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The Preparation of Steroid N-Hydroxysuccinimide Esters and Their Reactivities with Bovine Serum Albumin[1]

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The N-hydroxysuccinimide esters of testosterone and cortisol were prepared from the corresponding carboxylic acid derivatives by a carbodiimide method. These activated esters were covalently linked to bovine serum albumin in organic solvent/phosphate buffer (pH 7.4) employing various molar ratios of the steroid to protein. The results indicated that the activated ester method would be useful for enzyme labeling in enzyme immunoassay of steroids in respects of simplicity and reproducibility.

Keywords——enzyme labeling method; testosterone haptens; cortisol haptens; N-hydroxysuccinimide ester; activated ester; reaction with BSA

In recent years a number of enzyme immunoassays for steroid hormones employing several enzymes have been developed. The preparation of enzyme-labeled steroids has been carried out mostly by coupling the carboxyl group of a steroid derivative with the amino groups of an enzyme by the use of a mixed anhydride[3] or carbodiimide reaction. One of the important factors affecting sensitivity and reproducibility in an enzyme immunoassay is the procedure for the enzyme labeling, where the least loss of antigenecity and enzymic activity should be essential. However, these two techniques are not always satisfactory due to the inevitable limitations. The use of the N-hydroxysuccinimide ester for enzyme labeling in enzyme immunoassay of steroids appears to be convenient and capable to overcome the problems by simplifying the reaction procedure. From these points of view we have attempted to prepare the N-hydroxysuccinimide esters of testosterone and cortisol and examine their reactivities with bivone serum albumin (BSA) as a model experiment.

Materials and Methods

Synthesis of Steroid Carboxylic Acids[4]

4-(Carboxymethylmercapto)testosterone (5a)——To a solution of 4,5-epoxytestosterone (**9**)[5] (500 mg) in EtOH (7 ml) was added a solution of mercaptoacetic acid (300 mg) in 60% KOH (0.22 ml), and the reaction mixture was stirred at room temperature under a N_2 gas stream for 2 hr. After addition of water the resulting solution was extracted with AcOEt, and the aqueous layer was acidified with 1 N HCl. The precipitate formed was collected by filtration and dried. Recrystallization of the crude product from aqueous MeOH gave **5a** (460 mg) as colorless plates. mp 201—202°. $[\alpha]_D^{20}$ +113.3° (c=0.36, MeOH). NMR($CDCl_3$/CD_3OD (4:1)) δ: 0.82 (3H, s, 18-CH_3), 1.25 (3H, s, 19-CH_3), 3.27 (2H, s, -SCH_2CO-), 3.4—3.8 (2H, 6ξ- and 17α-H). *Anal.* Calcd. for $C_{21}H_{30}O_4S$: C, 66.63; H, 7.99. Found: C, 66.55; H, 8.03.

4-(2-Carboxyethylmercapto)testosterone (6a)——Treatment of **9** with mercaptopropionic acid was carried out in the manner as described in **5a**. Recrystallization of the crude product from aqueous MeOH gave

1) Part CXLIII of "Studies on Steroids" by T. Nambara; Part CXLII H. Hosoda, K. Tadano, S. Miyairi, and T. Nambara, *J. Steroid Biochem.*, in press.
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3) B.F. Erlanger, F. Borek, S.M. Beiser, and S. Lieberman, *J. Biol. Chem.*, **228**, 713 (1957).
4) All melting points were taken on a micro hot-stage apparatus and are uncorrected. Nuclear magnetic resonance (NMR) spectra were recorded on a JEOL Model PS-100 spectrometer at 100 MHz using tetramethylsilane as an internal standard. Abbreviation used s=singlet, d=doublet, t=triplet, and m=multiplet.
5) H.J. Ringold, E. Batres, O. Mancera, and G. Rosenkranz, *J. Org. Chem.*, **21**, 1432 (1956).

6a as colorless prisms. mp 156—159°/179—181°. $[\alpha]_D^{20}$ +119.2° (c=0.39, MeOH). NMR ($CDCl_3$) δ: 0.80 (3H, s, 18-CH_3), 1.25 (3H, s, 19-CH_3), 2.4—3.1 (4H, -SCH_2CH_2CO-), 3.5—4.0 (2H, 6ξ- and 17α-H). *Anal.* Calcd. for $C_{22}H_{32}O_4S \cdot 1/2H_2O$: C, 65.80: H, 8.28. Found: C, 65.66; H, 8.15.

Cortisol 3-(O-Carboxymethyl)oxime (7a)——A solution of cortisol 21-tetrahydropyranyl ether[6] (1.3 g), AcONa (720 mg) and carboxymethoxylamine·HCl (670 mg) in EtOH (20 ml) was stirred at room temperature for 2 hr. After evaporation of the solvent followed by dilution with water, the resulting solution was brought to pH 14 with 10% KOH and extracted with ether. The aqueous layer was acidified with 1 N HCl and extracted with AcOEt. The organic layer was washed with water, dried over anhydrous Na_2SO_4, and evaporated. The residue was dissolved in 50% AcOH (6 ml) and allowed to stand at room temperature for 5 hr. Upon addition of water the precipitate formed was collected by filtration and dried. Recrystallization of the crude product from aqueous MeOH gave **7a** (800 mg) as colorless needles. mp 217—220° (lit. mp 226—228°).[7]

Testosterone 3-(O-carboxymethyl)oxime (**1a**),[3] 17-hemisuccinate (**2a**), 4-hydroxytestosterone 4-hemisuccinate (**3a**),[8] 4-hemiglutarate (**4a**),[8] and cortisol 21-hemisuccinate (**8a**) were prepared according to the known methods.

General Procedure for Preparation of N-Hydroxysuccinimide Ester——To a solution of steroid carboxylic acids (**1a—8a**) (1 mmol) in 95% dioxane (3.5 ml) were added 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide·HCl (1.4 mmol) and N-hydroxysuccinimide (1.4 mmol), and the resulting solution was stirred at room temperature for 2 hr. After addition of water the reaction mixture was extracted with AcOEt. The organic layer was washed with water and dried over anhydrous Na_2SO_4. The solution was passed quickly through an Al_2O_3 (10 g) layer on a sintered-glass funnel, and the filtrate was evaporated under reduced pressure. The product was purified, if necessary, by chromatography on silica gel, and recrystallized from an appropriate solvent.

Testosterone 3-(O-Carboxymethyl)oxime N-Hydroxysuccinimide Ester (1b)——Colorless plates from EtOH as a mixture of geometric isomers (E/Z=2:1). mp 172—173.5°. $[\alpha]_D^{20}$ +108.0° (c=0.20, $CHCl_3$). *Anal.* Calcd. for $C_{25}H_{34}N_2O_6$: C, 65.48; H, 7.47; N, 6.11. Found: C, 65.28; H, 7.55; N, 6.05.

17-O-Hemisuccinoyltestosterone N-Hydroxysuccinimide Ester (2b)——Colorless needles from CH_2Cl_2/MeOH. mp 164—165°. $[\alpha]_D^{20}$ +81.3° (c=0.40, $CHCl_3$). *Anal.* Calcd. for $C_{27}H_{35}NO_7$: C, 66.78; H, 7.27; N, 2.88. Found: C, 66.67; H, 7.32; N, 2.83.

4-(Hemisuccinoyloxy)testosterone N-Hydroxysuccinimide Ester (3b)——Colorless semi-crystals. NMR ($CDCl_3$) δ: 0.79 (3H, s, 18-CH_3), 1.26 (3H, s, 19-CH_3), 2.84 (4H, s, succinimidyl), 3.01 (4H, m, -$COCH_2CH_2CO$-). 3.65 (1H, t, J=8 Hz, 17α-H).

4-(Hemiglutaroyloxy)testosterone N-Hydroxysuccinimide Ester (4b)——Colorless semi-crystals. NMR ($CDCl_3$) δ: 0.79 (3H, s, 18-CH_3), 1.26 (3H, s, 19-CH_3), 2.84 (4H, s, succinimidyl), 3.65 (1H, t, J=8 Hz, 17α-H).

4-(Carboxymethylmercapto)testosterone N-Hydroxysuccinimide Ester (5b)——Colorless leaflets from CH_2Cl_2/MeOH. mp 226.5—228°. $[\alpha]_D^{20}$ +90.1° (c=0.42, $CHCl_3$). *Anal.* Calcd. for $C_{25}H_{33}NO_6S$: C, 63.13; H, 6.99; N, 2.95. Found: C, 63.31; H, 7.23; N, 3.10.

4-(2-Carboxyethylmercapto)testosterone N-Hydroxysuccinimide Ester (6b)——Colorless plates from acetone/hexane. mp 154—156°. $[\alpha]_D^{20}$ +88.6° (c=0.44, MeOH). *Anal.* Calcd. for $C_{26}H_{35}NO_6S$: C, 63.78; H, 7.21; N, 2.86. Found: C, 63.38; H, 7.34; N, 2.87.

Cortisol 3-(O-Carboxymethyl)oxime N-Hydroxysuccinimide Ester (7b)——Colorless semi-crystals mainly constituted of E form. NMR ($CDCl_3$) δ: 0.96 (3H, s, 18-CH_3), 1.35 (3H, s, 19-CH_3), 4.30 and 4.65 (each 1H, d, J=18 Hz, 21-H), 4.45 (1H, m, 11α-H), 4.93 (2H, s, -OCH_2CO-), 5.68 (1H, s, 4-H).

21-O-Hemisuccinoylcortisol N-Hydroxysuccinimide Ester (8b)——Colorless semi-crystals. NMR ($CDCl)_3$ δ: 0.95 (3H, s, 18-CH_3), 1.45 (3H, s, 19-CH_3), 2.87 (4H, s, succinimidyl), 2.96 (4H, m, -$COCH_2CH_2CO$-), 4.51 (1H, m, 11α-H), 4.96 and 5.10 (each 1H, d, J=18 Hz, 21-H), 5.72 (1H, s, 4-H).

Reaction of N-Hydroxysuccinimide Ester with BSA——To a solution of BSA (30 mg) in 0.05 M phosphate buffer (pH 7.4) (0.7 ml for pyridine, 0.4 ml for dioxane and dimethylformamide) was added the N-Hydroxysuccinimide ester (**1b, 2b, 5b, 8b**) in the organic solvent (0.7 ml), and the reaction mixture was stirred at 4° for 12 hr. The resulting solution was dialyzed against cold running water overnight, and the turbid protein solution was brought to pH 4.5 with 1 N HCl. After addition of acetone and a small amount of NaCl the suspension was centrifuged at 3000 rpm for 20 min, and the procedure was repeated until the free steroid and N-hydroxysuccinimide were removed completely. The precipitate was dissolved in 20% pyridine and dialyzed in the manner as described above. Lyophilization of the solution afforded the steroid-BSA conjugate (*ca.* 30 mg) as fluffy powder.

Determination of Number of Steroid Molecules Linked to a BSA Molecule——Ultraviolet spectral measurements were carried out on a Hitachi Model 124 spectrophotometer. The solvent used was 0.05 M phosphate buffer (pH 7.4) or 0.1 N KOH. Spectral analysis and calculation were carried out by comparing the absorbance

6) H. Hosoda, S. Miyairi, and T. Nambara, in preparation.
7) A. Tsuji, M. Smulowtiz, J.S.C. Liang, and D.K. Fukushima, *Steroids*, **24**, 739 (1974).
8) H. Hosoda, K. Tadano, S. Miyairi, and T. Nambara, *J. Steroid Biochem.*, in press.

of the conjugate with those of BSA as controls in both solvents and by using the following constants: molecular weight of BSA, 65000; ε (nm) for **1b**, 24000 (251), for **2b** and **8b**, 16000 (251), and for **5b**, 10600 (252).

Results and Discussion

An initial effort was focused on the preparation of steroid carboxylic acids and their N-hydroxysuccinimide esters. The testosterone derivatives *i.e.* testosterone 3-(O-carboxymethyl)oxime (**1a**), 17-hemisuccinate (**2a**), 4-hydroxytestosterone 4-hemisuccinate (**3a**), 4-hemiglutarate (**4a**), and cortisol 21-hemisuccinate (**8a**) were prepared by the known methods. 4-Carboxymethylmercaptotestosterone (**5a**) and 4-carboxyethylmercaptotestosterone (**6a**) were synthesized from 4,5-epoxytestosterone (**9**) by the base-catalyzed ring opening[6] with mercaptoacetic acid and mercaptopropionic acid, respectively. The preparation of these compounds was aimed at the enzyme immunoassay of testosterone using the "bridge" heterologous system with antisera produced against the BSA conjugates of **3a** and **4a**.[8] The synthesis of cortisol 3-(O-carboxymethyl)oxime (**7a**) was undertaken by a modified procedure in order to increase the selective oxime formation at C-3. When cortisol 21-tetrahydropyranyl ether (**10**) was treated with 1.2 mol equivalent of carboxymethoxylamine in ethanol, the formation of 3-mono-oxime (**11**) occurred with high selectivity due to the steric hindrance between the tetrahydropyranyl and 20-keto groups. Upon hydrolysis with acetic acid **11** was converted

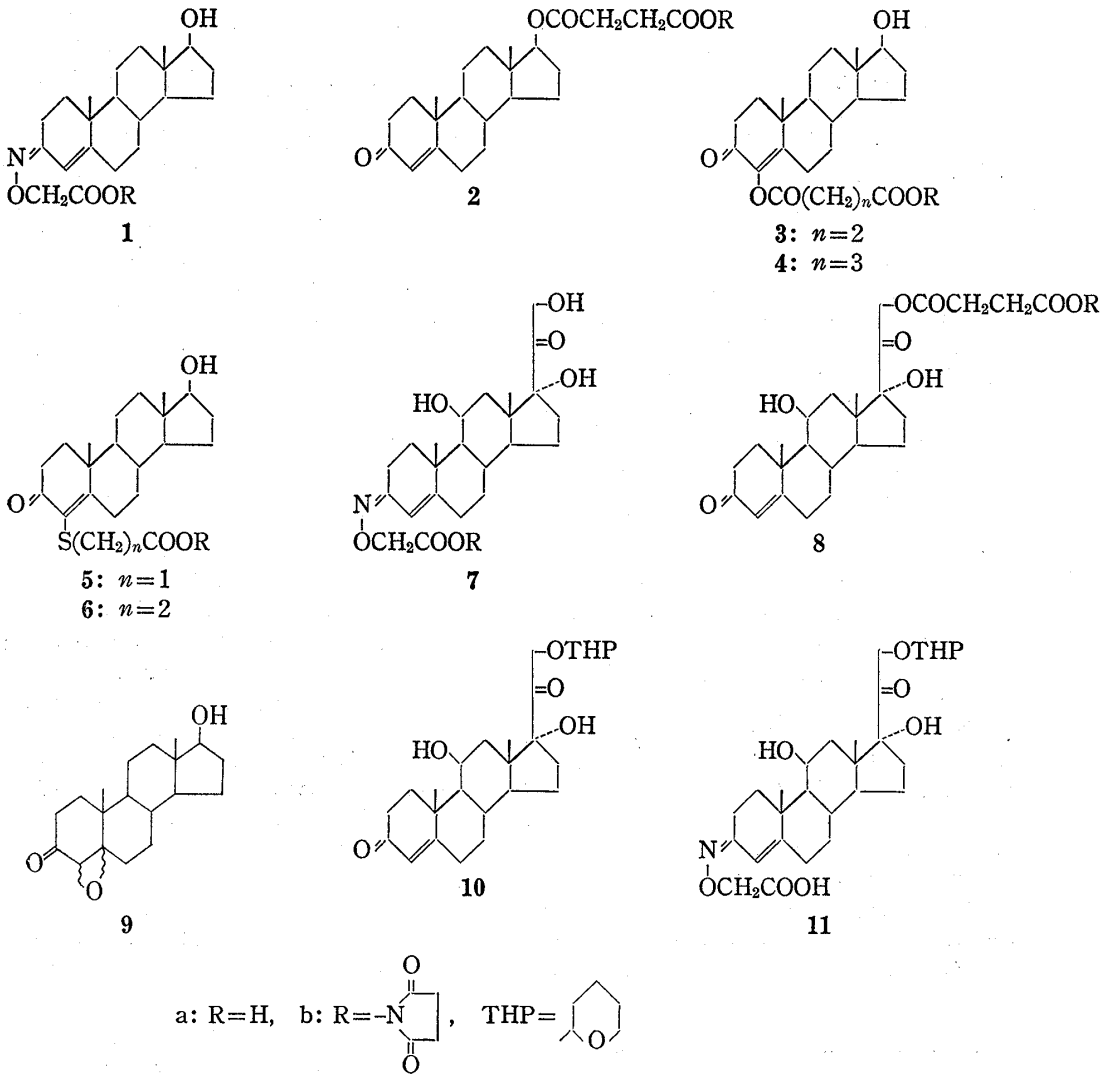

Chart 1

into **7a** in a fairly good yield. Being treated with N-hydroxysuccinimide in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide in 95% dioxane, steroid carboxylic acids (**1a**—**7a**) were transformed into the N-hydroxysuccinimide esters (**1b**—**7b**) in the yields of 50—80%. The activated esters were found to be stable when stored in the solid state at room temperature.

The reactivities of the selected derivatives (**1b**, **2b**, **5b**, **8b**) with the amino group of BSA in organic solvent/phosphate buffer (pH 7.4) were then investigated by employing various molar ratios of the steroid to the protein. Removal of the free steroid and liberated N-hydroxysuccinimide by dialysis and treatment with acetone was checked by thin-layer chromatography. The number of steroid residues incorporated in each BSA molecule was determined by the ultraviolet spectral analysis, and the results were listed in Table I and II.

Table I. Molar Ratio of Testosterone 17-Hemisuccinate incorporated in BSA[a]

Activated steroid[b]	Solvent			
	Pyridine		Dioxane	Dimethylformamide
60	33		26	26
30	18		18	19
20	—		16	—
10	8.9	9.0	8.8	—
5	4.5	4.7	4.2	—
3	2.7	2.8	2.1	—
1	1.1	1.1	—	—

a) The coupling reaction was carried out in organic solvent/phosphate buffer (pH 7.4). —; Not carried out.
b) N-Hydroxysuccinimide ester of 17-O-hemisuccinoyltestosterone was used in the various molar ratios to BSA.

Table II. Molar Ratio of Steroid Hapten incorporated in BSA[a]

Activated steroid[b]	Steroid			
	1	**2**	**5**	**8**
60	22	33	27	27
20	12	—	18	14
10	6.0	9.0	5.9	7.5
5	3.0	4.6	3.1	3.5
3	—	2.8	—	—

a) The coupling reaction was carried out in pyridine/phosphate buffer (pH 7.4). —; Not carried out.
b) N-Hydroxysuccinimide esters of steroid carboxylic acids were used.

In all the solvent systems used the reaction proceeded in the fashion comparable to the mixed anhydride and carbodiimide methods, and no significant difference in the reactivity was observed among them. When the molar ratio of sixty was taken, the steroid-BSA conjugates usable for the production of antisera were obtained. Furthermore, the results of the duplicate experiments in pyridine verified excellent reproducibility of the present method (Table I). The compound **2b** was much more reactive with BSA than the others, although such difference was not observed in the preliminary experiment with ethyl γ-aminobutyrate. The higher values obtained in the experiments with **5b** employing the molar ratios of sixty and twenty may be ascribable to low solubility and therefore slow hydrolysis into the free steroid. These results indicate that the rate of coupling reaction to a protein would be influenced by the steric effect of steroid molecule.

To the best of our knowledge this is the first reported synthesis of N-hydroxysuccinimide esters of steroids, although the preparation *in situ* has been reported by Cuatrecasas and his co-worker.[9] The activated ester method may be useful for enzyme immunoassay of steroids, since the coupling procedure is very simple and highly reproducible and the pH of the buffer solution can be varied in the range of 5 to 10.[10] Applications of this technique to enzyme labeling are being conducted in these laboratories, and the details will be reported in the near future.

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10) P. Cuatrecasas and I. Parikh, *Biochemistry*, **11**, 2291 (1972).