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Increased Stability of Peptidesulfonamide Peptidomimetics towards Protease Catalyzed Degradation

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Abstract—Replacement of amide bonds in peptides by sulfonamide moieties resulted in peptidosulfonamides with an increased stability towards protease catalyzed degradation. In addition to protection of the protease cleavage site, it was found that introduction of a sulfonamide also influenced the stability of adjacent amide bonds.

Introduction

Although peptides appear to be ideal starting points for the development of new drugs[1] because they can accommodate an infinite number of pharmacological profiles by permutation of the amino acid residues of which they are composed, the use of peptides as drugs is severely hampered, among others, by their rapid proteolytic degradation leading to a poor bioavailability. Therefore, one of the goals of design and synthesis of modified peptides and peptidomimetics is to obtain compounds with an improved stability towards degradation by proteases.[2–4]

Towards realizing this aim, peptidomimetics have been developed in which the amide moiety has been replaced by, for example, a carbamate,[5] a *N*-substituted amide leading to peptoids,[6] or a reduced amide bond.[7] In addition, α-amino acids have been replaced by β-amino acids,[8] resulting in compounds with an increased stability towards proteolytic degradation.[8]

Recently, we have published a positional scan of Leu-enkephaline in which systematically one peptide bond was replaced by a sulfonamide moiety ($\Psi CH_2SO_2N(H)$).[9] The binding activities of these peptidosulfonamides showed that replacement of the C-terminal amides resulted in nearly equipotent compounds but replacement of other amide bonds, that is, closer to the amino terminus resulted in a decreased, and ultimately complete loss of its inhibitory activity as was monitored by an inhibition ELISA for anti-β-endorphin monoclonal antibody.[10] In this paper, for the first time the reduction of the proteolysis rate of *peptido*sulfonamides,[11] when peptidosulfonamides derived from Leu-enkephaline or a TAP-substrate peptide were treated with pepsin, trypsine or Carlsberg (C.) subtilisin. Pepsin is an abundant aspartic protease which preferentially cleaves between phenylalanine, tyrosine or leucine as P^1 and leucine or phenylalanine as P^1 amino acid. Trypsin is a serine protease which prefers lysine or arginine as P^1 amino acid. The serine protease C. subtilisin prefers phenylanaline as P^1 amino acid.[12]

Results and Discussion

The pepsin catalyzed hydrolysis of the parent peptides H-TyrGlyGlyPheLeu-NH_2 (**1**) and H-TyrGlyGly-PheLeu-OH (**6**) and the corresponding peptidosulfonamides **2**–**5** and **7**–**9** (Fig. 1) was carried out using a 0.4 mg mL^{-1} solution of the appropriate peptidosulfonamide or peptide in a 50 mM formic acid buffer at 37 °C. The reaction was followed by taking aliquots of the reaction mixtures at different time intervals during 7 h, followed by analysis by HPLC.[13]

As is shown in both graphs, the parent Leu-enkephaline peptides **1** and **6** were rapidly degraded with half lives of 0.5 and 1.5 h, respectively. The peptidosulfonamides **5** and **9**, having a sulfonamide isostere between the two glycines, are far more stable towards pepsin catalyzed hydrolysis resulting in half lives that are three times those of their parent peptides. Moreover, Leu-enkephaline

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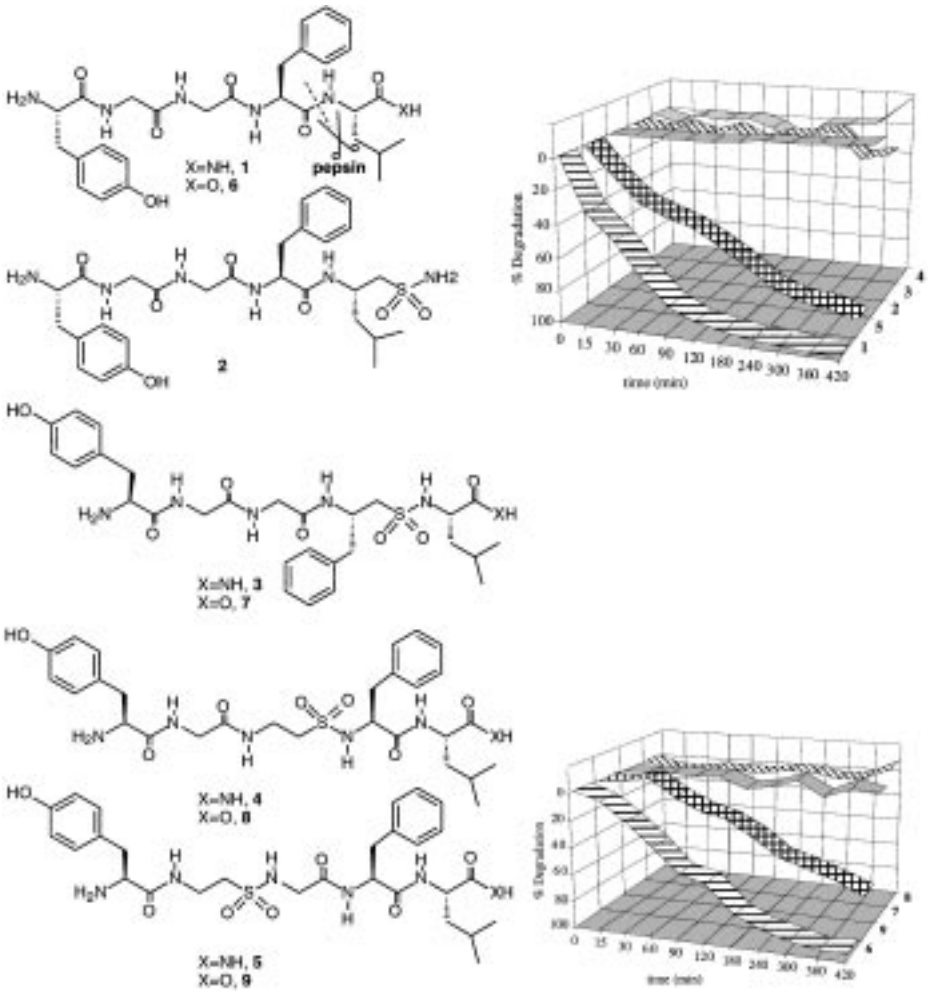

Figure 1. Peptidosulfonamides and hydrolysis by pepsin of Leu-enkephaline amide peptidosulfonamides (top graph) and Leu-enkephaline peptidosulfonamides (bottom graph).

derivatives having a sulfonamide between Gly^3-Phe^4 (**4** and **8**), Phe^4-Leu^5 (**3** and **7**) and C-terminal (**2**) are quite stable towards pepsin catalyzed degradation as no significant degradation of these peptidosulfonamides occurred within 7 h. These data show that the preferred cleavage site of pepsin (viz. Phe-Leu in compound **3** and **7**) is protected against proteolysis when this amide bond is substituted by a sulfonamide. In addition, substitution of an adjacent amide bond by a sulfonamide also increased the stability of the cleavage site (compounds **2**, **4**, **5**, **8**, and **9**).

Table 1. Half-lives of proteolysis by pepsin and IC_{50} values of peptidosulfonamide containing Leu-enkephaline derivatives

Leu-enkephalin derivative	Half-lives (h)	IC_{50} (μM)[9]
H-TyrGlyGlyPheLeu-NH_2 (**1**)	0.5	0.07
H-TyrGlyGlyPheLeu(ψCH_2SO_2)-NH_2 (**2**)	> 7	0.1
H-TyrGlyGlyPhe(ψCH_2SO_2NH)Leu-NH_2 (**3**)	> 7	0.9
H-TyrGlyGly(ψCH_2SO_2NH)PheLeu-NH_2 (**4**)	> 7	85
H-TyrGly(ψCH_2SO_2NH)GlyPheLeu-NH_2 (**5**)	1.5	75
H-TyrGlyGlyPheLeu-OH (**6**)	1.5	0.1
H-TyrGlyGlyPhe(ψCH_2SO_2NH)Leu-OH (**7**)	> 7	5
H-TyrGlyGly(ψCH_2SO_2NH)PheLeu-OH (**8**)	> 7	> 200
H-TyrGly(ψCH_2SO_2NH)GlyPheLeu-OH (**9**)	4.5	> 200

A comparison of the IC_{50} values by an inhibition ELISA for anti-β-endorphin monoclonal antibody[9,10] and the half-lives of the proteolytic degradation by pepsin of the Leu-enkephaline derived peptidosulfonamides (Table 1) revealed that peptidosulfonamides H-Tyr GlyGlyPhe-Leu(ψCH_2SO_2)-NH_2 (**2**), H-TyrGlyGlyPhe (ψCH_2SO_2-NH)Leu-NH_2 (**3**) and H-TyrGlyGlyPhe (ψCH_2SO_2NH)-Leu-OH (**7**) were much more slowly digested by pepsin (half lives > 7 h) and are, fortuitously, equipotent or only one order of magnitude less active as the corresponding parent peptides **1** and **6**. As such this represents a nice illustration of the validity of the peptidomimetic approach in which amide bonds are replaced by other moieties with the purpose to reduce degradation, while retaining the biological activity.

As part of our ongoing studies[14] towards potential inhibitors of TAP (transporter associated with antigen processing), we prepared peptidosulfonamides **11**–**13** (Fig. 2) to study the influence of backbone modification on the ability of TAP to transport these peptides through the membrane of the endoplasmic reticulum.*

*The effects of peptidosulfonamides **11**–**13** on TAP will be published elsewhere.

To investigate the influence of proteases on the degradation of these potential TAP inhibitors, compounds **11**–**13** and their corresponding peptide **10** were treated with trypsin or C. subtilisin.

The trypsin catalyzed degradation of peptidosulfonamides **11**–**13** and the corresponding peptide **10** was carried out using a 0.8 mg mL^{-1} solution of the appropriate peptidosulfonamide or peptide in a 100 mM Tris buffer containing 25 mM $CaCl_2$ at 37 °C, taking aliquots of the reaction mixtures at different time intervals during 7 h, followed by analysis by HPLC.[15]

As can be seen in Figure 2 and Table 2, peptide **10** was rapidly degraded by trypsin with an approximate half life of 12 min. Introduction of a sulfonamide moiety between Phe^7-Gly^8 and/or Phe^2-Gly^3 had an unexpected effect on the stability of these peptidosulfonamides towards trypsin treatment. Peptidosulfonamide **12** was somewhat faster degraded than the corresponding peptide **10**, whereas peptidomimetic **11**, having a sulfonamide between Phe^7-Gly^8, and peptidosulfonamide **13** bearing two sulfonamide moieties are actually more stable than the corresponding peptide **10**.

Digestion of peptidosulfonamides **11**–**13** and the corresponding peptide **10** by C. subtilisin was essentially carried out as described above for trypsin, now using a buffer of 35 mM HEPES (pH 7.8) at 37 °C.[16] Treatment of peptide **10** with C. subtilisin, which prefers phenylalanine as P^1 amino acid, resulted in cleavage at two sites.

The amide bond between Phe^7-Gly^8 was hydrolyzed faster (half-life of ca. 24 min) than the amide bond

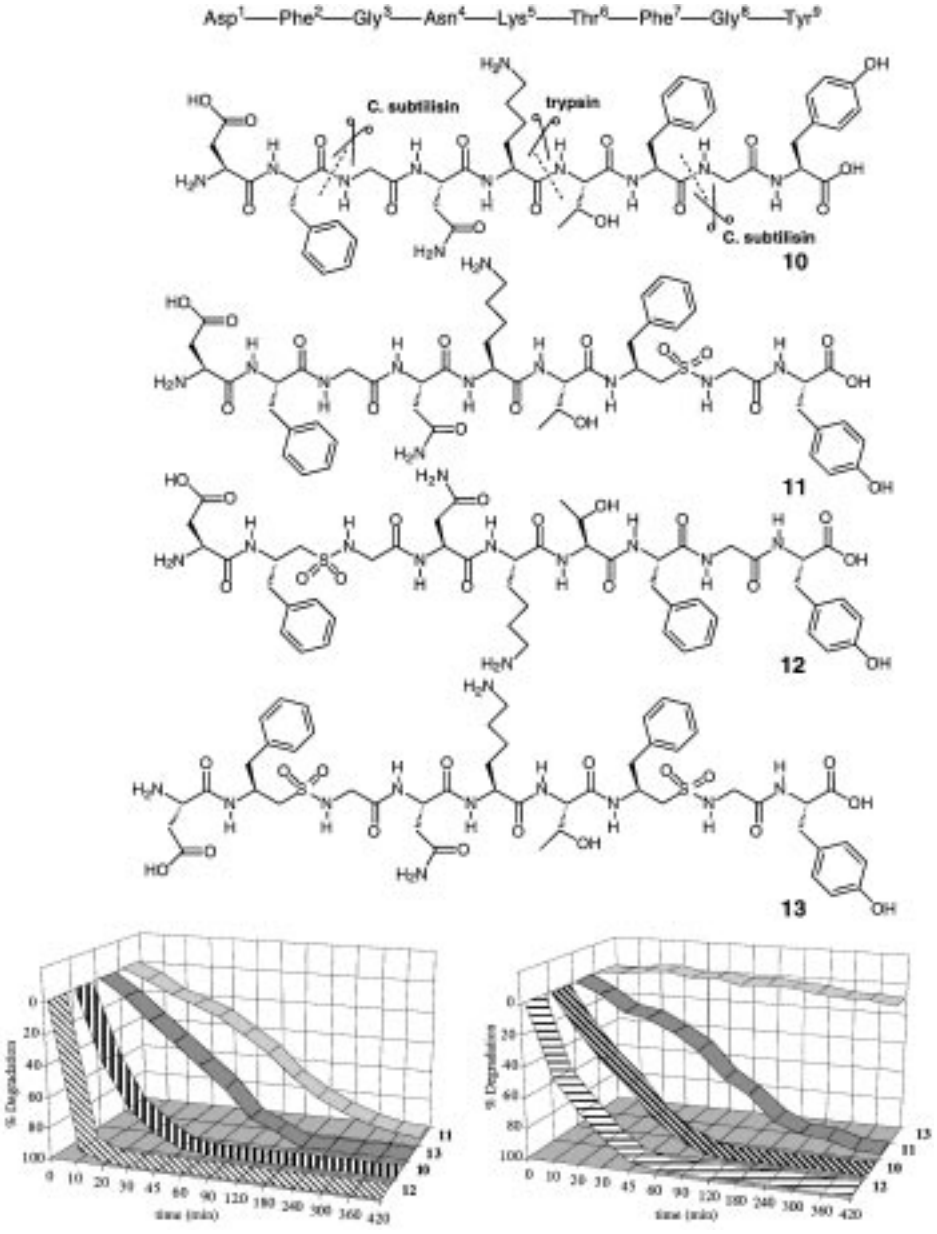

Figure 2. A TAP substrate peptide (**10**) and corresponding peptidosulfonamides (**11**–**13**) as well as graphic representation of trypsin (left) mediated and C. subtilisin (right) mediated hydrolysis.

Table 2. Protease catalyzed degradation of peptidosulfonamides **11**–**13** and the corresponding peptide **10**

	Half-life (min) trypsin	Half-life (min) C. subtilisin
H-AspPheGlyAsnLysThrPheGlyTyr-OH (**10**)	12	24 (Phe^7-Gly^8) 80 (Phe^2-Gly^3)
H-AspPheGlyAsnThrPhe(ψCH_2SO_2NH)GlyTyr-OH (**11**)	90	77 (Phe^2-Gly^3)
H-AspPhe(ψCH_2SO_2NH)GlyAsnLysThrPheGlyTyr-OH (**12**)	6	13 (Phe^7-Gly^8)
H-AspPhe(ψCH_2SO_2NH)GlyAsnLysThrPhe(ψCH_2SO_2NH)GlyTyr-OH (**13**)	43	>420

between Phe^2-Gly^3 (half-life of ca. 80 min). Introduction of a sulfonamide at latter position (peptidosulfonamide **12**, Fig. 2) resulted in a C. subtilisin catalyzed hydrolysis only between Phe^7-Gly^8 with a half-life of ca. 13 min. Evidently, when peptidosulfonamide **11**, having a sulfonamide moiety between Phe^7-Gly^8 was treated with C. subtilisin, only the amide bond between Phe^2-Gly^3 was hydrolyzed (half-life of 80 min). Substitution of both Phe-Gly amides by a sulfonamide moiety (peptidosulfonamide **13**) resulted in a peptidosulfonamide which was nearly stable towards treatment with C. subtilisin and no substantial degradation occurred.

Although there are no obvious pepsin cleavage sites present in the TAP-peptidosulfonamides **10**–**13**, we decided for comparison purposes with other TAP-peptidomimetics,[14] to subject **10**–**13** to treatment with pepsin. Surprisingly, TAP-peptide **10** and its corresponding sulfonamide **12** are digested by pepsin with half lives of ca. 70 and 40 min, respectively. Peptidosulfonamides **11** and **13** remained virtually unaffected by pepsin. The faster cleavage by pepsin of peptidosulfonamide **12** as compared to the parent peptide **10** is in agreement with the data shown in Table 2, where **12** is degraded faster by trypsin and subtilisin than peptide **10**. Mass spectrometry studies showed that pepsin had cleaved between Thr^6 and Phe^7 in both **10** and **12**.

In conclusion, we have found that substitution of an amide bond of the protease cleavage site by a sulfonamide moiety resulted in peptidosulfonamides with an increased stability towards protease catalyzed degradation. To our best knowledge this is the first time that such a favorable effect of a sulfonamide moiety in a *peptido*sulfonamide on its stability has been reported. Furthermore, it is duly expected that these peptidosulfonamide will also show an increased stability towards degradation by proteases encountered in biological fluids. Replacement of the scissile peptide bond with a sulfonamide moiety resulted in peptidosulfonamides with half-lives up to more than 7 h. Leu-enkephaline derivatives **3** and **7**, in which the preferred cleavage site between Phe^4 and Leu^5 is replaced by a sulfonamide moiety, is hardly degraded by pepsin. Furthermore, replacement of both the preferred cleavage sites of C. subtilisin (Phe-Gly) in peptide **10** by a sulfonamide moiety leading to peptidosulfonamide **13**, gave rise also to a peptidosulfonamide which was quite stable towards protease catalyzed degradation. Introduction of one sulfonamide moiety replacing a scissile peptide bond resulted in peptidosulfonamides **11** and **12**, which were degraded by C. subtilisin only at the remaining Phe-Gly amide bond.

Replacement of amide bonds by sulfonamide moieties *close to* the preferred cleavage site resulted in an increased stability of this scissile peptide bond towards proteolytic degradation. Introduction of a sulfonamide moiety between Gly^2 and Gly^3, Gly^3 and Phe^4 or at the C-terminus adjacent to the scissile Phe^4-Leu^5 amide bond in Leu-enkephaline (Table 1) or replacement of the amide bonds between Phe and Gly adjacent to the scissile Lys^5-Thr^4 peptide bond (Table 2) resulted in longer half-lives of protease catalyzed degradation.

Such a remote protection by a sulfonamide moiety of proteolytic cleavage of an amide bond seemed to have a distance dependence. When the sulfonamide moiety was introduced next to a pepsin sensitive peptide bond, i.e. **2**, **4** and **8**, these peptidosulfonamides are nearly stable towards proteolytic degradation. Introduction of the sulfonamide at a amide bond one additional amino acid removed from this site resulted in peptidosulfonamides, i.e. **5**, **9**, **11** and **13**, which were degraded by proteases but with significant longer half-lives when compared to the corresponding peptides (Tables 1 and 2). Introduction of a sulfonamide two amino acids removed from the trypsin cleavage site, that is, Lys^5-Thr^4 did not result in an increased stability of this peptidosulfonamide **12**. Perhaps, a sulfonamide bond substitution at a site distal to the scissile bond changes the conformation in the protease active site in such a way that bond cleavage is favored.

A similar remote effect of a single backbone modification on the degradation rate of neighboring amide bonds has also been described for replacement of peptide bonds by reduced amide bonds[7] and may be of significant importance for modulation of the degradation rates of peptides, especially when they are considered as possible starting structures in the development of new drugs.

Experimental

Synthesis

Leu-enkephalin **1**, Leu-enkephalin amide **6** and their corresponding peptidosulfonamides **2**–**5** and **7**–**9**, respectively were synthesized as was described earlier.[9] TAP-peptidosulfonamides **11**–**13** were also synthesized using this procedure for the solid-phase synthesis of peptidosulfonamides by a Fmoc-protocol employing Fmoc-amino acids and Fmoc amino sulfonyl chlorides.

^{1}H NMR spectra were recorded on a VARIAN G-300 spectrometer (300.1 MHz). ^{13}C NMR spectra were

recorded on a VARIAN G300-spectrometer (75.5 MHz). Fast atom bombardment (FAB) mass spectrometry was carried out using a Jeol JMS SX/SX 102A four-sector mass spectrometer, coupled with a HP-9000 data system. All peptides and their corresponding peptidosulfonamides were fully characterized by ^{1}H and ^{13}C NMR as well as mass spectrometry. Their purity was assessed by HPLC and generally greater than 95%. If necessary, the peptidosulfonamides were purified by preparative reverse phase HPLC. HPLC analysis was carried out on a Gilson automated HPLC system 205 with a 233XL autosampler and a 119 UV-Vis detector. Mass spectrometry was also used to identify the fragments of enzymatic cleavage.

Pepsin catalyzed hydrolysis of Leu-enkephaline amide (1), Leu-enkephaline sulfonamides 2–5, Leu-enkephaline (6), Leu-enkephaline sulfonamides 7–9, TAP peptide 20 and TAP peptidosulfonamides 11–13. 200 μL of a 10 mg mL^{-1} solution of the peptide or peptidosulfonamide was diluted with 4.8 mL of a 50 mM formic acid solution (pH 2.2). After incubation for 15 min at 37 °C, 2 units of pepsin (12 mg) were added. Samples (200 μL) were taken at 0, 15, 30, 45, 60, 90, 120, 180, 240, 300, 360, and 420 min, the reaction was stopped by dilution with acetonitrile (600 μL), centrifuged for 5 min, the supernatant was decanted and analyzed by HPLC using a C-8 column and as eluent a gradient of 100% water containing 0.1% TFA to 95% acetonitrile containing 0.1% TFA. The relative amount of the remaining peptide or peptidosulfonamide, normalized to an internal standard (formic acid) was determined by integration of the peak areas of the appropriate signals. The percentage of degradation and half-life of the pepsin catalyzed hydrolysis of the Leu-enkephalin peptides and peptidosulfonamides is shown in Figure 1 and Table 1, respectively.

Trypsin catalyzed hydrolysis of TAP substrate peptide 10 and TAP substrate sulfonamides 11–13. 52 μL of a 10 mg mL^{-1} solution of peptide or peptidosulfonamide was diluted with 565 μL of a 100 mM Tris buffer (pH 7.8) containing 0.1% DMSO as an internal standard and 25 mM $CaCl_2$. After addition of 32 μL of a trypsin solution (0.43 units mL^{-1}) the mixture was gently shaken at 37 °C. Aliquots (50 μL) were taken at 0, 10, 20, 30, 45, 60, 90, 120, 180, 240, 300, 360, and 420 min, the reaction was stopped by dilution with 1 H HCl solution (150 μL) and analyzed by HPLC as described above for compound **1**. The amount of peptide **10** and peptidosulfonamides **11**–**13**, normalized to the internal standard was determined by integration of the peak areas of the appropriate signals. The percentage of degradation and half-life of the trypsin catalyzed hydrolysis is shown in Figure 2 and Table 2, respectively.

C. Subtilisin catalyzed hydrolysis of TAP substrate peptide 10 and TAP substrate sulfonamides 11–13. Degradation, catalyzed by C. subtilisin, was carried out by adding a solution of C. subtilisin (32 μL, 0.05 units mL^{-1}) and HEPES buffer (565 μL, 35 mM, pH 7.8) containing 0.1% DMSO to a 10 mg mL^{-1} solution of TAP substrate peptide **10** (52 μL). Aliquots (50 μL) were taken at time intervals as described above for the trypsin catalyzed hydrolysis of **10** and the reaction was stopped by dilution with 1 H HCl solution (150 μL). The amount of peptide **10** and TAP substrate sulfonamides **11**–**13**, normalized to the internal standard (0.1% DMSO), were determined by integration of the peak areas of the appropriate signals. The percentage of degradation and half-life of the C. subtilisin catalyzed hydrolysis is shown in Figure 2 and Table 2, respectively.

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