Development of an Amino Acid Sequence and D/L-Configuration Determination Method of Peptide with a New Fluorescence Edman Reagent, 7-Methylthio-4-(2,1,3-benzoxadiazolyl) Isothiocyanate

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On the basis of the relationship between the fluorescence characteristics of the benzofurazan compounds and the Hammett constants (σ_p), a new fluorescence Edman reagent, 7-methylthio-4-(2,1,3-benzoxadiazolyl) isothiocyanate (MTBD-NCS) was designed and synthesized. MTBD-thiohydantoin (TH)-amino acid derivatives produced by the Edman sequencing method gave fluorescence, whereas other degradation byproducts such as MTBD-thiocarbamoyl (TC)- or carbamoyl (CA)-amino acids did not fluoresce. MTBD-NCS was applicable as an Edman sequencing reagent to the simultaneous determination of both the sequence and D/L-configuration of amino acids in peptides. Boron trifluoride (BF_3) and HCl/ methanol were adopted as the cyclization/cleavage and conversion reagents to suppress the amino acid residue racemization. The MTBD-TH-amino acids were separated on a reversed-phase column for amino acid sequencing, and their enantiomers were resolved on two types of polysaccharide-based chiral stationary phases for D/L-configuration determination. The method was successfully applied to the sequence and D/L-configuration determination of D-amino acid-containing peptide [D-Ala^2]-deltorphin II.

Proteins and peptides in living organisms have generally been assumed to consist of only L-amino acids. Recently, biologically active peptides containing D-amino acid have been found in eukaryotes such as frogs,[1,2] snails,[3,4] and spiders.[5] Deltorphin and its analogues, which bind to the μ-type opiate receptor, were D-amino acid-containing peptides isolated from the skin of frogs, and the configuration of D-amino acids in these peptides was essential for the biological activities.[1,2] On the other hand, D-amino acid-containing peptides related to diseases with aging have been found in mammals. The residues of D-amino acids in β-amyloid peptides of Alzheimer's disease[6] and crystallins of cataract[7,8] were observed, and the relationship between the D-amino acids and the diseases has extensively been discussed. However, usually only a small amount of these peptides is available; thus, the sensitive determination method of the sequence and the D/L-configuration of amino acid residues in peptides is desirable.

Amino acid sequence analysis of peptides and proteins using phenylisothiocyanate (PITC) was first reported by Edman;[9] i.e., N-terminal amino acids are derivatized with aryl isothiocyanate, cleaved, and cyclized to thiazolinone (TZ)-amino acids with anhydrous acid (trifluoroacetic acid, TFA), and then the TZ-amino acids are recyclized to aryl thiohydantoin (TH)-amino acids and identified with HPLC. Recentry, various fluorescence Edman reagents have been reported to enhance the detection sensitivity of TH-amino acids. These include fluorescein isothiocyanate (FITC),[10−12] 4-{[(5-(dimethylamino)-1-naphthyl)sulfonyl]amino}-phenyl isothiocyanate (dansylamino-PITC),[13] and 4-(3-isothiocyanatopyrrolidin-1-yl)-7-(*N,N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole (DBD-PyNCS).[14] However, these reagents are not yet routinely utilized as compared with PITC, since the strong

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fluorescence of the reagents themselves and degradation products sometimes interferes with the detection of the generated TH-amino acids. To overcome these disadvantages, we have reported a new Edman procedure using the fluorescent Edman reagents 7-*N*,*N*-dimethylaminosulfonyl-4-(2,1,3-benzoxadiazolyl) isothiocyanate (DBD-NCS),[15] 7-phenylsulfonyl-4-(2,1,3-benzoxadiazolyl) isothiocyanate (PSBD-NCS), and 7-methylsulfonyl-4-(2,1,3-benzoxadiazolyl) isothiocyanate (MSBD-NCS),[16] in which the TZ-amino acids generated by cleavage/cyclization reaction were detected fluorimetrically. These reagents do not fluoresce, and thus the excess reagents did not interfere with the detection of the TZ-amino acids. Further, we modified the procedure mentioned above with DBD-NCS to detect more stable fluorescent derivatives; DBD-TZ-amino acids were hydrolyzed to DBD-thiocarbamoyl (TC)-amino acids and oxidized to corresponding DBD-carbamoyl (CA)-amino acids, and detected fluorimetrically.[17] These gave us the sensitive methods for the determination of the sequence of the peptides.

The Edman procedure was difficult to apply to the D/L-configuration determination, since the amino acid derivatives liberated from the N-terminal of peptides had a tendency to racemize to a considerable extent.[14,18−21] We reported that the DBD-TZ-amino acids obtained using an aprotic acid, boron trifluoride (BF$_3$), as a cleavage/cyclization reaction catalyst were scarcely racemized, and BF$_3$ was used in an amino acid sequencing method for peptides to retain the D/L-configuration of amino acid residues.[22]

Since amino acid sequence analysis is currently accomplished on an automated sequencer, the methods applicable to the convenient vapor-phase sequencer are much more desirable for the simultaneous determination of both the sequence and D/L-configuration of amino acids in peptides. However, two methods using fluorescence Edman reagent DBD-NCS were difficult to apply to the automated sequencer because of the unstability of DBD-TZ-amino acids[15] or the increase in the reaction step of the oxidation to the DBD-CA-amino acids compared with the conventional Edman procedure.[17] Therefore, the development of a fluorescence Edman reagent, the TH-amino acid derivatives of which have fluorescence properties, is essential for the application to an automated sequencer.

In this study, we designed and synthesized a new class of fluorescence Edman reagent, 7-methylthio-4-(2,1,3-benzoxadiazolyl) isothiocyanate (MTBD-NCS) (Figure 1) on the basis of the relationship between the fluorescence characteristics of the benzofurazan compounds and the Hammett constants (σ_p). Using MTBD-NCS, we established a sensitive and simultaneous method for the sequence and configuration determination of peptides.

Figure 1. Structure of 7-methylthio-4-(2,1,3-benzoxadiazolyl) isothiocyanate (MTBD-NCS).

EXPERIMENTAL SECTION

Materials. The following materials were employed: amino acids, *S*β-(4-pyridylethyl)-L-cysteine, Asp-Ala, Pro-Leu, and [D-Ala^2]-Deltorphin II (Sigma, St. Louis, MO); Leu-Gly, Gly-Leu, β-Casomorphin-7 (Bovine), and Tyrosyl-Bradykinin (Peptide Institute, Osaka, Japan); sequencer-grade pyridine and boron trifluoride diethyl etherate complex (BF$_3$−Et$_2$O) (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan); 4-vinylpyridine, sequencer-grade trifluoroacetic acid (TFA), and hydrogen peroxide (H$_2$O$_2$) (Wako Pure Chemicals, Tokyo, Japan); and sodium perchlorate (anhydrous) and HPLC-grade acetonitrile (Kanto Chemical, Tokyo, Japan). Water was purified using a Milli-Q system (Millipore, Bedford, MA). All other reagents were of analytical or guaranteed reagent grade and used without further purification.

Apparatus. The following apparatus were used for the identification of synthesized compounds. Melting points were measured on a micro melting point apparatus (Yanagimoto, Tokyo, Japan) and uncorrected. Proton nuclear magnetic resonance (¹H NMR) spectra were obtained on a GSX-400 spectrometer (JEOL, Tokyo, Japan) with tetramethylsilane as the internal standard. Mass spectra were measured on an M-1200H mass spectrometer (Hitachi, Tokyo, Japan) with an atmospheric pressure chemical ionization system (APCI-MS) or an electrospray ionization system (ESI-MS).

HPLC. HPLC was carried out using an L-6200 intelligent pump, an L-4000H UV detector, an L-7480 fluorescence detector, an L-5020 column oven, a D-7500 integrator, and a D-2500 integrator (all from Hitachi, Tokyo, Japan). The analytes were monitored simultaneously by fluorescence and UV detectors, connected with different recorders. Fluorescence detection was performed at 523 nm with excitation at 391 nm. UV absorbance was monitored at 391 nm.

MTBD-TH-amino acids were separated using an ODS column (TSK gel ODS-80Ts, 250 × 4.6 mm i.d., 5 μm; Tosoh, Tokyo, Japan) for the identification of individual amino acids on sequencing analysis. The following conditions were used: eluent A, acetonitrile/water (26.5/73.5, v/v) containing 0.1% TFA; eluent B, acetonitrile/water (50/50, v/v) containing 0.1% TFA; gradient program, 0−20 min (eluent B composition, 0%), 20−35 min (B, 0−75%), 35−47 min (B, 75%), 47−52 min (B, 75−100%), 52−60 min (B, 100%). The flow rate was kept at 1.0 mL/min. The column temperature was maintained at 40 °C.

For identification of MTBD derivatives of amino acid or dipeptide, the following HPLC system (RP-HPLC) was also used: eluents A and B containing 0.1% TFA were 30/70 (v/v) and 70/30 (v/v) acetonitrile/water, respectively. The analytical column was ODS-80Ts, and the flow rate was 1.0 mL/min using gradient elution from 0 to 25 min (eluent B composition, 0−100%).

For enantiomeric separation of MTBD-TH-amino acids, two chiral stationary phases, i.e., a cellulose tris(4-methylbenzoate) coated on a silica gel support (CHIRALCEL OJ-R, 150 × 4.6 mm

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i.d., 5 μm; Daicel Co., Ltd., Osaka, Japan) and cellulose tris(3,5-dimethylphenyl carbamate) coated on a silica gel support (CHIRALCEL OD-RH, 150 × 4.6 mm i.d., 5 μm; Daicel), were used. The following chromatographic conditions were used: eluent A, 2.0 M sodium perchlorate/acetonitrile (70/30, v/v); eluent B, 2.0 M sodium perchlorate/acetonitrile (30/70, v/v); gradient program, 0–15 min (B, 0%), 15–45 min (B, 0–100%), 45–60 min (B, 100%). The flow rate was kept at 0.5 mL/min. For determination of the racemization ratio in the conversion reaction, the following HPLC system was used: eluents were 38/62 (v/v), 40/60 (v/v), and 35/65 (v/v) acetonitrile/water for TH-Pro, TH-Leu, and TH-Asp, respectively. The analytical column was CHIRALCEL OJ-R, and the flow rate was 0.5 mL/min using isocratic elution.

7-Methylthio-4-(2,1,3-benzoxadiazolyl) Isothiocyanate (MTBD-NCS). 4-Amino-7-methylthio-2,1,3-benzoxadiazole[16] (105 mg) was dissolved in 10 mL of acetonitrile, and 1.0 mL of 25% v/v thiophosgene in benzene was added slowly. The mixture was refluxed for 1 h. The reaction mixture was evaporated to dryness in vacuo, and the residue was chromatographed on silica gel (dichloromethane–hexane) to afford 10 mg of the corresponding isothiocyanate as a yellow powder (yield 87%), mp 113 °C. ^{1}H NMR (CDCl$_3$): δ 7.07 (1H, doublet, $J = 7.6$ Hz), 6.95 (1H, doublet, $J = 7.6$ Hz), 2.67 (3H, singlet). APCI-MS: m/z 223 (M$^-$). Anal. Calcd for C$_8$H$_5$N$_3$OS$_2$: C, 43.04; H, 2.26; N, 18.82. Found: C, 42.94; H, 2.26; N, 18.71%.

Preparation of Standard MTBD-TH-Amino Acids. Amino acids were dissolved in pyridine/water (1/1, v/v) (0.2–2.0 mM). MTBD-NCS (0.5 μmol) was dissolved in 20 μL of the solution, and the mixture was vortex-mixed and heated at 50 °C for 30 min. After the coupling reaction, the mixture was evaporated to dryness using a centrifugal evaporator (SPE-200, Shimadzu, Kyoto, Japan) at 50 °C for 15 min. For conversion to MTBD-TH-amino acids, 30 μL of 10 M HCl/methanol (1/10, v/v) was added to the residue, and the mixture was heated at 60 °C for 30 min and dried under a stream of nitrogen gas. For preparation of MTBD-TH-Asp and -Glu, 30 μL of 25% (v/v) TFA in water was used as the conversion reagent. The resultant residue was dissolved in the HPLC eluent and a portion of the solution was subjected to RP-HPLC. The collected fractions of MTBD-TH-DL- and -L-amino acids were evaporated to dryness and stored at −20 °C. MTBD-TH-dehydro-Ser (Δ-Ser) and TH-dehydro-Thr (Δ-Thr) were isolated as byproducts in the preparation of TH-Ser and TH-Thr, respectively. DL-PEC was prepared by the reaction of DL-Cys with 4-vinylpyridine.[23] DL-Cys was dissolved in pyridine/water (1/1, v/v) (0.1 mM). 4-Vinylpyridine (5 μL) was added to 20 μL of the solution, and the mixture was vortex-mixed and heated at 50 °C for 2 h. The excess reagent and byproducts were removed by washing two times with 200 μL of n-heptane/ethyl acetate (1/1, v/v). The aqueous phase was dried in a centrifugal evaporator at 50 °C. The resulting residue (DL-PEC) was subjected to the reaction with MTBD-NCS as described above. These MTBD-TH-amino acids were identified with APCI-MS by detecting the expected molecular mass: Ala, m/z 293 ([M − H]$^-$); Arg, m/z 380 ([M + H]$^+$); Asn, m/z 338 ([M + H]$^+$); Asp, m/z 339 ([M + H]$^+$); AspMe, m/z 353 ([M + H]$^+$); Δ-Ser, m/z 293 ([M + H]$^+$); Δ-Thr, m/z 307 ([M + H]$^+$); Gln, m/z 352 ([M + H]$^+$); Glu, m/z 353 ([M + H]$^+$); GluMe, m/z 367 ([M + H]$^+$); Gly, m/z 279 ([M − H]$^-$); His, m/z 361

([M + H]$^+$); Ile, m/z 337 ([M + H]$^+$); Leu, m/z 337 ([M + H]$^+$); Lys, m/z 352 ([M + H]$^+$); Met, m/z 355 ([M + H]$^+$); Phe, m/z 371 ([M + H]$^+$); Pro, m/z 321 ([M + H]$^+$); PEC, m/z 432 ([M + H]$^+$); Ser, m/z 311 ([M + H]$^+$); Thr, m/z 323 ([M − H]$^-$); Trp, m/z 410 ([M + H]$^+$); Tyr, m/z 387 ([M + H]$^+$); Val, m/z 323 ([M + H]$^+$).

Preparation of MTBD-TH-, MTBD-TC-, and MTBD-CA-Leu. MTBD-TH-Leu was prepared by the method described. MTBD-TC-Leu was prepared by the coupling of Leu and MTBD-NCS in pyridine/water (1/1, v/v) at 50 °C for 30 min (APCI-MS: m/z 355 ([M + H]$^+$)). MTBD-CA-Leu was prepared by the oxidation of MTBD-TC-Leu in 3% hydrogen peroxide at 50 °C for 10 min (APCI-MS: m/z 339 ([M + H]$^+$)). The identification of MTBD-TH-Leu was carried out not only by the molecular mass but also by the UV absorption maximum at 269 nm, which can be assigned to the TH ring, since MTBD-TZ derivatives have the same molecular mass as MTBD-TH derivatives. The UV spectra and fluorescence spectra of obtained derivatives in acetonitrile/water (1/1, v/v) were measured on a spectrophotometer (Ubest 50; JASCO, Tokyo, Japan) and a spectrofluorometer (F-4010; Hitachi), respectively.

Coupling Reaction. Dipeptides were dissolved in pyridine/water (1/1, v/v) (0.1 mM). A 200 nmol amount of a MTBD-NCS was dissolved in 20 μL of the solution, and the mixture was vortex-mixed and heated at 50 °C. An aliquot of the reaction mixture was withdrawn after appropriate time intervals. After the coupling reaction, the reaction mixture was washed three times with 100 μL of heptane/ethyl acetate (4/1, v/v). The aqueous phase was diluted with the HPLC eluent and subjected to RP-HPLC, and then MTBD-TC-dipeptides were detected with UV absorbance at 391 nm. These MTBD-TC-dipeptides were identified with ESI-MS for Asp-Ala and Leu-Gly derivatives or APCI-MS for Pro-Leu derivative by detecting the expected molecular mass: Asp-Ala, m/z 426 ([M − H]$^-$); Leu-Gly, m/z 410 ([M − H]$^-$); Pro-Leu, m/z 452 ([M + H]$^+$).

Cyclization/Cleavage Reaction by BF$_3$. The MTBD-TC derivatives of dipeptides were prepared by the reaction with MTBD-NCS (100 nmol) and dipeptide (0.1 mM) in pyridine/water (1/1, v/v) at 50 °C for 30 min. After the product was washed three times with 100 μL of ethyl acetate/heptane (4/1, v/v) and evaporation, 30 μL of 80 mM BF$_3$–Et$_2$O in acetonitrile was added to the residue, and the mixture was heated at 60 °C. An aliquot of the reaction mixture was withdrawn after appropriate time intervals. The reaction mixture was dried under nitrogen and converted to corresponding TH-amino acid by 25% (v/v) TFA in water at 60 °C for 30 min. The mixture was dried under nitrogen and dissolved in the HPLC eluent. The generated TH-amino acid was subjected to RP-HPLC and detected fluorimetrically.

Conversion Reaction. The MTBD-TC-dipeptides were cyclized and cleaved by 80 mM BF$_3$–Et$_2$O in acetonitrile at 60 °C for 30 min as described above. After the reaction mixture was dried under nitrogen, the resulting residue was dissolved in 30 μL of 25% (v/v) TFA in water or 10 M HCl/methanol (1/10, v/v) at 60 °C. An aliquot of the reaction mixture was withdrawn after appropriate time intervals and subjected to RP-HPLC and enantiomeric separation as described above.

Liquid-Phase Sequencing of Peptides with MTBD-NCS. The peptide was dissolved in pyridine/water (1/1, v/v) (0.1–0.2

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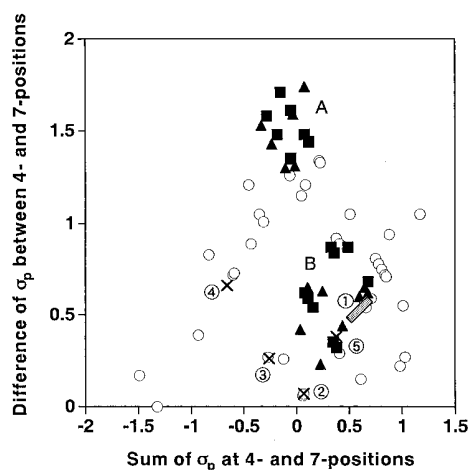
Figure 2. Design of fluorescent Edman reagent using the relationship between the fluorescence intensity of 4,7-disubstituted benzofurazan compounds and the Hammett substituent constants (σ_p) at the 4- and 7-positions. ①, ②, ③, ④, ⑤ denote MTBD-thiohydantoin, MTBD-NHCSNHC$_2$H$_5$, MTBD-NHCONHC$_2$H$_5$, MTBD-NH$_2$, and MTBD-NCS, respectively.

mM). A 500 nmol amount of MTBD-NCS was dissolved in 20 μL of the solution. The mixture was vortex-mixed and heated at 50 °C for 30 min. After the coupling reaction, the excess reagent and byproducts were removed by washing the mixture three times with 100 μL of ethyl acetate/heptane (1/6, v/v). The aqueous phase was evaporated to dryness using a centrifugal evaporator at 50 °C for 15 min, and 30 μL of 80 mM BF$_3$-Et$_2$O in acetonitrile was added to the residue. The mixture was heated at 60 °C for 30 min and dried under a stream of nitrogen. After the addition of 10 μL of water, the solution was extracted twice with 100 μL of ethyl acetate/heptane (4/1, v/v). The aqueous phase was dried in a centrifugal evaporator and subjected to the next cycle. The combined organic phase was dried under a stream of nitrogen, 30 μL of 25% (v/v) TFA in water or 10 M HCl/methanol (1/10, v/v) was added to the residue, and the mixture was heated at 60 °C for 30 min and dried under nitrogen. The resulting residue was dissolved in 100 μL of the HPLC eluent, and a 10 μL aliquot of the solution was subjected to the HPLC.

RESULTS AND DISCUSSION

Design of the Fluorescent Edman Reagent with a Benzofurazan Skelton. In the previous paper, we investigated the effects of the substituent group at the 4- and 7-positions on the fluorescence characteristics of the benzofurazan compounds and found the relationship between the fluorescence characteristics of these compounds and the Hammett substituent constants (σ_p)[24] of the substituent groups at the 4- and 7-positions.[25] Using this relationship, we can predict the fluorescence characteristics from the chemical structures of 4,7-disubstituted benzofurazan compounds and thus can design fluorescent reagents. Figure 2 shows the relationship obtained previously[25] between the fluorescence intensity of 4,7-disubstituted benzofurazan compounds and σ_p at the 4- and 7-positions. In this figure, the absicca and the ordinate

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Table 1. Fluorescence Characteristics of MTBD Derivatives

derivative	λ_{max}/nm	λ_{em}/nm	ratio[a]
MTBD-TC-Leu	412	581	3.2
MTBD-CA-Leu			0
MTBD-TH-Leu	391	523	165

[a] Ratio = fluorescence intensity (λ_{em})/UV absorption (λ_{max}).

represent the sum of σ_p values at the 4- and 7-positions and the difference of σ_p values at the 4- and 7-positions, respectively. The 70 4,7-disubstituted benzofurazan compounds were classified into three groups according to their relative fluorescence intensities (RFI; the fluorescence intensity of 4-amino-7-dimethylaminosulfonyl-2,1,3-benzoxadiazole was arbitrarily taken as 1.0) (RFI = 0−1, having no or weak fluorecece (○); RFI = 1−5, having moderate fluorescence (▲); RFI > 5, having strong fluorescence (■)). The fluorescent compounds, represented as closed squares and closed triangles, were gathered in two areas (A and B); in contrast nonfluorescent compounds scattered out of these areas. These results showed that the compounds in these areas are strongly fluorescent and the compounds out of these areas are nonfluorescent or weakly fluorescent. Accordingly to this relationship, we searched for a compound with its thiohydantoin derivatives within these areas and its thiocarbamoyl or carbamoyl derivatives outside these areas. Such a compound should be suitable as a fluorescent Edman reagent, since the byproducts generated in the conversion reaction are expected not to interfere with the fluorimetric detection of thiohydantoin derivatives.

As a candidate for such an Edman reagent, 7-methylthio-4-(2,1,3-benzoxadiazolyl) isothiocyanate (MTBD-NCS) was selected, since the MTBD-TH derivative (①) was plotted in the fluorescent area (the σ_p value was estimated as 0.5−0.6 from its structure, though the value for thiohydantoin was not reported) and the byproducts generated in the conversion reaction, the MTBD-TC derivative (②), the MTBD-CA derivative (③), and MTBD-NH$_2$ (④), were plotted out of the fluorescent area in Figure 2 (the σ_p values for −SCH$_3$, −NHCSC$_2$H$_5$, −NHCOC$_2$H$_5$, and −NH$_2$ were reported as 0.0, 0.07, −0.26, and −0.66, respectively),[24] though MTBD-NCS (⑤), the Edman reagent itself, was also predicted as fluorescent (the σ_p value for −NCS was 0.38).[24] Thus, MTBD-NCS was synthesized and used in this study.

Fluorescence Characteristics of MTBD Derivatives. The data of fluorescence spectra and UV spectra of the MTBD-Leu derivatives are summarized in Table 1. To evaluate the fluorescence quantum yields of these derivatives, the ratio of fluorescence intensity (at λ_{em}) to absorbance (at λ_{max}) was adopted.[26] As expected, MTBD-TH-Leu gave the highest quantum yield, whereas MTBD-TC-Leu, which was produced through the ring-opening step in the process of conversion to MTBD-TH-Leu, gave very low quantum yield. The major byproduct, MTBD-NH$_2$, in the sequencing analysis and MTBD-CA-Leu gave no fluorescence. DBD-NH$_2$ and MSBD-NH$_2$ gave high quantum yields, and those peaks were observed on the chromatograms,[15,16] whereas Edman previously observed that the PTC-amino acids showed a tendency

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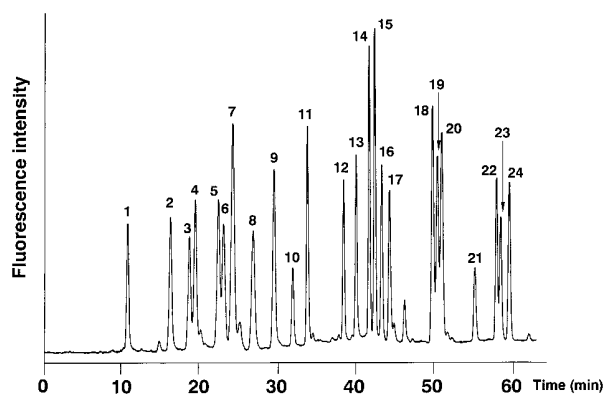
Figure 3. Chromatogram of MTBD-TH-amino acids. Each peak of MTBD-TH-amino acids corresponds to 70 (peak 21, Trp), 10 (2, Lys; 3, Arg; 4, Asn; 5, Ser; 6, Gln; 7, PEC; 8, Thr; 9, Asp; 10, Gly; 11, Glu; 12, Ala; 14, Tyr; 17, Δ-Thr; 18, Met; 19, Val; 24, Leu), 5 (1, His; 13, AspMe; 15, Δ-Ser; 16, GluMe; 22, Phe; 23, Ile), and 2 (20, Pro) pmol. Abbreviations: PEC, $S\text{-}\beta\text{-}(4\text{-pyridylethyl})$ cysteine; AspMe, aspartic acid β-methyl ester; GluMe, glutamic acid γ-methyl ester, Δ-Ser, dehydro-Ser; Δ-Thr, dehydro-Thr. HPLC conditions: column, TSK gel ODS-80Ts at 40 °C. Eluent A, acetonitrile/water (26.5/73.5, v/v) containing 0.1% TFA; eluent B, acetonitrile/water (50/50, v/v) containing 0.1% TFA; gradient program, 0−20 min (B, 0%), 20−35 min (B, 0−75%), 35−47 min (B, 75%), 47−52 min (B, 75−100%), 52−60 min (B, 100%); flow rate, 1.0 mL/min; detection Ex = 391 nm, Em = 523 nm.

Table 2. Capacity Factors (k_1') and Separation Factors (α) for MTBD-TH-Amino Acids on Chiral Stationary Phases[a]

amino acid	CHIRALCEL OJ-R		CHIRALCEL OD-RH	
	k_1'	α	k_1'	α
Ala	8.84 D	1.13	8.52 L	1.02
Arg	3.68 D	1.19	5.87 L	1.03
Asn	2.35 D	1.22	2.51 L	1.31
Asp[b]	9.16 D	1.13	8.72 L	1.02
Cys[c]	7.47 D	1.26	9.79	
ΔSer[d]	10.5		10.1	
ΔThr[d]	9.81		10.7	
Gln	1.62 D	1.30	3.00	
Glu[b]	9.31 D	1.13	9.74 L	1.02
Gly[d]	8.07		7.49	
His	1.29		2.54 D	1.10
Ile	10.6 D[e]	1.19	11.5 L	1.08
Leu	10.9 D	1.10	11.5 L	1.08
Lys	1.59 D	1.13	2.86 L	1.06
Met	10.6 D	1.11	11.1 L	1.09
Phe	10.7 D	1.08	11.8 L	1.03
Pro	9.45 L	1.05	10.3	
Ser	3.19 D	1.39	3.42 L	1.11
Thr	4.40 D	1.42	4.97 L	1.23
Trp	11.5 D	1.02	12.9	
Tyr	8.67 D	1.10	9.58 L	1.03
Val	9.69 D	1.26	10.1 L	1.04

[a] Conditions: Eluent A, 2.0 M sodium perchlorate/acetonitrile (70/30, v/v); eluent B, 2.0 M sodium perchlorate/acetonitrile (30/70, v/v); gradient program, 0−15 min (B, 0%), 15−45 min (B, 0−100%), 45−60 min (B, 100%); flow rate, 0.5 mL/min. [b] MTBD-TH-Asp and -Glu indicate those of methyl ester. [c] MTBD-TH-Cys indicates $S\beta$-(4-pyridylethyl)cysteine derivative. [d] Δ-Ser, Δ-Thr, and Gly have no chirality. [e] This is D-*allo*-Ile, which is of D-Ile at the β-carbon position.

toward oxidative desulfuration with formation of phenyl-CA-amino acids.[27] These results indicate that the byproducts generated in the sequencing do not interfere with the fluorimetric detection of MTBD-TH derivatives. In contrast to other fluorescent Edman reagents with benzofurazan structure,[16,26] we could not isolate the MTBD-TZ derivative.

Separation and Detection of MTBD-TH-Amino Acids on Reversed-Phase HPLC. Figure 3 shows the HPLC separation of a mixture of 24 MTBD-TH-amino acids. A good separation of all the MTBD-TH-amino acids was accomplished by gradient elution within 60 min. MTBD-TH-Asp and -Glu were detected as methyl esters of these MTBD-TH-amino acids, since the side chains of these amino acids were completely esterified in the conversion reaction using HCl/methanol.[27,28] MTBD-TH-Cys was detected as an $S\beta$-(4-pyridylethyl)cysteine derivative, since sequence analysis of cysteine-containing peptides or proteins is generally carried out after derivatization such as S-pyridylethylation.[29,30] MTBD-TH-Ser and -Thr were detected with two peaks similar to those of PTH-Ser and -Thr, indicating that the side chains of these amino acids were dehydrated during the conversion reaction. Next, the detection limits of the obtained MTBD-TH-amino acids were studied. MTBD-TH-Pro was most sensitively detected with the detection limit of 20 fmol on column (S/N = 3). The detection limits for the other TH-amino acids were 0.1 pmol to the sub-picomole level. These results were comparable to those of FITC (10 fmol),[11,12] DBD-Py-NCS (20 fmol),[14] and

MSBD-NCS (7 fmol).[16] However, the fact that the byproducts generated in the sequencing such as MTBD-TC- or -CA-amino acids and MTBD-NH_2 do not fluoresce gives the superiority of MTBD-NCS to others with regard to the avoidance of the interference in the fluorimetric detection.

Enantiomeric Separation of MTBD-TH-Amino Acids. To separate the enantiomers of MTBD-TH-amino acids, first, we examined β-cyclodextrin (β-CD)-bonded chiral stationary phases, since the enantiomers of PTH-, DBD-TZ-, and DBD-CA-amino acids were well separated on this column.[17,22,28] However, the enantiomeric separation of MTBD-TH-amino acids on a native β-CD column or a phenylcarbamoylated β-CD column with an acidic mobile phase was insufficient, although some enantiomers of MTBD-TH-amino acids were separated with a broad peak shape.

Next, we examined stationary phases of cellulose tris(4-methylbenzoate) (CHIRALCEL OJ-R) and cellulose tris(3, 5-dimethylphenyl carbamate) (CHIRALCEL OD-RH) coated on a silica gel support. These polysaccharide-based chiral stationary phases were very useful for the separation of enantiomers with benzofurazan structure.[31−33] The capacity factors and the separation factors for MTBD-TH-amino acids are shown in Table 2. The enantiomers of 18 MTBD-TH-amino acids, except for His, were separated on the OJ-R column, and those of 15 TH-amino acids, containing His, were separated on the OD-RH column. In this

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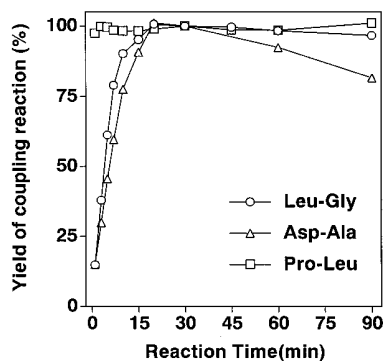(33) Ichihara, H.; Fukushima, T.; Imai, K. *Anal. Biochem.* **1999**, *269*, 379−85.

Figure 4. Time course for the coupling reaction of dipeptides with MTBD-NCS at 50 °C. The yield of the coupling reaction at 50 °C for 30 min was taken as 100%.
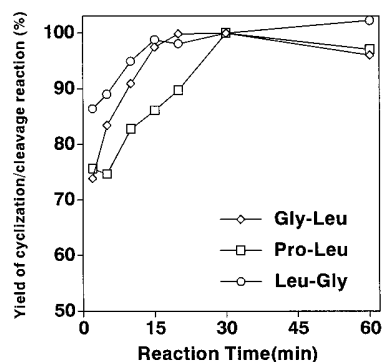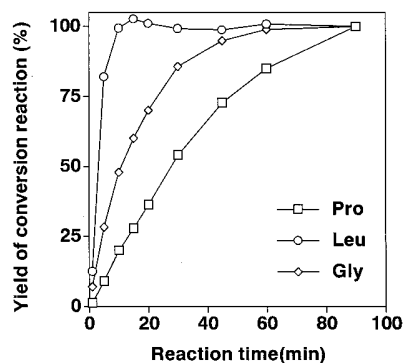

Figure 6. Yield of MTBD-TH-amino acids in the conversion reaction at 60 °C with 25% (v/v) TFA in water. The yield of the reaction for 90 min was taken as 100%.
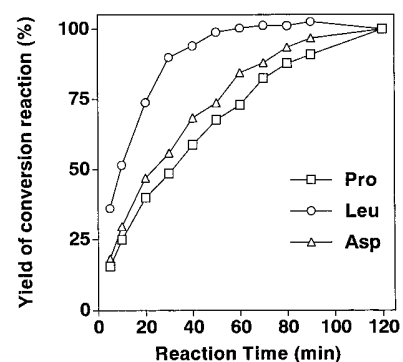

Figure 5. Time course for the cyclization/cleavage reaction of MTBD-TC-dipeptides. The yield of the reaction at 60 °C for 30 min was taken as 100%.

Figure 7. Yield of MTBD-TH-amino acids in the conversion reaction at 60 °C with 10 M HCl/methanol (1/10, v/v). The yield of the reaction for 120 min was taken as 100%.

manner, the enantiomeric separation of all TH-amino acids was achieved on these two different columns.

Coupling Reaction. The time course of the coupling reaction of MTBD-NCS and dipeptides is shown in Figure 4. The reaction was completed within 30 min at 50 °C at a concentration of the reagent 100 times greater than that of the peptide. Thus, a reaction time of 30 min was adopted in this study.

Cyclization/Cleavage Reaction. In our previous study, we reported that an aprotic acid, boron trifluoride (BF$_3$), facilitated the cyclization/cleavage reaction and gave the higher yields of DBD-TZ-amino acids and ATZ-amino acids than TFA, and these derivatized amino acids were scarcely racemized.[22] Therefore, BF$_3$ was employed as the cyclization/cleavage reagent in this study to suppress the amino acid residue racemization. MTBD-TZ-amino acids were further recyclized to MTBD-TH-amino acids. Figure 5 shows the time course of the cyclization/cleavage reaction. Pro-Leu, Gly-Leu, and Leu-Gly were selected as model peptides, since the N-terminus Pro and Gly residues of a peptide are known to be particularly resistant to cleavage.[27] The production of cleaved MTBD-TZ-amino acids reached a plateau within 30 min at 60 °C and also confirmed the disappearance of MTBD-TC-dipeptides (data not shown). A reaction time of 30 min at 60 °C was therefore adopted in this study.

Conversion Reaction. In the Edman sequencing method by PITC, ATZ-amino acids are converted to PTH-amino acids under strong acidic conditions, such as 25% TFA in water or HCl/methanol (1/10, v/v).[27,28] Then, 25% TFA and HCl/methanol were employed as the conversion reagents in this study. Leu-Gly, Gly-

Leu, Pro-Leu, and Asp-Ala were selected as model peptides for the optimization of the conversion reaction conditions, since the ATZ-Gly and -Pro were reported to resist the conversion reaction.[28] As shown in Figure 6, in the conversion reaction with 25% TFA, the cyclization/cleavage reaction product of MTBD-TC-Leu-Gly was converted to MTBD-TH-Leu within 15 min at 60 °C, whereas those of MTBD-Gly-Leu and -Pro-Leu were not converted completely even after 60 min and corresponding TC derivatives were still observed in the chromatogram with UV detection (data not shown). Although the conversion reaction was not completed, the reaction time of 30 min was adopted, since some amino acids, such as Ser and Thr, were labile and might be degraded during the long reaction period.

The degree of racemization of the cyclization/cleavage reaction product of PTC-peptides during the conversion to PTH-amino acids was examined as described in the previous study,[28] and we have reported that the racemization ratios of amino acids in HCl/methanol as conversion reagent were lower than in 20% TFA. In fact, the racemization ratios of the generated MTBD-TH-amino acids in this step with 25% TFA were high (ca. 45%) in our preliminary study. Therefore, HCl/methanol was selected as the conversion reagent in the sequence and D/L-configuration determination of peptides.

As shown in Figure 7, MTBD-TH-Leu was produced with good yield within 50 min, except for MTBD-TH-Pro and -Asp, at a concentration of HCl in methanol 10 times higher than that required to convert ATZ-amino acids to PTH-amino acids.[28] The side chains of MTBD-TH-Asp and -Glu were completely esterified
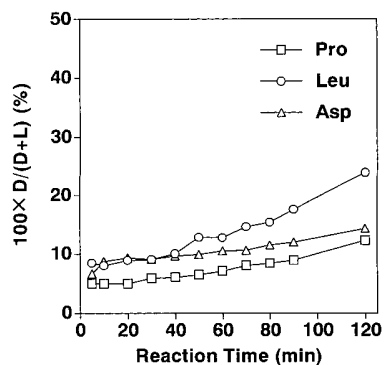
Figure 8. Racemization of MTBD-TH-amino acids in the conversion reaction at 60 °C with 10 M HCl/methanol (1/10, v/v).
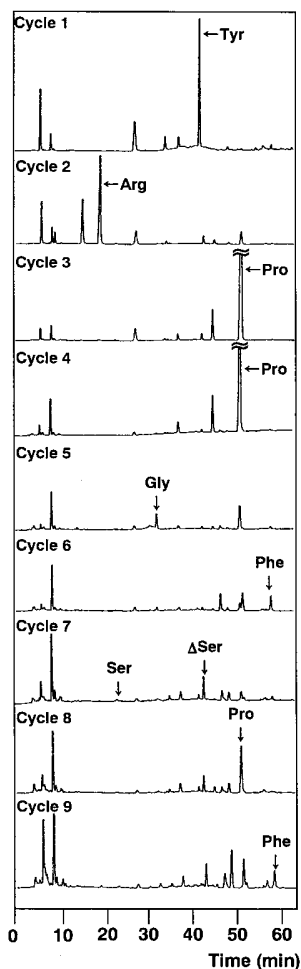

Figure 9. Chromatograms obtained from sequencing analysis of Tyrosyl-Bradykinin (1 nmol). HPLC conditions were the same as those described in the caption to Figure. 2.

Figure 10. Amino acid sequencing and D/L-configuration identification of [D-Ala²]-Deltorphin II (2 nmol). (A) sequence determination on the reversed phase HPLC system. HPLC conditions were the same as those described in the caption to Figure 2. (B) D/L-configuration determination on the chiral stationary phase HPLC system. HPLC conditions were the same as those described in Table 2.

as well as PTH-Asp and -Glu. The racemization ratios of amino acids in HCl/methanol were around 10% at 60 °C for 50 min, and the ratios increased gradually with reaction time (Figure 8). We made a compromise between the yield and the racemization ratio of the MTBD-TH-amino acids, and employed conversion reaction conditions of HCl/methanol at 60 °C for 60 min.

Liquid-Phase Sequencing of Peptides with MTBD-NCS. A manual sequencing procedure established as described above was applied to seque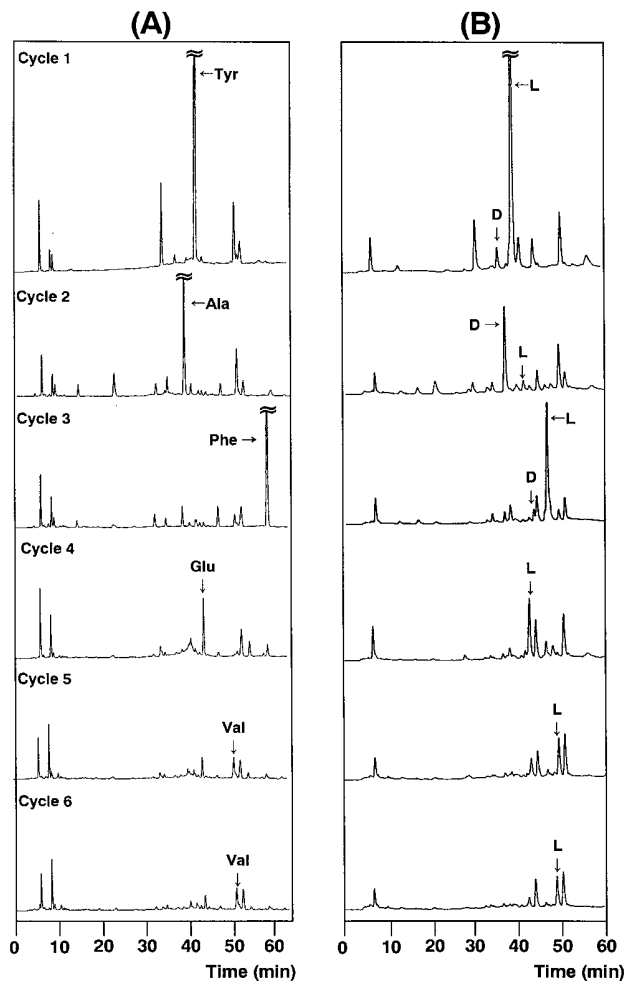ncing of peptides. Figure 9 shows the results of sequencing 1 nmol of Tyrosyl-Bradykinin. After the coupling reaction, the MTBD-TC-peptide was cyclized/cleaved by BF₃, and the generated MTBD-TZ-amino acid was further converted to MTBD-TH-amino acid by 25% TFA. From the chromatograms obtained in the respective cycles, the amino acid residues were easily identified, whereas the C-terminal amino acid Arg was not detected, presumably owing to the loss of MTBD-TC-Arg in the washing step. Some background peaks, the origin of which was not clear, were observed in the chromatograms in addition to those derived from the incomplete reaction in the previous cycles and had no influence on the identification of amino acid residues. The Ser residue (cycle 7) was identified by the peaks of both TH-Ser and TH-dehydro-Ser (Δ-Ser). The β-elimination reaction of the hydroxyl moiety of Ser might be caused by the conversion step, since the reaction was not observed with the cyclization/cleavage reaction in the BF₃ reagent.[22] The repetitive yield, which was calculated by comparison of the fluorescence intensities of Pro³ and Pro⁸, was 71%. The low repetitive yield could be improved by the double coupling method with PITC.

The sequece and D/L-configuration determination of D-amino acid-containing peptide was carried out by employing HCl/

methanol as the conversion reagent, instead of 25% TFA, to suppress the racemization. An aliquot of the resulting MTBD-TH-amino acids was first subjected to the reversed-phase HPLC system for amino acid sequencing, and the other part of the sample was subjected to the chiral stationary phase HPLC system, since the separation of individual MTBD-TH-amino acid enantiomers on chiral stationary phases was not successful. Figure 10 shows the results of sequencing and D/L identification of [D-Ala²]-Deltorphin II (2 nmol), which was a neuroactive D-amino acid-containing peptide analogous to the dermorphin family.[2] The amino acid sequence and D/L-configuration of the peptide were easily determined except for the C-terminal amino acid, and the Ala residue (cycle 2) was identified as D-form, although partial racemization was observed with the proposed method.

One of the major problems concerning the use of fluorescence Edman reagents is loss of the peptide in the washing step, since most of the reagents themselves and their byproducts give fluorescence and require relatively highly polar solvent as the washing solvent. For example, in the use of FITC, the loss of peptide, particulary short peptide, was inevitable, since FITC is hydrophilic and required more polar solvent to remove excess reagents and their byproducts.[10] Thus, they were used for sequencing analysis of only relatively large proteins such as insulin chain B. On the other hand, MTBD-NCS was used for sequencing of short peptides composed of less than 10 amino acids, and all amino acid residues were identified except for the C-terminal amino acid. Therefore, when the reagent is applied to the automated sequencer, the loss of the peptide in the washing step can be greatly diminished. BF₃, the cyclization/cleavage reagent, was already applied to the automated sequencer using PITC in our previous study.[34] Thus, MTBD-NCS could be easily applicable to such an automated sequencer, which is expected to require less amount of sample peptides compared with the conventional PITC system, since the sensitivity for MTBD-TH-amino acids is higher than that for PTH-amino acids.

In conclusion, a method for amino acid sequence and D/L-configuration determination using the new fluorescence Edman reagent MTBD-NCS was demonstrated. The generated MTBD-TH-amino acids fluoresced strongly, whereas other degradation byproducts such as MTBD-TC or -CA-amino acids did not fluoresce, giving its superiority to others with regard to the detectability of TH-amino acids. The combined use of MTBD-NCS and an automated sequencer will give us the sensitive and simultaneous determination method of amino acid sequence and D/L-configuration determination of peptides or proteins.

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