Title proposal: **4-Alkyliden-azetidinones modified with plant polyphenols: antibacterial and antioxidant properties.**

Daria Giacomini,[a]* Rosario Musumeci,[b] Paola Galletti,[a] Giulia Martelli,[a] Lorenzo Assennato,[a] Gianni Sacchetti,[c] Alessandra Guerrini,[c] Enrico Calaresu,[b] Marianna Martinelli,[b] and Clementina Cocuzza.[b]

Addresses

[a] Department of Chemistry "G. Ciamician", University of Bologna, Via Selmi 2, 40126 Bologna, Italy.

[b] Department of Medicine and Surgery, University of Milano-Bicocca, Via Cadore 48, 20900, Monza, Italy.

[c] Department of Life Sciences and Biotechnology – Pharmaceutical biology lab., UR7 Tecnopolo Terra&Acqua Tech, University of Ferrara, P.le Chiappini 3, 44123 Ferrara, Italy

Abstract

Antimicrobial resistance is one of the major and growing concerns in hospital- and community acquired infections, and new antimicrobial agents are therefore urgently required. It was reported that oxidative stress could contribute to the selection of resistant bacterial strains, since reactive oxygen species (ROS) revealed to be an essential driving force. In the present work 4-alkylidene-azetidinones, a new class of anti-bacterial agents, were functionalized with phytochemical polyphenolic acids such as protocatechuic, piperonyl, caffeic, ferulic, or sinapic acids and investigated as dual target antibacterial-antioxidant compounds. The best candidates showed good activities against multi resistant clinical isolates of MRSA (MICs 2-8 μL/mL). Among the new compounds, two revealed the best antioxidant capacity with TEAC-DPPH and TEAC-ABTS "results showing significantly better activity than Trolox®" oppure "being significantly more active than Trolox®".

Keywords:

Azetidinones, Gram-positive, Antibacterials, Lactams, Resistant bacteria, Antioxidant capacity

Highlights:

- Design and synthesis of new β-lactams targeting resistant bacteria.
- Synthesis of new 4-alkyliden-azetidinones decorated with phenolic esters.
- Antibacterial activity was tested on multidrug-resistant bacterial strains.
- Antioxidant potency of the new compounds was evaluated.

1. Introduction

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) play important roles in regulation of cell life [1, 2]. In general, moderate levels of ROS/RNS may function as signals to promote cell proliferation and survival, whereas severe increase of ROS/RNS can also induce cell death *in vitro* [3].

Oxidative stress is generated by an imbalance increase of ROS/RNS which can damage all classes of cellular macromolecules as proteins, lipids and nucleic acids with strong effects on human metabolic pathways and causing the onset of several disorders and diseases. In fact, oxidative stress is generally considered the starting point for the onset of several diseases (i.e. tissues chronic inflammation, cancers) and undoubtedly one of the major pathophysiological hallmarks of severe obstructive lung diseases, including chronic obstructive pulmonary disease and cystic fibrosis (CF) [4, 5]. In cystic fibrosis, a lethal genetic disease, the presence of oxidative stress is due to an increased production of ROS and to an impaired antioxidant status [6, 7] that particularly arise during chronic pulmonary infections.

In CF patients, a persistent colonization by pathogenic bacteria occurs and the repeated use of antibacterial agents selects for specific resistant strains such as multidrug resistant *Pseudomonas aeruginosa* (incidence 43%) and *Staphylococcus aureus* (incidence 73.1%) strains [8]. 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) [9-11]. Persistent MRSA infections represent an underestimated *new threat* to the CF community, as they are not only difficult to treat but also associated with factors complicating patient outcome as well as biofilm formation, Panton–Valentine leukocidin (PVL) production and decreased lung function, with an impairment of patient survival [12].

The involvement of oxidative stress in the mechanism of antibiotic-mediated cell death is unclear and subject to debate [13]. It has been shown that under aerobic conditions bactericidal antibiotics with specific targets in bacterial cell stimulate the production of harmful reactive oxygen species (ROS), which contribute to killing by these drugs [14]. On the contrary, several research groups provided convincing arguments against ROS-mediated killing of bacteria by antibiotics (Keren et al. 2013; Liu and Imlay 2013) [15, 16].

Moreover, it was recently reported that oxidative stress could contribute to the phenomenon of selection of pro-biofilm variants and H_2O_2-resistance, since ROS revealed to be an essential driving force for the selection of variants of *Pseudomonas aeruginosa* strains [17]. This evidence was also recently confirmed by clinical evaluations in patients affected by diabetes and/or related cardiovascular diseases, becoming more susceptible to bacterial infections (nosocomial infections and infections related to specific diseases) and less reactive to pharmacological treatments [18].

The described phenomena point to the need of new therapeutic strategies in targeting anti-oxidant pathways together with new antibacterial agents able to fight chronic infections caused by multidrug resistant bacterial strains. In this article, we report our recent efforts on this topic, as part of an ongoing project on the synthesis of dual-target monocyclic β-lactams, specifically designed to target resistant bacterial strains [19], and to have antioxidant activities [20, 21, 22].

We focused our attention on the synthesis of 4-alkyliden-azetidinones armed with polyphenolic residues as possible antibacterial-antioxidant dual-active compounds. In these derivatives a double C=C bond on the C-4 position of the β-lactam ring acts as a strong electron withdrawing group [23], thus conferring an increased aptitude toward ring-opening reactions by suitable enzymes. Indeed some 4-alkyliden-azetidinones were strong inhibitors of serine-dependent enzymes such as human leucocyte elastase [24], for the treatment of pulmonary emphysema, or matrix-metallo proteases (MMPs) involved in cancer invasion and metastasis [25, 26].

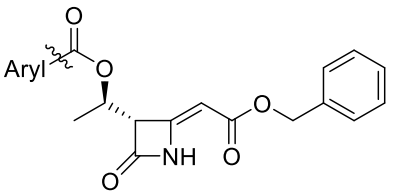
Concerning the antibacterial activity, some structural features resulted particularly important for valuable potency: the alkylidene function on the C4 position with a (*Z)* stereochemistry showed to favor the biological activity if compared to (*E*) stereoisomers; the presence of a benzyl ester strongly improved the potency in respect to the ethyl ester or the carboxylic acid [19]. Furthermore, it was observed that coupling a phenolic ester with *N*-methylthio-4-alkylidene-β-lactams resulted in inactivation [21], hence showing the need to include an unsubstituted-NH atom in these compounds.

Our aim was then to broad the scope of our previous studies by functionalizing the antibacterial-core structure of 4-alkylidenazetidin-2-ones with different polyphenolic moieties from some plant derived benzoic acids such as protocatechuic- or piperonyl acids at the C3 position as to efficiently combine in one structure both antioxidant and antibacterial activities. In a previous

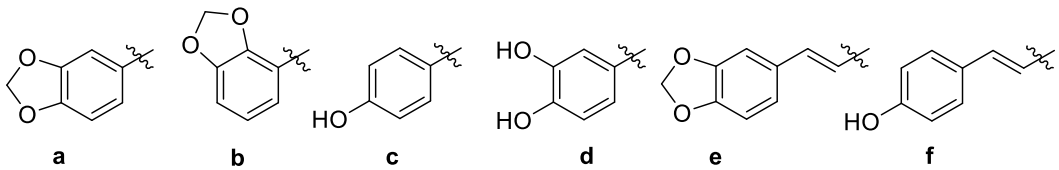
work it was found out that polyphenol-substituted β-lactams presented a decreased antioxidant activity if compared with their corresponding polyhydroxybenzoates, pointing out a steric hindrance phenomenon [22]. Hence, we considered that an extension of the side chain linking the phenolic aromatic ring to the rest of the structure might provide a greater exposure of the phenolic OH residues to affect the antioxidant activity without disturbing the antibacterial activity. Relying on the commonly occurring structures in natural phytochemical polyphenols we identified also the C6-C3 leitmotiv of hydroxy-cinnamic acids such as coumaric, caffeic, ferulic, or sinapic acids as promising groups (Chart 1) [27, 28, 29].

Hydroxycinnamic acids are a group of polyphenols highly abundant in food that may account for about one-third of the phenolic compounds in our diet. Moreover, they have gained an increasing interest in health because they are known to be potent antioxidants [30]. These compounds have been described as chain-breaking antioxidants acting through radical scavenging activity that is related to their hydrogen or electron donating capacity and to the ability to delocalize/stabilize the resulting phenoxyl radical within their structure.

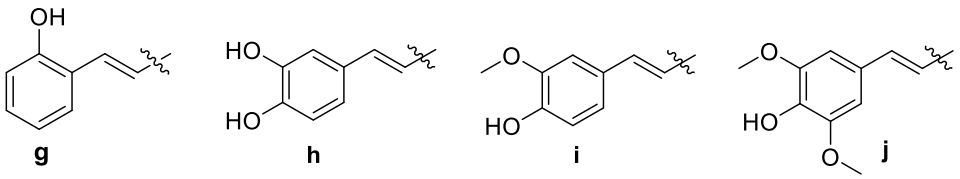
Moreover, in the study of the phenol-protection strategy we considered the methylenedioxy moiety, present in many natural product, as an interesting substructure to be held in the target compounds for evaluating its antibacterial activity. As a result, a number of novel compounds with good antibacterial activity against staphylococci, particularly multiresistant *S. aureus* (MRSA), and with antioxidant potency have been discovered and qualified for further *in vivo* evaluations.



1a-j



a **b** **c** **d** **e** **f**



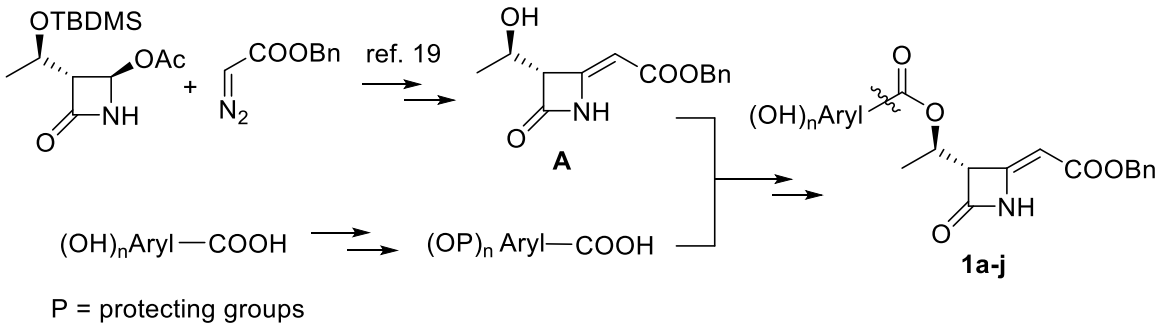
g **h** **i** **j**

Chart 1. Novel β-lactam derivatives synthesized and evaluated in this study

2. Results and Discussion

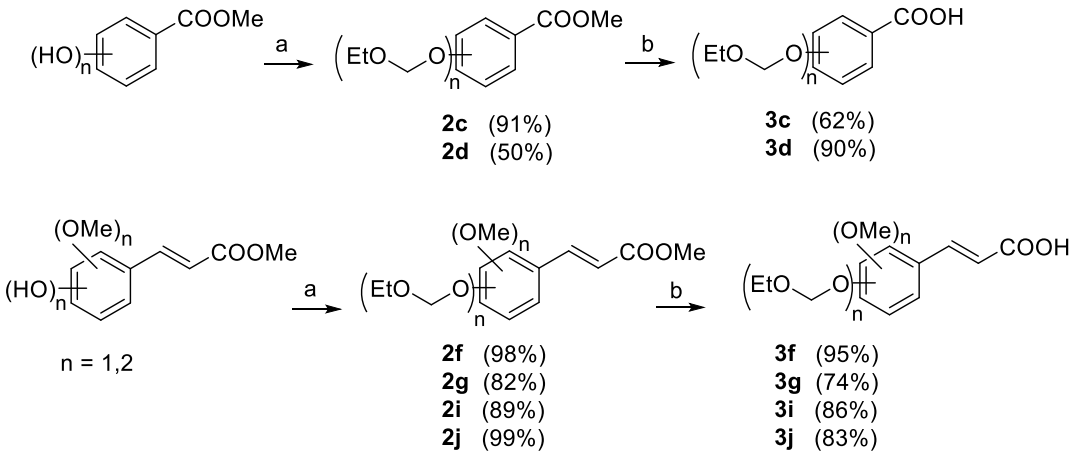
2.1 Synthesis of azetidinones

All target compounds were prepared with a convergent synthetic strategy that comprised the preparation of the 3-hydroxyethyl-4-alkylidene β-lactam **A**, obtained in turn from the commercially available (3*R*,4*R*)-4-acetoxy-3-[(1*R*)-1-(tert-butyldimethylsilyloxy) ethyl]azetidin-2-one and benzyl diazoacetate [23], and its esterification with polyphenolic acids duly protected on the phenolic OHs (Scheme 1).



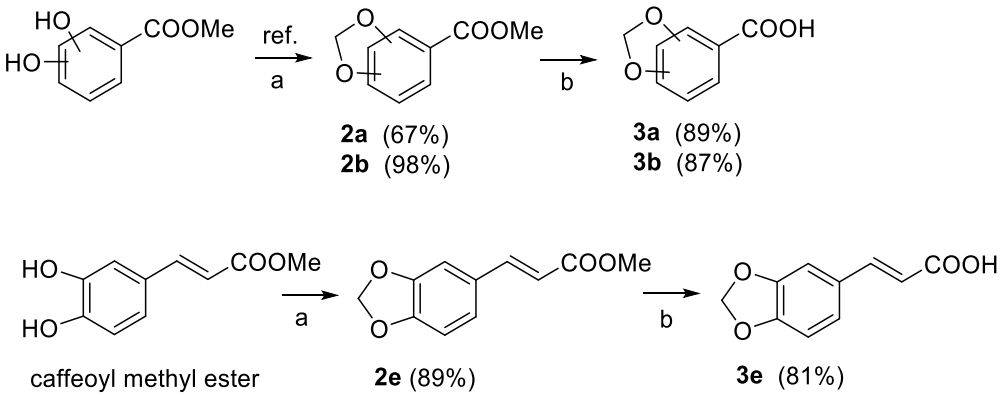
Scheme 1. Convergent synthesis of new 4-alkylidene β-lactams with polyphenolic residues.

The O-protected aryl acids were prepared in a two-step procedure starting from their corresponding methyl esters exploiting a phenolic oxygen protection with chloromethylethylether (EOM) followed by alkaline hydrolysis (Scheme 2), according to what previously optimized for the O-protected caffeic acid [20].



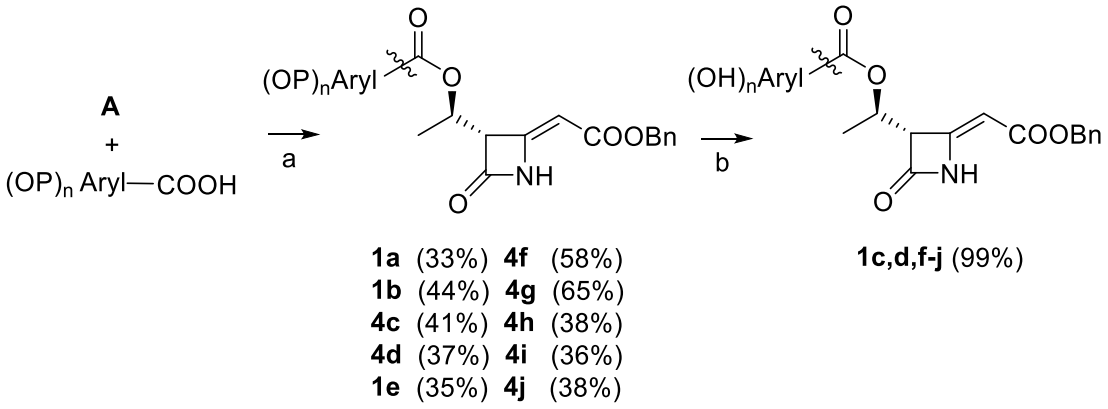
Scheme 2. Synthesis of O-protected phenolic acids; a) NaH, EOMCl, THF, 0°C to rt; b) NaOH 5M, THF/MeOH 1:1, 40°C. Yields of isolated products after flash chromatography reported in brackets. Letters in compound numbering referred to the substituents as in Chart 1 for target products.

The EOM ether turned out to be an effective protective group that can be easily inserted, and selectively removed at the late stage of the synthesis with a mild procedure without damaging the β-lactam ring in final compounds. The dioxymethylene-group was inserted on the suitable methyl esters (i.e. 2,3-dihydroxy-methyl-benzoate, 3,4-dihydroxy-methyl-benzoate), with diiodomethane and potassium carbonate as reported by Alam et al. (Scheme 3) [31].



Scheme 3. Synthesis of methylenedioxy-protected acids; a) CH_2I_2, K_2CO_3, DMF, 110°C; b) NaOH 5M, THF/MeOH 1:1, 40°C. Yields of isolated products after flash chromatography in brackets. Letters in compound numbering referred to the substituents as in Chart 1 for target products.

The 3-hydroxyethyl side chain of β-lactam **A** was then derivatized as phenolic or polyphenolic aromatic ester with the appropriate O-protected acid **3a-j** by treatment with *N,N'*-dicyclohexylcarbodiimide (DCC) and a catalytic amount of 4-dimethylaminopyridine (DMAP) in dichloromethane (Scheme 4) according to the Steglich procedure [32]. Yields were slightly affected by this step probably due to a low reactivity of the secondary alcohol of the intermediate **A** due to a steric hindrance that could obstruct the nucleophilic attack to the acid-DCC adduct. Esters **4c, 4d**, and **4f-j** were finally treated with trifluoroacetic acid (TFA) to eliminate the EOM protection [20], affording the target β-lactams **1c**, **1d**, and **1f-j** in quantitative yields.



Scheme 4. Synthesis of target compounds a) DCC, DMAP, DCM, 0°C to rt; b) CF_3COOH, DCM, 0°C to rt. Yields of isolated compounds are reported in brackets.

2.2 Antibacterial activity

Antibacterial activity screening of compounds **1a-j** was carried out against recent, well characterized clinical isolates. A selection of Gram-positive and Gram-negative bacterial pathogens used for the *in vitro* antimicrobial susceptibility testing included: 11 Gram-positive (8 staphylococci and 3 enterococci) and 5 Gram-negative strains.

Most of the bacterial strains were specifically selected to exhibit a multidrug-resistant phenotype against penicillins/cephalosporins, linezolid, or vancomycin. Antimicrobial activities of the compounds are listed in Table 2, with potency being showed as minimum inhibitory concentration values (MICs) expressed in μg/mL, and only MIC values equal to or less than 128 μg/mL against corresponding bacterial species were reported. Commercially available vancomycin and cefuroxime were used as reference compounds in antibacterial susceptibility testing.

The *in vitro* antimicrobial susceptibility analysis indicated antibacterial activity only against Gram-positive bacteria, while none of the tested compounds exhibited an appreciable antibiotic effect against studied Gram-negative bacteria (results not included in Table 2 as for them MIC values of tested compounds were > 128 μg/mL). Recently, lauryl ester of ferulic acid showed antibacterial activity rather toward *E. coli* than *S. aureus* in agar diffusion assays, but here the presence of ferulic moiety (compound **1i**) was not able to determine antibacterial activity against Gram-negative bacteria [33].

Table 1 MIC values (μg/mL) for compounds **1a-j**, vancomycin (VA) and cefuroxime (FUR) as reference compounds.

Organism ID	Phenotype	1a	1b	1c	1d	1e	1f	1g	1h	1i	1j	VA	FUR
S. aureus ATCC *29213*	CLSI control	2	16	2	16	>128	8	2	>128	4	>128	0.5	2
S. aureus SAU-1	LZD-R MRSA	2	2	2	16	>128	4	1	>128	4	8	1	>128
S. aureus 44674	MRSA	4	>128	>128	8	>128	4	>128	>128	4	8	4	8
S. aureus 69856	MSSA	8	>128	>128	4	>128	8	>128	>128	8	>128	0.5	1
S. aureus 39249	MSSA	4	>128	>128	4	>128	4	>128	>128	8	8	2	2
S. hominis α26	LZD-R MRSH	4	8	16	>128	128	4	2	>128	4	4	1	8
S. epidermidis G1027	LZD-R MRSE	4	>128	>128	16	>128	8	>128	>128	4	>128	2	32
S. epidermidis 3226	MSSE	8	>128	>128	16	>128	8	>128	>128	8	>128	1	0.5
E. faecalis ATCC *29212*	CLSI control	>128	>128	>128	128	>128	>128	>128	>128	>128	>128	2	>128*
E. faecalis 4150	all-S	>128	>128	>128	128	>128	64	>128	>128	8	>128	0.25	>128*
E. faecium VRE 2	LZD-R VRE	>128	>128	>128	128	>128	>128	>128	>128	>128	>128	>128	>128*

* enterococci are inherently resistant to cephalosporins such as cefuroxime.

However, the lack of activity against Gram-negative of the tested compounds confirmed a general trend of this class of monocyclic β-lactam derivatives as previously observed [19-21]. This inefficiency could be due to a reduced uptake through the outer membrane of Gram-negative bacteria, and unfortunately, the presence of one cathecol moiety, as in compounds reported here, was not sufficient to allow bacterial cell penetration via the siderophore receptors of Gram-negative bacteria [34, 35].

On the contrary, compounds **1a**, **1f** and **1i** produced significant antibacterial activity against the tested Gram-positive bacteria grouped in staphylococci with MIC values between 2 and 8 μg/mL (Table 1). Other compounds, such as **1b**, **1c 1g** and **1j** showed a selective activity towards some *S. aureus* resistant strains (MICs 1-16 μg/mL). Compound **1g** showed the most prominent activity against strains of *S. aureus* ATCC 29213, *S. aureus* SAU-1, and *S. hominis* α26, the latter two strains characterized by an extensive condition of multi drug resistance (MDR) and high resistance to linezolid (MIC values 2, 1, and 2 μg/mL, respectively). It is noteworthy that against

S. aureus SAU-1 **1g** was as active as vancomycin, whereas cefuroxime, a second-generation cephalosporin, were completely inactive.

Among the three methylenedioxy-derivatives, **1a** was the most active, **1e** completely inactive, whereas **1b** with an oxygen atom at an orto position, resulted in a selective activity against *S. aureus* ATCC 29213 and *S. aureus* SAU-1. The association of orto substitution *vs* selectivity was observed also for azetidinones **1f** vs **1g**.

The cathecol- and caffeoyl-derivatives **1d** and **1h** possessing two vicinal OH groups, are less or no active, whereas the monomethylether **1i** recovers an appreciable activity, as well as the coumaroyl derivative **1f** (against staphylococci MICs 4-8 μg/mL, respectively). This result is consistent with some data recently reported for some xanthone derivatives where a loss of antibacterial activity of OH phenolics compared to the corresponding isoprenyl ethers was reported [36]. Relying on the cinnamyl series, the most and the less lipophilic compounds **1e** and **1h** were both inactive, whereas compounds with an intermediate lipophilicity **1f**, **1g**, **1i**, and **1j** showed good activities.

Except for compounds **1f** and **1i,** active also against *E. faecalis* 4150 strain (MIC values = 64 and 8 μg/mL, respectively), all other compounds exhibited an exclusively anti-staphylococcal activity.

2.3 Antioxidant activity: TEAC-DPPH and TEAC-ABTS evaluations.

Antioxidant activity determination based on different approaches was carried out, considering the importance of a multidimensional evaluation of the antioxidant activity [37]. With the objective to ensure a better comparison of the results and covering a wider range of possible applications of novel molecules, the use of more than a single method – employing radicals such as DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) - is highly advisable [38]. Therefore, the β-lactams were then subjected to TEAC (trolox equivalent antioxidant capacity) assays, based on the ability of the compounds to scavenge the DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) radical cations, compared to the same scavenging ability of Trolox®, a water-soluble derivative of vitamin E with potent antioxidant properties [39].

Compounds **1b** and **1e** were not tested because the absence of OH groups precludes from antioxidant activity, as obtained for **1a** (Table 2). The presence of one OH as in compounds **1c**, **1f**, and **1g** was not enough to give radical-scavenging ability. The best performances were instead recorded with compounds with a cathecol group **1d** and **1h** in both the assays (Table 2). In particular, **1d** and **1h** showed antioxidant capacity with DPPH assay 2.6 and 1.7 times respectively more effective than that expressed by 1 mmol of Trolox® as reference compound. Analogously, **1d** and **1h** evidenced the same outcomes with ABTS test, even if with lower values than those of TEAC-DPPH. The quantitative difference is due to the different kind of radical employed, but it should be however pointed out that for both the assays the 1,2 dihydroxy groups provided in those azetidinones excellent values of antioxidant capacity. Moreover, the **1d** and **1h** data are comparable with those expressed by natural compounds known for their important applications thanks to their valuable antioxidant properties, i.e. ascorbic acid and α-tocopherol [40].

Table 2 Antioxidant activity of eight β-lactam derivatives based on DPPH and ABTS assays. The results are expressed as mmol of Trolox Equivalent Antioxidant Capacity (TEAC) ± Standard Deviation (SD).

Entry	Compound	TEAC-DPPH (mmol eq. Trolox ± SD)	TEAC-ABTS (mmol eq. Trolox ± SD)
1	**1a**	0.012 ± 0.001	nd
2	**1c**	0.007 ± 0.001	0.010 ± 0.001
3	**1d**	2.590 ± 0.220	1.300 ± 0.138
4	**1f**	0.003 ± 0.001	0.053 ± 0.006
5	**1g**	0.002 ± 0.001	0.143 ± 0.015
6	**1h**	1.670 ± 0.178	1.030 ± 0.110
7	**1i**	0.142 ± 0.015	0.284 ± 0.030
8	**1j**	0.191 ± 0.020	0.232 ± 0.025

nd = not detectable

Values obtained for the other compounds - ranging from 0.284 ± 0.030 to 0.002 ± 0.001 are very low and not significant for antioxidant applications, even though ferulic and sinapic esters performed a little bit better than the others, in line with results regarding some polyphenolic-β-lactams, as previously reported [20, 22].

It would be interesting now to compare the most active compounds reported here, with some monocyclic β-lactams with polyphenolic esters on the 3-hydroxylethyl side-chain previously studied

with reference to their antibacterial and antioxidant activities (Chart 2). Compounds **A** is a 4-alkylideneazetidinone syringoyl ester which showed a good antibacterial activity against MRSA (MICs range 1-64 μg/mL) strains [19], but the syringic moiety get scarce antioxidant potency as observed for compound **D** (TEAC 0.037 mmol equiv. TROLOX) [20]. Compounds **B** and **C** are good antioxidants (TEAC 1.23 and 0.98 mmol equiv. TROLOX, respectively) but have a lower antibacterial activity against MRSA (MICs 32-128 and 16-128 μg/mL, respectively) [20]. It is important to highlight that in this class of monocyclic β-lactam derivatives the antibacterial potency is mainly activated by the presence of a good electron withdrawing group on the C4 position of the β lactam ring such as 4-alkylidene- or 4-acetoxy groups. Whenever such substituents were absent, as in compounds **E** and **F**, the antibacterial potency was completely lost [41], notwithstanding the presence of cinnamoyl esters which in some other derivatives showed a certain potency [28]. Finally compounds **1a**, **1d**, and **1i** of the present work are good anti-MRSA agents with a narrower MIC range than compound **A**, and **1d** showed also a very good antioxidant capacity (TEAC 2.59 mmol equiv. TROLOX).

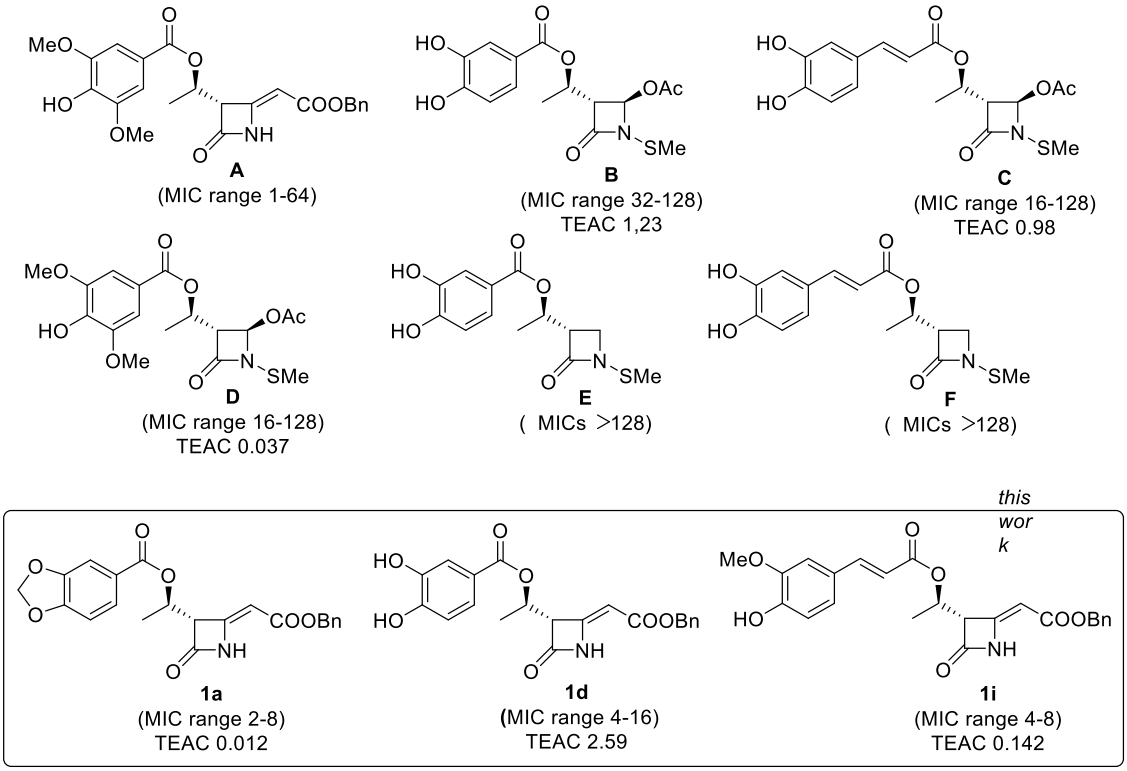


Chart 2. Best antibacterial and antioxidant activities of the compounds reported in this work in comparison with data obtained for monocyclic β-lactam derivatives previously studied [19, 20]. In bracket MIC ranges in μg/mL toward MRSA strains, and TEAC-DDPH expressed as mmol eq. Trolox.

3. Conclusions

The synthesis of new 4-alkyliden-β-lactams functionalized with some plant phenolic compounds was accomplished through a convergent synthetic strategy. The new derivatives were tested *in vitro* for antibacterial and antioxidant activities. Molecules with a good antibacterial potency against multi drug resistant staphylococcal strains were found. Moreover, compounds with a catechol moiety on the side-chain showed anti-radical capacity against DPPH and ABTS, with TEAC values 2.5 times higher than those known for compounds currently used as antioxidants (for e.g. Vitamin E, citric acid). The present study reinforces the idea that phytochemicals moieties can be used for the development of new 4-alkyliden-β-lactams that combine in one molecule the antibacterial and the antioxidant activities thus realizing dual-active compounds with a synergistic action for the treatment of those infections where the oxidative stress could increase specific resistant variants [42].

4. Experimental Section

4.1. General

Commercial reagents (reagent grade, >99%) were used as received without additional purification. Anhydrous solvents (CH_2Cl_2, THF, MeOH, DMF) were obtained commercially. All reactions were performed under an inert atmosphere (N_2) unless unspecified. ^{1}H and ^{13}C NMR spectra were recorded with an INOVA 400 instrument with a 5 mm probe. All chemical shifts are quoted relative to deuterated solvent signals (δ in ppm and J in Hz). Polarimetric Analyses were conducted on Unipol L 1000 "Schmidt-Haensch" Polarimeter at 598 nm. FTIR spectra: Brucker instrument, measured as films between NaCl plates; wave numbers are reported in cm^{-1}. The purities of the target compounds were assessed as being >95% using HPLC-MS. Elemental analysis were performed on a Thermo Flash 2000 CHNS/O Analyzer. HPLC-MS: Agilent Technologies HP1100 instrument, equipped with a ZOBRAX-Eclipse XDB-C8 Agilent Technologies column; mobile phase: H_2O/CH_3CN, 0.4 mL/min, gradient from 30 to 80% of CH_3CN in 8 min, 80% of CH_3CN until 25 min, coupled with an Agilent Technologies MSD1100 single-quadrupole mass spectrometer, full scan mode from m/z = 50 to 2600, in positive ion mode. Elemental analysis were performed on a Thermo Flash 2000 CHNS/O analyzer.

4.2 Synthesis

Starting polyhydroxy benzoic and polyhydroxy cinnamic methyl esters are all known and were prepared according to reported procedures with methanol and sulfuric acid as catalyst [43]. EOM-protected esters **2a**, **2b**, **2e** are known and were prepared by following a published procedure [31]; acids **3a**, **3b**, and **3e** are known; alcohol **A**, ester **2h** and acid **3h** were prepared as previously reported [20].

General procedure for EOM-protection (GP1). A solution of the desired methyl ester (1equiv) in THF (1.5mL/mmol) was added dropwise to a suspension of NaH (60% in mineral oil) in THF (5mL/mmol) at 0°C under inert atmosphere. After 10 min a solution of chloromethylethylether (EOMCl) in THF (2.2mL/mmol) 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 a saturated $NaHCO_3$ solution until pH 10 at 0°C and extracted with EtOAc (3 x 15 mL). The organic extracts were dried over Na_2SO_4 and concentrated to obtain the desired EOM-protected compound (**2c**, **2d**, **2f**, **2g**, **2i** or **2j**). The crude was purified by column chromatography only when specified.

General procedure for hydrolysis of methyl esters (GP2). A stirred solution of the desired methyl ester (**2c**, **2d**, **2f**, **2g**, **2i** or **2j**) in a mixture of THF/MeOH 4:1 (4mL/mmol) was treated with 5 M NaOH (1mL/mmol). The reaction mixture was heated at 40°C until consumption of the starting material. At completion, EtOAc (10 mL) was added and the organic phase was separated and discarded. The aqueous phase was then cooled to 0°C, adjusted to pH 5 with HCl 1M and then extracted with EtOAc (3 x 15 mL). The collected organic layers were dried on Na_2SO_4 and concentrated to afford the corresponding carboxylic acid (**3c**, **3d**, **3f**, **3g**, **3i** or **3j**).

General procedure for alcohol-acid coupling (GP3). To a solution of alcohol **A** (1equiv), in CH_2Cl_2 (10mL/mmol) under inert atmosphere, the desired acid (**3a-j**) (1.58equiv) and DMAP (0.2equiv) were added. The mixture was then cooled to 0°C and DCC (1.58equiv) was added; the system was allowed to reach room temperature in 15 min and left under stirring overnight. After 16h (TLC monitoring) the reaction mixture was quenched with water at 0°C and extracted with CH_2Cl_2 (3 x 15 mL). The collected organic phases were dried on Na_2SO_4 and evaporated. The residue was treated with EtOAc (2 x 20 mL) and filtered to separate dicycloexylurea precipitate. The solution was then evaporated and purified by flash-chromatography to afford the desired β-lactams functionalized with O-protected polyphenolic residues (**1a**, **1b**, **1e**, **4c**, **4d** or **4f-j**).

General procedure for EOM-deprotection (GP4). A stirred solution of the EOM-protected β-lactam (**4c**, **4d**, **4f**, **4g**, **4i**, **4h** or **4j**) in CH_2Cl_2 under inert atmosphere was treated with aliquots of trifluoroacetic acid at 0°C every 60 min. After reaction completion (TLC monitoring), the solvent and the trifluoroacetic acid were evaporated to obtain the phenolic compounds (**1c**, **1d**, **1f**, **1g**, **1i**, **1h** or **1j**).

4.2.1. Methyl 4-(ethoxymethoxy)benzoate (**2c**)

Following GP1, *p*-hydroxybenzoic acid methyl ester (395mg, 2.60mmol), NaH (208mg, 5.20mmol) and EOMCl (482µL, 5.20mmol) yielded compound **2c** (502mg, 91% yield) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.21 (t, *J* = 7.1 Hz, 3H), 3.72 (q, *J* = 7.1 Hz, 2H), 3.87 (s, 3H), 5.26 (s, 2H), 7.05 (d, *J* = 8.9 Hz, 2H), 7.97 (d, *J* = 8.9 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 15.0, 51.8, 64.5, 92.8, 115.6, 123.4, 131.4, 161.1, 166.7; HPLC-MS (ESI^+): R_t = 8.83 min; m/z = 211 $[M+H]^+$; IR (film, cm^{-1}): 2978, 1720, 1607, 1510, 1435, 1279.

4.2.2. Methyl 3,4-bis(ethoxymethoxy)benzoate (**2d**)

Following GP1, 3,4-dihydroxy-benzoic acid methyl ester (193mg, 1.15mmol), NaH (184mg, 4.60mmol) and EOMCl (427µL, 4.60mmol) yielded compound **2d** (163mg, 50% yield) as a colorless oil after flash chromatography purification (cyclohexane/EtOAc 3:1). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.18 – 1.25 (m, 6H), 3.72 – 3.81 (m, 4H), 3.88 (s, 3H), 5.32 (d, *J* = 9.6 Hz, 4H), 7.20 (d, *J* = 8.6 Hz, 1H), 7.66 – 7.71 (m, 1H), 7.80 – 7.83 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 15.0, 52.0, 64.5, 64.6, 93.6, 94.1, 115.1, 117.7, 123.9, 124.7, 146.6, 151.5, 166.6; HPLC-MS (ESI^+): R_t = 9.51 min; m/z = 285 $[M+H]^+$; IR (film, cm^{-1}): 2978, 1719, 1602, 1510, 1440, 1290.

4.2.3. (E)-methyl 3-(4-(ethoxymethoxy)phenyl)acrylate (**2f**)

Following GP1, methyl 3-(4-hydroxyphenyl)acrylate (523mg, 2.93mmol), NaH (235mg, 5.88mmol) and EOMCl (546µL, 5.88mmol) yielded compound 2f (701mg, 98% yield) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.20 (t, *J* = 7.0 Hz, 3H), 3.70 (q, *J* = 7.0 Hz, 2H), 3.77 (s, 3H), 5.22 (s, 2H), 6.31 (d, *J* = 16.0 Hz, 1H), 7.02 (d, *J* = 8.7 Hz, 2H), 7.44 (d, *J* = 8.7 Hz, 2H), 7.63 (d, *J* = 16.0 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 15.0, 51.4, 64.4, 92.8, 115.6, 116.4, 127.9, 129.5, 144.3, 159.1, 167.5; HPLC-MS (ESI^+): R_t = 10.79 min; m/z = 237 $[M+H]^+$; IR (film, cm^{-1}): 2950, 1714, 1634, 1604, 1511, 1434, 1230.

4.2.4. (E)-methyl 3-(2-(ethoxymethoxy)phenyl)acrylate (**2g**)

Following GP1, methyl (2E)-3-(2-hydroxyphenyl)-2-propenoate (539mg, 3.03mmol), NaH (242mg, 6.06mmol) and EOMCl (562µL, 6.06mmol) yielded compound **2g** (584mg, 82% yield) as a colorless oil after flash chromatography (cyclohexane/EtOAc 7:3). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.22 (t, *J* = 7.1 Hz, 3H), 3.75 (q, *J* = 7.1 Hz, 2H), 3.81 (s, 3H), 5.30 (s, 2H), 6.50 (d, *J* = 16.2 Hz, 1H), 6.99 – 7.03 (m, 1H), 7.19 (dd, *J* = 8.4, 0.8 Hz, 1H), 7.31 – 7.35 (m, 1H), 7.53 (dd, *J* = 7.7, 1.6 Hz, 1H), 8.03 (d, *J* = 16.2 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 15.1, 51.6, 64.6, 93.2, 114.8, 118.3, 121.7, 124.0, 128.4, 131.5, 140.1, 156.2, 167.8; HPLC-MS (ESI^+): R_t = 9.41 min; m/z = 237 $[M+H]^+$; IR (film, cm^{-1}): 2961, 1716, 1636, 1601, 1511, 1448, 1251.

4.2.5. (E)-methyl 3-(4-(ethoxymethoxy)-3-methoxyphenyl)acrylate (**2i**)

Following GP1, methyl 3-(4-hydroxy-3-methoxyphenyl)acrylate (139mg, 0.67mmol), NaH (67mg, 1.68mmol) and EOMCl (124µL, 1.34mmol) yielded compound 2i (159mg, 89% yield) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.17 (t, *J* = 7.1 Hz, 3H), 3.70 (q, *J* = 7.1 Hz, 2H), 3.74 (s, 3H), 3.85 (s, 3H), 5.25 (s, 2H), 6.28 (d, *J* = 15.9 Hz, 1H), 7.01 – 7.13 (m, 3H), 7.58 (d, *J* = 15.9 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 14.9, 51.4, 55.7, 64.3, 93.7, 110.2, 115.7, 115.8, 122.0, 128.4, 144.5, 148.6, 149.6, 167.3; HPLC-MS (ESI^+): R_t = 8.67 min; m/z = 267 $[M+H]^+$; IR (film, cm^{-1}): 2923, 1702, 1637, 1582, 1510, 1461, 1256.

4.2.6. (E)-methyl 3-(4-(ethoxymethoxy)-3,5-dimethoxyphenyl)acrylate (**2j**)

Following GP1, methyl 3-(4-hydroxy-3,5-dimethoxyphenyl)acrylate (470mg, 1.97mmol), NaH (197mg, 4.93mmol) and EOMCl (366µL, 3.94mmol) yielded compound **2j** (582mg, 99% yield) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.13 (t, *J* = 7.1 Hz, 3H), 3.72 (s, 3H), 3.79 (s, 6H), 3.81 (q, *J* = 7.1 Hz, 2H), 5.11 (s, 2H), 6.28 (d, *J* = 15.9 Hz, 1H), 6.68 (s, 2H), 7.53 (d, *J* = 15.9 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 14.8, 51.4, 55.8, 64.7, 96.4, 105.0, 116.9, 130.1, 136.5, 144.7, 153.4, 167.1; HPLC-MS (ESI^+): R_t = 8.72 min; m/z (%) = 297 $[M+H]^+$; IR (film, cm^{-1}): 2926, 1714, 1636, 1587, 1505, 1454, 1277.

4.2.7. 4-(ethoxymethoxy)benzoic acid (**3c**)

Following GP2, ester **2c** (210mg, 1.00mmol) yielded acid **3c** (121mg, 62% yield) as a white solid. ^{1}H NMR (400 MHz, CD_3OD) δ (ppm) 1.19 (t, *J* = 7.1 Hz, 3H), 3.72 (q, *J* = 7.1 Hz, 2H), 5.30 (s, 2H), 7.08 (d, *J* = 8.8 Hz, 2H), 7.96 (d, *J* = 8.8 Hz, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ (ppm) 15.5, 65.5, 93.9, 116.7, 125.0, 132.7, 162.7, 169.7; HPLC-MS (ESI^-): R_t = 5.85 min; m/z = 195 $[M-H]^+$; IR (film, cm^{-1}): 3418, 2983, 1688, 1603, 1427, 1291; m.p. = 118- 119 °C.

4.2.8. 3,4-bis(ethoxymethoxy)benzoic acid (**3d**)

3d. Following GP2, ester **2d** (163mg, 0.57mmol) yielded acid **3d** (138mg, 90% yield) as a waxy white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.18 – 1.24 (m, 6H), 3.72 – 3.80 (m, 4H), 5.31 (s, 2H), 5.34 (s, 2H), 7.22 (d, *J* = 8.6 Hz, 1H), 7.75 (d, *J* = 8.6 Hz, 1H), 7.88 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 15.0, 64.6, 64.7, 93.6, 94.1, 115.0, 118.2, 123.0, 125.5, 146.6, 152.2, 171.6; HPLC-MS (ESI^+): R_t = 10.02 min; m/z = 288 $[M+H_2O]^+$; IR (film, cm^{-1}): 3404, 2976, 1687, 1599, 1512, 1443, 1296.

4.2.9. (E)-3-(4-(ethoxymethoxy)phenyl)acrylic acid (**3f**)

Following GP2, ester **2f** (236mg, 1mmol) yielded acid **3f** (212mg, 95% yield) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.23 (t, *J* = 7.1 Hz, 3H), 3.73 (q, *J* = 7.1 Hz, 2H), 5.26 (s, 2H), 6.33 (d, *J* = 15.9 Hz, 1H), 7.06 (d, *J* = 8.6 Hz, 2H), 7.50 (d, *J* = 8.6 Hz, 2H), 7.74 (d, *J* = 15.9 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 15.1, 64.5, 92.9, 115.1, 116.5, 127.7, 130.0, 146.6, 159.5, 172.5; HPLC-MS (ESI^+): R_t = 6.76 min; m/z = 223 $[M+H]^+$; IR (film, cm^{-1}): 3424, 2973, 1670, 1627, 1603, 1511, 1429, 1264; m.p. = 152 - 154°C.

4.2.10. (E)-3-(2-(ethoxymethoxy)phenyl)acrylic acid (**3g**)

Following GP2, ester **2g** (236mg, 1mmol) yielded acid **3g** (164mg, 74% yield) as a waxy white solid. ^{1}H NMR (400 MHz, CD_3OD) δ (ppm) 1.16 (t, *J* = 7.1 Hz, 3H), 3.70 (q, *J* = 7.1 Hz, 2H), 5.27 (s, 2H), 6.49 (d, *J* = 16.2 Hz, 1H), 6.98 (t, *J* = 7.5 Hz, 1H), 7.15 (d, *J* = 8.3 Hz, 1H), 7.30 (t, *J* = 7.8 Hz, 1H), 7.55 (d, *J* = 7.7 Hz, 1H), 8.01 (d, *J* = 16.2 Hz, 1H); ^{13}C NMR (100 MHz, CD_3OD) δ (ppm) 15.4, 65.6, 94.4, 116.0, 119.5, 122.9, 125.1, 129.3, 132.6, 141.4, 157.3, 170.7; HPLC-MS (ESI^-): R_t = 6.96 min; m/z = 221 $[M-H]^+$; IR (film, cm^{-1}): 3419, 2974, 1677, 1634, 1601, 1510, 1435, 1271.

4.2.11. (E)-3-(4-(ethoxymethoxy)-3-methoxyphenyl)acrylic acid (**3i**)

Following GP2, ester **2i** (267mg, 1mmol) yielded acid **3i** (217mg, 86% yield) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.22 (t, *J* = 7.1 Hz, 3H), 3.77 (q, *J* = 7.1 Hz, 2H), 3.91 (s, 3H), 5.32 (s, 2H), 6.33 (d, *J* = 15.9 Hz, 1H), 7.08 – 7.21 (m, 3H), 7.73 (d, *J* = 15.9 Hz, 1H); ^{13}C NMR (100MHz, $(CD_3)_2CO$) δ (ppm) 15.4, 56.2, 64.9, 94.6, 111.9, 117.3, 117.4, 123.0, 129.8, 145.5, 149.7, 151.4, 167.9; HPLC-MS (ESI^+): R_t = 5.90 min; m/z = 253 $[M+H]^+$; IR (film, cm^{-1}): 3423, 2904, 1671, 1625, 1583, 1509, 1424, 1254; m.p. = 131 - 133°C.

4.2.12. (E)-3-(4-(ethoxymethoxy)-3,5-dimethoxyphenyl)acrylic acid (**3j**)

Following GP2, ester **2j** (237mg, 0.8mmol) yielded acid **3j** (187mg, 83% yield) as a white solid. ^{1}H NMR (400 MHz, $(CD_3)_2CO$) δ (ppm) 1.14 (t, *J* = 7.1 Hz, 3H), 3.85 (q, *J* = 7.1 Hz, 2H), 3.90 (s, 6H), 5.10 (s, 2H), 6.50 (d, *J* = 15.9 Hz, 1H), 7.03 (s, 2H), 7.61 (d, *J* = 15.9 Hz, 1H); ^{13}C NMR (100 MHz, $(CD_3)_2CO$) δ (ppm) 15.4, 56.2, 64.9, 94.6, 111.9, 117.2, 117.3, 123.0, 129.7, 145.5, 149.7, 151.4, 168.2; HPLC-MS (ESI^+): R_t = 6.44 min; m/z = 283 $[M+H]^+$; IR (film, cm^{-1}): 3423, 2921, 1675, 1627, 1449, 1250; m.p. = 110 - 112°C.

4.2.13. (R)-1-((S,Z)-2-(2-(benzyloxy)-2-oxoethylidene)-4-oxoazetidin-3-yl)ethylbenzo[d][1,3]dioxole-5-carboxylate (**1a**)

Following GP3, alcohol **A** (55mg, 0.21mmol) and acid **3a** (55mg, 0.33mmol) yielded compound **1a** (28mg, 33% yield) as a waxy solid after flash chromatography purification (CH_2Cl_2/acetone 99:1). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.50 (d, *J* = 6.4 Hz, 3H), 4.00 (d, *J* = 6.8 Hz, 1H), 5.14 (d, J_{AB} = 12.3 Hz, 1H), 5.19 (d, J_{AB} = 12.3 Hz, 1H), 5.25 (s, 1H), 5.45 (quintet, *J* = 6.4 Hz, 1H), 6.04 (s, 2H), 6.83 (d, *J* = 8.2 Hz, 1H), 7.30 – 7.39 (m, 5H), 7.42 (d, *J* = 1.5 Hz, 1H), 7.62 (dd, *J* = 1.5, 8.2 Hz, 1H), 8.65 (bs, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 18.0, 61.6, 66.2, 67.3, 90.6, 101.8, 108.1, 109.5, 123.5, 125.6, 128.2, 128.3, 128.6, 135.7, 147.8, 151.9, 152.1, 164.5, 164.6, 166.6; HPLC- MS (ESI^+) R_t = 10.08 min; m/z = 427 $[M+H_2O]^+$; IR (film, cm^{-1}): 3286, 2980, 1820, 1701, 1657, 1489, 1259; $[\alpha]^{20}_D$ = - 0.24 (c = 0.8, CH_2Cl_2); found C, 64.72; H, 4.65; N, 3.40%; $C_{22}H_{19}NO_7$ requires C, 64.54; H, 4.68; N, 3.42%.

4.2.14. (R)-1-((S,Z)-2-(2-(benzyloxy)-2-oxoethylidene)-4-oxoazetidin-3-yl)ethyl benzo[d][1,3]dioxole-4-carboxylate (**1b**)

Following GP3, alcohol **A** (35mg, 0.13mmol) and acid **3b** (34mg, 0.21mmol) yielded compound 1**b** (24mg, 44% yield) as a waxy solid after flash chromatography purification (CH_2Cl_2/acetone 99:1). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.53 (d, *J* = 6.4 Hz, 3H), 3.99 (d, *J* = 6.7 Hz, 1H), 5.17 (s, 2H), 5.29 (s, 1H), 5.46 (quintet, *J* = 6.4 Hz, 1H), 5.97 – 6.01 (m, 2H), 6.85 (t, *J* = 8.0 Hz, 1H), 6.96 (dd, *J* = 1.1, 7.7 Hz, 1H), 7.30 – 7.38 (m, 6H), 8.67 (bs, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 18.3, 61.5, 66.1, 67.7, 90.7, 101.8, 112.4, 112.5, 121.3, 122.5, 128.2, 128.3, 128.6, 135.8, 148.7, 148.8, 152.2, 163.2, 164.6, 166.7; HPLC-MS (ESI$^+$): R_t =10.00 min; m/z = 427 $[M+H_2O]^+$; IR (film, cm^{-1}): 3287, 2924, 1820, 1700, 1656, 1457, 1288; $[\alpha]^{20}_D$ = - 0.17 (c=1.2, CH_2Cl_2); found C, 64.69 ; H, 4.70; N, 3.41%; $C_{22}H_{19}NO_7$ requires C, 64.54; H, 4.68; N, 3.42%.

4.2.15. (R)-1-((S,Z)-2-(2-(benzyloxy)-2-oxoethylidene)-4-oxoazetidin-3-yl)ethyl 4-(ethoxymethoxy)benzoate (**4c**)

Following GP3, alcohol **A** (39mg, 0.15mmol) and acid **3c** (47mg, 0.24mmol) yielded compound **4c** (27mg, 41% yield) as a waxy solid after flash chromatography purification (CH_2Cl_2/acetone 99:1). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.22 (t, *J* = 7.0 Hz, 3H), 1.51 (d, *J* = 6.3 Hz, 3H), 3.73 (q, *J* = 7.0 Hz, 2H), 4.01 (d, *J* = 6.7 Hz, 1H), 5.15 (d, J_{AB} = 12.3 Hz, 1H), 5.19 (d, J_{AB} = 12.3 Hz, 1H), 5.26 (s, 1H), 5.27 (s, 2H), 5.48 (quintet, *J* = 6.3 Hz, 1H), 7.06 (d, *J* = 8.7 Hz, 2H), 7.32 – 7.40 (m, 5H), 7.96 (d, *J* = 8.7 Hz, 2H), 8.67 (bs, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 15.0, 18.1, 61.6, 64.6, 66.2, 67.1, 90.6, 92.8, 115.8, 122.9, 128.2, 128.3, 128.6, 131.6, 135.8, 152.2, 161.4, 164.6, 164.9, 166.7; HPLC-MS (ESI$^+$): R_t = 10.86 min; m/z = 457 $[M+H_2O]^+$; IR (film, cm^{-1}): 3300, 2926, 1821, 1702, 1656, 1606, 1509, 1270; $[\alpha]^{20}_D$ = - 0.10 (c=1.05, CH_2Cl_2).

4.2.16. (R)-1-((S,Z)-2-(2-(benzyloxy)-2-oxoethylidene)-4-oxoazetidin-3-yl)ethyl-3,4-bis(ethoxymethoxy) benzoate (**4d**)

Following GP3, alcohol **A** (60mg, 0.23mmol) and acid **3d** (98mg, 0.36mmol) yielded compound **4d** (44mg, 37% yield) as a yellow oil after flash chromatography purification (CH_2Cl_2/diethyl ether 95:5). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.20 (t, *J* = 7.0 Hz, 3H), 1.21 (t, *J* = 7.0 Hz, 3H), 1.51 (d, *J* = 6.4 Hz, 3H), 3.72 – 3.81 (m, 4H), 4.00 (d, *J* = 6.7 Hz, 1H), 5.13 (d, J_{AB} = 12.3 Hz, 1H), 5.18 (d, J_{AB} = 12.3 Hz, 1H), 5.26 – 5.33 (m, 5H), 5.43 (quintet, *J* = 6.4 Hz, 1H), 7.20 (d, *J* = 8.6 Hz, 1H), 7.30 – 7.35 (m, 5H), 7.65 (dd, *J* = 1.8, 8.6 Hz, 1H), 7.81 (d, *J* = 1.8 Hz, 1H), 8.76 (bs, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 14.99, 15.01, 18.2, 61.6, 64.5, 64.6, 66.1, 67.3, 90.6, 93.5, 94.0, 115.1, 117.7, 123.3, 124.7, 128.2, 128.3, 128.6, 135.7, 146.7, 151.7, 152.2, 164.7, 164.8, 166.6; HPLC-MS (ESI$^+$): R_t = 11.52 min; m/z = 531 $[M+H_2O]^+$; IR (film, cm^{-1}): 3287, 2976, 1822, 1704, 1658, 1603, 1509, 1263; $[\alpha]^{20}_D$ = - 0.12 (c= 0.6, CH_2Cl_2).

4.2.17. (E)-(R)-1-((S,Z)-2-(2-(benzyloxy)-2-oxoethylidene)-4-oxoazetidin-3-yl)ethyl 3-(benzo[d][1,3]dioxol-5-yl)acrylate (**1e**)

Following GP3, alcohol **A** (67mg, 0.25mmol) and acid **3e** (77mg, 0.40mmol) yielded compound **1e** (38mg, 35% yield) as a waxy solid after flash chromatography purification (CH_2Cl_2/acetone 98:2). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.46 (d, *J* = 6.4 Hz, 3H), 3.95 (d, *J* = 6.8 Hz, 1H), 5.15 (d, J_{AB} = 12.3 Hz, 1H), 5.18 (d, J_{AB} = 12.3 Hz, 1H), 5.27 (d, *J* = 0.6 Hz, 1H), 5.36 (quintet, *J* = 6.4 Hz, 1H), 6.01 (s, 2H), 6.23 (d, *J* = 15.9 Hz, 1H), 6.81 (d, *J* = 7.9 Hz, 1H), 6.98 – 7.03 (m, 2H), 7.32 – 7.37 (m, 5H), 7.59 (d, *J* = 15.9 Hz, 1H), 8.51 (bs, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 18.0, 61.5, 66.2, 66.7, 90.6, 101.6, 106.5, 108.5, 115.2, 124.7, 128.2, 128.3, 128.5, 128.6, 135.7, 145.5, 148.3, 149.8, 152.1, 164.7, 165.9, 166.7; HPLC-MS (ESI$^+$): R_t = 10.44 min; m/z = 392 [M-43]$^+$; IR (film, cm^{-1}): 3286, 2924, 1821, 1704, 1657, 1510, 1256; $[\alpha]^{20}{}_D$ = + 0.09 (c=1.0, CH_2Cl_2); found C, 66.57; H, 4.89; N, 3.20%; $C_{24}H_{21}NO_7$ requires C, 66.20; H, 4.86; N, 3.22%.

4.2.18. (E)-(R)-1-((S,Z)-2-(2-(benzyloxy)-2-oxoethylidene)-4-oxoazetidin-3-yl)ethyl-3-(4-ethoxymethoxy) phenyl)acrylate (**4f**)

Following GP3, alcohol **A** (67mg, 0.25mmol) and acid **3f** (89mg, 0.40mmol) yielded compound **4f** (67mg, 58% yield) as a waxy solid after flash chromatography purification (cyclohexane/EtOAc 3:1). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.22 (t, *J* = 7.1 Hz, 3H), 1.47 (d, *J* = 6.4 Hz, 3H), 3.73 (q, *J* = 7.1 Hz, 2H), 3.96 (d, *J* = 6.9 Hz, 1H), 5.14 (d, J_{AB} = 12.3 Hz, 1H), 5.18 (d, J_{AB} = 12.3 Hz, 1H), 5.25 (s, 2H), 5.27 (s, 1H), 5.37 (quintet, *J* = 6.4 Hz, 1H), 6.29 (d, *J* = 15.9 Hz, 1H), 7.04 (d, *J* = 8.7 Hz, 2H), 7.33 – 7.38 (m, 5H), 7.47 (d, *J* = 8.7 Hz, 2H), 7.65 (d, *J* = 15.9 Hz, 1H), 8.44 (bs, 1H); HPLC-MS (ESI$^+$): R_t = 11.57 min; m/z = 422 [M-43]$^+$; IR (film, cm^{-1}): 3286, 2924, 1821, 1704, 1658, 1454, 1256.

4.2.19. (E)-(R)-1-((S,Z)-2-(2-(benzyloxy)-2-oxoethylidene)-4-oxoazetidin-3-yl)ethyl 3-(2-(ethoxymethoxy) phenyl)acrylate (**4g**)

Following GP3, alcohol **A** (40mg, 0.15mmol) and acid **3g** (40mg, 0.18mmol) yielded compound **4g** (45mg, 65% yield) as a waxy solid after flash chromatography purification (cyclohexane/EtOAc 3:1). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.21 (t, *J* = 7.1 Hz, 3H), 1.48 (d, *J* = 6.4 Hz, 3H), 3.74 (q, *J* = 7.1 Hz, 2H), 3.97 (d, *J* = 7.0 Hz, 1H), 5.16 (d, J_{AB} = 12.3 Hz, 1H), 5.20 (d, J_{AB} = 12.3 Hz, 1H), 5.29 (s, 3H), 5.38 (quintet, *J* = 6.4 Hz, 1H), 6.48 (d, *J* = 16.2 Hz, 1H), 7.01 (t, *J* = 7.5 Hz, 1H), 7.19 (d, *J* = 8.3 Hz, 1H), 7.32– 7.36 (m, 6H), 7.53 (d, *J* = 7.7 Hz, 1H), 8.06 (d, *J* = 16.2 Hz, 1H), 8.78 (bs, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 15.0, 18.1, 61.5, 64.5, 66.1, 66.8, 90.5, 93.2, 114.9, 117.7, 121.7, 123.7, 128.2, 128.3, 128.4, 128.6, 131.7, 135.8, 141.0, 152.3, 156.2, 164.7, 166.1, 166.7; HPLC-MS (ESI$^+$): R_t = 11.74 min; m/z = 483 [M+H_2O]$^+$; IR (film, cm^{-1}): 3284, 2933, 1821, 1702, 1657, 1631, 1542, 1457, 1264; $[\alpha]^{20}{}_D$ = - 0.16 (c=1.0, CH_2Cl_2).

4.2.20. (E)-(R)-1-((S,Z)-2-(2-(benzyloxy)-2-oxoethylidene)-4-oxoazetidin-3-yl)ethyl3-(3,4-bis(ethoxy methoxy) phenyl) acrylate (**4h**)

Following GP3, alcohol **A** (65mg, 0.25mmol) and acid **3h** (116mg, 0.39mmol) yielded compound **4h** (51mg, 38% yield) as a yellow oil after flash chromatography purification (CH_2Cl_2/diethyl ether 95:5). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.20 – 1.25 (m, 6H), 1.46 (d, *J* = 6.3 Hz, 3H), 3.73 – 3.80 (m, 4H), 3.96 (d, *J* = 7.0 Hz, 1H), 5.14 (d, J_{AB} = 12.4 Hz, 1H), 5.19 (d, J_{AB} = 12.4 Hz, 1H), 5.27 (s, 1H), 5.30 (s, 2H), 5.31 (s, 2H), 5.37 (quintet, *J* = 6.3 Hz, 1H), 6.29 (d, *J* = 15.9 Hz, 1H), 7.12 – 7.19 (m, 2H), 7.32 – 7.37 (m, 6H), 7.61 (d, *J* = 15.9 Hz, 1H), 8.66 (bs, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 15.0, 15.1, 18.0, 61.5, 64.5, 64.6, 66.2, 66.8, 90.6, 93.7, 94.0, 115.5, 115.6, 115.9, 123.1, 123.5, 128.2, 128.3, 128.6, 135.7, 145.5, 147.4, 149.5, 152.1, 164.7, 165.9, 166.7; HPLC-MS (ESI$^+$): R_t = 12.10 min; m/z 557 $[M+H_2O]^+$; IR (film, cm^{-1}): 3266, 2973, 1820, 1701, 1655, 1638, 1510, 1251; $[\alpha]^{20}_D$ = + 0.06 (c= 1.0, CH_2Cl_2).

4.2.21. (E)-(R)-1-((S,Z)-2-(2-(benzyloxy)-2-oxoethylidene)-4-oxoazetidin-3-yl)ethyl 3-(4-(ethoxymethoxy)-3-methoxyphenyl)acrylate (**4i**)

Following GP3, alcohol **A** (40mg, 0.15mmol) and acid **3i** (61mg, 0.24mmol) yielded compound **4i** (27mg, 36% yield) as a waxy solid after flash chromatography purification (CH_2Cl_2/acetone 98:2). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.22 (t, *J* = 7.1 Hz, 3H), 1.47 (d, *J* = 6.4 Hz, 3H), 3.77 (q, *J* = 7.1 Hz, 2H), 3.92 (s, 3H), 3.97 (d, *J* = 6.8 Hz, 1H), 5.14 (d, J_{AB} = 12.3 Hz, 1H), 5.18 (d, J_{AB} = 12.3 Hz, 1H), 5.28 (s, 1H), 5.32 (s, 2H), 5.38 (quintet, *J* = 6.4 Hz, 1H), 6.29 (d, *J* = 15.9 Hz, 1H), 7.06 (s, 1H), 7.07 (d, *J* = 8.1 Hz, 1H), 7.18 (d, *J* = 8.1 Hz, 1H), 7.33 – 7.37 (m, 5H), 7.64 (d, *J* = 15.9 Hz, 1H), 8.47 (bs, 1H); ^{13}C NMR (100MHz, $(CD_3)_2CO$) δ (ppm) 15.4, 18.4, 56.2, 62.3, 65.0, 66.0, 67.5, 90.4, 94.6, 111.8, 116.5, 117.2, 123.5, 128.8, 129.0, 129.3, 129.5, 137.8, 146.2, 151.4, 153.5, 154.2, 166.2, 166.4, 166.7; HPLC-MS (ESI$^+$): R_t = 10.85 min; m/z = 452 $[M-43]^+$; IR (film, cm^{-1}): 3287, 2924, 1821, 1704, 1657, 1510, 1256.

4.2.22. (E)-(R)-1-((S,Z)-2-(2-(benzyloxy)-2-oxoethylidene)-4-oxoazetidin-3-yl)ethyl 3-(4-(ethoxymethoxy)-3,5-dimethoxyphenyl)acrylate (**4j**)

Following GP3, alcohol **A** (40mg, 0.15mmol) and acid **3j** (68mg, 0.24mmol) yielded compound **4j** (30mg, 38% yield) as a waxy solid after flash chromatography purification (CH_2Cl_2/acetone 98:2). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.21 (t, *J* = 7.1 Hz, 3H), 1.47 (d, *J* = 6.4 Hz, 3H), 3.87 (s, 6H), 3.88 (q, *J* = 7.1 Hz, 2H), 3.97 (d, *J* = 6.7 Hz, 1H), 5.15 (d, J_{AB} = 12.3 Hz, 1H), 5.18 (d, J_{AB} = 12.3 Hz, 1H), 5.19 (s, 2H), 5.28 (s, 1H), 5.38 (quintet, *J* = 6.4 Hz, 1H), 6.32 (d, *J* = 15.9 Hz, 1H), 6.75 (s, 2H), 7.33 – 7.36 (m, 5H), 7.61 (d, *J* = 15.9 Hz, 1H), 8.56 (bs, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 15.0, 17.9, 56.1, 61.5, 62.1, 65.0, 66.2, 66.8, 90.7, 96.6, 105.3, 116.5, 128.2, 128.4, 128.7, 130.0, 135.7, 136.8, 145.9, 152.0, 153.6, 164.5, 165.7, 166.7; HPLC-MS (ESI$^+$): R_t = 11.46 min; m/z = 543 $[M+H_2O]^+$; IR (film, cm^{-1}): 3289, 2925, 1736, 1649, 1584, 1504, 1454.

4.2.23. (R)-1-((S,Z)-2-(2-(benzyloxy)-2-oxoethylidene)-4-oxoazetidin-3-yl)ethyl 4-hydroxybenzoate (**1c**)

Following GP4, β-lactam **4c** (27mg, 61µmol) treated with TFA (126µL, 1.68mmol) yielded compound **1c** (23mg, 99% yield) as an oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.51 (d, *J* = 6.3 Hz, 3H), 4.02 (d, *J* = 6.5 Hz, 1H), 5.14 (d, J_{AB} = 12.0 Hz, 1H), 5.19 (d, J_{AB} = 12.0 Hz, 1H), 5.30 (d, *J* = 1.7 Hz, 1H), 5.47 (quintet, *J* = 6.3 Hz, 1H), 6.83 (d, *J* = 7.2 Hz, 2H), 7.31 – 7.40 (m, 5H), 7.89 (d, *J* = 7.2 Hz, 2H), 8.67 (bs, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 18.0, 61.6, 66.5, 67.1, 91.0, 115.4, 121.5, 128.2, 128.4, 128.7, 132.1, 135.4, 152.1, 160.6, 165.6, 165.7, 167.0; HPLC-MS (ESI$^+$): R_t = 8.64 min; m/z = 399 $[M+H_2O]^+$; IR (film, cm^{-1}): 3304, 2982, 1818, 1697, 1662, 1608, 1592, 1269; $[\alpha]^{20}_D$ = - 0.05 (c=1.00, CH_2Cl_2); found C, 66.20; H, 5.03; N, 3.65%; $C_{21}H_{19}NO_6$ requires C, 66.13; H, 5.02; N, 3.67%.

4.2.24. (R)-1-((S,Z)-2-(2-(benzyloxy)-2-oxoethylidene)-4-oxoazetidin-3-yl)ethyl 3,4-dihydroxybenzoate (**1d**)

Following GP4, β-lactam **4d** (36mg, 70µmol) treated with TFA (210µL, 2.85mmol) yielded compound **1d** (27mg, 99% yield) as an oil. ^{1}H NMR (400 MHz, $(CD_3)_2CO$) δ (ppm) 1.48 (d, *J* = 6.4 Hz, 3H), 4.22 (d, *J* = 6.3 Hz, 1H), 5.14 (d, J_{AB} = 12.4 Hz, 1H), 5.20 (d, J_{AB} = 12.4 Hz, 1H), 5.25 (s, 1H), 5.42 (quintet, *J* = 6.3 Hz, 1H), 6.89 (d, *J* = 8.3 Hz, 1H), 7.29 – 7.50 (m, 7H), 9.89 (bs, 1H); ^{13}C NMR (100 MHz, $(CD_3)_2CO$) δ (ppm) 18.4, 62.4, 65.9, 67.8, 90.4, 115.8, 117.2, 122.5, 123.4, 128.8, 128.9, 129.2, 137.8, 145.7, 151.1, 154.2, 165.5, 166.3, 166.6; HPLC-MS (ESI$^+$): R_t = 8.82 min; m/z = 415 $[M+H_2O]^+$; IR (film, cm^{-1}): 3356, 2925, 1815, 1694, 1657, 1604, 1524, 1293; $[\alpha]^{20}_D$ = - 1.27 (c= 0.9, MeOH); found C, ; H, ; N, %; $C_{21}H_{19}NO_7$ requires C, 63.47; H, 4.82; N, 3.52%.

4.2.25. (E)-(R)-1-((S,Z)-2-(2-(benzyloxy)-2-oxoethylidene)-4-oxoazetidin-3-yl)ethyl 3-(4-hydroxyphenyl) acrylate (**1f**)

Following GP4, β-lactam **4f** (50mg, 107µmol) treated with TFA (85µL, 1.13mmol) yielded compound **1f** (43mg, 99% yield) as an oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.46 (d, *J* = 6.4 Hz, 3H), 3.75 (bs, 1H), 3.97 (d, *J* = 6.6 Hz, 1H), 5.15 (d, J_{AB} = 12.4 Hz, 1H), 5.19 (d, J_{AB} = 12.4 Hz, 1H), 5.28 (s, 1H), 5.37 (quintet, *J* = 6.4 Hz, 1H), 6.25 (d, *J* = 15.9 Hz, 1H), 6.84 (d, *J* = 8.5 Hz, 2H), 7.33 – 7.38 (m, 5H), 7.40 (d, *J* = 8.5 Hz, 2H), 7.62 (d, *J* = 15.9 Hz, 1H), 8.53 (bs, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm) 18.0, 61.5, 66.3, 66.6, 90.7, 114.4, 115.9, 126.6, 128.2, 128.4, 128.6, 130.1, 135.7, 145.7, 152.1, 158.4, 164.9, 166.2, 166.7; HPLC-MS (ESI$^+$): R_t = 8.93 min; m/z = 364 $[M-43]^+$; IR (film, cm^{-1}): 3405, 3286, 2929, 1819, 1650, 1515, 1453, 1257, 1159, 1038, 832, 698; $[\alpha]^{20}_D$ = + 0.06 (c=1.0, CH_2Cl_2); found C, 67.85; H, 5.23; N, 3.42%; $C_{23}H_{21}NO_6$ requires C, 67.80; H, 5.20; N, 3.44%.

4.2.26. (E)-(R)-1-((S,Z)-2-(2-(benzyloxy)-2-oxoethylidene)-4-oxoazetidin-3-yl)ethyl 3-(2-hydroxyphenyl)acrylate (**1g**)

Following GP4, β-lactam **4g** (38mg, 82µmol) treated with TFA (120µL, 1.60mmol) yielded compound **1g** (33mg, 99% yield) as an oil. ^{1}H NMR (400 MHz, $(CD_3)_2CO$) δ 1.45 (d, *J* = 6.3 Hz, 3H), 4.16 (d, *J* = 6.6 Hz, 1H), 5.15 (d, J_{AB} = 12.5 Hz, 1H), 5.19 (d, J_{AB} = 12.5 Hz, 1H), 5.28 (s, 1H), 5.37 (quintet, *J* = 6.3 Hz, 1H), 6.64 (d, *J* = 16.1 Hz, 1H), 6.89 (t, *J* = 7.4 Hz, 1H), 6.97 (d, *J* = 8.1 Hz, 1H), 7.26 (t, *J* = 7.4 Hz, 1H), 7.30 – 7.40 (m, 5H), 7.59 (d, *J* = 7.6 Hz, 1H), 8.02 (d, *J* = 16.1 Hz, 1H), 9.84 (bs, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 18.0, 61.5, 66.4, 66.8, 90.9, 116.3, 117.0, 120.6, 121.2, 128.2, 128.4, 128.6, 129.1, 131.8, 141.6, 152.1, 155.5, 165.7, 166.9, 167.1; HPLC-MS (ESI^-): R_t = 11.74 min; m/z = 406 $[M-H]^+$; IR (film, cm^{-1}): 3386, 3282, 2934, 1822, 1702, 1662, 1629, 1604, 1458, 1249; $[\alpha]^{20}_D$ = - 0.14 (c=1.0, CH_2Cl_2); found C, 67.84; H, 5.23; N, 3.43%; $C_{23}H_{21}NO_6$ requires C, 67.80; H, 5.20; N, 3.44%.

4.2.27. (E)-(R)-1-((S,Z)-2-(2-(benzyloxy)-2-oxoethylidene)-4-oxoazetidin-3-yl)ethyl 3-(3,4-dihydroxyphenyl) acrylate (**1h**)

Following GP4, β-lactam **4h** (44mg, 82µmol) treated with TFA (490µL, 6.56mmol) yielded compound **1h** (34mg, 99% yield) as an oil. ^{1}H NMR (400 MHz, $(CD_3)_2CO$) δ (ppm) 1.43 (d, *J* = 6.4 Hz, 3H), 4.14 (d, *J* = 6.5 Hz, 1H), 5.15 (d, J_{AB} = 12.5 Hz, 1H), 5.19 (d, J_{AB} = 12.5 Hz, 1H), 5.26 (s, 1H), 5.33 (quintet, *J* = 6.4 Hz, 1H), 6.28 (d, *J* = 15.9 Hz, 1H), 6.87 (d, *J* = 8.2 Hz, 1H), 7.03 (dd, *J* = 1.6, 8.2 Hz, 1H), 7.16 (d, *J* = 1.6 Hz, 1H), 7.30 – 7.40 (m, 5H), 7.56 (d, *J* = 15.9 Hz, 1H),9.85 (bs, 1H); ^{13}C NMR (100 MHz, $(CD_3)_2CO$) δ (ppm) 18.5, 62.3, 66.0, 67.5, 90.4, 115.1, 115.3, 116.4, 122.7, 127.4, 128.8, 129.0, 129.3, 137.8, 146.3, 146.5, 149.0, 154.2, 166.2, 166.4, 166.7; HPLC-MS (ESI^+): R_t = 8.90 min; m/z = 441 $[M+H_2O]^+$; IR (film, cm^{-1}): 3331, 2925, 1816, 1698, 1657, 1604, 1515, 1260; $[\alpha]_D^{20}$= + 0.11 (c=0.6, MeOH); found C, 65.29; H, 5.04; N, 3.30%; $C_{23}H_{21}NO_7$ requires C, 65.24; H, 5.00; N, 3.31%.

4.2.28. (E)-(R)-1-((S,Z)-2-(2-(benzyloxy)-2-oxoethylidene)-4-oxoazetidin-3-yl)ethyl 3-(4-hydroxy-3-methoxyphenyl)acrylate (**1i**)

Following GP4, β-lactam **4i** (20mg, 40µmol) treated with TFA (150µL, 2.02mmol) yielded compound **1i** (17mg, 99% yield) as an oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.46 (d, *J* = 6.3 Hz, 3H), 3.93 (s, 3H), 3.96 (d, *J* = 6.8 Hz, 1H), 5.14 (d, J_{AB} = 12.4 Hz, 1H), 5.18 (d, J_{AB} = 12.4 Hz, 1H), 5.28 (s, 1H), 5.37 (quintet, *J* = 6.3 Hz, 1H), 6.26 (d, *J* = 15.9 Hz, 1H), 6.92 (d, *J* = 8.1Hz, 1H), 7.03 (s, 1H), 7.06 (d, *J* = 8.1Hz, 1H), 7.31 – 7.39 (m, 5H), 7.62 (d, *J* = 15.9 Hz, 1H), 8.53 (bs, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 18.0, 56.0, 61.5, 66.2, 66.7, 90.7, 109.3, 114.6, 114.7, 123.5, 126.7, 128.2, 128.4, 128.7, 146.0, 146.7, 148.2, 152.1, 164.7, 166.1, 166.4, 166.7; HPLC-MS (ESI^+): R_t = 9.16 min; m/z = 394 $[M-43]^+$; IR (film, cm^{-1}): 3397, 3293, 2930, 1819, 1702, 1659, 1514, 1454, 1267; $[\alpha]^{20}_D$ = + 0.13 (c=1.0, CH_2Cl_2); found C, 65.97; H, 5.32; N, 3.19%; $C_{24}H_{23}NO_7$ requires C, 65.90; H, 5.30; N, 3.20%.

4.2.29. (E)-(R)-1-((S,Z)-2-(2-(benzyloxy)-2-oxoethylidene)-4-oxoazetidin-3-yl)ethyl 3-(4-hydroxy-3,5-dimethoxyphenyl)acrylate (**1j**)

Following GP4, β-lactam **4j** (30mg, 57μmol) treated with TFA (210μL, 2.85mmol) yielded compound **1j** (26mg, 99% yield) as an oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.46 (d, *J* = 6.4 Hz, 3H), 3.92 (s, 6H), 3.97 (d, *J* = 6.8 Hz, 1H), 5.14 (d, J_{AB} = 12.4 Hz, 1H), 5.18 (d, J_{AB} = 12.4 Hz, 1H), 5.28 (s, 1H), 5.38 (quintet, *J* = 6.4 Hz, 1H), 6.27 (d, *J* = 15.8 Hz, 1H), 6.77 (s, 2H), 7.33 – 7.36 (m, 5H), 7.60 (d, *J* = 15.8 Hz, 1H), 8.54 (bs, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 18.0, 56.4, 61.6, 62.1, 66.2, 66.6, 90.7, 105.2, 114.9, 125.6, 128.3, 128.4, 128.7, 135.7, 137.3, 146.1, 147.2, 152.1, 160.0, 164.6, 165.9, 166.7; HPLC-MS (ESI^+): R_t = 9.04 min; m/z = 424 $[M-43]^+$; IR (film, cm^{-1}): 3398, 3286, 2923, 1819, 1702, 1657, 1514, 1457, 1255; $[\alpha]^{20}_D$ = + 0.11 (c=1.0, CH_2Cl_2); found C, 64.31; H, 5.41; N, 2.99%; $C_{25}H_{25}NO_8$ requires C, 64.23; H, 5.39; N, 3.00%.

4.3. Bacterial strains and antimicrobial susceptibility testing procedures

Representative Gram-positive and Gram-negative bacterial pathogens, hosted in the microbial Biobank (MicroMiB Biobank) of the Laboratory of Clinical Microbiology and Virology of the University of Milano-Bicocca, Monza, were used for the *in vitro* antimicrobial susceptibility testing; these included *Staphylococcus aureus*, *Staphylococcus hominis*, *Staphylococcus epidermidis*, *Enterococcus faecalis* and *Enterococcus faecium* as Gram-positive species and *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Escherichia coli* as Gram-negative species, respectively. In particular, laboratory stocks of methicillin-susceptible *S. aureus* (MSSA) strain No. 69856, *S. aureus* (MSSA) strain No. 39249, methicillin-resistant *S. aureus* (MRSA) strain No. 44674, linezolid and methicillin-resistant *S. aureus* (LIN-R MRSA) strain No. SAU-1, methicillin-susceptible *Staphylococcus epidermidis* (MSSE) strain No. 3226, linezolid and methicillin-resistant *S. epidermidis* (LIN-R MRSE) strain No. G1027, linezolid and methicillin-resistant *Staphylococcus hominis* (LIN-R MRSH) strain No. α26, *E. faecalis* strain No. 4150 and linezolid and vancomycin-resistant *Enterococcus faecium* (LIN-R VRE) strain No. VRE-2 were used as test strains. *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 were purchased from the American Type Culture Collection (Manassas, VA, USA) and used as additional test strains.

The *in vitro* antibacterial activity of the new synthetized compounds **1a-j** was studied by determining their minimum inhibitory concentrations (MICs) by means of the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [44]. All chemicals used in this study were of analytical grade. Vancomycin and cefuroxime (Sigma Aldrich, Italy) were used as reference antibiotic compounds for MIC determinations.

Briefly, serial 2-fold dilutions of each compound were made using the Mueller-Hinton broth in 96 wells microtitre plates. Dimethyl sulfoxide (DMSO) was used as solvent for all the synthetized

compounds. An equal volume of the bacterial inoculum ($1x10^6$ CFU/mL) was added to each well on the microtitre plate containing 0.05 mL of the serial antibiotic dilutions. The microtitre plate was then incubated at 37 °C for 18-24 h after which each well was analyzed for the presence of bacterial growth.

The MIC was defined as the lowest concentration of antimicrobial agent able to cause inhibition of bacterial growth as shown by the lack of visible turbidity of the culture medium. Standard strains *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212 were used as control and to MIC testing validation.

4.4. Trolox equivalent antioxidant capacity assay (TEAC)

Antioxidant capacity was checked through TEAC-DPPH and TEAC-ABTS assays, performed following the methods described by Cheng et al. [45] and Marecek et al. [46] and properly modified.

4.4.1. TEAC-DPPH

The DPPH (2,2-diphenyl-1-picrylhydrazyl; Sigma-Aldrich) working sample was prepared in the dark at room temperature dissolving the radical (0.208 mM) in a 50% acetone solution. The stock solution of Trolox® (0.160 mM) and the solutions of sample compounds **1a-j** (7.00 mM) were prepared following the same procedure. Trolox® and **1a-j** stock solutions were then serially diluted to obtain solutions progressively less concentrated (dilution ratio 1: 2) to be tested. To 100 µL of each of these solutions were added 100 µL of DPPH solution, and then each 200 µL sample was poured into a 96-microwells plate, incubated in agitation (100 rpm) for 40 min at room temperature in the dark. The DPPH acetone solution (200 µL) was used as negative control, while 200 µL of acetone solution (50%) was considered as blank sample. After incubation, the 96-microwells plate was checked with microplate reader (Microplate Reader 680 XR, Biorad) at 515 nm. The content of antioxidants in the sample is determined as inhibition percentage of DPPH radical related to the negative control sample (see the below reported formula). The total antioxidant capacity of **1a-j** compounds was then expressed as mmol equivalent of Trolox® (TEAC).

DPPH radical inhibition (%) = $\left(\frac{\text{An.control}-\text{Asample}}{\text{An.control}}\right) \text{x}100$

A_{sample}: absorbance value of **1a-j** sample compounds

$A_{n.control}$: absorbance value of negative control

4.4.2. TEAC-ABTS

An aqueous solution of the radical cation ABTS (ABTS•+; 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); Sigma-Aldrich) was prepared by mixing 10 mL of a 7.0 mM aqueous solution of ABTS with 0.5 mL of a 51.4 mM aqueous solution of $K_2S_2O_4$ (pH=5.0), and then incubated overnight in the dark with the object to achieve an absorbance of 0.700 ± 0.02 at 734 nm. Both the acetone (50%) stock solution of Trolox® (0.160 mM) and of those of sample compounds **1a-j** (7.0 mM) were prepared in the dark at room temperature. Trolox®; **1a-j** stock solutions were then serially diluted to obtain solutions progressively less concentrated (dilution ratio 1: 2) to be tested. To 10 µL of each of these solutions, 290 µL of radical cation ABTS aqueous solution (ABTS•+) were added and poured into a 96-microwells plate, incubated for 6 min at room temperature in the dark and then assayed with Microplate reader (Microplate Reader 680 XR, Biorad). The TEAC-ABTS antioxidant capacity was measured as reported for TEAC-DPPH assay.

Acknowledgements

This research was supported by University of Bologna (RFO 2016). D.G. would like to thank S. Forni, S. Cerofolini and M. Cirillo for the fruitful technical assistance.

References

[1] W. Dröge, Free radicals in the physiological control of cell function. Physiol. Rev. 82 (2002) 48-95.

[2] J.D. Lambeth, A.S. Neish, Nox Enzymes and New Thinking on Reactive Oxygen: A Double-Edged Sword Revisited. Annu. Rev. Pathol. Mech. Dis. 9 (2014) 119-145.

[3] D. Trachootham, W. Lu, M.A. Ogasawara, N. Rivera-Del Valle, P. Huang, Redox regulation of cell survival. Antioxid. Redox Signal. 10 (2008) 1343-1374.

[4] G. Folkerts, J. Kloek, R.B. Muijsers, F.P. Nijkamp, Reactive nitrogen and oxygen species in airway inflammation. Eur. J. Pharmacol. 429 (2001) 251–262.

[5] A.Hector, M. Griese, D. Hartl, Oxidative stress in cystic fibrosis lung disease: an early event, but worth targeting. Eur. Respir. J. 44 (2014) 17–19.

[6] P.Ø. Jensen, J. Lykkesfeldt, T. Bjarnsholt, H.P. Hougen, N. Høiby, O. Ciofu, Basic Clin. Pharmacol. Toxicol. 110 (2012) 353-358.

[7] F. Galli, A. Battistoni, R. Gambari, A. Pompella, A. Bragonzi, F. Pilolli, L. Iuliano, M. Piroddi, M. C. Dechecchi, G. Cabrini, Oxidative stress and antioxidant therapy in cystic fibrosis. Biochim. Biophys. Acta 1822 (2012) 690-713.

[8] W.C. Rutter,D.R. Burgess,D.S. Burgess, Increasing Incidence of Multidrug Resistance among Cystic Fibrosis Respiratory Bacterial Isolates. Microb. Drug Resist. 23 (2017) 51-55.

[9] C.H. Goss, M.S. Muhlebach, Review: *Staphylococcus aureus* and MRSA in cystic fibrosis. J. Cystic Fibrosis 10 (2011) 298–306.

[10] N.S. Harik, G. Com, X. Tang, M. Melguizo Castro, M.E. Stemper, J.L. Carroll, Clinical characteristics and epidemiology of methicillin-resistant *Staphylococcus aureus* (MRSA) in children with cystic fibrosis from a center with a high MRSA prevalence. Am. J. Infect. Contr. 44 (2016) 409-415.

[11] S. Razvi, L. Quittell, A. Sewall, H. Quinton, B. Marshall, L. Saiman, Respiratory microbiology of patients with cystic fibrosis in the United States, 1995 to 2005. Chest 136 (2009) 1554-1560.

[12] B.C. Kahl, Impact of Staphylococcus aureus on the pathogenesis of chronic cystic fibrosis lung disease. Int. J. Med. Microbiol. 300 (2010) 514–519.

[13] G. Smirnova, N. Muzyka, E. Lepekhina, O. Oktyabrsky, Roles of the glutathione‐ and thioredoxin‐dependent systems in the *Escherichia coli* responses to ciprofloxacin and ampicillin. Arch. Microbiol. 198 (2016) 913-921.

[14] M.A. Kohanski, D.J. Dwyer, B. Hayete, C.A. Lawrence, J. Collins, A common mechanism of cellular death induced by bactericidal antibiotics. Cell 130 (2007) 797-810

[15] I. Keren, Y. Wu, J. Inocencio, L.R. Mulcahy, K. Lewis, Killing by bactericidal antibiotics does not depend on reactive oxygen species. Science, 339 (2013) 1213-1216.

[16] Y. Liu, J.A. Imlay, Cell death from antibiotics without the involvement of reactive oxygen species. Science, 339 (2013) 1210-1213.

[17] S.L. Chua, Y. Ding, Y.Liu, Z. Cai, J. Zhou, S. Swarup, D.I. Drautz-Moses, S.C. Schuster, S. Kjelleberg, M. Givskov, L. Yang, Reactive oxygen species drive evolution of pro-biofilm variants in pathogens by modulating cyclic-di-GMP levels. Open Biol. 6 (2016) 160162.

[18] O.H. Aragon-Martinez, F. Martinez-Morales, M.A. Isiordia-Espinoza, D. Luque Contreras, J.R. Zapata Morales, M.L. Gonzalez-Rivera. Bacterial resistance and failure of

clinical cure could be produced by oxidative stress in patients with diabetes or cardiovascular diseases during fluoroquinolone therapy. Med. Hypotheses 103 (2017) 32–34.

[19] F. Broccolo, G. Cainelli, G. Caltabiano, C.E.A. Cocuzza, C.G. Fortuna, P. Galletti, D. Giacomini, G. Musumarra, R. Musumeci, A. Quintavalla, Design, synthesis, and biological evaluation of 4-alkyliden-β lactams: new products with promising antibiotic activity against resistant bacteria, J. Med. Chem. 49 (2006) 2804-2811.

[20] R. Cervellati, P. Galletti, E. Greco, C.E.A. Cocuzza, R. Musumeci, L. Bardini, F. Paolucci, M. Pori, R. Soldati, D. Giacomini, Monocyclic β-lactams as antibacterial agents: facing antioxidant activity of *N*-methylthio-azetidinones, Eur. J. Med. Chem. 60 (2013) 340-349.

[21] P. Galletti, C.E.A. Cocuzza, M. Pori, A. Quintavalla, R. Musumeci, D. Giacomini, Antibacterial agents and cystic fibrosis: synthesis and antimicrobial evaluation of a series of *N*-thiomethylazetidinones, ChemMedChem 6 (2011) 1919-1927.

[22] G. Cainelli, C. Angeloni, R. Cervellati, P. Galletti, D. Giacomini, S. Hrelia, R. Sinisi, New polyphenolic β-lactams with antioxidant activity, Chem. Biodiv. 8 (2008) 811-829.

[23] G. Cainelli, D. Giacomini, P. Galletti, A. Quintavalla, Synthesis of Novel 4-(2-Oxoethylidene)azetidin-2-ones by a Lewis acid mediated reaction of acyldiazo compounds, Eur. J. Org. Chem. (2003) 1765-1774.

[24] I. Dell'Aica, L. Sartor, P. Galletti, D. Giacomini, A. Quintavalla, F. Calabrese, C. Giacometti, E. Brunetta, F. Piazza, C. Agostini, S. Garbisa Inhibition of leukocyte elastase, polymorphonuclear chemoinvasion, and inflammation-triggered pulmonary fibrosis by a 4-alkyliden-β-lactam with a galloyl moiety , J. Pharm. Exper. Therap. 316 (2006) 539-546.

[25] G. Cainelli, P. Galletti, S. Garbisa, D. Giacomini, L. Sartor, A. Quintavalla 4-Alkyliden-β-lactams conjugated to polyphenols: Synthesis and inhibitory activity, Bioorg. Med. Chem. 13 (2005) 6120-6132.

[26] G. Cainelli, P. Galletti, S. Garbisa, D. Giacomini, L. Sartor A. Quintavalla, 4-Alkylidene-azetidin-2-ones: Novel Inhibitors of Leukocyte Elastase and Gelatinase, Bioorg. Med. Chem. 11 (2003) 5391-5399.

[27] M. Drăgan, C.D. Stan, A. Iacob, L. Profire , Assessment of *in vitro* antioxidant and antiinflammatory activities of new azetidin-2-one derivatives of ferulic acid. Farmacia, 2016, 64, 5.

[28] M. Andrade, S. Benfeito, P. Soares, D. Magalhães e Silva, J. Loureiro, A. Borges, F. Borges, M. Simões, Fine-tuning of the hydrophobicity of caffeic acid: studies on the antimicrobial activity against Staphylococcus aureus and Escherichia coli. RSC Adv. 5 (2015) 53915-53925.

[29] J. Guzman, Natural cinnamic acids, synthetic derivatives and hybrids with antimicrobial activity. Molecules 19 (2014) 19292-19349.

[30] J.Teixeira, A. Gaspar, E. M. Garrido, J. Garrido, F. Borges, Hydroxycinnamic Acid Antioxidants: An Electrochemical Overview. BioMed Research International Volume 2013, Article ID 251754

[31] A. Alam, Y. Takaguchi, H. Ito, T. Yoshida, S.Tsuboi Multi-functionalization of gallic acid towards improved synthesis of α- and β-DDB, Tetrahedron 61 (2005) 1909–1918.

[32] B. Neises, W. Steglich, Simple Method for the Esterification of Carboxylic Acids. Angew. Chem. Int. Ed. Engl. 17 (1978) 522-524.

[33] Y.G. Shi, Y. Wu, X.Y. Lu, Y.P.Ren, Q. Wang, C.M. Zhu, D. Yu, H. Wang, Lipase-catalyzed esterification of ferulic acid with lauryl alcohol in ionic liquids and antibacterial properties *in vitro* against three food-related bacteria. Food Chem. 220 (2017) 249-256.

[34] I.K. Schalk, G.L.A. Mislin, Bacterial iron uptake pathways: gates for the import of bactericide compounds. J. Med. Chem. 60 (2017) 4573-4576.

[35] C. Ji, R.E. Juárez-Hernández, M.J. Miller, Exploiting bacterial iron acquisition: siderophore conjugates. Future Med. Chem. 4 (2012) 297-313.

[36] S. Lin, J.J. Koh, T.T. Aung, F. Lim, J. Li, H. Zou, L. Wang, R. Lakshminarayanan, C. Verma, Y. Wang, D.T. Tan, D. Cao, R.W. Beuerman, L. Ren, S. Liu, Symmetrically substituted xanthone amphiphiles combat gram-positive bacterial resistance with enhanced membrane selectivity. J. Med. Chem. 60 (2017) 1362-1378.

[37] R. Martincic, I. Kuzmanovski, A. Wagner, M. Novi, Development of models for prediction of the antioxidant activity of derivatives of natural compounds. Analytica Chimica Acta 868 (2015) 23–35.

[38] A. Guerrini, G. Sacchetti, D. Rossi, G. Paganetto, M. Muzzoli, E. Andreotti, M. Tognolini M.E. Maldonado, R. Bruni, Bioactivities of piper aduncum l. and piper obliquum ruiz & pavon (piperaceae) essential oils from eastern Ecuador. Environ. Toxicol. Pharmacol. 27 (2009) 39–48

[39] R.M. Uppu, S.N. Murthy, W.A. Pryor, N.L. Parinandi. Free Radicals and Antioxidant Protocols (Methods in Molecular Biology) (Second Edition). Humana Press, 2010.

[40] A. Cilla, L. Bosch, R. Barberá, A. Alegría. Effect of processing on the bio-accessibility of bioactive compounds – A review focusing on carotenoids, minerals, ascorbic acid, tocopherols and polyphenols. J. Food Compos. Anal., ASAP (2017) doi.org/10.1016/j.jfca.2017.01.009

[41] D. Giacomini, R. Musumeci, C.E.A. Cocuzza unpublished results.

[42] S. Yin, B. Jiang, G. Huang, Y. Gong, B. You, Z. Yang, Y. Chen, J. Chen, Z. Yuan, M. Li, F. Hu, Y. Zhao, Y. Peng, Burn serum increases staphylococcus aureus biofilm formation via oxidative stress. Front. Microbiol. 8 (2017) art. n° 1191 doi: 10.3389/fmicb.2017.01191.

[43] C. Spatafora, V. Barresi, V. M. Bhusainahalli, S. Di Micco, N. Musso, R. Riccio, G. Bifulco, D. Condorelli, C. Tringali, Bio-inspired benzo[k,l]xanthene lignans: synthesis, DNA-interaction and antiproliferative properties. Org. Biomol. Chem. 12 (2014) 2686-2701.

[44] [CLSI] Clinical and Laboratory Standards Institute, Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved Standard Eighth Edition, CLSI document M07-A8, Clinical and Labo45ratory Standards Institute, Wayne, PA, 2008.

[45] Z. Cheng, J. Moore, L. Yu, High-Throughput Relative DPPH Radical Scavenging Capacity Assay. J. Agric. Food Chem. 54 (2006) 7429-7436.

[46] V. Marecek, A. Mikyska, D. Hampel, P. Cejka, J. Neuwirthova, A. Malachova, R. Cerkal. ABTS and DPPH methods as a tool for studying antioxidant capacity of spring barley and malt. J. Cereal Science 73 (2017) 40-45.