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

Yuta Murai,[a] Lei Wang,[a] Katsuyoshi Masuda,[b] Yasuko Sakihama,[a] Yasuyuki Hashidoko,[a] Yasumaru Hatanaka,[c] and Makoto Hashimoto*[a]

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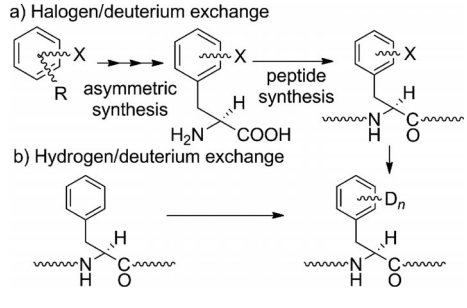
Novel hydrogen/deuterium exchange for aromatic α-amino acids and their corresponding peptides were performed through the use of deuterated trifluoromethanesulfonic acid (TfOD). Detailed analysis of the exchange revealed that equal hydrogen/deuterium exchange was observed for phenylalanine, and specific exchange at the *ortho*-positions of phenol for tyrosine was also detected. The stereochemistry of the aromatic α-amino acids was retained under the exchange conditions. The hydrogen/deuterium exchange properties for these aromatic α-amino acids are identical for peptides that contain several aromatic α-amino acids. The exchange proceeded significantly faster than previous methods. Detailed analysis of the exchange revealed that the method could be controlled by temperature, time, and the amount of reagent.

Introduction

Quantitative measurement of biologically active peptides and identification of the peptides involved are very important in proteomics. Many research groups have reported methodologies for the quantitative analysis of peptides or α-amino acids by incorporating stable isotopes with mass spectrometry (isotope dilution mass spectrometry, IDMS[1]). There are two major methods with which to prepare deuterated α-amino acids and biologically active peptides with the label on the aromatic moiety. Halogen-substituted aromatics have been converted into α-amino acids through asymmetric synthesis, subjected to peptide synthesis, then halogen/deuterium exchange was achieved by hydrogenation (Scheme 1a).[2] However, this approach involves multistep syntheses. An alternative, more direct approach is hydrogen/deuterium exchange,[3] which can be a cost-effective approach to the synthesis of deuterium-labeled 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 1b). Although several methods have been reported for hydrogen/deuterium exchange targeting aromatic α-amino acids and their constituent peptides, these methods require long reaction times,[4] high temperature, and the use of additives.[5] However, it is difficult to delineate details of time- and temperature-dependent effects for hydrogen/deuterium exchange reactions. We have been developing Friedel–Crafts reactions for carboxylic acid side chains of α-amino acids (Asp and Glu) and aromatics performed 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. Deuterated trifluoromethanesulfonic acid (TfOD) was used to study the reaction mechanism, especially the effects of stereochemistry at the α- and β-positions of the 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 protons in TfOD (Scheme 2).



Scheme 1. Synthetic approaches to deuterated α-amino acids and biologically active peptides.

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]

[a] Division of Applied Science, Graduate School of Agriculture, Hokkaido University,
Kita 9, Nishi 9, Kita-ku, Sapporo 060-8589, Japan
Fax: +81-117063849
E-mail: hasimoto@abs.agr.hokudai.ac.jp
Homepage: http://www.agr.hokudai.ac.jp/en/
[b] Suntory Institute for Bioorganic Research,
1-1-1 Wakayamadai, Shimamoto-cho, Mishima-gun, Osaka 618-8503, Japan
[c] Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama,
2630 Sugitani, Toyama 930-0194, Japan
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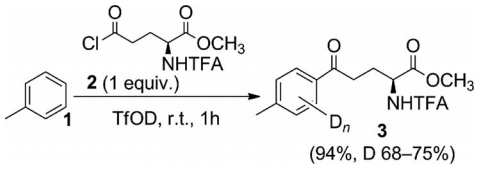

Scheme 2. Friedel–Crafts reaction and hydrogen/deuterium exchange on aromatic protons in TfOD.

It was reported that no deuterium was observed at the α-position, but no description was given for the effects on the aromatic hydrogen atoms. We studied the effects of TfOD on aromatic hydrogen atoms and found 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 for the first time on rapid hydrogen/deuterium exchanges for aromatic α-amino acids and their constituent peptides with TfOD, and found that this process could be controlled by changing the number of equivalents of reagent, the reaction time, and the reaction temperature.

Results and Discussion

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

Tyrosine was then treated with TfOD (40 equiv.) at 0 °C and room temperature. Mass analysis showed that less than two hydrogen atoms per molecule were exchanged with deuterium at 0 °C. The ^{1}H NMR measurement of the product revealed that the hydrogen atom was predominantly exchanged at the *ortho*-position (see the Supporting Information, Figure SI-3b). When performed at room temperature, it was observed by mass spectrometry that the exchange proceeded from two to four sites in a time-dependent manner until 9 h had elapsed (Figure 2a).

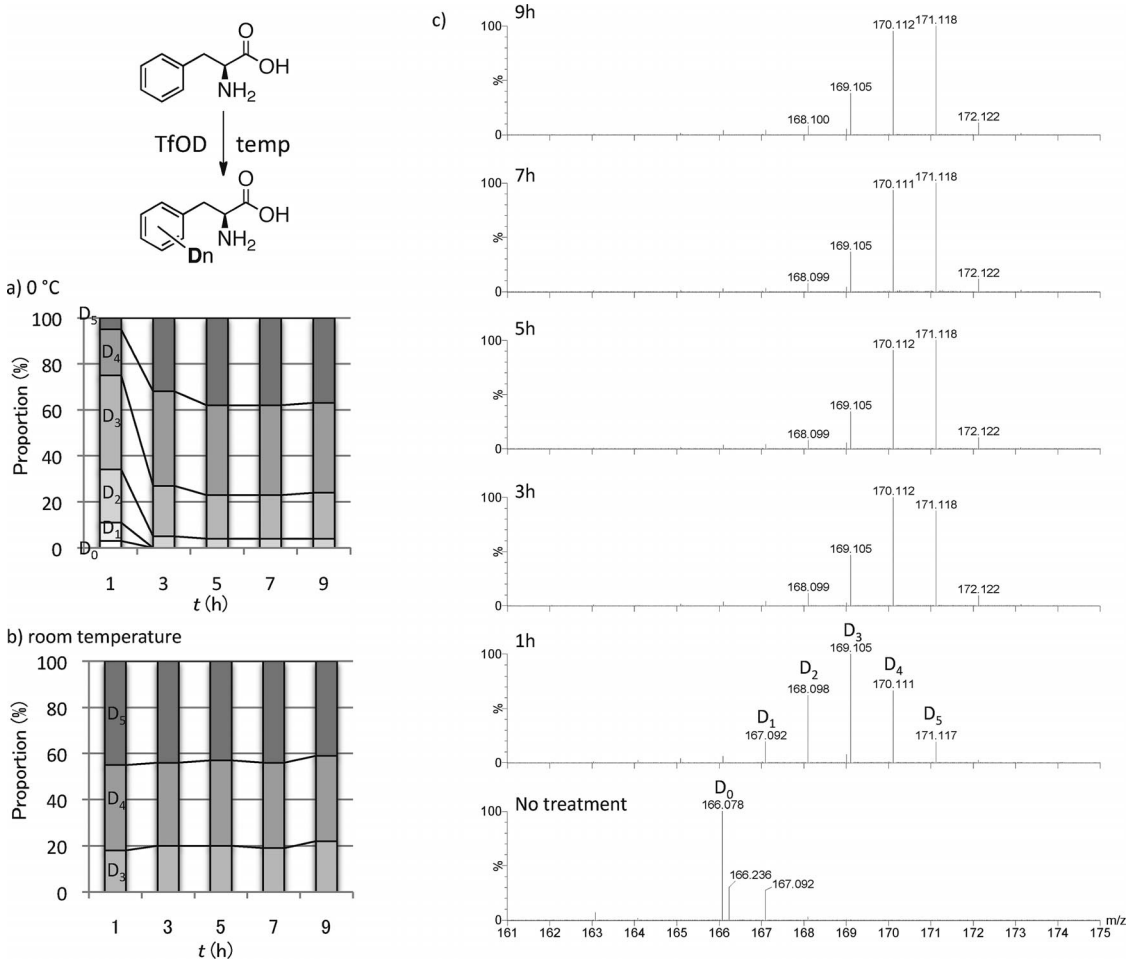

Figure 1. Hydrogen/deuterium exchange on aromatic rings of L-phenylalanine with TfOD at (a) 0 °C and (b) room temperature. D_0–D_5 indicate the number of incorporated deuterium atoms on the aromatic ring. (c) Typical mass spectrum of TfOD-treated L-phenylalanine at 0 °C.

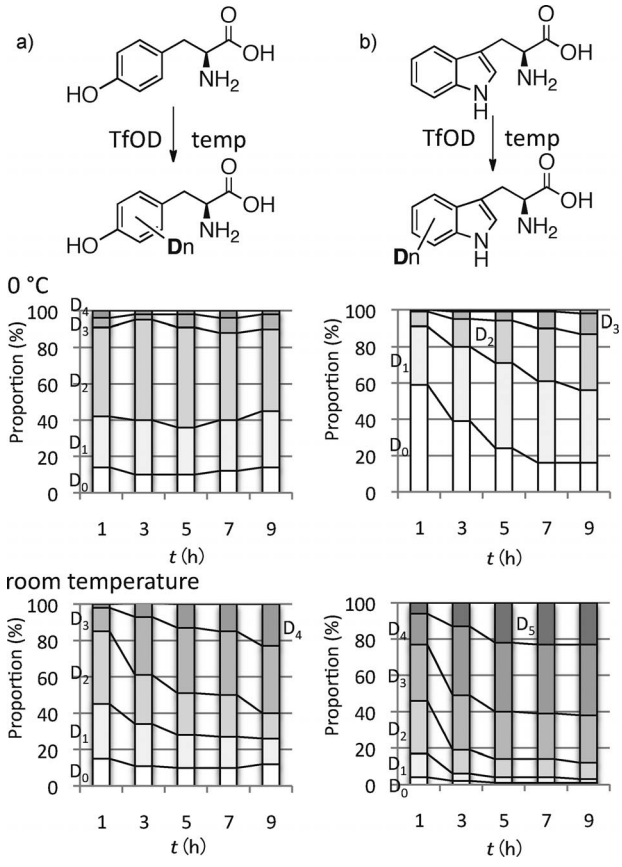

Figure 2. Hydrogen/deuterium exchange on aromatic rings of (a) L-tyrosine and (b) L-tryptophan with TfOD.

Treatment of tryptophan with TfOD (40 equiv.) showed three to five deuterium exchanges at room temperature. Complete exchange (five deuterated, D_5) was observed in nearly 20% of the examined sites. Treatment at 0 °C showed less than three exchanges over time in most cases (Figure 2b).

The hydrogen/deuterium exchange methods were applied to bioactive leucine-enkephalin (YGGFL), which consists of two different aromatic amino acids. Less than three exchanges were observed within 1 h upon exposure to 80 equiv. TfOD at 0 °C. The exchange ratio increased from less than three exchanges at 1 h to less than six exchanges at 9 h (Figure 3a).

Treatment with 80 equiv. TfOD at room temperature showed less than five and seven exchanges for 1 and 9 h, respectively (Figure 3b). The exchange ratios were improved with larger amounts of TfOD (200 equiv.) at room temperature. Less than six exchanges were observed within 1 h, and the ratio was increased to less than eight exchanges after 9 h (Figure 3c).

Deuterated peptides were subjected to MS/MS analysis to verify that deuterated sites were formed on each aromatic α-amino acid. MS/MS analysis of leucine-enkephalin showed immonium ions of Y (m/z = 136 $[M + H]^+$ as no exchanges) and F (m/z = 120 $[M + H]^+$ as no exchanges) under various conditions.[8] Analysis of the Y imminium ion indicated that less than two hydrogen/deuterium exchanges were observed under 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, no exchange and less than two exchanges for the ring of phenylalanine were observed within 1 and 9 h, respectively, at 0 °C. Less than three exchanges were observed within 1 h with 80 equiv. TfOD at room temperature, and the exchangeable sites increased to four at 9 h. A larger excess of TfOD (200 equiv.) at room temperature increased the exchange to a maximum of four and five exchanges at

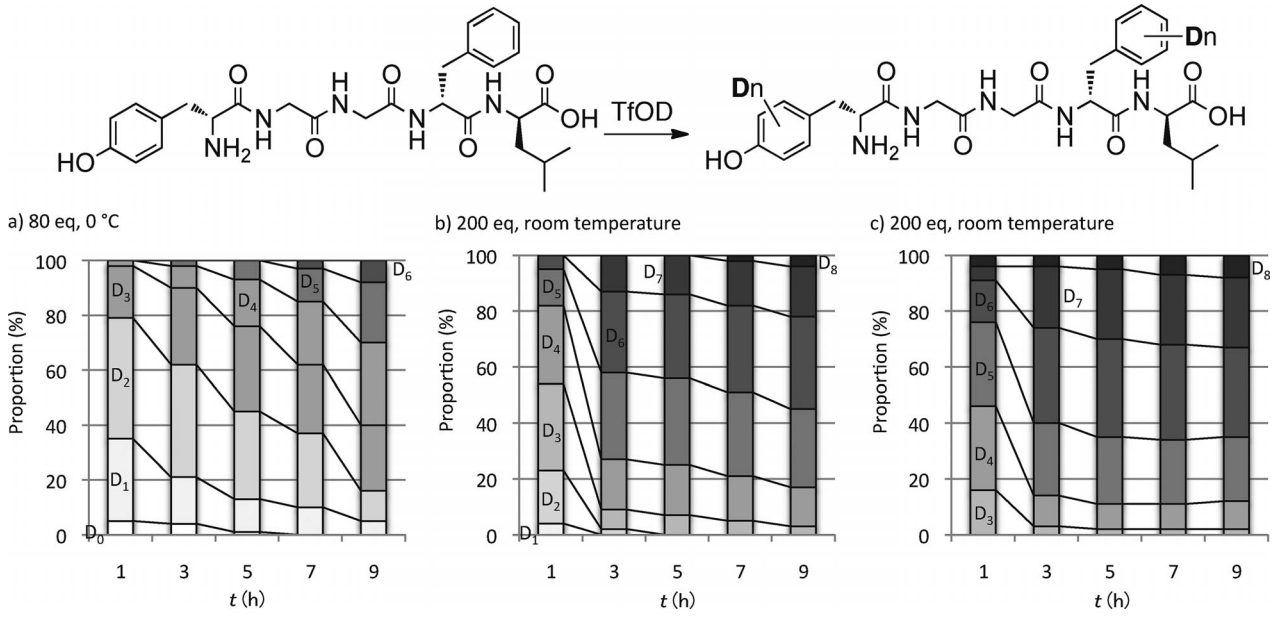

Figure 3. Hydrogen/deuterium exchange on aromatic rings of leucine-enkephalin with TfOD under various conditions.

1 and 9 h, respectively. Unfortunately, it was difficult to identify the hydrogen/deuterium exchange numbers accurately using MS/MS analysis due to a limitation in the resolution of the mass spectrometer.

The deuterated leucine-enkephalins obtained under various conditions were subjected to enzymatic digestions with chymotrypsin to ensure that labeled sites could be identified. 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 (Figure 4). The exchanged sample treated with 80 equiv. TfOD at 0 °C for 1 h afforded two and no deuterium exchanges for tyrosine (m/z = 184) and GGFL (m/z = 280), respectively.

On the other hand, the sample treated with 200 equiv. TfOD at room temperature for 9 h afforded two and up to five deuterium exchanges for tyrosine (m/z = 184) and GGFL (m/z = 283–285), respectively (see the Supporting Information, Figure SI-6). These results indicated that the hydrogen/deuterium exchange was favored in the order: *ortho* positions to the hydroxy group on tyrosine > hydrogen atoms of phenylalanine > *meta* positions to the hydroxy group on tyrosine.

Maculosin [cyclo(L-prolinyl-L-tyrosine)],[9] was also treated with TfOD (Figure 5). The cyclopeptide was exposed to 40 equiv. TfOD at either 0 °C or room temperature (see the Supporting Information, Figure SI-7). No cleavage of the cyclic peptide was observed during the exchange reaction; however, one and two hydrogen/deuterium exchanges were mainly observed under both conditions (Figure 5). These results are consistent with the deuteration of leucine-enkephalin.

Although TfOH is known as a reagent for the deglycosylation of glycoproteins for the analysis of protein moieties[10] and as a reagent for the cleavage of peptides in solid-phase synthesis,[11] decomposition of peptides has sometimes been observed. When Delta-sleeping inducing peptide (DSIP), WAGGDASGE,[12] was subjected to TfOD treatment at 0 °C, a maximum of three hydrogen/deuterium exchanges were observed within 1 h. Although further hydrogen/deuterium exchange for DSIP was observed in a time-dependent manner, dehydrated DSIP was also observed after 3 h. The amounts of DSIP and dehydrated DSIP were almost identical after 9 h (see the Supporting Information, Figure SI-8A). The degree of deuterium incorporation in both compounds was almost identical for each time period (see the Supporting Information, Figure SI-8B). The extent of dehydration of deuterated DSIP increased when the reaction was conducted at room temperature, and no deuterated DSIP was detected after 1 h. These results indicated that TfOD treatment promoted both hydrogen/deuterium exchange for the tryptophan residue and dehydration of DSIP. The hydrogen/deuterium exchange predominated at low temperature.

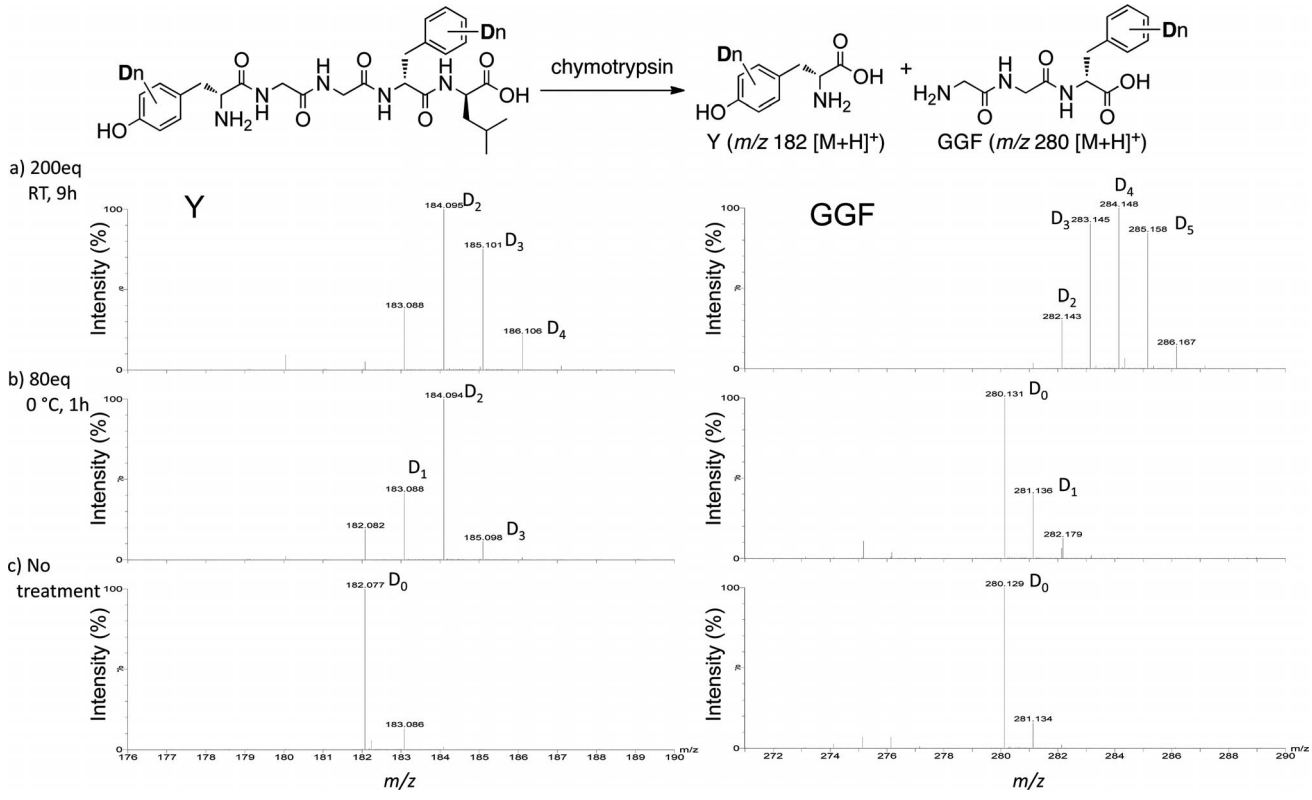

Figure 4. ESI mass spectra of chymotrypsin-digested leucine-enkephalin (YGGFL) treated with (a) TfOD (200 equiv.) at room temperature for 9 h, and (b) TfOD (8 equiv.) at 0 °C for 1 h. (c) Leucine-enephaline without TfOD treatment was analyzed as control digestion.

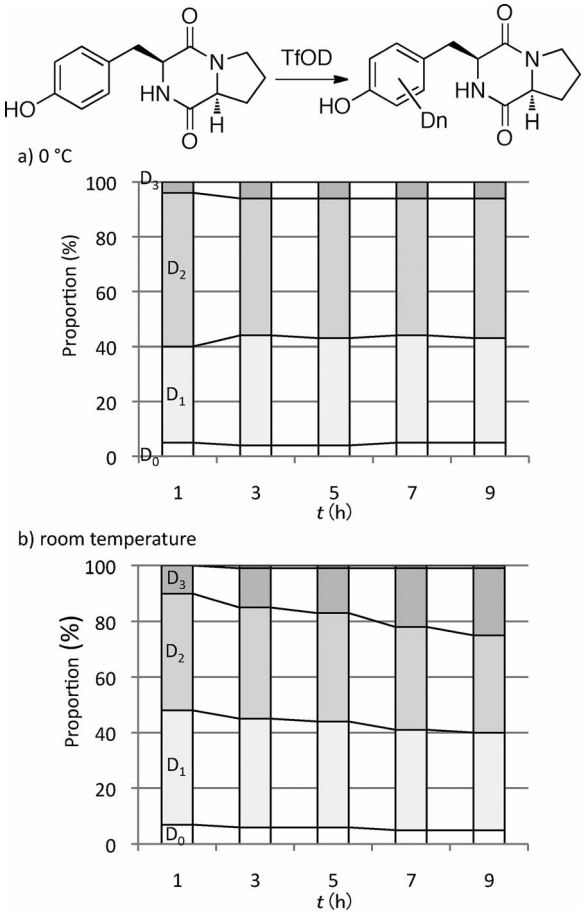

Figure 5. Extent of deuteration of the aromatic residue of maculosin in hydrogen/deuterium exchange with TfOD.

Conclusions

This first detailed analysis of hydrogen/deuterium exchange at aromatic α-amino acids with TfOD at low temperature (from 0 °C to room temperature) was examined. The reaction time for exchange was extremely fast compared with previous methods, and this encouraged us to examine which factors determine the deuterium content in α-amino acids and peptides. It was found that deuterium incorporation can be controlled by the amount of reagent, the reaction time, and the temperature. To the best of our knowledge, there has been no comprehensive study of hydrogen/deuterium exchange for aromatic rings in biologically active peptides. This new method is easier to apply to small amounts of peptides than current standard approaches and does not require special equipment. Hydrogen/deuterium exchange for aromatic rings of α-amino acids and peptides should encourage researchers to use IDMS techniques for the quantitative analysis of biologically active peptides. Further application of this hydrogen/deuterium exchange with TfOD for other aromatic compounds is underway. Our findings show that aromatic C–H bond activation can occur at low temperature, which could contribute to the establishment of new development processes.

Experimental Section

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

Friedel–Crafts Reaction of Toluene (1) and *N*-Trifluoroacetyl-L-Asp(Cl)-OMe (2) in TfOD: Compound **2**[6b] (31.0 mg, 0.113 mmol) and toluene (**1**; 12 μL, 0.113 mmol, 1 equiv.) were dissolved in TfOD (0.5 mL) at 0 °C, and the reaction mixture was stirred for 1 h at room temperature and poured into ice-cold H_2O (15 mL) and EtOAc (15 mL). The organic layer was washed with saturated aqueous NaCl and dried with $MgSO_4$, filtered, and then concentrated. The residue was purified by silica gel chromatography (EtOAc/*n*-hexane, 1:4) to yield **3** (34.8 mg, 0.105 mmol, 94%). $[\alpha]_D$ = +24.0 (c = 2.0, $CHCl_3$). The chirality was also verified by chiral HPLC (Chirobiotic T2; EtOH/H_2O, 10%). Deuterium incorporation was calculated to be 68–75% on the basis of ^{1}H NMR spectroscopic analysis (see the 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 equiv.) at the indicated temperature. The reaction mixture was diluted with H_2O (0.6 mL), and the solution was analyzed by ^{1}H NMR spectroscopy and ESI-TOF MS.

For Peptides: Peptide (9 μmol) was dissolved in TfOD (32 μL, 0.36 mmol for one aromatic α-amino acid) at the indicated temperature. The reaction mixture was diluted with H_2O (0.5 mL), and the solution was analyzed by ^{1}H NMR spectroscopy and ESI-TOF MS. The hydrogen/deuterium exchange samples were analyzed by ESI-TOF MS several times. No change in the analytical data for the sample was observed when measurements were repeated after storing the sample in a refrigerator for 1 month.

Chymotrypsin Digestion for Analysis of Hydrogen/Deuterium-Exchanged Leucine-Enkephaline: The hydrogen/deuterium-exchanged leucine-enkephalin was made neutral by addition of 1 M NaOH. To the solution, chymotrypsin (1/20 against mol-equiv.) was added. The mixture was incubated at 37 °C for 24 h, then subjected to ESI-TOF MS.

Supporting Information (see footnote on the first page of this article): MS and MSMS data of hydrogen/deuterium exchanged peptides.

Acknowledgments

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