

# Towards more Sustainable Peptide-based Antibiotics: Stable in Human Blood, Enzymatically Hydrolyzed in Wastewater?

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**Abstract:** The emergence and spread of antibiotic resistance is a major societal challenge and new antibiotics are needed to successfully fight bacterial infections. Because the release of antibiotics into wastewater and downstream environments is expected to contribute to the problem of antibiotic resistance, it would be beneficial to consider the environmental fate of antibiotics in the development of novel antibiotics. In this article, we discuss the possibility of designing peptide-based antibiotics that are stable during treatment (e.g. in human blood), but rapidly inactivated through hydrolysis by peptidases after their secretion into wastewater. In the first part, we review studies on the biotransformation of peptide-based antibiotics during biological wastewater treatment and on the specificity of dissolved extracellular peptidases derived from wastewater. In the second part, we present first results of our endeavour to identify peptide bonds that are stable in human blood plasma and susceptible to hydrolysis by the industrially produced peptidase Subtilisin A.

**Keywords:** Biotransformation · Environmental benign-by-design · Peptidase specificity · Peptide-based antibiotics · Wastewater



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## 1. Introduction

Synthetic organic chemicals play an important role in many aspects of modern life – including but not limited to health, hygiene, and food production. While we use organic chemicals to our benefit on a daily basis, there is another side to this coin: chemicals often find their way into environments where they do not belong and where they can harm the respective ecosystem.<sup>[1]</sup> One strategy to address this problem is to design environmentally benign chemicals that are stable and functional during their use phase *and* degraded when (or ideally before) they are released into the environment.<sup>[2–4]</sup>

Antibiotics are a class of chemicals for which we and others think that such a strategy should be further investigated.<sup>[5–7]</sup> While

there is no doubt that novel antibiotics are needed to successfully fight infectious diseases and that these antibiotics need to be stable in the system where they act on the pathogens (e.g. in human blood in the case of a systemic infection),<sup>[8,9]</sup> their release into downstream environments should be reduced as much as possible.<sup>[10]</sup> Important sources of antibiotics released into the environment include wastewater from hospitals, production facilities, and households.<sup>[11–13]</sup> It is expected that the presence of antibiotics in wastewater and natural environments contributes to the emergence and spread of antibiotic resistance – even at concentrations below the minimal inhibitory concentration.<sup>[10,14–16]</sup> Furthermore, the presence of antibiotics in natural systems can affect native microbial communities that provide important ecosystem functions.<sup>[17]</sup>

One possibility to reduce the release of antibiotics into the environment is to design them for rapid inactivation during wastewater treatment (Fig. 1).<sup>[6,7]</sup> Because the stability of an organic chemical depends on its molecular structure *and* on the characteristics of the environment, we are convinced that differences between environments can be exploited for the design of antibiotics that are stable in one system and rapidly inactivated in another system. For example, antibiotics could be biotransformed into products that no longer bear antimicrobial activity by enzymes present in wastewater. Such wastewater enzymes are either produced by microbial communities growing in tanks at wastewater treatment plants (WWTPs) or they enter the WWTP together with wastewater (e.g. enzymes that originate from the human digestive tract or laundry detergents). For enzymes produced by wastewater microbial communities, the location of enzymatic antibiotic

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inactivation is important in the context of antibiotic resistance:<sup>[18]</sup> while inactivation by intracellular enzymes or extracellular enzymes closely associated with the producing cell likely provides a competitive advantage to the producing organism, we expect that inactivation by dissolved extracellular enzymes reduces the selective pressure on the entire microbial community, and as a consequence reduces the spread of resistance. Therefore, a detailed understanding of the enzymology in the systems of interest is required for the design of degradable antibiotics.

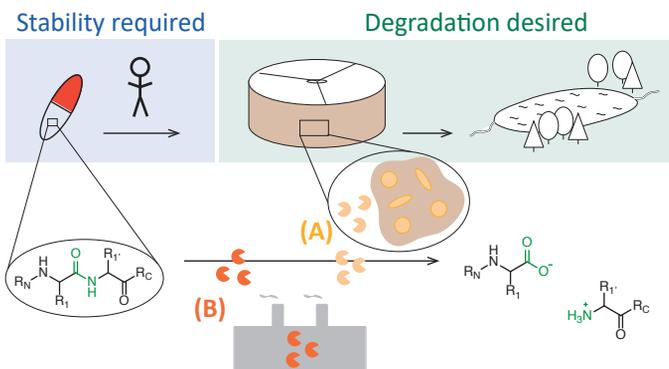


Fig. 1. Benign-by-design framework for peptide-based antibiotics. While stable during treatment (e.g. in human blood), antibiotics might be designed for rapid inactivation after secretion into wastewater. Options include the hydrolysis of antibiotics by dissolved extracellular peptidases inherently present in wastewater (A, see section 2) or by industrially produced peptidases (B, see section 3).  $R_N$ : N-terminus,  $R_C$ : C-terminus,  $R_1$  side chain of amino acid in P1 position,  $R_{1'}$ : side chain of amino acid residue P1' position.

## 2. Peptide-based Antibiotics and Wastewater Peptidases

Peptide-based antibiotics are particularly promising for such an environmental benign-by-design approach. First, a lot of research is currently conducted to identify, characterize, and further develop peptides with antimicrobial activity.<sup>[19–22]</sup> Second, antimicrobial peptides are highly specific for target pathogens and some peptides are promising with respect to resistance (*i.e.* reduced resistance emergence relative to antibiotics from other classes).<sup>[23,24]</sup> Third, established peptide synthesis techniques enable the modification of a peptide's amino acid sequence with relative ease and thus enable the systematic assessment of derivative structures.<sup>[19]</sup> Lastly, extracellular peptidases play an important role in wastewater metabolism (*i.e.* microorganisms secrete peptidases to make amino acids in proteins and peptides bioavailable) and may thus bear potential for the co-metabolic inactivation of peptide-based antibiotics.<sup>[6,25]</sup>

To assess the potential of wastewater peptidases for the biotransformation of antibiotics and to investigate where these peptidases are located within the wastewater matrix, we recently developed an approach based on experiments of Frølund *et al.* to fractionate wastewater enzymes into different pools.<sup>[6,26]</sup> In brief, we sampled wastewater from aeration tanks of full-scale WWTPs and derived enzyme pools containing either dissolved extracellular enzymes, all extracellular enzymes (*i.e.* including enzymes bound to the extracellular polymeric substance (EPS)), or all enzymes (*i.e.* including intracellular enzymes). We then incubated a set of commercially relevant antibiotics with these enzyme pools and used a method based on high-performance liquid chromatography coupled to high-resolution mass spectrometry (HPLC-HRMS) to study the enzymatic removal of these antibiotics and to identify occurring transformation products.

For the peptide-based antibiotic daptomycin, we found slow biotransformation by EPS-bound extracellular enzymes and no detectable biotransformation by dissolved extracellular enzymes.<sup>[6]</sup> The observation that peptides are not necessarily rapidly degraded by extracellular wastewater peptidases is supported by findings of Li *et al.*, who found that the concentration of vancomycin decreased only by approximately 50% during biological wastewater treatment (we note that other processes such as adsorption contribute to this removal and that only a fraction can thus be ascribed to biotransformation).<sup>[27]</sup> Because we think that dissolved extracellular enzymes are particularly promising with respect to antibiotic biotransformation, we used a rapid fluorescence-based assay to assess if this enzyme pool has the potential for peptide hydrolysis and we indeed detected substantial activity of dissolved extracellular peptidases.<sup>[6]</sup>

To learn something about the specificity of these dissolved extracellular wastewater peptidases, we incubated a set of model peptides with this enzyme pool and used a method based on HPLC-HRMS to monitor which peptides were rapidly removed and which products emerged.<sup>[7]</sup> From the resulting data, we identified a set of peptide bonds that were particularly susceptible to hydrolysis by dissolved extracellular peptidases, meaning that this peptide pool showed specificity as to the peptide bonds it cleaved. Furthermore, we found that a substantial number of the detected products occurred in incubations with enzymes from three different WWTPs – indicating that the peptidase specificity was conserved to some extent across wastewater microbial communities.

While these findings on the specificity of dissolved extracellular wastewater peptidases show promise for the identification of peptide bonds that could serve as predetermined 'breaking points' in antibiotics, many open points remain to be addressed. For example, the activity and specificity of peptidases need to be assessed across a wider range of WWTPs (including more different treatment processes, influent compositions, and geographic locations). If there is a substantial level of conserved peptidase specificity across WWTPs, it will be interesting to identify these peptidases and to assess their global distribution across wastewater microbial communities.<sup>[28]</sup> Furthermore, it will be interesting to study the transformation potential of extracellular peptidases in environments located upstream of WWTP aeration tanks (where antibiotics pass by before they reach WWTPs). Complementary, the stability of promising peptide bonds will need to be assessed in the system where the antibiotic is used during treatment (*e.g.* human blood).

## 3. Inactivating Antibiotics in Wastewater by Commercial Peptidases?

In addition to the idea that peptide-based antibiotics might be hydrolyzed by peptidases present in wastewater, we also started exploring the option of using industrial peptidases to inactivate peptide-based antibiotics released into wastewater. Enzyme-based wastewater treatment has been discussed for other enzyme-pollutant combinations before.<sup>[29,30]</sup> In our eyes, such an approach might particularly have potential to reduce the concentration of antibiotics in highly affected wastewaters such as those from hospitals and antibiotic production facilities. While the development of such an approach would certainly be a very challenging and interdisciplinary endeavour, we highlight that some peptidases (*e.g.* Subtilisin A – also referred to as Subtilisin Carlsberg) are already being industrially produced for large-scale applications, *e.g.* as laundry detergents.<sup>[31]</sup> In the following, we present findings from our first experiments exploring the potential of an approach based on industrial peptidases.

The goal of these experiments was to assess the specificity of Subtilisin A (*i.e.* to identify peptide bonds that are susceptible to hydrolysis by Subtilisin A) and to compare it to the specificity of

peptidases in human blood plasma (*i.e.* to find which of the identified bonds are stable in blood plasma).<sup>[32]</sup> Peptide bonds that fulfil both of these criteria would be promising ‘predetermined breaking points’ for the incorporation into peptide-based antibiotics. In search of such peptide bonds, we applied the methodological approach that we used in the study discussed above.<sup>[7]</sup> In brief, we used HPLC-HRMS to monitor the abundance of a set of model peptides (Table S1 in the Supplementary Information) during their incubation in a Subtilisin A solution and in human blood plasma that was either stabilized with citrate or with ethylenediaminetetraacetate (EDTA).

For incubations with Subtilisin A, we found that the peak area of certain peptides rapidly decreased, while it remained constant for other parent peptides (Fig. S1). This observation indicates that Subtilisin A might be more specific than previously expected.<sup>[33]</sup> For incubations in blood plasma, we found that the majority of peptides were stable in EDTA-stabilized plasma, while many peptides were rapidly removed in citrate-stabilized plasma (Fig. S2). This observation is consistent with previous findings and probably results from the fact that EDTA is a stronger chelator than citrate and that it therefore has a stronger inhibitive effect on metallopeptidases in blood plasma.<sup>[34–36]</sup> We note that experiments based on stabilized plasma, while relatively simple and thus widely used, should ideally be complemented with additional approaches that allow the assessment of metallopeptidases.<sup>[35]</sup>

To identify target peptide bonds that are susceptible to hydrolysis by Subtilisin A and stable in human blood plasma, we estimated rate constants of peptide removal in all incubation experiments (Fig. 2) and selected peptides for which the ratio of these estimates between Subtilisin A and plasma were highest (*i.e.*  $k_{\text{SubA}}/k_{\text{plasma}} > 4$ ). We note that we used rate constant estimates of

citrate-plasma incubations because they were higher than those of EDTA-plasma incubations. At the same time, we excluded peptides for which removal rate constant estimates in either citrate- or EDTA-stabilized plasma were larger than  $1 \text{ h}^{-1}$  (which corresponds to a lifetime of 1 h). This criterion was applied because target peptide bonds should be sufficiently stable in human blood. For the five peptides (*i.e.* #5, 20, 24, 25, and 40, Table S1) that fulfilled both criteria, we predicted all possible products resulting from the hydrolysis of one peptide bond and screened the HPLC-HRMS data for these products.

Amino acid sequences and analytical parameters of identified product peptides are provided in Table S2. To verify that target peptide bonds are stable towards non-enzymatic hydrolysis and towards hydrolysis by peptidases in blood plasma, we excluded products that were substantially present in buffer controls and plasma incubations (*i.e.* if peak areas in these incubations were less than 10-fold smaller than those in Subtilisin A incubations). For each of the five selected parent peptides, we picked the predominant product based on peak area progress curves (Fig. S3) and identified the amino acid pairs flanking the hydrolyzed peptide bonds (*i.e.* residues P1-P1'). We then assessed if these amino acid pairs (*i.e.* M-A, L-E, A-D, A-V, and E-A – single letter amino-acid abbreviations) occurred multiple times on the set of model peptides and if so, how rapidly the respective peptides were removed by Subtilisin A. We found that amino acid pairs L-E and A-D also occurred on parent peptides that were not susceptible to Subtilisin A hydrolysis (*i.e.*  $k_{\text{SubA}} < 1 \text{ h}^{-1}$  for parent peptides #22 and #3) and that these pairs were thus not sufficient to trigger hydrolysis. All parent peptides containing the remaining three amino acid pairs (*i.e.* M-A, A-V, E-A) were hydrolyzed by Subtilisin A and the respective products were de-

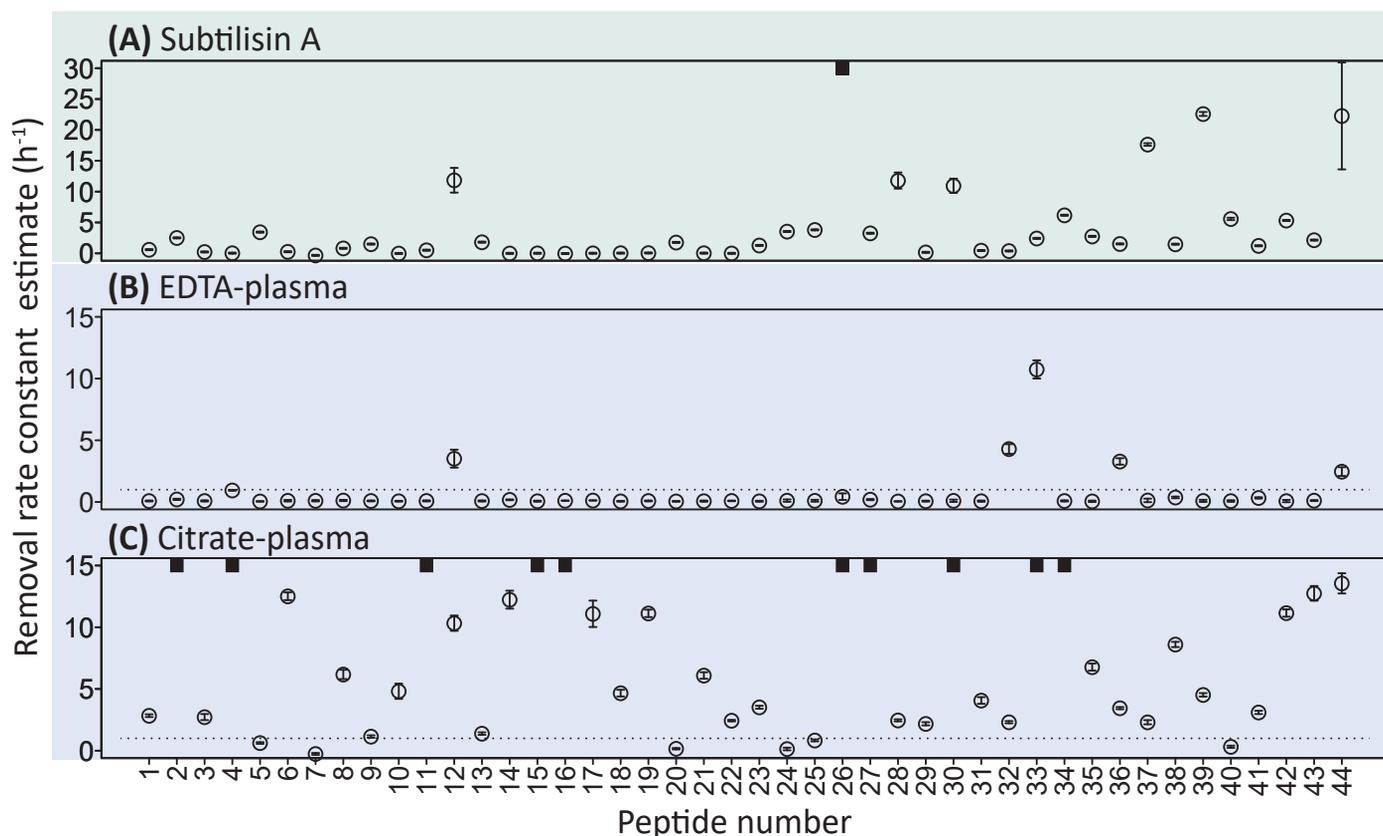


Fig. 2. Rate constant estimates of model peptide removal measured in Subtilisin A solution (A) and human blood plasma that was either stabilized with ethylenediaminetetraacetic acid (EDTA) (B) or citrate (C). Symbols and error bars represent means and standard deviations, respectively, of triplicate incubation experiments. Black squares indicate that we did not detect the respective peptide beyond the initial sampling time point and we thus assumed rate constants to be higher than for the fastest reactions for which we detected peptides at two or more time points. Dashed lines in (B) and (C) represent a rate constant of  $1 \text{ h}^{-1}$ .

tected in Subtilisin A incubations (Table S2), but not in plasma and buffer-control incubations.

Based on the process described above, we selected amino acid pairs M-A, A-V, and E-A for the next step. To assess if these amino acid pairs are sufficient to trigger hydrolysis by Subtilisin A, we designed three peptides that each contained one of these amino acid pairs in the centre of the peptide – flanked by parts of the sequence of peptide #7, which was persistent towards hydrolysis by Subtilisin A (Fig. 3). Incubation experiments with Subtilisin A revealed that the peak areas of these three peptides remained constant (Fig. S4) – suggesting that the peptides were not substantially hydrolyzed and that short amino acid patterns might not be sufficient to trigger hydrolysis by Subtilisin A (unlike other peptidases such as Trypsin). Alternatively, the flanking region of the peptide (*i.e.* the parts shaded in blue in Fig. 3) has a particularly strong suppression effect for the hydrolysis by Subtilisin A. To address this further, the hydrolyzability of the peptide bond candidates should be studied in other peptides – ideally directly in peptides with antimicrobial activity. Additional important questions towards wastewater treatment by industrial peptides include (i) where such a treatment could be applied (*e.g.* in pre-treatment tanks of hospital wastewater), (ii) whether peptidase-mediated inactivation occurs on a relevant time scale with respect to the hydrolytic retention times, and (iii) how peptidases are affected by other components (*i.e.* chemicals, particles, *etc.*) present in a system.

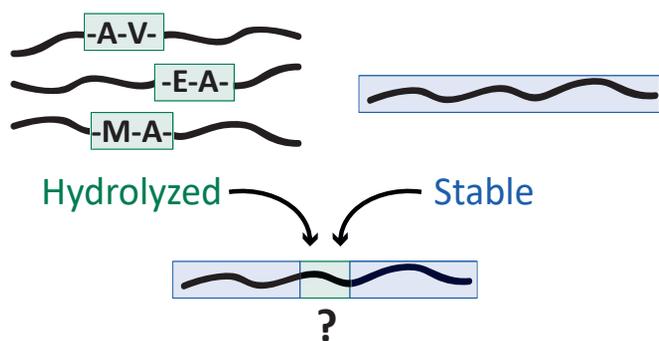


Fig. 3. Design of synthetic peptides. We combined amino acid pairs flanking peptide bonds for which we had detected rapid hydrolysis by Subtilisin A (*i.e.* A-V, E-A, and M-A; single letter amino acid abbreviations; green shading) with parts of model peptide #7 (stable towards hydrolysis by Subtilisin A; blue shading) and assessed the stability of the resulting peptides.

#### 4. Conclusions

Both ideas presented above constitute a first step towards a potential environmental benign-by-design approach for peptide-based antibiotics and many open points remain. The next step should be the identification/development of an antibiotic that can be inactivated by an enzyme – either industrially produced or of natural origin – in the extracellular solution. With such a system, one could assess the question whether inactivation of an antibiotic by dissolved extracellular enzymes is indeed beneficial with respect to the emergence and spread of resistance. Lastly, we note that while we discussed here the idea of designing environmentally benign chemicals based on insights from environmental biochemistry in the context of antibiotics, it can (and should) also be applied for other combinations of chemicals and receiving environments.

#### 5. Materials and Methods

**Materials.** Subtilisin A was purchased from Sigma-Aldrich (product number P8038). Pooled gender human blood plasma

(stabilized with sodium citrate or potassium EDTA) was purchased from BioIVT, West Sussex, UK (product identifiers: HUMANPL38NCPNN and HUMANPLK2PNN, respectively). BSA-derived model peptides were prepared as previously described.<sup>[17]</sup> In brief, BSA (Sigma-Aldrich, A2153) was digested with Trypsin (ThermoFisher, Pierce 90057) and the resulting peptides were cleaned using desalting spin columns (ThermoFisher, Pierce 89852). For this study, we digested 3 mg of BSA with 10 µg of Trypsin (ratio 300:1). Synthetic model peptides (95% purity, trifluoroacetate removed and replaced by acetate) were custom ordered from SynPeptide LTD, Shanghai, China. Information on additional chemicals used in this study are listed in the Supplementary Information.

**Blood plasma experiments.** The experimental protocol was adapted from Luther *et al.*<sup>[20]</sup> We thawed and centrifuged (10 min at 3'200 g) plasma immediately before the experiment and aliquoted the supernatant into 1.5 mL aliquots. We then spiked 15 µL of model peptide solution to these aliquots and to 1.5 mL of pH-buffered MQ water (pH 7.4, 5 mM potassium phosphate). To demonstrate that none of the detected peptides originated from plasma, we ran a control incubation to which we spiked a mock digest instead of model peptides. Triplicate incubations were conducted where not indicated differently. Plasma activity was confirmed by the removal of propantheline (Fig. S5).<sup>[20]</sup> After peptide spiking, we mixed solutions by inverting test vials and incubated them at 37 °C while horizontal shaking at 180 rpm. At sampling time points, we transferred 150 µL of incubation solution to 450 µL of acetonitrile containing 1% (v/v) formic acid, mixed the resulting solution by inverting the tube and by horizontally shaking it at 180 rpm for 2 min (room temperature), and centrifuged samples at 16'200 g for 1 min. We then transferred the supernatant into a fresh vial and dried the samples in a SpeedVac vacuum concentrator (60 °C for approximately 2 h). To resolubilize peptides, we added 150 µL of MQ water containing 0.1% (v/v) formic acid, let the resulting solution incubate for 30 min at RT, and transferred the solution into HPLC vials. Samples were stored at –20 °C until analysis. Experiments with blood plasma were conducted in a Biosafety Level-2 laboratory.

**Subtilisin A experiments.** Experiments were conducted analogous to the blood plasma experiments described above with the following differences: 1) model peptides were spiked to 1.5 mL of pH-buffered solutions (pH 7.4, 5 mM potassium phosphate) containing Subtilisin A (0.1 µg/mL) instead of plasma, 2) incubations were conducted at room temperature. Synthetic model peptides were incubated at a concentration of 100 µg/L.

**HPLC-HRMS analysis.** We analyzed peptides using HPLC (Thermo Ultimate 3000 equipped with a Thermo Acclaim PepMap C18 column (product number: 164572)) coupled to HRMS (Thermo QExactive Plus). We injected 25 µL of each sample and applied a flow rate of 40 µL/min using the following eluent gradient (A: MQ water; B: LCMS-grade methanol; both containing 0.1% (v/v) formic acid): 0–2 min: 2% B, 2–70 min: 2% B–95% B, 70–74 min: 95% B, 74–75 min: 95% B–2% B, 75–80 min: 2% B. Detection parameters were chosen as follows: full-scan: MS range: 160–1800 m/z, resolution: 70'000, AGC target: 10<sup>6</sup>, Maximum IT: 200 ms, positive electrospray ionization (tune data: capillary temperature: 275 °C, sheath gas: 15, aux gas: 5, spare gas: 0, and S-lens RF: 70), MS/MS acquisitions: Top10, resolution: 17'500, AGC target: 10<sup>5</sup>, Maximum IT: 100 ms, isolation window: 1.0 m/z, NCE (stepped): 20, 25, 30, dynamic exclusion time: 6 s). For data analysis, we used Skyline (version 20.1.0) as described previously.<sup>[17,37]</sup> Criteria for parent and product peptide identification were chosen as follows: m/z deviation < 2ppm, at least one MS/MS fragment with m/z deviation < 5ppm, reasonable chromatographic peak shape, matching isotope pattern (Skyline idotp > 0.9). For products, an additional

criterion was that the peak area either increased or first increased and then decreased during incubation.

### Supplementary Information

Supplementary information is available on <https://www.ingentaconnect.com/content/scs/chimia>

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