Preparation of Specific Antiserum to 17α-Estradiol 17-*N*-Acetylglucosaminide

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A specific enzyme immunoassay (EIA) method has been developed for 17α-estradiol 17-*N*-acetylglucosaminide as a model compound instead of 15α-hydroxyestrogen 15-*N*-acetylglucosaminides. Two new haptens, 3-(ω-carboxyalkyl) ether derivatives of 17α-estradiol 17-*N*-acetylglucosaminide, were synthesized and conjugated with bovine serum albumin (BSA). The EIA system was newly established using specific antiserum elicited against 3-(1-carboxypropyl) ether of 17α-estradiol 17-*N*-acetylglucosaminide (17NAG CPE)–BSA conjugate and β-galactosidase-labeled 17NAG CPE as a labeled antigen.

An appropriate dose–response curve of EIA for 17α-estradiol 17-*N*-acetylglucosaminide was obtained in the range of 20—1000 pg/tube. The specificity of EIA proved to be satisfactory in terms of cross-reactivities to related compounds including 15α-*N*-acetylglucosaminides. The proposed method will be applicable to the preparation of antisera for use in EIA of 15α-hydroxyestrogen 15-*N*-acetylglucosaminides.

Key words estrogen *N*-acetylglucosaminide; enzyme immunoassay; Koenigs–Knorr reaction; β-galactosidase; 17α-estradiol

Since the initial isolation of *N*-acetylglucosamine conjugate of 17α-estradiol (**1**) from rabbit urine after administration of estrone benzoate in 1964,[1] several *N*-acetylglucosamine conjugates have been isolated and characterized.[2–4] We have also reported the synthesis of 15α-hydroxyestrogen 15-*N*-acetylglucosaminides which might be important for monitoring fetal estrogens.[5] However, their physiological significance has not yet fully been investigated. In this respect, it is of interest that ursodeoxycholic acid 7-*N*-acetylglucosaminide was identified as one of the principal metabolites in human urine from a patient with primary biliary cirrhosis after administration of ursodeoxycholic acid. Development of an enzyme immunoassay (EIA) is desirable for determining the plasma and urine levels of steroids because of their extremely low levels. Numerous methods based upon the principle of immunoassay have been developed for the determination of estrogens including their glucuronic acid, sulfuric acid and *O*-methyl conjugates, and other steroid hormones in biological fluids.[6–8] With regard to steroids conjugated with *N*-acetylglucosamine, however, no attempts have been made to obtain specific antiserum required for the direct immunoassay. We thus prepared a specific antiserum to 17α-estradiol 17-*N*-acetylglucosaminide (**4**) since the synthesis of 15α-hydroxyestrogen 15-*N*-acetylglucosaminides involves tedious chemical proc-

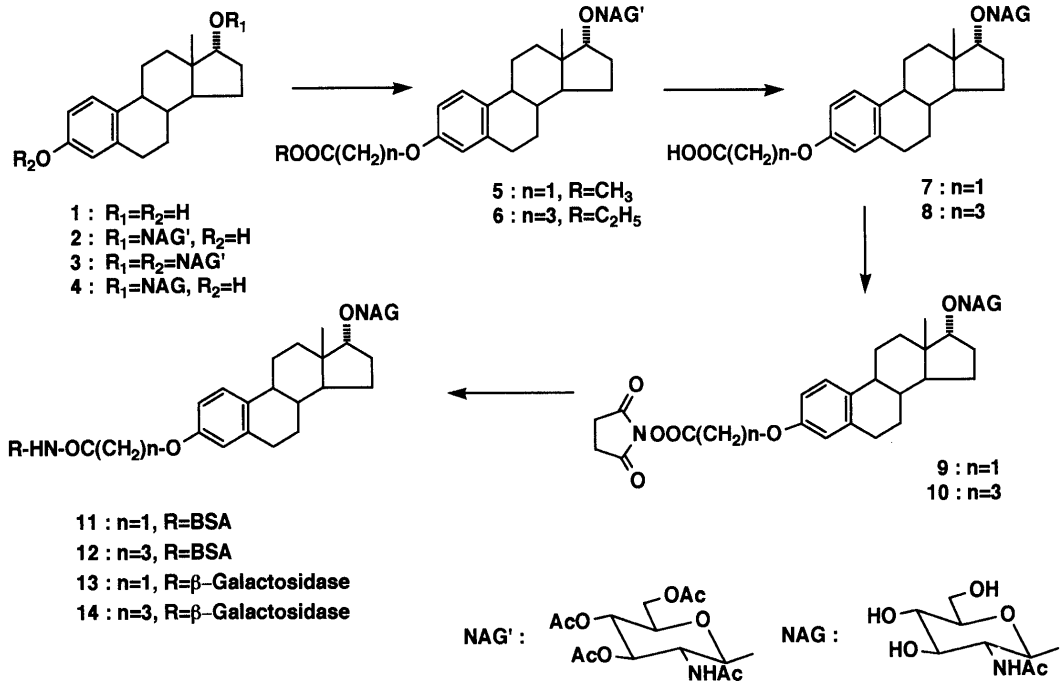

Fig. 1. Synthesis of Haptens and Immunogens of 17α-Estradiol 17-*N*-Acetylglucosaminide

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essing. This paper deals with the synthesis of derivatives of **4** having a carboxyl group at the C-3 position and the properties of antiserum elicited against these new hapten–bovine serum albumin (BSA) conjugates (Fig. 1).

MATERIALS AND METHODS

Apparatus Melting points were determined on a micro hot-stage apparatus and are uncorrected. IR spectra were run on a 215 Hitachi grating infrared spectrophotometer as KBr disks. ^{1}H-NMR spectra and 2 dimensional correlation spectroscopy (2D-COSY) spectra were measured at 300 MHz on a QE-300 NMR spectrometer (General Electric, Fremont, CA) using solvent lock system or at 500 MHz on a JEOL GX-500 NMR spectrometer (JEOL, Tokyo, Japan) with tetramethylsilane as an internal standard. Chemical shifts are expressed in ppm (δ) (s, singlet, d, doublet, t, triplet, m, multiplet). Visible spectral measurements were carried out on a Shimadzu model 2100 spectrophotometer (Kyoto, Japan).

Materials β-Galactosidase (EC 3.2.1.23) from *Escherichia coli* (320 units/mg protein), BSA and *o*-nitrophenyl-β-D-galactopyranoside were supplied by Sigma Chemical Co. (St. Louis, MO, U.S.A.). Anti-rabbit γ-globulin goat serum and normal rabbit serum were obtained from Daiichi Radioisotope Labs., Ltd. (Tokyo, Japan). These sera were diluted with 50 mM sodium phosphate buffer (pH 7.3) containing 0.1% BSA, 0.1% NaN_3 and 0.9% NaCl (buffer A).

3-Hydroxyestra-1,3,5(10)-trien-17α-yl-2′-acetamido-2′-deoxy- 3′,4′,6′-tri-*O*-acetyl-β-D-glucopyranoside (2), Estra-1,3,5(10)-trien-3,17α-di-yl-bis(2′-acetamido-2′-deoxy-3′, 4′,6′-tri-*O*-acetyl-β-D-glucopyranoside (3) To a solution of **1** (250 mg) in dehydrated $CHCl_3$ (20 ml) were added freshly prepared $CdCO_3$ (800 mg) and 2-acetamido-1α-chloro-1,2-dideoxy-3,4,6-tri-*O*-acetyl-D-glucopyranose (acetochloroglucosamine; 1.6 g) in four portions for 3 d and the suspension was stirred under reflux with occasional slow distillation of $CHCl_3$ to remove the moisture. The precipitate was removed by filtration and washed with $CHCl_3$. The filtrate and washings were combined and evaporated under reduced pressure to give an oily residue, which in turn was subjected to column chromatography on silica gel. Elution with hexane–AcOEt (1 : 3, v/v) and recrystallization of the eluate from acetone–hexane gave **2** (203 mg) as colorless needles. mp 245—246 °C (lit. mp 244—246 °C).[9] ^{1}H-NMR ($CDCl_3$) δ: 0.69 (s, 3H, 18-CH_3), 1.94 (s, 3H, $COCH_3$), 2.04 (s, 3H, $COCH_3$), 2.05 (s, 3H, $COCH_3$), 2.11 (s, 3H, $COCH_3$), 3.63—3.70 (m, 1H, 5′-H), 3.75 (d, 1H, J=5.4 Hz, 17β-H), 3.94 (q, 1H, J=9.8 Hz, 2′-H), 4.14—4.18 (m, 1H, 6′-H), 4.26—4.31 (m, 1H, 6′-H), 4.57 (d, 1H, J=8.1 Hz, 1′-H), 5.10 (t, 1H, J=9.5 Hz, 4′-H), 5.24 (t, 1H, J=9.8 Hz, 3′-H), 5.55 (d, 1H, J=8.4 Hz, NH), 6.54—6.68 (m, 2H, 2-, 4-H), 7.08 (d, 1H, J=8.7 Hz, 1-H). Eluate obtained from the more polar fraction gave a pale yellow solid, which in turn was chromatographed on silica gel with AcOEt to afford a colorless solid (97 mg). The crude product was reprecipitated from AcOEt–hexane to give a colorless solid (31 mg). mp 149—152 °C. IR: 1750, 1675, 1505, 1370, 1235, 1040 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 0.74 (s, 3H, 18-CH_3), 1.75 (s, 3H, $COCH_3$), 1.90 (s, 3H, $COCH_3$), 1.91 (s, 3H, $COCH_3$), 1.99 (s, 3H, $COCH_3$), 2.00 (s, 6H, $COCH_3 \times 2$), 2.10 (s, 3H, $COCH_3$), 2.11 (s, 3H, $COCH_3$), 3.14—3.19 (m, 2H, 2′-, 2″-H), 3.69—3.72 (m, 1H, 5′-H), 3.84—3.87 (m, 1H, 5″-H), 3.90 (d, 1H, J=6.2 Hz, 17β-H), 4.10—4.13 (m, 2H, 6″-H), 4.17—4.28 (m, 2H, 6′-H), 4.80 (s, 1H, NH), 4.97—5.01 (m, 2H, 4′-H, 4″-H), 5.50 (d, 1H, J=8.1 Hz, 1′-H), 5.68 (d, 1H, J=9.5 Hz, 1″-H), 5.82 (t, 1H, J=9.5 Hz, 3′-H), 5.88 (d, 1H, J=6.2 Hz, NH), 6.19 (t, 1H, J=9.7 Hz, 3″-H), 6.52—6.61 (m, 2H, 2-, 4-H), 7.10 (d, 1H, J=8.4 Hz, 1-H). *Anal.* Calcd for $C_{46}H_{62}N_2O_{18}\cdot H_2O$: C, 58.22; H, 6.80; N, 2.95. Found: C, 57.75; H, 6.89; N, 2.99.

3-(Methoxycarbonylmethyloxy)estra-1,3,5(10)-trien-17α-yl-2′-acetamido-2′-deoxy-3′,4′,6′-tri-*O*-acetyl-β-D-glucopyranoside (5) To a solution of **2** (100 mg) in dehydrated dimethyl sulfoxide (DMSO, 1 ml) were added methyl chloroacetate (25 μl) and K_2CO_3 (90 mg), and the whole was stirred at 60 °C for 2 h. The resulting solution was neutralized with 1 N HCl and poured into ice-cooled water (50 ml) and extracted with AcOEt (150 ml). The organic layer was washed with H_2O and dried over anhydrous Na_2SO_4. Evaporation of the solvent gave an oily residue, which was chromatographed on silica gel. Elution with $CHCl_3$ and recrystallization of the eluate from acetone–hexane gave **5** (70 mg) as colorless needles. mp 146—148 °C. IR: 1750, 1680, 1550, 1240, 1050 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.72 (s, 3H, 18-CH_3), 1.94 (s, 3H, $COCH_3$), 2.03 (s, 6H, $COCH_3 \times 2$), 2.10 (s, 3H, $COCH_3$), 3.64—3.70 (m, 1H, 5′-H), 3.76 (d, 1H, J=6.0 Hz, 17β-H), 3.75—3.81 (m, 1H, 2′-H), 4.12—4.29 (m, 2H, 6′-H), 4.60 (s, 2H, $COOCH_2O$), 4.68 (d, 1H, J=8.1 Hz, 1′-H), 5.07 (t, 1H, J=9.3 Hz, 4′-H), 5.32 (t, 1H, J=9.6 Hz, 3′-H), 5.49 (d, 1H, J=8.1 Hz, NH), 6.62—6.72 (m, 2H, 2-, 4-H), 7.22 (d, 1H, J=8.7 Hz, 1-H). *Anal.* Calcd for $C_{35}H_{47}NO_{12}$: C, 62.39; H, 7.03; N, 2.08. Found: C, 62.76; H, 7.09; N, 2.15.

3-[3-(1-Ethoxycarbonyl)propyloxy]estra-1,3,5(10)-trien-17α-yl-2′-acetamido-2′-deoxy-3′,4′,6′-tri-*O*-acetyl-β-D-glucopyranoside (6) To a solution of **2** (150 mg) in dehydrated DMSO (1.5 ml) were added ethyl 4-bromo-*n*-butyrate (70 μl) and K_2CO_3 (100 mg), and the whole was treated in a manner as described above. The crude product obtained was purified on a column of silica gel using $CHCl_3$ as a developing solvent. Recrystallization of the eluate from aqueous MeOH gave **6** (74 mg) as colorless needles. mp 109.5—112 °C. IR: 1730, 1670, 1540, 1250 cm^{-1}. ^{1}H-NMR (CD_3OD) δ: 0.72 (s, 3H, 18-CH_3), 1.22 (t, 3H, $CH_2\underline{CH_3}$), 1.88 (s, 3H, $COCH_3$), 1.96 (s, 3H, $COCH_3$), 1.98 (s, 3H, $COCH_3$), 2.05 (s, 3H, $COCH_3$), 4.62 (d, 1H, J=8.1 Hz, 1′-H), 4.95 (t, 1H, J=9.6 Hz, 4′-H), 5.22 (t, 1H, J=9.8 Hz, 3′-H), 6.55—6.65 (m, 2H, 2-, 4-H), 7.14 (d, 1H, J=8.7 Hz, 1-H). *Anal.* Calcd for $C_{38}H_{53}NO_{12}\cdot H_2O$: C, 62.20; H, 7.55; N, 1.91. Found: C, 62.64; H, 7.62; N, 1.89.

3-(Carboxymethyloxy)estra-1,3,5(10)-trien-17α-yl-2′-acetamido-2′-deoxy-β-D-glucopyranoside (7) To a solution of **5** (100 mg) in MeOH (5.5 ml) was added 1 N NaOH (560 μl) and the mixture was allowed to stand at room temperature for 1 h. The resulting solution was percolated through a column of Dowex 50W-X8 (H^+ form) and eluted with MeOH. Recrystallization of the eluate from MeOH

gave **7** (77 mg) as colorless needles. mp 239—240 °C. IR: 3425, 3300, 1735, 1600, 1570, 1235 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.62 (s, 3H, 18-CH_3), 1.73 (s, 3H, $COCH_3$), 4.23 (d, 1H, J=7.5 Hz, 1′-H), 4.55 (s, 2H, $COOCH_2O$), 6.52—6.63 (m, 2H, 2-, 4-H), 7.14 (d, 1H, J=8.4 Hz, 1-H). *Anal.* Calcd for $C_{28}H_{39}NO_9$: C, 63.01; H, 7.37; N, 2.63. Found: C, 62.98; H, 7.35; N, 2.68.

3-[3-(1-Carboxypropyloxy)]estra-1,3,5(10)-trien-17α-yl-2′-acetamido-2′-deoxy-β-D-glucopyranoside (8) To a solution of **6** (149 mg) in MeOH (4 ml) was added 1 N NaOH (1.2 ml) and the mixture was allowed to stand at room temperature overnight. The resulting solution was treated in a manner as described for **7**. Recrystallization of the crude product from aqueous MeOH gave **8** (73 mg) as colorless needles. mp 241—244 °C (dec.). IR: 3330, 2930, 1710, 1615, 1070, 1060 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.61 (s, 3H, 18-CH_3), 1.73 (s, 3H, $COCH_3$), 4.23 (d, 1H, J=8.1 Hz, 1′-H), 6.54—6.64 (m, 2H, 2-, 4-H), 7.12 (d, 1H, J=8.7 Hz, 1-H). *Anal.* Calcd for $C_{30}H_{43}NO_9 \cdot 1/2H_2O$: C, 63.13; H, 7.78; N, 2.45. Found: C, 63.01; H, 7.70; N, 2.52.

Conjugation of 7 with BSA (11) To a solution of **7** (72 mg) in DMSO (0.5 ml) were added *N*-hydroxysuccinimide (24 mg) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide·HCl (40 mg), and the whole was stirred at room temperature for 3 h. The resulting solution was diluted with AcOEt (150 ml), washed with satd. NaCl, and dried over anhydrous Na_2SO_4. The solution was passed quickly through Al_2O_3 (1.5 g) and Celite layer on a sintered-glass funnel, and the filtrate was evaporated down. The structure of the residue was characterized as the activated ester (**9**) by NMR spectrum. ^{1}H-NMR (CD_3OD) δ: 0.71 (s, 3H, 18-CH_3), 1.94 (s, 3H, $COCH_3$), 3.75 (s, 4H, CH_2CH_2), 4.40 (d, 1H, J=7.8 Hz, NH), 4.62 (s, 2H, OCH_2CO), 6.57 (d, 1H, J=3.0 Hz, 4-H), 6.64 (d, d, 1H, J=3.0, 8.7 Hz, 2-H), 7.17 (d, 1H, J=8.7 Hz, 1-H). A mixture of the activated ester in pyridine (2 ml) and BSA (207 mg) in 0.05 M phosphate buffer (pH 7.4, 2 ml) was stirred overnight at 4 °C. The resulting solution was dialyzed against cold running water for 3 d at 4 °C. Lyophilization of the resulting solution gave the hapten–BSA conjugate **11** (203 mg) as a fluffy powder.

Conjugation of 8 with BSA (12) A mixture of **8** (32 mg) in DMSO (0.4 ml) and *N*-hydroxysuccinimide (10 mg) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide·HCl (13 mg) was treated in a manner as described for **11**. Conjugation of the activated ester (**10**) with BSA (80 mg) followed by dialysis and lyophilization gave **12** (71 mg) as a fluffy powder.

Determination of Number of Steroid Haptens Conjugated to a BSA Molecule Spectrometric analysis was carried out by measuring the absorbance at 456 nm after hydrolysis of the hapten–BSA conjugates with 75% H_2SO_4 at 80 °C for 1 h, whereby the number of steroid molecules linked to a BSA molecule was determined to be 7 and 19, for **11** and **12**, respectively.

Preparation of Antiserum The hapten–BSA conjugate (1 mg) dissolved in sterile isotonic saline (0.5 ml) was emulsified with complete Freund's adjuvant (0.5 ml). The emulsion was injected into a domestic male albino rabbit intradermally at multiple sites along the back. This procedure was repeated once every fortnight. The antiserum prepared from blood by centrifugation at 2050 × ***g*** for 10 min was stored at 4 °C with 0.1% (w/v) NaN_3.

Preparation of β-Galactosidase-Labeled Antigen A dioxane solution of the *N*-succimidyl ester (**9**, **10**) was added to a solution of β-galactosidase (1 mg) in 50 mM phosphate buffer (pH 7.4, 0.2 ml) at a molar ratio (steroid to enzyme) of 64, 128, 192, 256 and 16, 24, 32, 64, respectively, at 0 °C, and the mixture was stirred at 4 °C overnight. After dialysis against cold 50 mM phosphate buffer (pH 7.3), an aliquot of the conjugate solution was transferred to a test tube; the solution was diluted with buffer A to a concentration of 500 μg/ml and stored at 4 °C. For the immunoassay procedure, the solution was diluted with buffer A containing 0.5% normal rabbit serum.

Procedure for EIA[10] The hapten–enzyme conjugate (100 ng, 0.1 ml) was mixed with **4**[9] (0 and 10 pg—10 ng, or 100 ng related compounds for the cross-reactivity test) in buffer A (0.1 ml) and diluted antiserum (0.1 ml) sequentially, and the mixture was allowed to stand at 4 °C for 4 h. To the mixture was added anti-rabbit γ-globulin goat serum (0.1 ml) diluted to 1:30 (v/v) with buffer A containing 0.3% (w/v) EDTA, and the whole was allowed to stand at 4 °C for 16 h. After dilution with 50 mM sodium phosphate buffer (pH 7.3, 1.5 ml) and centrifugation at 2050 × ***g*** for 10 min, the immune precipitate was collected by aspirating off the supernatant and washed with the phosphate buffer (1.5 ml) by repeating the procedure. The precipitate was then suspended in 1 ml of the phosphate buffer (pH 7.3) containing 0.12% (w/v) *o*-nitrophenyl-β-D-galactopyranoside, 0.2% (w/v) $MgCl_2$ and 0.7% (v/v) 2-mercaptoethanol, and then incubated at 37 °C for 1 h. The reaction was terminated by addition of 1 M Na_2CO_3 (2 ml), and the absorbance was measured at 420 nm.

Cross-Reactivity Test The specificity of the EIA system was assessed by cross-reactivity to seven **4** related compounds. The cross-reactivity was expressed as the percentage of the amount of **4** which reduced the enzyme activity in the immune precipitate by half to the amount of each compound.

RESULTS AND DISCUSSION

For obtaining antiserum specific for **4**, a carrier protein should be coupled to the steroid hapten through the C-3 position to allow the immune mechanism free access to the ring D structure. The ω-carboxyalkyl ether at C-3 appeared to be a pertinent derivative for coupling with free amino groups of BSA. It is documented in the literature that the bridge heterologous system rather than the homologous one is preferable in respect to assay sensitivity.[11] We therefore synthesized two haptens of **4** having a bridge of different length.

Synthesis of **2** has been reported by condensation of acetochloroglucosamine with 3-benzyl ether of **1** by the Koenigs–Knorr reaction and subsequent catalytic hydrogenolysis of the resulting product to remove the protecting group. As the steroidal phenolic group is usually less reactive than the steroidal alcoholic group towards Koenigs–Knorr reaction, **1** was submitted to

the condensation reaction without protecting a hydroxyl group at C-3. Chloroform was used as solvent since lower temperature seemed more favorable for this reaction to minimize the thermal decomposition of acetochloroglucosamine.[5] Besides the desired **2**, 3,17α-di-*N*-acetylglucosaminide (**3**) was obtained from the crude product after chromatographic separation. Since **2** was afforded in reasonable yield (37%) and these two compounds can be easily resolved, direct introduction of *N*-acetylglucosaminyl residue into **1** seems to be a favorable method for obtaining **2**. Treatment of **2** with methyl chloroacetate in the presence of potassium carbonate in dimethylsulfoxide furnished the 3-carbomethoxymethyl ether derivative (**5**) in 63% yield. Upon treatment with alkali **5** was converted to the 3-carboxymethyl ether (**7**). Similar reaction of **2** with ethyl 3-chloropropionate failed, forming no desired compounds. Even the use of more reactive 3-bromo- or 3-iodopropionic acid methyl esters proved to be unsuccessful. Since a halogen substituent at the β-position of a carbonyl group might be easily eliminated to form a double bond, the synthesis of another hapten with a longer bridge was undertaken. Treatment of **2** with ethyl 4-bromo-*n*-butyrate in a similar fashion afforded the expected 3-ether (**6**) in 42% yield. Subsequent alkaline hydrolysis gave **8** in 62% yield.

The next step was preparation of the BSA conjugates. The *N*-succinimidyl esters (**9**, **10**) prepared from **7** and **8** by condensation with *N*-hydroxysuccinimide in the presence of a water-soluble carbodiimide, were reacted with BSA, respectively. Subsequent dialysis of the reaction mixture gave the desired BSA conjugates (**11**, **12**). The molar ratios of steroid to BSA were determined to be 7 and 19 for **11** and **12**, respectively. The appropriate antiserum was thus obtained from rabbits 6 months after the initial administration of the immunogens.

The enzyme labeling was carried out by mixing the activated esters (**9**, **10**) in dioxane with the enzyme in phosphate buffer (pH 7.4). Various molar ratios of the steroid to enzyme, ranging from 64—256 for **9** and 16—64 for **10**, were used. The titer was evaluated by incubating various dilutions of antiserum with a constant amount of β-galactosidase-labeled antigen (**13**). The highest titer antiserum (anti-**12** antiserum) among antisera elicited in three rabbits was adopted for detailed characterization. Immunoreactivities of the enzyme-labeled antigens (**13** and **14**) obtained with various steroid/enzyme molar ratios at 1:1000 dilution of the anti-**12** antiserum are illustrated in Fig. 2. The binding ability increased with increasing molar ratio. No satisfactory immunoreactivity was attained even at a molar ratio of more than 250 with **13**, while satisfactory reactivity was obtained with **14** at a molar ratio of 20. It was previously reported that the heterologous system using the enzyme-labeled steroid obtained from a hapten having a bridge shorter than that used for antibody production resulted in an increase in assay sensitivity whereas the use of a longer bridge for enzyme labeling was not effective.[11] In this case, however, the homologous system using **12** and **14** showed higher sensitivity than the heterologous system using **12** and **13**. Since no difference was observed in the stability of **9** and **10** in the reaction mixture (0.05 M phosphate buffer) (data not shown), the lower sensitivity of **13** than **14** might be explained in part by lower reactivity of **9** with bulky β-galactosidase due to its shorter bridge. The lower binding molar ratio of **11** to BSA as compared with **12** might be ascribable to a similar situation. To determine an appropriate dilution of antiserum for use in EIA, an antiserum dilution curve was then constructed by using antiserum **12** and labeled antigen **14**. The binding ability was expressed for convenience as a percentage of that with 1 : 1000 dilution (Fig. 3). Based on these data, use of the antiserum diluted approximately 1 : 50000 seemed to be suitable. A typical dose–response curve for **4** obtained with the assay system using 100 ng of β-galactosidase is shown in Fig. 4. The amount of **4** needed to displace 50% of the bound label was 150 pg. The affinity constant (K_a) of this antibody was estimated to be $(1.97 \pm 0.25) \times 10^9\ \text{M}^{-1}$, as calculated by Scatchard analysis.

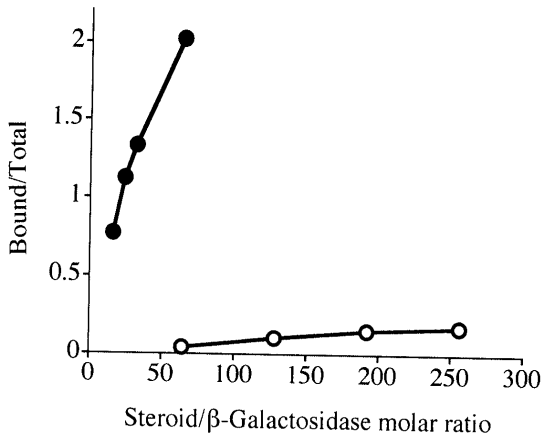

Fig. 2. Immunoreactivities of β-Galactosidase-Labeled Antigens (○—○, **9**; ●—●, **10**) with Anti-17α-estradiol 17-*N*-Acetylglucosaminide Antiserum (× 1000)

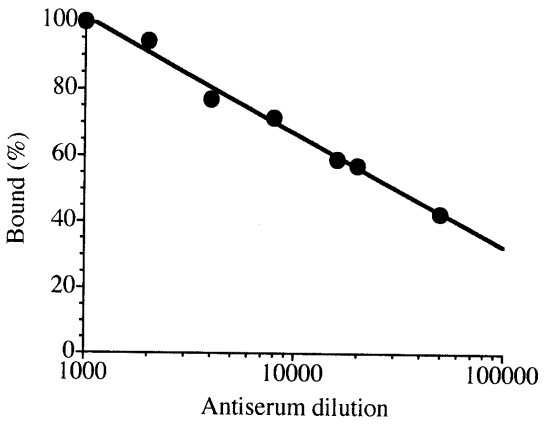

Fig. 3. An Antiserum Dilution Curve with the β-Galactosidase-Labeled Antigen

Specificity of the antibody was assessed by measuring the ability of various steroids to compete with **4** for binding to the antibody. The cross-reactivities of seven kinds of related steroids (estrone, estradiol, estriol, **1**, 15α-hydroxyestriol, 15α-hydroxyestradiol 15-*N*-acetylglucosaminide, and 15α-hydroxyestriol 15-*N*-acetylglucosaminide) in the EIA were all less than 0.15%.

Assessment of the standard assay was then carried out in the usual manner by checking the accuracy and pre-

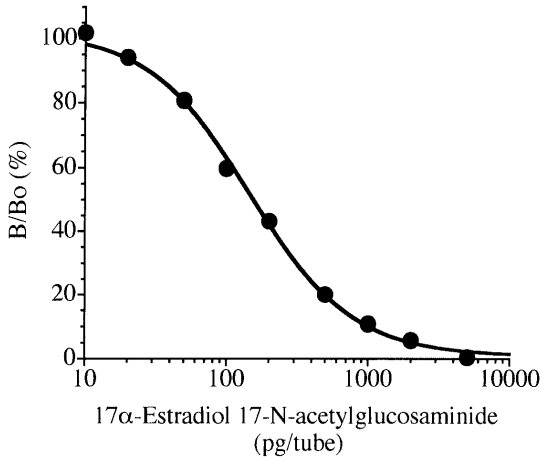

Fig. 4. Dose–Response Curve for EIA of 17α-Estradiol 17-*N*-Acetylglucosaminide Using β-Galactosidase-Labeled Antigen

Table 1. Accuracy and Precision of the EIA for Assessment as the Standard Assay to Determine 17α-Estradiol 17-*N*-Acetylglucosaminide

Spiked (pg/tube)	Assayed (pg/tube)	Accuracy (%)	RSD (%)
20.0	20.6	103	15.3
50.0	50.0	99.3	20.5
100	97.4	97.4	7.47
500	610	122	10.2
1000	1300	130	5.24

$n=7$. RSD, relative standard deviation.

cision, and the results were satisfactory as shown in Table 1. Thus the antiserum elicited in rabbits with **12** showed a high specificity. To the best of our knowledge this is the first report demonstrating the preparation of antisera against estrogen *N*-acetylglucosaminide. It is hoped that this model experiment will be applicable to the development of an EIA system of 15α-hydroxyestrogen 15-*N*-acetylglucosaminides used to clarify the physiological significance of these novel estrogen conjugates.

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REFERENCES

1) Layne D. S., Sheth N. A., Kirdani R. Y., *J. Biol. Chem.*, **239**, 3221—3225 (1964).
2) Arcos M., Lieberman S., *Biochemistry*, **6**, 2032—2039 (1967).
3) Sauer G., Matsui M., Bloch R., Liang J. S., Fukushima D. K., *J. Org. Chem.*, **34**, 3525—3530 (1969).
4) Jirku H., Kadner S., Levitz M., *Steroids*, **19**, 519—534 (1972).
5) Suzuki E., Namba S., Kurihara H., Goto J., Matsuki Y., Nambara T., *Steroids*, **60**, 277—284 (1995).
6) Hosoda H., Yokohama H., Ishii K., Ito Y., Nambara T., *Chem. Pharm. Bull.*, **31**, 4001—4007 (1983).
7) Nambara T., Niwa T., Shimada K., *J. Steroid Biochem.*, **21**, 199—203 (1984)
8) Berg F. D., Kuss E., *Arch. Gynecol. Obstet.*, **251**, 17—27 (1992).
9) Nambara T., Anjo T., Goya S., *Chem. Pharm. Bull.*, **19**, 2183—2185 (1971).
10) Miyairi S., Shimada H., Morita H., Awata N., Goto J., Nambara T., *J. Pharm. Biomed. Anal.*, **11**, 469—476 (1993).
11) Hosoda H., Kobayashi N., Nambara T., *Chem. Pharm. Bull.*, **31**, 953—958 (1983).