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Towards Gram-negative antivirulence drugs: New inhibitors of HldE kinase

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ABSTRACT

Gram-negative bacteria lacking heptoses in their lipopolysaccharide (LPS) display attenuated virulence and increased sensitivity to human serum and to some antibiotics. Thus inhibition of bacterial heptose synthesis represents an attractive target for the development of new antibacterial agents. HldE is a bifunctional enzyme involved in the synthesis of bacterial heptoses. Development of a biochemical assay suitable for high-throughput screening allowed the discovery of inhibitors **1** and **2** of HldE kinase. Study of the structure–activity relationship of this series of inhibitors led to highly potent compounds.

1. Introduction

Nowadays, it is estimated that 5–10% of patients are expected to develop a nosocomial infection during their stay in hospitals in the US and Europe. Considering their contribution to morbidity and mortality, these hospital-acquired infections therefore represent a public health problem with an increasing economic impact.[1–4]

Bacteria are the most common nosocomial pathogens, and their resistance to existing therapies is increasing at an alarming rate.[5,6] Gram-positive organisms such as MRSA (methicillin resistant *Staphylococcus aureus*) account for the majority of nosocomial infections. However, there has been in the last few years a dramatic increase in multi-drug resistant Gram-negative bacteria. Among those bacteria, extended-spectrum β-lactamase (ESBL) producing Enterobacteriaceae (e.g., *Escherichia coli*, *Proteus*, *Klebsiella*, *Enterobacter*) and *Pseudomonas aeruginosa* have become resistant to most of the currently available antibacterial agents. Although appropriate antimicrobial selection, surveillance systems, and effective infection-control procedures intend to limit the occurrence and spread of resistant pathogens, new effective therapies are urgently needed.[7,8]

Many nosocomial infections are caused by pathogens that take advantage of compromised host defenses, and some of these pathogens are normally constitutive of the normal flora of healthy humans. Therefore an interesting approach relies on targeting the factors affecting the pathogenicity of these bacteria. The so-called antivirulence strategy intends to increase the sensitivity of bacteria to the innate immunity components such as complement of human serum but also to classic antibiotics.[9,10]

Lipopolysaccharide (LPS) is one of the major constituent of the outer leaflet of the outer membrane of Gram-negative bacteria. LPS plays a major role in the host/pathogen interaction, helps to anchor proteins in the outer membrane and provides an efficient protective barrier against immune effectors.[11] Lipopolysaccharide consists of lipid A, a core oligosaccharide and a repeating polysaccharide O-antigen. The core oligosaccharide can be further divided into an inner core made of 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) and heptose residues, and an outer core comprising hexoses derivatives. The requisite LPS structure for cell viability is ReLPS: a LPS constituted only of lipid A and Kdo residues (deep-rough phenotype). Bacteria having the deep rough phenotype or a truncated LPS exhibit an increased sensitivity to antibiotics like erythromycin, novobiocin or rifampicin due to permeabilization of the outer membrane.[11,12] Furthermore, strains of *E. coli*, *Salmonella typhimurium* and *Neisseria meningitidis* harboring a ReLPS display attenuated virulence.[13–16]

Chemical inhibition of heptose synthesis or transfer should lead to bacteria having a truncated LPS. Such bacteria should display an increased sensitivity to host defenses and an increased permeability to antibiotics. Thus inhibition of heptose synthesis or transfer

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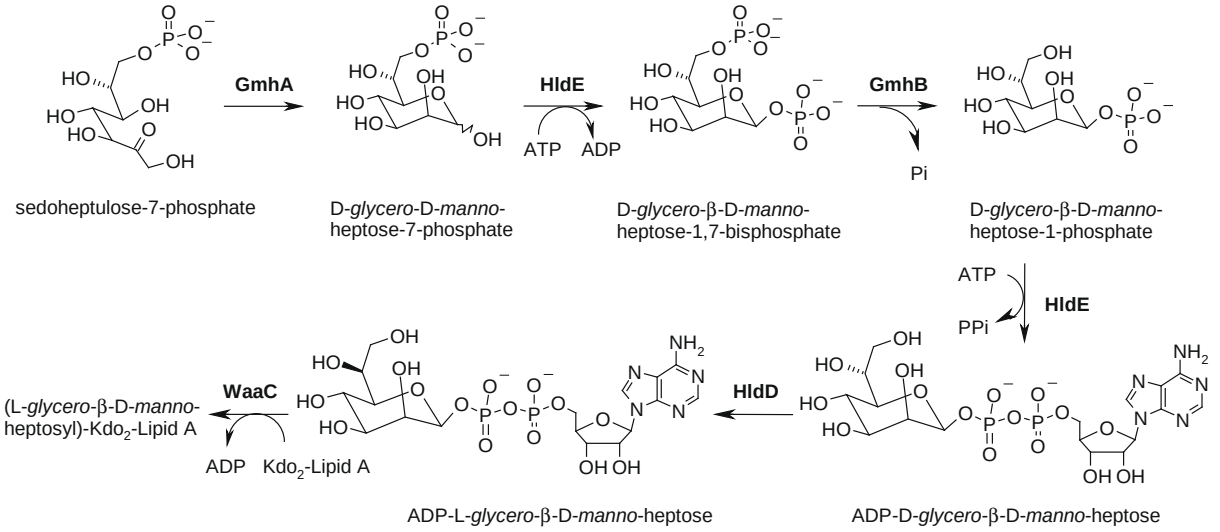


Scheme 1. Synthesis of ADP-L-*glycero*-β-D-*manno*-heptose from sedoheptulose-7-phosphate and incorporation into LPS.

represents an attractive target for the development of new Gram-negative antimicrobial agents with antivirulence and membrane permeabilizing properties.

We previously disclosed the structure of *E. coli* WaaC enzyme,[17] which catalyzes the addition of the first heptose molecule to a LPS Kdo residue, and the discovery of inhibitors of this enzyme.[18] Here we report our efforts towards inhibition of HldE (previously named RfaE), an enzyme involved in the biosynthesis of ADP-heptose.

2. Biochemistry

2.1. Biosynthesis of bacterial heptose

The bacterial synthesis of ADP-L-*glycero*-β-D-*manno*-heptose from sedoheptulose-7-phosphate in *E. coli* is presented in Scheme 1.[19] Sedoheptulose-7-phosphate is first obtained from the reaction of fructose-6-phosphate with ribose-5-phosphate catalyzed by transketolase TktA. Ketose-aldose isomerase GmhA transforms sedoheptulose-7-phosphate into D-*glycero*-D-*manno*-heptose-7-phosphate, which is next phosphorylated at the anomeric hydroxyl by HldE. At this step only the β anomer is formed. Next, dephosphorylation at position 7 is achieved by phosphatase GmhB, and adenylyl transfer to the phosphate group in position 1 catalyzed by HldE leads to ADP-D-*glycero*-β-D-*manno*-heptose. Inversion of the D-stereochemistry of the C6 hydroxyl by the epimerase HldD (previously named RfaD) affords ADP-L-*glycero*-β-D-*manno*-heptose which is next incorporated into LPS by the heptosyltransferases WaaC, WaaF and WaaQ if a third heptose molecule is present.

2.2. Biochemical characterization of HldE-kinase

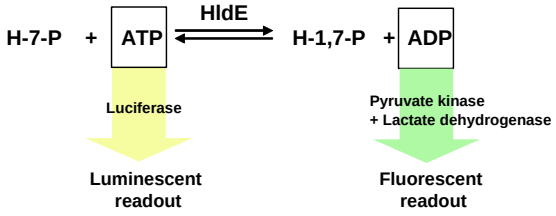
HldE in *E. coli* is a bifunctional cytoplasmic enzyme comprising 2 functional domains: a carbohydrate kinase and an adenylyltransferase one.[20] We focused in this work on the inhibition of the first one: the kinase activity. HldE is well conserved among Gram-negative bacteria as shown in Table 1. However, this enzyme is absent of genera producing LPS core devoid of heptose like *Acinetobacter*, *Moraxella* and *Chlamydia*.[21] In bacteria like *N. meningitidis*, the two catalytic activities found in HldE of *E. coli* are split on two separate enzymes HldA (kinase) and HldC (adenylyltransferase). Identity/similarity of HldE (kinase domain) with human enzymes is poor: a significant sequence similarity is essentially found with human ribokinase (40%). Inhibition of HldE-kinase has therefore a low potential for toxicity by interfering with human enzymes.

Table 1
Conservation of HldE protein among Gram-negative bacteria

Bacteria	Identity/similarity (%)
Escherichia coli	100/100
Shigella sonnei	99/99
Enterobacter sp. 638	93/98
Salmonella typhimurium LT2	93/97
Klebsiella pneumoniae MGH 78578	92/97
Yersinia pestis	86/92
Haemophilus influenzae	69/81
Pasteurella multocida	68/82
Pseudomonas aeruginosa	58/72
Neisseria meningitidis Z2491	50/66
Helicobacter pylori	38/58

A biochemical assay for HldE-kinase (*E. coli*) activity was set up using D-*glycero*-D-*manno*-heptose-7-phosphate (H-7-P) and ATP as substrates. Readout was either luminescent (ATP depletion) or fluorescent (ADP formation) depending on possible compound interferences (Scheme 2).

As for all carbohydrate kinases, the kinase activity requires a divalent cation as electrophilic activator. Here, Mg^{2+} and Mn^{2+} cations were shown to sustain HldE activity, the latter being approximately 3-fold more efficient than the former at the optimal concentration of 1 mM (Fig. 1A). As often observed within the carbohydrate kinase family,[22,23] a monovalent cation is absolutely required for catalysis: while Na^+ did not affect activity, K^+ strongly activated HldE-kinase with a Kd of 12 mM, and an optimal concentration of 25 mM (Fig. 1B). The optimal pH was 7.5. Surprisingly, DMSO (up to 15%) had a significant activating effect. We used a maximum of 5% DMSO in the assays for dissolution of compounds.



Scheme 2. Biochemical assays for HldE-kinase. H-7-P = D-*glycero*-D-*manno*-heptose-7-phosphate; H-1,7-P = D-*glycero*-β-D-*manno*-heptose-1,7-bisphosphate.

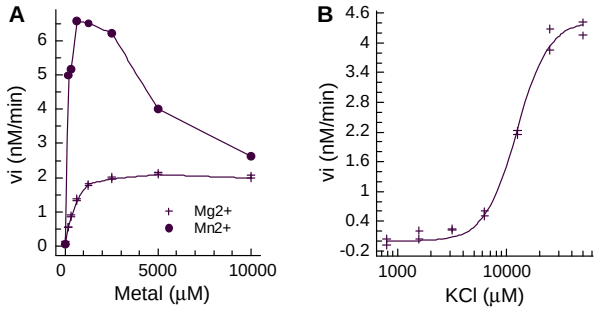


Figure 1. HldE-kinase initial velocity (vi): (A) Effect of the divalent cation added; (B) effect of K^+ ions.

Table 2
Steady-state kinetic parameters of HldE-kinase of *E. coli*

Entry	Compound	K_M[a] (μM)
1	ATP at [H-7-P] = 1.8 μM	147 ± 14
2	ATP at [H-7-P] = 0.2 μM	>0.8[b]
3	H-7-P at [ATP] = 50 μM	0.60 ± 0.07
4	H-7-P at [ATP] = 0.3 μM	0.23 ± 0.03

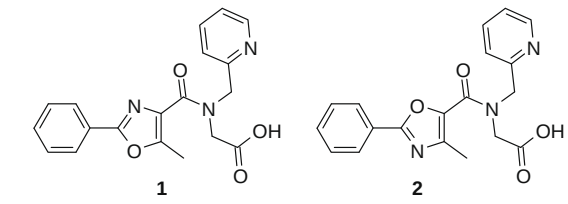
[a] Values ± standard deviations.
[b] No defined value could be determined due to rapid consumption of H-7-P at high ATP concentrations.

The steady-state kinetic parameters of HldE-kinase of *E. coli* were measured at pH 7.5, in the presence of 1 mM $MnCl_2$, 25 mM KCl and 5% DMSO (Table 2). We found a k_{cat} of 1714 ± 24 min^{-1} for the enzyme. The K_M of ATP for HldE-kinase was in the high micromolar range (Table 2, entry 1) as observed for *E. coli* ribokinase.[23] On the contrary, the K_M of H-7-P was below 1 μM (Table 2, entries 3 and 4). These values were extremely low compared to the K_M of D-ribose for the same ribokinase (280 μM).[23]

2.3. Identification and characterization of HldE-kinase inhibitors

The high-throughput screening (HTS) of 40,000 compounds from Mutabilis internal library was performed on HldE-kinase of *E. coli* using the luminescent assay. HTS was performed in the presence of 3 nM of HldE, 1 mM $MnCl_2$, 25 mM KCl and 5% DMSO at pH 7.5. In this assay, concentrations of ATP and H-7-P were both set at 0.2 μM in order to favor detection of substrate competitors. Concentration for each compound to be screened was 50 μM. This HTS campaign led to the identification of compounds **1** and **2** as inhibitors of HldE-kinase with IC_{50} of 51 ± 11 μM and 69 ± 16 μM, respectively (Scheme 3).

The mechanism of action of compound **2** was investigated in order to rule out non specific (promiscuous) inhibition. Inhibition kinetic of **2** was linear. It neither depended on order of addition nor on enzyme/inhibitor pre-incubation. The inhibition was neither shifted by Triton-X100 (100 μM to 1 mM) nor by H-7-P (up to 15 K_M) (data not shown). Finally, as shown in Figure 2, compound **2** behaved as a competitive reversible inhibitor with respect to ATP. The K_i of compound **2** determined using the fluorescent assay was 38 ± 3 μM. The only other published small molecule inhibitor of HldE reported to date is a sulfonic acid derivative with a slightly higher K_i of 63 μM for HldE-kinase of *E. coli*.[20b]



Scheme 3. Structure of HTS hits **1** and **2**.

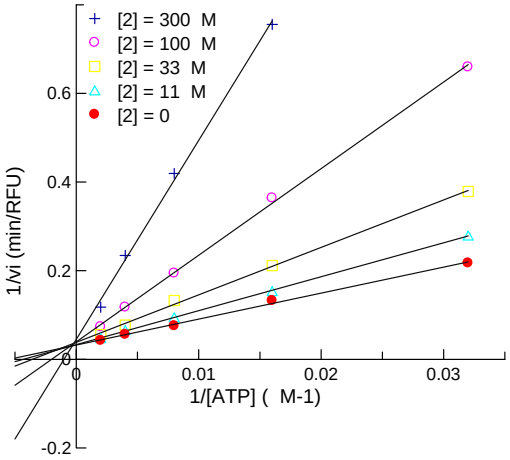


Figure 2. Competition between **2** and ATP on HldE-kinase measured in the presence of 1.8 μM of H-7-P substrate: double reciprocal plot (1/initial velocity vs 1/[ATP]) showing intersecting lines on *y*-axis at $1/V_{max}$.

Taken together, these data showed that compound **2** was not a promiscuous hit as analyzed by Feng and Shoichet:[24] this compound was a specific inhibitor of HldE-kinase, binding reversibly to its ATP site.

2.4. Selectivity of the HldE-kinase inhibitors

The selectivity of our inhibitors of HldE-kinase of *E. coli* (HldE-K) with respect to the ribokinase (RK) of *E. coli*, and to HldA (the equivalent of HldE-kinase for *N. meningitidis*) was studied. The goal was to investigate the ability of such chemical scaffold to recognize the ATP binding pocket of closely related enzymes: on the one hand HldA, an enzyme catalyzing the same reaction but with only 66% sequence similarity with respect to HldE-K, and on the other hand RK, an enzyme catalyzing another reaction, although belonging to the same carbohydrate kinase family (38% sequence similarity with respect to HldE-K). Comparison with human ribokinase was also envisaged in order to take into consideration potential interactions. RK, HldA and HldE share highly conserved amino-acids in the ATP binding site as depicted from the structure of *E. coli* RK[25] (Fig. 3). Therefore an ATP competitor like compound **2** could potentially interact with any of them.

A biochemical assay was set up for RK of *E coli* with D-ribose and ATP as substrates, and for HldA with H-7-P and ATP as substrates (see Section 6 for details). The K_M of ATP for both enzymes is presented in Table 3.

The IC_{50} of compounds **1** and **2** were measured on RK and were found to be higher than 300 μM. In this assay, the concentration of ATP was 3 μM, i.e., far below the apparent K_M of ATP in the same conditions (18 μM). Their IC_{50} is therefore a reasonable approximation of their affinity for the ATP site of RK.

On this basis, we concluded that the series related to **1** and **2** had the potential for being selective of HldE with respect to RK and was therefore of interest for chemical optimization. In order to study the structure–activity relationship (SAR) of these compounds, the synthesis of analogues was investigated. The synthesis of derivatives and their activity is described herein.

```
                    200       210       220       230       240       250       260       270       280
                     |         |         |         |         |         |         |         |         |
HldE_Ecoli          GATLLTPNLSEFEAVVGKCKTEEEIVERGMKLIADYELSALLVTRSEQGMSLLQPGKA-PLHMPTQAQEVYDVTGAGDTVIGV
HldA_Nmeningitidis  GATLITPNRAELKEVVGSWKNENDLTEKAQNLRRHLDLTAILLTRSEEGMTLFSEGE--PIYQPTRAQEVYDVSGAGDTVIAG
RK_Ecoli            LVDIITPNETEAEKLTGIRVENDEDAAKAAQVLHEKGIRTVLITLGSRG--VWASVNGEGQRVPGFRVQAVDTIAAGDTFNGA
RK_Human            LSDVFCCNESEAEILTGLTVGSAADAGEAALVLLKRGCQVVIITLGAEGCVVLSQTEPEPKHIPTEKVKAVDTTGAGDSFVGA
Consensus           l d itpNesEae ltG  v   eda kaa vl   g   vliTlg eG  vls  e ep h Pt  v avDttgAGDtf ga

                          290       300       310       320       330       340       350       360
                           |         |         |         |         |         |         |         |
HldE_Ecoli          LA--ATLAAGNSLEEACFFANAAAGVVVGKLGTSTVSPIELENAVRGRADTGFGVMTEEELKLAVAAARKRGEKVVMTNG
HldA_Nmeningitidis  MG--LGLAAGCTMPEAMYLANTAAGVVVAKLGTAVCSFAELTKALSGQSTM----------------------------
RK_Ecoli            LI--TALLEEKPLPEAIRFAHAAAAIAVTRKGAQPSVPWREEIDAFLDRQR----------------------------
RK_Human            LAFYLAYYPNLSLEDMLNRSNFIAAVSVQAAGTQSSYPYKKDLPLTLF------------------------------
Consensus           $a  lal    s$e#a  fanaaAa! V   Gtq s p   e  l l
```

Figure 3. Sequences alignment: HldE of *E. coli*, HldA of *N. meningitidis*, RK of *E. coli* and human are shown. Conserved sequence motifs found in the ATP binding site of *E. coli* RK[25] are underlined. Alignment done with Multalin.[26]

Table 3
Steady-state kinetic parameters of HldA and RK of *E. coli*

Entry	Enzyme	K_M of ATP[a] (μM)
1	HldA at [H-7-P] = 0.2 μM	>0.4[b]
2	RK at [D-ribose] = 3 μM	18 ± 6

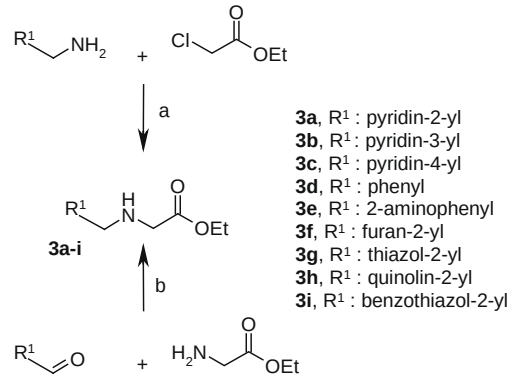
[a] Values ± standard deviations.
[b] No defined value could be determined due to rapid consumption of H-7-P at high ATP concentrations.

3. Chemistry

The synthesis of analogues of compounds **1** and **2** was first carried out by coupling an oxazole-carboxylic acid to a secondary amine. In order to avoid side reactions, the acidic function of the desired amines needed to be masked.

A set of secondary amines **3a–i** was prepared by nucleophilic substitution or reductive amination as depicted in Scheme 4. The 2-pyridinyl group R^1 of **1** and **2** was replaced by regioisomers or other heterocycles. The carboxylic acid of the final analogues was protected as an ethyl ester.

The synthesis of oxazole-carboxylic acids **4** and **6** with varying substituents on the phenyl ring was realized according to known procedures (Scheme 5).[27–29] Reaction of methyl 2-aminoacetoacetate hydrochloride with aroyl chlorides gave the corresponding amides which were cyclized using triphenylphosphine, iodine and triethylamine. Saponification led to oxazolecarboxylic acids **4**. Another route involved the thermal reaction of ethyl 2-chloroacetoacetate with aryl amides followed by saponification to afford oxazolecarboxylic acids **6**. Coupling of **4** and **6** to secondary amines **3a–i** was performed using EDAC as coupling agent. Saponification finally afforded analogues **5a–t** and **7a–c**. Compounds **5b**, **5f**, **5k**

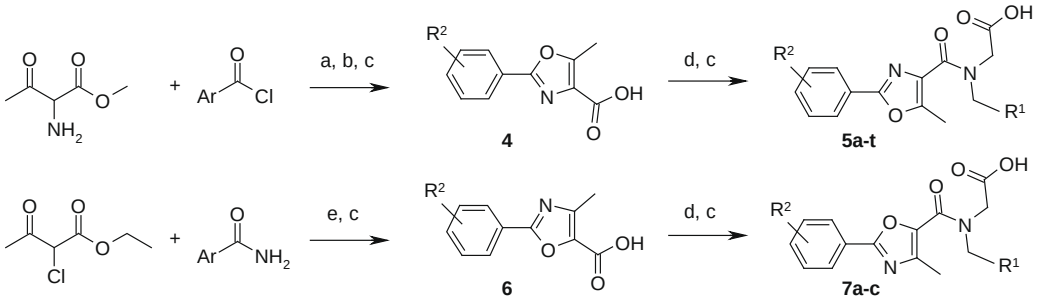


Scheme 4. Reagents and conditions: (a) NEt_3, CH_3CN, rt to 50 °C, overnight; (b) AcOH, $NaBH_3CN$, CH_2Cl_2, 0 °C to rt, overnight.

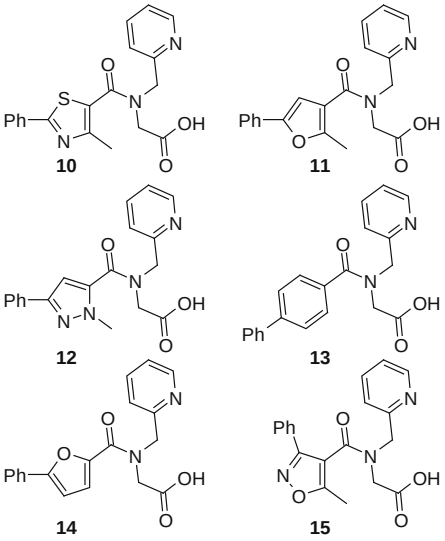
in which R^2 is NH_2 were obtained by reduction of the corresponding nitro groups of **5a**, **5e**, **5j**, respectively.

The replacement of the central oxazole ring by other heterocycles was also implemented. Compounds **10–15** (Scheme 6) were prepared from commercially or readily available carboxylic acids which were reacted with amine **3a** followed by saponification.

A straightforward synthetic pathway to thiazole derivatives **9a–h** was also devised from 2-bromo-4-methyl-1,3-thiazole-5-carboxylic acid (Scheme 7). Since activation of the carboxylic acid with oxalyl chloride led to partial replacement of the bromine atom by chlorine, oxalyl bromide was used. Coupling with amines **3a** and **3i** followed by one-pot Suzuki coupling and saponification afforded compounds **9a–h**.



Scheme 5. Reagents and conditions: (a) NEt_3, CH_2Cl_2, rt, overnight; (b) PPh_3, I_2, NEt_3, CH_2Cl_2, rt, overnight; (c) LiOH, THF, H_2O, rt, overnight; (d) EDAC, 4-DMAP, amine **3a–3i**, CH_2Cl_2, rt to 50 °C, overnight; (e) toluene, 120–140 °C, overnight.



Scheme 6. Structures of analogues **10**–**15**.

4. Results and discussion

The activity on HldE kinase of compounds synthesized was measured using the biochemical assay described previously. Results are reported in Tables 4–7.

As seen in Table 4, substituents were introduced on the phenyl group in different positions. Although it appeared difficult to deduce from the results a clear trend concerning the required steric and electronic properties of the best substituent in each position, some important observations were made. The amino group was the best substituent in *ortho* (**5b**) and *para* (**5k**) positions (Table 4, entries 4 and 14). In contrast it led to the greatest loss of activity in *meta* position (**5f**, Table 4, entry 8). An opposite behavior was observed for the nitro substituent: a great loss of activity was seen in *ortho* (**5a**) and *para* (**5j**) positions (Table 4, entries 3 and 13) whereas it displayed the best activity in *meta* position (**5e**, Table 4, entry 7). The trifluoromethyl group in *meta* (**5g**) position (Table 4, entry 9) gave only a slight increase in activity compared to **1**, however a loss of activity was observed in *ortho* (**5c**) and *para* (**5l**) positions (Table 4, entries 5 and 15). Introduction of a bromine in any position gave an increase of activity (**5d**, **5h**, **5m**, Table 4, entries 6, 10 and 16), the most significant effect being observed in *meta* position. The methoxy substituent in *meta* (**5i**) afforded a gain of activity (Table 4, entry 11), and in contrast a slight loss of activity in *para* position (**5n**, Table 4, entry 17). Similarly, as seen by the comparison between **2** and **7a** (Table 4, entries 2 and 12), introduction of a methoxy substituent in *meta* position also led to an increase of activity with this scaffold.

Analogues having a heteroaromatic ring (pyridinyl, pyrazinyl, furanyl) or an ethyl group instead of the phenyl ring were also prepared, but they were all inactive (IC_{50} > 300 μM). Therefore, we concluded that the phenyl ring was required for activity and that with respect to the IC_{50} of **1** at 51 μM, *meta* substitution by a nitro, bromine or a methoxy group gave the most significant gain in potency: 5.8 μM (**5e**), 10 μM (**5h**) and 15 μM (**5i**), respectively. In contrast, only a moderate increase of activity could be obtained with *ortho* and *para* substitutions by an amino group, at 31 μM (**5b**) and 22 μM (**5k**), respectively.

As seen in Table 5, the central oxazole ring could be replaced by other heterocycles such as thiazole (**10**), furane (**11**) or pyrazole (**12**) without affecting the potency (Table 5, entries 1–3). Compound **14** having no methyl group displayed an IC_{50} of 100 μM

Table 4
Influence of R2 on the inhibitory activity

Entry	Compound	R1	R2	IC_{50}[a] (μM)
1	**1**	Pyridin-2-yl	None	51 ± 11
2	**2**	Pyridin-2-yl	None	69 ± 16
3	**5a**	Pyridin-2-yl	*o*-NO_2	141 ± 24
4	**5b**	Pyridin-2-yl	*o*-NH_2	31 ± 11
5	**5c**	Pyridin-2-yl	*o*-CF_3	121 ± 30
6	**5d**	Pyridin-2-yl	*o*-Br	44 ± 10
7	**5e**	Pyridin-2-yl	*m*-NO_2	5.8 ± 1.6
8	**5f**	Pyridin-2-yl	*m*-NH_2	153 ± 40
9	**5g**	Pyridin-2-yl	*m*-CF_3	35 ± 11
10	**5h**	Pyridin-2-yl	*m*-Br	10 ± 2
11	**5i**	Pyridin-2-yl	*m*-OMe	15 ± 4
12	**7a**	Pyridin-2-yl	*m*-OMe	17 ± 4
13	**5j**	Pyridin-2-yl	*p*-NO_2	151 ± 30
14	**5k**	Pyridin-2-yl	*p*-NH_2	22 ± 3
15	**5l**	Pyridin-2-yl	*p*-CF_3	142 ± 9
16	**5m**	Pyridin-2-yl	*p*-Br	27 ± 3
17	**5n**	Pyridin-2-yl	*p*-OMe	75 ± 6

[a] Values ± standard deviations are means of three experiments.

Table 5
Influence of the central ring on the inhibitory activity

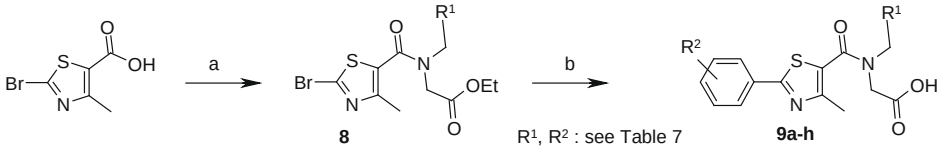
Entry	Compound	IC_{50}[a] (μM)
1	**10**	52 ± 14
2	**11**	42 ± 13
3	**12**	57 ± 15
4	**13**	>300
5	**14**	100 ± 20
6	**15**	>300

[a] Values ± standard deviations are means of three experiments.

Table 6
Influence of R1 on the inhibitory activity

Entry	Compound	R1	R2	IC_{50}[a] (μM)
1	**5o**	Pyridin-3-yl	None	>300
2	**5p**	Pyridin-4-yl	None	>300
3	**5q**	Phenyl	None	>300
4	**7b**	2-Aminophenyl	None	>300
5	**5r**	Furan-2-yl	None	>300
6	**5s**	Thiazol-2-yl	None	110 ± 10
7	**5t**	Quinolin-2-yl	*m*-OMe	12 ± 4
8	**7c**	Benzothiazol-2-yl	*m*-OMe	0.27 ± 0.07

[a] Values ± standard deviations are means of three experiments.



Scheme 7. Reagents and conditions: (a) $(COBr)_2$, CH_2Cl_2, DMF, 0 °C to rt, 2 h, then amine **3a** or **3i**, DIPEA, 0 °C to rt, overnight; (b) boronic acid or boronate ester, $PdCl_2$dppf, Cs_2CO_3, 1,4-dioxane, water, 110 °C, 2 days.

Table 7
Further R2 modifications in *meta* and *para* positions

Entry	Compound	R1	R2	IC_{50}[a] (μM)
1	**5u**	Pyridin-2-yl	*m*-Morpholinyl	70 ± 30
2	**5v**	Pyridin-2-yl	*m*-$NHCOCH_3$	28 ± 2
3	**5w**	Pyridin-2-yl	*m*-$NHCOC_3H_5$	14 ± 3
4	**9a**	Pyridin-2-yl	*m*-$COCH_3$	216 ± 75
5	**9b**	Pyridin-2-yl	*m*-NO_2, *p*-NH_2	13 ± 2
6	**9c**	Benzothiazol-2-yl	*m*-OMe	0.42 ± 0.04
7	**9d**	Benzothiazol-2-yl	*m*-NO_2	0.50 ± 0.09
8	**9e**	Benzothiazol-2-yl	*m*-SO_2Me	75 ± 2
9	**9f**	Benzothiazol-2-yl	*m*-OMe, *p*-NH_2	0.26 ± 0.06
10	**9g**	Benzothiazol-2-yl	[b]	0.48 ± 0.04
11	**9h**	Benzothiazol-2-yl	[b]	0.33 ± 0.04
12	**5x**	Benzothiazol-2-yl	*m*-Pyrazol-3-yl	0.11 ± 0.02

[a] Values ± standard deviations are means of three experiments.
[b] R2 substituents are fused with the phenyl ring, see Scheme 9 for structures corresponding to **9g** and **9h**.

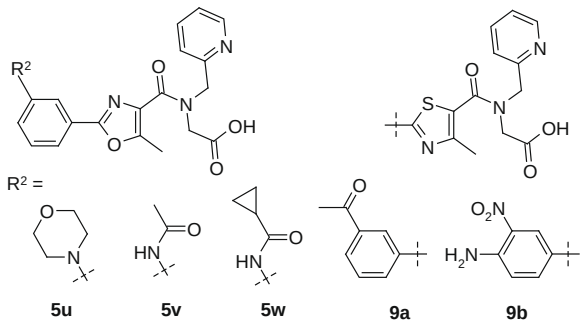
(Table 5, entry 5).When the substitution pattern was modified or the central ring replaced by a 6-membered ring as in **13** and **15**, a complete loss of activity was observed (Table 5, entries 4 and 6).

The carboxylic acid on one of the amide side chains of **1** and **2** proved to be essential for activity. Replacement by an amide ($CONH_2$), alcohol, tetrazole, or acylsulfonamide ($CO–NH–SO_2Me$) gave inactive analogues. Additionally, lengthening of this side chain by one methylene led to a fivefold decrease in activity (IC_{50} of 272 μM).

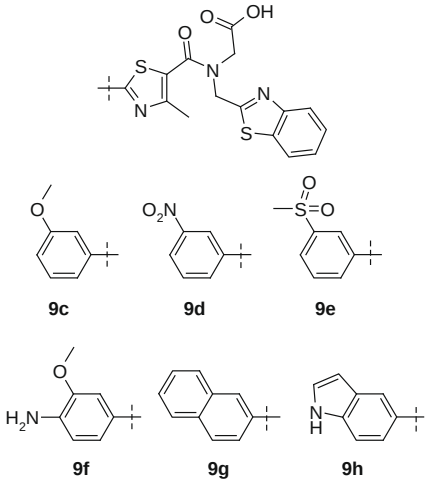
Considering the other amide side chain bearing the pyridyl group, the results presented in Table 6 show that removal (**5q**) or shift (**5o**, **5p**) of the pyridyl nitrogen provided inactive compounds (Table 6, entries 1–3). Placing the nitrogen out of the ring (**7b**) also proved deleterious for activity (Table 6, entry 4). Replacement of the 2-pyridinyl group by a 2-furanyl (**5r**) led to a complete loss of activity (Table 6, entry 5). In contrast the presence of a 2-thiazolyl (**5s**) instead of the 2-pyridinyl led only to a twofold loss of activity (table 6, entry 6), thus reinforcing the importance of the nitrogen and its position in R^1. Whereas the presence of an additional phenyl ring (**5t**) fused with the 2-pyridinyl only afforded a slight improvement compared to **5i** (Table 6, entry 7), it led to a tremendous gain in potency when it was fused with the 2-thiazolyl group, compound **7c** displaying an IC_{50} of 0.27 μM (Table 6, entry 8).

Substitution on the phenyl ring was further investigated, with a particular emphasis on the *meta* position. Morpholine derivative **5u** and amides **5v** and **5w** were prepared from intermediates in the synthesis of **5h** and **5f** (Scheme 8). Since the central oxazole ring could be replaced by a thiazole, the straightforward route depicted in Scheme 7 allowed the synthesis of analogues **9a**–**9h** (Schemes 8 and 9).

As seen in Table 7, replacement of the *meta* amino group of **5f** by a morpholine led to an increase of activity but remained less active than **1** (**5u**, Table 7, entry 1). Interestingly introduction of



Scheme 8. *Meta* and *meta–para* substitutions with R1 = pyridin-2-yl.



Scheme 9. *Meta* and *meta–para* substitutions with R1 = benzothiazol-2-yl.

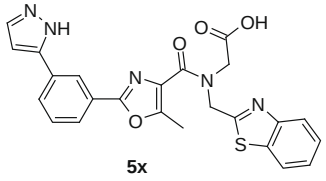
amide functionality in *meta* position (**5v**, **5w**, Table 7, entries 2 and 3) improved the activity. The most notable effect was observed with the cyclopropylcarboxamide substituted **5w**.

Replacement of the central oxazole ring of **7c** by a thiazole ring (**9c**) resulted in a slight loss of activity (Table 7, entry 6). Surprisingly, the nitro substituent was less potent than the methoxy group in *meta* position with this scaffold (**9d**, Table 7, entry 7). Attempts to replace the nitro group by other electron withdrawing substituents such as acetyl or methanesulfonyl groups generated poorly active compounds (**9a**, **9e**, Table 7, entries 4 and 8).

As could be expected, compound **9b** bearing *meta* nitro and *para* amino substituents was less active than **5e** (Table 7, entry 5), in part due to antagonistic electronic effects of the substituents. However compound **9f** with *meta* methoxy and *para* amino groups was more active than **9c** (Table 7, entry 9), resulting from additional effects of substituents.

Compounds **9g** and **9h** in which the phenyl ring was replaced by a naphthyl or indolyl ring also showed interesting activities with IC_{50} of 0.48 μM and 0.33 μM, respectively (Table 7, entries 10 and 11). These last results suggested that room was available around the phenyl ring, and in particular for a planar additional ring. Since the *meta* substitution on the phenyl ring had already given potent compounds with various substituents having different electronic properties, we decided to prepare compound **5x** with a pyrazole ring in *meta* position (Scheme 10). Compound **5x** was synthesized by Suzuki coupling between the corresponding *meta*-bromo ester derivative and 1*H*-pyrazole-3-boronic acid followed by saponification. This compound displayed the best activity of our series of inhibitors with an IC_{50} of 0.11 μM (Table 7, entry 12).

The IC_{50} of a subset of compounds towards HldE-kinase of *E. coli*, RK of *E. coli* and HldA of *N. meningitidis* was studied (Table 8). ATP concentration in each of the 3 assays (0.2 μM for HldE and HldA, 3 μM for RK) was below its apparent K_M in the same conditions (see Tables 2 and 3). The IC_{50} measured in these assays provided therefore a reasonable approximation of the relative



Scheme 10. Structure of **5x**.

Table 8
Comparison of IC_{50} of compounds on HldE-kinase (*E. coli*), HldA (*N. meningitidis*) and RK (*E. coli*)

Compound	HldE-K IC_{50}[a] (μM)	HldA IC_{50}[a] (μM)	RK IC_{50} (μM)
5e	5.8 ± 1.6	>300	>300
5i	15 ± 4	>300	>300
7c	0.27 ± 0.07	6.0 ± 0.9	>300
9d	0.50 ± 0.09	75 ± 9.5	>300
9g	0.48 ± 0.04	12 ± 0.7	>33[b]
9h	0.33 ± 0.04	8.5 ± 0.9	>100[b]

[a] Values ± standard deviations are means of three experiments.
[b] Signal quenching was observed above this concentration.

selectivity of the tested compounds towards the three enzymes. On the basis of the compounds tested, we could conclude that the series appeared selective with respect to RK. Actually, none of the compounds tested did inhibit RK significantly at doses as high as 300 μM. HldA on the contrary was inhibited but only by the most potent compounds and with a 10 to >100-fold higher IC_{50}: the series could therefore be potentially recognized by HldE/HldA enzymes of Gram-negative bacteria but not equally.

Compounds of this series displayed no antibacterial activity against strains of *E. coli* and other Gram-negative bacteria. Since HldE is not essential for survival of Gram-negative bacteria under normal growth conditions, further envisaged investigations include characterization of the effect of our inhibitors on LPS biosynthesis and on the sensitivity towards antibiotics of Gram-negative bacteria.

5. Conclusion

HldE is a bifunctional enzyme involved in the synthesis of bacterial heptoses constituents of LPS. Gram-negative bacteria lacking heptoses in their LPS display attenuated virulence and increased sensitivity to human serum and to some antibiotics. Thus HldE represents a target of interest for the development of new Gram-negative antimicrobial agents.

High-throughput screening on HldE-kinase activity of *E. coli* led to the discovery of inhibitors **1** and **2** with IC_{50} in the range of 50–70 μM. These compounds were shown to be competitive reversible inhibitors with respect to ATP in the reaction catalyzed by HldE-kinase. The study of the structure–activity relationship of this series resulted in a considerable improvement of potency with compound **5x** having an IC_{50} of 0.11 μM. Compounds of this series were also able to inhibit HldA of *N. meningitidis* but were completely inactive on ribokinase of *E. coli*. Considering the similarity between HldE of *E. coli* and HldA of *N. meningitidis*, this series is likely to be selective of HldE/HldA enzymes of Gram-negative bacteria.

As a result, this family of inhibitors represents a promising lead series for the development of new Gram-negative antimicrobial agents with antivirulence and membrane permeabilizing properties. Further chemical optimization and biological evaluation are currently underway; these results will be presented in due course.

6. Experimental

6.1. General

Reagents and enzymes (except HldE, HldA and RK) for biochemical assays were purchased from Sigma–Aldrich. Chemical reagents were obtained from Sigma–Aldrich, Acros, Alfa Aesar, Maybridge and solvents from Sigma–Aldrich or VWR, and were used without further purification. Anhydrous solvents were purchased from Sigma–Aldrich. Organometallic reagents were purchased from Strem Chemicals, Inc. All reactions were conducted under argon atmosphere in oven-dried glassware with magnetic stirring or in screwed-cap tubes with orbital shaking using an Advanced Chemtech PLS apparatus. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a 400 MHz Brüker instrument, and chemical shifts are reported in parts per million downfield from the internal standard tetramethylsilane (TMS). NMR analysis displayed amide rotamers for most compounds. The following abbreviations are used to indicate signal multiplicities: s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), dt (doublet of triplets), td (triplet of doublets), br (broad). *J* indicates the NMR coupling constant measured in Hertz. $CDCl_3$ is deuteriochloroform, DMSO-d_6 is hexadeuterio-dimethylsulfoxide, and CD_3OD is tetradeuteriomethanol. Mass spectra were obtained using electrospray (ES) ionization techniques on an Agilent 1100 Series LCMS. HPLC (analytical and preparative) were performed on an Agilent 1100 HPLC with DAD (Diode Array Detection). Preparative HPLC were performed at 0.7 mL/min on a ThermoElectron, Hypersil BDS C-18 column (250 × 4.6 mm, 5 μm) using a gradient of acetonitrile in water with 0.1% TFA (50% in acetonitrile to 100% and then back to 50%). Analtech Silica Gel GF and E. Merck Silica Gel 60 F-254 thin layer plates were used for thin layer chromatography. Flash chromatography was carried out on Flashsmart Pack cartridge, using silica gel columns prepacked with irregular silica 40–60 μm or spherical silica 20–40 μm. The meaning of certain abbreviations is given herein. ESI refers to electrospray ionization, HPLC refers to high pressure liquid chromatography, LCMS refers to liquid chromatography coupled with a mass spectrometer, M in the context of mass spectrometry refers to the molecular peak, MS refers to mass spectrometer, NMR refers to nuclear magnetic resonance, pH refers to potential of hydrogen, TFA refers to trifluoroacetic acid, DIPEA refers to *N*,*N*-diisopropylethylamine, dppf refers to 1,1′-bis(diphenylphosphino)ferrocene, EDAC refers *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride,4-DMAP refers to 4-(dimethylamino)pyridine, BINAP refers to 2,2′-bis (diphenylphosphino)-1,1′-binaphthalene, TLC refers to thin layer chromatography, Hepes refers to 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid, DTT refers to dithiothrietol, H-7-P refers to D-*glycero*-D-*manno*-heptose-7-phosphate.

6.2. Chemistry protocols

6.2.1. Representative procedure for the synthesis of amines 3a–i (Scheme 3, path a)

Under argon, a solution of 1,3-benzothiazol-2-ylmethylamine hydrochloride (600 mg, 3 mmol), ethyl chloroacetate (324 μL, 3 mmol) and triethylamine (916 μL, 6.6 mmol) in anhydrous acetonitrile (6 mL) was stirred at room temperature for 1.5 h, then at 50 °C overnight. The reaction mixture was evaporated under reduced pressure. The crude product was purified by flash chromatography on silica gel eluted with a gradient of methanol in dichloromethane (0–5%) to afford ethyl [(1,3-benzothiazol-2-ylmethyl)amino]acetate (**3i**, 436 mg, 58%) as a yellow oil. ^{1}H NMR ($CDCl_3$): 7.97 (d, *J* = 8.4 Hz, 1H), 7.87 (dd, *J* = 8.0 Hz and 0.8 Hz, 1H), 7.49–7.43 (m, 1H), 7.40–7.35 (m, 1H), 4.37 (s, 2H), 4.20 (q, *J* = 7.2 Hz, 2H), 3.63 (s, 2H), 1.27 (t, *J* = 7.2 Hz, 3H). ESI-MS *m*/*z* 251 $(M+H)^+$.

6.2.2. Representative procedure for the synthesis of amines 3a–i (Scheme 3, path b)

Under argon, acetic acid (275 μL, 4.8 mmol) was added to a solution of thiazole-2-carbaldehyde (500 mg, 4.4 mmol) and glycine ethyl ester hydrochloride (614 mg, 4.4 mmol) in anhydrous dichloromethane (20 mL). The reaction mixture was stirred for 3 h at room temperature. The solution was then cooled to 0 °C and sodium cyanoborohydride (418 mg, 6.6 mmol) was added portionwise. After completion of the addition, the reaction mixture

was allowed to warm to room temperature and kept stirring overnight. Then water was added at 0 °C and dichloromethane was removed under reduced pressure. The aqueous solution was extracted with ethyl acetate. The combined organic extracts were washed with water, brine, dried over sodium sulfate, filtered and evaporated. Purification by flash chromatography on silica gel eluted with a gradient of methanol in dichloromethane (0–5%) led to ethyl [(1,3-thiazol-2-ylmethyl)amino]acetate (**3g**, 120 mg, 14%) as a yellow oil. ^{1}H NMR ($CDCl_3$): 7.86 (d, J = 4.0 Hz, 1H), 7.30 (d, J = 4.0 Hz, 1H), 4.22 (q, J = 7.2 Hz, 2H), 4.2 (s, 2H), 3.50 (s, 2H), 1.31 (t, J = 7.2 Hz, 3H). ESI-MS m/z 201 $(M+H)^+$.

6.2.3. Representative procedure for the synthesis of compounds 7a–c (Scheme 4). ((1,3-benzothiazol-2-ylmethyl){[2-(3-methoxyphenyl)-4-methyl-1,3-oxazol-5-yl]carbonyl}amino)acetic acid (7c)

A solution of ethyl 2-chloroacetoacetate (1.45 mL, 10 mmol) and 3-methoxybenzamide (1.55 g, 10 mmol) in anhydrous toluene (3 mL) was stirred at 120 °C for 2 h, then at 140 °C for 2 h and at 120 °C overnight. An aqueous solution of ammonium chloride was added at room temperature and the reaction mixture was extracted with ethyl acetate. The combined organic extracts were dried over sodium sulfate, filtered and evaporated. Purification by flash chromatography on silica gel with a gradient of ethyl acetate in cyclohexane (5–30%) led to ethyl 2-(3-methoxyphenyl)-4-methyl-1,3-oxazole-5-carboxylate (1.28 g, 48%) as a white solid. ESI-MS m/z 262 $(M+H)^+$.

A solution of ethyl 2-(3-methoxyphenyl)-4-methyl-1,3-oxazole-5-carboxylate (1.0 g, 3.83 mmol) and lithium hydroxide (275 mg, 11.5 mmol) in water (10 mL) and THF (10 mL) was stirred at room temperature overnight. Then 1 N HCl was added and the reaction mixture was extracted with diethyl ether and ethyl acetate. The combined organic extracts were dried over sodium sulfate, filtered and evaporated. The solid obtained was washed with 5% ethyl acetate in cyclohexane and dried to give 2-(3-methoxyphenyl)-4-methyl-1,3-oxazole-5-carboxylic acid (845 mg, 94%) as a white solid. ESI-MS m/z 234 $(M+H)^+$.

Under argon, ethyl 1,3-benzothiazol-2-ylmethyl)amino]acetate (**3i**, 53.9 mg, 0.22 mmol) was added to a solution of 2-(3-methoxyphenyl)-4-methyl-1,3-oxazole-5-carboxylic acid (38.6 mg, 0.17 mmol), EDAC (64 mg, 0.33 mmol) and 4-DMAP (62 mg, 0.51 mmol) in anhydrous dichloromethane (3 mL). The resulting mixture was stirred at room temperature for 5 h and then at 50 °C overnight. Water was added and the reaction mixture was extracted with dichloromethane. Combined organic extracts were washed with 1 N HCl, then dried over sodium sulfate, filtered and evaporated. The crude product was purified by preparative TLC eluted with 5% methanol in dichloromethane to afford ethyl ((1,3-benzothiazol-2-ylmethyl){[2-(3-methoxyphenyl)-4-methyl-1,3-oxazol-5-yl]carbonyl}amino)acetate (57.7 mg, 75%) as a pale yellow oil. ESI-MS m/z 466 $(M+H)^+$.

Lithium hydroxide (14.8 mg, 0.62 mmol) was added to a solution of ethyl ((1,3-benzothiazol-2-ylmethyl){[2-(3-methoxyphenyl)-4-methyl-1,3-oxazol-5-yl]carbonyl} amino)acetate (57.7 mg, 0.12 mmol) in THF (1 mL) and water (1 mL). The reaction mixture was stirred at room temperature overnight. Then 1 N HCl was added and the reaction mixture was extracted with diethyl ether and ethyl acetate. The combined organic extracts were dried over sodium sulfate, filtered and evaporated. Crystallization in a mixture of dichloromethane and cyclohexane led to a solid which was filtered and dried to give **7c** (51 mg, 94%) as a pale yellow solid. ^{1}H NMR ($CDCl_3$), 2 rotamers in a roughly 1/3 ratio, each chemical shift is for both rotamers except when stated: 8.04 (d, J = 8.4 Hz, 1H, minor rotamer), 7.99 (d, J = 8.1 Hz, 1H, major rotamer), 7.89 (d, J = 7.5 Hz, 1H, major rotamer), 7.70 (d, J = 7.8 Hz, 1H, major rotamer), 7.62 (s, 1H, major rotamer), 7.55–7.41 (m, 2H), 7.37–7.26 (m, 2H of minor rotamer and 1H of both rotamers), 7.18–7.12 (m, 1H, minor rotamer), 7.02 (dd, J = 8.1 Hz and 1.8 Hz, 1H, major rotamer), 6.95–6.92 (m, 1H, minor rotamer), 5.40 (s, 2H, minor rotamer), 5.24 (s, 2H, major rotamer), 4.61 (s, 2H, major rotamer), 4.36 (s, 2H, minor rotamer), 3.87 (s, 3H, major rotamer), 3.60 (s, 3H, minor rotamer), 2.57 (3H). ESI-MS m/z 438 $(M+H)^+$.

6.2.4. [{[2-(3-methoxyphenyl)-4-methyl-1,3-oxazol-5-yl]carbonyl}(pyridin-2-ylmethyl)amino]acetic acid (7a)

This compound was prepared from ethyl [(pyridin-2-ylmethyl)amino]acetate (synthesized according to the procedure described in Section 6.2.1) and 2-(3-methoxyphenyl)-4-methyl-1,3-oxazole-5-carboxylic acid (following the procedure depicted in Section 6.2.3). ^{1}H NMR (DMSO-d_6), 2 rotamers in a 2/1 ratio, each chemical shift is for both rotamers except when stated: 8.74 (d, J = 5.2 Hz, 1H, major rotamer), 8.71 (d, J = 5.2 Hz, 1H, minor rotamer), 8.30–8.26 (m, 1H, major rotamer), 8.15–8.11 (m, 1H, minor rotamer), 7.86 (d, J = 8.0 Hz, 1H, major rotamer), 7.76 (d, J = 8.0 Hz, 1H, minor rotamer), 7.72 (t, J = 6.4 Hz, 1H, major rotamer), 7.62–7.60 (m, 1H), 7.51 (br s, 1H, major rotamer), 7.48 (t, J = 7.8 Hz, 1H, major rotamer), 7.29 (t, J = 7.8 Hz, 1H, minor rotamer), 7.17 (dd, J = 8.4 Hz and J = 2.0 Hz, 1H, major rotamer), 7.07 (dd, J = 8.4 Hz and J = 2.0 Hz, 1H, minor rotamer), 7.00 (br s, 1H, minor rotamer), 6.93 (d, J = 7.6 Hz, 1H, minor rotamer), 5.14 (s, 2H, minor rotamer), 4.98 (s, 2H, major rotamer), 4.67 (s, 2H, major rotamer), 4.20 (s, 2H, minor rotamer), 3.85 (s, 3H, major rotamer), 3.72 (s, 3H, minor rotamer), 2.42 (s, 3H). ESI-MS m/z 382 $(M+H)^+$.

6.2.5. {(2-aminobenzyl)[(4-methyl-2-phenyl-1,3-oxazol-5-yl)carbonyl]amino}acetic acid (7b)

This compound was prepared from ethyl [(2-aminobenzyl) amino]acetate (synthesized according to the procedure described in Section 6.2.1) and 4-methyl-2-phenyl-1,3-oxazole-5-carboxylic acid (synthesized according to the procedure described in Section 6.2.3). ^{1}H NMR (DMSO-d_6), 2 rotamers in a roughly 3/1 ratio, each chemical shift is for both rotamers except when stated: 8.31–8.30 (m, 1H, minor rotamer), 8.07–8.03 (m, 1H of both rotamers and 1H of major rotamer), 7.57–7.35 (m, 3H), 7.01–6.98 (m, 2H), 6.64–6.50 (m, 2H), 5.28 (br s, 2H, minor rotamer), 4.59 (br s, 2H, major rotamer), 3.81 (br s, 2H, major rotamer), 3.33 (2H of minor rotamer under water peak), 2.40 (s, 3H, major rotamer), 2.34 (s, 3H, minor rotamer). ESI-MS m/z 366 $(M+H)^+$.

6.2.6. Representative procedure for the synthesis of compounds 5a–t (Scheme 4). [{[2-(3-Bromophenyl)-5-methyl-1,3-oxazol-4-yl]carbonyl}(pyridin-2-ylmethyl)amino]acetic acid (5h)

Methyl 2-aminoacetoacetate hydrochloride (462 mg, 2.75 mmol) was added portionwise to a solution of 3-bromobenzoyl chloride (400 μL, 3 mmol) in anhydrous dichloromethane (10 mL) stirred under argon at room temperature. Then triethylamine (835 μL, 6 mmol) was added dropwise. The reaction mixture was stirred at room temperature overnight. Dichloromethane was added and the organic layer was successively washed with 1 N HCl, saturated $NaHCO_3$ and water. The organic layer was dried over sodium sulfate, filtered and evaporated. Purification by flash chromatography on silica gel eluted with a gradient of ethyl acetate in dichloromethane (0–5%) led to methyl 2-[(3-bromobenzoyl) amino]-3-oxobutanoate (454.9 mg, 53%) as a white solid. ESI-MS m/z 314 and 316 $(M+H)^+$.

To a solution of triphenylphosphine (760 mg, 2.90 mmol) and iodine (736 mg, 2.90 mmol) in anhydrous dichloromethane (85 mL) stirred under argon at room temperature were added triethylamine (805 μL, 5.80 mmol) and a solution of methyl 2-[(3-bromobenzoyl)amino]-3-oxobutanoate (454.9 mg, 1.45 mmol) in dichloromethane (15 mL). The resulting mixture was stirred at room temperature overnight. Then the organic solution was successively

washed with saturated $Na_2S_2O_3$, water, NH_4Cl and water again. The organic layer was dried over sodium sulfate, filtered and evaporated. Purification by flash chromatography on silica gel eluted with a gradient of ethyl acetate in dichloromethane (0–5%) gave methyl 2-(3-bromophenyl)-5-methyl-1,3-oxazole-4-carboxylate (344.6 mg, 80%) as a yellow solid. ESI-MS *m*/*z* 296 and 298 $(M+H)^+$.

The remaining steps (saponification, coupling with amine and saponification) were performed following the procedure described in Section 6.2.3. Purification by preparative HPLC afforded **5h** as its trifluoroacetic salt. ^1H NMR (CD_3OD), 2 rotamers in a roughly 1/2 ratio, each chemical shift is for both rotamers except when stated: 8.73–8.68 (m, 1H), 8.38–8.32 (m, 1H), 8.17 (s, 1H, major rotamer), 8.01–7.95 (m, 2H, minor rotamer, 1H, both rotamers), 7.81–7.75 (m, 1H), 7.68–7.60 (m, 1H, major rotamer, 1H, both rotamers), 7.44 (t, *J* = 8.0 Hz, 1H, major rotamer), 7.35 (t, *J* = 8.0 Hz, 1H, minor rotamer), 5.39 (s, 2H, minor rotamer), 5.03 (s, 2H, major rotamer), 4.83 (s, 2H, major rotamer), 4.35 (s, 2H, minor rotamer), 2.66 (s, 3H, minor rotamer), 2.64 (s, 3H, major rotamer). ESI-MS *m*/*z* 430 and 432 $(M+H)^+$.

6.2.7. [{[5-Methyl-2-(2-nitrophenyl)-1,3-oxazol-4-yl]carbonyl}(pyridin-2-ylmethyl)amino]acetic acid (5a)

This compound was prepared from ethyl [(pyridin-2-ylmethyl)amino]acetate (synthesized according to the procedure described in Section 6.2.1) and 5-methyl-2-(2-nitrophenyl)-1,3-oxazole-4-carboxylic acid (prepared according to the procedure described in Section 6.2.6). ^1H NMR ($CDCl_3$), 2 rotamers in a roughly 2/3 ratio, each chemical shift is for both rotamers except when stated: 8.54–8.53 (m, 1H), 8.13 (d, *J* = 8.0 Hz, 1H, major rotamer), 7.95 (t, *J* = 8.0 Hz, 1H), 7.83–7.79 (m, 1H), 7.74–7.56 (m, 3H of both rotamers and 1H of minor rotamer), 7.45–7.42 (m, 1H), 5.46 (s, 2H, minor rotamer), 4.93 (s, 2H, major rotamer), 4,79 (s, 2H, major rotamer), 4.24 (s, 2H, minor rotamer), 2.64 (s, 3H, minor rotamer), 2.57 (s, 3H, major rotamer). ESI-MS *m*/*z* 397 $(M+H)^+$.

6.2.8. [{[2-(2-Aminophenyl)-5-methyl-1,3-oxazol-4-yl]carbonyl}(pyridin-2-ylmethyl)amino]acetic acid (5b)

To a solution of [{[5-methyl-2-(2-nitrophenyl)-1,3-oxazol-4-yl]carbonyl}(pyridin-2-ylmethyl)amino]acetic acid (**5a**, 40 mg, 0.1 mmol) in anhydrous methanol (4 mL), was added ferric chloride (2 mg, 5% by weight) and activated charcoal (2 mg, 5% by weight). The reaction mixture was heated to 65 °C. Hydrazine hydrate (40 mg, 0.8 mmol) was added dropwise. The reaction mixture was refluxed overnight and then cooled to room temperature. Then the reaction mixture was filtered through a pad of Celite and the filtrate was concentrated. Purification of the crude product by preparative HPLC afforded **5b** as its trifluoroacetic acid salt (15 mg, 31%). ^1H NMR (CD_3OD), 2 rotamers in a 1/2 ratio, each chemical shift is for both rotamers except when stated: 8.83–8.78 (m, 1H), 8.56–8.52 (m, 1H), 8.19 (d, *J* = 8.0 Hz, 1H, major rotamer), 8.13 (d, *J* = 8.0 Hz, 1H, minor rotamer), 7.97–7.92 (m, 1H), 7.79 (d, *J* = 8.0 Hz, 1H, major rotamer), 7.68 (d, *J* = 8.0 Hz, 1H, minor rotamer), 7.24 (t, *J* = 8.0 Hz, 1H, major rotamer), 7.15 (t, *J* = 8.0 Hz, 1H, minor rotamer), 6.91 (d, *J* = 8.0 Hz, 1H, major rotamer), 6.78 (t, *J* = 8.0 Hz, 1H, major rotamer), 6.72–6.68 (m, 2H, minor rotamer), 5.42 (s, 2H, minor rotamer), 5.08 (s, 2H, major rotamer), 4.87 (s, 2H, major rotamer), 4.31 (s, 2H, minor rotamer), 2.63 (s, 3H, minor rotamer), 2.59 (s, 3H, major rotamer). ESI-MS *m*/*z* 367 $(M+H)^+$.

6.2.9. [({5-Methyl-2-[2-(trifluoromethyl)phenyl]-1,3-oxazol-4-yl}carbonyl)(pyridin-2-yl -methyl)amino]acetic acid (5c)

This compound was prepared from ethyl [(pyridin-2-ylmethyl)amino]acetate (synthesized according to the procedure described in Section 6.2.1) and 5-methyl-2-[2-(trifluoromethyl)phenyl]-1,3-oxazole-4-carboxylic acid (prepared according to the procedure described in Section 6.2.6). ^1H NMR ($CDCl_3$), 2 rotamers in a roughly 2/3 ratio, each chemical shift is for both rotamers except when stated: 8.56 (br s, 1H), 8.16 (d, *J* = 8.0 Hz, 1H, major rotamer), 8.03–7.95 (m, 1H), 7.80–7.45 (m, 1H of minor rotamer and 5H of both rotamers), 5.64 (s, 2H, minor rotamer), 5.01 (s, 2H, major rotamer), 4.89 (s, 2H, major rotamer), 4.28 (s, 2H, minor rotamer), 2.66 (s, 3H, minor rotamer), 2.63 (s, 3H, major rotamer). ESI-MS *m*/*z* 420 $(M+H)^+$.

6.2.10. [{[2-(2-Bromophenyl)-5-methyl-1,3-oxazol-4-yl]carbonyl}(pyridin-2-ylmethyl)amino]acetic acid (5d)

This compound was prepared from ethyl [(pyridin-2-ylmethyl)amino]acetate (synthesized according to the procedure described in Section 6.2.1) and 2-(2-bromophenyl)-5-methyl-1,3-oxazole-4-carboxylic acid (prepared according to the procedure described in Section 6.2.6). ^1H NMR ($CDCl_3$), 2 rotamers in a roughly 1/1 ratio, each chemical shift is for both rotamers except when stated: 8.48–8.43 (m, 1H), 8.02 (d, *J* = 8.0 Hz, 1H, one rotamer), 7.82 (t, *J* = 8.0 Hz, 1H, one rotamer), 7.70–7.59 (m, 2H), 7.51–7.38 (m, 1H of one rotamer and 1H of both rotamers), 7.35–7.16 (m, 1H of one rotamer and 2H of both rotamers), 5.39 (s, 2H, one rotamer), 4.85 (s, 2H, one rotamer), 4.78 (s, 2H, one rotamer), 4.08 (s, 2H, one rotamer), 2.61 (s, 3H, one rotamer), 2.56 (s, 3H, one rotamer). ESI-MS *m*/*z* 430 and 432 $(M+H)^+$.

6.2.11. [{[5-Methyl-2-(3-nitrophenyl)-1,3-oxazol-4-yl] carbonyl}(pyridin-2-ylmethyl) amino]acetic acid (5e)

This compound was prepared from ethyl [(pyridin-2-ylmethyl)amino]acetate (synthesized according to the procedure described in Section 6.2.1) and 5-methyl-2-(3-nitrophenyl)-1,3-oxazole-4-carboxylic acid (prepared according to the procedure described in Section 6.2.6).

^1H NMR (CD_3OD), 2 rotamers in a roughly 1/1 ratio, each chemical shift is for both rotamers except when stated: 8.76 (br s, 1H, one rotamer), 8.56–8.52 (m, 1H), 8.42 (br s, 1H, one rotamer), 8.39 (d, *J* = 8.0 Hz, 1H, one rotamer), 8.34–8.27 (m, 1H), 8.11 (d, *J* = 7.2 Hz, 1H, one rotamer), 7.95–7.87 (m, 1H), 7.75 (t, *J* = 8.0 Hz, 1H, one rotamer), 7.69 (t, *J* = 8.0 Hz, 1H, one rotamer), 7.65 (d, *J* = 8.0 Hz, 1H, one rotamer), 7.55 (d, *J* = 8.0 Hz, 1H, one rotamer), 7.41–7.35 (m, 1H), 5.26 (s, 2H, one rotamer), 4.93 (s, 2H, one rotamer), 4.61 (s, 2H, one rotamer), 4.23 (s, 2H, one rotamer), 2.67 (s, 3H). ESI-MS *m*/*z* 397 $(M+H)^+$.

6.2.12. [{[2-(3-Aminophenyl)-5-methyl-1,3-oxazol-4-yl]carbonyl}(pyridin-2-ylmethyl)amino]acetic acid (5f)

This compound was synthesized from **5e** following the procedure described in Section 6.2.8 for the synthesis of **5b.** Purification by preparative HPLC afforded **5f** as its trifluoroacetic acid salt. ^1H NMR (CD_3OD), 2 rotamers in a roughly 2/1 ratio, each chemical shift is for both rotamers except when stated: 8.78–8.76 (m, 1H), 8.49–8.45 (m, 1H), 8.12–8.10 (m, 1H), 7.93–7.85 (m, 2H), 7.59–7.42 (m, 3H), 5.49 (s, 2H, minor rotamer), 5.08 (s, 2H, major rotamer), 4.89 (s, 2H, major rotamer), 4.35 (s, 2H, minor rotamer), 2.66 (s, 3H, minor rotamer), 2.63 (s, 3H, major rotamer). ESI-MS *m*/z 367 $(M+H)^+$.

6.2.13. [({5-Methyl-2-[3-(trifluoromethyl)phenyl]-1,3-oxazol-4-yl}carbonyl)(pyridin-2-ylmethyl)amino]acetic acid (5g)

This compound was prepared from ethyl [(pyridin-2-ylmethyl)amino]acetate (synthesized according to the procedure described in Section 6.2.1) and 5-methyl-2-[3-(trifluoromethyl)phenyl]-1,3-oxazole-4-carboxylic acid (prepared according to the procedure described in Section 6.2.6). Purification by preparative HPLC afforded **5g** as its trifluoroacetic acid salt. ^1H NMR

($CDCl_3$), 2 rotamers in a roughly 1/3 ratio, each chemical shift is for both rotamers except when stated: 8.74 (br s, 1H), 8.30–8.25 (m, 1H), 8.20–8.15 (m, 2H, major rotamer), 8.02–7.95 (m, 1H), 7.85–7.52 (m, 3H of both rotamers and 2H of minor rotamer), 5.56 (s, 2H, minor rotamer), 5.10 (s, 2H, major rotamer), 4.90 (s, 2H, major rotamer), 4.34 (s, 2H, minor rotamer), 2.64 (s, 3H, minor rotamer), 2.61 (s, 3H, major rotamer). ESI-MS *m*/*z* 420 $(M+H)^+$.

6.2.14. [{[2-(3-Methoxyphenyl)-5-methyl-1,3-oxazol-4-yl]carbonyl}(pyridin-2-ylmethyl)amino]acetic acid (5i)

This compound was prepared from ethyl [(pyridin-2-ylmethyl)amino]acetate (synthesized according to the procedure described in Section 6.2.1) and 2-(3-methoxyphenyl)-5-methyl-1,3-oxazole-4-carboxylic acid (prepared according to the procedure described in Section 6.2.6). ^1H NMR ($CDCl_3$), 2 rotamers in a 1/3 ratio, each chemical shift is for both rotamers except when stated: 8.52–8.48 (m, 1H), 7.88–7.83 (m, 1H), 7.64 (s, 1H), 7.59 (d, *J* = 8.0 Hz, 1H), 7.51 (d, J = 8.0 Hz, 1H), 7.37–7.30 (m, 2H), 7.01–6.98 (m, 1H), 5.47 (s, 2H, minor rotamer), 4.86 (s, 2H, major rotamer), 4.72 (s, 2H, major rotamer), 4.27 (s, 2H, minor rotamer), 3.94 (s, 3H, major rotamer), 3.83 (s, 3H, minor rotamer), 2.63 (s, 3H, minor rotamer), 2.61 (s, 3H, major rotamer). ESI-MS *m*/*z* 382 $(M+H)^+$

6.2.15. [{[5-Methyl-2-(4-nitrophenyl)-1,3-oxazol-4-yl]carbonyl}(pyridin-2-ylmethyl)amino]acetic acid (5j)

This compound was prepared from ethyl [(pyridin-2-ylmethyl)amino]acetate (synthesized according to the procedure described in Section 6.2.1) and 5-methyl-2-(4-nitrophenyl)-1,3-oxazole-4-carboxylic acid (prepared according to the procedure described in Section 6.2.6). ^1H NMR (CD_3OD), 2 rotamers in a roughly 2/1 ratio, each chemical shift is for both rotamers except when stated: 8.83–8.78 (m, 1H), 8.52–8.49 (m, 1H), 8.39–8.12 (m, 4H of both rotamers and 1H of minor rotamer), 7.95–7.90 (m, 1H), 7.84–7.81 (m, 1H, major rotamer), 5.49 (s, 2H, minor rotamer), 5.10 (s, 2H, major rotamer), 4.85 (s, 2H, major rotamer), 4.37 (s, 2H, minor rotamer), 2.71 (s, 3H, minor rotamer), 2.67 (s, 3H, major rotamer). ESI-MS *m*/*z* 397 $(M+H)^+$.

6.2.16. [{[2-(4-Aminophenyl)-5-methyl-1,3-oxazol-4-yl]carbonyl}(pyridin-2-ylmethyl)amino]acetic acid (5k)

This compound was synthesized from **5j** following the procedure described in Section 6.2.8 for the synthesis of **5b.** ^1H NMR (CD_3OD), 2 rotamers in a roughly 3/2 ratio, each chemical shift is for both rotamers except when stated: 8.53 (br s, 1H), 7.95–7.91 (m, 1H), 7.73 (d, *J* = 8.0 Hz, 2H, major rotamer), 7.65 (d, *J* = 8.0 Hz, 1H, minor rotamer), 7.59 (d, *J* = 8.0 Hz, 1H, major rotamer), 7.49 (d, *J* = 8.0 Hz, 2H, minor rotamer), 7.43–7.39 (m, 1H), 6.72 (d, *J* = 8.0 Hz, 2H, major rotamer), 6.65 (d, *J* = 8.0 Hz, 2H, minor rotamer), 5.32 (s, 2H, minor rotamer), 4.87 (s, 2H, major rotamer), 4.70 (s, 2H, major rotamer), 4.24 (s, 2H, minor rotamer), 2.58 (s, 3H, major rotamer), 2.56 (s, 3H, minor rotamer). ESI-MS *m*/*z* 367 $(M+H)^+$.

6.2.17. [({5-Methyl-2-[4-(trifluoromethyl)phenyl]-1,3-oxazol-4-yl}carbonyl)(pyridin-2-ylmethyl)amino]acetic acid (5l)

This compound was prepared from ethyl [(pyridin-2-ylmethyl)amino]acetate (synthesized according to the procedure described in Section 6.2.1) and 5-methyl-2-[4-(trifluoromethyl)phenyl]-1,3-oxazole-4-carboxylic acid (prepared according to the procedure described in Section 6.2.6). ^1H NMR ($CDCl_3$) 2 rotamers in a roughly 1/3 ratio, each chemical shift is for both rotamers except when stated: 8.77 (br s, 1H), 8.38–8.32 (m, 1H), 8.16–8.14 (m, 1H), 8.06 (d, *J* = 8.0 Hz, 2H), 7.85–7.75 (m, 1H), 7.67 (d, *J* = 8.0 Hz, 2H), 5.82 (s, 2H, minor rotamer), 5.35 (s, 2H, major rotamer), 5.10 (s, 2H, major rotamer), 4.42 (s, 2H, minor rotamer), 2.70 (s, 3H). ESI-MS *m*/*z* 420 $(M+H)^+$.

6.2.18. [{[2-(4-Bromophenyl)-5-methyl-1,3-oxazol-4-yl]carbonyl}(pyridin-2-ylmethyl)amino]acetic acid (5m)

This compound was prepared from ethyl [(pyridin-2-ylmethyl)amino]acetate (synthesized according to the procedure described in Section 6.2.1) and 2-(4-bromophenyl)-5-methyl-1,3-oxazole-4-carboxylic acid (prepared according to the procedure described in Section 6.2.6). ^1H NMR ($CDCl_3$), 2 rotamers in a roughly 1/3 ratio, each chemical shift is for both rotamers except when stated: 8.51–8.50 (m, 1H), 7.93–7.89 (m, 3H), 7.60–7.53 (m, 3H), 7.42–7.37 (m, 1H), 5.50 (s, 2H, minor rotamer), 4.89 (s, 2H, major rotamer), 4.75 (s, 2H, major rotamer), 4.29 (s, 2H, minor rotamer), 2.61 (s, 3H). ESI-MS *m*/*z* 430 and 432 $(M+H)^+$.

6.2.19. [{[2-(4-Methoxyphenyl)-5-methyl-1,3-oxazol-4-yl]carbonyl}(pyridin-2-ylmethyl)amino]acetic acid (5n)

This compound was prepared from ethyl [(pyridin-2-ylmethyl)amino]acetate (synthesized according to the procedure described in Section 6.2.1) and 2-(4-methoxyphenyl)-5-methyl-1,3-oxazole-4-carboxylic acid (prepared according to the procedure described in Section 6.2.6). ^1H NMR ($CDCl_3$), 2 rotamers in a roughly 1/3 ratio, each chemical shift is for both rotamers except when stated: 8.47–8.46 (m, 1H), 7.94 (d, *J* = 8.0 Hz, 2H, major rotamer), 7.82 (t, *J* = 8.0 Hz, 1H, major rotamer), 7.74 (t, *J* = 8.0 Hz, 1H, minor rotamer), 7.65 (d, *J* = 8.0 Hz, 2H, minor rotamer), 7.49–7.46 (m, 1H), 7.32–7.23 (m, 1H), 6.94 (d, *J* = 8.0 Hz, 2H, major rotamer), 6.86 (d, *J* = 8.0 Hz, 2H, minor rotamer), 5.38 (s, 2H, minor rotamer), 4.84 (s, 2H, major rotamer), 4.70 (s, 2H, major rotamer), 4.17 (s, 2H, minor rotamer), 3.83 (s, 3H, major rotamer), 3.81 (s, 3H, minor rotamer), 2.57 (s, 3H, major rotamer), 2.54 (s, 3H, minor rotamer). ESI-MS *m*/*z* 382 $(M+H)^+$.

6.2.20. [[(5-Methyl-2-phenyl-1,3-oxazol-4-yl)carbonyl](pyridin-3-ylmethyl)amino]acetic acid (5o)

This compound was prepared from ethyl [(pyridin-3-ylmethyl)amino]acetate (synthesized according to the procedure described in Section 6.2.1) and 5-methyl-2-phenyl-1,3-oxazole-4-carboxylic acid (prepared according to the procedure described in Section 6.2.6). ^1H NMR (CD_3OD), 2 rotamers in a roughly 1/1 ratio, each chemical shift is for both rotamers except when stated: 8.68 (s, 1H, one rotamer), 8.59 (s, 1H, one rotamer), 8.51–8.47 (m, 1H), 8.03–8.01 (m, 3H, one rotamer), 7.90 (d, *J* = 7.6 Hz, 1H, one rotamer), 7.85 (d, *J* = 7.2 Hz, 2H, one rotamer), 7.49–7.44 (m, 4H), 5.19 (s, 2H, one rotamer), 4.85 (s, 2H, one rotamer), 4.48 (s, 2H, one rotamer), 3.98 (s, 2H, one rotamer), 2.65 (s, 3H). ESI-MS *m*/*z* 352 $(M+H)^+$.

6.2.21. [[(5-Methyl-2-phenyl-1,3-oxazol-4-yl)carbonyl](pyridin-4-ylmethyl)amino]acetic acid (5p)

This compound was prepared from ethyl [(pyridin-4-ylmethyl)amino]acetate (synthesized according to the procedure described in Section 6.2.1) and 5-methyl-2-phenyl-1,3-oxazole-4-carboxylic acid (prepared according to the procedure described in Section 6.2.6). ^1H NMR (CD_3OD), 2 rotamers in a roughly 1/1 ratio, each chemical shift is for both rotamers except when stated: 8.55–5.52 (m, 2H), 8.04–8.03 (m, 2H, one rotamer), 7.77 (d, *J* = 7.2 Hz, 2H, one rotamer), 7.51–7.40 (m, 5H), 5.23 (s, 2H, one rotamer), 4.87 (2H of one rotamer under water peak), 4.59 (s, 2H, one rotamer), 4.08 (s, 2H, one rotamer), 2.66 (s, 3H). ESI-MS *m*/*z* 352 $(M+H)^+$.

6.2.22. {Benzyl[(5-methyl-2-phenyl-1,3-oxazol-4-yl)carbonyl]amino}acetic acid (5q)

This compound was prepared from ethyl (benzylamino)acetate (synthesized according to the procedure described in Section 6.2.1) and 5-methyl-2-phenyl-1,3-oxazole-4-carboxylic acid (prepared according to the procedure described in Section 6.2.6). ^1H NMR (acetone-d_6), 2 rotamers in a roughly 2/1 ratio, each chemical shift is for both rotamers except when stated: 7.89–7.86 (m, 2H, major

rotamer), 7.82–7.79 (m, 2H, minor rotamer), 7.40–7.15 (m, 8H), 5.12 (s, 2H, minor rotamer), 4.68 (s, 2H, major rotamer), 4.46 (s, 2H, major rotamer), 3.93 (s, 2H, minor rotamer), 2.55 (s, 3H, major rotamer), 2.51 (s, 3H, minor rotamer). ESI-MS *m*/*z* 351 $(M+H)^+$.

6.2.23. {(2-Furylmethyl)[(5-methyl-2-phenyl-1,3-oxazol-4-yl)carbonyl]amino}acetic acid (5r)

This compound was prepared from ethyl [(2-furylmethyl)amino]acetate (synthesized according to the procedure described in Section 6.2.2) and 5-methyl-2-phenyl-1,3-oxazole-4-carboxylic acid (prepared according to the procedure described in Section 6.2.6). ^{1}H NMR (DMSO-d_6), 2 rotamers in a roughly 2/1 ratio, each chemical shift is for both rotamers except when stated: 7.94 (br s, 2H), 7.56–7.52 (m, 4H), 6.40 (s, 2H), 5.15 (s, 2H, minor rotamer), 4.71 (s, 2H, major rotamer), 4.51 (s, 2H, major rotamer), 4.07 (s, 2H, minor rotamer), 2.61 (s, 3H, major rotamer), 2.58 (s, 3H, minor rotamer). ESI-MS *m*/*z* 341 $(M+H)^+$.

6.2.24. [[(5-Methyl-2-phenyl-1,3-oxazol-4-yl)carbonyl] (1,3-thiazol-2-ylmethyl)amino]acetic acid (5s)

This compound was prepared from ethyl [(1,3-thiazol-2-ylmethyl)amino]acetate (**3i**, synthesized as in Section 6.2.2) and 5-methyl-2-phenyl-1,3-oxazole-4-carboxylic acid (prepared according to the procedure described in Section 6.2.6). ^{1}H NMR ($CDCl_3$), 2 rotamers in roughly 2/3 ratio, each chemical shift is for both rotamers except when stated: 8.00–7.97 (m, 2H), 7.81–7.76 (m, 1H), 7.48–7.41 (br s, 4H), 5.66 (s, 2H, minor rotamer), 5.12 (s, 2H, major rotamer), 4.73 (s, 2H, major rotamer), 4.28 (s, 2H, minor rotamer), 2.72 (s, 3H, minor rotamer), 2.69 (s, 3H, major rotamer). ESI-MS *m*/*z* 358 $(M+H)^+$.

6.2.25. [{[2-(3-Methoxyphenyl)-5-methyl-1,3-oxazol-4-yl]carbonyl}(quinolin-2-ylmethyl)amino]acetic acid (5t)

This compound was prepared from ethyl [(quinolin-2-ylmethyl)amino]acetate (synthesized according to the procedure described in Section 6.2.2) and 5-methyl-2-phenyl-1,3-oxazole-4-carboxylic acid (prepared according to the procedure described in Section 6.2.6). ^{1}H NMR (CD_3OD), 2 rotamers in a roughly 3/2 ratio, each chemical shift is for both rotamers except when stated: 9.01–8.97 (m, 1H), 8.30–8.21 (m, 2H), 8.15–8.07 (m, 2H), 7.92–7.88 (m, 1H), 7.62–7.60 (m, 2H, major rotamer), 7.42 (t, *J* = 8.0 Hz, 1H, major rotamer), 7.19 (t, *J* = 8.0 Hz, 1H, minor rotamer), 7.10–7.06 (m, 1H), 6.95–6.92 (m, 1H, minor rotamer), 6.85 (br s, 1H minor rotamer), 5.64 (s, 2H, minor rotamer), 5.25 (s, 2H, major rotamer), 4.97 (s, 2H, major rotamer), 4.48 (s, 2H, minor rotamer), 3.89 (s, 3H, major rotamer), 3.58 (s, 3H, minor rotamer), 2.66 (s, 3H, minor rotamer), 2.61 (s, 3H, major rotamer). ESI-MS *m*/*z* 432 $(M+H)^+$.

6.2.26. [({5-Methyl-2-(3-morpholin-4-ylphenyl)-1,3-oxazol-4-yl}carbonyl)(pyridin-2-ylmethyl)amino]acetic acid (5u)

Under argon, a solution of ethyl 2-(3-bromophenyl)-5-methyl-1,3-oxazole-4-carboxylate (500 mg, 1.61 mmol, prepared as in Section 6.2.6), morpholine (175 μL, 2 mmol), cesium carbonate (2.25 mmol, 735 mg), Pd_2dba_3 (29.5 mg, 0.03 mmol), BINAP (30.1 mg, 0.05 mmol) in degassed toluene (6.5 mL) was stirred at 110 °C for 2 days. The reaction mixture was filtered through a pad of Celite and evaporated. The crude product was purified by flash chromatography on silica gel with a gradient of ethyl acetate in cyclohexane (5–40%) to give ethyl 5-methyl-2-(3-morpholin-4-ylphenyl)-1,3-oxazole-4-carboxylate (330 mg, 65%). ESI-MS *m*/*z* 317 $(M+H)^+$.

The remaining steps (saponification, coupling with amine and saponification) were performed following the procedure described in Section 6.2.3. Purification by preparative HPLC afforded **5u** as its trifluoroacetic salt. ^{1}H NMR (CD_3OD), 2 rotamers in a roughly 1/1 ratio, each chemical shift is for both rotamers except when stated: 8.47 (s, 1H), 7.82 (br s, 1H), 7.59–7.46 (m, 2H), 7.31–7.16 (m, 3H), 7.06–7.02 (m, 1H), 5.17 (s, 2H, one rotamer), 4.87 (2H of one rotamer under water peak), 4.47 (s, 2H, one rotamer), 4.07 (s, 2H, one rotamer), 3.81 (s, 4H), 3.18 (s, 4H, one rotamer), 3.01 (s, 4H, one rotamer), 2.58 (s, 3H). ESI-MS *m*/*z* 437 $(M+H)^+$.

6.2.27. {[(2-{3-[(Cyclopropylcarbonyl)amino]phenyl}-5-methyl-1,3-oxazol-4-yl)carbonyl](pyridin-2-ylmethyl)amino}acetic acid (5w)

Under argon, cyclopropanecarbonyl chloride (17 μL, 0.2 mmol) was added to a solution of ethyl [{[2-(3-aminophenyl)-5-methyl-1,3-oxazol-4-yl]carbonyl}(pyridin-2-ylmethyl)amino]acetate (52 mg, 0.13 mmol, prepared as in Section 6.2.12) and triethylamine (54 μL, 0.4 mmol) in anhydrous dichloromethane (2 mL) cooled to 0 °C. The reaction mixture was allowed to warm to room temperature and stirred at room temperature for 30 min. The reaction was quenched with water. The layers were separated and the aqueous layer was extracted with dichloromethane. The combined organic layers were washed with brine, dried over anhydrous sodium sulfate, filtered and concentrated. The crude product was purified by flash chromatography on silica gel eluted with a gradient of methanol in dichloromethane (0–10%) to obtain ethyl {[(2-{3-[(cyclopropylcarbonyl)amino]phenyl}-5-methyl-1,3-oxazol-4-yl)carbonyl](pyridin-2-ylmethyl)amino}acetate (31 mg, 51%) as a colorless oil. ESI-MS *m*/*z* 463 $(M+H)^+$.

Saponification of ethyl {[(2-{3-[(cyclopropylcarbonyl) amino]-phenyl}-5-methyl-1,3-oxazol-4-yl)carbonyl] (pyridin-2-ylmethyl) amino}acetate performed according to the procedure described in Section 6.2.3 followed by purification by preparative HPLC led to **5w** as its trifluoroacetic acid salt. ^{1}H NMR (CD_3OD), 2 rotamers in a roughly 1/1 ratio, each chemical shift is for both rotamers except when stated: 8.83–8.79 (m, 1H, one rotamer), 8.78–8.72 (m, 1H, one rotamer), 8.49–8.41 (m, 1H), 8.31 (s, 1H, one rotamer), 8.23 (s, 1H, one rotamer), 8.11–8.07 (m, 1H), 7.89–7.84 (m, 1H), 7.77–7.75 (m, 1H, one rotamer), 7.65–7.63 (m, 1H, one rotamer), 7.46–7.35 (m, 2H), 5.47 (s, 2H, one rotamer), 5.05 (s, 2H, one rotamer), 4.87 (2H under water peak), 4.34 (s, 2H, one rotamer), 2.66 (s, 3H, one rotamer), 2.62 (s, 3H, one rotamer), 1.81–1.78 (m, 1H), 1.00 (br s, 4H, one rotamer), 0.91 (br s, 4H, one rotamer). ESI-MS *m*/*z* 435 $(M+H)^+$.

6.2.28. [({2-[3-(Acetylamino)phenyl]-5-methyl-1,3-oxazol-4-yl}carbonyl)(pyridin-2-ylmethyl)amino]acetic acid (5v)

This compound was prepared following the same procedure as in Section 6.2.27 using acetyl chloride instead of cyclopropanecarbonyl chloride. Final purification by preparative HPLC led to **5v** as its trifluoroacetic acid salt. ^{1}H NMR (CD_3OD), 2 rotamers in a roughly 1/1 ratio, each chemical shift is for both rotamers except when stated: 8.81–8.80 (m, 1H, one rotamer), 8.72 (br s, 1H, one rotamer), 8.53–8.43 (m, 1H), 8.25 (br s, 1H, one rotamer), 8.12–8.09 (m, 1H of both rotamers and 1H of one rotamer), 7.92–7.84 (m, 1H), 7.71–7.69 (m, 1H, one rotamer), 7.56–7.54 (m, 1H, one rotamer), 7.40–7.25 (m, 2H), 5.43 (s, 2H, one rotamer), 5.01 (s, 2H, one rotamer), 4.81 (s, 2H, one rotamer), 4.30 (s, 2H, one rotamer), 2.61 (s, 3H, one rotamer), 2.56 (s, 3H, one rotamer), 2.17 (s, 3H). ESI-MS *m*/*z* 409 $(M+H)^+$.

6.2.29. ((1,3-Benzothiazol-2-ylmethyl){[5-methyl-2-(3-(2*H*-pyrazol-3-yl)-phenyl)-1,3-oxazol-4-yl]carbonyl}amino)acetic acid (5x)

Under argon, a solution of ethyl ((1,3-benzothiazol-2-ylmethyl){[2-(3-bromo)-phenyl)-5-methyl-1,3-oxazol-4-yl]carbonyl} amino)acetate (50.5 mg, 0.10 mmol, prepared from ethyl [(1,3-thiazol-2-ylmethyl)amino]acetate **3i**, synthesized as in Section 6.2.2 and 2-(3-bromophenyl)-5-methyl-1,3-oxazole-4-carboxylic acid synthesized as in Section 6.2.6), 1*H*-pyrazole-3-boronic acid (22 mg, 0.20 mmol), sodium carbonate (31.1 mg, 0.29 mmol) and

$Pd(PPh_3)_4$ (34 mg, 0.03 mmol) in degassed anhydrous DMF (0.7 mL) was stirred overnight at 100 °C. Cold HCl 1 N was added and the solution was extracted with ethyl acetate. The combined organic extracts were dried over sodium sulfate, filtered and evaporated. Purification by preparative TLC eluted with 10% methanol in dichloromethane led to ethyl ((1,3-benzothiazol-2-ylmethyl){[5-methyl-2-(3-(2H-pyrazol-3-yl)-phenyl)-1,3-oxazol-4-yl]carbonyl}amino)acetate. The latter compound was dissolved in THF (1 mL) and water (1 mL). LiOH (9.4 mg, 0.39 mmol) was added and the reaction mixture was stirred overnight at room temperature. Then HCl 1N was added and the solution was extracted with diethyl ether and ethyl acetate. The combined organic extracts were dried over sodium sulfate, filtered and evaporated. The crude product was purified by preparative TLC eluted with 10% methanol in dichloromethane to afford **5x** (26.7 mg, 57%) as a white solid. ^{1}H NMR (DMSO-d_6), 2 rotamers in a roughly 2/1 ratio, each chemical shift is for both rotamers except when stated: 8.38 (br s, 1H, major rotamer), 8.32 (br s, 1H, minor rotamer), 7.81–7.43 (m, 8H), 6.81 (br s, 1H, major rotamer), 6.60 (br s, 1H, minor rotamer), 5.51 (s, 2H, minor rotamer), 5.07 (s, 2H, major rotamer), 4.68 (br s, 2H, major rotamer), 4.15 (br s, 2H, minor rotamer), 2.70 (s, 3H, minor rotamer), 2.64 (s, 3H, major rotamer). ESI-MS m/z 474 $(M+H)^+$.

6.2.30. Representative procedure for the synthesis of compounds 9a–h (Scheme 6). ((1,3-Benzothiazol-2-ylmethyl){[2-(1*H*-indol-5-yl)-4-methyl-1,3-thiazol-5-yl]carbonyl}amino)acetic acid (9h)

Under argon, to a solution of 2-bromo-4-methyl-1,3-thiazole-5-carboxylic acid (80.5 mg, 0.36 mmol) in anhydrous dichloromethane (2 mL) at 0 °C, were successively added a solution of oxalyl bromide (2 M solution in dichloromethane, 190 μL, 0.38 mmol) and *N*,*N*-dimethylformamide (1 drop). The reaction mixture was stirred for 2 h allowing the temperature to rise to room temperature. Then at 0 °C, a solution of ethyl [(1,3-benzothiazol-2-ylmethyl)amino]acetate (**3i**, 86.9 mg, 0.35 mmol) in dichloromethane (1 mL) was added followed by *N*,*N*-diisopropylethylamine (0.2 mL, 1.1 mmol). The resulting mixture was stirred overnight allowing the temperature to rise to room temperature. Water was added and the reaction mixture was extracted with dichloromethane. The combined organic extracts were dried over sodium sulfate, filtered and evaporated. The crude product was purified by flash chromatography on silica gel eluted with a gradient of ethyl acetate in cyclohexane (0–30%) to afford ethyl {(1,3-benzothiazol-2-ylmethyl)[(2-bromo-4-methyl-1,3-thiazol-5-yl)carbonyl]amino}acetate (124.5 mg, 79%) as a yellow oil. ESI-MS m/z 454 and 456 $(M+H)^+$.

Under argon, a solution of {(1,3-benzothiazol-2-ylmethyl)[(2-bromo-4-methyl-1,3-thiazol-5-yl)carbonyl]amino}acetate (35.2mg, 0.077 mmol), *tert*-butyl 5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-y)-1*H*-indole-1-carboxylate (35.6 mg, 0.10 mmol), cesium carbonate (50.5 mg, 0.15 mmol), and $PdCl_2$dppf (3.2 mg, 0.004 mmol) in degassed 1,4-dioxane (0.5 mL) and water (0.15 mL) was stirred at 110 °C for 2 days. The reaction mixture was filtered through a bed of Celite and rinsed with dichloromethane, methanol and ethyl acetate. The solvents were evaporated and the crude product was purified by preparative TLC eluted with 10% methanol in dichloromethane to give **9h** (9.6 mg, 27%) as a beige solid. ^{1}H NMR (CD_3OD): 8.23–8.13 (m, 1H), 8.04 (t, J = 7.2 Hz, 2H), 7.75–7.65 (m, 1H), 7.85 (t, J = 7.4 Hz, 1H), 7.50 (t, J = 7.6 Hz, 2H), 7.37 (d, J = 2.4 Hz, 1H), 6.60 (br s, 1H), 5.26–5.18 (m, 2H), 4.87 (2H under water peak), 2.58 (br s, 3H). ESI-MS m/z 463 $(M+H)^+$.

6.2.31. [{[2-(3-Acetylphenyl)-4-methyl-1,3-thiazol-5-yl]carbonyl}(pyridin-2-ylmethyl)amino]acetic acid (9a)

According to the procedure described in Section 6.2.30, this compound was prepared from 2-bromo-4-methyl-1,3-thiazole-5-carboxylic acid, ethyl [(pyridin-2-ylmethyl)amino]acetate (synthesized according to the procedure described in Section 6.2.1) and 3-acetylphenylboronic acid. ^{1}H NMR (CD_3OD) 2 rotamers in a 1/1 ratio, each chemical shift is for both rotamers except when stated: 8.46–8.34 (m, 2H), 8.08–7.98 (m, 2H), 7.83–7.71 (m, 1H), 7.55–7.45 and 7.33–7.26 (m, 3H), 4.84–4.80 (m, 2H), 4.20–4.16 (m, 2H), 2.57 (s, 3H, one rotamer), 2.55 (s, 3H, one rotamer), 2.40 (br s, 3H). ESI-MS m/z 410 $(M+H)^+$.

6.2.32. [{[2-(4-Amino-3-nitrophenyl)-4-methyl-1,3-thiazol-5-yl]carbonyl}(pyridin-2-ylmethyl)amino]acetic acid (9b)

According to the procedure described in Section 6.2.30, this compound was prepared from 2-bromo-4-methyl-1,3-thiazole-5-carboxylic acid, ethyl [(pyridin-2-ylmethyl)amino]acetate (synthesized according to the procedure described in Section 6.2.1) and 2-nitro-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)aniline. ^{1}H NMR (CD_3OD): 8.58–8.53 (m, 2H), 7.86–7.82 (m, 2H), 7.40–7.35 (m, 2H), 7.02 (d, J = 8.2 Hz, 1H), 4.87 (2H under water peak), 4.29–4.24 (m, 2H), 2.44 (s, 3H). ESI-MS m/z 428 $(M+H)^+$.

6.2.33. ((1,3-Benzothiazol-2-ylmethyl){[2-(3-methoxy phenyl)-4-methyl-1,3-thiazol-5-yl]carbonyl}amino)acetic acid (9c)

According to the procedure described in Section 6.2.30, this compound was prepared from {(1,3-benzothiazol-2-ylmethyl)[(2-bromo-4-methyl-1,3-thiazol-5-yl)carbonyl]amino}acetate and 3-methoxyphenylboronic acid. ^{1}H NMR (DMSO-d_6): 8.12 (d, J = 8.0 Hz, 1H), 7.99 (d, J = 8.4 Hz, 1H), 7.53–7.40 (m, 5H), 7.10–7.08 (m, 1H), 5.10 (br s, 2H), 4.27–4.17 (m, 2H), 3.83 (br s, 3H), 2.48 (s, 3H). ESI-MS m/z 454 $(M+H)^+$.

6.2.34. ((1,3-Benzothiazol-2-ylmethyl){[4-methyl-2-(3-nitrophenyl)-1,3-thiazol-5-yl]carbonyl}amino)acetic acid (9d)

According to the procedure described in Section 6.2.30, this compound was prepared from {(1,3-benzothiazol-2-ylmethyl)[(2-bromo-4-methyl-1,3-thiazol-5-yl)carbonyl]amino}acetate and 3-nitrophenylboronic acid. ^{1}H NMR (CD_3OD): 8.85–8.75 (m, 1H), 8.41–8.26 (m, 2H), 8.05 (t, J = 8.6 Hz, 2H), 7.82–7.75 (m, 1H), 7.59 (t, J = 7.4 Hz, 1 H), 7.51 (t, J = 7.6 Hz, 1H), 5.27–5.20 (m, 2H), 4.87 (2H under water peak), 2.63 (br s, 3H). ESI-MS m/z 469 $(M+H)^+$.

6.2.35. ((1,3-Benzothiazol-2-ylmethyl){[2-(3-methanesulfonyl-phenyl)-4-methyl-1,3-thiazol-5-yl]carbonyl}amino)acetic acid (9e)

According to the procedure described in Section 6.2.30, this compound was prepared from {(1,3-benzothiazol-2-ylmethyl)[(2-bromo-4-methyl-1,3-thiazol-5-yl)carbonyl]amino}acetate and 3-(methanesulfonyl)phenylboronic acid. ^{1}H NMR ($CDCl_3$): 8.44–8.36 (m, 1H), 8.06–7.75 (m, 4H), 7.60–7-30 (m, 3H), 5.25–5.05 (m, 2H), 4.35–4.15 (m, 2H), 3.09 (br s, 3H), 2.57 (br s, 3H). ESI-MS m/z 502 $(M+H)^+$.

6.2.36. ((1,3-Benzothiazol-2-ylmethyl){[2-(4-amino-3-methoxy phenyl)-4-methyl-1,3-thiazol-5-yl]carbonyl}amino)acetic acid acid (9f)

According to the procedure described in Section 6.2.30, this compound was prepared from {(1,3-benzothiazol-2-ylmethyl)[(2-bromo-4-methyl-1,3-thiazol-5-yl)carbonyl]amino}acetate and 2-methoxy-4-(4,4,5,5-tetramethyl-[1,3,2]dioxaborolan-2-yl)-aniline. ^{1}H NMR (CD_3OD): 8.01–7.97 (m, 2H), 7.53 (td, J = 8.0 Hz and 1.2 Hz, 1H), 7.45 (td, J = 8.0 Hz and 1.2 Hz, 1H), 7.39–7.26 (m, 2H), 6.72–6.70 (m, 1H), 5.16 (br s, 2H), 4.39 (br s, 2H), 3.90 (s, 3H), 2.48 (s, 3H). ESI-MS m/z 469 $(M+H)^+$.

6.2.37. ((1,3-Benzothiazol-2-ylmethyl){[4-methyl-2-(2-naphthyl)-1,3-thiazol-5-yl]carbonyl}amino)acetic acid (9g)

According to the procedure described in Section 6.2.30, this compound was prepared from {(1,3-benzothiazol-2-ylmethyl)

[(2-bromo-4-methyl-1,3-thiazol-5-yl)carbonyl]amino}acetate and 2-naphthylboronic acid. ^{1}H NMR (DMSO-d_6): 8.53–8.47 (m, 1H), 8.11–7.96 (m, 6H), 7.59 (br s, 2H), 7.52 (t, J = 7.6 Hz, 1H), 7.45 (t, J = 7.6 Hz, 1H), 5.13 (s, 2H), 4.30 (s, 2H), 2.50 (s, 3H). ESI-MS m/z 474 $(M+H)^+$.

6.2.38. [[(4-Methyl-2-phenyl-1,3-thiazol-5-yl)carbonyl] (pyridin-2-ylmethyl)amino]acetic acid (10)

The title compound was obtained from commercially available 4-methyl-2-phenyl-1,3-thiazole-5-carboxylic acid (Maybridge) and amine **3a**, following the same procedures as in Section 6.2.3 for coupling with amine and saponification. ^{1}H NMR (DMSO-d_6), 2 rotamers in a 2/1 ratio, each chemical shift is for both rotamers except when stated: 8.56–8.50 (m, 1H), 7.94–7.84 (m, 2H), 7.82–7.72 (m, 1H), 7.49 (br s, 3H), 7.38–7.34 (m, 1H, major rotamer), 7.32–7.22 (m, 1H of minor rotamer and 1H of both rotamers), 4.75 (br s, 2H, major rotamer), 4.70 (br s, 2H, minor rotamer), 3.92 (br s, 2H, minor rotamer), 3.68 (br s, 2H, major rotamer), 2.48 (s, 3H, minor rotamer), 2.42 (s, 3H, major rotamer). ESI-MS m/z 368 $(M+H)^+$.

6.2.39. [(2-Methyl-5-phenyl-3-furoyl)(pyridin-2-yl methyl)amino]acetic acid (11)

The title compound was obtained from commercially available 2-methyl-5-phenyl-3-furoic acid (Maybridge) and amine **3a**, following the same procedures as in Section 6.2.3 for coupling with amine and saponification. ^{1}H NMR (DMSO-d_6), 2 rotamers in a 2/1 ratio, each chemical shift is for both rotamers except when stated: 8.54 (br s, 1H, minor rotamer), 8.48 (br s, 1H, major rotamer), 7.80–7.73 (m, 1H), 7.64–7.55 (m, 2H), 7.42–7.24 (m, 5H), 6.97 (s, 1H, major rotamer), 6.84 (s, 1H, minor rotamer), 4.73 (br s, 2H), 3.89 (br s, 2H, minor rotamer), 3.79 (br s, 2H, major rotamer), 2.48 (br s, 3H). ESI-MS m/z 351 $(M+H)^+$.

6.2.40. [[(1-Methyl-3-phenyl-1*H*-pyrazol-5-yl)carbonyl] (pyridin-2-ylmethyl)amino]acetic acid (12)

The title compound was obtained from commercially available 1-methyl-3-phenyl-1*H*-pyrazole-5-carboxylic acid (Maybridge) and amine **3a**, following the same procedures as in Section 6.2.3 for coupling with amine and saponification. ^{1}H NMR (DMSO-d_6), 2 rotamers in a 1/1 ratio, each chemical shift is for both rotamers except when stated: 8.58 (d, 1H, J = 3.6 Hz, one rotamer), 8.55 (d, 1H, J = 4.4 Hz, one rotamer), 7.82–7.64 (m, 3H), 7.47–7.28 (m, 5H), 6.95 (br s, 1H, one rotamer), 6.82 (br s, 1H, one rotamer), 4.79 (br s, 2H), 4.24 (br s, 2H, one rotamer), 4.12 (br s, 2H, one rotamer), 3.88 (br s, 3H). ESI-MS m/z 351 $(M+H)^+$.

6.2.41. [(1,1′-Biphenyl-4-ylcarbonyl)(pyridin-2-ylmethyl)amino]acetic acid (13)

The title compound was obtained from commercially available 1,1′-biphenyl-4-carboxylic acid (Sigma–Aldrich) and amine **3a**, following the same procedures as in Section 6.2.3 for coupling with amine and saponification. ^{1}H NMR (DMSO-d_6) 2 rotamers in a roughly 1/1 ratio, each chemical shift is for both rotamers except when stated: 8.58 (br s, 1H, one rotamer), 8.53 (br s, 1H, one rotamer), 7.82–7.31 (m, 12 H), 4.76 (s, 2H, one rotamer), 4.66 (s, 2H, one rotamer), 4.02 (s, 2H, one rotamer), 3.85 (s, 2H, one rotamer). ESI-MS m/z 347 $(M+H)^+$.

6.2.42. [(5-Phenyl-2-furoyl)(pyridin-2-ylmethyl)amino]acetic acid (14)

The title compound was obtained from commercially available 5-phenyl-2-furoic acid (Sigma–Aldrich) and amine **3a**, following the same procedures as in Section 6.2.3 for coupling with amine and saponification. ^{1}H NMR (DMSO-d_6) 2 rotamers in a 2/1 ratio, each chemical shift is for both rotamers except when stated: 8.59 (br s, 1H, minor rotamer), 8.50 (br s, 1H, major rotamer), 7.82–7.69 (m, 2H), 7.46–7.26 (m, 6H), 7.13–7.04 (m, 2H), 5.01 (br s, 2H, minor rotamer), 4.74 (br s, 2H, major rotamer), 4.10 (br s, 2H, major rotamer), 3.97 (br s, 2H, minor rotamer). ESI-MS m/z 337 $(M+H)^+$.

6.2.43. [[(5-Methyl-3-phenylisoxazol-4-yl)carbonyl] (pyridin-2-ylmethyl)amino]acetic acid (15)

The title compound was obtained from commercially available 5-methyl-3-phenylisoxazole-4-carboxylic acid (Sigma–Aldrich) and amine **3a**, following the same procedures as in Section 6.2.3 for coupling with amine and saponification. ^{1}H NMR (DMSO-d_6), 2 rotamers in a roughly 1/1 ratio, each chemical shift is for both rotamers except when stated: 8.56 (d, J = 4.0 Hz, 1H, one rotamer), 8.43 (d, J = 4.0 Hz, 1H, one rotamer), 7.83–7.64 (m, 3H), 7.48–7.40 (m, 3H), 7.35–7.22 (m, 1H of both rotamers and 1H of one rotamer), 7.13 (d, J = 7.6 Hz, 1H, one rotamer), 4.58 (br s, 2H, one rotamer), 4.11 (br s, 2H, one rotamer), 3.71 (br s, 2H, one rotamer), 3.33 (2H of one rotamer under water peak), 2.45 (s, 3H, one rotamer), 2.48 (s, 3H, one rotamer). ESI-MS m/z 338 $(M+H)^+$.

6.3. Plasmid construction, expression and purification of HldE, HldA and RK

Genes encoding HldE, RK and HldA were amplified by PCR from the genomic DNA of *E. coli* O18:K1 C7 strain (*hldE* gene), *E. coli* K12 MG1655 strain (*ribokinase* gene) or *N. meningitidis* 2C43 strain (*hldA* gene) by using pfu polymerase (Promega) and the following primers: rk forward primer (CACCATGCAAAACGCAGGCAGC) and reverse primer (TCACCTCTGCCTGTCTAAAAATG), *hldA* forward primer (CACCATGTCCGCCAAGTTCCAACAAGAAAC) and reverse primer (CTACATTGTTGATTGCCCTGACAATGCCTT), *hldE* forward primer (CACCATGAAAGTAACGCTGC) and reverse primer (AACCGCTTTCCG GTTAGCCT). Blunt-ended PCR fragments were cloned into pET100 expression vector (Invitrogen) and resulting plasmids were used to transform *E. coli* Top10 (Invitrogen). Finally, purified plasmids were introduced into *E. coli* BL21 (Invitrogen) by transformation. Nucleotide sequencing of the various cloned fragment revealed no mutation.

Recombinant proteins were expressed and purified as previously described.[30] Briefly, exponential phase culture (OD_{600} = 0.5–0.7) were induced for expression by the addition of isopropyl-β-D-glucopyranoside (IPTG) (0.5 mM final concentration) and incubated for further 3 h. Bacteria containing over-expressed recombinant proteins were collected by centrifugation and bacterial lysis was achieved by sonication. After centrifugation and removal of bacterial debris or unbroken cell, Ni-NTA agarose (Sigma) was added to the supernatant (soluble fraction) and incubation at 4 °C was performed to maximize contact of recombinant proteins with gel matrix. His-tagged proteins were eluted stepwise by competition with imidazole (Sigma). The various fractions were analyzed by SDS–PAGE and fractions containing recombinant protein were pooled and concentrated by ultrafiltration (Amicon Ultra-15-Millipore). Protein concentration was determined by Bradford Method (Invitrogen). Protein solutions were adjusted to 50% glycerol and stored at −20 °C until used.

6.4. Activity assays

6.4.1. HldE-kinase luminescent assay

This assay was especially used for HTS and IC_{50} measurements.

The luminescent assay was based on the detection of ATP depletion by the luciferase/luciferin system. The assay buffer (AB) adjusted to pH 7.5 contained Hepes (50 mM), $MnCl_2$ (1 mM), KCl (25 mM), Triton-X100 (0.012%) and DTT (1 mM) in deionized water. The following components were added in a white polystyrene Costar plate up to a final volume of 31 μL:3 μL of inhibitor

dissolved in DMSO (or only DMSO), and 28 μL of HldE in AB. After 30 min of pre-incubation at room temperature, 29 μL of substrates mixture (ATP and H-7-P) in AB were added in each well to a final volume of 60 μL. This reaction mixture was then composed of HldE (3 nM), inhibitor to be tested (various concentrations), H-7-P (0.2 μM, in house synthesis)[31] and ATP (0.2 μM) in AB. After 40 min of incubation at room temperature, 200 μL of the revelation mixture (luciferase, D-luciferin and *N*-acetylcysteamine) were added to give a final volume of 260 μL, including the following constituents at the respective final concentrations: luciferase (4 nM), D-luciferin (30 μM) and *N*-acetylcysteamine (100 μM). Luminescence intensity was immediately measured on an Analyst-HT (Molecular Devices) or Microbeta 1450 (Wallac) and converted into inhibition percentages.

For IC_{50} determinations, the inhibitor is tested at 6–10 different concentrations, and the related inhibitions are fitted to a classical Langmuir equilibrium model using XLFIT® (IDBS).

6.4.2. HldE-kinase fluorescent assay

This assay was more specifically used for steady-state kinetics and ATP competition experiments.

The fluorescent assay was based on the detection of the product ADP by the coupled enzymatic assay using pyruvate kinase (PK) and lactate dehydrogenase (LDH). The assay buffer (AB) adjusted to pH 7.5 contained Hepes (50 mM), $MnCl_2$ (1 mM), KCl (25 mM), Triton-X100 (0.012%) and DTT (1 mM) in deionised water. The following components were added in a black polystyrene Costar plate up to a final volume of 50 μL:5 μL of inhibitor dissolved in DMSO (or only DMSO) and 45 μL HldE in AB. After 30 min of pre-incubation at room temperature, 50 μL of substrates-revelation mixture (ATP, H-7-P, pyruvate kinase, phosphoenolpyruvate, lactate dehydrogenase and NADH) in AB were added in each well to a final volume of 100 μL. This reaction mixture was then composed of HldE (66 pM), inhibitor to be tested (various concentrations), H-7-P (1 μM), ATP (50 μM), pyruvate kinase (5 u/mL), phosphoenolpyruvate (50 μM), lactate dehydrogenase (5 u/mL) and NADH (2.5 μM) in AB. Fluorescence intensity of NADH (λ_{ex} = 360 nm, λ_{em} = 520 nm) was immediately measured kinetically by a Fluostar Optima (BMG). All calculations/fits were done in Excel®using XLFIT® (IDBS).

Alternatively, H-7-P, inhibitor to be tested, ATP and NADH were varied on relevant scales in order to accommodate competition and saturation experiments.

6.4.3. HldA luminescent assay

This assay was basically identical to the HldE-kinase luminescent assay. HldE (3 nM) was replaced by HldA (20 nM), other components were used at the same concentrations as in the luminescent assay for HldE-kinase.

6.4.4. RK luminescent assay

This assay was basically identical to the HldE-kinase luminescent assay, with the following differences: H-7-P (0.2 μM) was replaced by D-ribose (3 μM), HldE (3 nM) by RK (0.6 nM), and a concentration of 3 μM ATP was used instead of 0.2 μM.

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