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Effect of Methyl-β-Cyclodextrin on the antimicrobial activity of a new series of poorly water-soluble benzothiazoles

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This work is dedicated to Prof. Nicolino De Laurentis, a friend and a scientist on occasion of his retirement

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Abstract

The antibacterial activity of the S-unsubstituted- and S-benzyl-substituted-2-mercapto-benzothiazoles **1**-**4** has been evaluated after complexation with Methyl-β-Cyclodextrin (Me-β-CD) or incorporation in solid dispersions based on Pluronic® F-127 and compared with that of the pure compounds. This with the aim to gain further insights on the possible mechanism(s) involved in the CD-mediated enhancement of antimicrobial effectiveness, a promising methodology to overcome the microbial resistance issue. Together with Differential Scanning Calorimetry, FT-IR spectroscopy and X-ray Powder Diffraction investigations, a molecular modeling study focused on compounds **2** and **4** showed that the S-unsubstituted compound **2**/Me-β-CD complex should be more stable than S-benzyl-substituted **4**/Me-β-CD. Only for **1**/Me-β-CD or, particularly, **2**/Me-β-CD complexes, the antibacterial

effectiveness was enhanced in the presence of selected bacterial strains. The results herein presented support the mechanisms focusing on the interactions of the bacterial membrane with CD complexes more than those focusing on the improvement of dissolution properties consequent to CD complexation.

Keywords: *Antimicrobial agents, Methyl-β-Cyclodextrin, PF-127, X-ray Powder Diffraction, Molecular Modelling*

2-Mercapto-6-nitrobenzothiazole (PubChem CID: 947375)

Methyl Betacyclodextrin (PubChem CID: 51051622)

Pluronic F127 (PubChem CID: 10154203)

Betacyclodextrin (PubChem CID: CID: 320761)

1. Introduction

The global diffusion of new microbial infections, as well as the continuously increasing multi-resistance of pathogens against many of the commonly used antibiotics, imposes a considerable effort to develop new antimicrobial agents or new formulation approaches of so called "classical antibiotic drugs" (Wijma, Huttner, Koch, Mouton, & Muller, 2018; Sportelli et al., 2017; Lu et al., 2014; Ancona et al., 2014). In this context, it has recently been evidenced that the multidrug-resistant Gram-negative bacteria represents an increasingly prevalent public health concern (Aliyu, Smaldone, & Larson, 2017). As part of our ongoing program on benzothiazole-nucleus containing antimicrobial agents, we focused our attention on the lipophilic 2-mercapto benzothiazoles **1-4** (Figure 1) of which the synthetic routes and activities were already reported (Franchini et al., 2009). Among them, compounds **1** and **2** showed high antibacterial activity against *S. aureus* and *E. coli*, with MIC values of 3.12 μg/mL and 25 μg/mL, respectively, whereas the replacement of the -SH group with a S-benzyl moiety, leading to compounds **3** and **4**, resulted in the loss of antibacterial activity.

On the other hand, we have also recently reported that the antimicrobial effectiveness of some lipophilic fluoro-substituted *N*-benzoyl-2-aminobenzothiazoles may be positively affected in the presence of natural or chemically modified cyclodextrins [CDs, *e.g.*, β-CD or 2-hydroxypropyl-β-cyclodextrin (HP-β-CD)] containing aqueous solutions (Catalano et al., 2013; Trapani et al., 2016). Our working hypothesis was that also the antibacterial activity of 2-mercapto benzothiazoles **1-4** might be favourably influenced by the presence of CDs. Such cyclic oligosaccharides are made up of six to eight dextrose units and are recognized as suitable solubilizing pharmaceutical excipients in oral and

injectable formulations. CDs can interact with poorly soluble drug molecules to form inclusion complexes enhancing their solubility (even up to 10^5 times) and bioavailability (Carrier, Miller, & Ahmed, 2007; Strickley, 2004). Natural cyclodextrins (α-, β- and γ-CD) are widely used, particularly the β-CD. However, since the latter CD exhibits relatively low solubility in water, various chemically modified β-CD derivatives have been synthesized in order to increase drug solubility, dissolution rate, bioavailability, and stability (Loftsson, & Brewster, 1996; Rajewski, & Stella, 1996; Szejtli, 1991; Uekama, & Otagiri, 1987).

In our previous work, to account for the observed CD-mediated enhancement of the antimicrobial effectiveness of the substituted *N*-benzoyl-2-aminobenzothiazoles, some mechanisms were elucidated (Trapani et al., 2016). Thus, we hypothesized that CDs can improve the activity of antibacterial agents not only by drug solubility enhancement consequent to the complexation, but also by modification of the bacterial membrane permeability or dissolution properties due to the interaction with CDs (Trapani et al., 2016). Hence, further work was necessary to draw more reliable conclusions.

With the aim to gain further insights in this context, in this paper we report the comparative effects on the antimicrobial effectiveness of compounds **1**-**4** of a hydrophilic CD, Methyl-β-Cyclodextrin (Me-β-CD) and an amphiphilic polymer as Pluronic® F-127 (PF-127), a non-ionic surfactant solubilizing agent *via* micelle formation (Figure 1). Me-β-CD was selected because it provided a peculiar increase in antimicrobial activity against Gram-negative strains in a series of β-lactam antibiotics when they were complexed with this CD (Athanassiou, Michaleas, Lada-Chitiroglou, Tsitsa, & Antoniadou-Vyza, 2003). The amphiphilic polymer PF-127 was used in order to prepare examples of the so-called "third generation solid dispersions" by which mainly the release rate of a poorly soluble drug may be improved when the carrier has surface activity (Vasconcelos, Sarmento, & Costa, 2007; Vasconcelos, Marques, das Neves, & Sarmento, 2016). Solid dispersions, indeed, are defined as mixtures of poor water soluble drugs with carriers providing a drug release profile determined by the carrier properties (Vasconcelos et al., 2007). Thus, this comparative study could allow us to gain information on the possible role played by dissolution properties as factor to be taken into consideration to account for the mentioned improvement of the antibacterial activity. It is noteworthy that PF-127 has been already used as drug carrier for a poorly water-soluble drug in solid dispersion technology (Irwan, Berania, & Liu, 2016). It should be also pointed out that the formulation approaches herein studied are mainly intended for oral administration route, where high patient compliance occurs (Drumonda, & Stegemanna, 2018; Trapani et al., 2004) since the solid dispersion strategy is essentially applied to improve oral bioavailability of poor water soluble drugs (Vasconcelos et al., 2007). For compounds **1**-**4**, both inclusion complexes with Me-β-CD and the corresponding solid dispersions in PF-127 were

prepared. The solid state characterization of these complexes and dispersions was performed by employing thermal analysis (Differential Scanning Calorimetry, DSC), FT-IR spectroscopy and X-Ray Powder Diffraction (XRPD).

The solubility data of compounds **1**-**4**, in the presence and without Me-β-CD or PF-127 were measured as well as the antibacterial activity against selected Gram positive and Gram negative bacterial strains was assessed. The results obtained are herein presented and discussed.

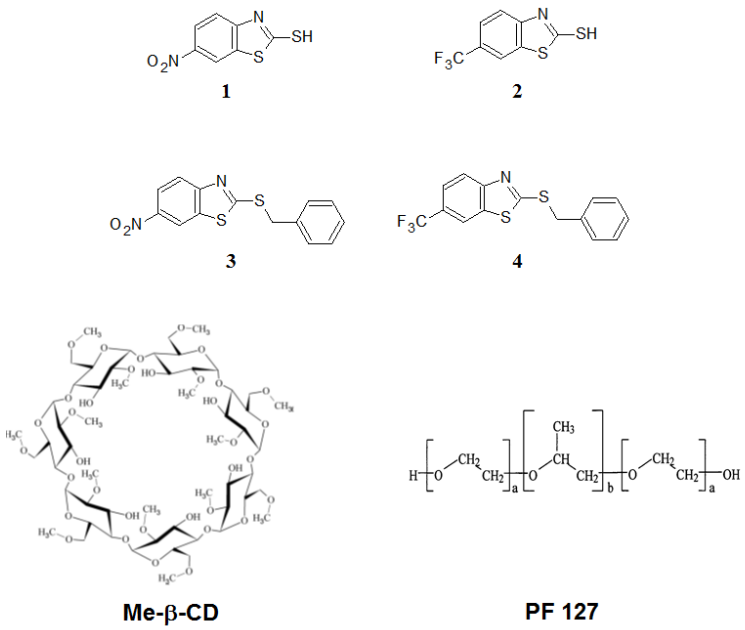

Figure 1. Chemical structures of 2-mercapto-benzothiazoles **1**-**4**, Me-β-CD and PF-127.

2. Materials and methods

The following chemicals were obtained from commercial sources and used as received. KBr and Dulbecco's modified PBS (D-PBS pH 7.4) were purchased from Sigma-Aldrich, Italy. Methyl-β-cyclodextrin (Me-β-CD, Mw 1320 Da, average substitution degree 1.8), was received as gift from Wacker Chemie (Italy) and kept in a desiccator until use. Lutrol 127 (poly(ethylene oxide)-poly(propylene oxide) - poly(ethylene oxide) (PEO-PPO-PEO) triblock copolymer, PF-127) was provided by BASF (Ludwigschafen, Germany). Ultrapure water (Carlo Erba, Italy) was used throughout the study. All other chemicals were reagent grade. Compounds **1-4** were prepared as previously described (Franchini et al., 2009).

2.1. Preparation of inclusion complexes and physical mixtures

1-**4**/Me-β-CD complexes were prepared in 20 mL of D-PBS by mixing the reactants (substrate : CD) in a 1:1 molar ratio at 25 °C and under magnetic stirring. All compounds were used at the concentration 0.4 mg/mL whereas Me-β-CD was employed at the concentration of 3 mg/mL for all tested compounds, excepted for compound **3** for which the CD concentration was set at 1.7 mg/mL.

After 24 h of equilibration at room temperature under light protection, the mixture was filtered (Millipore, 0.44 μm) and the solubility of each compound was determined spectrophotometrically at 300 nm wavelength (Perkin-Elmer Lambda Bio20) on the resulting filtrate. Solvents for calibration curves were constituted by ethanol for **1** and **2,** while a mixture of dioxane:water (7:3, v/v) was adopted for calibration curves of **3** and **4**. Linearity was checked over the range of concentrations tested and, in details, from 3 μg/mL to 100 μg/mL for **1**, from 3 μg/mL to 300 μg/mL for **2**, from 0.1 μg/mL to 15 μg/mL for **3** and from 0.05 μg/mL to 60 μg/mL for **4**.

Moreover, all the solutions obtained after filtration were freeze dried for 72 h using a Lio Pascal 5P (Milan, Italy), giving rise to powders used for following solid state and microbiological studies.

For lyophilized powders the Incorporation Degree (I.D.) was calculated as follows:

I.D. = weight of appropriate compound in the freeze dried mass/total weight of freeze dried product.

The weight of each compound in the freeze dried mass was determined after dissolution in D-PBS.

Moreover, only for compound **1** and **2**, physical mixtures with Me-β-CD were prepared by weighting CD and the appropriate compound at 1:1 molar ratio. Afterwards, the powders of Me-β-CD and **1** or Me-β-CD and **2** were gently mixed in a mortar at room temperature.

2.2. Preparation of solid dispersions

Solid dispersions were prepared by using the solvent evaporation method as manufacturing process (Vasconcelos et al., 2007; Vasconcelos et al., 2016) employing PF-127 as carrier and a ratio carrier:compound 10:1, w:w. Firstly, in a tube PF-127 was dissolved in water (2 mg/mL), whereas in a separate flask each compound was dissolved at the concentration of 0.2 mg/mL. Particularly, ethanol was adopted to solubilize all the compounds with the exception of **3** for which the mixture dioxane:ethanol (7:1, v/v) was required. Then, PF-127 was poured in the flask containing the compound and, to achieve the formation of the solid dispersion, the organic solvent was gently evaporated by a rotary evaporator (Rotavapor R-200, Buchi) at 70 °C. Afterwards, the solid dispersions were freeze-dried for 72 h (Lio Pascal 5P, Milan, Italy). The powders of solid dispersions so obtained were also used for following solid state and microbiological studies. Moreover, the Incorporation Degree (I.D.) of lyophilized powders of solid dispersions was also calculated as follows:

I.D. = weight of appropriate compound in the freeze dried mass/total weight of freeze dried product.

The solubility of each compound in the solid dispersion was evaluated by weighting 5 mg of the formulation containing PF-127 and dissolving it in 3 mL of D-PBS at 25 °C under magnetic stirring. After 24h of equilibration at room temperature under light protection, the mixture was filtered and the solubility of **1-4** was determined spectrophotometrically on the resulting filtrate.

2.3. Differential Scanning Calorimetry (DSC) and FT-IR studies

DSC runs were performed using a Mettler Toledo DSC 822e STARe 202 System equipped with a DSC MettlerSTARe Software. For DSC analysis, aliquots of about 5 mg of each product were placed in an aluminium pan and hermetically sealed. The scanning rate was of 5 °C/min under a nitrogen flow of 20 cm^3/min and the temperature range was from 25 to 275 °C. The calorimetric system was calibrated in transition temperature by using indium (99.9% purity) and following the procedure of the MettlerSTARe Software. Each experiment was carried out in triplicate to check the reproducibility.

The FT-IR spectroscopy analysis was performed for representative mixtures containing compounds **2** and **4** using a PerkinElmer 1600 FT-IR spectrometer (Perkin Elmer, Italy). To acquire FTIR spectra all samples were mixed with an appropriate amount of KBr. The range examined was 4,000–400 cm^{-1} with a resolution of 1 cm^{-1} (Trapani et al., 2016).

2.4. X-ray Powder Diffraction (XRPD)

X-ray powder diffraction data on selected samples were collected in air using a Panalytical Empyrean X-ray diffractometer with Bragg-Brentano geometry, large beta filter-Nickel detector, PIXcel3D and CuKα radiation (λ = 1.5418Å), operating at 40 kV/40 mA. Powder samples were deposited on a plexiglas sample holder. XRPD data were collected in the 2θ range 5-85°, with step size 0.0131° and step time 23.970s. Unit cell parameters were determined using the routine N-TREOR09 implemented in the EXPO2014 software (Altomare et al., 2013).

2.5. Molecular modeling of inclusion complexes

Molecular scaffold of **2**/β-CD and **4**/β-CD inclusion complexes were obtained according to our previous study (Trapani et al., 2016). Indeed, for the cyclodextrin moiety the X-ray puckering of the 2,7-dihydroxynaphthalene/β-CD complex (Anibarro, Gessler, Uson, Sheldrick, & Saenger, 2001) was used after the removal of the bounded aromatic molecule and all water atoms, whereas 2-mercapto benzothiazole structures were built with standard bond lengths and valence angles within Maestro (Schrödinger Release 2017-1: Maestro, Schrödinger, LLC, New York, NY, 2017) and afterwards submitted to AM1BCC charges calculation with the QUACPAC tool implemented in the OpenEye

software package (QUACPAC 1.7.0.2: OpenEye Scientific Software, Santa Fe, NM. http://www.eyesopen.com).

The initial poses into the β-CD core were assessed by dockings carried out with AutoDock ver. 4.2.5.1 (Morris et al., 1998). Ligand atoms and solvent molecules affinity maps were initially calculated using the water force field potential (Forli, & Olson, 2012) in a 0.375 Å spaced cubic box centered on β-CD and protruding by 80×80×80 Å around the oligosaccharide moiety, and thereafter ligands were docked by randomly translating and perturbing the benzothiazoles in a total of 10 LGA runs. The population size and the number of energy evaluations were set to 150 and 5000000 respectively. Further molecular dynamics carried out with Desmond (Bowers et al., 2006) started from the best pose according to Free Energy of Binding (FEB).

The inclusion complexes solvated with explicit water molecules were assembled using the Desmond system builder tool implemented in Maestro (Schrödinger Release 2017-1: Desmond Molecular Dynamics System, D. E. Shaw Research, New York, NY, 2018. Maestro-Desmond Interoperability Tools, Schrödinger, New York, NY, 2017). All simulations were performed on a NVDIA Quadro M4000 GPU at constant temperature (300 K) and pressure (1 bar) for a total of 480 ns, with a trajectory recording interval of 48 ps. Each collected structure frames were afterwards sampled for the data set calculations: the ligand excluded surface (LES) was calculated according to the following formula $SAS_{IC} - (SAS_{BTZ} + SAS_{CD})$ were SAS_{IC} is the solvent accessible surface as measured on the entire inclusion complex while SAS_{BTZ} and SAS_{CD} count for the extracted benzothiazole and oligosaccharide moieties, respectively.

2.6. Microbiological assays

The *in vitro* Minimum Inhibitory Concentrations (MICs, μg/mL) were assessed by the broth microdilution method, using 96-well plates, according to CLSI guidelines (CLSI, 2012).

Stock solutions of the tested compounds were prepared by setting the concentration at the maximum possible value. Then, the stock solutions were diluted 1:10 with Cation Adjusted Mueller Hinton Broth (Oxoid, Italy). Afterwards, twofold serial dilutions in the suitable test medium were carried out. The following bacteria strains, available as freeze-dried discs, belonging to the ATCC collection, were used: Gram-positive strains such as *S. aureus* 29213, *E. faecalis* 29212, *Bacillus subtilis* ATCC 6633, and Gram-negative one such as *E. coli* 25922. To preserve the purity of cultures and to allow the reproducibility, crio-vials of all microbial strains in the medium were set up and stored at –80 °C. Pre-cultures of each bacterial strain were prepared in Mueller Hinton Broth (MHB) and incubated at 37 °C for 3-5 h. The turbidity of bacterial cell suspension was calibrated to 0.5 McFarland Standard by

spectrophotometric method (OD_{625nm} 0.08-0.10), as indicated in CLSI protocol M7-A9 and, further, the standardized suspension was diluted (1:100) with MHB to reach 1-2 x 10^6 CFU/ml. All wells were seeded with 100 µL of inoculum and some wells contained only inoculated broth as control growth. The plates were incubated at 37 °C for 24 h, and the MIC values were recorded as the lowest concentration of compounds at which there was no optically detectable microorganism growth. The MICs were determined by using the assay repeated twice in triplicate. Throughout the study, norfloxacin was used as reference antibiotic.

2.7. Statistical analysis

Data are expressed as mean ± standard deviation (SD). Statistical significance from different experimental groups was determined by one-way ANOVA and differences were considered significant at 99 % level of confidence ($p < 0.05$) using GraphPad Prism v. 5.00 computer program (GraphPad Software, Inc. CA, USA) and Bonferroni's post-hoc test.

3. Results

*3.1. Solubility studies carried out on 2-mercapto benzothiazoles **1-4***

Table 1 shows the solubility data of compounds **1-4,** including intrinsic solubility *(i.e.*, the solubility of the compound alone in D-PBS), solubility after their complexation with Me-β–CD and incorporation degrees (I.D.) of complexes and solid dispersions as well as the calculated log P and the observed melting points of the 2-mercapto benzothiazoles. From the results reported in Table 1, it could be deduced that the solubility in D-PBS of S-unsubstituted compounds **1** and **2** was higher than the corresponding S-benzyl derivatives **3** and **4** in the **2** > **1** >> **3**,**4** rank order.

Table 1. Solubilities in D-PBS of pure compounds, in D-PBS of Compound/Me-β-CD complex as well as of Compound/solid dispersions, Incorporation Degree (I.D.) of complexes and solid dispersions, calculated lipophilicity and melting points of the studied mercapto-benzothiazoles **1**-**4**.

[a]Compd/Me-β-CD complex (1/1 mol. ratio). [b]Compd/PF-127 solid dispersion (1/10 weight ratio). [c]Calculated by MSKETCH software (ChemAxon).

Moreover, it was noted that the solubility of compounds **1** and **3** did not change in a statistically

Compoud	Solubility in D-PBS (μg/mL)	Solubility in D-PBS of Compd/Me-β-CD complex (μg/mL)[a]	I.D. (μg compound/mg freeze dried complex)	Solubility in D-PBS of Compound/solid dispersion (μg/mL)[b]	I.D. (μg compound/mg freeze dried solid dispersion)[b]	Calculated log P[c]
1	28.00 (± 2.22)	36.59 (±6.34)	6.68(±1.26)	71.24(±5.29)	40.73(±3.57)	2.83
2	230.65(± 40.64)	43.43 (±8.07)	66.90 (±7.20)	87.68(±9.99)	83.65(±5.60)	3.77
3	1.84 (± 0.12)	1.69 (± 0.02)	63.39 (±8.60)	36.30(±3.66)	23.28(±3.66)	4.99
4	1.46 (±0.01)	0.02 (± 0.01)	2.94 (±0.06)	0.072(±0.001)	1.99(±0.56)	5.92

significant manner after complexation with Me-β-CD ($p > 0.05$) compared to the corresponding in D-PBS. Instead, the presence of Me-β-CD negatively affects the solubility of compounds **2** and **4** since a notable decrease occurs when this CD was used. Interestingly, in the case of solid dispersions, the solubility of compounds **1** and **3** was enhanced by the PF-127 whereas a marked reduction was observed in the case of the compounds **2** and **4.** As for the incorporation degrees, the lowest values were observed for both **4**/Me-β-CD complex and **4**/PF-127 solid dispersion. In the other cases, I.D. values ranging from 6.68 to 83.65 μg compound/mg freeze dried complex with Me-β-CD or solid dispersion with PF-127 were detected (Table 1).

*3.2. Solid state characterization studies of 2-mercapto benzothiazoles **1**-**4**/Me-β-CD complexes and their solid dispersions with PF-127*

The solid state characterization of the 2-mercapto benzothiazoles **1**-**4**/Me-β-CD complexes and their solid dispersions with PF-127 was performed by DSC, FT-IR and XRPD to gain insights into the possible interactions between compounds and the excipients herein studied, *i.e.*, Me-β-CD and PF-127. The DSC profiles of the pure benzothiazoles **1**,**2** and **3**,**4** are reported in Figures 1S and 2S, respectively, together with those of the pure excipients, corresponding to Me-β-CD complexes and solid dispersions with PF-127. In the DSC thermograms of the benzothiazoles **2**-**4**, the endothermic melting peaks were detected, whereas in the case of compound **1** such peak was not observed since, as previously observed (Franchini et al., 2009), it melts with decomposition at a temperature > 240°C. Moreover, the more lipophilic compounds **3**,**4** melt at much more lower temperatures than those of the corresponding S-

unsubstitute-2-mercapto-benzothiazoles **1** and **2**. The DSC curve of the Me-β-CD showed a very broad peak centered at about 105 °C according to its amorphous nature and attributable to loss of water molecules (Wang et al., 2015) while in the thermogram of PF-127 a melting peak at 57 °C was detected. In the DSC curves of the **1**-**4**/Me-β-CD complexes these dehydration peaks of the CD were present, even though somewhat shifted or attenuated but, in any case, the endothermic melting peaks of compounds **1**-**4** were not detected (Figures 1S and 2S), suggesting that these complexes are at a significant degree of amorphous state. Instead, in the case of the **2**/Me-β-CD physical mixture (Figure 1S, panel B), the melting peak of compound **2** was shifted at lower temperature (about 85 °C lower compared to the pure compound **2**) indicating that this mixture should still possess a significant level of crystallinity. On the other hand, the DSC thermograms of **1**-**4**/PF-127 solid dispersions showed only the PF-127 a melting peak, although slightly shifted at lower temperature. Altogether, the DSC profiles of **1**-**4**/Me-β-CD complexes and of **1**-**4**/PF-127 solid dispersions were quite similar to those of Me-β-CD and PF-127, respectively.

The FT-IR of pure compound **2**, Me-β-CD, PF-127 as well as those of the corresponding **2**/Me-β-CD complex, and **2**/PF-127 solid dispersion are shown in Figure 2. The spectrum of the pure compound **2** showed a sharp absorption band at 1601 cm^{-1} attributable to the stretching of -C=N group. After complexation with Me-β-CD, the band at 1601 cm^{-1} disappeared and a new broad band at 1631 cm^{-1} resulted. Similarly, in the case of **2**/Me-β-CD physical mixture together with the disappearance of the band at 1601 cm^{-1} the presence of a new broad band at 1642 $cm^{-1}$was noted. On the other hand, the FT-IR spectrum of PF-127 in the pure form showed an absorption band at 1649 cm^{-1} which was slightly shifted at 1642 cm^{-1} following solid dispersion formation. From these results, it appears that compound **2** has stronger interactions with Me-β-CD leading to complex formation than PF-127 to give solid dispersion. Similar results were observed with compound **4** and the relative FT-IR spectra including those corresponding to **4**/Me-β-CD complex, and **4**/PF-127 solid dispersion are shown in Figure 3S.

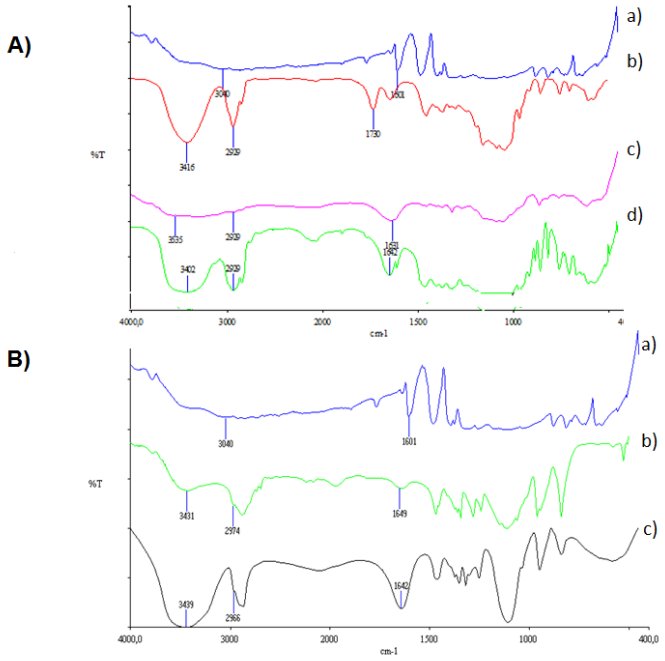

Figure 2. Panel A): FT-IR spectra of a) pure compound **2**, b) pure Me-β-CD, c) **2**/Me-β-CD complex, and d) **2**/Me-β-CD physical mixture. Panel B): FT-IR spectra of a) pure compound **2**, b) pure PF127, and c) solid dispersion **2**/PF127.

In Figure 3, the X-ray diffraction patterns for pure **4**, pure Me-β-CD, **2**/Me-β-CD and **4**/Me-β-CD are superimposed for comparative purposes. On the other hand, in Figure 3, X-ray diffractograms of **4**, PF-127 and solid dispersion **4**/PF-127 are compared. From Figure 3, it can be noticed that both the diffraction patterns of pure **2** and pure **4** show sharp diffraction peaks, pointing out a good crystallinity of the relevant compounds. Conversely, the spectrum of pure Me-β-CD is characterized by the occurrence of only two very broad bands centred at the Bragg angles 11.13 and 18.10°, respectively, indicating that the excipient is amorphous. Interestingly, the formation of the Me-β-CD complexes (Figure 3, bottom patterns) was found to alter the original crystal patterns of the **2** and **4** species. Indeed, sharp Bragg peaks occur at similar 2θ angles in both **2**/Me-β-CD and **4**/Me-β-CD/ patterns, excepted for the peak at 28.37° that only occurs in the **2**/Me-β-CD pattern. It is also noteworthy that patterns of both complexes also exhibit peaks at Bragg angles higher than 50° whereas the same region is flat in the XRD patterns of the pure molecules. This outcome could be related to some changes occurring in the crystal structure of the active principles as corroborated by the unit cell parameters derived from X-ray data and reported in Table 1S.

The analysis of the patterns in Figure 3 shows that the **4**/PF-127 XRD pattern resembles quite well that provided by PF-127 powder alone. In turn, the latter pattern is very close to that reported by Cavallari

(Cavallari, Fini, & Ceschel, 2013). Indeed, due to the adopted weight ratio, only two peaks of the **4** phase dispersed in the PF-127, located at 7.84 and 16.37° 2θ angles, are evident.

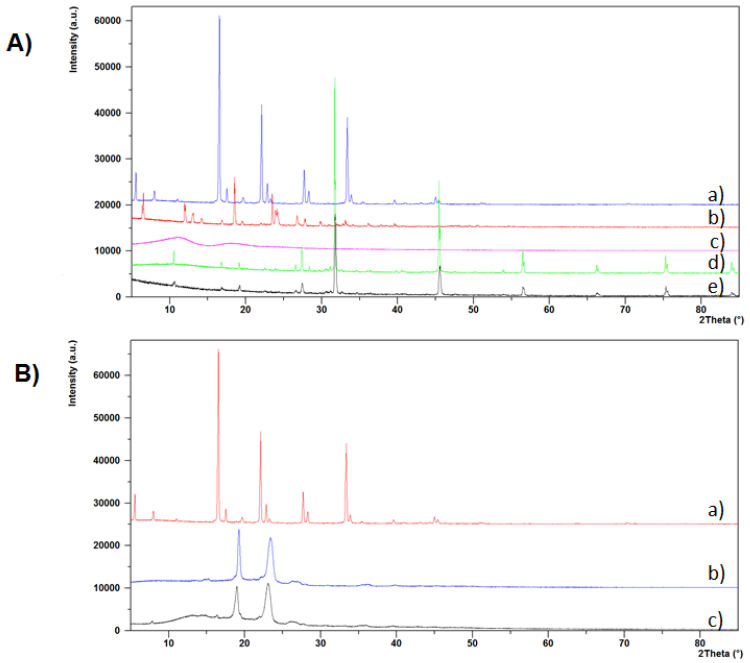

Figure 3. Panel A): Room temperature X-ray diffraction patterns of a) pure compound **4**, b) pure compound **2**, c) pure Me-β-CD, d) **4**/Me-β-CD complex, and e) **2**/Me-β-CD complex. Panel B): Room temperature X-ray diffraction patterns of a) pure compound **4**, b) pure PF127, and c) solid dispersion **4**/PF127.

3.3. Molecular modeling

To demonstrate whether the formation of inclusion complexes with CDs takes place, NMR studies are very useful (Trapani et al. 2016). However, in the cases herein examined, this approach could not be used even in the case of compound **2** characterized by the highest aqueous solubility in D-PBS (Table 1). The reason is that the poor aqueous solubility of the corresponding complex with Me-β-CD prevented the possibility to record a satisfactory NMR spectrum.

To confirm the inclusion complexation occurring between the guest molecules **2** and **4** in the host β-CD, a molecular modeling study was carried out. The observed binding mode suggests that both ligands might be easily incorporated by the β-CD, being the benzothiazole ring deeply included into the hydrophobic cavity of β-CD and surrounded by the oligosaccharide ring, nonetheless the substituents in position 2 and 6 of the 2-mercapto-benzothiazole nucleus are pointing towards the solvent. These dockings resulted in a similar estimated Free Energy of Binding (-4.25 and -4.91 kcal/mol for **2** and **4**,

respectively), suggesting that in both cases there are no steric or electrostatic hindrances, and starting from this facts deeper and fresh insights were achieved from the subsequent molecular dynamics runs. The root means square deviation (RMSD) along the analyzed trajectories and relative to the heavy atoms position suggests that **2** persists within the β-CD cavity much more stable than **4** as confirmed by a lower fluctuation calculated with respect to the initial frame of the mercapto derivative (RMSD mean values 0.743±0.263 and 1.648±0.462 in that order). As long as this evidence is proven, the most significant difference is indeed ascribed to the LES values as reported in Table 2.

Table 2. Free Energy of Binding (FEB) predicted from dockings and average values of the Root Mean Square Deviation (RMSD) and Ligand Excluded Surface (LES) calculated over the molecular dynamics run.

Compound	FEB	RMSD	LES
1	-4.25	0.743 ±0.263	347.726±24.764
2	-4.91	1.648 ±0.462	441.857 ±35.272

Very interestingly, the measured LES for **4** is in average, and with higher frequency, much more larger over the dynamic trajectory (Figure 4), and this might suggest that a particular moiety of the

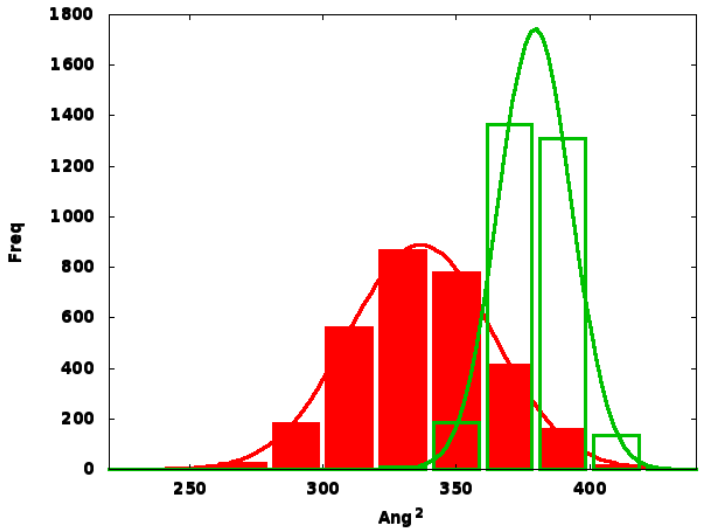

Figure 4. Distribution of the LES measured during the dynamic trajectory. Solid red and empty green histograms refer to **2** and **4** data, respectively.

ligand scaffold, most likely the benzyl moiety, is less enfold in the inclusion complex. Most likely this lower degree of incorporation could forbid a proper masking of its lipophilic mark, as instead evocated by Me-β-CD on **2** (see Figure 5).

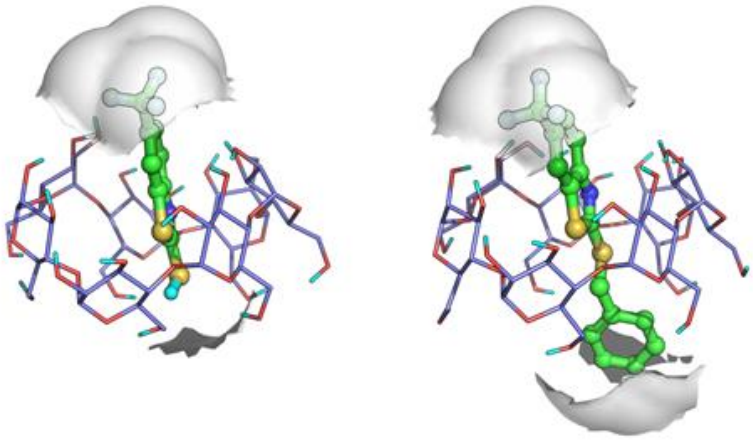

Figure 5. Ligand excluded surface of **2** (right) and **4** (left) inclusion complex endowing the lowest potential energy during the molecular dynamic trajectory.

3.4. Microbiological assays

All pure compounds herein presented, belonging to 2-mercapto-1,3-benzothiazoles series, had already showed high microbiological activity (**1** and **2**) against S. *aureus* and *E. coli*, while the corresponding S-benzyl derivatives (**3** and **4**) did not exhibit any activity (Franchini et al., 2009). Herein, the results arising from microbiological assays, and referred to complexes and solid dispersions, expressed as MIC (μg/mL), are reported in Table 3. The antimicrobial activities of compounds **1-4** after complexation with Me-β-CD and/or incorporation in PF-127 solid dispersions were measured *in vitro* against Gram positive (*Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212 and *Bacillus subtilis* ATCC 6633) and Gram negative (*Escherichia coli* ATCC 25922) bacterial strains. By evaluating the results for compound **1,** we observed that the complex with Me-β-CD is more active than the pure compound against all bacterial strains. In particular, it is 50 times more active against *E.coli* (0.50 *vs* 25 μg/mL), 78 times more active against *S. aureus* (0.16 *vs* 12.5 μg/mL), 150 times more active against *B. subtilis* (0.33 *vs* 50 μg/mL) and 74 times more active against *E. faecalis* (1.35 *vs* 100 μg/mL). However, by using **1**/Me-β-CD physical mixture or its solid dispersion with PF-127 brought all bacterial strains to the loss of the activity again.

Table 3. Antibacterial activities reported as MIC (μg/mL) of tested compounds in the presence of Me-β-CD or PF-127.

	E. coli ATCC 25922	*S. aureus* ATCC 29213	*E. faecalis* ATCC 29212	*B. subtilis* ATCC 6633
1	25	12.5	100	50
1/Me-β-CD[a]	0.50±0.32	0.16±0.035	1.35±0.35	0.33±0.06
1/Me-β-CD[b]	R	R	R	R
1/PF-127[c]	R	R	R	R
2	R	3.12	100	50
2/Me-β-CD[a]	3.19±1.40	2.26±1.62	3.71±0.49	1.56± 0.75
2/Me-β-CD[b]	R	12.8	R	R
2/PF-127[c]	R	R	R	R
3	R	R	R	R
3/Me-β-CD[a]	R	R	R	R
3/PF-127[c]	R	R	R	R
4	R	R	R	R
4/Me-β-CD[a]	R	R	R	R
4/PF-127[c]	R	R	R	R
NRF	0.06	0.5	2	0.125

[a] Inclusion complexes compound/Me-β-CD; [b] Physical mixtures compound/Me-β-CD; [c] Solid dispersion with PF-127; NRF, Norfloxacin; R, resistant.

The antibacterial screening revealed that the activity of compound **2** was significantly improved (about 27 times against *E. faecalis* and 32 times against *B. subtilis)* by complexation with Me-β-CD, while it doesn't change significantly in the case of *S. aureus* (2.26 vs 3.12 μg/mL). The most important result is the high activity exhibited by the complex **2**/Me-β-CD against the Gram negative *E. coli* (3.19 μg/mL), whereas such activity was absent in the case of **2** as it is. As for the **2**/Me-β-CD physical mixture, the only significant result is the activity of compound **2** on *S. aureus* that is, anyway, lower respect to **2** as it is (12.8 *vs* 3.12 μg/mL). However, once again the loss of the activity was observed by using **2**/PF-127 solid dispersion. Concerning the S-benzyl-2-mercapto benzothiazoles **3** and **4**, they showed no activity against all bacterial strains both as complexes with Me-β-CD and as solid dispersions.

4. Discussion

The main objective of the present work was to compare the antibacterial activity of compounds **1-4** after complexation with Me-β-CD or after incorporation in PF-127 based solid dispersion with that of

the pure drugs in order to gain information, among other, on the possible mechanism(s) involved in the CD-mediated enhancement of antimicrobial effectiveness showed by several antibacterial benzothiazole compounds, similarly to some classic antibiotics (Athanassiou et al., 2003; Trapani et al., 2016). The interest for this research area is motivated by the possibility that the adopted methodology may be a promising strategy to bypass the microbial resistance issue.

As for the solubility data of compounds **1**-**4** (Table 1), the rank order observed in D-PBS comprising a higher aqueous solubility of S-unsubstituted compounds **1** and **2** than the corresponding S-benzyl derivatives **3** and **4** can be easily accounted for by the higher lipophilicity of these latter compounds, as demonstrated by the calculated log P values (Table 1). The lower aqueous solubility of the nitro-2-mercapto-benzothiazole derivative **1** than the corresponding trifluoromethyl compound **2** may be rationalized taking into account the higher crystal lattice stability of the former compound as proved by its higher melting point and according to the General Solubility Equation (Walker, 2017). To explain the solubility trend of compounds **1**-**4** observed in the presence of Me-β-CD further appropriate experiments should be necessary, but it is out the scope of the present study. However, the results of the modeling studies constitute the major evidence that inclusion complexation of compounds **2** and **4** with Me-β-CD may occur. It should be evidenced that molecular modeling approaches are often used to investigate drug/CDs inclusion complexation (Yang et al., 2014). In our case, such studies proved that the benzothiazole ring of compounds **2** and **4** is deeply included into the inner cavity of β-CD and surrounded by the oligosaccharide ring, while the substituents in position 2 and 6 of the heterocyclic nucleus are pointing towards the solvent. Moreover, the complex with the S-unsubstituted benzothiazole compound **2** should be more stable of the corresponding S-benzylated **4** based on the most significant difference in ligand excluded surface (LES) observed (Table 2). Hence, it is possible that in the case of the S-unsubstituted nitro-derivative **1**, where essentially the solubility after complexation with Me-β-CD did not change compared to that observed in D-PBS, an inclusion complex with very low apparent stability constant ($K_{1:1}$) may be formed. In the case of the S-unsubstituted trifluoromethyl compound **2**, where the presence of Me-β-CD negatively affects the solubility characteristics, an inclusion complex with limited aqueous solubility characterized by B-type phase solubility profiles may take place (Loftsson, Hreinsdottir, & Masson, 2005). As for the S-benzyl substituted compound **3** a very unstable inclusion complex may occur, similarly to that observed for **4**. In addition to inclusion complexation between 2-mercapto benzothiazoles **1**-**4** and Me-β-CD, it should be also taken into account that interaction between these compounds and the hydrophilic outside surface of CDs leading to non-inclusion complexes might occur (de Jesus et al., 2012; Trapani et al., 2016).

Altogether, the solid state characterization studies on 2-mercapto benzothiazoles **1**-**4**/Me-β-CD complexes and their solid dispersions with PF-127 revealed that significant interactions take place between compounds and the mentioned excipients. In the case of **1**-**4**/PF-127 solid dispersions, the main interactions should be of hydrophobic type between these lipophilic molecules and the poly(propylene oxide) moieties of the carrier Pluronic® F-127 but also electrostatic interactions involving the oxygen atom of ethylene- and/or propylene oxide portions and compounds **1**-**4** could take place.

Concerning the microbiological results, it is evident that a substantial improvement of the antimicrobial activity compared to that of the pure compounds **1** or **2** was noted only when the complexes between the S-unsubstituted benzothiazoles compounds **1** or **2** and Me-β-CD were used (Table 3). Conversely, when physical mixtures between **1** or **2** and Me-β-CD or **1** or **2**/PF-127 solid dispersions were tested, a complete loss of antibacterial activity was found. Similarly, using the S-benzyl substituted compounds **3** and **4** the lack of antibacterial activity observed for the pure compounds **3** and **4** occurred also for the corresponding complexes with Me-β-CD or solid dispersions with PF-127. That is, with S-benzyl substituted compounds **3** and **4**, where very unstable inclusion complexes are used or with the corresponding solid dispersions, complete lack of antibacterial activity was observed like to the pure compounds **3** and **4**. Hence, in the series examined, an enhancement in antibacterial activity by complexation with Me-β-CD occurred only with the less lipophilic S-unsubstituted benzothiazoles compounds **1** or **2**. Moreover, our results clearly show that the improvement in antimicrobial effectiveness after complexation with Me-β-CD take place both towards Gram positive and Gram negative bacterial strains. In the case of compound **1**, the improvements observed with Gram positive strains were even greater than that observed with the Gram negative *E. coli* (*i.e.*, 78-, 150- and 74-times for *S. aureus* 29213, *E. faecalis* 29212 and *Bacillus subtilis* ATCC 6633, respectively, compared to 50-times for *E. coli* 25922). However, in the case of compound **2**, the improvements in antimicrobial effectiveness after complexation with Me-β-CD were, with the Gram positive strains, lower than those observed with compound **1** (*i.e.*, 27- and 32-times for *E. faecalis* 29212 and *Bacillus subtilis* ATCC 6633, respectively). Instead, with compound **2** a remarkable change was observed with the Gram negative strain *E. coli* 25922 which resulted fully resistant to the pure compound but sensitive enough to **2**/Me-β-CD complex. These results are partially in agreement with previous studies on a series of β-lactam antibiotics (Athanassiou et al., 2003), which showed that the increase in antibacterial activity after complexation with Me-β-CD was more substantial against Gram negative strains. Such literature suggestion is confirmed by our findings in the case of the S-unsubstituted benzothiazole compound **2** but not with **1**.

An important objective of this work was to gain information on the possible mechanism(s) of antibacterial activity enhancement after complexation with CDs and, in this regard, several proposals have been made (Athanassiou et al., 2003; Trapani et al., 2016) which essentially focus on two aspects. The first one is the improvement of dissolution properties arising from the complexation with CDs. Thus, the increase in aqueous solubility consequent to complexation may provide a higher drug concentration at the outer bacterial membrane bringing about an increased drug diffusion rate across this membrane. However, it seems that the results of the present study are not in agreement with this hypothesis since we noted that the complexes of the S-unsubstituted benzothiazoles compounds **1** or **2** with Me-β-CD did not provide an enhancement of aqueous solubility. On the other hand, considering that the solid dispersion technology leads to, in particular, an improvement of dissolution properties for poorly soluble drugs (Vasconcelos et al., 2007), also the complete lack of antibacterial activity observed using the systems **1**-**4**/PF-127 does not support that hypothesis. Furthermore, for compounds **1** and **3,** although the aqueous solubility was increased by their solid dispersion in PF-127, no antibacterial effect was noticed. The second aspect focused by the mentioned mechanisms concerns another scenario and precisely, the interactions of the bacterial membrane with CDs and the consequences of such interactions in terms of fluidity and permeability of membrane, transport across it and possible involvement of efflux proteins (Athanassiou et al., 2003; Fenyvesi et al., 2008; Trapani et al., 2016). It is well-known, indeed, that CDs can both improve and make difficult drug permeation through biological membranes and furthermore that the effects of membrane damage caused by dimethyl-β-CD can be P-glycoprotein (P-gp) related due to perturbation of the lipid environment of the pump (Trapani et al., 2014). Moreover, it cannot be ruled out that enhancement or decrease in antibacterial activity in the presence of CDs may be also bacterial strain-dependent. Our data seem to give support for the involvement of mechanism(s) belonging to this second scenario for which, due to the presence of different pathways, difficulties arise to draw reliable conclusions concerning each of them. From the results of the present study, it cannot be ruled out that the observed complete lack of antibacterial activity observed using the systems **1**-**4**/PF-127 may be related to the fact that Pluronic surfactants, as many other polymeric excipients, are characterized by P-gp inhibition (Kabanov, Batrakova, & Alakhov, 2002; Mandracchia et al., 2017; Trapani et al., 2014). It has been proposed that changing the level of P-gp molecules, an influence on intracellular trafficking exerted by some membrane proteins may occur (Fenyvesi et al., 2008).

4. Conclusions

The results of the present work confirm the working hypothesis that the *in vitro* antimicrobial activity of the S-unsubstituted-2-mercapto benzothiazoles **1** and **2** may be positively affected by complexation with Me-β-CD against both Gram positive and Gram negative bacterial strains. These outcomes do not confirm that observed in a series of β-lactam antibiotics, *i.e.*, that complexation with Me-β-CD provides a peculiar increase in antimicrobial activity against Gram-negative strains (Athanassiou et al., 2003). Conversely, with S-benzyl-substituted-2-mercapto benzothiazoles **3** and **4** no activity against all bacterial strains was observed after complexation with Me-β-CD. These last findings can be explained in terms of stability of the formed complex as proved, in particular, on the basis of a modeling study. Similarly, remarkable decrease or even complete loss of antibacterial activity was noted using **1**/ or **2**/Me-β-CD physical mixtures or **1**-**4**/PF-127 solid dispersions. As for the hypothesized pathways for which CDs can improve the activity of antibacterial agents, the results obtained lend support for mechanisms involving implications on fluidity, permeability of membrane, transport across it and possible involvement of efflux proteins more than the improvement of dissolution properties due to CD complexation. In this context, however, there are still unanswered questions to be solved just due to the presence of different pathways. In perspective, it should be evidenced that, for an appropriate use of the CD complexation methodology as a formulation strategy to bypass the microbial resistance problem, it is essential that our understanding on the mechanism(s) underlying the interactions CD-bacterial cell is notably improved. The achievement of this objective should represent an important step forward for the science of CDs since these oligosaccharides could find a useful application as excipient in medicine in the area of infectious diseases.

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