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DOI: 10.1039/C6CC10300F

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Scandium(III) triflate-promoted serine/threonine-selective peptide bond cleavage

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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Site-selective cleavage of peptide bonds is an important chemical modification that is useful not only for the structural determination of peptides, but also as an artificial modulator of peptide/protein function and properties. Here we report site-selective hydrolysis of peptide bonds at the Ser and Thr positions with a high conversion yield. This chemical cleavage relies on Sc(III)-promoted N,O-acyl rearrangement and subsequent hydrolysis. The method is applicable to a broad scope of polypeptides with various functional groups, including a post-translationally modified peptide that is unsuitable for enzymatic hydrolysis. The system was further extended to site-selective cleavage of a native protein, Aβ1-42, which is closely related to the onset of Alzheimer's disease.

Methods for site-selective peptide bond cleavage are valuable tools for the structural determination of proteins and peptides, chemical biology, and therapeutics.[1,2] Although several proteases (e.g., trypsin, chymotrypsin, and pepsin) promote the hydrolysis of peptide bonds at specific sites with high fidelity,[3,4] the scope of the scissile bonds is still narrow, and the substrates are limited, in principle, to genetically encoded amino acid sequences. Substrates comprising unnatural amino acids and/or amino acids with post-translational modifications are generally out of the scope of enzymatic reactions. In addition, proteases used for digestion contaminate the protein fragments, which could interfere with peptide mapping. Moreover, these proteases often require strict pH and temperature control.

As chemical methods for site-selective peptide/protein cleavage, cyanogen bromide in 70% formic acid (scissile bond: Met–Xaa), 2-nitro-5-thio-cyanobenzoic acid (scissile bond: Cys–Xaa),[5] and 2-iodosylbenzoic acid (scissile bonds: Trp–Xaa and Tyr–Xaa)[6] were used more than 30 years ago. The cleavage was triggered by a chemoselective reaction at a specific functional group in a side chain. Significant progress toward site-selective peptide bond cleavage using this type of mechanism has been reported in recent years.[7] For example, diacetoxyiodobenzene-mediated[8] and metal-catalysed [Ru(III)/$NaIO_4$[9] and Cu(I)/ketoABNO/O_2[10]] side-chain oxidations initiated peptide bond cleavage at Asn and Ser or Thr residues, respectively. Selective activation of side-chain functionalities at Ser and Glu residues using condensation reagents (e.g., *N,N'*-disuccinimidyl carbonate or PyBOP), followed by cyclisation of the main chain amide nitrogen atom and hydrolytic cleavage were also reported.[11] The functional group tolerance of these methods, however, remains challenging. Undesired oxidation of sensitive amino acid residues (e.g., His, Tyr, Trp, and Met) and non-selective activation of carboxylic acid functionalities of Asp side chains and the C-terminal are unavoidable. These side reactions often complicate interpretation of the data.

Hydrolysis of peptide bonds without modifying side-chain structures is more straightforward.[12] Although intensive studies have been devoted to metal-catalysed, site-selective peptide bond cleavage, its practical use for the structural determination of proteins remains undeveloped. For example, through selective interactions between soft metals and soft heteroatoms (S and N atoms) of side chains, Pd(II)[13] and Pt(II)[14] catalysts promote site-selective hydrolytic cleavage of peptide bonds proximate to the coordinating residues, His, Cys, and Met. Another approach providing selectivity utilises the reactivity of a side-chain hydroxy group at a Ser (or Thr) residue *via* N,O-acyl rearrangement (Fig. 1).[15] Lewis acid metal ions could promote this rearrangement and the successive hydrolysis of the thus-formed ester by polarising the carbonyl group.[16] Although the Zn(II) ion assists cleavage of a Xaa-Ser bond in dipeptides,[16,17] a His residue next to the Ser residue is required for the cleavage of longer peptides.[18] In Cu(II)-[19] and Ni(II)-promoted[20] hydrolysis, a neighbouring His is also involved in the cleavage of Xaa-Ser or -Thr

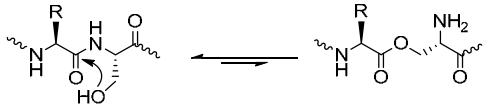

Fig. 1 N,O-Acyl rearrangement

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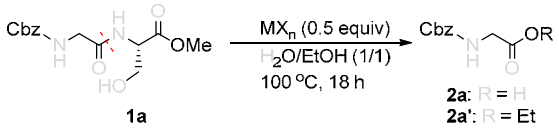
Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

bonds. Other metal ions often suffer from a limited substrate scope or lack of reliable site-specificity [e.g., Zr(IV)[21] and Mo(VI)[22]]. To enable artificial protease-like catalysis, a reliable and practical method with high generality for site-selective peptide/protein hydrolysis is highly demanded. We report herein Sc(III)-mediated Ser- or Thr-selective peptide cleavage, including a native protein, *via* N,O-acyl rearrangement and subsequent hydrolysis. This chemical and site-selective peptide bond cleavage exhibited a wide substrate scope, including a chemically modified (N-acetylated at a Lys side-chain) peptide that was not cleavable by a protease.

We aimed to develop a novel catalyst for site-selective cleavage of peptides at Ser or Thr residues that does not require the assistance of neighbouring coordination residues (e.g., His and Met). Hard metals could be attractive candidate promoters of peptide hydrolysis, because they preferentially interact with hard bases (e.g., a carbonyl oxygen atom). We therefore screened several metal promoters (50 mol%) in a model reaction for the cleavage of a dipeptide substrate, Cbz-Gly-Ser-OMe (Table 1). The reaction was conducted in aqueous medium with 50% ethanol to solubilize the substrate. $Zn(OTf)_2$ promoted the reaction in low yield (4%, entry 2). This is in stark contrast to the previously reported effective cleavage of unprotected dipeptides, H-Xaa-Ser-OH, in which a five-membered chelate was formed by coordination of the amino group at the N-terminal and the carbonyl oxygen atom at the cleavage site with a zinc ion.[17a] Poor yield was observed in the case of $Cu(OTf)_2$ (entry 3), while $NiCl_2$ was inactive for cleavage (entry 4). Further screening (entries 5-19) revealed that a Sc(III) ion afforded the highest yield for peptide cleavage (78%-86%, entries 17-19). The loading of $Sc(OTf)_3$ could be decreased to 10 mol%, affording 80% yield after a prolonged reaction time (entry 20). To the best of our knowledge, this is the first example of Lewis acidic Sc(III) ion-catalysed peptide bond hydrolysis (catalyst turnover number = 8.0).

Table 1. Catalyst screening for peptide bond cleavage reaction[a]



1a

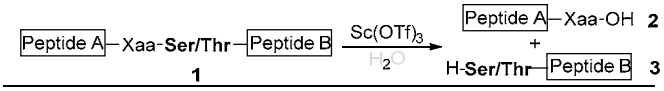
Entry	MX_n	Yield (%)[b]	Entry	MX_n	Yield (%)[b]
1	none	trace	11	$Sm(OTf)_3$	19
2	$Zn(OTf)_2$	4	12	$Eu(OTf)_3$	17
3	$Cu(OTf)_2$	22	13	$Er(OTf)_3$	16
4	$NiCl_2$	trace	14	$YbCl_3$	21
5	$MgCl_2$	trace	15	$Lu(OTf)_3$	17
6	$CaCl_2$	trace	16	YCl_3	12
7	$Pd(OAc)_2$	trace	17	$ScCl_3 \bullet H_2O$	78
8	AgOTf	11	18	$Sc(O_3SOC_{12}H_{25})_3$	81
9	$La(OTf)_3$	13	19	$Sc(OTf)_3$	86
10	$Ce(OTf)_3$	11	20[c]	$Sc(OTf)_3$	80

[a] Standard reaction conditions: a solution of substrate (150 mM) and MX_n (75 mM) was stirred at 100 °C for 18 h. [b] Combined yields of N-terminal fragments (Cbz-Gly-OH (**2a**) and Cbz-Gly-OEt (**2a'**)) calculated from the absorbance at 254 nm using reverse-phase HPLC. [c] Substrate (150 mM), MX_n (15 mM), t = 48 h. $Sc(O_3SOC_{12}H_{25})_3$ = scandium tris(dodecyl sulfate).[23]

We then applied the optimised conditions to longer peptides (Table 2). Except **1b** where the N-terminal is protected with a Cbz group (entry 1), 100% water was used as a solvent because most of the peptides/proteins were dissolved in water. Pentapeptide **1c** was cleanly cleaved at the Ser site in high yield without affecting the oxidatively sensitive Tyr side chain (97%, Table 2, entry 2 and Fig. 2a). We observed no side products derived from peptide bond cleavage at alternative sites. A biorelevant nonapeptide, bradykinin (**1d**), was also hydrolytically cleaved at the Ser site in high yield (85%, entry 3). HPLC analysis of the reaction profile revealed that the reaction was clean and well-defined (Fig. 2b); two fragments of both the N- and C-terminal sides were observed after the cleavage. An intermediate ester after N,O-acyl rearrangement was not observable, indicating that acyl rearrangement was the rate-determining step for the overall peptide cleavage reaction. When the Ser residue in **1d** was replaced with a Thr residue (**1e**), the bond cleavage was slower, giving both the N- and C-terminal fragments in 40% yield with the same reaction time (entry 3 *vs*. entry 4). The higher steric hindrance of a Thr side chain than a Ser side chain probably slowed down both the N,O-acyl rearrangement and the following ester hydrolysis processes.[25]

To further investigate the substrate scope of this method, six more derivatives of bradykinin were synthesised (Table 2, entries 5-10). In these peptides, the Phe residue adjacent to the Ser residue in the wild-type bradykinin was replaced by various amino acids, i.e., Asn (**1f**), Met (**1g**), His (**1h**), Tyr (**1i**), Trp (**1j**), and Lys (**1k**). Under the

Table 2. $Sc(OTf)_3$ promoted Ser/Thr-selective peptide cleavage.[a]



Entry	Substrate	Yield (%)[b]
1[c]	Cbz-Phe-Gly-**Ser**-OMe (**1b**)	90[d]
2[e]	H-Tyr-Ile-Gly-**Ser**-Arg-NH_2 (**1c**)	97
3	H-Arg-Pro-Pro-Gly-Phe-**Ser**-Pro-Phe-Arg-OH (**1d**, bradykinin)	85
4	H-Arg-Pro-Pro-Gly-Phe-**Thr**-Pro-Phe-Arg-OH (**1e**)	40
5[f]	H-Arg-Pro-Pro-Gly-Asn-**Ser**-Pro-Phe-Arg-OH (**1f**)	95
6	H-Arg-Pro-Pro-Gly-Met-**Ser**-Pro-Phe-Arg-OH (**1g**)	96
7	H-Arg-Pro-Pro-Gly-His-**Ser**-Pro-Phe-Arg-OH (**1h**)	91
8	H-Arg-Pro-Pro-Gly-Tyr-**Ser**-Pro-Phe-Arg-OH (**1i**)	88
9	H-Arg-Pro-Pro-Gly-Trp-**Ser**-Pro-Phe-Arg-OH (**1j**)	80
10	H-Arg-Pro-Pro-Gly-Lys-**Ser**-Pro-Phe-Arg-OH (**1k**)	97
11	H-Arg-Pro-Pro-Gly-Lys(Ac)-**Ser**-Pro-Phe-Arg-OH (**1l**)	90

[a] Standard conditions: substrate (2 mM) and $Sc(OTf)_3$ (200 mM) in H_2O were stirred at 80 °C for 40 h unless otherwise noted.[24] [b] Yields were determined based on the UV absorption intensity of the reverse-phase HPLC peak(s) corresponding to N-terminal fragment (entries 1 and 2), or N- and C-terminal fragments (entries 3-11), detected at 254 nm (entry 1) or 230 nm (entries 2–11). [c] Substrate (150 mM) and $Sc(OTf)_3$ (75 mM) in EtOH/H_2O (1:1) were stirred at 100 °C for 40 h. [d] Combined yield of Cbz-Phe-Gly-OH (**2b**), Cbz-Phe-Gly-OEt (**2b'**), and Cbz-Phe-Gly-OMe (**2b''**) [e] A mixture of substrates (0.5 mM) and $Sc(OTf)_3$ (50 mM) was stirred in H_2O at 60 °C for 52 h.[24] [f] Asn was hydrolysed to Asp during the reaction.

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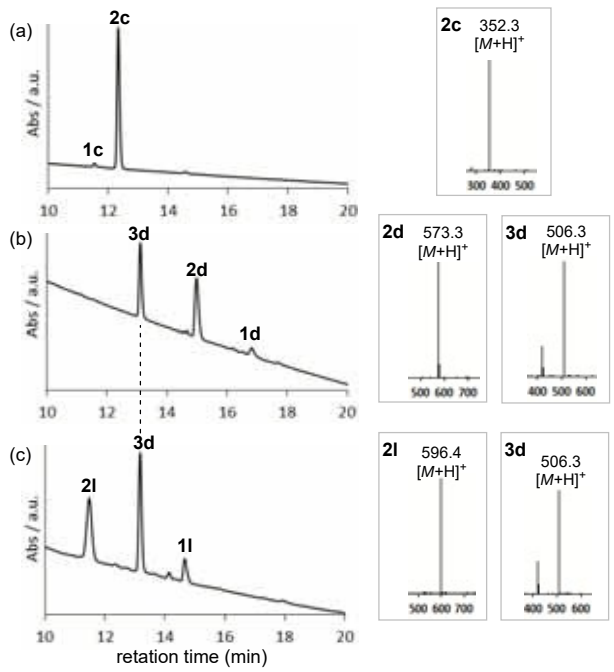

Fig. 2 HPLC charts of the reaction mixture of **1c**, **1d**, and **1l** under the conditions described in Table 2.

Sc(III)-mediated hydrolysis conditions, the Xaa-Ser peptide bond was cleanly and selectively cleaved in 80% to 97% yield. These results indicate that the side-chain functionalities of the amino acid neighbouring the Ser residue at the N-terminal side does not greatly participate in the cleavage reaction. No fragment peaks were derived from the cleavage proximate to the Met and His residues, in contrast to the previously reported soft metal Pd(II)-catalysed peptide cleavage (entries 6 and 7) [13] In contrast to the previously reported oxidative conditions,[8-10] oxidant-sensitive functionalities in Met (sulphide), His (imidazole), and Tyr (phenol) residues remained intact under the present Sc(III)-promoted conditions (entries 6-8), although Trp (indole) underwent oxidation to some extent (entry 9, Fig. S8).

We next studied whether the present method is applicable to a substrate that is resistant to enzymatic digestion. N-Acetylation of Lys side-chain amino group is a typical post-translational modification.[26] Therefore, a method for site-selective peptide bond cleavage applicable to substrates comprising N-acetylated Lys will be useful for the structural determination of post-translationally modified peptides and proteins. Enzymatic digestion of peptide **1l** comprising N-acetylated Lys did not proceed efficiently using endoproteinase Lys-C (Fig. S11), while digestion of unacetylated **1k** proceeded smoothly using the same enzyme (Fig. S10). In contrast, when **1l** was treated with an aqueous solution of $Sc(OTf)_3$, LC/MS spectroscopy of the reaction mixture at t = 40 h (Fig. 2c) revealed two products, H-Ser-Pro-Phe-Arg-OH (**3d**, retention time = 13.1 min) with an m/z of 506.3 Da (calcd. $[M+H]^+$ = 506.3) and H-Arg-Pro-Pro-Gly-Lys(Ac)-OH (**2l**, retention time = 11.5 min) with an m/z of 596.4 Da (calcd. $[M+H]^+$ = 596.4) (90%, Table 2, entry 11). This result demonstrates the advantage of the developed chemical method over enzymatic digestion in site-selective peptide bond cleavage of structurally modified peptides.

Finally, to demonstrate the substrate scope of the present method, we applied the method to cleavage of an Alzheimer's disease-related protein, Aβ1-42 (molecular weight = 4514.1 Da; Fig. 3a),[27] which comprises 42 amino acid residues, including two Ser (Ser8, Ser26). The starting material, Aβ1-42, was completely consumed after 20 h [Aβ1-42 (0.25 mM) and $Sc(OTf)_3$ (100 mM) in H_2O at 80 °C]. Analysis of the crude reaction mixture using MALDI-TOF-MS indicated that the major cleavage products were three fragments, Aβ1-7 (observed m/z = 889.7 Da), Aβ8-25 (observed m/z = 2064.0 Da), and Aβ1-25 (observed m/z = 2935.6 Da; Fig. 3b). Although the Aβ26-42 fragment was not observed by MALDI-TOF-MS due to its poor ionizability, its ESI mass signal was detected by LC-MS (observed m/z = 1599.0 Da). Four minor fragments, Aβ1-6 (observed m/z = 774.8 Da), Aβ8-23 (observed m/z = 1906.8 Da),

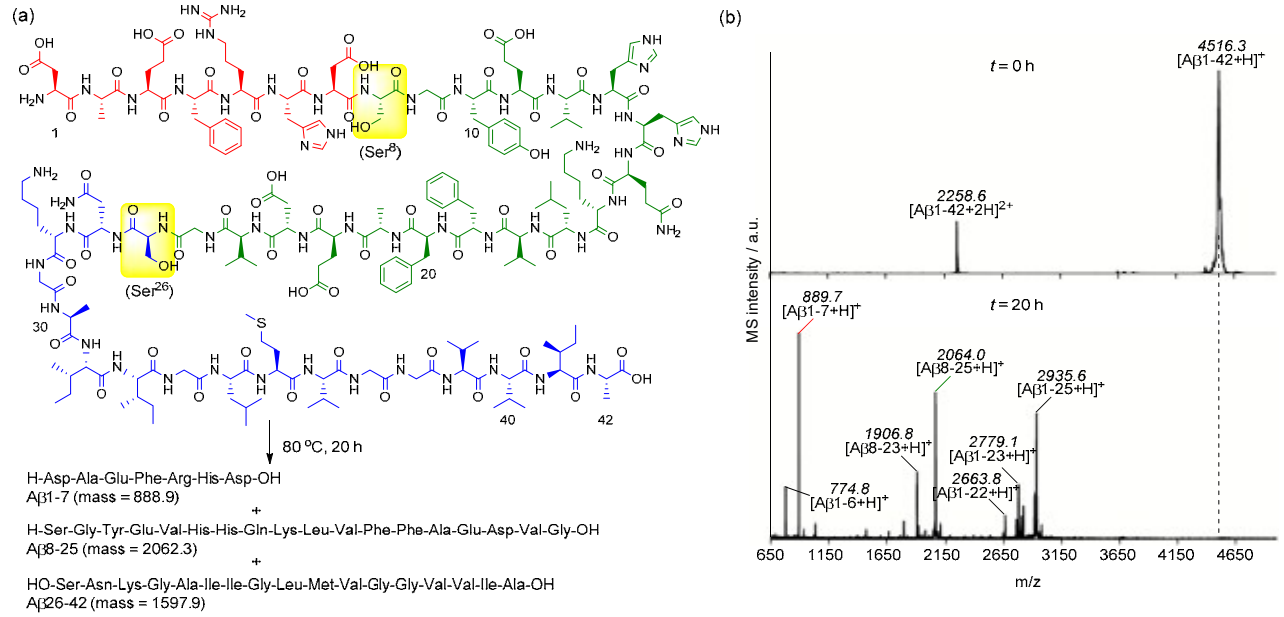

Fig. 3 (a) Site-selective hydrolysis of Aβ1-42. (b) MALDI-MS analysis of the reaction mixture at t = 0 and 20 h.

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Aβ1-23 (observed *m/z* = 2779.1 Da), and Aβ1-22 (observed *m/z* = 2663.8 Da), were also identified to be derived from cleavage at the Asp7 and Asp23 residues. This suggests that a hard carboxylate functional group also directs the hard metal ion Sc(III) for cleavage of the peptide bonds at Asp residues. Again, no hydrolysis was detected at the His and Met sites. The predominant production of the three expected fragments (Aβ1-7, Aβ8-25, and Aβ26-42) clearly demonstrated that the Ser-selectivity of the present method is reliable when using complex peptide/protein substrates.

In conclusion, we developed the first Sc(III)-promoted site-selective hydrolysis of peptide/protein chains at the Ser and Thr positions. Side-chain functionalities were little affected during the hydrolysis. Penta- to nona-peptide substrates, including post-translationally modified peptides (e.g., N-acetylated at a Lys residue side-chain), were site-selectively cleaved in high yields. The reaction conditions were also successfully applied to site-selective cleavage of a native protein, Aβ1-42, which is related to Alzheimer's disease. Due to its broad substrate scope, this method will be a useful complement to enzymatic degradation of peptides.

This work is supported by JST-ERATO KANAI Life Science Catalysis Project.

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24 Preparing the solution of **1c**-**1l** at the concentration higher than 0.5-2 mM was difficult due to the solubility limits (*vs*. **1a** and **1b**). The concentration of Sc(III) ion (> 50 mM) was important for complete substrate consumption within the indicated time periods. Accordingly, the concentration of Sc(III) ion exceeded that of the substrate.

25 A peak (11% yield) giving the same mass value as **1e** was observed at earlier retention time in HPLC analysis. Possibly it was the ester intermediate after N,O-acyl rearrangement. See Fig. S3 for detail.

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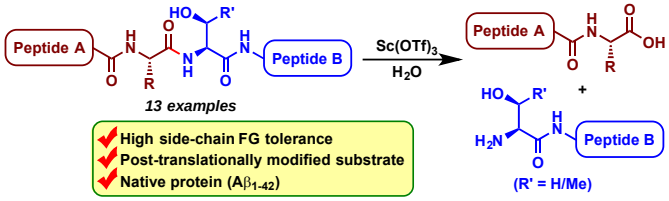

Site-selective hydrolysis of peptide bonds at Ser and Thr positions was promoted by Scandium(III) triflate with a high conversion yield.