Department of Pharmaceutical/Medicinal Chemistry[1], Institute of Pharmacy, Ernst-Moritz-Arndt-Universität Greifswald and Department of Pharmaceutical Chemistry[2], Albert-Ludwigs-Universität Freiburg, Germany

β-Lactam derivatives as enzyme inhibitors: N-substituted derivatives of (*S*)-4-oxoazetidine-2-carboxylate as inhibitors of elastase and papain

K. Achilles[1], M. Schneider[2], T. Schirmeister[2] and H.-H. Otto[1]

N-Alkyl and N-acyl substituted 4-oxoazetidine-2-carboxylates are synthesized and evaluated as inhibitors of the proteases porcine pancreatic elastase (PPE) and papain. The compounds are obtained by alkylation or acylation at the nitrogen of benzyl (*S*)-4-oxoazetidine-2-carboxylate, which is synthesized by a modified literature procedure. The enzymatic assays prove some derivatives to be effective inhibitors of PPE and/or papain. The N-BOC protected amino acid derivatives **10** and **13** inhibit PPE reversibly with K_I-values in the micromolar range. On the other hand, papain is inactivated irreversibly by benzyl (*S*)-2-(benzyloxycarbonyl)azetidin-1-acetate (**6**).

1. Introduction

Proteases, in particular the serine protease elastase and cysteine proteases, have been found involved in the pathogenesis of a large number of diseases [1]. Following the concept of development of protease inhibitors bearing a chemical reactive group allowing a nucleophilic attack at the active site of the enzyme, and a peptide moiety responsible for the recognition by the enzyme, we now report about syntheses and properties of some derivatives of benzyl (*S*)-2-oxoazetidine-4-carboxylate (**3**).

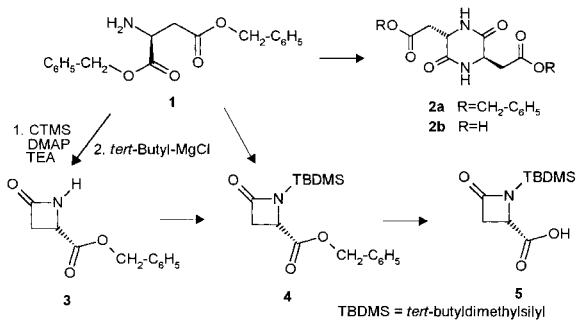
2. Investigations, results and discussion

2.1. Synthesis of the compounds

The first synthesis of **3** starting from (*S*)-4-vinylazetidin-2-one was reported by H. Pietsch [2] in 1976. We prefer the synthesis from dibenzyl aspartate (**1**) as described by Salzmann et al. [3]. However, the last two steps of the reported sequence, silylation at nitrogen and cyclization to the lactam ring, are, at least in our hands, completely unsatisfactory, and have therefore been modified.

The addition of a catalytic amount of 4-dimethylaminopyridine (DMAP) during *N*-silylation and, more important, the use of *tert*-butylmagnesium chloride instead of ethylmagnesium bromide during ring-closure make the preparation much shorter and more effective. Another side reaction during this synthesis is the dimerization of dibenzyl aspartate yielding (3*S*,6*S*)-dibenzyl 2,5-dioxopiperazine-3,6-diacetate (**2a**), which can be hydrogenated with H_2/Pd-C to the free diacid **2b** [4]. As reported by Pietsch, the hydrogenolysis of **3** yields the free acid which is only stable below −80 °C as it is destroyed by autoprotolysis. Therefore, **3** is first transformed to **4**, and then the free acid **5** becomes available in 75% yield [5]. The highest yields finally are obtained, when we prepare **4** directly from **1** by a reaction with *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide in analogy to a method of Baldwin [6].

Scheme 1

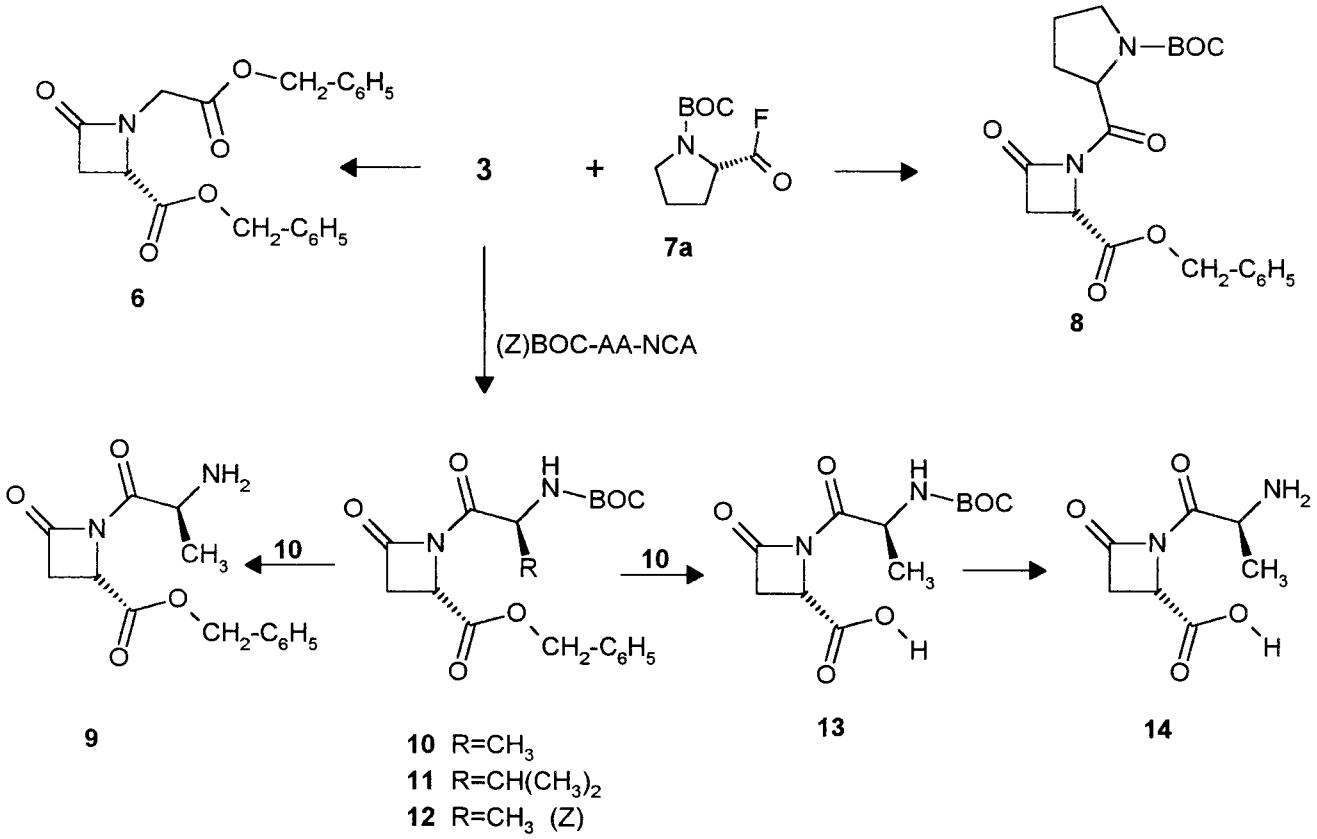


The *N*-alkylation of **3** with bromoacetates is not very successful using ptc-conditions due to several side-reactions such as ring-opening or hydrolysis of the benzyl ester. The yield is not higher than 22%, but finally, the alkylation with benzyl bromoacetate is achieved by using n-butyllithium in THF/TMEDA yielding **6**. However, alkylation with other esters and transformation into the free diacid fails.

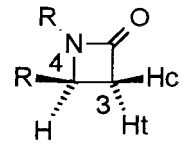
N-Acylation [7–10] of β-lactams is a well known process but causes very often severe problems especially when the combination acid chloride/triethylamine is used. To introduce amino acyl residues we tried different active esters without great success. Finally, we ended up with two methods. The synthesis of **8** is not possible with 1-(*N*-BOC-L-prolyl)pyrrolidine-2,5-dione (**7b**) but it is achieved by a reaction between freshly prepared N-BOC-proline fluoride (**7a**) and **3** in THF at −78 °C. Using similar conditions reactions between **3** and *N*-protected amino acid *N*-carboxyanhydrides (NCA) give **10**, **11** and **12** in yields of 18–71%. In all reactions we use BuLi as deprotonating agent, all reactions fail when triethylamine is used. Compound **10** prepared from **3** and BOC-L-alanine-NCA is hydrogenated in the presence of Pd/C-catalyst giving the β-lactam carboxylic acid **13**. Deprotection of **10** at nitrogen with hydrochloric acid yields the hydrochloride of **9**, and from **13** the hydrochloride of **14** is obtained as a very unstable and hygroscopic crystalline compound (Scheme 2).

All structures are clearly established by spectroscopic data. The IR spectra of **3** and **5** show two carbonyl bands at 1773 and 1736 cm^{-1}, and 1740 and 1690 cm^{-1}, while that of **4** only gives one band at 1759 cm^{-1}. The β-lactam carbonyl bands of **8** are detected at 1743 and 1685 cm^{-1}. The IR spectra of **10**–**12** are characterized by three strong carbonyl absorptions at about 1800, 1750 and 1710 cm^{-1}. Compound **9** is detected by the IR carbonyl bands at 1809, 1751, and 1720 cm^{-1}, **13** at 1806 and 1709 cm^{-1}, and the hydrochloride of **14** at 1808 and 1729 cm^{-1}.

Scheme 2



All ^{1}H NMR spectra show great similarity. The ABX spectra of the β-lactam ring shown above are found at δ = 3.03 to 3.26 (A part 3-H_t), δ = 3.31–3.62 (B part 3-H_c), and δ = 4.20–4.63 ppm (X part 4-H) with J_{AX} = 2.5–3.7, J_{BX} = 5.8–7.1, and J_{AB} = 15.0–16.5 Hz. The 300 MHz ^{1}H NMR spectrum of **3** shows additional couplings between N-H and the 3-H with $J_{N\text{-}H/3\text{-}Hc}$ = 2.1 Hz and $J_{N\text{-}H/3\text{-}Ht}$ = 1.5 Hz. The coupling between N-H and 4-H is not detectable [11, 12]. As can be deduced from the ^{1}H NMR spectra, no remarkable racemization occurs during the syntheses. The only exception is **8**, from which two diastereomers are detected in the NMR spectrum with a ratio of 6 : 4. But a separation of the diastereomers does not seem to be possible.



2.2. Enzyme inhibition

The values for the enzyme inhibition are obtained from the time-dependent decrease of enzyme activity monitored by the increase of UV-absorption at 405 nm due to the release of *p*-nitroaniline from the respective substrates: Ac-Ala-Ala-Ala-pNA for elastase, and *Nα*-benzoylarginine-*p*-nitroanilide·HCl (L-BAPA) for papain. In cases where no time-dependent inhibition is observed, the inhibitor constants K_I are calculated from Dixon plots. Lineweaver-Burke and Eadie-Hofstee plots indicate that the mode of inhibition of porcine pancreatic elastase (PPE) is competitive for the substrate. Alternatively, inactivation rates for time-dependent inhibition are determined by dilution assays according to Kitz and Wilson [13] or continuously as described by Tian and Tsou [14]. K_I-values [mM] characterizing the inhibition of PPE are found for **10**, 0.35, and for **13**, 0.98. The inactivation rates k_{2nd} [$M^{-1}min^{-1}$] characterizing the inhibition of papain in a continuous assay found are 40 (**6**) and 36 (**10**). In a dilution assay the following rates are determined: 90 (**6**), 50 (**10**) and 14 (**13**).

The highest inhibitory activity against PPE is found for the *N*-acylated compounds **10** and **13**. This is in good accordance with the results from Doherty et al. who found high inhibition constants for *N*-acylated β-lactams bearing a leaving group in position 4 [15]. All other compounds show no or only weak inhibitory activity against PPE. The *N*-alkylated compound **6**, and the *N*-acylated compounds **10** and **13** are weak inhibitors of papain. All compounds are irreversible inhibitors. Inactivation rates obtained from the dilution assays are comparable to those obtained from continuous assays.

3. Experimental

3.1. Chemistry

M.p: not corrected; Linström apparatus. IR spectra (cm^{-1}): Perkin-Elmer IR 841; in KBr, if not noted otherwise. NMR spectra: Varian U-300 (300 MHz) for ^{1}H; Varian U-300 (75.4 MHz) for ^{13}C; δ in ppm, J in Hz; ^{1}H values and ^{13}C values from spectra in $CDCl_3$, if not noted otherwise. Standard reference for all spectra in $CDCl_3$ or D_4-MeOH was the signal of the undeuterated solvent obtained in D_2O (not marked) or TMS, δ_{TMS} = 0 ppm (marked with *). UV spectra: Pharmacia Ultrospec III. Polarimeter: Perkin-Elmer 241. Mass Spectra: Finnigan MAT 312, MAT44S or TSQ-700. Elementary analyses: Pharmazeutisches Institut or Chemisches Laboratorium der Universität Freiburg. All the results were in an acceptable range. R_f values from CC (silica gel 60 Merck 7734). Tlc (Merck Alufolien, silica gel 60 F_{254}).

Tetrahydrofuran (THF) was stored with KOH, then refluxed with Na and benzophenone, and distilled prior to use. Other solvents were dried/puri-

fied according to literature procedures. Unless noted otherwise, moisture and air sensitive reactions were conducted under a dry N_2 atmosphere.
PPE (EC 3.4.21.36) was purchased from Serva (Nr. 20929), L-BAPA from Merck KGaA, Darmstadt, and papain from carica papya (EC 3.4.22.2), from Fluka. All enzymes were used without further purification. All substrates were purchased from Bachem.
Abbreviations: BuLi = n-Butyllithium, 1.6 M solution in n-hexane; EtOAc = Ethyl acetate; TEA = Triethylamine; CTMS = Chlorotrimethylsilane; DMAP = 4-Dimethylaminopyridine; TMEDA = Tetramethylethylendiamine; DCC = Dicyclohexylcarbodiimide; PPE = Porcine pancreatic elastase; ar = aromatic.

3.1.1. Dibenzyl L-aspartate (**1**) [16]

Yield 25 g (97%). – $[\alpha]_D^{25} = -32$ (c = 1.2, MeOH). – ^{1}H NMR* (80 MHz): $\delta = 1.73$ (s, 2 H, NH_2), 2.81 (mc, 2 H, CH_2), 3.87 (mc, 1 H, CH), 5.10, 5.13 (2s, 4 H, CH_2), 7.2–7.4 (m, 10 H, ar-H).

3.1.2. Dibenzyl (3S,6S)-2,5-dioxopiperazine-3,6-diacetate (**2a**) [4]

After some days, obtained as crystals from **1**. The crystals are separated and washed with diethyl ether. M.p 152 °C (EtOH). – $[\alpha]_D^{25} = -69.5$ (c = 1, $CDCl_3$). – IR: $\nu = 3189$, 3059 (NH), 1739 (C=O). – ^{1}H NMR*: $\delta = 2.90$, 3.13, 4.38 (ABX, 6H, $J_{AX} = 9.2$ Hz, $J_{BX} = 3.0$ Hz, $J_{AB} = 17.8$ Hz, $J_{XNH} = 1.2$ Hz), 5.15 (s, 4 H, 2 CH_2), 6.75 (s, 2 H, N-H), 7.30–7.38 (m, 10 H, ar-H). – MS-EI (70 eV): m/z (%) = 410 (20) [M^+], 319 (20) [$M^+ - C_7H_7$], 303 (10) [$M^+ - C_7H_7O$], 275 (50) [$M^+ - C_8H_7O_2$].
$C_{22}H_{22}N_2O_6$ (410.4)

3.1.3. (3S,6S)-2,5-Dioxopiperazine-3,6-diacetic acid (**2b**) [4]

1 g (2.4 mmol) of **2a** is dissolved in 100 ml of a mixture of EtOH and dioxane. Then 0.4 g of Pd-C is added, and the mixture is hydrogenated (ca. 3 h) until no further hydrogen is absorbed. The catalyst is separated, and the solvent evaporated. The crystalline residue is dried in vacuo. Yield 240 mg (87%). – M.p 254 °C. – $[\alpha]_D^{25} = -37$ (c = 1, DMSO). – IR: ν = 3336 (NH), 2981 (OH), 1750, 1708, 1675 (C=O). – ^{1}H NMR* (80 MHz, [d_6]DMSO): δ = 2.67 (m, 4 H, 2 CH_2), 4.27 (m, 2 H, 2 CH), 8.08 (s, 2 H, 2 N-H).

3.1.4. Benzyl (S)-4-oxoazetidine-2-carboxylate (**3**) [3, 6]

At 0 °C under N_2, 9.7 ml (70 mmol) of TEA, a catalytic amount of DMAP (≈ 50 mg), and 8.9 ml (70 mmol) of CTMS are added to a solution of 22.0 g (70 mmol) of **1** in 350 ml of diethyl ether. After stirring for 3 h, the mixture is warmed to RT. The precipitated hydrochloride is separated. Diethyl ether (700 ml) is added, and the solution is cooled to −5 °C under N_2. Then, 38.5 ml (77 mmol) of *tert*-butylmagnesium chloride (2 M solution in diethyl ether) is added dropwise, and the mixture is stirred vigorously for 30 min at −5 °C, and then 12 h at RT. With cooling, the solution is acidified with HCl-satd. diethyl ether. The precipitated salt is dissolved in 100 ml of H_2O, the phases are separated, and the aqueous layer is extracted with 100 ml of diethyl ether. The combined organic layers are dried (Na_2SO_4) and evaporated in vacuo. The residue is dissolved in 50 ml of MeOH, heated to 50 °C, and the solvent is removed in vacuo. The product is crystallized from diethyl ether. Yield: 5.1 g (32%). – Light yellow square crystals. – M.p 134–138 °C (isopropanol) (ref. [3] 141–143 °C). – $[\alpha]_D^{25} = -35.4$ (c = 1, MeOH) [6]. – IR: $\nu = 3202$ (NH), 1773, 1736 (C=O). ^{1}H NMR* [8]: $\delta = 3.07$, 3.31, 4.20 (ABX, $J_{AX} = 2.5$ Hz, $J_{BX} = 6.1$ Hz, $J_{AB} = 14.9$ Hz, $J_{ANH} = 1.5$ Hz, $J_{BNH} = 2.0$ Hz, 3 H, 3-H_t, 3-H_c, 4-H), 5.20 (s, 2 H, CH_2), 6.36 (bs, 1 H, NH), 7.40 (mc, 5 H, ar-H).
$C_{11}H_{11}NO_3$ (205.2)

3.1.5. Benzyl (S)-1-(tert-butyldimethylsilyl)-4-oxoazetidine-2-carboxylate (**4**)

a) At −78 °C, 3.75 ml (6 mmol) of BuLi are added to a solution of 1.02 g (5 mmol) of **3** in 50 ml THF. After 5 min, 0.9 g (6 mmol) of *tert*-butylchlorodimethylsilane in 5 ml THF is added, the mixture is stirred 1 h at −78 °C and 2 h at RT, then, a satd. solution of NH_4Cl is added, the organic layer is separated, and the aqueous layer is extracted three times with EtOAc. The combined organic layers are dried (Na_2SO_4), concentrated in vacuo, and the residue is purified by cc (silica gel 60, EtOAc/cyclohexane 1 : 1). Yield: 1.24 g (78%).
b) 660 mg (2.1 mmol) of **1**, 1.5 ml (6.3 ml) of *N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide, and 32 mg (0.21 mmol) of *tert*-butylchlorodimethylsilane in 10 ml CH_3CN are stirred for 30 min, the solvent is evaporated, and the excess of the silylation agent is removed in vacuo (1 mm Hg). The residue is dissolved in 20 ml of diethyl ether, cooled to 0 °C, and then, 1.2 ml of a 2 molar solution of *tert*-butylmagnesiumchloride in diethyl ether is added. The mixture is allowed to warm to RT, stirred for 12 h, and hydrolyzed with a satd. solution of NH_4Cl. The layers are separated, the organic layer is washed with H_2O and a satd. solution of NaCl, dried (Na_2SO_4), and concentrated in vacuo. The residue is purified by CC (silica gel 60, EtOAc/cyclohexane 1 : 1). Yield: 490 mg (73%). – Colorless viscous liquid. – $[\alpha]_D^{25} = -58$ (c = 1, chloroform) [6]. – IR (film): $\nu = 1759$ (C=O). – ^{1}H NMR* [11, 12]: $\delta = 0.06$ (s, 3 H, CH_3), 0.24 (s, 3 H, CH_3), 0.93 [s, 9 H, $(H_3C)_3C$], 3.06, 3.32, 4.06 (ABX, 3 H, $J_{AX} = 2.9$ Hz, $J_{BX} = 5.9$ Hz, $J_{AB} = 15.1$ Hz, 3-H_t, 3-H_c, 4-H), 5.18 (s, 2 H, CH_2), 7.38 (s, 5 H, ar-H).
$C_{17}H_{25}NO_3Si$ (319.5)

3.1.6. (S)-1-(tert-Butyldimethylsilyl)-4-oxoazetidine-2-carboxylic acid (**5**)

1 g (3.13 mmol) of **4** and 0.4 g Pd-C in 150 ml of EtOH are hydrogenated for 3 h at RT and normal pressure. The catalyst is separated and the solvent removed by distillation. Yield: 700 mg (98%). – Colorless crystals. – M.p 129–130 °C (THF). – $[\alpha]_D^{25} = -71.6$ (c = 1, chloroform). – IR: $\nu = 2932$ (CH), 1740 (C=O). – ^{1}H NMR*: $\delta = 0.16$ (s, 3 H, CH_3), 0.30 (s, 3 H, CH_3), 0.96 [s, 9 H, $(H_3C)_3C$], 3.15, 3.40, 4.07 (ABX, $J_{AX} = 2.8$ Hz, $J_{BX} = 6.0$ Hz, $J_{AB} = 15.1$ Hz, 3 H, 3-H_t, 3-H_c, 4-H), 8.30 (bs, 1 H, CO_2H).
$C_{10}H_{19}NO_3Si$ (229.4)

3.1.7. Benzyl (S)-1-[(benzyloxycarbonyl)methyl]-4-oxoazetidine-2-carboxylate (**6**)

At −78 °C, under N_2, 6.25 ml (10 mmol) of BuLi is added to a solution of 2.05 g (10 mmol) of **3** and 2.32 g (20 mmol) of TMEDA in 100 ml of THF. The mixture is stirred for 2 min, and then, 4.58 g (20 mmol) of benzyl bromoacetate is added. After stirring for 15 min at −78 °C, the mixture is warmed to RT. 100 ml of brine is added, and the layers are separated. The aqueous layer is extracted with EtOAc, and the combined organic layers are dried (Na_2SO_4), and concentrated in vacuo. The residue is dissolved in 100 ml of diethyl ether and subsequently washed with 3 × 100 ml of H_2O. The organic phase is dried ($MgSO_4$) and concentrated. The residue is purified by CC (silica gel, EtOAc). Yield: 2.08 g (59%). - Colorless liquid. – $[\alpha]_D^{25} = -33.7$ (c = 0.74, EtOH). – IR (film): $\nu = 3031$, 2958 (CH), 1746 (CO). – ^{1}H NMR: $\delta = 3.02$, 3.28, 4.45 (ABX, $J_{AX} = 2.7$ Hz, $J_{BX} = 5.9$ Hz, $J_{AB} = 14.7$ Hz, 3 H, 3-H_t, 3-H_c, 4-H), 3.85, 4.39 (AB, J = 18.3 Hz, 2 H, CH_2), 5.14 (AB, J = 14.4 Hz, 2 H, CH_2), 7.31 (m, 10 H, ar-H). – MS (70 eV): *m/z* (%) = 353 (0.4) [M^+], 276 (0.7) [M^+-Ph], 262 (0.5) [M^+-Bn], 218 (3.4) [M^+-CH_2COOBn], 91 (100). – HRMS: Calcd. 353.1263; found 353.1257.

3.1.8. N-(tert-Butoxycarbonyl)-L-proline fluoride (**7a**)[3]

At −10 °C, and under N_2, 377 µl (4.65 mmol) of pyridine and 1.2 ml (13 mmol) of cyanurfluoride are added to a solution of 1 g (4.65 mmol) of BOC-L-proline in 5 ml of dichloromethane and stirred for 1 h. 10 ml of CH_2Cl_2 and 10 g of crashed ice are added to the formed white suspension, the organic layer is separated, the aqueous layer is extracted with 10 ml of CH_2Cl_2, the combined organic layers are washed with ice-cold H_2O, dried (Na_2SO_4), and the solvent is removed at RT. Yield: 760 mg (81%). – Colorless liquid. – $[\alpha]_D^{25} = -34$ (c = 1, EtOAc). IR: $\nu = 2981$ (CH), 1851, 1752 (C=O).

3.1.9. 1-[N-(tert-Butoxycarbonyl)-L-prolyl]pyrrolidine-2,5-dione (**7b**)

At −10 °C, 2.15 g (10 mmol) of BOC-L-proline, 2.3 g (20 mmol) of *N*-hydroxysuccinimide, and 2.27 g of DCC in 100 ml of CH_2Cl_2 are stirred and finally warmed to RT. After 24 h, the precipitate (dicyclohexylurea) is separated by filtration, the solvent removed by distillation, and 100 ml of EtOAc is added to the residue. After stirring for 30 min, the precipitate is filtered off, the solvent removed by distillation, and the residue is purified by CC (silica gel 60, EtOAc). Yield: 2.8 g (90%). – M.p 133 °C. – IR: $\nu = 2985$ (CH), 1818, 1789, 1749, 1697 (C=O). – ^{1}H NMR: $\delta = 1.41$ [s, 9 H, $(H_3C)_3C$], 1.88-2.46 [m, 4 H, β-H, γ-H (pro)], 2.82 (s, 4 H, 2 CH_2), 3.40–3.64 [m, 2 H, δ-H(pro)], 4.56 [dd, 1 H, α-H(pro)].

3.1.10. Benzyl N-[N-(tert-butoxycarbonyl)prolyl]-4-oxoazetidine-2-carboxylate (**8**)

At −78 °C, 2.3 ml (3.7 mmol) of BuLi are added to a suspension of 750 mg (3.7 mmol) of **3** in 50 ml of THF. Then, 760 mg of **7a** is added, stirring is continued for 15 min at −78 °C, the mixture is warmed to RT, and hydrolyzed with a satd. solution of NaCl. The organic layer is separated, the aqueous layer twice extracted with EtOAc, the combined organic layers are dried (Na_2SO_4), and the solvent is removed in vacuo. The residue is purified by CC (silica gel 60, CH_2Cl_2/MeOH 6 : 1). Yield: 300 mg (20%). – Light yellow viscous liquid. – $[\alpha]_D^{25} = -82.9$ (c = 0.8, MeOH). – IR (film): $\nu = 3064$, 3032, 2976, (C–H), 1743, 1685 (C=O). – ^{1}H NMR ([d_4]methanol, values from 300 MHz ^{1}H-^{1}H-COSY spectra): Isomer 1 : $\delta = 1.44$ [s, 9 H, $C(CH_3)_3$], 1.76–2.02 [m, 3 H, β-H′(pro), γ-H(pro)], 2.15–2.34 [m, 1 H, β-H(pro)], 2.98, 3.33, 4.53 (ABX, $J_{AX} = 3.3$ Hz, $J_{BX} = 6.8$ Hz, $J_{AB} = 15.9$ Hz, 3 H, 3-H_t, 3-H_c, 4-H), 3.35–3.60 [m, 2 H, δ-H(pro)], 4.94 [ABX, $J_{AX} = 9.3$ Hz, $J_{BX} = 3.7$ Hz, 1 H, α-H(pro)], 5.13, 5.26 [AB, $J_{AB} = 12.3$ Hz, 2 H, CH_2(benzyl)], 7.25–7.38 (mc, 5 H, ar-H]. – Isomer 2: $\delta = 1.44$ [s, 9 H, $C(CH_3)_3$], 1.76–2.02 [m, 3 H, β-H′(pro), γ-H(pro)], 2.15–2.34 [m, 1 H, β-H(pro)], 3.04, 3.35, 4.50 (ABX,

$J_{AX} = 6.8$ Hz, $J_{BX} = 3.3$ Hz, $J_{AB} = -16.2$ Hz, 3 H, 3-H, 4-H), 3.35–3.60 [m, 2H, δ-H(pro)], 4.88 [ABX, $J_{AX} = 9.1$ Hz, $J_{BX} = 3.7$ Hz, 1 H, α-H(pro)], 5.15, 5.27 [AB, $J_{AB} = -12.1$ Hz, 2 H, CH_2(benzyl)], 7.25–7.38 (mc, 5 H, ar-H). – MS EI (70 eV/2 mA): *m/z* (%) = 402 (4) [M^+], 346 (56) [M-$C_4H_8^+$], 329 (16) [M-$C_4H_9O^+$], 301 (45) [M-$C_5H_9O_2^+$], 170 (72) [M-$C_9H_{16}NO_2^+$], 91 (100) [$C_7H_7^+$].
$C_{21}H_{26}N_2O_6$ (402.5)

3.1.11. Benzyl (S)-1-[(S)-alanyl]-4-oxoazetidine-2-carboxylate hydrochloride (**9**)

At 0 °C, 100 mg (0.27 mmol) of **10** is dissolved in 20 ml of with HCl satd. EtOAc, stirred for 1 h at 0 °C, then 12 h at RF. The solvent is removed in vacuo, and the residue is dried in vacuo for 24 h. Yield: 83 mg (98%). – Colorless solid. – M.p 123 °C. – $[\alpha]_D^{25} = -68.7$ (c = 1.04, EtOH). - IR: ν = 3493 (NH), 1809, 1751, 1720 (CO). – ^{1}H NMR ([d_4]methanol): δ = 1.53 (d, J = 7.1 Hz, 3 H, CH_3), 3.26, 3.58, 4.69 (ABX, $J_{AX} = 3.7$ Hz, $J_{BX} = 7.1$ Hz, $J_{AB} = 16.5$ Hz, 3 H, 3-H_t, 3-H_c, 4-H), 4.47 [q, J = 7.1 Hz, 1 H, α-H(ala)], 5.22, 5.26 (AB, J = 12.3 Hz, 2 H, CH_2), 7.38 (m, 5 H, ar-H). – ^{13}C NMR ([d_4]methanol): δ = 16.79 (CH_3), 28.67 [$C(CH_3)_3$], 41.79 (C-3), 51.71 (C-4), 80.70 [$C(CH_3)_3$], 164.46, 171.40 (CO). MS (FAB): m/z (%) = 277 (100) [M^+ + 1-HCl].
$C_{14}H_{17}ClN_2O_4$ (312.8)

3.1.12. General procedure for the N-acylation of **3** *with (Z)BOC-AA-NCA*

Under N_2, a solution of 6.8 mmol of **3** in 50 ml of THF is cooled to −78 °C, 4.7 ml (7.5 mmol) of BuLi is added, and the mixture is stirred for 10 min. A solution of the appropriate BOC-amino acid-NCA (6.8 mmol) in 10 ml of THF is added, and the reaction is completed by stirring at −78 °C for 1 h. The mixture is warmed to RT, and subsequently quenched with 100 ml of a satd. solution of NH_4Cl. The layers are separated, and the aqueous layer is extracted twice with 50 ml EtOAc each. The combined organic layers are dried (Na_2SO_4) and concentrated *in vacuo*. The product is purified by CC.

3.1.13. Benzyl (S)-1-[(S)-N-(tert-butoxycarbonyl)alanyl]-4-oxoazetidine-2-carboxylate (**10**)

Purified by CC (cyclohexane/EtOAc = 8 : 2). Yield: 1.82 g (71%). – $R_f = 0.20$. – Waxy solid. - M.p 119 °C. - $[\alpha]_D^{25} = -92.6$ (c = 1.04, EtOH). – IR: ν = 3331 (NH), 1793, 1745, 1719, 1713, 1671 (CO). – ^{1}H NMR: δ = 1.36 (d, J = 7.3 Hz, 3 H, CH_3), 1.41 [s, 9 H, $C(CH_3)_3$], 3.03, 3.35, 4.53 (ABX, $J_{AX} = 3.4$ Hz, $J_{BX} = 6.8$ Hz, $J_{AB} = 16.1$ Hz, 3 H, 3-H_t, 3-H_c, 4-H), 4.85 [m, 1 H, α-H(ala)], 5.02 (m, 1 H, N-H), 5.16, 5.25 (AB, J = 12.1 Hz, 2 H, CH_2), 7.35 (m, 5 H, ar-H).
$C_{19}H_{24}N_2O_6$ (376.4)

3.1.14. Benzyl (S)-1-[(S)-N-(tert-butoxycarbonyl)valyl]-4-oxoazetidine-2-carboxylate (**11**)

Purified by CC (cyclohexane/EtOAc = 8 : 2). Yield: 0.8 g (41%). – $R_f = 0.26$. – Colorless solid. – M.p 58 °C. – $[\alpha]_D^{25} = -64.6$ (c = 0.95, EtOH). – IR: ν = 3351 (NH), 1799, 1746, 1712, 1673 (CO). – ^{1}H NMR: δ = 0.85, 1.04 (2d, each J = 6.9 Hz, 3 H, CH_3), 1.43 [s, 9 H, $C(CH_3)_3$], 2.19 [m, 1 H, β-H(val)], 3.03, 3.35, 4.53 (ABX, $J_{AX} = 3.5$ Hz, $J_{BX} = 6.9$ Hz, $J_{AB} = 16.1$ Hz, 3 H, 3-H_t, 3-H_c, 4-H), 4.80 [m, 1 H, α-H(val)], 5.03 (m, 1 H, N-H), 5.19, 5.23 (AB, J = 12.2 Hz, 2 H, CH_2), 7.37 (m, 5 H, ar-H).
$C_{21}H_{28}N_2O_6$ (404.5)

3.1.15. Benzyl (S)-1-[(S)-N-(benzyloxycarbonyl)alanyl]-4-oxoazetidine-2-carboxylate (**12**)

Purified by CC (cyclohexane/EtOAc = 1 : 1). Yield: 0.74 g (37%). – R_f = 0.48. – Colorless liquid. – $[\alpha]_D^{25} = -45.1$ (c = 0.84, EtOH). – IR (film): ν = 3384 (NH), 1802, 1707 (CO). – ^{1}H NMR: δ = 1.40 (d, J = 7.1 Hz, 3 H, CH_3), 3.03, 3.35, 4.51 (ABX, $J_{AX} = 2.9$ Hz, $J_{AB} = 16.1$ Hz, 3 H, 3-H_t, 3-H_c, 4-H), 4.89 [m, 1 H, α-H(ala)], 5.10, 5.21 (2AB, each J = 12.2 Hz, 2 H, CH_2), 5.31 (m, 1 H, N-H), 7.32 (m, 10 H, ar-H).
$C_{22}H_{22}N_2O_6$ (410.4)

3.1.16. (S)-1-[(S)-N-(tert-Butoxycarbonyl)alanyl]-4-oxoazetidine-2-carboxylic acid (**13**)

A suspension of 1.22 g (3.2 mmol) of **10** in 150 ml of EtOH and 250 mg Pd/C (10%) is degassed several times and subsequently shaken in an H_2 atmosphere. The hydrogenation is completed when no further absorption is observed (~ 8 h). The catalyst is filtered off, and the solvent is evaporated in vacuo. Yield: 0.91 g (98%). – Colorless solid. – M.p. 158 °C. – $[\alpha]_D^{25} = -83.4$ (c = 0.94, EtOH). – IR: ν = 3412 (OH), 1806, 1709 (CO). – ^{1}H NMR: δ = 1.38 [d, 3 H, CH_3; s, 9 H, $C(CH_3)_3$], 3.13, 3.39, 4.50 (ABX, $J_{AX} = 3.5$ Hz, $J_{BX} = 6.8$ Hz, $J_{AB} = 16.3$ Hz, 3 H, 3-H_t, 3-H_c, 4-H), 4.81 [m, 1 H, α-H(ala)], 5.07 (m, 1 H, N-H), 6.58 [bs, 1 H, CO_2H].
$C_{12}H_{18}N_2O_6$ (286.3)

3.1.17 (S)-1-[(S)-Alanyl]-4-oxoazetidine-2-carboxylic acid hydrochloride (**14**)

From 120 mg (0.47 mmol) of **13** as described for **9**. Yield 86 mg (96%). – Very hygroscopic solid. – $[\alpha]_D^{25} = -75.8$ (c = 0.36, EtOH). – IR (film): ν = 3432 (OH), 1808, 1729 (CO). – ^{1}H NMR ([d_4]methanol): δ = 1.65 [d, J = 7.1 Hz, 3 H, CH_3), 3.23, 3.62, 4.63 (ABX, $J_{AX} = 3.7$ Hz, $J_{BX} = 7.1$ Hz, $J_{AB} = 16.3$ Hz, 3 H, 3-H_t, 3-H_c, 4-H), 4.54 [q, J = 7.1 Hz, 1 H, α-H(ala)]. – ^{13}C NMR ([d_4]methanol): δ = 15.97 (CH_3), 42.45 (C-3), 48.15 [α-C(ala)], 51.22 (C-4), 68.80 (CH_2), 129.42, 129.61, 129.66, 136.60 (ar-C), 164.25, 167.74, 169.84 (C=O). MS (FAB): m/z (%) = 187 (76) [M^+ +1-HCl].
$C_7H_{11}ClN_2O_4$ (222.6)

3.2. Enzyme kinetics

3.2.1. Elastase

The assays were performed at porcine pancreatic elastase concentrations of 7.5 μg/ml and substrate concentrations between 0.46–0.52 mM Ac-Ala-Ala-Ala-pNA in tris-buffer (0.1 M, pH 8.0). Stock solutions of elastase were prepared by dissolving the enzyme in 1 mM acetic acid. The rate of enzymatic hydrolysis was monitored at 405 nm continuously for 20 min via the release of *p*-nitroaniline at 25 °C at 405 nm ($\varepsilon = 9960\ l\ mol^{-1}\ cm^{-1}$). The K_m value was calculated to be 0.69 mM. Both substrate and inhibitor were dissolved in DMSO and all assays were performed with the same final concentration of DMSO (10–15%). Enzyme, inhibitor and substrate were mixed and the time-dependent increase in the UV-absorption was monitored over 20 min (final concentrations [I]: **8**: 0.70 mM; **10**: 0.38–0.72 mM; **13**: 0.91 mM. Without inhibitor no significant decrease in enzyme activity occurred during the time of the assay (steady-state conditions). For each inhibitor, two independent experiments were performed, each with at least three different inhibitor concentrations and two substrate concentrations, respectively.

3.2.2. Papain

The assays were performed at papain concentrations 1.3–3.5 μM and L-BAPA as substrate at concentrations of 1.37–1.80 mM. Solutions of papain were prepared freshly by incubating the enzyme (about 100 mg/10 ml = 30–35 μM) in 0.05 M sodium phophate buffer (pH 6.5), which contained 5 mM EDTA and 5 mM cysteine for 30 min at 25 °C. The K_m-value was determined as 2.5 mM from five independent experiments. The substrate was dissolved in 1 ml DMSO and diluted with buffer, the inhibitor was dissolved in DMSO. All assays were performed with the same final concentration of DMSO. The rate of enzymatic hydrolysis was monitored by the release of p-nitroaniline at 25 °C at 405 nm ($\varepsilon = 9960\ l\ mol^{-1}\ cm^{-1}$).

3.2.2.1. Dilution assay

Papain was incubated with various inhibitor concentrations (final concentrations [I]: **8**: 0.43–4.35 mM; **10**: 5.53 mM; **13**: 0.32–3.22 mM) for varying periods, 5–40 min, each 5–7 values. Following this preincubation with inhibitor, the substrate was added, and the remaining enzyme activity was monitored as described above. Without inhibitor, no significant decrease in enzyme activity was observed (steady-state conditions). The experiments were repeated with 3–7 inhibitor concentrations. Two independent assays were carried out for each inhibitor.

3.2.2.2. Continuous assay

Enzyme, inhibitor and substrate were mixed and the time-dependent increase in absorption was monitored at 405 nm over 20 min (final concentrations [I]: **8**: 0.11–0.57 mM; **10**: 0.37 mM; **13**: 1.1 mM). Without inhibitor, no significant decrease in enzyme activity occurred (steady-state conditions).
Kinetic constants were obtained by non-linear or linear regression analysis with the program GraFit® [17].

Part of the theses of K. A. and M. S., University of Freiburg, 1997 and 1996
[3] From the thesis of Till Röhrich, University of Freiburg 1993

Acknowledgements: We wish to thank Degussa AG, Frankfurt, for the generous support with amino acids, E. Merck KGaA, Darmstadt, for the support with chromatography materials, and the "Fonds der Chemischen Industrie" for financial support. T. S. gratefully acknowledges the continued support from the "Deutsche Forschungsgemeinschaft".

References

1 For detailled ref. see: Achilles, K.; Schirmeister, T.; Otto, H.-H.: Arch. Pharm. 2000, in press
2 Pietsch, H.: Tetrahedron Lett. **45**, 4053 (1976)

3 Salzmann, T. N.; Ratcliffe, R.; Christensen, B. G.; Bouffard, F. A.: J. Am. Chem. Soc. **102**, 6161 (1980)
4 Bergeron, R. J.; Phanstiel IV, O.; Yao, G. W.; Milstein, S.; Weimar, W. R.: J. Am. Chem. Soc. **116**, 8479 (1994)
5 Rehling, H.; Jensen, H.: Tetrahedron Lett. 2793 (1972)
6 Baldwin, J. E.; Adlington, R. M.; Gollins, D. W.; Schofield, C. J.: Tetrahedron **46**, 4733 (1990)
7 Bergmann, H.-J.; Otto, H.-H.: Arch. Pharm. **319**, 635 (1986)
8 Kricheldorf, H. R., Makro. Chem. **170**, 89 (1973)
9 Testa, E.; Pifferi, G.; Fontanella, L.; Aresi, V.: Liebigs Ann. Chem. **696**, 108 (1966)
10 Schlack, P. (Farbwerke Hoechst), DBP 1186065, 1965; C.A. **62**, 10382c (1965)
11 Meider, P. J.; Grabowski, E.: Tetrahedron Lett. **23**, 3868 (1982)
12 Moriconi, E.J.; Meyer, W. C.: J. Org. Chem. **36**, 2841 (1971)
13 Kitz, R., Wilson, I.: J. Biol. Chem. **237**, 3245 (1962)
14 Tsou, W. X.; Tian, C. L.: Biochemistry **21**, 1028 (1982)
15 Knight, W. B.; Green, B. G.; Cesabin, R. M.; Gale, P.; Maycock, A. L.; Weston, H.; Kuo, D. W.; Westler, W. M.; Dorn, C. P.; Finke, P. E.; Hagman, W. K.; Hale, J. J.; Liesch, J.; MacCoss, M.; Navia, M. A.; Shah, S. K.; Underwood, D.; Doherty, J. B.: Biochemistry **31**, 8160 (1992)
16 Bryant, P. M.; Young, G. T.: J. Chem. Soc. 3868 (1959)
17 GraFit®, Version 3.0, Erithacus Software Ltd., London 1992

Received March 10, 2000
Accepted March 24, 2000

Prof. Dr. Hans-Hartwig Otto
Pharmazeutische/Medizinische Chemie
Institut für Pharmazie
Friedrich-Ludwig-Jahn-Str. 17
D-17487 Greifswald