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Rapid and Controllable Hydrogen-deuterium Exchange on Aromatic Rings of α -Amino Acids and Peptides

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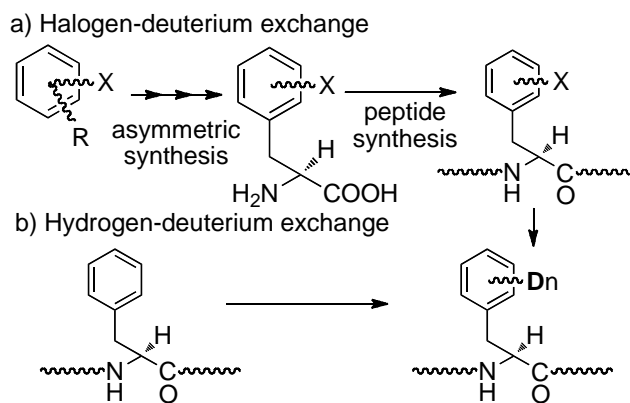
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Novel hydrogen-deuterium exchange for aromatic α -amino acids and their containing peptides were performed by deuterated trifluoromethanesulfonic acid (TfOD). Detail analysis of the exchange revealed that equal hydrogen-deuterium exchange was observed for phenylalanine and specific exchange at *ortho*-positions of phenol for tyrosine was also detected. Stereochemistry of aromatic α -amino acids retained during the exchange conditions.

These hydrogen-deuterium exchange properties for these aromatic α -amino acids are identical to peptides, which contained several aromatic α -amino acids. The exchange proceeded significantly faster than previous methods. Detail analysis of the exchange revealed that the method was controllable by temperature-, time- and reagent equivalent-dependent manner.

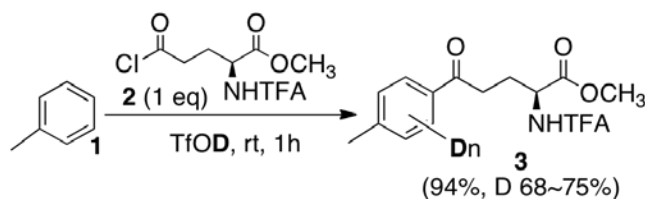
Introduction

Quantitative measurement of biologically active peptides and identification of the peptides are very important in proteomics. Many research groups have reported methodologies for the quantitative analysis of peptides or α -amino acids incorporating stable isotopes with mass spectrometry (isotope dilution mass spectrometry, IDMS^[1]). There are two major methods of preparation of deuterated α -amino acids and biologically active peptides on aromatic moiety. The halo substituted aromatics were converted to α -amino acid with asymmetric synthesis, subjected to peptide synthesis, then halo-deuterium exchange by hydrogenation (Scheme 1 a).^[2] The methods required multistep. The other one is hydrogen-deuterium exchange^[3], which can be cost-efficient alteration approach for peptides. Hydrogen-deuterium exchange reactions for aromatic protons in α -amino acids and peptides are one of the most efficient methods for the exchange (Scheme 1 b). Although several methods for hydrogen-deuterium exchange targeted aromatic α -amino acids and their constituent peptides have been reported, but these methods required long reaction time^[4] and high temperature with additives.^[5] It is difficult to reveal details of time- and temperature- dependent manners for the hydrogen-deuterium exchange reactions. We have been developing Friedel-Crafts reactions for carboxylic acids side chains of α -amino acids (Asp and Glu) and aromatics in trifluoromethanesulfonic acid (TfOH).^[6] TfOH plays a critical role as both the catalyst for the Friedel-Crafts acylation and as a good solvent for the α -amino acid derivatives. Trifluoromethanesulfonic acid-D (TfOD) was used to study the reaction mechanism,



Scheme 1. Friedel-Crafts reaction and hydrogen-deuterium exchange on aromatic proton in TfOD

especially the effects of stereochemistry at the α - and β - positions of α -amino acid skeleton. Although no deuterium incorporation was observed at the α - and β - positions, effective hydrogen-deuterium exchange was observed on the aromatic proton in TfOD (Scheme 2).



Scheme 2. Friedel-Crafts reaction and hydrogen-deuterium exchange on aromatic proton in TfOD

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TfOD has recently been used as a solvent to study the reaction mechanisms of the intramolecular cyclization of methyl 2-cyano-3-phenylpropionate at room temperature.^[7] It was reported that no deuterium was observed at the α -position, but no description was given for the effects of the aromatic hydrogen. Our observations given for the effects of the aromatic hydrogen. Our observations suggest that the good solubility and strong acidity of TfOH(D) effectively promoted hydrogen-deuterium exchange on aromatic α -amino acids. In this study, we focused first time on rapid hydrogen-deuterium exchanges for aromatic α -amino acids and their constituent peptides with TfOD, and controllable exchange by equivalents of reagent, reaction time and reaction temperature.

Results and Discussion

First, phenylalanine was dissolved in TfOD (40 eq) at 0 °C. Integrations of aromatic protons of NMR spectra decreased in a time dependent manner. After quenching the exchange with water, the product was analyzed using mass spectrometry. The incorporated deuterium was observed mainly in 2-4 sites during the first hour, then 3-5 sites after 3 hours. When the same exchange was run at room temperature, exchanges at 3-5 sites were observed within an hour (Figure 1).

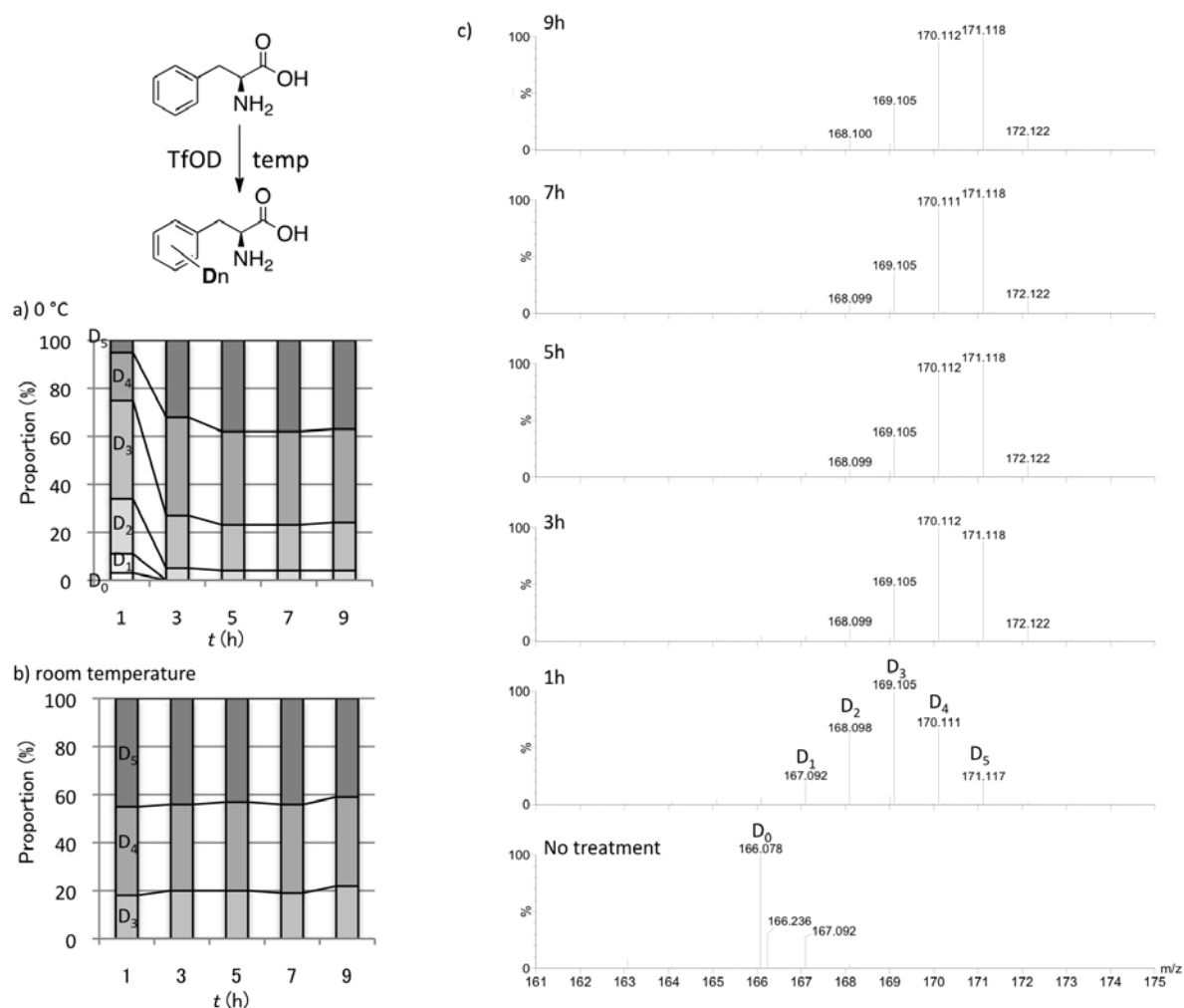


Figure 1. Hydrogen-deuterium exchange on aromatic rings of L-phenylalanine with TfOD at 0 °C and room temperature. (a) and (b); D₀-D₅ indicated incorporated number of deuterium on aromatic ring at 0 °C (a) and room temperature (b). (c); Typical mass spectrum of TfOD treated L-phenylalanine at 0 °C.

Tyrosine was treated with TfOD (40 eq) at 0 °C and room temperature. Mass analysis showed that less than two hydrogen were exchanged with deuterium at 0 °C. The NMR measurement of the product revealed that the hydrogen was predominantly exchanged at the ortho-position (SI, Figure SI-3b). When performed at room temperature, it was observed that the exchange proceeded from two to four sites in a time-dependent manner until nine hours elapsed by mass spectrometry (Figure 2 a).

Treatment of tryptophan with TfOD (40 eq) showed three to five deuterium exchanges at room temperature. Complete exchange (five deuterated, D₅) was observed in nearly 20% of the examined sites. Treatment at 0 °C showed less than three exchanges over time in most cases (Figure 2 b).

The hydrogen-deuterium exchange methods were applied to bioactive leucine-enkephalin (YGGFL), which consisted of two

different aromatic amino acids. Less than three exchanges were observed within an hour at 80 equivalents of TfOD at 0 °C. The exchange ratio increased from less than three exchanges at 1 hour to less than six exchanges at 9 hours (Figure 3 a).

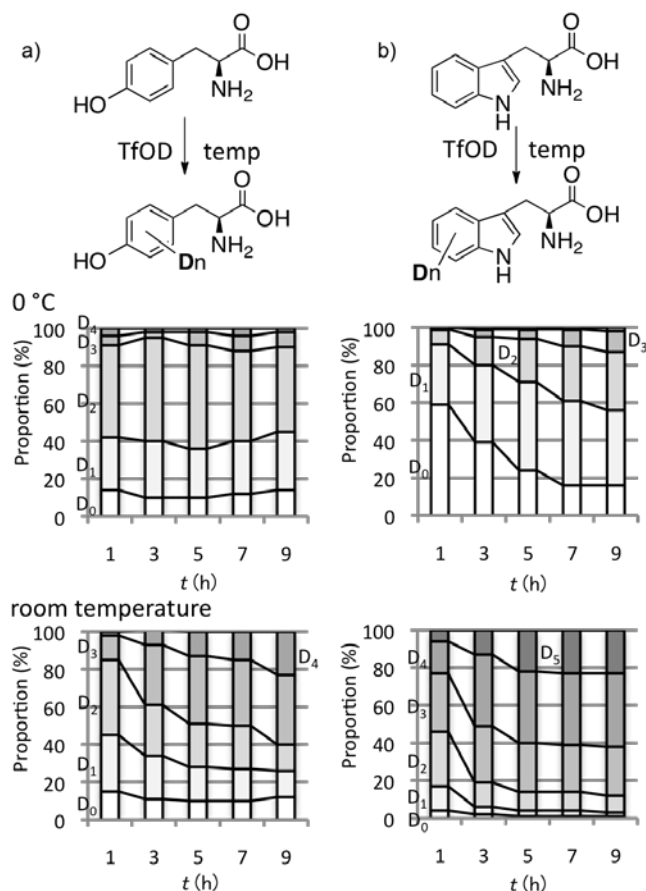


Figure 2. Hydrogen-deuterium exchange on aromatic rings of *L*-tyrosine (a) and *L*-tryptophan (b) with TfOD.

Treatment with 80 equivalents of TfOD at room temperature showed less than five and seven exchanges for 1 hour and 9 hours, respectively (Figure 3 b). The exchange ratios were improved with large amounts of TfOD (200 eq) at room temperature. Less than six exchanges were observed within 1 hour, and the ratio was increased to less than eight exchanges after 9 hours (Figure 3 c). Deuterated peptides were subjected to MS/MS analysis to prove that deuterated sites formed on each aromatic α -amino acid. MS/MS analysis of leucine-enkephalin showed imminium ions of “Y (m/z 136 $[M+H]^+$ as no exchanges)” and “F (m/z 120 $[M+H]^+$ as no exchanges)” at various conditions.^[8] Analysis of the “Y” imminium ion indicated that less than two hydrogen-deuterium exchanges were observed at all conditions (m/z 138). The result indicated that two ortho positions of the phenol moiety were favored for the exchanges. The meta positions of phenol were less favored for the exchanges than the ortho positions both at room temperature and at 0 °C. On the other hand, there were observed that no exchange and less than two exchanges for the ring of phenylalanine within 1 hour and 9 hours at 0 °C, respectively. Less than three exchanges were observed within 1 hour with 80 equivalents of TfOD at room temperature, and the exchangeable sites increased to four at 9 hours. A larger excess of TfOD (200 equivalents) at room temperature increased the exchanges to less than four and five exchanges at 1 hour and 9 hours, respectively. Unfortunately, it is difficult to identify the hydrogen-deuterium exchange numbers accurately using MS/MS analysis due to a limitation in the resolution of the mass spectrophotometer. The deuterated leucine-enkephalins with various conditions were subjected to enzymatic digestions with chymotrypsin to ensure that labeled sites could be observed. LC-MS analysis of the digested deuterated leucine-enkephalin showed identical sequences of tyrosine (m/z 182 $[M+H]^+$ as no exchanges) and GGFL (m/z 280 $[M+H]^+$ as no exchanges) peptides. The exchanged sample treated with 80 equivalents of TfOD at 0 °C for 1 hour afforded two and no deuterium exchanges for tyrosine (m/z 184) and GGFL (m/z 280), respectively.

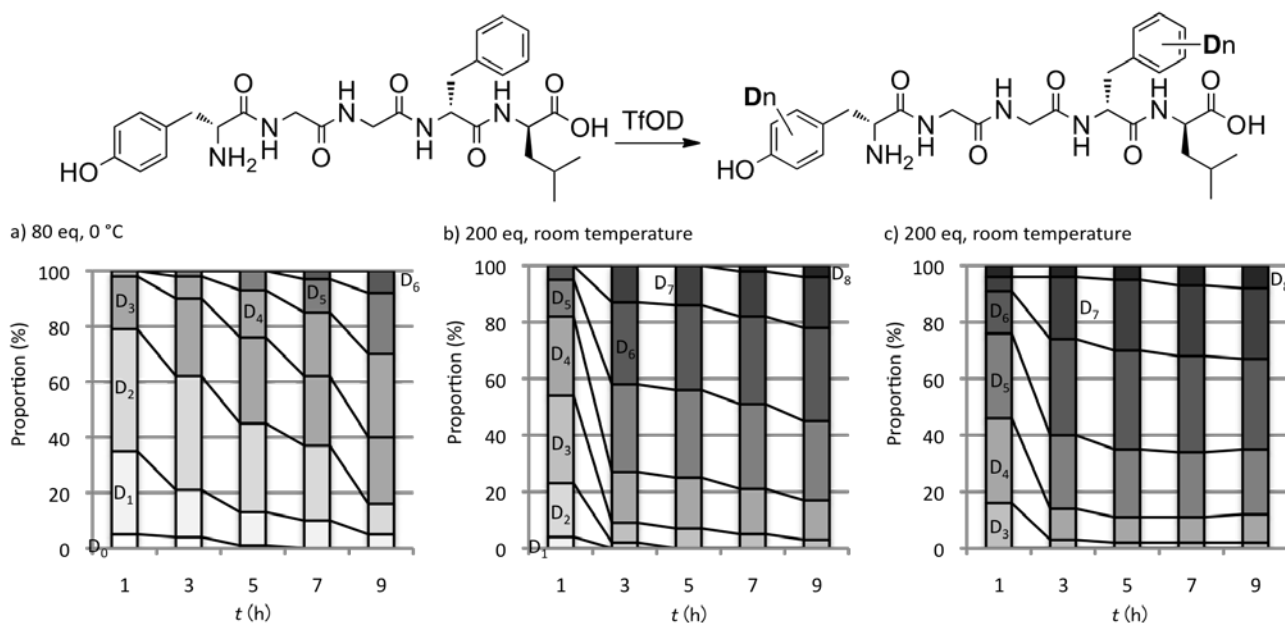


Figure 3. Hydrogen-deuterium exchanges on aromatic rings of leucine-enkephalin with TfOD in various conditions

On the other hand, the sample treated with 200 equivalents of TfOD at room temperature for 9 hours afforded two and up to five deuterium exchanges for tyrosine (m/z 184) and GGFL (m/z 283-285), respectively (SI, Figure SI-6). These results indicated that the

hydrogen-deuterium exchange was favored in the order described below: the ortho-positions to the hydroxyl group on tyrosine > hydrogen of phenylalanine > the meta-positions to the hydroxyl group on tyrosine.

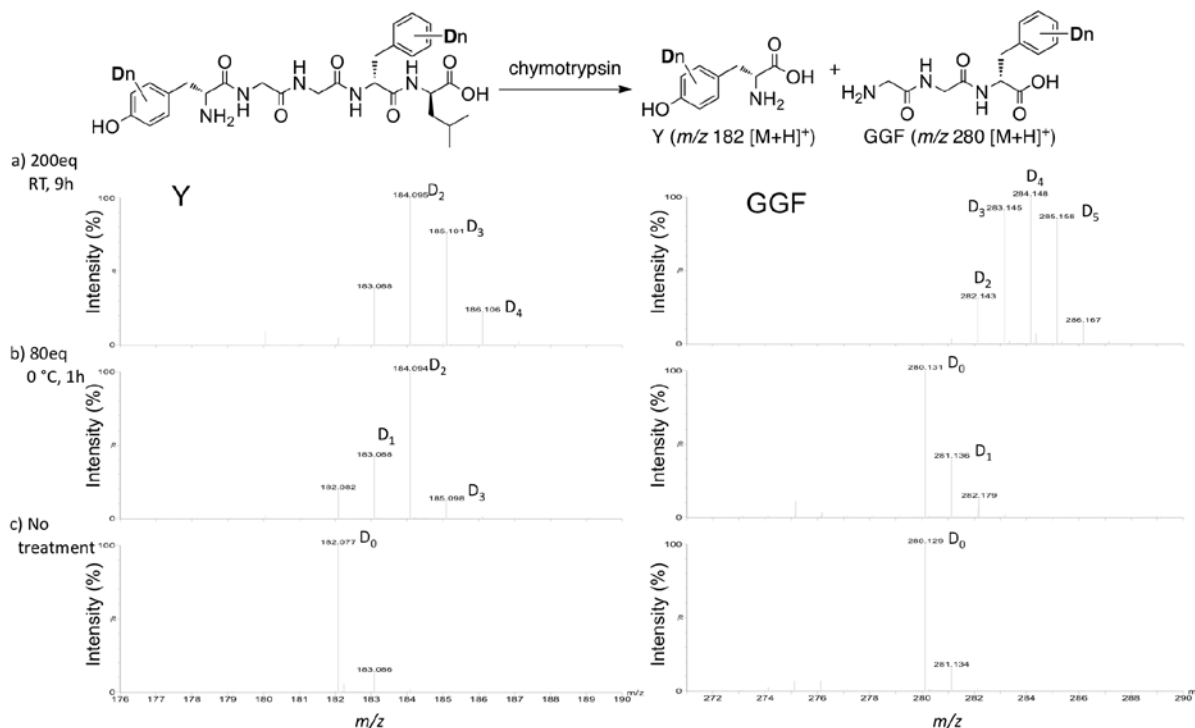


Figure 4. ESI-MS spectra of chymotrypsin digested leucine-enkephalin (YGGFL) treated with TfOD in 200 equivalents at room temperature for 9 h (a) and 8 equivalents at 0 °C for 1 h (b). Leucine-enkephalin without TfOD treatment was analyzed as control digestion (c).

Maculosin, cyclo(L-prolinyl-L-tyrosine),^[9] was also treated with TfOD. The cyclopeptide was exposed to 40 equivalents of TfOD at both 0 °C and room temperature (SI, Figure SI-7). No cleavage of the cyclic peptide was observed during the exchange reaction. One and two hydrogen-deuterium exchanges were observed mainly in both conditions (Figure 5). These results also consisted of deuteration results for leucine-enkephalin.

Although TfOH was known as a deglycosylated reagent for glycoprotein to analyze protein moieties^[10] and as a cleavage reagent for peptide solid phase synthesis,^[11] decomposition of peptides was sometimes observed. Delta-sleeping inducing peptide (DSIP), WAGGDASGE,^[12] was subjected to the TfOD treatment at 0 °C. Maximum three hydrogen-deuterium exchanges were observed within 1 hour. Although further hydrogen-deuterium exchange for DSIP was observed with time-dependent manner, dehydrated DSIP was also observed after 3 hours. The DSIP and dehydrated DSIP were almost identical after 9 hours (SI, Figure SI-8A). The degree of deuterium incorporation in both compounds was almost identical for each time period (SI, Figure SI-8B). The dehydration of deuterated DSIP was raised when the reaction was subjected at room temperature and no deuterated DSIP was detected in 1 hour. These results indicated that TfOD treatment promoted both hydrogen-deuterium exchange for tryptophan residue and dehydration of the DSIP. The hydrogen-deuterium exchange proceeded predominantly at low temperature.

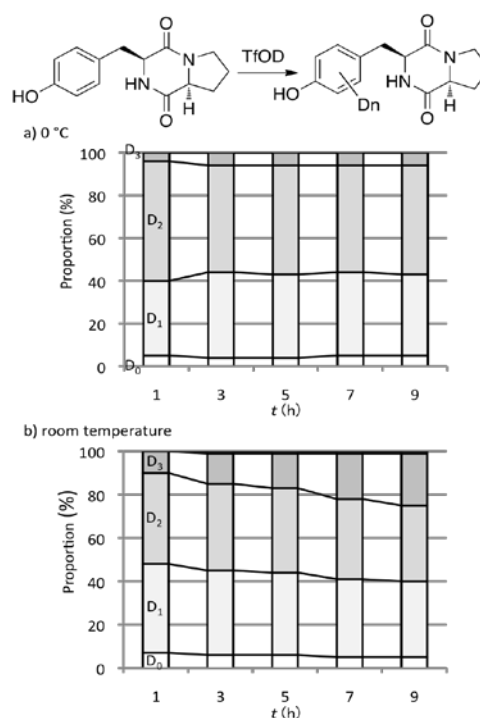


Figure 5. Proportions of deuterated number of maculosin in hydrogen-deuterium exchange with TfOD.

Conclusions

This first detailed analysis of hydrogen-deuterium exchange at aromatic α -amino acids and their constituted with TfOD at low temperature (from 0 °C to room temperature) was examined. The reaction time for an exchange was extremely fast compared with previous methods. The property encouraged us to reveal the regulations of the deuterium contents in α -amino acid and peptides. The deuterium incorporation can be controllable by reagent-, time- and temperature- dependent manner. In our best knowledge there has been no comprehensive study of hydrogen-deuterium exchange for aromatic ring in some biologically active peptides. This new method will be easier to apply for small amounts of peptides than current standard approaches and to handle with no special equipments for the exchange. Hydrogen-deuterium exchange for aromatic rings of α -amino acids and peptides will encourage many researchers to use IDMS techniques for the quantitative analysis of biologically active peptides. Further application with this hydrogen-deuterium exchange for other aromatic compound with TfOD is underway. Furthermore, our findings, which are aromatic C-H bond activation at low temperature, can be contributed to establish new development process.

Experimental Section

General Remarks: NMR spectra were measured with JEOL EX-280 spectrometer. ESI-TOF-MS data were obtained with a Waters UPLC ESI-TOF mass spectrometer. Trifluoromethanesulfonic acid-D (98 atom % D) and maculosin were purchased from Sigma-Aldrich. Chymotrypsin was purchased from Wako chemical. Leucine-enkephaline and Delta-sleeping inducing peptide (DSIP) were purchased from Peptide institute.

Friedel-Crafts reaction of toluene (1) and *N*-TFA-*L*-Asp(Cl)-OMe (2) in TfOD: Compound 2 (31.0 mg, 0.113 mmol) and toluene 1 (12 μ l, 0.113 mmol, 1 eq) were dissolved in TfOD (0.5 ml) at 0 °C. The reaction mixture was stirred for an hour at room temperature, and poured into ice-cold H₂O and AcOEt (15 / 15 ml). The organic layer was washed with saturated NaCl and dried over MgSO₄, filtrated, then concentrated. The residue was purified with silica gel chromatography (AcOEt : *n*-hexane = 1 : 4) to yield 3 (34.8 mg, 0.105 mmol, 94%). [α]_D +24.0 (c 2.0, CHCl₃). The chirality was also checked by chiral HPLC (Chirobiotic T2, 10% EtOH-H₂O). Deuterium incorporation was calculated between 68 to 75% from ¹H-NMR spectrum (supporting information Figure SI-1).

General procedures for hydrogen-deuterium exchange with TfOD:

For aromatic α -amino acids: Aromatic α -amino acid (0.25 mmol) was dissolved in TfOD (0.9 mL, 10 mmol, 40 eq.) at indicated temperature. The reaction mixture was diluted with H₂O (0.6 mL) followed by subject to ¹H-NMR and ESI-TOF mass analysis.

For peptides: Peptide (9 μ mol) was dissolved in TfOD (32 μ L, 0.36 mmol for one aromatic α -amino acid) at indicated temperature. The reaction mixture was diluted with H₂O (0.5 mL) followed by subject to ESI-TOF mass analysis. The hydrogen-deuterium exchange samples were subjected to ESI-TOF mass analysis for several times. There is no difference for the analytical data for the sample was stored at refrigerator within a month.

Chymotrypsin digestion for hydrogen-deuterium exchanged leucine-enkephaline : The hydrogen-deuterium exchanged leucine-enkephalin was made neutral by 1M NaOH. To the solution, chymotrypsin (1/20 against mol equivalent) was added. The mixture was incubated at 37 °C for 24 hours, then subjected to ESI-TOF mass analysis.

Supporting Information (see footnote on the first page of this article): Mass and MSMS spectra of hydrogen-deuterium exchanges peptides.

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