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# The Stability of Pseudopeptides Bearing Sulfoximines as Chiral Backbone Modifying Element towards Proteinase K

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Dedicated to Professor Dr. M. T. Reetz on occasion of his 60th birthday

**Abstract**—Incorporation of sulfoximines as backbone modifying element results in two new pseudopeptide bonds which display enhanced (bond A) and strongly reduced reactivity (bond B) towards hydrolysis by Proteinase K.


In recent years, peptides have extensively been modified in order to find new therapeutic agents for pharmaceutical applications.[1] Such modifications are required because natural peptides are often excreted rapidly or do not pass biological membranes resulting in a poor bioavailability.[2] Furthermore, replacing some (or even all) elements of the peptide backbone by unnatural linkages can enhance the stability against enzymatic amide bond cleavage.[3] An additional advantage of those peptide alternations stems from the fact that they often lead to significant stabilization of secondary structures.[1,2a,4]

In that respect, β-amino acids are particularly interesting. Their oligomerization leads to β-peptides which adopt stable well-defined secondary structures[5] and cannot be cleaved by peptidases[6] such as Pronase and Proteinase K.[7] Furthermore, some compounds were found to exhibit, among others, antimicrobial activity directly related to conformational stability.[8]

Another important modifying element is the sulfonamide moiety.[9] This group mimics the tetrahedral intermediate formed during peptide bond cleavage,[10] and certain compounds containing this modifying element are active as peptidase inhibitors.[11] Moreover, sulfonamide peptidomimetics reveal increased stability towards enzymatic degeneration,[12] and several examples of secondary structures induced by the sulfonamide group are known.[13]

Recently, we introduced sulfoximines as building blocks for pseudopeptides[14] and demonstrated that bis(sulfoximines) favor turn formation.[15] In this context it is also noteworthy, that several sulfoximines are highly biologically active leading to interesting pharmaceutical applications. Examples include buthionine sulfoximine (BSO), an additive in anti-cancer drugs,[16] and carboxypeptidase A inhibitors.[17]

Pseudopeptides with the sulfoximine-containing core fragment **I** can be regarded as structural analogues of β-amino acid- and sulfonamide-derived pseudopeptides **II** and **III**, respectively (Fig. 1). In this paper we describe the first peptidase cleavage experiments of pseudotripeptides containing such sulfoximine-based core **I.**

We initiated our study by synthesizing pseudotripeptides **3** (Scheme 1).[18] After carboxylation of sulfoximines **1**, the resulting pseudodipeptide ammonium salts

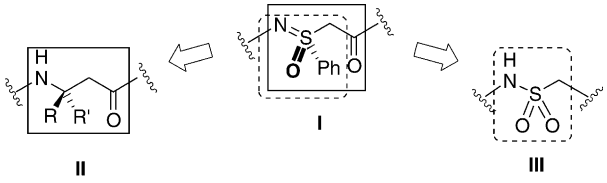


**Figure 1.** Comparison of pseudopeptide fragments containing a sulfoximine **I**, a β-amino acid **II** and a sulfonamide **III**.

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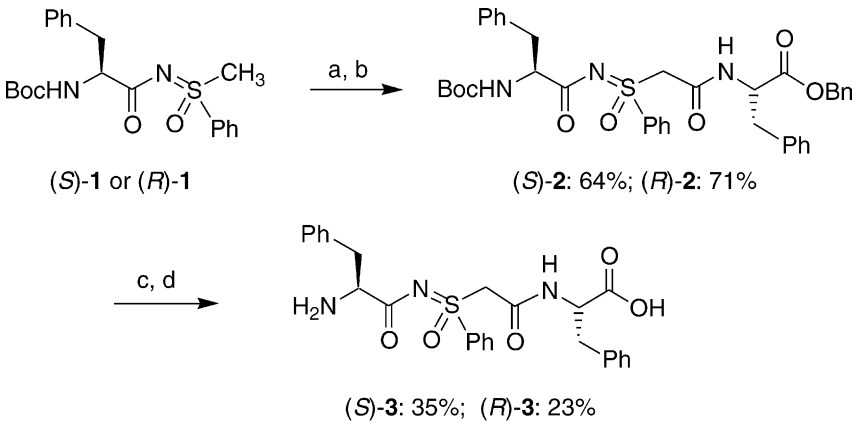
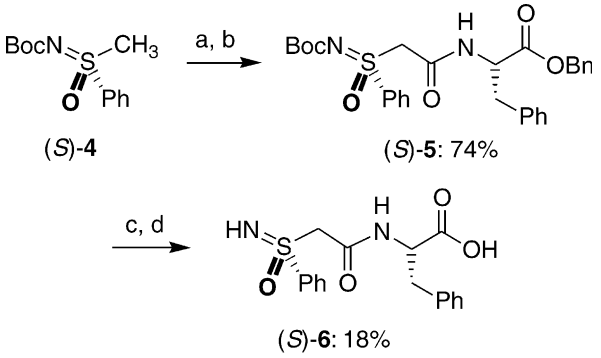



**Scheme 1.** Syntheses of pseudotripeptides (*S*)-**3** and (*R*)-**3**: (a) *n*BuLi (1.0 equiv), cyclohexylisopropylamine (1.0 equiv), THF, $CO_2$, −78 °C then 25 °C; (b) H-Phe-OBn·HCl (1.0 equiv), DCC (1.05 equiv), DMAP (0.12 equiv), $CH_2Cl_2$, 0 °C, 12 h; (c) Pd/C (15 mol %), $H_2$, ethyl acetate, 24 h; (d) TFA/$CH_2Cl_2$ (1:4), 4 h; yield after additional HPLC purification.




**Scheme 2.** Synthesis of pseudopeptide (*S*)-**6** (for a–d see the legend to Scheme 1).

(not shown) were coupled with C-terminal protected phenylalanine using conventional DCC activation affording **2** in good yields. Deprotection of **2** at the C- and N-terminus by hydrogenation and subsequent TFA treatment, respectively, gave pseudotripeptides H-Phe-(*S*)-SulfCO-Phe-OH [(*S*)-**3**] and H-Phe-(*R*)-SulfCO-Phe-OH [(*R*)-**3**].

In addition, pseudodipeptide (*S*)-**6** was synthesized (Scheme 2). Similar to the syntheses of compounds **3** BOC-protected sulfoximine (*S*)-**4**[19] was carboxylated and then coupled with phenylalanine benzyl ester to give protected pseudodipeptide (*S*)-**5**. C- and N-terminal deprotection of (*S*)-**5** yielded pseudodipeptide H-(*S*)-SulfCO-Phe-OH [(*S*)-**6**].

First NMR studies of sulfoximine-containing pseudopeptides **2** demonstrated that the $^{13}C$ NMR resonances of the carbonyl groups adjacent to the sulfoximine nitrogen are significantly different to those of the amide carbonyls in the molecules (located in the β-position of the sulfur atom). Thus, whereas the former give signals at about 180 ppm, the latter resonate at 160 ppm (Fig. 2). Both values are different to the amide carbonyl signals in the Phe trimer (H-Phe-Phe-Phe-OH), which give signals at about 170 ppm. Apparently, all carbonyl groups differ significantly in their electronic nature and consequently we expected distinct chemical properties, which we hoped to reveal in enzymatic cleavage studies.

For those enzymatic fragmentation reactions diastereomers (*S*)-**3** and (*R*)-**3** were treated with Proteinase K. This enzyme is a non-specific serine protease of the subtilisin family, which was isolated from the mould *Tritirachium album limber*.[20] It readily hydrolyzes proteins and has a low preference for peptide bond cleavage after aromatic and aliphatic amino acid residues.

First, pseudotripeptide H-Phe-(*S*)-SulfCO-Phe-OH [(*S*)-**3**] was incubated with Proteinase K, and the cleavage reaction was analyzed by reversed-phase HPLC and integration of the UV signal (210 nm) resulting from the starting material.[21] The fragmentation of (*S*)-**3** followed first-order kinetics (Fig. 3) with an observed rate constant $k_{obs}$ of 3.75 $h^{-1}$ (Table 1, entry 1). No cleavage was detected in a control experiment in which Proteinase K was omitted. This result was compared with a

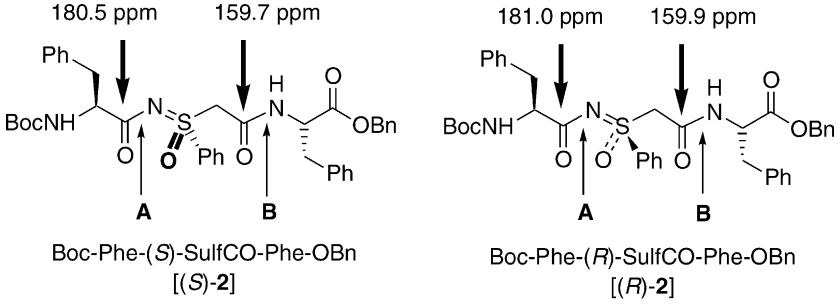



**Figure 2.** Characteristic $^{13}C$ NMR carbonyl resonances in sulfoximine-pseudopeptides (*S*)-**2** und (*R*)-**2** with two pseudopeptide bonds **A** and **B**.

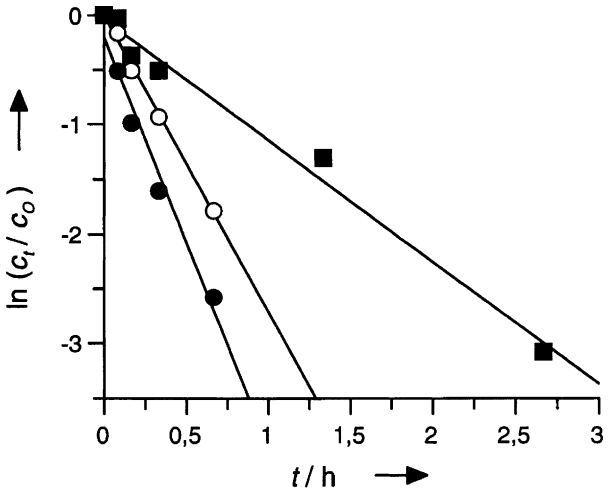



**Figure 3.** Kinetic analysis of substrate cleavage by Proteinase K. The ln of the substrate concentration at time $t$ ($c_t$) divided by the concentration at time zero ($c_o$) is plotted against the reaction time. ● = (*S*)-**3** (304 μM) and Proteinase K (3 mAU mL$^{-1}$); ○ = (*R*)-**3** (304 μM) and Proteinase K (3 mAU mL$^{-1}$); ■ = H-Phe-Phe-Phe-OH (218 μM) and Proteinase K (1.5 mAU mL$^{-1}$). One mAU (AU = Active Unit) is defined as the amount of Proteinase K required to release folin-positive amino acids and peptides corresponding to 1 μmol of tyrosine from hemoglobin at 37 °C in 1 min.

cleavage of the analogous natural tripeptide H-Phe-Phe-Phe-OH by Proteinase K. As can be seen from Table 1 (entry 2) H-Phe-Phe-Phe-OH was cleaved somewhat slower than sulfoximine-containing pseudotripeptide (*S*)-**3**.

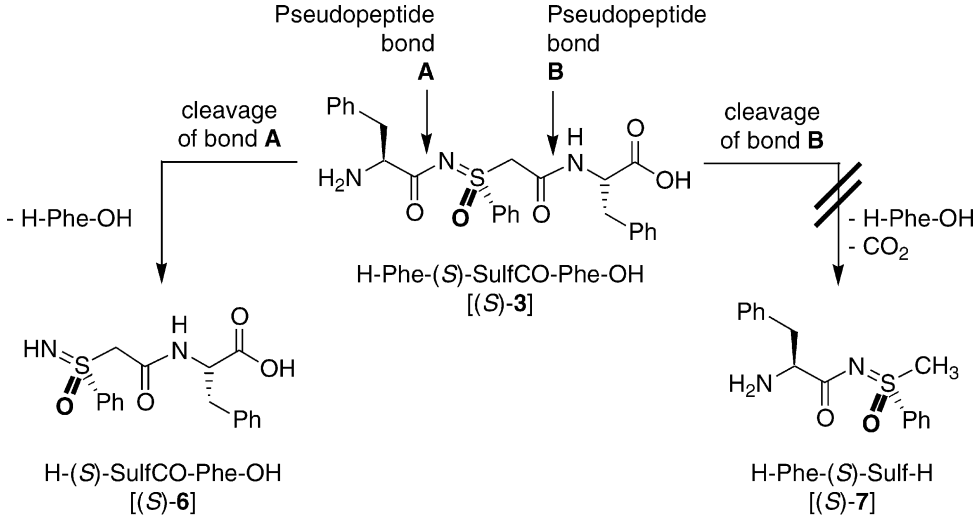
Most interestingly, the only products formed from the pseudo-tripeptide (*S*)-**3** (retention time 24.3 min) were phenylalanine (retention time 14.2 min) and the pseudodipeptide H-(*S*)-SulfCO-Phe-OH [(*S*)-**6**] (retention time 15.4–16.0 min, verified by co-injection with synthetic material).[21] The alternative product H-Phe-(*S*)-Sulf-H [(*S*)-**7**], synthesized by deprotection of (*S*)-**1** with TFA, retention time 27.8 min, which should be formed spontaneously by decarboxylation of the primary product, was not observed (Scheme 3). Thus, cleavage occurred exclusively at the pseudopeptide bond A and not at the pseudopeptide bond B (Scheme 3).[22]

In order to test the stereoselectivity of Proteinase K for the P1′ residue (the residue after the cleavage site), we investigated the cleavage of pseudotripeptide H-Phe-(*R*)-SulfCO-Phe-OH [(*R*)-**3**]. In (*R*)-**3** the configuration at sulfur is inverted compared to (*S*)-**3**, and (*R*)-**3** can be regarded as a tripeptide analogue with an internal

**Table 1.** Observed rate constants $k_{obs}$ for the cleavage of sulfoximine-containing pseudopeptides and natural peptides by Proteinase K

| Entry | Compd | [Substrate] (μM) | $k_{obs}$[a] ($h^{-1}$) | $t_{1/2}$ (min) | [Enzyme][b] (mAU mL$^{-1}$) | $k_{rel}$[c] |
|---|---|---|---|---|---|---|
| 1 | (*S*)-**3** | 304 | 3.75±0.36 | 11.1 | 3.0 | 1 |
| 2 | H-Phe-Phe-Phe-OH | 218 | 1.11±0.07 | 37.5 | 1.5 | 0.59 |
| 3 | (*R*)-**3** | 304 | 2.72±0.10 | 15.3 | 3.0 | 0.73 |
| 4 | H-Phe-Phe-OH | 262 | 0.06±0.01 | 693.1 | 30.0 | 0.002 |
| 5 | (*S*)-**6** | 262 | No cleavage | | 300.0 | |

[a]Errors are given as standard deviations.
[b]AU, Active Unit; see the note to Figure 3 for definition.
[c]Defined as $k_{obs}$ divided by the enzyme concentration relative to entry 1.




**Scheme 3.** Bonds in H-Phe-(*S*)-SulfCO-Phe-OH [(*S*)-**3**] that could be cleaved by Proteinase K. Only cleavage of the pseudopeptide bond **A** with the formation of H-(*S*)-SulfCO-Phe-OH [(*S*)-**6**] was observed.

unnatural β-D-amino acid analogue. Again cleavage occurred exclusively at the pseudopeptide bond A. The observed first order rate constant $k_{obs} = 2.72\ h^{-1}$ (Table 1, entry 3) was similar to that observed with the diastereoisomer (*S*)-**3**. This result can be rationalized based on the three-dimensional structure of Proteinase K in complex with a cleaved hexapeptide.[23] It seems as if both pseudotripeptides, (*S*)-**3** and (*R*)-**3**, could bind equally well after side-chain rotations on the surface of Proteinase K, and we assume that the primary structural determinate for cleavage of our pseudotripeptides is the N-terminal Phe residue (P1 residue).

Based on those results and in the light of the $^{13}C$ NMR data, we anticipated that the sulfoximine moiety should stabilize the amide bonds connecting the β-carbonyl group of the sulfoximine sulfur with the natural *N*-bound α-amino acid. This was tested in cleavage experiments of pseudodipeptide H-(*S*)-SulfCO-Phe-OH [(*S*)-**6**] in comparison with those of natural dipeptide H-Phe-Phe-OH. The latter was cleaved very slowly (Table 1, entry 4), which is in line with the results by Kraus et al.,[24] who suggested that small peptides were bad substrates for Proteinase K. With pseudodipeptide (*S*)-**6**, however, no cleavage was observed at all even in the presence of a 10-fold higher concentration of Proteinase K (Table 1, entry 5).

Taken together, our results demonstrate that the sulfoximine-pseudopeptide modification in (*S*)-**6** [and in (*S*)-**3** or (*R*)-**3**] stabilizes the following pseudopeptide bond B against enzymatic cleavage. Furthermore it is apparent that pseudopeptide bond A is readily cleaved by the enzyme. These observations make this class of pseudopeptides interesting candidates for a prodrug approach. For example, a released free N–H-sulfoximine moiety is very important in sulfoximine containing active compounds like methionine sulfoximine (MSO). MSO is a selective inhibitor of γ-glutamylcysteine synthetase and its free N–H group is phosphorylated by the enzyme.[25] Currently further investigations towards an understanding of the properties of the sulfoximine-pseudopeptide bonds A and B are in progress.

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18. The given absolute configurations refer to those at sulfur only. All natural α-amino acids have *S*-configuration. The structure for the abbreviation (*S*)-SulfCO is shown in Figure 1 (solid frame in **I**). The abbreviation Sulf stands for SulfCO without the carbonyl moiety.
19. Boc-Sulf-H [(*S*)-**4**] was synthesized according to literature[14a] and is the reaction product of phenyl methyl sulfoximine with $(Boc)_2O$ and KO*t*Bu.

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21. **Experimental**: Compounds **1**–**7** were prepared according to literature procedures for analogous substrates.[14] **Proteinase K fragmentation assay:** The cleavage reactions were performed in a buffer (1 mL, 30 mM Tris–HCl, 1 mM $CaCl_2$, pH 8.0) containing the pseudopeptides or peptides (see Table 1 for concentrations) and Proteinase K (Qiagen Proteinase K, see Table 1 for concentrations) at 37 °C. Aliquots were withdrawn after different incubation times, frozen in liquid nitrogen and stored at −20 °C before RP-HPLC analysis. Samples (60 μL) were thawed and directly injected onto a C-18 column (Prontosil 120-5-C18-AQ-5.0 μm, 250 mm×4.6 mm, Bischoff). Compounds were eluted with water (0–5 min) followed by linear gradient to 70% $CH_3CN$ (5–30 min) at a flow rate of 1 mL $min^{-1}$ and detected by UV-absorption at 210 nm. The following retention times $t_R$ were observed: H-Phe-OH (14.2 min), (*S*)-**6** (15.4–16.0 min), H-Phe-Phe-OH (18.5–19.2 min), (*R*)-**3** (20.7 min), H-Phe-Phe-Phe-OH (22.9 min) (*S*)-**3** (24.3 min), and (*S*)-**7** (27.8 min).
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