Preparation of Deuterated Methyl and Dimethyl Substituted Nicotinoylating Agents for Derivatization of the N-Terminal of Protein

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Methyl groups of 6-methylnicotinic acid and 2,6-dimethylnicotinic acid were deuterated by an H–D exchange reaction under conditions of 1% NaOD/D_2O on heating. With a condensation reaction between the D-labeled nicotinic acid derivative and *N*-hydroxysuccinimide with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, the nicotinoylating agents, 1-(6-methyl[D_3]nicotinoyloxy)succinimide (2c) and 1-(2,6-dimethyl-[D_6]nicotinoyloxy)succinimide (2f) were prepared. Both D-labeled nicotinoylating agents and their unlabeled counterparts quantitatively modified the N-terminal of protein.

Key words nicotinoylating agent; N-terminal; protein; mass spectrometry; D-label; differential expression analysis

Proteins are cell components with a variety of important functions such as serving as receptors, enzymes, hormones and immune factors. With a disruption of these functions, various diseases may develop. Therefore proteome analysis is increasingly carried out in medical and pharmaceutical research. For analyses of proteins, mass spectrometry is widely used, and recently, there has been rapid development of this technology. At the same time, studies on agents for modifying proteins (peptides) are also being conducted for the purpose of facilitating ionization and sequence analysis in mass spectrometry. Using stable isotope-labeled modifying agents, differential expression analysis of protein can be easily performed by mass spectrometry. The isotope-coded affinity tag (ICAT) method[1] reported by Gygi *et al.* is a well known method of this type. Münchback *et al.* reported the effective usage of the N-terminal-modifying agent, 1-(nicotinoyloxy)succinimide; in allowing for easier analysis of the b-ion of peptides in sequencing.[2] Using the ring proton-D_4-labeled 1-(nicotinoyloxy)succinimide, differential expression analysis of protein was also made possible.[2] However, D_4-labeled nicotinic acid, the starting material for the preparation of D_4-labeled 1-(nicotinoyloxy)succinimide, is extremely expensive. In this study, we describe the ease of preparing deuterium-labeled 6-methyl[D_3]nicotinic acid and 2,6-dimethyl[D_6]nicotinic acid, as well as 1-(6-methyl[D_3]nicotinoyloxy)succinimide and 1-(2,6-dimethyl[D_6]nicotinoyloxy)succinimide, by the condensation of D-labeled nicotinic acid derivatives and *N*-hydroxysuccinimide with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC). Other 1-(nicotinoyloxy)succinimide derivatives and analogs were also prepared using EDC, and their abilities in modifying the N-terminal of peptides were compared.

Results and Discussion

H–D Exchange of Nicotinic Acid Derivatives Hydrogen–deuterium exchange reactions of compounds **1b**, **d**, **e** (Chart 1) were carried out under conditions of 1% NaOD/D_2O at 180 °C, and the degrees of exchange at appropriate times were estimated from the decrease in the height of the peak for each proton of the compounds in the NMR spectrum. Figure 1 shows the degrees of exchange of 6-CH_3, 2-H and 4-H protons of 6-methylnicotinic acid (**1b**) and 2-CH_3, 4-H and 6-H protons of 2-methylnicotinic acid (**1d**). In compound **1b**, protons of the 6-CH_3 exchanged so rapidly and the reaction was almost complete at 2 h. The 2-H proton was also exchanged with time but the degree of the exchange was far lower than that of 6-CH_3. In compound **1d**, 2-CH_3 protons were exchanged with time, however, the degree of exchange was much lower than that of 6-CH_3 protons of **1b** and required more than 8 h for a complete exchange. Protons of 6-H were also exchanged very slowly, as for 2-H of **1b**. Protons of 4-H and 5-H of each of **1b** and **1d** were not exchanged under these conditions. Figure 2 showed the H–D exchange reaction of 2,6-dimethylnicotinic acid (**1e**). Protons of the 6-CH_3 group exchanged more rapidly than those of 2-CH_3 as

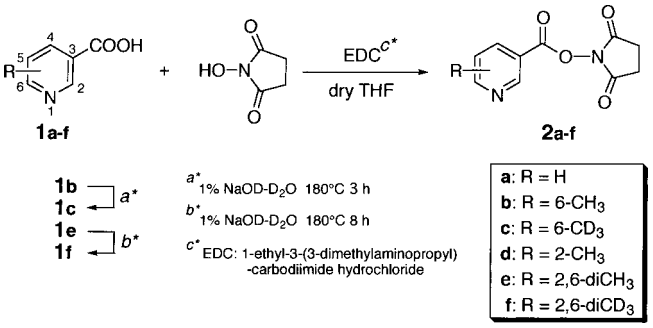

Chart 1

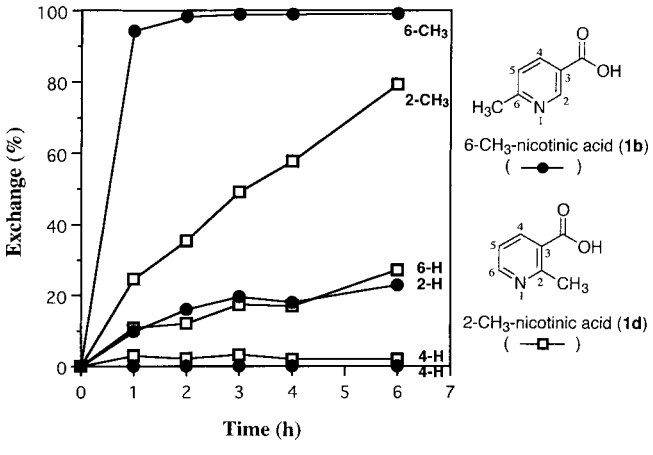

Fig. 1. H–D Exchange of 6-Methyl- and 2-Methylnicotinic Acids (**1b**, **d**)

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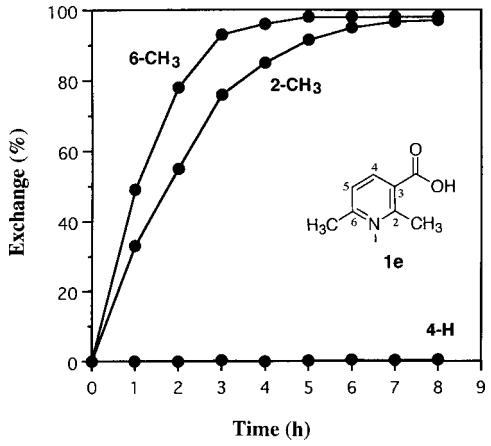

Fig. 2. H–D Exchange of 2,6-Dimethylnicotinic Acid (**1e**)

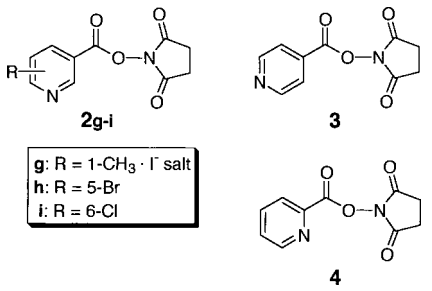

Chart 2

already shown in Fig. 1. After 8 h, the exchange reactions of both 6-CH_3 and 2-CH_3 groups were almost complete and formed **1f**. Under these conditions, protons of 4-H and 5-H were not exchanged. From these results, we decided to use **1f** for subsequent preparation of **2f**. For compound **1b**, although less than about 20% of the 2-H proton was also exchanged when the exchange of the 6-CH_3 was completed in a 3 h reaction (Fig. 1), the deuterium-labeled compound **1c** was also considered to be effective and used in the preparation of **2c**.

Preparation of 1-(Nicotinoyloxy)succinimide Derivatives and Analogs The condensation reaction between the carboxylic acid (**1** and precursors of **2h**, **2i**, **3** and **4**) and the *N*-hydroxysuccinimide was carried out using EDC (Charts 1, 2). The reaction with **1a**—**f** proceeded to a 56—85% yield. In order to obtain the pure products of **2e**, **f**, column chromatography was required. Products **2h**,[3] **2i**,[4] **3**[5] and **4**[5] were obtained in a 40—66% yield. For the condensation reaction, 1,3-dicyclohexylcarbodiimide (DCC) is often employed, however, the separation of the decomposed product, 1,3-dicyclohexylurea, is tedious. By using EDC, we could easily obtain **2a**[6] in as good a yield as 85%.

N-Terminal Derivatization of Bradykinin Bradykinin was treated with modifying agent **2** as described in Experimental and its mass spectra were taken with a matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF/MS). As an example, MALDI-TOF mass spectra of bradykinin before and after derivatization by **2e** and **2f** are shown in Fig. 3 (mass *m*/*z*: 1060.18, 1193.25 and 1199.30, respectively). The degrees of the derivatization of bradykinin by each modifying agent were calculated from the peak height of the derivatized and underivatized peptides as

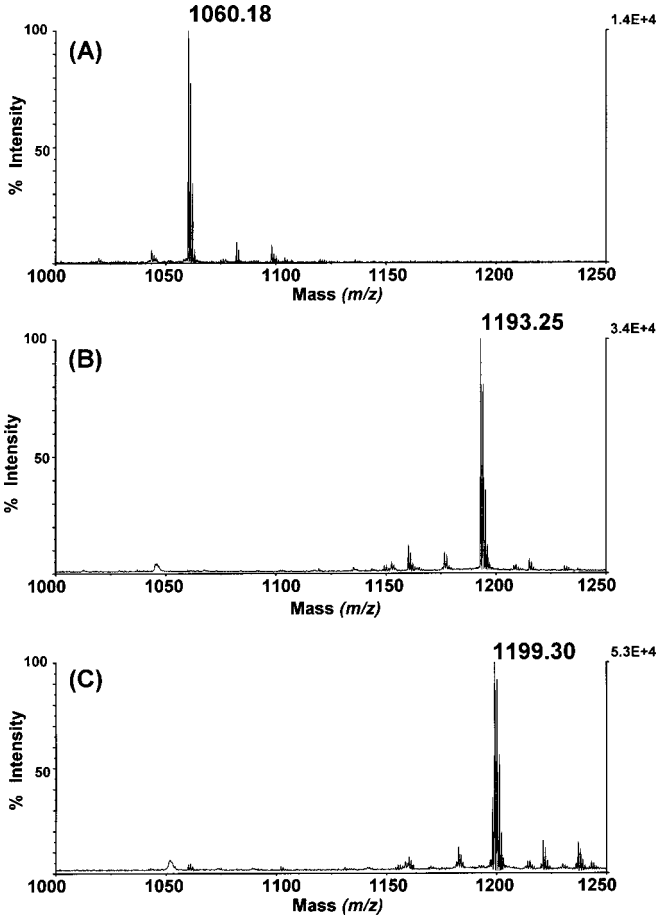

Fig. 3. MALDI-TOF Mass Spectra of Bradykinin

Before (A) and after derivatization by 1-(2,6-dimethylnicotinoyloxy)succinimide (**2e**) (B) and by 1-(2,6-dimethyl[D_6]nicotinoyloxy)succinimide (**2f**) (C).

Table 1. N-Terminal Derivatization of Bradykinin by Modifying Agent

Agent	Modification (%)
2a (R=H)	90—100
2b (R=6-CH_3)	90—100
2c (R=6-CD_3)	90—100
2d (R=2-CH_3)	90—100
2e (R=2,6-diCH_3)	90—100
2f (R=2,6-diCD_3)	90—100
2g (R=1-CH_3)	0
2h (R=5-Br)	42
2i (R=6-Cl)	10
3	13
4	86

summarized in Table 1. The modifications by agents **2a**—**f** all proceeded almost quantitatively. Other agents examined (**2g**—**i**, **3**) did not show a stronger degree of modification, except for compound **4** for which the degree was passable. Since compound **2g** has a cationic center, the derivatized peptide was expected to have a capacity for very strong ionization in mass spectrometry when this compound was introduced at the N-terminal of the peptide. However, the reaction for this peptide did not proceed under the several conditions employed.

Utility of the Modifying Agents 2a—f As already described above, the modifying agents **2a**—**f** showed an almost

quantitative ability to derivatize peptides. Therefore, by combinations of two desired modifying agents, one can select a mass difference of 3 Da (**2b**, **c**), 6 Da (**2e**, **f**), 14 Da (**2a**, **b**, or **2b**, **e**), *etc.* This also means that without using an expensive deuterated modifying agent, differential expression analysis can still be carried out. We already confirmed that more than 20% of the difference in protein expression can be detected with these agents. The precise scope and limitation of these nicotinoylating agents will be reported elsewhere.

Experimental

^{1}H-NMR spectra were recorded on a JEOL GSX 400 spectrometer, and chemical shifts were expressed in ppm using Me_4Si as the internal standard. Mass spectra were obtained with a JEOL JMS-DX300 spectrometer. Melting points were measured with a Yanagimoto micro-melting point apparatus and are uncorrected. Silica gel (Merck) was used for column chromatography. For analysis of peptides, a Voyager Elite MALDI-TOF/MS (Perseptive Biosystems) was used. 2,6-Dimethylnicotinic acid (**1e**) was prepared as reported.[7] Other nicotinic acid derivatives and analogs were purchased. Bradykinin (R-P-P-G-F-S-P-F-R, monoisotopic molecular weight 1059.56) was purchased from the Peptide Institute, Inc.

Preparation of Deuterium-Labeled Nicotinic Acid Derivatives (1c, f) Nicotinic acid derivative (**1b**, **e** 5.0 mmol) was dissolved in 10 ml of 1% NaOD–D_2O and heated at 180 °C in a sealed tube for an appropriate time. The degree of H–D exchange was checked by means of NMR spectra. After the reaction was completed, a small amount of Dowex 50W cation exchange resin (H^+ form) was added and the solution was stirred for 10 min. After the resin was removed by filtration, the filtrate was evaporated to dryness. Without further purification, these D-labeled nicotinic acids (**1c**, **f**) were used for subsequent synthesis of 1-(nicotinoyloxy)succinimide derivatives (**2c**, **f**) as described below.

6-Methyl[D_3]nicotinic Acid (**1c**): 6-Methylnicotinic acid (**1b**) was allowed to react for 3 h. Under these conditions, less than 20% of 2-H proton was also deuterated. Yield 76%. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 7.32 (1H, d, J=8.1 Hz, 5-H), 8.27 (1H, dd, J=2.2, 8.1 Hz, 4-H), 9.22 (1H, d, J=2.2 Hz, 2-H). Electron impact (EI)-MS m/z: 140 (M^+), 141 (M^+ of 2-D derivative).

2,6-Dimethyl[D_6]nicotinic Acid (**1f**): 2,6-Dimethylnicotinic acid (**1e**)[7] was allowed to react for 8 h. Yield 71%. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 7.14 (1H, d, J=8.1 Hz, 5-H), 8.27 (1H, d, J=8.1 Hz, 4-H). EI-MS m/z: 157 (M^+).

Synthesis of 1-(Nicotinoyloxy)succinimide Derivatives (2a—f) [General procedure] Nicotinic acid derivative (**1**, 4 mmol) was dissolved in 80 ml of dehydrated tetrahydrofuran (THF). *N*-Hydroxysuccinimide (4.8 mmol) and EDC (5.2 mmol) were then added to the solution and the mixture was stirred at room temperature for 2 d. The soluble part was separated from the viscous residue and the solvent was removed by evaporation. The residue was dissolved in 80 ml of $CHCl_3$ and washed with 80 ml of brine. After the $CHCl_3$ layer was separated and dried over anhydrous magnesium sulfate, the solvent was removed by evaporation to dryness. Recrystallization afforded the product.

1-(Nicotinoyloxy)succinimide (**2a**)[6]: Yield 85%.

1-(6-Methylnicotinoyloxy)succinimide (**2b**): Yield 66%. Recrystallization from AcOEt gave colorless plates. mp 164—165 °C. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 2.68 (3H, s, CH_3), 2.92 (4H, br s, CH_2×2), 7.32 (1H, d, J=7.9 Hz, 5-H), 8.27 (1H, dd, J=2.1, 7.9 Hz, 4-H), 9.21 (1H, d, J=2.1 Hz, 2-H). EI-MS m/z: 234 (M^+). *Anal.* Calcd for $C_{11}H_{10}N_2O_4$: C, 56.41; H, 4.30; N, 11.96. Found: C, 56.50; H, 4.39; N, 11.99.

1-(6-Methyl[D_3]nicotinoyloxy)succinimide (**2c**): Yield 67%. Recrystallization from AcOEt/hexane gave colorless plates. mp 165—167 °C. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 2.92 (4H, br s, CH_2×2), 7.32 (1H, d, J=8.1 Hz, 5-H), 8.27 (1H, dd, J=2.2, 8.1 Hz, 4-H), 9.22 (1H, d, J=2.2 Hz, 2-H). EI-MS m/z: 237 (M^+).

1-(2-Methylnicotinoyloxy)succinimide (**2d**): Yield 56%. Recrystallization from AcOEt gave colorless plates. mp 174—177 °C. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 2.87 (3H, s, CH_3), 2.93 (4H, br s, CH_2×2), 7.30 (1H, dd, J=4.9, 7.8 Hz, 5-H), 8.38 (1H, dd, J=1.7, 7.8 Hz, 4-H), 8.73 (d, 1H, J=1.7, 4.9 Hz, 6-H). EI-MS m/z: 234 (M^+). *Anal.* Calcd for $C_{11}H_{10}N_2O_4$: C, 56.41; H, 4.30; N, 11.96. Found: C, 56.27; H, 4.27; N, 11.98.

1-(2,6-Dimethylnicotinoyloxy)succinimide (**2e**): The product was purified by column chromatography (silica gel, AcOEt : hexane=1 : 1). Yield 66%. Recrystallization from AcOEt gave colorless plates. mp 134—136 °C. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 2.61 (3H, s, 6-CH_3), 2.82 (3H, s, 2-CH_3), 2.92 (4H, br s, CH_2×2), 7.19 (1H, d, J=8.1 Hz, 5-H), 8.28 (1H, d, J=8.1 Hz, 4-H). EI-MS m/z: 248 (M^+). *Anal.* Calcd for $C_{12}H_{12}N_2O_4$: C, 58.06; H, 4.87; N, 11.29. Found: C, 57.98; H, 4.95; N, 11.14.

1-(2,6-Dimethyl[D_6]nicotinoyloxy)succinimide (**2f**): The product was purified by column chromatography (silica gel, AcOEt : hexane=1 : 1). Yield 65%. Recrystallization from AcOEt/hexane gave colorless plates. mp 135—137 °C. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 2.92 (4H, br s, CH_2×2), 7.14 (1H, d, J=8.1 Hz, 5-H), 8.27 (1H, d, J=8.1 Hz, 4-H). EI-MS m/z: 254 (M^+).

Preparation of Other Modifying Agents (2g—i, 3, 4) Compound **2g** was prepared as reported.[6] Compounds **2h**,[3] **2i**,[4] **3**[5] and **4**[5] were prepared by a condensation procedure between carboxylic acid and *N*-hydroxysuccinimide using EDC as described for **2a**—**f**. For compound **3**, dimethylformamide (DMF) was used instead of THF as the solvent because of the low solubility in THF. The yields of **2h**, **2i**, **3** and **4** were 58%, 66%, 40% and 52%, respectively.

N-Terminal Derivatization of Bradykinin Solutions of bradykinin (100 pmol/μl in H_2O) and the modifying agent (1 mg of modifying agent was dissolved in 1 ml of 50 mM sodium phosphate buffer (pH 8.5)) were prepared. The bradykinin solution (20 μl) and the solution containing the modifying agent (60 μl) were mixed and left at room temperature for 20 min. Another 60 μl of modifying agent was added and the mixture was left for a further 2 h. Then, 60 μl of hydroxylamine solution (0.5 M in 50 mM sodium phosphate buffer (pH 8.5)) was added and the mixture was left for about 17 h. An aliquot was taken from this solution and subjected to MALDI-TOF/MS analysis.

MALDI-TOF/MS Analysis The sample was desalted using a ZipTip C_{18} (Millipore). As the matrix, α-cyano-4-hydroxycinnamic acid (CHCA) was used (1 mg of CHCA was dissolved in 100 μl of solution consisting of 60% ethanol–36% CH_3CN–4% H_2O v/v). The matrix solution (1 μl), sample solution (1 μl) and standard solution (1 μl) were mixed, and 1 μl of the mixture was taken and cocrystallized on a sample plate. The MALDI-TOF/MS was operated in delayed extraction reflector mode using an accelerating voltage of 20 kV, a pulse delay time of 100 ns, a grid voltage of 90—94%, a laser intensity of 1800, and a guide wire voltage of 0.05%. CHCA (dimer, monoisotopic $[2M+H]^+$ 379.0930) and ACTH (monoisotopic $[M+H]^+$ 2465.1989) were used as internal standards.

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