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Studies on Peptides. CXXV.[1,2] 3-(3-*p*-Methoxybenzylthiopropionyl)-thiazolidine-2-thione and Its Analogs as Reagents for the Introduction of the Mercapto Group into Peptides and Proteins

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A new SH-introducing reagent, 3-(3-*p*-methoxybenzylthiopropionyl)thiazolidine-2-thione (MTPTT), was prepared by condensation of 3-*p*-methoxybenzylthiopropionic acid and thiazolidine-2-thione using DCC. Reaction of MTPTT with peptides can be monitored by following the disappearance of the pale yellow color of the reagent. As an example, synthetic human growth hormone-releasing factor (hGRF-40) was treated with MTPTT, followed by 1 M trifluoromethanesulfonic acid–thioanisole/trifluoroacetic acid. The resulting HS–CH_2CH_2–CO–(hGRF-40) was conjugated with bovine serum albumin (BSA) by using a newly introduced heterobifunctional conjugating reagent, 2,4-dinitrophenyl *p*-(β-nitrovinyl)benzoate for antigen preparation. Four other similar SH-introducing reagents were also prepared.

Keywords——3-mercaptopropionic acid derivative as SH-introducing reagent; 3-acylthiazolidine-2-thione as carboxyl activator; *p*-methoxybenzyl as SH-protection; acetyl as SH-protection; deacetylation by hydroxylamine; de-*p*-methoxybenzylation by trifluoromethanesulfonic acid-thioanisole; *p*-(β-nitrovinyl)benzoic acid derivative as heterobifunctional conjugating reagent; SH-derivative of human growth hormone-releasing factor (hGRF); hGRF-BSA conjugate

Recently, a number of heterobifunctional conjugating reagents have been introduced to improve the radioimmunoassay of peptide hormones, as well as their enzyme immunoassay.[3] Most of these reagents are based upon the use of the maleimido function[4] to trap the mercapto group on the one hand and the *N*-hydroxysuccinimide ester function[5] to react with the amino group of peptides or proteins on the other. In combination with these reagents, a number of SH-introducing reagents have recently been investigated,[3] such as 2-acetylthiosuccinic anhydride,[6] *N*-[3-(2-pyridylthio)propionyloxy]succinimide,[7] 3-mercaptopropionyl hydrazide,[8] 3-(phenyldithio)propionimidate,[8] and *N*-(2-acetylthioacetyloxy)succinimide.[9]

We wish to present a new reagent, 3-(3-*p*-methoxybenzylthiopropionyl)thiazolidine-2-thione (MTPTT), for introduction of the mercapto group into peptides and proteins (Fig. 1). This reagent bears the acid-labile MBzl group[10] for S-protection and the thiazolidine-2-thione for carboxyl activation.[11,12] The reagent can be prepared easily by the usual DCC condensation[13] of 3-*p*-methoxybenzylthiopropionic acid[14] with thiazolidine-2-thione. The pale yellow color of the reagent disappears with the progress of aminolysis. By utilizing this advantageous property, the coupling reaction of MTPTT with peptides or proteins can easily be monitored. The S-MBzl group can be removed quickly and quantitatively by 1 M TFMSA–thioanisole/TFA,[15] whereas removal of other S-protecting groups, such as the S-acetyl group, requires much longer treatment with a base, such as hydroxylamine.[9] Excess of base may interfere with the subsequent reaction with bifunctional conjugating reagents.

As an example, aminolysis of MTPTT with alanine was performed in the presence of Et_3N. The reaction was completed within 4 h and the product was then treated with 1 M

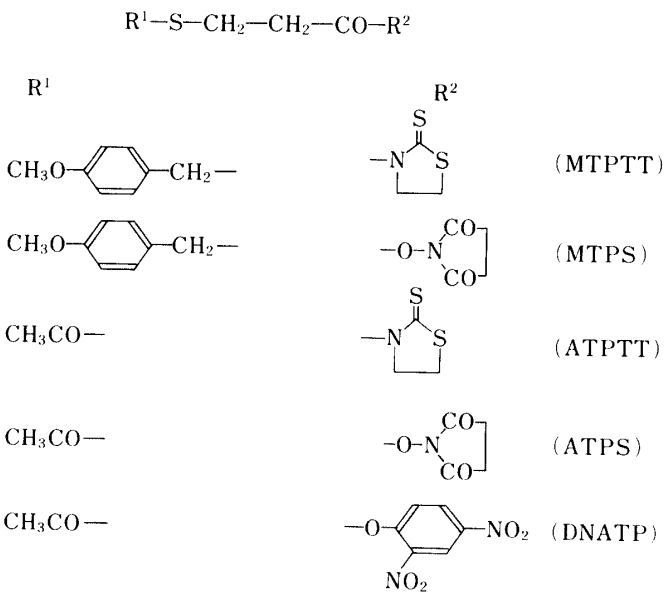

Fig. 1. Structures of the SH-Introducing Reagents

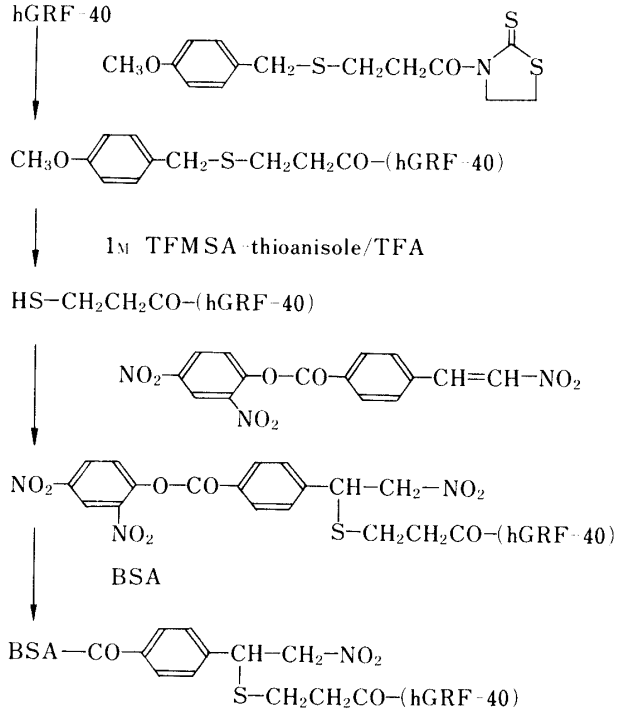

Fig. 2. Conjugation of hGRF-40 to BSA with the Aid of the SH-Introducing Reagent (MTPTT)

TFMSA–thioanisole/TFA in an ice-bath for 60 min to remove the S-MBzl group. It was confirmed that the mercapto group was quantitatively exposed, as measured by the use of Ellman's reagent.[16] After this model experiment, synthetic human growth hormone-releasing factor (hGRF-40)[17] was similarly treated with MTPTT followed by 1 M TFMSA–thioanisole/TFA, as shown in Fig. 2. In this case, the latter acid treatment was repeated once more to ensure complete S-deprotection. The resulting HS–CH_2–CH_2–CO–(hGRF-40) was allowed to react with 2,4-dinitrophenyl *p*-(*β*-nitrovinyl)benzoate in acidic media. Our previously introduced heterobifunctional conjugating reagent *N*-[*p*-(*β*-nitrovinyl)benzoyloxy]succinimide,[18] was modified by replacing its active ester function with a 2,4-dinitrophenyl ester function[19] to facilitate the aminolysis step. When examined by means of the Ellman test, it was found that the SH-addition reaction to the nitrovinyl moiety of the above reagent was completed within 30 min, as expected. Next, the adduct was allowed to react with bovine

serum albumin (BSA) at pH 7.5. Thin-layer chromatography (TLC) examination showed that this aminolysis was completed within 4 h, whereas the *N*-succinimide ester derivative required more than 8 h in this aminolysis step. The hGRF–BSA conjugate was easily isolated by gel-filtration on Sephadex G-25. After acid hydrolysis, hGRF used was found to be conjugated to BSA through the *p*-(2-nitroethyl)benzoyl bridge in more than 95% yield.

Similarly, we have prepared four analogs, *N*-(3-*p*-methoxybenzylthiopropionyloxy)-succinimide (MTPS), 3-(3-acetylthiopropionyl)thiazolidine-2-thione (ATPTT), *N*-(3-acetylthiopropionyloxy)succinimide (ATPS), and 2,4-dinitrophenyl 3-acetylthiopropionate (DNATP) as listed in Fig. 1. By using these reagents, including MTPTT, at the final step of a peptide synthesis, it is possible to introduce the mercapto group at the N-terminal position of desired peptides. The S-acetyl compounds, ATPTT, ATPS and DNATP, can be used when the TFMSA treatment is not ideal, depending on the nature of the peptides and proteins employed.

Experimental

Rf values in TLC performed on silica gel (Kieselgel G, Merck) refer to the following solvent systems (v/v); Rf_1 $CHCl_3$, Rf_2 $CHCl_3$–MeOH (10:0.5), Rf_3 $CHCl_3$–MeOH–AcOH (9:1:0.5), Rf_4 $CHCl_3$–MeOH–AcOH (20 : 0.5 : 0.2), Rf_5 *n*-BuOH–AcOH–AcOEt–H_2O (1 : 1 : 1 : 1).

Proton nuclear magnetic resonance (^{1}H-NMR) spectra were taken with a JEOL JNM-FX200 or JNM-PMX60 spectrometer, using tetramethylsilane as an internal standard. Mass spectra (MS) were taken with a JEOL 01SG-2 spectrometer. Ultraviolet (UV) absorption was measured with a Hitachi 200-20 spectrophotometer.

3-(3-*p*-Methoxybenzylthiopropionyl)thiazolidine-2-thione (MTPTT)——Thiazolidine-2-thione (170 mg, 1.1 eq) and DCC (300 mg, 1.1 eq) were added successively to a solution of 3-*p*-methoxybenzylthiopropionic acid[14] (300 mg, 1.31 mmol) in THF (5 ml). After being stirred at room temperature for 10 h, the mixture was filtered, the filtrate was concentrated and the residue was purified by column chromatography on silica gel (1.6 × 30 cm) with $CHCl_3$. The desired fractions (Rf_1 0.81) were combined, the solvent was removed by evaporation and the residue was recrystallized from $CHCl_3$ and hexane; yield 280 mg (65%), mp 39—40 °C. IR ν_{max}^{Nujol} cm^{-1}: 1690 (–CO). ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 2.75, 3.50 (2H, t, J=6.8 Hz, $-CH_2-$), 3.70 (2H, s, $-CH_2-$, benzyl), 3.79 (3H, s, CH_3O-), 4.55, 3.27 (2H, t, J=7.6 Hz, $-CH_2-$, thiazolidine ring), 6.84 (2H, d, J=8.4 Hz, aromatic), 7.23 (2H, d, J=8.4 Hz, aromatic). *Anal.* Calcd for $C_{14}H_{17}NO_2S_3$: C, 51.34; H, 5.23; N, 4.28. Found: C, 51.20; H, 5.11; N, 4.34.

***N*-(3-*p*-Methoxybenzylthiopropionyloxy)succinimide (MTPS)**——The title compound was prepared as described above by condensation of 3-*p*-methoxybenzylthiopropionic acid (300 mg, 1.31 mmol) and *N*-hydroxysuccinimide (166 mg, 1.1 eq) using DCC (298 mg, 1.1 eq). The product was purified by column chromatography on silica gel as described above followed by recrystallization from $CHCl_3$ and hexane; yield 255 mg (60%), mp 74—76 °C, Rf_1 0.65. IR ν_{max}^{Nujol} cm^{-1}: 1740, 1780, 1820 (–CO–, –COOR). ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 2.72—2.86 (4H, m, $-S-CH_2-CH_2-CO-$), 2.82 (4H, s, $-CH_2-CH_2-$, succinimide), 3.72 (2H, s, $-CH_2-$, benzyl), 3.79 (3H, s, CH_3O-), 6.85 (2H, d, J=8.8 Hz, aromatic), 7.24 (2H, d, J=8.8 Hz, aromatic). *Anal.* Calcd for $C_{15}H_{17}NO_5S$: C, 55.71; H, 5.30; N, 4.33. Found: C, 55.95; H, 5.38; N, 4.34.

3-(3-Acetylthiopropionyl)thiazolidine-2-thione (ATPTT)——The title compound was prepared as described above by condensation of 3-acetylthiopropionic acid[20] (500 mg, 3.37 mmol) and thiazolidine-2-thione (443 mg, 1.1 eq) using DCC (835 mg, 1.2 eq), and the product was purified by column chromatography on silica gel followed by recrystallization from $CHCl_3$ and hexane; yield 350 mg (42%), mp 59—61 °C. IR ν_{max}^{Nujol} cm^{-1}: 1700, 1670 (CO). ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 2.33 (3H, s, CH_3-), 3.19 (2H, t, J=6.8 Hz, $-S-CH_2-CH_2-CO-$), 3.58 (2H, t, J=6.8 Hz, $-S-CH_2-CH_2-CO-$), 3.32 (2H, t, J=7.8 Hz, $-CH_2-$, thiazolidine ring), 4.60 (2H, t, J=7.8 Hz, $-CH_2-$, thiazolidine ring). *Anal.* Calcd for $C_8H_{11}NO_2S_2$: C, 38.53; H, 4.45; N, 5.62. Found: C, 38.57; H, 4.47; N, 5.62.

***N*-(3-Acetylthiopropionyloxy)succinimide (ATPS)**——The title compound was prepared as described above by condensation of 3-acetylthiopropionic acid (500 mg, 3.37 mmol) and *N*-hydroxysuccinimide (430 mg, 1.1 eq) using DCC (770 mg, 1.1 eq), and the product was recrystallized from CH_2Cl_2 and hexane; yield 665 mg (80%), mp 52—54 °C. IR ν_{max}^{Nujol} cm^{-1}: 1819, 1720, 1680 (CO). ^{1}H-NMR (60 MHz, $CDCl_3$) δ: 2.30 (3H, s, CH_3-CO-), 2.76 (4H, s, $-CH_2-CH_2-$, succinimide), 2.86—3.23 (4H, m, $-S-CH_2-CH_2-CO-$). *Anal.* Calcd for $C_9H_{11}NO_5S$: C, 44.07; H, 4.52; N, 5.71. Found: C, 44.20; H, 4.51; N, 5.84.

2,4-Dinitrophenyl 3-Acetylthiopropionate (DNATP)——The title compound was prepared as described above by condensation of *S*-acetylthiopropionic acid (500 mg, 3.37 mmol) and 2,4-dinitrophenol (680 mg, 1.1 eq) using DCC (840 mg, 1.5 eq). The product was recrystallized from CH_2Cl_2–isopropyl ether; yield 415 mg (39%), mp 65.5—66 °C, Rf_2 0.93. ^{1}H-NMR (60 MHz, $CDCl_3$) δ: 2.33 (3H, s, acetyl), 2.97—3.18 (4H, m, $-CH_2-CH_2-$), 7.33 (1H, d like, aromatic), 8.23—8.40 (1H, m, aromatic), 8.73 (1H, d like, aromatic). *Anal.* Calcd for $C_{11}H_{10}N_2O_7S$: C, 42.04; H, 3.21;

N, 8.93. Found: C, 41.94; H, 3.05; N, 8.77.

***N*-(3-*p*-Methoxybenzylthiopropionyl)-L-alanine**——(a) A mixture of MTPTT (102 mg, 0.31 mmol) in DMF (2 ml) and L-alanine (30 mg, 1.1 eq) in H_2O (1 ml) was stirred in the presence of NMM (61 μl, 1.5 eq) at room temperature for 4 h, the pale yellow solution became colorless with the progress of the reaction. After evaporation of the solvent, the residue was acidified with 5% citric acid and extracted with AcOEt. The organic layer was washed with 5% citric acid and H_2O, dried over Na_2SO_4 and concentrated. Treatment of the residue with hexane afforded a powder, which was recrystallized from AcOEt and hexane; yield 75 mg (82%), Rf_3 0.66. $[\alpha]_D^{15}$ −18.6° (c=0.2, MeOH). Recovery of Ala in 6 N HCl hydrolysate: 99%. ^{1}H-NMR (200 MHz, DMSO-d_6) δ: 1.25 (3H, d, J=7.2 Hz, $-CH_3$), 2.50—2.58 (4H, m, $-CH_2-CH_2-$), 3.68 (2H, s, $-CH_2-$, benzyl), 3.73 (3H, s, CH_3O-), 4.19 (1H, dq, J_1=7.2 Hz, J_2=7.2 Hz $-CH-$), 6.87 (2H, d, J=8.6 Hz, aromatic), 7.23 (2H, d, J=8.6 Hz, aromatic), 8.21 (1H, d, J=7.2 Hz, $-NH-$). *Anal.* Calcd for $C_{14}H_{19}NO_4S$: C, 56.54; H, 6.44; N, 4.71. Found: C, 56.64; H, 6.53; N, 4.92.

(b) Starting with MTPS (100 mg, 0.31 mmol) and L-alanine (30 mg, 1.1 eq), a product identical with that obtained in (a) was isolated; yield 74 mg (81%), Rf_3 0.66.

Treatment of *N*-(3-*p*-Methoxybenzylthiopropionyl)-L-alanine with 1M TFMSA–Thioanisole/TFA——The above S-protected *N*-(3-mercaptopropionyl)-L-alanine (30 mg, 0.1 mmol) was treated in an ice-bath with 1 M TFMSA-thioanisole/TFA (1 ml, 10 eq) in the presence of *m*-cresol (53 μl, 5 eq). Periodically, an aliquot (15 μl each) was withdrawn from the solution and the mercapto group exposed was measured by means of the Ellman test (OD 412 nm): 81% after 40 min, 98% after 60 min.

***N*-(3-Acetylthiopropionyl)-L-phenylalanine**——(a) A mixture of ATPTT (300 mg, 1.2 mmol) in DMF (6 ml) and L-phenylalanine (218 mg, 1.1 eq) in H_2O (3 ml) was stirred in the presence of Et_3N (184 μl, 1.1 eq) at room temperature for 4 h; the pale yellow color of the solution disappeared with the progress of the reaction. The mixture, after being neutralized with AcOH, was concentrated and the residue was dissolved in AcOEt. The organic layer was washed with 5% citric acid and H_2O, dried over Na_2SO_4 and concentrated. Treatment of the residue with hexane gave a powder, which was recrystallized from AcOEt and hexane; yield 229 mg (65%), mp 132—135°C, Rf_3 0.68. $[\alpha]_D^{15}$ −2.8° (c=0.7, DMF). *Anal.* Calcd for $C_{14}H_{17}NO_4S$: C, 56.93; H, 5.80; N, 4.74. Found: C, 56.68; H, 5.94; N, 4.68.

(b) A mixture of ATPS (245 mg, 1 mmol) in DMF (5 ml) and L-phenylalanine (182 mg, 1.1 eq) in H_2O (3 ml) was stirred in the presence of NMM (144 μl, 1.1 eq) at room temperature for 4 h; the spot corresponding to ATPS disappeared on TLC. The product was isolated as described above and its identity with the sample obtained in (a) was confirmed by TLC; yield 266 mg (90%).

(c) Reaction of DNATP (314 mg, 1.0 mmol) with L-phenylalanine (182 mg, 1.1 eq) was similarly performed in the presence of NMM (144 μl, 1.1 eq). Within 2 h, the spot corresponding to DNATP had disappeared. The product was isolated as described above (yield 89%) and shown by TLC to be identical with the product obtained in (a).

Treatment of *N*-(3-Acetylthiopropionyl)-L-phenylalanine with Hydroxylamine——Under an N_2 atmosphere, the above S-protected *N*-(3-mercaptopropionyl)-L-phenylalanine (30 mg, 0.1 mmol) in DMF (1 ml) was mixed with a solution of hydroxylamine [prepared from 10 mg (0.15 mmol) of the HCl salt with 20 μl (0.15 mmol) of NMM] in phosphate buffer (100 μl) at pH 7.5 and the mixture was stirred at room temperature for 4 h; the spot corresponding to the starting material disappeared on TLC. The Ellman test revealed that *ca.* 85% of the mercapto group was exposed.

2,4-Dinitrophenyl *p*-(*β*-Nitrovinyl)benzoate——DCC (801 mg, 1.5 eq) was added to an ice-chilled mixture of *p*-(*β*-nitrovinyl)benzoic acid (500 mg, 2.59 mmol) and 2,4-dinitrophenol (524 mg, 1.1 eq) in DMF (15 ml). After being stirred at room temperature for 5 h, the solution was filtered, the filtrate was concentrated and the residue was

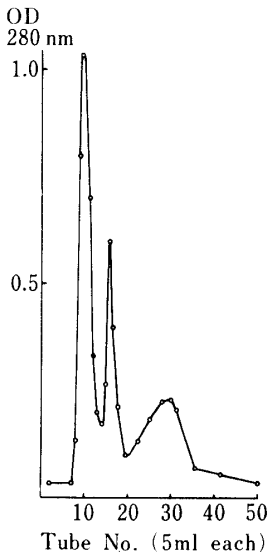

Fig. 3. Gel-Filtration of the hGRF-BSA Conjugated Product on Sephadex G-25

Table I. Amino Acid Ratios of the hGRF–BSA Conjugated Peptide

A.A.	(A) BSA	(B) GRF–BSA	(B)−(A)	(C) GRF theory	(B)−(A)/(C)
Asp	56.4	90.8	34.4	4	8.6
Thr	34.9	43.0	8.1	1	8.1
Ser	28.3	60.3	32.0	4	8.0
Glu	83.2	146.2	63.0	7	9.0
Pro	37.2	35.3	−1.9	0	0
Gly	18.2	44.8	26.6	3	8.9
Ala	52.9	89.4	36.5	4	9.1
Val	33.2	40.5	7.3	1	7.3
Met	4.3	12.5	8.2	1	8.2
Ile	13.3	27.7	14.4	2	7.2
Leu	64.6	97.6	33.0	4	8.3
Tyr	20.8	35.7	14.9	2	7.5
Phe	28.7	36.0	7.3	1	7.3
Lys	62.5	81.2	18.7	2	9.4
His[a)]	17.0	17.0	0	0	0
Arg	26.0	62.3	36.3	4	9.1
Trp	N.D.	N.D.	N.D.	0	0
Recov. (%)	77	72		Average	8.3

a) Diagnostic amino acid.

triturated with EtOH to afford a powder, which was recrystallized from DMF–$CHCl_3$; yield 625 mg (67%), mp 212—213 °C, Rf_4 0.87. *Anal.* Calcd for $C_{15}H_9N_3O_8$: C, 50.15; H, 2.53; N, 11.70. Found: C, 50.43; H, 2.54; N, 11.69.

Synthetic Human Growth Hormone-Releasing Factor (hGRF-40)–BSA Conjugate——A mixture of synthetic hGRF-40 (5.6 mg, 1.23 μmol) and MTPTT (10 mg, 3.05 μmol) in DMF (1.5 ml) was stirred at room temperature for 4 h; the yellow color of the solution disappeared with the progress of the reaction. Ether was added and the resulting powder was treated with 1 M TFMSA–thioanisole/TFA (200 μl) in the presence of *m*-cresol (10 μl) in an ice-bath for 60 min. Ether was added and the resulting powder was again treated with the above acid under the same conditions. The SH-compound thus obtained was precipitated with ether then dissolved in DMF (2 ml). To this solution, an aliquot (89 μl, 2 eq) of a solution of 2,4-dinitrophenyl *p*-(β-nitrovinyl)benzoate (10 mg) in DMF (1 ml) was added. The mixture was stirred at room temperature for 30 min, while the value obtained in the Ellman test (OD 412 nm) decreased from 0.080 to 0.005. Ether was added and the precipitate, after being washed with ether, was dissolved in DMF–H_2O (2 ml, 1 : 1). The pH of the solution was adjusted to 7.5 with NMM and then BSA (5.0 mg, 0.075 μmol) in H_2O (1 ml) was added. After being stirred at room temperature for 4 h, the mixture was applied to a column of Sephadex G-25 (1.8 × 60 cm), which was eluted with 1 N AcOH. The fractions corresponding to the front peak (Fig. 3, tube Nos. 8—14) were combined and the solvent was removed by lyophilization to give a fluffy powder; yield 7.70 mg. The materials obtained after lyophilization of peaks 2 and 3 were negligible. Acid hydrolysis of the product indicated that 8 molecules of hGRF were conjugated to one molecule of BSA as shown in Table I.

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