RESEARCH ARTICLE

[View Article Online](#)
[View Journal](#) | [View Issue](#) Cite this: *Med. Chem. Commun.*,
2018, 9, 1017

Synthesis and preliminary anti-inflammatory and anti-bacterial evaluation of some diflunisal aza-analogs†

Davide Carta,^{‡a} Paola Brun,^{id} ^{‡b} Matteo Dal Pra,^a Giulia Bernabè,^b
Ignazio Castagliuolo^b and Maria Grazia Ferlin ^{id} ^{*a}

Our aim was to identify new multi-target compounds endowed with both anti-inflammatory and anti-bacterial activities for treatment of human infections. Diflunisal, a nonsteroidal anti-inflammatory agent, has recently been repurposed for its anti-virulence properties against methicillin-resistant *Staphylococcus aureus*. Effective synthesis of some aza-analogs of the anti-inflammatory drug diflunisal was carried out following the route involving key oxazole intermediates to obtain *o*- and *m*-hydroxypyridinecarboxylic acid derivatives. The newly synthesized diflunisal aza-analogs did not exhibit cytotoxic activity up to 80 μ M and some of them exhibited anti-inflammatory activities, decreasing the levels of pro-inflammatory cytokines and prostaglandins induced by bacterial lipopolysaccharide in human primary macrophages. Ten of the diflunisal aza-analogs were found to have interesting antibacterial activity, sensitizing *S. aureus*, *Streptococcus pyogenes*, *Enterococcus faecium*, and *Pseudomonas aeruginosa* to the antibacterial effects of beta-lactam antibiotics and protein synthesis inhibitors.

Received 13th March 2018,
Accepted 14th April 2018

DOI: 10.1039/c8md00139a

rsc.li/medchemcomm

Introduction

Antibiotic resistance is nowadays recognized as one of the most important threats to global human health and novel intervention strategies are required to cope with or anticipate multiple mechanisms of drug resistance emerging in bacteria. Diflunisal, a well-known non-steroidal anti-inflammatory drug (NSAID), is currently used in many countries to treat painful conditions in patients suffering from rheumatoid arthritis and osteoarthritis,¹ but it has been recently repurposed as an anti-virulent agent for the treatment of *Staphylococcus aureus* osteomyelitis.^{2,3} The general anti-inflammatory mechanism of action of diflunisal has not been fully identified, but it has been demonstrated to act as a prostaglandin synthetase inhibitor, thus reducing prostaglandin levels at peripheral tissues and resulting in anti-inflammatory activity. Inhibition of prostaglandin synthetase, however, has been reported to increase the rate of thrombotic events, myocardial infarction, and stroke following administration of diflunisal. Besides the cardiovascular adverse effects, administration of diflunisal has been associated with increased risk of bleeding, ulceration and perforation of the stomach and intestine that, as with other NSAIDs, usually arise without any warning signs.

Diflunisal is a derivative of salicylic acid with a structure differing from that of the latter because of the presence of the 2,4-difluoro-phenyl substitution at the 5 position. Although the aza-isosteres of salicylic acid, namely the *o*-hydroxypyridinecarboxylic acids,⁴ are generally less potent than salicylic acid itself, like flufenamic acid, niflumic acid and triflocin (Fig. 1) they were reported to have anti-inflammatory activity comparable to the acid but with different metabolic properties and longer duration of the effects.

The renewed worldwide pharmacological interest in the multiple biological effects of diflunisal and our experience in the synthesis and study of hydroxypyridinecarboxylic acid derivatives (HP₂₃ and HP₂₄ in Fig. 1)^{5,6} prompted us to target the synthesis of some diflunisal aza-analogs and related pyridine compounds, all called diflunisal aza-analogs. Our aim was to identify new multi-target compounds endowed with both anti-inflammatory and antibacterial activities to treat human infections with improved pharmacokinetics and possibly reduced side effects. Thus, a multi-target approach is remarkable in the treatment of infections induced by multi-drug resistant bacteria as an alternative to the currently used combination therapies.^{7–9} Further, in the benzene ring, the switch from a C to a N atom, a classical isosteric modification,¹⁰ generally makes the aza-derivatives more water soluble in comparison with the parent compound, in this case the insoluble diflunisal (diflunisal, log *P* = 3.19, and the

^a Department of Pharmaceutical and, Pharmacological Sciences, University of Padova, 35131 Padova, Italy. E-mail: mariagrazia.ferlin@unipd.it^b Department of Molecular Medicine, University of Padova, 35131 Padova, Italy

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c8md00139a

‡ These authors contributed equally to this work.

Fig. 1 Structures of compounds mentioned in the text.

corresponding aza-analog, $\log P = 2.28$, ESI⁺). Also, previous studies on 3-hydroxy-4-pyridinecarboxylic acids reported pK_a values of the order of 1 for carboxylic function. All these features taken together might be a noteworthy improvement for drug formulations.

Concerning the synthesis, we followed a shorter and efficient pathway different from the one implemented by some authors in 1971 for the preparation of flufenisal aza-analogs (Fig. 1).¹¹ In the present work, we describe the synthesis of some diflunisal aza-analogs showing the distinctive bi-phenyl structure and other hydroxypyridinecarboxylic acid derivatives (Fig. 1) and their preliminary anti-inflammatory and antibacterial activities.

Results and discussion

Chemical synthesis

Oxazole derivatives have become increasingly important because of their use as intermediates for the preparation of new biological materials.¹² In the past we exploited the reactivity of oxazole as an azadiene in intramolecular cycloaddition Diels–Alder reactions with a dienophile to obtain several *o*-hydroxypyridinecarboxylic acid (HP) derivatives.⁵ It is known that the reaction between a 5-alkoxy-oxazole and an alkene

proceeds through a bicyclic adduct intermediate that, spontaneously but with different reaction times and amounts depending on 2 and 4 substitutions, transforms into the pyridine structure (Fig. 2).¹³

Hence, for the synthesis of diflunisal aza-analogs we have repurposed the same route involving key oxazole intermediates.

In Scheme 1, the key oxazoles 10–13 were obtained in almost quantitative yields starting from the amino acids phenylalanine 2 and phenylglycine 3 that were transformed into the respective ethyl ester hydrochlorides 4 and 5 by treatment with SOCl_2 in absolute ethanol under reflux for 4 h. The esters 4 and 5 were reacted with trimethyl-*o*-formate or trimethyl-*o*-acetate at refluxing for 2 h to give the corresponding formyl and acetyl-derivatives 6, 7 and 8, 9 (88–90% yields).¹⁴ Following a previously described procedure,¹⁵ compounds 6–9 were subjected to a cyclization reaction carried out with P_2O_5 and MgO in chloroform and in the presence of celite under reflux for 24 h. After work up of the reaction mixture, oxazole derivatives 10–13 were obtained in good yields (73–96%).

The next Diels–Alder reactions were performed with acrylic acid or ethyl acrylate to obtain HP compounds. Thus, 4-benzyl-oxazole derivative 10 was reacted with acrylic acid at room temperature to give 2-benzyl-*o*-HP 14 (30% yield).¹³ 4-Phenyl-oxazole derivative 11 was reacted with ethyl acrylate

Fig. 2 Oxazole synthesis of 3-hydroxy-4-pyridinecarboxylic acids.

Scheme 1 Synthesis of *o*- and *m*-hydroxypyridinecarboxylic acids derivatives **14** and **19–22**. Reagents and conditions: (a) SOCl₂, abs EtOH, 80 °C, 4 h, 95–99%; (b) trimethyl-*o*-formate or trimethyl-*o*-acetate, ref., 3 h; 88–90%; (c) P₂O₅, Celite, MgO, CHCl₃, ref., 24 h, 73–96%; (d) acrylic acid, rt, 0.5 h, 30%; (e) ethyl acrylate, 100 °C, 48 h, 20–35%; (f) NaOH 10% aq, MeOH, ref., 4 h, 95–98%.

at 100 °C for 24–48 h¹⁶ to give, after separation by flash chromatography, two 2-phenyl-isomers as ethyl esters: the *o*-HP derivative **15** (16%) and the *m*-HP derivative **17** (28%). The 2-methyl-4-benzyl-oxazole **12**, by reacting with ethyl acrylate, gave the corresponding ethyl ester *o*-HP derivative **16** (35%), while by the same reaction the 2-methyl-4-phenyl-oxazole **13** yielded the ethyl ester isomer *m*-HP **18** (19%). Finally, all the ethyl esters **15–18** were transformed into the corresponding acids by treatment with a mixture of 10% NaOH aqueous solution and methanol at reflux for 4 h, furnishing the *o*- and *m*-HPs **19–22** in almost quantitative yields.

In Scheme 2, the phenylalanine ethyl ester **4** (Scheme 1) and the commercial ethyl glycine and alanine ethyl esters **26** and **27** were reacted with benzoyl chloride derivatives **23–25** in CH₂Cl₂ at room temperature for 1 h to give amide derivatives **28–32** in almost quantitative yields (95–99%). All these last compounds were subjected to a cyclisation reaction carried out in diethyl ether/dioxane 4 : 1 in the presence of an excess of dehydrating PCl₅ for 1 h. After removal of the solvents, the mixture was left at 40 °C in the dark for 48 h and oxazole derivatives **33–37** were obtained in high yields (50–

90%).¹⁷ The following Diels–Alder reactions with compounds **33–37** were all performed in the presence of ethyl acrylate¹⁶ because they did not work with acrylic acid. Therefore, in all cases the ethyl esters of HP derivatives were obtained. As described in Scheme 2, 2-phenyl-oxazole **33** by reacting with ethyl acrylate gave the two *o*- and *m*-isomers HP **38** and **39** (15% and 21%, respectively). Similarly, oxazole compound **34** gave the two isomer HPs **40** and **41** (11% and 31%), whereas oxazoles **35–37** provided only the ethyl esters of *o*-HPs **46–48**. Finally, all the ethyl esters of HPs **38–41** and **46–48** were transformed to HP derivatives **42–45** and **49–51** by treatment with 10% NaOH aqueous solution and methanol for 4 h (80–90% yields).

Scheme 3 describes the route followed to obtain a quinoline compound showing the same structural elements as the diflunisal aza-analog **51** (Scheme 2) such as a pyridine ring, the *o*-hydroxy carboxylic acid groups and the two fluorines in the *meta* position between them on an aromatic ring, although in a different arrangement. The starting compound 2,4-difluoroaniline was reacted with diethyl ethoxymethylenemalonate for 3 h at 90 °C to yield the condensed product **52** that was subjected to thermal cyclisation in

Scheme 2 Synthesis of *o*- and *m*-hydroxypyridinecarboxylic acid derivatives **42–45** and **49–51**. Reagents and conditions: (a) Et₃N, CH₂Cl₂, rt, 1 h, 95–99%; (b) 1. PCl₅, Et₂O/diox., rt, 1 h; 2. 40 °C, dark, 48 h, 50–90%; (c) ethyl acrylate, 100 °C, 24–48 h, 20–40%; (d) 10% aq NaOH, MeOH, ref., 4 h, 80–90%.

Scheme 3 Synthesis of 6,8-difluoro-4-hydroxyquinoline-3-carboxylic acid (**54**). Reagents and conditions: (a) 90 °C, 3 h, 99%; (b) boiling Ph₂O, 15 min, 60%; (c) 10% aq NaOH, CH₃OH, ref., 4 h, 86.5%.

boiling diphenyl ether for 15 min to give the ethyl ester of the quinoline-4-hydroxy-3-carboxylic acid derivative **53** (60%).¹⁸ As before, this last ethyl ester was hydrolyzed to the corresponding acid by treatment with 10% NaOH aqueous solution and methanol (86.5%).

As a final step, the diflunisal aza-analogs **42**, **14** and **50**, prepared as in Schemes 1 and 2, were subjected to methylation with CH₃I in DMF and 10% NaOH aqueous solution at refluxing for 24 h (Scheme 4). The scope for an *N*-alkylation

was to obtain water soluble zwitterion compounds as previously found out for similar pyridine derivatives.⁵ In all cases only 1*N*-methylated compounds were obtained as sodium and iodine salts.

These intermediate salts were not isolated but were transformed directly into the zwitterions **55–57**. To do this, they were dissolved in water, treated with H₂O₂ and the iodine formed was removed by CHCl₃ extraction. The aqueous phase was acidified with 1 M HCl and the formed precipitate was

Scheme 4 Synthesis of *N*-methylated *o*-hydroxypyridinecarboxylic derivatives **55–57**. Reagents and conditions: (a) CH₃I, DMF, NaOH 10%, ref., 24 h, 20–70%; (b) H₂O₂, CHCl₃, H₂O, HCl 1 M.

subjected to reverse-phase flash chromatography to remove inorganics. In this way, pure zwitterion compounds 55–57 were obtained as proved by spectral evidence.

Biological activity

The cytotoxic activity of diflunisal aza-analogs was assessed on cultured human macrophages using the MTT assay. The compounds were tested at concentrations ranging from 10 nM to 100 μ M and the calculated IC_{50} values are listed in Table 1. All the aza-analogs show minimal cytotoxic activity, with IC_{50} values generally higher than 80 μ M whereas diflunisal showed a more evident cytotoxic activity (IC_{50} 53 μ M).

Diflunisal and aza-analogs were tested for anti-inflammatory activity by ELISA in cultured macrophages stimulated with LPS. Production of TNF- α and IL-1 β significantly increased in macrophages stimulated with LPS (404.1 ± 23.22 pg mL $^{-1}$ and 612.2 ± 30.1 pg mL $^{-1}$, respectively) as compared with unstimulated macrophages (TNF- α : 75.6 ± 10.4 ; IL-1 β : 216.9 ± 14.2 ; $P < 0.02$). The compounds were tested at concentrations ranging from 10 nM to 100 μ M and the results are reported in Table 2 as the lowest concentration able to reduce by 25% the production of pro-inflammatory cytokines triggered by LPS. As reported, compounds 19, 22, 43, 44, and 45 significantly ($P < 0.05$) reduced production of TNF- α and IL-1 β at 10 μ M, whereas the anti-inflammatory activity of compounds 51 and 54 was already evident at 1 μ M. Likewise, in human macrophages LPS stimulation induced secretion of the chemokine IL-8 (544.0 ± 29.7 pg mL $^{-1}$) as compared with unstimulated cells (176.9 ± 2.6 pg mL $^{-1}$; $P < 0.02$). Compounds 19, 44, 45, 51 and 54 significantly ($P < 0.05$) reduced by at least 25% the production of IL-8 induced by LPS (Table 2). Unstimulated human macrophages produced low levels of PGs (56.0 ± 3.6 pg mL $^{-1}$) which were significantly increased by LPS stimulation (632.9 ± 31.7 pg mL $^{-1}$; $P < 0.02$). As expected, diflunisal significantly reduced PGs production at 0.1 μ M ($P < 0.05$), whereas only com-

Table 2 Anti-inflammatory activity of diflunisal aza-analogs evaluated by ELISA. Data are reported as the lowest concentration (μ M) of compounds which significantly ($P < 0.05$) reduced by at least 25% the levels of cytokines triggered by LPS stimulation. n.d.: the anti-inflammatory activity was not detected in the range 10 nM–10 μ M. PGs: prostaglandins

Compound	TNF- α	IL-8	IL-1 β	PGs
14	n.d.	n.d.	n.d.	n.d.
19	10	10	10	n.d.
20	n.d.	n.d.	n.d.	n.d.
21	n.d.	n.d.	n.d.	n.d.
22	10	n.d.	10	n.d.
42	n.d.	n.d.	n.d.	n.d.
43	10	n.d.	10	10
44	10	10	10	n.d.
45	10	10	10	n.d.
49	n.d.	n.d.	n.d.	n.d.
50	n.d.	n.d.	n.d.	n.d.
51	1	1	1	10
54	1	10	1	10
55	n.d.	n.d.	n.d.	n.d.
56	n.d.	n.d.	n.d.	n.d.
57	n.d.	n.d.	n.d.	n.d.
Diflunisal	n.d.	n.d.	n.d.	0.1

pounds 43, 51, and 54 inhibited PGs release at 10 μ M. All the other tested compounds did not show anti-inflammatory activity. No significant increase in pro-inflammatory cytokines or PGs production were observed in human macrophages incubated with diflunisal aza-analogs without LPS (data not shown).

At up to 32 μ M final concentration, diflunisal or aza-analogs alone did not show an intrinsic antibacterial activity, as expected. The highest final concentrations of DMSO or aqueous NaOH caused no significant bacterial growth inhibition (data not shown).

Diflunisal aza-analogs were then investigated for their ability to potentiate the antibacterial activity of antibiotics. In a first screening test, the minimum inhibitory concentration (MIC) of antibiotics against *S. aureus*, *S. pyogenes*, *K. pneumoniae*, *E. faecium*, *P. aeruginosa*, and *E. coli* was assessed, and antibiotic susceptibility was defined as per clinical breakpoints.¹⁹

As reported with diflunisal,³ we tested diflunisal aza-analogs for their ability to potentiate the antibacterial activity, in combination with methicillin (MET), geneticin (GEN), ciprofloxacin (CPR), tetracycline (TET), and erythromycin (ERY) as representative antibiotics for different mechanisms of action. The bacterial strains were then incubated with each compound (final concentrations ranging from 0.25 μ M to 32 μ M) in combination with antibiotics at sub-inhibitory concentration (MIC/4). Data were compared with bacteria incubated with antibiotics (MIC/4) alone. As reported in Table 3, the diflunisal aza-analogs 19, 21, 22, 43, 44, 45, 51 and 54 significantly potentiated the antimicrobial activity of antibiotics in Gram-positive bacteria (*S. aureus*, *S. pyogenes*, *E. faecium*) and the effect was not evident in Gram-negative bacteria *K. pneumoniae* and *E. coli*, while interestingly it was evident in *P. aeruginosa*. In Gram-positive bacteria diflunisal aza-analogs showed the most important antibacterial effects

Table 1 Cytotoxic activity of diflunisal aza-analogs assessed *in vitro* on human macrophages

Compound	IC_{50} (μ M)
14	>100
19	>100
20	>100
21	>100
22	97.4
42	84.5
43	81.2
44	87.9
45	92.1
49	90.8
50	88.6
51	97.3
54	96.5
55	>100
56	91.4
57	96.0
Diflunisal	53

Table 3 Antibacterial activity of diflunisal aza-analogs in combination with antibiotics. Data are reported as minimum modulatory concentration (MMC, in μM) of diflunisal aza-analog required to obtain significant ($P < 0.05$) antibacterial activity in combination with sub-inhibitory concentration (MIC/4) of antibiotic. MET: methicillin; GEN: geneticin; CPR: ciprofloxacin; TET: tetracycline; ERY: erythromycin

Compounds	Activity against <i>S. aureus</i> MMC ₄ (μM)					Activity against <i>S. pyogenes</i> MMC ₄ (μM)				
	MET	GEN	CPR	TET	ERY	MET	GEN	CPR	TET	ERY
14	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
19	0.25	0.5	16	2	0.25	0.5	2	>32	1	2
20	>32	>32	>32	>32	>32	>32	>32	>32	16	>32
21	0.5	2	>32	0.25	2	1	8	>32	4	4
22	0.5	4	16	4	4	4	2	>32	4	4
42	16	>32	16	>32	>32	16	16	>32	>32	>32
43	4	8	16	2	2	2	4	>32	8	4
44	8	16	>32	1	4	1	2	>32	2	4
45	4	8	16	8	8	1	2	>32	4	8
49	16	16	>32	16	>32	>32	>32	>32	>32	>32
50	16	>32	>32	>32	>32	>32	>32	>32	>32	>32
51	0.5	2	16	2	4	2	8	>32	8	4
54	0.5	4	16	2	1	1	4	>32	4	8
55	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
56	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
57	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
Diflunisal	8	>32	>32	>32	>32	16	>32	>32	>32	>32

Compounds	Activity against <i>K. pneumoniae</i> MMC ₄ (μM)					Activity against <i>E. faecium</i> MMC ₄ (μM)				
	MET	GEN	CPR	TET	ERY	MET	GEN	CPR	TET	ERY
14	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
19	>32	>32	>32	>32	>32	1	4	>32	2	4
20	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
21	>32	>32	>32	>32	>32	0.5	8	>32	1	4
22	>32	>32	>32	>32	>32	0.5	8	>32	1	4
42	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
43	>32	>32	>32	>32	>32	2	8	>32	2	2
44	>32	>32	>32	>32	>32	2	8	>32	4	2
45	>32	>32	>32	>32	>32	1	4	>32	2	2
49	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
50	>32	>32	>32	>32	>32	16	>32	>32	>32	>32
51	>32	>32	>32	>32	>32	0.25	4	>32	4	4
54	>32	>32	>32	>32	>32	0.25	4	>32	4	4
55	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
56	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
57	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
Diflunisal	>32	>32	>32	>32	>32	16	>32	>32	>32	>32

Compounds	Activity against <i>P. aeruginosa</i> MMC ₄ (μM)					Activity against <i>E. coli</i> MMC ₄ (μM)				
	MET	GEN	CPR	TET	ERY	MET	GEN	CPR	TET	ERY
14	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
19	8	>32	4	>32	4	>32	>32	>32	>32	>32
20	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
21	16	>32	4	>32	4	>32	>32	>32	>32	>32
22	16	>32	4	>32	4	>32	>32	>32	>32	>32
42	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
43	8	>32	8	>32	8	>32	>32	>32	>32	>32
44	16	>32	8	>32	8	>32	>32	>32	>32	>32
45	16	>32	4	>32	8	>32	>32	>32	>32	>32
49	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
50	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
51	8	>32	4	>32	4	>32	>32	>32	>32	>32
54	8	>32	4	>32	4	>32	>32	>32	>32	>32
55	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
56	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
57	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
Diflunisal	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32

when used in combination with MET, a β -lactam that disrupts the synthesis of the peptidoglycan layer of the bacterial

cell wall, and with protein synthesis inhibitors (GEN, TET, ERY). In contrast, the combination with CPR, which

targets DNA gyrase, was ineffective. The same diflunisal aza-analogs increased inhibitory capacity against *P. aeruginosa* only when used in combination with CPR and ERY, antibiotics previously reported to interfere with virulence factors regulated by a quorum sensing system.²⁰

Conclusion

Following the multi-target approach in treatment of infections induced by multi-drug resistant bacteria, in this study we investigated the anti-inflammatory and antibacterial properties of some newly synthesized aza-analogs of the known diflunisal, which was taken as a reference compound. Regarding the anti-inflammatory activity, diflunisal was confirmed to inhibit the production of prostaglandins while in our experiments, it did not influence the pro-inflammatory cytokines TNF- α , IL-8, and IL-1 β , in LPS stimulated macrophages. In contrast, except for compounds 43, 51 and 54, diflunisal aza-analogs lost the inhibitory activity against prostaglandin synthetase but some of them (19, 22, 44 and 45) revealed interesting anti-inflammatory effects probably acting on the NF- κ B pathway (Table 2). This mechanism of aza-analog action might be advantageous by reducing gastrointestinal adverse effects. Nevertheless, 51 and 54 showed the best anti-inflammatory activity, since at concentrations ranging from 1 to 10 μ M they reduced the levels of both cytokines and PGs in human macrophages. However, their effects on PGs were 100-fold less than that of diflunisal (0.1 μ M). From a SAR point of view, it is possible to affirm that the presence of fluorine atoms on the phenyl moiety is crucial for a wide range of anti-inflammatory activity. The most interesting results were observed when we studied the antibacterial activity of diflunisal aza-analogs in combination with common antibiotics. Our results (Table 3) clearly showed that some diflunisal aza-analogs (19, 21, 22, 43, 44, 45, 51, and 54) significantly potentiated the antimicrobial activity of antibiotics targeting the synthesis of peptidoglycan or proteins in tested Gram-positive bacteria and to a lesser extent in *P. aeruginosa* whereas no intrinsic antibacterial activities were observed when the compounds were tested alone. The fluorine compounds 51 and 54 were again the most effective, exhibiting low values of minimum modulatory concentration (range 0.25–16 μ M). Important antibacterial activities were also observed for compounds 21 and 22 although they lack both fluorine atoms and the specific salicylic acid structure. The observed synergistic effects could be explained by several mechanisms such as alterations in superficial properties of bacteria induced by aza-analogs. Moreover, compelling evidence showed that bi-aryl compounds interfere with bacterial quorum sensing, a signaling mechanism controlling a bacterial phenotype through the exchange of autoinducer peptides (AIPs).³ We hypothesize that since the AIPs regulate bacterial virulence and survival,²¹ the use of the pyridine compounds lowered the dose of antibiotics required for the antibacterial effects, likely reducing the side effects and the selective pressure in the emergence of new resistant bacteria.²² In this per-

spective, and taking into account that most pyridine derivatives are more water soluble than the corresponding phenyl compounds (see diflunisal, $\log P = 3.19$, and the aza-analog 51, $\log P = 2.28$, ESI[†]) and less cytotoxic than diflunisal (Table 1), they represent a promising chemo type for the development of new multi-target agents able to sensitize bacteria to co-administered antibiotics.

Experimental section

Chemistry

¹H NMR spectra were recorded on Bruker 300 and 400 MHz spectrometers using the solvents indicated; chemical shifts are reported in δ (ppm) downfield from tetramethylsilane as internal reference. Coupling constants are given in hertz. In the case of multiplets, chemical shifts were measured starting from the approximate center. Integrals were satisfactorily in line with those expected based on compound structure. Mass spectra were obtained on a Mat 112 Varian Mat Bremen (70 eV) mass spectrometer and Applied Biosystems Mariner System 5220 LC/MS (nozzle potential 140 eV). Column flash chromatography was performed on Merck silica gel (250–400 mesh ASTM); chemical reactions were monitored by analytical thin-layer chromatography (TLC) on Merck silica gel 60 F-254 glass plates. Solutions were concentrated on a rotary evaporator under reduced pressure. The purity of new tested compounds was checked by HPLC using the HPLC SHIMADZU model LC-10 AT-VP instrument with a UV-vis A-VP detector. The analysis was performed with a flow of 1 mL min⁻¹, a C-18 column of dimensions 250 mm $\times$ 4.6 mm, a particle size of 5 μ m, and a loop of 10 μ L. The detector was set at 254 nm. The mobile phase consisted of phase A (Milli-Q H₂O, 18.0 MU, TFA 0.05%) and phase B (MeCN). Gradient elution was performed as reported: 0 min, % B = 0; 3 min, % B = 0; 33 min, % B = 30; 38 min, % B = 30; 39 min, % B = 0; 45 min, % B = 0. Starting materials were purchased from Sigma-Aldrich and Alfa Aesar, and solvents were from Carlo Erba, Fluka and Lab-Scan.

General procedure for the synthesis of amino acid derivatives (4 and 5). As a typical procedure, the synthesis of ethyl 2-amino-3-phenylpropanoate hydrochloride 4 is described in detail. Into a round-bottomed flask, 25 g (151 mmol) of phenylalanine (2) were suspended in absolute ethanol (150 mL). 13 mL (181 mmol) of SOCl₂ were added dropwise with stirring at 0 °C. The mixture was then refluxed for 4 h and monitored by TLC analysis (eluent BuOH/CH₃COOH/H₂O 3:1:1). At the end of the reaction, SOCl₂ was evaporated in a N₂ chamber and the mixture was left at room temperature overnight, leading to the formation of a white precipitate. The precipitate was filtered and dried to obtain 33.31 g of ester derivative.

Ethyl 2-amino-3-phenylpropanoate hydrochloride (4). Yield 96%; $R_f = 0.45$ (eluent BuOH/CH₃COOH/H₂O 3:1:1); ¹H NMR (300 MHz, CDCl₃): δ 7.29 (m, 5H, H-2', H-3', H-4', H-5' and H-6'), 4.13 (q, $J = 6.75$ Hz, 2H, OCH₂CH₃), 4.36 (s_b, 1H, H-2), 3.39 (m, $J = 5.53$ Hz, 2H, CH₂), 1.89 (s_b, 2H, NH₂), 1.18 (t, $J = 6.93$ Hz, 3H, OCH₂CH₃) ppm.

Ethyl 2-amino-2-phenylacetate hydrochloride (5). Compound 5 was prepared as for compound 4 by reacting commercial 2-phenylglycine (3) (15.0 g, 99.23 mmol) and SOCl_2 (14.2 mL, 119.08 mmol) in absolute ethanol. The reaction was monitored by TLC analysis (eluent $\text{BuOH}/\text{CH}_3\text{COOH}/\text{H}_2\text{O}$ 3:1:1) and at the end of the workup procedure, 21.40 g of a brownish solid were obtained. Yield 99%; R_f : 0.58 (eluent $\text{BuOH}/\text{CH}_3\text{COOH}/\text{H}_2\text{O}$ 3:1:1); ^1H NMR (300 MHz, CDCl_3): δ 7.53 (m, 2H, H-2' and H-6'), 7.36 (m, 2H, H-3' and H-5'), 7.35 (m, 1H, H-4'), 5.11 (s_b, 1H, H-2), 4.15 (q, J = 7.4 Hz, 2H, OCH_2CH_3), 1.89 (s_b, 2H, NH_2), 1.16 (t, J = 7.11 Hz, 3H, OCH_2CH_3) ppm.

General procedure for the synthesis of amide derivatives (6–9). As a typical procedure, the synthesis of ethyl 2-(formyl-amino)-3-phenylpropanoate 6 is described in detail. 5 g (21.76 mmol) of compound 4 were mixed with 7.16 mL (65.30 mmol) of trimethyl orthoformate and refluxed for 2 h. The reaction was monitored by TLC analysis (eluent *n*-hexane/ethyl acetate 1:1). At the end of the reaction, the mixture was cooled to room temperature and the solvents evaporated to yield 5.503 g of a brownish oil.

Ethyl 2-(formyl-amino)-3-phenylpropanoate (6). Yield 88%; R_f : 0.56 (eluent *n*-hexane/ethyl acetate 1:1); ^1H NMR (300 MHz, CDCl_3): δ 8.14 (s, 1H, CHO), 7.2 (m, 5H, H-2', H-3', H-4', H-5' and H-6'), 6.38 (d, J = 5.83 Hz, 1H, NH), 4.63 (t, J = 5.86, 1H, CH), 4.2 (q, J = 7.15 Hz, 2H, OCH_2CH_3), 3.14 (q_b, J = 2.97, 2H, H-3), 1.25 ppm (t, J = 7.13 Hz, 3H, OCH_2CH_3) ppm.

Ethyl 2-(formyl-amino)-2-phenylacetate (7). Compound 7 was prepared as for compound 6 by reacting previously synthesized compound 5 (5.0 g, 23.18 mmol) with trimethyl orthoformate (7.62 mL, 69.54 mmol), yielding 4.82 g of a brown oil. Yield 92%; R_f : 0.41 (eluent *n*-hexane/ethyl acetate 1:1); ^1H NMR (300 MHz, CDCl_3): δ 8.11 (s, 1H, CHO), 7.26 (m, 5H, H-2', H-3', H-4', H-5' and H-6'), 6.9 (d, J = 5.47 Hz, 1H, NH), 5.5 (d, J = 7.55 Hz, 1H, H-2), 4.1 (q, J = 7.18 Hz, 2H, OCH_2CH_3), 1.19 (t, J = 7.14 Hz, 3H, OCH_2CH_3) ppm.

Ethyl 2-(acetyl-amino)-3-phenylpropanoate (8). Compound 8 was prepared as for compound 6 by reacting previously synthesized compound 4 (5.0 g, 21.76 mmol) with trimethyl orthoacetate (8.21 mL, 65.30 mmol), yielding 5.08 g of a brownish residue. Yield 92%; R_f : 0.34 (eluent *n*-hexane/ethyl acetate 1:1); ^1H NMR (300 MHz, CDCl_3): δ 7.28 (m, 3H, H-2', H-4' and H-6'), 7.1 (m, 2H, H-3' and H-5'), 5.99 (d, J = 6.83 Hz, 1H, NH), 4.2 (q, J = 7.15 Hz, 2H, OCH_2CH_3), 4.85 (dd, J = 7.78 Hz and J = 5.84 Hz, 1H, H-2), 3.14 (q_b, J = 2.97, 2H, H-3), 1.97 (s, 3H, COCH_3), 1.24 (t, J = 7.14 Hz, 3H, OCH_2CH_3) ppm.

Ethyl 2-(acetyl-amino)-2-phenylacetate (9). Compound 9 was prepared as for compound 6 by reacting previously synthesized compound 5 (5.0 g, 23.18 mmol) with trimethyl orthoacetate (8.85 mL, 69.54 mmol), yielding 4.63 g of a transparent oil. Yield 90%; R_f : 0.41 (eluent *n*-hexane/ethyl acetate 1:1); ^1H NMR (300 MHz, CDCl_3): δ 7.26 (m, 5H, H-2', H-3', H-4', H-5' and H-6'), 6.67 (d, J = 6.78 Hz, 1H, NH), 5.5 (d, J = 7.37 Hz, 1H, H-2), 4.12 (q, J = 7.15 Hz, 2H, OCH_2CH_3), 1.93 (s, 3H, COCH_3), 1.12 (t, J = 7.14 Hz, 3H, OCH_2CH_3) ppm.

General procedure for the synthesis of oxazole derivatives (10–13). As a typical procedure, the synthesis of 4-benzyl-5-ethoxyoxazole 10 is described in detail. Into a 500 mL round-bottomed flask, 5.17 g (19.13 mmol) of celite (CaO)₃· Al_2O_3 and 5.17 g (128.18 mmol) of MgO were suspended in 150 mL of chloroform. With stirring, 17.18 g (120.53 mmol) of P_2O_5 were added quickly to the suspension. 5.50 g (24.87 mmol) of compound 6 were dissolved in 5 mL of chloroform and added dropwise to the mixture at room temperature. The mixture was refluxed for 24 h under N_2 atmosphere and monitored by TLC analysis (eluent *n*-hexane/ethyl acetate 1:1). At the end of the reaction, the mixture was cooled to room temperature and neutralized with a saturated NaHCO_3 solution until pH 7 was reached. The mixture was then extracted with chloroform and the combined organic layers were dried over sodium sulphate and evaporated under vacuum.

4-Benzyl-5-ethoxyoxazole (10). Yield 77%; R_f : 0.76 (eluent *n*-hexane/ethyl acetate 1:1); ^1H NMR (300 MHz, CDCl_3): δ 7.38 (s, 1H, H-2), 7.27 (m, 5H, H-2', H-3', H-4', H-5' and H-6'), 4.12 (q, J = 7.08 Hz, 2H, OCH_2CH_3), 3.75 (s, 2H, CH_2), 1.31 (t, J = 7.08 Hz, 3H, OCH_2CH_3) ppm.

5-Ethoxy-4-phenyloxazole (11). Compound 11 was prepared as for compound 10 by reacting compound 7 (4.83 g, 23.32 mmol) with 4.49 g (17.94 mmol) of celite (CaO)₃· Al_2O_3 , 4.48 g (120.18 mmol) of MgO and 14.87 g (113 mmol) of P_2O_5 , yielding 4.23 g of a brown-orange semi-solid residue. Yield 96%; R_f : 0.77 (eluent *n*-hexane/ethyl acetate 1:1); ^1H NMR (400 MHz, CDCl_3): δ 7.87 (m, 2H, H-2' and H-6'), 7.50 (s, 1H, H-2), 7.42 (m, 2H, H-3' and H-5'), 7.25 (dd, J = 7.45 Hz and J = 1.29 Hz, 1H, H-4'), 4.38 (q, J = 7.08 Hz, 2H, OCH_2CH_3), 1.48 (t, J = 7.08 Hz, 3H, OCH_2CH_3) ppm.

4-Benzyl-5-ethoxy-2-methyloxazole (12). Compound 12 was prepared as for compound 10 by reacting compound 8 (5.09 g, 21.62 mmol) with 4.49 g (17.94 mmol) of celite (CaO)₃· Al_2O_3 , 4.48 g (120.18 mmol) of MgO and 14.87 g (113 mmol) of P_2O_5 , yielding 2.07 g of a brown-orange semi-solid residue. Yield 44%; R_f : 0.70 (eluent *n*-hexane/ethyl acetate 1:1); ^1H NMR (300 MHz, CDCl_3): δ 7.27 (m, 4H, H-2', H-3', H-5' and H-6'), 7.19 (m, 1H, H-4'), 4.07 (q, J = 7.10 Hz, 2H, OCH_2CH_3), 3.74 (s, 2H, CH_2), 2.30 (s, 3H, CH_3), 1.24 (t, J = 7.14 Hz, 3H, OCH_2CH_3) ppm.

5-Ethoxy-2-methyl-4-phenyloxazole (13). Compound 13 was prepared as for compound 10 by reacting compound 9 (3.00 g, 13.56 mmol) with 2.82 g (10.43 mmol) of celite (CaO)₃· Al_2O_3 , 2.83 g (69.88 mmol) of MgO and 9.33 g (65.70 mmol) of P_2O_5 , yielding 2.02 g of a brown-orange semi-solid residue. Yield 73%; R_f : 0.80 (eluent *n*-hexane/ethyl acetate 1:1); ^1H NMR (300 MHz, CDCl_3): δ 7.79 (m, 2H, H-2' and H-6'), 7.35 (m, 2H, H-3' and H-5'), 7.22 (m, 1H, H-4'), 4.32 (q, J = 7.09 Hz, 2H, OCH_2CH_3), 2.40 (s, 3H, CH_3), 1.45 (t, J = 7.08 Hz, 3H, OCH_2CH_3) ppm.

Synthesis of 2-benzyl-3-hydroxypyridine-4-carboxylic acid (14). 4 g (19.7 mmol) of compound 10 were mixed with 2.81 mL (56 mmol) of acrylic acid at room temperature. After 10 minutes, a precipitate formed at the bottom of the flask. The precipitate was filtered under vacuum, washed with acetone

and dried to obtain 1.43 g of hydroxypyridine-4-carboxylic acid **14**. Yield 32%; R_f : 0.64 (eluent BuOH/CH₃COOH/H₂O 3:1:1); mp = 314–315 °C; ¹H NMR (400 MHz, D₂O/NaOD): δ 7.23 (m, J = 6.96 Hz, 1H, H-6), 7.11 (m, 2H, H-2' and H-6'), 7.01 (m, 3H, H-3', H-4' and H-5'), 6.77 (d, J = 4.84 Hz, 1H, H-5), 3.88 (s, 2H, CH₂) ppm; ¹³C NMR (101 MHz, D₂O/NaOD): δ 177.90 (COOH), 168.38 (COH), 152.77 (C2), 141.07 (C4), 136.46 (C1'), 131.30 (C6), 128.73 (C3' and C5'), 128.43 (C2' and C6'), 125.79 (C4'), 120.42 (C5), 37.26 (CH₂) ppm; IR (KBr): ν = 3423.00 (OH, bonded), 3068.10 (CH, aromatic), 2433.78 (CO–OH, bonded), 2137.88 (intermolecular H bond), 1680.00 (C=O), 1474.31 (C=N), 1474.31 (CH₂), 1230.52 and 1192.17 (C–OH); UV-vis (H₂O, 0.05% NaOH): 316.46 nm; fluorescence (H₂O, 0.05% NaOH): λ_{exc} = 316 nm, λ_{ems} = 431.96 nm; HRMS (ESI-MS, 140 eV): m/z [M + H]⁺ calculated for C₁₃H₁₂NO₃⁺, 230.0812; found, 230.0870; calculated for C₁₃H₁₁NNaO₃⁺, 252.0631; found, 252.0682; RP-C18 HPLC: t_R = 8.61 min, 96.16%.

General procedure for the synthesis of pyridine derivatives (15–18). As a typical procedure, the synthesis of the mixture composed by ethyl 3-hydroxy-2-phenylpyridine-4-carboxylate **15** and ethyl 5-hydroxy-6-phenylpyridine-3-carboxylate **17** is described in detail. Into a sealed tube, 1.0 g (5.29 mmol) of oxazole derivative **11** were mixed with 0.634 mL (5.81 mmol) of ethyl acrylate and the mixture was stirred at 100 °C for 48 h. The reaction was monitored by TLC analysis (eluent *n*-hexane/ethyl acetate 8:2), and at the end of the reaction, two fluorescent spots appeared. The excess ethyl acrylate was evaporated, and the mixture was separated by flash-column chromatography (silica gel, *n*-hexane/ethyl acetate 8:2) to yield 0.208 g of derivative **15** and 0.350 g of derivative **17**.

Ethyl 3-hydroxy-2-phenylpyridine-4-carboxylate (15). Yield 16%; R_f : 0.56 (eluent *n*-hexane/ethyl acetate 8:2); ¹H NMR (400 MHz, CDCl₃): δ 11.10 (s, 1H, OH), 8.31 (d, J = 5.00 Hz, 1H, H-6), 8.02 (m, 2H, H-2' and H-6'), 7.62 (d, J = 5.00 Hz, 1H, H-5), 7.47 (m, 2H, H-3' and H-5'), 7.41 (m, 1H, H-4'), 4.48 (q, J = 7.14 Hz, 2H, OCH₂CH₃), 1.45 (t, J = 7.12 Hz, 3H, OCH₂CH₃) ppm; ¹³C NMR (101 MHz, CDCl₃): δ 169.55 (COOCH₂CH₃), 154.06 (C2), 149.05 (C3), 139.84 (C6), 136.77 (C1'), 129.21 (C2' and C6'), 128.79 (C4'), 128.08 (C3' and C5'), 120.79 (C5), 118.68 (C4), 62.46 (OCH₂CH₃), 14.08 (OCH₂CH₃) ppm.

Ethyl 5-hydroxy-6-phenylpyridine-3-carboxylate (17). Yield 28%; R_f : 0.23 (eluent *n*-hexane/ethyl acetate 8:2); ¹H NMR (400 MHz, CDCl₃): δ 8.88 (d, J = 1.76 Hz, 1H, H-2), 7.86 (d, J = 1.80, 1H, H-4), 7.81 (m, 2H, H-2' and H-6'), 7.52 (m, 2H, H-3' and H-5'), 7.47 (m, 1H, H-4'), 5.90 (s_b, 1H, OH), 4.42 (q, J = 7.13 Hz, 2H, OCH₂CH₃), 1.42 (t, J = 7.14 Hz, 3H, OCH₂CH₃) ppm; ¹³C NMR (101 MHz, CDCl₃): δ 165.13 (COOCH₂CH₃), 149.64 (C6), 149.35 (C5), 142.99 (C2), 135.78 (C1'), 129.63 (C4'), 129.17 (C2' and C6'), 128.67 (C3' and C5'), 126.33 (C3), 124.41 (C4), 61.49 (OCH₂CH₃), 14.28 (OCH₂CH₃) ppm.

Ethyl 2-benzyl-3-hydroxy-6-methylpyridine-4-carboxylate (16). Compound **16** was prepared as for compound **15** by reacting oxazole derivative **12** (1.0 g, 4.60 mmol) with ethyl acrylate (0.552 mL, 5.06 mmol). After 48 h, the excess ethyl acrylate was evaporated, and the crude product was purified by flash-

column chromatography (silica gel, *n*-hexane/ethyl acetate 9:1) yielding 0.439 g of pyridinic ester derivative. Yield 35%; R_f : 0.40 (eluent *n*-hexane/ethyl acetate 9:1); ¹H NMR (400 MHz, CDCl₃): δ 10.45 (s_b, 1H, OH), 7.41 (m, 2H, H-2' and H-6'), 7.38 (s, 1H, H-5), 7.29 (m, 2H, H-3' and H-5'), 7.20 (m, 1H, H-4'), 4.42 (q, J = 7.14 Hz, 2H, OCH₂CH₃), 4.27 (s, 2H, CH₂), 2.54 (s, 3H, CH₃), 1.43 (t, J = 7.14 Hz, 3H, OCH₂CH₃) ppm; ¹³C NMR (101 MHz, CDCl₃): δ 169.31 (COOCH₂CH₃), 151.66 (C3), 151.05 (C2), 147.54 (C6), 139.15 (C1'), 129.03 (C2' and C6'), 128.23 (C3' and C5'), 126.10 (C4'), 119.15 (C5), 118.17 (C4), 62.12 (OCH₂CH₃), 38.89 (CH₂), 23.45 (CH₃), 14.09 (OCH₂CH₃) ppm.

Ethyl 5-hydroxy-2-methyl-6-phenylpyridine-3-carboxylate (18). Compound **18** was prepared as for compound **15** by reacting oxazole derivative **13** (1.0 g, 4.92 mmol) with ethyl acrylate (0.590 mL, 5.41 mmol). After 48 h, the excess ethyl acrylate was evaporated, and the crude product was purified by flash-column chromatography (silica gel, *n*-hexane/ethyl acetate 9:1) yielding 0.239 g of pyridinic ester derivative. Yield 19%; R_f : 0.41 (eluent *n*-hexane/ethyl acetate 9:1); ¹H NMR (300 MHz, CDCl₃): δ 7.81 (s, 1H, H-4), 7.80 (m, 2H, H-2' and H-6'), 7.51 (m, 2H, H-3' and H-5'), 7.45 (m, 1H, H-4'), 5.50 (s_b, 1H, OH), 4.40 (q, J = 7.14 Hz, 2H, OCH₂CH₃), 2.82 (s, 3H, CH₃), 1.43 (t, J = 7.12 Hz, 3H, OCH₂CH₃) ppm; ¹³C NMR (75 MHz, CDCl₃): δ 166.29 (COOCH₂CH₃), 149.27 (C2), 147.78 (C6), 147.41 (C5), 135.88 (C1'), 129.39 (C4'), 129.08 (C3' and C5'), 128.74 (C2' and C6'), 126.10 (C3), 125.04 (C4), 61.32 (OCH₂CH₃), 23.95 (CH₃), 14.27 (OCH₂CH₃) ppm.

General procedure for the synthesis of pyridine carboxylic acid derivatives (19–22). As a typical procedure, the synthesis of 3-hydroxy-2-phenylpyridine-4-carboxylic acid **19** is described in detail. Into a 50 mL round-bottomed flask, compound **15** (0.272 g) was dissolved in 5 mL of MeOH and 3 mL of 10% NaOH solution were added. The mixture was refluxed for 4 h and monitored by TLC analysis (eluent BuOH/CH₃COOH/H₂O 3:1:1). At the end of the reaction, MeOH was removed under vacuum and the water layer was acidified with 10% HCl to obtain a precipitate corresponding to the pyridine-4-carboxylic acid derivative **19**. The precipitate was filtered and dried to yield 0.236 g of a white solid.

3-Hydroxy-2-phenylpyridine-4-carboxylic acid (19). Yield 98%; R_f : 0.69 (eluent BuOH/CH₃COOH/H₂O 3:1:1); mp = 310–311 °C; ¹H NMR (400 MHz, D₂O/NaOD): δ 7.59 (m, J = 6.96 Hz, 2H, H-2' and H-6'), 7.53 (d, J = 4.76 Hz, 1H, H-6), 7.39 (m, J = 7.38 Hz, J = 1.41 Hz, 2H, H-3' and H-5'), 7.31 (m, J = 7.36 Hz and J = 1.38 Hz, 1H, H-4'), 7.00 (d, J = 4.76 Hz, 1H, H-5) ppm; ¹³C NMR (101 MHz, D₂O/NaOD): δ 177.88 (C-4), 157.71 (C-2), 150.69 (C-3), 140.08 (C-4), 138.71 (C-1'), 131.84 (C-6), 128.99 (C-2' and C-6'), 128.05 (C-3' and C-5'), 127.30 (C-4'), 121.23 (C-5) ppm; IR (KBr): $\tilde{\nu}$ (cm⁻¹) = 3462.05 (OH, bonded), 3106.95 (CH, aromatic), 2445.37 (CO–OH, bonded), 2059.46 (intermolecular H bond), 1670.32 (C=O), 1534.71 (C=N), 1401.54 (C–OH), 1239.16 and 1277.69 (C–OH); UV-vis (H₂O, 0.05% NaOH): 341.19 nm; fluorescence (H₂O, 0.05% NaOH): λ_{exc} = 342 nm, λ_{ems} = 456.06 nm; HRMS (ESI-MS, 140 eV): m/z [M + H]⁺ calculated

for $C_{12}H_{10}NO_3^+$, 216.0655; found, 216.0756; calculated for $C_{12}H_9NNaO_3^+$, 238.0475; found, 238.0531; RP-C18 HPLC: t_R = 7.00 min, 99%.

2-Benzyl-3-hydroxy-6-methylpyridine-4-carboxylic acid (20).

Compound 20 was prepared as for compound 19 by reacting compound 16 (0.439 g) with 10% NaOH solution (3 mL) to obtain 0.374 g of a white solid. Yield 95%; R_f : 0.75 (eluent BuOH/ CH_3COOH/H_2O 3:1:1); mp = 241–244 °C; 1H NMR (400 MHz, $D_2O/NaOD$): δ 6.95 (m, 2H, H-2' and H-6'), 6.85 (m, 3H, H-3', H-4' and H-5'), 6.49 (s, 1H, H-3), 3.71 (s, 2H, CH_2), 1.88 (s, 3H, CH_3) ppm; ^{13}C NMR (101 MHz, $D_2O/NaOD$): δ 177.85 (COOH), 168.35 (C3), 155.39 (C2), 151.16 (C6), 141.13 (C4), 139.74 (C1'), 137.25 (C4'), 128.30 (C2' and C6'), 125.70 (C-3' and C5'), 119.73 (C5), 37.53 (CH_2), 21.24 (CH_3) ppm; IR (KBr): $\tilde{\nu}$ (cm^{-1}) = 3412.41 (OH, bonded), 3082.88 (CH, aromatic), 2553.89 (CO–OH, bonded), 2083.48 (intermolecular H bond), 1670.19 (C=O), 1542.41 (C=N), 1352.43 (C–OH), 1279.59 and 1279.59 (C–OH); UV-vis (H_2O , 0.05% NaOH): 213.83 nm, 327.30 nm; fluorescence (H_2O , 0.05% NaOH): λ_{exc} = 342 nm, λ_{ems} = 429 nm; HRMS (ESI-MS, 140 eV): m/z [$M + H$] $^+$ calculated for $C_{14}H_{14}NO_3^+$, 244.0968; found, 244.1124; calculated for $C_{14}H_{13}NNaO_3^+$, 266.0788; found, 266.0905; RP-C18 HPLC: t_R = 8.39 min, 98.63%.

5-Hydroxy-6-phenylpyridine-3-carboxylic acid (21).

Compound 21 was prepared as for compound 19 by reacting compound 17 (0.350 g) with 10% NaOH solution (3 mL) to obtain 0.294 g of a white solid. Yield 95%; R_f : 0.86 (eluent BuOH/ CH_3COOH/H_2O 3:1:1); mp = 312–314 °C; 1H NMR (300 MHz, $D_2O/NaOD$): δ 8.18 (d, J = 1.78 Hz, 1H, H-2), 7.16 (d, J = 1.81 Hz, 1H, H-4), 7.11 (m, 2H, H-2' and H-6'), 6.92 (m, 2H, H-3' and H-5'), 6.72 (m, 1H, H-4') ppm; ^{13}C NMR (75 MHz, $D_2O/NaOD$): δ 169.13 (COOH), 150.12 (C2), 148.28 (C6), 140.19 (C2), 138.28 (C1'), 128.63 (C4'), 128.19 (C3' and C5'), 127.67 (C2' and C6'), 120.71 (C4), 119.31 (C3) ppm; IR (KBr): $\tilde{\nu}$ (cm^{-1}) = 3601.23 (OH, bonded), 3000.00 (CH, aromatic), 1686.23 (C=O), 1559.75 (C=N), 1382.02 (C–OH), 1301.64 and 1221.59 (C–OH); UV-vis (H_2O , 0.05% NaOH): 341.5; fluorescence (H_2O , 0.05% NaOH): λ_{exc} = 342 nm, λ_{ems} = 448.49 nm; HRMS (ESI-MS, 140 eV): m/z [$M + H$] $^+$ calculated for $C_{12}H_{10}NO_3^+$, 216.0655; found, 216.0734; calculated for $C_{12}H_9NNaO_3^+$, 238.0475; found, 238.0559; RP-C18 HPLC: t_R = 10.10 min, 95.55%.

5-Hydroxy-2-methyl-6-phenylpyridine-3-carboxylic acid (22).

Compound 22 was prepared as for compound 19 by reacting compound 18 (0.095 g) with 10% NaOH solution (3 mL) to obtain 0.083 g of a yellowish solid. Yield 98%; R_f : 0.65 (eluent BuOH/ CH_3COOH/H_2O 3:1:1); mp = 308–309 °C; 1H NMR (300 MHz, $D_2O/NaOD$): δ 7.31 (m, 2H, H-2' and H-6'), 7.20 (m, 2H, H-3' and H-5'), 7.13 (m, 1H, H-4'), 6.74 (s, 1H, H-4), 2.12 (s, 3H, CH_3) ppm; ^{13}C NMR (75 MHz, $D_2O/NaOD$): δ 175.28 (COOH), 168.28 (C3), 151.29 (C2), 147.88 (C6), 139.27 (C4), 135.28 (C1'), 129.03 (C2' and C6'), 127.87 (C3' and C5'), 127.41 (C4'), 125.61 (C5), 19.40 (CH_3) ppm; IR (KBr): $\tilde{\nu}$ (cm^{-1}) = 3611.16 (OH, bonded), 3000.00 (CH, aromatic), 1676.44 (C=O), 1553.14 (C=N), 1360.09 (C–OH), 1311.14 and 1229.51 (C–OH); UV-vis (H_2O , 0.05% NaOH): 336 nm; fluores-

cence (H_2O , 0.05% NaOH): λ_{exc} = 336 nm, λ_{ems} = 445 nm; HRMS (ESI-MS, 140 eV): m/z [$M + H$] $^+$ calculated for $C_{13}H_{12}NO_3^+$, 230.0812; found, 230.0843; calculated for $C_{13}H_{11}NNaO_3^+$, 252.0631; found, 252.0615; RP-C18 HPLC: t_R = 11.12 min, 96.64%.

General procedure for the synthesis of amide derivatives (28–32). As a typical procedure, the synthesis of ethyl 2-(benzamido) acetate 28 is described in detail. 1.40 g (10.03 mmol) of compound 27 were suspended in 30 mL of DCM, and then 3.07 mL (22 mmol) of Et_3N were added. The mixture was cooled to 0 °C and then 1.280 mL (11 mmol) of benzoyl chloride were added dropwise. The mixture was stirred for 1 h and monitored by TLC analysis (eluent *n*-hexane/ethyl acetate 1:1). At the end of the reaction, 25 mL of water were added, and the mixture was extracted with DCM. The organic layers were dried over sodium sulphate, filtered and evaporated. The obtained residue was dissolved with a mixture of *n*-hexane/ethyl acetate 1:1, and the obtained precipitate was filtered. The filtrate was evaporated to dryness to yield 2.02 g of a yellow solid.

Ethyl 2-(benzamido) acetate (28). Yield 97.6%; R_f : 0.52 (eluent *n*-hexane/ethyl acetate 1:1); 1H NMR (300 MHz, $CDCl_3$): δ 7.81 (m, 2H, H-2' and H-6'), 7.51 (m, 2H, H-3' and H-5'), 7.45 (m, 1H, H-4'), 6.67 (s_{br} , 1H, NH), 4.26 (q, J = 7.15 Hz, 2H, OCH_2CH_3), 4.24 (d, J = 5.09 Hz, 2H, H-2), 1.31 (t, J = 7.13 Hz, 3H, OCH_2CH_3) ppm.

Ethyl 2-(benzamido)-2-phenylacetate (29). Compound 29 was prepared as for compound 28 by reacting compound 5 (1.40 g, 6.49 mmol) with Et_3N (2.0 mL, 13.63 mmol) and benzoyl chloride (0.829 mL, 7.14 mmol) to yield 1.838 g of a white solid. Yield 99%; R_f : 0.29 (eluent *n*-hexane/ethyl acetate 8:2); 1H NMR (300 MHz, $CDCl_3$): δ 7.82 (m, 2H, H-2' and H-6'), 7.5 (m, 2H, H-2" and H-6"), 7.45 (m, 4H, H-3', H-5', H-3" and H-5"), 7.36 (m, 2H, H-4' and H-4"), 7.16 (d, J = 6.38, 1H, NH), 5.76 (d, J = 7.04 Hz, 1H, H-2), 4.26 (q, J = 7.15 Hz, 2H, OCH_2CH_3), 1.24 (t, J = 7.13, 3H, OCH_2CH_3) ppm.

Ethyl 2-(benzamido) propanoate (30). Compound 30 was prepared as for compound 28 by reacting compound 26 (1.53 g, 10 mmol) with Et_3N (3.06 mL, 22 mmol) and benzoyl chloride (1.28 mL, 11 mmol) to yield 2.212 g of a yellow solid. Yield 99%; R_f : 0.70 (eluent *n*-hexane/ethyl acetate 9:1); 1H NMR (300 MHz, $CDCl_3$): δ 7.78 (m, 2H, H-2' and H-6'), 7.46 (m, 2H, H-3' and H-5'), 7.45 (m, 1H, H-4'), 6.86 (d, J = 6.54, 1H, NH), 4.74 (quin, J = 7.17 Hz, 1H, H-2), 4.23 (q, J = 7.14 Hz, 2H, OCH_2CH_3), 1.49 (d, J = 7.15, 3H, CH_3), 1.27 (t, J = 7.13, 3H, OCH_2CH_3) ppm.

Ethyl 2-(3-methoxybenzamido) acetate (31). Compound 31 was prepared as for compound 28 by reacting compound 27 (1.40 g, 10 mmol) with Et_3N (3.06 mL, 22 mmol) and 3-methoxybenzoyl chloride (1.55 mL, 11 mmol) to yield 2.320 g of an orange solid. Yield 99%; R_f : 0.48 (eluent *n*-hexane/ethyl acetate 1:1); 1H NMR (300 MHz, $CDCl_3$): δ 7.81 (m, 2H, H-2' and H-6'), 7.51 (m, 2H, H-3' and H-5'), 6.9 (m, 1H, H-4'), 6.7 (s_{br} , 1H, NH), 4.25 (q, J = 7.16 Hz, 2H, OCH_2CH_3), 4.21 (d, J = 4.97 Hz, 2H, H-2), 3.73 (s, 3H, OCH_3), 1.31 (t, J = 7.14 Hz, 3H, OCH_2CH_3) ppm.

Ethyl 2-(2,4-difluorobenzamido) acetate (32). Compound 32 was prepared as for compound 28 by reacting compound 27 (1.40 g, 10 mmol) with Et₃N (3.06 mL, 22 mmol) and 2,4-difluorobenzoyl chloride (1.35 mL, 11 mmol) to yield 2.420 g of a yellowish solid. Yield 99 %; *R*_f = 0.60 (eluent *n*-hexane/ethyl acetate 1:1); ¹H NMR (300 MHz, CDCl₃) δ 8.13 (m, *J*_{H-F} = 8.9, *J*_{H-H} = 6.6 Hz, 1H, H-3'), 7.24 (s, 1H, NH), 7.03–6.96 (m, *J*_{H-F} = 7.68 Hz, *J*_{H-H} = 1.33 Hz, 1H, H-5'), 6.89 (m, *J*_{H-F} = 11.9, *J*_{H-H} = 8.5, 1H, H-6'), 4.28 (q, *J* = 7.15 Hz, 2H, OCH₂CH₃), 4.25 (dd, *J* = 4.95 Hz, *J* = 1.24 Hz, 2H, H-2), 1.31 (t, *J* = 7.1 Hz, 3H, OCH₂CH₃) ppm.

General procedure for the synthesis of oxazole derivatives (33–37). As a typical procedure, the synthesis of 5-ethoxy-2-phenyloxazole 33 is described in detail. 1.00 g (4.82 mmol) of compound 28 was dissolved in a mixture of dioxane/ethyl ether 1:4. At room temperature, with stirring, 1.00 g (4.82 mmol) of PCl₅ was added to the solution. The mixture was stirred for 1 h and then the solvents were evaporated. The obtained residue was left in the dark at 40 °C for 48 h. Then, the mixture was cooled to 0 °C and PCl₅ was neutralised with a saturated solution of NaHCO₃. The mixture was extracted with chloroform and the combined organic layers were dried and evaporated *in vacuo* to obtain 0.817 g a red liquid.

5-Ethoxy-2-phenyloxazole (33). Yield 98%; *R*_f = 0.61 (eluent *n*-hexane/ethyl acetate 1:1); ¹H NMR (300 MHz, CDCl₃): δ 7.32 (m, 2H, H-2' and H-6'), 7.58 (m, 2H, H-3' and H-5'), 7.45 (m, 1H, H-4'), 6.87 (s, 1H, H-4), 4.37 (q, *J* = 7.05 Hz, 2H, OCH₂CH₃), 1.54 (t, *J* = 7.01 Hz, 3H, OCH₂CH₃) ppm.

5-Ethoxy-2,4-diphenyloxazole (34). Compound 34 was prepared as for compound 33 by reacting compound 29 (1.00 g, 3.53 mmol) with PCl₅ (0.74 g, 3.53 mmol) to obtain 0.918 g of a red liquid. Yield 95%; *R*_f = 0.76 (eluent *n*-hexane/ethyl acetate 8:2); ¹H NMR (300 MHz, CDCl₃): δ 8.01 (m, 2H, H-2' and H-6'), 7.93 (m, 2H, H-2'' and H-6''), 7.44 (m, 6H, H-3', H-5', H-3'', H-5'', H-4', H-4''), 4.44 (q, *J* = 7.08 Hz, 2H, OCH₂CH₃), 1.52 (t, *J* = 7.08, 3H, OCH₂CH₃) ppm.

5-Ethoxy-4-methyl-2-phenyloxazole (35). Compound 35 was prepared as for compound 33 by reacting compound 30 (2.21 g, 11.12 mmol) with PCl₅ (2.32 g, 11.12 mmol) to obtain 2.160 g of a red liquid. Yield 97%; *R*_f = 0.84 (eluent *n*-hexane/ethyl acetate 1:1); ¹H NMR (300 MHz, CDCl₃): δ 8.38 (m, 2H, H-2' and H-6'), 7.59 (m, 2H, H-3' and H-5'), 7.53 (m, 1H, H-4'), 4.40 (q, *J* = 7.08 Hz, 2H, OCH₂CH₃), 2.41 (s, 3H, CH₃), 1.27 (t, *J* = 7.13, 3H, OCH₂CH₃) ppm.

5-Ethoxy-2-(3-methoxyphenyl)oxazole (36). Compound 36 was prepared as for compound 33 by reacting compound 31 (2.32 g, 9.79 mmol) with PCl₅ (2.04 g, 9.79 mmol) to obtain 2.05 g of a red liquid. Yield 95%; *R*_f = 0.52 (eluent *n*-hexane/ethyl acetate 1:1); ¹H NMR (300 MHz, CDCl₃): δ 8.04 (s, 1H, H-2'), 7.77 (d, *J* = 7.72 Hz, 1H, H-6'), 7.47 (t, *J* = 7.87, 1H, H-5'), 7.2 (d, *J* = 6.42 Hz, 1H, H-4'), 6.69 (s, 1H, H-4), 4.38 (q, *J* = 7.14 Hz, 2H, OCH₂CH₃), 3.69 (s, 3H, OCH₃), 1.55 (t, *J* = 6.77 Hz, 3H, OCH₂CH₃) ppm.

5-Ethoxy-2-(2,4-difluorophenyl)oxazole (37). Compound 37 was prepared as for compound 33 by reacting compound 32 (0.949 g, 3.90 mmol) with PCl₅ (0.812 g, 3.90 mmol) to obtain

0.456 g of a red liquid. Yield 52%; *R*_f = 0.81 (eluent *n*-hexane/ethyl acetate 1:1); ¹H NMR (300 MHz, CDCl₃): δ 7.90 (m, *J*_{H-F} = 12.16 Hz, *J*_{H-H} = 6.10 Hz, 1H, H-3'), 6.95–6.84 (m, 2H, H-5' and H-6'), 6.26 (s, 1H, H-4), 4.26 (q, *J* = 7.07 Hz, 2H, OCH₂CH₃), 1.47 (t, *J* = 7.06 Hz, 3H, OCH₂CH₃) ppm.

General procedure for the synthesis of pyridine derivatives (38–41 and 46–48). The synthesis of derivatives 38–41 and 46–48 follows the general procedure reported for the preparation of derivatives (15–18).

Synthesis of ethyl 5-hydroxy-2-phenylpyridine-4-carboxylate (38) and ethyl 5-hydroxy-2-phenylpyridine-3-carboxylate (39). Compounds 38 and 39 were prepared as described for compound 15 by reacting 0.817 g (4.32 mmol) of the oxazole derivative 33 with 0.520 mL (4.75 mmol) of ethyl acrylate to yield a crude product consisting of the two isomers 38 and 39. The two isomers were separated by flash-column chromatography (silica gel, *n*-hexane/ethyl acetate 1:1) to obtain 0.160 g of a yellow oil (38) and 0.224 g of a yellow solid (39). Compound 38: yield 15%; *R*_f = 0.84 (eluent *n*-hexane/ethyl acetate 1:1); ¹H NMR (300 MHz, CDCl₃): δ 10.34 (s, 1H, OH), 8.57 (d, *J* = 0.69 Hz, 1H, H-6), 8.05 (d, *J* = 0.66 Hz, 1H, H-3), 7.94 (m, 2H, H-2' and H-6'), 7.46 (m, 2H, H-3' and H-5'), 7.38 (m, 1H, H-4'), 4.50 (q, *J* = 7.14 Hz, 2H, OCH₂CH₃), 1.47 ppm (t, *J* = 7.12 Hz, 3H, OCH₂CH₃); ¹³C NMR (75 MHz, CDCl₃): δ 168.86 (COOCH₂CH₃), 154.68 (C2), 148.78 (C5), 141.40 (C6), 138.25 (C1'), 128.80 (C3' and C5'), 128.51 (C4'), 126.33 (C2' and C6'), 119.18 (C4), 118.42 (C3), 62.51 (OCH₂CH₃), 14.15 (OCH₂CH₃) ppm. Compound 39: yield 21%; *R*_f = 0.48 (eluent *n*-hexane/ethyl acetate 1:1); ¹H NMR (400 MHz, CDCl₃): δ 8.88 (d, *J* = 1.76 Hz, 1H, H-6), 7.86 (d, *J* = 1.76 Hz, 1H, H-4), 7.80 (m, 2H, H-2' e H-6'), 7.50 (m, 2H, H-3' and H-5'), 7.47 (m, 1H, H-4'), 5.86 (s_{br}, 1H, OH), 4.42 (q, *J* = 7.13 Hz, 2H, OCH₂CH₃), 1.42 (t, *J* = 7.14 Hz, 3H, OCH₂CH₃) ppm; ¹³C NMR (101 MHz, CDCl₃): δ 169.79 (COOCH₂CH₃), 150.27 (C2), 148.27 (C5), 142.95 (C6), 135.82 (C1'), 129.83 (C4'), 129.21 (C3' and C5'), 128.72 (C2' and C6'), 124.51 (C4), 119.27 (C1'), 61.47 (OCH₂CH₃), 14.55 (OCH₂CH₃) ppm.

Synthesis of ethyl 3-hydroxy-2,6-diphenylpyridine-4-carboxylate (40) and ethyl 5-hydroxy-2,6-diphenylpyridine-3-carboxylate (41). Compounds 40 and 41 were prepared as described for compound 15 by reacting 0.890 g (3.35 mmol) of the oxazole derivative 34 with 0.400 mL (3.69 mmol) of ethyl acrylate to yield a crude product consisting of the two isomers. The two isomers were separated by flash-column chromatography (silica gel, *n*-hexane/ethyl acetate 8:2) to obtain 0.117 g of a yellow semi-solid compound (40) and 0.335 g of a yellowish solid (41). Compound 40: yield 11%; *R*_f = 0.67 (eluent *n*-hexane/ethyl acetate 8:2); ¹H NMR (400 MHz, CDCl₃): δ 11.17 (s_b, 1H, OH), 8.23 (m, 2H, H-2'' and H-6''), 8.11 (s, 1H, H-5), 8.10 (m, 2H, H-2' and H-6'), 7.53 (m, 2H, H-3'' and H-5''), 7.50 (m, 2H, H-3' and H-5'), 7.48 (m, 1H, H-4'), 7.41 (m, 1H, H-4''), 4.54 (q, *J* = 7.14 Hz, 2H, OCH₂CH₃), 1.52 (t, *J* = 7.14 Hz, 3H, OCH₂CH₃) ppm; ¹³C NMR (101 MHz, CDCl₃): δ 169.69 (COOCH₂CH₃), 153.19 (OH), 147.97 (C6), 147.56 (C2), 138.66 (C1''), 137.07 (C1'), 129.54 (C2' and C6'), 129.46 (C2'' and C6''), 128.82 (C4'), 128.70 (C3'' and C5''), 128.36 (C4''), 126.33 (C2'' and C6''), 119.47 (C4), 117.29 (C5), 62.55

(OCH₂CH₃), 14.17 (OCH₂CH₃) ppm. Compound 41: yield 31%; *R*_f = 0.16 (eluent *n*-hexane/ethyl acetate 8:2); ¹H NMR (300 MHz, CDCl₃): δ 8.02 (s, 1H, H-4), 8.01 (m, 2H, H-2' and H-6'), 7.94 (m, 2H, H-2'' and H-6''), 7.53 (m, 2H, H-3'' and H-5''), 7.46 (m, 2H, H-3' and H-5'), 7.45 (m, 1H, H-4''), 7.43 (m, 1H, H-4'), 4.16 (q, *J* = 7.17 Hz, 2H, OCH₂CH₃), 1.26 (t, *J* = 7.13 Hz, 3H, OCH₂CH₃) ppm; ¹³C NMR (75 MHz, CDCl₃): δ 171.06 (COOCH₂CH₃), 167.38 (COH), 150.19 (C2), 149.27 (C6), 140.17 (C3), 136.63 (C1'), 135.92 (C1''), 128.76 (C4'), 128.72 (C2' and C6'), 128.56 (C3' and C5'), 128.25 (C3'' and C5''), 128.12 (C4''), 127.97 (C2'' and C6''), 127.23 (C4), 61.42 (OCH₂CH₃), 14.02 (OCH₂CH₃) ppm.

Ethyl 3-hydroxy-2-methyl-6-phenylpyridine-4-carboxylate (46). Compound 46 was prepared as for compound 15 by reacting the oxazole derivative 35 (0.790 g, 3.89 mmol) with ethyl acrylate (0.451 mL, 4.28 mmol). After 48 h, the excess ethyl acrylate was evaporated, and the crude product was purified by flash-column chromatography (silica gel, *n*-hexane/ethyl acetate 1:1) yielding 0.350 g of pyridinic ester derivative. Yield 35%; *R*_f 0.91 (eluent *n*-hexane/ethyl acetate 1:1); ¹H NMR (400 MHz, CDCl₃): δ 10.64 (s, 1H, OH), 7.96 (m, 2H, H-2' and H-6'), 7.92 (d, *J* = 0.48 Hz, 1H, H-3), 7.47 (m, 2H, H-3' and H-5'), 7.38 (m, 1H, H-4'), 4.50 (q, *J* = 7.13 Hz, 2H, OCH₂CH₃), 2.64 (s, *J* = 0.34, 3H, CH₃), 1.48 ppm (t, *J* = 7.14 Hz, 3H, OCH₂CH₃); ¹³C NMR (101 MHz, CDCl₃): δ 169.52 (COOCH₂CH₃), 153.15 (C2), 150.13 (C5), 147.27 (C6), 138.96 (C1'), 128.68 (C3' and C5'), 128.10 (C4'), 126.31 (C2' and C6'), 117.56 (C4), 116.16 (C3), 62.28 (OCH₂CH₃), 19.34 (CH₃), 14.15 (OCH₂CH₃) ppm.

Ethyl 5-hydroxy-2-(3-methoxyphenyl)pyridine-4-carboxylate (47). Compound 47 was prepared as for compound 15 by reacting oxazole derivative 36 (2.05 g, 9.35 mmol) with ethyl acrylate (1.12 mL, 10, 28 mmol). After 48 h, the excess ethyl acrylate was evaporated, and the crude product was purified by flash-column chromatography (silica gel, *n*-hexane/ethyl acetate 1:1) yielding 0.410 g of pyridinic ester derivative. Yield 16%; *R*_f 0.87 (eluent *n*-hexane/ethyl acetate 1:1); ¹H NMR (400 MHz, CDCl₃): δ 10.34 (s, 1H, OH), 8.56 (d, *J* = 0.56 Hz, 1H, H-6), 8.05 (d, *J* = 0.52 Hz, 1H, H-3), 7.54 (m, 1H, H-6'), 7.50 (m, 1H, H-2'), 7.37 (t, *J* = 7.98 Hz, 1H, H-5'), 6.93 (ddd, *J* = 8.17, *J* = 2.06 e *J* = 0.90 Hz, 1H, H-4'), 4.49 (q, *J* = 7.14 Hz, 2H, OCH₂CH₃), 3.89 (s, 3H, OCH₃), 1.47 (t, *J* = 7.14 Hz, 3H, OCH₂CH₃) ppm; ¹³C NMR (101 MHz, CDCl₃): δ 168.84 (COOCH₂CH₃), 160.14 (COCH₃), 154.78 (C2), 148.49 (C5), 141.28 (C6), 139.71 (C1'), 129.79 (C5'), 119.09 (C4), 118.58 (C3), 114.37 (C4'), 111.75 (C6'), 62.53 (OCH₃ e OCH₂CH₃), 14.16 (OCH₂CH₃) ppm.

Ethyl 2-(2,4-difluorophenyl)-5-hydroxypyridine-4-carboxylate (48). Compound 48 was prepared as for compound 15 by reacting the oxazole derivative 37 (0.215 g, 0.884 mmol) with ethyl acrylate (0.243 mL, 0.972 mmol). After 48 h, the excess ethyl acrylate was evaporated, and the crude product was purified by flash-column chromatography (silica gel, *n*-hexane/ethyl acetate 8:2) yielding 0.043 g of pyridinic ester derivative. Yield 20%; *R*_f = 0.73 (eluent *n*-hexane/ethyl acetate 8:2); ¹H NMR (300 MHz, CDCl₃): δ 8.68 (s, 1H, H-6), 8.34 (d, *J* = 1.5

Hz, 1H, H-3), 8.23 (td, *J* = 8.5, 6.5 Hz, 1H, H-3'), 8.04 (td, *J* = 8.8, 6.6 Hz, 1H, H-6'), 6.99–6.92 (m, 1H, H-5'), 4.32 (q, *J* = 7.1 Hz, 2H, OCH₂CH₃), 1.23 (t, *J* = 7.1 Hz, 3H, OCH₂CH₃) ppm; ¹³C NMR (101 MHz, CDCl₃): δ 168.14 ppm (COOCH₂CH₃), 162.99 (dd, *J* = 243.70 Hz, *J* = 8.90 Hz, C4'), 158.50 (d, *J* = 3.00 Hz, C2), 158.36 (dd, *J* = 248.72 Hz, *J* = 7.99 Hz, C2'), 147.27 (C5), 141.88 (C6), 137.72 (dd, *J* = 13.21 Hz, *J* = 3.89 Hz, C6'), 132.01 (dd, *J* = 9.00 Hz, *J* = 4.95 Hz, C5'), 123.69 (dd, *J* = 5.00 Hz, C3), 116.14 (d, *J* = 1.90 Hz, C4), 112.64 (dd, *J* = 21.60 Hz, *J* = 3.39 Hz, C5'), 105.13 (t, *J* = 26.50 Hz, C3'), 62.51 (OCH₃ e OCH₂CH₃), 14.15 (OCH₂CH₃) ppm.

General procedure for the synthesis of pyridine carboxylic acid derivatives (42–45 and 49–51). The synthesis of derivatives 42–45 and 49–51 follows the general procedure reported for the preparation of derivatives (19–22).

5-Hydroxy-2-phenylpyridine-4-carboxylic acid (42). Compound 42 was prepared as for compound 19 by reacting compound 38 (0.160 g) with 10% NaOH solution (3 mL) to obtain 0.112 g of a brownish solid. Yield 70%; *R*_f 0.69 (eluent BuOH/CH₃COOH/H₂O 3:1:1); mp = 314–315 °C; ¹H NMR (300 MHz, DMSO): δ 8.47 (s, 1H, H-6), 8.09 (s, 1H, H-3), 7.98 (m, *J* = 7.96 Hz, 2H, H-2' and H-6'), 7.47 (m, *J* = 7.58 Hz, 2H, H-3' and H-5'), 7.39 (m, *J* = 7.07 Hz and *J* = 2.06 Hz, 1H, H-4') ppm; ¹³C NMR (75 MHz, DMSO): δ 182.45 (COOH), 154.92 (C2), 146.69 (C5), 144.69 (C4), 140.18 (C6), 137.66 (C1'), 128.77 (C3' and C5'), 128.30 (C4'), 125.79 (C2' and C6'), 119.07 (C3) ppm; IR (KBr): $\tilde{\nu}$ (cm⁻¹) = 3432.40 (OH, bonded), 3029.21 (CH, aromatic), 2395.47 (CO–OH, bonded), 2115.97 (intermolecular H bond), 1647.32 (C=O), 1545.63 (C=N), 1492.63 (C–OH), 1259.66 and 1283.33 (C–OH); UV-vis (H₂O, 0.05% NaOH): 254.33, 337.27 nm; fluorescence (H₂O, 0.05% NaOH): λ_{exc} = 337 nm, λ_{ems} = 445 nm; HRMS (ESI-MS, 140 eV): *m/z* [M + H]⁺ calculated for C₁₂H₁₀NO₃⁺, 216.0655; found, 216.0670; calculated for C₁₂H₉NNaO₃⁺, 238.0475; found, 238.0483; RP-C18 HPLC: *t*_R = 7.69 min, 97%.

3-Hydroxy-2,6-diphenylpyridine-4-carboxylic acid (43). Compound 43 was prepared as for compound 19 by reacting compound 40 (0.117 g) with 10% NaOH solution (3 mL) to obtain 0.104 g of a yellowish solid. Yield 98%; *R*_f 0.81 (eluent BuOH/CH₃COOH/H₂O 3:1:1); mp = 119–120 °C; ¹H NMR (300 MHz, D₂O/NaOD): δ 7.58 (m, *J* = 7.88 Hz, 2H, H-2'' and H-6''), 7.53 (m, *J* = 7.59 Hz, 2H, H-2' and H-6'), 7.28 (s, 1H, H-5), 7.24 (m, 2H, H-3'' and H-5''), 7.23 (m, 2H, H-3' and H-5'), 7.17 (m, 1H, H-4''), 7.12 ppm (m, 1H, H-4'); ¹³C NMR (75 MHz, D₂O/NaOD): δ 177.61 (COOH), 168.30 (COH), 157.65 (C6), 150.60 (C2), 140.24 (C4), 139.93 (C1''), 139.47 (C1'), 129.03 (C3'' and C5''), 128.68 (C3'' and C5''), 128.16 (C3' and C5'), 127.88 (C2' and C6'), 127.28 (C4''), 127.06 (C4'), 119.08 (C5) ppm; UV-vis (H₂O, 0.05% NaOH): 224.86, 360.23 nm; fluorescence (H₂O, 0.05% NaOH): λ_{exc} = 360 nm, λ_{ems} = 455 nm; HRMS (ESI-MS, 140 eV): *m/z* [M + H]⁺ calculated for C₁₈H₁₄NO₃⁺, 292.0968; found, 292.1117; calculated for C₁₈H₁₃NNaO₃⁺, 314.0788; found, 314.0863; RP-C18 HPLC: *t*_R = 17.07 min, 98%.

5-Hydroxy-2-phenylpyridine-3-carboxylic acid (44). Compound 44 was prepared as for compound 19 by reacting

compound **39** (0.224 g) with 10% NaOH solution (3 mL) to obtain 0.226 g of a brown solid. Yield 98%; R_f : 0.72 (eluent BuOH/CH₃COOH/H₂O 3:1:1); mp = 312–314 °C; ¹H NMR (400 MHz, D₂O/NaOD): δ 7.69 (s, 1H, H-6), 7.54 (m, J = 7.24 Hz, 2H, H-2' and H-6'), 7.35 (s, 1H, H-4), 7.27 (m, J = 7.66 Hz, 2H, H-3' and H-5'), 7.15 (m, J = 7.36 Hz, 1H, H-4') ppm; ¹³C NMR (101 MHz, D₂O/NaOD): δ 176.60 (COOH), 159.78 (C5), 142.64 (C2), 140.52 (C1'), 139.77 (C6), 129.14 (C3' and C5'), 127.19 (C4'), 125.38 (C2' and C6'), 120.84 (C4), 119.70 (C3) ppm; IR (KBr): $\tilde{\nu}$ (cm⁻¹) = 3560.20 (OH), 3000.00 (CH, aromatic), 1656.54 (C=O), 1543.56 (C=N), 1399.83 (C–OH), 1259.23 and 1212.69 (C–OH); UV-vis (H₂O, 0.05% NaOH): 253.49, 337.39 nm; fluorescence (H₂O, 0.05% NaOH): λ_{exc} = 337 nm, λ_{ems} = 428.93 nm; HRMS (ESI-MS, 140 eV): m/z [M + H]⁺ calculated for C₁₂H₁₀NO₃⁺, 216.0655; found, 216.0736; calculated for C₁₂H₉NNaO₃⁺, 238.0475; found, 238.0555; RP-C18 HPLC: t_R = 7.67 min, 95.2%.

5-Hydroxy-2,6-diphenylpyridine-3-carboxylic acid (45). Compound **45** was prepared as for compound **19** by reacting compound **41** (0.335 g) with 10% NaOH solution (3 mL) to obtain 0.293 g of a brown solid. Yield 96%; R_f : 0.94 (eluent BuOH/CH₃COOH/H₂O 3:1:1); mp = 149–151 °C; ¹H NMR (300 MHz, D₂O/NaOD): δ 7.23 (m, J = 8.09 Hz, 2H, H-2' and H-6'), 7.11 (m, J = 8.24 Hz, 2H, H-2'' and H-6''), 6.95 (m, 2H, H-3' and H-5'), 6.80 (m, 2H, H-3'' and H-5''), 6.73 (m, 1H, H-4''), 6.72 (m, 1H, H-4'), 6.44 (s, 1H, H-4) ppm; ¹³C NMR (75 MHz, D₂O/NaOD): δ 177.66 (COOH), 165.34 (C5), 151.28 (C2), 150.27 (C6), 136.27 (C1'), 135.92 (C1''), 129.98 (C2' and C6'), 128.48 (C3' and C5'), 128.44 (C2'' and C6''), 128.15 (C3'' and C5''), 128.11 (C4'), 128.02 (C4''), 127.36 (C3), 125.26 (C4) ppm; UV-vis (H₂O, 0.05% NaOH): 329.44 nm; fluorescence (H₂O, 0.05% NaOH): λ_{exc} = 329 nm, λ_{ems} = 437.67 nm; HRMS (ESI-MS, 140 eV): m/z [M + H]⁺ calculated for C₁₈H₁₄NO₃⁺, 292.0968; found, 292.1080; calculated for C₁₈H₁₃NNaO₃⁺, 314.0788; found, 314.0933; RP-C18 HPLC: t_R = 12.84 min, 99%.

3-Hydroxy-2-methyl-6-phenylpyridine-4-carboxylic acid (49). Compound **49** was prepared as for compound **19** by reacting compound **46** (0.350 g) with 10% NaOH solution (3 mL) to obtain 0.302 g of a yellow solid. Yield 96%; R_f : 0.74 (eluent BuOH/CH₃COOH/H₂O 3:1:1); mp = 300–302 °C; ¹H NMR (400 MHz, D₂O/NaOD): δ 7.28 (m, J = 7.16 Hz, 2H, H-2' and H-6'), 7.02 (m, J = 7.66 Hz, 2H, H-3' and H-5'), 6.95 (d, J = 0.40 Hz, 1H, H-3), 6.91 (m, J = 7.94 Hz, J = 1.24 Hz, 1H, H-4'), 1.93 (s, J = 0.23, 3H, CH₃) ppm; ¹³C NMR (101 MHz, D₂O/NaOD): δ 177.36 (COOH), 168.29 (C5), 157.82 (C2), 151.67 (C6), 139.34 (C4), 134.83 (C1'), 128.64 (C3' and C5'), 126.94 (C4'), 125.63 (C2' and C6'), 112.89 (C3), 19.35 (CH₃) ppm; IR (KBr): $\tilde{\nu}$ (cm⁻¹) = 3503.88 (OH, bonded), 3074 (CH, aromatic), 2607.89 (CO–OH, bonded), 2048.53 (intermolecular H bond), 1670.21 (C=O), 1534.17 (C=N), 1458.46 (CH₃); UV-vis (H₂O, 0.05% NaOH): 253.72 nm, 342.10 nm; fluorescence (H₂O, 0.05% NaOH): λ_{exc} = 342 nm, λ_{ems} = 451.96 nm; HRMS (ESI-MS, 140 eV): m/z [M + H]⁺ calculated for C₁₃H₁₂NO₃⁺, 230.0812; found, 230.0854; calculated for C₁₃H₁₁NNaO₃⁺, 252.0631; found, 252.0672; RP-C18 HPLC: t_R = 7.50 min, 98.8%.

5-Hydroxy-2-(3-methoxyphenyl)pyridine-4-carboxylic acid (50). Compound **50** was prepared as for compound **19** by reacting compound **47** (0.417 g) with 10% NaOH solution (3 mL) to obtain 0.334 g of a yellow solid. Yield 91%; R_f : 0.87 (eluent BuOH/CH₃COOH/H₂O 3:1:1); mp = 240 °C; ¹H NMR (400 MHz, D₂O/NaOD): δ 7.50 (s, 1H, H-6), 7.15 (s, 1H, H-3), 6.89 (m, 2H, H-2' and H-6'), 6.80 (m, 1H, H-5'), 6.42 (m, J = 6.92 Hz, J = 2.24 Hz, 1H, H-4'), 6.36 ppm (s, 3H, OCH₃); ¹³C NMR (101 MHz, D₂O/NaOD): δ 176.45 (COOH), 168.33 (C3'), 160.22 (C2), 158.81 (C5), 142.83 (C6), 140.81 (C4), 135.77 (C1'), 129.89 (C5'), 119.97 (C3), 118.27 (C2'), 112.59 (C4'), 110.76 (C6'), 55.27 (OCH₃) ppm; UV-vis (H₂O, 0.05% NaOH): 219.79, 257.44, 340.04 nm; fluorescence (H₂O, 0.05% NaOH): λ_{exc} = 342 nm, λ_{ems} = 455 nm; HRMS (ESI-MS, 140 eV): m/z [M + H]⁺ calculated for C₁₃H₁₂NO₄⁺, 246.0761; found, 246.0841; calculated for C₁₃H₁₁NNaO₄⁺, 268.0580; found, 268.0604; RP-C18 HPLC: t_R = 8.87 min, 98.8%.

2-(2,4-Difluorophenyl)-5-hydroxypyridine-4-carboxylic acid (51). Compound **51** was prepared as for compound **19** by reacting compound **48** (0.043 g) with 10% NaOH solution (3 mL) to obtain 0.037 g of a yellow solid. Yield 97%; R_f : 0.71 (eluent BuOH/CH₃COOH/H₂O 3:1:1); ¹H NMR (400 MHz, D₂O/NaOD): δ 7.76 (d, J = 0.5 Hz, 1H, H-6), 7.44 (m, J_{H-H} = 8.86 Hz, J_{H-F} = 6.72 Hz, 1H, H-3'), 7.27 (dd, J = 2.1 Hz, J = 0.5 Hz, 1H, H-3), 6.90 (m, H-4H' and H-6') ppm; ¹³C NMR (101 MHz, D₂O/NaOD): δ 104.13 (t, J = 26.40 Hz, C3'), 111.54 (dd, J = 21.30 Hz, J = 3.38 Hz, C5'), 122.59 (d, J = 4.99 Hz, C3), 123.83 (dd, J = 13.11 Hz, J = 3.91 Hz, C1'), 131.01 (dd, J = 8.98 Hz, J = 4.65 Hz, C6'), 136.22 (d, J = 1.82 Hz, C4), 142.78 (C6), 159.26 (dd, J = 249.82 Hz, J = 7.88 Hz, C2'), 159.60 (dd, J = 249.82 Hz, J = 7.88 Hz, C2'), 161.89 (dd, J = 242.82 Hz, J = 8.80 Hz, C4'), 168.40 (C5), 176.71 (COOH) ppm; UV-vis (H₂O, 0.05% NaOH): λ_{max} = 250.11, 330.46 nm; fluorescence λ_{exc} = 342 nm, λ_{ems} = 433.03 nm; HRMS (ESI-MS, 140 eV): m/z [M + H]⁺ calculated for C₁₂H₈F₂NO₃⁺, 252.0467; found, 252.0520; calculated for C₁₂H₇F₂NNaO₃⁺, 274.0286; found, 274.0337; RP-C18 HPLC: t_R = 10.59 min, 98.83%.

Synthesis of diethyl 2-((2,4-difluorophenylamino)-methylene)malonate (52). Into a 100 mL round-bottomed flask, 1.00 g (7.74 mmol) of 2,4-difluoroaniline was mixed with 1.565 mL (7.74 mmol) of diethyl 2-(ethoxymethylene)-malonate. The mixture was stirred at 90 °C for 3 h and the reaction was monitored by TLC analysis (eluent *n*-hexane/ethyl acetate 1:1). At the end of the reaction, the mixture was evaporated under vacuum to obtain 2.32 g of condensation product. Yield 99%; R_f : 0.78 (eluent *n*-hexane/ethyl acetate 1:1); ¹H NMR (300 MHz, CDCl₃): δ 11.00 (d, J = 13.24 Hz, 2H, N-H), 8.14 (d, J = 13.46 Hz, 1H, C-H), 7.24 (ddt, J = 5.39 Hz, J = 9.49 Hz, 1H, H-3), 6.92 (m, J = 6.99 Hz, 2H, H-5 and H-6), 4.28 (ddq, J = 7.12 Hz, 4H, OCH₂CH₃), 1.38 (ddt, J = 7.12 Hz, 6H, OCH₂CH₃) ppm.

Synthesis of ethyl 6,8-difluoro-4-hydroxyquinoline-3-carboxylate (53). In a two-necked round-bottomed flask, 15 mL of diphenyl ether were heated to boiling. An amount of 2.32 g of derivative **52** was then added portion wise, and the resulting mixture was refluxed for 15 min. After cooling to

room temperature, a precipitate started to precipitate from the mixture. The separated precipitate was collected by filtration and washed many times with diethyl ether and dried, obtaining 1.460 g of a pink solid. Yield 60%; R_f : 0.48 (eluent chloroform/MeOH 95:5); ^1H NMR (400 MHz, DMSO- d_6): δ 8.40 (s, 1H, H-2), 7.81 (m, J = 10.96 Hz, J = 2.76 Hz, 1H, H-7), 7.66 (m, J = 9.07 Hz, J = 1.46, 1H, H-5), 4.23 (q, J = 7.09 Hz, 2H, OCH_2CH_3), 1.28 ppm (t, J = 7.10 Hz, 3H, OCH_2CH_3); ^{13}C NMR (101 MHz, DMSO- d_6): δ 172.03 (dd, J = 2.85 Hz, COH), 164.80 ($\text{COOCH}_2\text{CH}_3$), 158.39 (dd, J = 245.59 Hz, J = 11.29 Hz, C8), 152.83 (dd, J = 253.18 Hz, J = 12.24 Hz, C6), 145.23 (C2), 129.96 (dd, J = 7.22 Hz, J = 1.47 Hz, C4a), 126.14 (dd, J = 12.75 Hz, J = 2.02 Hz, C8a), 110.31 (C3), 108.16 (dd, J = 29.17 Hz, J = 21.14 Hz, C7), 106.50 (dd, J = 22.83 Hz, J = 4.22 Hz, C5), 62.32 (OCH_2CH_3), 14.72 (OCH_2CH_3) ppm.

Synthesis of 6,8-difluoro-4-hydroxyquinoline-3-carboxylic acid (54). Into a 50 mL round-bottomed flask, compound 53 (1.00 g) was dissolved in 5 mL of MeOH and 3 mL of 10% NaOH solution were added. The mixture was refluxed for 4 h and monitored by TLC analysis (eluent chloroform/MeOH, 95:5). At the end of the reaction, MeOH was removed under vacuum and the water layer was acidified with HCl 10% to obtain a precipitate corresponding to the carboxylic acid derivative 54. The precipitate was filtered and dried to yield 0.865 g of a white solid. Yield 87%; R_f : 0.11 (eluent chloroform/MeOH 95:5); mp = 284–285 °C; ^1H NMR (400 MHz, DMSO- d_6): δ 14.73 (s_{br} , 1H, OH), 13.87 (s_{br} , 1H, COOH), 8.63 (s, 1H, H-2), 8.00 (m, J = 9.90 Hz, J = 2.76 Hz, 1H, H-7), 7.78 (m, J = 8.74 Hz, J = 2.74 Hz, J = 1.54 Hz, 1H, H-5) ppm; ^{13}C NMR (101 MHz, DMSO- d_6): δ 177.33 ppm (dd, J = 3.06 Hz, OH), 166.12 (COOH), 159.14 (dd, J = 247.78 Hz, J = 11.22 Hz, C8), 153.11 (dd, J = 254.91 Hz, J = 12.78 Hz, C6), 145.44 (C2), 127.19 (dd, J = 9.19 Hz, J = 2.10 Hz, C4a), 126.73 (dd, J = 14.18 Hz, J = 2.36 Hz, C8a), 109.66 (dd, J = 29.73 Hz, J = 21.13 Hz, C3), 108.45 (C3), 106.04 (dd, J = 23.16 Hz, J = 4.19 Hz, C5) ppm; IR (KBr): $\tilde{\nu}$ (cm^{-1}) = 1690.00 (C=O), 1490.20 (C=N), 1389.89 (C–OH), 1315.54 and 1284.36 (C–OH), 1119.33 (C–F); UV-vis (H_2O , 0.05% NaOH): 228.28 nm, 303.90 nm; HRMS (ESI-MS, 140 eV): m/z [$\text{M} + \text{H}$] $^+$ calculated for $\text{C}_{10}\text{H}_6\text{F}_2\text{NO}_3^+$, 226.031; found, 226.0414; calculated for $\text{C}_{10}\text{H}_5\text{F}_2\text{NNaO}_3^+$, 248.013; found, 248.0232; RP-C18 HPLC: t_R = 9.75 min, 97.04%.

General procedure for the synthesis of *N*-methylated derivatives (55–57). As a typical procedure, the synthesis of 2-benzyl-3-hydroxy-1-methylpyridin-1-ium-4-carboxylate 55 is described in detail. 0.200 g (0.872 mmol) of compound 14 were dissolved in 4 mL of DMF. The resulting suspension was stirred at room temperature and 10% NaOH (7.5 mL) was added dropwise until complete dissolution of the solid was achieved (pH 9–10). Methyl iodide, 0.163 g (2.62 mmol, d = 2.28 g mL^{-1}), was added under stirring and the mixture was heated to 150 °C for 24 h and monitored by TLC analysis (eluent $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ 1:1). Once the starting material disappeared, the solvent was removed under reduced pressure obtaining a coloured solid which was dissolved in boiling water (50 mL) and 5% H_2O_2 (1 mL) was added. Then, the iodine was exhaustively extracted with CHCl_3 (5 $\times$ 15 mL) in a

separating funnel. The mixture was separated, and the aqueous phase was acidified with 1 M HCl to obtain the precipitation of the desired methylated compound, which was filtered and dried. The crude product was purified by flash-column chromatography (Biotage Isolera Spektra, RP-C18, eluent $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ 1:1) to yield 0.071 g of a yellow solid.

2-Benzyl-3-hydroxy-1-methylpyridin-1-ium-4-carboxylate (55). Yield 36%; R_f : 0.36 (eluent $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ 1:1); mp = 260–262 °C; ^1H NMR (300 MHz, $\text{D}_2\text{O}/\text{NaOD}$): δ 7.36 (m, J = 6.03 Hz, 1H, H-6), 7.16 (m, 2H, H-3' and H-5'), 7.13 (m, 1H, H-4'), 7.10 (m, 1H, H-5), 6.97 (m, 2H, H-2' and H-6'), 4.24 (s, 2H, CH_2), 3.72 (s, 3H, CH_3) ppm; ^{13}C NMR (75 MHz, $\text{D}_2\text{O}/\text{NaOD}$): δ 174.75 (C4), 168.33 (C3), 148.15 (C2), 141.96 (C4), 135.91 (C1'), 128.93 (C3' and C5'), 128.29 (C6), 127.87 (C2' and C6'), 126.79 (C4'), 121.48 (C5), 45.34 (CH_3), 37.45 (CH_2) ppm; IR (KBr): $\tilde{\nu}$ (cm^{-1}) = 3422.04 (OH, bonded), 3086.09 (CH, aromatic), 1653.88 (C=O), 1474.71 (C=N), 1376.11 (C–OH), 1302.11 and 1246.64 (C–OH); UV-vis (H_2O , 0.05% NaOH): 214.31, 321.85 nm; HRMS (ESI-MS, 140 eV): m/z [$\text{M} + \text{H}$] $^+$ calculated for $\text{C}_{14}\text{H}_{13}\text{NO}_3^+$, 244.0968; found, 244.1166; calculated for $\text{C}_{14}\text{H}_{12}\text{NNaO}_3^+$, 266.0788; found, 266.0927; RP-C18 HPLC: t_R = 8.70 min, 97.7%.

2-Phenyl-5-hydroxy-1-methylpyridin-1-ium-4-carboxylate (56). Compound 56 was prepared as for compound 55 by reacting compound 38 (0.045 g) with CH_3I (0.039 mL) to obtain 0.037 g of a yellow solid. Yield 70%; R_f : 0.43 (eluent $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ 1:1); mp = 165 °C; ^1H NMR (300 MHz, $\text{D}_2\text{O}/\text{NaOD}$): δ 7.68 (s, 1H, H-6), 7.49 (m, 3H, H-3', H-4' and H-5'), 7.40 (m, 2H, H-2' and H-6'), 7.29 (s, 1H, H-3), 3.82 (s, 3H, CH_3) ppm; ^{13}C NMR (75 MHz, $\text{D}_2\text{O}/\text{NaOD}$): δ 182.45 (COOH), 154.92 (C2), 146.69 (C5), 144.69 (C4), 140.18 (C6), 137.66 (C1'), 128.77 (C3' and C5'), 128.30 (C4'), 125.79 (C2' and C6'), 119.07 (C3) ppm; IR (KBr): $\tilde{\nu}$ (cm^{-1}) = 3422.37 (OH, bonded), 2980.59 (CH, aromatic), 2775.42 (CH_3), 2446.10 (CO–OH, bonded), 1647.96 (C=O), 1458.47 (C=N), 1354.99 (C–OH), 1299.27 and 1262.20 (C–OH); UV-vis (H_2O , 0.05% NaOH): 326.87 nm; HRMS (ESI-MS, 140 eV): m/z [$\text{M} + \text{H}$] $^+$ calculated for $\text{C}_{13}\text{H}_{12}\text{NO}_3^+$, 230.0812; found, 230.0934; calculated for $\text{C}_{13}\text{H}_{11}\text{NNaO}_3^+$, 252.0631; found, 252.0750; RP-C18 HPLC: t_R = 6.52 min, 97.9%.

5-Hydroxy-2-(3'-methoxyphenyl)-1-methylpyridin-1-ium-4-carboxylate (57). Compound 57 was prepared as for compound 55 by reacting compound 41 (0.122 g) with CH_3I (0.093 mL) to obtain 0.059 g of an orange solid. Yield 46%; R_f : 0.40 (eluent $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ 1:1); mp = 140–143 °C; ^1H NMR (300 MHz, $\text{D}_2\text{O}/\text{NaOD}$): δ 7.55 (s, 1H, H-6), 7.28 (t, J = 8.02 Hz, 1H, H-5'), 7.14 (s, 1H, H-3), 6.94 (dd, J = 8.18 Hz, J = 2.28 Hz, 1H, H-4'), 6.88 (m, J = 1.5 Hz, 1H, H-6'), 6.85 (m, 1H, H-2'), 3.68 (s, 3H, CH_3), 3.64 (s, 3H, OCH_3) ppm; ^{13}C NMR (75 MHz, $\text{D}_2\text{O}/\text{NaOD}$): δ 173.72 (COOH), 164.27 (C5), 161.24 (C3'), 154.23 (C2), 140.11 (C4), 139.43 (C5), 139.27 (C1'), 129.08 (C5'), 127.78 (C2'), 126.94 (C6'), 121.72 (C3), 120.76 (C4'), 62.74 (OCH_3), 48.25 (CH_3) ppm; IR (KBr): $\tilde{\nu}$ (cm^{-1}) = 3412.41 (OH, bonded), 3082.88 (CH, aromatic), 2553.89 (CO–OH, bonded), 2083.48 (intermolecular H bond), 1670.19 (C=O), 1542.41 (C=N), 1352.43 (C–OH), 1279.59 (C–OH), 1279.59

and 1229.93 (C–O–C); UV-vis (H_2O , 0.05% NaOH): 216.14, 325.17 nm; HRMS (ESI-MS, 140 eV): m/z $[\text{M} + \text{H}]^+$ calculated for $\text{C}_{14}\text{H}_{14}\text{NO}_4^+$, 260.0917; found, 260.1043; calculated for $\text{C}_{14}\text{H}_{13}\text{NNaO}_3^+$, 282.0737; found, 282.0879; RP-C18 HPLC: t_R = 7.57 min, 95.9%.

Biology

Isolation and culture of human monocyte derived macrophages

Human macrophages were differentiated from peripheral blood mononuclear cells (PBMCs) obtained from buffy-coat preparations of whole human blood. The buffy-coat preparations were obtained from Hospital of Padova under the protocol approved by the University of Padova Ethics Committee. The buffy-coat was mixed at a ratio of 1:1 (vol/vol) with sterile RPMI 1640 medium (Gibco; Life Technologies, Monza, Italy) and layered over Ficoll-Paque PLUS (GE Healthcare; Sigma, Milan, Italy). After centrifugation (1200 rpm, 30 min, at 20 °C without brake), PBMCs were separated by collecting the ring of cells located at the interface of the two phases. Cells were washed by centrifugation (1600 rpm, 10 min) in sterile RPMI 1640 medium, pooled, resuspended in RPMI 1640 supplemented with 1% penicillin–streptomycin and 10% FBS (all provided by Life Technologies), and counted. PBMCs were seeded (3×10^5 cells per well) on 96-well tissue-culture plates (Corning, Sigma) and cultured for 3 h in a 5% CO_2 , humidified, 37 °C incubator. Floating cells consisting of mainly lymphocytes were then removed by aspiration. Attached cells were extensively washed with RPMI 1640 and differentiated in mature macrophages by incubation for 10 days with complete medium supplemented with recombinant human granulocyte-macrophage colony-stimulating factor (rh GM-CSF, 2 ng mL^{-1} ; ImmunoTools; Friesoythe, Germany). The culture medium was renewed every 3 days.

Cytotoxicity assay

Diffunilal was dissolved in dimethyl sulfoxide (DMSO; Sigma); aza-analogs of diffunilal were dissolved in 5 N NaOH to obtain a stock solution of 100 mM. Compounds were then diluted in sterile water. For the cytotoxic assay, mature macrophages were treated for 24 h with compounds at final concentrations ranging from 0 to 100 μM . Controls received the highest final DMSO and NaOH concentrations used. Treatment compounds were removed, and cells were cultured for an additional 48 h. Then, MTT solution (50 $\mu\text{g}/100 \mu\text{L}$; Sigma) was added. Cells were incubated for 4 h at 37 °C and formazan crystals were then solubilized in 100 μL of 10% SDS (w/vol) and 0.01 N HCl. The absorbance was recorded 16 h later at 590 nm using a micro plate reader (Sunrise; Tecan, Switzerland). IC_{50} values were determined in three separate experiments, each performed in duplicate.

Cytokine and prostaglandin measurement

Differentiated macrophages were incubated in serum-free medium for 24 h with compounds at final concentrations

ranging from 0 to 10 μM with or without 100 ng mL^{-1} lipopolysaccharide (LPS from *Salmonella enterica* serotype Typhimurium; Sigma). At the end of incubation, the conditioned medium was collected and stored at –80 °C. Levels of tumor necrosis factor (TNF)- α , interleukin (IL)-8, and IL-1 β were measured in the conditioned medium using commercially available enzyme-linked immunosorbent assay kits (ELISA, Affymetrix eBioscience; Prodotti Gianni, Milan, Italy) and developed using 3,3',5,5'-tetramethylbenzidine (TMB). Optical densities were measured at 450 nm using a micro plate reader (Sunrise). The sensitivity of the assays ranged from 5 to 15 pg mL^{-1} . Prostaglandins (PGs) were screened in the conditioned medium using the Prostaglandin Screening ELISA kit (Cayman Chemical; Vinci-Biochem, Vinci, Italy). The sensitivity of the assay was approximately 30 pg mL^{-1} . All experiments were performed in triplicate.

Bacterial strains and antibiotics

Staphylococcus aureus ATCC 33592, *Streptococcus pyogenes* ATCC 14289, *Klebsiella pneumoniae* ATCC 13883, *Enterococcus faecium* ATCC 700221, *Pseudomonas aeruginosa* ATCC BAA-2108, and *Escherichia coli* ATCC 43894 were purchased from ATCC (LGC Standards; Milan, Italy) and cultured at 37 °C in Mueller–Hinton broth (MHB) or agar. Methicillin (MET), geneticin (GEN), ciprofloxacin (CPR), tetracycline (TET), and erythromycin (ERY) were purchased from Sigma and dissolved in sterile water.

Antimicrobial susceptibility test

The antimicrobial activities of antibiotics, diffunilal, and aza-analogs of diffunilal were tested through determination of the minimum inhibitory concentration (MIC) standardized by the Clinical and Laboratory Standards Institute.²³ The bacterial inoculum was grown in 10 mL of MHB at 37 °C for 16 h. The concentration of bacteria was adjusted to 0.5 McFarland standard. Bacteria were diluted in MHB and dispensed in 96-well microtiter plates (final bacterial concentration 1×10^4 CFU per well). The tested compounds were added at final concentrations ranging from 0.25 μM to 32 μM and plates were incubated at 37 °C for 16 h. The plates were at first visually inspected. Bacterial growth was recorded using a micro plate reader (Sunrise) by quantifying the adsorption at 620 nm. In addition, bacteria were diluted and used to inoculate MH agar plates. Uninoculated control wells, wells inoculated without compounds, and wells inoculated and incubated with the highest final DMSO and NaOH concentrations were included in each test. All experiments were performed at least in triplicate.

Assessment of interaction of antibiotics and diffunilal aza-analogs

In a separate set of experiments, the antimicrobial activity of diffunilal and aza-analogs were evaluated in combination with sub-inhibitory concentrations (MIC/4) of antibiotics. Experiments were performed as described above and bacterial

growth was monitored by recording the adsorption at 620 nm and by counting bacterial colony growth on MH agar plates. The inhibitory activity was reported as minimum modulatory concentration (MMC₄), which means the lowest concentrations of aza-analog required to achieve antibacterial activity in combination with the sub-inhibitory concentration (MIC/4) of the antibiotic.²⁴ All experiments were performed at least in triplicate.

Statistical analysis

Data were analyzed by two-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison post hoc test using GraphPad Prism (version 6.0). *P* values <0.05 were considered statistically significant.

Conflicts of interest

The authors declare no competing interest.

Acknowledgements

Synthesis by M. G. Ferlin and biological evaluation by P. Brun were supported by financial grants from the University of Padova, Italy.

Notes and references

- 1 C. Stone, C. Van Arman, V. Lotti, D. Minsker, E. Risley, W. Bagdon, D. Bokelman, R. Jensen, B. Mendlowski, C. Tate, H. Peck, R. Zwickley and S. McKinney, *Br. J. Clin. Pharmacol.*, 1977, **4**, 19S–29S; R. N. Brogden, R. C. Heel, G. E. Pakes, T. M. Speight and G. S. Avery, *Drugs*, 1980, **19**, 84–106.
- 2 A. S. Hendrix, T. J. Spoonmore, A. D. Wilde, N. E. Putnam, N. D. Hammer, D. J. Snyder, S. A. Guelcher, E. P. Skaar and J. E. Cassat, *Antimicrob. Agents Chemother.*, 2016, **60**, 5322–5330.
- 3 V. Khodaverdian, M. Pesho, B. Truitt, L. Bollinger, P. Patel, S. Nithianantham, G. Yu, E. Delaney, E. Jankowsky and M. Shoham, *Antimicrob. Agents Chemother.*, 2013, **57**, 3645–3652.
- 4 D. Williams and T. Lemke, *Foye's Principles of Medicinal Chemistry*, Lippincott Williams & Wilkins, Philadelphia, 2002.
- 5 P. Brun, A. Dean, V. Di Marco, P. Surajit, I. Castagliuolo, D. Carta and M. G. Ferlin, *Eur. J. Med. Chem.*, 2013, **62**, 486–497.
- 6 F. N. Penas, D. Carta, G. Dmytrenko, G. A. Mirkin, C. P. Modenutti, Á. C. Cevey, M. J. Rada, M. G. Ferlin, M. E. Sales and N. B. Goren, *Front. Immunol.*, 2017, **8**, 1738.
- 7 D. C. Swinney and J. Anthony, *Nat. Rev. Drug Discovery*, 2011, **10**, 507–519.
- 8 L. M. Espinoza-Fonseca, *Bioorg. Med. Chem.*, 2006, **14**, 896–897.
- 9 J. N. Pendleton, S. P. Gorman and B. F. Gilmore, *Expert Rev. Anti-Infect. Ther.*, 2013, **11**, 297–308.
- 10 G. A. Patani and E. J. LaVoie, *Chem. Rev.*, 1996, **96**, 3147–3176.
- 11 G. L. Walford, H. Jones and T. Y. Shen, *J. Med. Chem.*, 1971, **14**, 339–343.
- 12 H. Z. Zhang, Z. L. Zhao and C. H. Zhou, *Eur. J. Med. Chem.*, 2018, **144**, 444–492.
- 13 J. I. Levin and L. M. Laakso, in *Oxazoles: Synthesis, Reactions, and Spectroscopy, Part A*, John Wiley & Sons, Inc., Hoboken, NJ, USA, 2003, pp. 417–472.
- 14 T. Chancellor and A. Morton, *Synthesis*, 1994, **10**, 1023–1025.
- 15 R. G. F. Giles and C. P. Taylor, *ARKIVOC*, 2002, 149–165.
- 16 K. E. Benenato, A. L. Choy, M. R. Hale, P. Hill, V. Marone and M. Miller, Hydroxamic acid derivatives as gram-negative antibacterial agents, WO2010/100475A1, Astrazeneca, UK, 2010.
- 17 D. J. Collins, T. C. Hughes and W. M. Johnson, *Aust. J. Chem.*, 1996, 463–468.
- 18 G. X. Li, Z. Q. Liu and X. Y. Luo, *Eur. J. Med. Chem.*, 2010, **45**, 1821–1827.
- 19 European Committee on Antimicrobial Susceptibility Testing, 2013, Breakpoint tables for interpretation of MICs and zone diameters, version 3.1, 2013. http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/Breakpoint_table_v_3.1.pdf.
- 20 M. E. Skindersoe, M. Alhede, R. Phipps, L. Yang, P. O. Jensen, T. B. Rasmussen, T. Bjarnsholt, T. Tolker-Nielsen, N. Høiby and M. Givskov, *Antimicrob. Agents Chemother.*, 2008, **52**, 3648–3663.
- 21 K. Reuter, A. Steinbach and V. Helms, *Perspect. Med. Chem.*, 2016, **8**, 1–15.
- 22 C. A. Deryke, Y. L. Su, J. L. Kuti and D. P. Nicolau, *Drugs*, 2006, **66**, 1–14.
- 23 CLSI, *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically, Approved Standard, 9th ed.*, CLSI document M07-A9, Clinical and Laboratory Standards Institute, 950 West Valley Road, Suite 2500, Wayne, Pennsylvania 19087, USA, 2012.
- 24 F. Fontaine, A. Hequet, A. S. Voisin-Chiret, A. Bouillon, A. Lesnard, T. Cresteil, C. Jolivald and S. Rault, *J. Med. Chem.*, 2014, **57**, 2536–2548.