Studies on Heterobifunctional Cross-Linking Reagents, 6-Maleimidohexanoic Acid Active Esters

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6-Maleimidohexanoic acid *N*-hydroxysuccinimide ester has been used widely for preparation of enzyme immunoconjugates as a unique heterobifunctional cross-linking reagent. Its heterobifunctional reactivity is good, but its ester portion hydrolyzes easily in the presence of water. Several 6-maleimidohexanoic acid active esters (6-maleimidohexanoic acid 4-nitrophenyl ester, 6-maleimidohexanoic acid *N*-hydroxy-5-norbornene-endo-2,3-dicarboximide ester, and 6-maleimidohexanoic acid pentafluorophenyl ester) were prepared and their reactivity and stability in an aqueous media were tested. Of the synthetic esters, the pentafluorophenyl ester exhibited the highest reactivity and stability in aqueous media.

Key words heterobifunctional cross-linking reagent; maleimidohexanoic acid ester; peptide

6-Maleimidohexanoic acid *N*-hydroxysuccinimide ester (MHSu)[1—3] is a heterobifunctional reagent cross-linking between amino and sulfhydryl groups. It is usually coupled initially to molecules containing primary or secondary amines through amide bond, followed by specific coupling with sulfhydryl-containing molecules through thioether linkage. MHSu is used mostly in aqueous media. MHSu has been used as a unique and useful reagent for preparation of hapten conjugate and enzyme immunoconjugates.

We recently reported preparation of a peptide-poly(ethylene glycol) (PEG) transporter tool for carrying adenovirus vector into cells.[4] In that study, synthetic (Ac-Tyr-Gly-Gly-Arg-Gly-Asp-Thr-Pro-βAla)$_2$Lys-PEG-βAla-Cys-NH$_2$ was reacted with MHSu and the resulting material, [(Ac-Tyr-Gly-Gly-Arg-Gly-Asp-Thr-Pro-βAla)$_2$Lys-PEG-βAla-Cys-NH$_2$]-6-succinimidohexanoic acid *N*-hydroxysuccinimide ester (I), was purified by HPLC. 0.05% Trifluoroacetic acid (TFA) in a mixture of acetonitrile and water was used as an eluent. The peak corresponding to I was observed beside the peak corresponding to [(Ac-Tyr-Gly-Gly-Arg-Gly-Asp-Thr-Pro-βAla)$_2$Lys-PEG-βAla-Cys-NH$_2$]-6-succinimido-hexanoic acid (II). The peak corresponding to I was collected, lyophilized and analyzed by analytical HPLC. An extra peak corresponding to II was observed beside the peak corresponding to I. During purification and analytical procedures, the *N*-hydroxysuccinimide ester portion of I may have been hydrolyzed to produce II. An eluent without TFA was also used for HPLC purification and HPLC analysis, but the extra peak corresponding to II was always detected in the purified I by analytical HPLC suggesting that the *N*-hydroxysuccinimide ester portion of I may be easily hydrolysed in the presence of water.

Here hydrolysis of MHSu is examined. MHSu was dissolved in a mixture of acetonitril and phosphate buffer (pH 6.4 or 7.4) and the solution was kept at 20 °C. An aliquot was removed and analyzed by HPLC every 1.5 h. The peak corresponding to 6-maleimidohexanoic acid (MHA) increased and the result showed that MHSu is hydrolyzed easily in the presence of water at both pH 6.4 and 7.4.

To obtain more stable and reactive 6-maleimidohexanoic acid active esters, the following active esters were prepared and examined for their reactivity and stability to water: 6-maleimidohexanoic acid 4-nitrophenyl ester (MHNp), 6-maleimidohexanoic acid *N*-hydroxy-5-norbornene-endo-2,3-dicarboximide ester (MHNb), and 6-maleimidohexanoic acid pentafluorophenyl ester (MHPf).

Preparation of 6-maleimidohexanoic acid active esters was performed by coupling of 6-maleimidohexanoic acid and a corresponding hydroxyl compound with diisopropylcarbodiimide (DIC) in ethyl acetate (AcOEt). The products were purified by silica gel column chromatography using chloroform as an eluent.

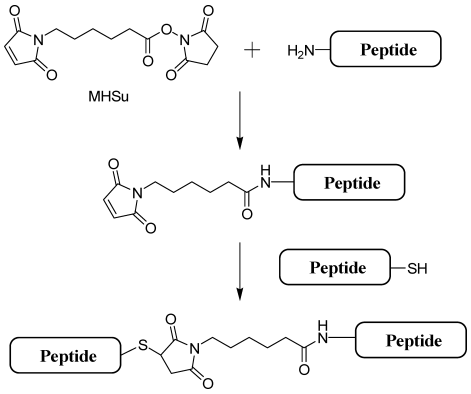

Fig. 1. Heterobifunctinal Reaction of MHSu

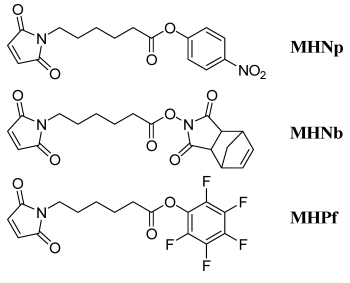

Fig. 2. New Synthetic Heterobifunctional Reagents

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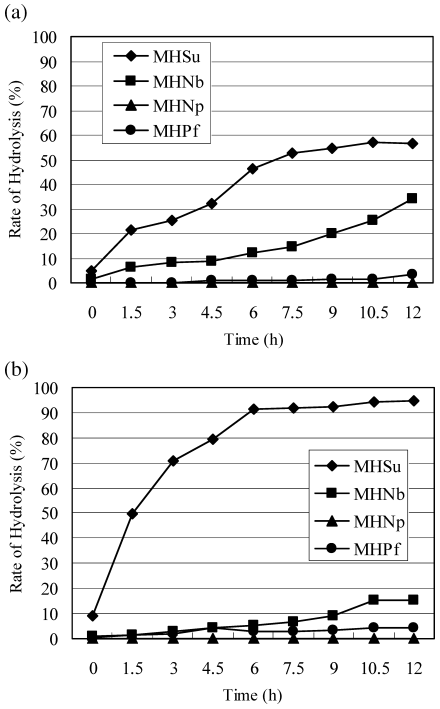

Fig. 3. Hydrolysis of 6-Maleimidohexanoic Acid Esters at pH 6.4 (a) and 7.4 (b)

To study the stability of synthetic 6-maleimidohexanoic acid esters, hydrolysis of synthetic 6-maleimidohexanoic acid esters was examined as described above for the hydrolysis test of MHSu by HPLC, and the results are shown in Fig. 3.

Among synthetic 6-maleimidohexanoic acid esters, MHNp was most stable: hydrolysis was not observed even after 12 h at both pH 6.4 and 7.4. MHPf was relatively stable: 5% of hydrolysis was observed after 12 h at the both pH 6.4 and 7.4. MHNb was relatively stable compared to MHSu: 34% and 15% hydrolysis was observed at pH 6.4 and 7.4.

Reactivity of each synthetic 6-maleimidohexanoic acid active ester was examined by coupling reaction of 6-maleimidohexanoic acid active esters (0.1 mmol) and proline amide (H-Pro-NH_2, 0.11 mmol) to form 6-maleimodohexanoylproline amide in a mixture of acetonitrile and phosphate buffer (pH 7.0) at 25 °C. The reaction was checked by HPLC and the results are shown in Fig. 4.

Of the esters, MHPf exhibited the highest reactivity and the others (MHNb, MHNp, MHSu) showed nearly equal reactivity. MHPf was the best reagent, based on the hydrolysis test and the reactivity test. MHNp was stable to hydrolysis, but its reactivity was slower than that of MHPf. MHNb was more stable to hydrolysis, but its reactivity was almost the same compared to MHSu.

MHPf, MHNb and MHNp were prepared and their stability to hydrolysis and reactivity to H-Pro-NH_2 were examined. Among them, MHPf exhibited relatively good stability to hydrolysis and superior reactivity for aminolysis. MHPf will be a promising heterobifunctional cross-linking reagent for conjugating an amino compound and a thiol compound.

Experimental

The RP-HPLC was conducted with a Waters 600E on an Inertsil ODS-3 column using gradient systems of CH_3CN/H_2O containing 0.05% trifluoroacetic acid. TOF-MS spectra were measured with a Shimadzu/Kratos Kompact MALDI IV mass spectrometer using sinapic acid as a matrix. Melting Points were determined on a Yanagimoto micro-melting point apparatus. Column chromatography was performed on silica gel 60 (BW-127ZH, Fuji Silica Chemical Co.). ^{1}H-NMR spectra were recorded on a Bruker 400 MHz spectrometer. Solvent system for ascending thin-layer chromatography on Silica gel G (type 60, Merck) is $CHCl_3$/acetic acid (95/5).

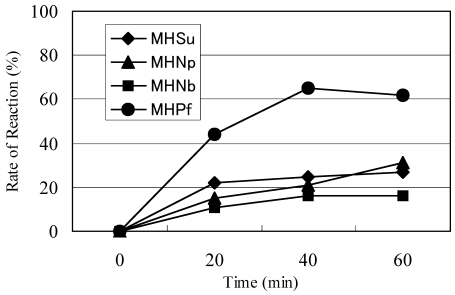

Fig. 4. Reactivity of 6-Maleimidohexanoic Acid Esters with H-Pro-NH_2

Table 1. Yields and TOF-MS Data of MHa-Pro-NH_2

	MHSu method	MHNp method	MHNb method	MHPf method
Yield	27%	31%	16%	62%
TOF-MS *m/z* (Calcd 307.2)	308.1	308.4	308.3	307.8

Preparation of 6-Maleimidohexanoic Acid 4-Nitrophenyl Ester (MHNp) 6-Maleimidohexanoic acid (422 mg, 2 mmol) and 4-nitrophenol (306 mg, 2.2 mmol) were dissolved in AcOEt (10 ml) and the solution was cooled in an ice-bath. DIC (0.4 ml, 2.2 mmol) was added to the solution and the mixture was stirred for 4 h. The solvent was removed *in vacuo* and the residue was purified by silica gel column (2×41 cm) chromatography using chloroform as an eluent. The material was recrystallized from isopropyl ether. Yield 490 mg (74%), mp 73—74 °C. MS *m/z*: 333.3 ($M+H^+$), TLC: *Rf* 0.70. *Anal.* Calcd for $C_{16}H_{16}N_2O_6$; C, 57.83; H, 4.85; N, 8.43. Found; C, 57.82; H, 4.83; N, 8.49. ^{1}H-NMR ($CDCl_3$) δ: 8.27(2H, d, ArH), 7.27 (2H, d, ArH), 6.73 (2H, s, maleimido), 3.55 (2H, t, $-CH_2^{\varepsilon}-$), 2.59 (2H, t, $-CH_2^{\alpha}-$), 1.79 (2H, m, $-CH_2^{\delta}-$), 1.65 (2H, m, $-CH_2^{\beta}-$), 1.43 (2H, m, $-CH_2^{\gamma}-$).

Preparation of 6-Maleimidohexanoic Acid Pentafluorophenyl Ester (MHPf) Prepared in the same way described above for preparation of MHNp. Yield 701 mg (93%), mp 59 °C. MS *m/z*: 378.8 ($M+H^+$), TLC: *Rf* 0.68. *Anal.* Calcd for $C_{16}H_{12}NO_4F_5$; C, 50.94; H, 3.21; N, 3.71. Found; C, 51.08; H, 3.47; N, 3.75. ^{1}H-NMR ($CDCl_3$) δ: 6.69 (2H, s, maleimide), 3.55 (2H, t, $-CH_2^{\varepsilon}-$), 2.66 (2H, t, $-CH_2^{\alpha}-$), 1.80 (2H, m, $-CH_2^{\delta}-$), 1.65 (2H, m, $-CH_2^{\beta}-$), 1.43 (2H, m, $-CH_2^{\gamma}-$).

Preparation of 6-Maleimidohexanoic Acid *N*-Hydroxy-5-norbornene-2,3-dicarboximide Ester (MHNb) Prepared in the same way described above for preparation of MHNp. Yield 738 mg (95%), mp 111—113 °C. MS *m/z*: 373.9 ($M+H^+$), TLC: *Rf* 0.69. *Anal.* Calcd for $C_{19}H_{20}N_2O_6$; C, 61.28; H, 5.41; N, 7.52. Found; C, 60.62; H, 5.38; N, 7.50. ^{1}H-NMR ($CDCl_3$) δ: 6.69 (2H, s, maleimide), 6.20 (2H, s, norbornene H-2,3), 3.52 (2H, t, $-CH_2^{\varepsilon}-$), 3.44 (2H, s, norbornene H-1,4), 3.31 (2H, s, norbornene H-5,6), 2.53 (2H, t, $-CH_2^{\alpha}-$), 1.75 (2H, m, $-CH_2^{\delta}-$), 1.62 (2H, m, $-CH_2^{\beta}-$), 1.53 (2H, d, norbornene H-7), 1.38 (2H, m, $-CH_2^{\gamma}-$).

Stability Test of 6-Maleimidohexanoic Acid Active Esters in Aqueous Medium Each 6-maleimidohexanoic acid active ester (1 mg) was dissolved in acetonitrile (100 μl) and mixed with 0.1 mol/l phosphate buffer (pH 6.4 or pH 7.4, 900 μl) and the mixture was kept at 20 °C. An aliquot of the solution was removed and subjected to HPLC to examine hydrolysis of the 6-maleimidohexanoic ester. Optical density of each aliquot at 220 nm was measured.

Preparation of 6-Maleimidohexanoylproline Amide Using 6-Maleimidohexanoic Acid Active Esters in Aqueous Medium Each 6-maleimidohexanoic acid active ester (0.1 mmol) was dissolved in acetonitrile (4 ml) and the solution was mixed with H-Pro-NH_2 (12.5 mg, 0.11 mmol) dissolved in 0.1 mol/l phosphate buffer (pH 7.0, 2 ml). The mixture was stirred at 20 °C and an aliquot of the mixture was subjected to HPLC to examine for-

mation of 6-maleimidohexanoyl-Pro-NH_2(MHa-Pro-NH_2) every 20 min. Optical density of each aliquot at 220 nm was measured. After 1 h, the solvent was removed *in vacuo* and the residue was purified by HPLC. Yields and mass spectra data of products are shown in Table 1.

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