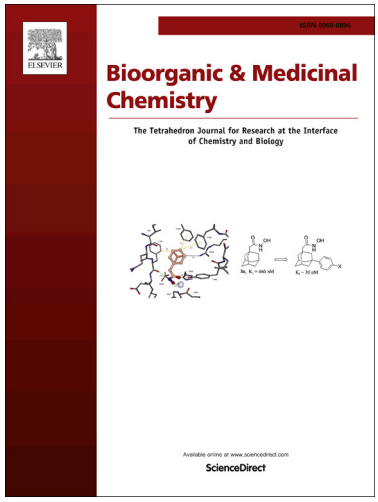

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Synthesis and antimicrobial activity of chloramphenicol-polyamine conjugates

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ABSTRACT

A series of chloramphenicol (CAM) amides with polyamines (PAs), suitable for structure-activity relationship studies, were synthesized either by direct attachment of the PA chain on the 2-aminopropane-1,3-diol backbone of CAM, previously oxidized selectively at its primary hydroxyl group, or from chloramphenicol base (CLB) through acylation with succinic or phthalic anhydride and finally coupling with a PA. Conjugates **4** and **5**, in which the CLB moiety was attached on N4 and N1 positions, respectively, of the N^8,N^8-dibenzylated spermidine through the succinate linker, were the most potent antibacterial agents. Both conjugates were internalized into *E. coli* cells by using the spermidine-preferential uptake system and caused decrease in protein and polyamine content of the cells. Noteworthy, conjugate **4** displayed comparable activity to CAM in MRSA or wild-type strains of *Staphylococcus aureus* and *Escherichia coli*, but superior activity in *E. coli* strains possessing ribosomal mutations or expressing the CAM acetyltransferase (*cat*) gene. Lead compounds, and in particular conjugate **4**, have been therefore discovered during the course of the present work with clinical potential.

Keywords:

Chloramphenicol
Polyamines
Polyamine conjugates
Polyamine uptake system
Antibacterial activity

1. Introduction

Chloramphenicol (CAM) was isolated in 1947 from *Streptomyces venezuele* and two years after was introduced in clinical practice [1]. Its molecule consists of a *p*-nitrobenzene moiety, an 2-amino-1,3-propanediol moiety, and a dichloroacetyl tail (Fig. 1). CAM is active against a wide range of bacteria, usually behaving as a bacteriostatic drug, although it exhibits bactericidal activity against the three most common causes of meningitis, *Haemophilus influenza*, *Streptococcus pneumonia*, and *Neisseria meningitides* [2]. CAM inhibits protein synthesis by binding to the peptidyl transferase (PTase) center of the bacterial ribosome and abrogating essential ribosomal functions, like peptide-bond formation [3], termination of translation [4], and translational accuracy [5].

Resistance or decreased sensitivity to CAM is frequently mediated by mutations/modifications in residues of the ribosomal binding site [6-9], as well as by decreased influx [10] or/and over-expression of efflux pumps [11], and acetylation of hydroxyl groups in the 2-amino-1,3-propanediol moiety of CAM [12]. However the most important concerns, that limit the utility of this antibiotic, relate with its adverse effects, like neurotoxicity [13], bone marrow depression, and in some cases severe aplastic anaemia [14]. Intensive research on the adverse effects of CAM has led to the conclusion that the toxicity of CAM may have a mitochondrial basis [15], particularly in cases in which underlying mitochondrial defects accentuate CAM binding to mitochondrial ribosomes [16].

Elucidation of the X-ray structures of CAM bound to bacterial 50S ribosomal subunits have revealed all the ribosomal residues implicated in the drug binding [17-19]. Most of them are consistent with findings from mutational studies [6-9] and footprinting analysis [20-22], and agree with kinetic evidence suggesting that CAM primarily binds adjacently to the A-site of the PTase center, blocking the accommodation of the 3´-aminoacyl end of tRNA within the catalytic region [3]. Two

nucleotides, A2058 and A2059 (*Escherichia coli* numbering is followed throughout the text), placed outside the PTase center, seem to influence the binding of the drug by allosteric effects [22] or constitute a secondary binding site of low affinity [23]. Interestingly, crystallographic study on 50S ribosomal subunits from *Deinococcus radiodurans* in complex with CAM revealed two putative cationic centers within the PTase center, involved in the stabilization of the ribosome-CAM complex [17]. One of these centers, regarded as a Mg^{2+} ion, was found to coordinate the interaction between the 3-OH group of CAM and the carbonyl oxygen at position 4 of U2506. At the resolution level of this study, however, the possibility of cationic center being either a monovalent ion or a polyamine (PA) could not be precluded. In fact, kinetic analysis using a cell-free system derived from *E. coli* has indicated that PAs enhance the CAM potency [3]. On the other hand, photoaffinity labeling experiments have revealed that the binding site of CAM on the ribosome is one of the preferred cross-linking sites of PAs [24].

The above information prompted us to design and synthesize a series of CAM-PA conjugates (CAM-PACs), expecting that the PA-conjugated portion could strengthen the binding of CAM to the ribosome. Moreover, decorating CAM with PAs could facilitate the transport of CAM into the bacterial cells by exploiting their capacity of destabilizing the liposaccharide layer of the outer bacterial membrane [10] or/and acting as a vector system by taking advantage of the bacterial uptake system for PAs [25]. Previous studies from our group and other investigators have indicated that the uptake system for PAs in bacteria consists of two members belonging to the ATP binding cassette (ABC) transporters, characterized by substrate-specificity but also by high tolerance for the import of non-native PAs [25-27].

The synthesized series of CAM-PACs **1-9** is depicted in Figure 1. In these conjugates, the PA chain is either attached directly on the 2-aminopropane-1,3-diol backbone of CAM, by selectively oxidizing the primary hydroxyl group of CAM, or using a dicarboxylic acid linker (succinic or phthalic acid) replacing the dichloroacetyl part of CAM. With these particular conjugates, we wanted to examine the effect of (a) the site of attachment of the PA chain on the CAM antibacterial activity (e.g. compounds **2** and **7**) and at the same time to verify if a PA attached to the 3-position of the 2-aminopropane-1,3-diol chain could mediate interaction of the CAM-PAC with the U2506 nucleotide of the PTase center, (b) the size and the number of the free amino functions of the PA chain (compounds **1**-**3**), (c) the site of

attachment of CAM on the PA chain (compounds **4** and **5**), (d) increasing the lipophilicity of the PA chain by incorporating benzyl groups on N atoms (compounds **3** and **4**), and (e) the nature and the flexibility of the linker (compounds **1** and **6**). We also included in this study two analogues of CAM in which the dichloroacetyl part of the molecule has been replaced by the 1,2,4-triazole-3-carboxylate unit, which is either directly connected to the 2-amino group (amide **8**) or through a β-alanine spacer (bisamide **9**). With these compounds we wished to examine the effect of replacing the two chlorine atoms of CAM by N atoms and further evaluate whether removing this replacement away from the 2-aminopropane-1,3-diol main chain would have any amplifying effect on the antibacterial activity of the parent compound.

Recently, we published on the effects of CAM-PACs **1-9** on peptide bond formation catalyzed by the ribosome in a cell-free system derived from *E. coli*, the growth of Gram- negative and Gram-positive bacteria, as well as on the viability of human, healthy and cancer cells [28]. We found that compounds **4** and **5** exhibited higher activity than CAM in inhibiting the in vitro peptide-bond formation, compounds **1-3** and **8** displayed activity comparable to those of CAM, while the rest were essentially inactive. Although compounds **4** and **5** displayed better activity than CAM against CAM-resistant *E. coli* strains, they were less active against wild-type *Streptococcus aureus* and *E. coli* cells. Compounds **2**, **3** and **9** showed a little activity against *S. aureus*, while the rest were quite inactive against both bacteria. This fact revealed that penetration of the cellular envelope or/and efflux mechanisms may be significant obstacles to the effectiveness of PA-CAM conjugates acting as antibacterials. However, compound **4** compared to CAM possessed stronger toxicity against human leukemic cells, and lower toxicity against healthy human leukocytes, thus highlighting the potentiality of compound **4** as a safe antibacterial and anticancer agent**.** In the present study, we focus on the synthesis of CAM-PACs **1-9**, the mechanisms by which they penetrate the bacterial outer and inner cellular membranes, and the effects of efflux pumps and the CAM acetyltransferase activity on their efficacy. Unexpectedly, we found that compounds **4** and **5**, once administrated to *E coli* cells, cause additionally severe depletion in intracellular polyamine pools, a finding suggesting that these compounds might have additional targets for their inhibitory effects beyond their impact on peptide-bond formation.

2. Results and Discussion

2.1. Chemistry

The synthesis of CAM-PACs **1-5** is depicted in Scheme 1. It involves a one-pot acylation of the commercially available chloramphenicol base (CLB) with succinic anhydride followed by coupling with the appropriate selectively protected, with the amino protecting groups triphenylmethyl (Trt) or butoxycarbonyl (Boc) or trifluoroacetyl (Tfa), PA derivatives **10**-**13**, in the presence of the coupling agent *O*-(benzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate (HBTU) and ethyldiisopropylamine. With the exception of PA derivative **14**, which was synthesised from **13** through N^4-trifluoroacetylation and trifluoroacetic acid (TFA)-mediated N^1-detritylation, the preparation of the other required PA derivatives **10** [29], **11** [30], **12** [29] and **13** [31] have been described in the literature. From the thus obtained bisamides, the protecting groups were routinely removed by TFA-mediated acidolysis or saponification to provide the anticipated compounds **1**-**5** (see experimental section). Similarly, acylation of CLB with phthalic anhydride, followed by coupling with PA derivative **10** gave the anticipated bisamide, which upon TFA-mediated deprotection provided conjugate **6**. On the other hand, conjugate **8** was readily obtained from coupling of CLB with the commercially available 1,2,4-triazole-3-carboxylic acid (TCA). Finally, coupling of CLB with 'active' ester Trt-β-Ala-OSu [32] followed by TFA-mediated deprotection and further coupling with TCA gave conjugate **9**.

The synthesis of conjugate **7** is depicted in Scheme 2. It involved selective protection of the primary hydroxyl function of CAM with the bulky *tert*-butyldimethylsilyl (TBDMS) group, followed by protection of the secondary hydroxyl group with the acetyl (Ac) group to afford the fully protected compound **15**. Selective removal of the TBDMS group gave compound **16**, which upon oxidation of the primary hydroxyl function, provided the corresponding stable carboxylic acid **17** in 55% total yield. It should be noted that direct selective oxidation of the primary hydroxyl group of CAM or N-Boc [33, 34] protected CLB has been reported but leads to the corresponding acids in low to moderate yields. In particular the acid from CAM could be only purified and isolated as its corresponding methyl ester [33]. Acid **17** was then coupled with SPM derivative **11** using the 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI)/ 1-hydroxybenzotriazole (HOBt) methodology to give the fully protected intermediate **18**. From this

compound, CAM-PAC **7** was finally obtained upon sequential deprotection, first of the secondary hydroxyl group, and then of the amino functions of the PA chain.

2.2. Biological evaluation

The antibacterial activities of PA-CAMCs against a panel of CAM-sensitive and CAM-resistant bacteria are shown in Table 1. Compounds **4** and **5** were the most active against *S. aureus* and *E. coli* strains of wild-type, followed by compounds **3**, and **2**, however only against the *S. aureus* strain. It is evident that replacement of hydrogen atoms by benzyl groups in the N8-amino function of the spermidine (SPD) tail of compounds **3,** a modification that enhances the lipophilicity of the PA chain and possibly its potency to adopt stacking interactions with nucleotide bases of ribosomal RNA, has prominent influence on its antibacterial activity and recognition by the PA-transporters (compare conjugates **3** and **4**). Indeed, footprinting analysis and Molecular Dynamics (MD) simulations have revealed that incorporation of benzyl rings at the polyamine end of compound **4** provides additional stability on the binding to the ribosome through π-stacking interactions of the benzyl rings with U2585 and U2586 of 23S rRNA [28]. It is yet known that a periplasmic component of the SPD/spermine (SPM)-preferential uptake system, the PotD protein, specifically recognizes PAs analogues with intact aminopropane portion(s) [25, 26], while benzyl substitution at the N4 and N9 amino groups of SPM contributes favorably to the affinity of acyl-PA analogues for the transporter [27]. Therefore, it is tempting to assume that CAM-PACs **4** and **5** receive better recognition by the PotD protein, than the non-benzylated ones **2**, **3**, and **7**. Also, compound **4** is more active than **5**, probably because it possesses an intact aminopropane portion. The position of polyamine tail and the CAM scaffold, through which the two molecules are coupled, appears to be of paramount importance. Thus, attachment of SPM at position 3 of the 2-aminopropane-1,3-diol moiety of CAM, following oxidation of the hydroxymethyl to the carboxyl group, instead of position 2 (the amino function functionalized by a succinate linker), led to diminution of activity (compare compounds **2** and **7**). Interestingly, the length of the PA chain and the number of the free amino functions that PA chain carries seem to play critical roles for activity. Thus, comparison of the IC_{50} values for conjugates **1**-**5** reveals that the SPD chain offers better opportunities for activity, followed by the SPM chain, whereas PUT chain is associated with less activity.

The design of compound **7** was based on X-ray crystallographic studies in crystals of *Deinococcus radiodurans* 50S ribosomal subunit complexed with CAM, in which the *p*-nitrobenzene moiety of CAM is directed away from the catalytic center, while two putative Mg^{2+} ions, Mg-C1 and Mg-C2, mediate interactions between the 3OH and one of the oxygens of the *p*-NO_2 of CAM with U2506 and U2500 of 23S rRNA, respectively, holding the drug in place [17]. Unfortunately, the activity of compound **7** in our study was much lower than expected (Table 1). In fact, a recent crystallographic study conducted in *E. coli* ribosomes is in controversy with the previous one [18]; such Mg^{2+} ions are not detected, while the orientation of the drug is rotated approximately by 180^{o}, thus placing the *p*-nitrobenzene ring in a position stacking on C2452 and orienting 3OH away from U2506. Therefore, our results are more consistent with the second model as well as with previous SAR-studies indicating that the conformation and integrity of 2-aminopropane-1,3-diol moiety is of great importance for the activity of CAM [1].

The loss of activity observed for compound **6** denotes the influence of the nature and the flexibility of the linker on the activity of compound (compare compounds **1** and **6**); less flexible and more lipophilic linker results to complete loss of activity. Finally, replacement of the dichloroacetyl moiety of CAM by the 1,2,4-triazole-3-carboxylate unit resulted in a product (compound **8**) exhibiting worse activity, when compared to the parent compound. Removing this replacement away from the 2-aminopropane-1,3-diol moiety resulted in compound **9** that displayed mostly weak activity, but still remaining lower than those of CAM.

In a closed system, like a cell-free system, an important contributor to the efficacy of a drug is its affinity for the target. Drug and target are at equilibrium, and thus affinity constants precisely quantify the concentration of the encounter complex, provided that drug-inactivating and/or target-modifying enzymes are absent or inactive. However, the cell is an open system in which drug concentration and the target itself fluctuate over time. Assuming that the function and concentration of target remain unalterable, the drug concentration depends on the relative activities of uptake and efflux systems. The cytoplasm also must not contain drug-modifying enzymes. For instance, *E. coli* possesses more than seven efflux transporters that remove a broad range of antibiotics and other toxic compounds from the intracellular space. One well-studied efflux pump is the AcrA-AcrB-TolC tripartite pump system which is assembled by the outer membrane protein TolC, the periplasmic protein

AcrA, and the inner membrane protein AcrB [35]. Deletions in *acrAB* and/or *tolC* genes result in increased susceptibility of *E. coli* to a panel of compounds, including CAM [36]. To investigate the effect of this efflux system on our compounds, we determined the IC_{50} values against an *E. coli* strain BL21 DE3 lacking the *tolC* gene that codes the TolC protein. As shown in Table 1, this efflux system does not seem to affect the antibacterial activity of CAM-PACs, as the IC_{50} values regarding this strain were similar to those of wild-type *E. coli*. However, *E. coli* BL21 DE3 (Δ*tolC*) strain was approximately 3-fold more sensitive to CAM. Nevertheless, the probability that CAM-PACs are extruded through another set of efflux systems of RND family [37], cannot be excluded with absolute certainty.

Regarding now the internalization of a drug into the bacterial cell, the first barrier that should be overcome is the outer membrane. This requirement explains our finding that the IC_{50} values for CAM and CAM-PACs measured in *S. aureus* are smaller than those measured in *E. coli* (Table 1). *S. aureus,* as a representative of Gram-positive bacteria, possessing a simpler and more permeable outer membrane than those existing in *E. coli,* appears higher susceptibility to the tested compounds. This prompted us to examine the activity of CAM-PACs against three multi-drug resistant (MDR) MRSA isolates, GRE2272, GRE2372, and GRE2691. The resistance of these isolates against a variety of antibiotics, in addition to methicillin, is reported in Table S2. Interestingly, all of them showed similar or better susceptibility to compound **4**, than to CAM. Given that compound **4** is better tolerated than CAM by human leukocytes [28], this conjugate seems to be a well promising lead candidate in designing efficacious drugs against MDR Gram-positive bacteria.

CAM utilizes channel proteins, like pore-forming porins, for access to the periplasm in Gram-negative bacteria, like *E. coli* [10]. Conjugation of CAM with PAs should be excluded from the passage, since PA portion(s) increase the size of the drug. Apart from steric hindrance, PA portions, due to their polycationic nature, may bind to internal, negatively charged regions of porins and block penetration [38]. On the other hand, compounds possessing polycationic components can destabilize the liposaccharide layer of the outer membrane and cross the outer-membrane barrier, by using a self promoted uptake pathway [37]. Moreover, the PA portion(s) may endow CAM-PACs with the ability to penetrate the bacterial inner membrane, by utilizing the polyamine uptake system, a group of polyamine carriers which pertain to the ATP-binding cassette transporter family [25]. To test this hypothesis, *S. aureus* or *E*

coli cells grown in M9 medium were exposed to an IC_{50} dose of each CAM-PAC, at which 50% of growth inhibition was observed. Interestingly, $10\times IC_{50}$ exogenously added polyamines were able to significantly salvage normal growth of bacterial cells exposed to an IC_{50} dose of compounds **2-5** (Fig. 2). In contrast, when exogenous polyamines were added to *S. aureus* or *E coli* cells exposed to an IC_{50} dose of CAM, no rescue was observed. It is mentioned that the concentration used for each polyamine was much lower than the maximum one tolerated by *S. aureus* or *E coli* cells [39]. We rationalized the competition observed as being due to the relative propensity of compounds **2-5** to use the polyamine transport system for cell entry (see also next paragraph). Except for compound **2**, all the rest CAM-PACs of this subgroup competed more efficiently with putrescine and spermidine for the polyamine uptake system, than with spermine. This finding tempted us to assume that CAM-PACs are mainly internalized into the cell through the transporter encoded by the pPT104 operon, which constitutes the spermidine-preferential uptake system [25]. The molecular demands for the selective delivery of PA conjugates into eukaryotic cells are slightly different than those required by the prokaryotic PA-uptake system [40]. Nevertheless, the most effective member of CAM-PACs in bacteria, compound **4**, was also able to penetrate eukaryotic cells. As detected previously [28], compound **4** compared to CAM possesses enhanced toxicity against human cancer cells, and lower toxicity against healthy human cells. This was attributed to the highly active PA-transporters (PAT) existing in cancer cells [41].

The rescue in growth observed when CAM-PACs and exogenous polyamines are simultaneously added in the medium of bacterial culture may arise either due to a competition between them for the polyamine uptake system or to an increase of the protein synthesis rate, owing to the enrichment of the intracellular environment in polyamines. To differentiate between these two possibilities and to confirm that CAM-PACs act on their target after entry into the cells, *E. coli* cells at an A_{560} of 0.020 were grown at 37^{o}C in M9 medium supplemented with compound **4** or **5** at concentrations equal to their respective IC_{50}. In parallel, cells were grown in the absence of CAM-PACs (control cultures). The cells were harvested when the control cultures reached an A_{560} of 0.800, washed and then homogenized in 0.6 M $HClO_4$. After centrifugation, the supernatant was analyzed by RP-HPLC, while the pellet was extracted in 0.1 M NaOH and analyzed for protein content. Using this analytical approach, we were able to detect micromolar quantities of compounds **4** and **5** in the

cellular extracts of *E. coli* (Table 2, Fig. S1). Although much less than the exogenous doses, the intracellular concentrations of CAM-PACs detected were adequate to substantially inhibit protein synthesis, given that the inhibition constant (K_i*) values in the puromycin reaction, previously calculated for these compounds [28], are less than 1 μM (Table S1). Consistently, inhibition of cell growth in the presence of compounds **4** or **5** was accompanied by remarkable decrease in the protein content of cells (Table 2). Surprisingly, compounds **4** and **5** once administrated to *E. coli* cells interfered with polyamine metabolism, as detected by changes measured in the intracellular polyamine content. Putrescine and spermidine intracellular pools were depleted by around 75% and 80%, respectively, at the time of cell harvesting, i.e. 3 h after the addition of compound **4**, whereas the corresponding depletions when compound **5** used as the challenging agent reached the values 90% and 95%, respectively (Table 2). In contrast to some studies reporting liberation of the appended polyamine from the drug scaffold once administrated within cells [42], we failed to detect N^8,N^8-dibenzylspermidine in the cellular extracts by RP-HPLC (Fig. S2), and therefore we suggest that conjugates **4** and **5** are not decomposed in the cellular environment to any significant extent. Nevertheless, polyamines are essential for cell growth and protein synthesis [43], and therefore the revealed effects on polyamine content suggest that CAM-PACs may directly exhibit an additional mode of toxicity. The origin of this additional effect of CAM-PACs is not clear at present.

It should be noted that, conjugate **4** was more efficient than CAM in inhibiting the growth of A2058G and A2503C mutants of *E. coli*, while both mutations failed to provide any growth advantage to *E. coli* cells against compound **5**, similar to that conferred against CAM (Table 1) [7,44,45]. It should be mentioned that nucleoside A2058 along with A2059 participate in the formation of a hydrophobic crevice at the entrance to the exit tunnel. As previously revealed [22,46], even minor changes in the structure or conformation of these nucleosides can affect functions of the PTase center and response to antibiotics, via allosteric effects transmitted through a signal exchange network including A2503 and A2062. Therefore, it is perceived why mutations A2058G and A2503G confer resistance to *E. coli* against CAM. However, when compound **5** is used as antibacterial, the signal exchange network is interrupted at the level of nucleoside A2062 (Fig. S3A), i.e. no hydrogen bond is formed between A2062 and compound **5,** a fact that impairs the transmission of signals to the PTase center. On the other hand, compound **4** preserves this hydrogen bond and also

displays π-π interaction with U2585 (Fig. S3B), thus limiting the flexibility of this nucleoside. As proposed by others, U2585 is implicated in the binding of peptidyl-tRNA to the P-site [47,48]. In addition, Agmon et al suggested that loss of the flexibility of U2585 may be detrimental for peptide-bond formation, given that U2585 along with A2602 control the spiral rotation of the acceptor 3′-acceptor end of a new-bound aminoacyl-tRNA (A/T site) as seeking out its functional orientation towards the P-site bound peptidyl-tRNA [49].

Last but not least, compounds **4** and **5** exhibited higher activity than CAM in inhibiting the growth of Rosetta(DE3)pLysS *E. coli* cells expressing the *cat* gene (Table 1). Production of CAM acetyltransferase, encoded by the *cat* gene, is a major mechanism by which bacteria become resistant to CAM. This enzyme catalyzes transfer of the acetyl moiety from acetyl coenzyme A to CAM [50]. The O-acetoxy derivatives of CAM are devoid of antibiotic activity, because they do not bind to bacterial ribosomes [50]. The sensitivity of Rosetta(DE3)pLysS *E. coli* cells against **4** and **5** suggests that these compounds are not so good substrates for CAM acetyltransferase compared to CAM. This was confirmed by calculating the specificity of CAM and compounds **4** and **5** for CAM acetyltransferase, using the ratio V_{max}/K_m to benchmark the capacity of each drug to be used as acceptor of acetyl groups. As shown in Table 3 and Fig. S4, compound **4** behaves less efficiently as substrate of CAM acetyltransferase, compared to CAM or compound **5**, and therefore is more capable of combating this type of CAM resistance.

3. Conclusions

A series of CAM-PACs (**1**-**9**) were readily synthesized from either CAM, through indirect oxidation of its primary hydroxyl function, or from CLB through N-acylation with succinic or phthalic anhydride, followed by coupling with suitably protected polyamines and finally, routine O- and/or N-deprotection. The study of their antibacterial activity against a variety of bacterial strains and in comparison to CAM revealed the structural characteristics which are necessary for optimum activity, namely the presence of the hydroxymethyl unit in the 2-amino-1,3-diol CAM subunit, the preference for a more flexible aliphatic dicarboxylic acid linker and the N^8,N^8-dibenzyl-SPD as the PA chain, preferably acylated at position N4. The most active conjugate **4**, incorporating these characteristics, presented comparable antibacterial activity to CAM against MRSA or wild-type *S. aureus* and *E. coli* cells, and superior activity against *E. coli* mutants that exhibit resistance against CAM. Given that

antibiotic resistance is considered to be one of the major global public health threats, compounds that are capable to combat resistant strains acquire great importance.

Although oligo-amines, like polymyxins and aminoglycosides, are relatively neurotoxic and nephrotoxic because of their polycationic nature at physiological pH (7.4) [51,52], natural polyamines are ubiquitous and essential components of cells. Due to their cellular origin, numerous derivatives and analogues of polyamines have been synthesized and some of them are presently in early phases of clinical trials [53]. Nevertheless, high extracellular polyamine levels can lead to toxicity and neurotoxicity. Despite that direct cytotoxic actions are observed only at mM concentrations [54], any potential toxic effects of polyamine conjugates against healthy human cells must be avoided. Encouragingly, a previous study of CAM-PACs has indicated that these compounds are well tolerated by human leukocytes [28]. Certainly, further work has to be performed to refute the suspicion that toxic properties may be shared by CAM-PACs.

Lead compounds, and in particular conjugate **4**, have been therefore discovered during the course of the present work with clinical potential. We are currently in the process of exploiting the mechanisms through which these conjugates exert their antibacterial effects and designing better compounds, taking into consideration results from related molecular modeling studies and using conjugate **4** as the lead compound.

4. Experimental

4.1. Materials and analytical methods

Melting points were determined with a Buchi SMP-20 apparatus and are uncorrected. IR spectra were recorded as KBr pellets, where necessary, on a Perkin Elmer 16PC FT-IR spectrophotometer. ^{1}H NMR spectra were obtained at 400.13 MHz and ^{13}C NMR spectra at 100.62 MHz on a Bruker DPX spectrometer. Chemical shifts are reported in δ units, parts per million (ppm) downfield from TMS. Electron-spray ionization (ESI) mass spectra were recorded at 30V, on a Micromass-Platform LC spectrometer using MeOH as solvent. The MALDI high-resolution MS (HRMS) experiments were carried out in an Applied Biosystems MALDI-TOF/TOF A/B 4700 Proteomics Analyzer instrument using dithranol as matrix. Microanalyses were performed on a Carlo Erba EA 1108 CHNS elemental analyzer in the Laboratory of Instrumental Analysis of the University of Patras. Flash column chromatography (FCC) was performed on Merck silica gel 60 (230-400 mesh) and TLC on 60 Merck

$60F_{254}$ films (0.2 mm) precoated on aluminium foil. Spots were visualized with UV light at 254 nm and ninhydrine solution or charring agents. All solvents were dried and/or purified according to standard procedures prior to use. All reagents employed in the present work were purchased from either Aldrich or Alfa-Aesar. The synthesis and characterization of compounds **13** and **14** are described in the supplementary data section.

4.2. General procedure for the synthesis of CAM-PACs 1-6

Succinic (0.1 g, 1.0 mmol) or phthalic (0.15 g, 1.0 mmol) anhydride was added to a solution of CLB (0.21 g, 1.0 mmol) in DMF (3.0 mL). The reaction mixture was stirred at ambient temperature for 1.5h and the consumption of CLB was checked by TLC using as eluant $CHCl_3$/MeOH (1:1). Following completion of the reaction, the resulting solution was cooled to 0 ^oC, the appropriate PA derivative (1.1 mmol) was added followed by HBTU (0.46 g, 1.2 mmol) and finally $^iPr_2NEt$ (0.52 mL, 3.0 mmol). The reaction mixture was stirred at ambient temperature for the indicated time and diluted with EtOAc. The organic phase was washed twice with a 5% aq. solution of $NaHCO_3$ and twice with H_2O, dried over Na_2SO_4 and evaporated to dryness. The protected CAM-PACs were obtained in pure form following FCC purification.

4.2.1. N^1-(4-Aminobutyl)-N^4-((1*R*,2*R*)-1,3-dihydroxy-1-(4-nitrophenyl)-propan-2-yl)succinamide (1)

The N-protected compound **1** was synthesized according to the above general procedure using **10** (0.36 g, 1.1 mmol) as the PA derivative. Reaction time: 1h; Yield: 0.47 g (75%); White foam; R_f ($CHCl_3$/MeOH 95:5): 0.16; IR (KBr, cm^{-1}): 3381, 3350, 2934, 1647, 1519, 1351, 753, 712; MS (ESI, 30eV): *m/z* 663.30 [M+K], 647.43 [M+Na], 625.39 [M+H], 243.16 [Trt]; HRMS (m/z): $[M+H]^+$ calcd for $C_{36}H_{41}N_4O_6$, 625.3026. Found, 625.3046; ^1H NMR ($CDCl_3$): δ 8.10 (d, J = 8.8 Hz, 2H), 7.51 (d, J = 8.8 Hz, 2H), 7.43-7.38, 7.27-7.21 and 7.18-7.13 (three m, 15H), 7.09 (d, J = 8.8 Hz, 2H), 6.28 (t, J = 5.6 Hz, 1H), 5.10 (d, J = 3.2 Hz, 1H), 4.09 (unresolved dddd, 1H), 3.76 (dd, J = 5.2 and 11.6 Hz, 1H), 3.66 (dd, J = 4.4 Hz and 11.6 Hz, 1H), 3.08 (unresolved q, 2H), 2.47-2.36 (m, 2H), 2.35-2.24 (m, 2H), 2.12 (unresolved t, 2H), 1.46-1.39 (m, 4H); ^{13}C NMR ($CDCl_3$): δ 173.3, 172.5, 149.1, 147.2, 145.9 (three C), 128.6 (six C), 127.8 (six C), 126.9 (two C), 126.3 (three C), 123.4 (two C), 72.6, 70.9, 62.8, 56.4, 43.3, 39.7, 31.3 (two C), 28.0, 27.2.

The thus obtained N-protected conjugate (0.4 g, 0.64 mmol) was treated at ambient temperature with a 30% TFA solution in CH_2Cl_2 (2 mL) for 1h. The resulting solution was evaporated to dryness and the residue was triturated with Et_2O to afford conjugate **1**, as its corresponding trifluoroacetate salt. Yield: 0.29 g (90%); white solid; IR (KBr, cm^{-1}): 3395, 3025, 3000-2700, 1676, 1542, 1445; MS (ESI, 30eV): *m/z* 405.46 [M+Na], 383.41 [M+H], 365.47 [(M+H)-H_2O]; HRMS (m/z): $[M+H]^+$ calcd for $C_{17}H_{27}N_4O_6$, 383.1931. Found, 383.1959; ^{1}H NMR (*d*$_4$-MeOH): δ 8.17 (d, *J* = 8.4 Hz, 2H), 7.62 (d, *J* = 8.4 Hz, 2H), 5.07 (d, *J* = 3.0 Hz, 1H), 4.12 (dt, *J* = 3.0 and 6.6 Hz, 1H), 3.74 (dd, *J* = 6.6 and 10.8 Hz, 1H), 3.54 (dd, *J* = 6.6 and 10.8 Hz, 1H), 3.23-3.08 (m, 2H), 2.91 (t, *J* = 7.2 Hz, 2H), 2.48-2.28 (m, 4H), 1.63 (quintet, *J* = 7.2 Hz, 2H), 1.54 (quintet, *J* = 7.2 Hz, 2H); ^{13}C NMR (*d*$_4$-MeOD): δ 174.9 (two C), 152.2, 148.6, 128.6 (two C), 124.2 (two C), 72.1, 62.8, 58.0, 40.4, 39.5, 32.2, 32.0, 27.4, 25.8.

4.2.2. N^1-(3-(4-(3-Aminopropylamino)butylamino)propyl)-N^4-((1*R*,2*R*)-1,3 dihydroxy-1-(4-nitrophenyl)-propan-2-yl)succinamide (2)

Then N-protected compound **2** was synthesized according to the above general procedure using **11** (0.55 g, 1.1 mmol) as the PA derivative. Reaction time: 2h; Yield: 0.66 g (83%); colorless oil; R_f ($CHCl_3$/MeOH 97:3): 0.19; IR (thin film, $CHCl_3$, cm^{-1}): 3480 3448, 2922, 1670, 1636, 1418, 1162, 728; MS (ESI, 30eV): *m/z* 835.37 [M+K], 819.31 [M+Na], 797.39 [M+H]; HRMS (m/z): $[M+H]^+$ calcd for $C_{38}H_{65}N_6O_{12}$, 797.4660. Found, 797.4697; ^{1}H NMR ($CDCl_3$): δ 8.16 (d, *J* = 8.8 Hz, 2H), 7.58 (d, *J* = 8.8 Hz, 2H), 7.05 (br.s, 1H), 6.93 (br.s., 1H), 5.17 (d, *J* = 4.0 Hz, 1H), 4.07-4.00 (m, 1H), 3.86 (dd, *J* = 4.0 and 11.6 Hz, 1H), 3.76 (dd, *J* = 4.0 and 11.2 Hz, 1H), 3.27-3.18 (m, 4H), 3.17-3.03 (m, 8H), 2.50-2.32 (m, 4H), 1.69-1.57 (m, 4H), 1.49-1.45 (m, 4H), 1.42 (s, 27H); ^{13}C NMR ($CDCl_3$): δ 173.5, 172.4, 156.5, 156.1 (two C), 149.1, 147.3, 127.0 (two C), 123.4 (two C), 80.0 (two C), 79.2, 73.3, 63.1, 56.9, 46.9 (two C), 44.0, 43.6, 37.6, 36.1, 31.8, 31.6, 28.4 (nine C), 28.0, 27.7, 25.9 (two C).

The thus obtained N-protected conjugate (0.6 g, 0.75 mmol) was treated with a 50% TFA solution in CH_2Cl_2 (4 mL) for 2h. The reaction mixture was evaporated to dryness and the residue was triturated with Et_2O to afford conjugate **2**, as its corresponding tristrifluoroacetate salt. Yield: 0.58 g (92%); colorless oil; IR (thin film, $CHCl_3$, cm^{-1}): 3396, 3030-2700, 1684, 1674, 1654, 1202, 1132, 722; MS (ESI, 30eV): *m/z* 519.45 [M+Na], 497.47 [M+H]; HRMS (m/z): $[M+H]^+$ calcd for

$C_{23}H_{41}N_6O_6$, 497.3088. Found, 497.3042; ^{1}H NMR (d_4-MeOH): δ 8.18 (d, J = 8.8 Hz, 2H), 7.79 (d, J = 9.2 Hz, 1H), 7.63 (d, J = 8.8 Hz, 2H), 5.01 (d, J = 2.8 Hz, 1H), 4.12 (unresolved ddt, 1H), 3.75 (dd, J = 6.4 and 10.8 Hz, 1H), 3.53 (dd, J = 6.4 Hz and 10.8 Hz, 1H), 3.13 (t, J = 7.6 Hz, 2H), 3.10-3.03 and 3.03- 2.95 (two m, 10H), 2.51-2.30 (m, 4H), 2.08 (quintet, J = 7.6 Hz, 4H), 1.86-1.77 (m, 4H); ^{13}C NMR (d_4-MeOD): δ 176.6, 175.1, 152.7, 149.1, 129.0 (two C), 124.7 (two C), 72.5, 63.2, 58.4, 48.8, 48.6, 46.7, 46.4, 38.4, 37.2, 32.4, 32.3, 28.2, 25.9, 24.8, 24.7.

4.2.3. N^1-(4-Aminobutyl)-N^1-(3-aminopropyl)-N^4-((1*R*,2*R*)-1,3-dihydroxy-1-(4-nitrophenyl)propan-2-yl)succinamide (3)

The N-protected compound **3** was synthesized according to the above general procedure using **12** (0.69 g, 1.1 mmol) as the PA derivative. Reaction time: 2h; Yield: 0.67 g (72%); white foam; R_f ($CHCl_3$/MeOH 97:3): 0.27; IR (KBr, cm^{-1}): 3394, 3320, 3056, 3018, 2932, 2856, 1620, 1518, 1488, 1448, 1346, 1216, 750, 706; MS (ESI, 30eV): *m/z* 946.34 [M+Na], 924.42 [M+H], 682.45 [(M+H)-Trt], 243.47 [Trt]; ^{1}H NMR ($CDCl_3$): δ 8.14 (d, J = 8.8 Hz, 2H), 7.54 (d, J = 8.8 Hz, 2H), 7.48-7.41, 7.30-7.22 and 7.21-7.14 (three m, 30H), 6.80 (br.s, 1H), 5.13 (d, J = 3.2 Hz, 1H), 4.02-3.96 (m, 1H), 3.82 (dd, J = 4.0 and 11.6 Hz, 1H), 3.71 (dd, J = 4.0 and 11.6 Hz, 1H), 3.41-3.20 (m, 2H), 3.18-3.04 (m, 2H), 2.47-2.28 (m, 4H), 2.21-2.08 (m, 4H), 1.73-1.60 (m, 2H), 1.49-1.39 (m, 4H); ^{13}C NMR ($CDCl_3$): δ 173.9, 172.0, 149.2, 147.2, 146.8 (six C), 128.6 (twelve C), 127.8 (twelve C), 127.0 (six C), 126.5 (two C), 123.4 (two C), 73.2, 71.0 (two C), 63.0, 56.8, 48.0, 46.4, 44.3, 43.3, 31.6, 30.1, 28.8, 26.5, 25.8.

The thus obtained N-protected conjugate (0.6 g, 0.65 mmol) was treated with a 30% TFA solution in CH_2Cl_2 (4 mL) for 2h. The reaction mixture was evaporated to dryness and the residue was triturated with Et_2O to afford conjugate **3**, as its corresponding bistrifluoroacetate salt. Yield: 0.40 g (93%); white foam; IR (KBr, cm^{-1}): 3390, 3000-2700, 1681, 1642, 1531, 1461, 1347, 1202, 1150, 840, 722; MS (ESI, 30eV): *m/z* 462.39 [M+Na], 440.48 [M+H]; HRMS (m/z): $[M+H]^+$ calcd for $C_{20}H_{34}N_5O_6$, 440.2509. Found, 440.2532; ^{1}H NMR (d_6-DMSO): δ 8.15 (d, J = 8.4 Hz, 2H), 7.89-7.70 (m, 6H), 7.58 (d, J = 8.4 Hz, 2H), 5.00 (unresolved d, 1H), 3.95 (m, 1H), 3.54 (unresolved dd, 1H), 3.32-3.22 (m, 3H), 3.21-3.14 (m, 2H), 2.84-2.75 (m, 2H), 2.73-2.64 (m, 2H), 2.40-2.17 (m, 4H), 1.80-1.65 (m, 2H), 1.56-1.41 (m, 4H); ^{13}C NMR (d_6-DMSO): δ 172.4, 171.9, 152.4, 146.7, 127.9 (two C), 123.2 (two C), 69.9, 60.9, 56.4, 46.9, 44.4, 42.2, 39.0, 37.0, 30.7, 28.1, 25.9, 24.7.

4.2.4. N^1-(3-Aminopropyl)-N^1-(4-dibenzylamino)butyl)-N^4-((1*R*,2*R*)-1,3-dihydroxy-1-(4-nitrophenyl)-propan-2-yl)succinamide (4)

The N-protected compound **4** was synthesized according to the above general procedure using **13** (0.62 g, 1.1 mmol) as the PA derivative. Reaction time: 2h; Yield: 0.62 g (72%); white foam; R_f (PhMe/EtOAc 2:8): 0.27; IR (KBr, cm^{-1}): 3352, 2927, 1618, 1522, 1490, 1346, 1072, 748, 698; MS (ESI, 30eV): *m/z* 884.49 [M+Na], 862.32 [M+H], 620.41 [(M+H)-Trt], 243.41 [Trt]; HRMS (m/z): $[M+H]^+$ calcd for $C_{53}H_{60}N_5O_6$, 862.4544. Found, 862.4576; ^{1}H NMR ($CDCl_3$,): δ 8.14 (d, J = 8.8 Hz, 2H), 7.54 (d, J = 8.8 Hz, 2H), 7.46-7.14 (m, 25H), 6.70 (d, J = 6.4 Hz, 1H), 5.14 (d, J = 4.4 Hz, 1H), 3.98-3.89 (m, 1H), 3.84 (unresolved dd, 1H), 3.71 (unresolved dd, 1H), 3.55 (br. s, 4H), 3.36-3.07 (m, 4H), 2.61-2.20 (m, 6H), 2.16-2.01 (m, 2H), 1.68-1.56 (m, 2H), 1.52-1.36 (m, 4H); ^{13}C NMR ($CDCl_3$,): δ 174.1, 171.9, 149.0, 147.2, 146.8 (three C), 128.8 (two C), 128.6 (four C), 128.5 (six C), 127.9 (six C), 127.8 (four C), 126.9 (three C), 126.4 (two C), 126.3 (two C), 123.4 (two C), 73.5, 71.0, 63.2, 58.6 (two C), 57.0, 47.7, 46.3, 44.2, 41.2, 31.7, 29.7, 28.9, 26.2, 25.5.

The thus obtained N-protected conjugate (0.55 g, 0.64 mmol) was treated with a 30% TFA solution in CH_2Cl_2 (4 mL) for 1h. The reaction mixture was evaporated to dryness and the residue was triturated with Et_2O to afford conjugate **4**, as its corresponding bistrifluoroacetate salt. Yield: 0.51 g (94%); white foam; IR (KBr, cm^{-1}): 3386, 3000-2700, 1676, 1641, 1552, 1458, 1348, 1202, 1141, 836, 722; MS (ESI, 30eV): *m/z* 642.41 [M+Na], 620.48 [M+H]; HRMS (m/z): $[M+H]^+$ calcd for $C_{34}H_{46}N_5O_6$, 620.3448. Found, 620.3455; ^{1}H NMR (d_6-DMSO): δ 8.23 (d, J = 8.0 Hz, 1H), 8.12 (d, J = 8.8 Hz, 2H), 7.47 (d, J = 8.4 Hz, 2H), 7.89-7.73 (m, 3H), 7.59 (d, J = 8.8 Hz, 2H), 7.54-7.39 (m, 10H), 5.00 (unresolved d, 1H), 4.41-4.13 (m, 5H), 3.98 (unresolved dd, 1H), 3.56 (unresolved dd, 1H), 3.35-3.10 (m, 4H), 2.96-2.85 (m, 2H), 2.75-2.65 (m, 2H), 2.39-2.23 (m, 4H), 1.81-1.66 (m, 2H), 1.46-1.26 (m, 4H); ^{13}C NMR (d_6-DMSO): δ 172.3, 171.9, 158.8 (q, J = 32.7 Hz, $CF_3\mathit{C}O_2^-$), 148.2, 146.7, 131.5 (two C), 130.0 (two C), 129.3 (four C), 128.2 (two C), 127.9 (two C), 127.8 (two C), 123.2 (two C), 117.3 (q, J = 295.8 Hz, $\mathit{C}F_3CO_2^-$), 69.9, 60.9, 56.7 (two C), 56.4, 46.6, 44.3, 42.1, 38.7, 37.0, 30.6, 27.9, 25.9, 25.7.

4.2.5. N^1-(3-(4-(Dibenzylamino)butylamino)propyl)-N^4-((1*R*,2*R*)-1,3-dihydroxy-1-(4-nitrophenyl)-propan-2-yl)succinamide (5)

The N-protected compound **5** was synthesized according to the above general procedure using **14** (0.57 g, 1.1 mmol) as the PA derivative. Reaction time: 1h; Yield: 0.47 g (65%); colorless oil; R_f ($CHCl_3$/MeOH 9:1): 0.33; IR (thin film, $CHCl_3$, cm^{-1}): 3408, 3331, 3037, 2938, 2854, 1618, 1552, 1490, 1346, 1215, 1072, 748, 698; MS (ESI, 30eV): *m/z* 754.37 [M+K], 738.41 [M+Na], 716.39 [M+H]; HRMS (m/z): $[M+H]^+$ calcd for $C_{36}H_{45}F_3N_5O_7$, 716.3271. Found, 716.3297; ^{1}H NMR ($CDCl_3$): δ 8.18 (d, *J* = 8.8 Hz, 2H), 7.58 (d, *J* = 8.8 Hz, 2H), 7.45-7.23 (m, 10H), 6.89 (br.s, 1H), 6.63 (br.s, 1H), 5.18 (d, *J* = 3.6 Hz, 1H), 4.09-4.00 (m, 1H), 3.88 (dd, *J* = 4.0 Hz and 9.6 Hz, 1H), 3.76 (dd, *J* = 4.0 Hz and 9.6 Hz, 1H), 3.62 (br. s, 4H), 3.36 (t, *J* = 6.8 Hz, 2H), 3.24 (q, *J* = 6.4Hz, 2H), 3.18-3.07 (m, 2H), 2.61-2.35 (m, 6H), 1.68 (quintet, *J* = 6.4 Hz, 2H), 1.62-1.46 (m, 4H); ^{13}C NMR ($CDCl_3$): δ 173.4, 172.6, 148.9, 147.3, 137.7 (two C), 129.0 (four C), 128.4 (four C), 126.9 (four C), 123.4 (two C), 73.3, 63.1, 58.5 (two C), 56.9, 52.5, 47.5, 44.0, 36.2, 31.7, 31.6, 28.7, 26.9, 26.3.

Finally, water (1 mL) and K_2CO_3 (0.46 g, 3.36 mmol) were added to a solution of the thus obtained N^4-protected conjugate (0.4 g, 0.56 mmol) in MeOH (15 mL). The resulting suspension was refluxed at 65 ^{o}C for 1h. Upon completion of deprotection, salts were filtered off and discarded, the filtrate was evaporated to dryness and the residue was taken up in EtOAc. The organic phase was washed twice with brine, dried over Na_2SO_4 and evaporated under vacuo. Pure compound **5** was obtained following FCC purification. Yield: 0.20 g (59%); colorless oil; R_f ($CHCl_3$/MeOH 8:2): 0.12; IR (thin film, $CHCl_3$, cm^{-1}): 3296, 2934, 2808, 1644, 1520, 1449, 1346, 1076, 750, 700; MS (ESI, 30eV): *m/z* 620.48 [M+H]; HRMS (m/z): $[M+H]^+$ calcd for $C_{34}H_{46}N_5O_6$, 620.3448. Found, 620.3483; ^{1}H NMR ($CDCl_3$): δ 8.13 (d, *J* = 8.8 Hz, 2H), 7.54 (d, *J* = 8.8 Hz, 2H), 7.36-7.24 and 7.23-7.18 (two m, 10H), 7.10 (t, *J* = 4.8 Hz, 1H), 7.02 (d, *J* = 8.4 Hz, 1H), 5.12 (d, *J* = 2.8 Hz, 1H), 4.06 (unresolved ddt, 1H), 3.81 (dd, *J* = 4.0 Hz and 9.6 Hz, 1H), 3.71 (dd, *J* = 4.0 Hz and 9.6 Hz, 1H), 3.52 (br. s, 4H), 3.37-3.25 (m, 2H), 3.18-3.08 (m, 2H), 2.57 (t, *J* = 6.4 Hz, 2H), 2.48-2.26 (m, 6H), 1.65-1.36 (m, 6H); ^{13}C NMR ($CDCl_3$,): δ 173.1, 172.4, 149.4, 147.2, 139.8 (two C), 128.7 (four C), 128.1 (four C), 126.9 (two C), 126.8 (two C), 123.3 (two C), 73.0, 63.1, 58.3 (two C), 56.3, 53.0, 49.0, 47.4, 38.3, 31.7, 31.6, 28.2, 27.3, 24.8.

4.2.6. *N*^{1}-(4-Aminobutyl)-*N*^{2}-((1*R*,2*R*)-1,3-dihydroxy-1-(4-nitrophenyl)-propan-2-yl)phthalamide (6)

The N-protected CLB conjugate was synthesized according to the above general procedure using **10** (0.36 g, 1.1 mmol) as the PA derivative. Reaction time: 2h; Yield: 0.57 g (85%); white foam; R_f ($CHCl_3$/MeOH 95:5): 0.26; IR (KBr, cm^{-1}): 3395, 3294, 2921, 1656, 1515, 1337, 757, 707; MS (ESI, 30eV): *m/z* 695.48 [M+Na], 673.50 [M+H]; HRMS (m/z): $[M+H]^+$ calcd for $C_{40}H_{41}N_4O_6$, 673.3026. Found, 673.3042; ^1H NMR ($CDCl_3$): δ 8.14 (d, J = 8.8 Hz, 2H), 7.49 (d, J = 8.8 Hz 2H), 7.46-7.39, 7.34-7.21 and 7.19-7.05 (three m, 20H), 6.82 (unresolved t, 1H), 5.10 (d, J = 2.8 Hz, 1H), 4.09 (unresolved ddt, 1H), 3.89 (dd, J = 2.8 and 11.6 Hz, 1H), 3.72 (dd, J = 3.6 Hz and 11.6 Hz, 1H), 3.42 (s, 1H), 3.27 (q, J = 5.6 Hz, 2H), 2.17 (t, J = 5.6 Hz, 2H), 1.60-1.47 (m, 4H); ^{13}C NMR ($CDCl_3$,): δ 170.1, 169.2, 148.8, 147.3, 145.8 (three C), 135.5, 134.1, 130.6, 130.0, 129.0, 128.6 (six C), 128.2, 127.8 (six C), 126.8 (two C), 126.3 (three C), 123.4 (two C), 73.4, 71.0, 63.1, 57.2, 43.4, 40.3, 28.0, 27.2.

The thus obtained N-protected conjugate (0.5 g, 0.74 mmol) was treated with a 30% TFA solution in CH_2Cl_2 (2 mL) for 1h. The reaction mixture was evaporated to dryness and the residue was triturated with Et_2O to afford conjugate **6**, as its corresponding trifluoroacetate salt. Yield: 0.38 g (95%); white solid; IR (KBr, cm^{-1}): 3390, 3000-2700, 1651, 1520, 840, 730; MS (ESI, 30eV): *m/z* 453.32 [M+Na], 431.41 [M+H]; HRMS (m/z): $[M+H]^+$ calcd for $C_{21}H_{27}N_4O_6$, 431.1931. Found, 431.1967; ^1H NMR (d_4-MeOH): δ 8.22 (d, J = 8.8 Hz, 2H), 7.72 (d, J = 8.8 Hz, 2H), 7.52-7.47 (m, 3H), 7.41-7.36 (m, 1H), 5.13 (d, J = 4.0 Hz, 1H), 4.31 (ddd, J = 4.0, 5.6 and 6.4 Hz, 1H), 3.81 (dd, J = 5.6 Hz and 11.2 Hz, 1H), 3.67 (dd, J = 6.4 Hz and 11.2 Hz, 1H), 3.35-3.35 (2H under solvent), 3.00-2.92 (m, 2H), 1.71 (quintet, J = 6.4 Hz, 2H), 1.64 (quintet, J = 6.4 Hz, 2H); ^{13}C NMR (d_4-MeOH): δ 172.1, 171.7, 151.9, 148.9, 137.2, 136.8, 131.5, 131.4, 129.0, 128.9, 128.8 (two C), 124.4 (two C), 72.5, 62.9, 59.0, 40.4, 39.7, 27.3, 25.6.

4.3. (1*R*,2*R*)-3-((tert-butyldimethylsilyl)oxy)-2-(2,2-dichloroacetamido)-1-(4-nitrophenyl)propyl acetate (15)

To a suspension of CAM (0.97 g, 3.0 mmol) and imidazole (0.25 g, 3.6 mmol) in CH_2Cl_2 (10 mL) TBDMSCl (0.54 g, 3.6 mmol) was added and the reaction mixture was stirred at ambient temperature for 30 min. Then, Et_3N (0.84 mL, 6.0 mmol) and Ac_2O (0.57 mL, 6.0 mmol) were added and the reaction mixture was stirred for additional 12h. The reaction mixture was diluted with CH_2Cl_2 and the organic phase was washed once with 5% cold aq. solution $NaHCO_3$ and twice with H_2O, dried over

Na_2SO_4 and evaporated under vacuum to dryness. The residue was subjected to FCC purification to give intermediate **15**. Yield: 1.29 g (90%); colorless oil; R_f (PhMe/EtOAc 95:5): 0.16; IR (thin film, $CHCl_3$, cm^{-1}): 3314, 2948, 2930, 2858, 1748, 1682, 1608, 1526, 1472, 1348, 1226, 1118, 840, 778; MS (ESI, 30eV): *m/z* 519.27 and 517.25 [M+K], 503.39 and 501.38 [M+Na], 481.44 and 479.40 [M+H] 421.33 and 419.44 [M-AcOH]; HRMS (m/z): $[M+H]^+$ calcd for $C_{19}H_{29}Cl_2N_2O_6Si$, 479.1172. Found, 479.1161; ^1H NMR ($CDCl_3$): δ 8.24 (d, J = 8.8 Hz, 2H), 7.58 (d, J = 8.8 Hz, 2H), 6.96 (d, J = 9.2 Hz, 1H), 6.12 (d, J = 8.0 Hz, 1H), 5.91 (s, 1H), 4.34 (dddd, J = 2.4, 4.0, 8.0 and 9.2 Hz, 1H), 3.62 (dd, J = 4.0 and 10.4 Hz, 1H), 3.42 (dd, J = 2.4 and 10.4 Hz, 1H), 2.12 (s, 3H), 0.93 (s, 9H), 0.06 and 0.05 (two s, 6H); ^{13}C NMR ($CDCl_3$): δ 170.0, 164.0, 148.1, 144.2, 128.2 (two C), 123.9 (two C), 73.5, 66.3, 61.3, 55.0, 25.8 (three C), 20.9, 18.2, -5.6 and -5.5 (two C).

4.4. (1*R*,2*R*)-2-(2,2-dichloroacetamido)-3-hydroxy-1-(4-nitrophenyl)propyl acetate (16)

A solution of **15 (**1.2 g, 2.5 mmol) in AcOH/H_2O (2:1, 12.5 mL) was stirred at ambient temperature for 12h and then diluted with EtOAc. The organic phase was washed once with 5% cold aq. solution $NaHCO_3$ and twice with H_2O, dried over Na_2SO_4 and evaporated to dryness to give pure compound **16**, following FCC purification. Yield: 0.87 g (95%); white solid; m.p: 116-117 ^oC; R_f (PhMe/EtOAc 1:1): 0.13; IR (KBr, cm^{-1}): 3461, 3272, 2916, 1743, 1684, 1520, 1348, 1237, 1059; MS (ESI, 30eV): *m/z* 405.24 and 403.19 [M+K], 389.21 and 387.26 [M+Na], 307.33 and 305.32 [M-AcOH]; ^1H NMR ($CDCl_3$): δ 8.24 (d, J = 8.8 Hz, 2H), 7.60 (d, J = 8.8 Hz, 2H), 6.96 (d, J = 9.2 Hz, 1H), 6.22 (d, J = 6.8 Hz, 1H), 5.90 (s, 1H), 4.37 (dddd, J = 4.0, 4.8, 6.8 and 9.2, 1H), 3.70 (dd, J = 4.8 and 11.2 Hz, 1H), 3.55 (dd, J = 4.0 and 11.2 Hz, 1H), 2.17 (s, 3H); ^{13}C NMR ($CDCl_3$): δ 170.2, 164.4, 148.0, 143.9, 127.9 (two C), 124.0 (two C), 73.0, 66.2, 61.1, 55.3, 21.0. Anal. ($C_{13}H_{14}Cl_2N_2O_6$) calcd: C, 42.76; H, 3.86; N, 7.67. Found: C, 42.97; H, 3.70; N, 7.49.

4.5. (2*S*,3*R*)-3-Acetoxy-2-(2,2-dichloroacetamido)-3-(4-nitrophenyl)propanoic acid (17)

To a round bottom-flask containing MeCN (16 mL) at 0 ^oC, H_5IO_6 (1.12 g, 4.93 mmol) was added and the mixture was stirred for 15 min. Then, the resulting cold mixture was transferred to a flask containing compound **16** (0.8 g, 2.19 mmol) and finally a catalytic amount of PCC (*ca.* 10 mg) was added. The resulting mixture was

stirred at 0 ^{o}C for 30 min and then at ambient temperature for further 1h. Subsequently, it was diluted with EtOAc and the organic phase was washed once brine, once with saturated aqueous solution $NaHSO_3$ and once again with brine, then dried over Na_2SO_4 and evaporated to dryness. Pure Acid **17** was obtained after FCC purification. Yield: 0.53 g (64%); white solid, m.p. 104-106 ^{o}C ; R_f ($CHCl_3$/MeOH 8:2): 0.14; IR (KBr, cm^{-1}): 3600-2800, 1732, 1694, 1606, 1520, 1376, 1350, 1234, 1046, 856, 812, 716; MS (ESI, 30eV): *m/z* 781.01 and 779.01 [2M+Na], 419.35 and 417.21 [M+K], 403.19 and 401.24 [M+Na], 359.42 and 357.28 [M-CO_2]; ^{1}H NMR (d_4-MeOD): δ 8.20 (d, *J* = 8.8 Hz, 2H), 7.63 (d, *J* = 8.8 Hz, 2H), 6.50 (d, *J* = 3.2 Hz, 1H), 6.37 (s, 1H), 4.87 (d, *J* = 3.2 Hz, 1H), 2.18 (s, 3H); ^{13}C NMR (d_4-MeOD): δ 172.0, 169.8, 164.8, 147.6, 144.9, 127.2 (two C), 123.1 (two C), 75.1, 65.9, 57.9, 19.4. Anal. ($C_{13}H_{12}Cl_2N_2O_7$) calcd: C, 41.18; H, 3.19; N, 7.39. Found: C, 41.25; H, 2.92; N, 7.12.

4.6. N^1-((2*S*,3*R*)-3-Acetoxy-2-(2,2-dichloroacetamido)-3-(4-nitrophenyl) propano-yl)-N^4,N^9,N^{12}-tri(tert-butoxycarbonyl)spermine (18)

To an ice-cold solution of acid **17** (0.38 g, 1.0 mmol), PA derivative **11** (0.60 g, 1.2 mmol), HOBt (0.16 g, 1.2 mmol) and Et_3N (0.15 mL, 1.1 mmol) in THF (1.5 mL), EDCI (0.38 g, 2.0 mmol) was added portion-wise over 1.5 h. Upon addition, the resulting mixture was stirred at ambient temperature for 12h. Then, it was evaporated under vacuo and the residue was partitioned between EtOAc and a 5% aq. solution of citric acid. The organic phase was washed with a 5% aq. solution $NaHCO_3$ and brine, dried over Na_2SO_4 and evaporated to dryness. Compound **18** was obtained pure following FCC purification. Yield: 0.52 g (60%); colorless oil; R_f (PhMe/EtOAc 35:65): 0.23; MS (ESI, 30eV): *m/z* 903.11 and 901.16 [M+K], 887.25 and 885.24 [M+Na], 865.31 and 863.27 [M+H], 765.28 and 763.27 [(M-Boc)+H]; HRMS (m/z): $[M+H]^+$ calcd for $C_{38}H_{61}Cl_2N_6O_{12}$, 863.3725. Found, 863.3764; ^{1}H NMR (d_4-MeOH): δ 8.29-8.24 (m, 1H), 8.22 (d, *J* = 8.4 Hz, 2H), 7.64 (d, *J* = 8.4 Hz, 2H), 7.41-7.37 (m, 1H), 7.32-7.26 (m, 1H), 6.34 (s, 1H), 6.30 (unresolved d, 1H), 4.90 (unresolved dd, 1H), 3.26-3.06 (two m, 10H), 3.03 (t, *J* = 6.8 Hz, 2H), 2.13 (s, 3H), 1.68 (unresolved quintet, 2H), 1.51-1.40 (m, 33H); ^{13}C NMR (d_4-MeOH): δ 168.1, 166.2, 166.1, 155.5, 154.6, 154.5, 146.4, 143.0, 126.2 (two C), 121.7 (two C), 78.1, 78.0, 77.0, 72.5, 64.3, 64.0, 46.5 (under the solvent), 45.3, 44.4, 42.1, 36.4, 36.0, 25.9 (six C), 25.8 (three), 25.6, 24.3, 24.0, 23.6, 17.8.

4.7. (2*S*,3*R*)-N-(3-(4-(3-aminopropylamino)butylamino)propyl)-2-(2,2-dichloro-acetamido)-3-hydroxy-3-(4-nitrophenyl)propanamide (7)

To a solution of intermediate **18 (**0.43 g, 0.5 mmol) in MeOH/H_2O (5.0 mL, 9:1), K_2CO_3 (0.14 g, 1.0 mmol) was added. The reaction mixture was refluxed for 2 h and then evaporated to a minimum volume, taken up in $CHCl_3$ and washed twice with H_2O. The organic layer was dried over Na_2SO_4 and evaporated to dryness. The deacylated intermediate was purified by FCC. Yield: 0.33 g (80%); colorless oil; R_f (PhMe/EtOAc 35:65): 0.21; MS (ESI, 30eV): *m/z* 861.25 and 859.24 [M+K], 845.23 and 843.11 [M+Na], 823.28 and 821.28 [M+H]; HRMS (m/z): $[M+H]^+$ calcd for $C_{36}H_{59}Cl_2N_6O_{11}$, 821.3619. Found, 821.3668.

To an ice-cold solution of the deacetylated compound (0.30 g, 0.37 mmol) in CH_2Cl_2 (1 mL), TFA (1 mL) was added. The reaction mixture was stirred at ambient temperature for 1h, evaporated to dryness, triturated with Et_2O and refrigerated. The supernatant solution was decanted to afford conjugate **7** as the corresponding tris-trifluoroacate salt. Yield: 0.29 g (90%); colorless oil; MS (ESI, 30eV): *m/z* 545.41 and 543.32 [M+Na], 523.30 and 521.28 [M+H]; HRMS (m/z): $[M+H]^+$ calcd for $C_{21}H_{35}Cl_2N_6O_5$, 521.2046. Found, 521.2089; ^{1}H NMR (d_6-DMSO): δ 8.86 (br. s, 2H), 8.74 (d, *J* = 8.8 Hz, 1H), 8.67 (br.s, 2H), 8.20 (d, *J* = 8.8 Hz, 2H), 7.99 (br.s, 3H), 7.65 (d, *J* = 8.8 Hz, 2H), 7.62 (t, *J* = 8.8 Hz, 1H), 6.60 (s, 1H), 5.23 (d, *J* = 3.0 Hz, 1H), 4.51 (dd, *J* = 3.0 and 8.8 Hz, 1H), 3.03-2.81 (two m, 12H), 1.90 (quintet, *J* = 8.0 Hz, 2H), 1.73 (unresolved quintet, 2H), 1.67-1.56 (m, 4H); ^{13}C NMR (d_6-DMSO): δ 168.9, 164.1, 158.9 (q, *J* = 32.3 Hz, $CF_3CO_2^-$), 150.2, 147.1, 128.1 (two C), 123.4 (two C), 117.2 (q, *J* = 295.5 Hz, $CF_3CO_2^-$), 72.3, 66.8, 59.2, 46.6, 46.5, 45.0, 44.3, 36.6, 36.4, 26.3, 24.2, 23.1, 23.0.

4.8. *N*-((1*R*,2*R*)-1,3-Dihydroxy-1-(4-nitrophenyl)propan-2-yl)-1*H*-1,2,4-triazole-3-carboxamide (8)

To an ice-cold solution of CLB (0.21 g, 1.0 mmol) in DMF (1.2 mL), TCA (0.14 g, 1.2 mmol), ^{i}Pr$_2$NEt (0.52 mL, 3.0 mmol) and PyBrOP (0.67 g, 1.44 mmol) were added sequentially. The reaction mixture was stirred at ambient temperature for 2 h and then diluted with EtOAc. The organic phase was washed twice with a 5% aq. solution of $NaHCO_3$ and thrice with H_2O, dried over Na_2SO_4 and evaporated to dryness to afford an oily residue. Conjugate **8** was purified with FCC. Yield: 0.25 g (80%); white foam; R_f ($CHCl_3$/MeOH 8:2): 0.26; IR (KBr, cm^{-1}): 3122, 3028, 2927,

2840, 1655, 1541, 1426, 1334, 1256, 1062, 807, 739,; MS (ESI, 30eV): *m/z* 637.19 [2M+Na], 346.33 [M+K], 330.39 [M+Na], 308.41 [M+H]; HRMS (m/z): $[M+H]^+$ calcd for $C_{12}H_{14}N_5O_5$, 308.0995. Found, 308.0948; ^{1}H NMR (d_6-DMSO, 40 ^{o}C): δ 8.40 (s, 1H), 8.13 (d, J = 8.8 Hz, 2H), 7.68 (d, J = 9.2 Hz, 1H), 7.62 (d, J = 8.8 Hz, 2H), 7.61 (s, 1H), 7.00 and 6.99 (two s, 2H), 5.13 (d, J = 2.4 Hz, 1H), 4.22-4.15 (ddt, J = 2.4, 7.6 and 9.2 Hz, 1H), 3.66 (1H, dd, J = 7.6 and 10.8 Hz), 3.52 (1H, dd, J = 7.6 and 10.8 Hz); ^{13}C NMR (d_6-DMSO, δ): 172.6, 158.1, 152.0, 146.9, 135.6, 127.6 (2C), 123.5 (2C), 69.4, 61.0, 56.7.

4.9. *N*-(3-((1*R*,2*R*)-1,3-Dihydroxy-1-(4-nitrophenyl)propan-2-ylamino)-3-oxo-propyl)-1*H*-1,2,4-triazole-3-carboxamide (9)

This compound was obtained through a three-step sequence, as described below:

4.9.1. N-Acylation of CLB using 'active' ester Trt-β-Ala-OSu

To an ice-cold solution of CLB (0.21 g, 1.0 mmol) in DMF (1.2 mL), Trt-*β*-Ala-OSu (0.47 g, 1.1 mmol) and ^{i}Pr$_2$NEt (0.52 mL, 3.0 mmol) were added. The reaction mixture was stirred at ambient temperature for 1h and then diluted with EtOAc. The organic phase was washed twice with a 5% aq. solution of $NaHCO_3$ and thrice with H_2O, dried over Na_2SO_4 and evaporated to dryness to afford an oily residue. The acylated CLB was purified with FCC. Yield: 0.47 g (89%); white foam; R_f ($CHCl_3$/MeOH 97:3): 0.1; IR (KBr, cm^{-1}): 3416, 3226, 3056, 2926, 2880, 1670, 1616, 1516, 1344, 1262, 1082, 746, 706; MS (ESI, 30eV): *m/z* 564.36 [M+K], 548.36 [M+Na], 526.38 [M+H], 243.41 [Trt]; ^{1}H NMR ($CDCl_3$): δ 8.10 (d, J = 8.6 Hz, 2H), 7.51 (d, J = 8.6 Hz, 2H), 7.42-7.36, 7.30-7.24 and 7.23-7.18 (three m, 15H), 5.18 (d, J = 3.2 Hz, 1H), 4.17 (dt, J = 3.2 and 4.8 Hz, 1H), 3.89 (1H, dd, J = 4.6 and 11.2 Hz), 3.82 (1H, dd, J = 4.6 and 11.2 Hz), 2.53-2.26 (m, 4H); ^{13}C NMR ($CDCl_3$): δ 173.2, 162.6, 148.9, 147.3 (three C), 128.6 (six C), 128.1 (six C), 127.0 (two C), 126.8 (three C), 123.5 (two C), 73.3, 71.9, 63.5, 56.0, 40.1, 36.5.

4.9.2. Removal of the Trt protecting group

The thus obtained N-protected intermediate (0.4 g, 0.76 mmol) was treated with a 30% TFA solution in CH_2Cl_2 (2 mL) for 1h. The reaction mixture was evaporated to dryness and the residue triturated with Et_2O to afford the corresponding detritylated compound in the form of its trifluoroacetate salt. Yield: 0.29 g (95%); white foam; IR

(KBr, cm^{-1}): 3390, 3000-2700, 1676, 1522, 1458, 1202, 1135; MS (ESI, 30eV): *m/z* 284.42 [M+H].

4.9.3. Coupling with TCA

To an ice-cold solution of the above obtained salt (0.25 g, 0.63 mmol) in DMF (0.8 mL), $^{i}Pr_2NEt$ (0.33 mL, 1.89 mmol) was added. The reaction mixture was stirred at 0 ^{o}C for 10 min and then TCA (0.064 g, 0.57 mmol) and PyBrOP (0.32 g, 0.68 mmol) were added sequentially. The mixture was stirred at ambient temperature for 1h and then it was diluted with EtOAc. The organic phase was washed once with a 5% aq. solution of $NaHCO_3$ and thrice with H_2O, dried over Na_2SO_4 and evaporated to dryness. Compound **9** was purified with FCC. Yield: 0.16 g (75%); white foam; R_f ($CHCl_3$/MeOH 9:1): 0.12; IR (KBr, cm^{-1}): 3117, 3041, 2931, 1648, 1540, 1060; MS (ESI, 30eV): *m/z* 417.45 [M+K], 401.24 [M+Na], 379.28 [M+H]; HRMS (m/z): $[M+H]^+$ calcd for $C_{15}H_{19}N_6O_6$, 379.1366. Found, 379.1398; ^1H NMR (d_6-DMSO): δ 9.27 (t, J = 5.2 Hz, 1H), 8.30 (s, 1H), 8.14 (d, J = 8.8 Hz, 2H), 7.68 (d, J = 9.2 Hz, 1H), 7.57 (d, J = 8.8 Hz, 2H), 5.82 (br. s, 1H), 5.00 (1H, unresolved d), 4.84 (br. s, 1H), 4.02-3.94 (m, 1H), 3.54 (unresolved dd, 1H), 3.29 (dd, J = 6.0 and 10.4 Hz, 1H), 3.20 (q, J = 6.8 Hz, 2H), 2.36-2.20 (m, 2H); ^{13}C NMR (d_6-DMSO): δ 170.1 (two C), 156.9, 156.3, 152.4, 146.8, 127.8 (two C), 123.2 (two C), 69.8, 60.9, 56.4, 36.4, 34.3.

4.10. Biological evaluation

4.10.1. Antibacterial assays

The antibacterial activity of CAM-PACs was assessed against CAM-sensitive *S. aureus* and *E. coli* strains, as well against two CAM-resistant strains of *E. coli* lacking chromosomal *rrn* alleles, but containing pKK35 plasmids possessing mutated 23S rRNA (A2058G or A2503C), kindly offered by Prof. A.S. Mankin (University of Illinois). *E. coli ΔtolC* strain BL21 DE3 with impaired a proton-dependent MDR efflux pump causing multidrug resistance, AcrAB-TolC, offered by Dr D.N. Wilson (University of Munich), was included in our study to test if this mechanism of resistance affects the efficacy of CAM-PACs. Three methicillin-resistant *S. aureus* (MRSA) isolates belonging to the ST80 clone that carries the staphylococcal cassette chromosome *mec* type A and exhibits multi drug resistance behavior (Table S2 in Supplementary Data section), were kindly offered by Prof. I. Spiliopoulou-Sdougkou (National Reference Laboratory for Staphylococci, School of Medicine, University of

Patras, Greece). *E. coli strain*, Rosetta(DE3)pLysS, containing rare tRNA genes and a chloramphenicol resistant gene in the pLysS plasmid, was purchased from Novagen. CAM was tested as a reference compound. The *in vitro* antibacterial activity was determined as previously described [28], by calculating the IC_{50} from bacterial cultures grown in LB (Luria Broth) medium in the absence or the presence of drugs at different concentrations. Since the tested compounds differ in molecular mass, IC_{50} values were expressed in μM. Molecular mass of each compound was unequivocally determined by MS spectrometry. The activity of CAM acetyltransferase (CAT) using CAM or CAM-PACs as acceptor substrates was assayed by using enzyme from *E. coli*, acetyl coenzyme A as donor substrate, and 5,5′-dithiobis(2-nitobenzoic acid) purchased from Sigma-Aldrich, following the manufacturer' protocols (http://www.sigmaaldrich.com/technical-documents/protocols/biology/enzymatic-assay-of-chloramphenicol-acetyltransferase.html). The V_{max} and K_m values of CAT were determined by fitting the substrate concentrations [S] and the obtained ΔA_{412nm}/min (V_o) values into Equation 1,

$$V_o = V_{max}[S]/(K_m+[S]) \qquad \text{(Equation 1)}$$

and then by dividing the V_{max} value with 0.0136 to convert its units in $\mu M \cdot min^{-1}$.

4.10.2. Quantification of the intracellular levels of polyamines and compounds 4 and 5

Polyamines and compounds **4** and **5** in *E. coli* cells were determined by reverse phase high-performance liquid chromatography (RP-HPLC-UV). For these assays, 100 mg cells (wet weight), isolated from *E. coli* cultures grown at 37°C in M9 medium (48 mM Na_2HPO_4, 22 mM KH_2PO_4, 9 mM NaCl, 19 mM NH_4Cl) enriched with 0.03 mM $FeCl_3$, 0.1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.01 mM vitamin B_1, 0.6% glucose and 0.1% casamino acids and supplemented with compound **4** or **5** at concentrations equal to their IC_{50} (9.4 μM and 35.5 μM, respectively), were washed and then homogenized in 0.6 M $HClO_4$. Cultures grown in the absence of compounds **4** and **5** were used as a reference.The homogenate was incubated in ice for 1h, and then centrifuged at 12,000×g for 20 min at 4°C. The pellet was extracted twice with 1 mL 0.6 M $HClO_4$ and recentrifuged. The three supernatants were pooled and used to determine the levels of polyamines and compounds **4** and **5,** while the pellet was hydrolyzed o/n in 1 ml of 0.1 M NaOH and used for protein determination [55]. Each

supernatant, after dansylation and extraction of the dansyl derivatives by toluene [56], was dried, redissolved in 10 mM phosphate buffer pH 4.4 and fractionated by RP-HPLC (Waters 600 HPLC system equipped with a tunable UV absorbance detector Waters 486), using a C_{18}-Symmetry, 3.5-µm, 75 mm×4.6 mm column obtained from Waters, as previously described [57]. Polyamines and N^8,N^8-dibenzylspermidine, a putative degradation product of compounds **4** and **5**, were identified by comparing retention times. 1,6-Diaminohexane was used as internal standard. Verification of the identity of each peak was further done by U.V. and mass spectrometry. The detection limits for PUT, SPD, and N^8,N^8-dibenzylspermidine were 52 pmol, 18 pmol, and 16 pmol at 258 nm, respectively. For determination of the intracellularly accumulated compounds **4** and **5**, the cellular extracts were directly fractionated by RP-HPLC, using the same C_{18}-Symmetry as above, and the fractions were analyzed by optical scanning at 275 nm. The detection limits for compounds **4** and **5** were 25 pmol and 30 pmol at 275 nm, respectively. The coefficient of variation (C.V.) of the intra-assays ranged from 0.05 to 0.07, while those of the inter-assays ranged from 0.13 to 0.17. Data processing was made, using the Millenium 32 software-Waters, and the results were expressed as µmol /g wet weight.

4.10.3. Statistical analysis

All biological and biochemical experiments were carried out at least three times with two replicates per time, and the data were expressed as means ± standard deviation. Significant differences between mean values were measured at $p < 0.05$ by the F-Scheffe test (SPSS program 20.0 for Windows).

Footnotes

Abbreviations used: Boc, tert-butoxycarbonyl; CAM, chloramphenicol; CAM-PACs, CAM-polyamine conjugates; CLB, chloramphenicol base; EDCI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; HBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; MRSA, methicillin resistant *S. aureus*; PA, polyamine; PTase, peptidyl transferase; PUT, putrescine; rRNA, ribosomal RNA; SPD, spermidine; SPM, spermine; TBDMS, *tert*-butyldimethylsilyl; TCA, 1,2,4-triazole-3-carboxylic acid; Tfa, trifluoroacetyl; TFA, trifluoroacetic acid; Trt, triphenylmethyl; WT, wild-type

Conflict of interest

All authors declare no conflict of interest.

Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/.....

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Legends to Figures and Schemes

Figure 1. Structures of CAM, natural PAs and synthesized CAM-PA conjugates.

Figure 2. Rescue of the growth of *S. aureus* and *E. coli* by exogenously added polyamines, when bacteria were dosed at a IC_{50} concentration of each CAM-PACs **2-5**. *S. aureus* and *E. coli* cells at an A_{560} of 0.020 were grown in M9 medium in the absence (white bars) or the presence of each CAM-PAC, added at a concentration equal to their respective IC_{50} (white bars with lined pattern). CAM was used as a reference compound. Similar cultures containing exogenous putrescine (light gray bars), spermine (dark gray bars), or spermidine (black bars) at $10\times IC_{50}$ were also monitored until the optical density of the control culture (grown in the absence of drug) reached the value 0.800 at 560 nm. Values are expressed as a percentage of optical density measured in control cultures.

*Significant different from the value measured in cells grown in the presence of each CAM-PAC at IC_{50} ($p < 0.05$).

Scheme 1. Synthesis of CAM-PA conjugates **1**-**9**. *Reagents and reaction conditions*: (i) succinic anhydride, DMF, 25 ^{o}C, 1.5 h; (ii) **10** or **11** or **12** or **13** or **14**, HBTU, $^{i}Pr_2NEt$, 0 ^{o}C, 15 min then 25 ^{o}C, 1-2 h, yields for the two steps: 65-83 %; (iii) CF_3CO_2H-CH_2Cl_2 (1:1), 0 ^{o}C, 5 min then 25 ^{o}C, 1 h, 90-95%; (iv) K_2CO_3, MeOH, H_2O, 65 ^{o}C, 1 h, 59% (for **5)**; (v) phthalic anhydride, DMF, 25 ^{o}C, 10 h; (vi) **10**, HBTU, $^{i}Pr_2NEt$, 0 ^{o}C, 15 min then 25 ^{o}C, 1 h, 85% for the two steps; (vii) TCA, PyBrOP, $^{i}Pr_2NEt$, DMF, 25 ^{o}C, 2 h 80% (for **8**), 75% (for **9**); (viii) Trt-βAla-OSu, $^{i}Pr_2NEt$, DMF, 25 ^{o}C, 1 h, 89%.

Scheme 2. Synthesis of acid **17** and CAM-PA conjugate **7**. *Reagents and reaction conditions*: (i) $^{t}BuMe_2SiCl$, imidazole, CH_2Cl_2, 25 ^{o}C, 30 min, then Ac_2O, Et_3N, 25 ^{o}C, 12 h, 90%; (ii) AcOH-H_2O (2:1), 25 ^{o}C, 12 h, 95%; (iii) H_5IO_6, PCC (cat.), MeCN, 0 ^{o}C, 30 min then 25 ^{o}C, 1 h, 64%; (iv) **11**, EDCI, HOBt, THF, 0 ^{o}C, 1.5 h, then 25 ^{o}C, 12 h, 60%; (v) (a) K_2CO_3, MeOH-H_2O (9:1), reflux, 2 h, 80%, (b) CF_3CO_2H-CH_2Cl_2 (1:1), 0 ^{o}C, 5 min then 25 ^{o}C, 1 h, 90%.

Table 1. Determination of IC_{50} for CAM and CAM-PACs, indicating the concentration of each compound needed to inhibit the growth of wild-type and mutant *S. aureus* and *E. coli* cells by half [a]

Compound	IC_{50} (μM)								
	MRSA (GRE2272)	MRSA (GRE2372)	MRSA (GRE2691)	*S. aureus*[b] (WT)	*E. coli*[b] (WT)	*E. coli*[b] (A2058G)	*E. coli*[b] (A2503C)	*E. coli* (Δ*tolC*)	*E. coli* Rosetta(DE3)pLysS (Cam^R)
CAM	8.0 ± 0.9	6.0 ± 0.8	14.5 ± 1.2	3.1 ± 0.3	6.2 ± 0.5	15.5 ± 1.3	24.7 ± 2.3	2.0 ± 0.5	110.0 ± 5.7
1	>200	>200	>200	>200	>200	>200	>200	>200	>200
2	>200	>200	>200	45.3 ± 5.5	>100	>100	>100	>100	>200
3	>100	>100	>200	12.7 ± 1.0	>150	>150	>150	>100	>100
4	7.0 ± 0.9	9.0 ± 0.8	10.0 ± 0.9	4.7 ± 0.5	9.4 ± 0.8	9.4 ± 1.0	9.4 ± 0.9	9.0 ± 1.2	19.0 ± 2.1
5	>100	>100	>200	13.7 ± 1.2	35.5 ± 3.6	32.3 ± 3.0	37.1 ± 3.1	33.4 ± 3.8	42.5 ± 5.1
6	>200	>200	>200	>200	>200	>200	>200	>200	>200
7	>200	>200	>200	>200	>200	>200	>200	>200	>200
8	>100	>100	>100	>100	>300	>300	>300	>300	>300
9	>100	>100	>100	66.0 ± 4.6	>200	>200	>200	>200	>200

[a]Data represent the mean±SD values obtained from three independently performed experiments, with two replicates per experiment. *E. coli* TA531 cells lacking chromosomal *rrn* alleles, but containing pKK35 plasmids possessing wild-type 23S rRNA displayed the same IC_{50} value for each drug, like those of wild-type (WT) *E. coli* K12 cells. [b]Data in these columns have been obtained from ref. 28 and are presented here for the sake of comparison.

Table 2. Protein, polyamine, and CAM-PAC content in *E. coli* cells grown in the presence or absence of compounds **4** and **5**[a]

Parameter assayed	Cells grown in the absence of CAM-PACs	Cells grown in the presence of comp. **4**	Cells grown in the presence of comp. **5**
Culture optical density (560 nm) at the time of harvesting	0.800 ± 0.070	0.416 ± 0.030*	0.440 ± 0.035*
Protein (mg/g w.w.)	57.00 ± 6.35	13.10 ± 1.92*	21.8 ± 3.00*
Putrescine (μmol/g mg/g w.w.)	7.60 ± 1.20	1.14 ± 0.15*	0.46 ± 0.08*
Spermidine (μmol/g mg/g w.w.)	1.63 ± 0.50	0.47 ± 0.05*	0.35 ± 0.03*
Compound **4** (μM)[b]		1.06 ± 0.12	
Compound **5** (μM)[b]			3.96 ± 0.47

[a]The cells used for these assays were grown in polyamine deficient M9 medium in the presence of compound **4** or **5** added at a dose equal to their individual IC_{50}, and harvested when the control culture reached 0.800 at 560 nm. The cells were then collected by centrifugation and extracted with 0.6 M $HClO_4$. The supernatants were assayed for polyamines, compound **4**, and compound **5** by RP-HPLC chromatography**,** while the pellets were extracted in 0.1 M NaOH and analyzed for protein.

[b]The concentrations of compounds **4** and **5** were estimated in nmol/g wet weight and then expressed in μM by assuming a cell density equal to 1 g/ml.

*Significant different from the value measured in cells grown in the absence of CAM-PACs ($p < 0.05$).

Table 3. Kinetic constants for CAM acetyltransferase reaction using CAM or compounds **4** and **5** as substrates [a]

Compound	Constant: V_{max} ($\mu M \cdot min^{-1}$)[b]	K_m (μM)[c]	V_{max}/K_m (min^{-1})
CAM	8.23 ± 0.29	59.26 ± 5.32	0.139 ± 0.013
4	1.54 ± 0.06	37.74 ± 4.95	0.041 ± 0.005
5	4.56 ± 0.29	53.45 ± 9.19	0.085 ± 0.016

[a] The reaction was carried out in 3 ml of 94 mM Tris/HCl pH 7.8, containing 0.083 mM 5,5´-dithio-bis(2-nitrobenzoic acid), 0.16 mM acetyl coenzyme A, 25 units CAM acetyltransferase, and 0.154 mM either CAM or CAM-PAC. The product of the enzymatic reaction, coenzyme A, reacted with 5,5´-dithio-bis(2-nitrobenzoic acid) to yield 5-thio-2-nitrobenzoate that absorbs at 412 nm, with a micromolar extinction coefficient equal to 0.0136.

[b]To express the V_{max} in $\mu M \cdot min^{-1}$, the obtained values of ΔA_{412nm}/min were divided by 0.0136 (http://www.sigmaaldrich.com/technical-documents/protocols/biology/enzymatic-assay-of-chloramphenicol-acetyltransferase.html).

[c]The K_m value refers to the acetyl- acceptor substrate.

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1	>200	>200	>200	>200	>200	>200	>200	>200	>200
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3	>100	>100	>200	12.7 ± 1.0	>150	>150	>150	>100	>100
4	7.0 ± 0.9	9.0 ± 0.8	10.0 ± 0.9	4.7 ± 0.5	9.4 ± 0.8	9.4 ± 1.0	9.4 ± 0.9	9.0 ± 1.2	19.0 ± 2.1
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7	>200	>200	>200	>200	>200	>200	>200	>200	>200
8	>100	>100	>100	>100	>300	>300	>300	>300	>300
9	>100	>100	>100	66.0 ± 4.6	>200	>200	>200	>200	>200

[a]Data represent the mean±SD values obtained from three independently performed experiments, with two replicates per experiment. *E. coli* TA531 cells lacking chromosomal *rrn* alleles, but containing pKK35 plasmids possessing wild-type 23S rRNA displayed the same IC_{50} value for each drug, like those of wild-type (WT) *E. coli* K12 cells. [b]Data in these columns have been obtained from ref. 28 and are presented here for the sake of comparison.

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[a]The cells used for these assays were grown in polyamine deficient M9 medium in the presence of compound **4** or **5** added at a dose equal to their individual IC_{50}, and harvested when the control culture reached 0.800 at 560 nm. The cells were then collected by centrifugation and extracted with 0.6 M $HClO_4$. The supernatants were assayed for polyamines, compound **4**, and compound **5** by RP-HPLC chromatography**,** while the pellets were extracted in 0.1 M NaOH and analyzed for protein.

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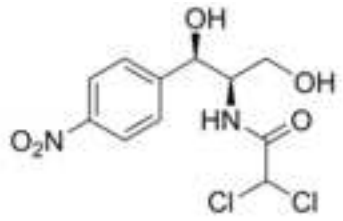
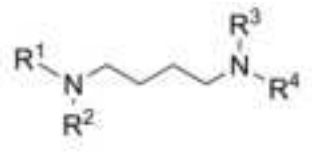
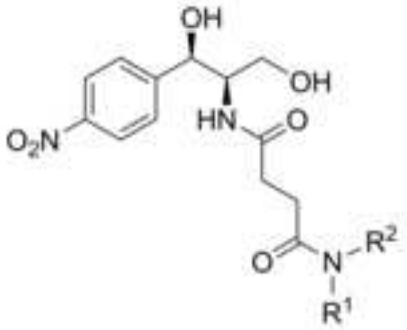
[c] The K_m value refers to the acetyl- acceptor substrate.



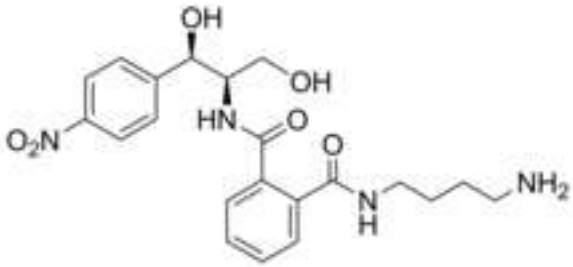
CAM



PUT : $R^1 = R^2 = R^3 = R^4 = H$
SPD : $R^1 = R^2 = R^3 = H$, $R^4 = (CH_2)_3NH_2$
SPM : $R^1 = R^4 = (CH_2)_3NH_2$, $R^2 = R^3 = H$



1 : $R^1 = H$, $R^2 = (CH_2)_4NH_2$
2 : $R^1 = H$, $R^2 = (CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$
3 : $R^1 = (CH_2)_3NH_2$, $R^2 = (CH_2)_4NH_2$
4 : $R^1 = (CH_2)_3NH_2$, $R^2 = (CH_2)_4N(CH_2Ph)_2$
5 : $R^1 = H$, $R^2 = (CH_2)_3NH(CH_2)_4N(CH_2Ph)_2$



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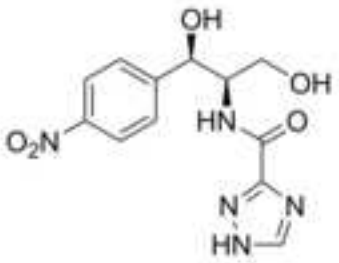

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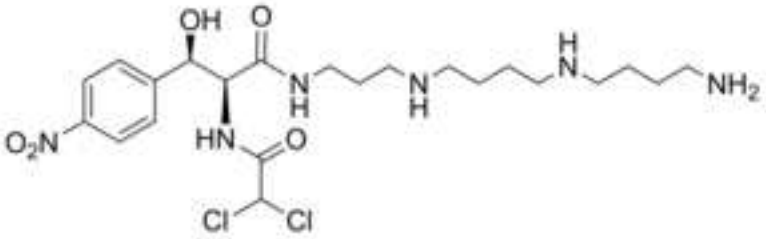

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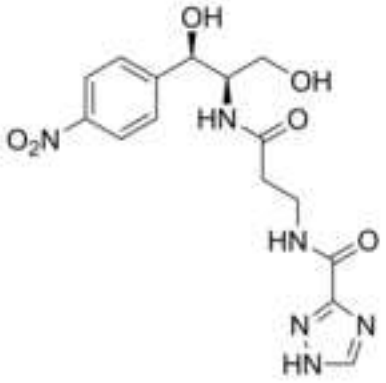

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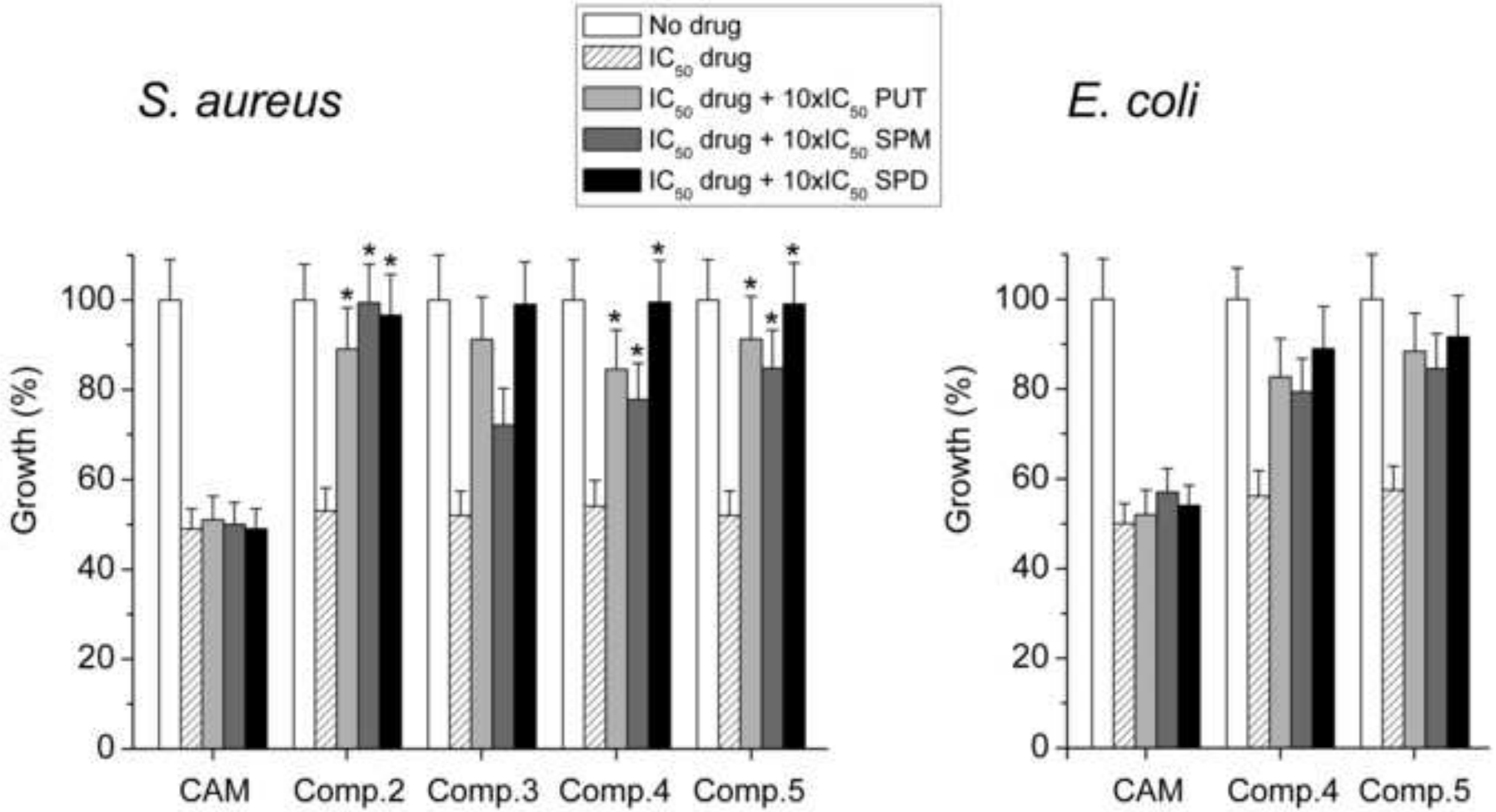
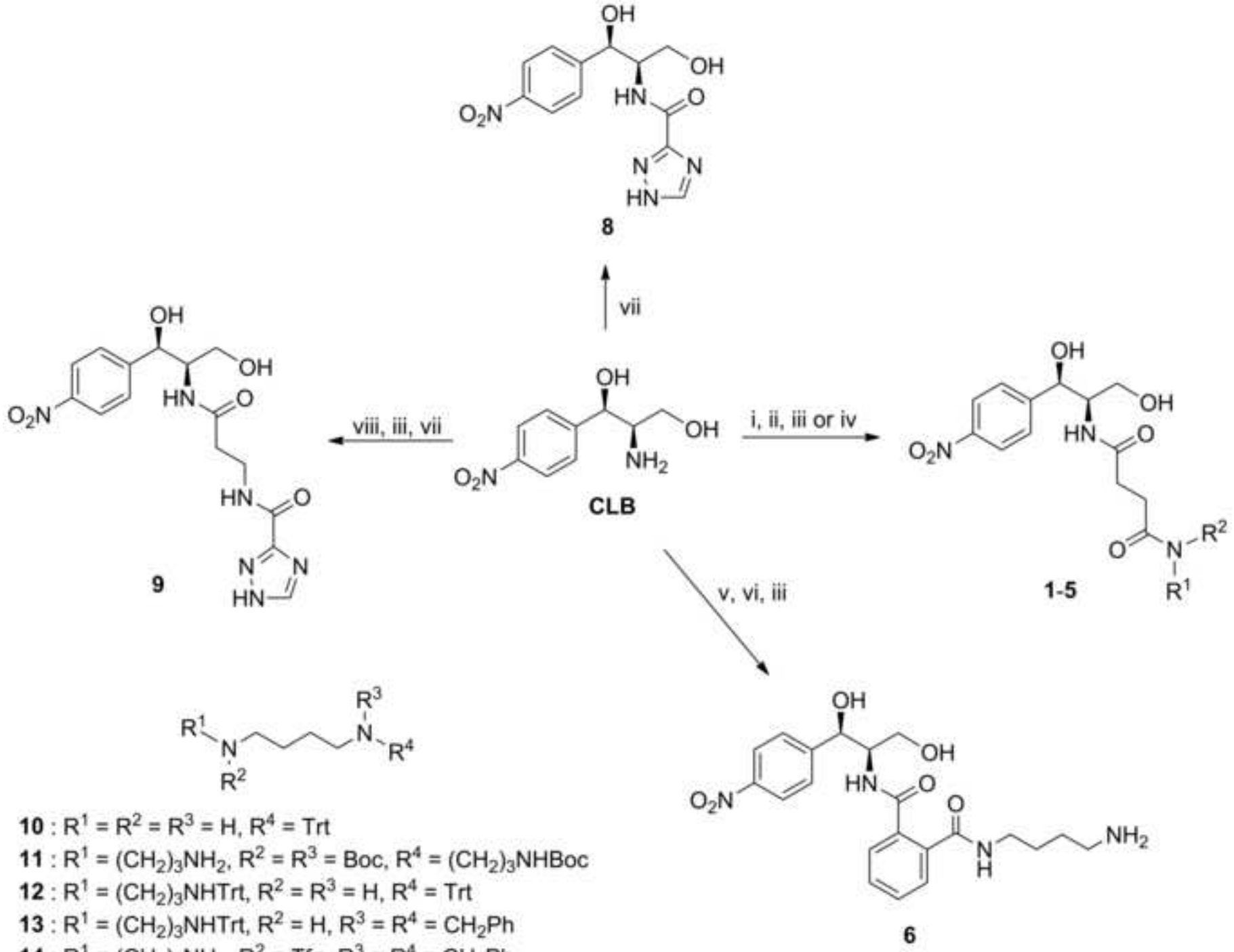




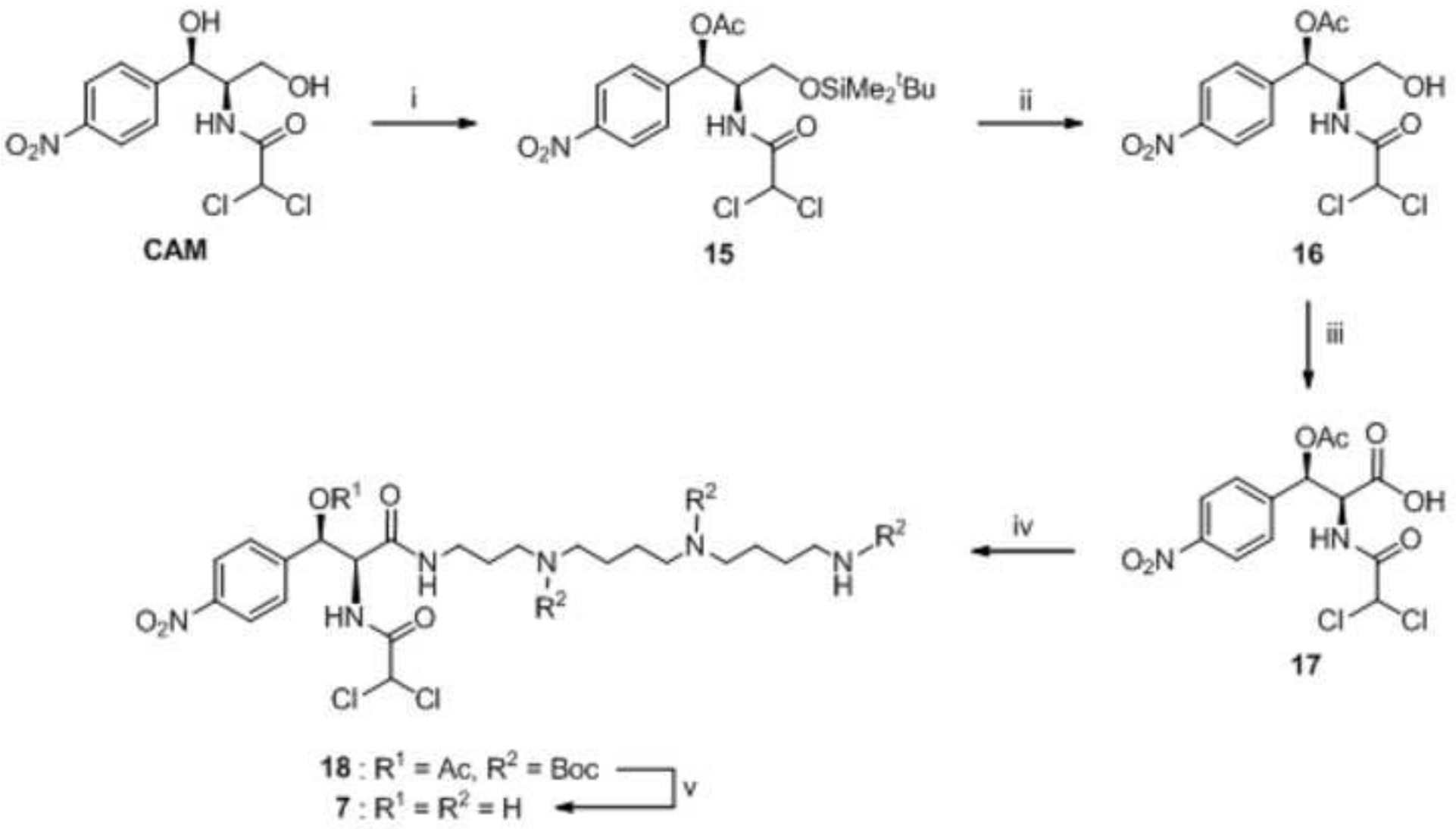
10 : $R^1 = R^2 = R^3 = H$, $R^4 = Trt$

11 : $R^1 = (CH_2)_3NH_2$, $R^2 = R^3 = Boc$, $R^4 = (CH_2)_3NHBoc$

12 : $R^1 = (CH_2)_3NHTrt$, $R^2 = R^3 = H$, $R^4 = Trt$

13 : $R^1 = (CH_2)_3NHTrt$, $R^2 = H$, $R^3 = R^4 = CH_2Ph$

14 : $R^1 = (CH_2)_3NH_2$, $R^2 = Tfa$, $R^3 = R^4 = CH_2Ph$


OH
OH
HN
O
O_2N
Cl
Cl
CAM
i
OAc
$OSiMe_2{}^tBu$
15
ii
16
iii
17
iv
OR^1
R^2
18 : R^1 = Ac, R^2 = Boc
7 : R^1 = R^2 = H
v