

Generation of an *S*-Peptide via an *N–S* Acyl Shift Reaction in a TFA Solution

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An *N–S* acyl shift reaction of thiol-containing peptides in a trifluoroacetic acid (TFA) solution was confirmed by a combination of ^{13}C NMR spectroscopy, reversed-phase (RP) HPLC, and MS analyses. A model peptide containing a cysteine residue was transformed into an *S*-peptide in a TFA solution. The *S*-peptide was quickly transformed to the original peptide during RP-HPLC analysis even when an eluent that contained 0.1% TFA was used. A peptide that had an *N*-2-mercapto-4,5-dimethoxybenzyl (Dmmb) group was also transformed into an *S*-peptide, forming a thioester bond with the thiol of the Dmmb group by TFA treatment. The generated *S*-peptide was isolated by directly injecting the reaction mixture into a RP-HPLC and was readily converted to the corresponding 2-sulfoethyl thioester via an intermolecular thiol exchange reaction with sodium 2-mercaptoethanesulfonate. The 2-sulfoethyl peptide thioester is widely used as a building block in polypeptide synthesis. The *N–S* acyl shift reaction observed in peptides with or without an auxiliary group provides a new route for the preparation of peptide thioesters.

N–S acyl shift reactions may occur at a cysteine residue in peptides to some extent under acidic conditions. However, the *N–S* acyl shift reaction has not been studied in detail. In 1966, Sakakibara et al. referred to a possible *N–S* acyl shift reaction at a cysteine residue during the treatment of a protected cysteine-containing peptide with anhydrous hydrogen fluoride.[1]

Vizzavona et al. recently reported that a peptide, prepared by extended chemical ligation using an *N*-2-mercapto-4,5-dimethoxybenzyl (Dmmb) group as an auxiliary,[2] generated an earlier eluting compound on reversed-phase (RP) HPLC when the ligated peptide was treated with a trifluoroacetic acid (TFA) cleavage solution.[3] They attempted to analyze this more polar compound but failed. Judging from the chemical behavior of the compound, they presumed that it was probably an *S*-peptide that formed a thioester bond with the thiol group in the auxiliary (Fig. 1).

Peptide thioesters are used as building blocks in protein synthesis. An *N–S* acyl shift reaction is a key step in the protein splicing reaction,[4,5] which has been successfully applied to the biological preparation of peptide thioesters.[6] If it were possible to achieve an *N–S* acyl shift reaction of a peptide bond triggered by chemical process such as TFA treatment, such a reaction would represent a new method for preparing a peptide thioester as well as for the chemical modification of proteins.

In a previous report,[7] we described a new procedure for the preparation of peptide thioesters, in which a TFA-treated peptide resin that contained a Dmmb group-attached amide bond released a peptide thioester in the presence of a thiol compound, such as 2-mercaptoethanesulfonic acid.

In the present paper, we provide evidence to show that a thioester bond is formed via an *N–S* acyl shift reaction in a TFA solution at a cysteine residue and also at the Dmmb group-attached amide bond in peptides. We also report the details of the *N–S* acyl shift reaction at a cysteine residue in a peptide. Furthermore, we show that a 2-sulfoethyl thioester is formed in the reaction of the TFA-treated Dmmb group-attached peptide with sodium 2-mercaptoethanesulfonate.

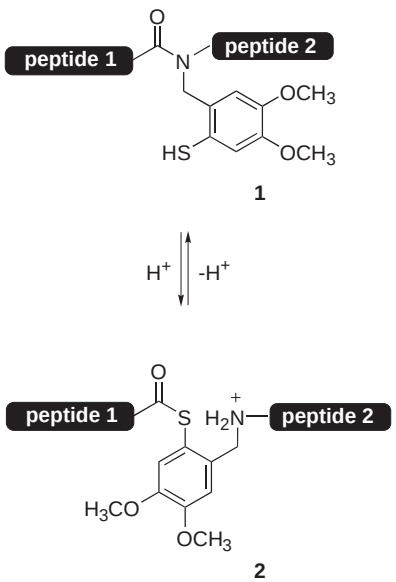

Fig. 1. Possible *S*-peptide formation under acidic conditions.

Results and Discussion

Analysis of the Compound Generated from a Cysteine-Containing Peptide in a TFA Solution. Fmoc–Ile–Ala–

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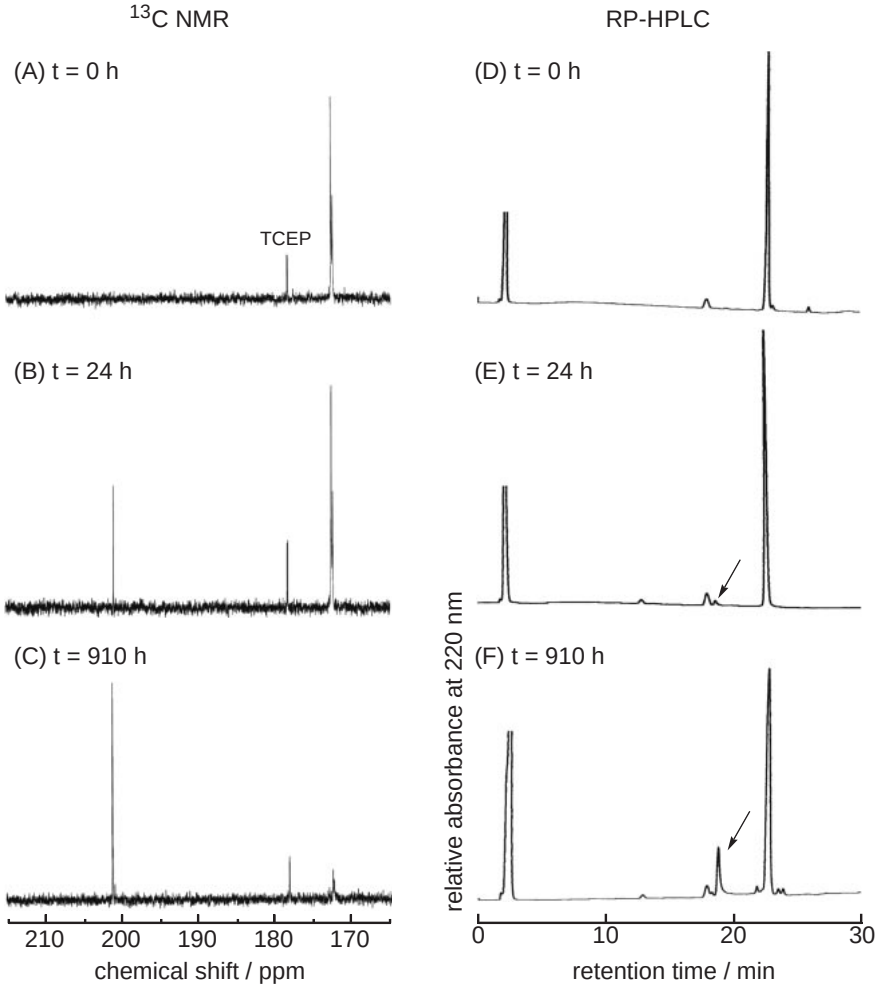

Fig. 2. Analysis of the product generated from Fmoc–Ile–Ala–Gly(1-^{13}C)–Cys–Arg–NH_2 (**3**) in a TFA solution containing $CDCl_3$ (29%) and TCEP (0.5%) (*v*/*v*/*w*). (A), (B), and (C): ^{13}C NMR spectra of peptide **3** in the TFA solution after 0-h, 24-h, and 910-h storage, respectively. (D), (E), and (F): RP-HPLC elution profiles of peptide **3** in the TFA solution after 0-h, 24-h, and 910-h storage, respectively. The arrow indicates the peak at the retention time of 18.5 min. HPLC conditions; column: YMC-Pack ProC18 (4.6 × 150 mm), eluent: linear gradient of acetonitrile in 0.1% TFA from 20 to 50% over 30 min, flow rate: 1.0 mL min^{-1}.

Gly(1-^{13}C)–Cys–Arg–NH_2 (**3**) was prepared by 9-fluorenylmethoxycarbonyl (Fmoc) solid-phase peptide synthesis (SPPS) and purified by RP-HPLC after cleavage of the peptide using Reagent B (TFA/phenol/water/triisopropylsilane, 88/5/5/2, *v*/*v*/*v*/*v*),[8] and the formulation was confirmed by electron spray ionization mass spectrometry (ESI-MS) and amino acid analysis after acid hydrolysis. Peptide **3** was dissolved in a solution containing TFA (71%), $CDCl_3$ (29%), and tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (0.5%) (*v*/*v*/*w*). The progress of the reaction was monitored by using ^{13}C NMR spectroscopy. An aliquot of the peptide solution was subjected to RP-HPLC analysis, and the eluted solutions with a UV absorbance at 220 nm were collected. The molecular mass number of the material in each fraction was determined by ESI-MS. In the ^{13}C spectrum, signals were observed at 172.5 and 172.7 ppm corresponding to the carbonyl carbon of the amide bond just after the peptide was dissolved in a TFA solution, as shown in panel A in Fig. 2. Panel D shows the RP-HPLC elution profile for the same sample. A major peak that was eluted at 22.8 min had exactly the same retention time and molecular mass number as those of peptide **3**. After 24 h treatment of peptide **3** with a TFA solution, a new signal appeared at 201.8 ppm. This chemical shift was assigned to the carbonyl carbon of a thioester. The signal intensity ratio of the carbonyl carbons between the amide and thioester was about 11 to 1. An RP-HPLC analysis of the sample that was treated for 24 h showed a new peak with a retention time of 18.5 min, indicated by an arrow in panel E. The peptide corresponding to this new peak had the same mass number as that of peptide **3**. However, the proportion of the peak area toward that of peptide **3** was less than one fiftieth. The proportion of the ^{13}C-signal intensity between the amide carbonyl carbon and the thioester reached a nearly constant value of 1 to 4 after the storage of peptide **3** in a TFA solution for 910 h. However, the proportion of the newly generated peak area to that of peptide **3** was still less than a fifth in RP-HPLC. These results indicate that the newly generated compound is *S*-peptide **4**, as shown in Fig. 3, which would be formed via an *N*–*S* acyl shift reaction, and that peptide **4** is rapidly transformed to the original peptide even in a solution that contains 0.1 vol % of TFA in aqueous acetonitrile. Furthermore, 80% of the total peptide was *S*-peptide **4** at equilibrium in a mixed solvent of TFA (71%), $CDCl_3$ (29%), and

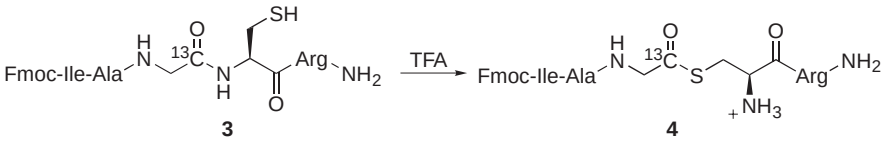

Fig. 3. *S*-Peptide formation via an *N*–*S* acyl shift reaction of peptide **3** in a TFA solution.

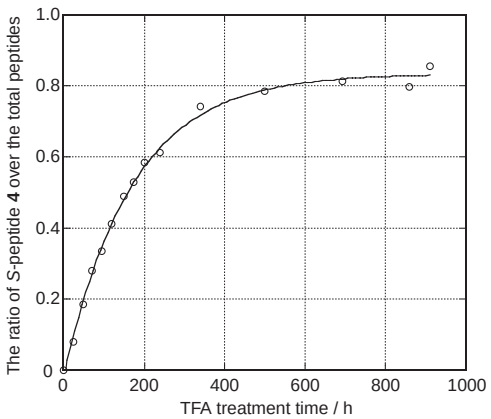

Fig. 4. Time course of the ratio of *S*-peptide **4** over the total peptides in a mixture of TFA (71%), $CDCl_3$ (29%), and TCEP (0.5%) (*v*/*v*/*w*).

TCEP (0.5%) (*v*/*v*/*w*) as shown in Fig. 4.

Analysis of the Compound Generated from a Dipeptide Having a Dmmb Group on Amide Bond in a TFA Solution. Fmoc–Gly(1-^{13}C)–D,L-(Dmmb)Ala–OMe (**8**) was prepared as a model compound in an attempt to detect the thioester form of the model peptide by ^{13}C NMR and RP-HPLC. Peptide **8** was prepared according to the scheme shown in Fig. 5 in an overall yield of 27%.

Peptide **8** was dissolved in a mixture of TFA (86%), $CDCl_3$ (14%), and TCEP (0.5%) (*v*/*v*/*w*). The solution was subjected to ^{13}C NMR and RP-HPLC analyses. The ^{13}C signal of the glycine(1-^{13}C) in peptide **8** was observed at 172.8 ppm (Fig. 6A). Peptide **8** was eluted at 21.5 min by RP-HPLC (Fig. 6D). After storage of the TFA solution for 24 h, new ^{13}C signals at 204.6 and 205.3 ppm appeared (Fig. 6B). After 48 h, the ^{13}C signal of the amide in peptide **8** had nearly disappeared (Fig. 6C). The new signals at 204.6 and 205.3 ppm are consistent with the chemical shift of a carbonyl carbon of a thiophenyl ester. These results indicate that a carbonyl carbon in the amide bond of peptide **8** formed a thioester with the thiol in the Dmmb group via an *N*–*S* acyl shift reaction. The two signals at 204.6 and 205.3 ppm indicate that two conformers are present in the NMR time scale, the ratio of which changed depending on the temperature of the TFA solution.

On RP-HPLC analysis, new peaks with retention times of 12.3 and 14.2 min appeared after storage of the peptide solution for 24 h (Fig. 6E). On ESI-MS analysis, the molecular mass number of the compound in the fraction eluting at 12.3 min was the same as that of amide **8**. The compound that was eluted earlier than peptide **8** on RP-HPLC must be the compound generated via the same reaction as was observed by Vizzavona et al.[3] Thus, the compound must be **9** (Fig. 7). The molecular mass number of the compound in the fraction eluting at 14.2 min coincided with that of Fmoc–Gly(1-^{13}C)–D,L-Ala–OMe (**10**). This compound was produced via acidolysis of the Dmmb group. The ^{13}C-signal at 173.4 ppm is assigned as the signal of the amide carbonyl carbon of **10**.

After a 48 h storage, the ratio of ^{13}C signal intensities became nearly constant. Judging from the ^{13}C signal intensities, the ratio of compounds **8** to **9** in the TFA solution was 1 to 4 (Fig. 6C). The RP-HPLC elution profile of the sample stored for 48 h showed roughly the same ratio of compounds **8** to **9** (Fig. 6F). This indicates that the thioester **9** is much more stable than the thioester produced from the –Gly–Cys– sequence.

Reactivity of Compound 9. To obtain additional evidence that compound **9** is, in fact, a thioester, the fraction at 12.3 min was isolated, freeze-dried and mixed with sodium 2-mercaptoethanesulfonate in a neutral buffer. The reaction mixture was subjected to RP-HPLC after 2 h, and the fraction was analyzed by ESI-MS. Mass analysis indicated that the thioester,

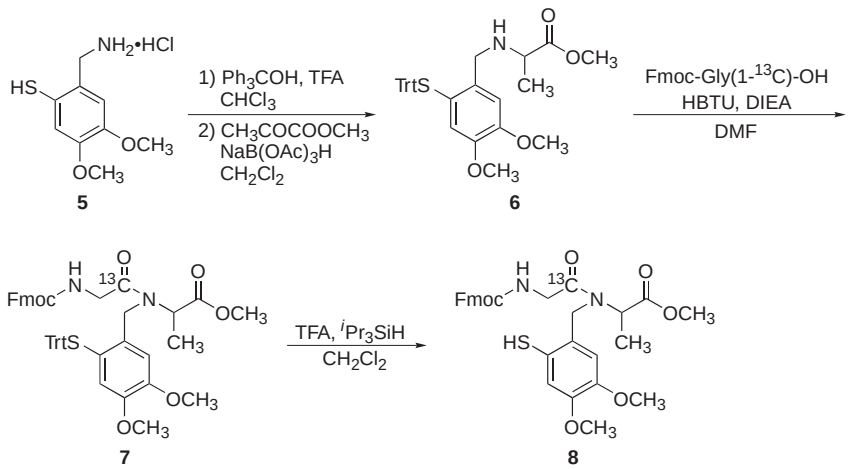

Fig. 5. Synthetic route of ^{13}C-labeled peptide **8**.

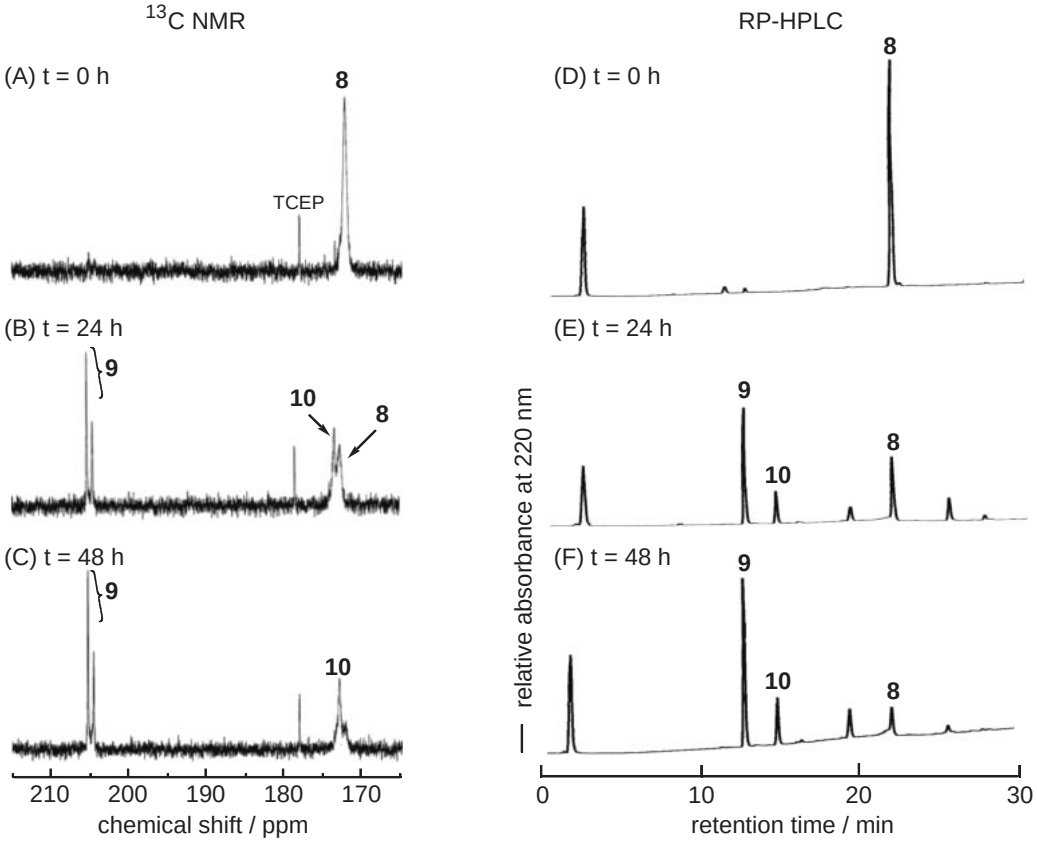

Fig. 6. Analysis of the product generated from peptide **8** in a TFA solution containing $CDCl_3$ (14%) and TCEP (0.5%) ($v/v/w$). (A), (B), and (C): ^{13}C NMR spectra of peptide **8** in the TFA solution after 0-h, 24-h, and 48-h storage, respectively. (D), (E), and (F): RP-HPLC elution profiles of peptide **8** in the TFA solution after 0-h, 24-h, and 48-h storage, respectively. HPLC conditions; column: YMC-Pack ProC18 (4.6 × 150 mm), eluent: linear gradient of acetonitrile in 0.1% TFA from 30 to 90% over 30 min, flow rate: 1.0 mL min^{-1}.

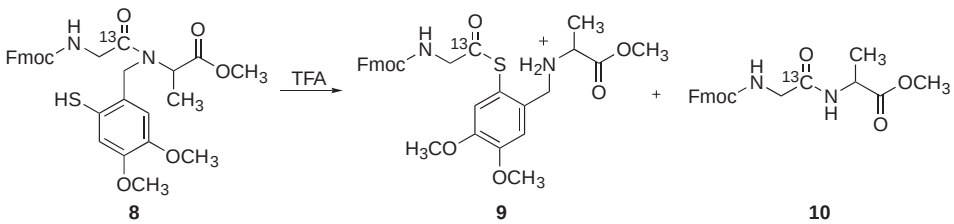

Fig. 7. *S*-Peptide formation from peptide **8** in a TFA solution. The Dmmb group was partly cleaved by acidolysis.

Fmoc–Gly(1-^{13}C)–$SCH_2CH_2SO_3H$, was present. At the same time, peptide **8** was detected by RP-HPLC. These observations indicate that compound **9** is a thioester. Thus, it can be safely concluded that the Dmmb group mediates thioester-bond formation via an *N–S* acyl shift reaction in the TFA solution.

Preparation of a Peptide Thioester from a Peptide Having a Dmmb Group on Its Backbone Amide. Val–Ala–Val–Phe–Val–Gly(1-^{13}C)–D,L-(Dmmb)Ala–NH_2 (**13**) was prepared according to the scheme shown in Fig. 8. Fmoc–Gly(1-^{13}C)–D,L-[Dmmb(Trt)]Ala–OH (**11**), obtained by the hydrolysis of peptide **7**, was introduced to 4-(α-amino-2,4-dimethoxybenzyl)phenoxymethyl polyethylene glycol polystyrene resin (H_2N–SAL–PEG resin). Peptide chain elongation was carried out by using Fmoc SPPS to give Val–Ala–Val–Phe–Val–Gly(1-^{13}C)–D,L-[Dmmb(Trt)]Ala–NH–SAL–PEG resin (**12**). Peptide **13** was obtained by treating resin **12** with Reagent B, followed by purification by RP-HPLC, in an 18% yield, which was based on the amino group in the Fmoc–NH–SAL–PEG resin. Peptide **13** was eluted at 23.8 min as an overlapping peak, reflecting D,L-Ala.

Peptide **13** was treated with TFA containing 0.5% TCEP (w/v) for 30 h (Fig. 9A). The newly generated peptide **14**, which was eluted at 18.9 min, with a molecular mass number of 844.3 was isolated by injecting the reaction mixture into a RP-HPLC. After adding sodium 2-mercaptoethanesulfonate to the isolated fraction, it was lyophilized. The resulting powder was dissolved in a mixture of sodium phosphate buffer (pH 7.8) and acetonitrile (1:1, v/v), and analyzed by RP-HPLC after stirring for 2 h (Fig. 9B). The peptide thioester, Val–Ala–Val–Phe–Val–Gly(1-^{13}C)–$SCH_2CH_2SO_3H$ (**15**), was

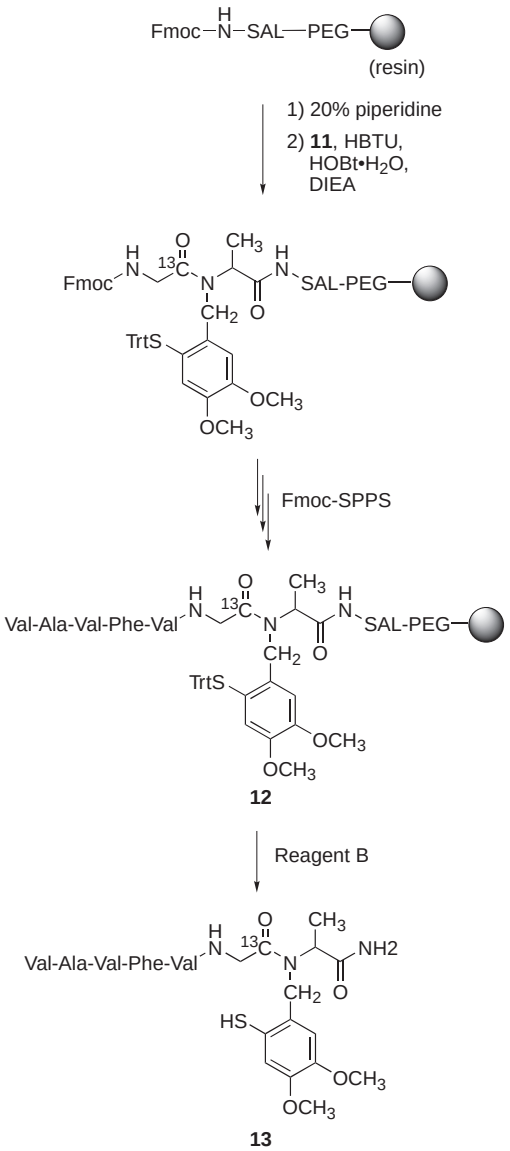

Fig. 8. Synthetic route of Dmmb containing peptide **13**.

eluted at 17.5 min, and the formulation was confirmed by ESI-MS. The peptide thioester was isolated by RP-HPLC, and the fraction containing **15** was freeze-dried. This peptide thioester **15** readily reacted with cysteine methyl ester in a solution of Tris-tricine buffer (pH 8.2) and acetonitrile (1:1, *v*/*v*) to yield Val–Ala–Val–Phe–Val–Gly(1-^{13}C)–Cys–OMe (**16**) (Fig. 9C).

Conclusion

An *N*–*S* acyl shift reaction occurs in a cysteine-containing peptide and peptides with a thiol-containing ligation auxiliary in a TFA solution. A peptide containing a –Gly–Cys– sequence was transformed into the corresponding *S*-peptide with a conversion rate of ca. 80% and that a peptide with Dmmb attached also formed an *S*-peptide. However, the Dmmb group was gradually decomposed when the peptide was stored in a TFA solution, as observed by ^{13}C NMR spectroscopy. The quantitative estimation of the *S*-peptide ratio by RP-HPLC analysis was difficult because of the rate of the *S*–*N* acyl shift reaction. The *S*-peptide generated from a Dmmb-attached peptide was converted to the corresponding 2-sulfoethyl peptide thioester, which is widely used as a building block in polypeptide synthesis. This work will provide deeper insight into the chemical nature of the cysteine residue and thiol-containing auxiliary groups in peptides and will help in the development of a new methodology in peptide and protein chemistry.

Experimental

General. Acetonitrile was HPLC grade. Other solvents were reagent grade. Amino acid derivatives used were of the L-configuration, unless otherwise noted. Fmoc–amino acid derivatives were purchased from the Peptide Institute Inc. 4-(*N*-*t*-Butoxycarbonyl–alaninyl–oxymethyl)phenylacetoamidemethyl resin (Boc–Ala–OCH_2–Pam resin) were purchased from Applied Biosystems Inc. 4-(α-9-Fluorenylmethoxycarbonylamino-2,4-dimethoxybenzyl)-phenoxy resin (Fmoc–Rink amide resin) were purchased from Novabiochem. The Fmoc–NH–SAL–PEG resin was purchased from Watanabe Chemical Ind., Ltd. All other chemicals were commercially available and were used without further purification.

Melting points were measured on a Yanaco micro melting point apparatus and are uncorrected. NMR spectra were recorded on a Bruker Avance™ 400 (^{1}H, 400 MHz, ^{13}C, 100 MHz) spectrometer, and chemical shifts (δ) are expressed in parts per million relative to tetramethylsilane. Splitting patterns are designated as: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad. ESI-MS spectra were recorded on a Thermo Finnigan LCQ™ DECA XP spectrometer. Peptide yields were determined by amino acid analyses, which were performed on a Hitachi L-8500 amino acid analyzer after hydrolysis with constantly boiling point HCl (Nacalai Tesque) at 110 °C for 24 h in an evacuated sealed tube. Silica-gel column chromatography was carried out on E. Merck silica gel 60 (230–400 mesh). HPLC was carried out on a reversed phase column (YMC-Pack ProC18, (4.6 × 150 mm) (YMC Co., Ltd.), Cosmosil 5C18-AR-II (10 × 250 mm), or Cosmosil 5C18-AR-II (20 × 250 mm) (Nacalai Tesque)) using a linear increasing gradient of acetonitrile in water/0.1% TFA and peptides were detected by an absorbance measurement at 220 nm. A disposable ODS cartridge, TOYOPACK ODS-M, was purchased from Tosoh.

Fmoc–Ile–Ala–Gly(1-^{13}C)–Cys–Arg–NH_2 (3). Starting from the Fmoc–Rink amide resin (0.43 mmol g^{-1} resin, 465 mg), Fmoc–Ile–Ala–Gly(1-^{13}C)–Cys(Trt)–Arg(Pbf)–Rink amide resin was prepared manually following Fmoc SPPS. Each synthetic cycle consisted of the removal of Fmoc groups with a 20% piperidine solution of 1-methyl-2-pyrrolidinone (NMP), washing with NMP (×5), coupling of the Fmoc–amino acid (0.80 mmol), which was preactivated by mixing for 3 min with 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (0.72 mmol), 1-hydroxybenzotriazole (HOBt) (0.8 mmol), and *N*,*N*-diisopropylethylamine (DIEA) (1.6 mmol) in a mixture of DMF (1.6 mL) and NMP (0.8 mL), washing with NMP (×3), end capping with an NMP solution containing 10% acetic anhydride and 5% DIEA, and washing with NMP (×3). After each coupling step, the completion of the coupling reaction was confirmed by using a ninhydrin color test. The Fmoc–amino acids used were Fmoc–Arg(Pbf)–OH, where Pbf is 2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-sulfonyl group, Fmoc–Cys(Trt)–OH, where Trt is trityl, Fmoc–Ala–OH and Fmoc–Ile–OH. Fmoc–Gly-(1-^{13}C)–OH was introduced by mixing Fmoc–Gly(1-^{13}C)–OH (72 mg, 0.24 mmol), HBTU (87 mg, 0.23 mmol), HOBt•H_2O (37 mg, 0.24 mmol), and DIEA (77 μL, 0.44 mmol) in DMF (1.6 mL).

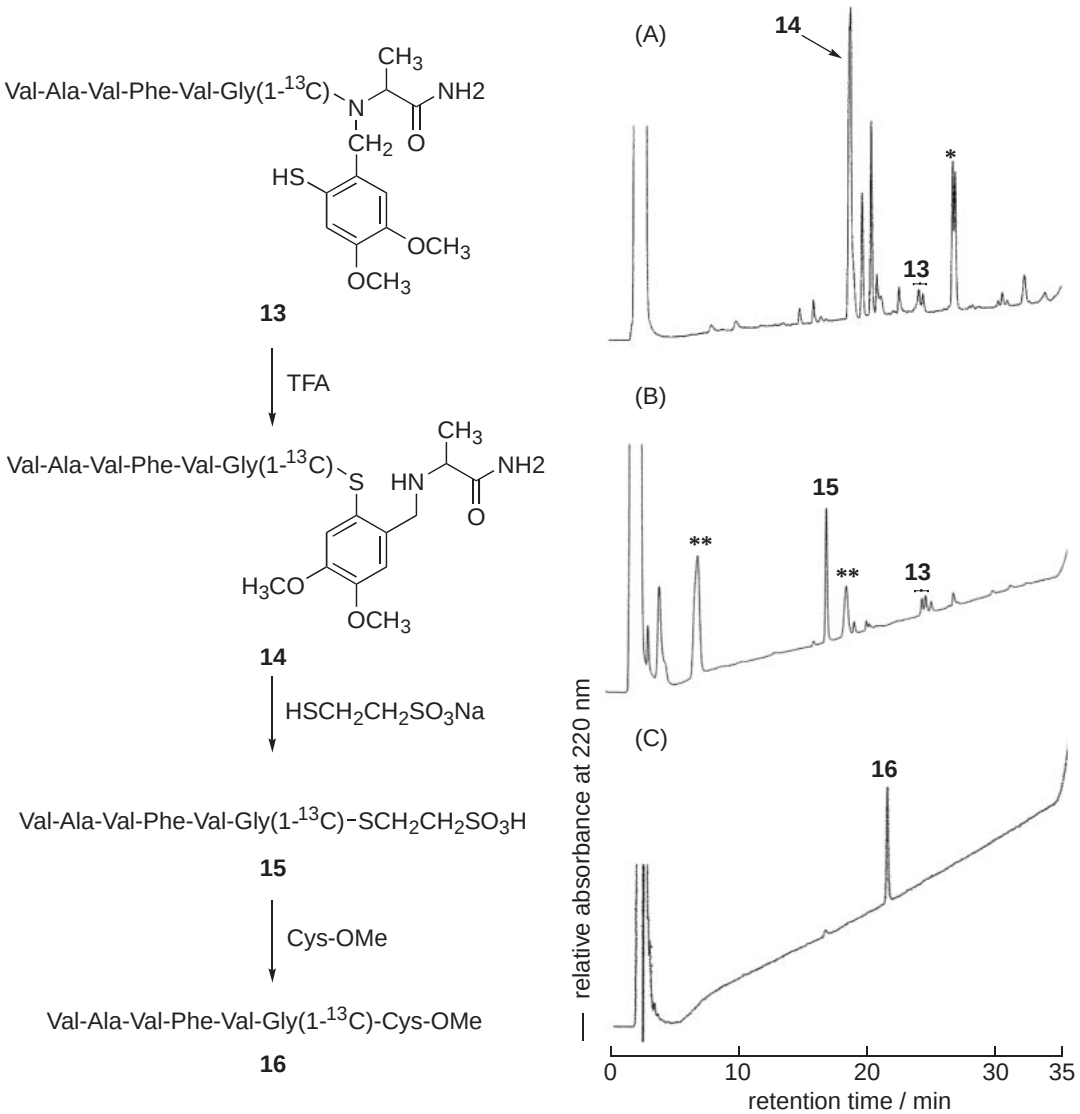

Fig. 9. Generation of peptide **16** from peptide **13** via an *N–S* acyl shift reaction followed by an intermolecular thiol exchange reaction (left), and RP-HPLC elution profiles of reaction mixtures (right). (A) Generation of peptide thioester **14** from peptide **13** via an *N–S* acyl shift reaction; (B) generation of 2-sulfoethyl thioester **15** from peptide thioester **14** via an intermolecular thiol exchange reaction; (C) generation of peptide **16** from peptide thioester **15** via native chemical ligation.[9] Peak ∗ contained a peptide with a higher molecular mass number than original peptide **13** by 1.0. Peaks ∗∗ were not peptides, whose molecular mass number were not detected more than 700 by ESI-MS. HPLC conditions; column: YMC-Pack ProC18 (4.6 × 150 mm), eluent: linear gradient of acetonitrile in 0.1% TFA from 10 to 40% over 30 min, flow rate: 1.0 mL min^{-1}.

Fmoc–Ile–Ala–Gly(1-^{13}C)–Cys(Trt)–Arg(Pbf)–Rink amide resin was obtained in a yield of 658 mg.

An aliquot of the protected peptide resin (195 mg) was mixed with 2.0 mL of reagent B, consisting of 88% TFA, 5% PhOH, 5% H_2O, and 2% $^{i}Pr_3SiH$ ($v/v/v/v$), and stirred for 2 h. Ether was added to the reaction mixture. The precipitate that formed was washed with ether three times and dissolved in aq acetonitrile. The resin-containing suspension was passed through a disposable ODS cartridge, and the filtrate was freeze-dried to give a crude peptide (40 mg). This crude material was purified by RP-HPLC (column: Cosmosil 5C18-AR-II (20 × 250 mm)) to give labeled peptide **3** (24.6 mg, 41% yield based on the amino group on the Rink amide resin); ESI-MS, m/z 741.6, calcd for $C_{35}H_{50}N_9O_7S$: $(M + H)^+$, 741.4; amino acid analysis: $Gly_{1.2}Ala_1Cys_{n.d.}Ile_{0.6}Arg_{1.2}$.

D,L-(4,5-Dimethoxy-2-tritylthiobenzyl)Ala–OMe (6). 2-Mercapto-4,5-dimethoxybenzylamine hydrochloride (**5**) (2.00 g, 7.81 mmol) was dissolved in chloroform (35 mL). To the solution, triphenylmethyl alcohol (2.13 g, 8.18 mmol) and TFA (1.16 mL, 15.6 mmol) were added, followed by stirring for 3 h at room temperature. A saturated aq solution of $NaHCO_3$ (40 mL) was then added. The reaction mixture was concentrated in vacuo. The product was extracted with chloroform (50 mL × 2), and the combined organic layers were washed with brine (20 mL) and dried over Na_2SO_4. The solvent of the organic layer was evaporated under reduced pressure to give a solid crude material (4.39 g). This was dissolved in chloroform and precipitated with ether to yield a white solid (3.17 g). This solid (2.65 g) was dissolved in dichloromethane (DCM) (30 mL) and methyl pyruvate (674 mg, 6.60 mmol) was added to the solution. After stirring for 15 min, sodium triacetoxyhydroborate (3.18 g, 15.0 mmol) was added to

the solution. The mixture was stirred for 4 h under Ar at room temperature. A saturated aq solution of $NaHCO_3$ (30 mL) was then added. The solution was concentrated in vacuo, and the product was extracted with EtOAc (200 mL) from the solution. The organic layer was successively washed with a saturated aq solution of $NaHCO_3$ (50 mL × 2) and brine (20 mL), and the extract was dried over Na_2SO_4. The organic layer was concentrated in vacuo. The crude product (4.65 g) was chromatographed over silica gel using a mixture of methanol and chloroform (5:95, v/v) to yield the product (2.51 g, 79%) as a white solid after evaporating the eluent. An aliquot of the product was dissolved in dioxane, and a 1 M HCl solution in dioxane, followed by ether, was added. The crystals were subjected to analysis: mp 96.8–98.5 °C; ^{1}H NMR ($CDCl_3$) δ 1.19 (d, 3H, J = 6.9 Hz, –CH(C*H*$_3$)–), 3.01 (d, 1H, J = 13.0 Hz, –C*H*H–NH$_2$–), 3.08 (d, 1H, J = 13.0 Hz, –CH*H*–NH$_2$–), 3.19 (q, 1H, J = 6.9 Hz, –C*H*(CH$_3$)–CO), 3.38 (s, 3H, –COOC*H*$_3$), 3.68 (s, 3H, –OC*H*$_3$), 3.87 (s, 3H, –OC*H*$_3$), 6.63 (s, 1H), 6.81 (s, 1H), 7.20–7.30 (m, 15H); ^{13}C NMR ($CDCl_3$) δ 18.2, 49.2, 51.7, 55.5, 55.8, 56.0, 70.8, 112.0, 118.7, 123.5, 126.8, 127.5, 130.2, 138.9, 144.3, 147.1, 149.8, 175.9; ESI-MS m/z 527.8, calcd for $C_{32}H_{34}NO_4S$: $(M + H)^+$, 528.2; Anal. Found: C, 72.80; H, 6.41; N, 2.62%, calcd for $C_{32}H_{34}ClNO_4S$: C, 72.84; H, 6.30; N, 2.65%.

Fmoc–Gly(1-^{13}C)–D,L-(4,5-Dimethoxy-2-tritylthiobenzyl)-Ala–OMe (7). To a solution of Fmoc–Gly(1-^{13}C)–OH (58.5 mg, 0.196 mmol) and HBTU (69.3 mg, 0.183 mmol) in DMF (500 μL), DIEA (66.0 μL, 0.380 mmol) was added, and the solution was stirred for 5 min. Compound **6** (51.5 mg, 0.0976 mmol) was added to the reaction mixture, followed by stirring for 17 h under an Ar atmosphere. The solution was concentrated in vacuo. The solid residue was dissolved in EtOAc (50 mL), and the organic layer was successively washed with 5% aq citric acid (20 mL × 2), sat. aq $NaHCO_3$ (20 mL × 2), and brine (5 mL), and dried over Na_2SO_4. The filtrate was concentrated in vacuo to give a crude product (118 mg). The crude material was purified by silica-gel column chromatography using a mixture of hexane:EtOAc (70:30, v/v) as an eluent to yield 57.8 mg (73%) of ^{13}C-labeled compound **7** as a white solid: ESI-MS, m/z 830.1, calcd for $C_{48}{}^{13}CH_{46}N_2NaO_7S$: $(M + Na)^+$, 830.3.

Fmoc–Gly(1-^{13}C)–D,L-(4,5-Dimethoxy-2-mercaptobenzyl)-Ala–OMe (8). To a solution of **7** (40.5 mg, 50.1 μmol) in DCM (200 μL), triisopropylsilane (51.3 μL, 250 μmol) and TFA (200 μL) were added. The reaction mixture was stirred at room temperature for 1 h and then concentrated in vacuo. The product was isolated by RP-HPLC (column: Cosmosil 5C18-AR-II (20 × 250 mm)) and lyophilized to give 12.9 mg (46%) of **8** as a white solid: ESI-MS, m/z 566.2, calcd for $C_{29}{}^{13}CH_{33}N_2O_7S$: $(M + H)^+$, 566.2.

Fmoc–Gly(1-^{13}C)–D,L-(4,5-Dimethoxy-2-tritylthiobenzyl)-Ala–OH (11). To a stirred ice cold solution of **7** (619 mg, 0.766 mmol) in THF (15 mL), 0.5 M aq solution of lithium hydroxide (1.53 mL, 0.765 mmol) was added dropwise over 30 min. The reaction mixture was stirred for 30 min, and a 0.5 M aq solution of lithium hydroxide (0.770 mL, 0.385 mmol) was then added dropwise to the reaction solution, in an ice bath, over 15 min and 1.0 M aq HCl was added until the pH of the solution reached to 4.0. The reaction mixture was concentrated in vacuo, and H_2O (10 mL) was added to dissolve the residue. The product was extracted from the aqueous layer with chloroform (20 mL × 2), and the combined organic layer was washed with brine (5 mL) and dried over Na_2SO_4. The organic layer was concentrated in vacuo, and the solid residue (826 mg) was purified by silica-gel column chromatography using a mixture of AcOH, hexane, and EtOAc (1:40:60, $v/v/v$) as an eluent to give 223 mg (36%) of **11** as a white solid: ESI-MS, m/z 816.6, calcd for $C_{47}{}^{13}CH_{44}N_2NaO_7S$: $(M + Na)^+$, 816.3.

Val–Ala–Val–Phe–Val–Gly(1-^{13}C)–D,L-(Dmmb)Ala–NH$_2$ (13). Fmoc–NH–SAL–PEG resin (0.26 mmol g^{-1}, 385 mg, 0.10 mmol) was washed with NMP (2 min × 3), treated with 20% piperidine in NMP (v/v) (5 min × 2, 10 min × 1) and washed with NMP (1 min × 5). The resin was mixed with a solution, which was prepared by mixing dipeptide **11** (83 mg, 0.11 mmol), HBTU (38 mg, 0.10 mmol), HOBt·H_2O (16 mg, 0.11 mmol), and DIEA (36 μL, 0.21 mmol) in DMF, for 17 h. The obtained resin, Fmoc–Gly-(1-^{13}C)–D,L-[Dmmb(Trt)]Ala–NH–SAL–PEG resin, was washed with NMP (1 min × 5), treated with 10% Ac_2O, 5% DIEA in NMP (v/v), and washed with NMP (1 min × 3). Then, the peptide chain elongation was carried out on a model 433A peptide synthesizer (Applied Biosystems, Inc.) using the FastMoc 0.25 mmol MonPrevPk protocol with end capping with acetic anhydride/HOBt/DIEA. The amino acids were introduced by a double coupling protocol. After completion of the peptide elongation, the peptide resin was extensively washed with methanol and dried in vacuo to give 448 mg of peptide resin **12**.

An aliquot of peptide resin **12** (101 mg) was stirred with Reagent B for 2 h. Ether was added to the reaction mixture, followed by stirring for 1 h. The resulting precipitate was washed with ether three times and then dissolved in 50% aq acetonitrile. The solution was passed through a disposable ODS cartridge, and the eluted solution was lyophilized to give a white powder (18 mg). This compound was purified by RP-HPLC (column: Cosmosil 5C18-AR-II (10 × 250 mm)) to give Val–Ala–Val–Phe–Val–Gly(1-^{13}C)–D,L-(Dmmb)Ala–NH$_2$ (**13**) (18% based on Fmoc–NH–SAL–PEG resin); ESI-MS, m/z 844.4, calcd for $C_{40}{}^{13}CH_{63}N_8O_9S$: $(M + H)^+$, 844.4; amino acid analysis: Gly_1-$Ala_{1.1}Val_{3.2}Phe_{1.3}$.

Val–Ala–Val–Phe–Val–Gly(1-^{13}C)–SCH$_2$CH$_2$SO$_3$H (15). Peptide **13** (0.5 mg) was treated with a 200 μL of a TFA solution containing 0.5% TCEP (v/w) by stirring for 30 h. The reaction mixture was injected directly onto an RP-HPLC (column: YMC-PACK ProC18 (4.6 × 150 mm)). After the addition of sodium 2-mercaptoethanesulfonate (1 mg, excess) to the fraction at 18.9 min (Fig. 9), the mixture was freeze-dried to give white powder. The powder was dissolved in 100 μL of a mixture of 0.2 M sodium phosphate buffer (pH 7.8) and acetonitrile (1:1, v/v), and the reaction mixture was stirred for 2 h and subjected to RP-HPLC to isolate the product, peptide thioester **15**; ESI-MS, m/z 716.3, calcd for $C_{30}{}^{13}CH_{51}N_6O_9S_2$: $(M + H)^+$, 716.3.

Val–Ala–Val–Phe–Val–Gly(1-^{13}C)–Cys–OMe (16). To a stirred solution of peptide thioester **15**, which was obtained by lyophilizing the elution at 17.5 min (Fig. 9B), in 100 μL of a mixture of 0.1 M Tris-tricine buffer (pH 8.2), containing 6 M guanidine and 0.02 M TCEP, and acetonitrile (1:1, v/v) was added Cys–OMe·HCl (1 mg, excess). The reaction mixture was stirred for 1 h and then subjected to RP-HPLC to give the product, peptide **16**; ESI-MS, m/z 709.3, calcd for $C_{32}{}^{13}CH_{54}N_7O_8S$: $(M + H)^+$ 709.4.

^{13}C NMR Spectra Measurement of Fmoc–Ile–Ala–Gly-(1-^{13}C)–Cys–Arg–NH$_2$ (3) in a TFA Solution. Fmoc–Ile–Ala–Gly(1-^{13}C)–Cys–Arg–NH$_2$ (4.0 mg) was dissolved in a mixture of TCEP (3.5 mg), $CDCl_3$ (200 μL), and TFA (500 μL). The ^{13}C NMR measurement was carried out at 300 K.

^{13}C NMR Spectra Measurement of Fmoc–Gly(1-^{13}C)–D,L-(Dmmb)Ala–OMe (8) in a TFA Solution. Fmoc–Gly(1-^{13}C)–D,L-(Dmmb)Ala–OMe (4.2 mg) was dissolved in a mixture of TCEP (3.5 mg), $CDCl_3$ (100 μL), and TFA (600 μL). The ^{13}C NMR measurement was carried out at 300 K.

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Supporting Information

Analytical data, such as melting point, chemical shift, elemental analysis, of non-labeled compounds **7′**, **8′**, and **11′**. The structures of ^{13}C-labeled compound **7**, **8**, and **11** were confirmed by comparing with **7′**, **8′**, and **11′**. This material is available free of charge on the web at http://www.csj.jp/journals/bcsj/.

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