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**Studies on Peptides. CXXII.^{1,2)} *N*-Succinimidyl-*p*-(2-nitrovinyl)-benzoate
and Its *m*-Isomer, as Heterobifunctional Conjugating
Reagents for Immunoassay**

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N-Succinimidyl-*p*- and *m*-(2-nitrovinyl)-benzoates (SNVB) were introduced as heterobifunctional conjugating reagents for immunoassay. These reagents smoothly react with SH-compounds, such as cysteine or mercaptopropionic acid, in acidic media, then with alanine under slightly basic conditions to yield the corresponding benzoylalanine derivatives. On the basis of these model experiments, the feasibility of practical use of these reagents is discussed.

Keywords — *N*-succinimidyl-*p*-(2-nitrovinyl)-benzoate; *N*-succinimidyl-*m*-(2-nitrovinyl)-benzoate; cysteine addition to nitrovinyl function; heterobifunctional conjugating reagent; cross-linking reagent; radioimmunoassay; enzyme immunoassay

In radioimmunoassay (RIA) and enzyme immunoassay (EIA), the reagents commonly used for conjugation of hapten-peptides to high-molecular-weight carriers, such as glutaraldehyde,³⁾ toluene-2,4-diisocyanate⁴⁾ and carbodiimide derivatives,⁵⁾ produce a heterogeneous mixture of antigens, because of extensive random inter- and intramolecular self-couplings of both components.

Recently, various heterobifunctional reagents have been devised to produce homogeneous antigens with unmodified antigenic determinants in the peptide moieties and consequently to improve RIA techniques for peptide hormones, as well as EIA techniques.⁶⁾ Such reagents generally bear one function which reacts readily in a specific way with the thiol group and one which does so with the amino group. The former is the maleimide function⁷⁾ in most instances and the pyridylthio⁸⁾ or the iodoacetyl function⁹⁾ as an alternative. For the latter, the *N*-hydroxysuccinimide ester¹⁰⁾ is preferred. Thus, among various reagents, *N*-hydroxysuccinimide ester of *m*-maleimidobenzoic acid¹¹⁾ or *N*-(4-carboxycyclohexylmethyl)-maleimide¹²⁾ seems to be an attractive conjugating reagent for RIA and EIA.

A number of heterobifunctional crosslinking reagents can be designed by combination of specific SH reagents and various sophisticated active esters currently available in peptide and protein chemistries. As an example, we wish to present an easily available conjugating reagent for RIA and EIA. *N*-Succinimidyl-*p*-(2-nitrovinyl)-benzoate [I], introduced here, has two functional groups, the nitrovinyl function to trap the SH group¹³⁾ and the aforementioned active ester function to react with the amino group of peptides and proteins.

The starting material, *p*-(2-nitrovinyl)-benzoic acid, is a known compound,¹⁴⁾ which can be prepared by condensation of nitromethane and terephthalaldehydic acid. The usual DCC condensation^{5a)} of this acid and *N*-hydroxysuccinimide afforded the desired reagent [I] as a crystalline compound, which was fully characterized by various spectral methods and elemental analysis. Its typical ultraviolet (UV) absorption at 303 nm due to the 2-nitrovinyl

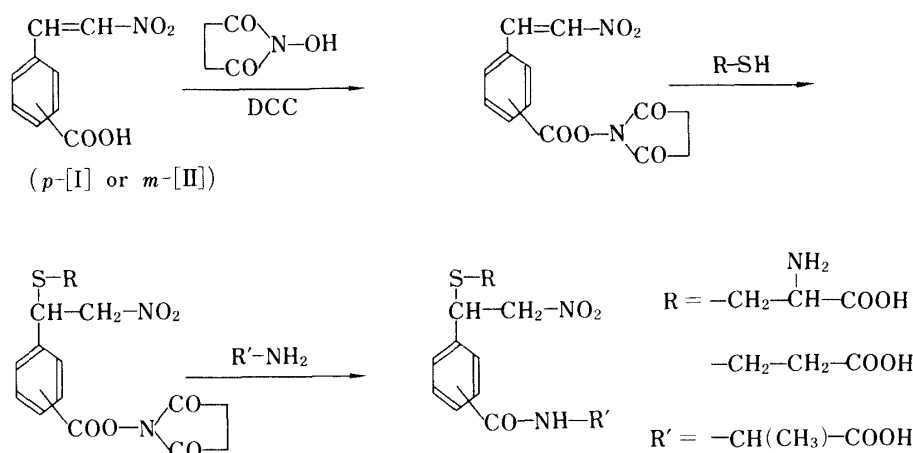


Fig. 1. Model Experiments with *N*-Succinimidyl-*p*-(2-nitrovinyl)-benzoate [I] and Its *m*-Isomer [II]

moiety is a useful guide for monitoring the SH-addition to the reagent.

Base-catalyzed addition of Boc-cysteine to β -nitrostyrene was reported to produce *N* $^{\alpha}$ -Boc-S-(2-nitro-1-phenylethyl)-cysteine.^{13c)} Michael type addition of cysteine to the reagent [I] proceeded quantitatively (Fig. 1), even in acidic pH, within 30 min, while the succinimide ester function remained intact. The UV max. of the adduct shifted from 303 to 230 nm. For confirmation, [I] was allowed to react with another SH-compound, mercaptopropionic acid, at pH 3.0. The reaction again proceeded quantitatively within 30 min and the isolated adduct was fully characterized.

Next, aminolysis of the above cysteine adduct, as well as the mercaptopropionic acid adduct, with alanine was performed under slightly basic conditions. After overnight reaction, the benzoylalanine derivatives were isolated by gel-filtration and characterized by nuclear magnetic resonance (NMR) spectroscopy, acid hydrolysis, and elemental analysis. Reagent [I] behaved as a bifunctional reagent trapping two different components, as expected. Addition of SH compounds to the vinyl function is so quick that the active ester function remained intact. However, the rate of aminolysis of this *p*-substituted benzoic acid active ester seemed somewhat slower than expected. The isolated yield remained at *ca.* 30% in both cases.

Thus, the *m*-substituted analog of [I], *N*-succinimidyl-*m*-(2-nitrovinyl)-benzoate [II], was similarly prepared (Fig. 1). Addition of mercaptopropionic acid to [II] proceeded as smoothly and quickly as to [I], and little difference in the rate of aminolysis with alanine was observed between the *m*- and *p*-isomers. In such a model system, we also observed on thin layer chromatography (TLC) that the rate of aminolysis of the above-mentioned *N*-hydroxysuccinimide ester of *m*-maleimidobenzoic acid¹¹⁾ with alanine was not very different from that of [I].

The possibility cannot be excluded that increasing basicity in the aminolysis step may cause partial elimination of the SH-function^{13d)} of the adducts of [I] and [II]. Therefore, the use of NMM or pH 7.5 phosphate buffer, rather than Et_3N , may be preferable with these reagents.

In view of the properties of the two functional groups of these reagents, it seems reasonable to carry out the following two consecutive reactions in order to prepare hapten-carrier conjugates with little disruption of immunogenic functions: firstly, quick addition of an SH-compound to the nitrovinyl function in acidic media, and secondly aminolysis of the *N*-hydroxysuccinimide ester with protein, such as human serum albumin or bovine serum albumin (which has 60 amino functions). Such multiple amino functions of albumin may improve the aminolysis of the active ester function of these reagents. On the other hand, in the

case of the above-mentioned *m*-maleimido-derivative,^{11,12)} aminolysis followed by SH addition reaction was recommended. This procedure seems unsuitable for our reagents, because of the instability of the nitrovinyl function under basic conditions.

Various excellent SH-introducing reagents, such as *S*-acetylmercaptosuccinic anhydride,¹⁵⁾ are now available. With the aid of such reagents, any hapten (peptide) can be converted to an SH-derivative. Recently, we introduced a new SH-reagent, 3-(*S*-*p*-methoxybenzylmercaptopropionyl)-thiazolidine-2-thione and with the aid of this reagent, we converted synthetic human growth hormone releasing factor (hGRF 1-40-OH)¹⁶⁾ to the corresponding SH-compound. This derivatized hGRF was then conjugated to bovine serum albumin with reagent [I]. These results will be reported in a subsequent paper.

Experimental

R_f values in TLC performed on silica gel (Kieselgel G, Merck) refer to the following solvent systems; *R_f*₁ CHCl₃-MeOH (10:0.5), *R_f*₂ CHCl₃-MeOH-AcOH (9:1:0.5), *R_f*₃ *n*-BuOH-AcOH-AcOEt-H₂O (1:1:1:1).

Proton nuclear magnetic resonance (¹H-NMR) spectra were taken with a JEOL JNM-FX200 (200 MHz) instrument using tetramethylsilane as an internal standard. Mass spectra were taken with a JEOL JMS-01SG-2 machine. UV absorption was measured with a Hitachi model 200-20 spectrophotometer.

***N*-Succinimidyl-*p*-(2-nitrovinyl)-benzoate [I, *p*-SNVB]**—DCC (1.18 g, 5.69 mmol) was added to an ice-chilled mixture of *p*-(2-nitrovinyl)-benzoic acid¹⁴⁾ (1.0 g, 5.18 mmol) and HOSu (0.66 g, 5.69 mmol) in THF (10 ml) and the mixture, after being stirred at room temperature for 5 h, was filtered. The filtrate was concentrated and the residue was treated with EtOH to afford a solid, which was precipitated twice from CHCl₃-MeOH (10:1) with EtOH; yield 0.49 g (33%), mp 222–223 °C, *R_f*₁ 0.73 [Ce(SO₄)₂ stained]. MS *m/e*: 290 (M⁺). UV^{CHCl₃-MeOH(10:1)}_{max} nm (ε): 303 (25460). IR $\nu_{\text{max}}^{\text{KBr}}$ cm⁻¹: 1790, 1720 (CO, COOR) 1630 (C=C). ¹H-NMR (DMSO-*d*₆) δ: 2.92 (4H, s, -CH₂-CH₂-), 8.13 (2H, d, *J* = 8 Hz, aromatic), 8.19 (2H, d, *J* = 8 Hz, aromatic), 8.25 (1H, d, *J* = 13 Hz, vinyl), 8.39 (1H, d, *J* = 13 Hz, vinyl). Anal. Calcd for C₁₃H₁₀N₂O₆: C, 53.80; H, 3.47; N, 9.65. Found: C, 53.87; H, 3.44; N, 9.56.

Cysteine Adduct of *p*-(2-Nitrovinyl)-benzoyl-alanine—A mixture of [I] (100 mg, 0.34 mmol) in DMF (2 ml) and cysteine·HCl (60 mg, 0.34 mmol) in H₂O (2 ml) was stirred at pH 1.5 at room temperature for 30 min; the starting material disappeared completely on TLC and the UV max. shifted from 303 to 230 nm. The solvent was evaporated off and the residue was treated with H₂O. The resulting powder (*R_f*₃ 0.68; recovery of cysteine in 6N HCl hydrolysate, 90%) was dissolved in DMF and mixed with a solution of alanine (153 mg, 5 eq) in H₂O in the presence of NMM (35 μl, 1 eq). After being stirred at room temperature overnight, the solution (*R_f*₃ 0.60 together with a small amount of the starting material) was concentrated and the residue was subjected to gel-filtration on Sephadex G-25 (2.3 × 135 cm) using 0.5N AcOH as an eluant. Individual fractions (5.4 ml each) were examined for UV absorption at 300 nm. The fractions corresponding to the front main peak (tube Nos. 76–81) were collected and the solvent was removed by lyophilization to give a white fluffy powder; yield 23.6 mg (17.8%), mp 151 °C (dec.), *R_f*₃ 0.60. Amino acid ratios in 6N HCl hydrolysate: Ala: 1/2Cys = 1:0.88 (recovery 90%) UV^{0.5N AcOH}_{max} nm (ε): 230 (13900). ¹H-NMR (DMSO-*d*₆) δ: 1.38 (3H, d, *J* = 7.2 Hz, -CH₃(Ala)), 2.90 (2H, d, *J* = 5.2 Hz, -CH₂-(Cys)), 3.50–3.44 (1H, m, -CH-(Cys)), 4.40 (1H, dq, *J*₁ = 7.2 Hz, *J*₂ = 7.2 Hz, -CH-(Ala)), 8.67 (1H, d, *J* = 7.2 Hz, -NH-(Ala)). Anal. Calcd for C₁₅H₁₉N₃O₇S·H₂O: C, 44.66; H, 5.25; N, 10.42. Found: C, 44.91; H, 5.06; N, 9.95.

Mercaptopropionic Acid Adduct of *N*-Succinimidyl-*p*-(2-nitrovinyl)-benzoate—A mixture of the reagent [I] (500 mg) in THF (5 ml) and mercaptopropionic acid (225 μl, 1.5 eq) in pH 3.0, 0.1 M phosphate buffer (2 ml) was stirred at room temperature for 30 min; the spot corresponding to reagent [I] disappeared on TLC. The organic phase was separated, washed with H₂O and concentrated. Treatment of the residue with EtOH afforded a powder, which was recrystallized from THF and EtOH; yield 563 mg (82%), mp 151–154 °C, *R_f*₁ 0.16. UV^{MeOH}_{max} nm (ε): 238 (20870). ¹H-NMR (DMSO-*d*₆) δ: 2.65–2.78 (4H, m, -S-CH₂-CH₂-), 2.90 (4H, s, -CH₂-CH₂-), 4.85 (1H, dd, *J*₁ = 9 Hz, *J*₂ = 7 Hz, -CH-), 5.20 (1H, dd, *J*₁ = 14 Hz, *J*₂ = 7 Hz, -CH₂-NO₂), 5.29 (1H, dd, *J*₁ = 14 Hz, *J*₂ = 9 Hz, -CH₂-NO₂), 7.75 (2H, d, *J* = 8.3 Hz, aromatic), 8.09 (2H, d, *J* = 8.3 Hz, aromatic). Anal. Calcd for C₁₆H₁₆N₂O₈S: C, 48.48; H, 4.07; N, 7.07. Found: C, 48.75; H, 4.08; N, 7.12.

Mercaptopropionic Acid Adduct of *p*-(2-Nitrovinyl)-benzoyl-alanine—The above *S*-propionic acid adduct (200 mg, 0.51 mmol) in THF (4 ml) was mixed with H-Ala-OH (67 mg, 1.5 eq) in pH 7.5, 0.1 M phosphate buffer (2 ml), and the mixture was stirred at room temperature for 2 h. The aqueous phase was separated and applied to a column of Sephadex G-25 (2.3 × 135 cm), which was eluted with 0.5N AcOH. Individual fractions (7 ml each) were collected, and examined for UV absorption at 295 nm. The solvent of the desired fractions was removed by lyophilization to give a fluffy powder, yield, 60 mg (32%), mp 44–46 °C, *R_f*₃ 0.85. Ala recovery in 6N HCl hydrolysate: 93%. UV^{MeOH}_{max} nm (ε): 230 (16310). ¹H-NMR (DMSO-*d*₆) δ: 1.39 (3H, d, *J* = 7.2 Hz, -CH₃), 2.56–2.77 (4H, m, -S-CH₂-CH₂-), 4.41 (1H, d of q, *J*₁ = 7.2 Hz, *J*₂ = 7.2 Hz, -CH-), 4.74 (1H, t, *J* = 8.2 Hz, -CH-CH₂-NO₂), 5.19 (2H, d, *J* =

8.2 Hz, $-\text{CH}_2-\text{NO}_2$), 7.55 (2H, d, $J=8.1$ Hz, aromatic), 7.85 (2H, d, $J=8.1$ Hz, aromatic), 8.67 (1H, d, $J=7.2$ Hz, $-\text{NH}-$). Anal. Calcd for $\text{C}_{15}\text{H}_{18}\text{N}_2\text{O}_7\text{S} \cdot 1/2\text{H}_2\text{O}$: C, 47.48; H, 5.05; N, 7.38. Found: C, 47.99; H, 4.89; N, 7.27.

***N*-Succinimidyl-*m*-(2-nitrovinyl)-benzoate [II, *m*-SNVB]**—Starting with *m*-(2-nitrovinyl)-benzoic acid,¹⁷⁾ the title compound was prepared in the same manner as described for reagent [I] and recrystallized from AcOEt and EtOH; yield 83%. mp 207–209 °C, R_f 0.78. UV $^{\text{CHCl}_3}_{\text{max}}$ nm (ϵ): 300 (16700). IR $\nu^{\text{Nujol}}_{\text{max}}$ cm^{-1} : 1790, 1720 (CO, COOR), 1630 (C=C). $^1\text{H-NMR}$ (DMSO- d_6) δ : 2.93 (4H, s, $-\text{CH}_2-\text{CH}_2-$), 7.77–8.57 (4H, m, aromatic), 8.29 (1H, d, $J=13.8$ Hz, vinyl), 8.39 (1H, d, $J=13.8$ Hz, vinyl). Anal. Calcd for $\text{C}_{13}\text{H}_{10}\text{N}_2\text{O}_6$: C, 53.80; H, 3.47; N, 9.65. Found: C, 54.05; H, 3.55; N, 9.41.

Mercaptopropionic Acid Adduct of *m*-(2-Nitrovinyl)-benzoyl-alanine—In the same way as described above, the mercaptopropionic acid adduct was prepared by mixing reagent [II] (340 mg, 1.17 mmol) in THF (4 ml) and mercaptopropionic acid (102 μl , 1.0 eq) in pH 3.0, 0.1 M phosphate buffer (2 ml) for 30 min. The organic phase was separated, washed with H_2O and mixed with H-Ala-OH (209 mg, 2 eq) in pH 7.5, 0.1 M phosphate buffer (5 ml). After being stirred at room temperature for 2 h, the mixture was applied to a column of Sephadex G-25 (2.3×130 cm), which was eluted with 0.5 N AcOH. Individual fractions (6 ml each) were examined for UV absorption at 330 nm. The desired fractions (tube Nos. 76–85) were collected and the solvent was removed by lyophilization to give a fluffy powder; yield 63 mg (15%), mp 48–50 °C, R_f 0.85. Ala recovery in 6 N HCl hydrolysate: 84%. $^1\text{H-NMR}$ (DMSO- d_6) δ : 1.42 (3H, d, $J=7.4$ Hz, $-\text{CH}_3$), 2.51–2.80 (4H, m, $-\text{S}-\text{CH}_2-\text{CH}_2-$) 4.44 (1H, dq, $J_1=7.4$ Hz, $J_2=7.4$ Hz, $-\text{CH}-$), 4.74 (1H, t, $J=8.0$ Hz, $-\text{CH}-\text{CH}_2-\text{NO}_2$), 5.19 (2H, d, $J=8.0$ Hz, $-\text{CH}_2-\text{NO}_2$), 7.40–7.94 (4H, m, aromatic), 8.68 (1H, d, $J=7.4$ Hz, $-\text{NH}-$). Anal. Calcd for $\text{C}_{15}\text{H}_{18}\text{N}_2\text{O}_7\text{S}$: C, 48.64; H, 4.90; N, 7.56. Found: C, 48.41; H, 4.88; N, 7.64.

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References and Notes

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