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RESEARCH ARTICLE

Dipeptide-derived nitriles containing additional electrophilic sites: Potentially irreversible inhibitors of cysteine proteases

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Abstract

Heterocyclic and open-chain dipeptide-derived nitriles have been synthesized, containing an additional electrophilic center enabling the subsequent covalent modification of the thioimidate nitrogen formed *in situ* at the active site of the enzyme. The inhibitory potential of these nitriles against the cysteine proteases papain and cathepsins L, S, and K was determined. The open-chain dipeptide nitriles **8** and **10** acted as moderate reversible inhibitors, but no evidence for an irreversible inhibition of these enzymes was discernable.

Keywords: *Cysteine proteases; nitriles; mechanism-based inactivators; cyclizations; cascade reactions*

Abbreviations: Ac-, acetyl-; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; dec, decomposition; DMF, N,N-dimethyl formamide; DMSO, dimethyl sulfoxide; DTT, (±)-threo-2,3-dihydroxy-1,4-butanedithiol; d, doublet; EDTA, ethylenediamine tetraacetic acid; EI, electron impact ionization; ESI, electrospray ionization; m, multiplet; Me, methyl; Mec, 4-methylcoumarin-7-yl; mp, melting point; MS, mass spectrometry; n.d., not determined; neg, negative mode; NMR, nuclear magnetic resonance; Np, para-nitrophenyl; pos, positive mode; RDA, retro-Diels-Alder fragmentation; s, singlet; sat, saturated; t, triplet; THF, tetrahydrofurane; Z-, benzyloxycarbonyl-

Introduction

Among the large and heterogeneous class of cysteine proteases, the papain-like cysteine proteases have attracted remarkable interest in recent research, not only due to their vital role in various human physiological and pathological processes but also because of their importance as virulence factors in different human-pathogenic parasites[1]. Common characteristics of papain-like cysteine proteases are the active site thiol and the importance of the S^2–P^2 interaction as the main determinant for substrate specificity. In the field of inhibition of these enzymes by low-molecular weight synthetic substances, the focus is directed toward developing potent reversible inhibitors as they have a lower risk of side effects and toxicity during the long-term treatment of chronic diseases. However, compounds that act by irreversible covalent modification of the active site cysteine are of great value, as the inhibition of the enzyme is complete and insurmountable[2]. Irreversible inhibitors are therefore attractive candidates for treating microbial infections and valuable tools for elucidating the *in vivo* functions of the enzyme. Compounds of different classes have been reported as irreversible inactivators of cysteine proteases[3], including peptide-derived epoxides[2,4] and related aziridines[5], diazoketones[6], and Michael acceptors such as vinyl sulfones and peptidyl ene diones[7–9]. The inherent chemical reactivity of these agents limits their applicability due to unspecific reactions with other biomolecules.

The class of peptide nitriles has received considerable attention because they interact in a covalent-reversible manner with the thiol of the active site to form an enzyme-bound thioimidate[10]. As the cyano group represents a soft electrophile, they are generally selective for cysteine proteases over other proteases[11]; however, exceptions have been observed for instance in the case of the serine protease dipeptidyl peptidase IV[12]. To combine the benefits of the nitriles with those of irreversible inhibitors, we envisaged

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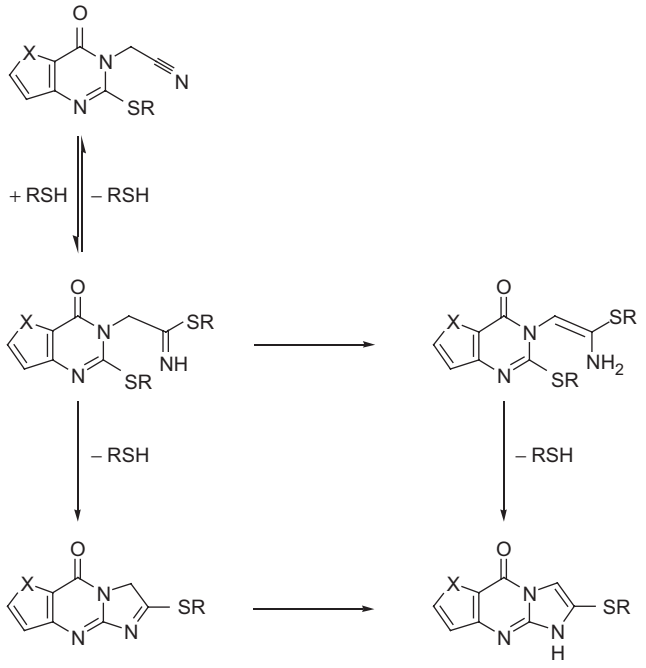


Figure 1. Reaction of 2-alkylthio-4-oxo-quinazolineacetonitriles and analogous thieno[3,2-*d*]pyrimidineacetonitriles with aliphatic thiols to imidazo-fused tricyclic systems by trapping of thioimidates; X = CH=CH or S[13].

the development of compounds capable of facilitating an intramolecular "trapping" of the enzyme-bound thioimidate adduct. This irreversible "trapping" of the temporary thioimidate can be accomplished by the introduction of a nucleofuge in a suitable position relative to the cyano group. The attack of the thioimidate nitrogen then leads to the formation of a five (or six)-membered heterocyclic ring. 2-Alkylthio-4-oxo-quinazoline-3-acetonitriles and the analogous thieno[3,2-*d*] pyrimidine-3-acetonitriles (Figure 1) are molecules that react with thiols in this manner[13]. Such reactions, where the initial step generates the functionality for the following one, belong to the so called domino or cascade reactions. The particular conversion depicted in Figure 1 matches the anionic type of cascade reaction, according to the classification introduced by Tietze[14]. As these nitriles are devoid of cysteine protease-inhibiting activity, probably due to the lack of structural elements that can be recognized by the enzyme's subsites, it was intended to apply the concept to compounds of more peptidic character, which is reported herein.

Materials and methods

General methods and materials

Melting points were determined on a Büchi 510 oil bath apparatus and are not corrected. Thin layer chromatography was performed on aluminum-supported plates of silica gel 60. Optical rotations were determined on a PerkinElmer 241 polarimeter. ^{1}H NMR spectra (500 MHz) and ^{13}C NMR spectra (125 MHz) were recorded on a Bruker Avance 500. Mass spectra were obtained on an API 2000 spectrometer from Applied Biosystems (ESI; sprayed from a 10^{-5} M solution in 2 mM NH_4OAc/MeOH 1:1; volumetric flow rate 10 µL/min) and on an A.E.I. MS-50 spectrometer (EI; 70 eV). Enzymatic activity of papain and cathepsins was measured using a Varian Cary Bio 50 UV/Vis spectrophotometer and a PerkinElmer luminescence spectrometer LS 50 B, respectively. Papain was purchased from Sigma-Aldrich, Steinheim, Germany. Cathepsin L from bovine liver and recombinant human cathepsin S were purchased from Calbiochem, Darmstadt, Germany. Recombinant human cathepsin K (expressed in *Pichia pastoris*) was a gift from D. Brömme[15]. The substrates, Z-Phe-Arg-NHNp, Z-Phe-Arg-NHMec, Z-Val-Val-Arg-NHMec, and Z-Leu-Arg-NHMec, were from Bachem, Bubendorf, Switzerland. The amino acid derivatives were purchased from Novabiochem, Läufelfingen, Switzerland and Bachem, Bubendorf, Switzerland, as well as Fluka, Deisenhofen, Germany. DTT, Brij 35 P, and Triton X-100 were obtained from Fluka; CHAPS was from Sigma, Germany. Diethyl 2-acetamido-2-benzylmalonate was prepared according to reference [16]. Mathematical data analyses were done with the programs Grafit 4 (Erithacus Software) and GraphPad Prism 4 (GraphPad Software).

Synthesis

5-Acetamido-5-benzyl-thiobarbituric acid (1)

Diethyl 2-acetamido-2-benzylmalonate (13.11 g, 45 mmol) was added to a solution of sodium (1.04 g, 45 mmol) in dry ethanol (60 mL). After addition of thiourea (3.43 g, 45 mmol), the mixture was refluxed for 37 h. The precipitated sodium salt of the product (8.32 g, 22 mmol) was filtered off, dissolved in water, and treated with 2 N HCl (11 mL) under ice cooling. The precipitate was filtered with suction and dried over P_4O_{10} to obtain sufficiently pure material, yield 52%, mp 283–290°C (dec) (lit.[17] 288°C (dec) (H_2O)); ^{1}H NMR (400 MHz; DMSO-d_6): 1.87 (s, 3H, CH_3CO), 3.34 (s, 2H, PhCH_2C), 6.99–7.04 (m, 2H, 2′-H, 6′-H), 7.24–7.30 (m, 3H, 3′-H, 4′-H, 5′-H), 9.29 (s, 1H, CONHC), 12.18 (s, 2H, 2 × CONHCS); ^{13}C NMR (100 MHz; DMSO-d_6): 21.24 (*C*H_3CO), 42.44 (Ph*C*H_2C), 63.11 (C-5), 128.11 (C-4′), 128.61, 129.82 (C-3′, C-5′, C-2′, C-6′), 131.63 (C-1′), 168.61, 169.98 (C-4, C-6, CH_3*C*ONH), 178.17 (C-2). Anal. found: C, 50.59; H, 4.98; N, 13.28. $C_{13}H_{13}N_3O_3S \times H_2O$ requires: C, 50.48; H, 4.89; N, 13.58%.

(±)-2-Methylthio-5-acetamido-5-benzyl-1H,5H-pyrimidine-4,6-dione (2)

Methyl iodide (0.69 mL, 11 mmol) and 1 N NaOH (11 mL) were added to a suspension of **1** (2.90 g, 10 mmol) in acetone (25 mL). After stirring for 5 h at room temperature, the organic solvent was removed *in vacuo*. The precipitate was filtered off, washed with H_2O, and dried. The crude product was recrystallized from acetone/H_2O, yield 77 %, mp 285–294°C (dec); ^{1}H NMR (400 MHz; DMSO-d_6): 1.85 (s, 3H, CH_3CO), 2.18 (s, 3H, CH_3S), 3.04 (s, 2H, PhCH_2C), 6.97–7.04 (m, 2H, 2′-H, 6′-H), 7.20–7.29 (m, 3H, 3′-H, 4′-H, 5′-H), 9.10 (s, 1H, CH_3CONH), 11.98 (s, 1H, 1-H); ^{13}C NMR (100 MHz, DMSO-d_6): 13.47 (*C*H_3S), 21.68 (*C*H_3CO), 43.22 (Ph*C*H_2C), 65.66 (C-5), 128.07 (C-4′), 128.36, 130.24 (C-3′, C-5′, C-2′, C-6′),

132.41 (C-1′), 169.65, 171.80 (C-4, C-6, CH_3CONH), 176.61 (C-2). Anal. found: C, 55.07; H, 4.95; N, 13.76. $C_{14}H_{15}N_3O_3S$ requires: C, 54.77; H, 5.12; N, 13.35%.

(±)-1-Cyanomethyl-2-methylthio-5-acetamido-5-benzyl-1H,5H-pyrimidine-4,6-dione (3)

A solution of tetrabutylammonium bromide (0.065 g, 0.2 mmol) in 1 N NaOH (2.0 mL) and H_2O (4 mL) was added to a solution of **2** (0.6 g, 2.0 mmol) in dichloromethane (6 mL). After addition of bromoacetonitrile (0.15 mL, 2.2 mmol) the mixture was stirred for 12 h at room temperature. Subsequently, the phases were separated and the aqueous phase was extracted with dichloromethane (2 × 20 mL). The combined organic layers were washed with H_2O (2 × 15 mL) and brine (15 mL), dried over Na_2SO_4, and evaporated *in vacuo* to obtain pure **3**, yield 57%, mp 200°C (dec); ^{1}H NMR (500 MHz; $CDCl_3$): 1.88 (s, 3H, C*H*$_3$CO), 2.26 (s, 3H, C*H*$_3$S), 3.08 (d, ^2J = 12.6 Hz, 1H, PhC*H*HC), 3.18 (d, ^2J = 12.6 Hz, 1H, PhCH*H*C), 4.82 (s, 2H, NC*H*$_2$CN), 6.94–6.98 (m, 2H, 2′-H, 6′-H), 7.22–7.30 (m, 3H, 3′-H, 4′-H, 5′-H), 9.41 (br s, 1H, CH_3CONH); ^{13}C NMR (125 MHz; $CDCl_3$): 15.34 (S*C*H$_3$), 21.22 (*C*H$_3$CO), 31.25 (N*C*H$_2$CN), 42.89 (Ph*C*H$_2$C), 65.88 (C-5), 114.12 (NCH$_2$*C*N), 128.05 (C-4′), 128.41, 129.74 (C-3′, C-5′, C-2′, C-6′), 131.56 (C-1′), 169.84, 170.00, 170.20 (C-4, C-6, CH_3CONH), 173.77 (C-2); MS (EI) *m*/*z* (%): 344 (3, $M^{+\cdot}$), 189 (100, RDA: [dienophile]$^{+\cdot}$), 156 (57, RDA: [diene + H]$^+$), 147 (18, [dienophile-C_2H_2O]$^{+\cdot}$), 91 (42, [C_7H_7]$^+$). Anal. found: C, 53.18; H, 5.20; N, 15.11; $C_{16}H_{16}N_4O_3S$ requires: C, 55.80; H, 4.68; N, 16.27%.

***N*-(tert-Butoxycarbonyl)-methionine amide (4)**

*General procedure for amides **4**, **6**, and **9*** Boc-Met-OH (4.00 g, 16 mmol) was dissolved in THF (15 mL) and cooled to –25°C. Subsequently, *N*-methylmorpholine (4.87 mL, 16 mmol) and isobutyl chloroformate (2.11 mL, 16 mmol) were sequentially added under vigorous stirring. Immediately after the formation of the white precipitate of *N*-methylmorpholine hydrochloride, 25% NH_3 (6 mL, 80 mmol) was added. The resulting mixture was allowed to warm to room temperature. After 2 h, THF was removed under reduced pressure and the residual aqueous mixture was diluted with a small volume of H_2O, adjusted to pH 1 (10% $NaHSO_4$), and extracted with ethyl acetate (3 × 50 mL). The combined organic layers were washed with H_2O (20 mL), sat. $NaHCO_3$ (2 × 20 mL), and brine (20 mL), dried over Na_2SO_4 and evaporated to dryness to obtain pure **4**, yield 86%, mp 122–123°C (lit.[18] 118–119°C); ^{1}H NMR (500 MHz; DMSO-d_6): 1.37 (s, 9H, ((CH$_3$)$_3$C), 1.69–1.78 (m, 1H, SC*H*HCH$_2$), 1.80–1.89 (m, 1H, SCH*H*CH$_2$), 2.02 (s, 3H, C*H*$_3$S), 2.36–2.47 (m, 2H, CH$_2$C*H*$_2$CH), 3.89–3.96 (m, 1H, NHC*H*CO), 6.80 (d, ^3J = 8.2 Hz, 1H, N*H*CHCO), 6.94 (s, 1H, CON*H*H), 7.20 (s, 1H, CONH*H*); ^{13}C NMR (125 MHz; DMSO-d_6): 14.76 (*C*H$_3$S), 28.32 ((*C*H$_3$)$_3$C), 29.95 (S*C*H$_2$CH$_2$), 31.87 (CH$_2$*C*H$_2$CH), 53.53 (NH*C*HCO), 78.15 ((CH$_3$)$_3$*C*O), 155.50 (O*C*ONH), 173.87 (CH*C*ONH$_2$). Anal. found: C, 46.41; H, 8.42; N, 10.94; $C_{10}H_{20}N_2O_3S \times 1/2\,H_2O$ requires: C, 46.67; H, 8.22; N, 10.89%.

Methionine amide hydrochloride (5)

Compound **4** (1.0 g, 4.0 mmol) was dissolved in 4 M HCl/dioxane (20 mL) under an atmosphere of argon. After a few minutes the product precipitated. The mixture was kept at room temperature for 1 h. The solvent was removed *in vacuo*, and the residue suspended in petroleum ether, filtered off, and dried in a desiccator, yield 97%, mp 221°C (dec) (lit.[19] mp 189–190°C); ^{1}H NMR (500 MHz; DMSO-d_6): 1.98–2.04 (m, 2H, SCH$_2$*C*H$_2$), 2.05 (s, 3H, C*H*$_3$S), 2.49–2.54 (m, 2H, CH$_2$C*H*$_2$CH), 3.79 (t, ^3J = 6.1 Hz, 1H, H$_3$N$^+$C*H*CO), 7.51 (s, 1H, CON*H*H), 8.02 (s, 1H, CONH*H*), 8.35 (br s, 3H, *H*$_3$N$^+$CHCO); ^{13}C NMR (125 MHz; DMSO-d_6): δ 14.61 (*C*H$_3$S), 28.49 (S*C*H$_2$CH$_2$), 30.88 (CH$_2$*C*H$_2$CH), 51.68 (H$_3$N$^+$*C*HCO), 170.03 (CH*C*ONH$_2$); Anal. found: C, 31.87; H, 8.16; N, 15.49; $C_5H_{12}N_2OS \times HCl$ requires: C, 32.52; H, 7.09; N: 15.17%.

***N*-(tert-Butoxycarbonyl)-phenylalanyl-methionine amide (6)**

Preparation was done according to the general procedure given for compound **4** using 0.93 g Boc-Phe-OH (3.5 mmol). Instead of NH_3, a solution of **5** (0.7 g, 3.8 mmol) in H_2O (0.5 mL) and 1 N NaOH (3.8 mL) was used, yield 99%, mp 187°C; ^{1}H NMR (500 MHz; DMSO-d_6): 1.30 (s, 9H, (C*H*$_3$)$_3$C), 1.72–1.82 (m, 1H, SC*H*HCH$_2$), 1.90–1.99 (m, 1H; SCH*H*CH$_2$), 2.02 (s, 3H, C*H*$_3$S), 2.33–2.47 (m, 2H, CH$_2$C*H*$_2$CH), 2.75 (dd, ^2J = 13.6 Hz, ^3J = 10.1 Hz, 1H, PhC*H*HCH), 2.97 (dd, ^2J = 13.8 Hz, ^3J = 4.6 Hz, 1H, PhCH*H*CH), 4.11–4.18 (m, 1H), 4.25–4.35 (m, 1H) (NHC*H*CO-Met, NHC*H*CO-Phe); 6.99 (d, ^3J = 8.4 Hz, 1H, N*H*CHCO-Phe), 7.06 (s, 1H, CON*H*H), 7.15–7.21 (m, 2H, 2′-H, 6′-H), 7.22–7.28 (m, 4H, 3′-H, 4′-H, 5′-H, CONH*H*), 7.89 (d, ^3J = 8.2 Hz, 1H, N*H*CHCO-Met); ^{13}C NMR (125 MHz; DMSO-d_6): 14.76 (S*C*H$_3$), 28.25 ((*C*H$_3$)$_3$C), 29.58 (S*C*H$_2$CH$_2$), 32.18 (CH$_2$*C*H$_2$CH), 37.15 (Ph*C*H$_2$CH), 51.75, 56.04 (NH*C*HCO-Met, NH*C*HCO-Phe), 78.30 ((CH$_3$)$_3$*C*O), 126.31 (C-4′), 128.15 (C-3′, C-5′), 129.32 (C-2′, C-6′), 138.26 (C-1′), 155.45 (OCONH), 171.66, 172.98 (CH*C*ONH, *C*ONH$_2$). Anal. found: C, 54.34; H, 9.24; N, 11.94; $C_{19}H_{29}N_3O_4S \times H_2O$ requires: C, 55.13; H, 7.56; N, 10.16%.

***N*-(tert-Butoxycarbonyl)-phenylalanyl-methionine nitrile (7)**

To a solution of **6** (0.80 g, 2.0 mmol) in DMF (5 mL), cyanuric chloride (0.30 g, 2.0 mmol) was added as a solid under ice cooling. After stirring for 2 h at room temperature, ice-cold sat. $NaHCO_3$ (10 mL) was added and the resulting mixture was extracted with ethyl acetate (3 × 20 mL). The combined organic layers were washed with H_2O (3 × 10 mL) and brine (10 mL), dried over Na_2SO_4, and evaporated *in vacuo*. The obtained residue was recrystallized from *n*-hexane/ethyl acetate, yield 60%, mp 127–128°C; ^{1}H NMR (500 MHz; $CDCl_3$): 1.40 (s, 9H, (C*H*$_3$)$_3$C), 1.96–2.03 (m, 2H, SC*H*$_2$CH$_2$), 2.05 (s, 3H, C*H*$_3$S), 2.49–2.57 (m, 2H, CH$_2$C*H*$_2$CH), 3.03 (br s, 1H, PhC*H*HCH), 3.04 (br s, 1H, PhCH*H*CH), 4.25–4.31 (m, 1H, NHC*H*CO), 4.93–5.01 (m, 1H, NHC*H*CN), 5.04 (br s, 1H, N*H*CHCO), 6.72 (d, ^3J = 8.2 Hz, 1H, N*H*CHCN), 7.15–7.19 (m, 2H, 2′-H, 6′-H); 7.22–7.27 (m, 1H, 4′-H); 7.28–7.33 (m, 2H, 3′-H, 5′-H); ^{13}C NMR (125 MHz; $CDCl_3$): 15.34 (*C*H$_3$S),

28.23 ((*C*H$_3$)$_3$C), 29.50 (S*C*H$_2$CH$_2$), 31.67 (NH*C*HCN), 38.00, 39.50 (Ph*C*H$_2$CH, CH$_2$*C*H$_2$CH), 55.79 (NH*C*HCO), 80.72 ((CH$_3$)$_3$*C*O), 117.54 (NHCH*C*N), 127.25 (C-4′), 128.89, 129.25 (C-3′, C-5′, C2′, C-6′), 136.00 (C-1′), 155.54 (O*C*ONH), 171.06 (CH*C*ONH); MS (ESI) *m*/*z* (%) (pos): 416 (7, [M + K]$^+$), 400 (11, [M + Na]$^+$), 395 (67, [M + NH$_4$]$^+$), 378 (83, [M + H]$^+$), 322 (100, [M-(CH$_3$)$_2$CCH$_2$ + H]$^+$), 278 (94, [M-(CH$_3$)$_2$CCH$_2$-CO$_2$ + H]$^+$) (neg): 436 (7, [M + CH$_3$COO]$^-$), 376 (100, [M – H]$^-$), 302 (72, [M-*t*BuOH-H]$^-$). Anal. found: C, 59.96; H, 7.66; N; 10.95; $C_{19}H_{27}N_3O_3S$ requires: C, 60.45; H, 7.21; N, 11.13%.

N-(tert-Butoxycarbonyl)-phenylalanyl-S-methylmethionine nitrile iodide (8)

Methyl iodide (0.16 mL, 2.60 mmol) was added to a solution of **7** (0.10 g, 0.26 mmol) in ethyl acetate (1 mL). The mixture was stirred for 3 days at room temperature under the exclusion of light. The residue obtained after concentration *in vacuo* was treated with dry ether. The precipitate was filtered off and washed carefully with ether, yield 36%; ^{1}H NMR (500 MHz; DMSO-d_6): 1.31 (s, 9H, (C*H*$_3$)$_3$C), 2.19–2.37 (m, 2H, S$^+$C*H*$_2$CH$_2$), 2.81 (dd, ^2J = 13.6 Hz, ^3J = 9.5 Hz, 1H, PhC*H*HCH), 2.88 (s, 3H, C*H*$_3$CH$_3$S$^+$), 2.91 (s, 3H, CH$_3$C*H*$_3$S$^+$), 2.95 (dd, ^2J = 13.7 Hz, ^3J = 5.2 Hz, 1H, PhCH*H*CH), 3.20–3.35 (m, 2H, CH$_2$C*H*$_2$CH), 4.05–4.13 (m, 1H, NHC*H*CO), 4.94–5.02 (m, 1H, NHC*H*CN), 7.13 (d, ^3J = 7.6 Hz, N*H*CHCO), 7.16–7.30 (m, 5H, Ph-H), 8.88 (d, ^3J = 7.9 Hz, 1H, N*H*CHCN); ^{13}C NMR (125 MHz, DMSO-d_6): 24.72 (*C*H$_3$CH$_3$S$^+$), 24.75 (CH$_3$*C*H$_3$S$^+$), 26.55 (NH*C*HCN), 28.28 ((*C*H$_3$)$_3$C), 37.02, 38.33, 38.69 (S$^+$*C*H$_2$CH$_2$, Ph*C*H$_2$CH, CH$_2$*C*H$_2$CH), 56.03 (NH*C*HCO), 78.44 ((CH$_3$)$_3$*C*O), 118.41 (NHCH*C*N), 126.52 (C-4′), 128.26 (C-3′, C-5′), 129.29 (C-2′, C-6′), 137.70 (C-1′), 155.51 (O*C*ONH), 172.28 (CH*C*ONH); MS (ESI) *m*/*z* (%) (pos): 392 (100, $C_{20}H_{30}N_3O_3S^+$) (neg): 127 (100, I$^-$).

N-(tert-Butoxycarbonyl)-phenylalanyl-asparagine amide (9)

Preparation was done according to the general procedure given for compound **4** using 0.80 g Boc-Phe-OH (3.0 mmol). Instead of NH$_3$, a solution of H-Asn-NH$_2$ × HCl (0.50 g, 3.0 mmol) in H$_2$O (0.5 mL) and 1 N NaOH (3.0 mL) was used. Differing from the general procedure, the precipitate obtained after the removal of THF was treated with H$_2$O (5 mL) and the suspension acidified to pH 1 (10% NaHSO$_4$). The solid was filtered off and washed with H$_2$O (2 × 15 mL), sat. NaHCO$_3$ (15 mL), and H$_2$O (15 mL) and dried in a desiccator over P$_4$O$_{10}$, yield 79%, mp 179–181°C (dec); ^{1}H NMR (500 MHz; DMSO-d_6): 1.30 (s, 9H, (C*H*$_3$)$_3$C), 2.45 (dd, ^2J = 15.5 Hz, ^3J = 6.1 Hz, 1H, H$_2$NCOC*H*HCH), 2.51 (dd, ^2J = 15.8 Hz, ^3J = 6.1 Hz, 1H, H$_2$NCOCH*H*CH), 2.73 (dd, ^2J = 13.7 Hz, ^3J = 10.5 Hz, 1H, PhC*H*HCH), 2.98 (dd, ^2J = 14.0 Hz, ^3J = 4.2 Hz, PhCH*H*CH), 4.07–4.15 (m, 1H), 4.38–4.53 (m, 1H) (2 × NHC*H*CO), 6.86 (s, 1H, CONH*H*H), 6.97 (s, 1H, CONH*H*), 7.03 (d, ^3J = 8.2 Hz, 1H, N*H*CHCO-Phe), 7.05 (s, 1H, CONH*H*H), 7.13–7.30 (m, 5H, Ph-H), 7.33 (s, 1H, CONH*H*), 8.11 (d, ^3J = 7.9 Hz, 1H, N*H*CHCO-Asn); ^{13}C NMR (125 MHz; DMSO-d_6): 28.27 ((*C*H$_3$)C), 36.87, 37.15 (CO*C*H$_2$CH-Asn, Ph*C*H$_2$CH-Phe), 49.65, 56.25 (NH*C*HCO-Asn, NH*C*HCO-Phe), 78.48 ((CH$_3$)$_3$*C*O), 126.30 (C-4′), 128.16, 129.32 (C-3′, C-5′, C-2′, C-6′), 138.29 (C-1′), 155.61 (O*C*ONH), 171.45, 171.96, 172.87 (CONH, CH*C*ONH$_2$, CH$_2$*C*ONH$_2$). Anal. found: C, 52.73; H, 7.24; N, 13.11; $C_{18}H_{26}N_4O_5 \times 2\,H_2O$ requires: C, 52.16; H, 7.30; N, 13.52%.

N-(tert-Butoxycarbonyl)-phenylalanyl-cyanoalanine nitrile (10)

To a solution of **9** (0.80 g, 2.1 mmol) in DMF (10 mL), cyanuric chloride (0.62 g, 4.2 mmol) was added as a solid under ice cooling. After stirring for 2 h at room temperature, ice-cold sat. NaHCO$_3$ (10 mL) was added and the resulting mixture was extracted with ethyl acetate (3 × 20 mL). The combined organic layers were washed with H$_2$O (3 × 10 mL) and brine (10 mL), dried over Na$_2$SO$_4$ and evaporated *in vacuo*, yield 99%, mp 117–119°C, $[\alpha]_D^{20} = -8.53$ (c = 1.17, MeOH); ^{1}H NMR (500 MHz; CDCl$_3$): 1.39 (s, 9H, (C*H*$_3$)$_3$C), 2.89 (d, ^3J = 6.1 Hz, 2H, CHC*H*$_2$CN), 2.97 (dd, ^2J = 13.8 Hz, ^3J = 7.8 Hz, 1H, PhC*H*HCH), 3.02–3.11 (m, 1H, PhCH*H*CH), 4.32–4.43 (m, 1H, NHC*H*CO), 5.05–5.11 (m, 1H, NHC*H*CN), 5.23 (d, ^3J = 6.3 Hz, 1H, N*H*CHCO), 7.14 (d, ^3J = 6.9 Hz, 2H, 2′-H, 6′-H), 7.21–7.26 (m, 1H, 4′-H), 7.27–7.31 (m, 2H, 3′-H, 5′-H), 7.74 (br s, 1H, N*H*CHCN); ^{13}C NMR (125 MHz; CDCl$_3$): 22.63 (CH*C*H$_2$CN), 28.26 ((*C*H$_3$)$_3$C), 37.22, 37.95 (NH*C*HCN, Ph*C*H$_2$CH), 55.71 (NH*C*HCO), 81.00 ((CH$_3$)$_3$*C*O), 114.55, 115.39 (CH$_2$*C*N, CH*C*N), 127.29 (C-4′), 128.83, 129.16 (C-3′, C-5′, C-2′, C-6′), 135.84 (C-1′), 155.75 (O*C*ONH), 171.93 (CH*C*ONH); MS (ESI) *m*/*z* (%) (pos): 381 (8, [M + K]$^+$), 365 (15, [M + Na]$^+$), 360 (62, [M + NH$_4$]$^+$), 343 (22, [M + H]$^+$), 287 (100, [M-(CH$_3$)$_2$CCH$_2$ + H]$^+$), 243 (75, [M-(CH$_3$)$_2$CCH$_2$-CO$_2$ + H]$^+$) (neg): 341 (100, [M – H]$^-$), 267 (64, [M-*t*BuOH – H]$^-$). Anal. found: C, 62.22; H, 6.96; N, 16.08; $C_{18}H_{22}N_4O_3$ requires: C, 63.14; H, 6.48; N, 16.36%.

Inhibition experiments

Papain

Enzyme activities were determined by spectrophotometric detection of the product *p*-nitroaniline at 25°C in a multicell holder (parallel measurements for each single inhibitor determination; final volume 1 mL) at a wavelength of 405 nm. A 4 mM stock solution of the chromogenic substrate Z-Phe-Arg-NHNp was prepared in DMSO; the final concentration was 200 µM (= 0.207K_m)[20]. The assay medium was 0.1 M sodium phosphate pH 6.5, 2.5 mM EDTA, 300 µM DTT, and 12% DMSO. Stock solutions of the inhibitors were prepared in DMSO. In the absence of inhibitor, 70 µL of DMSO was added to the cuvette. A papain stock solution was prepared in 1 mM HCl. For daily activation, the papain stock solution was diluted 1:100 in 0.1 M sodium phosphate pH 6.5, 2.5 mM EDTA, and 15 mM DTT and incubated at 25°C for 1 h. The activated enzyme was kept on ice. After thermal equilibration, the reaction was initiated by addition of the enzyme (20 µL); its final concentration catalyzed the conversion of the substrate at a rate of 1–2 µM/min. Progress curves were monitored over 10 min. Rates were determined for eight different inhibitor concentrations including the control in duplicate.

Cathepsins

Enzyme activities were calculated from kinetic measurements performed by fluorimetric detection of the product 7-amino-4-methylcoumarin at 37°C in a stirred cuvette. The wavelengths for excitation and emission were 360 nm and 440 nm, respectively. The reaction volume of the assay was 2 mL. To assay cathepsin L, Z-Phe-Arg-NHMec was used as substrate at a concentration of 10 μM (= 1.89K_m)[20] in 100 mM sodium phosphate pH 6.0, 100 mM NaCl, 5 mM EDTA, 0.01% Brij 35, 25 μM DTT, and 1% DMSO. For cathepsin S, Z-Val-Val-Arg-NHMec was chosen as substrate at a concentration of 40 μM (= 2.08 K_m)[20] in 50 mM potassium phosphate pH 6.5, 50 mM NaCl, 2 mM EDTA, 0.01% Triton X-100, 25 μM DTT, and 1% DMSO. In the cathepsin K assay, Z-Leu-Arg-NHMec was used as substrate at a concentration of 20 μM (= 3.23K_m)[20] in 100 mM sodium citrate pH 5.0, 100 mM NaCl, 1 mM EDTA, 0.01% CHAPS, 25 μM DTT, and 1% DMSO. Stock solutions of the substrates were prepared in DMSO in a 1000-fold higher concentration than the final concentrations. Dilutions were done in the corresponding assay medium without DTT and DMSO. Stock solutions of the inhibitors and dilutions were prepared in DMSO. In the absence of inhibitor, 20 μL of DMSO was added to the cuvette. The enzyme dilutions were daily prepared from a stock with the corresponding assay medium without DMSO, containing 5 mM DTT (the complete amount of DTT required for the final concentration noted above), and kept on ice. After thermal equilibration, 10 μL of the enzyme solution was added and product formation was monitored over 5 min. The inhibition constants were obtained from measurements at 10–12 different inhibitor concentrations and duplicate or triplicate measurements in the absence of the inhibitor.

Determination of kinetic parameters for the inhibition of cysteine proteases

The progress curves of the cysteine protease-catalyzed reactions in the presence of the dipeptide nitriles **7**, **8**, and **10** were nearly linear, with a slight deviation from linearity—also in the reactions without inhibitor—in the case of cathepsin S. The apparent inhibition constant K_i' was determined by fitting:

$$v = v_0/(1+[I]/K_i')$$

to the experimental data, where v is the rate, v_0 is the rate in absence of inhibitor, and [I] is the inhibitor concentration[21]. The true inhibition constant K_i was calculated by correction of K_i' according to:

$$K_i = K_i'/(1+[S]/K_m)$$

where [S] is the substrate concentration and K_m is the Michaelis constant[22].

Structure calculations

The structure of the L-phenylalanine-derived enantiomer of **3** was calculated in the program Sybyl 7.3 using the Tripos force field. A simulated annealing was performed by heating to 700 K for 1000 fs followed by cooling to 200 K within 1000 fs. Low-energy conformers were selected and their structure subsequently energetically minimized using the Powell gradient method (0.05 kcal/mol, 1000 iterations). A low-energy conformation with values of –69° and +128° for ϕ and ψ, respectively, was selected and subjected to molecular dynamics simulation at different temperatures of 100 K, 300 K, 600 K, and 800 K. The chosen parameters were as follows: ensemble: NTV (constant temperature and volume); initial velocity: Boltzmann; time length: 1000 fs; coupling: 100 fs; step: 1 s; number of maximum iterations: 100. Each simulation was followed by energy minimization as described above. After each dynamics/minimization run the dihedral angles were measured, yielding average values for ϕ and ψ of –(80 ± 10)° and +(124 ± 3)°, respectively.

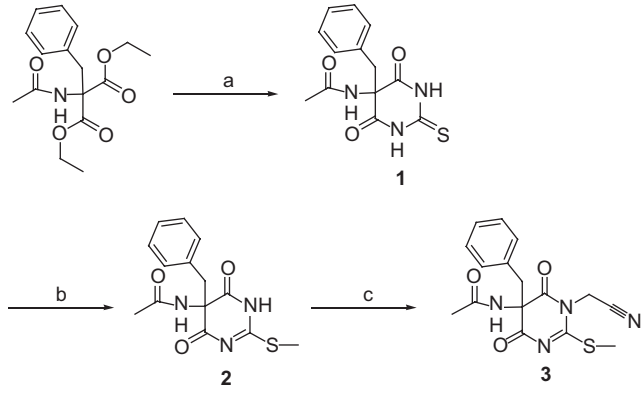
The structure of the corresponding open-chain *N*-acetyl-phenylalanyl-glycine nitrile was generated by constraining the dihedral angles to ϕ = –139° and ψ = +135°, typical values for the extended β-strand conformation[23], and energy minimization as described above.

Results and discussion

Synthesis

A heterocyclic mimetic of *N*-acetyl-phenylalanyl-glycine nitrile was designed by connecting the α-carbon of phenylalanine with the peptide bond nitrogen by a -CO–N=C(SCH_3)- chain (Scheme 1). To afford this heterocyclic dipeptide derivative **3**, the synthesis started with the preparation of the thiobarbituric acid **1** by cyclocondensation of thiourea and diethyl 2-acetamido-2-benzylmalonate. This intermediate was *S*-methylated to obtain **2** in good yield. Compound **2** was converted to the final product **3** by *N*-alkylation with bromoacetonitrile under phase transfer catalysis. The chemoselective alkylation at the *N*-atom was confirmed by NMR as well as by EI-MS due to the occurrence of the retro-Diels-Alder fragmentation of the molecular ion of **3** (see "Materials and methods").

The synthesis of the open-chain dipeptide nitrile **8** containing a methionine-derived dimethylsulfonium moiety is



Scheme 1. Synthesis of compound **3**. Reagents and conditions: (a) $(NH_2)_2CS$, NaOEt, EtOH, reflux; (b) 1 N NaOH, CH_3I, acetone, room temperature; (c) $BrCH_2CN$, $(nBu)_4NBr$, 1 N NaOH, H_2O/CH_2Cl_2.

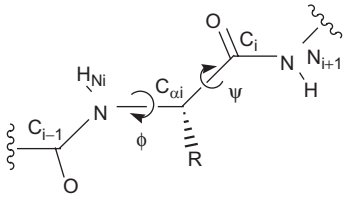
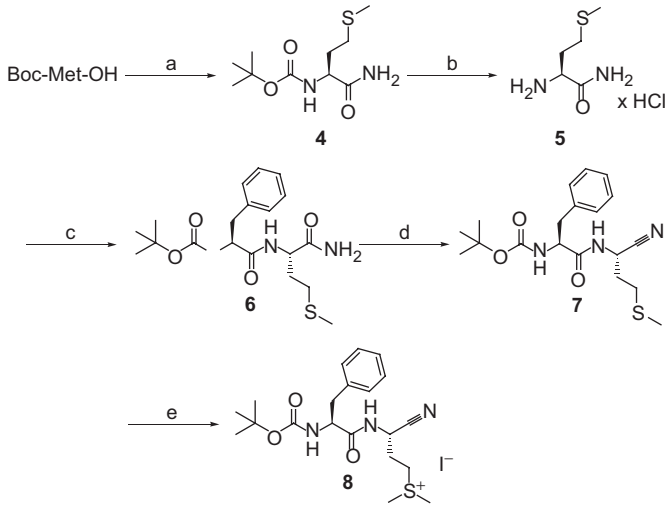


Figure 2. The dihedral angles ϕ and ψ at the peptidic backbone. ϕ is defined by the atoms C_{i-1}, N_i, $C\alpha_i$, and C_i and ψ by N_i, $C\alpha_i$, C_i, and N_{i+1}.



Scheme 2. Synthesis of compound **8**. Reagents and conditions: (a) (i) *N*-methylmorpholine, $ClCO_2CH_2CH(CH_3)_2$, THF; (ii) 25% NH_3, −25°C to room temperature; (b) 4 N HCl/dioxane, room temperature; (c) (i) Boc-Phe-OH, *N*-methylmorpholine, $ClCO_2CH_2CH(CH_3)_2$, THF; (ii) **7** in 1 N $NaOH/H_2O$, −25°C to room temperature; (d) $(ClCN)_3$, DMF, room temperature; (e) CH_3I, ethyl acetate, room temperature.

outlined in Scheme 2. The route involved the formation of the Boc-protected methionine amide **4** and deprotection to the amino acid amide **5**. Coupling of **5** with Boc-Phe-OH and treatment of the resulting dipeptide amide **6** with cyanuric chloride in DMF[24] gave the dipeptide nitrile **7**. The dimethylsulfonium derivative **8** was finally obtained by *S*-methylation of **7** with methyl iodide in moderate yield.

The dipeptide-derived dinitrile **10** (Scheme 3) was prepared by the same methodology as that applied for the preparation of **8**. Dehydratization of the two primary amide groups in **4** with cyanuric chloride in DMF afforded **10** in very good yield.

Inhibitory activity

The heterocyclic nitrile **3** was prepared to combine the dipeptide nitrile pharmacophore and the structural requirements for a thioimidate trapping, i.e. the cyanomethyl group and a neighboring methylthio group attached to an sp^2 hybridized carbon. Despite its dipeptide nitrile structure, compound **3** did not show any inhibitory activity against papain. This can be explained by different reasons. The first is that the amide bond between the P^2 and P^1 positions lacks a proton for hydrogen bonding, and the second is that the "nonpeptidic" parts of the pyrimidine ring may lead to steric hindrance in the active site of the enzyme. Another

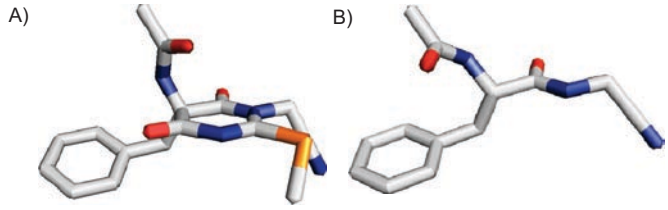


Figure 3. (A) Three-dimensional representation of the L-phenylalanine-derived enantiomer of compound **3**. The values for the dihedral angles are ϕ = −80° and ψ = +124°. (B) For comparison, the corresponding open-chain *N*-acetyl-phenylalanyl-glycine nitrile in the extended, bioactive conformation (ϕ = −139°, ψ = +135°) is shown. Images prepared with PyMOL[29].

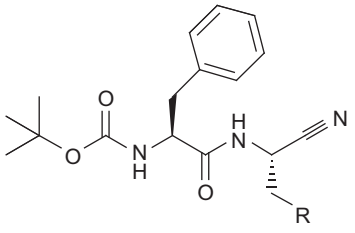
explanation for the lack of inhibitory activity concerns the conformational rigidity of the compound. It is widely accepted that proteases generally recognize their substrates and inhibitors in an extended β-strand conformation[25]. The conformation of the main chain of a peptide is described by the dihedral angles ϕ and ψ (Figure 2). The pyrimidine ring in compound **3** restricts the possible values of the dihedral angles. Simulated annealing[26] and molecular dynamics simulations[27] for the L-phenylanine-derived enantiomer of **3** suggested for ϕ and ψ average values of −80° and +124°, respectively. This matches approximately the *I* + 1 position of a β-II turn ($\phi_{i+1} = -60°$, $\psi_{i+1} = +120°$)[28]. Therefore, one can conclude that the peptidic backbone in compound **3** is restricted in a rather bended conformation, which cannot be recognized by the proteases (Figure 3). A decrease in the cysteine protease-inhibiting activity as a result of rigidization was also observed with similarly constrained aldehyde and vinyl sulfone inhibitors[30]. However, the fact that the dihedral angles meet those of the β-II turn makes this type of compound interesting for the design of turn mimetics.

To enable the inhibitors to adopt the bioactive conformation, open-chain dipeptide nitriles containing the electrophilic center in the side chain of the P^1 position were designed. This was promising, as the P^1 position in papain-like cysteine proteases is mostly solvent exposed, and therefore a broad range of functionalities should be tolerated there[31]. The electrophiles considered were a second cyano group in the 1,2-position as realized in the cyanoalanine nitrile **10** and the γ-carbon attached to a positively charged sulfur atom in the methionine-derived sulfonium salt **8**. The cyanoalanine nitrile was selected as dielectrophile because it has been demonstrated that succinic dinitrile reacts with mercaptoacetic acid to form the corresponding 2-alkylthio-5-aminopyrrole, according to Figure 4[32]. In the methionine-derived sulfonium-salt **8**, the nitrogen of the cyano group could attack the γ-carbon in an S_N2 reaction after formation of the thioimidate, with the active-site thiol leading to an enzyme-bound five-membered cyclic thioimidate. According to Baldwin's rules on ring closing reactions, this represents a 5-*exo-tet* process, which—as all *exo-tet* ring closures—is favored[33]. Methionine-derived sulfonium salts have been successfully applied to the synthesis of pyrrolinones[34].

The three dipeptide nitriles **7**, **8**, and **10** were evaluated against the cysteine proteases papain and cathepsin L, S, and K (Table 1). As expected, derivative **7**, containing no electrophilic group in the P^1 side chain, showed a reasonable inhibition against all enzymes in the higher nanomolar to the lower micromolar range. This confirms that the Phe moiety in P^2, the N-terminal Boc-group as well as the Met residue, as one among a number of possible residues in P^1, can be well adopted by all considered enzymes. Typical for peptide-derived nitriles, its inhibition behavior was time-independent[11,20].

Compounds **8** and **10** were also potent inhibitors of cysteine proteases, with K_i values of similar magnitude (Table 1). However, as the dipeptide nitriles **8** and **10** were designed as mechanism-based inactivators, a two-step mechanism for their interactions with the enzymes was expected. The first step should comprise the reversible formation of the thioimidate by reaction of the active-site thiol with the cyano group, followed by the second, irreversible intramolecular reaction of the thioimidate nitrogen with the carbon-electrophile in the P^1 side chain. However, the evaluation of the two side chain-modified dipeptides revealed linear progress curves for each inhibitor concentration (Figure 5). The sulfonium salt **8** showed similar inhibition constants when compared to its nonmethylated counterpart **7**. In contrast, the inhibitory activity of the dinitrile **10** was significantly weaker than that of **7**, especially for the cathepsins L and K, as well as

Table 1. Inhibition of cathepsin L, cathepsin S, cathepsin K, and papain by dipeptide nitriles with electrophilic side chains in the P1 position.



		K_i (μM)			
Compound	R	Bovine cathepsin L	Human cathepsin S	Human cathepsin K	Papain
7	CH_2SCH_3	0.27 ± 0.02	0.65 ± 0.03	3.6 ± 0.6	0.25 ± 0.01
8	$CH_2S^+(CH_3)_2$	0.52 ± 0.07	0.73 ± 0.03	3.8 ± 0.3	n.d.
10	C≡N	0.90 ± 0.02	0.84 ± 0.04	6.3 ± 0.5	1.1 ± 0.1

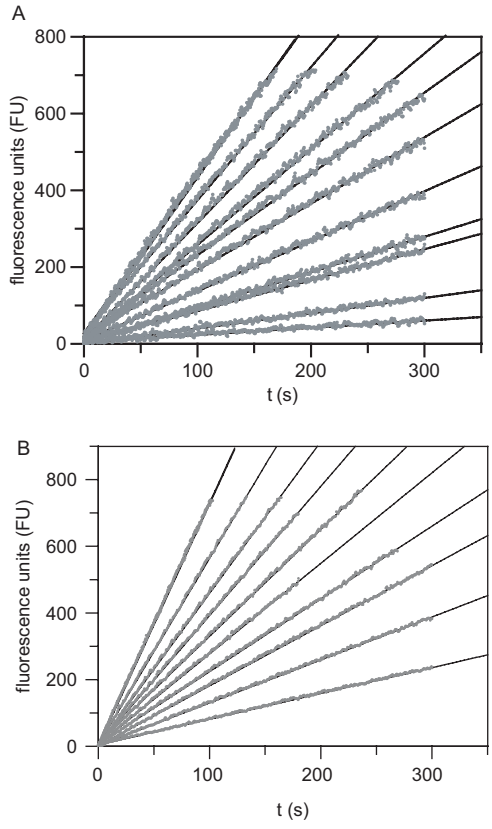
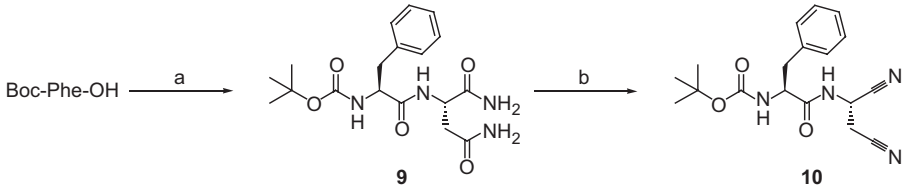


Figure 5. (A) Cathepsin S-catalyzed hydrolysis of Z-Val-Val-Arg-NHMec (40 μM) in the presence of increasing concentrations of compound **8** (from top to bottom: 0, 0, 0.3, 0.5, 1.0, 2.0, 3.0, 5.0, 7.0, 10, 20, 100 μM) in 50 mM potassium phosphate pH 6.5, 50 mM NaCl, 2 mM EDTA, 0.01% Triton X-100, 25 μM DTT, 1% DMSO, 37°C. (B) Cathepsin L-catalyzed hydrolysis of Z-Phe-Arg-NHMec (10 μM) in the presence of increasing concentrations of compound **10** (from top to bottom: 0, 0, 1, 2, 3, 4, 5, 7, 10, 15, 50 μM) in 0.1 M sodium phosphate pH 6.0, 0.1 M NaCl, 5 mM EDTA, 0.01% Brij 35, 25 μM DTT, 1% DMSO, 37°C. The reactions were initiated by addition of the enzyme. Fluorescence emission at 440 nm was measured after excitation at 360 nm. Fluorescence units (FU) were corrected for background fluorescence.



Scheme 3. Synthesis of compound **10**. Reagents and conditions: (a) (i) *N*-methylmorpholine, $ClCO_2CH_2CH(CH_3)_2$, THF; (ii) H-Asn-NH_2 × HCl, H_2O, 1 N NaOH, –25°C to room temperature; (b) $(ClCN)_3$, DMF, room temperature.

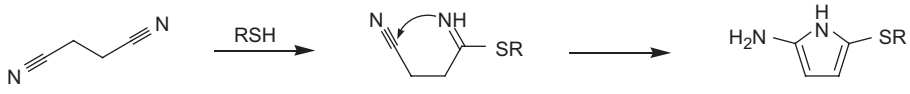


Figure 4. Formation of 2-alkylthio-5-aminopyrroles from succinic dinitrile and thioles (R = CH_2COOH) according to Elnagdi et al.[32].

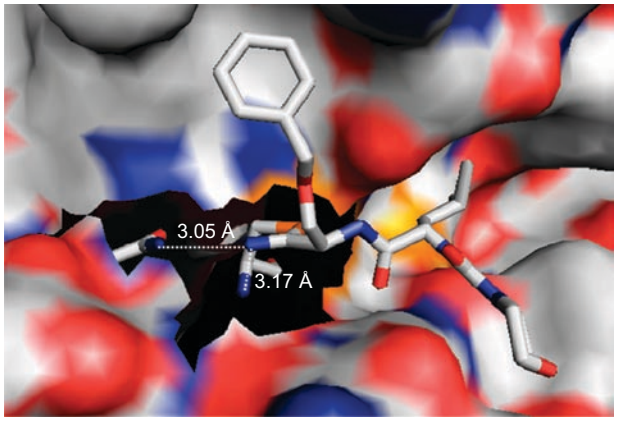


Figure 6. Crystal structure of the complex between cathepsin S and morpholinocarbonyl-leucyl-*O*-benzylserine nitrile. The thioimidate nitrogen is in hydrogen bond distance to the side chain nitrogen of Gln 19 and the main chain nitrogen of Cys 25. PDB entry 1MS6[37], image prepared with PyMOL[29].

papain. This is probably due to the fact that the cyanomethyl residue in P^1 is shorter than the Met-derived side chains of the derivatives **7** and **8** in the according position, and thus the interactions to the P^1 or possibly the $P^{1\prime}$ binding sites are less attractive than in the case of **10**. The linearity of the substrate conversion observed in the inhibition experiments for the compounds **8** and **10** indicates that no covalent trapping of the thioimidate adduct occurred, as irreversible inhibition is generally associated with a slow two-step mechanism leading to steady-state rates equaling zero[35,36]. Therefore, it can be concluded that a further covalent modification of the thioimidate nitrogen by a nearby electrophile cannot be achieved in the nitrile-derived enzyme-inhibitor complexes.

This might be because the thioimidate adduct—despite the fact that it is planar—is stabilized by hydrogen bond contacts between the nitrogen and the NH of the side chain of Gln 19, similar to the stabilizing interaction between the tetrahedral oxyanion intermediate and the oxyanion hole formed by the backbone NH of Cys 25 and the side chain amide group of Gln 19. Indeed, such a stabilization of the enzyme-bound thioimidate becomes evident from a published crystal structure of cathepsin S complexed with a dipeptide nitrile, morpholinocarbonyl-leucyl-*O*-benzylserine nitrile[37]. The nitrogen atom of the thioimidate group formed from the cyano group by attack of the active-site thiol is in hydrogen bond distances of 3.17 Å and 3.05 Å to the backbone nitrogen of Cys 25 and the side chain nitrogen of Gln 19, respectively (Figure 6). Such information from protein crystallography and our kinetic results indicate the limitations of an approach to irreversibly inhibit cysteine proteases by applying thioimidate-trapping cascade reactions.

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***Declaration of interest*:** The authors report no conflicts of interest.

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