Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 17 (2007) 2047–2054

Diazenedicarboxamides as inhibitors of D-alanine-D-alanine ligase (Ddl)

Andreja Kovač,[a] Vita Majce,[b] Roman Lenaršič,[b] Sergeja Bombek,[b] Julieanne M. Bostock,[c] Ian Chopra,[c] Slovenko Polanc[b,*] and Stanislav Gobec[a,*]

[a]*University of Ljubljana, Faculty of Pharmacy, Aškerčeva 7, 1000 Ljubljana, Slovenia*
[b]*University of Ljubljana, Faculty of Chemistry and Chemical Technology, Aškerčeva 5, 1000 Ljubljana, Slovenia*
[c]*Institute of Molecular and Cellular Biology and Antimicrobial Research Centre, University of Leeds, Leeds LS 9JT, UK*

Received 24 November 2006; revised 27 December 2006; accepted 5 January 2007
Available online 17 January 2007

Abstract—D-Alanine-D-alanine ligase (Ddl) catalyzes the biosynthesis of an essential bacterial peptidoglycan precursor D-alanyl-D-alanine and it represents an important target for development of new antibacterial drugs. A series of semicarbazides, aminocarbonyldiazenecarboxylates, diazenedicarboxamides, and hydrazinedicarboxamides was synthesized and screened for inhibition of DdlB from *Escherichia coli*. Compounds with good inhibitory activity were identified, enabling us to deduce initial structure–activity relationships. Thirteen diazenedicarboxamides were better inhibitors than D-cycloserine and some of them also possess antibacterial activity, which makes them a promising starting point for further development.

The emergence of bacterial resistance to antibiotic therapy has become a global health threat.[1] To overcome this problem, new antibacterial agents directed toward novel targets have to be developed. The best known and most validated target for antibacterial therapy is the system of enzymes responsible for the construction of peptidoglycan.[2,3] The late extracellular stages of bacterial peptidoglycan biosynthesis are inhibited by β-lactam and glycopeptide antibiotics. In contrast, the early intracellular biosynthetic steps have so far received only marginal attention as potential drug targets.[1]

Peptidoglycan is an essential macromolecular component of the cell wall of both Gram-positive and Gram-negative bacteria. Its main function is to provide structural integrity by withstanding the internal osmotic pressure. The glycan chains are composed of alternating units of *N*-acetylglucosamine (Glc*N*Ac) and *N*-acetylmuramic acid (Mur*N*Ac). The carboxyl group of Mur*N*Ac residues is substituted in most bacteria by a peptide unit, L-alanyl-γ-D-glutamyl-*meso*-diaminopimeloyl(or L-lysyl)-D-alanine.[3] The final intracellular peptidoglycan precursor UDP-Mur*N*Ac-pentapeptide is assembled by successive addition of L-Ala, D-Glu, m-dpm or L-Lys, and D-Ala-D-Ala to UDP-Mur*N*Ac by the action of Mur ligases (MurC, MurD, MurE, and MurF, respectively). D-Ala-D-Ala ligase (Ddl) is responsible for supplying the MurF substrate, the D-Ala-D-Ala dipeptide.[3] In *Escherichia coli*, two isoforms of Ddl exist with 35% amino-acid sequence homology and similar catalytic efficiencies and substrate recognition features: DdlA and DdlB.[4] In the present work, we focused our attention on DdlB, which is a more extensively characterized enzyme than DdlA. For example, the crystal structures of the wild-type *E. coli* DdlB and Y216F mutant derivate have been previously reported.[5,6] In contrast, no crystal structures are currently available for the DdlA isoform. However, both DdlA and DdlB show similar susceptibility to known inhibitors of D-Ala-D-Ala ligase validating their potential as novel antibacterial targets.[4]

The dimerization of D-alanine begins with an attack on the first D-alanine by the γ-phosphate of adenosine triphosphate (ATP) to give an acylphosphate. This is followed by attack by the amino group of the second D-alanine, which eliminates the phosphate and yields the product D-alanyl-D-alanine.[7,8] In Ddl mutants, this reaction preferentially uses other amino-acid substrates

Keywords: D-Alanine ligase; Inhibitors; Antibacterials.
* Corresponding authors. Tel.: +386 1 47 69 585; fax: +386 1 42 58 031; e-mail addresses: slovenko.polanc@fkkt.uni-lj.si; gobecs@ffa.uni-lj.si

0960-894X/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2007.01.015

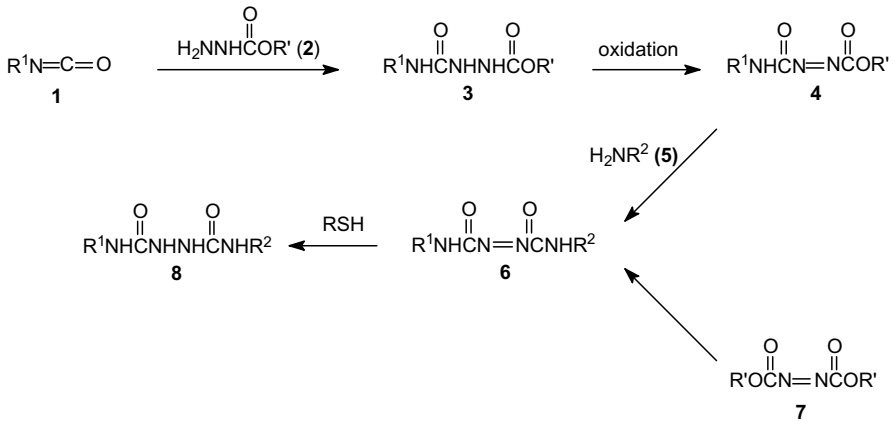
with a D-configuration: VanA and VanB use D-lactate and VanC uses D-serine.[9] This modification of the D-Ala-D-Ala terminus leads to reduction in the binding affinity of vancomycin and confers vancomycin resistance.[9]

As Ddl is essential for bacteria development and has no human counterpart, it is an important target for development of new antibacterial drugs.[10] The most important inhibitor of Ddl is no doubt a structural analog of D-alanine, the antitubercular agent D-cycloserine.[4,11,12] A series of phosphinates, phosphonates, and phosphonamidates have been developed as transition-state analog inhibitors or as analogs of D-alanyl phosphate.[13–16] It was shown that transition-state mimetics can be phosphorylated by Ddl and inhibit the reaction by tight binding to the enzyme after this phosphorylation. Although their antibacterial activities are low, they enabled the crystallographic determination of complexes of *E. coli* DdlB with ADP/phosphorylated phosphinate (pdb code 2DLN)[5] and ADP/phosphorylated phosphonate (pdb code 1IOV).[6] Using the de novo structure-based molecular design software SPROUT, a cyclopropane derivative was developed as an inhibitor of DdlB.[17] Recently, an allosteric inhibitor of D-alanine-D-alanine ligase from *Staphylococcus aureus* was discovered by high-throughput screening, and it was co-crystallized with the enzyme.[10] In addition, the crystal structure of the apo form of Ddl from *Thermus caldophilus* has just been resolved, providing insight into the substrate-induced conformational changes, which could be important for inhibitor design.[18]

As a part of our efforts toward the discovery of new small molecule inhibitors of the early steps of peptidoglycan biosynthesis,[17,19,20] we screened our in-house bank of compounds against DdlB from *E. coli*. We found that some diazenedicarboxamides inhibited the enzyme, prompting us to synthesize a small focused library of structurally related compounds and to evaluate their enzyme inhibitory and antibacterial activities.

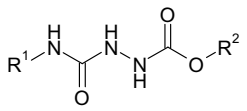
Unsymmetrical diazenedicarboxamides are usually prepared from alkyl aminocarbonyldiazenecarboxylates. The latter are easily available by the oxidation of the corresponding 1,4-disubstituted semicarbazides (Scheme 1). Thus, the addition of alkyl hydrazinecarboxylate **2** to the isocyanate **1** results in the formation of 1,4-disubstituted semicarbazide **3**.[21] Oxidation of **3** to give **4** can be performed with either *N*-bromosuccinimide/pyridine[22] or ceric(IV) ammonium nitrate (CAN).[23] The most convenient route to unsymmetrical diazenedicarboxamides **6** ($R^1 \neq R^2$) is a substitution of the alkoxy group in the diazene **4** employing a primary amine **5** as a nucleophile.[24] On the other hand, the synthesis of symmetrical diazenedicarboxamides of type **6** ($R^1 = R^2$) involves treatment of dialkyl diazenedicarboxylates **7** with two equivalents of the appropriate primary amine **5**. A reduction of any diazenedicarboxamide with various thiols leads to the formation of product **8**.[25]

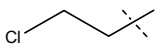
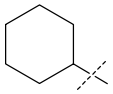
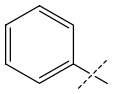
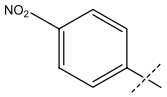
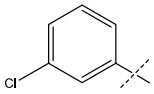
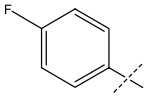
Target compounds **3**, **4**, **6**, and **8** were tested for inhibitory activity on DdlB from *E. coli*.[35] The results are presented as residual activities (RA) of the enzyme in the presence of 500 μM of each compound (Tables 1, 2, and 4), and for the more active compounds, as IC_{50} values (Table 3). In a series of semicarbazides (**3**, Table 1), aminocarbonyldiazenecarboxylates (**4**, Table 3), and hydrazinedicarboxamides (**8**, Table 4), only some aminocarbonyldiazenecarboxylates displayed moderate inhibition at 500 μM, while the remainder were inactive. In contrast, very potent inhibitors were obtained in the series of diazenedicarboxamides (**6**, compounds in Table 3 are ordered according to their synthesis). With the systematic variation of substituents R^1 and R^2, we were able to deduce some initial structure–activity relationships (SARs). In the set of compounds where the R^1 substituent is 2-chloroethyl, all compounds were good inhibitors of DdlB, with IC_{50} values between 119 and 236 μM (compounds **6aA**, **6aB**, and **6aD**). Similar inhibitory activity was observed also for cyclohexyl derivative **6bD**. Promising activities were obtained if the R^1 substituent is phenyl and R^2 is 2-, 3- or 4-picolyl (compounds **6cB**, **6cC**, and **6cD**; IC_{50} values 111, 36, and 106 μM,



Scheme 1. Synthesis of semicarbazides (**3**), aminocarbonyldiazenecarboxylates (**4**), diazenedicarboxamides (**6**), and hydrazinedicarboxamides (**8**).

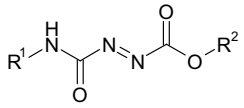
Table 1. DdlB inhibitory activity of semicarbazides **3**

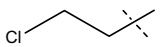
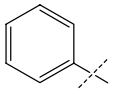
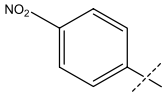
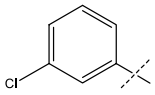
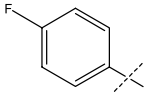
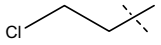


Compound	R^1	R^2	% inhibition at 500 μM[a]
3a[26]		Me	5
3b[27]		Me	1
3c[28]		Et	1
3d[29]		Et	1
3e[30]		Et	0
3f		Et	0

[a] Results represent means of two independent experiments. Standard deviations were within ±10% of the means.

Table 2. DdlB inhibitory activity of aminocarbonyldiazenecarboxylates **4**



Compound	R^1	R^2	% inhibition at 500 μM[a]
4a[26]		Me	7
4c[31]		Et	1
4d[32]		Et	40
4e[33]		Et	33
4f[33]		Et	37
4g		*t*-Bu	7

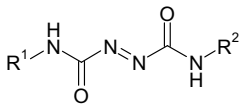
[a] Results represent means of two independent experiments. Standard deviations were within ±10% of the means.

respectively). Compound **6cE** with phenyl and the more flexible *N*,*N*-dimethylamino-2-ethyl residue was completely inactive. The best inhibitors were those where the R^1 is a substituted phenyl. Introduction of an *m*-chloro substituent to R^1 of compound **6cC** resulted in a 2-fold increase in DdlB inhibition (compound **6eC**, IC_{50} = 15 μM). If the halogen atom is introduced either to the *meta* or *para* position of **6cD**, the activity also improves (IC_{50} values of compounds **6eD** and **6fD** were 33 and 73 μM, respectively). Compounds **6hA** and **6iA** where R^1 is 4-alkylphenyl and R^2 is 2-chloroethyl show very good DdlB inhibition (IC_{50} values 49 and 25 μM, respectively). If we compare the activities of these two compounds with inhibitors **6aB** and **6aD**, we can conclude that in a series of 2-chloroethyl derivatives, inhibition is optimized if the second substituent of the diazenedicarboxamide core is 4-alkylphenyl rather than picolyl. The combination of 4-*t*-butylphenyl (as R^1) and 4-picolyl (as R^2) substituents yielded an inactive compound, **6jD**. When both substituents R^1 and R^2 were 3-picolyl, the IC_{50} value of the symmetrical derivative **6kC** remained above 120 μM. When we compare the inhibitory activities of our diazenedicarboxylates with the positive control, we saw that six of our compounds are one order of magnitude more potent as inhibitors than D-cycloserine. In our assay, the IC_{50} value for D-cycloserine was 314 μM.

To investigate the possible binding mode, a representative inhibitor, compound **6cB**, was docked into the DdlB active site (pdb code 1IOV), using AutoDock 3.0 with the Lamarckian genetic algorithm.[36] AutoDock calculated that the inhibitor could have a novel binding mode as it occupies the binding sites of the phosphorylated phosphinate inhibitor and partly also of ADP (Fig. 1). As the binding site that accommodates the phosphorylated phosphinate is too small to bind our diazenedicarboxylates, the predicted binding pose is not surprising. In addition, the carbonyl groups of the inhibitor could interact with Mg^{2+} ions present in the active site of the enzyme. The predicted final docked energy was −13.37 kcal/mol.

Compounds that exhibited DdlB inhibitory activities were further tested for their antimicrobial activities (Table 3).[37] The minimal inhibitory concentrations (MICs) of each compound were determined against *E. coli* 1411,[38] and SM1411,[39] an *acrAB* deficient derivative of 1411 that exhibits increased susceptibility to a range of antimicrobial agents,[40,41] and *S. aureus* 8325-4.[42] 2-Chloroethyl derivatives **6aA**, **6aB**, **6hA**, and **6iA**, and the symmetrical 3-picolyl derivative **6kC** did not prevent the growth of any of the bacteria strains under investigation. Compound **6aD** prevented the growth of *E. coli* 1411 and *E. coli* SM1411 at 128 μg/mL, but not of *S. aureus* 8325-4. All of the remaining DdlB inhibitors

Table 3. DdlB inhibitory activity of diazenedicarboxamides **6**



6

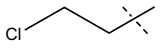
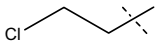
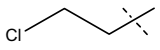
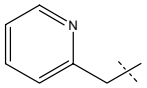
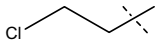
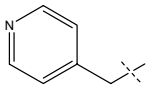
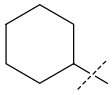
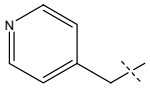
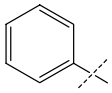
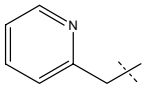
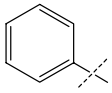
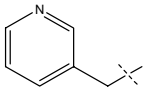
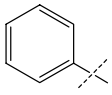
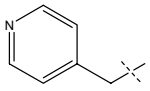
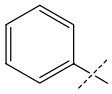
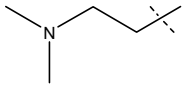
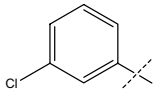
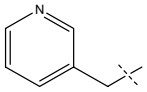
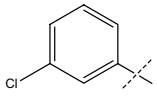
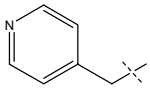
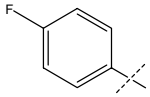
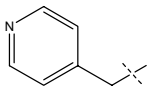
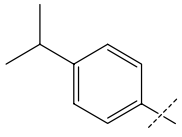
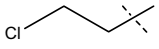
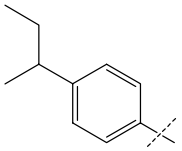
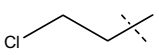
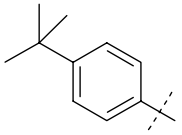
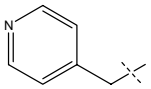
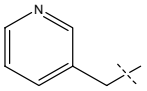
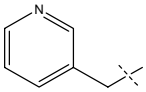
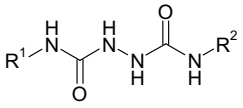
Compound	R^1	R^2	IC_{50}[a] (μM)	MIC[a] (μg/mL)		
				E. coli 1411	*E. coli* 1411 AcrAB$^-$	*S. aureus* 8325-4
6aA[34]			133	>256	>256	>256
6aB			119	>256	>256	>256
6aD			236	128	128	>256
6bD			121	256	256	128
6cB[32]			111	64	64	64
6cC			36	64	64	128
6cD			106	64	32	128
6cE			>500	ND	ND	ND
6eC			15	64	64	256
6eD			33	64	64	64
6fD			73	64	64	128
6hA			49	>256	>256	>256

Table 3 (*continued*)

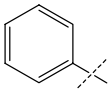
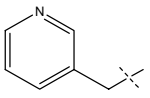
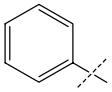
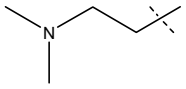
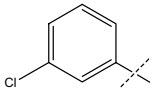
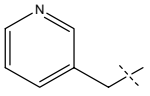
Compound	R^1	R^2	IC_{50}[a] (μM)	MIC[a] (μg/mL)		
				E. coli 1411	*E. coli* 1411 AcrAB$^-$	*S. aureus* 8325-4
6iA			25	>256	>256	>256
6jD			>500	ND	ND	ND
6kC			123	>256	>256	>256
D-cycloserine[b]			314	16	16	32

[a] Results represent means of two independent experiments. Standard deviations were within ±10% of the means. ND, not done.
[b] Positive control.

Table 4. DdlB inhibitory activity of hydrazinedicarboxamides **8**



8

Compound	R^1	R^2	% inhibition at 500 μM[a]
8cC			0
8cE			2
8eC			3

[a] Results represent means of two independent experiments. Standard deviations were within ±10% of the means.

prevented the growth of all three bacteria strains, with MICs between 32 and 256 μg/mL. The greater activity seen against *E. coli* may be due to increased uptake of the compound into these cells or to differences in the susceptibility of the Ddl enzymes in *E. coli* and *S. aureus*. It is interesting to note that the best MICs were obtained for the compounds where both substituents of the diazenedicarboxamide core are aromatic. We can also see that there is no strict correlation between DdlB inhibitory activities and in vitro antimicrobial activities. For this reason we have initiated further studies to investigate the modes of antimicrobial action of our inhibitors.

To conclude, we report the synthesis and activity of a series of new diazenedicarboxamides as inhibitors of DdlB from *E. coli*. Thirteen compounds were better inhibitors than D-cycloserine, and five of them had IC_{50} values below 50 μM and MIC values as low as 64 μg/mL against *E. coli* and *S. aureus*. As Ddl is essential and universal in prokaryotes, we predict antimicrobial activity against a wide range of bacteria. These inhibitors are structurally distinct from both ADP and D-alanine, so we expect that they inhibit Ddl by a novel binding mode. As our diazenedicarboxamides also have in vitro antimicrobial activities, they constitute a promising starting point for further investigations.

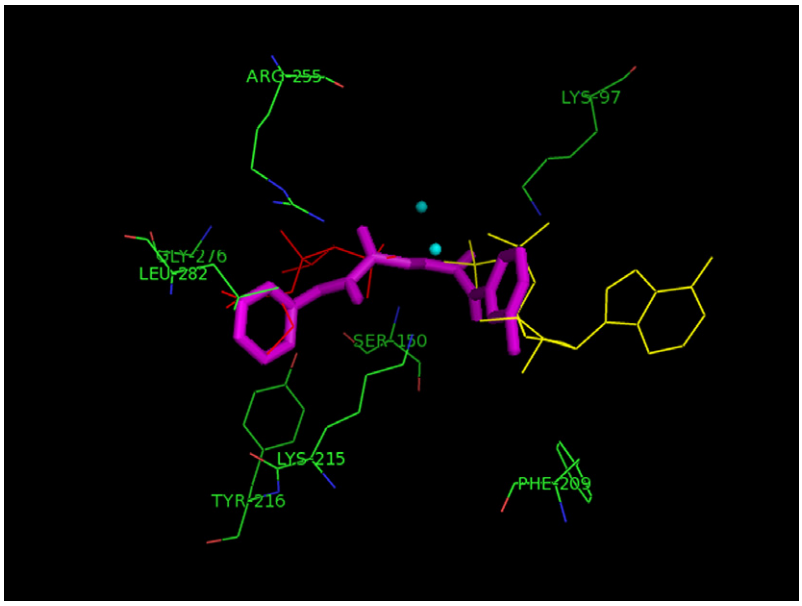


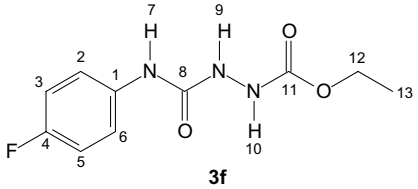
Figure 1. Superimposition of the computer model of compound **6eC** (in magenta) on the X-ray structure of phosphorylated phosphinate inhibitor (in red), ADP (in yellow) and Mg^{2+} (in cyan) bound to DdlB. The highest ranked position of the inhibitor, as calculated by AutoDock 3.0, is presented.

Acknowledgments

This work was supported by the European Union FP6 Integrated Project EUR-INTAFAR (Project No. LSHM-CT-2004-512138) under the thematic priority of Life Sciences, Genomics and Biotechnology for Health, the British Society of Antimicrobial Chemotherapy and the British Council. The support from the Ministry of Higher Education, Science and Technology of the Republic of Slovenia and the Slovenian Research Agency (P1-0208, P1-0230, J1-6693) is also acknowledged. The authors thank Dr. Chris Berrie for critical reading of the manuscript.

References and notes

1. Courvalin, P.; Davies, J. *Curr. Opin. Microbiol.* **2003**, *6*, 425.
2. Labischinski, H.; Maidhof, H. In *Bacterial Cell Wall*; Ghuysen, J.-M., Hakenbeck, R., Eds.; Elsevier Science B.V.: Amsterdam, 1994; p 23.
3. van Heijenoort, J. *Nat. Prod. Rep.* **2001**, *18*, 503.
4. Zawadzke, L. E.; Bugg, T. D. H.; Walsh, C. T. *Biochemistry* **1991**, *30*, 1673.
5. Fan, C.; Moews, P. C.; Walsh, C. T.; Knox, J. R. *Science* **1994**, *266*, 439.
6. Fan, C.; Park, I.-S.; Walsh, C. T.; Knox, J. R. *Biochemistry* **1997**, *36*, 2531.
7. Walsh, C. T. *J. Biol. Chem.* **1989**, *164*, 2393.
8. Shi, Y.; Walsh, C. T. *Biochemistry* **1995**, *34*, 2768.
9. Arthur, M.; Reynolds, P.; Courvalin, P. *Trends Microbiol.* **1996**, *4*, 401.
10. Liu, S.; Chang, J. S.; Herberg, J. T.; Horng, M.-M.; Tomich, P. K.; Lin, A. H.; Marotti, K. R. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 15178.
11. Neuhaus, F. C.; Lynch, J. L. *Biochemistry* **1964**, *3*, 471.
12. Strominger, J. L.; Ito, E.; Threnn, R. H. *J. Am. Chem. Soc.* **1960**, *82*, 998.
13. Parsons, W. H.; Patchett, A. A.; Bull, H. G.; Schoen, W. R.; Taub, D.; Davidson, J.; Combs, P. L.; Springer, J. P.; Gadebusch, H.; Weissberger, B.; Valiant, M. E.; Mellin, T. N.; Busch, R. D. *J. Med. Chem.* **1988**, *31*, 1772.
14. Chakravarty, P. K.; Greenlee, W. J.; Parsons, W. H.; Patchett, A. A.; Combs, P.; Roth, A.; Busch, R. D.; Mellin, T. N. *J. Med. Chem.* **1989**, *32*, 1886.
15. Lacoste, A. M.; Chollet-Gravey, A. M.; Vo Quang, L.; Vo Quang, Y.; Le Goffic, F. *Eur. J. Med. Chem.* **1991**, *26*, 255.
16. Ellsworth, B. A.; Tom, N. J.; Bartlett, P. A. *Chem. Biol.* **1996**, *3*, 37.
17. Besong, G. E.; Bostock, J. M.; Stubbings, W.; Chopra, I.; Roper, D. I.; Lloyd, A. J.; Fishwick, C. W. G.; Johnson, A. P. *Angew. Chem. Int. Ed.* **2005**, *44*, 2.
18. Lee, J. H.; Na, Y.; Song, H.-E.; Kim, D.; Park, B.-H.; Rho, S.-H.; Im, Y. J.; Kim, M.-K.; Kang, G. B.; Lee, D.-S.; Eom, S. H. *Proteins* **2006**, *64*, 1078.
19. Štrancar, K.; Blanot, D.; Gobec, S. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 343.
20. Humljan, J.; Kotnik, M.; Boniface, A.; Šolmajer, T.; Urleb, U.; Blanot, D.; Gobec, S. *Tetrahedron* **2006**, *67*, 10980.
21. *Procedure for the preparation of aminocarbonylhydrazinecarboxylates: synthesis of ethyl (4-fluorophenyl)aminocarbonylhydrazinecarboxylate (***3f***).*

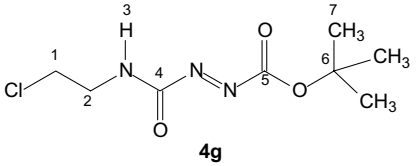


3f

A solution of 4-fluorophenyl isocyanate (0.675 mL, 6 mmol) in acetonitrile (4 mL) was added drop-wise to a stirred solution of ethyl hydrazinecarboxylate (0.625 g, 6 mmol) in acetonitrile (4 mL) at 0 °C. The suspension was stirred for 10 min at 0 °C and then for 10 min at rt. Solid material was filtered off and washed with diethyl ether (10 mL), to provide **3f** (1.369 g, 95% yield): mp 190–191.5 °C (methanol); IR 3260, 3060, 2980, 1730, 1680, 1630, 1560, 1240, 1030, 850 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.19 (t, 3H, J = 7.1 Hz, H-13), 4.06 (q, 2H, J = 7.1 Hz, H-12) 7.08 (m, 2H, H-3, H-5), 7.48 (m, 2H, H-2, H-6), 8.02 (s, 1H, H-7), 8.75 (s, 1H) and 8.90 (s, 1H):

H-9 and H-10; ^{13}C NMR (DMSO-d_6) δ 14.5 (C-13), 60.5 (C-12), 115.0 (d, J = 22.4 Hz, C-3, C-5), 120.3 (d, J = 5.2 Hz, C-2, C-6), 136.0 (d, J = 2.3 Hz, C-1), 155.7 and 156.9: C-8 and C-11, 157.3 (d, J = 238.0 Hz, C-4); MS (EI) m/z 241 (M^+, 13), 137 (32), 110 (36), 104 (100), 83 (25). Anal. Calcd for $C_{10}H_{12}FN_3O_3$ (241.22): C, 49.79; H, 5.01; N, 17.42. Found: C, 49.72; H, 4.99; N 17.69.

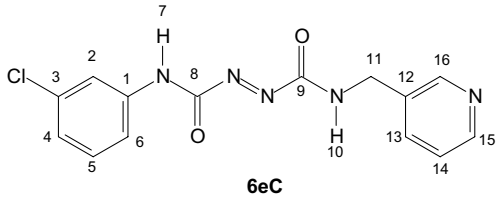
22. *Typical preparation of aminocarbonyldiazenecarboxylates: synthesis of t-butyl (2-chloroethyl)aminocarbonyldiazenecarboxylate (***4g***).*



4g

NBS (979 mg; 5.5 mmol) was slowly added at rt to a stirred mixture of *t*-butyl (2-chloroethyl)aminocarbonylhydrazinecarboxylate (1.19 g, 5 mmol) and pyridine (0.81 mL, 10 mmol) in CH_2Cl_2 (7 mL). After continuous stirring at rt for 30 min, HCl (1:1, 15 mL) was added and two phases were separated. A CH_2Cl_2 solution was treated successively with 5% aqueous solution of $Na_2S_2O_3$ (7 mL), saturated solution of $NaHCO_3$ (2× 7 mL), and water (15 mL), then dried over anhydrous Na_2SO_4 and evaporated to dryness to give **4g** (1.14 g, 97% yield): mp 73–74 °C (dichloromethane/diethyl ether); IR 3266, 3059, 2991, 1763, 1734, 1564, 1537, 1435, 1372, 1255, 1194, 1144, 1057, 957, 825 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.63 (s, 9H, H-7), 3.75 (m, 2H, H-2), 3.83 (m, 2H, H-1), 6.75 (broad, 1H, H-3); ^{13}C NMR ($CDCl_3$) δ 27.7 (C-7), 42.6 and 42.7: C-1 and C-2, 87.1 (C-6), 159.0 and 160.0: C-4 and C-5; MS (FAB) m/z 236 (M^++H, 5), 57 (100). Anal. Calcd for $C_8H_{14}ClN_3O_3$ (235.67): C, 40.77; H, 5.99; N, 17.83. Found: C, 40.78; H, 6.09; N 17.64.

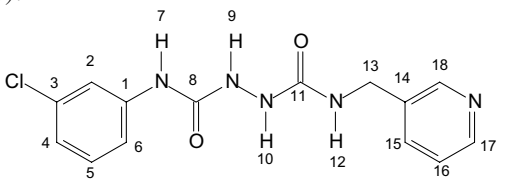
23. Košmrlj, J.; Kočevar, M.; Polanc, S. *J. Chem. Soc., Perkin Trans. 1* **1998**, 3917.
24. *Procedure for the preparation of diazenedicarboxamides: synthesis of N-(3-chlorophenyl)-N′-(3-picolyl)diazene-1,2-dicarboxamide (***6eC***).*



6eC

Ethyl (3-chlorophenyl)aminocarbonyldiazenecarboxylate (1.534 g, 6 mmol) was slowly added to the stirred solution of 3-picolylamine (0.61 mL, 6 mmol) in acetonitrile (2 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 15 min. The solid material was filtered off and washed with acetonitrile to give the diazene **6eC** (1.412 g, 74%): mp 129–130 °C (ethyl acetate); IR 3338, 2939, 1741, 1713, 1546, 1504, 1428, 1189 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 4.54 (d, 2H, J = 6.0 Hz, H-11), 7.27 (ddd, 1H, J_1 = 8.0 Hz, J_2 = 2.1 Hz, J_3 = 2.0 Hz, H-6), 7.41 (ddd, 1H, J_1 = 7.8 Hz, J_2 = 4.8 Hz, J_3 = 0.9 Hz, H-14), 7.45 (dd, 1H, J_1 = 8.2 Hz, J_2 = 8.0 Hz, H-5), 7.66 (ddd, 1H, J_1 = 8.2 Hz, J_2 = 2.1 Hz, J_3 = 2.0 Hz, H-4), 7.78 (ddd, 1H, J_1 = 7.8 Hz, J_2 = 2.4 Hz, J_3 = 1.7 Hz, H-13), 7.86 (dd, 1H, J_1 = 2.1 Hz, J_2 = 2.0 Hz, H-2), 8.52 (dd, 1H, J_1 = 4.8 Hz, J_2 = 1.7 Hz, H-15), 8.60 (dd, 1H, J_1 = 2.4 Hz, J_2 = 0.9 Hz, H-16), 9.63 (t, 1H, J = 6.0 Hz, H-10), 11.55 (s, 1H, H-7); ^{13}C NMR (DMSO-d_6) δ 41.2 (C-11), 118.1 and 119.0: C-2 and C-6, 123.6 (C-4), 124.7 (C-14), 130.9 (C-5), 133.4 and 133.6: C-3 and C-13, 135.4 (C-12), 138.9 (C-1), 148.6 and 148.9: C-15 and C-16, 158.2 and 161.7: C-8 and C-9; MS (FAB) 318 (M^++H, 30), 123 (22), 107 (33). Anal. Calcd for $C_{14}H_{12}ClN_5O_2$ (317.73): C, 52.92; H, 3.81; N, 22.04. Found: C, 53.14; H, 3.88; N, 21.72.

25. *Reduction of diazenedicarboxamides: synthesis of N-(3-chlorophenyl)-N′-(3-picolyl)hydrazine-1,2-dicarboxamide (***8eC***).*



8eC

A suspension of the diazene **6eC** (794 mg, 2.5 mmol) in acetone (10 mL) was treated with 1-thioglycerol (0.505 mL, 5.2 mmol). The reaction mixture was stirred at rt for 15 min. The solid material was filtered off and washed with acetone to provide the product **8eC** (770 mg; 96% yield): mp 206–208 °C (methanol); IR 3299, 3226, 2925, 1667, 1594, 1542, 1427, 1324, 1259 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 4.27 (d, 2H, J = 6.1 Hz, H-13), 6.99 (ddd, 1H, J_1 = 7.9 Hz, J_2 = 2.1 Hz, J_3 = 2.0 Hz, H-6), 7.18 (t, 1H, J = 6.1 Hz, H-12), 7.26 (dd, 1H, J_1 = 8.3 Hz, J_2 = 7.9 Hz, H-5), 7.32 (ddd, 1H, J_1 = 7.8 Hz, J_2 = 4.8 Hz, J_3 = 0.8 Hz, H-16), 7.40 (m, 1H, H-4), 7.68 (ddd, 1H, J_1 = 7.8 Hz, J_2 = 2.3 Hz, J_3 = 1.8 Hz, H-15), 7.74 (dd, 1H, J_1 = 2.1 Hz, J_2 = 2.0 Hz, H-2), 7.90 (s, 1H) and 8.08 (s, 1H): H-9 and H-10, 8.43 (dd, 1H, J_1 = 4.8 Hz, J_2 = 1.8 Hz, H-17), 8.50 (m, 1H, H-18), 8.93 (s, 1H, H-7); ^{13}C NMR (DMSO-d_6) δ 40.4 (C-13), 117.0 and 118.0: C-2 and C-6, 121.4 (C-4), 123.2 (C-16), 130.1 (C-5), 132.9 (C-15), 134.8 and 135.9: C-3 and C-14, 141.3 (C-1), 147.8 and 148.6: C-17 and C-18, 155.8 and 158.7: C-8 and C-11; MS (FAB) 320 (M^++H, 24), 107 (30), 69 (84). Anal. Calcd for $C_{14}H_{14}ClN_5O_2{\cdot}\frac{1}{4}$MeOH: C, 52.22; H, 4.61; N, 21.37. Found: C, 52.15; H, 4.61; N, 21.50.

26. Bombek, S.; Požgan, F.; Kočevar, M.; Polanc, S. *J. Org. Chem.* **2004**, *69*, 2224.
27. Bombek, S.; Požgan, F.; Kočevar, M.; Polanc, S. *New J. Chem.* **2005**, *29*, 948.
28. Zinner, G.; Deucker, W. *Arch. Pharm.* **1961**, *249*, 370.
29. Strickler, J. C.; Pirkle, W. H. *J. Org. Chem.* **1966**, *31*, 3444.
30. Bollbuck, G.; Stroh, H. H.; Barnikow, G. *J. Prakt. Chem.* **1971**, *313*, 773.
31. Knight, G. T.; Loadman, M. J. R.; Saville, B.; Wildgoose, J. *J. Chem. Soc., Chem. Commun.* **1974**, 193.
32. Pieters, L.; Košmrlj, J.; Lenaršič, R.; Kočevar, M.; Polanc, S. *ARKIVOC* **2001**, 42 (Part V).
33. Lenaršič, R.; Kočevar, M.; Polanc, S. *J. Org. Chem.* **1999**, *64*, 2558.
34. Kraebel, C. M.; Davis, S. M. *J. Chem. Eng. Data* **1969**, *14*, 133.

35. The D-Ala-adding activity of DdlB ligase was monitored by the detection of orthophosphate generated during the reaction based on the colorimetric malachite green method described by Walsh, A. et al. *J. Bacteriol.* **1999**, *181*, 5395. Assays were performed at 37 °C in a mixture (final volume: 50 μl) containing 38.5 mM Hepes, pH 8.0, 3.25 mM $MgCl_2$, 6.5 mM $(NH_4)_2SO_4$, 700 μM D-Ala, 500 μM ATP, purified DdlB (diluted in 20 mM Hepes, pH 7.2, and 1 mM dithiothreitol), and 500 μM test compound (IC_{50} values were determined for a range of inhibitor concentrations). All compounds were soluble in the assay mixture containing 5% DMSO. After 30 min of incubation, 100 μL Biomol® reagent was added. After 5 min, absorbance was read at 650 nm. Residual activity was calculated with respect to a similar assay without inhibitor. To exclude possible non-specific (promiscuous) inhibitors, representative

compounds **3c**, **4c**, **6aD**, **6bD**, **6cC**, **6cD**, **6eD**, **6fD**, **6iA**, and **6jD** were tested also in the presence of Tween (0.003%), Triton X-114 (0.005%), and SDS (420 μM), as described by McGovern, S. L. et al. *J. Med. Chem.* **2003**, *46*, 4265, and Ryan, A. J. et al. *J. Med. Chem.* **2003**, *46*, 3448. No significant differences were found when compared to measurements without detergents.

36. Morris, G. M.; Goodsell, D. S.; Halliday, R. S.; Huey, R.; Hart, W. E.; Belew, R. K.; Olson, A. J. *J. Comp. Chem.* **1998**, *19*, 1639.
37. Minimal inhibitory concentrations (MICs) of compounds were determined by broth microdilution in IsoSensitest broth (Oxoid, Basingstoke, UK) using an inoculum of 10^4 cells per mL for *E. coli* or 10^6 cells per mL for *S. aureus*. The antimicrobial agents were prepared in a 2-fold dilution series in 50% dimethyl sulfoxide (Sigma–Aldrich, Dorset, UK). Microwell plates with 96 wells (Nunc, Fisher Scientific, Loughborough, UK) containing the antimicrobial agent and bacterial suspension were incubated for 16 h at 37 °C in a Spectramax 384 plus microwell plate reader (Molecular Devices, Abingdon, UK), running SOFTmax PRO 3.1.1 software. Optical density readings (600 nm) were taken at 10 min intervals. Plates were shaken for 30 s before each reading. The MIC was taken as the lowest concentration of antimicrobial agent that prevented growth.
38. Miller, K.; O'Neill, A. J.; Chopra, I. *J. Antimicrob. Chemother.* **2002**, *49*, 925.
39. O'Neill, A. J.; Bostock, J. M.; Morais Moita, A.; Chopra, I. *J. Antimicrob. Chemother.* **2002**, *50*, 839.
40. Sulavik, M. C.; Houseweart, C.; Cramer, C.; Jiwani, N.; Murgolo, N.; Greene, J.; DiDomenico, B.; Shaw, K. J.; Miller, G. H.; Hare, R.; Shimer, G. *Antimicrob. Agents Chemother.* **2001**, *45*, 1126.
41. Xiong, L. Q.; Kloss, P.; Douthwaite, S.; Andersen, N. M.; Swaney, S.; Shinabarger, D. L.; Mankin, A. S. *J. Bacteriol.* **2000**, *182*, 5325.
42. Novick, R. *Virology* **1967**, *33*, 155.