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Synthesis and evaluation of chloromethyl sulfoxides as a new class of selective irreversible cysteine protease inhibitors

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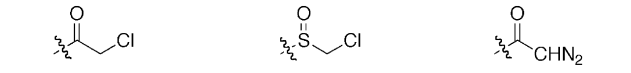
Abstract—The synthesis and biological evaluation of a new class of selective irreversible cysteine protease inhibitors is described. A set of amino acid based chloromethyl sulfoxides was prepared and they were found to inhibit irreversibly the cysteine protease papain. They were selective for cysteine proteases since no inhibition was found for the serine protease chymotrypsin.

1. Introduction

Cysteine proteases are important targets in medicinal chemistry, as they have been implicated in different diseases.[1] Therefore, there is a continuous demand for new and more selective cysteine protease inhibitors. Inhibitors are also needed as activity-based probes for tracking protease activities in cells, tissues and whole animals.[2] Many types of reversible and irreversible inhibitors have been described.[3,4] The more selective inhibitors of cysteine proteases, that is, inhibitors with a strong preference for cysteine versus serine proteases, are typically irreversible inhibitors. Among the irreversible inhibitors are diazomethyl ketones (Fig. 1), epoxides (E64), Michael acceptors and chloromethyl ketones (Fig. 1).[1,3,4] All these inhibitors contain an electrophilic group which can react with the thiol function present in the active site of a cysteine protease. The reactivity of this electrophilic group greatly determines the selectivity and reaction rate of formation of the covalent inhibitor–enzyme complex. With respect to this, peptidyl chloromethanes containing the electrophilic chloromethyl ketone moiety, (Fig. 1) are known to react irreversibly with cysteine proteases, but also with serine proteases.[4]



Chloromethyl Ketone Chloromethyl Sulfoxide Diazomethyl Ketone

Figure 1.

It is expected that by lowering the reactivity of the chloromethyl ketone moiety the selectivity can be improved. We were wondering whether replacement of the carbonyl by a sulfoxide would reduce the reactivity sufficiently to uncover new selective irreversible cysteine protease inhibitors. This replacement would require a chloromethyl sulfoxide moiety (Fig. 1), which as far as we know has not been described in the literature for incorporation in protease inhibitors. Amino acid based chloromethyl sulfoxides are known compounds and have been used for example in the synthesis of mono oxodithioacetal containing natural products and derivatives, that is, sparsomycin and γ-glutamyl marasmin.[5a–d] In this synthesis the chloromethyl sulfoxide moiety was used for reaction with sodium methylmercaptide[5a] and with other thiolates.[5c] In the literature there is one procedure described[5e] in which a few alcohols were reacted with chloromethyl methyl sulfoxide. However, these reactions were slow (18 h) and needed elevated temperatures (50 °C). If chloromethyl sulfoxides indeed are capable of inhibiting cysteine proteases, they might become a new class of selective irreversible inhibitors of cysteine proteases.

Abbreviations: ESI-MS, electrospray ionization mass spectrometry; Boc, *tert*-butyloxycarbonyl; BOP, (1*H*-benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DTT, dithiothreitol; D*i*PEA, *N*,*N*-diisopropylethylamine; TFA, trifluoroacetic acid; TMS, trimethylsilyl.
Keywords: Chloromethyl sulfoxide; Cysteine protease; Irreversible inhibitor; Papain; Amino acid.
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In addition, by altering the amino part of the chloromethyl sulfoxide containing inhibitor, their specificity and binding affinity might be tuned towards inhibition of specific enzymes.

2. Results and discussion

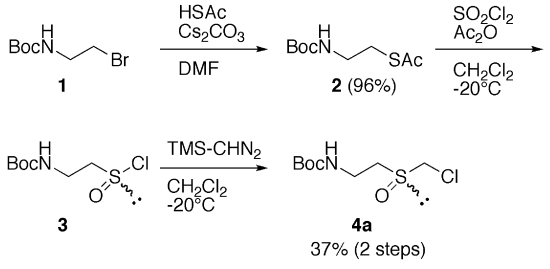
2.1. Chemistry

It was decided to start with the most simple and easier accessible chloromethyl sulfoxide derived from glycine. To this end 2-(Boc-amino)ethyl bromide (**1**)[6] was reacted with in situ generated cesium thioacetate to give thioacetate **2** in high yield (Scheme 1).

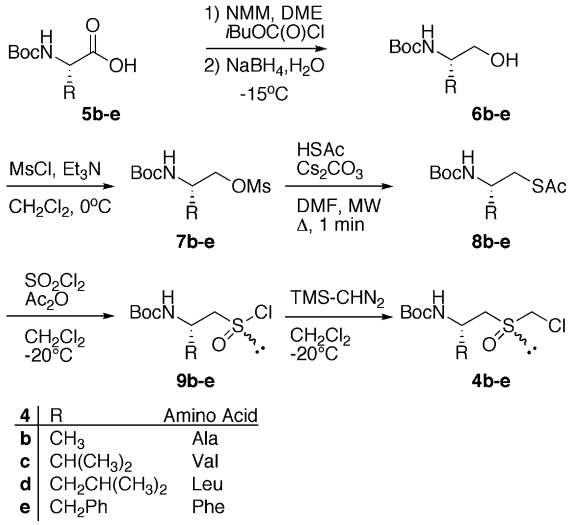
The thioacetate (**2**) was oxidized to the sulfinyl chloride (**3**) by treatment with sulfuryl chloride and acetic anhydride.[7] The crude sulfinyl chloride (**3**) was directly used in a reaction with diazomethane in dichloromethane to yield the chloromethyl sulfoxide (**4a**).[5a–d,8] Due to problems with the commercial availability of the starting material (diazogen) of diazomethane, it was decided to perform the reaction with TMS-diazomethane (Scheme 1), which is safe and not explosive and can be stored for longer periods.[9a] TMS-diazomethane has been used in the literature for different types of reactions,[9] but not for the synthesis of chloromethyl sulfoxides. The purity and yield (37%) of **4a** obtained with TMS-diazomethane were almost equal to the reaction with diazomethane, and comparable to the yield reported in the literature.[5d]

After preparation of the glycine derived chloromethyl sulfoxide, the next step was the preparation of chloromethyl sulfoxides derived from amino acids with different side chains. To this end Boc-protected amino acids (Ala, Val, Leu, Phe) (**5b–e**) were used as the starting materials which were reduced to their corresponding alcohols (**6b–e**) (Scheme 2) using the method described by Rodriguez et al.[10] by reduction ($NaBH_4$) of the in situ prepared mixed anhydride.

Alcohols **6b–e** were obtained in high yields and were converted to mesylates **7b–e** also in good to high yields (after crystallization, Table 1). Introduction of the thioacetate group, was performed using the procedure used for **2** (Scheme 1), by reaction with in situ generated cesium thioacetate in DMF at rt overnight. Unfortunately the yields were low, variable (5–50%)



Scheme 1. Synthesis of the glycine derived chloromethyl sulfoxide.



4	R	Amino Acid
b	CH_3	Ala
c	$CH(CH_3)_2$	Val
d	$CH_2CH(CH_3)_2$	Leu
e	CH_2Ph	Phe

Scheme 2. Synthesis of chloromethyl sulfoxides **4b–e** from Boc-protected amino acids.

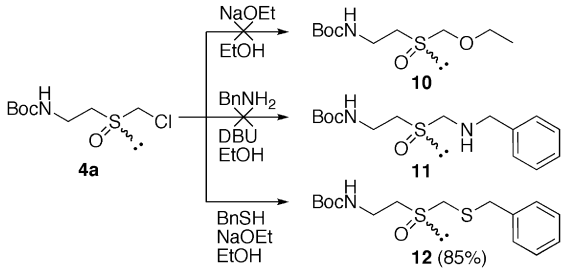
Table 1. Yields (%) for the synthesis of chloromethyl sulfoxides **4b–e** from Boc-protected amino acids

Amino acid	6	7	8	4	Overall yield
Boc-Ala-OH (**b**)	89	90	82	19	12
Boc-Val-OH (**c**)	95	69	94	20	12
Boc-Leu-OH (**d**)	96	75	85	20	12
Boc-Phe-OH (**e**)	64	83	96	27	14

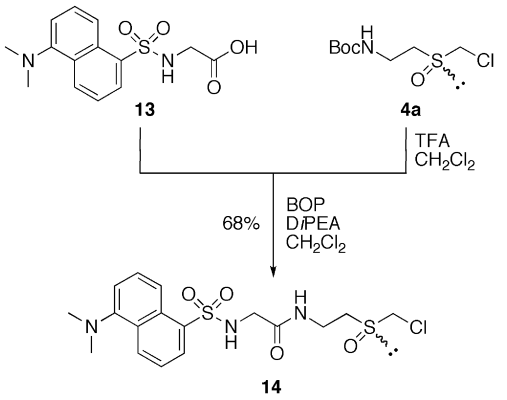
and the relatively large by-product formation caused tedious purifications. In order to decrease reaction time and by-product formation it was decided to perform the reaction in a microwave oven (Scheme 2). Initial experiments, which were carried out in a domestic microwave oven using a low power setting, were already successful. After 4 cycles of only 15 s TLC showed completion of the reaction and by-product was absent. Thioacetates **8b–e** could now be prepared in high yields (82–96%) (Table 1) and—if necessary—easily purified by silica gel column chromatography. For comparison thioacetate **8e** was also prepared using a dedicated microwave reactor. After irradiation for only 20 s at 140 °C thioacetate **8e** was isolated in a comparable yield (88%). Next, thioacetates (**8b–e**) were oxidized to sulfinyl chlorides **9b–e**, analogous to the preparation of **3**. The crude almost pure sulfinyl chlorides (**9b–e**) were used directly in the reaction with TMS-diazomethane in dichloromethane to afford the chloromethyl sulfoxides (**4b–e**), albeit in low yields (19–27%) (Table 1), but still comparable or better than the yields reported in the literature.[9c,d]

Before evaluation of chloromethyl sulfoxides **4a–e** in biological assays, we were interested in obtaining an indication of their reactivity towards different nucleophiles (Scheme 3).

When chloromethyl sulfoxide **4a** was treated with sodium ethoxide in ethanol no product was found after stirring for 40 h, only starting material was visible on TLC. Treatment with benzylamine in ethanol with



Scheme 3. Reactions of chloromethyl sulfoxides **4a** with different nucleophiles.



Scheme 4. Synthesis of dansyl-labeled chloromethyl sulfoxide **14**.

DBU gave similar results, even when the reaction was stirred at 50 °C. As expected product formation was found after reaction with the sodium thiolate of benzyl mercaptan in ethanol. After stirring for 16 h at rt more than half of chloromethyl sulfoxide **4a** was consumed. After a total of 80 h, and addition of more reagent, mono oxodithioacetal **12** was obtained in 85% yield after purification. These simple test-reactions clearly showed the preference of a chloromethyl sulfoxide (**4a**) for a sulfur nucleophile and as a consequence, its potential to react selectively with the cysteine thiol of a cysteine protease, as opposed to the serine-hydroxyl of a serine protease. In addition, it is expected that no reaction will take place with the abundantly present lysine amino groups.

For biological evaluation a (fluorescently) labeled chloromethyl sulfoxide was needed. It was decided to attach a dansyl-group to the N-terminus of chloromethyl sulfoxide **4a**. Since the dansyl-group is relatively bulky, a glycine spacer was introduced. Thus, the Boc-group in chloromethyl sulfoxide **4a** was removed by TFA, followed by coupling of dansylglycine **13** and labeled chloromethyl sulfoxide **14** was obtained in 68% yield (Scheme 4).

2.2. Biological evaluation

Papain was chosen as a target enzyme for evaluation of the chloromethyl sulfoxides. Papain is the most widely studied member of the cysteine protease class of enzymes. First, papain (6.86 μM) was activated with DTT in a phosphatebuffer (0.05 M, pH = 6.5) and subsequently incubated with chloromethyl sulfoxide **14** (10 μM) for 1 h at room temperature. After centrifugation of the solution through a filter with a cut off of 5 kDa, the fluorescence of the filtrate was measured. A significant difference in the fluorescence was found between the chloromethyl sulfoxide containing sample and a control sample without papain which had received the same treatment. The difference in fluorescence indicated that the chloromethyl sulfoxide (**14**) was capable of binding of ca. 40% of the papain which was a very promising result. However it was also possible that chloromethyl sulfoxide **14** binds reversibly to papain. To this end the cut off filter was extensively washed with the phosphate buffer. Since hardly any fluorescence was measured in the concentrated filtrate, it appeared that chloromethyl sulfoxide **14** was bound irreversibly to papain.

The next step was to evaluate the chloromethyl sulfoxides in an activity-based enzyme assay with papain using Bz-L-Arg-pNA as a substrate. Although a relatively high

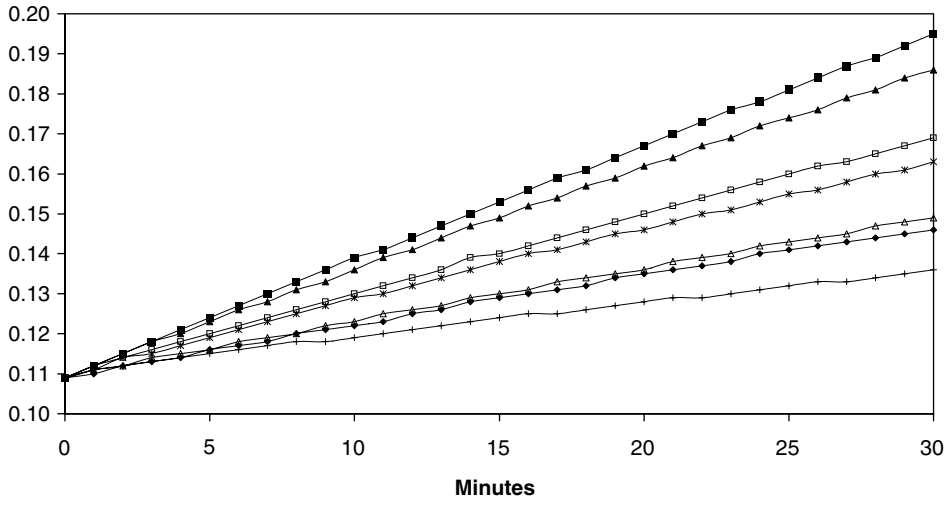


Figure 2. Progress curve of a papain enzyme assay for comparison of the inhibitory activity of chloromethyl sulfoxides **4a**–**e** and **14** (■, control; ▲, **4a**; □, **4b**; ∗, **4c**; △, **14**; ♦, **4d**; +, **4e**). [E] = 800 nM, [S] = 1.0 mM, [I] = 500 μM.

concentration of 500 μM was required for sufficient inhibition, we were enraptured that our chloromethyl sulfoxides could indeed decrease the activity of papain. Next, chloromethyl sulfoxides **4a**–**e** were tested for their inhibitory activity. No large differences were expected since the S1 subsite of papain is not very selective. Although it is reported that this site shows some preference for Arg and Lys side chains,[11] most amino acid side chains are accepted. However, when chloromethyl sulfoxides **4a**–**e** were compared in this assay, some remarkable selectivity's were found (Fig. 2). Glycine derived chloromethyl sulfoxide **4a** showed no inhibition while dansyl-labeled chloromethyl sulfoxide **14** (Scheme 4) was clearly one of the best inhibitors.

Phenylalanine derived chloromethyl sulfoxide **4e** showed the highest inhibition. High inhibition was also found for valine derived chloromethyl sulfoxide **4c**. Since the S1 subsite of papain does not accept the side chain of valine,[12,13] the isopropyl side chain of this chloromethyl sulfoxide (**4c**) is presumably binding in the S2 subsite of papain instead of the S1 subsite. In addition, it is known from the literature that the S2 subsite of papain has a preference for the side chains of phenylalanine and valine residues.[13] Since dansyl containing chloromethyl sulfoxide **14** was one of the best inhibitors it is likely that its aromatic part also binds to the S2 subsite. Therefore, we surmised that chloromethyl sulfoxide **15**, containing an N-terminal phenylalanine residue, would display inhibitory behavior similarly to **14**. Chloromethyl sulfoxide **15** was obtained in high yield (89%, Fig. 5) by the same procedure as was used for the synthesis of **14** (Scheme 4). Unfortunately, chloromethyl sulfoxide **15** displayed poor inhibition, possibly indicating that the phenylalanine side chain cannot be properly positioned to bind in the S2 subsite. Based on the found affinities, together with the known preference for the papain subsites, it is assumed that the side chains of chloromethyl sulfoxides **4b**–**e** bind to the S2 subsite instead of the S1 subsite of papain.

Molecular modeling corroborated this assumption. The three dimensional structure of the diazomethyl ketone inhibitor Z-LFG-CH_2-papain covalent complex has been determined by X-ray crystallography.[14] This inhibitor is suitable for comparison with chloromethyl sulfoxide inhibitor **4e** since it contains a phenylalanine residue which binds to the S2 subsite of papain (Fig. 3).

Using the program Yasara Structure, molecular modeling of the **4e**-papain complex clearly showed that the side chain of chloromethyl sulfoxide **4e** binds to the same (S2) pocket of papain (A) as the diazoketone inhibitor (B). Although only the S(Cα)R(S═O) diastereomer covalently bound to papain (Fig. 3a) is shown here, both diastereoisomers of **4e** were found to be able to bind in a similar way to the active site of papain.

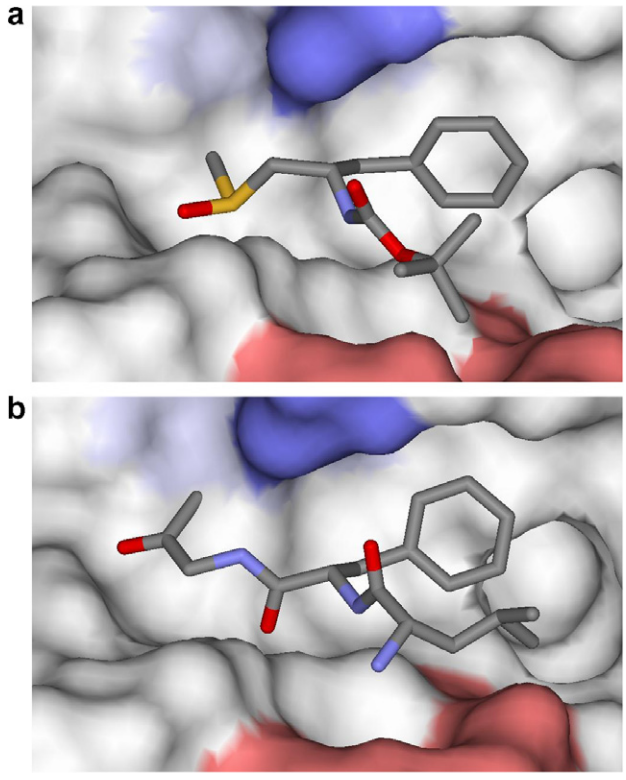


Figure 3. Comparison of covalent bound **4e**-papain (a) with a crystal structure of Z-LFG-CH_2-papain[14] (b).

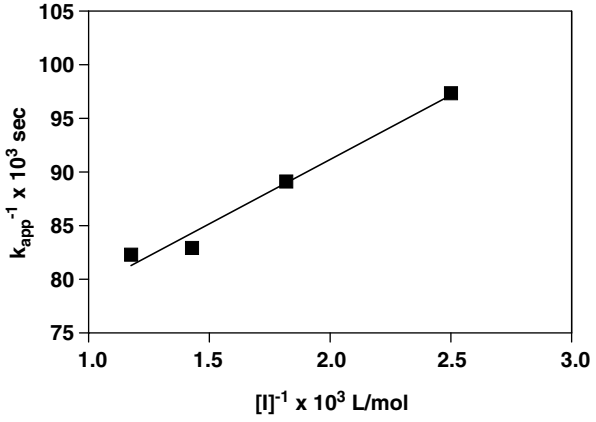


Figure 4. The dependence of κ upon the concentration of inhibitor **4e**, plotted as reciprocals in accordance to Kitz and Wilson kinetics.[15]

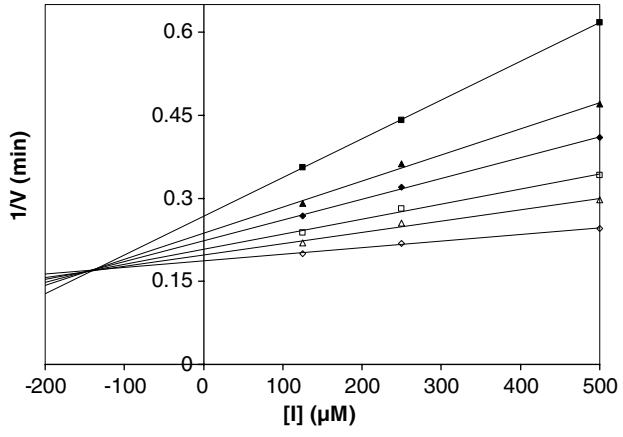


Figure 5. A Dixon plot of inhibition of papain by chloromethyl sulfoxide **4e** ([S] (mM): ■, 1.0; ▲, 1.2; ♦, 1.4; □, 1.6; △, 1.8; ◊, 2.0).

Table 2. Inhibitor constants of chloromethyl sulfoxides **4a**–**e**, **14** and **15** (R-Ψ[$CH_2S(O)$]–CH_2Cl)[a]

Chloromethyl sulfoxide	R	K_i (μM)
4a	Boc-Gly	No inhibition
15	Boc-Phe-Gly	3671 ± 558
4b	Boc-Ala	1426 ± 149
4c	Boc-Val	543 ± 21
4d	Boc-Leu	485 ± 51
14	Ds-Gly-Gly	471 ± 78
4e	Boc-Phe	142 ± 5[b]

[a] K_i values were determined using Dixon plots.[16]
[b] Determination using Kitz and Wilson kinetics[15] gave K_i = 179 μM.

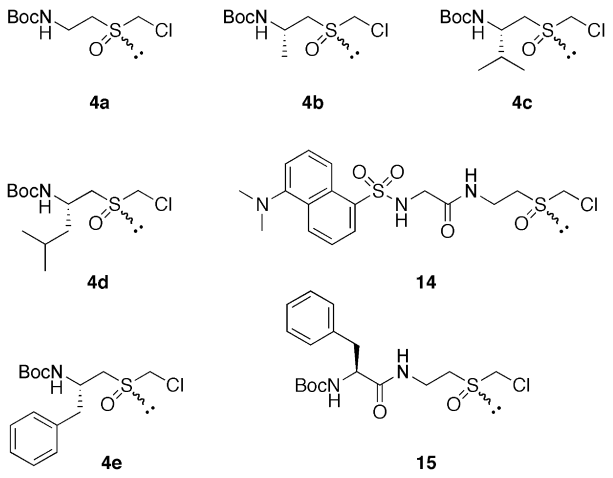


Figure 6. Structures of chloromethyl sulfoxides **4a**–**e**, **14** and **15**.

For determination of the inhibition constants and inactivation rates, the chloromethyl sulfoxides were subjected to time-dependent assays according to Kitz and Wilson.[15] In the first assays no significant irreversible binding was observed after 90 min, indicating that the chloromethyl sulfoxides were not bound covalently to the active site and thus were binding as reversible inhibitors. Fortunately, after 4 h the binding was found to be indeed irreversible. As expected the rate constant for the conversion of the reversible complex to the irreversibly inhibited enzyme (k_3) was low (1.5×10^{-5} s^{-1}) (Fig. 4).

The low reaction rate reflects to insufficient reactivity of the thiol in the active site of papain towards the chloromethyl sulfoxide moiety and/or to low reactivity of the chloromethyl sulfoxide. Another explanation is that the chloromethyl sulfoxide moiety of the reversibly bound inhibitor is not properly positioned with respect to the active site thiol, thus making formation of a covalent bond less likely.

Since all prepared chloromethyl sulfoxides showed no significant irreversible binding in the first hour it was decided to determine the inhibitor constants K_i using Dixon plots (Fig. 5), which normally are used for reversible inhibition.[16]

The K_i values found (Table 2) for all prepared chloromethyl sulfoxides (Fig. 6) were in agreement with the results from the comparison assay (Fig. 1).

The best K_i (142 μM) was obtained for phenylalanine derived chloromethyl sulfoxide **4e**, which was comparable to the K_i determined by the time-dependent assay (179 μM). Although this K_i is modest, we think that this result is encouraging and that affinity for the active site can be improved by modification of the N-terminus, for example by introduction of (an) amino acid residue(s). In addition, it is possible that one of both diastereoisomers, which so far could not be separated, has a higher affinity and therefore better K_i. Lineweaver–Burk plots indicated that the mode of inhibition by the chloromethyl sulfoxides was competitive for the substrate.

To determine whether the chloromethyl sulfoxides were selective towards cysteine proteases, an enzyme assay was performed with the serine protease chymotrypsin, which clearly showed that none of the chloromethyl sulfoxides were capable of inhibition of this protease.

3. Conclusions

A series of amino acid based chloromethyl sulfoxides and N-terminal modified chloromethyl sulfoxides have been successfully synthesized in moderate to high yields. The synthesis of the intermediate thioacetates was optimized using the microwave leading to high yields and very short reaction times. Instead of the highly toxic and explosive diazomethane, the stable and safe TMS-diazomethane was found to be equally suitable in the synthesis of the chloromethyl sulfoxides from the corresponding sulfinyl chlorides.

The described chloromethyl sulfoxides were capable of irreversible inhibition of the cysteine protease papain. In addition, the affinities were found to be dependent on the side chain of the inhibitor, which points at the possibilities for tuning the selectivity. The chloromethyl sulfoxides were also found to be selective cysteine protease inhibitors, since no inhibition of the serine protease chymotrypsin was observed. Although relatively modest affinities and rate constants were found, we believe that these chloromethyl sulfoxides might become a new important class of irreversible cysteine protease inhibitors of which the affinity can be further improved by using different amino acid side chains and/or by extending the N terminus. Currently, we are preparing small libraries of N-terminal functionalized amino acid based chloromethyl sulfoxides for finding inhibitors with higher affinities for biologically more relevant cysteine proteases. Under present investigation is also the influence of the backbone length on the rate constant.

4. Experimental

Boc-protected amino acids were purchased from Advanced Chemtech Europe Ltd (France). Thioacetic acid was distilled and used within 4 weeks. Peptide grade solvents for synthesis were purchased from Biosolve (The Netherlands) and were stored on molecular sieves (4 Å). Reactions were carried out at ambient temperature unless stated otherwise. TLC analysis was per-

formed on Merck pre-coated silicagel 60 F-254 (0.25 mm) plates. Spots were visualized with UV light, ninhydrin or Cl_2-TDM.[17] Solvents were evaporated under reduced pressure at 40 °C. Column chromatography was performed on ICN silicagel 60 (32–63 μm). Melting points were measured on a Büchi Schmelzpunktbestimmungsapparat (according to Dr. Tottoli) and are uncorrected. Electrospray mass spectra were recorded on a Shimadzu LCMS-QP-8000 spectrometer, or a Finnigan LCQ Deca XP MAX spectrometer. Elemental analyses were carried out at Kolbe Mikroanalytisches Laboratorium (Mülheim an der Ruhr, Germany). Microwave assisted reactions were carried out in either a Biotage Initiator or in a SMC (E70-TFA, 650 W) domestic microwave oven. ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded on a Varian G-300 spectrometer. Chemical shifts are reported in ppm relative to TMS (0 ppm) or DMSO (2.50 ppm) for the ^{1}H NMR and to $CDCl_3$ (77 ppm) or DMSO (39.5 ppm) for the ^{13}C NMR spectra as internal standards. ^{13}C NMR spectra were recorded using the attached proton test (APT) pulse sequence. HSQC spectra of **12** and **14** and a HMBC spectrum of **12** were recorded on a Varian Inova-500 spectrometer.

4.1. Alcohols 6b–d: general procedure

Alcohols **6b–d** were synthesized according to Rodriquez et al.[10] The described method afforded the alcohols in high yields (Table 1).

4.2. Mesylates 7b–e: general procedure

Mesylates **7b–e** were prepared following an earlier described protocol.[18] All products were recrystallized from EtOAc/hexanes and obtained as white fluffy crystals. All spectroscopic data were consistent with the literature.[19]

4.3. Glycine derived thioacetate 2

Thioacetic acid (3.8 mL, 53.8 mmol) and cesium carbonate (9.45 g, 29 mmol) were dissolved in DMF and this solution was added to Boc-(2-bromoethyl)amine[6] (10.0 g, 44.8 mmol). The flask was wrapped in aluminum foil and stirred overnight at rt. After evaporation of the DMF, ethylacetate (500 mL) was added. The organic layer was washed with $NaHCO_3$(2.5%), water, brine and dried (Na_2SO_4). After concentration and coevaporation with chloroform, the product was obtained as a brownish oil (9.43 g, 96%). Spectroscopic data were consistent with the literature.[20]

4.4. Thioacetates 8b–e: general procedure

A solution of thioacetic acid (1.12 mL, 15.8 mmol) and cesium carbonate (2.69 g, 8.25 mmol) in DMF (60 mL) was added to the mesylate (**7b–e**, 15.0 mmol) in a pressure vessel. The closed vessel was subjected to microwave irradiation in either a domestic microwave oven (four or five times 15 s at the defrost setting) or in a dedicated microwave reactor (in 40 s from rt to 140 °C, 20 s at 140 °C). The formed white precipitate was filtered off and DMF was evaporated in vacuo. The residue was dissolved in EtOAc, washed with 5% $NaHCO_3$, water, 1 N $KHSO_4$, water and brine. Drying (Na_2SO_4) and concentration in vacuo afforded the crude thioacetate which was purified by column chromatography. All thioacetates were obtained as pale orange oils, which crystallized either directly or overnight. Spectroscopic data for **8b**, **c**, and **e** were consistent with the literature.[5c,d]

4.4.1. Alanine derived thioacetate 8b. The scale of the reaction was 15.0 mmol and **8b** was obtained (2.87 g, 82%) after column chromatography (hexanes/CH_2Cl_2/EtOAc, 15:4:1).

4.4.2. Valine derived thioacetate 8c. The scale of the reaction was 15.0 mmol and **8c** was obtained (3.67 g, 94%) after column chromatography (hexanes/CH_2Cl_2/EtOAc, 10:4:1).

4.4.3. Leucine derived thioacetate 8d. The scale of the reaction was 15.0 mmol and **8d** was obtained (3.51 g, 85%) after column chromatography (hexanes/CH_2Cl_2/EtOAc, 5:4:1).

R_f = 0.51 (hexanes/CH_2Cl_2/EtOAc, 5:4:1). Mp = 51 °C.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.91 (2d, J = 1.9 Hz, 6H, CH(CH_3)$_2$), 1.32 (m, 2H, CH_2CH(CH_3)$_2$), 1.43 (s, 9H, C(CH_3)$_3$), 1.66 (m, 1H, CH_2CH(CH_3)$_2$), 2.35 (s, 3H, SC(O)CH_3), 2.96 (dd, J_{gem} = 13.7 Hz, J_{vic} = 6.9 Hz, 1H, NCHCH^a), 3.13 (dd, J_{gem} = 13.7 Hz, J_{vic} = 4.7 Hz, 1H, NCHCH^b), 3.83 (m, 1H, NCH), 4.47 (d, J = 8.8 Hz, 1H, NH).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 21.9, 22.8 (CH_2CH(CH_3)$_2$), 24.6 (CH_2CH(CH_3)$_2$), 28.1 (C(CH_3)$_3$), 30.3 (SC(O)CH_3), 34.2 (CH_2CH(CH_3)$_2$), 43.2 (SCH_2), 48.3 (NCH), 78.8 (C(CH_3)$_3$), 155.2 (C(O)C(CH_3)$_3$), 195.3 (SC(O)CH_3).

ESI-MS: m/z = 176.50 [M-Boc+H]$^+$, 220.33 [M-t-Bu+H]$^+$, 276.33 [M+H]$^+$, 298.50 [M+Na]$^+$.

4.4.4. Phenylalanine derived thioacetate 8e. The scale of the reaction was 15.0 mmol (domestic microwave oven) and **8e** was obtained and used without further purification (4.45 g, 96%). With a dedicated microwave reactor the scale was 2.0 mmol and **8e** was obtained (545 mg, 88%) after column chromatography (hexanes/CH_2Cl_2/EtOAc, 5:4:1).

4.5. Chloromethyl sulfoxides 4a–e: general procedure

A solution of the thioacetate (**8a–e**) (12.3 mmol) in dry dichloromethane (25 mL) was cooled under a nitrogen atmosphere to −20 °C. Acetic anhydride (1.16 mL, 12.3 mmol) was added all at once and sulfuryl chloride (1.99 mL, 24.6 mmol) was added dropwise. After stirring for 1 h at approximately −10 °C, the reaction was warmed up to rt. The mixture was concentrated in vacuo and the residue was dissolved in dry dichloromethane (15 mL). TMS-diazomethane (2 M in hexanes) (12.3 mL, 24.6 mmol) was cooled under a nitrogen

atmosphere to −20 °C and the dissolved residue was added to the cooled TMS-diazomethane dropwise. After 2 h stirring, the excess TMS-diazomethane was decomposed by addition of a few drops of acetic acid until no more nitrogen gas evolved. Concentration in vacuo followed by column chromatography afforded the chloromethyl sulfoxides (**4a**–**e**). All chloromethyl sulfoxides were obtained as white solids. Spectroscopic data for **4a**–**c**, **e** were consistent with the literature.[5c,d]

4.5.1. Glycine derived chloromethyl sulfoxide 4a. The scale of the reaction was 9.0 mmol and **4a** was obtained (790 mg, 37%) after column chromatography (EtOAc/CH_2Cl_2, 1:1). Glycine derived chloromethyl sulfoxide **4a** was also prepared using freshly prepared diazomethane. The same procedure was followed and similar yields were found.

4.5.2. Alanine derived chloromethyl sulfoxide 4b. The scale of the reaction was 12.3 mmol and **4b** was obtained (596 mg, 19%) after column chromatography (EtOAc/CH_2Cl_2, 3:7).

4.5.3. Valine derived chloromethyl sulfoxide 4c. The scale of the reaction was 14.0 mmol and **4c** was obtained (787 mg, 20%) after column chromatography (EtOAc/CH_2Cl_2, 25:75).

4.5.4. Leucine derived chloromethyl sulfoxide 4d. The scale of the reaction was 10.3 mmol and **4d** was obtained (610 mg, 20%) after column chromatography (EtOAc/CH_2Cl_2 gradient from 5:95 to 20:80).

R_f = 0.53 (EtOAc). Mp = 139 °C.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.93, 0.95 (2d, J = 4.4 Hz, 6H, $CH(CH_3)_2$), 1.47, 1.71 (2m, 3H, $CH_2CH(CH_3)_2$), 1.43 (s, 9H, $C(CH_3)_3$), 2.88 (dd, J_{gem} = 13.5 Hz, J_{vic} = 8.9 Hz, 0.5H, $NCHCH_2$), 3.00 (dd, J_{gem} = 13.2 Hz, J_{vic} = 3.7 Hz, 0.5H, $NCHCH_2$), 3.14 (dd, J_{gem} = 13.2 Hz, J_{vic} = 7.7 Hz, 0.5H, $NCHCH_2$), 3.31 (dd, J_{gem} = 13.5 Hz, J_{vic} = 3.8 Hz, 0.5H, $NCHCH_2$), 3.92, 4.10 (2m, 1H, NCH), 4.40, 4.51 (2d, J = 11.0 Hz, 1H, $S(O)CH_2Cl$), 4.64, 4.74 (2d, 1H, $S(O)CH_2Cl$), 4.70, 5.03 (s d, 1H, NH).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 21.9, 22.6 ($CH_2CH(CH_3)_2$), 24.7, 24.9 ($CH_2CH(CH_3)_2$), 28.2 ($C(CH_3)_3$), 43.2, 44.0 ($CH_2CH(CH_3)_2$), 45.0, 45.2 (NCH), 55.4, 56.7 ($NCHCH_2$), 56.7, 56.9 ($S(O)CH_2Cl$), 79.9 ($C(CH_3)_3$), 155.4 ($C(O)C(CH_3)_3$).

ESI-MS: m/z = 198.63, 200.88 $[M\text{-Boc}+H]^+$, 242.39, 244.40 $[M\text{-}t\text{-Bu}+H]^+$, 298.37, 300.31 $[M+H]^+$, 320.49, 322.43 $[M+Na]^+$: due to chlorine isotopes.

Anal. Calcd for $C_{12}H_{24}ClNO_3S$: C, 48.39; H, 8.12; N, 4.70. Found: C, 48.46; H, 8.03; N, 4.72.

4.5.5. Phenylalanine derived chloromethyl sulfoxide 4e. The scale of the reaction was 14.3 mmol and **4e** was obtained (1.29 g, 27%) after column chromatography (EtOAc/CH_2Cl_2, 25:75).

4.6. Mono oxodithioacetal 12

To a solution of chloromethyl sulfoxide **4a** (73 mg, 0.30 mmol) in dry methanol was added benzyl mercaptan (39 μL, 0.33 mmol) and a solution of sodium methoxide in methanol (76 μL, 30% w/w). After stirring overnight (20 h) at rt the conversion was 50% according to TLC. Additional portions of benzyl mercaptan and sodium methoxide solution were added and after 2 h the conversion was already 80%. After additional stirring for ca, 2.5 days, the reaction mixture was concentrated in vacuo. Column chromatography (DCM/EtOAc, 1:1) afforded mono oxodithioacetal **12** as a white solid (84 mg, 85%).

R_f = 0.43 (EtOAc). Mp = 87 °C.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.45 (s, 9H, $C(CH_3)_3$), 2.86 (dt, J_{gem} = 13.2 Hz, J_{vic} = 5.5 Hz, 1H, $NCH_2CH^aS(O)$), 3.13 (dt, J_{gem} = 13.2 Hz, J_{vic} = 6.6 Hz, 1H, $NCH_2CH^bS(O)$), 3.67 (m, 4H, NCH_2, SCH_2Ph), 3.92 (s, 2H, $S(O)CH_2S$), 5.13 (bs, 1H, NH), 7.34 (m, 5H, Ar–CH).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 28.3 ($C(CH_3)_3$), 35.2 (NCH_2), 37.0 (SCH_2Ph), 50.9 (4 lines) ($S(O)CH_2S$), 50.7 (2 lines) ($NCH_2CH_2S(O)$), 79.8 ($C(CH_3)_3$), 129.2, 127.3, 128.8, 136.3, 136.4 (Ar—C), 155.8 (C═O (Boc). ESI-MS: m/z = 351.95 $[M+Na]^+$.

Anal. Calcd for $C_{15}H_{23}NO_3S_2$: C, 54.68; H, 7.04; N, 4.25; S, 19.46. Found: C, 54.78; H, 6.98; N, 4.20; S, 19.40.

4.7. Ds-Gly-GlyΨ[$CH_2S(O)$]-CH_2Cl (14)

Chloromethyl sulfoxide **4a** (483 mg, 2.00 mmol) was Boc-deprotected by treatment with TFA/DCM (10 mL, 1:1) for 5 min. After evaporation, the residue was dissolved in dichloromethane (10 mL). A solution of dansyl-glycine[21] (678 mg, 2.20 mmol) in dichloromethane (150 mL) was added to the Boc-deprotected chloromethyl sulfoxide solution. BOP (973 mg, 2.20 mmol) and D*i*PEA (660 μL, 4.00 mmol) were added, and the reaction was stirred overnight at rt with the flask covered with aluminum foil. If necessary more D*i*PEA was added to maintain a basic pH. After stirring for an additional hour followed by evaporation in vacuo, the residue was dissolved in EtOAc and washed with 1 N $KHSO_4$, water and brine. The organic layer was dried (Na_2SO_4) and concentrated in vacuo. Column chromatography (MeOH/EtOAc, 5:95) and trituration using MeOH, afforded **14** as a yellow/greenish solid (585 mg, 68%).

R_f = 0.43 (10% MeOH/EtOAc). Mp = 87 °C.

^{1}H NMR (300 MHz, DMSO-d_6) δ, 2.83 (s, 6H, $N(CH_3)_3$), 2.85–2.93 (m, 2H, NCH_2CH_2), 3.30–3.39 (m, 2H, NCH_2CH_2), 3.44 (s, 2H, $NCH_2C(O)$), 4.77 (d, J = 11.3 Hz, 1H, $S(O)\text{–}CH^a\text{–}Cl$), 4.96 (d, J = 11.3 Hz, 1H, $S(O)\text{–}CH^b\text{–}Cl$), 7.26, 7.60, 8.11, 8.29, 8.46 (m, 6H, Ds-Ar–CH).

^{13}C NMR (75 MHz, DMSO-d_6) δ: 32.7 (NCH_2CH_2), 45.3 (N$(CH_3)_2$), 49.2 (NCH_2CH_2), 58.3 (S(O)–CH_2–Cl), 115.4, 119.4, 123.8, 128.1, 128.4, 129.7 (Ar–CH), 129.3, 136.0, 151.5 (Ar–C), 168.4 (C(O)).

The signals of NCH_2C(O) were found in the proton spectra but were absent in the carbon spectra. From a HSQC-measurement the (NCH_2C(O))-signal was determined at 45.3 ppm.

ESI-MS: m/z = 432.25, 434.25 $[M+Na]^+$: due to chlorine isotopes.

4.8. Boc-Phe-GlyΨ[CH_2S(O)]-CH_2Cl (15)

Chloromethyl sulfoxide **15** was synthesized according to the preparation of **14** on a scale of 0.40 mmol. The product was isolated after workup by crystallization (EtOAc/hexanes) as a white solid (143 mg, 89%).

R_f = 0.30 (EtOAc). Mp = 125 °C.

^{1}H NMR (300 MHz, $CDCl_3$) δ, 1.40 (s, 9H, C$(CH_3)_3$), 2.84–3.11 (m, 4H, CH_2Ph, NCH_2CH_2), 3.59–3.78 (m, 2H, NCH_2), 4.32 (m, 1H, NCH), 4.39 (dd, J_{gem} = 11.3 Hz, J_{vic} = 5.0 Hz, 1H, S(O)–CH^a–Cl), 4.47 (dd, J_{gem} = 11.3 Hz, J_{vic} = 4.1 Hz, 1H, S(O)–CH^b–Cl), 5.07 (bd, 1H, BocNH), 6.77 (bs, 1H, CH_2N*H*C═O), 7.17–7.33 (m, 5H, Ar–CH).

^{13}C NMR (75 MHz, $CDCl_3$) δ: 28.2 (C(CH_3)$_3$), 33.2, 33.4 (NCH_2), 38.6 (CH_2Ph), 49.2 (S(O)CH_2CH_2), 55.7 (NCH), 56.5 (CH_2Cl), 80.1 (C$(CH_3)_3$), 126.9, 128.6, 129.3, 136.6 (Ar—C), 155.3 (C═O (Boc)), 172.2 (NHCH*C*═O).

ESI-MS: m/z = 411.53, 413.29 $[M+Na]^+$: due to chlorine isotopes.

4.9. Fluorescence based binding test with papain

An aqueous solution of papain (7.2 μM, 500 μL) was buffered with 25 μL sodium phosphate buffer (1.0 M) containing EDTA (30 mM) and DTT (40 mM) yielding a final 50 mM sodium phosphate buffer at a pH of 6.5 containing 1.5 mM EDTA and 2 mM DTT. To this mixture and the reference mixture without Papain, 2.2 μL of inhibitor **14** (2.38 mM in DMSO) was added, yielding a concentration of 10 μM. After incubation for 1 h at RT, 450 μL of each sample was subjected to ultra filtration with a filter with a cut off of 5000 Da in a centrifuge at 10,000 rpm. This treatment was continued with the same buffer without Papain or probe until the volume of the filtrate was 1.5 mL, the filtrate of each sample was measured with an excitation wavelength of 321 nm and an emission wavelength of 564 nm to monitor the decrease of **14**.

4.10. Enzyme assays

4.10.1. General remarks for kinetic experiments. Buffer solutions were prepared using distilled water. Stock assay solutions were centrifuged before use. Papain and chymotrypsin were purchased from Sigma and were used without further purification. Bz-L-Arg-pNA and Bz-L-Tyr-pNA were purchased from Bachem (Switzerland). Kinetic enzyme assays were done in a μQuant Biotek plate reader for 1 h at rt at 405 nm. All assays were done in triplicate or quadruplicate.

4.10.2. Papain assays. A colorimetric assay in 96-well plates was used. In this assay a phosphate buffer (0.10 M, pH 6.5) containing EDTA (1.5 mM) and DTT (2.0 mM) was used, and Bz-L-Arg-pNA as a substrate. The optimal papain and substrate concentrations were found to be 800 nM and 1.0 mM. The chloromethyl sulfoxide was first pre-incubated for 1 h with the activated papain before addition of the substrate.

Papain was dissolved in buffer without DTT (8.16 μM) and after shaking for 5 min the solution was centrifuged. The substrate Bz-L-Arg-pNA was first dissolved in DMSO and then diluted twice (50.0 mM) with buffer without DTT. The chloromethyl sulfoxides were dissolved in DMSO and diluted twice (25.0 mM) with buffer without DTT. In a typical assay to each well was added inhibitor solution (4.0 μL), buffer (172.0 μL) and enzyme solution (20.0 μL). For the controls a DMSO/buffer without DTT solution (1:1) was used instead of the inhibitor solution. After 1 h pre-inhibition a sample (98.0 μL) was taken from each well and added to wells containing substrate solution (2.0 μL), and subsequently the liberation *p*-nitroaniline from Bz-L-Arg-pNA was measured at 405 nm during 1 h. Final concentrations in the wells were: enzyme: 800 nM; substrate: 1.0 mM; inhibitor: 500 μM.

For the assays for determination of the K_i's with Dixon plots[15] a similar procedure was used. Stock solutions of the substrate and inhibitors were prepared using only DMSO and with different concentrations. Substrate concentrations were 200, 180, 160, 140, 120 and 100 mM, and inhibitor concentrations were 100, 50 and 25 mM in the stock solutions (for **4e**: 50, 25 and 12.5 mM). To each well was added inhibitor solution (2.0 μL), substrate solution (2.0 μL), buffer (176.0 μL) and enzyme solution (20.0 μL). For the controls DMSO was added instead of inhibitor solution. The liberation of *p*-nitroaniline was directly measured for 1 h. Final concentrations in the wells were: enzyme: 800 nM; substrate: 1.0–2.0 mM; inhibitor: 0.25, 0.50 or 1.00 mM (for **4e**: 0.125, 0.25 or 0.50 mM).

For the time-dependent assays[15] the stock solution of the substrate was 100 mM in DMSO, and the stock solutions of the inhibitor were 40, 55, 70, 85 and 100 mM. To vials containing inhibitor solution (10 μL) or DMSO (10 μL, controls) was added buffer (880 μL) and enzyme solution (100 μL). The rate of irreversible inhibition was followed by withdrawing samples (99 μL) at different time intervals. These samples were added to wells containing substrate solution (1.0 μL) and the remaining enzyme activity was measured.

4.10.3. Chymotrypsin assay. The following stock solutions were prepared: buffer: sodium phosphate (50 mM, pH 7.0); enzyme: chymotrypsin was dissolved

in buffer (1.1 μM); substrate: Bz-L-Tyr-pNA was dissolved in DMSO (5.0 mM); inhibitors: the chloromethyl sulfoxides were dissolved in DMSO (20.0 mM). To each well was added inhibitor solution (10.0 μL) and enzyme solution (180.0 μL). For the controls DMSO was used instead of the inhibitor solution. After 1 h pre-inhibition a sample (95.0 μL) was taken from each well and added to wells containing substrate solution (5.0 μL), and subsequently the liberation *p*-nitroaniline was monitored during 30 min. Final concentrations in the wells were: enzyme: 1.0 μM; substrate: 0.25 mM; inhibitor: 1.0 mM.

4.11. Molecular modeling

Molecular modeling was performed using the program Yasara Structure (version 6.6.20; www.yasara.org). In the crystal structure of papain covalently bound to the diazomethyl ketone inhibitor (Z-LFG-CH_2-N_2) was used (Pdb-file 1KHQ),[14] the bound diazomethyl ketone inhibitor was replaced by chloromethyl sulfoxide **4e** followed by minimization without changing the atomic coordinates of the enzyme. The following settings were used: Yamber2 force field; PME electrostatics with a cutoff at 7.8 Å; simulated annealing; the simulation cell was 5 Å bigger than papain; no extra water molecules were added, only the water molecules which were already present in the original crystal structure were used. The static minimization was done from 300 K until the temperature dropped below 10 K. The minimized complexes were visualized using the program DS ViewerLite 5.0. The enzyme surface was colored according to electrostatic surface potential: red is negatively charged; blue is positively charged.

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