Synthesis of pseudoxazolones and their inhibition of the 3C cysteine proteinases from hepatitis A virus and human rhinovirus-14

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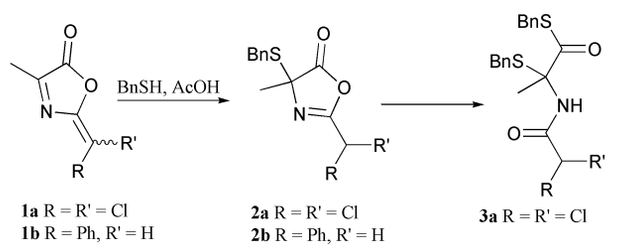
The hepatitis A virus (HAV) and human rhinovirus (HRV) are important pathogens that belong to the picornavirus family. All picornaviruses have a 3C proteinase that processes an initially biosynthesised precursor protein and is crucial for viral maturation and replication. Monophenyl and diphenyl pseudoxazolones were prepared by cyclisation–elimination of *N*-α-chloroacyl amino acids or by condensation of *p*-substituted benzamides with glyoxylic acid followed by dehydrative cyclisation. Such pseudoxazolones are good time-dependent inhibitors of the HAV and HRV 3C proteinases with IC_{50} values in the micromolar range. Mechanistic insights into the mode of inhibition of the pseudoxazolones were obtained from mass spectrometry and gHMQC NMR spectroscopy of the HAV 3C enzyme–inhibitor complex using a pseudoxazolone labelled at the α-carbon with ^{13}C, **13b**(α-^{13}C). The results indicate that HAV 3C proteinase was inactivated *via* the formation of a thioether bond by nucleophilic attack of the enzyme thiolate at the imine position of the pseudoxazolone **13b**(α-^{13}C).

Introduction

Picornaviruses are small, positive-sense, single stranded RNA viruses that cause a wide variety of diseases in humans and animals.[1] Members of this family include important pathogens such as the human rhinovirus (HRV), hepatitis A virus (HAV), poliovirus (PV) and foot and mouth disease virus (FMDV).[1] HAV produces an acute form of infectious hepatitis, is most commonly found in developing countries (although occasional cases still occur elsewhere) and is usually spread through the contamination of food or water.[2] Human rhinovirus is the major cause of common cold in humans, and because more than 100 serotypes exist, development of a vaccine against HRV is considered problematic.[1] The picornaviral RNA genome encodes for a single large polyprotein (~250 kDa) precursor which is cleaved by the viral 2A and/or 3C proteinases to generate the structural and nonstructural viral components.[1,3] In HRV the first cleavage is mediated by the 2A proteinase and then further processed by the 3C proteinase. However, in HAV the 3C proteinase is the sole enzyme involved in the initial as well as all secondary cleavages.[1] Because of the importance of the 2A and 3C proteinases in viral replication and the conservation of active site residues among a wide range of serotypes, these enzymes are attractive targets for drug design and development.

Although the 3C enzymes are cysteine proteinases, X-ray crystal structures of HAV 3C,[4] HRV 3C[5] and PV 3C[6] have revealed that these enzymes are structurally related to the chymotrypsin-like serine proteinases rather than the cysteine proteinases of the papain family. The structure consists of two β-barrel domains with nearly identical topology. The 3C proteinases cleave specifically after a glutamine residue at the P_1 site,[7] and an additional four to five residues around the scissile bond contribute to substrate recognition. The active site cysteine (Cys-172 in HAV, Cys-146 in HRV-14) acts as the nucleophile which is assisted by a general acid–base catalyst (His-44 in HAV, His-40 in HRV-14) with the formation of a tetrahedral intermediate stabilized by an electrophilic oxyanion hole.[1] Several classes of inhibitors have been reported for HAV and HRV 3C proteinases[8] by our group and others. These include peptide aldehydes,[9] halomethyl ketones,[10] azapeptides,[11] α,β-unsaturated compounds,[12] benzamides,[13] iodoacetamides,[14] azodicarboxamides,[15] β-lactones[16] and keto-glutamines.[17]

Pseudoxazolones **1a** and **1b** are known to add one thiol at the imine position to give adduct **2a** and **2b** (Fig. 1), and futher



1a R = R' = Cl
1b R = Ph, R' = H
2a R = R' = Cl
2b R = Ph, R' = H
3a R = R' = Cl

Fig. 1 Reaction of pseudoxazolones with benzyl thiol.

addition of thiol at the carbonyl position of **2a** gives the thioester **3a**.[18] These observations led us to believe that pseudoxazolones could be used to inhibit thiol-containing enzymes, in particular, cysteine proteinases. In a recent communication we reported initial work on the inhibition of HAV and HRV 3C proteinases by pseudoxazolones.[19] In the present study we investigate the interaction of additional pseudoxazolones with these enzymes and examine the mode of inhibition. Mass spectrometric analysis of the enzyme–inhibitor complex reveals the formation of a covalent 1 : 1 enzyme–inhibitor adduct. Examination of the enzyme–inhibitor complex generated from ^{13}C-labelled monophenyl glycine pseudoxazolone **13b**(α-^{13}C) using gHMQC NMR spectroscopy indicates that the enzyme thiol adds to the imine rather than the carbonyl moiety of the inhibitor.

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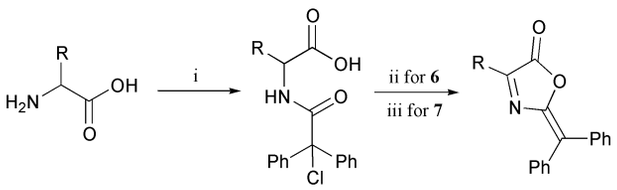
Results and discussion

Synthesis and enzyme inhibition of pseudoxazolones

The diphenyl pseudoxazolones of glycine **6** and alanine **7** were our initial targets. These compounds are prepared using a modified literature procedure as outlined in Scheme 1.[20]



4 R = H
5 R = Me

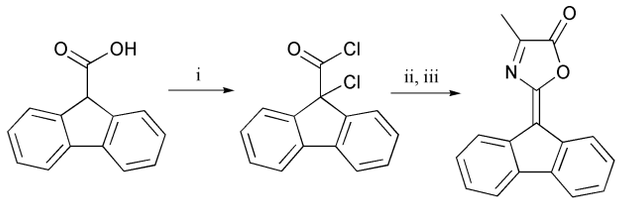
6 R = H
7 R = Me

Scheme 1 *Reagents and conditions*: (i) 2-chloro-2,2-diphenylacetyl chloride, propylene oxide, ethyl acetate, Δ; (ii) DCC, propylene oxide, CH_3CN, 80% over 2 steps; (iii) Ac_2O, pyridine, 68% over 2 steps.

Reaction of glycine **4** and alanine **5** with 2-chloro-2,2-diphenylacetyl chloride gives the corresponding chloro adducts which are cyclized with acetic anhydride–pyridine or DCC followed by elimination of HCl to afford the desired compounds **6** and **7**, respectively.

These pseudoxazolones were tested as inhibitors of the HAV and HRV-14 3C proteinases. Assay of HAV 3C proteinase employs an overexpressed C24S mutant in which a non-essential surface cysteine is substituted by serine. This mutant enzyme displays catalytic parameters indistinguishable from the native proteinase.[21] Removal of this surface cysteine is useful as it prevents enzyme dimerisation through disulfide formation. The HAV 3C activity is monitored using a fluorimetric assay at an enzyme concentration of approximately 0.1 μM with Dabcyl-GLRTQSFS-Edans as the substrate. Cleavage at the carbonyl of the glutamine residue of this peptide, which has a fluorescent moiety and a quenching group at opposite ends, results in increased fluorescence. The HRV 3C assays use the enzyme from serotype 14 at a concentration of approximately 0.4 μM, and the activity is conveniently monitored using a continuous UV assay in the presence of EALFQ-pNA as the substrate.[22] This assay measures the increased absorbance at 405 nm due to release of free *p*-nitrophenylanilide (pNA). In both cases the enzyme is incubated with the appropriate inhibitor for 5 min and the assay is initiated by the addition of substrate. Testing of the diphenyl pseudoxazolone **6** against HAV 3C gives a time dependent inhibition with an IC_{50} of 33 μM (Table 1). However, the alanine derivative **7** displays less potency with an IC_{50} > 100 μM. Presumably the extra methyl group provides steric bulk that hinders interaction with the nucleophilic cysteine thiolate of the enzyme. The HRV 3C proteinase also displays the same type of behaviour. However, one limitation is the low stability of the pseudoxazolones in phosphate buffer at pH 7.5, (*e.g.* **6** has a half-life of 40 min). The pseudoxazolones are relatively easily hydrolysed under the slightly basic conditions used in the assay.

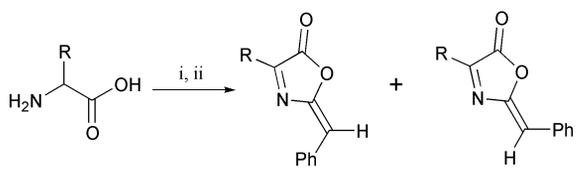
Our next goal was to investigate the analogue **10**, since the planar fluorenone system would be expected to display different chemical behaviour than **6** or **7**, wherein the two mobile phenyl rings are unlikely both to be simultaneously coplanar with the pseudoxazolone. This could potentially lead to improved inhibition and/or chemical stability. The alanine derivative is readily synthesized as depicted in Scheme 2. Reaction of fluorene-9-carboxylic acid **8** with thionyl chloride gives the α-chloro acyl chloride **9**,[23] which upon reaction with alanine followed by cyclization with TFAA affords the desired compound **10**. The glycine derivative could also be prepared but is quite unstable. Although **10** has better aqueous stability ($t_{½}$ = 68 min) than **6** or **7**, testing with HAV and HRV 3C proteinases did not show a great improvement in inhibition with an IC_{50} of 68 μM for the former enzyme.



8 **9** **10**

Scheme 2 *Reagents and conditions*: (i) $SOCl_2$, Δ, 29%; (ii) alanine **5**, propylene oxide, EtOAc; (iii) TFAA, 0 °C, 76% over 2 steps.

To examine the structural influences and to potentially identify better inhibitors, the known[20b,c] monophenyl pseudoxazolones of glycine **13a**,**b** and alanine **14a**,**b** were prepared as shown in Scheme 3. Condensation of glycine **4** or alanine **5** with



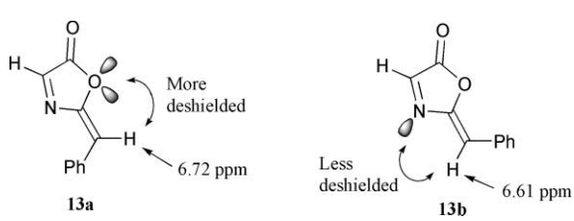
4 R = H
5 R = Me
11 R = $(CH_2)_3CONH_2$
12 R = $(CH_2)_2CONH_2$

13a R = H, 3%
14a R = Me, 38%
15a R = $(CH_2)_3CONH_2$, 7%
16a R = $(CH_2)_2CONH_2$, 9%

13b R = H, 14%
14b R = Me, 38%
15b R = $(CH_2)_3CONH_2$, 11%
16b R = $(CH_2)_2CONH_2$, 10%

Scheme 3 *Reagents and conditions*: (i) DL-2-chloro-2-phenylacetyl chloride, NaOH, H_2O, 0–5 °C; (ii) Ac_2O, pyridine; the yields reported are over two steps (unoptimised).

DL-2-chloro-2-phenylacetyl chloride gives the corresponding chloro acids, which cyclise with acetic anhydride and pyridine to afford the pseudoxazolones **13a**,**b** and **14a**,**b**, respectively. Although these compounds have previously been reported as a mixture of *E* and *Z* isomers,[20b,c] in both cases we were able to separate the pure geometrical isomers by flash column chromatography for full characterisation. Assignments of the *E* and *Z* isomers are made based on the proton (^1H) chemical shift of the olefinic hydrogen. The *E* isomer (*e.g.* **13a**, Fig. 2) has its olefinic



13a **13b**

Fig. 2 ^1H Chemical shift of the glycine pseudoxazolone olefinic proton in acetone-d_6 (300 MHz).

proton aligned with the oxazolone ring oxygen and is more deshielded whereas the *Z* isomer (*e.g.* **13b**) is aligned with the imine nitrogen and is therefore less deshielded. These observations are confirmed by two X-ray crystal structures of **13b** and **14a**, which define the geometry of the double bond (Fig. 3).[19] The NMR analysis could then be used to unambiguously assign the *E* and *Z* configuration of the other monophenyl pseudoxzolones described below. Although interconversion of the *E* and *Z* double bond is possible, this was not observed for the monophenyl pseudoxazolones.

Enzymatic testing with **13a** and **13b** shows time-dependent inhibition with IC_{50} values of 6 μM and 4 μM for HAV and 16 and 17 μM for HRV 3C, respectively. The minor disparity in inhibition levels for the two enzymes may be due primarily to different concentrations of enzyme (0.1 μM for HAV and 0.4 μM for HRV 3C) because of the limitations of the UV assay used in the latter. Although **13a** and **13b** give similar levels of inhibition, compounds **14a** and **14b** are much weaker inhibitors

Table 1 Inhibition data for HAV 3C, HRV 3C and half-life of the pseudoxazolones in phosphate buffer at pH 7.5

Compound	HAV 3C IC_{50}/μM [a]	HRV 3C IC_{50}/μM [b]	Aqueous hydrolysis $t_{½}$/min [c]
6	33	38	40
7	>100	>100	15
10	68	>100	68
13a	6	16	28
13b	4	17	16
14a	26	43	17
14b	>100	>100	14
15a	43	>100	29
15b	>100	>100	13
16a	47	>100	24
16b	>100	>100	12
21a	3	11	17
21b	4	13	19
22a	20	N.d.[d]	21
22b	10	N.d.[d]	39

[a] Fluorimetric assay conditions: 0.1 μM HAV 3C, 10 μM Dabcyl-GLRTQSFS-Edans, 2 mM EDTA, 0.1 mg cm^{-3} BSA, 100 mM KH_2PO_4–K_2HPO_4, pH 7.5, 1% DMF, 5 min pre-incubation of the enzyme with inhibitor. [b] Continuous UV assay conditions: 0.4 μM HRV 3C, 250 μM EALFQ-pNA, 50 mM HEPES, 150 mM NaCl, 1 mM EDTA, pH 7.5, 1% DMF, 5 min pre-incubation of the enzyme with inhibitor. [c] Pseudoxazolone hydrolysis half-life in phosphate buffer at pH 7.5. [d] N.d. = not determined.

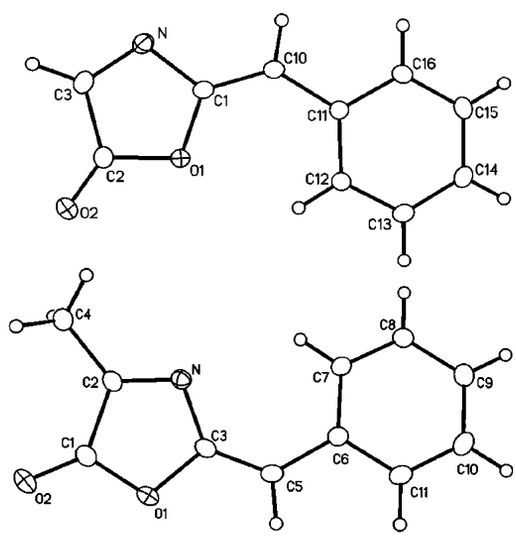
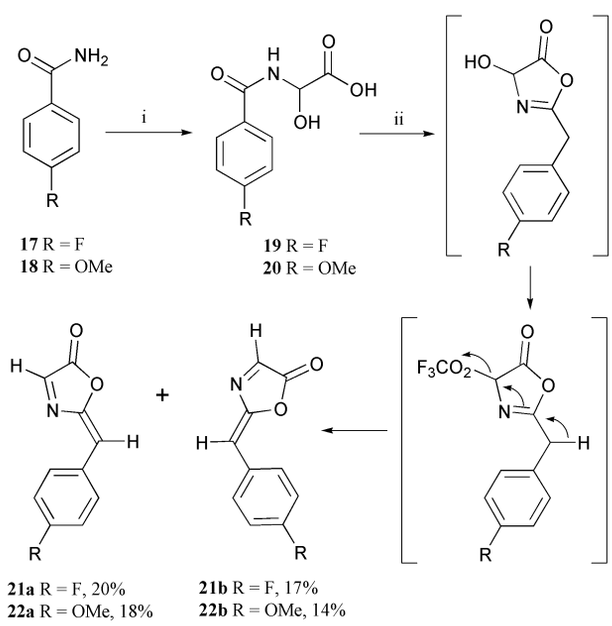


Fig. 3 Crystal structure of **13b** (top) and **14a** (bottom).

with IC_{50} values of 26 μM and >100 μM, respectively, for HAV 3C. The enzyme appears to prefer the *E* over the *Z* isomer when there is substitution at the imine carbon.

Since the HAV and HRV 3C proteinases require a glutamine residue at the P_1 site for recognition of the peptide substrate, our next approach was to incorporate a glutamine side chain on the monophenyl pseudoxazolone to potentially enhance the recognition and binding of the inhibitors. Although substitution at the imine position decreases the potency of the inhibitor (*cf.* **13** *vs.* **14**), it seemed worthwhile to evaluate the effect of such a glutamine side chain as it is a key recognition element for 3C proteinases.[4–6] Both the homoglutamine **15a**,**b** and glutamine **16a**,**b** side chain analogues were prepared as described in Scheme 3. However, testing of **15a**,**b** and **16a**,**b** gave no improved inhibition against HAV and HRV 3C. Again the same trend is observed: the *E* isomers **14a** and **15a** are better inhibitors than the corresponding *Z* monophenyl pseudoxazolones **14b** and **15b**.

Having determined that the monophenyl glycine pseudoxazolones are the most potent, the effect of electron withdrawing (*e.g.* fluoro) and electron donating (*e.g.* methoxy) substituents on the phenyl ring was investigated. Since derivatives of 2-chloro-2-phenylacetyl chloride with substituents such as fluoro or methoxy in the side chain can be difficult to generate, a new preparative route was used for these pseudoxazolones. A hydroxy group at the α-position of an *N*-acylglycine (*e.g.* **19** and **20**) can, in principle, be converted to a leaving group *in situ* to assist cyclisation. Thus, reaction of amide **17** or **18** with glyoxylic acid[24] gives the corresponding hydroxy acids **19** and **20**, respectively (Scheme 4). Cyclization



Scheme 4 *Reagents and conditions*: (i) glyoxylic acid monohydrate, acetone, Δ; (ii) TFAA, CH_2Cl_2, RT; the yields reported are over two steps (unoptimised).

with TFAA followed by elimination of the activated α-hydroxy group as trifluoroacetate affords the desired *p*-fluoro **21a**, **21b** and the *p*-methoxy **22a**, **22b** derivatives, respectively.

Incubation of these compounds with HAV and HRV 3C proteinases displayed potent time-dependent inhibition with IC_{50} values in the range 3 to 13 μM for **21a** and **21b** (Table 1). The *p*-methoxy derivatives **22a** and **22b** are also effective against HAV 3C, with IC_{50} values of 20 and 10 μM, respectively. The latter compounds were not tested against the HRV 3C enzyme because of their strong absorbance at 405 nm, the wavelength used to detect the release of *p*-nitroanilide from the substrate upon enzymatic cleavage. Although no significant increase in aqueous stability is observed for the compounds **21a**, **21b**, **22a** and **22b**, these results demonstrate that additional functionality

on the phenyl ring is well tolerated by the enzymes. Potentially, such pseudoxazolone systems with appropriate functional groups to assist recognition can be applied to inhibition of other cysteine proteinases and thiol-containing enzymes.

Mechanism of inhibition

As mentioned above, enzyme inactivation may involve covalent modification of the active site cysteine residue by nucleophilic addition of the thiolate to the imine moiety of the pseudoxazolone. To examine the influence of extraneous thiols, **13a** and **13b** were tested against HAV 3C in the presence of dithiothreitol (DTT). These pseudoxazolones show complete loss of inhibitory activity in the presence of a tenfold excess of DTT, thereby indicating that the added thiol reacts rapidly with these compounds. This clearly demonstrates that such compounds are unlikely to be useful drug candidates without considerable modification. However, they can serve as valuable biochemical tools and important information may be gained by structural analysis of enzyme–inhibitor adducts.

Further insight on the formation of a covalent adduct was obtained by analysis of the enzyme–inhibitor complex by mass spectrometry. Both HAV and HRV 3C were incubated with **13b** in DMF for 1 h. The mixtures were dialysed and subjected to MALDI-TOF mass spectrometry. The HAV 3C mass spectrum showed peaks at 23871(±10) for the enzyme alone and 24066(±10) for the enzyme–inhibitor complex (Fig. 4). This

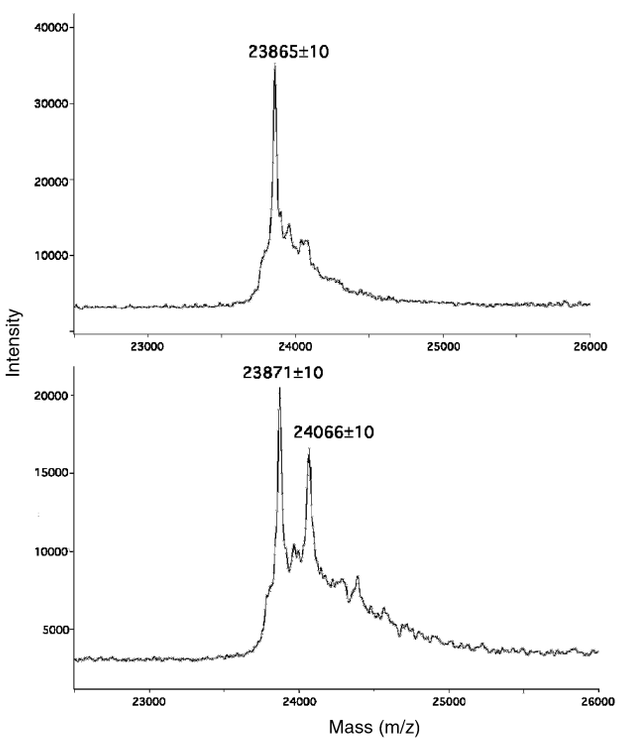


Fig. 4 MALDI-TOF mass spectra of HAV 3C + DMF (top) and HAV 3C + **13b** in DMF (bottom).

mass difference of 195 is within experimental error for the incorporation of the inhibitor **13b** (MW 173) plus water (MW 18). In a control experiment the HAV 3C was incubated with DMF containing no inhibitor, and this showed a mass of 23865(±10) (Fig. 4). The same type of result was obtained with HRV 3C, which gave a mass of 19995(±10) for the enzyme alone and 20184(±10) for the enzyme–inhibitor complex, with a mass difference of 189 corresponding to the addition of inhibitor **13b** plus water.

These results clearly support the formation of a covalent adduct with the enzyme and the inhibitor, rather than a non-covalent tight-binding complex, which would be expected

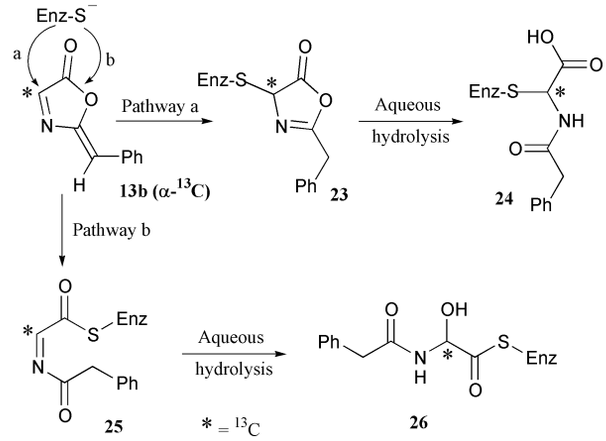
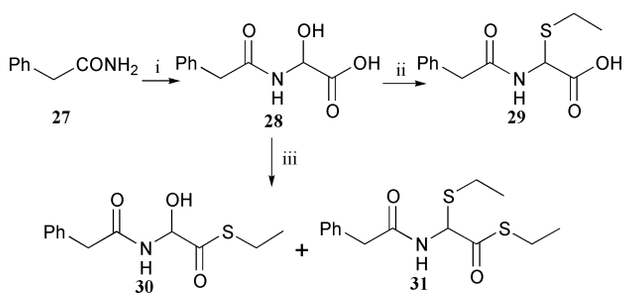


Fig. 5 Possible modes of inhibition of cysteine proteinase by the glycine monophenyl pseudoxazolone **13b**(α-^{13}C).

to dissociate upon extensive dialysis. A possible mode of inactivation of cysteine proteinase is shown in Fig. 5. Addition of the enzyme thiolate at the imine position of the inhibitor **13b** (pathway a) would form a thioether-substituted lactone **23**, which could add water to generate the thioether-acid **24**. However, the possibility of addition of the enzyme thiolate at the carbonyl of the oxazolone system cannot be ignored as it is also a reactive site. Initial condensation at this position would produce the thioester **25** (pathway b), which could subsequently add water at the imine position to form the hydroxy-thioester **26**. In each pathway, there is also the less likely prospect that attack by water could be replaced by reaction with another nucleophilic group on the enzyme.

To determine the type of adduct formed in the enzyme–inhibitor complex, compound **13b**(α-^{13}C) was prepared from [2-^{13}C]glycine as described in Scheme 1. In addition, model compounds such as the hydroxy acid **28**, thioether **29**, and thioester **30** were made (Scheme 5) to assist NMR analysis of



Scheme 5 *Reagents and conditions*: (i) glyoxylic acid monohydrate, acetone, Δ, 49%; (ii) EtSH, AcOH, H_2SO_4, 69%; (iii) EDCI, DMAP, NEt_3, EtSH, CH_2Cl_2 (12% for **30** and 6% for **31**).

the enzyme–inhibitor complex. Reaction of phenyl acetamide **27** with glyoxylic acid[24] gives the hydroxy acid **28** which upon reaction with ethanethiol under acidic condition affords the thioether **29**.[25] Alternatively, activation of the hydroxy acid **28** with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) followed by coupling with ethanethiol, produces the hydroxy-thioester **30** and the thioester **31** as a side product. The chemical shifts for the α-carbon of **13b**(α-^{13}C), **28**, **29** and **30** are shown in Fig. 6. The gHMQC spectrum of the pseudoxazolone **13b**(α-^{13}C) alone shows a cross peak at δ_C 148 ppm for the α-labelled carbon (Fig. 7). Upon formation of the enzyme–inhibitor [HAV 3C-**13b**(α-^{13}C)] complex a new peak appeared at δ_C 57 ppm for the α-carbon. This corresponds to the formation of a thioether (as opposed to a thioester), in accord with the α-carbon chemical shift observed for the thioether **29** (δ_C 54 ppm) under identical conditions.

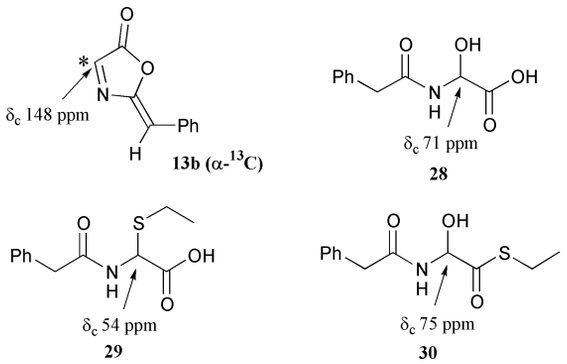


Fig. 6 α-Carbon chemical shifts for model compounds in DMF-d_7 and Na_3PO_4–D_2O at pD = 7.5. A 600 MHz Varian Inova NMR spectrometer was used to acquire the gHMQC spectra.

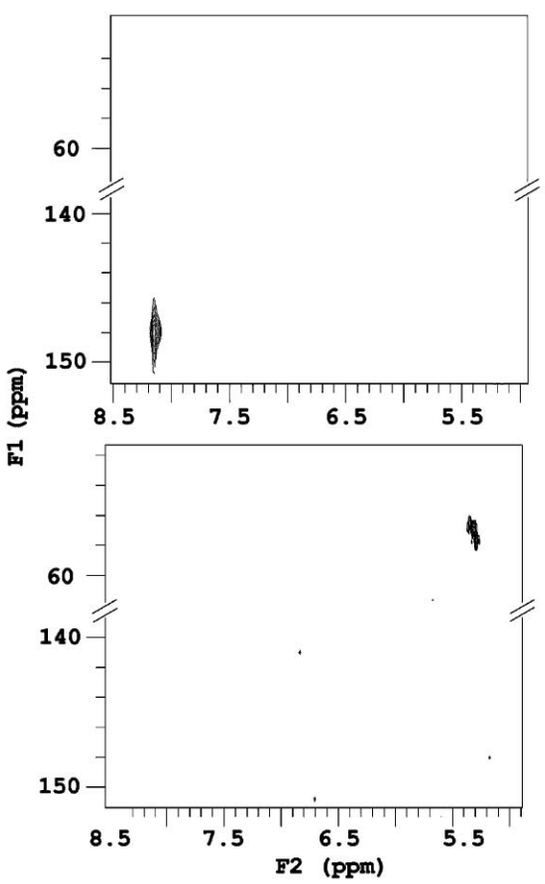


Fig. 7 Expansions of the gHMQC spectra of the inhibitor **13b**(α-^{13}C) alone (top) and the enzyme–inhibitor complex [HAV 3C-**13b**(α-^{13}C)] (bottom) in DMF-d_7 and Na_3PO_4–D_2O, pD = 7.5 at 600 MHz.

Conclusions

In summary, we have shown that the pseudoxazolones are potent time-dependent inhibitors of the HAV 3C and HRV 3C proteinases at micromolar levels. The monophenyl pseudoxazolones were found to be more effective than the diphenyl analogues, and the glycine derivatives are in turn more active than the pseudoxazolones with substitution at the α-position. Using X-ray crystallographic analysis and ^{1}H NMR spectroscopy, it is possible to assign the geometrical isomers of the monophenyl pseudoxazolones. A new pathway to access substituted phenyl derivatives of the pseudoxazolones has been devised starting from a phenyl acetamide and glyoxylic acid. Although the pseudoxazolones display low stability in phosphate buffer at pH 7.5 ($t_{½}$ = 12–68 min), further chemical modification could generate more stable compounds. Mass spectrometry, and gHMQC NMR spectroscopy experiments using ^{13}C labelled pseudoxazolone **13b**(α-^{13}C) demonstrate that the HAV 3C enzyme forms a covalent adduct with the inhibitor *via* initial creation of a thioether bond. Because of their reactivity towards thiols (*e.g.* dithiothreitol), these pseudoxaxolones are unlikely to be drug candidates, but they can be used as biochemical tools and to assist structural analysis of proteins (*e.g.* crystallographic studies), including other cysteine proteinases and thiol-dependent enzymes.

Experimental

General methods and enzyme assays

Most general procedures and instrumentation have been previously described.[26] Both HAV an HRV 3C proteinases were expressed and purified according to previously described procedures.[21a,27] The HAV 3C proteinase assays employed a C24S mutant in which the nonessential surface cysteine was replaced by serine and which displays catalytic parameters similar to the wild type enzyme.[21a] The purity of the enzyme samples was greater than 90% as determined by SDS-PAGE analysis (data not shown).[21a,27] The HAV 3C proteinase concentration was determined spectrophotometrically ε_{280} = 1.2 mg cm^{-3}. Cleavage reactions (700 μl) were performed at 30 °C in a solution containing 100 mM KH_2PO_4–K_2HPO_4 at pH 7.5, 2 mM EDTA, 0.1 mg cm^{-3} bovine serum albumin (BSA), 10 μM fluorogenic substrate Dabcyl-GLRTQSFS-Edans (Bachem), 0.1 μM HAV 3C proteinase and 1% DMF.[21,22] The activity was monitored by a fluorimetric assay similar to one described for HRV 3C proteinase.[22] Increase in fluorescence (λ_{ex} 336 nm, λ_{em} 472 nm) was continuously monitored using a Shimadzu RF5301 spectrofluorimeter. In the case of HRV, the 3C proteinase from serotype 14 was used and the concentration was determined using the BSA assay. HRV 3C assays (1 cm^3) were performed at 30 °C in a solution containing 50 mM HEPES, 150 mM NaCl, 1 mM EDTA, pH 7.5, 1% DMF, 0.4 μM HRV 3C and 250 μM EALFQ-pNA (Bachem) as the substrate. The increase in absorbance at λ 405 nm was measured using a GBC Cintra 40 UV spectrometer against a blank containing buffer and the appropriate inhibitor. In both cases, the proteinases were dialysed against reaction buffer to remove DTT immediately prior to use. For proteinase inhibition studies, the initial 3 minutes of the reaction were used for calculation purposes. Inhibitor stock solutions were prepared at 10 mM in DMF and serial dilutions made in DMF. At least five different inhibitor concentrations were examined along with a control sample containing no inhibitor under the conditions described above. The proteinase activity in the presence of the specified inhibitor was expressed as a percentage of that obtained from the respective control samples. For inhibitors displaying dose-dependent inhibition of the proteinase activity, IC_{50} values were determined from plots of the relative proteinase activity *versus* the log of inhibitor concentration. IC_{50} values were not determined for compounds showing weak inhibition. The sensitivity of inhibitors **13a** and **13b** to dithiothreitol (DTT) was evaluated using reactions similar to those described above, but with the addition of up to 0.1 mM molar concentrations of DTT to the inhibitor-containing mixture followed by the addition of enzyme. X-Ray crystallographic data for **13b** and **14a** have been reported in our initial communication[19] and deposited to the Cambridge Structural Database, CCDC deposition numbers 172532 and 172533.

Rate of hydrolysis of pseudoxazolones in phosphate buffer

The hydrolysis was followed using a Hewlett Packard 8452A diode array spectrometer. A solution containing 100 mM KH_2PO_4–K_2HPO_4 at pH 7.5, 2 mM EDTA and 100 μM of the appropriate inhibitor in DMF was prepared, and the disappearance of the pseudoxazolones imine band (~350 nm) was monitored over a 1 h period (the $t_{½}$ data reported are within ±10% error).

Mass spectrometry of HAV and HRV 3C-13b inhibitor complexes

The enzymes were dialysed separately against a solution containing 2 mM EDTA and 100 mM KH_2PO_4–K_2HPO_4 at pH 7.5 to remove DTT using a Centriprep-10 (Amicon) centricon ultrafiltration unit. The dialysed enzymes (~0.2 mM) were incubated with 10 equivalents of **13b** and 1% DMF at 25 °C for 1 h with mixing. The enzyme–inhibitor complexes were then dialysed against deionised H_2O for 1 h to a volume of approximately 300 µl. In addition, a control parallel experiment was performed on the enzyme alone without inhibitor **13b**. Mass spectrometric analysis was performed on a Voyager Elite MALDI-TOF instrument (Applied Biosystems) equipped with delayed extraction and an ion mirror (reflectron). Sinapinic acid was used as the matrix.

$^1H/^{13}C$ gHMQC spectroscopy of model compounds, 13b(α-^{13}C), 28–30, HAV 3C and HAV 3C-13b(α-^{13}C) inhibitor complex

Solutions of individual compounds **13b**(α-^{13}C) and **28**–**30** in 20% DMF-d_7 in 20 mM Na_3PO_4–D_2O at pD 7.5 were prepared to give a total volume of 700 µl each. Prior to use, DTT was removed from the enzyme preparation by dialysis with a Centriprep-10 (Amicon) centricon ultrafiltration unit with 20 mM Na_3PO_4–D_2O at pD 7.5. The resulting enzyme solution (0.5 mM) was incubated with the inhibitor **13b**(α-^{13}C) (5 mM) and 1% DMF-d_7 for 1 h. The enzyme–inhibitor complex was dialysed against Na_3PO_4–D_2O at pD 7.5 several times for 3 h, and concentrated to a volume of approximately 700 µl. Model compounds **13b**(α-^{13}C) and **28**–**30**, HAV 3C alone and the HAV 3C-**13b**(α-^{13}C) enzyme inhibitor complex were analysed by gHMQC NMR using a 600 MHz Varian Inova instrument. The parameters for model compound **13b**(α-^{13}C): temperature: 27 δC, solvent: D_2O, number of transients: 1, number of increments: 512, number of data points: 3000, acquisition time: 0.250 s, sweep width in *F*2: 6001 Hz, sweep width in *F*1: 29996 Hz. The same parameters as above were used for the HAV 3C-**13b**(α-^{13}C) enzyme inhibitor complex except the number of transients = 16 and $^1H,^{13}C$ decoupling was applied.

2-(Diphenylmethylene)-2*H*-oxazol-5-one 6

2-Chloro-2,2-diphenylacetyl chloride (6.6 g, 25.0 mmol) was dissolved in dry EtOAc (100 cm^3) under an argon atmosphere. Propylene oxide (1.75 cm^3, 25.0 mmol) was added, followed by glycine **4** (1.7 g, 22.5 mmol). The mixture was heated to reflux and stirred overnight. The reaction mixture was cooled to room temperature, filtered and concentrated *in vacuo*. Recrystallization from EtOAc–petroleum ether gave the α-chloro-diphenylacetylglycine as a white solid (6.48 g, 94%). A portion of this compound (1.0 g, 3.30 mmol) was dissolved in dry MeCN (40 cm^3) under an argon atmosphere. 1,3-Dicyclohexylcarbodiimide (0.75 g, 3.6 mmol) was added, followed by propylene oxide (0.70 g, 9.9 mmol). The mixture was stirred overnight, filtered and concentrated *in vacuo*. Purification by flash chromatography (SiO_2, petroleum ether–diethyl ether 3 : 1) gave the title compound **6** (0.70 g, 85%) as a yellow solid: mp 111–113 °C; ν_{max}($CHCl_3$ cast)/cm^{-1} 3057 (CH=), 1792 (C=O), 1770 (C=N), 1099; δ_H(300 MHz, $CDCl_3$) 7.36–7.55 (m, 10H, *Ph*), 7.74 (s, 1H, C*H*=N); δ_C(75 MHz, $CDCl_3$) 128.1, 128.4, 129.6 (Ar–CH), 129.7 (Ph_2C=C), 130.0, 131.7, 132.2 (Ar–CH), 136.1, 136.3 (Ar–C quaternary), 146.9 (*C*H=N), 153.3 (*C*=CPh_2), 163.7 (*C*O_2); *m*/*z* (EI) 249.0790 (M^+). $C_{16}H_{11}O_2N$ requires 249.0786; Found: C, 76.70; H, 4.34; N, 5.46. $C_{16}H_{11}O_2N$ requires C, 77.10; H, 4.45; N, 5.62%.

2-(Diphenylmethylene)-4-methyl-2*H*-oxazol-5-one 7

Alanine **5** (2.0 g, 22.5 mmol) was suspended in ethyl acetate (50 cm^3) containing propylene oxide (1.75 cm^3, 25.0 mmol). To the mixture was added a solution of 2-chloro-2,2-diphenylacetyl chloride (6.6 g, 25.0 mmol) in EtOAc (50 cm^3). After heating at reflux under argon for 24 h a homogenous solution was obtained. The solvent was removed *in vacuo* and the reaction mixture was cooled to 0 °C and treated with TFAA (7.5 cm^3, 53 mmol). Within 10 minutes of the TFAA addition a large amount of bright orange precipitate formed. The mixture was quenched with 100 cm^3 of ice–water and the crude solid recovered was recrystallized from acetonitrile–isopropyl alcohol to yield bright yellow crystals (4.0 g, 68% over 2 steps); mp 158–159 °C, lit.,[20a] mp 155–156 °C; ν_{max}(µscope)/cm^{-1} 3055 (CH=), 1767 (C=O), 1727 (C=N), 1625, 1537, 1490; δ_H(300 MHz, $CDCl_3$) 2.40 (s, 3H, CH_3), 7.36–7.45 (m, 10H, *Ph*); δ_C(75 MHz, $CDCl_3$) 13.9 (*C*H_3), 125.9 (Ph_2C=C), 128.1, 128.3, 129.1, 129.3, 131.4, 132.0 (Ar–CH), 136.5, 136.6 (Ar–C quaternary), 151.4 (*C*=CPh_2), 157.6 (*C*=N), 164.2 (*C*O_2); *m*/*z* (EI) 263.0946 (M^+). $C_{17}H_{13}O_2N$ requires 263.0946.

9-Chloro-9*H*-fluorene-9-carbonyl chloride 9

The title compound was prepared using the method of Stolle *et al.*[23b] 9*H*-Fluorene-9-carboxylic acid (5.0 g, 23.8 mmol) and thionyl chloride (17 cm^3, 238 mmol) were combined and heated to reflux under argon. After 48 h a small aliquot of the mixture was removed and analysed by ^1H NMR to look for the disappearance of the 9-H proton, indicating reaction completion. The excess thionyl chloride was removed *in vacuo* and the residue recrystallized from benzene to yield yellow crystals (1.81 g, 29%); mp 110–112 °C, lit.,[23] mp 113 °C; δ_H(300 MHz, $CDCl_3$) 7.40 (t, *J* 7.6 Hz, 2H, *Ar*), 7.50 (t, *J* 7.5 Hz, 2H, *Ar*), 7.62 (d, *J* 7.6 Hz, 2H, *Ar*), 7.72 (d, *J* 7.5 Hz, 2H, *Ar*); *m*/*z* (EI) 261.9952 (M^+). $C_{14}H_8O^{35}Cl_2$ requires 261.9952.

2-Fluoren-9-ylidene-4-methyl-2*H*-oxazol-5-one 10

Alanine **5** (300 mg, 3.4 mmol) was suspended in ethyl acetate (15 cm^3) containing propylene oxide (0.30 cm^3, 4.0 mmol). To the mixture was added a solution of 9-chloro-9*H*-fluorene-9-carbonyl chloride **9** (987 mg, 3.75 mmol) in EtOAc (15 cm^3). After heating at reflux under argon for 24 hours the mixture was bright orange with a precipitate present. After solvent removal *in vacuo* the residue was redissolved in CH_2Cl_2 and cooled to 0 °C. The solution was stirred under Ar, during which TFAA (1.0 cm^3, 7.0 mmol) was added. After warming to room temperature over 2 h the mixture was quenched with 30 cm^3 aqueous 5% $NaHCO_3$. After being washed with H_2O (3 × 50 cm^3) and brine (50 cm^3), the organic layer was dried over sodium sulfate. Solvent removal *in vacuo* yielded a bright orange solid which was recrystallized from toluene to yield the title compound (0.68 g, 76%, over 2 steps); mp 255 °C (decomp.); ν_{max}(µscope)/cm^{-1} 3059, 1882, 1777, 1652, 1604, 1447, 1376, 1357, 1290; δ_H(300 MHz, $CDCl_3$) 2.55 (s, 3H, CH_3), 7.24–7.40 (m, 4H, *Ar*), 7.60–7.68 (m, 2H, *Ar*), 8.12–8.18 (d, *J* 7.8 Hz, 1H, *Ar*), 8.40–8.46 (d, *J* 7.8 Hz, 1H, *Ar*); δ_C(75 MHz, $CDCl_3$) 14.2 (*C*H_3), 119.4 (Ar_2C=C), 120.1, 120.2, 127.5, 127.6, 127.7, 127.9, 129.7, 130.3 (Ar–CH), 135.2, 135.8, 141.3, 141.7 (Ar–C quaternary), 151.1 (Ar_2C=*C*), 158.4 (*C*=N), 163.3 (*C*O_2); *m*/*z* (EI) 261.0790 (M^+). $C_{17}H_{11}O_2N$ requires 261.0791.

13a (*E*)- and 13b (*Z*)-2-Benzylidene-2*H*-oxazol-5-one

The title compounds were prepared by a modification of the procedure of King *et al.*[20b] To a solution of glycine **4** (0.93 g, 23.0 mmol) in H_2O (7.5 cm^3), THF (3 cm^3) and NaOH (1.09 g, 27.30 mmol) at 0–5 °C was added DL-2-chloro-2-phenylacetyl chloride (2.0 cm^3, 13.0 mmol). The reaction mixture was stirred for 30 min then warmed to room temperature over 30 min. The mixture was basified with 1 M NaOH and then washed with EtOAc (2 × 15 cm^3). The aqueous layer was acidified to pH 1 with 4 M HCl, extracted with EtOAc (3 × 15 cm^3) and dried over $MgSO_4$. Evaporation of the solvent followed by recrystallization of the residue from MeOH–diethyl ether–hexane

furnished the α-chloro acid as a white solid (0.84 g, 37%). A portion of the acid (0.5 g, 2.2 mmol) was dissolved in acetic anhydride (20 cm^3) and pyridine (5 cm^3). The mixture was stirred for 1 h after which it was evaporated *in vacuo*. The crude product was taken up in EtOAc (15 cm^3) and washed successively with 1 M HCl (2 × 10 cm^3) and then saturated aq. $NaHCO_3$ (2 × 10 cm^3). The EtOAc extract was dried over $MgSO_4$ and concentrated *in vacuo*. The crude product was purified by column chromatography (SiO_2, Et_2O–hexane 4 : 1) to afford the title products which were recrystallized from hexane to give yellow crystalline solids.

Data for **13a**: (0.03 g, 9%, over 2 steps); mp 94–96 °C; ν_{max}($CHCl_3$ cast)/cm^{-1} 3065 (CH=), 1837, 1789, (C=O), 1670 (C=N), 1602, 1498, 1087; δ_H(300 MHz, acetone-d_6) 6.72 (d, *J* 2.0 Hz, 1H, C*H*=C), 7.36–7.49 (m, 3H, *Ph*), 7.94–7.99 (m, 2H, *Ph*), 8.12 (d, *J* 2.0 Hz, 1H, C*H*=N); δ_C(125 MHz, acetone-d_6) 113.6 (*C*H=C), 126.7, 130.4, 132.0 (Ar–CH), 133.5 (Ar–C quaternary), 151.4 (*C*H=N), 156.6 (*C*=CH), 163.5 (CO_2); *m*/*z* (EI) 173.0477 (M^+). $C_{10}H_7O_2N$ requires 173.0476.

Data for **13b**: (0.14 g, 37%, over 2 steps); mp 92–94 °C; ν_{max}($CHCl_3$ cast)/cm^{-1} 3036 (CH=), 1838, 1780 (C=O), 1672 (C=N), 1508, 1451, 1361, 1284, 1088; δ_H(300 MHz, acetone-d_6) 6.62 (s, 1H, C*H*=C), 7.38–7.52 (m, 3H, *Ph*), 7.86–7.8 (m, 2H, *Ph*), 8.05 (d, *J* 1.0 Hz, 1H, C*H*=N); δ_C(125 MHz, acetone-d_6) 114.5 (*C*H=C), 129.9, 130.8, 131.8 (Ar–CH), 133.3 (Ar–C quaternary), 148.7 (*C*H=N), 156.0 (*C*=CH), 164.3 (CO_2); *m*/*z* (EI) 173.0477 (M^+). $C_{10}H_7O_2N$ requires 173.0479.

13a(α-^{13}C) (*E*)- and 13b(α-^{13}C) (*Z*)-(4-^{13}C)-2-Benzylidene-*2H*-oxazol-5-one

These compounds were prepared as that described for **13a** and **13b**. Reaction of (2-^{13}C)glycine (1.0 g, 13.2 mmol), NaOH (1.06 g, 26.30 mmol) and DL-2-chloro-2-phenylacetyl chloride (2.23 cm^3, 14.5 mmol) gave the corresponding chloro acid which was cyclized with acetic anhydride (30 cm^3) and pyridine (6 cm^3). Purification by column chromatography (SiO_2, Et_2O–hexane 4 : 1) followed by recrystallization from hexane gave the title products as yellow crystalline solids.

Data for **13a**(α-^{13}C): (82.1 mg, 4%, over 2 steps); mp 93–94 °C; ν_{max}(μscope)/cm^{-1} 3326, 3061 (CH=), 1788 (C=O), 1668 (C=N), 1650, 1552, 1529, 1499; δ_H(400 MHz, CD_2Cl_2) 6.61 (d, *J* 2.0 Hz, 1H, C*H*=C), 7.38–7.48 (m, 3H, *Ph*), 7.83 (dd, $^1J_{13C-H}$ 204.4, 2.1 Hz, 1H, ^{13}C*H*=N) 7.86–7.90 (m, 2H, *Ph*); *m*/*z* (EI) 174.0510 (M^+). $^{13}CC_9H_7O_2N$ requires 174.0512.

Data for **13b**(α-^{13}C): (0.38 g, 17%, over 2 steps); mp 94–95 °C; ν_{max}(μscope)/cm^{-1} 3523, 3080 (CH=), 3061, 1816, 1788 (C=O), 1680 (C=N), 1650, 1572; δ_H(400 MHz, CD_2Cl_2) 6.52 (s, C*H*=C), 7.37–7.49 (m, 3H, *Ph*), 7.76 (dd, $^1J_{13C-H}$ 204.9, 1.0 Hz, 1H, ^{13}C*H*=N), 7.79–7.84 (m, 2H, *Ph*); *m*/*z* (EI) 174.0510 (M^+). $^{13}CC_9H_7O_2N$ requires 174.0511.

14a (*E*)- and 14b (*Z*)-2-Benzylidene-4-methyl-*2H*-oxazol-5-one

The title compounds were prepared as described for **13a** and **13b**. Reaction of alanine **5** (1.0 g, 11.2 mmol), NaOH (0.93 g, 23.0 mmol) and DL-2-chloro-2-phenylacetyl chloride (2.0 cm^3, 13.1 mmol) gave the corresponding chloro acid which was cyclized with acetic anhydride (30 cm^3) and pyridine (6 cm^3). Purification by column chromatography (SiO_2, 100% CH_2Cl_2) gave the title products which were recrystallized from hexane to give yellow crystalline solids.

Data for **14a**: (778 mg, 38%, over 2 steps); mp 145–146 °C; ν_{max}(μscope)/cm^{-1} 3066 (CH=), 1774 (C=O), 1651 (C=N), 1451, 1377, 1368, 1222; δ_H(300 MHz, $CDCl_3$) 2.44 (d, *J* 0.92 Hz, 3H, CH_3), 6.40 (s, 1H, C*H*=C), 7.29–7.42 (m, 3H, *Ph*), 7.82–7.88 (m, 2H, *Ph*); δ_C(75 MHz, $CDCl_3$) 14.0 (*C*H_3), 111.2 (*C*H=C), 128.6, 129.3, 130.9 (Ar–CH), 132.5 (Ar–C quaternary), 153.7 (CH=*C*), 159.9 (*C*=N), 163.3 (CO_2); *m*/*z* (EI) 187.0633 (M^+). $C_{11}H_9O_2N$ requires 187.0629.

Data for **14b**: (782 mg, 38%, over 2 steps); mp 126–127 °C; ν_{max}(μscope)/cm^{-1} 3052 (CH=), 2946, 1785 (C=O), 1659 (C=N), 1492, 1379, 1350, 1290, 1235; δ_H(300 MHz, $CDCl_3$) 2.40 (d, *J* 0.82 Hz, 3H, CH_3), 6.32 (s, 1H, C*H*=C), 7.31–7.44 (m, 3H, *Ph*), 7.74–7.78 (m, 2H, *Ph*); δ_C(75 MHz, $CDCl_3$) 13.7 (*C*H_3), 112.4 (*C*H=C), 129.0, 129.8, 130.9 (Ar–CH), 132.3 (Ar–C quaternary), 134.5 (CH=*C*), 156.9 (*C*=N), 163.6 (CO_2); *m*/*z* (EI) 187.0633 (M^+). $C_{11}H_9O_2N$ requires 187.0635.

15a (*E*)- and 15b (*Z*)-2-Benzylidene-4-(3-carbamoylpropyl)-*2H*-oxazol-5-one

The title compounds were prepared as described for **13a** and **13b**. Reaction of L-homoglutamine **11** (210 mg, 1.31 mmol), NaOH (0.12 g, 2.89 mmol) and DL-2-chloro-2-phenylacetyl chloride (0.23 cm^3, 1.44 mmol) gave the corresponding chloro acid. This acid was cyclized with acetic anhydride (20 cm^3) and pyridine (5 cm^3). Purification by column chromatography (SiO_2, EtOAc–hexane 2 : 1) gave the title products which were recrystallized from EtOAc–hexane to give yellow solids.

Data for **15a**: (25 mg, 7%, over 2 steps); mp 146–148 °C; ν_{max}(μscope)/cm^{-1} 3419 (N–H), 3214, 3064 (CH=), 2957 (C–H), 1778 (C=O), 1649 (C=N), 1487, 1449, 1364, 1089; δ_H(300 MHz, acetone-d_6) 1.96 (quintet, *J* 7.2 Hz, 2H, CH_2CH_2), 2.20 (t, *J* 7.2 Hz, 2H, CH_2C=N), 2.69 (t, *J* 7.6 Hz, 2H, CH_2CO), 6.62 (s, 1H, C*H*=C), 6.64 (br s, 1H, NH_2), 7.22–7.48 [m, 4H, 1 × H(NH_2), 3 × H(*Ph*)], 7.90 (d, *J* 7.1 Hz, 2H, *Ph*); δ_C(75 MHz, acetone-d_6) 22.1 (*C*H_2CH_2), 28.1 (*C*H_2C=N), 34.8 (*C*H_2CO), 110.3 (*C*H=C), 129.5, 129.7, 131.6 (Ar–CH), 133.9 (Ar–C quaternary), 154.9 (*C*=CH), 163.5 (*C*=N), 164.4 (CO_2), 174.5 (*C*ONH_2); *m*/*z* (EI) 258.1004 (M^+). $C_{14}H_{14}O_3N_2$ requires 258.1002.

Data for **15b**: (37 mg, 11%, over 2 steps); mp 194–196 °C; ν_{max}(μscope)/cm^{-1} 3442 (N–H), 3188 (CH=), 1778 (C=O), 1680, 1447, 1415, 1331, 1160, 1066; δ_H(300 MHz, DMSO-d_6) 1.92 (quintet, *J* 7.2 Hz, 2H, CH_2CH_2), 2.16 (t, *J* 7.2 Hz, 2H, CH_2C=N), 2.65 (t, *J* 7.3 Hz, 2H, CH_2CO), 6.28 (br s, 1H, NH_2), 6.54 (s, 1H, C*H*=C), 6.74 (br s, 1H, NH_2), 7.24–7.49 [m, 4H, 1 × H(NH_2), 3 × H(*Ph*)], 7.78 (d, *J* 7.5 Hz, 2H, *Ph*); δ_C(75 MHz, $CDCl_3$) 20.9 (*C*H_2CH_2), 26.7 (*C*H_2C=N), 33.9 (*C*H_2CO), 109.9 (*C*H=C), 127.9, 128.9, 130.2 (Ar–CH), 132.5 (Ar–C quaternary), 152.9 (*C*=CH), 160.6 (*C*=N), 163.3 (CO_2), 173.6 (*C*ONH_2); *m*/*z* (EI) 258.1004 (M^+). $C_{14}H_{14}O_3N_2$ requires 258.1003.

16a (*E*)- and 16b (*Z*)-2-Benzylidene-4-(2-carbamoylethyl)-*2H*-oxazol-5-one

These compounds were prepared as described for **13a** and **13b**. Reaction of L-glutamine **12** (2.0 g, 13.69 mmol), NaOH (1.09 g, 27.30 mmol) and DL-2-chloro-2-phenylacetyl chloride (2.39 cm^3, 15.06 mmol) gave the corresponding chloro acid (1.68 g, 41%). A portion of this acid (0.3 g, 1.0 mmol) was cyclized with acetic anhydride (20 cm^3) and pyridine (5 cm^3). The crude product was purified by column chromatography (SiO_2, EtOAc–hexane 2 : 1) to afford the title products which were recrystallized from CH_2Cl_2–Et_2O to give yellow crystalline solids.

Data for **16a**: (52 mg, 9%, over 2 steps); mp 160–161 °C; ν_{max}(μscope)/cm^{-1} 3509 (N–H), 3459, 3211 (CH=), 2919 (C–H), 2849, 1759 (C=O), 1659 (C=N), 1614, 1413, 1220; δ_H(300 MHz, DMSO-d_6) 2.62 (t, *J* 6.9 Hz, 2H, CH_2C=N), 2.88 (t, *J* 6.9 Hz, 2H, CH_2CO), 6.62 (s, 1H, C*H*=C), 6.86 (br s, 1H, NH_2), 7.31–7.49 [m, 4H, 1 × H(NH_2), 3 × H(*Ph*)], 7.91 (d, *J* 8.1 Hz, 2H, *Ph*); δ_C(75 MHz, DMSO-d_6) 23.6 (*C*H_2C=N), 30.9 (*C*H_2CO), 109.2 (*C*H=C), 129.0, 129.1, 130.7 (Ar–CH), 132.9 (Ar–C quaternary), 153.6 (*C*=CH), 162.7 (*C*=N), 163.9 (CO_2), 172.8 (*C*ONH_2); *m*/*z* (ES) 267.0746 (MNa^+). $C_{13}H_{12}O_3N_2Na$ requires 267.0748.

Data for **16b**: (58 mg, 10%, over 2 steps); mp 160–161 °C; ν_{max}(μscope)/cm^{-1} 3352 (N–H), 3179 (CH=), 3070, 2940 (C–H), 2803, 1782 (C=O), 1657 (C=N), 1490, 1447, 1161; δ_H(300 MHz,

DMSO-d_6) 2.56 (t, *J* 7.5 Hz, 2H, CH_2C=N), 2.83 (t, *J* 7.1 Hz, 2H, CH_2CO), 6.52 (s, 1H, C*H*=C), 6.85 (br s, 1H, NH_2), 7.34–7.51 [m, 4H, 1 × H(NH_2), 3 × H(*Ph*)], 7.75 (d, *J* 7.2 Hz, 2H, *Ph*); δ_C(75 MHz, DMSO-d_6) 23.2 (*C*H_2C=N), 30.9 (*C*H_2CO), 110.1 (*C*H=C), 129.3, 129.5, 130.5 (Ar–CH), 132.7 (*C*H_2CO), 153.0 (*C*=CH), 161.0 (*C*=N), 163.5 (*C*O_2), 172.7 (*C*ONH_2); *m*/*z* (ES) 267.0746 (MNa^+). $C_{13}H_{12}O_3N_2Na$ requires 267.0749; Found: C, 63.56; H, 4.74; N, 11.20. $C_{13}H_{12}O_3N_2$ requires C, 63.93; H, 4.95; N, 11.47%.

21a (*E*)- and 21b (*Z*)-2-(4-Fluorobenzylidene)-*2H*-oxazol-5-one

A solution of 4-fluorophenylacetamide **17** (0.33 g, 3.59 mmol) and glyoxylic acid monohydrate (0.33 g, 3.59 mmol) in acetone (15 cm^3) was refluxed at 65 °C for 4 h until a homogeneous solution was obtained. The solvent was evaporated *in vacuo* and the residue diluted with water (10 cm^3), extracted with EtOAc (3 × 10 cm^3) and dried over $MgSO_4$. The solvent was removed under reduced pressure and the white solid **19** obtained was dissolved in CH_2Cl_2 (15 cm^3) and trfluoroacetic anhydride was added (0.92 cm^3, 6.54 mmol). The reaction mixture was stirred for 0.5 h at room temperature after which the yellow solution was concentrated *in vacuo* and the residue purified by column chromatography (SiO_2, Et_2O–petroleum ether 1 : 3) to afford the title products which were recrystallized from Et_2O–hexane to give yellow crystalline solids.

Data for **21a**: (108 mg, 20%, over 2 steps); mp 200 °C (decomp.); ν_{max}(μscope)/cm^{-1} 3077 (CH=), 2945, 1802 (C=O), 1666 (C=N), 1605, 1099; δ_H(300 MHz, acetone-d_6) 6.74 (d, *J* 1.7 Hz, 1H, C*H*=C), 7.18–7.26 (m, 2H, *Ph*), 8.02–8.08 (m, 2H, *Ph*), 8.14 (d, *J* 2.0 Hz, 1H, C*H*=N); δ_C(125 MHz, acetone-d_6) 112.4 (*C*H=C), 116.6 (d, $^2J_{C-F}$ 22.5 Hz, Ar–CH), 129.9 (Ar–C quaternary), 134.1 (d, $^3J_{C-F}$ 8.8 Hz, Ar–CH), 151.2 (*C*H=N), 156.3 (*C*=CH), 163.2 (d, $^1J_{C-F}$ 250.0 Hz, Ar–C–F quaternary), 163.4 (*C*O_2); *m*/*z* (EI) 191.0383 (M^+). $C_{10}H_6O_2NF$ requires 191.0383.

Data for **21b**: (90 mg, 17%, over 2 steps); mp 128–129 °C; ν_{max}(μscope)/cm^{-1} 3109 (CH=), 3069, 2986, 1826, 1765 (C=O), 1654 (C=N), 1607; δ_H(300 MHz, acetone-d_6) 6.62 (s, 1H, C*H*=C), 7.22–7.32 (m, 2H, *Ph*), 7.88–7.96 (m, 2H, *Ph*), 8.01 (s, 1H, C*H*=N); δ_C(125 MHz, acetone-d_6) 113.2 (*C*H=C), 116.9 (d, $^2J_{C-F}$ 22.5 Hz, Ar–CH), 129.9 (Ar–C quaternary), 134.0 (d, $^3J_{C-F}$ 8.5 Hz, Ar–CH), 151.2 (*C*H=N), 155.8 (*C*=CH), 163.2 (d, $^1J_{C-F}$ 249.1 Hz, Ar–C–F quaternary), 164.2 (*C*O_2); *m*/*z* (EI) 191.0383 (M^+). $C_{10}H_6O_2NF$ requires 191.0386.

22a (*E*) and 22b (*Z*)-2-(4-Methoxybenzylidene)-*2H*-oxazol-5-one

These compounds were prepared as described for **21a** and **21b**. Reaction of glyoxylic acid monohydrate (0.21 g, 3.33 mmol) and 4-methoxyphenylacetamide **18** (0.5 g, 3.03 mmol) gave the hydroxy acid **20** which was recrystallized from acetone–hexane to give a white solid (0.37 g, 51%). This hydroxy acid was cyclized with TFAA (0.86 cm^3, 6.54 mmol) to give the title products after purification by column chromatography (SiO_2, Et_2O–hexane 1 : 4). The products were recrystallized from Et_2O–hexane to give **22a** as an orange and **22b** as a yellow crystalline solid.

Data for **22a**: (110 mg, 18%, over 2 steps); mp 230 °C (decomp.); ν_{max}(μscope)/cm^{-1} 3098 (CH=), 3064, 2937 (C–H), 2836, 2360, 1763 (C=O), 1646 (C=N), 1601; δ_H(300 MHz, acetone-d_6) 3.84 (s, 3H, OCH_3), 6.67 (d, 1H, *J* 2.0 Hz, C*H*=C), 6.98–7.08 (m, 2H, *Ph*), 7.92–7.98 (m, 2H, *Ph*), 8.02 (d, 1H, *J* 2.0 Hz, C*H*=N); δ_C(125 MHz, acetone-d_6) 55.8 (*C*H_3), 113.9, 115.4 (Ar–CH), 126.1 (Ar–C quaternary), 133.8 (*C*H=C), 151.2 (*C*H=N), 155.4 (*C*=CH), 162.2 (*C*O_2), 163.7 (Ar–C–OMe quaternary); *m*/*z* (EI) 203.0582 (M^+). $C_{11}H_9O_3N$ requires 203.0582.

Data for **22b**: (83 mg, 14%, over 2 steps); mp 108–110 °C; ν_{max}(μscope)/cm^{-1} 3098 (CH=), 2937, 2909 (C–H), 2097, 2836, 1890, 1760 (C=O), 1651 (C=N), 1606, 1511; δ_H(300 MHz, acetone-d_6) 3.88 (s, 3H, OCH_3), 6.58 (s, 1H, C*H*=C), 7.02–7.11 (m, 2H, *Ph*), 7.80–7.84 (m, 2H, *Ph*), 7.85 (d, 1H, *J* 1.0 Hz, C*H*=N); δ_C(125 MHz, acetone-d_6) 55.8 (*C*H_3), 114.9, 115.9 (Ar–CH), 125.9 (Ar–C quaternary), 133.8 (*C*H=C), 146.4 (*C*H=N), 154.9 (*C*=CH), 162.3 (*C*O_2), 164.6 (Ar–C–OMe quaternary); *m*/*z* (EI) 203.0582 (M^+). $C_{11}H_9O_3N$ requires 203.0582.

2-Hydroxy-2-(2-phenylacetamido)acetic acid 28

This known literature compound was prepared using a modified procedure of Ben-Ishai *et al.*[24] A solution of phenylacetamide (5.0 g, 37.0 mmol) and glyoxylic acid monohydrate (3.75 g, 40.70 mmol) in acetone (100 cm^3) was refluxed at 65 °C for 4 h until a homogeneous solution was obtained. The solvent was evaporated *in vacuo* and the residue diluted with $NaHCO_3$ (50 cm^3) and washed with EtOAc (2 × 20 cm^3). The aqueous layer was acidified to pH 1 with 4 M HCl, extracted with EtOAc (3 × 50 cm^3) and dried over $MgSO_4$. The white solid obtained after concentration was recrystallized from CH_2Cl_2–hexane to give a white crystalline solid (3.78 g, 49%); mp 91–92 °C; lit.,[24] mp 90–91 °C; δ_H(400 MHz, CD_3OD) 3.58 (s, 2H, CH_2Ph), 5.58 (s, 1H, C*H*OH), 7.12–7.36 (m, 5H, *Ph*); *m*/*z* (ES) 132.0586 (MNa^+). $C_{10}H_{11}O_4NNa$ requires 232.0583.

2-Ethylsulfanyl-2-(2-phenylacetamido)acetic acid 29

This compound was prepared using a similar procedure to that described by Davies *et al.*[25] To a solution of the hydroxy acid **28** (0.5 g, 2.39 mmol) in glacial acetic acid (5 cm^3) was added conc. H_2SO_4 followed by EtSH (0.71 cm^3, 9.67 mmol). The mixture was stirred for 12 h after which water (10 cm^3) was added and the mixture extracted with EtOAc (3 × 10 cm^3). The combined organic extracts were extracted with saturated aq. Na_2CO_3 (20 cm^3) and washed with EtOAc (2 × 10 cm^3). The aqueous layer was acidified to pH 1 with 1 M HCl, re-extracted with EtOAc (3 × 10 cm^3) and dried over $MgSO_4$. The product obtained after concentration *in vacuo* was recrystallized from Et_2O–hexane to give a solid (0.42 g, 69%); mp 118–120 °C; ν_{max}(μscope)/cm^{-1} 3321 (O–H), 3028 (CH=), 2968 (C–H), 2930, 2565, 1713 (C=O, acid), 1594 (C=O, amide), 1530, 1495; δ_H(300 MHz, $CDCl_3$) 1.20 (t, *J* 7.5 Hz, 3H, CH_3), 2.58 (q, *J* 7.5 Hz, 2H, SCH_2), 3.62 (s, 2H, CH_2Ph), 5.49 (d, *J* 8.3 Hz, 1H, C*H*OH), 6.42 (d, *J* 8.3 Hz, 1H, N*H*), 7.20–7.38 (m, 5H, *Ph*), 9.58 (br s, 1H, CO_2H); δ_C(100 MHz, $CDCl_3$) 14.6 (*C*H_3), 25.5 (*C*H_2S), 43.4 (Ph*C*H_2), 53.5 (*C*HS), 127.7, 129.1, 129.3 (Ar–CH), 133.6 (Ar–C quaternary), 171.1 (*C*ONH), 171.8 (*C*O_2H); *m*/*z* (ES) 276.0670 (MNa^+). $C_{12}H_{15}O_3NSNa$ requires 276.0671; Found: C, 56.85; H, 5.89; N, 5.55. $C_{12}H_{15}O_3NS$ requires C, 56.90; H, 5.97; N, 5.53%.

2-Hydroxy-2-(2-phenylacetamido)thioacetic acid *S*-ethyl ester 30 and 2-ethylsulfanyl-2-(2-phenylacetamido)thioacetic acid *S*-ethyl ester 31

To a solution of hydroxy acid **28** (0.5 g, 2.39 mmol) in CH_2Cl_2 (25 cm^3) at 0 °C was added EDCI (0.51 g, 2.63 mmol), DMAP (29.2 mg, 0.24 mmol), EtSH (0.18 cm^3, 2.39 mmol) and NEt_3 (0.33 cm^3, 2.39 mmol) in sequential order. The reaction mixture was stirred at 0 °C for 2 h and then diluted with saturated aq. $NaHCO_3$ (20 cm^3). The CH_2Cl_2 layer was separated, washed with 1 M HCl (20 cm^3) and dried over $MgSO_4$. Purification of the crude product by column chromatography (SiO_2, Et_2O–hexane 1 : 1) furnished the title products as solids.

Data for **30**: (75.0 mg, 12%); ν_{max}(μscope)/cm^{-1} 3287 (O–H), 3030 (CH=), 2969 (C–H), 2930, 1663 (C=O, amide), 1522, 1495, 1453; δ_H(400 MHz, CD_2Cl_2) 1.72 (t, *J* 7.5 Hz, 3H, CH_3), 2.89 (q, *J* 7.5 Hz, 2H, SCH_2), 3.60 (s, 2H, CH_2Ph), 5.49 (d, *J* 7.3 Hz, 1H, C*H*OH), 6.58 (br s, 1H, N*H*), 7.22–7.40 (m, 5H, *Ph*); δ_C(100 MHz, CD_2Cl_2) 14.6 (*C*H_3), 23.9 (*C*H_2S), 43.7 (Ph*C*H_2), 77.5 (*C*HOH), 127.7, 129.2, 129.8 (Ar–CH), 134.4 (Ar–C

quaternary), 172.3 (*C*ONH), 198.9 (*C*OS); *m*/*z* (CI) 271.3 (MNH_4^+, 0.11%).

Data for **31**: (42 mg, 6%); mp 59–60 °C; ν_{max}(μscope)/cm^{-1} 3309 (N–H), 3084 (CH=), 3029, 2965 (C–H), 2928, 2868, 1677 (C=O, amide), 1648, 1600, 1514, 1494; δ_H(400 MHz, CD_2Cl_2) 1.28–1.34 (m, 6H, 2 × CH_3), 2.44–2.62 (m, 2H, SCH_2), 2.84 (q, *J* 7.3 Hz, 2H, SCH_2), 3.60 (s, 2H, CH_2Ph), 5.49 (d, *J* 7.3 Hz, 1H, C*H*S), 6.58 (br s, 1H, N*H*), 7.22–7.40 (m, 5H, *Ph*); δ_C(125 MHz, CD_2Cl_2) 14.6 (*C*H$_3$), 14.7 (*C*H$_3$), 24.3 (*C*H$_2$S), 25.2 (*C*H$_2$S), 43.7 (Ph*C*H$_2$), 60.7 (*C*HS), 127.7, 129.3, 129.7 (Ar–CH), 134.9 (Ar–C quaternary), 170.2 (*C*ONH), 195.7 (*C*OS); *m*/*z* (EI) 297.0857 (M^+). $C_{14}H_{19}O_2NS_2$ requires 297.0860.

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