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Thiirancarboxamides as Inhibitors of Papain

Derivatives of the thiirancarboxylic acid building-block containing a peptide bond were synthesised and screened against the model cysteine protease papain. The most active of the series showed a second-order rate constant of inactivation comparable to that of the parent compound. The insertion of a peptide moiety seems to compensate the lack of a free carboxylate interacting with the histidinium ion at the enzyme's active site.

Keywords: Thiirane; Episulfide; Cysteine protease; Inhibitor; Papain

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Introduction

Cysteine proteases belong to a heterogeneous family of peptidases almost ubiquitous in nature [1]. Their key role in the life cycle of certain viruses [2–4], bacteria [5], and protozoa [6] offers a starting point for the design of cysteine protease inhibitors as therapeutic agents.

Lysosomal and cytoplasmatic cysteine proteases in mammals (cathepsins and calpains) fulfil specific house-keeping functions. An abnormal expression or an imbalance between proteases and endogenous inhibitors seems to be associated with a number of diseases such as malignancy of certain tumours, e.g. in prostate carcinoma [7], progression of rheumatoid arthritis [8], destruction of muscle fiber in sarcoid myopathy [9], neurodegenerative processes as in Alzheimer's disease [10] and Huntington's disease [11], ischemic neuronal death [12], calcium-triggered cell death [13], and atrial fibrillation [14].

Therefore, the urgency of new therapeutically active compounds is reflected by the strong efforts done lately in the development of more potent cysteine protease inhibitors. Three-membered heterocycles rank among the most promising irreversible cysteine protease inhibitors, due to their sensitivity against the thiolate at the active site of the enzyme.

Since the discovery of the naturally occuring irreversible inhibitor E-64, epoxysuccinyl peptides have represented a fruitful field for the development of potent cysteine protease inhibitors: Loxistatin reached phase III in the clinical trial, but was stopped later because of side-effects [15].

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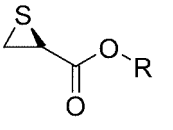
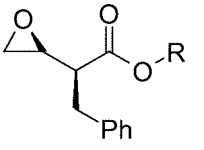
Replacement of the oxirane ring with an aziridine gave rise to new classes of peptidic inhibitors: the ring nitrogen offered a third position for derivatisation and, therefore, the possibility to influence activity and selectivity of those inhibitors.

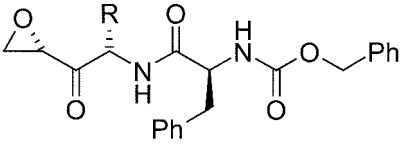
Thiirane is structurally related to oxirane and aziridine, being susceptible to ring-opening by nucleophiles as



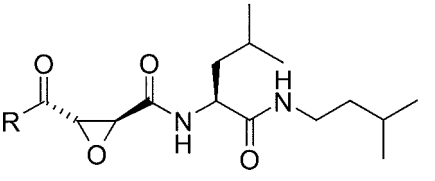
1a, R=H
1b, R=Me
1c, R=Et



2a, R=Me
2b, R=Bzl



3a, R=Me
3b, R=CH_2CH_2Ph



4a, R=OH
4b, R=NH_2
4c, R=OEt
4d, R=Me

Figure 1. Thiirane and epoxide derivatives as cysteine protease inhibitors.

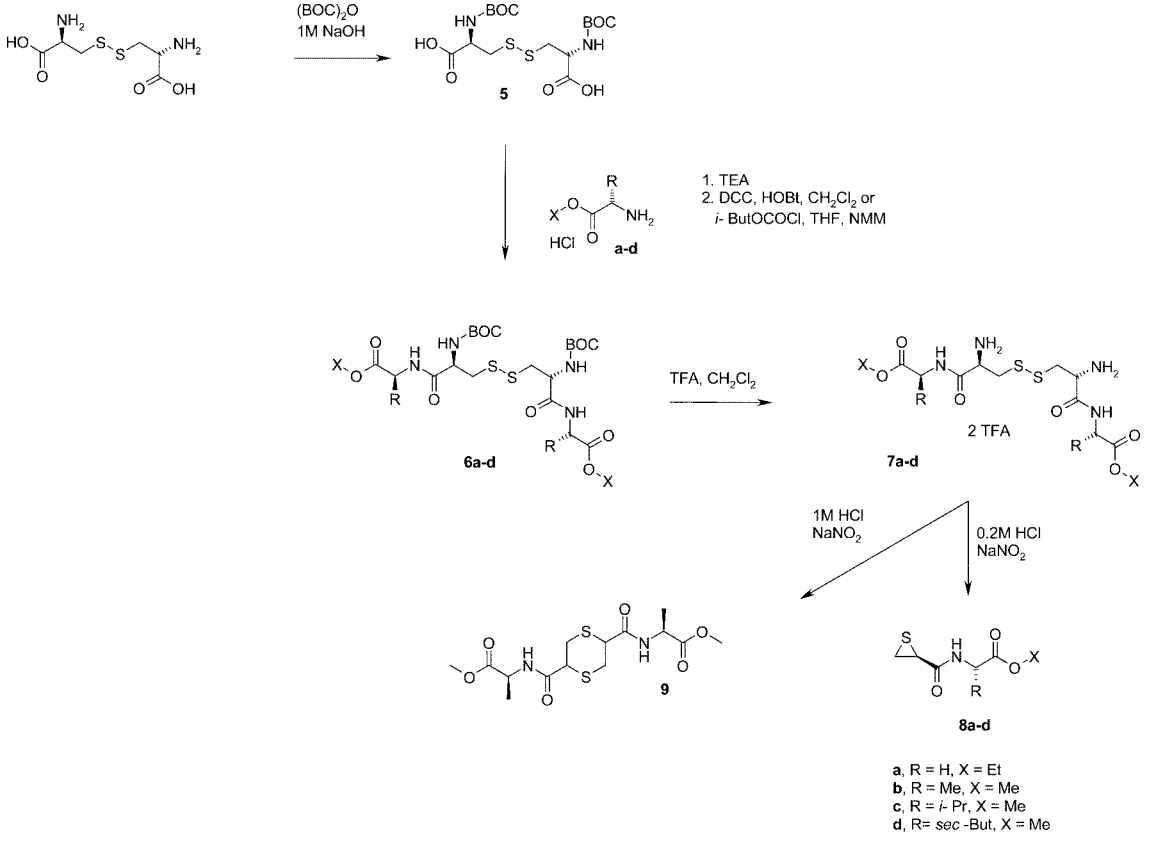
well. However, it shows a slower reaction rate of ring opening under either acidic or basic conditions, when compared with oxirane and aziridine [16-18], due to less ring strain and to lower electronegativity of the sulphur.

As reported previously [19], (*S*)-thiirancarboxylic acid (**1a**, Figure 1) irreversibly inactivates the cysteine protease papain. (*S*)-methyl- and ethyl thiirancarboxylate (**1b**, **1c**) showed a non time-dependent inhibition. A strong ionic interaction between the inhibitor and the imidazolium ion at the active site justified the importance of the free carboxylic acid for good inhibition. When the reaction rate of the (*S*)-aziridine-2-carboxylic acid is compared with that of its amide, the lack of ionic interactions affecting the inactivation rates of papain is evident [20].

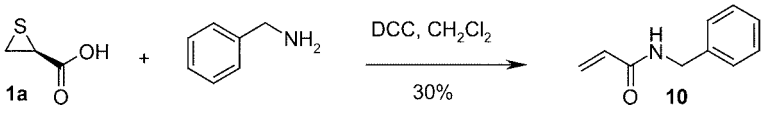
A direct comparison with an epoxide analogue is not possible, since no oxirane-2-carboxylate was tested so far against cysteine proteases; however, methyl- and benzyl esters of the homologue *(S,S)*-2-benzyl-3,4-epoxybutanoic acid (BEBA) (**2a**, **2b**, Figure 1) irreversibly inactivate papain (k_{2nd} 18 M^{-1} s^{-1} and 49 M^{-1} s^{-1}, respectively) [21] and epoxyketone derivates with a peptidic chain (**3a**, **3b**) were reported to be potent irreversible inhibitors of cruzain [22].

Indeed, the impact of an amide bond on the affinity and the alkylating capability of the thiirane-2-carboxylic acid is of interest.

Mechanistic studies on the inactivation of papain by epoxysuccinyl inhibitors suggested that a free carboxylate in those structures may not be an absolute



Scheme 1. Syntheses of thiirancarboxamides **8a**–**d**.



Scheme 2. Reaction of thiirancarboxylic acid with benzyl amine/DCC.

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requirement for a good enzyme-inhibitor interaction. The alkylation constant values (*ki*) of amide, ester, and ketone (**4b**, **4c**, **4d**, Figure 1) derivates of EP-475 (**4a**) are comparable to that of the parent compound (**4a**), indicating the need for at least a carbonyl group for hydrogen bonding to the enzyme [23].

In order to explore how the inhibitory activity of the thiirancarboxylic acid building block can vary by substituting the free acid with a secondary amide group, we synthesised a series of thiirancarboxamides with size-increasing side chains. In this paper, we discuss the inhibition constants of these compounds found for the inhibition of the model enzyme papain.

Results and discussion

(L)-(*R,R*)-Cystine was protected with *t*BOC following a known procedure [24] and then coupled with the appropriate L-amino acid by the symmetric anhydride approach with dicyclohexylcarbodiimide/*N*-hydroxybenzotriazole (Scheme 1).

This step ran with pretty good yields between 60 and 82%. Alternatively, the *(S,S)*-isoleucine derivative was synthesised by the asymmetric anhydride approach with isobutylchloroformate. After cleavage of the *t*BOC group with trifluoroacetic acid, the synthesis of the episulfides was carried out in the presence of nitrous acid, running with inversion of configuration, according to a mechanism proposed by Owen and Leone [25]. The end product (**8a**–**d**) was isolated in good overall yield. Carrying out the reaction at lower pH values led to dithiane (**9**) via dimerisation [26]. Attempts, to obtain **8a**–**d** by peptide coupling of *(S)*-thiirancarboxylic acid (**1a**) with the appropriate amino acid esters using different standard coupling procedures (DCC; EEDQ; DPPA; pentafluorophenyl ester of the thiirancarboxylic acid; acid chloride of the thiirancarboxylic acid) failed. The reaction of thiirancarboxylic acid with DCC and benzylamine as model amine to test several coupling methods led to the formation of benzyl acrylamide (**10**) (Scheme 2).

The second-order rates of inactivation (k_{2nd}) shown in Table 1 were determined for the inhibition of papain by the inhibitors **8a**–**d**; the method was described previously [19, 27].

Thiirancarboxamides (**8a**–**d**) irreversibly inactivate papain (Figures 2, 3). The second-order rate constant for inhibition of papain by the most potent of the series shows the same order of magnitude as the parent compound **1a** (Table 1). These products completely lack any negatively charged moiety that could interact with the histidinium ion at the enzyme's active site.

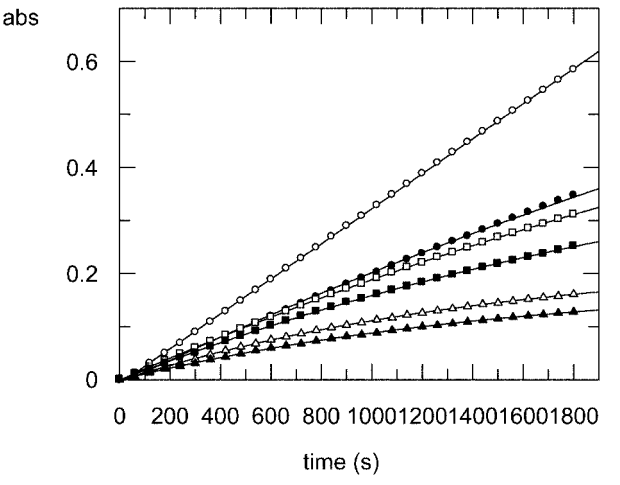

Figure 2. Continuous assays with papain and derivative **8d**. Progress curves of the hydrolysis of the substrate L-BAPA (absorption *versus* time) at increasing inhibitor concentrations: ○ without inhibitor; ● [I] = 0.037 mM; □ [I] = 0.124 mM; ■ [I] = 0.249 mM; △ [I] = 0.747 mM; ▲ [I] = 0.871 mM.

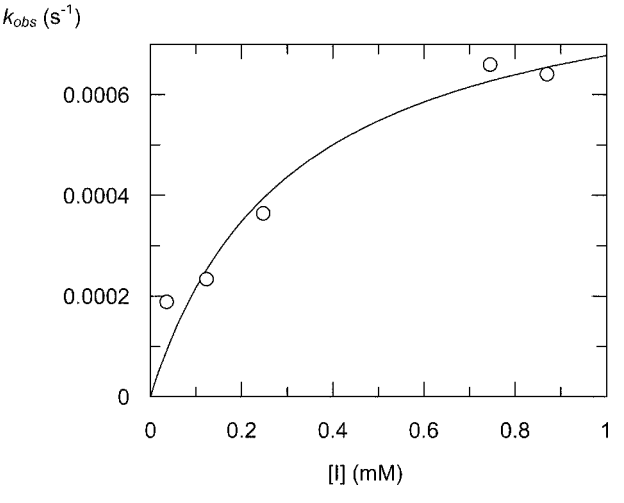

Figure 3. Inactivation of papain with **8d**. Plot of pseudo first-order inactivation rates k_{obs} *versus* inhibitor concentration.

This kind of interaction is known to be important for the inhibitory activity of epoxysuccinyl peptides and probably for the thiirancarboxylic acid (**1a**).

The insertion of a peptidic moiety, however, positively influences the affinity of the inhibitor for the active site of the enzyme. This suggests that an electrostatic interaction of the thiirane carboxylate moiety is not an absolute requirement if another part of the molecule can stabilize the enzyme-inhibitor interaction.

Table 1. Second-order rate constants determined for the inhibition of papain by thiirancarboxamides **8a**–**d**[a].

	R	X	AA	k_i (s^{-1})	K_i (mM)	k_{2nd} (M^{-1} min^{-1})
8a	H	Et	Gly	0.0008	1.93	24
8b	Me	Me	Ala	[b]	[b]	33
8c	*i*-Pr	Me	Val	0.0012	0.47	156
8d	*sec*-But	Me	Ile	0.0009	0.2	234
1a[19]						222

[a] Assays were conducted in 0.05 M phosphate buffer, pH 6.5, 5 mM EDTA and 5 mM cysteine, at 30 °C.
[b] Measurements were limited to the linear range, with [I] $\ll K_i$. Therefore, only the second-order rate constant could be obtained.

The lipophilicity of the molecules grows with the size of the side-chain. A correspondance between the improvement of the inhibitory activity and the size of the amino acid residue is observed. This can be explained with a better fitting of the molecule into papain's active site by positioning the hydrophobic side-chain in the S2 subsite which is known to accept hydrophobic residues like Leu and Ile, respectively. This higher affinity is reflected by the decreasing K_i-values of the compounds while the k_i-values remain approximately constant. According to these results we propose a binding mode in which the Ile side chain of inhibitor **8d** is located in the S2 subsite of papain consisting of the amino acids Trp69, Tyr67, Phe207, Pro68, Ala160, Val133, and Val157 (papain numbering) [1].

Acknowledgments

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Experimental

General methods

NMR spectra were recorded on a Bruker Avance-400 instrument (Bruker, Rheinstetten, Germany). *J* values are given in Hz. Mass spectra were recorded on a Finnigan MAT 8200 (Finnigan, San Jose, CA, USA) under electron impact (EI) or chemical ionisation (CI) conditions. IR spectra were recorded on a Perkin-Elmer 841 instrument (Perkin-Elmer, Ueberlingen, Germany). All melting points were determined on a Buechi type 530 (Buechi, Flawil, Switzerland) and the values are uncorrected. Optical rotations were determined using a Perkin-Elmer type 241.

Column chromatography was performed with Merck silica gel 60 (0.063–0.200). Analytical TLC was performed on alumina sheets coated with silica gel 60 F254 (Merck, Darmstadt, Germany). Anhydrous reactions were conducted under an atmosphere of dry nitrogen. Anhydrous solvents were prepared following literature standard procedures. Other solvents were of reagent grade. All other reagents were used as received. Solvents were removed by rotary evaporation.

(R,R)-{2-tert-Butoxycarbonylamino-3-[2-tert-butoxycarbonylamino-2-(ethoxycarbonylmethylcarbamoyl)-ethyldisulfanyl]-propionylamino}-acetic acid ethyl ester (**6a**)

To a stirred solution of *(R,R)-bis*-BOC-cystine **5** (0.55 g, 1.25 mmol) in dry dichloromethane, 1-hydroxybenzotriazole (0.337 g, 2.5 mmol) and a solution of *N,N*-dicyclohexylcarbodiimide (0.515 g, 2.5 mmol) in dichloromethane were added. A solution of glycine ethyl ester, freshly prepared by dropwise addition of triethylamine (1.095 g, 25 mmol) to an ice-cold and stirred solution of glycine ethyl ester hydrochloride (0.349 g, 2.5 mmol) in dry DMF (15 cm^3), was then added. The mixture was stirred overnight, filtered off, and the residue was washed with dichloromethane (15 cm^3). The organic phases were evaporated, the residue dissolved in ethyl acetate, washed with 2 M hydrochloric acid (2 × 25 cm^3), brine (2 × 15 cm^3), dried with magnesium sulphate, and evaporated. The crude peptide was purified by column chromatography on silica gel. Eluent: ethyl acetate/cyclohexane 3/2. Yield: 60 %.

(S,R,S,R)-2-{2-tert-Butoxycarbonylamino-3-[2-tert-butoxycarbonylamino-2-(1-ethoxycarbonylethylcarbamoyl)-ethyldisulfanyl]-propionylamino}-propionic acid methyl ester (**6b**)

The reaction was performed as for (**6a**) using the amino acid *(S)*-Ala. Eluent: ethyl acetate/cyclohexane 1/2. Yield: 63 %.

(S,R,S,R)-2-{2-tert-Butoxycarbonylamino-3-[2-tert-butoxycarbonylamino-2-(1-ethoxycarbonyl-2-methylpropionylcarbamoyl)-ethyldisulfanyl]-propionylamino}-3-methylbutyric acid methyl ester (**6c**)

The reaction was performed as for (**6a**) using the amino acid *(S)*-Val. Eluent: ethylacetate. Yield: 82 %.

(S,S,R,S,S,R)-2-{2-tert-Butoxycarbonylamino-3-[2-tert-butoxycarbonylamino-2-(1-ethoxycarbonyl-2-methylbutylcarbamoyl)-ethyldisulfanyl]-propionylamino}-3-methylpentanoic acid methyl ester (**6d**)

To a stirred and cooled (−15 °C) solution of *(R,R)-bis*-BOC-cystine **5** (2.0 g, 4.54 mmol) in dry tetrahydrofuran (20 cm^3) *N*-methylmorpholine (1.049 mL, 9.45 mmol) and then isobutylchloroformate (1.297 cm^3, 10 mmol) were added successively. After 5 min a solution of *(S,S)*-isoleucine methyl ester hydrochloride (1.645 g, 9.09 mmol) was added. The mixture was stirred for 5 min at −15 °C and then allowed to stand 20 hours at room temperature (rt). The solvent was evaporated under vacuum. The residue was taken up in ethyl acetate and extracted with water. The organic phase was washed successively with 20 cm^3 of a saturated sodium bicarbonate solu-

tion, 20 cm^3 of water, 20 cm^3 of 1[TH]M hydrochloric acid, and 20 cm^3 of water. The solution was dried with magnesium sulphate and then concentrated under vacuum. The peptide was purified by chromatography on silica gel. Eluent: ethyl acetate. The product was recrystallized from ethyl acetate (315 mg). Yield: 10%.

General procedure for the cleavage of the BOC group

To a vigorously stirred ice cold solution of **6a**–**d** (0.43 mmol) in 5 cm^3 dichloromethane, 5 cm^3 trifluoracetic acid were added dropwise. The solution was stirred for 15 min at 0°C and then 5 min at rt. The solvent was evaporated and the products (**7a**–**d**) were used for the next step without further purification.

(S)-Thiirancarbonylamino acetic acid ethyl ester (**8a**)

To a suspension of **7a** (1.070 g, 1.678 mmol) in 9 mL of 0.2 M hydrochloric acid, sodium nitrite (463 mg, 6.714 mmol) was added. The mixture was stirred for one hour at rt. Then, the solution was extracted with chloroform (2 × 10 cm^3), the organic phase dried with magnesium sulphate, and evaporated under vacuum at 30°C. The crude product was purified twice by column chromatography on silica gel to give 190 mg (60%) of a white solid. Eluents: ethyl acetate/petroleum ether 1/1 and ethyl acetate/petroleum ether 1/2.

δ_H (400 MHz; $CDCl_3$; Me_4Si) 1.27 (3H, t); 2.64 (2H, dd, J_{AB} 11.6, J_{AX} 6.8); 3.38 (dd, 1H, J_{AX} 6.8, J_{BX} 5.3); 4.00 (1H, d, J 5.3); 4.20 (2H, q); 6.64 (1H br d); δ_C ($CDCl_3$) 13.6, 23.3, 30.7, 42.2, 63.1, 163.5, 171.8; ν_{max}/cm^{-1} (FT-IR): 3302 (m), 3062 (w), 2963 (m), 1737 (s), 1655 (s), 1532 (s), 1205 (s); mp 75–78°C from ethyl acetate/petroleum ether; $[\alpha]_D^{20} = -1.05$ (c 2 in $CHCl_3$); Anal. Found: C 44.78; H 6.16; N 7.35. Calc. for $C_7H_{15}N_2O_3S$: C 44.43; H 5.86; N 7.40%; *m/z* (CI, react. gas NH_3) 207.1 ($M^+ + NH_4^+$, 100%); 190 (2.4); 175.2 (12.0).

(S,S)-Thiirancarbonyl-2-amino propionic acid methyl ester (**8b**)

The reaction was performed as for (**8a**). Yield: 27% as colourless crystals. Eluent: ethyl acetate/cyclohexane 1/2.

δ_H (400 MHz; $CDCl_3$; Me_4Si) 1.35 (3H, d, J 7.3); 2.57 (2H, dd, J_{AX}, 6.8, J_{AB} 11.3); 3.29 (1H, dd J_{AX} 6.8, J_{BX} 5.0); 3.67 (3H, s); 4.48 (1H, dq, J 7.3); 6.59 (1H, br d); δ_C ($CDCl_3$): 16.2, 24.8, 32.1, 51.9, 62.0, 168.0, 174.7; ν_{max}/cm^{-1} (FT-IR): 3323 (m), 3049 (w), 1734 (s), 1652 (s), 1532 (s), 1217 (s); mp 65–68°C from ethyl acetate/cyclohexane; $[\alpha]_D^{20}$ −1.0 (c 2 in $CHCl_3$) m/z (EI) 189.0 (M^+, 3.9%); 130 (21.5); 103 (15.6); HRMS 189.046 (189.046 calc. for $C_7H_{11}NO_3S$).

(S,S)-3-Methyl-2-(thiirancarbonylamino)-butyric acid methyl ester (**8c**)

The reaction was performed as for (**8a**). Yield: 10% as a colourless viscous oil. Eluents: ethyl acetate/cyclohexane 1/5 and ethyl acetate/cyclohexane 3/5.

δ_H (400 MHz; $CDCl_3$; Me_4Si) 0.9 (6H, dd, J_{AX} 6.8, J_{MX} 21.8); 2.17 (1H, m, J_{AX} 6.8, J_{AX} 4.8, J_{MX} 21.8); 2.64 (2H, dd, J_{AX} 6.8, J_{AB} 12.1); 3.38 (1H, dd, J_{AX} 6.8, J_{BX} 5.1); 3.71 (3H, s); 4.47 (1H, dd, J 4.8, J 9.1); 6.6 (1H, br s); δ_C ($CDCl_3$): 18.0, 19.3, 24.6, 31.4, 32.5, 52.5, 57.6, 169.2, 172.3; ν_{max}/cm^{-1} (FT-IR): 3293 (m), 3078 (w), 1746 (s), 1649 (s), 1554 (s), 1373 (w), 1199 (s); $[\alpha]_D^{20}$ +2.8 (c 2 in $CHCl_3$); *m/z* (EI) 217.1 (M^+, 6.71%); 184 (12.8); 158 (54.0); HRMS 217.078 (217.077 calc. for $C_9H_{15}NO_3S$).

(S,S,S)-3-Methyl-2-(thiirancarbonylamino)-pentanoic acid methyl ester (**8d**)

The reaction was performed as for (**8a**). Yield: 14% as colourless viscous oil. Eluent: ethyl acetate/cyclohexane 1/1. δ_H (400 MHz; $CDCl_3$; Me_4Si) 0.91 (6H, m); 1.15 (1H, m); 1.40 (1H, m); 1.91 (1H, m); 2.64 (2H, dd, J_{AX} 6.8, J_{AB} 16.9); 3.37 (1H, dd, J_{AX} 6.8, J_{BX} 5.3); 3.71 (3H, s), 4.51 (1H, dd, J 4.8, J 8.8); 6.63 (1H, br d); δ_C ($CDCl_3$): 11.9; 15.8; 24.7; 25.5; 32.6; 38.0; 52.5; 57.0; 169.1; 172.3; ν_{max}/cm^{-1} (FT-IR): 3302 (m), 3082 (w), 2963 (m), 1740 (s), 1656 (s), 1459-1436 (m), 1532 (s), 1205 (s); $[\alpha]_D^{20}$ +0.6 (c 4 in $CHCl_3$); *m/z* (EI) 231.1 (M^+, 1.9%); 172.1 (9.0); 102 (1.77).

Abbreviations

AA amino acid, BEBA *(S,S)*-2-benzyl-3,4-epoxybutanoic acid, L-BAPA L-benzoyl arginyl *p*-nitroanilide, DCC dicyclohexylcarbodiimide, DPPA diphenyl phosphorazidate; EEDQ 1-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline; HOBt *N*-hydroxybenzotriazole, NMM N-methyl morpholine, TEA triethylamine, E-64 *(S,S)*-1-[N-[[(L-3-*trans*-carboxyoxiran-2-yl)-carbonyl]-L-leucyl]amino]-4-guanidinobutane, EDTA ethylenedinitrilotetraacetic acid.

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[26] (^{1}H NMR) 1.41 (3H, d, 3.22 (1H, d, J_{AX} 5.3 and J_{AB} 14.15), 3.37 (1H, dd, J_{BX} 8.08 J_{BA} 14.15), 3.75 (3H, s), 4.62 (1H J5.3 and 8.08), 4.68 (1H, q), 7.61 (1H, br d). The product was not further characterized. .

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