Studies on structure/antimicrobial activity relationship of cyclam derivatives

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Abstract

In this work, cyclam derivatives of the type $H_4[H_3BnCyclam]Cl_4$, $H_2[H_2Bn_2Cyclam](CH_3COO)_2$, $H_4[H_2Bn_2Cyclam]Cl_4$ and $H_4[H_4Cyclam]Cl_4$ were prepared. These compounds were tested against the Gram-positive *Staphylococcus aureus* and the Gram-negative *Escherichia coli*, *Burkholderia contaminans* and *Pseudomonas aeruginosa* bacteria, to gain insights into the relationship between molecular structure and antimicrobial activity of these compounds. The most effective cyclam derivatives against the bacterial strains tested were the *trans*-disubstituted cyclams displaying fluorinated groups in the *para*-position of the benzyl moieties, for which Minimal Inhibitory Concentration (MIC) values within the range 5 – 18 µg/mL were registered for *E. coli* and S. *aureus*. An inconsistent wider range of MIC values were registered for *P. aeruginosa*, while for *B. contaminans* MIC values for the compounds tested were higher than 512 µg/mL. No significant differences in the antimicrobial activity of the compounds were observed when testing their acetate or chloride salts. For *E. coli*, the highest value estimated for the frequency of spontaneous emergence of resistance to the most effective cyclam derivatives was 6×10^{-8}. This work evidenced that the presence of an increasing number of pendant arms in the cyclam backbone greatly enhances its antimicrobial activity and decrease its solubility in water. The compound bearing 4 pendant arms was not soluble in water and its antimicrobial activity could not be tested.

. **Keywords: Tetraazamacrocycles, MIC, Drug resistance, Novel drugs, Chemical Synthesis**

1 Introduction.

The rise of microorganisms resistant to the existing antimicrobials is considered a great threat to public health. Most of the currently available antimicrobials were developed and introduced between 1940s and 1960s, the era known as the golden age of antimicrobial chemotherapy [1]. Since Sir Alexander Fleming discovered penicillin in 1928 and its introduction in the market in 1945 that antimicrobials are used to treat infectious diseases [2]. Unfortunately, it is possible to observe a direct link between antimicrobials usage and emergence of resistant microorganisms to that same drug. The misuse and overuse of antimicrobials led to the rise of resistant microorganisms that is observed today [3]. This event, associated with a lack of investment in the field of antimicrobial research constitutes a serious problem that urgently needs an answer [4].

Antimicrobial resistance is threatening modern medicine, and the infections caused by a particular group of bacterial pathogens called ESKAPE are particularly threatening [5]. ESKAPE is an acronym that stands for *Enterococcus faecium*, *Staphylococus aureus*, *Klebsiella pneumoniae*, *Acinobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species [6]. This group was identified as major causes of life-threatening nosocomial infections worldwide [5].

Efforts are being done to answer the present lack of new antimicrobials crisis [7]. Previous work from the research groups headed by the advisors of this thesis has pointed out cyclam derivatives as potential new antimicrobials [8]. Cyclams are versatile compounds that can be used to create a wide array of derivatives by linking *trans*-positioned chemical groups in the molecule backbone [8].

The objective of this study is to expand the knowledge on tetraazamacrocycles as potential new antimicrobials. To achieve this goal, cyclam derivatives were synthesized and chemically characterized, and their antimicrobial activity was tested against the ESKAPE species *E. coli*, *S. aureus* and *P. aeruginosa*. *B. contaminans* [9], a member of the *Burkholderia cepacia* complex that comprises opportunistic pathogens able to cause respiratory infections in cystic fibrosis patients [10] was also included in this study.

2 Materials and Methods

General considerations for chemical synthesis and characterization.

Compound 16d was prepared as previously described [8]. All reagents were of commercial grade and used without further purification.

NMR spectra were recorded in Bruker AVANCE II 300 or 400 MHz spectrometers referenced internally to residual proton-solvent (1H) or solvent (13C) resonances, and reported relative to tetramethylsilane (0 ppm). 19F NMR was referenced to external CF3COOH (-76.55 ppm). 1H-13C{1H} HSQC and 1H-1H COSY NMR experiments were used to assign all proton and carbon resonances. Elemental analyses were performed at the Laboratório de Análises of IST.

Synthesis of the cyclam derivatives

Cyclam (1) was prepared according to a published procedure [11].

Compound 2 was prepared according to a previously described method [12].

Compound 3: Compound **2** (1.02 g, 2.04 mmol) was dissolved in a minimum volume of dimethylformamide. K_2CO_3 (0.70 g, 5.06 mmol) and 4-(trifluoromethyl)benzyl bromide (0.49 g, 2.05 mmol) was added. The reaction mixture was left stirring overnight. A saturated solution of $KHCO_3$ and brine were added and the product extracted with small portions of chloroform. The organic phases were combined and dried with anhydrous $MgSO_4$. After filtration, the solvent was evaporated under reduced pressure and the product was obtained with a 98 % yield (1.32 g, 2.00 mmol). ^{1}H NMR ($CDCl_3$, 300.1 MHz, 296 K): δ (ppm) 7.50 (d, $^3J_{H-H}$ = 8 Hz, 2H, *o-Ph* or *m-Ph*), 7.34 (d, $^3J_{H-H}$ = 8 Hz, 2H, *o-Ph* or *m-Ph*), 3.53 (s, 4H, PhC*H*$_2$N), 3.30 (overlapping, 12H total, 6H, [C3]*C*H$_2$N and 6H, [C2]*C*H$_2$N), 2.56 (m, 2H [C2]*C*H$_2$N), 2.34 (m, 2H [C3]*C*H$_2$N), 1.84 (m, 2H, CH$_2$C*H*$_2$CH$_2$), 1.64 (m, 2H, CH$_2$C*H*$_2$CH$_2$), 1.41-1.22 (br, 27H, C*H*$_3$). ^{13}C {^{1}H} NMR ($CDCl_3$, 100.6 MHz, 296 K): δ (ppm) 155.8 (*C*O), 155.6 (overlapping, *C*O), 143.4 (*i-Ph*), 129.2 (*o-Ph* or *m-Ph*), 128.0 (t, $^2J_{C-F}$ = 93 Hz, *p-Ph*), 124.6 (q, $^1J_{C-F}$ = 273 Hz, *C*F$_3$), 122.5 (*o-Ph* or *m-Ph*), 79.7 (*C*(CH$_3$)$_3$), 79.6 (*C*(CH$_3$)$_3$), 79.5 (*C*(CH$_3$)$_3$), 59.5 (PhC*H*$_2$N), 53.5 ([C2]*C*H$_2$N), 51.9 ([C3]*C*H$_2$N), 47.9 - 46.3 (overlapping, [C2]*C*H$_2$N and [C3]*C*H$_2$N), 28.5 - 28.4 (overlapping (CH$_2$*C*H$_2$CH$_2$) and (*C*H$_3$). ^{19}F NMR (D_2O/$(CD_3)_2CO$, 282.4 MHz, 296K): δ (ppm) -62.4 (s, C*F*$_3$). MS (CH_3CN, ESI): *m/z* 501.20 [M-^{CF3}PHCH$_2$+H]$^+$, 659.21 [M+H]$^+$

Compound 6: Compound **3** (0.66 g, 1.00 mmol) was dissolved in dichloromethane and HCl at 37% was added until the pH ≈ 1. The solution was left stirring overnight at room temperature. The solvent was evaporated under reduced pressure affording the product as white solid in 47% yield (0.29 g, 0.47 mmol). ^{1}H NMR (D_2O/$(CD_3)_2CO$, 300.1 MHz, 296 K): δ (ppm) 7.87 (d, $^3J_{H-H}$ = 9 Hz, 2H, *o-Ph* or *m-Ph*), 7.78 (d, $^3J_{H-H}$ = 9 Hz, 2H, *o-Ph* or *m-Ph*), 4.48 (s, 2H, PhC*H*$_2$N), 3.72-3.68 (overlapping, 6H total, [C2]*C*H$_2$N), 3.60 (m, 2H [C2]*C*H$_2$N), 3.52-3.47 (overlapping, 6H total, [C3]*C*H$_2$N), 3.36 (m, 2H [C3]*C*H$_2$N), 2.33 (m, 4H, CH$_2$C*H*$_2$CH$_2$). ^{13}C {^{1}H} NMR (D_2O/$(CD_3)_2CO$, 75.5 MHz, 296 K): δ (ppm) 135.1 (*i-Ph)* , 131.7 (*o-Ph*), 131.2 (q, $^2J_{C-F}$ = 32 Hz, *p-Ph*), 126.4 (d, $^3J_{C-F}$ = 3 Hz, *m-Ph*), 124.2 (q, $^1J_{C-F}$ = 272 Hz, *C*F$_3$), 57.4 (PhCH$_2$N), 48.7 ([C3]*C*H$_2$N), 46.3 ([C2]*C*H$_2$N), 43.0 ([C3]*C*H$_2$N), 42.4 (overlapping, [C3]*C*H$_2$N), 39.6 (overlapping, [C2]*C*H$_2$N), 39.1 ([C2]*C*H$_2$N), 20.0 (CH$_2$*C*H$_2$CH$_2$), 19.7 (CH$_2$*C*H$_2$CH$_2$). ^{19}F NMR (D_2O/$(CD_3)_2CO$, 282.4 MHz, 296K): δ (ppm) -62.6 (s, C*F*$_3$). Anal. Calcd for $C_{18}H_{33}Cl_4F_3N_4.H_2O$: C, 41.39; H, 6.75; N, 10.73. Found: C, 41.29; H, 6.60; N, 10.68.

Compound 7 was synthesized according to the method previously described by Royal et al [13].

Compound 8: Compound **7** (5.0 g, 22.3 mmol) was dissolved in a minimum volume of acetonitrile and two equivalents of 4-(methyl)benzyl bromide (9.1 g, 49.2 mmol) were added. The solution was stirred overnight at room temperature, resulting in a white precipitate that was filtered and dried under reduced pressure. The product was obtained as a white solid in 47% yield (6.2 g, 10.4 mmol). ^{1}H NMR (D_2O/$(CD_3)_2CO$, 300.1 MHz, 296 K): δ (ppm) 7.81 (d, $^3J_{H-H}$ = 9 Hz, 4H, *o-Ph* or *m-Ph*), 7.62 (d, $^3J_{H-H}$ = 9 Hz, 4H, *o-Ph* or *m-Ph*), 5.94 (d, $^3J_{H-H}$ = 9 Hz, 2H, NC*H*$_2$N), 5.12 (d, $^3J_{H-H}$ = 15Hz, 2H, PhC*H*$_2$N), 4.94-4.86 (overlapping, 4H total, 2H, [C2]C*H*$_2$N and 2H, PhC*H*$_2$N), 4.05-3.97 (overlapping, 4H total, 2H, [C2]C*H*$_2$N and 2H, NC*H*$_2$N), 3.78 (m, 2H, [C3]C*H*$_2$N), 3.65-3.56 (overlapping, 4H total, 2H, [C3]C*H*$_2$N and 2H, [C3]C*H*$_2$N), 3.34 (m, 2H, [C2]C*H*$_2$N), 3.24 (m, 2H, [C2]C*H*$_2$N), 2.96-2.79 (overlapping, 4H total, 2H, [C3]C*H*$_2$N and 2H, CH$_2$C*H*$_2$CH$_2$), 2.65 (s, 6H, C*H*$_3$), 2.21 (m, 2H, CH$_2$C*H*$_2$CH$_2$). ^{13}C {^{1}H} NMR (D_2O/$(CD_3)_2CO$, 75.5 MHz, 296 K): δ (ppm) 141.4 (*i-Ph* or *p-Ph*), 133.4 (*o-Ph* or *m-Ph*), 130.3 (*o-Ph* or *m-Ph*), 123.6 (*i-Ph* or *p-Ph*), 77.1 (NCH$_2$N), 63.0 (PhCH$_2$N), 59.9 ([C3]*C*H$_2$N), 51.7 ([C3]*C*H$_2$N), 48.0 (overlapping, [C2]*C*H$_2$N and [C2]*C*H$_2$N), 20.9 (CH$_2$*C*H$_2$CH$_2$), 19.9 (*C*H$_3$). Anal. Calcd for $C_{28}H_{42}Br_2N_4$: C, 56.57; H, 7.12; N, 9.42. Found: C, 56.51; H, 6.85; N, 9.50.

Compound 9: Compound **7** (0.42 g, 1.89 mmol) was dissolved in a minimum volume of acetonitrile and two equivalents of 1-(bromomethyl)-4-(2,2,2-trifluoroethyl)benzene (1.00 g, 3.97 mmol) were rapidly added. The mixture was stirred overnight at temperature room. The white precipitate formed was filtered and dried under reduced pressure affording **7** in 52% yield (0.72 g, 0.98 mmol). ^{19}F NMR (D_2O/$(CD_3)_2CO$, 282.4 MHz, 296K): δ (ppm) -65.8 (s, CH$_2$C*F*$_3$). Anal. Calcd for $C_{30}H_{40}Br_2F_6N_4O_4.(H_2O)$: C, 48.14; H, 5.66; N, 7.49. Found: C, 47.64; H, 5.38; N, 7.32

Compound 12: Compound **8** (6.0 g, 10.1 mmol) was hydrolyzed in an aqueous NaOH solution (3M) for 4h under stirring at room temperature. The product was

extracted with small portions of chloroform that were combined and dried with $MgSO_4$ anhydrous. Evaporation of the solvent to dryness gave an oil that was converted into a white solid after successive freeze-trituration-pump-thaw cycles. Compound **10** was obtained with a 92% yield (3.8 g, 9.3 mmol). ^{1}H NMR ($CDCl_3$, 400.1 MHz, 296 K): δ (ppm) 7.19 (d, $^3J_{H-H}$ = 8 Hz, 4H, *o-Ph* or *m-Ph*), 7.10 (d, $^3J_{H-H}$ = 8 Hz, 4H, *o-Ph* or *m-Ph*), 3.69 (s, 4H, PhCH_2N), 2.80 (s, 2H, N*H*), 2.73 (m, 4H, [C3]CH_2N), 2.68 (m, 4H, [C2]CH_2N), 2.55 (m, 4H, [C2]CH_2N), 2.51 (m, 4H, [C3]CH_2N), 2.29 (s, 6H, CH_3), 1.83 (m, 4H, $CH_2CH_2CH_2$).^{13}C {^{1}H} NMR ($CDCl_3$, 100.6 MHz, 296 K): δ (ppm) 136.6 (*i-Ph* or *p-Ph*), 134.2 (*i-Ph* or *p-Ph*), 129.7 (*o-Ph* or *m-Ph*), 128.9 (*o-Ph* or *m-Ph*), 57.5 (Ph*C*H_2N), 54.2 ([C2]*C*H_2N), 51.8 ([C3]*C*H_2N), 50.5 ([C3]*C*H_2N), 47.9 ([C2]*C*H_2N), 26.1 ($CH_2CH_2CH_2$), 21.8 (*C*H_3). Anal. Calcd for $C_{26}H_{40}N_4$: C, 76.42; H, 9,87; N, 13.71. Found: C, 76.06; H, 9.91; N, 13.69.

Compound 13: Compound **13** was prepared by the same procedure described for **12** using **9** instead of **8**. The product was obtained as a white solid in 52% yield (0.28 g, 0.51 mmol). ^{1}H NMR ($CDCl_3$, 400.1 MHz, 296 K): δ (ppm) 7.29 (d, $^3J_{H-H}$ = 8 Hz, 4H, *o-Ph* or *m-Ph*), 7.21 (d, $^3J_{H-H}$ = 8 Hz, 4H, *o-Ph* or *m-Ph*), 3.71 (s, 4H, PhCH_2N), 3.31 (q, 4H, $^3J_{H-F}$ = 11 Hz, CH_2CF_3), 2.71-2.52 (overlapping, 18H total, 2H, NH, 8H, [C3]CH_2N and 8H, [C2]CH_2N), 1.84 (m, 4H, $CH_2CH_2CH_2$). ^{13}C {^{1}H} NMR ($CDCl_3$, 100.6 MHz, 296 K): δ (ppm) 137.6 (*i-Ph* or *p-Ph*), 130.1 (*o-Ph* or *m-Ph*), 129.9 (*o-Ph* or *m-Ph*), 129.0 (*i-Ph* or *p-Ph*), 125.9 (q, $^1J_{H-F}$ = 279 Hz, CH_2CF_3), 57.6 (Ph*C*H_2N), 54.3 ([C2]*C*H_2N), 51.7 ([C3]*C*H_2N), 50.1 ([C2]*C*H_2N or [C3]*C*H_2N), 47.9 ([C2]*C*H_2N or [C3]*C*H_2N), 40.0 (q, $^2J_{H-F}$ = 29 Hz, *C*H_2CF_3), 26.1 ($CH_2CH_2CH_2$). ^{19}F NMR ($CDCl_3$), 376.5 MHz, 296K): δ (ppm) -65.9 (s, C*F*$_3$). Anal. Calcd for $C_{28}H_{38}F_6N_4$: C, 61.75; H, 7.03; N, 10.29. Found: C, 60.82; H, 6.75; N, 10.00.

Compound 16a: Compound **12** (1.00 g, 2.45 mmol) was dissolved in a small volume acetonitrile and 1 mL of glacial acetic acid was added to the solution. This mixture was refluxed for 1h and the solvent was evaporated under reduced pressure resulting in **16a** in 98% yield (1.27 g, 2.40 mmol). ^{1}H NMR ($CDCl_3$, 300.1 MHz, 296 K): δ (ppm) 10.74 (overlapping, 6H total, 4H, NH_2^+ and 2H, CH_3COOH), 7.12 (d, $^3J_{H-H}$ = 6 Hz, 4H, *o-Ph* or *m-Ph*), 7.02 (d, $^3J_{H-H}$ = 9 Hz, 4H, *o-Ph* or *m-Ph*), 3.79 (s, 4H, PhCH_2N), 3.16-3.14 (overlapping, 8H total, 4H, [C3]CH_2N and 4H, [C2]CH_2N), 2.75 (m, 4H, [C2]CH_2N), 2.66 (m, 4H, [C3]CH_2N), 2.32 (s, 6H, CH_3), 1.99 (overlapping, 16H total, 4H, $CH_2CH_2CH_2$, 6H, CH_3COO$^-$ and 6H, CH_3COOH). ^{13}C{^{1}H} NMR ($CDCl_3$, 75.5 MHz, 296 K): δ (ppm) 176.6 (*C*OO), 137.3 (*i-Ph*), 130.9 (*p-Ph*), 130.8 (*o-Ph* or *m-Ph*), 129.0 (*o-Ph* or *m-Ph*), 52.7 (Ph*C*H_2N), 51.3 ([C3]*C*H_2N), 48.7 ([C2]*C*H_2N), 48.3 ([C3]*C*H_2N), 45.6 ([C2]*C*H_2N), 22.8 ($CH_2CH_2CH_2$), 22.6 (*C*H_3COO), 21.2 (*C*H_3). Anal. Calcd for $C_{30}H_{48}N_4O_4.(CH_3COOH)_2$: C, 62.94; H,8.70; N,8.64. Found: C, 62.26; H, 9.17; N, 8.46.

Compound 16b: This compound was prepared using a similar protocol described for **16a** but using **13**. Compound **16b** was obtained as a white solid in 84% yield (0.29 g, 0.43 mmol). ^{1}H NMR ($CDCl_3$, 400.1 MHz, 296 K): δ (ppm) 10.28 (overlapping, 6H total, 4H, NH_2^+ and 2H, COO*H*), 7.25 (d, $^3J_{H-H}$ = 8 Hz, 4H, *o-Ph* or *m-Ph*), 7.16 (d, $^3J_{H-H}$ = 8 Hz, 4H, *o-Ph* or *m-Ph*), 3.83 (s, 4H, PhCH_2N), 3.34 (m, $^3J_{H-F}$ = 11 Hz, 4H, CH_2CF_3), 3.10 (overlapping, 8H total, 4H, [C2]CH_2N and 4H, [C3]CH_2N), 2.76 (m, 4H, [C2]CH_2N), 2.68 (m, 4H, [C3]CH_2N), 2.00-1.96 (overlapping, 16H total, 4H, $CH_2CH_2CH_2$, 6H, CH_3COO$^-$ and 6H, CH_3COOH). ^{13}C{^{1}H} NMR ($CDCl_3$,100.6 MHz, 296 K): δ (ppm) 176.7 (*C*OO), 134.6 (*i-Ph*), 131.0 (*o-Ph* or *m-Ph*), 130.2 (*o-Ph* or *m-Ph*), 129.6 (m, $^3J_{H-F}$ = 3 Hz, *p-Ph*), 125.8 (q, $^1J_{H-F}$ = 277 Hz, CH_2CF_3), 53.3 (PhCH_2N), 51.1 ([C3]*C*H_2N), 49.1 ([C2]*C*H_2N), 48.2 ([C3]*C*H_2N or [C2]*C*H_2N), 45.9 ([C3]*C*H_2N or [C2]*C*H_2N), 40.0 (q, $^2J_{H-F}$ = 29 Hz, *C*H_2CF_3), 23.2 ($CH_2CH_2CH_2$), 22.6 (*C*H_3COO). ^{19}F NMR (D_2O/$(CD_3)_2CO$, 376.5 MHz, 296 K): δ (ppm) -65.8 (s, CH_2CF_3) ^{19}F NMR ($CDCl_3$, 376.5 MHz, 296 K): δ (ppm) -65.9 (s, CH_2CF_3) Anal. Calcd for $C_{32}H_{46}F_6N_4O_4.(CH_3COOH)_2$: C, 55.09; H, 6.94; N, 7.14. Found: C, 55.00; H, 6.86; N, 7.01.

Compound 16c: 4,11-bis(4-(2,2,2-rifluoroethyl)benzyl)-1,4,8,11-tetraazacyclotetradecane (0.35 g, 0.92 mmol) was dissolved in a small volume acetonitrile and 1 mL of glacial acetic acid was added to the solution. This mixture was refluxed for 1h and the solvent was evaporated under reduced pressure resulting in **16c** in 84% yield (0.48 g, 0.77mmol). ^{1}H NMR ($CDCl_3$, 400.1 MHz, 296 K): δ (ppm) 10.82 (overlapping, 6H total, 4H, NH_2^+ and 2H, CH_3COOH), 7.31-7.29 (overlapping, 6H total, 2H, *p-Ph* and 4H, *m-Ph*), 7.21 (d, $^3J_{H-H}$ = 8 Hz, 4H, *o-Ph*), 3.97 (s, 4H, PhCH_2N), 3.25 (m, 4H, [C3]CH_2N), 3.20 (m, 4H, [C2]CH_2N), 2.78 (m, 4H, [C2]CH_2N), 2.74 (m, 4H, [C3]CH_2N), 2.00-1.96 (overlapping, 16H total, 4H, $CH_2CH_2CH_2$, 6H, CH_3COO$^-$ and 6H, CH_3COOH). ^{13}C{^{1}H} NMR ($CDCl_3$,100.6 MHz, 296 K): δ (ppm) 176.5 (*C*OO), 134.0 (*i-Ph*), 130.8 (*o-Ph*), 128.4 (*m-Ph*), 127.7 (*p-Ph*), 53.9 (PhCH_2N), 51.9 ([C3]*C*H_2N), 48.5 (overlapping, [C2]*C*H_2N and [C3]*C*H_2N), 45.5 ([C2]*C*H_2N), 22.8 ($CH_2CH_2CH_2$), 22.5 (*C*H_3COO). Anal. Calcd for $C_{28}H_{44}N_4O_4.(CH_3COOH)_2$: C, 61.91; N, 9.03; H, 8.44. Found: C, 61.85; N, 9.81; H, 8.40.

Compound 17a: Compound **12** (0.80g, 1.96mmol) was dissolved in a minimum volume of ethanol and HCl at 37% was added until the solution reached pH≈1. The white precipitated formed was filtered, washed with ethanol and dried under reduced pressure affording the product in 91% yield (0,99 g, 1.79mmol). ^{1}H NMR (D_2O/C_6D_5N, 300.1 MHz, 296 K): δ (ppm) 6.51(d, $^3J_{H-H}$ = 6 Hz, 4H, *o-Ph* or *m-Ph*), 6.51 (d, $^3J_{H-H}$ = 6 Hz, 4H, *o-Ph* or *m-Ph*), 2.92 (m, 4H, [C3]CH_2N), 2.83 (overlapping, 8H total, 4H, [C2]CH_2N and 4H, PhCH_2N), 2.09 (m, 4H, [C2]CH_2N), 1.96 (m, 4H, [C3]CH_2N), 1.42 (m, 4H, $CH_2CH_2CH_2$) 1.37 (s, 6H, CH_3).^{13}C{^{1}H} NMR (D_2O/C_6D_5N, 75.5 MHz, 296 K): δ (ppm) 139.9 (*i-Ph* or *p-Ph*), 133.2 (*i-Ph* or *p-Ph*), 132.7 (*o-Ph* or *m-Ph*), 131.6 (*o-Ph* or *m-Ph*), 57.3 ([C3]CH_2N), 53.9 ([C3]CH_2N), 53.0 ([C2]CH_2N), 50.9 (PhCH_2N), 47.5 ([C2]CH_2N), 24.2 ($CH_2CH_2CH_2$), 22.7 (*C*H_3).Anal. Calcd

for $C_{26}H_{44}Cl_4N_4.H_2O$: C, 54.55; H, 8.10; N, 9.79. Found: C, 54.41; H, 8.30; N, 9.57.

Compound 17b: Compound **17b** was obtained quantitatively as a white solid using the procedure described for the preparation of **17a** but using **13**. ^{1}H NMR (D_2O/$(CD_3)_2CO$, 300.1 MHz, 296 K): δ (ppm) 7.61 (d, $^3J_{H-H}$ = 8 Hz, 4H, *o-Ph* or *m-Ph*), 7.54 (d, $^3J_{H-H}$ = 8 Hz, 4H, *o-Ph* or *m-Ph*), 4.45 (s, 4H, PhCH_2N), 3.72 (overlapping, 8H total, [C2]CH_2N), 3.61 (q, $^3J_{H-F}$ = 11 Hz, 4H, CH_2CF_3), 3.51-3.46 (overlapping, 8H total, [C3]CH_2N), 2.31 (m, 4H, $CH_2CH_2CH_2$). ^{13}C{^{1}H} NMR (D_2O/$(CD_3)_2CO$, 75.5 MHz, 296 K): δ (ppm) 133.1 (*i-Ph*), 131.8 (*o-Ph* or *m-Ph*), 131.7 (*o-Ph* or *m-Ph*), 129.5 (*p-Ph*), 126.4 (q, $^1J_{H-F}$ = 277 Hz, CH_2CF_3), 58.5 (PhCH_2N), 48.2 ([C3]CH_2N), 45.2 ([C2]CH_2N), 42.4 ([C3]CH_2N), 39.0 (q, $^2J_{H-F}$ = 29 Hz, CH_2CF_3), 38.2 ([C2]CH_2N), 18.7 ($CH_2CH_2CH_2$). ^{19}F NMR (D_2O/$(CD_3)_2CO$, 282.4 MHz, 296 K): δ (ppm) -65.7 (s, CH_2CF_3). Anal. Calcd for $C_{28}H_{42}Cl_4F_6N_4.(H_2O)_2$: C, 46.29; N, 7.71; H, 6.38. Found: C, 46.29; N, 747; H, 6.24.

Compound 21: Cyclam (0.20g, 1.00mmol) was dissolved in 10 mL of a NaOH solution (1M). An acetonitrile solution of 4-(trifluoromethyl)benzyl bromide (0.98g, 4.10mmol) was added and the reaction mixture was left stirring for 2 hours. The white precipitate formed was filtered off and dried under reduced pressure. Compound **21** was obtained in 84% yield (0.70 g, 0,84mmol). ^{1}H NMR ($CDCl_3$, 400.1 MHz, 296 K): δ (ppm) 7.50 (m, 8H, *o-Ph* or *m-Ph*), 7.39 (m, 8H, *o-Ph* or *m-Ph*), 3.48 (m, 8H, PhCH_2N), 2.62 (m, 8H, [C2]CH_2N), 2.54 (m, 8H, [C3]CH_2N), 1.79 (m, 4H, $CH_2CH_2CH_2$). ^{13}C{^{1}H} NMR ($CDCl_3$, 100.6 MHz, 296 K): δ (ppm) 129.2 (*o-Ph* or *m-Ph*), 125.2 (*o-Ph* or *m-Ph*), 58.9 (PhCH_2N), 51.7 ([C2]CH_2N or [C3]CH_2N), 50.7 ([C2]CH_2N or [C3]CH_2N), 24.5 ($CH_2CH_2CH_2$). ^{19}F NMR ($CDCl_3$, 282.4 MHz, 296 K): δ (ppm) -62.4 (s, CF_3) Anal. Calcd for $C_{42}H_{44}F_{12}N_4$: C, 60.57; N, 6.73; H, 5.33. Found: C, 59.03; N, 6.72; H, 5.34.

Compound 23: Compound **23** was obtained quantitatively as a white solid with the reaction of cyclam with HCl in ethanol. ^{1}H NMR (D_2O/$(CD_3)_2CO$, 400.1 MHz, 296 K): δ (ppm) 3.59 (m, 8H, [C2]CH_2N), 3.44 (m, 8H, [C3]CH_2N), 2.25 (m, 4H, $CH_2CH_2CH_2$). ^{13}C {^{1}H} NMR (D_2O/$(CD_3)_2CO$, 100.6 MHz, 296 K): δ (ppm) 40.4 ([C3]CH_2N), 37.3 ([C2]CH_2N), 17.5 ($CH_2CH_2CH_2$). Anal. Calcd for $C_{10}H_{28}Cl_4N_4.(H_2O)_2$: C, 31.43; H, 8.44; N, 14.66. Found: C, 31.34; H, 7.59; N, 14.56.

Bacterial strains and media

The strains used were *Escherichia coli* ATCC 2592, *Burkholderia contaminans* IST408, *Pseudomonas aeruginosa* 477 and *Staphylococcus aureus* Newman. All these strains are human clinical isolates. When in use, *Escherichia coli* ATCC 2592 and *Staphylococcus aureus* Newman were maintained in Lennox Broth (LB) solid medium, while *Burkholderia contaminans* IST408 and *Pseudomonas aeruginosa* 477 were maintained in Pseudomonas Isolation Agar (PIA) plates.

Minimum inhibition Concentration Assays

Minimal inhibitory concentration (MIC) assays were performed in Mueller Hinton Broth (Sigma) (MHB) using a microdilution assay, based on previously described methods[14]. Optical densities (OD) were determined at 640nm using a U-2000 Spectrophotometer (HITACHI) and the absorbance of microbial cultures in microtiter plates were measured at 600 nm in a SPECTROstar^{Nano} microplate reader (BMG LABTECH).

Briefly, bacteria were grown in MHB liquid medium overnight with orbital agitation (250 rpm) at 37 ºC. The cultures were then diluted in fresh MHB to an OD of 0.05 and incubated for 5h under the same conditions. Cultures were then diluted to an OD of 0.02 and 100µL aliquots of these cell suspensions were inoculated in 96-well polystyrene microtiter plate containing 100µL of MHB supplemented with different concentrations of each compound under study, achieved by serial 1:2 dilutions starting at 512 µg/mL to 0.5 µg/mL. Compounds were prepared with distilled water and filtered with a 0.22 µm sterile filter. As positive control an aliquot of 100 µL of 1x concentrated MHB and 100 µL of the bacteria inocula (OD_{640nm}= 0.02) were used, while for negative controls aliquots of 200 µL of sterile MHB 1x concentrated was used. Additionally the compounds sterility was also tested. After the inoculation, the microtiter plates were incubated at 37 ºC for 20h and bacterial growth was assessed by determining the OD of cultures at 600nm using a SPECTROstar^{Nano} microplate reader. Experiments were carried out at least four times.

Bacteriostatic /Bactericidal tests

To assess the bacteriostatic or bactericidal activity of the compounds 20 µL aliquots the 96-well polystyrene microtiter plates used in the MIC assays were collected from the wells exhibiting the highest concentration of compound with bacterial growth and the two subsequent wells with no evident growth. These aliquots were serially diluted 1:10 until 10^{-7}. 10 µL of each serial dilution were sport inoculated in LB solid medium. The spot inoculated plaques were incubated at 37ºC for 18h. After incubation, the plaques were photographed using Biorad Imaging System and the CFUs were enumerated.

Emergence of resistance assays

The assays, to assess the frequency of spontaneous resistance to the compounds under study, were done based on published procedure[15]. Since the procedure used larger plates, the conditions needed to be adjust to the ones used in this study. Plates were prepared with 50 mL of solid MHB and contained the compounds at concentrations four times higher than the MIC calculated

for each strain. The number of bacteria inoculated was adjusted 1-9 x 10^9, for this purpose.

MHB was inoculated with an isolated colony of each bacterial strain and incubated overnight at 37°C with orbital agitation (250 rpm). Afterwards aliquots of the cultures were collected, and diluted them in 20mL of MHB to obtain an OD_{640} of 0.05. These cultures were further incubated at 37°C with orbital agitation (250 rpm) for five hours. After 5 hours, the OD_{640} was measured in order to determinate the volume of culture correspondent to the CFUs desired. These volumes were centrifuged in microtubes using a microcentrifuge (8000 rpm; 5min). The supernatant was discarded and the pellets were ressuspended in 300 µL 0.9% NaCl solution and spread onto the surface of square plates, followed by incubation at 37°C for 5 days.

To assess the precise number of CFUs plated separate assays were done. For this purpose aliquots of the cultures used to inoculate the plates with the compounds, at the same optical density, were used. These aliquots were serially diluted 1:10 until concentration $1x10^{-7}$ was reached and all concentrations were plated in MHB solid medium and incubated overnight at 37ºC. The colonies formed were counted, after 24h of incubation.

3 Results and Discussion

Chemical synthesis and characterization of compounds

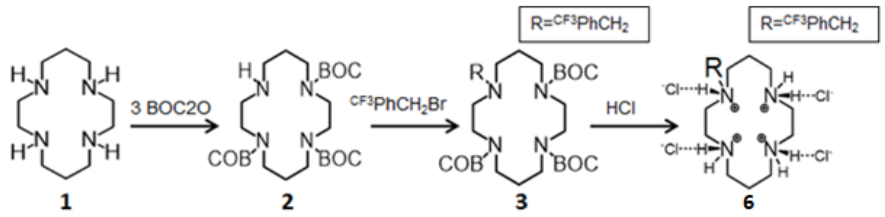
Cyclam derivatives with a varying number of pendant arms on the macrocyclic ring were prepared in order to test if the number and chemical nature of the pendant arms had any effect on the antimicrobial activity of these compounds. Since the compounds must be soluble in water for the MIC assays, their neutral forms were converted into the corresponding acetate and/or chloride salts in order to increase their solubility.

To synthesize the mono-substituted cyclam salt (**6**) it was necessary to protect three of the four nitrogen atoms of the cyclam ring in order to selectively functionalize only one of them. As so, protection was done using *tert*-butylcarbamate groups (Boc) by reaction of cyclam with di-*tert*-butyldicarbonate (Boc_2O) leading to the formation of $H(Boc)_3$Cyclam (**2**) (see **Scheme 1**). The subsequent alkylation reaction with 4-(trifluoromethyl)benzyl bromide led to the formation of ($^{4\text{-}CF3}PhCH_2$)$(Boc)_3$Cyclam (**3**). Compound **3** was dissolved in ethanol and concentrated HCl (37%) was added, which gave the chloride salt $H_4[H_3(^{4\text{-}CF3}PhCH_2)$Cyclam$]Cl_4$ (**6**)

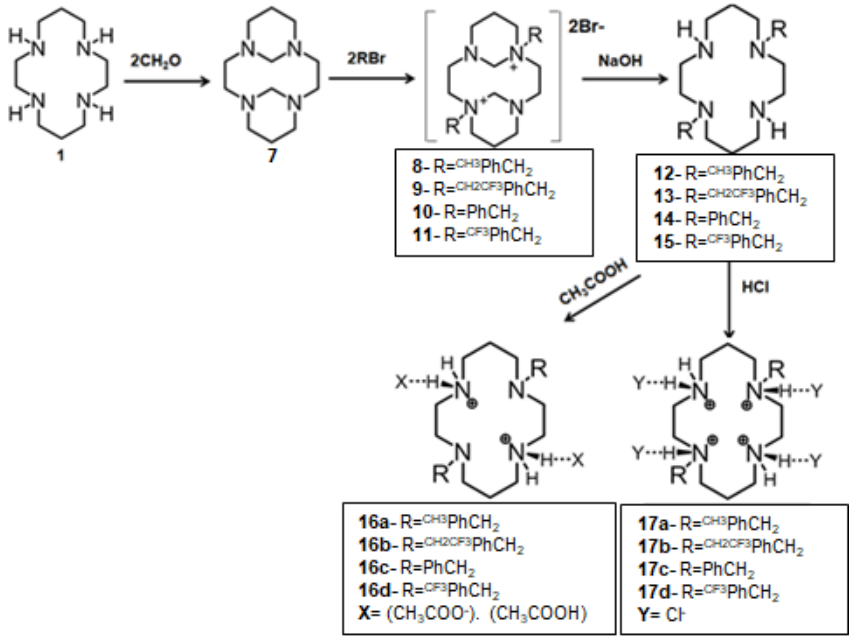
The ^{1}H NMR spectrum of **6** reveals ten multiplets corresponding to the methylene protons of the macrocycle backbone integrating to two protons each and one singlet that correspond to the two benzylic protons of the pendant arm due to the C_1 symmetry of the compound. In addition, two doublets integrating to two protons each appear in the aromatic region of the spectra. The NH_2^+ protons are absent because the spectrum was recorded in D_2O, revealing proton exchange. The $^{13}C\{^1H\}$ NMR spectrum of **6** displays ten different resonances for the macrocycle carbons and one for the benzylic carbon of the pedant arm, as expected. Additional resonances due to aromatic rings and the CF_3 group are also present. Despite the multiplicity observed in the ^{1}H and $^{13}C\{^1H\}$ NMR spectra did not correspond to a C_1 symmetry due to overlapping of the resonances, its confirmation was based on 2D NMR experiments (COSY and HSQC). The ^{19}F NMR spectrum shows a singlet due to the CF_3 group at -62.6 ppm.

trans-disubstituted cyclams of formula H_2Bn_2Cyclam (Bn = $^{4\text{-}CH3}PhCH_2$, 12, $^{4\text{-}CH2CF3}PhCH_2$, 13, $PhCH_2$, 14 and $^{4\text{-}CF3}PhCH_2$, 15) were prepared as shown in Scheme 2. Conversion of cyclam (**1**) into 1,4,8,11-tetraazatricyclo[9.3.1.14,8]hexadecane (**7**) was achieved by reaction with two equivalents of formaldehyde. Compound **7** displays two methylene cross-bridges between adjacent nitrogen atoms that point to opposite faces of the macrocycle. In this species, due to the stereochemical conformation imposed by the methylene cross-bridge, the deepest negative potential of the cyclam ring corresponds only to one pair of *trans* nitrogen atoms. These atoms are not only the less sterically hindered but they also have the electron lone pair pointing out of the macrocycle backbone; this structure is crucial for the second step of the reaction as it directs the nucleophilic attack and controls the selectivity [16]. In this way, the formation of **8-11** was attained by reaction of **7** with two equivalents of the appropriate benzyl bromide. All compounds are slightly hygroscopic solids that can hydrolyze in air. This behavior is more pronounced in 9 which hampered its characterization by NMR. Full hydrolysis of **8-11** in basic conditions affords the correspondent neutral compounds **12-15**. In acetic acid medium, compounds **12**-**15** were converted into the corresponding acetate salts **16** that were obtained in high yields after taking the solution to dryness. On the other hand, the chloride salts were obtained by addition of HCl to a solution of the compounds in ethanol that cause them to precipitate (see **Scheme 2**). Therefore salts **17** were obtained in very high yields due to the nature of the reaction.

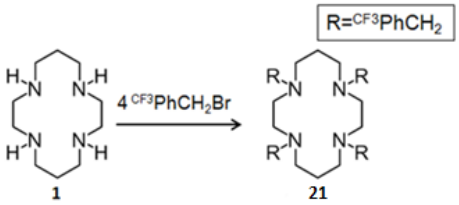
The ^{1}H NMR spectra of compounds **16** and **17** are similar to the ones described for **12**-**15**, showing five multiplets corresponding to the methylene protons of the macrocycle backbone integrating to four protons each and one singlet that corresponds to the four benzylic protons of the pendant arms. In addition, one set of resonances appear in the aromatic region of the spectra. In compounds **16a** and **17a** the protons of the CH_3 groups show up as singlets at



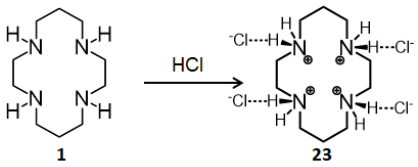
Scheme1



Scheme 2



Scheme 3



Scheme 4

2.25 and 1.32ppm, respectively. In **16b** and **17b** the CH_2CF_3 groups appear as a quartet with $^3J_{H-F}$=11Hz at 3.34 and 3.32ppm, respectively. The NH_2^+ and COOH protons overlap at 10.74 and 10.28 ppm in the spectra of **16a** and **16b** respectively. These protons are absent if the spectra are performed in D_2O, revealing proton exchange. The $^{13}C\{^1H\}$ NMR spectra of **16** and **17** are also similar to the ones described for **12**-**15** displaying five different resonances for the macrocycle and one for the benzylic carbon of the pedant arm as expected. Additional resonances due to aromatic rings are also presented.

Contrasting to the reactions to produce mono- and di-substituted cyclams**,** the production of the tetra-substituted derivative (**21**) revealed to be challenging. To obtain this compound several reactions were performed, with various conditions (*e.g.* different stoichiometries, reaction time, temperature and base) that are classically described in literature for the desired transformation. Surprisingly, none of them was able to give the final product affording a racial mixture of products. To obtain the compound **21**, cyclam was reacted with 4.2 equivalents of 4-(trifluoromethyl)benzyl bromide in a mixture of acetonitrile and an aqueous solution of NaOH (1M) in a 1:1 ratio as shown in **Scheme 3**.

The product precipitated out of solution and with a very high yield.

The convertion of the cyclam in its chloride salt form **23** was really straightforward using the same method used to obtain the chloride salts (see **Scheme 4**). This reaction had a very high yield as expected. The NMR spectra of **23** is consistent with a tetraprotonated cyclam salt revealing only three resonances in both proton and carbon spectra. These signals correspond to the CH2 groups of the [C2] and [C3] chains of the cyclam ring.

MIC assays

In the assays done, it was not possible to determine a MIC value for *B. contaminans* IST 408 for any compound synthesized and therefore it is possible to conclude that the MIC values of the compounds for this strain are higher than 512µg/mL. Therefore the compounds presented in these study are not candidates to treat infectious diseases caused by this pathogen.

Table 1| MIC values obtained from the Gompertz model for the compounds 16 and 17. Towards *E. coli*, *S. aureus* and *P. aeruginosa.*

	***E.coli ATCC* 25922**	***S. aureus* Newman**	***P.aeruginosa* 477**
	MIC for Acetate salt (µg/mL)		
16a	55	49	9
16b	10	10	164
16c	80	206	19
16d	7	8	15
	MIC for Chloride salt (µg/mL)		
17a	43	119	25
17b	16	18	20
17c	124	80	26
17d	5	5	70

Results presented on **Table 1** and **Figure 1** show that, the two most active *trans*-disubstituted derivatives compounds were those containing the CF_3 group variations. In fact, the MIC values estimated for *E. coli* ATCC 25922 and *S. aureus* Newman were respectively 10 µg/mL and 10 µg/mL for **16b**, 16 µg/mL and 18 µg/mL for **17b**, 7 µg/mL and 8 µg/mL for **16d** and 5 µg/mL and 5 µg/mL for **17d** . Since the similarities of these four compounds are the presence of a CF_3 group attached to the phenyl ring, in the case of the **16b** and **17b** compounds with a CH_2 linker between, our results indicate that the CF_3 is a group that greatly enhances the antimicrobial activity for these strains. In addition, since the MIC values of **16b, 17b, 16d** and **17d** are very similar, our results suggest that the CH_2 linker group in **16b** and **17b** does not significantly affect the activity. These results also point to the importance of the fluorine atoms in the pendant arms of the compounds. However, in a previous study, the presence of perfluorinated phenyl groups in the pendant arms of the cyclam ring did not increase the antimicrobial activity of the compound [8]. Therefore the group CF_3 must be required, most probably in that position of the phenyl ring for the enhanced antimicrobial activity observed in this study.

For the compounds **16a**, **16c**, **17a** and **17c** a higher MIC values for *E. coli* and *S. aureus* were obtained, compared to **16b**, **16d**, **17b** and **17d** (**Table 1**). It is also possible to observe the importance of a group attached to the carbon in para-position of the phenyl ring since compounds **16a**, **16b** and **16d** have lower MIC values than **16c** and this behavior is also possible to observe in compounds **17** (**Figure 1**).

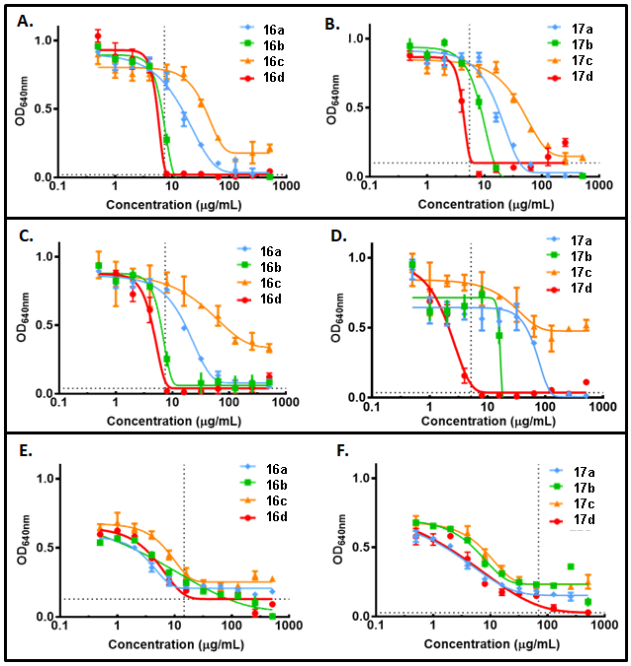


Figure 1| OD $_{640nm}$ adjusted to Gompertz model to determine MIC values for compounds 16 (A. C. and E.) and 17 (B., D, and E) *for E. coli* (A. and B.), *S. aureus* (C. and D.) *and P. aeruginosa* (E. and F.).

For *Pseudomonas aeruginosa* 477 the results obtained are not consistent. As shown in **Table 2**, the MIC values estimated for this strain were lower than it was expected. When observing the microtiter plates no growth could be detected only in the wells containing concentrations of 512 µg/mL of **16b**, **16d**, **17b** and **17d**. This indicates that the MIC values for these should be between 512µg/mL and 256µg/mL and not the values presented in **Table 1**. For the other compounds, growth was detected in all wells and therefore it should not be possible to calculate the MIC value. These results suggest that the method used is not the most effective to determined MIC values for the behavior that *P. aeruginosa* shows.

In conclusion, the results obtained with *trans*-disubstituted cyclams show that the compounds with the highest antimicrobial activity were those with a CF_3 group, most probably attached at para-position since the non-inclusion of a methyl or trifluormethyl group at that position dramatically reduces the compound activity. In addition, results presented in **Table 1** also indicate that the change from acetate to chloride salts did not significantly affect the activity of the compounds. The minimal difference that it is possible to observe in the MIC values of the compounds is probably due to the fact that the acetate salt has a higher molecular mass than the chloride salt and therefore for the same mass concentration there will be more molecules of **16** than **17**. Furthermore **16** has, also acetic acid co-crystallized as shown above

Cyclam derivatives with varying number of pendant arms were synthesized to assess the effect of the number of pendant arms. For this purpose, the pendant arm used in **16d** and **17d** was selected since it led to be the most active compounds. Besides that, since acetic acid is not strong enough to create acetate salt for tetra-substituted cyclam, chloride salts were used for the purpose of testing the effect of the number of pendant arms

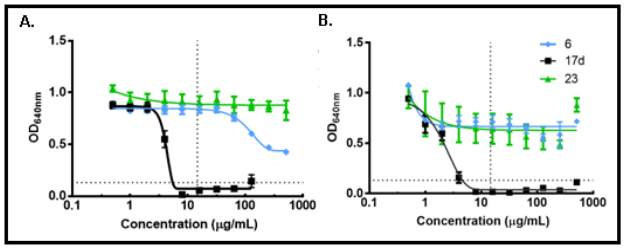


Figure 2| Optical density of *E. coli* (A.) and *S. aureus* (B.) after incubation for 24h at 37ºC in the presence of the indicated concentrations of 6, 17d or 23

The tetra-substituted cyclam was not soluble in water its antimicrobial activity was not possible to be assessed. For the remainder compounds tested, results show that the reduction of the number of pendant arms significantly reduced the antimicrobial activity. Furthermore, **23** had no antimicrobial activity within the range of concentrations towards the strains tested. The MIC values determined for compound **6** were 235 µg/mL and 261 µg/mL for *E. coli* and *P. aeruginosa*, respectively, and >512 µg/mL for *S. aureus* and *B. contaminans*. **Figure 2** shows a decline in bacterial growth with the increasing of compound concentration *E.coli*. **Figure 2** also depicts the difference in activity between the compounds with none, one and two pendant arms. The results, obtained for *E.coli* and *P. aeruginosa* with compounds **6**, **17d** and **23,** show us that the number of pendant arms is important for the activity of the compound.

Bactericidal/ Bacteriostatic assays

In this study only results from *E. coli* and *S. aureus* were presented since *P. aeruginosa* and *B. contaminans* seemed to be insensitive to the compounds. Also, the compounds with higher antimicrobial activity and probable candidates for the development of antimicrobial drug were selected.

The results obtained suggest that these compounds have bactericidal activity since there was no growth at concentrations equal or higher than the MIC. The exceptions were the results obtained with compounds **16a** for *S. aureus* and **17c** for *E. coli*, suggesting that they present a bacteriostatic activity. Giving that the other compounds behave as bactericidal, it is possible that some problems occurred during experiments. Further experiments are required to better characterize the antimicrobial activity of the compounds.

Spontaneous emergence of resistance to cyclam derivatives

Experiments were conducted to determine the frequency of spontaneous emergence of resistance, for the compounds **16b**, **16d**, **17b** and **17d**, which were found as the most active towards *E. coli* and *S. aureus*. These assays were made only for *E. coli* and *S. aureus* due to the lack of confidence in the MIC values determined for *P. aeruginosa*. As explained above for these assays, cells were incubated at 37ºC for a week in the presence of a concentrations of compounds equal to 4x the MICs determined (**Figure 3**).

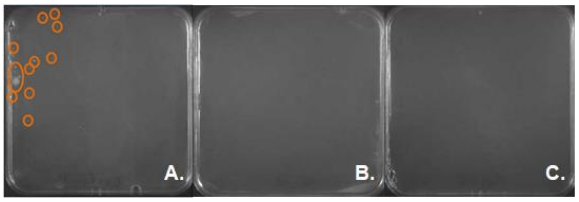


Figure 3| *E. coli* exposed to a concentration 4x MIC of 16b (A.), 16d (B.) and 17d (C.). 2.2 x10^8 CFU were plated and incubated for 37ºC for a week.

In the case of experiments performed for *S. aureus* with compounds **16b**, **16d**, **17b** and **17d,** the initial CFUs used were too high, and a lane of bacteria capable of growing on the surface of the plates appeared after 24h of incubation. No individual colonies were observed. Further experiments are required with a lower number of initial cells.

In the case of experiments carried out for *E. coli* it was possible to use adequate number of initial CFUs. No colonies were obtained for compounds **16d** and **17d**. Since the plates were inoculated with a number of CFUs of 2.2 x10^8 results obtained

indicate a frequency of emergence of spontaneous resistance lower than 4.5 x10^{-9}. For compound **16b** some colonies were observed which indicate the growth of spontaneous resistant colonies. The frequency of resistance emergence for this compound and this strain was 5.9 x10^{-8}.

Locke *et al*. in a study done in 2009, with *S. aureus* against compounds Lineolid (LZD) and Torezolid (TR-700) obtained a frequency of spontaneous resistance in the order of 10^{-9} and10^{-10}, respectively [15]. Despite the strain not being the same, the frequency of resistance obtained for *E. coli* to the compounds **16d** and **17d** are in the same order of values to the ones obtained by Locke *et al*. which suggests that the compounds are effective and should not lead to the development of resistance strains easily. As for compound **16b**, despite the value of frequency being a little higher it should not be excluded as a good candidate for drug development. This type of assays are a critical component for drug development since a compound for which bacteria exhibit a high frequency of spontaneous emergence of resistance does not constitute an appropriate therapeutic since bacteria can create resistance easily, requiring other therapeutics.

4. Conclusion

Cyclam derivatives were recently shown by Alves *et al.* as a new family of compounds with antimicrobial activity [8]. In particular, these authors showed that a cyclam derivative with a CF_3 group bound to a benzyl moiety presented MIC values of 9, 261 and 15µg/mL towards *E. coli*, *P. aeruginosa* and *S. aureus*, respectively.

In this work, cyclam derivatives with the CF_3 group bound to a benzyl moiety and replaced by CH_3 bound to a benzyl moiety (compound **16a** and **17a**), by CH_2CF_3 bound to a benzyl moiety (compound **16b** and **17b**), or substituted with only the benzyl group (compound **16c** and **17c**) were synthesized. These cyclam derivatives were conceived to gain knowledge on the role played by the fluorine presence and position on the molecule antimicrobial activity. In addition, compounds were prepared as acetate salts (**16**) or chloride salts (**17**) to investigate any possible role of the counter ion in the compound's activity.

The studies revealed in first hand that the anion do not change the activity of the compounds as minor differences were observed between an acetate (**16**) and chloride salts (**17**). These minor differences can be explained by the different molecular mass of the compounds and also by the error window of the assays. Since the synthesis of chloride salts were simpler and led to higher yields, these salts are better candidates for mass production. In addition, acetate salts are hygroscopic turning the quantification of cyclam derivatives acetate salts more difficult.

The work developed showed the importance of fluorine atoms in the pedant group since the compounds without fluorine had lower antimicrobial activity, as indicated by the higher MIC values for *E. coli* and *S. aureus*. We have also investigated the effects of the CF_3 group alone (**16b** and **17b**) or linked by CH_2 to the same carbon (**16d** and **17d**) on the antimicrobial activity of compounds. The differences in MIC values obtained for **16d**, **17d** and **16b**, **17b** suggest that the space between the CF_3 moiety and the remaining of the molecule did not affect activity, at least when the space corresponds to 1 carbon atom. To further study the effect of spacing between CF_3 moiety and the phenyl ring it would be interesting to synthesize a compound with a larger spacer. As for the other two *trans*-substituted cyclams, the results show us the importance of a methyl moiety attached to the phenyl ring since **16c** and **17c** had the lower activity towards both *E. coli* and *S. aureus*.

During this work, the determination of MIC values for *P. aeruginosa* was more difficult to achieve than for the other bacterial strains. Furthermore, it should be stated that there is a lack of confidence about the results presented, since variations in results were obtained. The observation of the microtitter plates showed that *P. aeruginosa* did not growth in concentrations higher than 256 µg/mL for **16d**, **17d** and **16b**, **17b** while growth were observed for all the other compounds, at all concentrations tested. This might be the result of the expression of several efflux pumps encoded in the organism's genome, or due to subpopulations with different levels of resistance to the compounds tested. It is also possible that the Gompertz model is not the best to determine MIC values for *P. aeruginosa* since the organism presents a slow decrease of OD with increasing concentrations of compounds.

The present work also shows that the number of pedant arms impacts the antimicrobial activity of the cyclam derivatives. The results obtained thus far allow us to conclude that the activity decreases with the decrease of pedant arms and vice-versa. To further complete this study, the synthesis of a tri-substituted cyclam could give us interesting results and further confirm the hypothesis made. In that scope, the tetra-substituted compound was synthesized. However the compound was not soluble and could not be tested. It is possible that the complexation of the tetra-substituted cyclam with a metal ion could turn the compound more soluble in water and make possible the characterization of its antimicrobial activity.

During this work attempts to synthesize a cyclen and a *trans*-disubstituted cyclen were carried out, but that was not possible. The synthesis of the *trans*-substituted cyclen with the particular pedant arm studied in this work seems to be complicated. Despite that, future work should encompass the synthesis of cyclen derivatives and the characterization of their antimicrobial activity. Such results are expected to further our knowledge on the relationship between the size of the macrocycle and its antimicrobial activity. This discovery could potentially give rise to novel families of compounds with enhanced antimicrobial activities.

5. Aknowledgments

I would like to deeply thank my thesis supervisor Professor Jorge Humberto Leitão of Instituto Superior Técnico- Universidade de Lisboa, for the support, for his patience, motivation and knowledge. A great thanks to my thesis co- supervisor, Dr. Luis Gonçalo Alves of of Instituto Superior Técnico- Universidade de Lisboa, for his guidance in the chemistry part of this work which was crucial. I would also like to acknowledge Professor Ana Margarida Martins and Dr. Silvia Sousa, of Instituto Superior Técnico- Universidade de Lisboa, for their contribution on the development of this master's project. The work developed in this Master's project was funded by FCT-Fundação para a Ciência e a Tecnologia (Portugal) through projects UID/QUI/00100/2013, UID/BIO/04565/2013, Programa Operacional Regional de Lisboa 2020 (Project N. 007317) and the NMR Network (IST-UTL Node) for facilities.

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