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Original article

Monocyclic β-lactams as antibacterial agents: Facing antioxidant activity of *N*-methylthio-azetidinones

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Dedicated to Prof. Gianfranco Cainelli on the occasion of his 80th birthday.

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ABSTRACT

A series of *N*-methylthio-β-lactams with antibacterial activity were thoroughly evaluated as antioxidants. We found that only the presence of a polyphenolic moiety anchored to the β-lactam ring ensured an adequate antioxidant potency. New compounds, efficiently combining in one structure antioxidant and antibacterial activity, may provide a promising basis for the development of new leads useful in adverse clinical conditions such as in cystic fibrosis patients, in whom colonization by MRSA and epithelial damage by chronic pulmonary oxidative stress take place.

1. Introduction

β-Lactam antibiotics are still the main class of agents used to treat bacterial infections [1]. However, notwithstanding the pivotal role of penicillins and cephalosporins as first choice antibiotics, the treatment of bacterial infections has been hindered by the increasing emergence of multidrug-resistant microorganisms, which today represents one of the greatest challenges in the clinical management of infectious diseases [2]. Whereas antibiotic resistance was once largely confined to hospitals and long-term care facilities, it has now emerged in community settings and represents one of the most pressing global public health concerns [3].

Staphylococcus aureus is a major human pathogen causing significant morbidity and mortality in both community- and hospital-acquired infections. Many staphylococcal infections are associated with multiple recurrences and with the development of resistance to β-lactam antibiotics. This is particularly important in patients with chronic diseases such as cystic fibrosis (CF) where persistent colonization by pathogenic bacteria occurs and the repeated use of antibacterial agents selects for specific resistant strains. A rise in *S. aureus* infections has been reported in CF patients, with an increase in the prevalence of highly virulent, methicillin-resistant *S. aureus* strains (MRSA) [4].

CF is characterized by a chronic inflammatory process even in the absence of bacterial pathogens, and by the recruitment of activated neutrophils. Under pathophysiological conditions, activated neutrophils and epithelial cells release highly reactive molecules in the extracellular space, like reactive oxygen species (ROS) and reactive nitrogen species (RNS) in order to attack and eliminate invasive pathogens [5]. However, in CF, several lines of evidence show that the defence systems are ineffective. In CF an increased production of ROS may be associated with cell dysfunction and with disease progression [6]. Thus, the presence of oxidative stress in CF due to an increased production of ROS and to an impaired antioxidant status, particularly during the chronic pulmonary infections, points to new therapeutic possibilities in targeting anti-oxidant pathways [7].

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As part of an interdisciplinary research project, involving the synthesis of new monocyclic β-lactams specifically designed to target resistant Gram-positive bacterial strains, a series of new azetidinones with polyphenolic side chains were evaluated for both their antibacterial [8] and antioxidant activities [9]. As a general trend, to activate the antioxidant potency of the tested compounds to a significant extent the presence of phenolic OH resulted necessary, with the exception of 4-alkylidene-thiophenol derivative (**1**) which had no phenolic residues but retained a certain antioxidant activity.

More recently a series of *N*-methylthio-azetidinones were evaluated *in vitro* against Gram-positive and Gram-negative clinical isolates and in particular on MRSA and MSSA strains isolated from patients with cystic fibrosis [10]. The combination of an *N*-SMe group and a benzyl ester on the 4-alkylidene-side chain, or electron-withdrawing groups, such as OAc, on the C-4 position of the β-lactam ring, appeared to strengthen the potency against Gram-positive bacteria. This study reports the evaluation of physicochemical parameters, such as the antioxidant activity and redox potentials, of some *N*-methylthio-azetidinones with antibacterial potency. The aim of this ongoing project was in fact to develop dual targeting molecules with elements incorporated in their structure that would allow dual antibacterial and antioxidant activities in order to contrast unfavourable clinical conditions, such as those resulting from bacterial colonization of the lung associated with extensive epithelial damage brought about by chronic pulmonary oxidative stress.

2. Results and discussion

Beside bicyclic β-lactam antibiotics such as penicillins, cephalosporins, and carbapenems, monocyclic compounds (azetidinones) emerged for their multiple and interesting biological activities [11]. The discovery of monobactams (Aztreonam) demonstrated for the first time that a conformationally constrained bicyclic structure was not necessary for antibacterial activity [12].

Turos and co-workers reported for the first time the synthesis and the biological evaluation as antibacterial agents of some monocyclic β-lactams with an alkylthio group on the β-lactam nitrogen atom [13]. These compounds have been found to be highly selective towards *Staphylococcus* spp. and *Bacillus* spp. Moreover, unlike penicillins, which inhibit cell wall crosslinking enzymes, *N*-thiolated lactams are characterized by a bacteriostatic activity and act through a different mechanistic model [14]. Recently some monocyclic *N*-methylthio-azetidinones were even reported as selective inhibitors of Histone deacetylases (HDACs) [15]. In that case the presence of an *N*-methylthio group had a key role in providing to the new β-lactams a stringent isoform selectivity.

2.1. Synthesis of β-lactams

All 15 monocyclic β-lactams investigated here are depicted in Fig. 1. Compounds **1**, **2**, **3**, **4**, **5**, **7**, **8**, **9**, and **10** were prepared according to previously reported procedures [10,16]. *N*-Methylsulfoxide-4-acetoxy-azetidin-2-one **6** (Scheme 1) was prepared starting from commercial 4-acetoxy-azetidin-2-one which was treated with LiHMDSA followed by addition of mesylchloride. Analogously, *N*-methylthio-azetidin-2-one **11** was obtained treating commercial azetidin-2-one with LiHMDSA and then *S*-methyl methanethiosulfonate (Scheme 1).

Compounds **12**–**15** were prepared starting from the commercial (3*R*,4*R*)-4-acetoxy-3-[(1*R*)-1-(*tert*-butyldimethylsilyloxy)-ethyl]-azetidin-2-one. The starting compound was *N*-thio methylated to afford compound **3** using Et_3N as a base because LiHMDSA or LDA led to the formation of significant amounts of by-products. Compound **3** was then deprotected on the 3-[*O*-(*tert*-butyldimethylsilyl)]-ethyl side chain using $BF_3 \cdot Et_2O$ in CH_3CN to give the corresponding alcohol **4** (Scheme 2) [16]. Treatment of **4** with the appropriate *O*-protected benzoic acid **19**, **21** or **22**, DCC or EDC as coupling reagents and in the presence of a catalytic amount of DMAP in dichloromethane, gave compounds **12**, **16** and **17**.

As regards the benzoic acids, methyl 4-(ethoxymethoxy)-3,5-dimethoxybenzoic acid **19** and (*E*)-3-(3,4-bis(ethoxymethoxy) phenyl)acrylic acid **21** were prepared starting from syringic methyl ester or caffeic methyl ester, respectively through protection of the phenolic oxygen atoms with chloromethylethylether followed by alkaline hydrolysis of methyl esters **18** and **20** (Scheme 3). Finally, compounds **12** and **17** were treated with trifluoroacetic acid (TFA)

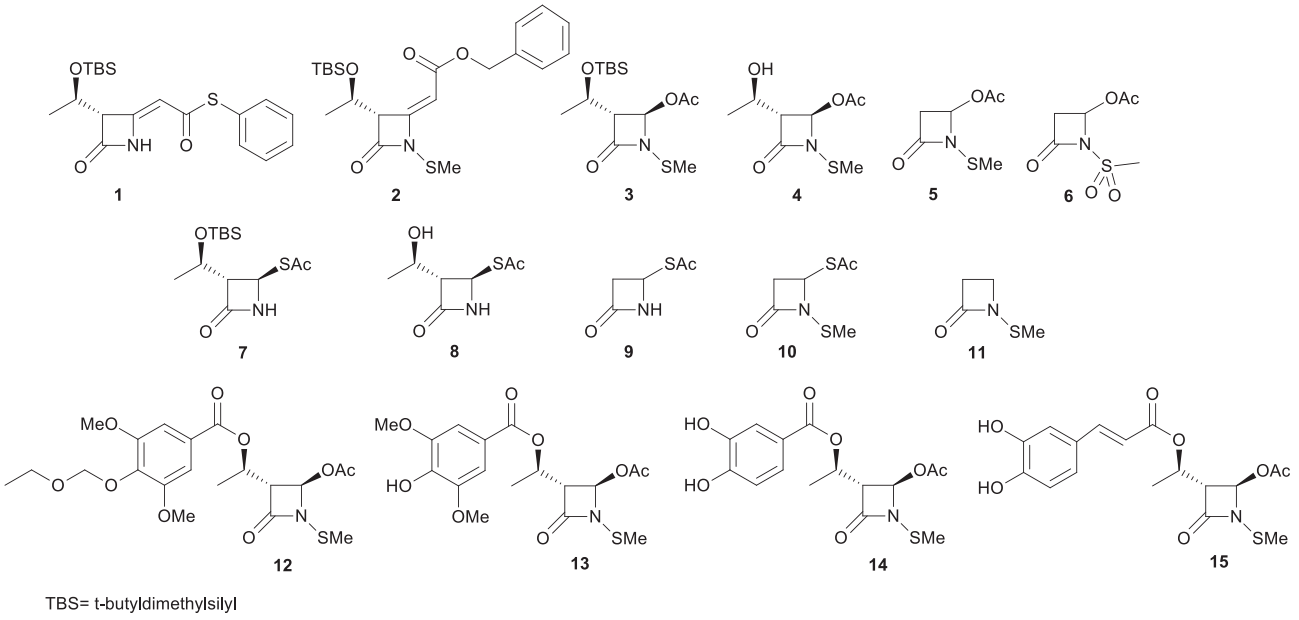
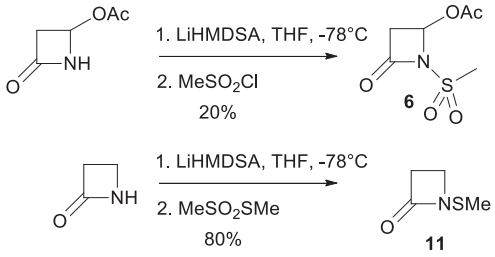


Fig. 1. β-Lactam compounds tested for the antioxidant activity.



Scheme 1. Synthesis of β-lactams **6**, and **11**.

to eliminate the phenolic protection and to give compounds **13** and **15**. The ethoxymethylether as protective group was not effective in the synthesis of 3,4-dihydroxybenzoic ester **14** which was successfully obtained using *t*-butyldimethylsilyl to protect the phenolic positions in compound **16**.

2.2. Antioxidant activity

Antioxidant activity determination based on different approaches was carried out, in light of the importance of a multidimensional evaluation of the antioxidant activity [17]. The β-lactams were then subjected to four antioxidant assays: the Briggs–Rauscher (BR) oscillating reaction [18,19], the Trolox Equivalent Antioxidant Capacity (TEAC) assay [20a], the DPPH (2,2-diphenyl-1-picrylhydrazyl DPPH• stable radical) test [21,22] and the Ferric Reducing Antioxidant Power (FRAP) method [23] (see Experimental section and Supplementary data for details).

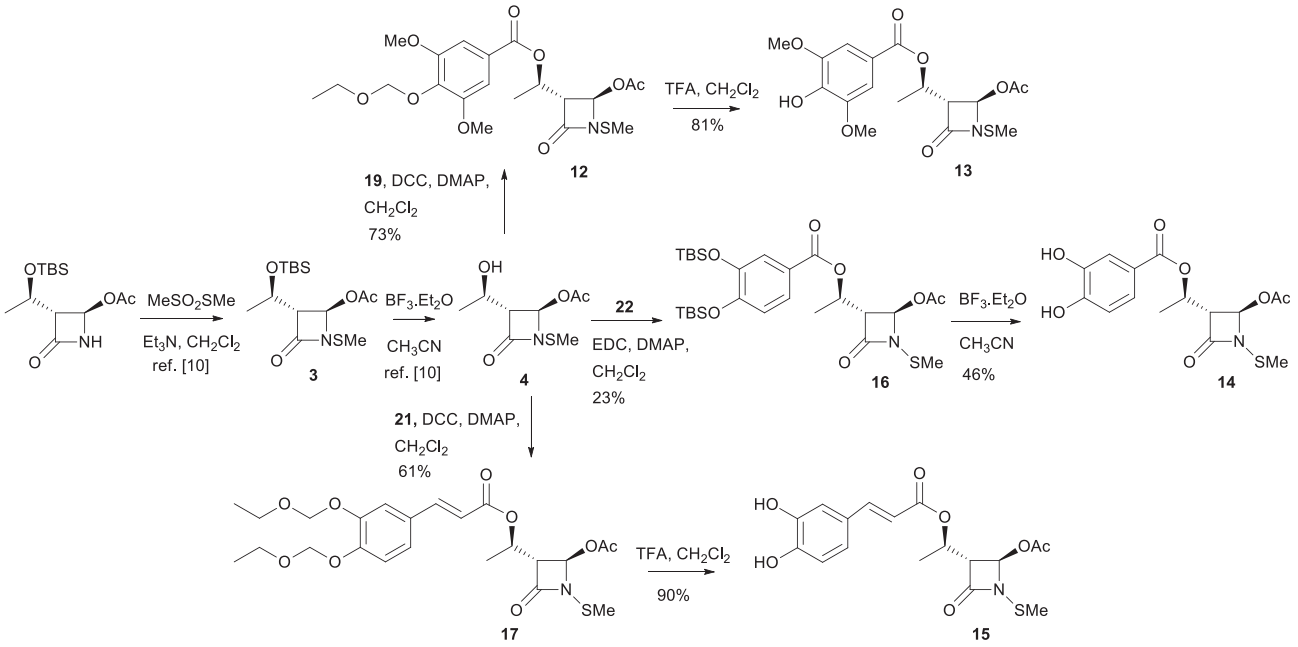
The majority of the synthesized β-lactams did not show appreciable antioxidant activity with BR, TEAC, DPPH, or FRAP methods; moreover compounds **2**, **3**, and **10** were also tested with the Folin–Ciocalteu (FC) reagent [24] to check if the *N*-methylthio-β-lactam skeleton has some reductive properties, but the result was negative. N-unsubstituted compounds **8** and **9** gave also negative results to antioxidant tests. Only the *N*-methylthio-β-lactams **12**–**15** with phenolic moieties were active and showed values in Table 1.

TEAC values for compounds **14** and **15** are in line with those of some polyphenolic-β-lactams we previously reported, ranging from 0.40 to 2.0 mM equiv Trolox [9]. The DPPH and FRAP values are in satisfactory agreement with the TEAC ones taking into account the different chemistry of the methods. For comparison, the TEAC values for ascorbic acid (vitamin C) and α-tocopherol (vitamin E) are 1.05 and 0.97 respectively [20a], the DPPH values are 1.1 and 0.5 respectively [20b], and the FRAP values for the two vitamins is 2.0 [23]. As expected compound **12** doesn't shows antioxidant activity with these three methods because it does not contain phenolic OH groups in the molecule (negative control). Compound **13** is a very low active antioxidant than the other two phenolic substituted molecules, a possible interpretation is given in next paragraph.

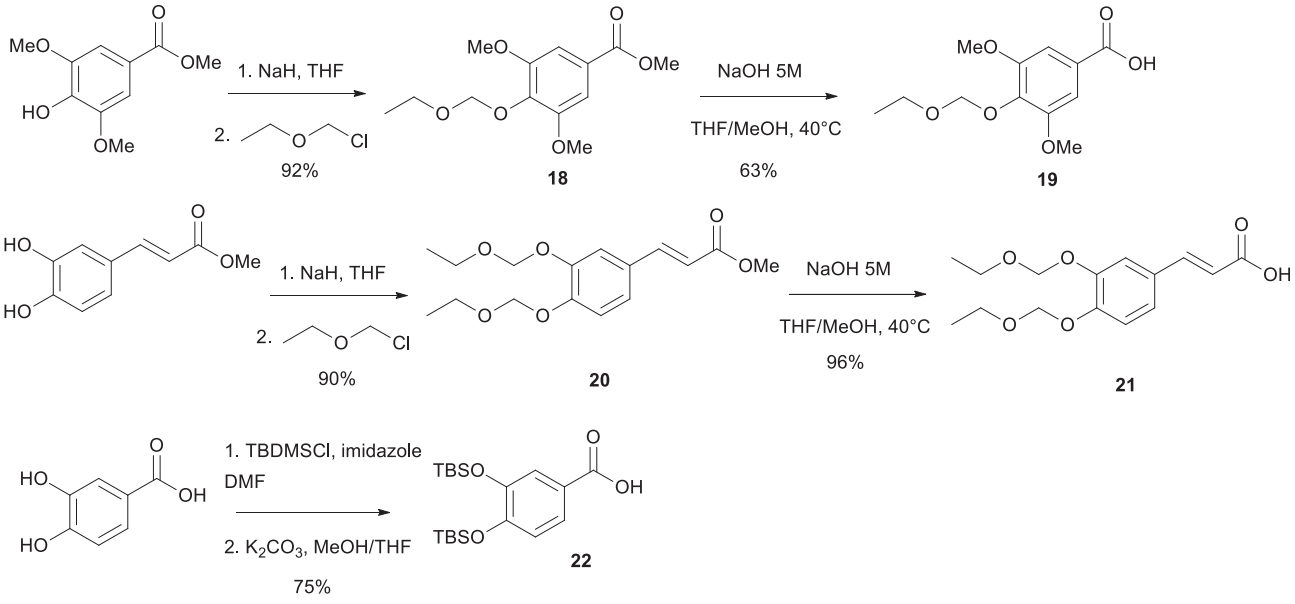
The BR values for all compounds are surprisingly higher than those of some polyphenolic-β-lactams previously reported, ranging from 0.021 to 0.28 μM equiv Re [9]. We suspect that these unusual results are due to parallel reaction of oxidation of sulphur in the *N*-SMe group by acidic iodate that compete with the HOO• scavenging reaction. On the contrary this parallel reaction doesn't occur with the usual *N*-unsubstituted β-lactams.

2.3. Structure–activity relationships

As a general trend, to activate the antioxidant potency of the tested compounds, the presence of two or three phenolic OH groups are necessary [25]. A theoretical method to calculate the Bond Dissociation Enthalpies (BDE) for molecules belonging to the class of polyphenols and to correlate them with their free radical scavenging activities has been reported by Wright et al. [26]. These authors also proposed empirical additivity rules that take into account the electronic, H-bond and conjugation effects of substituents in the phenol parent molecule to evaluate the BDE of a given phenolic OH group. In this way a value of BDE or $\Delta BDE = (BDE_{comp} - BDE_{\Phi\text{-}OH})$ can easily be calculated from the data reported in Ref. [26] Table 4. It was found that the number of phenolic OH is not so important and that it is the strategic placing of these groups with respect to other substituents that determine the strength of the OH



Scheme 2. Synthesis of β-lactams **13**, **14**, **15**, **16**, and **17**.



Scheme 3. Synthesis of acids **19**, **22** and **21**.

group and then the antioxidant activity. From the data reported in Ref. [26] Table 4, we calculated the ΔBDE values for two model compounds **A** and **B** in comparison with the free radical scavenging activity for compounds **13** and **14** (Fig. 2).

As can be seen from Fig. 2, results for the model match well with the experimental data, thus justifying the very low free radical scavenger potency of compound **13** with respect to that of **14**.

2.4. Voltammetry

It is well known that thioethers can be oxidized to sulfoxides and further to sulfones by H_2O_2 or by many other oxidizing agents [27]. The negative results of the antioxidant assays gave evidence that in the experimental conditions the *N*-methylthio group was not prone to be oxidized. To interpret these results we decided to perform some cyclic voltammetry experiments on the two molecules **9** and **11** chosen as models. In the first molecule the bivalent sulphur atom is bonded to C4 atom of the ring while in the second molecule the bivalent sulphur atom is bonded to the nitrogen atom of the beta lactam ring. Aim of the investigation was to measure the ability of such model compounds to undergo oxidation to evaluate their antioxidant activity through the measure of the corresponding standard potentials. We also submitted to cyclic voltammetry the thioester **1** which has no antibiotic activity and that in a previous work [9] showed some antioxidant activity both in the BR or TEAC tests (acidic and neutral pH method, respectively), and explained with a possible generation of an intermediate radical cation $R_2S^{\bullet+}$. The voltammograms are reported in Fig. 3.

All species displayed in acetonitrile a very similar CV behaviour showing, in the positive potential region, a main anodic peak at about 0.9 V attributed to the reversible oxidation of the pristine species. Weak-to-strong adsorption effects were observed in the CV pattern, leading in most cases to fouling of the electrode surface and thus to a highly irreversible behaviour. In the case of gold electrodes (Fig. 3), however, a more reproducible behaviour was observed, although adsorption was still present and was responsible for the asymmetric pattern of the CV curve and the apparent larger anodic currents (forward scan) with respect to the cathodic ones (reverse scan). This was particularly evident in the case of compound **1**. In such a case, in fact, the growth of an intense anodic

Table 1
Results of antioxidant and antibacterial tests.

Compd.	BR (μM equiv Resorcinol)	TEAC (mmol equiv TROLOX)	DPPH (mmol equiv TROLOX)	FRAP (mmol equiv Fe)	Antimicrobial activity on MRSA from CF[a]		
					MIC range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)
4	na[b]	na	na	na	32->128[c]	64[c]	>128[c]
5	na	na	na	na	32–64[c]	32[c]	64[c]
8	na	na	na	na	16–128[c]	64[c]	128[c]
9	na	na	na	na	16–64[c]	32[c]	64[c]
10	na	na	na	na	16–32[c]	16[c]	32[c]
12	0.70[d]	na	Negligible	na	nd[b]	nd	nd
13	1.8 ± 0.3	0.037 ± 0.001	0.0091 ± 0.0002	0.19 ± 0.02	16–128	64	128
14	0.38 ± 0.02	1.23 ± 0.02	1.96 ± 0.13	1.91 ± 0.07	32 to >128	128	>128
15	1.95 ± 0.06	0.98 ± 0.03	1.23 ± 0.03	1.98 ± 0.04	16–128	64	64
FOT[e]					1 to >128	128	>128
CTX[e]					1 to >128[c]	8[c]	>128[c]

[a] *S. aureus* American Type Culture Collection ATCC 29213, and ATCC 43300 were used as control strains.
[b] na = no activity; nd = not determined.
[c] See Ref. [10].
[d] Result of only one measure at 14.7 μM in mixture.
[e] FOT = cefotaxime; CTX = ceftriaxone were used as reference antibiotics.

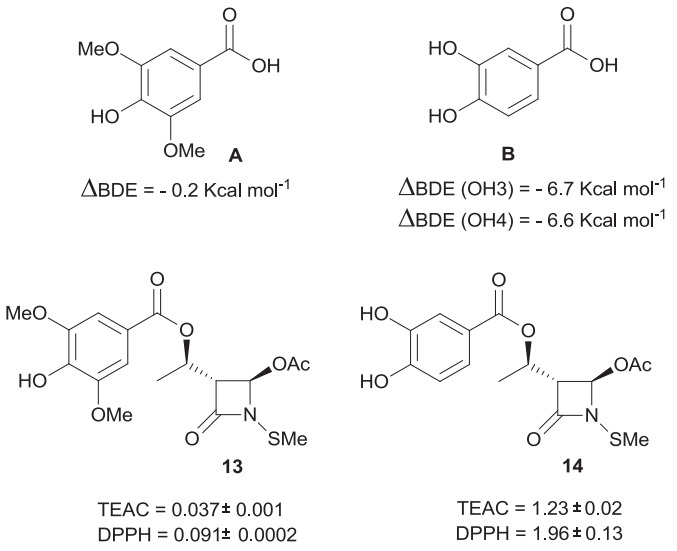


Fig. 2. ΔBDE for two model compounds **A** and **B** in comparison with the free radical scavenging activity for compounds **13** and **14**.

shoulder located at less positive potentials than the main peak suggests relatively strong adsorption of the product of the anodic oxidation onto the gold surface [28]. Functional groups within the molecule in question, which may give rise to this phenomenon are many. In addition to sulphur, phenyl groups may effectively promote the adsorption on gold, as well as the carboxyl groups, thus making identification of the exact nature of the process rather difficult. In spite of the adsorption effects on the CV curve morphology, an evaluation of the standard potential for all species was possible: 0.82, 0.86 and 0.88 V for compounds **9**, **11** and **1**, respectively.

On the basis of such results, and assuming that potentials determined under the aprotic and relatively apolar conditions of CV (Fig. 3) may be directly used for comparisons in the aqueous media, all the investigated compounds should not show appreciable antioxidant activity towards TEAC, DPPH or FRAP antioxidant methods. The redox potential of the couples $ABTS^{\bullet+}$/ABTS (TEAC); $DPPH^{\bullet}$/DPPH, $(TPTZ)_2Fe^{3+}/(TPTZ)_2/Fe^{2+}$ (FRAP) are in fact 0.68 V [17], 0.28 V [29], and 0.77 V [30], respectively, then $ABTS^{\bullet+}$, $DPPH^{\bullet}$, $(TPTZ)_2Fe^{3+}$ can thermodynamically be reduced by compounds with lower reduction potential. This is likely applicable to all *N*-methylthio-β-lactams synthesized here, except for compounds **13**, **14** and **15**. These compounds in fact contain phenolic moiety in the side chain, and many phenolic compounds have lower oxidation potential thus reacting with $ABTS^{\bullet+}$, $DPPH^{\bullet}$ and $(TPTZ)_2Fe^{3+}$ [30].

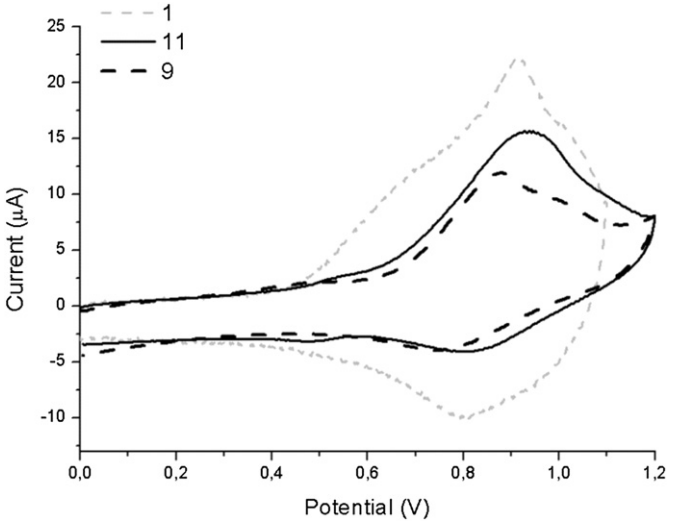


Fig. 3. Current potential diagrams for compounds **1**, **9**, and **11**.

Finally, the low antioxidant activity of compound **1** observed in a previous work [9] cannot be attributed, on the basis of the standard potential value reported above, to the occurrence of outer-sphere electron transfer between the sulphur atom (donor) and the antioxidant test reagents ($HOO^{\bullet}$, $ABTS^{\bullet+}$, $(TPTZ)_2Fe^{3+}$ acceptors) since the energy driving force would be largely unfavourable. On the other hand, in the actual chemical system the formation of a complex between the β-lactam and the test reagents can take place. Thus, an intimate interaction of some sort between the partners might render the electron transfer a thermodynamically more accessible process [31] in a fashion akin to the inner-sphere mechanism previously evidenced in the reactions of Grignard reagents with benzophenone [32].

In line with such a hypothesis, compound **1** displays the strongest adsorption behaviour on the gold surface among the investigated species and, because of the free energy of adsorption, its oxidation (inner-sphere process) becomes significantly easier than that involving the free species in solution (outer-sphere process).

2.5. Antibacterial activity

In order to evaluate the potential antibacterial activity of the new β-lactam derivatives with anti-oxidant properties, the minimum inhibitory concentrations (MICs) of compounds **13**–**15** were determined *in vitro* against 45 clinical strains of MRSA isolated from CF patients. Preliminary results (see Table 1) on the antibacterial activity demonstrated compounds **13** and **15** to be the most active with MIC values ranging from 16 to 128 mg/L against the tested isolates; in particular compound **15** showed MIC_{50} and MIC_{90} values equal to 64 mg/L.

Table 1 summarizes the antioxidant and antibacterial data of exemplificative new β-lactams. Compounds **4**, **5**, **8**, **9**, and **10** notwithstanding the potency against resistant *S. aureus* strains [10] and the presence of a sulphur atom in the structure did not present a sufficient antioxidant activity. The dual activity is present in case of compounds **13**, **14**, **15** in which the presence of phenolic residues on the hydroxyethyl-side chain switched on the antioxidant potency thus conjugated with an encouraging result of antibacterial activity.

3. Conclusions

In summary, the antioxidant behaviour of a series of monocyclic *N*-methylthio-β-lactams was deeply investigated with several methods: BR, TEAC, DPPH, and FRAP. Cyclic voltammetry measurements of three model azetidinones with sulphur substituents revealed redox potential in the range 0.82–0.88 eV thus explaining the impossibility of an *N*-methylthio group of an azetidinone to undergo oxidative reactions with the methods above indicated. However the presence of a phenolic moiety on a side chain on the β-lactams **13**–**15** activated the ability to be radical scavenger against $HOO^{\bullet}$, $ABTS^{\bullet+}$, and $DPPH^{\bullet}$.

Preliminary results on the antibacterial activity of these new compounds against clinical strains of MRSA isolated from CF patients gave promising results in terms of MIC values.

These encouraging results prompt us to currently work on the synthesis of a library of antioxidant *N*-methylthio-4-acetoxy-azetidinones with polyphenolic residues on the side chain which will

be tested on methicillin-resistant strains of *S. aureus* isolated from CF patients. Establishment of a structure–activity relationship (SAR) profile will add to the development of new leads capable of counteracting the adverse conditions in CF patients due to persistent colonization by resistant bacteria and extensive epithelial damage by chronic pulmonary oxidative stress. We are obviously aware that no chemical test can mimic what happens in the human body, however these tests may provide useful information in view of a possible development of new compounds as drugs with two synergistic pharmacological properties: antibacterial and antioxidant activity.

4. Experimental

4.1. General

All reactions were performed under an inert atmosphere (N_2). Commercial reagents (reagent grade, >99%) were used as received without additional purification. Anhydrous solvents (CH_3CN, CH_2Cl_2, THF) were obtained commercially.

$HClO_4$ was analyzed by titration versus a standard 0.1 M NaOH solution (from Merck). H_2O_2 was standardized daily by manganometric analysis. All stock solutions were prepared with doubly distilled, deionized water.

^1H and ^{13}C NMR values were recorded on an INOVA 400, or a GEMINI 200 instrument with a 5 mm probe. All chemical shifts have been quoted relative to deuterated solvent signals, δ in parts per million and J in hertz. FT-IR: Nicolet 380 measured as films between NaCl plates, wavenumbers reported in cm^{-1}. TLC: Merck 60 F_{254}. Column chromatography: Merck silica gel 200–300 mesh. GC–MS: Agilent Technologies, column HP5 5% Ph-Me Silicon. MS: Agilent Technologies MSD1100 single-quadrupole mass spectrometer, EI voltage 70 eV, gradient from 50 °C to 280 °C in 30 min. HPLC–MS, HPLC: Agilent Technologies HP1100, column ZOBRAX-Eclipse XDB-C8 Agilent Technologies, mobile phase: H_2O/CH_3CN, gradient from 30% to 80% of CH_3CN in 8 min, 80% of CH_3CN until 25 min, 0.4 mL/min MS: Agilent Technologies MSD1100 single-quadrupole mass spectrometer, full-scan mode from m/z 50 to m/z 2600, scan time 0.1 s in positive ion mode, ESI spray voltage 4500 V, nitrogen gas 35 psi, drying gas flow 11.5 mL/min, fragmentor voltage 20 V. Elemental analysis was performed on a Thermo Flash 2000 CHNS/O Analyzer, and they were within ±0.4% of the theoretical values. A Shimadzu UV-1601 PC spectrophotometer was used for spectrophotometric measurements.

4.2. Synthesis of β-lactams

Azetidinones **1** [33], and **2**, **3**, **4**, **5**, **7**, **8**, **9**, **10**, were prepared as previously reported [10].

4.2.1. 1-(Methylsulfonyl)-4-oxoazetidin-2-yl acetate (*6*)

A 1 M solution of LiHMDSA (1.1 mL, 1.1 mmol) was added to a solution of 4-acetoxy-2-azetidinone (129 mg, 1.0 mmol) in THF (10 mL) at −78 °C under inert atmosphere, followed by methanesulfonyl chloride (194 μL, 2.5 mmol). The solution was allowed to warm to room temperature and was monitored by TLC. After 3 h the reaction was quenched with aqueous NH_4Cl (15 mL) and extracted with EtOAc (3 × 10 mL). The organic extracts were dried over Na_2SO_4 and the residue was purified by flash-chromatography (cyclohexane/ethylacetate: 60/40) to afford product **6** (42 mg, 20%) as a pale yellow oil. Found C, 34.92; H, 4.51; N, 6.90; S, 15.32%; $C_6H_9NO_5S$ requires C, 34.78; H, 4.38; N, 6.76; S, 15.48%. ν_{max}/cm^{-1} 2970, 2917, 1757 and 1161. δ_H (400 MHz, $CDCl_3$) 2.17 (3H, s, CH_3CO_2), 3.10 (1H, dd, J = 2.0 and 16.4 Hz, CHC*H*H), 3.23 (3H, s, SO_2CH_3), 3.54 (1H, dd, J = 4.8 and 16.4 Hz, CHCH*H*), 6.47 (1H, dd, J = 2.0 and 4.8 Hz, C*H*CHH). δ_C (100 MHz, $CDCl_3$) 20.7, 42.5, 45.7, 75.6, 161.0, 169.3. HPLC–MS (ESI): R_t = 2.47 min, m/z: 225 $[M + H_2O]^+$, 230 $[M + Na]^+$, 246 $[M + K]^+$, 437 $[2M + Na]^+$.

4.2.2. 1-(Methylthio)azetidin-2-one (*11*)

LiHMDSA (2.2 mL 1 M solution in THF, 2.2 mmol) was added to a solution of 2-azetidinone (142 mg, 2 mmol) in THF (14 mL) at −78 °C under inert atmosphere, followed shortly by *S*-methyl methanethiosulfonate (514 μL, 5 mmol). The solution was allowed to warm to room temperature and was monitored by TLC. After 3 h, the reaction was quenched with aqueous NH_4Cl (15 mL) and extracted with EtOAc (3 × 10 mL). The organic extracts were dried over Na_2SO_4 and the residue was purified by flash-chromatography (cyclohexane/ethylacetate: 75/25) to afford product **11** (187 mg, 80%) as a pale yellow oil. Found C, 40.83; H, 5.98; N, 11.87; S, 27.15%; C_4H_7NOS requires C, 41.00; H, 6.02; N, 11.95; S, 27.37%. ν_{max}/cm^{-1} 2970, 2917, 1757 and 1161. δ_H (200 MHz, $CDCl_3$) 2.41 (3H, s, SMe), 3.04 (2H, t, J = 4.8 Hz, CH_2), 3.40 (2H, t, J = 4.8 Hz, CH_2). δ_C (100 MHz, $CDCl_3$) 21.9, 38.7, 43.0, 170.9. HPLC-MS (ESI): R_t = 1.97 min, m/z: 118 $[M + H]^+$, 135 $[M + H_2O]^+$, 140 $[M + Na]^+$, 257 $[2M + Na]^+$. GC–MS (EI, 70eV): R_t = 8.04 min, m/z: 117 (40%, M^+), 75 (100, M − $COCH_2$), 60 (50, M − $COCH_2$ − Me).

4.2.3. (R)-1-((2R,3R)-2-Acetoxy-1-(methylthio)-4-oxoazetidin-3-yl)ethyl-4-(ethoxymethoxy)-3,5-dimethoxybenzoate (*12*)

To a solution of **4** (117 mg, 0.53 mmol), in CH_2Cl_2 (20 mL) at 0 °C under inert atmosphere, the acid **19** (216 mg, 0.84 mmol), DMAP (13 mg, 0.11 mmol) and DCC (174 mg, 0.84 mmol) were added. After 10 min the mixture was allowed to warm to room temperature. After 70 h the reaction mixture was washed with cool water and extracted with CH_2Cl_2 (3 × 15 mL). The collected organic phases were dried on Na_2SO_4 and evaporated. The residue was treated with EtOAc and filtered. The solid was discarded whereas the solvent was evaporated and purified by flash-chromatography (cyclohexane/EtOAc: 70/30) obtaining product **12** (177 mg, 73%) as a white syrup. Found C, 52.58; H, 5.94; N, 3.11; S, 6.89%; $C_{20}H_{27}NO_9S$ requires C, 52.51; H, 5.95; N, 3.06; S, 7.01%. $[\alpha]_D^{22}$ −3.25 (*c* 1.6 in $CHCl_3$). ν_{max}/cm^{-1} 3323, 2926, 2850, 1789, 1755, 1716 and 1126. δ_H (400 MHz, $CDCl_3$) 1.17 (3H, t, J = 7.2 Hz, CH_3CH_2), 1.48 (3H, d, J = 6.4 Hz, CH_3CHOCO), 2.14 (3H, s, CH_3CO_2), 2.47 (3H, s, SCH_3), 3.46 (1H, dd, J = 1.2 and 6.4 Hz, *CH*CHO), 3.84 (2H, q, J = 7.2 Hz, CH_3CH_2), 3.87 (6H, s, 2 × OCH_3), 5.20 (2H, s, OCH_2O), 5.43 (1H, quintet, J = 6.4 Hz, CH_3CHO), 6.21 (1H, d, J = 1.2 Hz, *CH*OAc), 7.22 (2H, s, arom.). δ_C (50 MHz, $CDCl_3$) 14.8, 18.2, 20.8, 22.8, 56.1, 64.1, 64.9, 66.9, 81.5, 96.4, 106.7, 125.1, 138.9, 153.0, 164.9, 167.3, 169.6. HPLC–MS (ESI): R_t = 8.62 min, m/z: 475 $[M + H_2O]^+$.

4.2.4. (R)-1-((2R,3R)-2-Acetoxy-1-(methylthio)-4-oxoazetidin-3-yl)ethyl-4-hydroxy-3,5-dimethoxy benzoate (*13*)

A stirred solution of **12** (76 mg, 0.17 mmol) in CH_2Cl_2 under inert atmosphere was treated with aliquots of trifluoroacetic acid (12 μL, 0.17 mmol) every 15 min until the disappearing of the starting material. After the completion of the reaction, the solvent and the trifluoroacetic acid were evaporated to obtain **13** (55 mg, 81%) as a yellow oil. Found C, 51.03; H, 5.37; N, 3.44; S, 7.89%; $C_{17}H_{21}NO_8S$ requires C, 51.12; H, 5.30; N, 3.51; S, 8.03%. $[\alpha]_D^{22}$ −36.3 (*c* 0.52 in $CHCl_3$). ν_{max}/cm^{-1} 3412, 2938, 2850, 1783, 1756, 1711 and 1116. δ_H (400 MHz, $CDCl_3$) 1.50 (3H, d, J = 6.4 Hz, CH_3CHOCO), 2.16 (3H, s, CH_3CO_2), 2.49 (3H, s, SCH_3), 3.48 (1H, dd, J = 1.2 and 6.0 Hz, *CH*CHOCO), 3.94 (6H, s, 2 × OCH_3), 5.44 (1H, quintet, J = 6.4 Hz, CH_3CHO), 5.97 (1H, s, OH), 6.23 (1H, d, J = 1.2 Hz, *CH*OAc), 7.26 (2H, s, arom.). δ_C (100 MHz, $CDCl_3$) 18.3, 20.8, 22.9, 56.4, 64.2, 66.7, 81.5, 106.7, 120.6, 139.5, 146.6, 165.2, 167.5, 169.7. HPLC-MS (ESI): R_t = 5.68 min, m/z: 417 $[M + H_2O]^+$, 422 $[M + Na]^+$, 821 $[2M + Na]^+$.

*4.2.5. (R)-1-((2R,3R)-2-Acetoxy-1-(methylthio)-4-oxoazetidin-3-yl)ethyl-3,4-bis((tert-butyldimethylsilyl)oxy)benzoate (**16**)*

To a solution of **4** (121 mg, 0.55 mmol) in CH_2Cl_2 (8.2 mL), compound **22** (210 mg, 0.55 mmol) and DMAP (67 mg, 0.55 mmol) were added. The mixture was then cooled to 0 °C, EDC (105 mg, 0.55 mmol) was added and the system was allowed to reach rt in 10 min. After 30 h, the reaction was quenched with water and some drops of HCl (1 M), extracted with CH_2Cl_2, dried on Na_2SO_4 and evaporated. The silylated intermediate **16** was obtained after flash-chromatography (CH_2Cl_2/Et_2O 99/1) in 23% yield (73 mg) as an oil. $[\alpha]_D^{22}$ −13.8 (*c* 0.5 in CH_2Cl_2). ν_{max}/cm^{-1} 3427, 2930, 2858, 1793, 1758, 1719 and 1120. δ_H (400 MHz, $CDCl_3$) 0.22 (6H, s, SitBu*Me*$_2$), 0.23 (6H, s, SitBu*Me*$_2$), 0.99 (9H, s, Si*t*BuMe_2), 1.00 (9H, s, Si*t*BuMe_2), 1.47 (3H, d, *J* = 6.0 Hz, C*H*$_3$CHOCO), 2.17 (3H, s, C*H*$_3$CO$_2$), 2.47 (3H, s, SC*H*$_3$), 3.47 (1H, dd, *J* = 1.6 and 6.0 Hz, C*H*CHO), 5.45 (1H, quintet, *J* = 6.0 Hz, CH$_3$C*H*O), 6.26 (1H, d, *J* = 1.2 Hz, CHOAc), 6.82–6.84 (1H, m, Ar), 7.46–7.49 (2H, m, Ar). δ_C (100 MHz, $CDCl_3$) −4.2, −4.1, −4.1, −4.0, 18.3, 18.4, 18.5, 20.9, 22.8, 25.8, 25.9, 64.1, 66.3, 81.0, 120.4, 122.3, 122.9, 123.6, 146.8, 152.0, 165.0, 167.5, 169.8. HPLC–MS (ESI): R_t = 26.6 min, *m*/*z*: 601 $[M + H_2O]^+$, 606 $[M + Na]^+$, 1190 $[2M + Na]^+$.

*4.2.6. (R)-1-((2R,3R)-2-Acetoxy-1-(methylthio)-4-oxoazetidin-3-yl)ethyl3,4-dihydroxy benzoate (**14**)*

To a solution of **16** (47 mg, 0.08 mmol) in anhydrous CH_3CN (1.64 mL) under inert atmosphere and at 0 °C $BF_3{\cdot}Et_2O$ (23 μL, 0.182 mmol) was added dropwise. After 30 min the ice-bath was removed, and after 45 min at rt the reaction was quenched with a pH = 6 phosphate buffer solution 0.1 M, extracted with CH_2Cl_2, dried on Na_2SO_4 and evaporated. The crude was finally triturated to afford the desired product **14** in 46%yield (13 mg) as a light yellow oil. Found C, 50.96; H, 4.91; N, 3.96; S, 8.95%; $C_{15}H_{17}NO_7S$ requires C, 50.70; H, 4.82; N, 3.94; S, 9.02%. $[\alpha]_D^{22}$ −13.7 (*c* 0.25 in CH_2Cl_2). ν_{max}/cm^{-1} 3371, 2983, 2929, 1781, 1761, 1713, 1602 and 1294. δ_H (400 MHz, $CDCl_3$) 1.47 (3H, d, *J* = 6.0 Hz, C*H*$_3$CHOCO), 2.18 (3H, s, C*H*$_3$CO$_2$), 2.47 (3H, s, SC*H*$_3$), 3.49 (1H, dd, *J* = 1.2 and 4.8 Hz, C*H*CHO), 5.46 (1H, quintet, *J* = 6.0 Hz, CH$_3$C*H*O), 6.28 (1H, d, *J* = 1.2 Hz, CHOAc), 6.86 (1H, *J* = 8.4 Hz, Ar), 7.44 (1H, dd, *J* = 2.0 and 8.4 Hz, Ar), 7.50 (1H, d, *J* = 2.0 Hz, Ar). δ_C (100 MHz, $CDCl_3$) 18.3, 20.9, 22.7, 64.0, 66.3, 80.8, 114.8, 116.5, 121.8, 123.5, 143.4, 149.2, 165.3, 168.2, 170.1. HPLC–MS (ESI): R_t = 5.0 min, *m*/*z*: 373 $[M + H_2O]^+$, 378 $[M + Na]^+$, 733 $[2M + Na]^+$.

*4.2.7. (E)-(R)-1-((2R,3R)-2-Acetoxy-1-(methylthio)-4-oxoazetidin-3-yl)ethyl-3-(3,4-bis(ethoxymethoxy)phenyl)acrylate (**17**)*

To a solution of **4** (68 mg, 0.31 mmol) in CH_2Cl_2 (11.6 mL), compound **21** (145 mg, 0.49 mmol) and DMAP (7.6 mg, 0.06 mmol) were added. The mixture was then cooled to 0 °C, DCC (101 mg, 0.49 mmol) was added and the system was allowed to reach rt in 15 min. After 65 h, the reaction was quenched with water, extracted with CH_2Cl_2, dried on Na_2SO_4, evaporated and triturated with AcOEt to separate dicycloexylurea precipitate. The solution was then evaporated and purified by flash-chromatography (cyclohexane/ethylacetate 90/10 to 80/20) to afford the product in 61% yield (94 mg) as an oil. Found C, 55.78; H, 6.32; N, 2.68; S, 6.30%; $C_{23}H_{31}NO_9S$ requires C, 55.52; H, 6.28; N, 2.82; S, 6.44%. $[\alpha]_D^{22}$ −7.4 (*c* 0.84 in CH_2Cl_2). ν_{max}/cm^{-1} 3327, 2977, 2930, 1789, 1757, 1711, 1635, 1599, 1510, 1437, 1251 and 1159. δ_H (400 MHz, $CDCl_3$) 1.21–1.28 (6H, m, 2 × OCH$_2$C*H*$_3$), 1.44 (3H, d, *J* = 6.4 Hz, C*H*$_3$CHO), 2.17 (3H, s, C*H*$_3$CO$_2$), 2.51 (3H, s, SC*H*$_3$), 3.42 (1H, dd, *J* = 1.6 and 6.0 Hz, C*H*CHO), 3.74–3.81 (4H, m, 2 × OCH$_2$C*H*$_3$), 5.30 (2H, s, OCH$_2$O), 5.31 (2H, s, OCH$_2$O), 5.37 (1H, quintet, *J* = 6.0 Hz, CH$_3$C*H*O), 6.22 (1H, d, *J* = 1.6 Hz, CHOAc), 6.25 (1H, d, *J* = 16.0 Hz, CH=C*H*CO), 7.13 (1H, dd, *J* = 2.0 and 8.0 Hz, arom), 7.19 (1H, d, *J* = 8.0 Hz, arom), 7.37 (1H, d, *J* = 2.0 Hz, arom), 7.53 (1H, d, *J* = 16.0 Hz, C*H*=CHCO). δ_C (100 MHz, $CDCl_3$) 15.0, 15.1, 18.3, 20.9, 22.7, 64.1, 64.5, 64.6, 66.1, 80.9, 93.8, 94.1, 115.5, 115.7, 116.0, 123.4, 128.4, 145.3, 147.5, 149.5, 165.9, 167.5, 169.8. HPLC–MS (ESI): R_t = 9.9 min, *m*/*z*: 515 $[M + H_2O]^+$, 520 $[M + Na]^+$, 1017 $[2M + Na]^+$.

*4.2.8. (E)-(R)-1-((2R,3R)-2-Acetoxy-1-(methylthio)-4-oxoazetidin-3-yl)ethyl-3-(3,4-dihydroxyphenyl)acrylate (**15**)*

To a solution of **17** (94 mg, 0.19 mmol) in CH_2Cl_2 (23 mL) under inert atmosphere aliquots of TFA (64.5 μL, 0.869 mmol) were added every 15 min until the disappearing of the starting material (TLC monitoring). The solution was evaporated and the crude was purified by flash-chromatography (cyclohexane/ethylacetate 40/60). The product obtained from the collected fractions was finally triturated with pentane to afford the desired product **15** as a white solid in 90% yield (65 mg). Found C, 53.89; H, 5.13, N 3.55; S, 8.37%; $C_{17}H_{19}NO_7S$ requires C, 53.53; H, 5.02; N, 3.67; S, 8.41%; mp: 53–58 °C. $[\alpha]_D^{22}$ −19.3 (*c* 1.11 in CH_2Cl_2). ν_{max}/cm^{-1} 3391, 2933, 1755, 1716, 1605, 1515, 1445, 1397, 1260 and 1056. δ_H (400 MHz, $CDCl_3$) 1.44 (3H, d, *J* = 6.0 Hz, C*H*$_3$CHOCO), 2.19 (3H, s, C*H*$_3$CO$_2$), 2.52 (3H, s, SC*H*$_3$), 3.44 (1H, dd, *J* = 1.2 and 5.2 Hz, C*H*CHO), 5.38 (1H, quintet, *J* = 6.0 Hz, CH$_3$C*H*O), 6.18 (1H, d, *J* = 16.0 Hz, C*H*=CHCO), 6.23 (1H, d, *J* = 1.2 Hz, CHOAc), 6.87 (1H, d, *J* = 8.0 Hz, arom), 6.97 (1H, dd, *J* = 2.0 and 8.4 Hz, arom), 7.06 (1H, d, *J* = 1.6 Hz, arom), 7.54 (1H, d, *J* = 16.0 Hz, CH=C*H*CO). δ_C (100 MHz, $CDCl_3$) 18.2, 20.9, 22.7, 63.8, 65.9, 80.8, 114.1, 114.3, 115.4, 122.4, 126.9, 144.2, 146.1, 147.0, 166.5, 168.5, 170.1. HPLC–MS (ESI): R_t = 5.6 min, *m*/*z*: 399 $[M + H_2O]^+$, 404 $[M + Na]^+$, 785 $[2M + Na]^+$.

*4.2.9. Methyl 4-(ethoxymethoxy)-3,5-dimethoxybenzoate (**18**)*

A solution of methyl 4-hydroxy-3,5-dimethoxybenzoate (1.24 g, 5.9 mmol) in THF (10 mL) was added dropwise to a suspension of NaH (60% in mineral oil, 306 mg, 7.6 mmol) in THF (20 mL) at 0 °C under inert atmosphere. After 10 min a solution of chloromethylethylether (1.1 mL, 11.75 mmol) in THF (7 mL) was added dropwise and the reaction mixture was allowed to warm to room temperature and monitored by TLC. After 2 h the reaction was quenched with aqueous NH_4Cl (30 mL) and extracted with EtOAc (3 × 20 mL). The organic extracts were dried over Na_2SO_4 and concentrated to obtain product **18** (1.46 g, 92%) as pale yellow solid. Found C, 57.62; H, 6.80%; $C_{13}H_{18}O_6$ requires C, 57.77; H, 6.71%; mp: 52.7–55.6. ν_{max}/cm^{-1} 2926, 1720, 1592 and 1128. δ_H (200 MHz, $CDCl_3$) 1.21 (3H, t, *J* = 7.0 Hz, C*H*$_3$CH$_2$), 3.88 (2H, q, *J* = 7.0 Hz, CH$_3$C*H*$_2$), 3.90 (6H, s, 2 × OC*H*$_3$), 3.92 (3H, s, CO$_2$C*H*$_3$), 5.23 (2H, s, OC*H*$_2$O), 7.31 (2H, s, arom). δ_C (100 MHz, $CDCl_3$) 14.6, 51.8, 55.8, 64.6, 96.2, 106.4, 125.3, 138.4, 152.8, 166.3. HPLC–MS (ESI): R_t = 7.81 min, *m*/*z*: 271 $[M + H]^+$, 293 $[M + Na]^+$, 309 $[M + K]^+$.

*4.2.10. Methyl 4-(ethoxymethoxy)-3,5-dimethoxy benzoic acid (**19**)*

A stirred solution of **18** (730 mg, 2.7 mmol) in 10 mL of a mixture of THF/MeOH: 90/10 was treated with 5 M NaOH (2.7 mL). The reaction mixture was heated at 40 °C for 4 h and monitored by TLC. At completion, EtOAc (10 mL) was added and the organic phase was separated and discarded. The aqueous phase was then cooled to 0 °C and adjusted to pH 6 with aqueous NH_4Cl. The mixture was extracted with EtOAc (3 × 15 mL), dried on Na_2SO_4 and concentrated to afford **19** (432 mg, 63%) as a white solid. Found C, 56.18; H, 6.34%; $C_{12}H_{16}O_6$ requires C, 56.24; H, 6.29%; mp: 107.7–109.4 °C. ν_{max}/cm^{-1} 3398, 2924, 1716, 1589 and 1120. δ_H (400 MHz, $CDCl_3$) 1.22 (3H, t, *J* = 7.2 Hz, C*H*$_3$CH$_2$), 3.89 (2H, q, *J* = 7.2 Hz, CH$_3$C*H*$_2$), 3.92 (6H, s, 2 × OC*H*$_3$), 5.26 (2H, s, OC*H*$_2$O), 7.37 (2H, s, arom). δ_C (50 MHz, $CDCl_3$) 14.8, 56.0, 64.9, 96.3, 107.1, 124.5, 139.3, 153.0, 171.5. HPLC–MS (ESI): R_t = 1.53 min, *m*/*z*: 279 $[M + Na]^+$, 535 $[2M + Na]^+$.

*4.2.11. (E)-Methyl 3-(3,4-bis(ethoxymethoxy)phenyl)acrylate (**20**)*

A solution of caffeic acid methyl ester [34] (540 mg, 2.78 mmol) in THF (6 mL) was added dropwise into a solution of NaH

(60% dispersion in oil, 289 mg, 7.2 mmol) in THF (15 mL) under inert atmosphere and at 0 °C. After 10 min a solution of chloromethyl ethyl ether (1.01 mL, 11.1 mmol) in THF (7 mL) was added dropwise. After 2 h the solution was allowed to rt and quenched by adding a saturated NH_4Cl solution, extracted with CH_2Cl_2, dried over Na_2SO_4 and evaporated. The product **20** was obtained in 90% yield (776 mg) after flash-chromatography (cyclohexane/ethylacetate 90/10) as a colourless oil. Found C, 62.12; H, 7.38%; $C_{16}H_{22}O_6$ requires C, 61.92; H, 7.15%. ν_{max}/cm^{-1} 2977, 2900, 1717, 1634, 1600, 1582, 1511, 1436, 1393, and 1105. δ_H (400 MHz, $CDCl_3$) 1.21–1.27 (6H, m, 2 × OCH_2CH_3), 3.74–3.81 (4H, m, 2 × OCH_2CH_3), 3.81 (3H, s, OCH_3), 5.30 (1H, s, OCH_2O), 5.31 (1H, s, OCH_2O), 6.33 (1H, d, J = 16.0 Hz, C*H*=CHCO), 7.15 (1H, dd, J = 2.0 and 8.0 Hz, arom), 7.19 (1H, d, J = 8.4 Hz, arom), 7.39 (1H d, J = 1.6 Hz, arom), 7.63 (1H, d, J = 16.0 Hz, CH=C*H*CO). δ_C (100 MHz, $CDCl_3$) δ 14.9, 15.0, 51.4, 64.3, 64.4, 93.6, 94.0, 115.5, 115.9, 116.0, 123.1, 128.5, 144.4, 147.4, 149.2, 167.4. HPLC–MS (ESI): R_t = 9.2 min, m/z: 311 $[M + H]^+$, 333 $[M + Na]^+$, 349 $[M + K]^+$.

4.2.12. (E)-3-(3,4-Bis(ethoxymethoxy)phenyl)acrylic acid (*21*)

A stirred solution of **20** (310 mg, 1 mmol) in 3.8 mL of a mixture of THF/MeOH 90/10 was treated with 1 mL NaOH 5 M, warmed at 40 °C and stirred for 4 h. After completion, AcOEt (6 mL) was added and discharged. The aqueous phase was treated with HCl (1 M), extracted with AcOEt, the organic phase was dried over Na_2SO_4 and evaporated to afford the desired product in 96% yield (284 mg) as a white solid. Found C, 60.52; H, 6.95%; $C_{15}H_{20}O_6$ requires C, 60.80; H, 6.80%; mp: 101–103 °C. ν_{max}/cm^{-1} 2976, 1683, 1626, 1596, 1514, 1418, 1244 and 1114. δ_H (400 MHz, $CDCl_3$) δ 1.22–1.29 (6H, m, 2 × OCH_2CH_3), 3.75–3.85 (4H, m, 2 × CH_3CH_2O), 5.31 (1H, s, OCH_2O), 5.33 (1H, s, OCH_2O), 6.35 (1H, d, J = 16.0 Hz, C*H*=CHCO), 7.18 (1H, dd, J = 1.6 and 8.8 Hz, 1H, arom), 7.22 (1H, d, J = 8.8 Hz, arom), 7.42 (1H, d, J = 1.6 Hz, arom), 7.73 (1H, d, J = 16.0 Hz, CH=C*H*CO). δ_C (50 MHz, $CDCl_3$) 15.0, 15.1, 64.5, 64.6, 93.8, 94.2, 115.5, 115.8, 116.0, 123.7, 128.3, 146.8, 147.5, 149.7, 172.1. HPLC–MS (ESI): R_t = 1.90 min, m/z: 297 $[M + Na]^+$, 319 $[2M + Na]^+$, 335 $[M + K]^+$.

4.2.13. 3,4-Bis((tert-butyldimethylsilyl)oxy)benzoic acid (*22*)

To a solution of 3,4-dihydroxy-benzoic acid (500 mg, 3.24 mmol) in DMF (5.8 mL) under inert atmosphere, imidazole (1.983 g, 29.16 mmol) and then TBSCl (2.200 g, 14.6 mmol) were added. After 66 h the reaction was quenched at 0 °C by adding water, and then extracted with Et_2O (10 × 4 mL). The organic phases were washed twice with water and brine, dried over Na_2SO_4 and evaporated. To the persilylated intermediate (2.049 g, 4.12 mmol) in a 6 mL MeOH/THF 40/60, a solution of K_2CO_3 (231 mg, 1.67 mmol) in water (2.2 mL) was added. After 12 h the mixture was quenched a 0 °C by adding a saturated solution of citric acid and extracted with Et_2O. The organic phases were washed with water and brine, dried over Na_2SO_4 and evaporated. The product was finally obtained by flash-chromatography (CH_2Cl_2/MeCN: 90/10) in 75% yield (930 mg) as a white solid. Found C, 60.12; H, 9.10%; $C_{19}H_{34}O_4Si_2$ requires C, 59.64; H, 8.96%; mp: 152–154 °C. δ_H (400 MHz, $CDCl_3$) δ 0.24 (6H, s, SitBuMe_2), 0.25 (6H, s, SitBuMe_2), 1.00 (9H, s, Si*tBu*Me_2), 1.01 (9H, s, Si*tBu*Me_2), 6.88 (1H, d, J = 8.0 Hz, arom), 7.60 (1H, d, J = 2.0 Hz, arom), 7.63 (1H, dd, J = 1.6 and 8.8 Hz, 1H, arom). δ_C (50 MHz, $CDCl_3$) −4.1, −4.0, 18.4, 18.5, 25.8, 25.9, 120.5, 122.4, 122.7, 124.4, 146.8, 152.5, 171.2.

4.3. Antioxidant activity methods

4.3.1. BR assay

Relative antioxidant activity (r.a.c.) with respect to a substance chosen as standard, resorcinol (Re) in our case, is determined on the basis of concentrations of sample and resorcinol that give the same t_{inhib}; r.a.c. is expressed as μM resorcinol equivalents [18]. Oscillatory behaviours V(Pt) were followed potentiometrically by using the couple bright platinum electrode (Hamilton 238945) – Ag/AgCl double junction electrode (Ingold 373-90-WTE-ISE-S7) connected to a multimeter (WTW pH 540 GLP), controlled by a PC. Reacting mixtures were thermostated at 25.0 ± 0.1 °C. The straight line of the standard was checked before each series of measurements.

More details on the experimental procedure are reported in Refs. [18,19,35].

4.3.2. TEAC assay [20a]

The radical cation is preformed by reaction between ABTS and $K_2S_2O_8$ in PBS medium, pH = 7.4. The mixture was kept in the dark until the reaction was complete and absorbance at 734 nm stable. Spectroscopic measurements were performed in triplicate at four concentrations of the β-lactams in DMSO. Suitably diluted solutions of the standard (Trolox) were treated in the same way. In brief, 3.0 mL of diluted $ABTS^{\bullet+}$ solution and 30 μL of sample were mixed in a photometric cuvette and absorbance was measured at 734 nm at exactly 6 min after the mixing of the reagents (T = 30.0 ± 0.1 °C). A blank with DMSO was measured in the same way. The difference between the absorbance of the blank and the sample gave ΔE6 (E6blank – E6sample = ΔE6). Data (ΔE6 vs conc. (mM)) are well fitted by straight lines through the origin (following the Lambert–Beer law). Then, the relative antioxidant activity with respect to Trolox (TEAC) was obtained by the ratio:

$$\text{TEAC} = m(\text{smp})/m(\text{Trolox})$$

where m(smp) and m(Trolox) are the slopes of the straight lines of the sample and the standard respectively. Standard error of this ratio is calculated in the usual way.

Here also the straight line of the standard was always checked before each series of measurements.

4.3.3. DPPH assay

The principle of this method is the decolorization of the stable radical 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) by antioxidants [21,22]. The $DPPH^{\bullet}$ is intensely purple coloured (λ_{max} = 512–530 nm) due to the presence of the picric group (1,3,5-nitrobenzene) and is stabilized by either the steric hindrance of the aromatic substituents and the delocalization of the unpaired electron on the whole structure. The disappearance of this radical due to the addition of an antioxidant H-donor, may be evaluated spectrophotometrically, at its absorption maximum in methanol (515 nm). The measured decrease in absorbance at 515 nm is an expression of the antioxidant capacity of a sample [36]. The extent of the decolorization is a function of concentration and time. Trolox was used as the standard. In practice 2.9 mL of $DPPH^{\bullet}$ methanolic solution was placed in a cuvette and the initial absorbance, A_0, was measured. Then 100 μL was added and mixed; after 15 min the final absorbance, A_f, was measured. The percentage of inhibition was calculated according to the following equation (%) Inhibition = $(1 - A_f/A_0) \times 100$. The same procedure was repeated with the standard. Data (%inhib vs conc. (mM)) are well fitted by straight lines through the origin (following the Lambert–Beer law). Then, the relative antioxidant activity with respect to Trolox was obtained by the ratio: DPPH = m(smp)/m(Trolox).

4.3.4. FRAP assay [23]

2,4,6-Tris(2-pyridyl)-s-triazine-Fe^{3+} (FRAP reagent) was prepared by mixing suitable amounts of TPTZ and $FeCl_3$ solutions in aqueous acetate buffer. In brief, 3.0 mL of FRAP reagent and 100 μL of suitably diluted sample were mixed in a photometric cuvette and absorbance was measured after exactly 4 min after the mixing

($T = 25.0 \pm 0.1^{\circ}$). The absorbance of the blue Fe^{2+} complex is measured at 593 nm (Shimadzu UV-1601 PC spectrophotometer). Four or five different sample concentrations were tested and the straight line Abs vs. conc. was then compared with that of the standard ($FeSO_4$). The Lambert–Beer law is followed, then the ratio: FRAP = m(smp)/m(Fe^{2+}) gives the relative ferric reducing activity (mmol equiv Fe^{2+}).

4.4. Cyclic voltammetry

All electrochemical experiments were conducted in acetonitrile from Sigma–Aldrich (>99.5% over molecular sieves) after careful deaeration of the reaction mixture with argon; in all instances a silver wire was used as a pseudo-reference electrode, while a platinum coil was used as a counter electrode. Voltammetric analysis was performed with platinum, gold and glassy carbon working electrodes (Ø = 2 mm) using a function generator AMEL mod 568 equipped with a digital oscilloscope.

4.5. Bacterial strains selection and antibacterial activity evaluation

4.5.1. Bacterial isolates

A collection of 45 recently isolated and well characterized for the antimicrobial susceptibility phenotype MRSA strains recovered from CF patients was tested. All the strains were isolated at the Department of Pediatrics, CF Center, Fondazione IRCCS Cà Granda Ospedale Maggiore Policlinico, University of Milano.

4.5.2. Antimicrobial susceptibility determinations

The *in vitro* susceptibility of methicillin-resistant isolates of *S. aureus* to new antimicrobial compounds was evaluated by means of the broth microdilution method for the determination of the minimum inhibitory concentrations (MIC), in accordance with the Clinical and Laboratory Standards Institute (CLSI; formerly National Committee for Clinical Laboratory Standards) guidelines [37].

Briefly, serial two-fold dilutions were made of the compounds in 96-well plates in order to obtain concentrations ranging from of 0.06–128 mg/L in cation-adjusted Muller–Hinton broth (DIFCO). An equal volume of 1×10^6 CFU/mL (Colony Forming Unit/mL) bacterial inoculum was added to each well of the microtitre plate containing 0.05 mL of the serial antibiotic concentrations. The microtitre plate was then incubated overnight at 37 °C and subsequently analysed for the presence of visible bacterial growth. MIC was defined as the lowest concentration of the tested compound able to inhibit visible growth of the microorganism after overnight incubation. All the tested compounds were solubilized in 100% DMSO and appropriate dilutions were set up to prevent its toxicity. Positive strain controls without antimicrobial compounds, controls with DMSO and uninoculated media were run parallel to the tested compounds under the same conditions.

S. aureus American Type Culture Collection (ATCC) 29213 (MSSA) and ATCC 43300 (MRSA) were used as control strains. Cefotaxime sodium salt was used as the reference antibiotic for MIC experiments validation. Breakpoints for resistance were those recommended by the CLSI [38].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.ejmech.2012.12.024.

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