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Hydrogen-Deuterium Exchange of Cross-Linkable α-Amino Acid Derivatives in Deuterated Triflic Acid

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In this paper we report here a hydrogen-deuterium exchange (H/D exchange) of cross-linkable α-amino acid derivatives with deuterated trifluoromethanesulfonic acid (TfOD). H/D exchange with TfOD was easily applied to ω-catechol containing phenylalanine (DOPA) within an hour. A partial H/D exchange was observed for trifluoromethyldiazirinyl (TFMD) phenylalanine derivatives. N-Acetyl-protected natural aromatic α-amino acids (Tyr and Trp) were more effective in H/D exchange than unprotected ones. The N-acetylated TFMD phenylalanine derivative afforded slightly higher H/D exchange than unprotected derivatives. An effective post-deuteration method for cross-linkable α-amino acid derivatives will be useful for the analysis of biological functions of bioactive peptides and proteins by mass spectrometry.

Key words: hydrogen-deuterium exchange; aromatic α-amino acid; cross-link; photophore; triflic acid
**Introduction**

Cross-linking reagents are valuable tools for investigating ligand-biomolecule interactions.\(^1\) Based on the progress of the mass analysis, stable isotope-labeled bioactive ligands are attractive tools in the field.\(^2\) Deuterium is one of the simplest stable isotopes and can be utilized for post-introductions to bioactive compounds. Many attempts for deuterium introduction in the aromatic portions of compounds have been reported. Hydrogen-deuterium exchange (H/D exchange)\(^3\)\(^-\)\(^5\) at a specific position and universal hydrogen-deuterium exchange in acidic condition\(^6\) and/or metal catalysis\(^7\) on aromatics have been reported in the literature. Although aromatic α-amino acids were considered to be the substrate for H/D exchange, the α-amino acid skeleton sometimes inhibited to apply previous H/D exchange methods due to their solubility in many organic solvent.\(^8\),\(^9\)

Recently we developed a method for effective H/D exchange for natural aromatic α-amino acids and their containing peptides with trifluoromethanesulfonic acid-d (TfOD) at a temperature lower than room temperature without a side effect on stereochemistry. The exchange can be monitored regularly and controlled by adjusting the reaction temperature and reagent ratio.\(^10\) We report in this paper the post-H/D exchange for cross-linkable aromatic α-amino acid derivatives with TfOD under mild conditions.

**Results and Discussion**

3,4-Dihydroxy-L-phenylalanine (L-DOPA, 1) has catechol moiety on the benzene ring, which can be utilized as a cross-linkable functional group in the presence of periodate.\(^11\)\(^-\)\(^13\) The hydroxyl groups in the molecule might enhance the H/D exchange due to their electron-donating properties. H/D exchange of about 90% takes place at three exchangeable sites on the benzene ring at 0 °C or room temperature within 20 min in TfOD as see in
the $^1$H-NMR measurement (Fig. 1). The degree of exchange did not change with longer incubation time. ESI-TOF mass analysis also indicated that three hydrogen atoms exchanged with deuterium (m/z 201 and 198 with and without deuterium exchange, respectively). It has been reported that the H/D exchange of 1 in 37% DCl/D$_2$O was conducted at 50 °C for 24 h.\textsuperscript{14) Our H/D exchange with TfOD proceeded very swiftly even though it was conducted at low temperature. The H/D exchange ratio for 1 was enhanced significantly than that for L-tyrosine, which favored H/D exchange at the 3-position. All C-H bonds on the aromatic moiety of 1 were placed at $\sigma$ or $p$ positions against two hydroxyl groups. These characters promoted H/D exchange for DOPA swiftly even though the reaction was conducted at 0 °C.

Photoaffinity labeling is the most attractive method for creating a cross-link between ligand and biomolecule interactions.\textsuperscript{15, 16) Photoreactive aromatic $\alpha$-amino acid derivatives have been used to investigate bioactive peptides interactions.\textsuperscript{17-20) Three common photophores, arylazide, benzophenone and diazirine, were utilized for the photoaffinity labeling. The H/D or tritium exchange for photoreactive aromatic $\alpha$-amino acid derivatives has not yet been reported.

L-Phe(4-azido) (2) was treated with TfOD at room temperature and the reaction mixture afforded a complicated mixture within a half hour. It has been reported that the azide group easily reacts in TfOH \textsuperscript{21) and no improvement was observed in the exchange reaction conducted at 0 °C (Fig. 2 A).

L-Phe(4-benzoyl) (3) was stable in the treatment with TfOD at room temperature for a day. However, low incorporation (less than 8% per aromatic proton) was observed in $^1$H-NMR measurement. The low incorporation resulted in no significant detectable differences on the mass spectrum compared to untreated samples. These results indicated that the
carbonyl group acted as the electron withdrawing group to the benzene ring and hampered the H/D exchange reaction at room temperature (Fig. 2 B).

L-Phe(4-TFMD) (4) was subjected to H/D exchange with TfOD. It was observed that the diazirinyl ring was decomposed within 1 h at room temperature. The result was consistent with the previous reports of instability of the diazirinyl ring in strong acidic conditions over room temperature.22,23) No decomposition of the diazirine ring was observed when the reaction was conducted at 0 °C for 5 h. The H/D exchange proceeded in a time-dependent manner and the H/D exchange ratio was observed up to 35% (35% and 10 % for the 2- and 3- position, respectively) in 1H-NMR measurement (Fig. 2 C). Further reaction at the same temperature caused decomposition of diazirinyl ring.

To improve the H/D exchange ratio for compound 4, we used N-acetyl-protected aromatic α-amino acid derivatives in the H/D exchange. N-Ac-L-Tyr (6) was subjected to treatment with TfOD at room temperature. H/D exchange at both, the 2- and 3- position of 6 was more rapid than that of unprotected L-Tyr (5). It was observed that 90% H/D exchange occurred within 3 h at the 3-position and over 24 hours at the 2-position for 5 at room temperature (Fig. 3 A). The exchange rate was drastically improved using 6 for over 90% exchange, within 1 h and 6 h at the 3- and 2- position, respectively (Fig. 3 B). The same tendencies were observed for L-Trp derivatives. The H/D exchange for aromatic protons of Trp was observed to be up to 80% at room temperature. Although unprotected, L-Trp (7) took over 8 h and N-Ac-L-Trp (8) took less than 3 h (Fig. 3 C). These results indicate that the exchange rate for N-acetyl-protected aromatic α-amino acids is faster than that for unprotected compounds.

Based on the above observations, N-Ac-L-DOPA (9), N-Ac-L-Phe(4-benzoyl) (10) and N-Ac-L-Phe(4-TFMD) (11) were subjected to
H/D exchange in TfOD. For DOPA derivative 9, the N-protection did not cause improvement in H/D exchange drastically because unprotected 1 is a good substrate for our H/D exchange. Compound 9 was easily deuterated by over 90% at 0 °C within 20 min (Fig. 4 A). No improvement was observed for the treatment of 10 at room temperature with ¹H-NMR analysis (less than 8% exchange per aromatic proton) (Fig. 4 B). The results indicated that the electron-withdrawing properties of the carbonyl group were the main factor for a lower H/D exchange ratio.

For TFMD derivative 11, H/D exchange at room temperature promoted decomposition of the diazirinyl ring within 1 h, which was consistent with previous results. On the other hand, H/D exchange conducted at 0 °C promoted higher H/D exchange at both the 2⁻ (up to 35%) and 3⁻ (up to 70%) position for 5 h than unprotected L-Phe(4-TFMD) (4) (Fig. 4 C). N-Protection improved H/D exchange with TfOD for the unfavored substrate due to the prevention of neutralization of TfOD by amino groups.

We described post-deuteration (H/D exchange) of cross-linkable aromatic α-amino acid derivatives in TfOD at low temperature. The exchange conditions were beneficial due to the introduction of deuterium at the final steps, reaction at low temperature, and the detectable progress of the reaction. These reactions were not easy to perform using previously used methods. The advanced synthetic methods will be utilized to elucidate ligand-biomolecule interaction with mass spectrometry.

**Experimental**

General methods. ¹H-NMR spectra were measured by a Jeol EX 270 spectrometer for determining the hydrogen-deuterium exchange ratio. MS data were obtained with a Waters LCT Premier XE instrument. ESI-TOF-MS data were obtained with a Waters UPLC ESI-TOF mass spectrometer. Trifluoromethanesulfonic acid-D (98 atom % D) was purchased from Sigma-Aldrich. L-DOPA was purchased from Wako
chemicals. Photoreactive L-phenylalanine derivatives were synthesized by methods described in a previous study.\textsuperscript{24)}

General procedures for hydrogen-deuterium exchange with TfOD. Cross-linkable aromatic $\alpha$-amino acid derivatives (0.25 mmol) were dissolved in TfOD (0.9 mL, 10 mmol) at a specific temperature. The reaction mixture was diluted with $\text{H}_2\text{O}$ (0.6 mL) followed by $^1\text{H}$-NMR and ESI-TOF mass analysis.

General procedures for acetylation of aromatic $\alpha$-amino acid derivatives. Aromatic $\alpha$-amino acid derivatives (0.4 mmol) were dissolved in methanol (2 mL) and acetic anhydride (0.8 mmol) was added dropwise at 40 °C. The reaction mixture was stirred at same temperature for overnight and concentrated. The residue was subjected to silica-column chromatography (CH$_2$Cl$_2$:CH$_3$OH= 4:1, then ethyl acetate:CH$_3$OH= 1:1) to afford pure $N$-acetyl compounds in 60-75% yield.

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**References and Notes**


Legend to Schemes

Fig. 1. Time-course H/D exchange for L-DOPA (1) treated with TfOD at 0 °C by 1H-NMR spectrum in D2O

Fig. 2. H/D exchange of photophore containing aromatic α-amino acid.
A) L-Phe(4-azido) (2), B) L-Phe(4-benzoyl) (3) and C) L-Phe(4-TFMD) (4) at 0 °C
Time-course H/D exchange ratio for 2- and 3- position of compound 4 was presented by open-square and circle, respectively.

Fig. 3. Time-course H/D exchange of L-Tyr (5), N-Ac-L-Tyr (6), L-Trp (7) and N-Ac-L-Trp (8).
A, B) Time-course H/D exchange ratio for 2- and 3- position of compound 5 or 6 was presented by open-square and circle, respectively. C) Time-course H/D exchange ratio for indole protons of compound 7 or 8 was presented by open-triangle and diamond, respectively

Fig. 4. H/D exchange of photophore containing N-acetyl aromatic α-amino acid. A) N-Ac-L-DOPA (9), N-Ac-L-Phe(4-benzoyl) (10) and B) N-Ac-L-Phe(4-TFMD) (11) at 0 °C.
A) Time-course H/D exchange ratio for 2-, 5- and 6- position of compound 9 was presented by open-square, triangle and circle, respectively.
C) Time-course H/D exchange ratio for 2- and 3- position of compound 11 was presented by open-square and circle, respectively.
Fig. 1.

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