CO-HydrGly-$NHC_{12}H_{25}$, (**3Bc**).* Starting from **3Ac** (1.15 mmol, 631 mg) and following the general procedure, compound **3Bc** was obtained in 88% yield (1.01 mmol, 462 mg) as a white waxy solid. R_f = 0.14 (AcOEt). M.p. = 58-60 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.88 (t, *J* = 7.0 Hz, 3H), 1.25 (s, 18H), 1.43 (s, 9H), 1.46-1.56 (m, 2H), 2.08 (s, 3H), 3.22 (q, *J* = 6.6 Hz, 2H), 3.99 (bs, 2H), 4.17 (bs, 2H), 5.30 (bs, 1NH), 7.62 (bs, 1NH), 9.46 (bs, 1NH) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.2, 20.8, 22.8, 27.0, 28.5, 29.3, 29.4, 29.5, 29.68, 29.74, 29.76, 29.79, 32.0, 39.9, 41.8, 53.9, 80.1, 156.2, 168.2, 171.1, 171.7 ppm. LCMS: *m/z* = 457.4 $[M+H]^+$, 479.4 $[M+Na]^+$.

*4.2.5.4 tert-Butyl (2-(2-acetyl-1-(2-(octadecylamino)-2-oxoethyl)hydrazinyl)-2-oxoethyl)carbamate, CH_3CO-HydrGly-$NHC_{18}H_{37}$, (**3Bd**).* Starting from **3Ad** (1.00 mmol, 633 mg) and following the general procedure, compound **3Bd** was obtained in 91% yield (0.91 mmol, 492 mg) as a white waxy solid. R_f = 0.32 (AcOEt). M.p. = 77-79 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.88 (t, *J* = 7.0 Hz, 3H), 1.25 (s, 30H), 1.43 (s, 9H), 1.47-1.55 (m, 2H), 2.09 (s, 3H), 3.24 (q, *J* = 6.6 Hz, 2H), 3.98 (bs, 2H), 4.23 (bs, 2H), 5.28 (bs, 1NH), 7.54 (bs, 1NH), 9.27 (bs, 1NH) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.2, 20.8, 22.8, 27.1, 28.5, 29.3, 29.4, 29.5, 29.70, 29.77, 29.83, 32.0, 39.9, 41.8, 53.9, 80.1, 156.2, 168.2, 171.1, 171.7 ppm. LCMS: *m/z* = 541.5 $[M+H]^+$, 563.5 $[M+Na]^+$.

*4.2.5.5 tert-Butyl (2-(2-octanoyl-1-(2-oxo-2-(propylamino)ethyl)hydrazinyl)-2-oxoethyl)carbamate, C_7H_{15}CO-HydrGly-NHC_3H_7, (**3Ca**).* Starting from **3Aa** (1.00 mmol, 423 mg) and following the general procedure, compound **3Ca** was obtained in 90% yield (0.901 mmol, 373 mg) as a white waxy solid. R_f = 0.4 (cyclohexane/AcOEt 2:8). M.p. = 76-78°C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.88 (t, *J* = 7.0 Hz, 3H), 0.90 (t, *J* = 7.4 Hz, 3H), 1.22-1.37 (m, 8H), 1.43 (s, 9H), 1.48-1.57 (m, 2H), 1.63-1.70 (m, 2H), 2.24-2.30 (m, 2H), 3.19 (q, *J* = 6.6 Hz, 2H), 3.70-4.30 (m, 2H), 4.12 (bs, 2H), 5.30 (bs, 1NH), 7.71 (bs, 1NH), 9.37 (bs, 1NH) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 11.4, 14.0, 22.4, 22.6, 25.1, 28.3, 28.9, 29.2, 31.6, 33.8, 41.3, 41.7, 54.1, 79.7, 156.1, 168.2, 171.7, 174.3 ppm. LCMS: *m/z* = 414.3 $[M+H]^+$, 436.3 $[M+Na]^+$.

*4.2.5.6 tert-Butyl (2-(2-octanoyl-1-(2-(octylamino)-2-oxoethyl)hydrazinyl)-2-oxoethyl)carbamate, C_7H_{15}CO-HydrGly-NHC_8H_{17}, (**3Cb**).* Starting from **3Ab** (0.899 mmol, 443 mg) and following the general procedure, compound **3Cb** was obtained in 88% yield (0.790 mmol, 383 mg) as a white waxy solid. R_f = 0.47 (AcOEt). M.p. = 77-79 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.86-0.89 (m, 6H), 1.21-1.36 (m, 18H), 1.43 (s, 9H), 1.47-1.54 (m, 2H), 1.61-1.72 (m, 2H), 2.25 (t, *J* = 7.4 Hz, 2H), 3.22 (q, *J* = 6.6 Hz, 2H), 3.54-4.45 (m, 2H), 4.18 (bs, 2H), 5.25 (bs, 1NH), 7.12 (bs, 1NH), 8.74 (bs, 1NH) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.1, 14.2, 22.7, 22.8, 25.2, 27.0, 28.4, 29.0, 29.27, 29.34, 31.7, 31.9, 34.0, 39.8, 41.7, 54.1, 80.0, 156.2, 168.2, 171.7, 174.3 ppm. LCMS: *m/z* = 485.4 $[M+H]^+$, 507.4 $[M+Na]^+$.

*4.2.5.7 tert-Butyl (2-(1-(2-(dodecylamino)-2-oxoethyl)-2-octanoylhydrazinyl)-2-oxoethyl)carbamate, C_7H_{15}CO-HydrGly-NH$C_{12}H_{25}$, (**3Cc**).* Starting from **3Ac** (390 μmol, 214 mg) and following the general procedure, compound **3Cc** was obtained in 83% yield (324 μmol, 175 mg) as a white amorphous solid. R_f = 0.35 (AcOEt). M.p. = 81-83 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.86-0.89 (m, 6H), 1.21-1.38 (m, 26H), 1.43 (s, 9H), 1.47-1.59 (m, 2H), 1.62-1.73 (m, 2H), 2.24-2.33 (m, 2H), 3.20-3.31 (m, 2H), 3.63-4.50 (m, 2H), 4.23 (bs, 2H), 5.26 (bs, 1NH), 7.56 (bs, 1NH), 9.03 (bs, 1NH) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.17, 14.24, 22.7, 22.8, 25.2, 27.1, 28.5, 29.1, 29.3, 29.36, 29.44, 29.5, 29.7, 29.78, 29.80, 31.8, 32.0, 34.0, 39.9, 41.8, 54.1, 80.0, 156.1, 168.2, 171.7, 174.3 ppm. LCMS: *m/z* = 541.5 $[M+H]^+$,563.5 $[M+Na]^+$.

*4.2.5.8 tert-Butyl (2-(1-(2-(octadecylamino)-2-oxoethyl)-2-octanoylhydrazinyl)-2-oxoethyl)carbamate, C_7H_{15}CO-HydrGly-NH$C_{18}H_{37}$, (**3Cd**).* Starting from **3Ad** (525 μmol, 332 mg) and following the general procedure, compound **3Cd** was obtained in 83% yield (437 μmol, 273 mg) as a white amorphous solid. R_f = 0.75 (AcOEt). M.p. = 83-85 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.86-0.89 (m, 6H), 1.22-1.36 (m, 38H), 1.43 (s, 9H), 1.48-1.59 (m, 2H), 1.63-1.73 (m, 2H), 2.25-2.35 (m, 2H), 3.22-3.31 (m, 2H), 3.70-4.48 (m, 2H), 4.27 (bs, 2H), 5.26 (bs, 1NH), 7.58 (bs, 1NH), 9.02 (bs, 1NH) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.18, 14.24, 22.7, 22.8, 25.2, 27.1, 28.5, 29.1, 29.3, 29.36, 29.44, 29.48, 29.73, 29.79, 29.84, 31.8, 32.1, 34.0, 39.9, 41.8, 54.0, 80.0, 156.1, 168.2, 171.7, 174.2 ppm. LCMS: *m/z* = 625.5 $[M+H]^+$, 647.5 $[M+Na]^+$.

*4.2.5.9 tert-Butyl (2-(2-dodecanoyl-1-(2-oxo-2-(propylamino)ethyl)hydrazinyl)-2-oxoethyl)carbamate, $C_{11}H_{23}$CO-HydrGly-NHC_3H_7, (**3Da**).* Starting from **3Aa** (954 μmol, 403 mg) and following the general procedure, compound **3Da** was obtained in 94% yield (897 μmol, 422 mg) as a white amorphous solid. R_f = 0.33 (cyclohexane/AcOEt 2:8). M.p. = 69-71 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.88 (t, *J* = 7.0 Hz, 3H), 0.90 (t, *J* = 7.4 Hz, 3H), 1.20-1.38 (m, 16H), 1.43 (s, 9H), 1.46-1.58 (m, 2H), 1.60-1.72 (m, 2H), 2.27 (t, *J* = 7.4 Hz, 2H), 3.14-3.24 (m, 2H), 3.60-4.30 (m, 4H), 5.31 (bs, 1NH), 7.79 (bs, 1NH), 9.48 (bs, 1NH) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 11.4, 14.2, 22.5, 22.7, 25.2, 28.4, 29.35, 29.38, 29.5, 29.66, 29.67, 32.0, 33.9, 41.4, 41.7, 54.3, 79.8, 156.1, 168.3, 171.8, 174.3 ppm. LCMS: *m/z* = 471.4 $[M+H]^+$, 493.4 $[M+Na]^+$.

*4.2.5.10 tert-Butyl (2-(2-dodecanoyl-1-(2-(octylamino)-2-oxoethyl)hydrazinyl)-2-oxoethyl)carbamate, $C_{11}H_{23}$CO-HydrGly-NHC_8H_{17}, (**3Db**).* Starting from **3Ab** (627 μmol, 309 mg) and following the general procedure, compound **3Db** was obtained in 88% yield (553 μmol, 299 mg) as a white amorphous solid. R_f = 0.38 (cyclohexane:AcOEt 2:8). M.p. = 67-69 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.86-0.89 (m, 6H), 1.19-1.38 (m, 26H), 1.43 (s, 9H), 1.45-1.54 (m, 2H), 1.59-1.73 (m, 2H), 2.24-2.28 (m, 2H), 3.21 (q, *J* = 6.2 Hz, 2H), 3.62-4.33 (m, 2H), 4.15 (bs, 2H), 5.27 (bs, 1NH), 7.52 (bs, 1NH), 9.19 (bs, 1NH) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.20, 14.22, 22.76, 22.79, 25.2, 27.0, 28.5, 29.29, 29.35, 29.36, 29.42, 29.46, 29.6, 29.7, 29.8, 31.9, 32.0, 34.0, 39.9, 41.8, 54.0, 80.0, 156.2, 168.2, 171.7, 174.2 ppm. LCMS: *m/z* = 541.3 $[M+H]^+$, 563.3 $[M+Na]^+$.

*4.2.5.11 tert-Butyl (2-(2-dodecanoyl-1-(2-(dodecylamino)-2-oxoethyl)hydrazinyl)-2-oxoethyl)carbamate, $C_{11}H_{23}$CO-HydrGly-NH-$C_{12}H_{25}$, (**3Dc**).* Starting from **3Ac** (600 μmol, 329 mg)

and following the general procedure, compound **3Dc** was obtained in 86% yield (516 μmol, 308 mg) as a white amorphous solid. R_f = 0.44 (AcOEt). M.p. = 63-65 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.86-0.89 (m, 6H), 1.19-1.38 (m, 34H), 1.43 (s, 9H), 1.46-1.55 (m, 2H), 1.60-1.70 (m, 2H), 2.26 (t, *J* = 7.4 Hz, 2H), 3.21 (q, *J* = 6.2 Hz, 2H), 3.59-4.35 (m, 2H), 4.15 (bs, 2H), 5.27 (bs, 1NH), 7.50 (bs, 1NH), 9.16 (bs, 1NH) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.2, 22.8, 25.3, 27.1, 28.5, 29.3, 29.45, 29.48, 29.50, 29.6, 29.73, 29.76, 29.79, 29.82, 32.1, 34.0, 39.9, 41.8, 54.0, 80.0, 156.2, 168.2, 171.7, 174.2 ppm. LCMS: *m/z* = 597.6 $[M+H]^+$, 619.6 $[M+Na]^+$.

*4.2.5.12 tert-Butyl (2-(2-dodecanoyl-1-(2-(octadecylamino)-2-oxoethyl)hydrazinyl)-2-oxoetyl)carbamate, $C_{11}H_{23}$CO-HydrGly-NH$C_{18}H_{35}$, (**3Dd**).* Starting from **3Ad** (341 μmol, 216 mg) and following the general procedure, compound **3Dd** was obtained in 70% yield (238 μmol, 162 mg) as a white amorphous solid. R_f = 0.32 (AcOEt). M.p. = 45-47 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.86-0.89 (m, 6H), 1.20-1.37 (m, 46H), 1.43 (s, 9H), 1.46-1.54 (m, 2H), 1.59-1.72 (m, 2H), 2.26 (t, *J* = 7.4 Hz, 2H), 3.18-3.23 (m, 2H), 3.62-4.30 (m, 2H), 4.12 (bs, 2H), 5.29 (bs, 1NH), 7.62 (bs, 1NH), 9.30 (bs, 1NH) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.2, 22.8, 25.2, 27.1, 28.5, 29.3, 29.45, 29.48, 29.6, 29.75, 29.78, 29.80, 29.83, 32.0, 34.0, 39.9, 41.8, 54.2, 79.9, 156.2, 168.2, 171.7, 174.3 ppm. LCMS: *m/z* = 658.6 $[M+H]^+$, 680.6 $[M+Na]^+$.

*4.2.5.13 tert-Butyl (2-oxo-2-(1-(2-oxo-2-(propylamino)ethyl)-2-stearoylhydrazinyl)ethyl)carbamate, $C_{17}H_{35}$CO-HydrGly-NHC_8H_{17}, (**3Ea**).* Starting from **3Aa** (265 μmol, 112 mg) and following the general procedure, compound **3Ea** was obtained in 70% yield (186 μmol, 103 mg) as a white amorphous solid. R_f = 0.42 (AcOEt). M.p. = 63-65 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.88 (t, *J* = 7.0 Hz, 3H), 0.92 (t, *J* = 7.4 Hz, 3H), 1.20-1.38 (m, 28H), 1.43 (s, 9H), 1.51-1.60 (m, 2H), 1.62-1.73 (m, 2H), 2.29 (t, *J* = 7.4 Hz, 2H), 3.23 (q, *J* = 6.6 Hz, 2H), 3.96 (bs, 2H), 4.22 (bs, 2H) 5.28 (bs, 1NH), 7.78 (bs, 1NH), 9.25 (bs, 1NH) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 11.5, 14.2, 22.6, 22.8, 25.3, 28.5, 29.40, 29.43, 29.47, 29.6, 29.78, 29.82, 32.0, 34.0, 41.5, 41.8, 54.2, 80.0, 156.2, 168.3, 171.8, 174.3 ppm. LCMS: *m/z* = 532.4 $[M+H]^+$, 554.4 $[M+Na]^+$.

*4.2.5.14 tert-Butyl (2-(1-(2-(octylamino)-2-oxoethyl)-2-stearoylhydrazinyl)-2-oxoethyl)carbamate, $C_{17}H_{35}$CO-HydrGly-NHC_8H_{17}, (**3Eb**).* Starting from **3Ab** (451 μmol, 222 mg) and following the general procedure, compound **3Eb** was obtained in 86% yield (389 μmol, 243 mg) as a white amorphous solid. R_f = 0.52 (cyclohexane:AcOEt 3:7). M.p. = 125-127 °C. ^{1}H NMR (400 MHz,

$CDCl_3$): δ 0.86-0.89 (m, 6H), 1.18-1.38 (m, 38H), 1.43 (s, 9H), 1.45-1.54 (m, 2H), 1.58-1.72 (m, 2H), 2.25-2.29 (m, 2H), 3.18-3.23 (m, 2H), 3.64-4.40 (m, 2H), 4.12 (bs, 2H), 5.31 (bs, 1NH), 7.73 (bs, 1NH), 9.45 (bs, 1NH) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.2, 22.76, 22.80, 25.2, 27.0, 28.5, 29.30, 29.36, 29.44, 29.47, 29.6, 29.78, 29.83, 31.95, 32.04, 34.0, 39.9, 41.8, 54.1, 80.0, 156.2, 168.2, 171.7, 174.2 ppm. LCMS: *m/z* = 625.6 $[M+H]^+$, 647.6 $[M+Na]^+$.

*4.2.5.15 tert-Butyl (2-(1-(2-(dodecylamino)-2-oxoethyl)-2-stearoylhydrazinyl)-2-oxoethyl)carbamate, $C_{17}H_{35}$CO-HydrGly-NH$C_{12}H_{25}$, **(3Ec)**.* Starting from **3Ac** (304 μmol, 167 mg) and following the general procedure, compound **3Ec** was obtained in 80% yield (242 μmol, 165 mg) as a white amorphous solid. R_f = 0.46 (cyclohexane:AcOEt 2:8). M.p. = 60-63 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.86-0.89 (m, 6H), 1.18-1.38 (m, 46H), 1.43 (s, 9H), 1.45-1.54 (m, 2H), 1.59-1.72 (m, 2H), 2.21-2.35 (m, 2H), 3.16-3.28 (m, 2H), 3.64-4.36 (m, 2H), 4.16 (bs, 2H), 5.29 (bs, 1NH), 7.69 (bs, 1NH), 9.34 (bs, 1NH) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.2, 22.8, 25.3, 27.1, 28.5, 29.3, 29.45, 29.49, 29.6, 29.7, 29.79, 29.85, 32.1, 34.0, 39.9, 41.8, 53.9, 80.0, 156.2, 168.2, 171.7, 174.2 ppm. LCMS: *m/z* = 658.6 $[M+H]^+$, 680.6 $[M+Na]^+$.

*4.2.5.16 tert-Butyl (2-(1-(2-(octadecylamino)-2-oxoethyl)-2-stearoylhydrazinyl)-2-oxoethyl)carbamate, $C_{17}H_{35}$CO-HydrGly-NH$C_{18}H_{37}$, **(3Ed)**.* Starting from **3Ad** (348 μmol, 220 mg) and following the general procedure, compound **3Ed** was obtained in 80% yield (278 μmol, 213 mg) as a white waxy solid. R_f = 0.82 (AcOEt). M.p. = 89-90 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.86-0.89 (m, 6H), 1.19-1.37 (m, 46H), 1.43 (s, 9H), 1.45-1.56 (m, 2H), 1.59-1.72 (m, 2H), 2.21-2.35 (m, 2H), 3.21 (q, *J* = 6.4 Hz, 2H), 3.56-4.40 (m, 2H), 4.16 (s, 2H), 5.29 (bs, 1NH), 7.62 (bs, 1NH), 9.31 (bs, 1NH) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.2, 22.8, 25.3, 27.1, 28.5, 29.3, 29.45, 29.51, 29.65, 29.75, 29.81, 29.87, 32.1, 34.1, 39.9, 41.8, 53.8, 80.1, 156.2, 168.2, 171.7, 174.1 ppm. LCMS: *m/z* = 787.8 $[M+Na]^+$.

*4.2.5.17 tert-Butyl (2-(2-benzoyl-1-(2-(octylamino)-2-oxoethyl)hydrazinyl)-2-oxoethyl)carbamate, tBuCO-HydrGly-NHC_8H_{17}, **(3Fb)**.* Starting from **3Ab** (899 μmol, 443 mg) and following the general procedure, compound **3Fb** was obtained in 92% yield (828 μmol, 383 mg) as a white amorphous solid. R_f = 0.16 (AcOEt). M.p. = 128-130 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.87 (t, *J* = 7.0 Hz, 3H), 1.19-1.34 (m, 10H), 1.41 (s, 9H), 1.46-1.57 (m, 2H), 3.21-3.29 (m, 2H), 3.68-4.56 (m, 2H), 4.32 (bs, 2H), 5.29 (bs, 1NH), 7.23 (bs, 1NH), 7.48 (t, *J* = 7.4 Hz, 2ArH), 7.60 (t, *J* = 7.4 Hz, 1ArH), 7.88 (d, *J* = 7.4 Hz, 2ArH), 9.67 (bs, 1NH) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.2, 22.8, 27.1, 28.4, 29.30, 29.33,

29.36, 31.9, 39.9, 41.9, 54.2, 80.0, 128.0, 128.9, 130.7, 133.2, 156.2, 167.7, 168.3, 172.1 ppm. LCMS: *m/z* = 463.3 $[M+H]^+$, 485.3 $[M+Na]^+$.

*4.2.5.18 tert-Butyl (2-(2-benzoyl-1-(2-(dodecylamino)-2-oxoethyl)hydrazinyl)-2-oxoethyl)carbamate, PhCO-HydrGly-NHC$_{12}$H$_{25}$, (**3Fc**).* Starting from **3Ac** (425 μmol, 233 mg) and following the general procedure, compound **3Fc** was obtained in 85% yield (361 μmol, 187 mg) as a white amorphous solid. R_f = 0.56 (AcOEt). M.p. = 123-125 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.87 (t, *J* = 7.0 Hz, 3H), 1.21-1.31 (m, 18H), 1.40 (s, 9H), 1.46-1.56 (m, 2H), 3.21-3.26 (m, 2H), 3.71-4.47 (m, 2H), 4.29 (bs, 2H), 5.31 (bs, 1NH), 7.48 (t, *J* = 7.4 Hz, 2ArH + 1NH), 7.59 (t, *J* = 7.4 Hz, 1ArH), 7.89 (d, *J* = 7.4 Hz, 2ArH), 9.87 (bs, 1NH) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.2, 22.8, 27.1, 28.4, 29.3, 29.4, 29.5, 29.70, 29.76, 29.79, 32.0, 40.0, 41.9, 54.2, 80.0, 128.0, 128.9, 130.7, 133.2, 156.2, 167.7, 168.4, 172.1 ppm. LCMS: *m/z* = 519.4 $[M+H]^+$, 541.4 $[M+Na]^+$.

*4.2.5.19 tert-Butyl (2-(1-(2-(octylamino)-2-oxoethyl)-2-pivaloylhydrazinyl)-2-oxoethyl)carbamate, tBuCO-HydrGly-NHC$_8$H$_{17}$, (**3Gb**).* Starting from **3Ab** (510 μmol, 251 mg) and following the general procedure, compound **3Gb** was obtained in 89% yield (454 μmol, 201 mg) as a colorless pitchy compound. R_f = 0.22 (cyclohexane:AcOEt 3:7). M.p. = 121-123 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.87 (t, *J* = 7.0 Hz, 3H), 1.20-1.31 (m, 19H), 1.42 (s, 9H), 1.44-1.54 (m, 2H), 3.15-3.27 (m, 2H), 3.73 (bs, 1H), 4.10 (bs, 3H), 5.31 (bs, 1NH), 7.75 (bs, 1NH), 9.36 (bs, 1NH) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.2, 22.7, 27.0, 27.2, 28.5, 29.3, 31.9, 38.4, 39.8, 41.7, 54.1, 79.9, 156.1, 168.2, 171.8, 179.1 ppm. LCMS: *m/z* = 443.3 $[M+H]^+$, 465.2 $[M+Na]^+$.

*4.2.5.20 tert-Butyl (2-(1-(2-(dodecylamino)-2-oxoethyl)-2-pivaloylhydrazinyl)-2-oxoethyl)carbamate, tBuCO-HydrGly-NHC$_{12}$H$_{25}$, (**3Gc**).* Starting from **3Ac** (672 μmol, 369 mg) and following the general procedure, compound **3Gc** was obtained in 90% yield (606 μmol, 302 mg) as a colorless waxy solid. R_f = 0.49 (AcOEt). M.p. = 115-117 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.88 (t, *J* = 7.0 Hz, 3H), 1.20-1.33 (m, 27H), 1.43 (s, 9H), 1.46-1.55 (m, 2H), 3.20-3.30 (m, 2H), 3.5-4.37 (m, 4H), 5.26 (bs, 1NH), 7.50 (bs, 1NH), 9.09 (bs, 1NH) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.2, 22.7, 27.0, 27.1, 28.4, 29.2, 29.36, 29.40, 29.6, 29.7, 32.0, 38.3, 39.7, 41.6, 54.5, 79.6, 156.0, 168.2, 171.9, 179.2 ppm. LCMS: *m/z* = 499.4 $[M+H]^+$, 521.4 $[M+Na]^+$.

*4.2.5.21 tert-Butyl (2-(2-(2-naphthoyl)-1-(2-(octylamino)-2-oxoethyl)hydrazinyl)-2-oxoethyl)carbamate, 1-naphthylCO-HydrGly-NHC$_8$H$_{17}$, (**3Hb**).* Starting from **3Ab** (400 μmol, 197 mg) and following the general procedure, compound **3Hb** was obtained in 91% yield (365 μmol, 187 mg) as a white amorphous solid. R_f = 0.28 (cyclohexane/AcOEt 3:7). M.p. = 186-188 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.84-0.90 (m, 3H), 1.17-1.33 (m, 10H), 1.41 (s, 9H), 1.46-1.57 (m, 2H), 3.21-3.26 (m, 2H), 3.92-4.95 (m, 2H), 4.41 (bs, 2H), 5.31 (bs, 1NH), 7.23 (bs, 1NH), 7.49 (t, *J* = 7.4 Hz, 1ArH), 7.54-7.61 (m, 2ArH), 7.77 (d, *J* = 6.2 Hz, 1ArH), 7.89 (d, *J* = 8.6 Hz, 1ArH), 8.01 (d, *J* = 8.2 Hz, 1ArH), 8.34 (d, *J* = 8.2 Hz, 1ArH), 9.33 (bs, 1NH) ppm. ^{13}C NMR (100 MHz, DMSO-d_6): δ 13.9, 22.1, 26.4, 28.2, 28.6, 28.7, 29.0, 31.2, 38.6, 41.1, 51.1, 78.0, 124.80, 124.84, 126.48, 126.53, 127.2, 128.5, 129.8, 130.5, 131.3, 133.2, 155.8, 166.9, 167.8, 170.9 ppm. LCMS: *m/z* = 503.3 $[M+H]^+$, 525.3 $[M+Na]^+$.

*4.2.5.22 tert-Butyl (2-(2-(2-naphthoyl)-1-(2-(dodecylamino)-2-oxoethyl)hydrazinyl)-2-oxoethyl)carbamate, 1-naphthylCO-HydrGly-NHC$_{12}$H$_{25}$, (**3Hc**).* Starting from **3Ac** (322 μmol, 177 mg) and following the general procedure, compound **3Hc** was obtained in 87% yield (280 μmol, 159 mg) as a white waxy solid. R_f = 0.40 (DCM/MeOH 95:5). M.p. = 178-180 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.86-0.89 (m, 3H), 1.18-1.35 (m, 18H), 1.41 (s, 9H), 1.46-1.56 (m, 2H), 3.18-3.28 (m, 2H), 3.91-4.56 (m, 2H), 4.41 (bs, 2H), 5.32 (bs, 1NH), 7.41 (bs, 1NH), 7.40 (t, *J* = 7.4 Hz, 1ArH), 7.54-7.60 (m, 2ArH), 7.77 (d, *J* = 6.3 Hz, 1ArH), 7.89 (d, *J* = 7.4 Hz, 1ArH), 8.00 (d, *J* = 8.2 Hz, 1ArH), 8.35 (d, *J* = 8.2 Hz, 1ArH), 9.49 (bs, 1NH) ppm. ^{13}C NMR (100 MHz, DMSO-d_6): δ 13.9, 22.0, 26.3, 28.2, 28.66, 28.73, 28.90, 28.95, 29.00, 31.2, 38.6, 41.1, 51.0, 78.0, 124.8, 126.44, 126.47, 127.2, 128.4, 129.8, 130.5, 131.2, 133.1, 155.8, 166.8, 167.8, 170.9 ppm. LCMS: *m/z* = 569.5 $[M+H]^+$, 591.5 $[M+Na]^+$.

*4.2.6 General procedure for the synthesis of hydrochlorides **4***

Notes: representative procedure referred to 1 mmol of starting compound. Compound **4Ba**, due to very high solubility in water, was not submitted to the extractive procedure and was used as crude product, after azeotropic removal of TFA, in biological assays. The suitable compound was dissolved in dry DCM (3 mL), then TFA (1 mL) was added and the reaction was stirred at room temperature for 30 minutes. All the volatile species were removed at reduced pressure at room temperature, then traces of TFA were azeotropically removed by addition of DCM (1-2 mL) to the residue and further evaporation under vacuum (procedure repeated three times). The residue was diluted with DCM (10 mL) and a saturated solution of Na_2CO_3 (3 mL), then the phases were separated and the aqueous one was newly extracted with DCM (10 mL). After separation, the second organic phase was washed with a

saturated solution of Na_2CO_3 (3 mL). The combined organic phases were dried over anhydrous Na_2SO_4, the solvent was evaporated under vacuum at room temperature and the residue was purified by flash chromatography on silica gel, using the suitable mixtures of distilled DCM and distilled MeOH as the eluents. After concentration at reduced pressure and room temperature, the pure free amine was dissolved in dry DCM (3 mL), 3 M HCl in dry methanol (0.35 mL) was added and the mixture was evaporated under vacuum at room temperature, obtaining the desired pure hydrochlorides. Occasionally, if very careful smelling indicated that an excess HCl was still present, the residue was dissolved again in dry DCM (1-2 mL) and evaporated under vacuum.

*4.2.6.1 2-(2-((Benzyloxy)carbonyl)-1-(2-oxo-2-(propylamino)ethyl)hydrazinyl)-2-oxoethan-1-aminium chloride, BnOCO-HydrGly-NHC$_3$H$_7$ · HCl, (**4Aa**).* Starting from **3Aa** (263 μmol, 111 mg) and following the general procedure, compound **4Aa** was obtained in 98% yield (257 μmol, 92.2 mg) as a colorless waxy solid. R_f = 0.38 (free amine, DCM/MeOH 9:1). ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.84 (t, *J* = 7.4 Hz, 3H), 1.36-1.45 (m, 2H), 3.03 (q, *J* = 7.0 Hz, 2H), 3.56-4.65 (m, 4H), 5.15 (s, 2H), 7.33-7.41 (m, 5ArH), 8.01-8.18 (m, 4NH), 10.19 (bs, 1NH) ppm. t'$_R$ = 3.45 min (%2-propanol = 35.17%). HPLC purity: 98.2%. LCMS: *m/z* = 323.2 [M-Cl]$^+$, 345.2 [M-HCl+Na]$^+$. Elemental analysis: Anal. Calcd for $C_{15}H_{23}ClN_4O_4$: C, 50.21; H, 6.46; N, 15.61. Found: C, 50.08; H, 6.48; N, 15.59.

*4.2.6.2 2-(2-((Benzyloxy)carbonyl)-1-(2-(octylamino)-2-oxoethyl)hydrazinyl)-2-oxoethan-1-aminium chloride, BnOCO-HydrGly-NH-C$_8$H$_{17}$ · HCl, (**4Ab**).* Starting from **3Ab** (355 μmol, 175 mg) and following the general procedure, compound **4Ab** was obtained in 90% yield (320 μmol, 137 mg) as a colorless waxy solid. R_f = 0.31 (free amine, DCM/MeOH 95:5). ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.85 (t, *J* = 7.0 Hz, 3H), 1.24 (s, 10H), 1.34-1.43 (m, 2H), 3.05 (q, *J* = 6.6 Hz, 2H), 3.56-4.64 (m, 4H), 5.15 (s, 2H), 7.32-7.41 (m, 5ArH), 8.01 (t, *J* = 5.4 Hz, 1NH), 8.09-8.17 (m, 3NH), 10.19 (bs, 1NH) ppm. t'$_R$ = 3.45 min (%2-propanol = 51.38%). HPLC purity: 99.1%. LCMS: *m/z* = 393.3 [M-Cl]$^+$, 415.2 [M-HCl+Na]$^+$. Elemental analysis: Anal. Calcd for $C_{20}H_{33}ClN_4O_4$: C, 56.00; H, 7.75; N, 13.06. Found: C, 56.13; H, 7.77; N, 13.04.

*4.2.6.3 2-(2-((Benzyloxy)carbonyl)-1-(2-(octylamino)-2-oxoethyl)hydrazinyl)-2-oxoethan-1-aminium chloride, BnOCO-HydrGly-NHC$_{12}$H$_{25}$ · HCl, (**4Ac**).* Starting from **3Ac** (155 μmol, 85.2 mg) and following the general procedure, compound **4Ac** was obtained in 88% yield (136 μmol, 66.1 mg) as a colorless waxy solid. R_f = 0.37 (free amine, DCM:MeOH 9:1). ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.85

(t, *J* = 7.0 Hz, 3H), 1.24 (s, 18H), 1.34-1.42 (m, 2H), 3.05 (q, *J* = 6.6 Hz, 2H), 3.37-4.60 (m, 4H), 5.15 (s, 2H), 7.32-7.41 (m, 5ArH), 8.01 (t, *J* = 5.6 Hz, 1NH), 8.06-8.17 (m, 3NH), 10.17 (bs, 1NH) ppm. t'_R = 21.21 min (%2-propanol = 61.82%). HPLC purity: 97.1%. LCMS: *m/z* = 449.3 $[M\text{-}Cl]^+$, 471.3 $[M\text{-}HCl+Na]^+$. Elemental analysis: Anal. Calcd for $C_{24}H_{41}ClN_4O_4$: C, 59.43; H, 8.52; N, 11.55. Found: C, 59.40; H, 8.53; N, 11.55.

*4.2.6.4 2-(2-((Benzyloxy)carbonyl)-1-(2-(octadecylamino)-2-oxoethyl)hydrazinyl)-2-oxoethan-1-aminium chloride, BnOCO-HydrGly-NHC$_{18}$H$_{37}$ · HCl, (**4Ad**).* Starting from **3Ad** (152 μmol, 96.2 mg) and following the general procedure, compound **4Ad** was obtained in 82% yield (125 μmol, 71.0 mg) as a white waxy solid. R_f = 0.16 (free amine, DCM:MeOH 95:5). ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.85 (t, *J* = 7.0 Hz, 3H), 1.23 (s, 30H), 1.33-1.43 (m, 2H), 3.05 (q, *J* = 6.5 Hz, 2H), 3.59-4.60 (m, 4H), 5.15 (s, 2H), 7.32-7.41 (m, 5ArH), 8.01 (t, *J* = 5.5 Hz, 1NH), 8.10-8.20 (m, 3NH), 10.19 (bs, 1NH) ppm. t'_R = 30.94 min (%2-propanol = 76.41%). HPLC purity: 97.2%. LCMS: *m/z* = 533.4 $[M\text{-}Cl]^+$, 555.4 $[M\text{-}HCl+Na]^+$. Elemental analysis: Anal. Calcd for $C_{30}H_{53}ClN_4O_4$: C, 63.30; H, 9.39; N, 9.84. Found: C, 63.25; H, 9.42; N, 9.85.

*4.2.6.5 2-(2-Acetyl-1-(2-oxo-2-(propylamino)ethyl)hydrazinyl)-2-oxoethanaminium 2,2,2-trifluoroacetate, CH$_3$CO-HydrGly-NHC$_3$H$_7$ · CF$_3$CO$_2$H, (**4Ba**).* Starting from **3Ba** (275 μmol, 90.8 mg), compound **4Ba** was obtained as crude trifluoroacetate product in 95% yield (95.7 mg of crude product, corresponding to about 261 μmol, 90.0 mg, of pure product considering the HPLC purity, 95%) as a pale yellow oil, through evaporation under vacuum of the volatile species after reaction with TFA/DCM. R_f = 0.15 (free amine, DCM:MeOH = 8:2). ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.84 (t, *J* = 7.4 Hz, 3H), 1.37-1.46 (m, 2H), 1.92 (s, 3H), 3.04 (q, *J* = 6.5 Hz, 2H), 3.48-4.77 (m, 4H), 7.98-8.24 (m, 4NH), 10.59 (s, 1NH) ppm. t'_R = 0.26 min (%2-propanol = 30.39%). HPLC purity: 95.1%. LCMS: *m/z* 231.2 $[M\text{-}CF_3CO_2^-]^+$, 253.1 $[M\text{-}CF_3CO_2H+Na]^+$. Elemental analysis: Anal. Calcd for $C_{11}H_{19}F_3N_4O_5$: C, 38.37; H, 5.56; N, 16.27. Found: C, 38.39; H, 5.58; N, 16.25.

*4.2.6.6 2-(2-Acetyl-1-(2-(octylamino)-2-oxoethyl)hydrazinyl)-2-oxoethan-1-aminium chloride, CH$_3$CO-HydrGly-NHC$_8$H$_{17}$ · HCl, (**4Bb**).* Starting from **3Bb** (357 μmol, 143 mg) and following the general procedure, compound **4Bb** was obtained in 68% yield (242 μmol, 81.6 mg) as a white waxy solid. R_f = 0.13 (free amine, DCM:MeOH 9:1). ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.86 (t, *J* = 6.8 Hz, 3H), 1.24 (s, 10H), 1.34-1.44 (m, 2H), 1.92 (s, 3H), 3.06 (q, *J* = 6.4 Hz, 2H), 3.43-4.88 (m, 4H), 8.02-8.24 (m,

4NH), 10.60 (s, 1NH) ppm. t'$_R$ = 9.29 min (%2-propanol = 43.94%). HPLC purity: 96.0%. LCMS: *m/z* = 301.2 $[M\text{-}Cl]^+$, 323.2 $[M\text{-}HCl+Na]^+$. Elemental analysis: Anal. Calcd for $C_{14}H_{29}ClN_4O_3$: C, 49.92; H, 8.68; N, 16.63. Found: C, 49.89; H, 8.69; N, 16.66.

*4.2.6.7 2-(2-Acetyl-1-(2-(dodecylamino)-2-oxoethyl)hydrazinyl)-2-oxoethan-1-aminium chloride, CH_3CO-HydrGly-$NHC_{12}H_{25}$ · HCl, (**4Bc**).* Starting from **3Bc** (175 μmol, 79.8 mg) and following the general procedure, compound **4Bc** was obtained in 70% yield (123 μmol, 48.5 mg) as a white waxy solid. R_f = 0.42 (free amine, DCM:MeOH 7:3). ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.85 (t, *J* = 6.8 Hz, 3H), 1.24 (s, 18H), 1.34-1.44 (m, 2H), 1.92 (s, 3H), 3.06 (q, *J* = 6.4 Hz, 2H), 3.41-4.72 (m, 4H), 8.03-8.24 (m, 4NH), 10.62 (s, 1NH) ppm. t'$_R$ = 17.47 min (%2-propanol = 56.21%). HPLC purity: 99.6%. LCMS: *m/z* = 357.3 $[M\text{-}Cl]^+$, 379.3 $[M\text{-}HCl+Na]^+$. Elemental analysis: Anal. Calcd for $C_{18}H_{37}ClN_4O_3$: C, 55.02; H, 9.49; N, 14.26. Found: C, 54.80; H, 9.50; N, 14.29.

*4.2.6.8 2-(2-Acetyl-1-(2-(octadecylamino)-2-oxoethyl)hydrazinyl)-2-oxoethan-1-aminium chloride, CH_3CO-HydrGly-$NHC_{18}H_{37}$ · HCl, (**4Bd**).* Starting from **3Bd** (262 μmol, 142 mg) and following the general procedure, compound **4Bd** was obtained in 89% yield (233 μmol, 111 mg) as a white waxy solid. R_f = 0.29 (free amine, DCM:MeOH 85:15). ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.85 (t, *J* = 6.8 Hz, 3H), 1.23 (s, 30H), 1.34-1.43 (m, 2H), 1.91 (s, 3H), 3.05 (q, *J* = 6.4 Hz, 2H), 3.39-4.73 (m, 4H), 8.03-8.25 (m, 4NH), 10.64 (s, 1NH) ppm. t'$_R$ = 27.99 min (%2-propanol = 71.99%). HPLC purity: 99.1%. LCMS: *m/z* = 441.4 $[M\text{-}Cl]^+$, 463.3 $[M\text{-}HCl+Na]^+$. Elemental analysis: Anal. Calcd for $C_{24}H_{49}ClN_4O_3$: C, 60.42; H, 10.35; N, 11.74. Found: C, 60.39; H, 10.31; N, 11.77.

*4.2.6.9 2-(2-Octanoyl-1-(2-oxo-2-(propylamino)ethyl)hydrazinyl)-2-oxoethan-1-aminium chloride, $C_7H_{15}CO$-HydrGly-NHC_3H_7 · HCl, (**4Ca**).* Starting from **3Ca** (192 μmol, 79.5 mg) and following the general procedure, compound **4Ca** was obtained in 95% yield (182 μmol, 64.0 mg) as a white waxy solid. R_f = 0.47 (free amine, DCM:MeOH 8:2). ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.82-0.87 (m, 6H), 1.24 (s, 8H), 1.36-1.45 (m, 2H), 1.47-1.57 (m, 2H), 2.13-2.21 (m, 2H), 3.01-3.06 (m, 2H), 3.39-4.68 (m, 4H), 7.97-8.29 (m, 4NH), 10.63 (s, 1NH) ppm. t'$_R$ = 5.24 min (%2-propanol = 37.86%). HPLC purity: 97.0%. LCMS: *m/z* = 315.2 $[M\text{-}Cl]^+$, 337.2 $[M\text{-}HCl+Na]^+$. Elemental analysis: Anal. Calcd for $C_{15}H_{31}ClN_4O_3$: C, 51.35; H, 8.91; N, 15.97. Found: C, 51.46; H, 8.89; N, 15.91.

*4.2.6.10 2-(2-Octanoyl-1-(2-(octylamino)-2-oxoethyl)hydrazinyl)-2-oxoethan-1-aminium chloride, C_7H_{15}CO-HydrGly-NHC_8H_{17} · HCl, (**4Cb**).* Starting from **3Cb** (192 μmol, 93.2 mg) and following the general procedure, compound **4Cb** was obtained in 87% yield (167 μmol, 70.4 mg) as a white waxy solid. R_f = 0.28 (free amine, DCM:MeOH 1:1).). ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.84-0.87 (m, 6H), 1.19-1.29 (m, 18H), 1.35-1.41 (m, 2H), 1.48-1.56 (m, 2H), 2.11-2.19 (m, 2H), 2.99-3.14 (m, 2H), 3.40-4.62 (m, 4H), 7.98-8.25 (m, 4NH), 10.58 (s, 1NH) ppm. t'$_R$ = 17.78 min (%2-propanol = 56.67%). HPLC purity: 98.2%. LCMS: *m/z* = 385.3 $[M-Cl]^+$, 407.3 $[M-HCl+Na]^+$. Elemental analysis: Anal. Calcd for $C_{20}H_{41}ClN_4O_3$: C, 57.06; H, 9.82; N, 13.31. Found: C, 57.00; H, 9.84; N, 13.35.

*4.2.6.11 2-(1-(2-(Dodecylamino)-2-oxoethyl)-2-octanoylhydrazinyl)-2-oxoethan-1-aminium chloride, C_7H_{15}CO-HydrGly-NH$C_{12}H_{25}$ · HCl, (**4Cc**).* Starting from **3Cc** (135 μmol, 73.1 mg) and following the general procedure, compound **4Cc** was obtained in 84% yield (113 μmol, 53.8 mg) as a white waxy solid. R_f = 0.06 (free amine, DCM:MeOH 9:1). ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.84-0.87 (m, 6H), 1.19-1.31 (m, 26H), 1.34-1.44 (m, 2H), 1.46-1.57 (m, 2H), 2.11-2.19 (m, 2H), 2.99-3.13 (m, 2H), 3.37-4.65 (m, 4H), 7.91-8.24 (m, 4NH), 10.59 (s, 1NH) ppm. t'$_R$ = 24.01 min (%2-propanol = 66.02%). HPLC purity: 98.7%. LCMS: *m/z* = 441.4 $[M-Cl]^+$, 463.3 $[M-HCl+Na]^+$. Elemental analysis: Anal. Calcd for $C_{24}H_{49}ClN_4O_3$: C, 60.42; H, 10.35; N, 11.74. Found: C, 60.28; H, 10.32; N, 11.72.

*4.2.6.12 2-(1-(2-(Octadecylamino)-2-oxoethyl)-2-octanoylhydrazinyl)-2-oxoethan-1-aminium chloride, C_7H_{15}CO-HydrGly-NH$C_{18}H_{37}$ · HCl, (**4Cd**).* Starting from **3Cd** (174 μmol, 108.8 mg) and following the general procedure, compound **4Cd** was obtained in 81% yield (141 μmol, 79.2 mg) as a white waxy solid. R_f = 0.09 (free amine, DCM:MeOH 95:5). ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.84-0.88 (m, 6H), 1.23 (s, 38H), 1.34-1.42 (m, 2H), 1.47-1.56 (m, 2H), 2.11-2.19 (m, 2H), 2.98-3.14 (m, 2H), 3.27-4.66 (m, 4H), 7.97-8.14 (m, 4NH), 10.57 (s, 1NH) ppm. t'$_R$ = 33.05 min (%2-propanol = 79.58%). HPLC purity: 97.1%. LCMS: *m/z* = 525.5 $[M-Cl]^+$, 547.4 $[M-HCl+Na]^+$. Elemental analysis: Anal. Calcd for $C_{30}H_{61}ClN_4O_3$: C, 64.20; H, 10.95; N, 9.98. Found: C, 64.23; H, 10.99; N, 9.97.

*4.2.6.13 2-(2-Dodecanoyl-1-(2-oxo-2-(propylamino)ethyl)hydrazinyl)-2-oxoethan-1-aminium chloride, $C_{11}H_{23}$CO-HydrGly-NHC_3H_7 · HCl, (**4Da**).* Starting from **3Da** (157 μmol, 73.8 mg) and following the general procedure, compound **4Da** was obtained in 84% yield (132 μmol, 53.8 mg) as a white waxy solid. R_f = 0.40 (free amine, AcOEt:MeOH 8:2). ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.82-0.87 (m, 6H), 1.24 (s, 8H), 1.36-1.46 (m, 2H), 1.47-1.57 (m, 2H), 2.17 (t, *J* = 7.4 Hz, 2H), 3.01-3.06 (m, 2H), 3.32-

4.75 (m, 4H), 8.00-8.27 (m, 4NH), 10.60 (s, 1NH) ppm. t'$_R$ = 16.63 min (%2-propanol = 54.95%). HPLC purity: 97.3%. LCMS: *m/z* = 371.3, $[M\text{-}Cl]^+$, 393.3 $[M\text{-}HCl+Na]^+$. Elemental analysis: Anal. Calcd for $C_{19}H_{39}ClN_4O_3$: C, 56.07; H, 9.66; N, 13.77. Found: C, 56.12; H, 9.69; N, 13.76.

*4.2.6.14 2-(2-Dodecanoyl-1-(2-(octylamino)-2-oxoethyl)hydrazinyl)-2-oxoethan-1-aminium chloride, $C_{11}H_{23}CO$-HydrGly-NHC_8H_{17} · HCl, (**4Db**).* Starting from **3Dd** (169 μmol, 91.5 mg) and following the general procedure, compound **4Db** was obtained in 89% yield (150 μmol, 71.8 mg) as a white waxy solid. R_f = 0.08 (free amine, DCM:MeOH 9:1). ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.84-0.87 (m, 6H), 1.18-1.29 (m, 26H), 1.34-1.43 (m, 2H), 1.46-1.56 (m, 2H), 2.11-2.20 (m, 2H), 3.00-3.12 (m, 2H), 3.32-4.79 (m, 4H), 7.98-8.24 (m, 4NH), 10.59 (s, 1NH) ppm. t'$_R$ = 23.46 min (%2-propanol = 65.19%). HPLC purity: 97.4%. LCMS: *m/z* = 441.4 $[M\text{-}Cl]^+$, 463.4 $[M\text{-}HCl+Na]^+$. Elemental analysis: Anal. Calcd for $C_{24}H_{49}ClN_4O_3$: C, 60.42; H, 10.35; N, 11.74. Found: C, 60.46; H, 10.33; N, 11.72.

*4.2.6.15 2-(2-Dodecanoyl-1-(2-(dodecylamino)-2-oxoethyl)hydrazinyl)-2-oxoethan-1-aminium chloride, $C_{11}H_{23}CO$-HydrGly-$NHC_{12}H_{25}$ · HCl, (**4Dc**).* Starting from **3Dc** (253 μmol, 151 mg) and following the general procedure, compound **4Dc** was obtained in 82% yield (207 μmol, 111 mg) as a colorless waxy solid. R_f = 0.11 (free amine, DCM:MeOH 9:1). ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.84-0.87 (m, 6H), 1.24 (s, 34H), 1.33-1.43 (m, 2H), 1.46-1.56 (m, 2H), 2.11-2.20 (m, 2H), 2.94-3.16 (m, 2H), 3.36-4.69 (m, 4H), 7.99-8.17 (m, 4NH), 10.59 (s, 1NH) ppm. t'$_R$ = 29.14 min (%2-propanol = 73.71%). HPLC purity: 97.2%. LCMS: *m/z* = 497.4 $[M\text{-}Cl]^+$, 519.5 $[M\text{-}HCl+Na]^+$. Elemental analysis: Anal. Calcd for $C_{28}H_{57}ClN_4O_3$: C, 63.07; H, 10.77; N, 10.51. Found: C, 62.92; H, 10.77; N, 10.53.

*4.2.6.16 2-(2-Dodecanoyl-1-(2-(octadecylamino)-2-oxoethyl)hydrazinyl)-2-oxoethanaminium chloride, $C_{11}H_{23}CO$-HydrGly-$NHC_{18}H_{37}$ · HCl, (**4Dd**).* Starting from **3Dd** (110 μmol, 74.8 mg) and following the general procedure, compound **4Dd** was obtained in 97% yield (107 μmol, 66.3 mg) as a colorless waxy solid. R_f = 0.10 (free amine, DCM:MeOH 9:1). ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.84-0.87 (m, 6H), 1.19-1.29 (s, 46H), 1.34-1.43 (m, 2H), 1.46-1.56 (m, 2H), 2.11-2.20 (m, 2H), 2.96-3.16 (m, 2H), 3.36-4.67 (m, 4H), 8.00-8.23 (m, 4NH), 10.57 (s, 1NH) ppm. t'$_R$ = 35.93 min (%2-propanol = 83.90%). HPLC purity: 98.2%. LCMS: *m/z* = 581.6 $[M\text{-}Cl]^+$, 603.5 $[M\text{-}HCl+Na]^+$. Elemental analysis: Anal. Calcd for $C_{34}H_{69}ClN_4O_3$: C, 66.14; H, 11.27; N, 9.07. Found: C, 66.06; H, 11.25; N, 9.08.

*4.2.6.17 2-Oxo-2-(1-(2-oxo-2-(propylamino)ethyl)-2-stearoylhydrazinyl)ethanaminium chloride, $C_{17}H_{35}$CO-HydrGly-NHC_3H_7 · HCl, (**4Ea**).* Starting from **3Ea** (81.3 μmol, 45.1 mg) and following the general procedure, compound **4Ea** was obtained in 96% yield (77.0 μmol, 37.8 mg) as a white waxy solid. R_f = 0.1 (free amine, DCM:MeOH 9:1). ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.82-0.87 (m, 6H), 1.23 (s, 28H), 1.37-1.46 (m, 2H), 1.46-1.57 (m, 2H), 2.11-2.19 (m, 2H), 3.04 (q, J = 6.3 Hz, 2H), 3.39-4.68 (m, 4H), 8.01-8.26 (m, 4NH), 10.59 (s, 1NH) ppm. t'$_R$ = 26.49 min (%2-propanol = 69.74%). HPLC purity: 95.8%. LCMS: *m/z* = 455.4 $[M\text{-}Cl]^+$, 477.4 $[M\text{-}HCl+Na]^+$. Elemental analysis: Anal. Calcd for $C_{25}H_{51}ClN_4O_3$: C, 61.14; H, 10.47; N, 11.41. Found: C, 60.94; H, 10.46; N, 11.37.

*4.2.6.18 2-(1-(2-(Octylamino)-2-oxoethyl)-2-stearoylhydrazinyl)-2-oxoethan-1-aminium chloride, $C_{17}H_{35}$CO-HydrGly-NHC_8H_{17} · HCl, (**4Eb**).* Starting from **3Eb** (81.9 μmol, 51.2 mg) and following the general procedure, compound **4Eb** was obtained in 80% yield (65.9 μmol, 37.0 mg) as a white waxy solid. R_f = 0.35 (free amine, DCM:MeOH 9:1). ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.83-0.87 (m, 6H), 1.23 (s, 38H), 1.33-1.43 (m, 2H), 1.45-1.56 (m, 2H), 2.11-2.19 (m, 2H), 2.96-3.14 (m, 2H), 3.40-4.62 (m, 4H), 8.00-8.26 (m, 4NH), 10.61 (s, 1NH) ppm. t'$_R$ = 32.24 min (%2-propanol = 78.36%). HPLC purity: 96.3%. LCMS: *m/z* = 525.5 $[M\text{-}Cl]^+$, 547.4 $[M\text{-}HCl+Na]^+$. Elemental analysis: Anal. Calcd for $C_{30}H_{61}ClN_4O_3$: C, 64.20; H, 10.95; N, 9.98. Found: C, 64.44; H, 10.93; N, 9.96.

*4.2.6.19 2-(1-(2-(Dodecylamino)-2-oxoethyl)-2-stearoylhydrazinyl)-2-oxoethanamium chloride, $C_{17}H_{35}$CO-HydrGly-NH$C_{12}H_{25}$ · HCl, (**4Ec**).* Starting from **3Ec** (44.0 μmol, 30.0 mg) and following the general procedure, compound **4Ec** was obtained in 85% yield (37.3 μmol, 23.0 mg) as a colorless pitchy compound. R_f = 0.09 (free amine, DCM:MeOH 95:5). ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.83-0.87 (m, 6H), 1.23 (s, 46H), 1.33-1.43 (m, 2H), 1.45-1.56 (m, 2H), 2.11-2.19 (m, 2H), 2.93-3.19 (m, 2H), 3.41-4.67 (m, 4H), 7.99-8.26 (m, 4NH), 10.60 (s, 1NH) ppm. t'$_R$ = 35.81 min (%2-propanol = 83.72%). HPLC purity: 99.5%. LCMS: *m/z* = 581.5 $[M\text{-}Cl]^+$, 603.5 $[M\text{-}HCl+Na]^+$. Elemental analysis: Anal. Calcd for $C_{34}H_{69}ClN_4O_3$: C, 66.14; H, 11.27; N, 9.07. Found: C, 66.25; H, 11.24; N, 9.04.

*4.2.6.20 2-(1-(2-(Octadecylamino)-2-oxoethyl)-2-stearoylhydrazinyl)-2-oxoethan-1-aminium chloride, $C_{17}H_{35}$CO-HydrGly-NH$C_{18}H_{37}$ · HCl, (**4Ed**).* Starting from **3Ed** (141 μmol, 108 mg) and following the general procedure, compound **4Ed** was obtained in 82% yield (116 μmol, 81.4 mg) as a white waxy solid. R_f = 0.40 (free amine, DCM:MeOH 9:1). ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.84 (s, 6H), 1.10-1.53 (m, 62H), 2.08-2.22 (m, 2H), 2.92-3.24 (m, 2H), 3.42-4.66 (m, 4H), 7.97-8.26 (m, 4NH), 10.58 (s,

1NH) ppm. t'$_R$ = 41.78 min (%2-propanol = 92.67%). HPLC purity: 98.5%. LCMS: *m/z* = 665.6 [M-Cl]$^+$, 687.6 [M-HCl+Na]$^+$. Elemental analysis: Anal. Calcd for $C_{40}H_{81}ClN_4O_3$: C, 68.48; H, 11.64; N, 7.99. Found: C, 68.43; H, 11.67; N, 8.02.

*4.2.6.21 2-(2-Benzoyl-1-(2-(octylamino)-2-oxoethyl)hydrazinyl)-2-oxoethan-1-aminium chloride, PhCO-HydrGly-NHC$_8$H$_{17}$ · HCl, (**4Fb**).* Starting from **3Fb** (183 μmol, 84.7 mg) and following the general procedure, compound **4Fb** was obtained in 97% yield (178 μmol, 71.0 mg) as a colorless waxy solid. R_f = 0.12 (free amine, DCM:MeOH 9:1). ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.84 (t, *J* = 6.8 Hz, 3H), 1.16-1.28 (m, 10H), 1.33-1.43 (m, 2H), 3.00-3.14 (m, 2H), 3.25-4.70 (m, 4H), 7.50-7.55 (m, 2ArH), 7.62-7.66 (m, 1ArH), 7.87-7.92 (m, 2ArH), 8.04-8.26 (m, 4NH), 11.19 (s, 1NH) ppm. t'$_R$ = 12.27 min (%2-propanol = 48.41%). HPLC purity: 100%. LCMS: *m/z* = 363.2 [M-Cl]$^+$, 385.2 [M-HCl+Na]$^+$. Elemental analysis: Anal. Calcd for $C_{19}H_{31}ClN_4O_3$: C, 57.20; H, 7.83; N, 14.04. Found: C, 57.24; H, 7.85; N, 14.00.

*4.2.6.22 2-(2-Benzoyl-1-(2-(dodecylamino)-2-oxoethyl)hydrazinyl)-2-oxoethan-1-aminium chloride, PhCO-HydrGly-NHC$_{12}$H$_{25}$ · HCl, (**4Fc**).* Starting from **3Fc** (183 μmol, 94.9 mg) and following the general procedure, compound **4Fc** was obtained in 89% yield (163 μmol, 74.2 mg) as a colorless waxy solid. R_f = 0.12 (free amine, DCM:MeOH 9:1). ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.85 (t, *J* = 6.8 Hz, 3H), 1.15-1.29 (m, 18H), 1.33-1.43 (m, 2H), 3.00-3.14 (m, 2H), 3.47-4.87 (m, 4H), 7.48-7.55 (m, 2ArH), 7.60-7.66 (m, 1ArH), 7.87-7.92 (m, 2ArH), 8.06-8.27 (m, 4NH), 11.2 (s, 1NH) ppm. t'$_R$ = 19.70 min (%2-propanol = 59.55%). HPLC purity: 98.9%. LCMS: *m/z* = 419.3 [M-Cl]$^+$, 441.3 [M-HCl+Na]$^+$. Elemental analysis: Anal. Calcd for $C_{23}H_{39}ClN_4O_3$: C, 60.71; H, 8.64; N, 12.31. Found: C, 60.70; H, 8.61; N, 12.36.

*4.2.6.23 2-(1-(2-(Octylamino)-2-oxoethyl)-2-pivaloylhydrazinyl)-2-oxoethan-1-aminium chloride, t-BuCO-HydrGly-NHC$_8$H$_{17}$ · HCl, (**4Gb**).* Starting from **3Gb** (255 μmol, 113 mg) and following the general procedure, compound **4Gb** was obtained in 96% yield (245 μmol, 92.9 mg) as a colorless pitchy compound. R_f = 0.49 (free amine, DCM:MeOH 8:2). ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.85 (t, *J* = 6.8 Hz, 3H), 1.15 (s, 9H), 1.19-1.29 (m, 10H), 1.33-1.43 (m, 2H), 3.00 (bs, 1H), 3.11 (bs, 1H), 3.37-3.50 (m, 1H), 3.60-3.75 (m, 1H), 3.82-3.98 (m, 1H), 4.41-4.56 (m, 1H), 8.02-8.28 (m, 4NH), 10.37 (s, 1NH) ppm. t'$_R$ = 11.69 min (%2-propanol = 47.54%). HPLC purity: 98.1%. LCMS: *m/z* =

343.3 $[M\text{-}Cl]^+$, 365.3 $[M\text{-}HCl+Na]^+$. Elemental analysis: Anal. Calcd for $C_{17}H_{35}ClN_4O_3$: C, 53.88; H, 9.31; N, 14.79. Found: C, 53.93; H, 9.30; N, 14.83.

*4.2.6.24 2-(1-(2-(Dodecylamino)-2-oxoethyl)-2-pivaloylhydrazinyl)-2-oxoethan-1-aminium chloride, t-BuCO-HydrGly-$NHC_{12}H_{25}$ · HCl, (**4Gc**).* Starting from **3Gb** (222 μmol, 111 mg) and following the general procedure, compound **4Gc** was obtained in 94% yield (208 μmol, 90.5 mg) as a colorless waxy solid. R_f = 0.07 (free amine, DCM:MeOH 9:1). ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.85 (t, J = 6.8 Hz, 3H), 1.15 (s, 9H), 1.19-1.30 (m, 18H), 1.33-1.43 (m, 2H), 2.92-3.19 (m, 2H), 3.37-3.51 (m, 1H), 3.61-3.73 (m, 1H), 3.82-3.95 (m, 1H), 4.42-4.54 (m, 1H), 8.00-8.27 (m, 4NH), 10.35 (s, 1NH) ppm. t'$_R$ = 19.77 min (%2-propanol = 59.66%). HPLC purity: 98.9%. LCMS: *m/z* = 399.3 $[M\text{-}Cl]^+$, 421.3 $[M\text{-}HCl+Na]^+$. Elemental analysis: Anal. Calcd for $C_{21}H_{43}ClN_4O_3$: C, 57.98; H, 9.96; N, 12.88. Found: C, 58.04; H, 9.98; N, 12.90.

*4.2.6.25 2-(2-(1-Naphthoyl)-1-(2-(octylamino)-2-oxoethyl)hydrazinyl)-2-oxoethan-1-aminium chloride, 1-NaphthylCO-HydrGly-NHC_8H_{17} · HCl, (**4Hb**).* Starting from **3Hb** (246 μmol, 126 mg) and following the general procedure, compound **4Hb** was obtained in 88% yield (216 μmol, 96.8 mg) as a colorless pitchy compound. R_f = 0.48 (free amine, DCM:MeOH 8:2). ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.88 (t, J = 6.8 Hz, 3H), 1.17-1.34 (m, 10H), 1.39-1.48 (m, 2H), 3.00-3.25 (m, 2H), 3.78-4.91 (m, 4H), 7.58-7.67 (m, 3ArH), 7.78-7.80 (m, 1ArH), 8.03-8.08 (m, 1ArH), 8.15-8.21 (m, 4NH), 8.22-8.38 (m, 2ArH), 11.30 (s, 1NH) ppm. t'$_R$ = 14.37 min (%2-propanol = 51.56%). HPLC purity: 98.0%. LCMS: *m/z* = 413.3 $[M\text{-}Cl]^+$, 435.2 $[M\text{-}HCl+Na]^+$. Elemental analysis: Anal. Calcd for $C_{23}H_{33}ClN_4O_3$: C, 61.53; H, 7.41; N, 12.48. Found: C, 61.48; H, 7.39; N, 12.47.

*4.2.6.26 2-(2-(1-Naphthoyl)-1-(2-(dodecylamino)-2-oxoethyl)hydrazinyl)-2-oxoethan-1-aminium chloride, 1-NaphthylCO-HydrGly-$NHC_{12}H_{25}$ · HCl, (**4Hc**).* Starting from **3Hc** (190 μmol, 108 mg) and following the general procedure, compound **4Hc** was obtained in 79% yield (150 μmol, 75.8 mg) as a colorless pitchy compound. R_f = 0.37 (free amine, DCM:MeOH 8:2). ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.84 (t, J = 6.8 Hz, 3H), 1.19-1.27 (m, 18H), 1.36-1.45 (m, 2H), 2.98-3.20 (m, 2H), 3.79-4.85 (m, 4H), 7.55-7.63 (m, 3ArH), 7.75-7.77 (m, 1ArH), 7.99-8.05 (m, 1ArH), 8.12-8.19 (m, 4NH), 8.21-8.34 (m, 2ArH), 11.27 (s, 1NH) ppm. t'$_R$ = 21.60 min (%2-propanol = 62.40%). HPLC purity: 98.8%. LCMS: *m/z* = 469.3 $[M\text{-}Cl]^+$, 491.3 $[M\text{-}HCl+Na]^+$. Elemental analysis: Anal. Calcd for $C_{27}H_{41}ClN_4O_3$: C, 64.20; H, 8.18; N, 11.09. Found: C, 64.29; H, 8.21; N, 11.05.

*4.2.7 General procedure for the synthesis of compounds **5Ab** and **5Ac***

Note: representative procedure referred to 1 mmol of starting compound. To a solution of compound **3Ab** or **3Ac** dissolved in dry THF (5 mL) under inert atmosphere at 0 °C, LHMDS 1.0 M in THF (1.01 mL, 1.01 mmol) and CH_3I (65.4 μL, 1.05 mmol) were sequentially added. The mixture was stirred at 0 °C for 10 min and subsequently at room temperature for 2 h, then HCl 1 M (1.05 mL) was added. The volatile species were removed under vacuum at room temperature. The residue was diluted with AcOEt (50 mL) and water (20 mL), then the phases were separated and the organic layer was washed with additional water (5 mL). The aqueous phases were newly extracted with 50 mL di AcOEt, then the second organic phase was washed with 5 mL water. The combined organic phases were dried over anhydrous Na_2SO_4, the solvent was evaporated under vacuum and the residue was purified by flash chromatography on silica gel (DCM/MeOH mixtures as eluents), obtaining the pure compound.

*4.2.7.1 Benzyl 2-(2-((tert-butoxycarbonyl)glycyl)-1-methyl-2-(2-(octylamino)-2-oxoethyl)hydrazinecarboxylate, BnOCO-Hydr(Me)Gly-NHC$_8$H$_{17}$, (**5Ab**).* Starting from **2Ab** (3.00 mmol, 1.48 g) and following the general procedure, compound **5Ab** was obtained in 87% yield (2.61 mmol, 1.32 g) as a colorless pitchy compound. R_f = 0.33 (DCM:MeOH 95:5). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.87 (t, *J* = 6.6 Hz, 3H), 1.20-1.33 (m, 10H), 1.44 (s, 9H), 1.41-1.50 (m, 2H), 3.12-3.26 (m, 2H), 3.24 (s, 3H), 3.65-4.43 (m, 4H), 5.14-5.23 (m, 3H), 7.30-7.42 (m, 5ArH+1NH) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.2, 22.7, 27.0, 28.4, 29.3, 31.9, 36.6, 39.7, 41.8, 51.8, 69.4, 80.1, 128.4, 128.9, 135.0, 155.9, 167.1, 171.5 ppm. LCMS: *m/z* = 507.3 $[M+H]^+$, 529.3 $[M+Na]^+$.

*4.2.7.2 Benzyl 2-(2-((tert-butoxycarbonyl)glycyl)-2-(2-(dodecylamino)-2-oxoethyl)-1-methylhydrazinecarboxylate, BnOCO-Hydr(Me)Gly-NHC$_{12}$H$_{25}$, (**5Ac**).* Starting from **2Ac** (3.01 mmol, 1.65 g) and following the general procedure, compound **5Ac** was obtained in 90% yield (2.72 mmol, 1.53 g) as a white amorphous solid. R_f = 0.12 (cyclohexane:AcOEt 1:1). M.p. = 74-76 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.87 (t, *J* = 6.6 Hz, 3H), 1.18-1.34 (m, 18H), 1.44 (s, 11H), 3.11-3.29 (m, 2H), 3.24 (s, 3H), 3.68-4.39 (m, 4H), 5.14-5.23 (m, 3H), 7.28-7.47 (m, 5ArH+1NH) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.2, 22.7, 26.9, 28.4, 29.3, 29.4, 29.63, 29.67, 29.69, 29.71, 32.0, 36.5, 39.7, 41.8, 51.7, 69.4, 80.0, 128.3, 128.8, 135.0, 155.9, 167.0, 171.5 ppm. LCMS: *m/z* = 563.4 $[M+H]^+$, 585.3 $[M+Na]^+$.

*4.2.8 General procedure for the synthesis of compounds **5Bb**, **5Bc**, **5Gb**, and **5Gc***

Note: representative procedure referred to 1 mmol of starting compound. To a solution of compound **5Ab** or **5Ac** dissolved in dry DCM (2 mL) under inert atmosphere at room temperature, Pd/C (100 mg) and formic acid (76 µL, 2 mmol) were sequentially added and the mixture was stirred for 1 hour. The volatile species were removed under vacuum at room temperature, then DCM (20 mL) was added and the reaction mixture was filtered through Celite, washing with DCM (3 × 10 mL). The organic phase was washed with a saturated solution of Na_2CO_3 (5 mL), then the aqueous phase was newly extracted with DCM (25 mL) and, after separation, the second organic phase was washed with a saturated solution of Na_2CO_3 (5 mL). The combined organic phases were dried over anhydrous Na_2SO_4, the solvent was evaporated under vacuum at room temperature and the free hydrazide intermediate was directly submitted to the following acylation reaction or stored at -18 °C. To a solution of the suitable free hydrazide intermediate in dry DCM (5 mL) under inert atmosphere at room temperature, pyridine (121 µL, 1.5 mmol, for the synthesis of **5Bb** and **5Bc**; 145 µL, 1.8 mmol, for the synthesis of **5Gb** and **5Gc**) was added, followed by dropwise addition of the suitable acyl chloride (acetyl chloride, 78.5 µL, 1.1 mmol, for the synthesis of **5Bb** and **5Bc**; pivaloyl chloride, 185 µL, 1.5 mmol, for the synthesis of **5Gb** and **5Gc**). The solution was stirred at room temperature for 1 h (**5Bb** and **5Bc**) or 20 h (**5Gb** and **5Gc**), then all the volatile species were removed in vacuo at room temperature and the residue was diluted with AcOEt (30 mL) and water (5 mL). After separation, the organic phase was washed with HCl 1 M (2 × 3 mL), saturated aqueous sodium carbonate (5 mL) and water (5 mL). The aqueous phases were sequentially extracted with additional 30 mL of ethyl acetate, then the second organic phase was washed with HCl 1 M (2 × 3 mL), saturated aqueous sodium carbonate (5 mL) and water (5 mL). The combined organic phases were dried over anhydrous sodium sulfate and evaporated under vacuum, then the crude product was purified by column chromatography on silica gel (cyclohexane/AcOEt mixtures as eluents), to give the pure compound.

*4.2.8.1 tert-Butyl (2-(2-acetyl-2-methyl-1-(2-(octylamino)-2-oxoethyl)hydrazinyl)-2-oxoethyl)carbamate, CH_3CO-Hydr(Me)Gly-NHC_8H_{17}, (**5Bb**).* Starting from **5Ab** (505 µmol, 256 mg) and following the general procedure, compound **5Bb** was obtained in 87% yield (439 µmol, 182 mg) as a colorless pitchy compound. R_f = 0.10 (cyclohexane:AcOEt 2:8). ^{1}H NMR (400 MHz, $CDCl_3$, three conformers in a 64:28:8 ratio): δ 0.85-0.88 (m, 3H), 1.19-1.34 (m, 10H), 1.43 (s, 9H), 1.45-1.55 (m, 2H), 2.08 (s, 3H, 28%), 2.19 (s, 3H, 64%+8%), 3.17-3.24 (m, 2H + 3H, 28%+8%), 3.35 (s, 3H, 64%), 3.68 (dd, *J* = 3.5 Hz, 17.6 Hz, 1H, 64%+8%), 3.73 (d, *J* = 15.6 Hz, 1H, 28%), 3.85 (d, *J* = 16.8 Hz, 1H,

64%), 3.89-3.94 (m, 1H, 8%, + 2H, 28%), 4.05 (dd, *J* = 6.2 Hz, 17.6 Hz, 1H, 64%+8%), 4.25 (d, *J* = 18.0 Hz, 1H, 8%), 4.31 (d, *J* = 16.8 Hz, 1H, 64%), 4.48 (d, *J* = 15.6 Hz, 1H, 28%), 5.19 (bs, 1NH, 28%), 5.23 (bs, 1NH, 64%+8%), 6.28 (bs, 1NH, 28%), 7.63 (bs, 1NH, 64%), 8.73 (bs, 1NH, 8%) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, three conformers in a 64:28:8 ratio, only the major conformer is given): δ 14.1, 21.6, 22.7, 26.9, 28.4, 29.2, 31.8, 38.1, 39.7, 41.6, 51.7, 80.1, 155.9, 167.1, 170.7, 171.0 ppm. LCMS: *m/z* = 415.3 $[M+H]^+$, 437.3 $[M+Na]^+$.

*4.2.8.2 tert-Butyl (2-(2-acetyl-1-(2-(dodecylamino)-2-oxoethyl)-2-methylhydrazinyl)-2-oxoethyl)carbamate, CH_3CO-Hydr(Me)Gly-$NHC_{12}H_{25}$, (**5Bc**).* Starting from **5Ac** (547 μmol, 308 mg) and following the general procedure, compound **5Bc** was obtained in 86% yield (470 μmol, 221 mg) as a colorless pitchy compound. R_f = 0.29 (AcOEt). ^{1}H NMR (400 MHz, $CDCl_3$, three conformers in a 62:31:7 ratio): δ 0.87 (t, *J* = 6.8 Hz, 3H), 1.20-1.35 (m, 18H), 1.44 (s, 9H), 1.45-1.55 (m, 2H), 2.09 (s, 3H, 31%), 2.20 (s, 3H, 62%+7%), 3.17-3.24 (m, 2H + 3H, 31%+7%), 3.35 (s, 3H, 62%), 3.66-3.75 (m, 1H, 62%+7%, + 1H, 31%), 3.85 (d, *J* = 16.8 Hz, 1H, 62%), 3.87-3.94 (m, 1H, 7%, + 2H, 31%), 4.05 (dd, *J* = 4.6 Hz, 17.6 Hz, 1H, 62%+7%), 4.25 (d, *J* = 18.0 Hz, 1H, 7%), 4.33 (d, *J* = 16.8 Hz, 1H, 62%), 4.48 (d, *J* = 15.2 Hz, 1H, 31%), 5.17 (bs, 1NH, 31%), 5.21 (bs, 1NH, 62%+7%), 6.23 (bs, 1NH, 31%), 7.65 (bs, 1NH, 62%), 8.74 (bs, 1NH, 7%) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, three conformers in a 62:31:7 ratio, only the major conformer is given): δ 14.2, 21.6, 22.8, 27.0, 28.4, 29.3, 29.4, 29.5, 29.66, 29.70, 29.75, 32.0, 38.2, 39.8, 41.6, 51.7, 80.2, 156.0, 167.1, 170.9, 170.9 ppm. LCMS: *m/z* = 471.4 $[M+H]^+$, 493.3 $[M+Na]^+$.

*4.2.8.3 tert-Butyl (2-(2-methyl-1-(2-(octylamino)-2-oxoethyl)-2-pivaloylhydrazinyl)-2-oxoethyl)carbamate, tBuCO-Hydr(Me)Gly-NHC_8H_{17}, (**5Gb**).* Starting from **5Ab** (419 μmol, 212 mg) and following the general procedure, compound **5Gb** was obtained in 81% yield (339 μmol, 155 mg) as a colorless pitchy compound. R_f = 0.12 (cyclohexane:AcOEt 2:8). ^{1}H NMR (400 MHz, $CDCl_3$, two conformers in a 90:10 ratio, all the assignable signals are given): δ 0.84-0.87 (m, 3H), 1.17-1.30 (m, 10H), 1.31 (s, 9H), 1.42 (s, 9H), 1.44-1.52 (m, 2H), 3.12-3.26 (m, 2H), 3.30 (s, 3H, 10%), 3.45 (s, 3H, 90%), 3.57 (dd, *J* = 3.5 Hz, 17.6 Hz, 1H), 3.95 (d, *J* = 16.8 Hz, 1H), 4.00 (dd, *J* = 6.3 Hz, 17.6 Hz, 1H), 4.16 (d, *J* = 16.8 Hz, 1H), 4.99 (bs, 1NH, 10%), 5.23 (bs, 1NH, 90%), 7.56 (bs, 1NH, 90%), 8.73 (bs, 1NH, 10%) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, two conformers in a 90:10 ratio, only the major conformer is given): δ 14.2, 22.7, 27.0, 27.5, 28.4, 29.34, 29.36, 31.9, 38.7, 39.7, 41.5, 52.2, 80.1, 156.0, 167.3, 171.1, 178.0 ppm. LCMS: *m/z* = 457.3 $[M+H]^+$, 479.3 $[M+Na]^+$.

*4.2.8.4 tert-Butyl (2-(1-(2-(dodecylamino)-2-oxoethyl)-2-methyl-2-pivaloylhydrazinyl)-2-oxoethyl)carbamate, tBuCO-Hydr(Me)Gly-NHC$_{12}$H$_{25}$, (**5Gc**).* Starting from **5Ac** (604 μmol, 340 mg) and following the general procedure, compound **5Gc** was obtained in 92% yield (550 μmol, 282 mg) as a colorless pitchy compound. R_f = 0.50 (AcOEt). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.87 (t, *J* = 6.8 Hz, 3H), 1.19-1.29 (m, 18H), 1.33 (s, 9H), 1.43 (s, 9H), 1.44-1.54 (m, 2H), 3.13-3.29 (m, 2H), 3.46 (s, 3H), 3.58 (dd, *J* = 3.1 Hz, 17.6 Hz, 1H), 3.95 (d, *J* = 16.4 Hz, 1H), 4.02 (dd, *J* = 6.0 Hz, 17.6 Hz, 1H), 4.19 (d, *J* = 16.4 Hz, 1H), 5.21 (bs, 1NH), 7.54 (bs, 1NH) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.3, 22.8, 27.0, 27.56, 27.61, 28.5, 29.40, 29.45, 29.49, 29.7, 29.8, 32.1, 38.7, 39.7, 41.5, 52.2, 80.3, 156.0, 167.3, 171.1, 177.9 ppm. LCMS: *m/z* = 513.4 $[M+H]^+$, 535.4 $[M+Na]^+$.

*4.2.9 General procedure for the synthesis of N-methyl hydrazide hydrochlorides **6***

The procedure is the same previously reported for the synthesis of hydrochlorides **4**.

*4.2.9.1 2-(2-((Benzyloxy)carbonyl)-2-methyl-1-(2-(octilamino)-2-oxoethyl)hydrazinyl)-2-oxoethanaminium chloride, BnOCO-Hydr(Me)Gly-NHC$_8$H$_{17}$ · HCl, (**6Ab**).* Starting from **5Ab** (201 μmol, 102 mg) and following the general procedure, compound **6Ab** was obtained in 82% yield (165 μmol, 73.1 mg) as a colorless pitchy compound. R_f = 0.13 (free amine, DCM:MeOH 95:5). ^{1}H NMR (400 MHz, DMSO-d_6, mixture of conformers): δ 0.85 (t, *J* = 7.0 Hz, 3H), 1.24 (s, 10H), 1.32-1.46 (m, 2H), 3.02-3.15 (m, 2H), 3.21 (s, 3H), 3.48-3.73 (m, 1H), 3.78-4.15 (m, 2H), 4.40-4.52 (m, 1H), 5.00-5.26 (m, 2H), 7.27-7.43 (m, 5ArH), 8.05 (t, *J* = 5.5 Hz, 1NH), 8.10-8.33 (m, 4NH) ppm. t'$_R$ = 13.77 min (%2-propanol = 50.66%). HPLC purity: 96.4%. LCMS: *m/z* = 407.3 $[M-Cl]^+$, 429.2 $[M-HCl+Na]^+$. Elemental analysis: Anal. Calcd for $C_{21}H_{35}ClN_4O_4$: C, 56.94; H, 7.96; N, 12.65. Found: C, 57.02; H, 7.99; N, 12.64.

*4.2.9.2 2-(2-((Benzyloxy)carbonyl)-1-(2-(dodecylamino)-2-oxoethyl)-2-methylhydrazinyl)-2-oxoethanaminium chloride, BnOCO-HydrGly(Me)-NH-C$_{12}$H$_{25}$ · HCl, (**6Ac**).* Starting from **5Ac** (200 μmol, 112.8 mg) and following the general procedure, compound **6Ac** was obtained in 97% yield (194 μmol, 96.7 mg) as a colorless waxy solid. R_f = 0.72 (free amine, DCM:MeOH 8:2). ^{1}H NMR (400 MHz, DMSO-d_6, mixture of conformers): δ 0.85 (t, *J* = 7.0 Hz, 3H), 1.23 (s, 18H), 1.32-1.44 (m, 2H), 2.98-3.15 (m, 2H), 3.21 (s, 3H), 3.48-3.74 (m, 1H), 3.83-4.13 (m, 2H), 4.43-4.52 (m, 1H), 5.00-5.20 (m, 2H), 7.30-7.41 (m, 5ArH), 8.04 (t, *J* = 5.5 Hz, 1NH), 8.08-8.30 (m, 4NH) ppm. t'$_R$ = 20.66 min

(%2-propanol = 60.99%). HPLC purity: 99.4%. LCMS: *m/z* = 463.3 $[M\text{-}Cl]^+$, 485.3 $[M\text{-}HCl+Na]^+$. Elemental analysis: Anal. Calcd for $C_{25}H_{43}ClN_4O_4$: C, 60.16; H, 8.68; N, 11.23. Found: C, 60.11; H, 8.69; N, 11.25.

*4.2.9.3 2-(2-Acetyl-2-methyl-1-(2-(octylamino)-2-oxoethyl)hydrazinyl)-2-oxoethanaminium chloride, CH_3CO-HydrGly(Me)-NHC_8H_{17} · HCl, (**6Bb**).* Starting from **5Bb** (80.1 μmol, 33.2 mg) and following the general procedure, compound **6Bb** was obtained in 75% yield (60.4 μmol, 21.1 mg) as a colorless pitchy compound. R_f = 0.16 (free amine, DCM:MeOH 9:1). ^{1}H NMR (400 MHz, DMSO-d_6, mixture of 3 major conformers in about 1:1:1 ratio and a minor conformer): δ 0.86 (t, *J* = 6.8 Hz, 3H), 1.24 (s, 10H), 1.34-1.46 (m, 2H), 1.88 (s, 3H, major conf.), 2.02 (s, 3H, major conf.), 2.07 (s, 3H, minor conf.), 2.10 (s, 3H, major conf.), 2.90 (s, 3H, major conf.), 3.02-3.16 (m, 2H), 3.10 (s, 3H, major conf.), 3.16 (s, 3H, minor conf.), 3.31 (s, 3H, major conf.), 3.40-3.51 (m, 2H, major conf.), 3.70 (d, *J* = 16.4 Hz, 1H, major conf.), 3.74-3.94 (m, 1H+1H two major conf. and minor conf.), 4.00 (d, *J* = 16.0 Hz, 1H, major conf.), 4.12 (d, *J* = 17.2 Hz, 1H, major conf.+minor conf.), 4.41 (d, *J* = 16.4 Hz, 1H, major conf.), 4.42 (d, *J* = 16.0 Hz, 1H, major conf.), 4.49 (d, *J* = 17.2 Hz, 1H, major conf.), 8.05-8.39 (m, 4NH) ppm. t'_R = 9.11 min (%2-propanol = 43.67%). HPLC purity: 98.6%. LCMS: *m/z* = 315.3 $[M\text{-}Cl]^+$, 337.2 $[M\text{-}HCl+Na]^+$. Elemental analysis: Anal. Calcd for $C_{15}H_{31}ClN_4O_3$: C, 51.35; H, 8.91; N, 15.97. Found: C, 51.49; H, 8.88; N, 15.94.

*4.2.9.4 2-(2-Acetyl-1-(2-(dodecylamino)-2-oxoethyl)-2-methylhydrazinyl)-2-oxoethanaminium chloride, CH_3CO-HydrGly(Me)-$NHC_{12}H_{25}$ · HCl, (**6Bc**).* Starting from **5Bc** (83.1 μmol, 39.1 mg) and following the general procedure, compound **6Bc** was obtained in 78% yield (64.6 μmol, 26.3 mg) as a colorless pitchy compound. R_f = 0.42 (free amine, DCM:MeOH 8:2). ^{1}H NMR (400 MHz, DMSO-d_6, mixture of 3 major conformers in about 1:1:1 ratio and a minor conformer): δ 0.85 (t, *J* = 6.8 Hz, 3H), 1.24 (s, 18H), 1.33-1.44 (m, 2H), 1.88 (s, 3H, major conf.), 2.01 (s, 3H, major conf.), 2.07 (s, 3H, minor conf.), 2.10 (s, 3H, major conf.), 2.90 (s, 3H, major conf.), 3.01-3.15 (m, 2H), 3.10 (s, 3H, major conf.), 3.16 (s, 3H, minor conf.), 3.31 (s, 3H, major conf.), 3.70 (d, *J* = 16.4 Hz, 1H, major conf.), 3.74-3.95 (m, 1H+1H two major conf. and minor conf.), 4.00 (d, *J* = 16.0 Hz, 1H, major conf.), 4.11 (d, *J* = 17.2 Hz, 1H, major conf.+minor conf.), 4.41 (d, *J* = 16.4 Hz, 1H, major conf.), 4.42 (d, *J* = 16.0 Hz, 1H, major conf.), 4.48 (d, *J* = 17.2 Hz, 1H, major conf.), 8.03-8.34 (m, 4NH) ppm. t'_R = 17.05 min (%2-propanol = 55.58%). HPLC purity: 97.4%. LCMS: *m/z* = 371.3 $[M\text{-}Cl]^+$, 393.3 $[M\text{-}HCl+Na]^+$. Elemental

analysis: Anal. Calcd for $C_{19}H_{39}ClN_4O_3$: C, 56.07; H, 9.66; N, 13.77. Found: C, 56.02; H, 9.66; N, 13.82.

*4.2.9.5 2-(2-Methyl-1-(2-(octylamino)-2-oxoethyl)-2-pivaloylhydrazinyl)-2-oxoethanaminium chloride, tBuCO-HydrGly(Me)-NHC$_8$H$_{17}$ · HCl, (**6Gb**).* Starting from **5Gb** (180 μmol, 82.3 mg) and following the general procedure, compound **6Gb** was obtained in 98% yield (176 μmol, 69.2 mg) as a colorless pitchy compound. R_f = 0.40 (free amine, DCM:MeOH 8:2). ^{1}H NMR (400 MHz, DMSO-d_6, two conformers in about 7:3 ratio): δ 0.85 (t, *J* = 7.0 Hz, 3H), 1.12 (s, 9H, minor conf.), 1.23 (s, 9H, major conf.), 1.24 (s, 10H), 1.34-1.46 (m, 2H), 2.86-3.21 (m, 2H+2H, minor conformer), 3.39 (bs, 3H, minor conf.), 3.43 (s, 3H, major conf.), 3.66 (d, *J* = 16.4 Hz, 1H, major conf.), 3.88-4.13 (m, 2H, major conf., + 1H, minor conf.), 4.46 (d, *J* = 16.4 Hz, 1H, major conf.), 4.58-4.77 (m, 1H, minor conf.), 8.10 (t, *J* = 5.6 Hz, 3NH, minor conf.), 8.14 (bs, 3NH, major conf.), 8.28 (bs, 1NH, major conf.), 8.38 (t, *J* = 5.2 Hz, 1NH, minor conf.) ppm. t'$_R$ = 11.66 min (%2-propanol = 47.49%). HPLC purity: 95.3%. LCMS: *m/z* = 357.3 $[M\text{-}Cl]^+$, 379.3 $[M\text{-}HCl+Na]^+$. Elemental analysis: Anal. Calcd for $C_{18}H_{37}ClN_4O_3$: C, 55.02; H, 9.49; N, 14.26. Found: C, 55.10; H, 9.50; N, 14.26.

*4.2.9.6 2-(1-(2-(Dodecylamino)-2-oxoethyl)-2-methyl-2-pivaloylhydrazinyl)-2-oxoethanaminium chloride, tBuCO-HydrGly(Me)-NHC$_{12}$H$_{25}$ · HCl, (**6Gc**).* Starting from **5Gc** (185 μmol, 94.9 mg) and following the general procedure, compound **6Gc** was obtained in 98% yield (181 μmol, 81.4 mg) as a colorless waxy solid. R_f = 0.53 (free amine, DCM:MeOH 8:2). ^{1}H NMR (400 MHz, DMSO-d_6, two conformers in about 7:3 ratio): δ 0.85 (t, *J* = 7.0 Hz, 3H), 1.14 (s, 9H, minor conf.), 1.23 (s, 9H, major conf.), 1.24 (s, 18H), 1.34-1.45 (m, 2H), 2.98-3.21 (m, 2H+2H, minor conformer), 3.40 (bs, 3H, minor conf.), 3.43 (s, 3H, major conf.), 3.66 (d, *J* = 16.4 Hz, 1H, major conf.), 3.81-4.27 (m, 2H, major conf., + 1H, minor conf.), 4.46 (d, *J* = 16.4 Hz, 1H, major conf.), 4.57-4.71 (m, 1H, minor conf.), 8.09 (t, *J* = 5.2 Hz, 3NH), 8.24 (bs, 1NH, major conf.), 8.34-8.37 (m, 1NH, minor conf.) ppm. t'$_R$ = 18.93 min (%2-propanol = 58.40%). HPLC purity: 98.8%. LCMS: *m/z* = 413.3 $[M\text{-}Cl]^+$, 435.4 $[M\text{-}HCl+Na]^+$. Elemental analysis: Anal. Calcd for $C_{22}H_{45}ClN_4O_3$: C, 58.84; H, 10.10; N, 12.48. Found: C, 58.78; H, 10.09; N, 12.53.

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- Novel amphiphilic α-hydrazido acid derivatives were synthesized
- Good activity was assessed towards Gram-positive and Gram-negative bacteria
- Some analogues exhibited good therapeutic indices
- A non-disrupted amphiphilicity is required for antimicrobial activity
- Either the antibacterial or the hemolytic activity relies on overall lipophilicity

Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: