Comparison of four fluorescence Edman reagents with benzofurazan structure for the detection of thiazolinone amino acid derivatives

Akira Toriba, Tomofumi Santa, Takayuki Iida and Kazuhiro Imai*

Graduate School of Pharmaceutical Sciences, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-0033, Japan

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Two newly synthesized fluorescence Edman reagents with the benzofurazan structure, 7-phenylsulfonyl-4-(2,1,3-benzoxadiazolyl) isothiocyanate (PSBD-NCS) and 7-methylsulfonyl-4-(2,1,3-benzoxadiazolyl) isothiocyanate (MSBD-NCS), were compared with 7-aminosulfonyl-4-(2,1,3-benzoxadiazolyl) isothiocyanate (ABD-NCS) and 7-*N,N*-dimethylaminosulfonyl-4-(2,1,3-benzoxadiazolyl) isothiocyanate (DBD-NCS) for peptide and protein sequence analysis by the generation of fluorescent 2,1,3-benzoxadiazolylthiazolinone (TZ)-amino acids. The effects of the substituent group at the *para* position to the isothiocyanate moiety of these reagents on the rate of the cyclization/cleavage reaction, the repetitive yield and the fluorescence quantum yield and stability of TZ amino acids were investigated. MSBD-TZ-amino acids were most sensitively detected and the detection limit for MSBD-TZ-Pro was 7 fmol (S/N = 3). ABD-NCS afforded the highest repetitive yield in the sequencing analysis. Fewer interfering peaks were observed in the chromatogram with DBD-NCS.

Introduction

Amino acid sequence analysis of peptides and proteins using phenyl isothiocyanate (PITC) was first reported by Edman;[1] *N*-terminal amino acids are derivatized with aryl isothiocyanate, cleaved and cyclized to thiazolinone (TZ)-amino acids with anhydrous acid, and then the TZ-amino acids are recyclized to aryl thiohydantoin (TH)-amino acids and identified by HPLC. Since then, various techniques have been introduced to improve the detection sensitivity.[2–4] Although the introduction of the gas-phase sequencers substantially reduced the amounts of sample peptides and proteins required, the automated sequencers are still unable to analyze peptides and proteins at the sub-picomole level.

On the other hand, various fluorescence Edman reagents have been reported to enhance the detection sensitivity of the final product, TH-amino acid. These include fluorescein isothiocyanate (FITC),[5,6] 4-{[(5-(dimethylamino)-1-naphthyl)sulfonyl]-amino}phenyl isothiocyanate (dansylamino-PITC)[7] and 4-(3-isothiocyanatopyrrolidin-1-yl)-7-(*N,N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole (DBD-PyNCS).[8] However, these reagents are not yet routinely utilized as compared with PITC, since (1) the bulkiness of the fluorophore lowers the derivatization and cleavage reaction yield, (2) the hydrophobic fluorophore interferes with the simple removal of excess reagents without loss of the sample peptides, (3) only about 50% of the generated TZ-amino acids are converted to TH-amino acids and (4) the strong fluorescence of the reagents themselves sometimes interferes with the detection of the generated TH-amino acids.

To overcome these disadvantages, we have recently reported a new Edman procedure using the fluorescence Edman reagent, 7-*N,N*-dimethylaminosulfonyl-4-(2,1,3-benzoxadiazolyl) isothiocyanate (DBD-NCS),[9–11] in which DBD-TZ-amino acids generated by the cleavage/cyclization reaction were detected fluorimetrically and the step of the conversion reaction to TH-amino acid was eliminated to simplify the sequencing process. Furthermore, DBD-NCS itself did not fluoresce and thus the interfering peak derived from the excess reagents did not interfere with the detection of the TZ-amino acids.

In a previous paper, we reported that the fluorescence of the generated 2,1,3-benzoxadiazolyl-TZ-amino acids was more intense when a stronger electron-withdrawing group is present at the *para* position to the isothiocyanate moiety.[10] DBD-TZ-amino acid, which had the strongest electron-withdrawing *para* substituent at that time, gave the strongest fluorescence intensity. Therefore, in this study, two new benzofurazan fluorescence Edman reagents with stronger electron-withdrawing substituents, which have larger Hammett substituent constants (σ_p)[12] than that of DBD-NCS, namely 7-phenylsulfonyl-4-(2,1,3-benzoxadiazolyl) isothiocyanate (PSBD-NCS) and 7-methylsulfonyl-4-(2,1,3-benzoxadiazolyl) isothiocyanate (MSBD-NCS), were synthesized and applied to sequencing analysis. Furthermore, we examined the effects of the *para*-substituent group on the fluorescence quantum yield and stability of TZ-amino acids, the rate of the cyclization/cleavage reaction and the repetitive yield of the sequencing analysis utilizing these fluorescence Edman reagents, PSBD-NCS, MSBD-NCS, DBD-NCS and 7-aminosulfonyl-4-(2,1,3-benzoxadiazolyl) isothiocyanate (ABD-NCS)[10,13] (Table 1).

Experimental

Materials

The following materials were employed: β-casomorphin-7 (bovine), Gly–Leu and Leu–Gly (Peptide Institute, Osaka, Japan); Pro–His–Leu (Backem, Bubenford, Switzerland); Tyr–Val, Pro–Leu, Phe–Gly and leucine (Leu) (Sigma, St. Louis, MO, USA); sequencer-grade trifluoroacetic acid (TFA) (Wako, Osaka, Japan); sequencer-grade pyridine (Tokyo Chemical Industry, Tokyo, Japan); and HPLC-grade acetonitrile (Kanto Chemical, Tokyo, Japan). Water was purified using a Milli-Q

system (Millipore, Bedford, MA, USA). All other reagents were of analytical-reagent grade and used without further purification.

Apparatus

The following apparatus was used for the identification of synthesized compounds. Melting-points were measured on a micro melting-point apparatus (Yanagimoto, Tokyo, Japan) and are uncorrected. Proton nuclear magnetic resonance (^{1}H NMR) spectra were obtained on a GSX-400 spectrometer (JEOL, Tokyo, Japan) with tetramethylsilane as the internal standard (abbreviations used: s, singlet; d, doublet; t, triplet; m, multiplet). Mass spectra were measured on an M-1200H mass spectrometer (Hitachi, Tokyo, Japan) with an atmospheric pressure chemical ionization system (APCI-MS). HPLC was carried out using an L-7100 intelligent pump an L-4000H UV detector, an L-7480 fluorescence detector and a D-7500 integrator (all from Hitachi).

2,1,3-Benzoxadiazolyl isothiocyanates

PSBD-NCS and MSBD-NCS were synthesized as described below and ABD-NCS and DBD-NCS were synthesized as described previously.[13]

4-Amino-7-phenylsulfonyl-2,1,3-benzoxadiazole

4-Amino-7-phenylthio-2,1,3-benzoxadiazole (180 mg), synthesized as described previously,[14] and 400 mg of *m*-chloroperoxybenzoic acid were dissolved in 5 ml of dichloromethane and the mixture was stirred at room temperature for 5 h. The reaction mixture was poured into 100 ml of 1 mol dm^{-3} Na_2CO_3 solution and extracted twice with 100 ml of dichloromethane. The organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was chromatographed on silica gel (dichloromethane) to afford 155 mg of the corresponding amine as a yellow powder, mp 216–217 °C. ^{1}H NMR ($CDCl_3$): δ 8.16 (2H, t), 8.11 (1H, d, J = 8.0 Hz), 7.49–7.56 (3H, m), 6.40 (1H, d, J = 8.0 Hz), 5.28 (2H, s, br). APCI-MS: *m/z* 276 ([M + H]$^+$).

7-Phenylsulfonyl-4-(2,1,3-benzoxadiazolyl) isothiocyanate (PSBD-NCS)

4-Amino-7-phenylsulfonyl-2,1,3-benzoxadiazole (150 mg) was dissolved in 15 ml of acetonitrile and 1.5 ml of 25% v/v thiophosgene in benzene was added slowly. The mixture was refluxed for 5 h. The reaction mixture was evaporated to dryness *in vacuo* and the residue was chromatographed on silica gel (ethyl acetate–hexane) to afford 50 mg of the corresponding isothiocyanate as a yellow powder, mp 149–150 °C. ^{1}H NMR ($CDCl_3$): δ 8.19–8.25 (3H, m), 7.56–7.64 (3H, m), 7.20 (1H, d, J = 8.0 Hz). APCI-MS: *m/z* 317 (M$^-$).

Table 1 Structures of *para*-substituted 2,1,3-benzoxadiazolyl isothiocyanates

Reagent[a]	R	Hammett constant (σ_p)
ABD-NCS	SO_2NH_2	0.60
DBD-NCS	$SO_2N(CH_3)_2$	0.65
PSBD-NCS	$SO_2C_6H_5$	0.68
MSBD-NCS	SO_2CH_3	0.72

[a] ABD-NCS = 7-aminosulfonyl-4-(2,1,3-benzoxadiazolyl) isothiocyanate; DBD-NCS = 7-*N,N*-dimethylaminosulfonyl-4-(2,1,3-benzoxadiazolyl) isothiocyanate; PSBD-NCS = 7-phenylsulfonyl-4-(2,1,3-benzoxadiazolyl) isothiocyanate; MSBD-NCS = 7-methylsulfonyl-4-(2,1,3-benzoxadiazolyl) isothiocyanate.

4-Amino-7-methylthio-2,1,3-benzoxadiazole

4-Methylthio-7-nitro-2,1,3-benzoxadiazole (180 mg), synthesized as described previously,[14] was dissolved in 5 ml of dichloromethane. After addition of 1 ml of concentrated hydrochloric acid, methanol was added to the mixture until it became homogeneous. After addition of 120 mg of iron powder, the mixture was stirred vigorously for 30 min. After the iron had been removed by filtration, the reaction mixture was poured into 100 ml of a 1 mol dm^{-3} NaOH solution and extracted twice with 100 ml of dichloromethane. The organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was chromatographed on silica gel (dichloromethane–hexane) to afford of 50 mg of the corresponding amine as a red powder, mp 104–105 °C. ^{1}H NMR ($CDCl_3$): δ 7.15 (1H, d, J = 8.0 Hz), 6.32 (1H, d, J = 8.0 Hz), 4.54 (2H, s, br), 2.57 (3H, s). APCI-MS: *m/z* 180 ([M − H]$^-$)

4-Amino-7-methylsulfonyl-2,1,3-benzoxadiazole

4-Amino-7-methylthio-2,1,3-benzoxadiazole (46 mg) and 150 mg of *m*-chloroperoxybenzoic acid were dissolved in 5 ml of dichloromethane and the mixture was stirred at room temperature for 5 h. The reaction mixture was poured into 100 ml of 1 mol dm^{-3} Na_2CO_3 solution and extracted twice with 100 ml of dichloromethane. The organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was chromatographed on silica gel (dichloromethane) to afford of 39 mg of the corresponding amine as a deep orange powder, mp 238–239 °C. ^{1}H NMR [$CDCl_3$–$(CD_3)_2SO$ (9 + 1, v/v)]: δ 7.95 (1H, d, J = 8.0 Hz), 6.42 (1H, d, J = 8.0 Hz), 4.76 (2H, s, br), 3.26 (3H, s). APCI-MS: *m/z* 212 ([M − H]$^-$).

7-Methylsulfonyl-4-(2,1,3-benzoxadiazolyl) isothiocyanate (MSBD-NCS)

4-Amino-7-methylsulfonyl-2,1,3-benzoxadiazole (35 mg) was dissolved in 15 ml of acetonitrile and 1.5 ml of 25% v/v thiophosgene in benzene was added slowly. The mixture was refluxed for 5 h. The reaction mixture was evaporated to dryness *in vacuo* and the residue was chromatographed on silica gel (ethyl acetate–hexane) to afford 10 mg of the corresponding isothiocyanate as a yellow powder, mp 148–149 °C. ^{1}H NMR ($CDCl_3$): δ 8.14 (1H, d, J = 8.0 Hz), 7.24 (1H, d, J = 8.0 Hz), 3.83 (3H, s). APCI-MS: *m/z* 255 (M$^-$).

HPLC conditions

2,1,3-Benzoxadiazolyl-TZ-amino acids were separated using two columns in tandem, *i.e.*, an ODS column (TSK gel ODS-80Ts, 250 × 4.6 mm id, 5 μm; Tosoh, Tokyo, Japan) and a phenyl function bonded porous silica gel column (YMC-Pack Ph, 250 × 4.6 mm id, 5 μm; YMC, Kyoto, Japan) for sequencing analysis. Isocratic elution of TZ-amino acids was employed with acetonitrile–water (50 + 50, v/v for ABD-TZ-amino acids, 55 + 45 v/v for MSBD-TZ-amino acids; 60 + 40 v/v for DBD-TZ-amino acids and 65 + 35 v/v for PSBD-TZ-amino acids) containing 10 mmol dm^{-3} formic acid at a flow

rate of 0.5 ml min^{-1}. Fluorescence detection was performed at the maximum excitation and emission wavelength for the respective TZ-Leu (see Table 2).

TZ-amino acids and 2,1,3-benzoxadiazolylthiocarbamoylated (TC)-amino acids were also determined under the following conditions (RP-HPLC): eluents A and B containing 10 mmol dm^{-3} formic acid were 30 + 70 v/v and 70 + 30 v/v acetonitrile–water, respectively, and the analytical column was ODS-80Ts using gradient elution from 0 to 55 min (eluent B composition, 0–100 %) at a flow rate of 1.0 ml min^{-1}.

Preparation of standard 2,1,3-benzoxadiazolyl-TZ-amino acids

Dipeptides were dissolved in pyridine–water (1 + 1 v/v) (0.2–2.0 mmol dm^{-3}). A 100 nmol amount of a benzoxadiazolyl isothiocyanate (ABD-NCS, DBD-NCS, PSBD-NCS or MSBD-NCS) was dissolved in 20 μl of the solution and the mixture was vortex mixed and heated at 50 °C for 20 min. After the coupling reaction, the excess reagent and by-products were removed by washing three times with 200 μl of heptane–dichloromethane (4 + 1 v/v). The aqueous phase was evaporated to dryness using a centrifugal evaporator (SPE-200; Shimadzu, Kyoto, Japan) at 50 °C for 15 min and 40 μl of trifluoroacetic acid (TFA) were added to the residue. The mixture was heated at 50 °C for 10 min and dried under a stream of nitrogen. After the cleavage/cyclization reaction, 20 μl of water were added to the residue and the solution was extracted twice with 100 μl of ethyl acetate. The combined organic phase was dried under a stream of nitrogen. The resulting residue was dissolved in the HPLC eluent and immediately analyzed.

Characteristics of 2,1,3-benzoxadiazolyl-TZ-amino acids

TZ-Leu prepared as described above was collected by RP-HPLC and dried in a centrifugal evaporator at 50 °C. The residue was dissolved in acetonitrile and the solution was subjected to LC-MS. The fluorescence intensity and UV absorption of the solution were measured with a spectrofluorimeter (F-4010; Hitachi) and a spectrophotometer (Ubest 50; JASCO, Tokyo, Japan), respectively.[10] Further, TZ-Leu fractions were subjected to conversion reaction and hydrolysis to obtain 2,1,3-benzoxadiazolyl-TH-Leu and -TC-Leu, respectively. The molecular masses and UV spectra of the TH- and TC-Leu fractions were measured.[9]

Stability of 2,1,3-benzoxadiazolyl-TZ-amino acids

The residue of TZ-Leu prepared as described above was dissolved in acetonitrile–water (1 + 1 v/v) and kept at room temperature. Aliquots of the solution were subjected to HPLC at appropriate time intervals. The observed rate constant (k_{obs}) for the disappearance of TZ-Leu was calculated as described previously.[9]

Table 2 Characteristics of 2,1,3-benzoxadiazolyl-TZ-Leu

Derivative	m/z^a	λ_{max}/nm	λ_{em}/nm	Ratio[b]
ABD-TZ-Leu	370	384	522	1880
DBD-TZ-Leu	398	386	524	380
PSBD-TZ-Leu	431	483	525	2100
MSBD-TZ-Leu	369	386	519	3630

[a] Measured by APCI-MS. [b] Ratio of fluorescence intensity (λ_{em}) to UV absorption (λ_{max}).

Coupling reaction with benzoxadiazolyl isothiocyanate

Leu was dissolved in pyridine–water (1 + 1 v/v) (0.1 mmol dm^{-3}). A 100 nmol amount of a benzoxadiazolyl isothiocyanate (ABD-NCS, DBD-NCS, PSBD-NCS or MSBD-NCS) was dissolved in 20 μl of the solution and the mixture was vortex mixed and heated at 50 °C, samples being withdrawn after appropriate time intervals. After the coupling reaction, 50 μl of 1 mol dm^{-3} hydrochloric acid solution were added to the mixture and the solution was extracted twice with 100 μl of ethyl acetate. The combined organic phase was dried under a stream of nitrogen and dissolved in the HPLC eluent. The generated TC-Leu was subjected to RP-HPLC and the UV detection was carried out at 385 nm.

Cyclization/cleavage reaction by TFA

Pro–His–Leu was dissolved in pyridine–water (1 + 1 v/v) (0.1 mmol dm^{-3}). The 2,1,3-benzoxadiazolyl-TC derivatives of Pro–His–Leu were prepared by reaction with ABD-NCS, DBD-NCS, PSBD-NCS and MSBD-NCS in a manner similar to that described above. TFA was added to the residue and the mixture was heated at 50 °C, samples being withdrawn after appropriate time intervals. The resulting TZ-Pro was subjected to RP-HPLC.

Liquid phase sequencing of β-casomorphin-7 with 2,1,3-benzoxadiazolyl isothiocyanates

The peptide was dissolved in pyridine–water (1 + 1 v/v) (0.1 mmol dm^{-3}). A 100 nmol amount of a benzoxadiazolyl isothiocyanate (ABD-NCS, DBD-NCS, PSBD-NCS or MSBD-NCS) was dissolved in 20 μl of the solution and the mixture was vortex mixed and heated at 50 °C for 20 min. After the coupling reaction, the excess reagent and by-products were removed by washing three times with 200 μl of heptane–dichloromethane. As the sequencing cycle proceeded, the dichloromethane concentration was decreased, *i.e.*, those in cycles 1, 2, 3, 4, 5 and 6 were 20, 10, 5, 0, 0 and 0% v/v, respectively. The aqueous phase was evaporated to dryness using a centrifugal evaporator at 50 °C for 15 min and 40 μl of TFA were added to the residue. The mixture was heated at 50 °C for 10 min and dried under a stream of nitrogen. After the cleavage/cyclization reaction, 20 μl of water were added to the residue and the solution was extracted twice with 100 μl of ethyl acetate. 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. The resulting residue was dissolved in 1 ml of the HPLC eluent and 0.5% of the solution was subjected to HPLC.

Results and discussion

Characteristics of 2,1,3-benzoxadiazolyl-TZ-amino acids

The molecular masses, fluorescence spectra and UV spectra of four TZ-Leu fractions isolated are summarized in Table 2. The identification of TZ-Leu was carried out not only by the molecular mass but also by the absence of a UV absorption maximum at 266 nm that can be assigned to the TH ring since TH derivatives have the same molecular mass as TZ derivatives.

To compare the fluorescence quantum yields of TZ-Leu derivatives, we used the ratio of fluorescence intensity (at λ_{em}) to absorbance (at λ_{max})[10] as shown in Table 2. The ratios for PSBD-TZ-Leu and MSBD-TZ-Leu were about six and ten times higher than that of DBD-TZ-Leu, respectively, and it was

noted that the fluorescence quantum yield of TZ-amino acid increases with increasing electron-withdrawing activity, as was expected. With all the reagents, TH-Leu and TC-Leu derivatives gave no fluorescence.

Stability of 2,1,3-benzoxadiazolyl-TZ-amino acids

In an aprotic solvent such as acetonitrile, DBD-TZ-amino acids were extremely stable in comparison with a protic solvent such as water or methanol.[9] Hence TZ-amino acids were stored in acetonitrile. Next, the stability of TZ-amino acids in the HPLC eluent, acetonitrile–water (1 + 1 v/v), at room temperature was examined. Fig. 1 shows the relationship between σ_p of the *para*-substituent group of the isothiocyanate moiety and the observed rate constant (k_{obs}) for the disappearance of TZ-Leu. A linear relationship between σ_p and k_{obs} for the disappearance of TZ-Leu was obtained. An electron-withdrawing group is considered to promote the hydrolysis of TZ ring to the corresponding TC-amino acids. However, even the MSBD-TZ-amino acid containing the largest σ_p *para*-substituent group did not cause any problems in the sequencing analysis, since the $t_{1/2}$ of MSBD-TZ-Leu was 3.7 h, which was sufficient for HPLC detection.

Coupling reaction with 2,1,3-benzoxadiazolyl isothiocyanate

Four benzoxadiazolyl isothiocyanates were reacted with Leu and the reaction mixtures were subjected to RP-HPLC at intervals to observe the effects of a substituent group on the coupling reaction. The reaction was nearly completed in about 20 min and no difference in the reactivity of the benzoxadiazolyl isothiocyanates examined was observed under these conditions (Fig. 2).

Cyclization/cleavage reaction by TFA

We have reported that the use of an aprotic acid, boron trifluoride (BF_3), facilitated the cyclization/cleavage reaction and provided higher yields of TZ-amino acids than TFA without racemization of the chiral center of amino acid.[11,16–18] We used TFA since it has usually been used as the cyclization/cleavage reagent and the racemization of TZ-amino acids was not examined in this study. In order to observe the effect of the substituent group on the reaction, the time course of the yield of the TZ-Pro generated from TC-Pro–His–Leu was examined, since the *N*-terminal Pro–His linkage of a peptide is known to be particularly resistant to cleavage.[2]

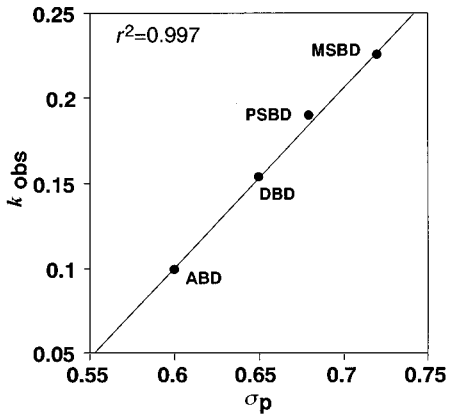

Fig. 1 Relationship between the Hammett *para*-substituent constants (σ_p) of benzoxadiazolyl isothiocyanates and the observed rate constants (k_{obs}) for the disappearance of TZ-Leu. The σ_p values of the *para*-substituent groups are taken from ref. 12.

The production of MSBD-TZ-Pro and PSBD-TZ-Pro required a longer reaction time, since MSBD and PSBD bear a strong electron-withdrawing substituent group and the electron density at the sulfur atom seems to be small[15] (Fig. 3). The yield of TZ-Pro decreased after attainment of the maximum yield. This was presumably due to the conversion to TH-Pro by trace amounts of contaminant water and/or decomposition of the generated TZ-Pro in TFA. The yield of other TZ-amino acids decreased significantly with the reaction time, completely cleaving the respective Pro–His linkage. Therefore, a reaction time of 10 min, which did not cause carryover and a decrease in the repetitive yield in sequencing analysis, was selected in subsequent work.

Detection of 2,1,3-benzoxadiazolyl-TZ-amino acids

In general, the fluorescence intensity of compounds containing a benzofurazan moiety increases with increasing the content of organic solvent in the HPLC eluent. Furthermore, TZ-amino acids were extremely stable in an aprotic solvent such as acetonitrile. Therefore, two analytical columns, *i.e.*, an ODS column and a phenyl bonded silica gel column, were used in tandem as in previous work.[9] PSBD-TZ-amino acids were the most hydrophobic and were strongly retained on the columns with the eluent of the highest acetonitrile content. It is worth

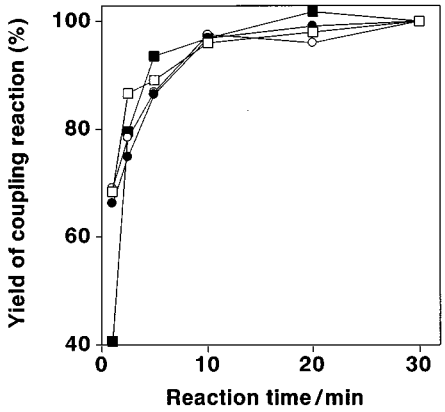

Fig. 2 Time course for the coupling reaction of Leu (2 nmol) with benzoxadiazolyl isothiocyanates at 50 °C. The yield of the coupling reaction at 50 °C after 30 min was taken as 100%. (●) ABD-NCS; (□) DBD-NCS; (■) PSBD-NCS and (○) MSBD-NCS.

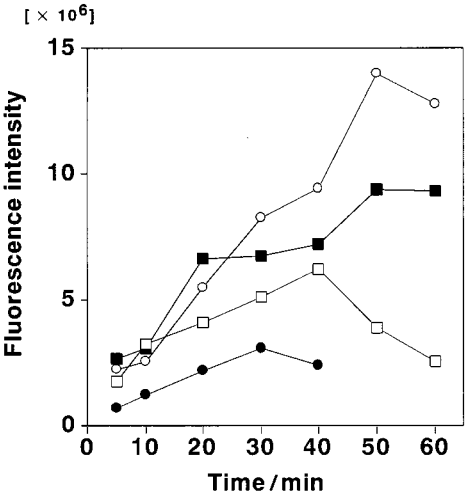

Fig. 3 Time course for the cyclization/cleavage reaction of 2,1,3-benzoxadiazolyl-TC-Pro–His–Leu at 50 °C. (●) ABD-NCS; (□) DBD-NCS; (■) PSBD-NCS; and (○) MSBD-NCS. The 2,1,3-benzoxadiazolyl-TC derivatives of Pro–His–Leu were prepared by the reaction of Pro–His–Leu (2 nmol) with benzoxadiazolyl isothiocyanates.

noting that TZ-Pro, particularly MSBD-TZ-Pro, was most sensitively detected among the TZ-amino acids. The detection limits for MSBD-TZ-Pro, PSBD-TZ-Pro, DBD-TZ-Pro and ABD-TZ-Pro were 7, 10, 15 and 30 fmol (S/N = 3), respectively, and those of the other TZ-amino acids were from 0.1 pmol to the sub-picomole level. The suggestion that TZ-amino acids containing an electron-withdrawing group would be more sensitively detected was confirmed. Since the most sensitive reagents previously reported were FITC (10 fmol)[6] and DBD-PyNCS (20 fmol),[8] it appears that the sensitivity of the TZ derivatives is slightly better than or comparable to them.

Liquid phase sequencing of β-casomorphin-7 with 2,1,3-benzoxadiazolyl isothiocyanate

β-Casomorphin-7 was adopted as a model peptide in this study. β-Casomorphin-7 is a short peptide composed of hydrophobic amino acid residues and would be easily lost in the washing step, hence being suitable for the comparison of the repetitive yields of the sequencing analysis with these reagents. Fig. 4 shows the results of the sequence analysis using ABD-NCS, DBD-NCS and MSBD-NCS. From the chromatograms obtained in the respective cycles, the peptide sequence was identified as Tyr–Pro–Phe–Pro–Gly–Pro, whereas the *C*-terminal amino acid, Ile, was not detected, presumably owing to the production of TC- or TH-Ile. As shown in Table 3, ABD-NCS gave the highest repetitive yield, which was calculated by comparison of the fluorescence intensities of Pro^2 and Pro^6, among these three reagents. These results suggest that the

Table 3 Analysis of peptides by the manual sequencing method with 2,1,3-benzoxadiazolyl isothiocyanates

Reagent	Repetitive yield (%)[a]
ABD-NCS	83
DBD-NCS	80
MSBD-NCS	76

[a] Mean values from 2 or 3 experiments.

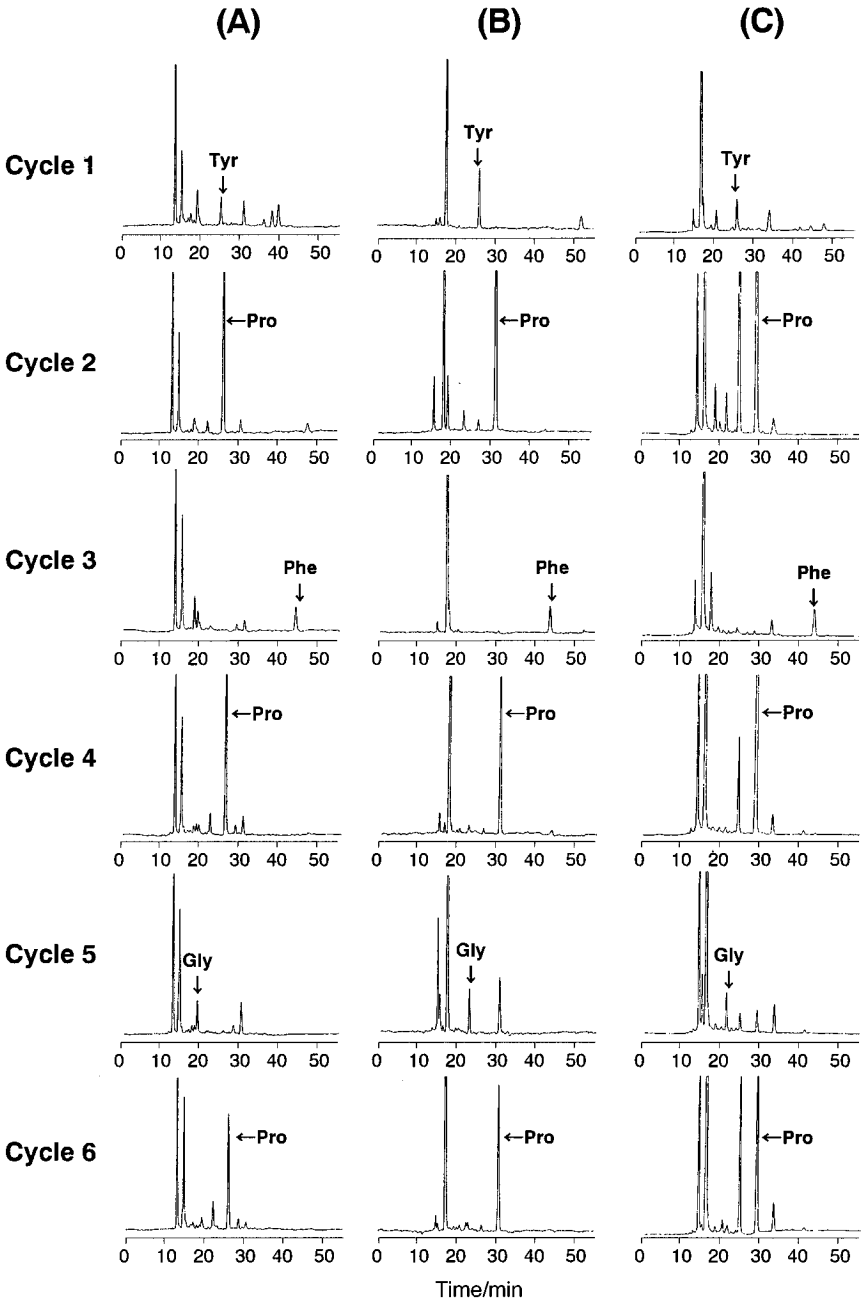

Fig. 4 Chromatograms obtained from sequencing analysis of β-casomorphin-7 (2 nmol) with (A) ABD-NCS, (B) DBD-NCS and (C) MSBD-NCS.

repetitive yields might be lowered with increase in the electron-withdrawing activity of the substituent group, although the differences in the repetitive yields were not large.

When the Pro residue was determined (cycles 2, 4, and 6) in sequencing with MSBD-NCS [Fig. 4(C)], an unknown peak (t_R = 24.3 min) derived from MSBD-TZ-Pro increased during the cyclization/cleavage reaction. This suggests that MSBD-TZ-Pro was significantly unstable in TFA. MSBD-NCS, which gave the most fluorescent TZ-amino acids but gave a slightly low repetitive yield, is considered to be applicable to the identification of the trace amounts of a short peptide. The Gly (cycle 5) and Pro (cycle 6) in the sequence analysis using PSBD-NCS afforded a small amount of the corresponding TZ-amino acids, and the repetitive yield calculated from the fluorescence intensities of Pro^2 and Pro^4 was 40% (data not shown). It is considered that the loss of the peptide in the washing step was significant owing to the hydrophobicity of the PSBD-TC-peptide. There seem to be a few disadvantages for DBD-NCS with regard to the stability and sensitivity of TZ-amino acids, the rate of the cyclization/cleavage reaction and the repetitive yield. Furthermore, fewer interfering peaks were observed in the chromatograms compared with other reagents.

In conclusion, the *para*-substituent group of benzoxadiazolyl isothiocyanate was demonstrated to affect the fluorescence quantum yield, stability and retention in the reversed-phase HPLC of TZ-amino acids, the rate of the cyclization/cleavage reaction and the hydrophobicity of the reagent. DBD-NCS was the most appropriate reagent, and the development of a double coupling method with PITC and an automated sequencing method is in progress.

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