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Discovery of Novel 5-Benzylidenerhodanine and 5-Benzylidenethiazolidine-2,4-dione Inhibitors of MurD Ligase†

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We have designed, synthesized, and evaluated 5-benzylidenerhodanine- and 5-benzylidenethiazolidine-2,4-dione-based compounds as inhibitors of bacterial enzyme MurD with *E. coli* IC_{50} in the range 45−206 μM. The high-resolution crystal structure of MurD in complex with (*R*,*Z*)-2-(3-[{4-([2,4-dioxothiazolidin-5-ylidene]methyl)phenylamino}methyl)benzamido)pentanedioic acid [(*R*)-**32**] revealed details of the binding mode of the inhibitor within the active site and provides a good foundation for structure-based design of a novel generation of MurD inhibitors.

1. Introduction

Infectious diseases remain the leading cause of death in low-income countries and the second leading cause of death worldwide.[1] The escalating rate of bacterial resistance to currently available antibiotics is a well-known problem, and the need for novel antibacterial drugs is increasingly important clinically.[2] The cell wall of bacteria remains a viable source of targets for novel antibacterials.[3] Peptidoglycan is an essential bacterial cell-wall polymer, unique to prokaryotic cells and therefore a prime target for the selective targeting of microbial vital pathways. Its main function is to preserve cell integrity by withstanding the internal osmotic pressure and maintaining a defined cell shape.[4] Biosynthesis of peptidoglycan is a multistage process, which begins in the cytoplasm, where the monomeric peptidoglycan building block is formed. This is then translocated through the cellular membrane to its outer side and incorporated in the peptidoglycan macromolecule. A large number of antibiotics in clinical use, e.g., β-lactams and glycopeptides, act by inhibiting the later, extracellular steps of peptidoglycan biosynthesis. Enzymes involved in the earlier steps of the biosynthesis of cytoplasmic peptidoglycan precursor are more difficult drug targets that have been subjected to many HTS efforts to no avail.[5] Mur ligases (MurC, MurD, MurE and MurF)[a] constitute a group of cytoplasmic enzymes that catalyze a series of reactions, starting from UDP-*N*-acetylmuramic acid (UDP-Mur*N*Ac) to yield UDP-*N*-acetylmuramoylpentapeptide (UDP-Mur*N*Ac-pentapeptide) by sequentially adding L-Ala (MurC), D-Glu (MurD), *meso*-diaminopimelic acid or L-Lys (MurE), and the D-Ala-D-Ala dipeptide (MurF).[6] The kinetic properties and the catalytic mechanism of Mur ligases, which catalyze the formation of a peptide bond with concomitant cleavage of ATP into ADP and inorganic phosphate, are well-known. The enzymes operate by essentially similar chemical mechanisms. This entails activation of the C-terminal carboxyl group of the nucleotide substrate to an acyl phosphate intermediate, followed by nucleophilic attack by the amino group of the condensing amino acid or dipeptide, elimination of phosphate, and subsequent peptide bond formation.[6] All Mur ligases have the same three-domain topology. The N-terminal and central domains bind UDP precursor and ATP, respectively, while the incoming amino acid or dipeptide binds to the C-terminal part.[7,8] Sequence alignments of Mur ligase orthologues and paralogues show relatively low homology, with the exception of residues comprising the active sites.[9,10] Therefore, the key features to be considered in the design of Mur ligases inhibitors are the conserved binding motifs and the common chemical mechanism of MurC-F. Despite extensive research on the discovery of new inhibitors of Mur ligases,[5,6,11,12] to date, none has found therapeutic application, probably because of problems associated with bacterial cell entry.[3] Most known MurD inhibitors contain D-glutamic acid, suggested to be an essential structural element of a potent inhibitor.[13−17] It has been demonstrated that MurD displays a high substrate stereospecificity toward D-Glu[18,19] and that the binding site for D-Glu is conserved across different bacterial species.[10] Rhodanine and thiazolidine-2,4-dione rings are well-known scaffolds in medicinal chemistry and have been incorporated in compounds displaying a variety of pharmacological effects, including antibacterial, antifungal, and antidiabetic activity.[20] Rhodanine derivatives have also been reported as inhibitors of Mur

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[a] Abbreviations: UDP, uridine 5′-diphosphate; UMA, UDP-*N*-acetylmuramoyl-L-alanine; UMAG, UDP-*N*-acetylmuramoyl-L-alanyl-D-glutamate; MurC, UDP-*N*-acetylmuramate:L-alanine ligase; MurD, UDP-*N*-acetylmuramoyl-L-alanine:D-glutamate ligase; MurE, UDP-*N*-acetylmuramoyl-L-alanyl-D-glutamate:*meso*-diaminopimelate ligase; MurF, UDP-*N*-acetylmuramoyl-L-alanyl-γ-D-glutamyl-*meso*-diaminopimelate:D-alanyl-D-alanine ligase.

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ligases.[13,21,22] In the search for novel peptidoglycan biosynthesis inhibitors we published a preliminary report on MurD inhibitory activity of novel compounds that combine glutamic acid and 5-benzylidenerhodanine or 5-benzylidenethiazolidine-2,4-dione structural elements (Figure 1). These were designed based on the 2,4-diaminoquinazoline-containing highest ranked compound from virtual screening.[13] (*R*)-**21**, which was the most potent in the series and inhibited MurD with an IC_{50} of 174 μM, stimulated us to undertake an intensive structure modification program with the aim of improving MurD inhibition.

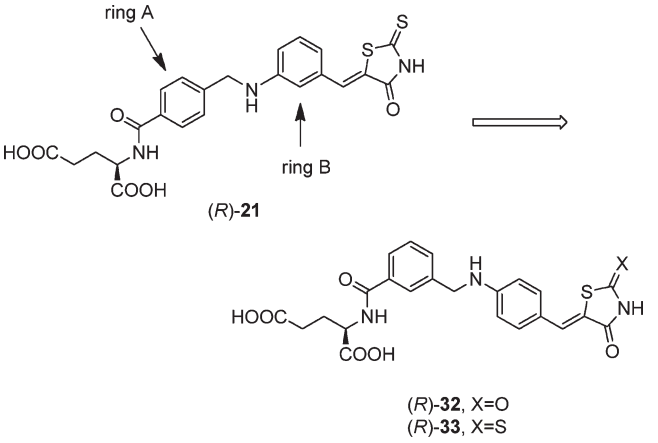


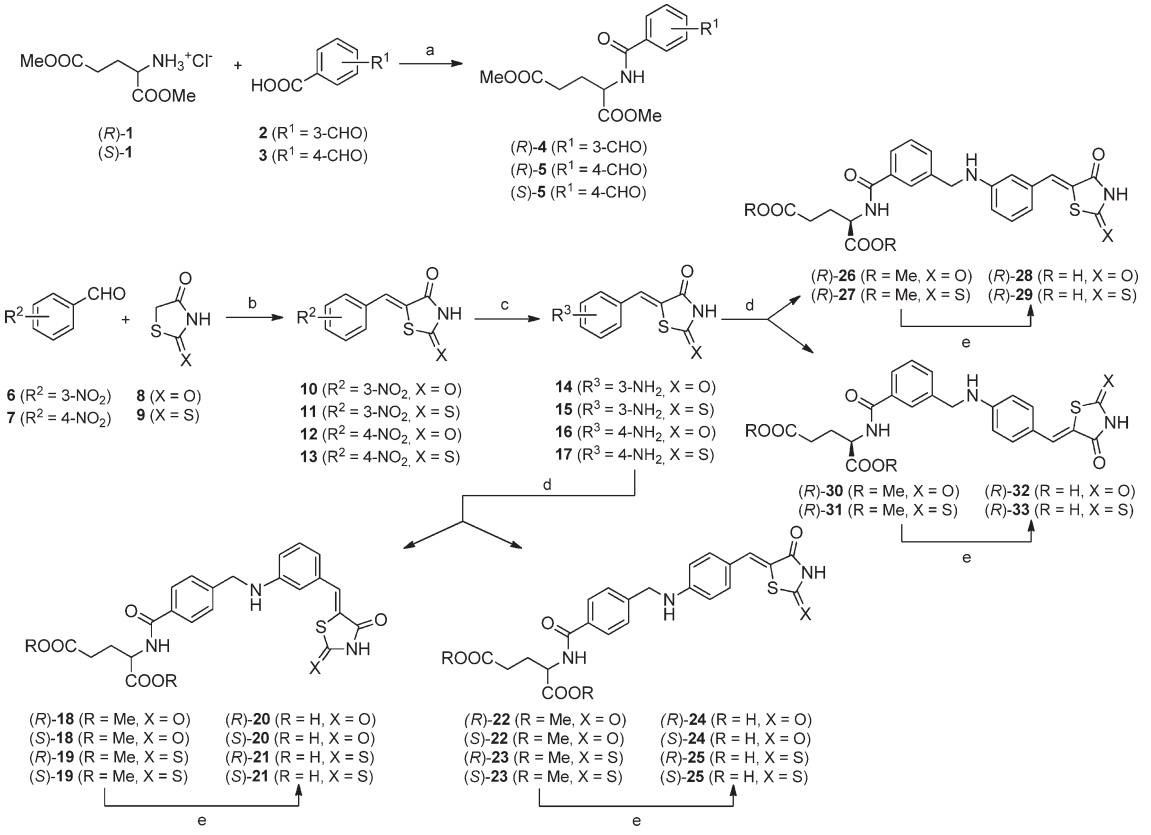
Figure 1. Design of improved MurD inhibitors by optimization of the initial hit compound (*R*)-**21**.

We report here a full account of the synthesis and biochemical evaluation of a series of new 5-benzylidenerhodanine- and 5-benzylidenethiazolidine-2,4-dione-based analogues as inhibitors of MurD. We examined the effects of (i) different substitution patterns in rings A and B, (ii) the replacement of glutamic acid, (iii) the distance between the glutamic acid and thiazolidine moieties on MurD inhibition, and (iv) the difference between thiazolidine-2,4-dione- and rhodanine-based derivatives. This endeavor resulted in (*R*)-**32** and (*R*)-**33** with improved inhibitory potency (IC_{50} = 85 and 45 μM, respectively). Compound (*R*)-**32** was cocrystallized with MurD, and the crystal structure of the complex was determined to 1.5 Å resolution. The details of the binding mode of (*R*)-**32** in the MurD active site will help us to understand the structure−activity relationships and provide a good foundation for structure-based design of a novel generation of MurD inhibitors.

2. Results and Discussion

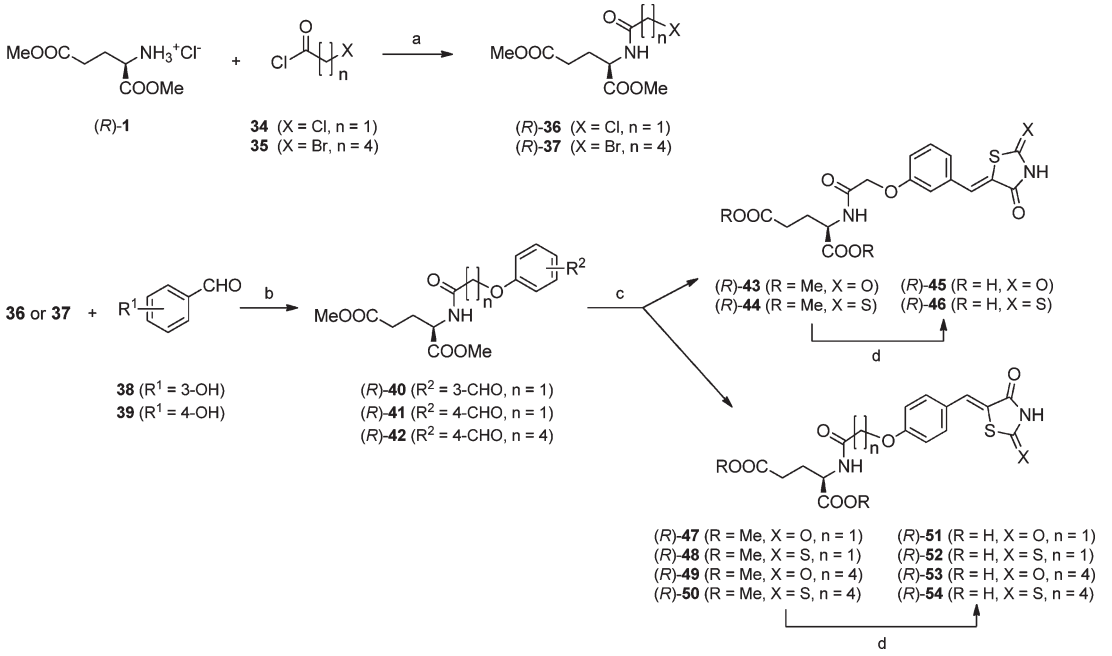
2.1. Design. Encouraged by the promising MurD inhibitory activity of (*R*)-**21**, whose docking into the MurD active site suggested binding of D-glutamic acid residue in the D-Glu pocket of the enzyme and the positioning of the rhodanine moiety in the region normally occupied by the diphosphate group of UDP,[13] we started a systematic structure modification program to increase the potency by optimizing the interactions of the inhibitor with the enzyme active site. To this end, four structural types of compound, comprising meta- or para-substitution patterns in rings A and B, were

Scheme 1. Synthesis of 5-Benzylidenerhodanine and 5-Benzylidenethiazolidine-2,4-dione Derivatives (*R*)-**20**, (*S*)-**20**, (*R*)-**21**, (*S*)-**21**, (*R*)-**24**, (*S*)-**24**, (*R*)-**25**, (*S*)-**25**, (*R*)-**28**, (*R*)-**29**, (*R*)-**32**, and (*R*)-**33**[a]



[a] Reagents and conditions: (a) EDC/HOBt, DMF, room temp, 24 h; (b) piperidine, AcOH, EtOH, microwave, 150 °C, 20 min. (c) For the preparation of **14** and **16**: H_2/Pd−C, MeOH/THF, room temp, 5 h. For the preparation of **15** and **17**: $SnCl_2$, EtOH, reflux, 2 h. (d) (*R*)-**4**, (*R*)-**5**, or (*S*)-**5**, $NaCNBH_3$, DMF, room temp, 24 h; (e) 2.2 M LiOH, MeOH/H_2O, room temp, 15 h.

Scheme 2. Synthesis of 5-Benzylidenerhodanine and 5-Benzylidenethiazolidine-2,4-dione Derivatives with an Alkanediyl Linker[a]



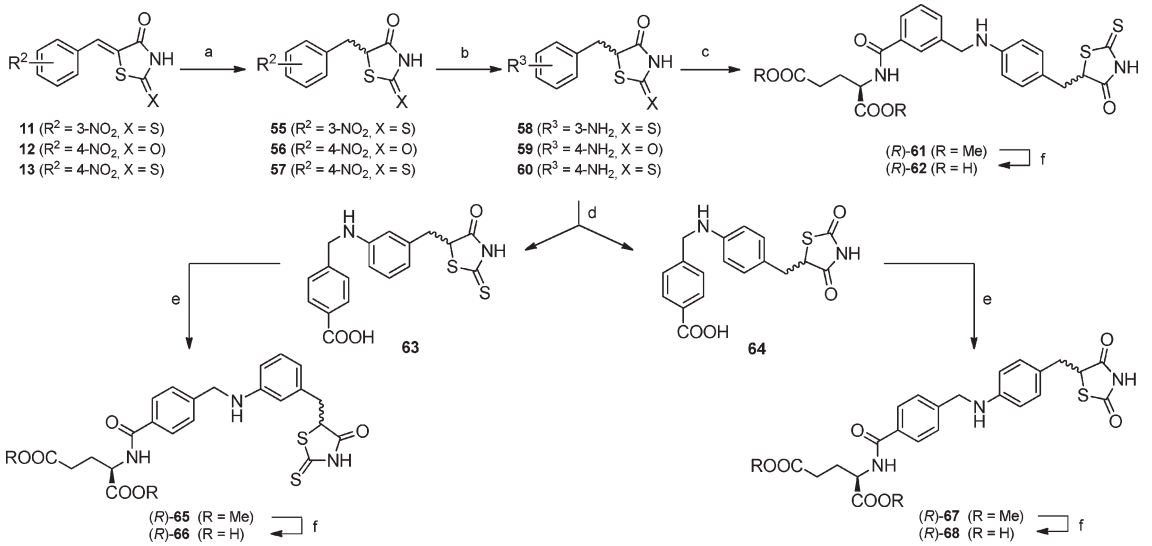
[a] Reagents and conditions: (a) Et_3N, CH_2Cl_2, 0 °C to room temp, 3 h; (b) K_2CO_3, KI, CH_3CN, 85 °C, 3 h; (c) **8** or **9**, piperidine, AcOH, EtOH, microwave, 150 °C, 20 min; (d) 2.2 M LiOH, MeOH/water, room temp, 15 h.

conceived. *R*- and *S*-enantiomeric series were first envisaged and later limited to (*R*)-glutamic acid derivatives because of the small difference in observed inhibitory potency of enantiomers. Rhodanine and thiazolidine-2,4-dione compounds were devised to assess the role of an exocyclic sulfur substituent for binding to the enzyme. Since rhodanine or thiazolidine-2,4-dione moieties and glutamic acid were considered crucial for interaction with the MurD active site and since the mode of binding of this class of MurD inhibitors was not known at the beginning of our studies, (*R*)-**53** and (*R*)-**54** with a more flexible linker, together with (*R*)-**45**, (*R*)-**46**, (*R*)-**51**, and (*R*)-**52** with a shorter linker between glutamic acid and thiazolidine moieties, were also considered. Compared to (*R*)-**32** and (*R*)-**33**, the number of bonds between the amide group and benzylidene moiety was retained in (*R*)-**53** and (*R*)-**54**, while the number of rotatable bonds was increased by 2. This modification gave (*R*)-**53** and (*R*)-**54** more conformational freedom and allowed shorter distances between glutamic acid and thiazolidine moieties. To further assess the role of conformational freedom on the interactions between the inhibitor and MurD, (*R*)-**62**, (*R*)-**66** and (*R*)-**68** possessing a reduced exocylic carbon−carbon double bond were also taken into consideration. These compounds can be regarded as more flexible analogues of (*R*)-**33**, (*R*)-**21**, and (*R*)-**24**, respectively, with a loss of conjugation and planarity in the eastern part of the molecules. Finally, analogues of (*R*)-**21** in which D-glutamic acid was replaced with L-lysine were designed to explore possible ionic interactions with active site amino acids possessing anionic side chains.

2.2. Chemistry. The syntheses of both enantiomers of compounds **20** and **21** with para-substitution in ring A and meta-substitution in ring B, *R*- and *S*-isomers of **24** and **25** possessing para-substitution in both phenyl rings, (*R*)-**28** and (*R*)-**29** with meta-substitution in both aromatic rings, and (*R*)-**32** and (*R*)-**33** with meta-substitution in ring A and para-substitution in ring B are outlined in Scheme 1. In the first step, 5-(nitrobenzylidene)thiazolidine-2,4-diones **10** and **12** and 5-(nitrobenzylidene)rhodanines **11** and **13** were obtained in good yields via Knoevenagel condensation of nitrobenzaldehyde **6** or **7** with thiazolidine-2,4-dione (**8**) or rhodanine (**9**) in a microwave reactor. The presence of only one signal for the methyne proton at 7.7−8.0 ppm in ^{1}H NMR spectra of **10**−**13** suggested that a single isomer was present, which was assigned *Z*-configuration with the aid of an ^{1}H-coupled ^{13}C NMR spectrum,[23] as described previously.[24] The exclusive formation of the thermodynamically stable *Z*-isomers of **10**−**13** is in agreement with literature reports for similar compounds.[24−26] The reduction of the nitro group in **10** and **12** was achieved through catalytic hydrogenation, using Pd−C as a catalyst. Reduction of rhodanine derivatives **11** and **13**, which could not be transformed into amines **15** and **17** by catalytic hydrogenation, was achieved with tin(II) chloride. In the next step, *R*- and *S*-isomers of **18**, **19**, **22**, and **23** and *R*-isomers of **26**, **27**, **30** and **31** were obtained by sodium cyanoborohydride effected reductive amination of amines **14**−**17** with benzaldehydes (*R*)-**4**, (*R*)-**5**, and (*S*)-**5**. The latter compounds were prepared via EDC/HOBt promoted coupling of L- and D-glutamic acid methyl esters **1** with carboxybenzaldehydes **2** and **3**. Finally, hydrolysis of methyl esters with aqueous lithium hydroxide solution afforded targets (*R*)-**20**, (*S*)-**20**, (*R*)-**21**, (*S*)-**21**, (*R*)-**24**, (*S*)-**24**, (*R*)-**25**, (*S*)-**25**, (*R*)-**28**, (*R*)-**29**, (*R*)-**32**, and (*R*)-**33**.

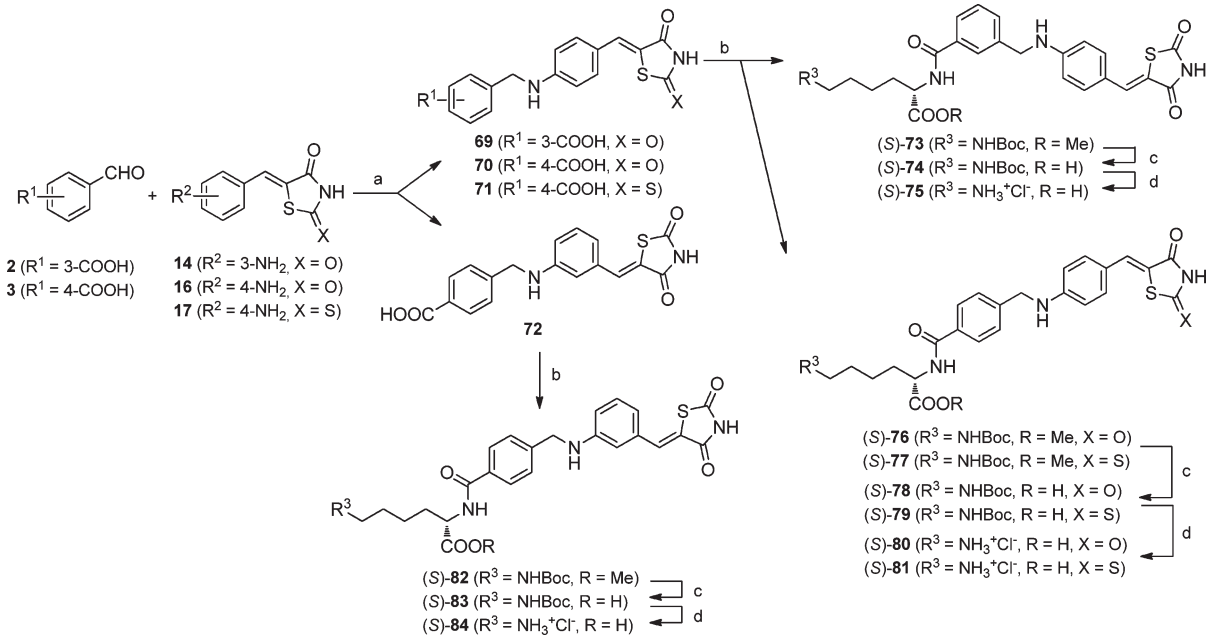
The synthesis of (*R*)-**45**, (*R*)-**46**, and (*R*)-**51**−(*R*)-**54** with a more flexible alkanediyl linker between glutamic acid and thiazolidine moieties, which would allow shorter distances between the two residues, is outlined in Scheme 2. The reaction of D-glutamic acid dimethyl ester [(*R*)-**1**] with acyl halides **34** and **35** gave (*R*)-**36** and (*R*)-**37**, respectively, which upon heating with hydroxylbenzaldehyde **38** or **39** in the presence of potassium iodide and potassium carbonate yielded (*R*)-**40**−(*R*)-**42**. Knoevenagel condensation of (*R*)-**40**−(*R*)-**42** with thiazolidine-2,4-dione (**8**) or rhodanine (**9**) in a microwave reactor afforded (*R*)-**43**, (*R*)-**44**, and (*R*)-**47**−(*R*)-**50**, which, after hydrolysis with aqueous lithium hydroxide solution,

Scheme 3. Synthesis of Reduced 5-Benzylrhodanine and 5-Benzylthiazolidine-2,4-dione Derivatives (*R*)-**62**, (*R*)-**66**, and (*R*)-**68**[a]



[a] Reagents and conditions: (a) diethyl 2,6-dimethyl-1,4-dihydro-3,5-pyridinedicarboxylate, silica gel 60, toluene, 100 °C, 24 h. (b) For the preparation of **59**: H_2/Pd−C, MeOH/THF, room temp, 5 h. For the preparation of **58** and **60**: $SnCl_2$, EtOH, reflux, 2 h. (c) (*R*)-**4**, $NaCNBH_3$, MeOH, room temp, 24 h; (d) **3**, $NaCNBH_3$, MeOH, room temp, 24 h; (e) (*R*)-**1**, EDC/HOBt, DMF, room temp, 24 h; (f) 2,2 M LiOH, MeOH/water, room temp, 15 h.

Scheme 4. Synthesis of Derivatives with a Lysine Residue[a]



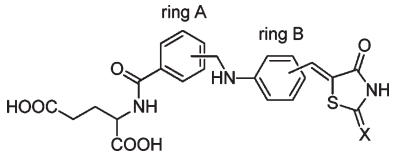
[a] Reagents and conditions: (a) for the preparation of **69**, **70** and **72**: $NaCNBH_3$, MeOH, room temp, 24 h. For the preparation of **71**: $NaCNBH_3$, DMF, room temp, 24 h. (b) *N*-Boc-L-Lys-OMe, EDC/HOBt, DMF, room temp, 24 h; (c) 2.2 M LiOH, MeOH/H_2O, room temp, 15 h; (d) $HCl_{(g)}$, AcOH, room temp, 0.5 h.

gave target compounds (*R*)-**45**, (*R*)-**46**, and (*R*)-**51**-(*R*)-**54**. For the synthesis of 5-benzylthiazolidin-4-one derivatives (*R*)-**62**, (*R*)-**66**, and (*R*)-**68** with reduced exocyclic double bond (reduced analogues of (*R*)-**33**, (*R*)-**21**, and (*R*)-**24**) the crucial step was the reduction of **11**−**13** using diethyl 2,6-dimethyl-1,4-dihydro-3,5-pyridinedicarboxylate (Hantsch ester)[27] and activated silica gel[28] to yield products **55**−**57**, which were further converted into final compounds (*R*)-**62**, (*R*)-**66**, and (*R*)-**68** using the synthetic strategy described in Scheme 3.

The synthesis of (*S*)-**75**, (*S*)-**80**, (*S*)-**81**, and (*S*)-**84**, in which D-glutamic acid was replaced with L-lysine, is shown in Scheme 4. Compounds **69**−**72** were obtained by reductive amination of amines **14**−**17** with carboxybenzaldehydes **2** and **3**, using sodium cyanoborohydride as a reducing agent. EDC/HOBt-promoted coupling of **69**−**72** with *N*-Boc-L-lysine methyl ester afforded the *S*-isomers of **73**, **76**, **77**, and **82**, which were converted into products (*S*)-**75**, (*S*)-**80**, (*S*)-**81**, and (*S*)-**84** upon hydrolysis with aqueous lithium hydroxide solution and subsequent cleavage of the Boc protecting group with gaseous hydrochloric acid in glacial acetic acid.

2.3. Biological Activity. Both isomers of targets **20**, **21**, **24**, and **25**, *R*-isomers of **28**, **29**, **32**, **33**, **45**, **46**, **51**−**54**, **62**, **66**, and

Table 1. MurD Inhibitory Potencies of 5-Benzylidenerhodanine- and 5-Benzylidenethiazolidine-2,4-dione-Based Target Compounds (Scheme 1)



compd	X	substitution ring A	substitution ring B	MurD RA[a] (%)
(*R*)-**20**	O	1,4	1,3	85
(*S*)-**20**	O	1,4	1,3	87
(*R*)-**21**	S	1,4	1,3	$IC_{50} = 174 \pm 2\ \mu M$
(*S*)-**21**	S	1,4	1,3	$IC_{50} = 206 \pm 3\ \mu M$
(*R*)-**24**	O	1,4	1,4	70
(*S*)-**24**	O	1,4	1,4	88
(*R*)-**25**	S	1,4	1,4	74
(*S*)-**25**	S	1,4	1,4	68
(*R*)-**28**	O	1,3	1,3	87
(*R*)-**29**	S	1,3	1,3	39
(*R*)-**32**	O	1,3	1,4	$IC_{50} = 85 \pm 3 (35 \pm 1)^b\ \mu M$
(*R*)-**33**	S	1,3	1,4	$IC_{50} = 45 \pm 1 (49 \pm 1)^b\ \mu M$

[a] Residual activity of the enzyme. Concentration of inhibitor was 250 μM. Results represent the mean of two independent experiments. Standard deviations were within ±10% of the mean. For IC_{50} determination assays were run in triplicate. [b] Radioactivity assay.

68, and *S*-isomers of **75**, **80**, **81**, and **84** were tested for their inhibitory activities on *E. coli* MurD using the Malachite green assay[29] for detecting orthophosphate generated during the enzymatic reaction (Table 1 and Supporting Information Table 2). The results are presented as residual activities (RAs) of the enzyme in the presence of 250 or 500 μM of each compound and as IC_{50} values for the most active compounds. Some of the dimethyl ester precursors of target compounds, i.e., (*R*)-**18**, (*S*)-**18**, (*R*)-**19**, (*S*)-**19**, (*R*)-**22**, (*S*)-**22**, (*R*)-**23**, and (*S*)-**23**, have also been tested against MurD but were found to be essentially noninhibitory, with residual activities in the range 86−98% (data not shown). These results were expected, considering the high affinity of the enzyme for D-Glu, and demonstrate the importance of the free carboxylate groups which, in the case of the MurD product UMAG, form several hydrogen bonds and electrostatic interactions with amino acid residues in the MurD active site.[7]

The results in Table 1 show no significant changes in inhibitory potency upon replacing D-Glu by L-Glu. This is in accordance with steady-state kinetics and X-ray diffraction studies, which demonstrated that the L-Glu moiety of N-substituted Glu derivatives can bind into the D-Glu-binding site of MurD in a manner similar to that of D-Glu in UMAG.[15] The results (Table 1) demonstrate that a change in the substitution pattern on both phenyl rings has an important effect on MurD inhibitory potency. Whereas extended structures with para-substitution on both phenyl rings [(*R*)-**24**, (*S*)-**24**, (*S*)-**25**, (*R*)-**25**] displayed only weak inhibition, compounds with meta-substitution in ring A [(*R*)-**32**, (*R*)-**33**], meta-substitution in ring B [(*R*)-**21**, (*S*)-**21**], or meta-substitution in both rings [(*R*)-**29**] were found to be promising inhibitors of MurD. The most potent MurD inhibitors of the series were (*R*)-**32** and (*R*)-**33** with the Malachite green assay IC_{50} of 85 and 45 μM, respectively. Although it appears that rhodanine derivatives are generally better inhibitors than thiazolidine-2,4-dione-type compounds [cf. (*R*)-**21**, (*S*)-**21** vs (*R*)-**20**, (*S*)-**20**, and (*R*)-**29** vs (*R*)-**28**], in the most active subset of compounds with a meta, para substitution pattern the inhibitory potencies of rhodanine (*R*)-**33** and thiazolidine-2,4-dione (*R*)-**32** were comparable (45 μM compared to 85 μM). These results were confirmed by additionally testing (*R*)-**32** and (*R*)-**33** for MurD inhibition in a radioactivity assay[30] which gave IC_{50} of 35 and 49 μM, respectively.

The reduction of the exocyclic double bond in (*R*)-**21**, (*R*)-**24**, and (*R*)-**33** to give (*R*)-**66**, (*R*)-**68**, and (*R*)-**62** reduced MurD inhibitory activity (Supporting Information, Table 2). Compounds with flexible alkyl linkers replacing the benzylamine moiety were devoid of inhibitory activity with the exception of (*R*)-**46**, for which a weak inhibition was observed. The absence of inhibitory activity in both classes of compounds is probably due to an increased internal entropy term because of additional rotational freedom in the more flexible molecules, whereas in (*R*)-**62**, (*R*)-**66**, and (*R*)-**68**, the loss of conjugation may also play a role. (*S*)-**75**, (*S*)-**80**, (*S*)-**81**, and (*S*)-**84**, in which glutamic acid was replaced with L-Lys, were inactive as MurD inhibitors, providing further evidence for the importance of the glutamic acid γ-carboxylate for interaction with the enzyme active site (Supporting Information Table 2).

Antimicrobial activity of (*R*)-**21** and (*R*)-**33** against two Gram-positive (*S. aureus* ATCC 29213, *E. faecalis* ATCC 29212) and two Gram-negative (*E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853) bacteria was evaluated. For both compounds the minimal inhibitory concentrations against all four bacteria tested were found to be greater than 128 μg/mL. Inactivity of (*R*)-**21** and (*R*)-**33** against tested bacteria is probably due to problems associated with bacterial cell entry.

2.4. X-ray Crystallography. The crystal structure of MurD in complex with compound (*R*)-**32** was solved to 1.5 Å resolution (PDB code 2x5o) to determine the binding mode of the inhibitor within the MurD active site (Figure 2). In the MurD-(*R*)-**32** complex the D-Glu moiety occupies the same site as the D-Glu residue of the product UMAG of the enzymatic reaction catalyzed by MurD.[7] The α-carboxyl group of the D-Glu moiety of the inhibitor forms a charge-based interaction with N^{ξ} of Lys348 and is additionally hydrogen-bonded with a conserved water molecule W2526 which is further hydrogen-bonded to O^{γ} of Thr321 and the carboxyl group of Asp182. The carboxyl group of the D-Glu side chain is held in place by hydrogen bonds with Ser415 and Phe422. One of the D-Glu side chain carboxylic oxygen atoms forms hydrogen bonds with both the backbone nitrogen and the O^{γ} of Ser415, whereas the other oxygen atom interacts with the backbone nitrogen of Phe422 and also with water molecule W2528. The latter is further hydrogen-bonded to water molecule W2530, which is in direct contact with the amide nitrogen of the D-Glu moiety of the inhibitor, forming an extended ring-type system. The carbonyl oxygen in the amide bond is hydrogen-bonded with W2532 and with guanidine nitrogen of Arg186. Additional interactions between the amino group in the para position of the 5-benzylidenethiazolidine-2,4-dione ring and W2527 also contribute to recognition within the active site. The water molecule W2527 is hydrogen-bonded to W2138 which itself interacts with the O^{γ} of Ser71 and W2033 that is hydrogen-bonded to the backbone carbonyl group of Leu13. The crystal structures of MurD in complex with small-molecule inhibitors have been solved before,[14,15] enabling the comparison of their binding modes with that of (*R*)-**32**. The increase of affinity observed for (*R*)-**32** can be explained by additional

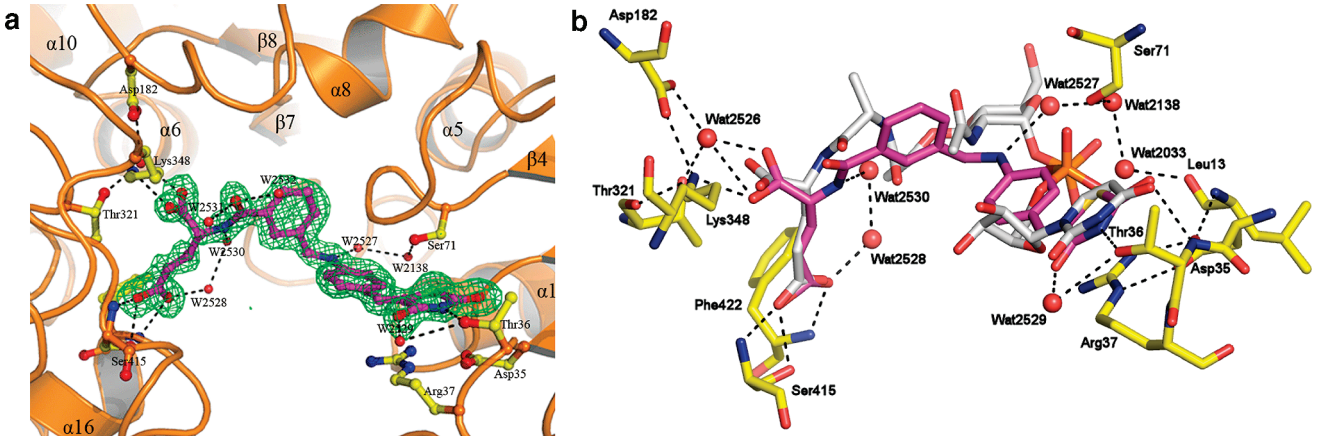


Figure 2. (a) Binding mode of compound (*R*)-**32** in the active site of *E. coli* MurD. The $F_o - F_c$ residual map is contoured at 3σ (green). (b) Superposition of compound (*R*)-**32** (pink) and UMAG (gray) in the MurD active site.

interactions, especially in the uracyl-binding pocket, which is occupied by the thiazolidine-2,4-dione ring of the inhibitor. Superposition of (*R*)-**32** and UMAG in the MurD active site is represented in Figure 2b. The ring nitrogen forms a hydrogen bond with the O^{γ} of Thr36, and the carbonyl oxygen at position 2 interacts with the Thr36 backbone NH group. The other carbonyl oxygen of the thiazolidine-2,4-dione ring interacts, via water molecule W2529, with the O^{γ} of Thr36. In addition, the thiazolidine-2,4-dione ring participates in interplane stacking with a salt bridge formed between Asp35 and Arg37. The crystal structure of (*R*)-**32** in complex with MurD also reveals that the exocyclic double bond exists in the *Z*-configuration, which has been reported as the thermodynamically stable configuration of similar compounds.[24−26]

3. Conclusion

We have prepared a series of novel 5-benzylidenerhodanine and 5-benzylidenethiazolidine-2,4-dione derivatives as promising inhibitors of MurD ligase, with IC_{50} in the range 45−206 μM for MurD from *E. coli*. The crystal structure of the complex of MurD with (*R*)-**32**, one of the most potent MurD inhibitors in the series, reveals its binding mode in the enzyme active site and makes (*R*)-**32** an excellent candidate for further structure-based optimization toward an effective novel anti bacterial drug.

4. Experimental Section

4.1. Colorimetric Inhibition Assay. The compounds were tested for their ability to inhibit the addition of D-Glu to UDP-Mur*N*Ac-L-Ala catalyzed by MurD from *E. coli*. Detection of the orthophosphate generated during the reaction was based on the colorimetric Malachite green method, as described,[29] with slight modifications. A mixture with a final volume of 50 μL contained 50 mM Hepes, pH 8.0, 3.25 mM $MgCl_2$, 6.5 mM $(NH_4)_2SO_4$, 0.005% Triton X-114, 80 μM UDP-Mur*N*Ac-L-Ala, 100 μM D-Glu, 400 μM ATP, purified MurD from *E. coli*[31] (diluted with 20 mM Hepes, pH 7.2, 5 mM dithiothreitol), and 500 or 250 μM of the tested compound dissolved in DMSO. The final concentration of DMSO was 5% (v/v). The reaction mixture was incubated at 37 °C for 15 min, then quenched with 100 μL of Biomol reagent. Absorbance at 650 nm was measured after 5 min. Residual activities were calculated with respect to control assays without the compounds and with DMSO. IC_{50} values, the concentrations of the compounds at which the residual activities were 50%, were determined by measuring the residual activities at seven different compound concentrations.

4.2. Radioactivity Inhibition Assay. Selected compounds were tested for inhibition of the addition of the radiolabelled amino-acid substrate D-[^{14}C]Glu to UDP-Mur*N*Ac-L-Ala catalyzed by MurD, using a mixture (final volume, 50 μL) containing 0.1 M Tris-HCl, pH 8.6, 5 mM $MgCl_2$, 5 mM ATP, 25 μM UDP-Mur*N*Ac-L-Ala, 25 μM D-[^{14}C]Glu (50,000 cpm), 5% (v/v) DMSO, 30 μM Tween 20, purified MurD (diluted in 20 mM potassium phosphate, pH 7.0, 1 mM dithiothreitol, 1 mg/mL BSA), and 250 μM of each test compound. The mixture was incubated at 37 °C for 30 min, and the reaction was stopped by adding 10 μL of glacial acetic acid. The resulting mixture was lyophilized and taken up in water (~10 μL). The radioactive substrate and product were separated by TLC on LK6D silica gel plates (Whatman), which were developed in 1-propanol/ammonium hydroxide/water 6:3:1 (v/v) and quantified with a radioactivity scanner (model Multi-Tracemaster LB285, Berthold-France, Thoiry, France). IC_{50} values were determined by measuring the residual activities at seven different compound concentrations.

4.3. Determination of Antibacterial Activity. With the macrodilution method, the susceptibility of four standard strains (*E. coli* ATCC 25922, *P. aeruginosa* 27853, *S. aureus* ATCC 29213, and *E. faecalis* ATCC 29212) to compounds (*R*)-**21** and (*R*)-**33** was tested.

Compounds (*R*)-**21** and (*R*)-**33** (10 mg) were dissolved in 5 mL of dimethyl sulfoxide (DMSO) to get a stock solution of the concentration 2 mg/mL. Working solutions were made by serially diluting stock solution in cation-adjusted Mueller–Hinton broth (CAMHB) as described by Amsterdam and Barry.[32,33] Briefly, 34 mL of CAMHB was added to 5 mL of stock to obtain a 256 μg/mL concentration and filter sterilized. The compound was further serially diluted to obtain 14 dilutions down to the lowest concentration of 0.031 μg/mL[33] and stored frozen for a maximum of 2 weeks. Just prior to bacterial inoculation, 0.5 mL dilutions in range of the compounds were pipetted into 13 × 100 mm screw cap tubes. The inoculum was prepared in such a way that four colonies of a fresh overnight culture on a nonselective agar plate were inoculated into saline. The turbidity was adjusted to match that of 0.5 McFarland standard (approximately 10^8 CFU/mL). A portion of a standardized suspension was diluted to approximately 1:1000 (10^5 CFU/mL). Afterwards, 0.5 mL of this dilution was added within 30 min to each tube containing 0.5 mL of the tested compound diluted in CAMHB and incubated at 35 °C for 18–24 h. After inoculum was added, dilutions of 0.016–128 μg/mL of the compound were achieved. The broth not containing any compound was inoculated as a growth control. If the compound inhibited bacterial growth, it was considered a potential antimicrobial agent. The lowest concentration of antimicrobial agent that resulted in complete inhibition of visible growth represented

the minimal inhibitory concentration (MIC). Quality control of the methods performance was done by testing *S. aureus* ATCC 29213 and gentamicin. Dilutions of antibiotic were made in the same way as for tested compound, and the MICs obtained were in the range proposed by Clinical Laboratory Standards Institute.[34]

4.4. Crystallization, Preparation of the Inhibitor Complexes, and Data Collection. Crystals of the conformationally closed MurD ligase were obtained by cocrystallizing the 6 × His-tagged enzyme at 293 K with UMA and a nonhydrolyzable analogue of ATP (AMP-PNP)[7,15,35] with the vapor-diffusion method and hanging-drop system in Linbro plates. An amount of 2 μL of protein solution drops (8.9 mg/mL purified enzyme, 20 mM HEPES, pH 7.4, 200 mM NaCl, 5 mM dithiothreitol, 1 mM UMA, and 5 mM AMP-PNP) was mixed with 2 μL of reservoir solution (0.1 M HEPES, pH 7.5, 1.8–1.9 M ammonium sulfate, 7–8% (w/v), PEG 400, and 100 mM NaCl). Crystals were grown in 4–6 days. They were subsequently incubated with 1 mM concentration of compound (*R*)-**32**, with 5% (v/v) DMSO. After 6 h of soaking, crystals were rapidly frozen in liquid nitrogen using Paratone oil as a cryoprotectant. The data set was collected at the European Synchrotron Radiation Facility (ESRF, Grenoble, France) ID13-EH2 beamline. The atomic coordinates and the related experimental data were deposited at Protein Data Bank under PDB ID code 2x5o.

4.5. Chemistry. Chemicals were obtained from Acros, Aldrich Chemical Co., and Fluka and used without further purification. THF was kept over sodium and distilled immediately prior to use. Analytical TLC was performed on silica gel Merck 60 F_{254} plates (0.25 mm), using visualization with UV light and ninhydrin. Column chromatography was carried out on silica gel 60 (particle size 240–400 mesh). HPLC analyses were performed on an Agilent Technologies 1100 instrument with a G1365B UV–vis detector, a G1316A thermostat, and a G1313A autosampler, using a Phenomenex Luna 5 μm C18 column (4.6 mm × 150 mm). The eluent consisted of trifluoroacetic acid (0.1% in water) and methanol or acetonitrile. Microwave-assisted reactions were performed using a CEM Discover microwave reactor (CEM Corp.). Melting points were determined on a Reichert hot stage microscope and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded at 300 and 75 MHz on a Bruker AVANCE DPX300 spectrometer in $CDCl_3$ or DMSO-d_6 solution, with TMS as the internal standard. Spectra were assigned using gradient COSY, HSQC, and ^{1}H-coupled ^{13}C NMR experiments. IR spectra were recorded on a Perkin-Elmer 1600 FT-IR spectrometer. Mass spectra were obtained using a VGAnalytical Autospec Q mass spectrometer. Optical rotations were measured on a Perkin-Elmer 1241 MC polarimeter. The reported values for specific rotation are average values of 10 successive measurements using an integration time of 5 s. Since chiral 5-benzylidenerhodanines and 5-benzylidenethiazolidine-2,4-diones showed ellipticity and circular dichroism, their specific rotation values are not reported. Microanalyses were performed on a Perkin-Elmer C, H, N analyzer 240 C. Analyses indicated by the symbols of the elements were within 0.4% of the theoretical values. The purity of the tested compounds was established to be ≥95%.

4.5.1. Synthesis of (*R*)-32. (*R*)-Dimethyl 2-(3-Formylbenzamido)-pentanedioate [(*R*)-4]. A solution of H-Glu(OMe)-OMe·HCl (0.500 g, 2.36 mmol), 3-carboxybenzaldehyde (**2**, 0.426 g, 2.83 mmol), and HOBt (0.488 g, 3.07 mmol) in DMF (25 mL) was prepared and the pH adjusted to 8 with *N*-methylmorpholine. EDC (0.634 g, 3.31 mmol) was added and the reaction mixture stirred overnight at room temperature. The solvent was evaporated in vacuo and the oily residue dissolved in ethyl acetate (60 mL) and washed successively with 10% citric acid (3 × 20 mL), saturated aqueous $NaHCO_3$ solution (3 × 20 mL), and brine (20 mL). The organic phase was dried over Na_2SO_4, filtered and the solvent evaporated under reduced pressure. The crude product was purified with flash column chromatography using ethyl acetate/petroleum ether (2:1) as eluent. Yield, 25% (0.181 g); white solid; mp 66−69 °C; $[\alpha]_D^{23}$ +1.3 (*c* 0.235, DMF); IR (KBr) ν = 3412, 3312, 2954, 2849, 1734, 1702, 1636, 1524, 1337, 1415, 1374, 1304, 1265, 1200, 1166, 1104, 1080, 980, 945, 902, 882, 829, 814, 780, 767, 749, 678, 668, 650 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.97−2.21 (m, 2H, $CHCH_2\underline{CH_2}$), 2.48 (t, 2H, J = 7.5 Hz, $CHCH_2\underline{CH_2}$), 3.59 (s, 3H, $C\underline{H_3}$), 3.66 (s, 3H, CH_3), 4.48−4.55 (m, 1H, $C\underline{H}$), 7.73 (t, 1H, J = 7.7 Hz, H-5), 8.10 (dt, 1H, ^3J = 7.7 Hz, ^4J = 1.4 Hz, Ar-H-4/6), 8.19 (dt, 1H, ^3J = 7.7 Hz, ^4J = 1.4 Hz, Ar-H-4/6), 8.42 (t, 1H, J = 1.4 Hz, Ar-H-2), 9.01 (d, 1H, J = 7.5 Hz, NH), 10.09 (s, 1H, CHO); MS (ESI) m/z (%) = 308 (MH^+). Anal. ($C_{15}H_{17}NO_6$) C, H, N.

(*Z*)-5-(4-Nitrobenzylidene)thiazolidine-2,4-dione (12). To a suspension of thiazolidine-2,4-dione (**8**, 0.616 g, 5.26 mmol) and 4-nitrobenzaldehyde (**7**, 0.870 g, 5.78 mmol) in absolute ethanol (5 mL) in a 10 mL process vial, glacial acetic acid (25 μL, 0.26 mmol) and piperidine (26 μL, 0.26 mmol) were added. The vial was sealed, placed in a microwave reactor, and heated at 150 °C for 20 min (max power 30 W). The mixture was cooled and the precipitate filtered off and dried. Yield, 85% (1.12 g); yellow crystals; mp 274−278 °C (lit.[36] 280 °C); IR (ATR) ν = 3187, 3113, 3047, 2731, 1747, 1704, 1669, 1606, 1591, 1525, 1342, 1311, 1143, 1007, 902, 844 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 7.86 (d, 2H, J = 8.7 Hz, Ar-H-2,6), 7.90 (s, 1H, CH), 8.34 (d, 2H, J = 8.7 Hz, Ar-H-3,5), 12.8 (s, 1H, NH); MS (EI) m/z = 250 (M^+).

(*Z*)-5-(4-Aminobenzylidene)thiazolidine-2,4-dione (16). (*Z*)-5-(4-Nitrobenzylidene)thiazolidine-2,4-dione (**12**, 0.500 g, 2.00 mmol) was dissolved in a mixture of tetrahydrofuran (15 mL) and methanol (15 mL), 10% Pd−C (150 mg) was added, and the mixture was stirred under hydrogen atmosphere for 5 h. The catalyst was filtered off and the solvent removed under reduced pressure. Yield, 95% (0.418 g); yellow crystals; mp 225−228 °C (lit.[36] 231 °C); ^{1}H NMR (DMSO-d_6) δ 6.08 (br s, 2H, NH_2), 6.66 (d, 2H, J = 8.7 Hz, Ar-H-3,5), 7.28 (d, 2H, J = 8.7 Hz, Ar-H-2,6), 7.60 (s, 1H, CH), 12.20 (br s, 1H, NH).

(*R*,*Z*)-Dimethyl 2-(3-((4-((2,4-Dioxothiazolidin-5-ylidene)methyl)-phenylamino)methyl)benzamido)pentanedioate [(*R*)-30]. To a stirred solution of amine **16** (0.750 g, 3.41 mmol) in anhydrous DMF (40 mL), benzaldehyde (*R*)-**4** (1.048 g, 3.41 mmol) and $NaCNBH_3$ (0.258 g, 4.09 mmol) were added. The mixture was stirred overnight at room temperature under an argon atmosphere. The solvent was evaporated under reduced pressure and the crude product recrystallized from ethyl acetate. Yield, 32% (0.558 g); yellow crystals; mp 148−153 °C; IR (KBr) ν = 3583, 3488, 3434, 2850, 2040, 1718, 1639, 1561, 1541, 1510, 1456, 1341, 1292, 1185, 1147, 812, 626 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.95−2.19 (m, 2H, $CHCH_2\underline{CH_2}$), 2.45 (t, 2H, J = 7.5 Hz, $CHCH_2\underline{CH_2}$), 3.58 (s, 3H, CH_3), 3.64 (s, 3H, CH_3), 4.41−4.50 (m, 3H, $CH_2\underline{NH}$, $CHCH_2CH_2$), 6.72 (d, 2H, J = 8.7 Hz, Ar-H-2,6), 7.28 (t, 1H, J = 6.0 Hz $CH_2\underline{NH}$), 7.33 (d, 2H, J = 8.7 Hz, Ar-H-3,5), 7.44 (t, 1H, J = 7.5 Hz, Ar-H-5′), 7.52 (d, 1H, J = 7.5 Hz, Ar-H-4′), 7.60 (s, 1H, CH=C), 7.77 (d, 1H, J = 7.5 Hz, Ar-H-6′), 7.88 (s, 1H, Ar-H-2′), 8.74 (d, 1H, J = 7.5 Hz, CONH), 12.28 (br s, 1H, CONHCO); MS (ESI−) m/z (%) = 510 ([M − H]$^-$, 100), 478 (5), 439 (20), 407 (20), 281 (30), 255 (30). Anal. ($C_{25}H_{25}N_3O_7S$) C, H, N.

(*R*,*Z*)-2-(3-((4-((2,4-Dioxothiazolidin-5-ylidene)methyl)phenyl-amino)methyl)benzamido)pentanedioic Acid [(*R*)-32]. To a stirred solution of the methyl ester (*R*)-**30** (0.115 g, 0.226 mmol) in MeOH/water (1:1) (10 mL), 2.2 M LiOH (0.41 mL, 0.902 mmol) was added. The mixture was stirred overnight at room temperature. The solution was neutralized with 1 M HCl and concentrated under reduced pressure. The residual aqueous solution was acidified with 1 M HCl to pH 2 and the product extracted with ethyl acetate (3 × 15 mL). The combined organic phases were washed with brine (2 × 15 mL), dried over Na_2SO_4, filtered and the solvent evaporated under reduced pressure. Yield, 73% (79.8 mg); yellow crystals; mp 125−128 °C; IR (KBr) ν = 3412, 3237, 2776, 2044, 1719, 1685, 1639, 1618, 1574, 1561, 1538, 1524, 1421, 1329, 1291, 1185, 1148, 1027, 815, 690, 610 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.89−2.16

(m, 2H, $CHCH_2\underline{CH_2}$), 2.36 (t, 2H, J = 7.5 Hz, $CHCH_2\underline{CH_2}$), 4.37−4.43 (m, $\underline{3H}$, $\underline{CH_2}NH$, $\underline{CH}CH_2CH_2$), 6.72 (d, 2H, J = 8.7 Hz, Ar-H-2,6), 7.27 (t, $\underline{1H}$, J = 5.7 Hz, $CH_2\underline{NH}$), 7.33 (d, 2H, J = 8.7 Hz, Ar-H-3,5), 7.44 (t, 1H, J = 7.7 Hz, Ar-H-5′), 7.51 (d, 1H, J = 7.7 Hz, Ar-H-4′), 7.60 (s, 1H, CH═C), 7.78 (d, 1H, J = 7.7 Hz, Ar-H-6′), 7.89 (s, 1H, Ar-H-2′), 8.58 (d, 1H, J = 7.5 Hz, CONH), 12.32 (br s, 3H, CONHCO, 2 × COOH); MS (ESI−) m/z (%) = 482 ([M − H]$^-$, 100), 464 (3), 353 (22). Anal. ($C_{23}H_{21}N_3O_7S$) C, H, N.

4.5.2. Synthesis of (*R*)-46. (*R*)-Dimethyl 2-(2-Chloroacetamido)pentanedioate [(*R*)-36]. To a solution of H-Glu(OMe)-OMe·HCl [(*R*)-**1**)] (1.00 g, 4.72 mmol) and triethylamine (1.97 mL, 14.18 mmol) in dichloromethane (30 mL), cooled to 0 °C on an ice bath, a solution of chloroacetyl chloride (414 μL, 5.20 mmol) in dichloromethane (5 mL) was added dropwise. The mixture was allowed to reach room temperature and stirred for 3 h, after which it was washed with water (2 × 15 mL), 10% citric acid (2 × 15 mL), and brine (2 × 10 mL), dried over Na_2SO_4, and concentrated under reduced pressure. Yield, 90% (1.07 g); colorless oil; $[\alpha]_{546}^{23}$ +37.0 (*c* 0.319, DMF); IR (neat) ν = 3319, 3008, 2957, 2851, 2090, 1738, 1732, 1682, 1539, 1436, 1372, 1338, 1219, 1176, 1123, 1087, 1015, 985, 825, 778 cm^{-1} (lit.[37,38] 3317, 2956, 1733, 1667, 1528, 1206, 1170 cm^{-1}); ^{1}H NMR (DMSO-d_6) δ 1.81–2.09 (m, 2H, $CHC\underline{H_2}CH_2$), 2.39 (t, 2H, J = 7.5 Hz, $CHCH_2\underline{CH_2}$), 3.59 (s, 3H, CH_3), 3.64 (s, 3H, CH_3), 4.11 (s, 2H, CH_2Cl), 4.28–4.36 (m, 1H, $\underline{CH}CH_2CH_2$), 8.63 (d, 1H, J = 7.5 Hz, NH).

(*R*)-Dimethyl 2-(2-(3-Formylphenoxy)acetamido)pentanedioate [(*R*)-40]. (*R*)-**36** (252 mg, 1.00 mmol), 3-hydroxybenzaldehyde (**38**, 122 mg, 1.00 mmol), potassium carbonate (276 mg, 2.00 mmol), and potassium iodide (199 mg, 1.20 mmol) were suspended in acetonitrile (10 mL) and heated at 85 °C for 3 h. The solvent was evaporated, the residue dissolved in ethyl acetate (20 mL), washed with water (2 × 10 mL), and brine (2 × 10 mL), dried over Na_2SO_4, and concentrated under reduced pressure. Yield, 89% (300 mg); colorless oil; $[\alpha]_{546}^{23}$ +22.1 (*c* 0.348, DMF); IR (neat) ν = 3355, 3067, 3002, 2955, 2850, 2736, 2091, 1717, 1616, 1594, 1539, 1488, 1456, 1436, 1386, 1374, 1260, 1173, 1082, 1062, 1016, 996, 870, 792, 744, 683 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.84–2.12 (m, 2H, $CHC\underline{H_2}CH_2$), 2.35 (t, 2H, J = 7.5 Hz, $CHCH_2\underline{CH_2}$), 3.58 (s, 3H, $C\underline{H_3}$), 3.63 (s, 3H, CH_3), 4.34–4.42 (m, 1H, $C\underline{H}CH_2CH_2$), 4.63 (d, 1H, AB system, ^2J = 15.0 Hz, H_A from $C\underline{H_2}O$), 4.69 (d, 1H, AB system, ^2J = 15.0 Hz, H_B from CH_2O), 7.29–7.33 (m, 1H, Ar-H), 7.44 (d, 1H, J = 2.7 Hz, Ar-H), 7.52–7.59 (m, 2H, 2 × Ar-H), 8.55 (d, 1H, J = 7.5 Hz, NH), 9.98 (s, 1H, CHO); MS (ESI) m/z (%) = 360 (MNa^+, 100), 338 (MH^+, 63), 278 (50), 246 (48), 218 (45). HRMS for $C_{16}H_{19}NO_7Na$: calculated 360.1059; found 360.1068. Anal. ($C_{16}H_{19}NO_7$) C, H, N.

(*R*,*Z*)-Dimethyl 2-(2-(3-((2-Oxo-4-thioxothiazolidin-5-ylidene)methyl)phenoxy)acetamido)pentanedioate [(*R*)-44]. (*R*)-**44** was prepared from (*R*)-**40** (500 mg, 1.48 mmol) and rhodanine (**9**, 179 mg, 1.35 mmol) according to the procedure described in the synthesis of **12**. Yield, 57% (348 mg); yellow crystals; mp 121–124 °C; IR (KBr) ν = 3417, 2920, 2849, 1721, 1641, 1436, 1296, 1254, 1213, 1176, 1059, 776 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.85–2.13 (m, 2H, $CHC\underline{H_2}CH_2$), 2.35 (t, 2H, J = 7.5 Hz, $CHCH_2\underline{CH_2}$), 3.58 (s, 3H, $C\underline{H_3}$), 3.63 (s, 3H, CH_3), 4.34–4.42 (m, 1H, $C\underline{H}CH_2CH_2$), 4.61 (d, 1H, AB system, ^2J = 15.6 Hz, H_A from $CH_2\underline{O}$), 4.66 (d, 1H, AB system, ^2J = 15.6 Hz, H_B from CH_2O), 7.10 (dd, 1H, ^3J = 8.0 Hz, ^4J = 2.3 Hz, Ar-H-6), 7.15 (s, 1H, Ar-H-2), 7.22 (d, 1H, J = 7.8 Hz, Ar-H-4), 7.48 (dd, 1H, J_1 = 8.0 Hz, J_2 = 7.8 Hz, Ar-H-5), 7.61 (s, 1H, CHC), 8.55 (d, 1H, J = 7.8 Hz, CONH), 13.84 (br s, 1H, CSNHCO); MS (ESI) m/z (%) = 475 (MNa^+, 100), 453 (MH^+, 90), 421 (43), 393 (50), 184 (50), 77 (70). Anal. ($C_{19}H_{20}N_2O_7S_2$) C, H, N.

(*R*,*Z*)-2-(2-(3-((2-Oxo-4-thioxothiazolidin-5-ylidene)methyl)-phenoxy)acetamido)-pentanedioic acid [(*R*)-46]. (*R*)-**46** was prepared by hydrolysis of (*R*)-**44** (200 mg, 0.442 mmol) according to the procedure described in the synthesis of (*R*)-**32**. Yield, 75% (141 mg); yellow crystals; mp 195–198 °C; IR (KBr) ν = 3371, 3200, 2925, 2850, 1716, 1606, 1574, 1549, 1491, 1422, 1275, 1207, 1063, 678 cm^{-1}. ^{1}H NMR (DMSO-d_6) δ 1.80–2.10 (m, 2H, $CHC\underline{H_2}CH_2$), 2.27 (t, 2H, J = 7.5 Hz, $CHCH_2\underline{CH_2}$), 4.27–4.34 (m, 1H, $C\underline{H}CH_2CH_2$), 4.63 (s, 2H, CH_2O), 7.11 (dd, 1H, ^3J = 8.1 Hz, ^4J = 2.3 Hz, Ar-H-6), 7.17–7.22 (m, 2H, Ar-H-2,4), 7.47 (t, 1H, J = 8.1 Hz, Ar-H-5), 7.61 (s, 1H, CHC), 8.38 (d, 1H, J = 8.1 Hz, CONH), 12.44 (br s, 2H, 2 × COOH), 13.84 (br s, 1H, CSNHCO); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 26.95 (CH_2), 30.95 (CH_2), 51.90 ($\underline{C}HNH$), 67.60 (CH_2O), 117.20 (Ar-C/CHC), 118.26 (Ar-C/$\underline{C}$HC), 123.97 (Ar-C/CHC), 127.01 (Ar-C/CHC), 131.38 (Ar-C/CHC), 132.20 (Ar-C/CHC), 135.19 (Ar-C/CHC), 159.11 (Ar-C/CHC), 168.46 (CO), 170.26 (CO), 173.78 (CO), 174.61 (CO), 196.59 (CS); MS (ESI) m/z (%) = 423 ([M − H]$^-$, 100), 369 (25), 336 (80), 302 (35). HRMS for $C_{17}H_{15}N_2O_7S_2$: calculated 423.0321; found 423.0332. Anal. ($C_{17}H_{16}N_2O_7S_2$) C, H, N.

4.5.3. Synthesis of (*R*)-66. 5-(3-Nitrobenzyl)-2-thioxothiazolidin-4-one (55). To a stirred suspension of **11** (1.98 g, 7.45 mmol) in toluene (100 mL) was added diethyl 2,6-dimethyl-1,4-dihydro-3,5-pyridinedicarboxylate (2.45 g, 9.69 mmol) and silica gel 60 (7.45 g, 1 g/mmol), previously activated by heating at 120 °C for 5 h. The mixture was heated to 100 °C for 24 h under an argon atmosphere in the dark, cooled, and filtered. The filter cake was rinsed with ethyl acetate. The combined filtrate and rinse were evaporated to dryness. The residue was redissolved in ethyl acetate (100 mL), washed with 1 M HCl (3 × 50 mL), and brine (50 mL), dried over Na_2SO_4, filtered and the solvent evaporated under reduced pressure. The crude product was purified by flash column chromatography using dichloromethane/methanol (40:1) as an eluent. Yield, 84% (1.68 g); yellow crystals; mp 165–168 °C; IR (KBr) ν = 3068, 2874, 1718, 1581, 1529, 1454, 1349, 1307, 1269, 1234, 1208, 1103, 1083, 939, 908, 833, 814, 736, 722, 680, 662, 585, 509 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 3.40 (dd, 1H, AB system, ^2J = 14.1 Hz, ^3J = 7.9 Hz, H_A from Ar-$C\underline{H_2}$CH), 3.52 (dd, 1H, AB system, ^2J = 14.1 Hz, ^3J = 5.4 Hz, H_B from Ar-$C\underline{H_2}$CH), 5.12 (dd, 1H, J_1 = 7.9 Hz, J_2 = 5.4 Hz, SCHCO), 7.63 (dt, 1H, ^3J = 7.7 Hz, ^4J = 0.8 Hz, Ar-H-5), 7.73 (d, 1H, J = 7.7 Hz, Ar-H-6), 8.12–8.16 (m, 2H, Ar-H-2,4), 13.17 (s, 1H, CSNHCO); MS (ESI) m/z (%) = 267 ([M − H]$^-$, 100). HRMS (ESI) for $C_{10}H_7N_2O_3S_2$: calculated 266.9898; found 266.9888.

5-(3-Aminobenzyl)-2-thioxothiazolidin-4-one (58). To a stirred solution of **55** (1.71 g, 6.38 mmol) in ethanol (100 mL), tin(II) chloride (6.05 g, 31.9 mmol) was added, and the mixture was heated under reflux for 2 h. The solvent was evaporated under reduced pressure. The residue was dissolved in saturated aqueous $NaHCO_3$ solution (100 mL) and the product extracted with ethyl acetate (5 × 50 mL). The combined organic extracts were washed with brine (2 × 50 mL), dried over Na_2SO_4 and the solvent removed under reduced pressure. Yield, 96% (1.46 g); yellow crystals; mp 71–74 °C; IR (KBr) ν = 3414, 2368, 2345, 1618, 1431, 1296, 1254, 1220, 1182, 1066, 863, 778, 723, 618, 475 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 2.96 (dd, 1H, AB system, ^2J = 14.0 Hz, ^3J = 9.7 Hz, H_A from Ar-$C\underline{H_2}$CH), 3.23 (dd, 1H, AB system, ^2J = 14.0 Hz, ^3J = 4.3 Hz, H_B from Ar-$C\underline{H_2}$CH), 4.93 (dd, 1H, J_1 = 9.7 Hz, J_2 = 4.3 Hz, SCHCO), 6.36–6.46 (m, 3H, Ar-H-2,4,6), 6.95 (t, 1H, J = 7.6 Hz, Ar-H-5), signals for Ar-NH_2 and CSNHCO not seen; MS (ESI) m/z (%) = 239 (MH^+, 33), 108 (85), 77 (100). HRMS (ESI) for $C_{10}H_{11}N_2OS_2$: calculated 239.0313; found 239.0311.

4-((3-((4-Oxo-2-thioxothiazolidin-5-yl)methyl)phenylamino)-methyl)benzoic Acid (63). A suspension of amine **58** (0.751 g, 3.15 mmol) and 4-carboxybenzaldehyde (**3**, 0.543 g, 3.62 mmol) in methanol (60 mL) was heated at 65 °C for 2 h and then cooled to room temperature. $NaCNBH_3$ (257 mg, 4.10 mmol) was added and the mixture stirred overnight under an argon atmosphere. The solvent was evaporated under reduced pressure. The residue was dissolved in ethyl acetate (40 mL) and washed with water (2 × 20 mL) and brine (20 mL). The organic phase was dried over Na_2SO_4, filtered and the solvent removed under

reduced pressure. The crude product was purified with flash column chromatography using dichloromethane/methanol (20:1) as an eluent. Yield, 45% (0.528 g); yellow crystals; mp 79–81 °C; IR (KBr) ν = 2852, 1686, 1605, 1426, 1222, 1176, 1069, 844, 763, 694, 670, 498 cm^{-1}. ^{1}H NMR (DMSO-d_6) δ 2.97 (dd, 1H, AB system, ^2J = 14.0 Hz, ^3J = 9.8 Hz, H_A from Ar-CH_2CH), 3.23 (dd, 1H, AB system, ^2J = 14.0 Hz, ^3J = 4.2 Hz, H_B from Ar-CH_2CH), 4.32 (s, 2H, CH_2NH), 4.93 (dd, 1H, J_1 = 9.8 Hz, J_2 = 4.2 Hz, SCHCO), 6.36–6.46 (m, 4H, Ar-H-2,4,6, CH$_2$NH), 6.97 (t, 1H, ^3J = 7.7 Hz, Ar-H-5), 7.45 (d, 2H, J = 8.2 Hz, Ar-H-3′,5′), 7.89 (d, 2H, J = 8.2 Hz, Ar-H-2′,6′), 12.79 (br s, 1H, COOH), 13.13 (br s, 1H, CSNHCO); MS (ESI) m/z (%) = 373 (MH$^+$, 94), 355 (100). Anal. ($C_{18}H_{16}N_2O_3S_2$) C, H, N.

(2*R*)-Dimethyl 2-(4-((3-((4-Oxo-2-thioxothiazolidin-5-yl)-methyl)phenylamino)methyl)benzamido)pentanedioate [(*R*)-65]. A solution of H-Glu(OMe)-OMe·HCl [(*R*)-**1**] (212 mg, 1.00 mmol), **63** (372 mg, 1.00 mmol), and HOBt (191 mg, 1.20 mmol) in DMF (25 mL) was prepared and pH adjusted to 8 with *N*-methylmorpholine. EDC (249 mg, 1.47 mmol) was added and the mixture stirred overnight at room temperature. The solvent was evaporated under reduced pressure. The residue was dissolved in ethyl acetate (50 mL) and washed successively with 10% citric acid (3 × 15 mL) and brine (2 × 10 mL). The organic phase was dried over Na_2SO_4, filtered and the solvent evaporated under reduced pressure. The crude product was purified by flash column chromatography using dichloromethane/methanol (20:1) as an eluent. Yield, 22% (117 mg); yellow crystals; mp 114–116 °C; IR (KBr) ν = 3419, 3340, 3251, 2948, 1742, 1637, 1607, 1526, 1499, 1437, 1337, 1281, 1217, 1181, 1108, 1070, 1018, 986, 837, 768, 699, 500 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.94–2.18 (m, 2H, CHCH_2CH$_2$), 2.45 (t, 2H, J = 7.5 Hz, CHCH$_2$CH_2), 2.97 (dd, 1H, AB system, ^2J = 14.1 Hz, ^3J = 9.8 Hz, H_A from Ar-CH_2CH), 3.24 (dd, 1H, AB system, ^2J = 14.1 Hz, ^3J = 4.3 Hz, H_B from Ar-CH_2CH), 3.58 (s, 3H, CH$_3$), 3.64 (s, 3H, CH$_3$), 4.32 (d, 2H, J = 5.0 Hz, CH_2NH), 4.42–4.49 (m, 1H, CHCH$_2$CH$_2$), 4.93 (dd, 1H, J_1 = 9.8 Hz, J_2 = 4.3 Hz, SCHCO), 6.33 (t, 1H, J = 6.2 Hz, CH$_2$NH), 6.38–6.44 (m, 2H, Ar-H-4,6), 6.48 (s, 1H, Ar-H-2), 6.97 (t, 1H, J = 7.8 Hz, Ar-H-5), 7.44 (d, 2H, J = 8.3 Hz, Ar-H-3′,5′), 7.82 (d, 2H, J = 8.3 Hz, Ar-H-2′,6′), 8.66 (d, 2H, J = 7.5 Hz, CONH), 13.14 (br s, 1H, CSNHCO); MS (ESI) m/z (%) = 530 (MH$^+$, 100). Anal. ($C_{25}H_{27}N_3O_6S_2$) C, H, N.

(2*R*)-2-(4-((3-((4-Oxo-2-thioxothiazolidin-5-yl)methyl)phenylamino)methyl)benzamido)pentanedioic Acid [(*R*)-66]. (*R*)-**66** was prepared from (*R*)-**65** (100 mg, 0.189 mmol) according to the procedure described in the synthesis of (*R*)-**32**. Yield, 96% (91 mg); yellow crystals; mp 89–91 °C; IR (KBr) ν = 2926, 2060, 1735, 1607, 1542, 1438, 1184, 1071, 696 cm^{-1}. ^{1}H NMR (DMSO-d_6) δ 1.90–2.13 (m, 2H, CHCH_2CH$_2$), 2.35 (t, 2H, J = 7.4 Hz, CHCH$_2$CH_2), 2.97 (dd, 1H, AB system, ^2J = 14.2 Hz, ^3J = 9.7 Hz, H_A from Ar-CH_2CH), 3.24 (dd, 1H, AB system, ^2J = 14.2 Hz, ^3J = 4.2 Hz, H_B from Ar-CH_2CH), 4.31 (s, 2H, CH_2NH), 4.36–4.43 (m, 1H, CHCH$_2$CH$_2$), 4.94 (dd, 1H, J_1 = 9.7 Hz, J_2 = 4.2 Hz, SCHCO), 6.37–6.44 (m, 2H, Ar-H-4,6), 6.49 (s, 1H, Ar-H-2), 6.97 (t, 1H, J = 7.8 Hz, Ar-H-5), 7.44 (d, 2H, J = 8.2 Hz, Ar-H-3′,5′), 7.83 (d, 2H, J = 8.2 Hz, Ar-H-2′,6′), 8.52 (d, 2H, J = 7.7 Hz, CONH), 12.46 (br s, 2H, COOH), 13.15 (s, 1H, CSNHCO), signal for CH$_2$NH not seen; MS (ESI) m/z (%) = 500 ([M – H]$^-$, 100). Anal. ($C_{23}H_{23}N_3O_6S_2$·0.25EtOAc) C, H, N.

4.5.4. Synthesis of (*S*)-75. (*Z*)-3-((4-((2,4-Dioxothiazolidin-5-ylidene)methyl)phenylamino)methyl)benzoic Acid (69). The suspension of amine **16** (220 mg, 2.11 mmol) and 3-carboxybenzaldehyde (**2**, 365 mg, 2.43 mmol) in methanol (60 mL) was heated at 65 °C for 2 h, then cooled to room temperature. $NaCNBH_3$ (172 mg, 2.74 mmol) was added and the mixture stirred overnight under an argon atmosphere. The solid was filtered off, washed with methanol, and dried in air. Yield, 70% (0.523 mg); yellow crystals; mp 270–275 °C; IR (KBr) ν = 3551, 3415, 3333, 3237, 3133, 3037, 2788, 2040, 1721, 1688, 1639, 1616, 1573, 1521, 1493, 1454, 1421, 1336, 1292, 1218, 1182, 1160, 1080, 1020, 975, 911, 820, 796, 755, 694 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 4.44 (d, 2H, J = 6.0 Hz, CH_2NH), 6.71 (d, 2H, J = 8.7 Hz, Ar-H-2′,6′), 7.27–7.34 (m, 3H, CH$_2$NH, Ar-H-3′,5′), 7.47 (t, 1H, J = 7.7 Hz, Ar-H-5), 7.59–7.61 (m, 2H, CHC, Ar-H-4), 7.82 (d, 1H, J = 7.7 Hz, Ar-H-6), 7.94 (s, 1H, Ar-H-2), 12.29 (br s, 1H, CONHCO/COOH), 12.92 (br s, 1H, CONHCO/COOH); MS (ESI) m/z (%) = 353 ([M – H]$^-$, 72), 325 (10), 311 (10), 282 (100), 255 (15), 218 (30). HRMS for $C_{18}H_{13}N_2O_4S$: calculated 353.0596; found 353.0603. Anal. ($C_{18}H_{14}N_2O_4S$·0.2H$_2$O) C, H, N.

(*S,Z*)-Methyl 6-(*tert*-Butoxycarbonylamino)-2-(3-((4-((2,4-dioxothiazolidin-5-ylidene)methyl)phenylamino)methyl)benzamido)-hexanoate [(*S*)-73]. A solution of H-Lys(Boc)-OMe·HCl (352 mg, 1.19 mmol), carboxylic acid **69** (400 mg, 1.13 mmol), and HOBt (215 mg, 1.35 mmol) in DMF (25 mL) was prepared and the pH adjusted to 8 with *N*-methylmorpholine. EDC (281 mg, 1.47 mmol) was added and the mixture stirred overnight at room temperature. The solvent was evaporated under reduced pressure. The residue was dissolved in ethyl acetate (50 mL) and washed successively with 10% citric acid (3 × 15 mL) and brine (2 × 10 mL). The organic phase was dried over Na_2SO_4, filtered and the solvent evaporated under reduced pressure. The crude product was crystallized from ethyl acetate. Yield, 40% (270 mg); yellow crystals; mp 125–130 °C; IR (KBr) ν = 3551, 3475, 3413, 2940, 2771, 2066, 1734, 1718, 1684, 1636, 1617, 1560, 1577, 1540, 1522, 1337, 1288, 1225, 1184, 1149, 1018, 820, 756, 632, 608 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.29–1.35 (m, 13H, *t*-Bu, CH$_2$CH$_2$CH$_2$CH$_2$CH), 1.78–1.82 (m, 2H, CH$_2$CH$_2$CH$_2$CH$_2$-CH), 2.89–2.91 (m, 2H, CH$_2$CH$_2$CH$_2$CH$_2$CH), 3.64 (s, 3H, CH$_3$), 4.36–4.43 (m, 3H, CH$_2$NHAr, CH$_2$CH$_2$CH$_2$CH$_2$CH), 6.70–6.75 (m, 3H, BocNH/CH$_2$NHAr, Ar-H-2′,6′), 7.26–7.34 (m, 3H, BocNH/CH$_2$NHAr, Ar-H-3′,5′), 7.44 (t, 1H, J = 7.5 Hz, Ar-H-5), 7.52 (d, 1H, J = 7.5 Hz, Ar-H-4), 7.61 (s, 1H, CHC), 7.78 (d, 1H, J = 7.5 Hz, Ar-H-6), 7.88 (s, 1H, Ar-H-2), 8.67 (d, 1H, J = 7.2 Hz, CONH), 12.28 (br s, 1H, CONHCO); MS (ESI) m/z (%) = 595 ([M – H]$^-$, 100), 524 (2), 495 (3), 397 (3), 265 (20). HRMS for $C_{30}H_{35}N_4O_7S$: calculated 595.2226; found 595.2211. Anal. ($C_{30}H_{36}N_4O_7S$·0.5H$_2$O) C, H, N.

(*S,Z*)-6-(*tert*-Butoxycarbonylamino)-2-(3-((4-((2,4-dioxothiazolidin-5-ylidene)methyl)phenylamino)methyl)benzamido)hexanoic Acid [(*S*)-74]. (*S*)-**74** was prepared by hydrolysis of (*S*)-**73** (200 mg, 0.335 mmol) according to the procedure described in the synthesis of (*R*)-**32** (instead of 4.0 equiv of LiOH, only an amount of 2.5 equiv was used). Yield, 96% (187 mg); yellow crystals; mp 110–115 °C; IR (KBr) ν = 3545, 3414, 3236, 2976, 2931, 2757, 2042, 1637, 1617, 1590, 1540, 1364, 1330, 1283, 1184, 1147, 1020, 820, 745, 607 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.30–1.39 (m, 13H, *t*-Bu, CH$_2$CH$_2$CH$_2$CH$_2$CH), 1.74–1.80 (m, 2H, CH$_2$CH$_2$CH$_2$CH$_2$CH), 2.87–2.92 (m, 2H, CH$_2$CH$_2$CH$_2$CH$_2$-CH), 4.30–4.43 (m, 3H, CH$_2$NHAr, CH$_2$CH$_2$CH$_2$CH$_2$CH), 6.70–6.77 (m, 3H, BocNH/CH$_2$NHAr, Ar-H-2′,6′), 7.26–7.34 (m, 3H, BocNH/CH$_2$NHAr, Ar-H-3′,5′), 7.43 (t, 1H, J = 7.5 Hz, Ar-H-5), 7.51 (d, 1H, J = 7.5 Hz, Ar-H-4), 7.60 (s, 1H, CHC), 7.78 (d, 1H, J = 7.5 Hz, Ar-H-6), 7.88 (s, 1H, Ar-H-2), 8.52 (d, 1H, J = 7.5 Hz, CONH), 12.28 (br s, 1H, CONHCO/COOH), 12.56 (br s, 1H, CONHCO/COOH); MS (ESI) m/z (%) = 581 ([M – H]$^-$, 100), 555 (1), 538 (1), 507 (10), 265 (20). HRMS for $C_{29}H_{33}N_4O_7S$: calculated 581.2070; found 581.2075. Anal. ($C_{29}H_{34}N_4O_7S$) C, H, N.

(*S,Z*)-5-Carboxy-5-(3-((4-((2,4-dioxothiazolidin-5-ylidene)-methyl)phenylamino)methyl)benzamido)pentan-1-aminium Chloride [(*S*)-75]. A solution of (*S*)-**74** (146 mg, 0.250 mmol) in glacial acetic acid (10 mL) was saturated with gaseous HCl and stirred at room temperature for 1 h. The solvent was removed under reduced pressure, and the solid was filtered off and washed with diethyl ether. Yield, 67% (87 mg); yellow crystals; mp 165–170 °C; IR (KBr) ν = 3552, 3475, 3414, 3235, 2934, 2737, 1734, 1700, 1636, 1617, 1323, 1289, 1186, 1151, 1014, 822, 750, 687, 610 cm^{-1}. ^{1}H NMR (DMSO-d_6) δ 1.34–1.64 (m, 4H,

$CH_2CH_2CH_2CH_2CH$), 1.78–1.86 (m, 2H, $CH_2CH_2CH_2CH_2$-CH), 2.74–2.80 (m, 2H, $CH_2CH_2CH_2CH_2CH$), 4.34–4.43 (m, 3H, CH_2NHAr, $CH_2CH_2CH_2CH_2CH$), 6.73 (d, 2H, J = 8.7 Hz, Ar-H-2′,6′), 7.33 (d, 2H, J = 8.7 Hz, Ar-H-3′,5′), 7.43 (t, 1H, J = 7.5 Hz, Ar-H-5), 7.52 (d, 1H, J = 7.5 Hz, Ar-H-4), 7.60 (s, 1H, CHC), 7.79–7.92 (m, 5H, NH_3^+, Ar-H-2,6), 8.52 (d, 1H, J = 7.5 Hz, CONH), 12.29 (br s, 1H, CONHCO), broad signals for COOH and CH_2NHAr not seen; MS (ESI) m/z (%) = 481 ($[M - HCl - H]^-$, 100), 470 (1), 438 (3), 410 (3), 286 (12), 255 (15), 212 (22). HRMS for $C_{24}H_{25}N_4O_5S$: calculated 481.1546; found 481.1542. Anal. ($C_{24}H_{27}ClN_4O_5S \cdot 2.2H_2O$) C, H, N.

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Supporting Information Available: Experimental procedures and characterization of intermediate and target compounds; IR and ^{13}C spectra of representative compounds; results from elemental analysis and HRMS. This material is available free of charge via the Internet at http://pubs.acs.org.

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