Pergamon

Bioorganic & Medicinal Chemistry 9 (2001) 2035–2044

BIOORGANIC & MEDICINAL CHEMISTRY

Design, Synthesis, and Evaluation of α-Ketoheterocycles as Class C β-Lactamase Inhibitors

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Received 29 January 2001; accepted 16 March 2001

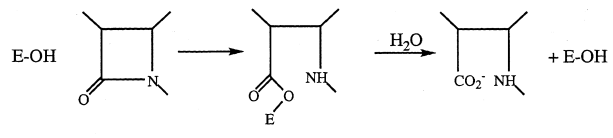
Abstract—A series of specific α-ketoheterocycles (benzoxazole, thiazole, imidazole, tetrazole, and thiazole-4-carboxylate) has been synthesized in order to assess their potential as β-lactamase inhibitors. The syntheses were achieved either by construction of the heterocycle (benzoxazole) from an appropriate α-hydroxyimidate, followed by oxidation of the alcohol, or by direct reaction of methyl phenaceturate with a lithiated heterocycle. The properties of these compounds in aqueous solution are described and their inhibitory activity against β-lactamases assessed. They did inhibit the class C β-lactamase of *Enterobacter cloacae* P99 but not the TEM β-lactamase. The most effective inhibitor of the former enzyme ($K_i = 0.11$ mM) was 5-(phenylacetylglycyl) tetrazole, probably because it is an anion at neutral pH. Interpretation of the results was aided by computational models of the tetrahedral adducts. Most of the compounds also inhibited α-chymotrypsin but not porcine pancreatic elastase.

Introduction

In recent years, resistance to antibiotics has been increasing due to the use, overuse and misuse of broad spectrum antibiotics and the ability of microbes to exchange resistance genes.[1,2] One of the most common forms of bacterial resistance to the β-lactam antibiotics comes from a group of enzymes called β-lactamases that catalyzes the hydrolysis of β-lactam antibiotics (Scheme 1) rendering them ineffective.[3]

Over the last 10 years, the prevalence of bacterial resistance to third generation cephalosporins has increased rapidly because of high levels of expression of class C β-lactamases.[4,5] Although originally found chromosomally encoded, these enzymes have recently been found on plasmids,[6] thus raising the chance that the resistance gene may spread to other species. The class C β-lactamases (also known as cephalosporinases) have become a major concern in the medical community[7–10] owing to their broad specificity for third generation cephalosporins. The β-lactamase inhibitors used clinically at present, clavulanate, sulbactam and tazobactam, are ineffective against class C β-lactamases and thus have not been used in combination therapy with β-lactam antibiotics against resistant organisms.[11] In the absence of any suitable commercial inhibitor of class C β-lactamases, there has been a pressing need in the research community to produce specific inhibitors against these enzymes. This work reports the synthesis and kinetics studies, in conjunction with molecular modeling, of specific α-ketoheterocycles as a new class of potential inhibitors of class C β-lactamases.

A wide variety of inhibitors of serine proteases have been designed and synthesized over the last 50 years.[12] These include covalent modifying reagents, including, recently, β-lactams,[13] and transition state analogue precursors such as peptidyl trifluoromethyl ketones and peptidylboronic acids.[14,15] Elastase inhibitors of this type, for example, have been sought for the treatment of emphysema.[16,17] Recently Edwards et al. have described peptidyl α-ketoheterocycle analogues as inhibitors of human neutrophil elastase (HNE).[18–20] These compounds are believed to form anionic tetrahedral adducts with the active site serine hydroxyl group (Scheme 2)



acyl-enzyme intermediate

Scheme 1.

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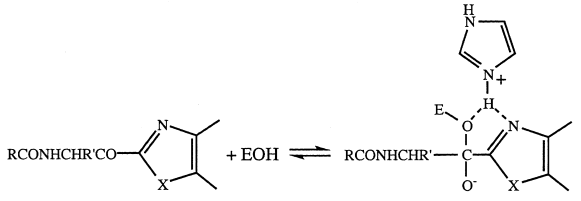
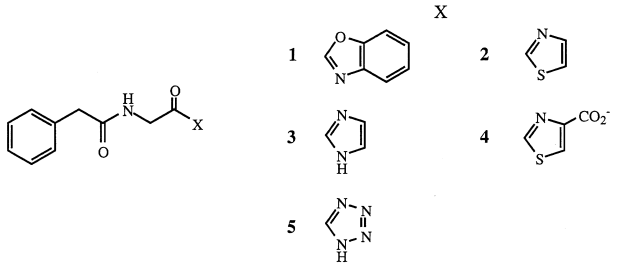
0968-0896/01/$ - see front matter
PII: S0968-0896(01)00107-9

and thus generate transition state analogue structures. Furthermore, the crystal structure of an elastase inhibitor complex showed that His 57, the active site general acid/base catalyst, is involved as a general acid in stabilizing the tetrahedral adduct by forming a bifurcated hydrogen bond to both the active site Ser 195 O_γ and the heterocyclic nitrogen atom (Scheme 2).[18] We therefore decided to investigate the potential of this class of inhibitor against β-lactamases. We thought it possible that such inhibitors might trap the general acid/base catalysts of β-lactamase active sites. The identity of such catalysts has long been controversial.[21] The following compounds **1**–**5** (Scheme 3) were synthesized and evaluated as inhibitors, primarily of a class C β-lactamase. They contain a combination of an electrophilic α-ketoheterocycle and the amido side chain favored by β-lactamases.



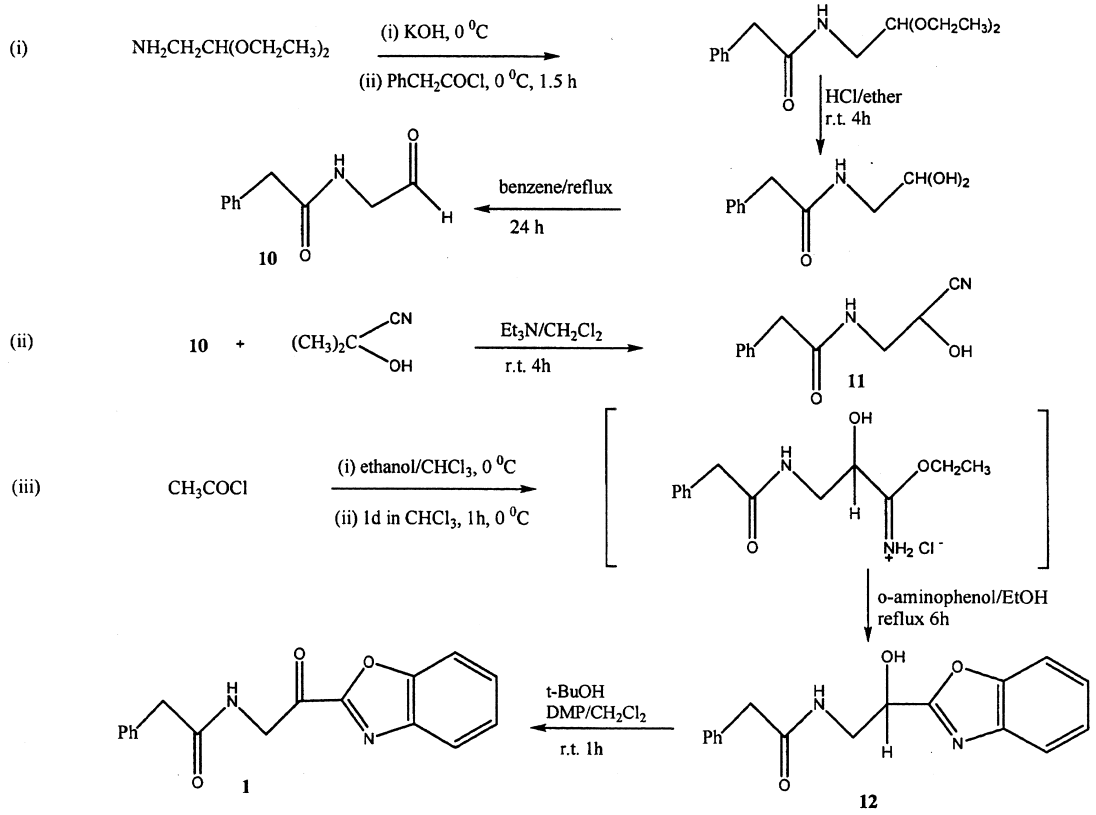
Scheme 2.



Scheme 3.

Results and Discussion

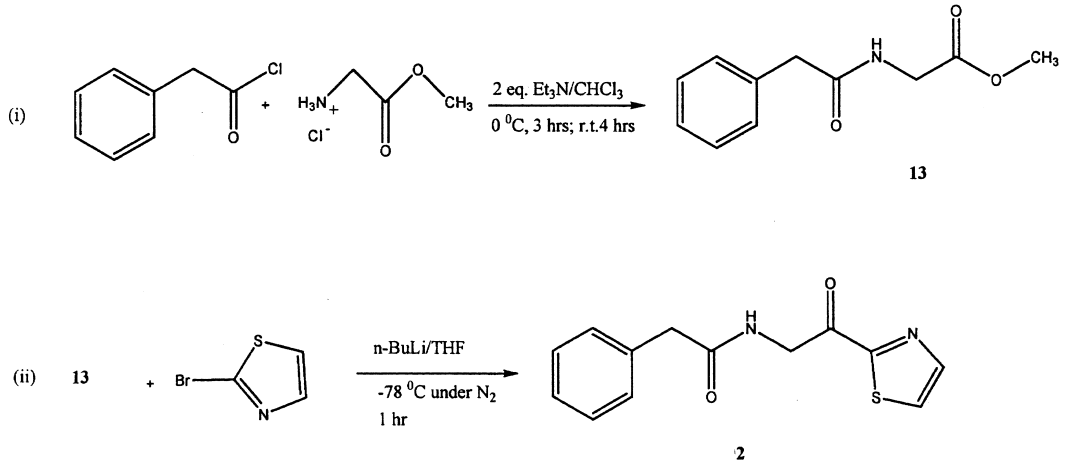
The heterocycles **1**–**5** were synthesized by variations of established methods (Schemes 4–8). Thus, the benzoxazole **1** was prepared from phenylacetylaminoacetaldehyde by construction of the oxazole ring and oxidation of the resulting alcohol **12**, essentially as described by Edwards et al.[18,20] The remaining compounds **2**–**5** were prepared by reaction of the appropriate lithiated heterocycle with methyl phenaceturate.[19,22] Thus, for the synthesis of **2**, 2-lithiothiazole was generated from *n*-butyl lithium and 2-bromothiazole, and for that of **4**, the 2-lithio derivative was prepared directly from thiazole-4-carboxylic acid. Protected heterocycles were employed in the remaining two cases, 1-SEM-imidazole for **3**[23,24] and 1-BOM-tetrazole for **5**.[25] Reaction of the lithio derivatives of these protected heterocycles with methyl phenaceturate yielded the required products in *N*-protected form. After some experimentation with alternative methods,[23–25] it was found that both protecting groups could be removed by treatment with trifluoroacetic acid under the appropriate conditions, as described in Experimental.



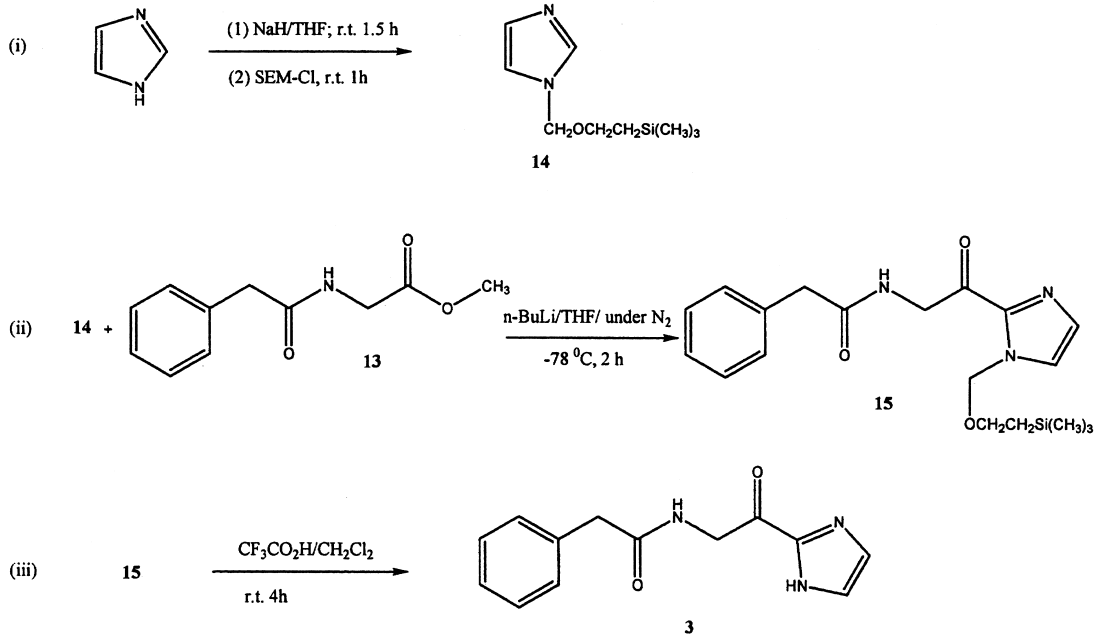
Scheme 4.

The properties of the heterocycles believed to be important to the inhibitory power of **1**–**5** are their electron-withdrawing ability (σ_I) and the basicity of the β-N atom.[26] High electron-withdrawing ability (large positive σ_I values) would promote nucleophilic attack by the active site nucleophile and high basicity of the β-N would attract adjacent general acids (Scheme 2); together these properties should both contribute to a strong inhibitor. Consideration of the values of these parameters for the heterocycles chosen (Table 1) suggests that the benzoxazole **1** and the tetrazole **5** should be the best inhibitors. The behavior of these compounds in solution however can be complicated, with the possibilities of keto, enol, enolate and hydrated keto forms to be considered. For example, the benzoxazole **1** exists in the solid state mainly in the keto form (Scheme 9; an infra-red spectrum shows an intense $\nu_{C=0}$ at 1720 cm^{-1}). In acetonitrile solution, ^1H NMR spectroscopy indicates that the keto form is also dominant, with a two proton doublet at δ 4.80 corresponding to the methylene group adjacent to the keto group; uv absorption spectra show that this species absorbs maximally at 291 nm ($\varepsilon = 13{,}250\ M^{-1}\ cm^{-1}$). In DMSO, however, the keto form converts essentially completely over an h into the enol **6**, characterized by a vinylic proton doublet at δ 7.75 and a λ_{max} of 326 nm ($\varepsilon = 16{,}300\ M^{-1}\ cm^{-1}$) in the NMR and UV absorption spectra, respectively. A spectrophotometric pH titration of the enol indicated that its pKa in water is 10.2; the maximal absorption of the enolate was observed at 344 nm ($\varepsilon = 17{,}600\ M^{-1}\ cm^{-1}$).

The solubility of **1** in water was insufficient to obtain a ^1H NMR spectrum in that solvent alone. Spectra in acetonitrile/water mixtures show the presence of two compounds, the keto form and a second form, most likely the hydrate **8**, the latter characterized by a keto-methylene resonance at δ 3.43, that is 1.37 ppm upfield



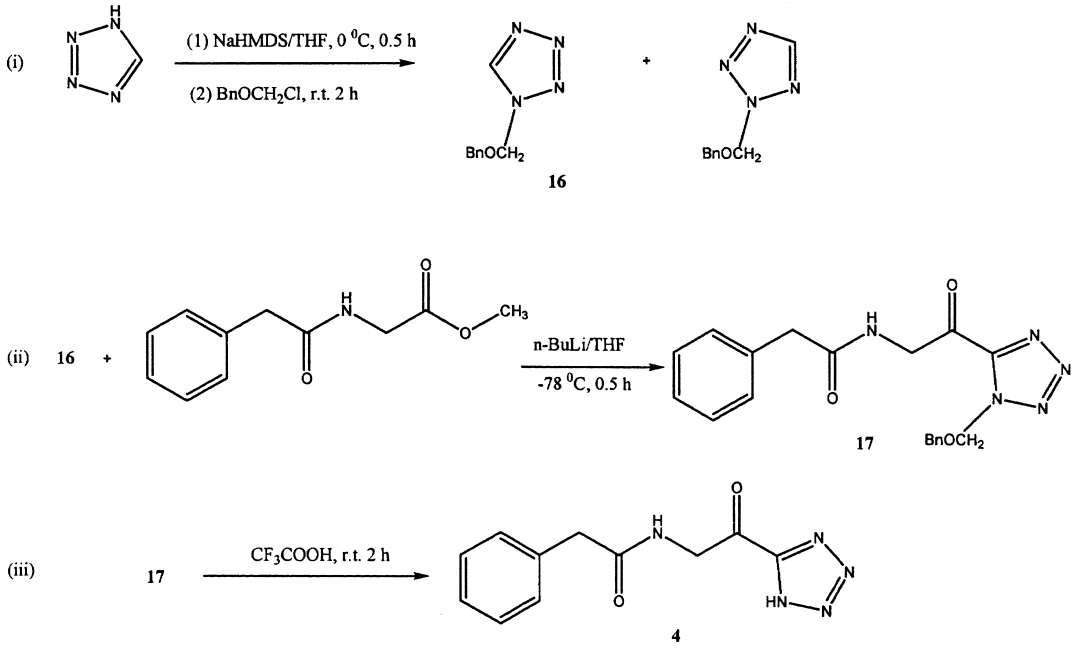
Scheme 5.



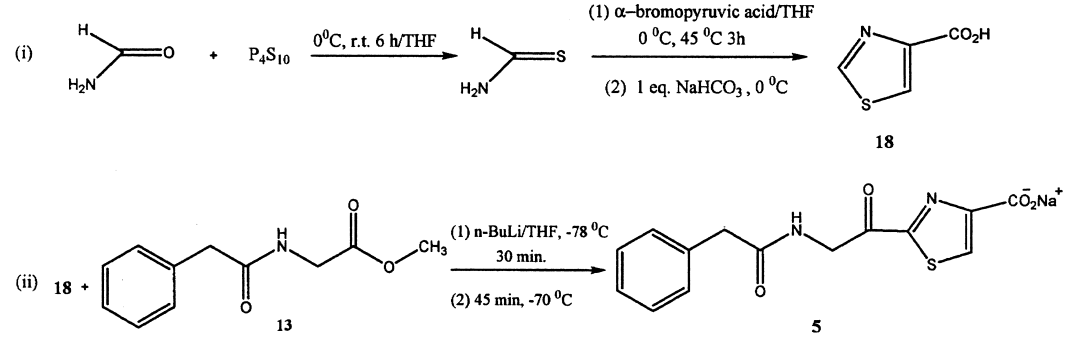
Scheme 6.

from its position in the keto compound, and UV absorptions peaks at 271, 278, and 301 nm.

In 50:50 acetonitrile/water, the hydrate comprises ca. 15% of the total. This proportion increased as the acetonitrile content of the solvent decreased. Absorption spectra in pure water indicate a hydrate content of around 50%. The presence of this hydrate attests to the electron-withdrawing power (σ_I) of the benzoxazole substituent (Table 1). This is also indicated by the exchange of the ketomethylene protons with solvent deuterons in ^2H_2O; in 50:50 acetonitrile/^2H_2O, this



Scheme 7.

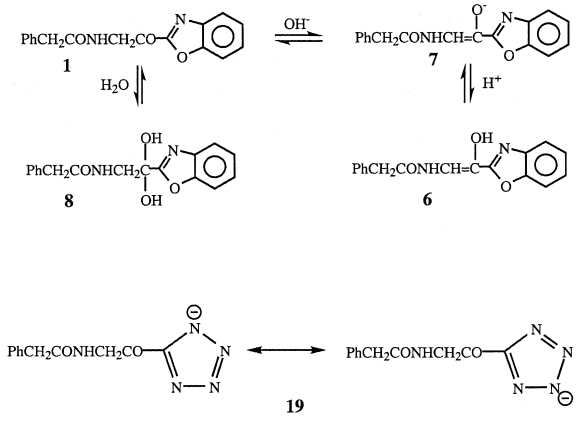


Scheme 8.

Table 1. Inhibition of the P99 β-lactamase and α-chymotrypsin by the α-ketoheterocycles **1**–**5**; electronic properties of the heterocycles

Inhibitor	K_i (mM)		σ_I[a]	β-N charge[a]
	P99 β-Lactamase	α-Chymotrypsin		
1	[b]	[b]	0.41	−0.62
2	0.55±0.06	0.34[c]	0.34	−0.43
3	1.5±0.1	0.77[c]	0.27	−0.53
4	[b]	1.1[c]	—	—
5	0.11±0.03	0.35[c]	0.49	−0.40[d]

[a]Taken from ref 26.
[b]No inhibition observed at 1 mM inhibitor.
[c]Single point estimates.
[d]Refers to neutral tetrazole.



Scheme 9.

process was complete in 2 h. The presence of the hydrate, however, would decrease the concentration of the presumed inhibitory form (keto) in solution.

^{1}H NMR spectra of **2**–**4** in ^2H_2O show the presence of essentially non-hydrated keto compounds. The smaller amount of hydrate in these cases presumably reflects the lower electron-withdrawing power of the heterocycles concerned (Table 1). The NMR spectrum of **3** in 1 M ^{2}HCl however shows the presence of 85% hydrate. The electron withdrawing power of the imidazole of **3** has presumably been significantly increased by protonation; the pKa of 2-acetylimidazolium is reported to be 3.12.[27] Although the σ_I value of the 5-tetrazolyl substituent is the largest of the heterocycles in Table 1, the heterocycle is also acidic to the extent that **5** probably exists essentially completely as a tetrazole anion at pH 7; the pK_a of tetrazole is 4.9[28] and that of a 5-acyl-tetrazole must be lower. An anionic tetrazole moiety in **5** should decrease the electrophilicity of the keto-carbonyl with respect to that of the neutral form, but increase the basicity of the β-N.

The results of experiments to determine the effectiveness of **1**–**5** as class C β-lactamase inhibitors are given in Table 1. These compounds were found to be fast reversible inhibitors of modest potency; their inhibitory ability did not further increase with time. The thiazole derivative **2** was more effective than the imidazole, in accord with the higher σ_I of the latter compound rather than its smaller β-N charge. The effectiveness of **3** decreased at lower pH, demonstrating that the inhibitory form was not the cation.

The tetrazole **5** is the best β-lactamase inhibitor of the five compounds. Its K_i only increased slightly to 0.28 mM at pH 5, suggesting that the inhibitory species at pH 7.5 was in fact the tetrazole anion **19**. This would probably have the least electrophilic carbonyl group, but would have the most basic nitrogen and also a negative charge. The latter point may well be the dominant effect here. Essentially all good substrates and inhbitors of the P99 β-lactamase (and, indeed, of most β-lactamases) are negatively charged. The active site has significant complementary positive charge, provided by the conserved lysine residues, 67 and 315, and the α-2 helix dipole.[29,30]

A computational model of **5** (as the anion **19**) bound as a tetrahedral carbonyl adduct at the active site was constructed, based on the crystal structure of an analogous phosphonate inhibitor as described in Experimental. The

Table 2. Structural parameters from molecular dynamics of enzyme-inhibitor complexes

Structural parameter	Distance (Å) or Angle (deg)[a]				
	1	**2**	**3**	**4**	**5**
Tyr 150 O_ζ-ligand N_β distance	4.3±0.5	3.4±0.5	3.7±0.5	3.6±0.8	3.1±0.2[c]
Tyr 150 O_ζ-Ser 64 O_γ distance	4.9	2.9	2.8	3.1	3.1
Tyr 150 C_ϵ–O_ζ-ligand N_β angle	83	93	79	83	107[c]
Ser 64 O_γ–C_{tet}-ligand C_α–N_β dihedral	32±17	27±35	80±23	5±21	−70±21[c]
ligand O^--Ser 64 N distance[b]	3.0	2.9	2.9	3.0	2.9

[a]Average and standard deviation of 100 snapshots from 10-20 psec.
[b]Major oxyanion hole interaction.
[c]The N_β closer to Tyr 150 O_ζ.

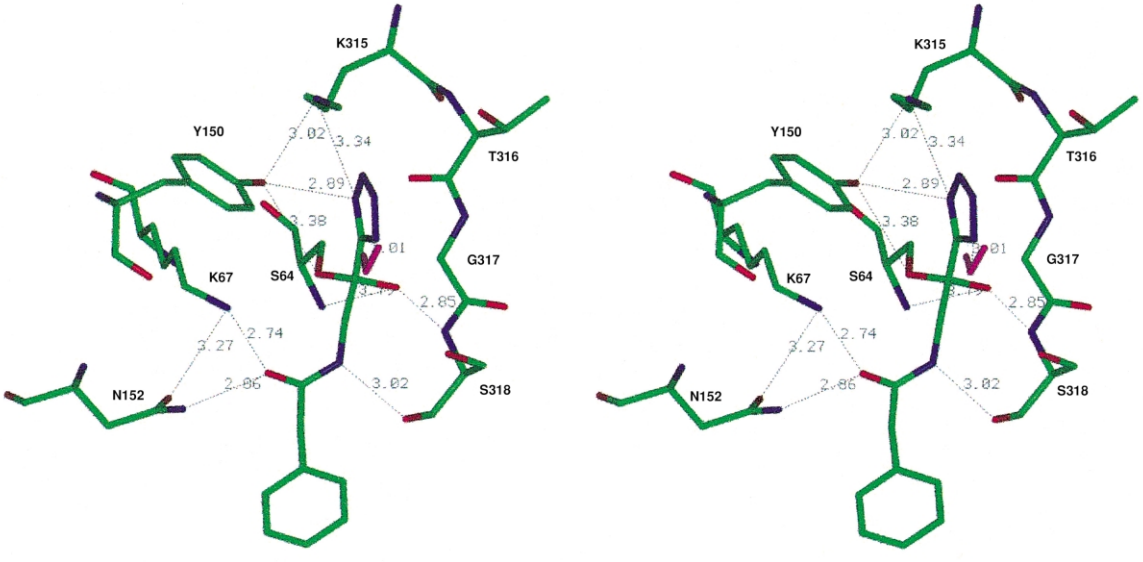


Figure 1. Stereo view of the optimized tetrahedral adduct of the anion of the tetrazole **5** with the P99 β-lactamase.

conformation of the bound inhibitor, and in particular the conformation of the heterocycle at the active site, was investigated by means of a 20 ps molecular dynamics run. Some important structural information derived from these runs, both for the tetrazole and also for the other heterocycles, as discussed below, is given in Table 2. It was found, in most snapshots of the tetrazole complex, that the hydroxyl oxygen of Tyr 150 was within hydrogen-bonding distance of the β-N of the tetrazole ring and both of these moieties were close to the ammonium group of Lys 315. (A water molecule was also generally found associated with the β-N of the tetrazole.) Although, the Tyr 150 hydroxyl was generally within hydrogen-bonding distance, it was directed, as indicated by the orientation of the tetrazole ring (Ser 64 O_γ–C_{tet}-ligand C_α–N_β dihedral angle; Table 2), not so much at a tetrazole nitrogen lone pair but rather directly to the negatively charged ring of the heterocycle. It was also noticed that the hydroxyl group of Thr 316 was within hydrogen-bonding distance of one of the γ-N atoms of the tetrazole ring in many of these snapshots. Figure 1 shows an energy-minimized structure derived from a typical snapshot. As well as the features mentioned above, this shows the tetrahedral oxyanion in its hole and the usual disposition of the amido side chain with respect to active site functional groups.[29–31] One distinct difference however, both in the minimized structure and in most of the dynamics snapshots, is that Lys 67 seems to have pulled away from the hydroxyl of Tyr 150, the side-chain carbonyl of Asn 152, and Ser 64 O_γ, and taken a position apparently hydrogen-bonded to the side chain carbonyl of the ligand.

Similar dynamics runs with the thiazole and imidazole inhibitors, **2** and **3**, found the heterocyclic rings in similar positions to that shown for the tetrazole in Figure 1 but with Tyr 150 and Lys 67 more closely attending Ser 64 O_γ than the heterocycle (Table 2). The average Tyr 150 O_ζ-ligand β-N distance is also less favorable than in the tetrazole case (Table 2). This apparently weaker interaction between heterocycle and Tyr 150, although probably offset to some degree by the greater electrophilicity of the carbonyl group in these cases, may explain the poorer performance of **2** and **3** as inhibitors with respect to **5**. In all of these cases, a barrier to the anticipated optimal orientation of the heterocycle, as shown in Scheme 2, was the eclipsing of the Ser 64 O_γ–C_{tet} bond by the C=N bond of the heterocycle. This is overcome, apparently, in elastase, but not so in the P99 β-lactamase, as indicated by the average Ser 64 O_γ–C_{tet}-ligand C_α–N_β dihedral angles (Table 2).

Model-building suggested that a carboxylate group added to the 4-position of an imidazole or thiazole might interact favorably with the Lys 315 ammonium ion and the Thr 316 hydroxyl group in a similar way to the carboxylate of a substrate;[31] thus, compound **5** was prepared. Unfortunately, it was not an effective inhibitor (Table 1). A major factor leading to this result is probably the high dehydration energy of the carboxylate group. 3-(Phenylacetamido)-pyruvic acid is also a poor inhibitor of this enzyme and probably for the same reason.[29] A molecular dynamics study (20 ps) of the complex of **5** with the β-lactamase suggested that although the carboxylate could indeed interact strongly with Lys 315 and Thr 316, bringing about a significant degree of tethering of the heterocyclic ring into a conformation appropriate for interaction with Tyr 150, the ligand did not remain as firmly bound as was the tetrazole (e.g., see the Y150-β-N distance in Table 2).

A final surprise was the ineffectiveness of the benzoxazole **1** as a β-lactamase inhibitor. The 2-benzoxazolyl substituent appears to represent an attractive combination of electron-withdrawing power and β-N basicity (Table 1). Indeed, benzoxazole analogues were among the most powerful elastase inhibitors prepared by Edwards et al.[19] The likely reason for the failure of **1** as a P99 β-lactamase inhibitor was demonstrated by molecular modeling. A dynamics run suggested that it may not be possible to accomodate the benzene ring of the heterocycle at the active site and also maintain the important hydrogen-bonding contacts mentioned above between the active site and the ligand. In fact, the side chains of Lys 315 and Tyr 150 moved away from the ligand as the simulation proceeded (see the Tyr 150-β-N distance in Table 2). The dynamics showed that the benzoxazole was rather less mobile at the active site than the monocyclic heterocycles, probably because of its greater size which led to more steric interactions with the protein surrounding the active site. Unfortunately, it was not able, even in this less mobile state, to interact productively with Tyr 150 and Lys 315. The rigidity of the tetrahedral adduct also suggests that the benzoxazole might have greater difficulty in binding as the ketone and reacting with the nucleophilic serine than would the smaller heterocycles. These factors probably lead to the inhibitory impotence of **1**. The α-ketoheterocycles **1**–**5** had some inhibitory activity against α-chymotrypsin (Table 1), but none against elastase. They were also ineffective against the class A TEM β-lactamase and the *Streptomyces* R61 DD-peptidase. The lesser effectiveness of **1**–**5** against the latter two enzymes than against the P99 β-lactamase mimics the relative activity of phosphonate monoesters against these enzymes.[32]

The results described in this paper appear to elaborate earlier indications[29] that specific electrophilic carbonyl compounds are, at best, only modest inhibitors of β-lactamases. These compounds have also, to date, not demonstrated any activity against DD-peptidases. Computational studies suggest that, in general, tetrahedral adducts at the active site of the P99 β-lactamase without an electronegative heteroatom α to the tetrahedral atom do not interact strongly with the enzyme.[29] There is, in fact, no direct evidence that **1**–**5** do actually form tetrahedral adducts at the β-lactamase active site. Inhibition, where it occurs, may just involve non-covalent interaction with the enzyme. The latter would presumably be enhanced by the negative charge on the tetrazole anion of **5**. Although the incorporation of a carboxylate, such as is found in β-lactamase substrates, into β-lactamase inhibitors seems an appropriate strategy, and one which has succeeded with boronates,[33] even a rationally placed carboxylate, as in **4**, was unable

to enhance the activity of the heterocyclic ketones studied in this work. It seems unlikely at this time that carbonyl compounds will prove to be useful against β-lactam recognizing enzymes.

Experimental

Enzymes and substrates

The class C β-lactamase of *Enterobacter cloacae* P99 and the class A TEM-2-β-lactamase from *Escherichia coli* W3310 were purchased from the center for Applied Microbiology and Research (Porton Down, Wiltshire, UK) and used as received. The *Streptomyces* R61 DD-peptidase was the generous gift of Dr. J.-M. Frère of the University of Liege, Liege, Belgium. Bovine α-chymotrypsin and its substrate *N*-succinyl-alanyl-alanyl-prolyl-phenylalanyl-*p*-nitroanilide, porcine pancreatic elastase (PPE) and its substrate *N*-succinyl-alanyl-alanyl-prolyl-leucyl-*p*-nitroanilide were purchased from Sigma Chemical Co.

Analytical and kinetic methods

Absorption spectra and spectrophotometric reaction rates, all at 25 °C, were measured with a Hewlett-Packard HP8452A spectrophotometer. Kinetics experiments with the P99 β-lactamase were performed in 20 mM MOPS buffer (pH 7) and 20 mM pyridine buffer (pH 5) using nitrocefin (Unipath, Ogdensburg, NY, USA) as the substrate. The activities of **1**–**5** as competitive inhibitors of the P99 β-lactamase (K_i values) were determined by measuring initial rates of product appearance at 482 nm as a function of inhibitor concentration, and fitting the data to a simple competitive inhibition equation. Similar experiments with bovine α-chymotrypsin were performed spectrophotometrically at 410 nm using *N*-succinyl-alanyl-alanyl-prolyl-phenylalanyl-*p*-nitroanilide as the substrate ($K_m = 0.043$ mM) in 0.1 M Tris–HCl, 0.01 mM $CaCl_2$ buffer at pH 7.8.[34] The porcine pancreatic elastase assays were performed using *N*-succinyl-alanyl-alanyl-prolyl-leucyl-*p*-nitroanilide substrate in 0.1 M Tris, 0.01 M $CaCl_2$ buffer at pH 7.8 as described by Largman et al.[35] The absence of time dependence of activity loss of the β-lactamase in the presence of **1**–**5** was demonstrated by measuring the enzyme activity in incubation mixtures of enzyme and inhibitor as a function of time: small aliquots were removed at appropriate intervals after mixing and assayed against nitrocefin.

Molecular modeling

A previously described general procedure for molecular modeling was adopted.[29] The starting point for these studies was the crystal structure of the class C β-lactamase from *E. cloacae* P99 with a phosphonate inhibitor bound covalently to the active site serine residue; the structure with the arylacetamido side chain closer to the β-3 strand was chosen.[30] The simulations were performed on a IBM 3CT computer with INSIGHT II 97.0 (MSI Technologies, Inc., San Diego, CA, USA). Atomic charges on the protein at pH 7.0 were assigned by the software. The charges on the inhibitor molecule were generated by MNDO calculations (MOPAC 6.0 module) on a peptide-bound inhibitor. The enzyme-inhibitor complex was hydrated with a 15 Å sphere with water molecules centered at the active site Ser 64 O_γ. A temperature of 300 K and a dielectric constant of 1 were employed in all the simulations. The molecular dynamics simulations in a CV force field were run for 20 ps with coordinates saved every 100 steps. The trajectories obtained in the simulation were analyzed in the Analysis module of INSIGHT II. Energy minimization was also performed in the CV force field as described.[29]

Synthesis

^1H and ^{13}C NMR spectra were obtained from a Varian 300 MHz spectrometer and IR spectra were recorded on a Perkin–Elmer BX FTIR V2.0 spectrophotometer. Thin-layer chromatography was performed on 250 μ silica gel plates (Analtech, Newark, DE, USA). Column chromatography were carried out on silica gel (grade 9385, 230–400; Merck), Sephadex LH-20 (bead size 20–100 μ, Sigma) and Sephadex G-10 (40–120 μ, Sigma). Mass spectra were obtained from the Mass Spectrometry Laboratory, School of Chemical Sciences, University of Illinois, IL, USA. Elemental analysis were performed by Desert Analytics, Tucson, AZ, USA.

2-(Phenylacetylglycyl) benzoxazole (1)

The synthesis of 2-(phenylacetylglycyl) benzoxazole is outlined in Scheme 4.

Phenylacetylaminoacetaldehyde (10). This was prepared by the procedure of Brutshy et al.[36] from aminoacetaldehyde diethyl acetal and phenylacetyl chloride. The product was crystallized from benzene/petroleum ether, collected by filtration, and dried under vacuum. It was characterized by its mp, 108–110 °C (uncorr) [lit.[36] 113.5–115.5 °C (corr)] and ^1H NMR spectrum [(300 MHz, DMSO-d_6), δ 3.65 (s, 2H), 4.2 (t, 2H), 6.1 (br s, 1H), 7.25–7.45 (m, 5H)].

Phenylacetylaminoacetaldehyde cyanohydrin (11). A solution of phenylacetylaminoacetaldehyde (10 g, 56 mmol) in methylene chloride (177.3 mL) under nitrogen was treated with acetone cyanohydrin (15.54 mL) and triethylamine (4.71 mL). The resulting mixture was stirred for four h. After evaporation of solvent, the residue was taken up in ethyl acetate, washed with brine and dried over $MgSO_4$. The crude product was purified using flash column chromatography on silica gel with a hexane/ethyl acetate (1:1) mixture as solvent. The product was isolated as a pale, white brittle solid in a yield of 57% (6.56 g); mp 57–58 °C, ^1H NMR (300 MHz, $CDCl_3$) δ 3.45 (dt, $J = 14.2$, 6.2 Hz, 1H), 3.63 (s, 2H), 4.6 (br s, 1H), 5.4 (br s, 1H), 6.4 (br s, 1H), 7.2–7.4 (m, 5H).

1-(2-benzoxazolyl)-2-(phenylacetylamino) ethanol (12). A mixture of chloroform (10.4 mL) and absolute ethanol (9.5 mL) under nitrogen at 0 °C was treated with acetyl chloride (10.5 mL) dropwise over 15 min. To this was

added phenylacetylaminoacetaldehyde cyanohydrin (**11**), 1.0 g (4.9 mmol), in chloroform (10.4 mL) and the reaction mixture was stirred for an hour. The solvent was evaporated while keeping the temperature at 25 °C or below. The crude imidate, dissolved in anhydrous ethanol (24.6 mL), was refluxed with *o*-aminophenol (0.59 g, 5.4 mmol) for 6 h. After the solvent had been removed by evaporation, the residue was dissolved in ethyl acetate, washed successively with 1N NaOH, 1 N HCl, saturated $NaHCO_3$ and brine, dried over $MgSO_4$, and evaporated to dryness in vacuo. The brownish crude material thus obtained was recrystallized from hot benzene/petroleum ether (20–40 °C). The product was isolated as a colorless granular solid in a yield of 85% (1.0 g), mp 110–112 °C, ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.6 (s, 2H), 3.7–3.8 (m, 1H), 3.9–4.0 (m, 1H), 5.1 (m, 1H), 7.15 (m, 2H), 7.25 (m, 3H), 7.5 (m, 1H), 7.65 (m, 1H). ^{13}C NMR (300 MHz, $CDCl_3$) δ 44 (CH_2), 45 (CH_2), 68 (COH), 111 (ArCH), 121 (ArCH), 125 (ArCH), 126 (ArCH), 135 (ArCH), 141 (ArCN), 151 (ArCO), 166, 173 (CO, CN).

2-(phenylacetylglycyl) benzoxazole (1). *tert*-Butyl alcohol (0.11 mL) was added to a suspension of 1-(2-benzoxazolyl)-2-(phenylacetylamino)ethanol (**12**) (0.366 g, 1.25 mmol) and Dess–Martin periodinane (1,1,1-triacetoxy-1,1,-dihydro-1,2-benziodoxol-3(1H)-one)[37] (1.48 g, 3.5 mmol) in dichloromethane (8.6 mL) and the resulting slurry stirred at room temperature for 1 h. The reaction mixture was then partitioned between ethyl acetate and saturated $Na_2S_2O_3$ (1:1), washed with saturated $NaHCO_3$ and brine, dried over $MgSO_4$ and evaporated to dryness. Recrystallization of the crude product from benzene afforded a colorless solid (0.34 g; 94%). It was characterized as follows: mp 150 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.71 (s, 2H), 4.93 (d, J = 5.4 Hz, 2H), 6.25 (br s, 1H), 7.3–7.4 (m, 5H), 7.48 (t, J = 7.8 Hz, 1H), 7.57 (t, J = 7.8 Hz, 1H), 7.67 (d, J = 8.1 Hz, 1H), 7.89 (d, J = 8.1 Hz, 1H); ^{13}C NMR (300 MHz, DMSO-d_6) δ 43 (CH_2), 44 (CH_2), 115 (ArCH), 120 ArCH), 125 (ArCH), 126 (ArCH), 127 (ArCH), 128 (ArCH), 129 (ArCH), 136 (ArCH), 146 (ArCH), 171 and 172 (CONH and C=N), 195 (CO); ν_{max} (KBr) 1720 cm^{-1} (C=O). Anal. calcd for $C_{17}H_{14}N_2O_3$: C, 69.40; H, 4.75; N, 9.52. Found: C, 69.30; H, 4.82; N, 9.57.

2-(Phenylacetylglycyl) thiazole (2)

The synthesis of 2-(phenylacetylglycyl) thiazole is outlined in Scheme 5.

Phenylacetylglycine methyl ester (13). To a stirred ice cold solution of glycine methyl ester hydrochloride (20 g, 159 mmol) in $CHCl_3$ (440 mL) was added 44.3 mL (318 mmol) of ice cold triethylamine. To this reaction mixture 21.0 mL (159 mmol) of phenylacetyl chloride in 100 mL $CHCl_3$ was added dropwise at 0 °C and the mixture stirred continuously for 2 h. The reaction mixture was then allowed to come to room temperature and stirred for a further 4 h. The organic layer was washed with water, dried over $MgSO_4$ and evaporated to dryness. Recrystallization of the crude product from diisopropyl ether gave a white crystalline solid in 52% (15.5 g) yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.5 (s, 2H), 3.63 (s, 3H), 3.86 (d, J = 6.2 Hz, 2H), 7.2–7.4 (m, 5H), 8.5 (br t, 1H).

2-(Phenylacetylglycyl) thiazole (2). Under an atmosphere of nitrogen, to a cold (−78 °C), stirred solution of *n*-butyl lithium [15.2 mmol; 9.5 mL of a 1.6 M solution in hexane (Aldrich)] in THF (18.3 mL) was added dropwise a solution of 2-bromothiazole [1.37 mL, 15.2 mmol (Aldrich)] in the same solvent (18.3 mL). After the yellow solution had been stirred at −78 °C for 30 min, a solution of phenylacetylglycine methyl ester (**13**) (1.44 g, 6.94 mmol) in 18.5 mL THF was added slowly. The mixture was stirred at −78 °C for 1 h. The reaction was quenched by the addition of 30 mL satd. $NaHCO_3$ and 75 mL water. Solvent THF was removed by rotary evaporation and the residual mixture was treated with 125 mL of ethyl acetate. The organic layer was washed with water, brine, dried over Na_2SO_4, and evaporated to dryness. The crude product was purified by silica gel flash column chromatography and eluted with 2% methanol in chloroform. Recrystallization from diisopropyl ether gave a white crystalline solid in a yield of 4% (60 mg).The product was characterized by mp 103–104 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.72 (s, 2H), 4.92 (d, J = 5.01 Hz, 2H), 7.22–7.44 (m, 5H), 7.74 (d, J = 2.9 Hz, 1H), 8.06 (d, J = 3.0 Hz, 1H), 6.04 (br t, 1H, NH); ν_{max} (KBr) 1705 cm^{-1} (C=O). Anal. calcd for $C_{13}H_{12}N_2O_2S$: C, 59.98; H, 4.65; N, 10.65; S, 12.32. Found: C, 60.23; H, 4.43; N, 10.65; S, 12.30.

2-(Phenylacetylglycyl) imidazole (3)

The synthesis of 2-(phenylacetylglycyl) imidazole is outlined in Scheme 6.

1-[2′-(trimethylsilyl)ethoxy]methyl-2-(phenylacetylglycyl)-imidazole (15). To a cold (−78 °C), stirred solution of *n*-butyl lithium [25.2 mmol; 15.8 mL of a 1.6 M solution in hexane (Aldrich)] under nitrogen atmosphere in THF (30.4 mL) was added dropwise a solution of 1-SEM-imidazole (**14**)[24] (5 g, 25.22 mmol) in the same solvent (25 mL). After the solution had been stirred at −78 °C for 1 h, a solution of phenylacetylglycine methyl ester (**13**) (2.39 g, 11.51 mmol) in 30.4 mL THF was added slowly. The mixture was stirred for 1 h at −78 °C. The reaction was quenched with 50 mL of saturated $NaHCO_3$. The reaction mixture was taken into 125 mL of water and the THF was removed by rotary evaporation. The residual aqueous layer was extracted with ethyl acetate. The organic layer was washed with water (2×), brine (2×), dried over Na_2SO_4 and evaporated to dryness. The crude product, a dark tan oil, was further purified by silica gel flash column chromatography using a ethyl acetate/hexane (4:1) mixture to elute the colorless product in 42% (1.8 g) yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0 (s, 9H), 0.95 (t, J = 5.8 Hz, 2H), 3.55 (t, J = 6.8 Hz, 2H), 3.70 (s, 2H), 4.82 (br s, 1H), 5.74 (s, 2H), 6.4 (br s, 1H), 7.2–7.44 (m, 7H).

2-(Phenylacetylglycyl)imidazole (3). 1-SEM-2-(phenylacetylglycyl)imidazole (**15**) (50 mg, 0.13 mmol) was stirred at room temperature with 3 mL of trifluoroacetic

acid/methylene chloride (2:1) and reaction was followed by monitoring the disappearance of starting material on TLC [R_f = 0.5 with ethyl acetate/hexane (4:1)]. After 4 h, the reaction was stopped and the solvents were evaporated to dryness to give the product in protonated form. This was taken into 10 mL ethyl acetate and washed with 5 mL saturated $NaHCO_3$. The aqueous layer was further washed twice with 10 mL ethyl acetate. The organic layers were combined, dried over Na_2SO_4 and evaporated to dryness. Final purification was achieved by silica gel flash column chromatography with an ethyl acetate/hexane mixture (17:3) as solvent, followed by recrystallization from CH_3CN to give pale yellow product in 56% yield (18 mg). Mp 160–161 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.7 (s, 2H), 4.83 (d, J = 5.2 Hz, 2H), 7.22–7.42 (m, 7H); ν_{max} (KBr) 1697.7 cm^{-1} (C=O); MS (ESI+): calcd for $(C_{13}H_{13}N_3O_2)^+$ 244.1, found 244.1.

5-(Phenylacetylglycyl)tetrazole sodium salt (4)

The synthesis of 5-(phenylacetylglycyl)tetrazole is outlined in Scheme 7.

1-benzyloxymethyl-5-(phenylacetylglycyl)tetrazole (17). To a stirred cold (−78 °C) solution of 4 g (21.1 mmol) 1-benzyloxymethyltetrazole (**16**)[25] in 100 mL THF under a nitrogen atmosphere was added *n*-butyl lithium (21.1 mmol; 12.9 mL of 1.6 M solution in hexane) dropwise. After the solution had been stirred for 0.5 h, a solution of phenylacetylglycine methyl ester (2.18 g, 10.5 mmol) in 50 mL THF was added slowly and the mixture was stirred for 40 min. The reaction mixture was allowed to come to room temperature and the reaction was quenched with 100 mL saturated NH_4Cl. THF was selectively evaporated and the aqueous layer was extracted with 150 mL (3×) ethyl acetate. The organic layers were combined, washed with 200 mL brine, dried over $MgSO_4$, and evaporated to dryness. This crude product was partially purified by silica gel flash column chromatography using acetone/hexane mixture (4:6) and, although still contaminated with a little unreacted ester, was directly used in the next step of the synthesis. ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.70 (s, 2H), 6.01 (s, 2H), 7.3–7.5 (m, 5H), 8.63 (s, 1H).

5-(phenylacetylglycyl) tetrazole sodium salt (4). The product (**17**) from the previous step (100 mg) was stirred with 5 mL trifluoroacetic acid (TFA) at room temperature for 5 h. After evaporation of the TFA, the crude product was dissolved in water and the pH was brought to 7.5 with the help of $NaHCO_3$. The aqueous layer was first extracted with 20 mL (3×) ethyl acetate and then freeze-dried. Final purification was achieved by aqueous Sephadex G-10 column chromatography, yielding a white hygroscopic solid in 23% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.6 (s, 2H), 4.6 (d, J = 5.01 Hz, 2H), 7.22–7.36 (m, 5H), 8.34 (br s, 1H); MS (ESI-): calcd for $(C_{11}H_{10}O_2N_5)^-$ 244.2, Found 244.1

Sodium 2-(phenylacetylglycyl)thiazole-4-carboxylate (5)

The synthesis of sodium 2-(phenylacetylglycyl)thiazole-4-carboxylate is outlined in Scheme 8.

Thiazole-4-carboxylic acid (18). This compound was obtained by a small modification of the synthetic route followed by Jagoe et al.[38] To a stirred solution of 9.5 g (21.1 mol) of formamide in 95 mL THF, which was cooled to 0 °C, was added all at once 9.5 g (21.4 mmol) of solid P_4S_{10}. After this addition, the ice bath was removed and the reaction was stirred at room temperature for 6 h. From the resultant mixture, 35 mL of THF solution was decanted into a clean dry flask. The solution was cooled to 0 °C and 10.5 g (63.0 mmol) of solid 3-bromopyruvic acid hydrate (Aldrich) was added all at once. The reaction mixture was then heated at 45–50 °C for 5.5 h. After the mixture was cooled to room temperature, the product, which precipitated, was collected by vacuum filtration and washed with acetone to give 7.1 g (53% yield) of thiazole-4-carboxylic acid hydrobromide. This was converted to the neutral form by treatment with one equivalent of $NaHCO_3$ at 0 °C. The crude product was purified by recrystallization from pentanol to give a colorless powder in 27% (3.59 g) yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.53 (s, 1H), 9.17 (s, 1H).

Sodium 2-(phenylacetylglycyl) thiazole-4-carboxylate (5). To a cold (−78 °C) stirred solution of *n*-butyl lithium (35.2 mmol; 22 mL of 1.6 M solution in hexane) in 20 mL THF was added dropwise a solution of 1.5 g (11.6 mmol) thiazole-4-carboxylic acid (**18**) in 45 mL THF. After the solution had been stirred at −78 °C for 0.5 h, a solution of 1.19 g (5.7 mmol) phenylacetylglycine methyl ester (**13**) in 20 mL THF was added slowly . The reaction mixture was stirred for 45 min at −78 °C and for a further 30 min after removing it from the −78 °C bath. The reaction was quenched with the successive addition of saturated $NaHCO_3$ solution (30 mL) and water (70 mL). THF was preferentially evaporated and the aqueous layer was washed with 350 mL ethyl acetate. After the pH of the aqueous layer was reduced to 1.5 with HCl, the solution was extracted with 100 mL ethyl acetate (3×). The organic layer was then washed with 100 mL water and 100 mL brine, dried over $MgSO_4$ and evaporated to dryness to give the crude product. Purification was achieved using (i) Sephadex LH-20 column chromatography with methanol as solvent and (ii) G-10 aqueous column chromatography of the sodium salt to give the final product in 1.5% yield (26 mg). ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.58 (s, 2H), 4.74 (d, J = 4.7 Hz, 2H,), 7.22–7.39 (m, 5H), 8.20 (s, 1H), 8.52 (br t, 1H); MS (ESI+1): calcd for ($C_{14}H_{11}N_2O_4SNa$) 327.05, found 327.2.

Acknowledgements

This research was supported by the National Institutes of Health.

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