An Inversion of Configuration of Threonine and Allothreonine in the *N,O*-Acyl Migration Reaction with Concentrated Sulfuric Acid[1)]

Tateaki WAKAMIYA, Yuzo TARUMI, and Tetsuo SHIBA

Department of Chemistry, Faculty of Science, Osaka University, Toyonaka, Osaka 560

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On the *N,O*-acyl migration reaction of β-hydroxy-α-amino acids with concentrated sulfuric acid, a configuration of the carbon atom carrying hydroxyl group was found to be inverted through the reaction. Thus, threonine residue in peptide was converted to allothreonine residue and *vice versa* in the selective chemical cleavage of threonine peptide employing the *N,O*-migration with concentrated sulfuric acid. The reaction mechanism involving S_Ni elimination of *O*-sulfate formed as an intermediate was suggested. The inversion did not occur in the *N,O*-acyl migration reaction with other mineral acids than sulfuric acid.

For the selective chemical cleavage of peptide bonds at β-hydroxy-α-amino acid residue, the *N,O*-acyl migration reaction in strong acids has been studied by many workers.[2)] Although the mechanism of the rearrangement had been discussed by Bergmann *et al.*[3)] and Elliott *et al.*,[2b,4)] a stereochemistry of the reaction has never been clarified yet. In our structural study on the antibiotics tuberactinomycins, we noticed that *threo*-γ-hydroxy-β-lysine was converted to *erythro* form in the *N,O*-migration reaction with concentrated sulfuric acid.[5)] The similar inversion may occur in the chemical cleavage reaction of threonine peptides with concentrated sulfuric acid. In this study, we investigated the *N,O*-acyl migration reaction in threonine peptides with concentrated sulfuric acid from the view point of the configurational change. Consequently, it was found in this reaction that threonine residue was converted to allothreonine residue, and reversely, allothreonine residue changed to threonine residue both in good yields. This fact has been overlooked hitherto in the sequential analysis of peptide using *N,O*-migration reaction with concentrated sulfuric acid. Furthermore, it was proved that this inversion took place certainly at β-carbon atom and not at α-carbon atom of threonine from results of ORD spectra of allothreonines which were obtained from the migration products of several acyl-L-threonine derivatives or L-threonine peptides.

In addition, the migration reaction of acyl derivatives of threonine or allothreonine was also examined, attempting to exploit a novel method for epimerization of threonine or allothreonine. Of the acyl derivatives tested, such as acetyl, benzoyl, benzyloxycarbonyl-threonine and so on, some of them participated to the migration reaction, though the yields were not satisfactory.

Experimental

Materials. The threonine peptides, *i.e.*, DL-alanyl-DL-threonine, benzyloxycarbonylglycyl-DL-threonine, and glycyl-L-threonyl-L-phenylalanine were prepared either by saponification or hydrogenolytic debenzyloxycarbonylation of the corresponding derivatives which had been early synthesized in our laboratory.[6)] The peptides involving DL-amino acids are actually mixtures of diastereoisomers. However, use of such compounds does not disturb the estimation of the epimerization on β-carbon atom of threonine or allothreonine. Benzyloxycarbonylglycyl-L-threonine and benzyloxycarbonyl-L-alanyl-L-threonine were prepared through the active ester method and isolated as dicyclohexylammonium salts. The corresponding allothreonine peptides were also synthesized by the active ester or by the dicyclohexylcarbodiimide method as mentioned below.

Benzyloxycarbonylglycyl-DL-allothreonine. To a solution of DL-allothreonine (1.19 g, 0.01 mol) and sodium hydrogencarbonate (1.85 g, 0.022 mol) in 15 ml of water, a solution of *p*-nitrophenyl benzyloxycarbonylglycinate (3.16 g, 0.01 mol) in 20 ml of dioxane was added. After stirring at room temperature for 50 hr, the reaction mixture was evaporated *in vacuo*. The residue was dissolved in water and insoluble material was filtered off. The filtrate was acidified with 6 M HCl and extracted with ethyl acetate. Ethyl acetate layer was washed with water and dried over anhydrous sodium sulfate. After evaporation of ethyl acetate, the oily residue was crystallized by treatment with petroleum ether. It was recrystallized from ethyl acetate, yield 1.90 g (61.5%), mp 130 °C.

Found: C, 54.35; H, 5.89; N, 8.96%. Calcd for $C_{14}H_{18}O_6N_2$: C, 54.19; H, 5.85; N, 9.03%.

Benzyloxycarbonyl-L-alanyl-DL-allothreonine. This compound was prepared from *p*-nitrophenyl benzyloxycarbonyl-L-alaninate (3.34 g, 0.01 mol) and DL-allothreonine (1.19 g, 0.01 mol) in the same procedure as mentioned above. The product was isolated as a dicyclohexylammonium salt which was recrystallized from ethanol and ether, yield 2.79 g (55.2%).

Found: C, 63.70; H, 8.50; N, 8.35%. Calcd for $C_{27}H_{43}O_6N_3$: C, 64.13; H, 8.57; N, 8.31%.

Benzyloxycarbonyl-DL-allothreonine. DL-Allothreonine (3.00 g, 0.025 mol) was benzyloxycarbonylated according to the synthesis of benzyloxycarbonyl-L-threonine.[7)] The oily product was crystallized as a dicyclohexylammonium salt, yield 9.80 g (89.3%). It was recrystallized from ethanol–ether–petroleum ether, mp 147—149 °C.

Found: C, 66.14; H, 8.83; N, 6.40%. Calcd for $C_{24}H_{33}O_5N_2$: C, 66.33; H, 8.81; N, 6.45%.

Benzyloxycarbonyl-DL-allothreonyl-L-phenylalanine Methyl Ester. Benzyloxycarbonyl-DL-allothreonine dicyclohexylammonium salt (4.50 g, 1.04 mmol) was coupled with methyl L-phenylalaninate hydrochloride (2.24 g, 1.04 mmol) by the dicyclohexylcarbodiimide method in dimethylformamide. The product was recrystallized from ethyl acetate and petroleum ether, yield 4.05 g (94.0%).

Found: C, 63.63; H, 6.38; N, 6.84%. Calcd for $C_{22}H_{26}O_6N_2$: C, 63.75; H, 6.32; N, 6.74%.

DL-Allothreonyl-L-phenylalanine Methyl Ester Hydrobromide. Benzyloxycarbonyl-DL-allothreonyl-L-phenylalanine methyl ester (1.70 g, 0.41 mmol) was dissolved in 5 ml of 30% hydrogen bromide in glacial acetic acid, and anhydrous ether

was added after one hour. The oily product was triturated with anhydrous ether several times and dried over sodium hydroxide *in vacuo*, yield 1.45 g (oil, almost quantitatively).

Benzyloxycarbonyl-L-alanyl-DL-allothreonyl-L-phenylalanine Methyl Ester. Benzyloxycarbonyl-L-alanine (0.60 g, 0.27 mmol) was condensed with DL-allothreonyl-L-phenylalanine methyl ester prepared from its hydrobromide (0.75 g, 0.21 mmol) through the dicyclohexylcarbodiimide method in tetrahydrofuran. The product was recrystallized from ethyl acetate and petroleum ether, yield 0.71 g (73.3%).

Found: C, 61.73; H, 6.71; N, 8.74%. Calcd for $C_{25}H_{31}O_7N_3$: C, 61.84; H, 6.44; N, 8.66%.

Benzyloxycarbonylglycyl-DL-allothreonyl-L-phenylalanine Methyl Ester. Benzyloxycarbonylglycine (0.52 g, 0.25 mmol) was condensed with DL-allothreonyl-L-phenylalanine methyl ester prepared from its hydrobromide (0.70 g, 0.19 mmol) in the similar manner to that mentioned above. The product was recrystallized from ethyl acetate and petroleum ether, yield 0.70 g (76.5%).

Found: C, 59.91; H, 6.29; N, 8.69%. Calcd for $C_{24}H_{29}O_7N_3 \cdot 1/2\,H_2O$: C, 59.99; H, 6.29; N, 8.75%.

Acyl Threonine and Acyl Allothreonine. Acyl derivatives of threonine and allothreonine were synthesized according to the convenient procedure. The melting points of the products are summarized in Table 1.

Table 1. Melting points of acylthreonine and acylallothreonine

Acyl group	Mp (°C)	
	Threonine[a]	Allothreonine[a]
CH_3CO	132—133	136—137
	131—132[b]	
$ClCH_2CO$	121—122	151—152[c]
CF_3CO	118—120	85—87
C_6H_5CO	145—146	177—178
p-ClC_6H_4CO	185—187	192—193
$C_6H_5OCH_2CO$	142—143	144—145
C_2H_5OCO	157—159[c]	138—139[c]
	169—170[b]	
$C_6H_5CH_2OCO$	103—104[b]	147—149[c]

a) DL-Forms were used except b). b) L-Forms were used. c) Isolated as a dicyclohexylammonium salt.

Preparation of O-Sulfate of Threonine and Allothreonine. L-Threonine (1.00 g) was dissolved in 20 ml of concentrated sulfuric acid and allowed to stand for 10 days in a sealed tube. The solution was poured into cold ether. Precipitate was washed with ether several times by decantation and finally filtered off. The crude product was powdered and dissolved in water. The solution was passed through a Dowex 50 W×8 (H^+ form) column. Acidic eluate was concentrated *in vacuo* and the residue was recrystallized from water and acetone to give monohydrate as fine prisms, yield 1.40 g (85%), mp 149—150 °C.

Found: C, 22.00; H, 5.07; N, 6.33; S, 14.71%. Calcd for $C_4H_9O_6NS \cdot H_2O$: C, 22.12; H, 5.10; N, 6.45; S, 14.76%.

DL-Allothreonine (1.00 g) gave fine prisms of *O*-sulfate by the similar procedure, yield 1.35 g (82%), mp 220—230 °C (decomp.).

Found: C, 23.99; H, 4.66; N, 7.03; S, 15.98%. Calcd for $C_4H_9O_6NS$: C, 24.12; H, 4.55; N, 7.03; S, 16.10%.

Ninhydrin Reagent. Ninhydrin reagent was prepared by addition of stannous chloride dihydrate (20 mg) and 0.5 M citrate buffer (5 ml) to a solution of ninhydrin (380 mg) in methylcellosolve (95 ml).

N,O-*Migration Reaction.* Sample of each 100—200 mg of peptide or acyl derivative of threonine or allothreonine was dissolved in 5—6 ml of concentrated sulfuric acid. The solution was allowed to stand at 20—25 °C for 10—30 days in a sealed tube. The reaction was stopped by pouring the reaction mixture into a plenty of cold ether in the case of peptide or into 25—30 ml of 2 M hydrochloric acid in the case of the acyl derivative.

Paper Chromatographic Separation of Threonine and Allothreonine. Precipitate from the migration product of peptide obtained as mentioned above was hydrolyzed with 6 M hydrochloric acid at 110 °C for 8 hr. Each hydrolyzate was spotted on Toyo Roshi No. 51 filter paper (10×40 cm) on a line of 7 cm length after thorough removal of hydrochloric acid by evaporation. On the other hand, the aqueous acidic solution of the migration product of acyl derivative was heated at 110 °C for 8 hr. Each hydrolyzate was passed through a column of Amberlite IR-45 (OH^- form) and a neutral eluate containing threonine and allothreonine was concentrated *in vacuo*. The residue was dissolved in a small amount of water and spotted on filter paper as mentioned above. Three sheets of the papers for each one sample were developed by use of R-solvent, *i.e.*, supernatant of *n*-butanol–water–acetone–28% ammonium hydroxide (40:30:5:5 v/v). For satisfactory separation of threonine, allothreonine, and alanine, it was necessary to repeat the development a few times.

Estimation of Inversion Rate.[8] Ninhydrin reagent was sprayed to the developed paper, and then the colored parts of threonine and allothreonine were cut off separately. The combined pieces of paper taken were sufficiently wetted with 0.1 M sodium hydroxide solution in a test tube (13×150 mm) and the tube was placed in a vacuum desiccator overnight on concentrated sulfuric acid. The pH was then brought to 5 with 0.2 M citric acid solution. Ninhydrin reagent (1.0 ml) was added to each tube which was shaken for 5—10 min and kept in a boiling water bath for 25 min. After cooling, distilled water (3 ml) was added, and the extract was poured into volumetric flask of 10 ml. Complete extraction of the colored material from the paper was carried out by treatment with acetone (3 ml). Finally the volume in the flask was adjusted to 10 ml with distilled water. The color strength was estimated using a Hitachi 124 Spectrophotometer at 570 nm. As reference in the colorimetry, a blank part of the same paper of an equal area was treated in the similar manner.

Measurement of ORD Spectra. Each hydrolyzate of migration products of acetyl-L-threonine, ethyloxycarbonyl-L-threonine, benzyloxycarbonylglycyl-L-threonine, benzyloxycarbonyl-L-alanyl-L-threonine, and benzyloxycarbonylglycyl-L-threonyl-L-phenylalanine was chromatographed on Toyo Roshi No. 51 filter paper (40×40 cm, eight sheets). The development was repeated three times each after drying and a part of allothreonine was cut off. Allothreonine was extracted with water from the paper and recrystallized from water and ethanol. ORD spectra were obtained with a JASCO Model ORD/UV-5 in water.

Results and Discussion

The results in the migration reactions of threonine and allothreonine peptides were listed in Table 2, in which the degrees of inversion were summarized as relative ratios of threonine to allothreonine.

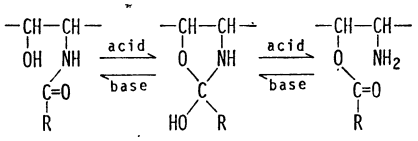
As can be seen in Table 2, the configuration on β-carbon atom of both threonine and allothreonine

TABLE 2. RATIOS OF THREONINE AND ALLOTHREONINE RESIDUE IN INVERSION REACTIONS

Peptide[a)]	Reaction days	Ratio (%) Allo-threonine	Ratio (%) Threo-nine
Z-Ala-Thr-OH[b)]	30	78	22
H-Ala-Thr-OH[b)]	30	82	18
Z-Gly-Thr-OH[b)]	30	71	29
Z-Gly-Thr-OCH_3[b)]	30	69	31
Z-Gly-Thr-Phe-OH[c)]	30	77	23
H-Gly-Thr-Phe-OH[c)]	30	77	23
Z-Gly-Ath-OH[d)]	10	0	100
Z-Ala-Ath-OH[d)]	10	25	75
	30	0	100
Z-Gly-Ath-Phe-OCH_3[d)]	10	0	100
Z-Ala-Ath-Phe-OCH_3[d)]	10	9	91
	45	0	100

$Z = C_6H_5CH_2OCO-$

a) Amino acids used are of DL-forms in peptides b) and of L-forms in peptides c). In peptides d), alanine and phenylalanine are of L-forms and allothreonine (Ath) is of DL-form.



(I)

Fig. 1. Reaction mechanism of rearrangement *via* hydroxyoxazolidine (I).

peptides was inverted in a fairly good yield through the migration reaction in concentrated sulfuric acid. This newly found fact is very important not only stereochemically, but also for a reaction mechanism of *N,O*-acyl migration. Bergmann had first suggested a plausible way *via* hydroxyoxazolidine intermediate (I) for the *N,O*-acyl migration reaction in strong acids (Fig. 1).[3a)] According to this mechanism, the configuration on β-carbon atom carrying hydroxyl group should be retained. There has never been reported that the inversion occurred through the acyl migration reaction of peptides and proteins hitherto investigated and the above mechanism seemed to be accepted in many studies of protein chemistry. On the other hand, there are known some examples in which an inversion of the configuration actually took place in acyl migration reaction. Thus *N*-acyl-(−)-ephedrine was converted to *O*-acyl-(+)-ψ-ephedrine by treatment with hydrochloric acid.[9)] However, this is a special case where the so-called neighboring group effect between methyl and phenyl groups is participated. Such a steric effect cannot be operated at threonine or allothreonine peptides.

In other examples, Elliott pointed out a possibility that an inversion of configuration may occur on an asymmetric carbon atom carrying the hydroxyl group in a reaction of acyl amino alcohol with thionyl chloride through oxazoline ring formation (Fig. 2).[4)] Furthermore, Elliott attempted to reveal a mechanism of acyl migration reaction in concentrated sulfuric acid, however he could not manifest the real reaction mechanism so far.[2b)]

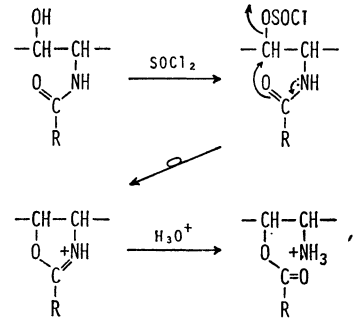


Fig. 2. Reaction mechanism of rearrangement by thionyl chloride accompanied with inversion.

From our experimental results in this study, a configurational change of β-carbon atom of threonine or allothreonine in peptide chain on the acyl migration reaction with concentrated sulfuric acid was demonstrated at the first time. There are suggested two important facts to support the inversion mechanism. First, *O*-sulfate of serine or threonine was readily isolated on simple treatment of these amino acids with concentrated sulfuric acid.[10)] Secondly, Fasman also observed the formation of *O*-sulfate before ester formation in the acyl migration reaction of poly-DL-serine in concentrated sulfuric acid.[11)] Taking these facts into consideration, we now propose the most plausible reaction mechanism as follows (Fig. 3).

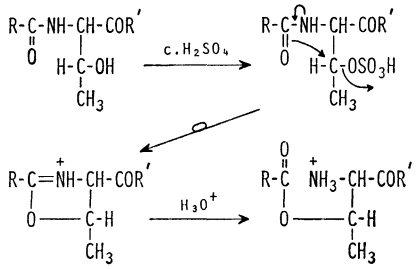


Fig. 3. Proposed mechanism of acyl migration reaction in concentrated sulfuric acid.

According to this mechanism, the *N,O*-acyl migration reaction in concentrated sulfuric acid is initiated by a formation of *O*-sulfate which is known as a strong electron-withdrawing group. A synchronous reaction of S_Ni type by an elimination of *O*-sulfate and an attack of oxygen of carbonyl group resulted in a formation of an oxazoline intermediate which is finally hydrolyzed to *O*-acyl derivative.

We have to examine a possibility that an acyl amino acids may be converted in concentrated sulfuric acid to an oxazolone derivative which causes a racemization at α-carbon atom according to the well-known mechanism. Actually, benzoyl and *p*-chlorobenzoyl derivatives of threonine and allothreonine gave 4-ethylidene-2-phenyloxazolone and 2-*p*-chlorophenyl-4-ethylidene-oxazolone respectively, under the condition employed in the migration reaction, while the formation of such oxazolone compound was never recognized in another

acyl derivatives, *e.g.*, acetyl, ethyloxycarbonyl or benzyloxycarbonylthreonine. If the apparent configurational changes in the latter cases should be due to the racemization at α-carbon atom through the oxazolone formation, allothreonine produced from L-threonine residue must be of D-form. However, measurements of ORD spectra of allothreonine obtained from the migration products of acetyl-L-threonine, ethyloxycarbonyl-L-threonine, benzyloxycarbonylglycyl-L-threonine, benzyloxycarbonyl-L-alanyl-L-threonine, and benzyloxycarbonylglycyl-L-threonyl-L-phenylalanine ascertained the complete retention of the configuration of α-carbon atom in threonine in all cases. The possibility of the inversion through racemization of oxazolone derivatives can also be excluded by the fact that threonine or allothreonine was not obtained by the hydrolysis of the unsaturated oxazolones mentioned above.

Table 3. Inversion rates of acyl derivatives of threonine and allothreonine

Acyl groups	Yields (%) of inversion from	
	Acylthreonine	Acylallothreonine
CH_3CO	16	11
$ClCH_2CO$	22	18
CF_3CO	1	13
$C_6H_5OCH_2CO$	9	18
C_2H_5OCO	39	30
$C_6H_5CH_2OCO$	3	7

Contrary to the peptide migration, an inversion of simple acyl derivatives of threonine and allothreonine occurred only in a low yield (Table 3). The reaction rate of the acyl migration reaction is known to be very slow even in the threonine peptides.[2m,12] Therefore, it may be considered that either the reaction of the acyl derivative proceeded in an extreme slow rate or a simple hydrolysis preceded to the slow migration reaction.

It can be now concluded that the acyl migration reaction with concentrated sulfuric acid leads to a complete inversion of the configuration on β-carbon atom of β-hydroxy-α-amino acids in peptides as far as the migration reaction takes place. In addition, it was confirmed that the inversion occurs only in concentrated sulfuric acid but not in another mineral acids, *e.g.*, hydrochloric acid, hydrobromic acid, and anhydrous hydrogen fluoride.

In view of wide applications of *N,O*-acyl migration reaction of threonine residue with concentrated sulfuric acid for determination of primary structures of peptides or proteins, the stereochemistry of β-carbon atom in threonine must be taken into consideration hereafter based on the new findings in this study.

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References and Footnotes

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