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ATP competitive inhibitors of D-alanine–D-alanine ligase based on protein kinase inhibitor scaffolds

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Abstract—D-Alanine–D-alanine ligase (DDl) is an essential enzyme in bacterial cell wall biosynthesis and an important target for developing new antibiotics. Here, we describe a new approach to identify new inhibitor scaffolds for DDl based on similarity in the ATP binding region of different kinases and DDl. After an initial screening of several protein kinase inhibitors, we found that the Brutons's tyrosine kinase inhibitor LFM-A13, an analog of the Leflunomide metabolite A771726, inhibits DDl with a K_i of 185 μM. A series of malononitrilamide and salicylamide derivatives of LFM-A13 has been synthesized to confirm the validity of this scaffold as an inhibitor of DDl.
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1. Introduction

Bacteria resist hypotonic shock and cell lysis through the biosynthesis of a peptidoglycan-containing cell wall which gives the cell shape and structural strength and insulates the organism from external osmotic pressure. Most antibiotics exploit the vulnerability of the assembly of this extra cellular polymer. For example, the cross-linking transpeptidation is a major target for β-lactam antibiotics (penicillines, cephalosporines). Glycopeptides such as vancomycin and teicoplanin target the peptidoglycan cell wall of Gram-positive bacteria by selectively binding to the D-alanyl–D-alanine termini of peptidoglycan precursors and preventing their cross linking to adjacent strands.[1,2] However, bacteria are continually mutating and resistance has evolved to every antibiotic ever placed into clinical practice, irrespective of their chemical class or the molecular target of the drug. More strikingly, first signs of alarm appear with increasing resistance to vancomycin which had been reserved as a drug of 'last resort', used only after treatment with other antibiotics had failed. Bacteria with reduced vancomycin susceptibility such as vancomycin-intermediate *Staphylococcus aureus* (VISA) need higher levels of vancomycin to be killed and are thought to be an intermediate stage to the development of bacteria that are fully resistant.[3] There consequently remains a strong need for novel antibiotics, particularly directed against multiresistant Gram-negative bacteria.

1.1. DDl as potential target for new antibiotics

D-Alanine–D-alanine ligase (DDl) (E.C. 6.3.2.4) catalyzes the ATP-dependent assembly of the dipeptide D-alanyl–D-alanine (D-ala–D-Ala) which is an essential building block for bacterial cell wall biosynthesis.[4] DDl consists of four domains, whose interfaces create binding sites for D-Ala and ATP, respectively. The formation of D-Ala–D-Ala proceeds via a two-step mechanism. First, a D-Ala unit is phosphorylated by ATP to produce an intermediate which subsequently will react with the second D-Ala moiety to form the dipeptide. For *Escherichia coli* two isoforms of DDl, DDlA, and DDlB, have been described and share a sequence homology of 35%.[5] D-Ala–D-Ala is common to both Gram-negative and Gram-positive organisms and highly conserved among prokaryotes and has no eukaryotic counterparts. Therefore, DDl has emerged as an attractive target to develop novel antibiotics.[5]

Keywords: D-Ala–D-ala ligase; Kinase inhibitor; Antibiotics.
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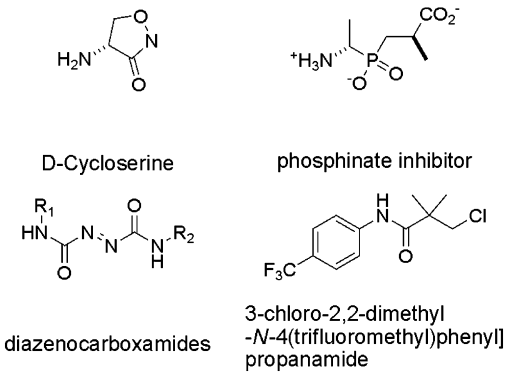
D-cycloserine, a structural analog of D-Ala, is the only small molecule known to inhibit DDl activity and to result in an antibiotic effect (Scheme 1). D-Cycloserine inhibits enzyme activity as a D-Ala competitive inhibitor with a reported K_i of 27 μM and is proposed to bind to the first D-Ala binding site.[5–7] However, its poor selectivity and toxicity limits its use.[6,8] Other inhibitors of DDl such as phosphonate and phosphinate dipeptides have been described as transition-state analogs that bind to the enzymes active site and inhibit enzyme function with IC_{50} values in the low micromolar range. However, these molecules failed to show significant antibacterial activity.[9–11] Using a de novo structure-based molecular design software SPROUT, a new inhibitor for DDlB was recently designed.[12] In another approach, 3-chloro-2,2-dimethyl-*N*-4-trifluoromethyl)phenyl-propan-amide was found as an inhibitor of *Staphylococcus aureus* DDl in a high–throughput screen. This molecule does not interfere with direct binding of ATP or D-Ala but binds to a hydrophobic pocket near the first D-Ala binding site.[13] Finally, a small family of diazenedicarboxamides were developed as inhibitors of DDlB with activities in the micromolar range.[14]

1.2. A link between protein and lipid kinases and DDl

In order to find new inhibitor scaffolds for DDl, we drew from earlier investigations[15–17] and based our search on the similarity that exists between the ATP binding domains of various kinases and the glutathione synthase fold (ATP-grasp) to which DDl belongs. Preliminary studies reported similarities between DDl and protein kinases, such as casein kinase (CK1), AMPc, cyclin dependent kinase 2 (CDK2), the Src family tyrosine kinase Hck, the insulin receptor tyrosine kinase (IRK), phosphorylase kinase (PhK), the extra cellular signal regulated protein kinase (ERK2), and lipid kinases such as PI3K.[18–20] Structural alignments of the ATP binding sites of DDl and different classes of kinases highlight a conserved topology of the co-factor binding site as well as the same relative orientation of the adenine part of ATP (Fig. 1). These common features served as starting points toward our investigations of novel DDl inhibitors.



Scheme 1. Structures of reported DDl inhibitors.

2. Results and discussion

2.1. Kinase inhibitors as starting points for new inhibitors of DDl

Over the last decades, kinases have evolved as a major class of target proteins in medicinal chemistry and chemical biology. In order to shut down unwanted kinase activities, a plethora of small organic molecules has since been developed to inhibit kinase function by targeting the ATP binding pocket of the kinase domain.[21–25] Typically, such inhibitors bind to the ATP binding site of the kinase through the formation of 2–3 hydrogen bonds and through hydrophobic interactions in and around the region occupied by the adenine ring of ATP.[†] Although some selective ATP competitive kinase inhibitors have been developed, the highly conserved nature of the ATP binding region often results in inhibitor scaffolds with poor kinase selectivity. This conserved nature of ATP binding sites among other protein classes and the wealth of ATP competitive kinase inhibitors could serve as a starting point for the development of inhibitors that target proteins other than kinases.

2.2. Biochemical screening

Based on the described similarities between the ATP binding sites of DDl and protein and lipid kinases we developed a new approach to find new inhibitor scaffolds binding to the ATP binding site of DDl. A collection of 27 different ATP competitive kinase inhibitors was selected and screened at mM concentrations against DDl measuring residual activity. ATP competitive kinase inhibitors identified as DDl inhibitors are given in Table 1 (complete list of compounds tested is provided as supporting information).

Under the conditions tested, DDl was better inhibited by the EGFR kinase inhibitors Tyrphostin-47 (T-47), Tyrphostin-51 (T-51) and by the Bruton's tyrosine kinase inhibitor LFM-A13. Slight inhibition could be detected for the AMPc inhibitor HA-1004, the CDK2 inhibitor Olomoucine, and the Src family tyrosine kinase inhibitor Piceatannol. Interestingly, the known PI3K inhibitors Wortmannin, and LY294002 were also found to inhibit DDl.

Detailed inhibition studies were then performed for the most potent DDl inhibitors identified in the initial screen. DDl enzyme inhibition was measured at different inhibitor and ATP concentrations in order to determine K_i values and the type of inhibition. For K_i determinations, parallel series of experiments were made under the same conditions in the presence of various inhibitor concentrations and data were analyzed by using Lineweaver–Burk plots (Table 1). Tyrphostin T-47, Olomoucine, LY29004, and LFM-A13 were found to be ATP competitive inhibitors of DDl. However, the Tyrphostin

[†] For our contributions to the development of kinase inhibitors see Refs. 26–29.

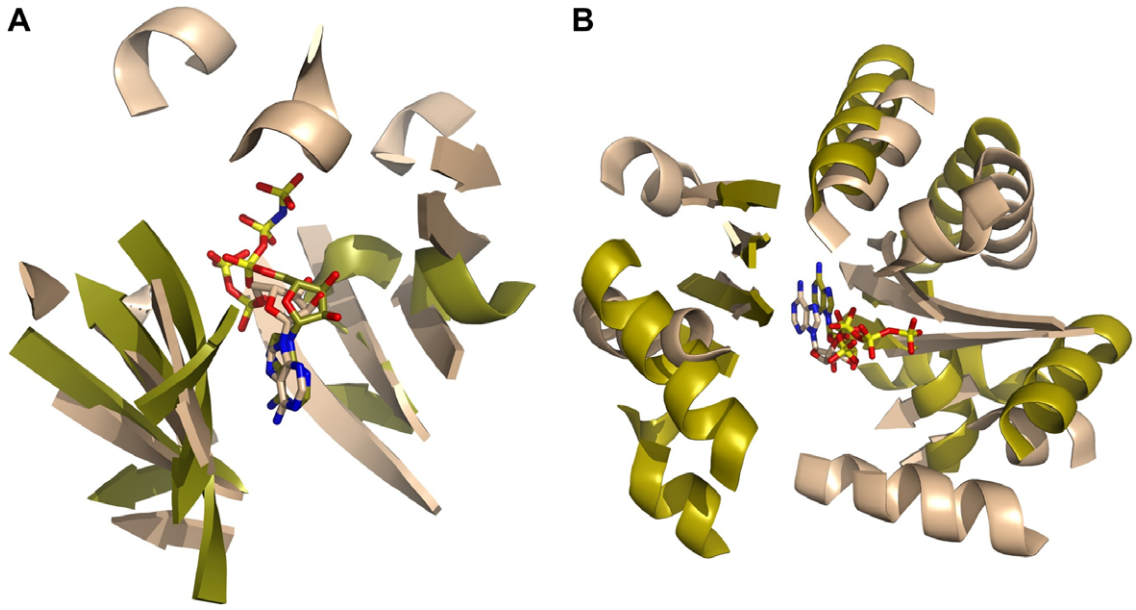
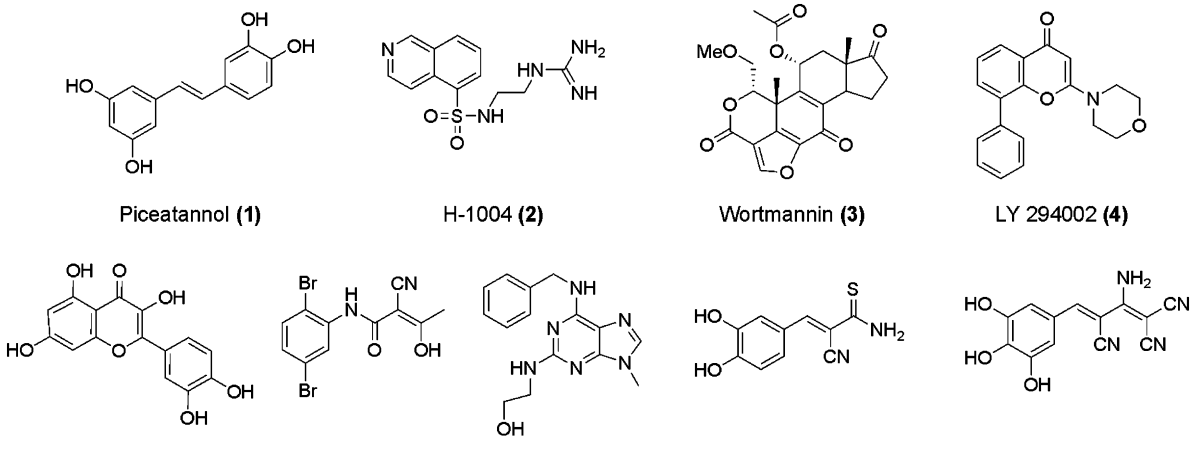


Figure 1. Structural alignments of the ATP binding sites of DDl and various kinases. (A) Structural alignment of the ATP binding sites of DDl (PDB code 2DLN)[9] in salmon and the protein kinase Hck (PDB code 1AD5)[46] in green. (B) Structural alignment of the ATP binding sites of DDl (PDB code 2DLN) in salmon and lipid kinase PI3K-γ (PDB code 1E8X[47]) in green. The alignments highlight similar binding modes for the ligands ADP (DDl), AMP-PNP (Hck), and ATP (PI3K-γ). Structural alignments were performed using DaliLite.[48] Images were generated using PyMol (http://www.pymol.org).

Table 1. Kinase inhibitors that showed inhibition of DDl



Compound	Target	IC_{50} values of kinase inhibition (μM)	Scaffold	DDl residual activity	K_i values for DDl inhibition
1	$p56^{lck}$, syk		I	84.63 ± 7.5	
2	PKA, PKG		II	85.3 ± 12.86	
3	PI3K, PKC		III	81.37 ± 4.4	
4	PI3K	10[49]	IV	83.94 ± 3.5	1.4 mM
	CK2	6.9[49,50]			(ATP competitive)
5	PI3K, PKC		IV	77.00 ± 14.9	
6	BTK	2.5	V	41.19 ± 15.5	185 μM
	Jak, Tec, Plk				(ATP competitive)
7	CDK2/cycB,A,E	7	VI	79.7 ± 10.3	1.8 mM
	ERK1/MAPK	25		(ATP competitive)	
8	EGFR, PDGFR	2.4–3.5	VII	51.79 ± 19.08	290 μM
	IRK	640			(ATP competitive)
9	EGFR	0.8	VII	61.92 ± 2.44	Mixed inhibition

Scaffold I (natural product, resverastrol analog, phenolic stilbene), scaffold II (sulfonamide), scaffold III (natural product, stereoidal furanoids), scaffold IV (flavonoid), scaffold V (leflunomide), scaffold VI (purine), scaffold VII (Tyrphostins).

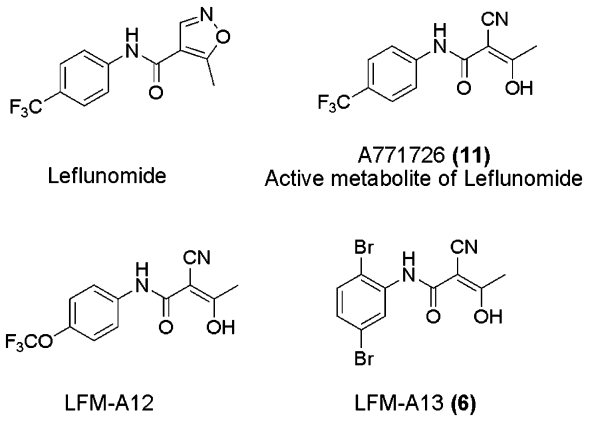
(Solubility of compounds could be increased by adding BSA (1 mg/ml) or chaps instead of 0.01% of the detergent NP-40). K_i values were determined for the best inhibitors.

T-51, although a structural homolog of T-47, showed the profile of a mixed inhibitor for DDl. Interestingly, mixed inhibition has also been reported for T-51 when screened against kinase activity.[30–32] The Tyrphostins were originally designed on the basis of the polyphenol erbstatin,[33] a mixed ATP and substrate competitive kinase inhibitor. T-51 and T-47 are reported as potent inhibitors of EGFR and PDGFR with IC_{50} values in the low micromolar range.[30–32] The malononitrilamide LFM-A13 belongs to a series of compounds that were synthesized based on the active Leflunomide metabolite A771726[34] (Scheme 2).

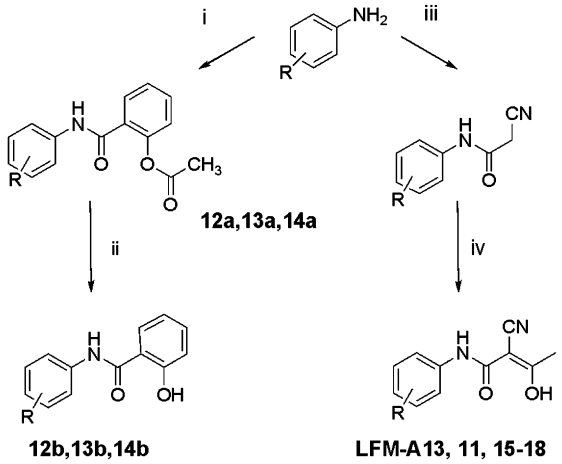
Leflunomide is an antirheumatic drug (Arava®) known to inhibit dihydroorotate dehydrogenase. However, its metabolite A771726 (**11**) has been hypothesized to exert immunosuppressive activity by the inhibition of several protein kinases such as PDGFR,[35] EGFR[36,37], and to prevent phosphorylation of Jak1, and Jak3 that are necessary for Interleukin-2 receptor signaling.[38] Several analogs of A771726 such as LFM-A12 and LFM-A13 were also reported to inhibit EGFR,[35] Bruton's tyrosine kinase (BTK),[34] Jak2,[39] Tec[40], and Polo-like kinases (Plk).[41]

2.3. Synthesis

Based on the findings that the binding of ATP to DDl can be inhibited by ATP competitive kinase inhibitors of the malononitrilamide class in the micromolar range, we developed a focused library based on LFM-A13 to establish further structure–activity relationships. To explore substitution patterns at the aniline moiety, compounds **11** and **15–18** were synthesized according to published procedures.[34] Briefly, different substituted anilines were coupled to cyanoacetic acid in the presence of diisopropylcarbodiimide followed by treatment with NaH and acylation with acetylchloride (Scheme 3). Simplified salicylic acid derivatives such as **12–14** were synthesized by coupling of anilines to acetylsalicylic acid and further deprotected to investigate whether the β-keto nitrile group is needed for DDl inhibitory activity. Such analogs are thought to mimic a planar conformation that is considered the active conformation of



Scheme 2. Leflunomide, active metabolites of leflunomide A771726 and analogs.



Scheme 3. Synthesis of Leflunomide analogs. Reagents and conditions: (i) acetylsalicylic acid chloride, NEr_3, DCM, o/n; (ii) imidazole, MeOH, overnight; (iii) cyanoacetic acid, DIC, THF, 24 h, rt; (iv) NaH, acetyl chloride, THF, 0 °C 30 min, then o/n at rt.

A771726[42,43] and stabilized by an intramolecular hydrogen bond. Finally, the extended analog of LFM-A13 **19**, which is further functionalized with a carboxylate and the simplified analog **20**, that retains the aryl substitution, however, lacks the nitrile group and the enol and thus is not able to form the discussed intramolecular hydrogen bond were synthesized to study the structural features required for inhibition.

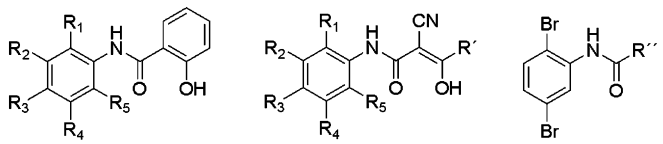
The prepared derivatives were evaluated for their ability to inhibit recombinant DDl. Compounds were screened in a DDl assay that was carried out in the presence of 250 μM ATP and 500 μM D-Ala. Orthophosphate was detected with malachite green. Structure–activities are provided in Table 2.

The inhibitory activity of LFMA13 could be retained by the salicylic derivative **12** and slightly increased by **15, 18**. Compound **18** with CF_3-substituents in the 2- and 5-position showed higher activity compared to LFM-A13 (bromo-substitution in 2,5-position). Similar results were obtained for the 2-chloro-6-methyl derivative **15**. However, neither A771726 (CF_3 in the 4-position) nor the dimethyl- (**16**) or trimethyl-analog **17** showed any DDl inhibiting activity. Changes in the aliphatic region of the molecules were not tolerated and the extended analogs **19** and the carbamate **20** were not active.

3. Conclusions

Using structural alignments of the ATP binding sites of the bacterial ligase DDl and protein and lipid kinases in complex with ATP analogs, we rationalized that inhibition of DDl activity may be achieved by ATP competitive kinase inhibitors. We tested a series of commercially available kinase inhibitors and found LFM-A13 and Tyrphostine derivatives to inhibit DDl enzyme activity. Based on the initial screening results we synthesized a series of malononitrilamide and salicylamide derivatives

Table 2. Activity of leflunomide analogs on DDl



A
Salicylic acid

B
Leflunomide metabolite
(malononitrilamides)

C

Molecule No.	Class	Substitution							DDl Residual activity (%)	K_i (μM)
		R_1	R_2	R_3	R_4	R_5	R'	R''		
11	B			CF_3			CH_3		114.52 ± 10.7	
12	A	Br			Br				65.43 ± 10.7	
13	A	CH_3				CH_3			Not soluble	
14	A	CH_3		CH_3		CH_3			Not soluble	
LFM-A13	B	Br			Br		CH_3		41.19 ± 15.5	185
15	B	Cl				CH_3	CH_3		46.15 ± 3.14	215
16	B	CH_3				CH_3	CH_3		95 ± 13	
17	B	CH_3		CH_3		CH_3	CH_3		116.37 ± 7.25	
18	B	CF_3			CF_3		CH_3		58.61 ± 0.3	60
19	B	Br			Br		CH_2COOH		88.10 ± 12.45	
20	C							$COCH_3$	112.02 ± 8.24	

and were able to confirm the validity of these scaffolds as inhibitors of DDl. From this investigation we gained a better understanding of the structural requirements and limitations necessary for the preparation of ATP competitive DDl inhibitors. The compounds in this study may serve as starting points for the development of bi-substrate inhibitors that incorporate both an ATP competitive and a substrate competitive moiety. Bisubstrate inhibitors that block the ATP and D-Ala binding sites should exhibit enhanced selectivity and potency profiles by preferentially inhibiting DDl over kinases.

4. Experimental

4.1. General

Unless otherwise noted, chemicals were obtained from Aldrich, Acros, and Fluka and were used without further purification. The following materials were obtained from Gerbu Biochemicals: EDTA, IPTG, and ampicillin. The following materials were obtained from Serva: HEPES, PMSF, and DTT. $MgCl_2$ and KCl were obtained from J.T. Baker and $(NH_4)_2SO_4$ from Riedel de Häen. Kinase inhibitors were obtained from Sigma, Tocris or Biomol.

All solvents were distilled by standard procedures. All reactions were performed under argon with freshly distilled and dried solvents. Analytical chromatography was performed by using Merck silica gel 60 F_{254} aluminum plates. Flash chromatography was performed by using Acros silica gel (0.035–0.07 mm). ^1H and ^{13}C NMR spectroscopic data were recorded on a Bruker DRX 500 or Varian Mercury VX 400 spectrometer at RT. NMR spectra were calibrated to the solvent signals of $CDCl_3$ (δ = 7.26 and 77.00 ppm), CD_3OD (δ = 3.31 and 49.05 ppm), DMSO-d_6 (δ = 2.50 and 39.43 ppm) CD_3CN (δ = 1.94 and 1.24 ppm) and the following abbreviations are used to indicate signal multiplicities: s (singlet), d (doublet), t (triplet), q (quartet), quint (quintet), sext (sextet), sept (septet), br (broad), ap (apparent). LCMS was performed on a Hewlett–Packard 1100 series connected to a Finnigan LCQ ESI-spectrometer using a gradient of 20% acetonitrile to 100% acetonitrile in 10 min. Preparative HPLC was performed on a Hewlett–Packard Agilent Series 1100 System equipped with a Nucleodur C18 gravity 5 μm column from Macherey&Nagel. GCMS (EI) analysis was performed on a Hewlett–Packard 6890 series gas chromatograph connected to a Hewlett–Packard 5973 series mass spectrometer; column: H&W 19091s-102 HP-5mS, capillary: 25.0U201mU0.33mmm nominal. IR spectra were measured on a Bruker Vector 22 spectrometer with an A527 diffuse reflectance head from Spectra Tech.

4.2. Expression and purification

The plasmid W3110/pTB2 containing the *E. coli* DDlB gene was a gift of C.T. Walsh (Havard Medical School, Boston). Expression and purification of *E. coli* DDlB was performed according to the modified protocol of Zawadzke et al.[5] All steps were performed at 4 °C unless otherwise specified. Enzyme activity was monitored by detection of orthophosphate using malachite green. Protein concentration was determined by the method of Bradford.[44] The standard column buffer consisted of 50 mM HEPES, 5 mM $MgCl_2$, 1 mM EDTA and 5 mM DTT, pH 7.2. BL21(DE3)RIL cells were transformed with W3110/pTB2 in and grown at 25 °C in LB medium containing ampicillin to an OD_{595} of 0.6, whereupon IPTG was added to a final concentration

of 1 mM for induction. Cells were then grown for 4 h at 25 °C and harvested by centrifugation at 7000 rpm for 10 min. The cell pellet (ca. 8 g) was resuspended in 20 mL of lysis buffer (100 mM HEPES, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, and 1 mM PMSF, pH 7.2) and was passed through a microfluidizer (Microfluidics Corporation, Newton, MA). Cell debris was removed by centrifugation at 10,000 rpm for 30 min. Powdered ammonium sulfate was gradually added to the supernatant to a concentration of 50% saturation, and the solution was stirred for 45 min. The solution was cleared by centrifugation at 10,000 rpm for 30 min, and ammonium sulfate was added to the supernatant to a concentration of 50% saturation. After being stirred for additional 45 min, the precipitate was removed by centrifugation at 10,000 rpm for 30 min. The precipitate was resuspended in column buffer and the resuspended pellet dialysed (Spectrum regenerated Cellulose MWCO 5000 kDa) over night against column buffer. After centrifugation (15 min, 10,000 rpm) the supernatant was applied to a Source Q30 (XK6/20, GE Healthcare) with 2 mL/min and washed with column buffer (5 mL/min). Protein was eluted at 4.0 mL/min with a gradient up to 300 mM KCl in column buffer. The activity eluted at ~150 mM KCl. The purified protein appeared as a 32-kDa band on SDS–polyacrylamide gel (15 %).

4.3. D-Alanine–D-alanine ligase activity

The assay was carried out in a 96-well format at a total reaction volume of 50 μL at 37 °C. The reaction mixture contained the following components (final concentration): 100 mM Hepes (pH 7.4), 10 mM KCl, 10 mM $MgCl_2$, 250 μM ATP, 500 μM D-Ala, (2 mM) DDlB (0.13 μg), and compound (2 mM). The antibiotic D-cycloserine, a known substrate competitive inhibitor of DDl was used as positive control (K_i for DDlB 27 μM). Compounds were pre-incubated with all assay components except the substrates for 30 min. D-Ala and ATP were added to start the reaction. After 15 min, DDlB activity was monitored by detection of orthophosphate using malachite green[45] (80 μL of malachite green-molybdate solution were added (2.45 mM malachite oxalate salt, 16 mM of ammonium molybdate tetrahydrate in 4 N HCl), followed by 10 μL of 34% sodic citrate solution). The plates were read at 620 nm after additional 20 min. K_i determinations were performed under similar conditions using D-Ala (32 mM), ATP (600 μM, 300 μM, 200, μM, 100 μM) and inhibitor (1 mM, 2 mM, 3 mM, 4 mM) with 15 min incubation at 25 °C.

4.4. Chemistry

All syntheses were performed by following literature procedures.[34,43]

4.4.1. General procedure A. Step (i): Acetylsalicylic acid (1.6 equiv) was added to thionyl chloride (3.2 equiv), and the suspension was refluxed until complete dissolution of the acid (1 h). The resulting yellow solution was then cooled to room temperature, and remaining thionyl chloride was evaporated under reduced pressure. Crude acyl chloride was dissolved in chloroform, and the solvent was removed under reduced pressure (3 times). The oily residue was redissolved in dichloromethane and the resulting solution slowly added at 0 °C to a mixture of substituted anilines (1 equiv) and triethylamine (3.3 equiv) in dichloromethane. The resulting mixture was stirred overnight at room temperature, followed by successive washing of the organic phase with 1 N HCl, 1 N NaOH, and water, drying with anhydrous magnesium sulfate and evaporation under reduced pressure. The crude product was purified by flash chromatography on silica gel (eluent, cyclohexane/ethyl acetate, 9:1) to afford compounds **12a**–**14a.**

Step (ii): Imidazole (4 equiv) was added to a 0.03 M solution of **12a–14a** (1 equiv) in methanol and was stirred at room temperature overnight. The resulting white precipitate was filtered off and dried under vacuum.

4.4.2. General procedure B. Steps (iii) and (iv): Cyanoacetic acid (1.6 equiv) and substituted aniline (1 equiv) were dissolved in THF (25 mL) and stirred at 0 °C. Diisopropylcarbodiimide (DIC) was added over 5 min. The reaction was stirred for 24 h at room temperature. AcOEt was added and the organic phase was sequentially washed with a 1 N HCl solution, with brine, dried over anhydrous magnesium sulfate, filtered, and the solvent was removed under reduced pressure. Finally, the crude solid product was recrystallized from ethyl alcohol or used without further purification. Sodium hydride (2 equiv) was added slowly to a solution of amide in tetrahydrofuran (1 equiv; 0.3 M) at 0 °C. After stirring for 30 min at 0 °C, acetyl chloride (1.1 equiv) was added to the reaction mixture. The reaction was continued overnight and then quenched by the addition of acetic acid. The mixture was poured into water containing hydrochloric acid to precipitate the crude product, which was collected by filtration and washed with water. The final pure product was obtained in some cases by recrystallization.

4.5. *N*-(2,5-Dibromophenyl)-2-acetoxybenzamide (12a)

Following general procedure A (Step 1), compound **12a** was obtained in 32% yield. Selected data: ^{1}H NMR (400 MHz, $CDCl_3$): 2.37 (s, 3 H), 7.15 (dd, 1H, *J* 3.6, 8.8 Hz), 7.20 (dd, 1H, *J* 0.8, 8.8 Hz), 7.38 (dt, 1H, *J* 1.6, 6.4 Hz), 7.40 (d, 1H, *J* 8.8 Hz), 7.55 (dt, 1H, *J* 2, 7.6 Hz), 7.93 (dd, 1H, *J* 1.6, 7.6 Hz), 8.62 (bs, 1H), 8.78 (d, 1H, *J* 2.4 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): 21.3, 111.5, 122.1, 123.5, 124.5, 126.5, 127.5, 128.2, 130.3, 132.8, 133.2, 136.9, 148.0, 163.3, 168.7; LC-MS (ESI): calcd for $C_{15}H_{11}{}^{79}Br_2NO_3$ 411.92 $[M+H]^+$, found 411.74 $[M+H]^+$, 371.98 [M-acetyl+H]$^+$, R_t 10.05 min; IR (KBr): 582, 1028, 1084, 1190, 1656, 1761, 3305.

4.6. *N*-(2,6-Dimethylphenyl)-2-acetoxybenzamide (13a)

Following general procedure A (Step 1), compound **13a** was obtained in 41% yield. Selected data: ^{1}H NMR (400 MHz, $CDCl_3$): 2.29 (s, 6H), 2.31 (s, 3H), 7.15 (m,

4H), 7.35 (dt, *J* 7.58, 0.96 Hz, 1H), 7.46 (bs, 1H), 7.53 (dt, *J* 7.84, 1.65 Hz, 1H), 7.86 (dd, *J* 7.70, 1.66 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): 18.1, 20.8, 26.7, 123.1, 126.0, 127.2, 128.3, 128.5, 129.5, 132.0, 133.5, 135.4, 148,2, 164.1, 169.3; ESI-MS: calcd for $C_{17}H_{18}NO_3$ 284.13 $[M+H]^+$, found 284.00 $[M+H]^+$, 242.77 $[M\text{-acetyl}+H]^+$; IR (KBr): 751, 914, 1191, 1131, 1521, 1649, 1765, 2965, 3223.

4.7. *N*-(2,4,6-Trimethylphenyl)-2-acetoxybenzamide (14a)

Following general procedure A (Step 1), compound **14a** was obtained in 28% yield. Selected data: ^{1}H NMR (400 MHz, $CDCl_3$): 2.20 (s, 6H), 2.27 (s, 3H), 2.30 (s, 3H), 6.29 (s, 2H), 7.15 (dd, *J* 8.13, 1.12 Hz, 1H), 7.30 (dt, *J* 7.57, 1.15 Hz, 1H), 7.50 (ddd, *J* 8.12, 7.50, 1.70 Hz, 1H), 7.52 (bs, 1H), 7.78 (dd, *J* 7.69, 1.69 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): 18.0, 20.7, 20.9, 123.1, 126.0, 128.4, 128.7, 129.2, 130.8, 131.6, 135.0, 136.8, 148.1, 164.3, 169.2; LC-MS (ESI): calcd for $C_{18}H_{21}NO_3$298.14 $[M+H]^+$, found 594.68 $[2M+H]^+$, 297.95 $[M+H]^+$, 256.20 $[M\text{-acetyl}+H]^+$, R_t 10.94 min; IR (KBr): 713, 1011, 1198, 1764, 2976, 3278.

4.8. *N*-(2,5-Dibromophenyl)-2-hydroxybenzamide (12b)

Following general procedure A (Step 2), compound **12b** was obtained in 12% yield. Selected data: ^{1}H NMR (400 MHz, DMSO-d_6): 7.01 (t, 1H, *J* 8 Hz), 7.04 (d, 1H, *J* 8 Hz), 7.29 (dd, 1H, *J* 2.4, 8.4 Hz), 7.45 (dt, 1H, *J* 1.6, 8.4 Hz), 7.66 (d, 1H, *J* 8.4 Hz), 8.03 (dd, 1H, *J* 1.6, 8 Hz), 8.66 (d, 1H, *J* 2.4 Hz), 10.91 (bs, 1H), 11.90 (bs, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): 112.1, 112.7, 116.9, 117.7, 119.7, 120.7, 121.6, 124.9, 127.9, 130.7, 134.0, 137.9, 143.9, 154.0, 156.4, 164.0; ESI-MS: calcd for $C_{13}H_8{}^{79}Br_2NO_2$ 367.89 $[M-H]^-$, found 367.93 $[M-H]^-$; IR (KBr): 682, 1025, 1082, 1229,1399, 1608, 3198.

4.9. *N*-(2,6-Dimethylphenyl)-2-hydroxybenzamide (13b)

Following general procedure A (Step 2), compound **13b** was obtained in 40% yield. Selected data: ^{1}H NMR (400 MHz, DMSO-d_6): 2.19 (s, 6H), 6.96 (ddd, *J* 6.22, 3.51, 0.97 Hz, 1H), 7.14, (s, 2H), 7.50–7.43 (m, 1H), 8.05 (dd, *J* 8.19, 1.63 Hz, 1H), 10.06 (bs, 1H), 12.32 (bs, 1H); ^{13}C NMR (100 MHz, CH_3CN): 18.3, 115.1. 118.8, 119.7, 127.9, 128.5, 128.9, 134.6, 135.4, 137.2, 162.6, 170.2; LC-MS (ESI): calcd for $C_{15}H_{16}NO_2$ 242.12 $[M+H]^+$, found 242.15 $[M+H]^+$, R_t 3.43 min.

4.10. *N*-(2,4,6-Trimethylphenyl)-2-hydroxybenzamide (14b)

Following general procedure A (Step 2), compound **14b** was obtained in 38% yield. Selected data: ^{1}H NMR (400 MHz, DMSO-d_6): 2.14 (s, 6H), 2.26 (s, 3H), 6.95 (m, 3H), 7.46 (dt, *J* 7.81, 1,21 Hz, 1H), 8.05 (dd, *J* 8.12, 1.41 Hz, 1H), 9.98 (bs, 1H), 12.39 (bs, 1H); ^{13}C NMR (100 MHz, CD_3CN): 17.9, 20.9, 118.8, 119.7, 127.8, 129.5, 131.9, 135.3, 136.8, 138.1, 162.5; LC-MS (ESI): calcd for $C_{16}H_{18}NO_2$ 256.13 $[M+H]^+$, found 256.18 $[M+H]^+$, R_t 10.12 min.

4.11. *N*-(2-Chloro-6-methyl)phenyl)-2-cyano-3-hydroxybut-2-enamide (15)

Following general procedure B, compound **15** (80 mg, 35%) was obtained. ^{1}H NMR (400 MHz, DMSO-d_6): 2.19 (s, 3H), 2.28 (s, 3H), 7.25 (q, *J* 7.70 Hz, 1H), 7.25 (s, 1H), 7.37 (dd, *J* 6.29, 2.95 Hz, 1H), 10.16 (bs, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): 18.9, 23.3, 43.8, 46.8, 122.8, 125.7, 127.5, 129.0, 129.7, 132.5, 133.5, 177.5, 195.5; ^{13}C NMR (100 MHz, CD_3CN) 19.1, 22.8, 23.9, 81.0, 117.2, 128.7, 130.2, 130.7, 130.8, 132.9, 132.2, 170.3, 190.9; LC-MS (ESI): calcd for $C_{12}H_{11}ClN_2O_2$ 251.06 $[M+H]^+$, found 251.11 $[M+H]^+$; R_t 7.11 min; ESI-MS 249.07 $[M-H]^-$.

4.12. *N*-(2,6-Dimethyl)phenyl)-2-cyano-3-hydroxybut-2-enamide (16)

Following General procedure B, product **16** was obtained (0.31 g; 67%). Selected data: ^{1}H NMR (400 MHz, CD_3CN): 2.22 (s, 6H), 2.32 (s, 3H), 7.15 (m, 3H), 8.03 (s, 1H); ^{13}C NMR (100 MHz, CD_3CN): 18.2, 22.2, 80.2, 116.9, 118.2, 128.9, 133.6, 137.8, 169.7, 190.1; ESI-MS: calcd for $C_{13}H_{13}N_2O_2$ 229.10 $[M-H]^-$, found 229.13 $[M-H]^-$.

4.13. *N*-(2,6-Trimethyl)phenyl)-2-cyano-3-hydroxybut-2-enamide (17)

Following general procedure B, product **17** was obtained (0.085 g; 20%). Selected data: ^{1}H NMR (400 MHz, DMSO-d_6): 2.11, 2.13 (2 s, 9H), 2.25, 2.28 (2 s, 3H), 6.93 (m, 2H); ESI-MS: calcd for $C_{14}H_{17}N_2O_2$ 245.13 $[M+H]^+$, found 245.87 $[M+H]^+$, 262.98 $[M+H_2O]^+$.

4.14. *N*-(2,5-Bis(trifluoromethyl)phenyl)-2-cyano-3-hydroxybut-2-enamide (18)

Following general procedure B, product **18** was obtained (0.19 g; 90%) after recrystallization from toluene. Selected data: ^{1}H NMR (400 MHz, CD_3CN): 2.36 (s, 3H), 7.98 (d, *J* 8.25 Hz, 1H), 7.82 (d, *J* 8.26 Hz, 1H). 8.14 (s, 1H), 8.33 (bs, 1H); ^{13}C NMR (100 MHz, CD_3CN): 23.0, 55.5, 55.9, 117.1, 118.9, 123.2, 123.4, 125.7, 125.8, 126.0, 126.1, 126.9, 126.9, 129.7, 129.6, 130.0, 130.1, 170.3, 191.0; GC/MS: calcd for $C_{12}H_8F_6N_2O_2$ 338.05, found *m/z* 338, 296, 229. ESI-MS: calcd for $C_{12}H_9F_6N_2O_2$ 339.06 $[M+H]^+$, found 338.93 $[M+H]^+$, 355.27 $[M+NH_3]^+$.

4.15. 4-Cyano-5-(2,5-dibromophenylamino)-3-hydroxy-5-oxopent-3-enoic acid (19)

General procedure B was applied with a slight modification: ethylmalonyl chloride (1.1 equiv.) was used instead of acetyl chloride to afford compound **19** (0.49 g; 60%). ^{1}H NMR (400 MHz, DMSO-d_6): 3.98 (s, 2 H), 7.35 (dd, *J* 8.57, 2.42 Hz, 1H), 7.61 (d, *J* 8.58 Hz, 1H), 7.83 (d, *J* 2.40 Hz, 1H), 9.97 (bs, 1H);^{13}C NMR (100 MHz, CD_3CN): 32.6, 35.0, 45.1, 49.8, 69.4, 119.9, 126.7, 130.8, 131.9, 133.7, 134.5, 134.9, 141.9, 159.8, 166.7, 184.7; IR (KBr): 1035, 1400, 1531, 1580, 1671, 2190, 2928, 3279; GC/MS: *m/z* 405, 318, 251, 237; ESI-MS: 403.27 $[M-H]^-$.

4.16. Methyl 2,5-dibromophenylcarbamate (20)

2,5-Dibromoaniline (0.8 g; 3.2 mmol) was dissolved in pyridine (5.23 mL; 65 mmol) and stirred at 0 °C. Methylchloroformiate (0.38 mL; 4.9 mmol) was then slowly added at 0 °C and the reaction was stirred at room temperature for 4 h. AcOEt (5 mL) and water (5 mL) were added to the reaction and the organic phase was subsequently washed with 1 M $CuSO_4$, 1 M HCl, and brine, dried over anhydrous $MgSO_4$, filtered, and the solvent was removed under reduced pressure. Compound **20** (0.72 g; 73%) was isolated by column chromatography using a mixture of 9:1 cyclohexane/AcOEt as eluent. ^{1}H NMR (400 MHz, $CDCl_3$): 3.81 (s, 3H), 7.06 (dd, *J* 8.52, 2.34 Hz, 1H), 7.12 (bs, 1H), 7.36 (d, *J* 8.53 Hz, 1H), 8.38 (d, *J* 2.13 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): 52.7, 109.7, 110.7, 122.1, 122.8, 127.1, 133.2, 136.2, 136.9, 152.1, 153.3; GCMS: calcd for $C_8H_7{}^{79}Br_2NO_2$ 306.88, found *m/z* 307, 250, 228, 171.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.02.046.

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