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17β-Hydroxy-11α-(3′-sulfanylpropyl)oxy-estra-1,3,5(10)-trien-3-yl sulfamate – a novel hapten structure: Toward the development of a specific enzyme immunoassay (EIA) for estra-1,3,5(10)-triene-3-yl sulfamates

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Abstract

The title compound **17** has been synthesized for the use as hapten in the development of a competitive enzyme immunoassay for estrogen sulfamates. The synthesis started from estradiol diacetate **2**. Oxyfunctionalization at C-11 to give 11α-hydroxy steroid **8** was accomplished by hydroboration/alkaline hydrogen peroxide oxidation of the 9(11)-dehydro derivative **7,** which was obtained from compound **2** via 9-hydroxylation with dimethyldioxirane. After transformation of compound **8** into the allyl ether **9**, the side chain was thio-functionalized at the ω-position affording the thioate **11** in two steps. Selective silylether deprotection at position 3 followed by sulfamoylation gave the sulfamate **19,** which in turn was demasked at position 17 and treated with sodium borohydride/aluminum chloride to liberate the side chain thiol. Alternatively, title compound **17** was synthesized via the disulfides **13–16**. For the preparation of the immunogen the title compound **17** was coupled to bovine gamma globulin in a two-step procedure using an amine and thiol specific bifunctional crosslinker. The immunization of rabbits resulted in the formation of antibodies which clearly discriminated the sulfamoylated estrogens from the non-esterified estrogens. The use of a biotinylated hapten derivative as a tracer in combination with a streptavidin-peroxidase-tetramethylbenzidine based detection system allowed the measurement of estradiol 3-sulfamate (**1**) in the range of about 1 to 1000 pg/well.

Keywords: Steroid thiols; Steroid disulfides; Estrogen sulfamates; Steroid hapten; Immunoassay for estrogens; Hydroboration; Alkaline hydrogen peroxide oxidation

1. Introduction

Steroidal estrogens esterified at position 3 by sulfamic acid (estrogen sulfamates), have a potential to overcome the liver barrier without alteration of important hepatic functions if administered orally. A confirmation of the current animal experiments by clinical trials may lead to new drugs with fundamental benefits in the hormone replacement therapy in women or in the therapy of prostate cancer [1,2]. Recently, clinical Phase I studies have been started, using estradiol-3-sulfamate (**1**, J 995) (Scheme 1) as a first selected estrogen sulfamate.

Pharmacokinetic studies in rats with ^{3}H-labeled sulfamate **1** have shown that almost 98% of ^{3}H-levels in the blood were detected in the erythrocyte fraction, whereas only 2% were found in the plasma. Upon oral administration, the main part of the erythrocyte-bound steroid proved to be estrone 3-sulfamate (**2**, J 994). However, after intravenous (i.v.) administration, estradiol 3-sulfamate (**1**) was the dominant species [3].

Sulfamate **1** has been found to be incapable of specific binding to uterine cytosol and of displacing estradiol from the estrogen receptor at high concentrations. Thus, estradiol 3-sulfamate (**1**) and its metabolite estrone sulfamate (**2**)

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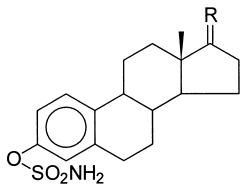


1: R = α-H, β-OH
2: R = O

Scheme 1. Estrone sulfamate (J 994) and estradiol 3-sulfamate (J 995).

appear to be prodrugs which exert estrogenic effects only after cleavage of the sulfamate moiety. As yet, the site(s) and the mechanism of the estradiol/estrone liberation from sulfamates **1**/**2** are unknown. However, to fully understand the pharmacokinetic behavior of estradiol 3-sulfamate (**1**), a detailed insight into the complex bioactivating process is essential. As a result, pharmacokinetic studies as part of our ongoing clinical trials of compound **1** called for a highly sensitive and specific immunoassay allowing us to quantitatively detect picomolar amounts of estra-1,3,5 (10)-trien-3-yl sulfamates in the presence of the corresponding estra-1,3,5 (10)-trien-3-ols.

Immunogens obtained by conjugating estra-1,3,5 (10)-trienes with a protein by means of a 6-(O)-carboxymethyl oxime moiety often fail to produce antisera with the desired specificity which may be due to E-/Z-isomerism of the oxime and conformational distortion of the B-ring by the sp2 hybridized C-6 [4,5]. Antisera directed against the estradiol-6-(O)-carboxymethyl oxime, in general, are specific to the D-ring of the steroid molecule (only little cross-reaction with estrone- and estriol-derivatives), but less specific to the A-ring showing substantial cross-reaction with C-3 conjugated estradiol or other A-ring substituted derivatives (Schumacher M, unpublished results and Refs. [6] and [7]). This also proved to be true for some antisera used in commercially available radioimmunoassays of estradiol which displayed a considerable cross-reactivity with sulfamate **1** (Elger W, unpublished results). However, coupling A-ring aromatic steroids to the protein through non-rigid carboxyalkyl ether linkages was shown to give immunogens capable of generating highly specific antisera [5] (Schumacher M, unpublished results and Ref. [8]). With this background, we decided to couple sulfamate **1** to the protein carrier through a (3′-sulfanyl) propanoxy spacer attached at 11α-position. On three counts, the hapten **17** (Scheme 4) was thought to meet the requisites for generating highly selective and sensitive antibodies upon conjugation with a suitable carrier protein. First, the spacer being arranged far from position 3 was assumed to facilitate the differentiation between the 3-sulfamate group and the phenolic hydroxy function. Second, the highly flexible spacer bound to the sp^3 hybridized C-11 in an equatorial arrangement was considered to closely mimic unmodified sulfamate **1**. Third, the ω-sulfanyl group of the spacer offered the possibility of a defined coupling of the hapten molecule to the carrier protein which is a prerequisite for obtaining sulfamate specific antibodies.

We report here on our efforts in the synthesis of the 11α-(3′-sulfanylalkyl)oxy substituted compound **1** (**17**) [9] and in the characterization of specific antibodies raised against a bovine γ-globulin conjugate derived from hapten **17**.

2. Experimental

2.1. Reagents and equipment

All reactions were run under argon protection, and, if essential, with strict protection from moisture. Solvents and chemicals (Sigma-Aldrich Chemie GmbH, Deisenhofen/Germany, Merck KGaA, Darmstadt/Germany) were reagent grade or analytical grade and were used without further purification. Estradiol 3-sulfamate, estrone sulfamate, 17α-estradiol 3-sulfamate, and estriol 3-sulfamate were prepared as described previously [10,11], as were estra-1,3,5 (10)-trien-3,17β-diyl 17-pentanoate, 3-sulfamate [11], estrone (N-acetyl)sulfamate, estradiol 3-(N-acetyl)sulfamate, and estriol 3-(N-acetyl)sulfamate [12]. Estradiol 3-pentanoate, estradiol, 17α-estradiol, ethinylestradiol, sodium estradiol 3-sulfate, and estradiol 3-glucuronide were purchased from Sigma-Aldrich Chemie GmbH, Deisenhofen (Germany). Organic solutions were dried with anhydrous sodium sulfate or magnesium sulfate monohydrate. Evaporation in vacuo was carried out with a rotary evaporator. Chromatography means flash chromatography on Kieselgel 60 (Merck-Darmstadt, 0.04–0.063 mm); eluents were given in volume proportions. Melting points (m.p.) were measured with a Boetius equipment and are uncorrected. Optical rotations were taken with a Jasco DIP-1000 equipment. Unless otherwise stated, chloroform was used as solvent, c = 1 g/100 ml, t = +20°C. ^{1}H NMR spectra were recorded on a Varian 300. Unless otherwise stated, deuteriochloroform was used as solvent. Chemical shifts were reported as δ-values in ppm downfield from tetramethylsilane as internal standard, J was given in Hz. The spectral data were recorded as δ (coupling pattern, J, proton number). Mass spectra (EI) were taken with an MS AMD 402 (BE configuration) of AMD- Intectra, Harpstedt. Mass spectrometry by electrospray ionization (ESI) was run with a VG-Quattro of Pfizens, VG Biotech, Altricham. Acronyms in the text were used according to Ref. [13].

2.2. 9α-Hydroxy-estra-1,3,5 (10)-triene-3,17β-diyl diacetate (4)

To a solution of estra-1,3,5 (10)-triene-3,17β-diyl 3,17-diacetate **3** (10 g, 28 mmol) in dichloromethane (200 ml) were added water (220 ml), sodium hydrogen carbonate (64 g), acetone (177 ml), and tetrabutyl ammonium hydrogen

sulfate (0.1 g). The suspension was cooled to +12°C and oxone (133 g) was added over a period of 2.5 h with vigorous stirring. The suspension was then stirred for another 3.5 h at 15°C–20°C. The reaction mixture was filtered and the separated organic layer was evaporated in vacuo. Purification of the residue by chromatography (eluent: toluene/ethyl acetate 10:1) and crystallization from methanol afforded compound **4** (8.36 g, 80%) identical in all respects with reported data [14].

*2.3. Estra-1,3,5(10),9(11)-tetraene-3,17β-diol (**6**)*

Compound **4** (10 g, 27 mmol) was dissolved in dichloromethane (200 ml). The solution was cooled to −20°C and stirred with 70% aqueous sulfuric acid (1 ml) for 2 h. The mixture was then treated with saturated sodium hydrogen carbonate solution until neutral. The organic phase was dried and evaporated in vacuo. The resulting estra-1,3,5 (10)9(11)-tetraene-3,17β-diyl diacetate **5** (9 g, 25.4 mmol) was dissolved in methanol (133 ml), potassium hydroxide (6.65 g, 118.5 mmol) was added and the solution was stirred at 40°C for 4 h. The main part of the methanol was distilled off in vacuo and to the resulting solution 1 N aqueous hydrochloric acid was added until neutral. On addition of water (300 ml), the precipitated crystals were filtered off and recrystallized from methanol to give compound **6** (4 g, 65%). The substance retained water tenaciously (1.7%), even upon prolonged drying in vacuo. m.p. 186–191°C. $[\alpha]_D$ + 132°. ^{1}H nmr (d_6-DMSO): 9.22 (s, 3-OH), 7.41 (d, 8.8, H-1) 6.54 (dd, 8.8, 2.6, H-2), 6.45 (d, 2.6, H-4), 6.03 (d, 4.9, H-11), 4.57 (d, 4.7, 17-OH), 3.63 (td, 8.5, 4.9, H-17), 2.72 (m, H-6), 0.68 (s, H-18). ms: (EI) m/z 270.0 $(M)^+$. $C_{18}H_{22}O_2$ (270.37) calculated: with 1.7% water C 79.19 H 8.20 found C 78.81 H 8.28.

*2.4. 3,17β-Bis-(t-butyldimethylsilyl)oxy-estra-1,3,5(10),9(11)-tetraene (**7**)*

To a solution of compound **6** (20 g, 74 mmol) in dimethylformamide (200 ml) and pyridine (200 ml) was added *t*-butyl-dimethylsilylchloride (55.76 g, 370 mmol). The mixture was stirred at 60–70°C for 3 h, then cooled to +10°C, poured into saturated aqueous sodium hydrogen carbonate solution (1-l), and extracted with toluene. The combined extracts were concentrated in vacuo and the residue was purified by chromatography (eluent: toluene), followed by crystallization from methanol to afford compound **7** (31.45 g, 85%). m.p. 130–132°C. $[\alpha]_D$ + 79°. ^{1}H nmr: 7.46 (d, 8.9, H-1), 6.62 (dd, 8.9, 2.8, H-2), 6.54 (d, 2.8, H-4), 6.11 (m, H-11), 3.72 (t, 8.4, H-17), 2.79 (m, H-6), 0.97 (s, -C$(CH_3)_3$), 0.90 (s, -C$(CH_3)_3$), 0.77 (s, H-18), 0.18 (s, Si-CH_3), 0.05 (s, Si-CH_3), 0.03 (s, Si-CH_3). ms: (EI) m/z 498.33661 $(M)^+$. $C_{30}H_{50}O_2Si_2$ (498.90) calculated C 72.23 H 10.10 found C 72.31 H 10.08.

*2.5. 3,17β-Bis-(t-butyldimethylsilyl)oxy-estra-1,3,5 (10)-trien-11α-ol (**8**)*

At 50°C, a stirred solution of compound **7** (21.8 g, 43.7 mmol) in tetrahydrofuran (100 ml) was treated with borane-dimethyl sulfide complex (12.5 ml, 130 mmol) for 1 h. Upon cooling to 0°C, the reaction was then carefully quenched with water (20 ml). Subsequently, a cold (5°C) aqueous mixture of sodium hydroxide (9.6 g, 240 mmol) and hydrogen peroxide (30%, 40 ml) in water (80 ml) was added. The mixture was stirred for another 1 h at room temperature and then extracted several times with n-hexane. The combined organic fractions were washed with water, dried and evaporated in vacuo to dryness. The residue was purified by chromatography (eluent: toluene/cyclohexane 1:1), followed by crystallization from ethyl acetate/methanol to yield compound **8** (16.87 g, 74.5%). m.p. 141°C-146°C. $[\alpha]_D$-46°. ^{1}H nmr: 7.74 (d, 8.7, H-1), 6.64 (dd, 8.7, 2.8, H-2), 6.57 (d, 2.8, H-4), 4.21 (m, H-11), 3.67 (t, 8.1, H-17), 2.76 (t, 6.7, H-6), 2.23 (dd, 11.6, 5.0, H-12), 2.11 (t, 9.5, H-9), 0.97 (s, -C$(CH_3)_3$), 0.89 (s, -C$(CH_3)_3$), 0.73 (s, H-18), 0.19 (s, Si-CH_3), 0.04 (s, Si-CH_3), 0.02 (s, Si-CH_3). ms: (EI) m/z 516.34582 $(M)^+$. $C_{30}H_{52}O_3Si_2$ (516.92) calculated C 69.71 H 10.14 found C 69.75 H 10.15.

*2.6. 3,17β-Bis-(t-butyldimethylsilyl)oxy-11α-(prop-2′-enyl)oxy-1,3,5 (10)-triene (**9**)*

At 0°C, a solution of compound **8** (62.9 g, 122 mmol) and allyl bromide (88.5 g, 730 mmol) in tetrahydrofuran (400 ml) was treated portionwise with potassium *t*-butoxide (41 g, 365 mmol). Two min after completion of addition, the reaction was quenched with saturated aqueous ammonium chloride solution (100 ml). The mixture was extracted with toluene, the combined extracts were washed with water, dried, evaporated in vacuo, and the residue was purified by crystallization from methanol to give compound **9** (43 g, 63.5%). m.p. 121°C–124°C. $[\alpha]_D$-56°. ^{1}H nmr: 7.53 (d, 8.8, H-1), 6.60 (dd, 8.8, 2.8, H-2), 6.54 (d, 2.8, H-4), 6.03 (m, H-3′), 5.34 (dq, 17.1, 1.6, H-4′), 5.18 (dq, 10.3, 1.7, H-4′), 4.25 (ddt, 12.7, 5.9, 1.1, H-2′), 4.25 (ddt, 12.7, 5.4, 1.6, H-2′), 3.86 (td, 10.1, 4.7, H-11), 3.66 (t, 8.4, H-17), 2.76 (dd, 6.8, 6.3, H-6), 2.39 (dd, 12.2, 5.5, H-12), 2.30 (t, 10.1, H-9), 1.12 (dd, 11.2, 10.5, H-12), 0.97 (s, -C$(CH_3)_3$), 0.90 (s, -C$(CH_3)_3$), 0.71 (s, H-18), 0.18 (s, Si-CH_3), 0.05 (s, Si-CH_3), 0.03 (s, Si-CH_3). ms: m/z 556.37500 $(M)^+$. $C_{33}H_{56}O_3Si_2$ (556.98) calculated C 71.16 H 10.13 found C 71.20 H 10.15.

*2.7. 3,17β-Bis-(t-butyldimethylsilyl)oxy-11α-(3′-hydroxypropyl)oxy-estra-1,3,5 (10)-triene (**10**)*

Compound **9** (43 g, 77 mmol), dissolved in tetrahydrofuran (300 ml), was allowed to react with 9-borabicyclo [3.3.1]nonane (37.7 g, 154.4 mmol) for 1 h at 65°C with

stirring. After that, the solution was cooled to 0°C and the reaction was quenched with water (30 ml). 3 N aqueous sodium hydroxide solution (200 ml) and aqueous hydrogen peroxide solution (200 ml, 30%) were added and the mixture was stirred at room temperature for 30 min. The product was isolated by extraction with n-hexane, chromatography (eluent: cyclohexane/methyl *t*-butyl ether 10:1) and crystallization from methanol/water to give compound **10** (42.6 g, 96%). m.p. 88°C–91°C. $[\alpha]_D$-57°. ^{1}H NMR: 7.40 (d, 8.3, H-1), 6.63 (dd, 8.3, 2.7, H-2), 6.55 (d, 2.7, H-4), 3.89 (m, H-1′), 3.80 (m, H-3′), 3.80 (m, H-11), 3.68 (m, H-17), 3.68 (m, H-1′), 2.76 (t, 7.1, H-6), 2.42 (dd, 11.9, 4.9, H-12), 0.98 (s, -C(CH_3)$_3$), 0.90 (s, -C(CH_3)$_3$), 0.70 (s, H-18), 0.19 (s, Si-CH_3), 0.06 (s, Si-CH_3), 0.03 (s, Si-CH3). ms: (EI) m/z 574.38702 $(M)^+$. $C_{33}H_{58}O_4Si_2$ (575.00) calculated C 68.93 H 10.17 found C 68.80 H 10.12.

*2.8. S-3-[3′,17′β-Bis-(t-butyldimethylsily)oxy-estra-1′,3′,5′(10′)-trien-11′α-yl]oxypropyl ethanethioate (**11**)*

A 0°C cold solution of triphenylphosphine (20.69 g, 78.6 mmol) and diisopropyl azo-dicarboxylate (15.89 g, 78.6 mmol) in tetrahydrofuran (200 ml) was stirred for 30 min. Then, in turn, a solution of compound **10** (22.6 g, 39.3 mmol) in tetrahydrofuran (150 ml) and thioacetic S-acid (5.97 g, 78.6 mmol) in tetrahydrofuran (15 ml) were added. The resulting mixture was stirred for another 45 min and, after that, evaporated in vacuo. The residue was treated with n-hexane/methyl *t*-butyl ether and the crystals (triphenylphosphane oxide) were filtered off. The filtrate was evaporated in vacuo and the residue was purified by chromatography (eluent:toluene) followed by crystallization from methanol to afford compound **11** (22.5 g, 90.4%). m.p. 77°–80°C. $[\alpha]_D$-44°. ^{1}H nmr: 7.37 (d, 8.6, H-1), 6.62 (dd, 8.6, 2.8, H-2), 6.55 (d, 2.8, H-4), 3.76 (m, H-1′), 3.76 (m, H-11), 3.66 (t, 8.4, H-17), 3.52 (dt, 9.1, 5.7, H-1′), 2.99 (t, 7.1, H-3′), 2.75 (t, 7.0, H-6), 2.38 (dd, 12.1, 5.1, H-12), 2.34 (s, -CO-CH_3), 0.98 (s, -C(CH_3)$_3$), 0.91 (s, -C(CH_3)$_3$), 0.70 (s, H-18), 0.19 (s, Si-CH_3), 0.06 (s, Si-CH_3), 0.03, (s, Si-CH_3). ms: m/z (EI) 632.37347 $(M)^+$. $C_{35}H_{60}O_4S$ Si_2 (633.10) calculated C 66.40 H 9.55 S 5.06 found C 66.38 H 9.55 S 5.20.

*2.9. 3,17β-Bis-(t-butyldimethylsilyl)oxy-11α-(3′-sulfanylpropyl)oxy-estra-1,3,5 (10)-triene (**12**)*

To a stirred solution of compound **11** (13 g, 20.5 mmol) in tetrahydrofuran (150 ml) 1 N lithium aluminium hydride solution in tetrahydrofuran (50 ml, 50 mmol) was added dropwise. After 2 h, the reaction was quenched with ethyl acetate (100 ml) and water. The organic phase was separated, washed with water, dried, and evaporated in vacuo. The residue was purified by chromatography (eluent:cyclohexane/methyl tert-butyl ether 10:1) followed by crystallization from methanol to give compound **12** (10 g, 82.5%). m.p. 102°C–103°C. $[\alpha]_D$-53°. ^{1}H nmr: 7.39 (d, 8.6, H-1), 6.62 (dd, 8.6, 2.7, H-2), 6.55 (d, 2.7, H-4), 3.80 (m, H-1′), 3.80 (m, H-11), 3.66 (t, 8.0, H-17), 3.58 (dt, 9.3, 6.1, H-1′), 2.75 (t, 6.7, H-6), 2.69 (d, 6.7, H-3′), 2.65 (d, 6.7, H-3′), 2.40 (dd, 11.8, 5.1, H-12), 2.23 (t, 9.6, H-9), 1.06 (t, 11.8, H-12), 0.98 (s, -C(CH_3)$_3$), 0.90 (s, -C(CH_3)$_3$), 0.70 (s, H-18), 0.19 (s, Si-CH_3), 0.06 (s, Si-CH_3), 0.03 (s, Si-CH_3). ms: (EI) m/z 590.36621 $(M)^+$. $C_{33}H_{58}O_3S$ Si_2 (591.06) calculated C 67.06 H 9.89 S 5.42 found C 66.83 H 9.89 S 5.43.

*2.10. Bis-3-[3′,17′β-bis-(t-butyldimethylsilyl)oxy-estra-1′,3′,5′(10′)-trien-11′α-yl]oxypropyl-disulfane (**13**)*

A stirred suspension of compound **12** (14 g, 23.7 mmol) in dichloromethane (150 ml) and saturated aqueous sodium hydrogen carbonate solution (150 ml) was treated dropwise with a solution of iodine (3.18 g, 2.45 mmol) in ethanol (250 ml) until the iodine color did no longer disappear. The organic phase was separated, dried, and evaporated in vacuo. After trituration with methanol compound **13** (14 g, nearly quantitative yield) was obtained. m.p. 83°–90°C. $[\alpha]_D$-46°. ^{1}H nmr: 7.38 (d, 8.3, H-1), 6.62 (dd, 8.3, 2.9, H-2), 6.55 (d, 2.9, H-4), 3.80 (m, H-1′), 3.80 (m, H-11), 3.65 (t, 8.5, H-17), 3.56 (m, 9.3, 6.1, H-1′), 2.80 (t, 6.6, H-6), 2.75 (t, 6.6, H-3′), 2.40 (dd, 12.2, 5.0, H-12), 2.23 (t, 9.7, H-9), 1.06 (t, 12, H-12), 0.97 (s, -C(CH_3)$_3$), 0.90 (s, -C(CH_3)$_3$), 0.70 (s, H-18), 0.19 (s, Si-CH_3), 0.06 (s, Si-CH_3), 0.03 (s, Si-CH_3). ms: (EI) m/z 1180.1 (M^+). $C_{66}H_{114}O_6S_2Si_4$ (1180.11) calculated C 67.18 H 9.74 S 5.43 found C 66.95 H 9.70 S 5.62.

*2.11. Bis-3-[17′β-(t-butyldimethylsilyl)oxy-3′hydroxy-estra-1′,3′,5′(10′)-trien-11′α-yl]oxypropyl-disulfane (**14**)*

A mixture of compound **13** (14 g, 11.8 mmol) in a solution of tetrabutylammonium fluoride (7.6 g, 24 mmol) in tetrahydrofuran (200 ml) was stirred at room temperature for 30 min. Saturated sodium hydrogen carbonate solution (50 ml) was then added, the organic solvent was evaporated in vacuo and the suspension extracted with n-hexane. The organic fractions were combined, washed with water, dried, and evaporated in vacuo. Purification of the residue by chromatography (eluent:toluene/ethyl acetate 10: 1) followed by crystallization from methanol gave compound **14 (**6 g, 93.2%). m.p. 154°C–157°C. $[\alpha]_D$-42°. ^{1}H nmr: 7.40 (d, 8.3, H-1), 6.64 (dd, 8.3, 2.6, H-2), 6.55 (d, 2.6, H-4), 5.25 (s, 3-OH), 3.80 (m, H-1′), 3.80 (m, H-11), 3.65 (t, 8.2, H-17), 3.56 (m, H-1′), 2.80 (t, 7.0, H-6), 2.73 (t, 6.6, H-3′), 2.39 (dd, 12.0, 5.0, H-12), 2.23 (t, 9.9, H-9), 1.07 (t, 11.0, H-12), 0.90 (s, -C(CH_3)$_3$), 0.69 (s, H-18), 0.06 (s, Si-CH_3), 0.03 (s, Si-CH_3). ms: (EI) m/z 952.5 $(M)^+$. $C_{54}H_{86}O_6S_2Si_2$ (951.58) calculated C 68.16 H 9.11 S 6.74 found C 68.01 H 9.07 S 6.95.

*2.12. Bis-3-[17′β-(t-butyldimethylsilyl)oxy-3′-sulfamoyloxy-estra-1′,3′,5′ (10′)-trien-11′α-yl]oxypropyl-disulfane (**15**)*

To a stirred solution of compound **14** (0.48 g, 0.5 mmol) in dichloromethane (15 ml) were added in turn 2,6-di-tert-butyl-pyridine (1.2 ml, 5.44 mmol) and sulfamoyl chloride (1.74 g, 15.06 mmol) at room temperature. Stirring was continued for 3 h. The reaction was then quenched with water, the organic phase was separated, and the aqueous phase re-extracted with dichloromethane. The combined organic phases were washed with saturated aqueous sodium hydrogen carbonate solution and water until neutral, dried, and evaporated in vacuo to give an oil which was purified by chromatography (eluent:cyclohexane/ethyl acetate 5:2) to give semi-crystalline compound **15** (0.145 g, 26%). ^{1}H nmr: 7.54 (d, 8.8, H-1), 7.08 (dd, 8.8, 2.6, H-2), 7.02 (d, 2.6, H-4), 5.08 (s, -NH_2), 3.80 (m, H-1′), 3.80 (m, H-11), 3.66 (t, 8.0, H-17), 3.51 (m, H-1′), 2.79 (t, 6.4, H-6), 2.40 (dd, 11.9, 4.9, H-12), 2.26 (t, 9.7, H-9), 1.07 (t, 10.4, H-12), 0.90 (s, -$C(CH_3)_3$), 0.68 (s, H-18), 0.05 (s, Si-CH_3), 0.03 (s, Si-CH_3). ms: (ESI^-) m/z 1107.9 $(M-H)^-$; (ESI^+) m/z 1126.7 ($M+NH_4$), 1131.3 (M+Na).

*2.13. Bis-3-(17′β-hydroxy-3′-sulfamoyloxy-estra-1′,3′,5′(10′)-trien-11′α-yl)oxypropyl-disulfane (**16**)*

Compound **15** (0.43 g, 0.39 mmol) was dissolved in a mixture of acetic acid, tetrahydrofuran, and water (3:2:1, 60 ml). The solution was allowed to stand 6 days at room temperature. Afterwards, the solvents were distilled off in vacuo and the resulting oil was partitioned between water and ethyl acetate. The organic phase was washed with saturated aqueous sodium hydrogen carbonate solution and water, dried, and evaporated in vacuo. Purification of the residue by chromatography (eluent:cyclohexane/ethyl acetate 1:2) gave compound **16** (0.2 g, 58.6%) as a foam. ^{1}H nmr (d_6-DMSO): 7.89 (s, -NH_2), 7.52 (d, 8.4, H-1), 7.01 (dd, 8.4, 2.5, H-2), 6.98 (d, 2.5, H-4), 4.61 (d, 4.5, -OH), 3.74 (m, H-1′), 3.74 (m, H-11), 3.53 (m, H-17), 3.53 (m, H-1′), 2.77 (t, 6.7, H-6), 2.42 (dd, 11.4, 4.8, H-12), 2.19 (t, 9.1, H-9), 1.00 (t, 10.4, H-12), 0.62 (s, H-18). ms: (ESI^-) m/z 879.2 $(M-H)^-$; (ESI^+) m/z 898.8 $(M+NH4)^+$, 903 $(M+Na)^+$.

*2.14. 17β-Hydroxy-11α-(3′-sulfanylpropyl)oxy-estra-1,3,5(10)-trien-3-yl sulfamate (**17**)*

*From compound **20** by reduction with sodium borohydride / aluminum chloride*: To a 0°C cold solution of compound **20** (0.45 g, 0.93 mmol) and sodium borohydride (0.3 g, 7.93 mmol) in diglyme (15 ml) anhydrous aluminum chloride (0.473 g, 3.55 mmol) was added portionwise. After the addition was finished, the reaction mixture was stirred for an additional h at 23°C. Afterwards, the reaction was quenched with saturated aqueous sodium hydrogen carbonate solution (20 ml) at 0°C and the resulting mixture extracted with ethyl acetate. The combined fractions were washed with saturated aqueous sodium chloride solution, dried, and evaporated in vacuo. The residue was purified by twofold chromatography (eluent:cyclohexane/ethyl acetate 1:2) followed by precipitating the compound with n-hexane from an acetone solution to give compound **17** (0.2 g, 49%) as a white amorphous powder. Purity by HPLC: 99.1 area %. $[\alpha]_D$-17° (pyridine). ^{1}H nmr (d_6-DMSO): 7.88 (s, -NH_2), 7.53 (d, 8.4, H-1), 7.03 (dd, 8.4, 2.7, H-2), 6.98 (d, 2.7, H-4), 4.60 (d, 4.9, -OH), 2.91 (t, 7.4, H-3′), 2.77 (t, 7.3, H-6), 2.44 (dd, 11.6, 4.8, H-12), 1.01 (t, 11.1, H-12), 0.63 (s, H-18). ms: (EI) m/z 441.16668 $(M)^+$; (ESI^-) m/z 440.5 $(M-H)^-$, 881.0 (2 $M-H)^-$; (ESI^+) m/z 442.5 $(M+H)^+$, 459.6 $(M+NH_4)^+$, 464.5 $(M+Na)^+$. $C_{21}H_{31}NO_5S_2$ (441.60) calculated C 57.12 H 7.08 N 3.17 found C 57.11 H 7.35 N 3.17. *From compound **20** by reduction with borane-dimethyl sulfide complex*: To a 0°C cold solution of compound **20** (0.62 g, 1.28 mmol) in tetrahydrofuran (3 ml) was added borane-disulfide complex (2.5 ml, 26.4 mmol). The solution was stirred for 3 h at room temperature, followed by cautious addition of water (15 ml) at 0°C. The mixture was then extracted with ethyl acetate. The combined fractions were washed with water until neutral, dried, and evaporated in vacuo. The residue was purified by column chromatography (eluent:cyclohexane/chloroform/methanol 45:45:10) and precipitated from an acetone solution with n-hexane to yield compound **17** (0.32 g, 57%). Purity by HPLC: 93.2 area %. The spectra proved to be identical in all respects with those of compound **17** prepared by sodium borohydride/aluminum chloride reduction. From disulfane **16**: Compound **16** (0.1 g, 0.11 mmol) dissolved in diglyme (1.7 ml) was reduced with sodium borohydride (0.035 g, 0.92 mmol) in the presence of anhydrous aluminum chloride (57 mg, 0.43 mmol) as described for the reduction of thioacetate **20**. The product was purified by chromatography (eluent:cyclohexane/ethyl acetate 1:4) followed by precipitation with n-hexane from an acetone solution to yield compound **17** (0.050 g, practically quantitative yield). Purity by HPLC: 92 area %. The spectra proved to be identical in all respects with those of compound **17** prepared from thioacetate **20**.

*2.15. S-3-[17′β -(t-butyldimethylsilyl)oxy-3′-hydroxy-estra-1′,3′,5′(10′)-trien-11′α-yl]oxypropyl ethanethioate (**18**)*

Following the same procedure as used to prepare compound **14**, compound **11** (5.51 g, 8.7 mmol) was reacted with 1 eq. tetrabutylammonium fluoride in tetrahydrofuran solution to afford compound **18** (2.8 g, 62%) as a foam. $[\alpha]_D$-46°. ^{1}H nmr: 7.40 (d, 8.6, H-1), 6.63 (dd, 8.6, 2.9, H-2), 6.55 (d, 2.9, H-4), 3.76 (m, H-1′), 3.76 (m, H-11), 3.66 (t, 8.1, H-17), 3.51 (dt, 9.3, 6.1, H-1′), 3.01 (t, 6.8, H-3′), 2.75 (t, 6.7, H-6), 2.38 (dd, 11.9, 5.3, H-12), 2.34 (s, -Ac), 1.07 (dd, 11.5, 11.2, H-12), 0.91 (s, -$C(CH_3)_3$), 0.70

(s, H-18), 0.06 (s, Si-CH_3), 0.03 (s, Si-CH_3). ms: (EI) m/z 518.29052 $(M)^+$. $C_{29}H_{46}O_4S$ Si (518.84) calculated C 67.14 H 8.94 S 6.18 found C 67.15 H 8.95 S 6.33.

*2.16. S-3-[17′β-(t-butyldimethylsilyl)oxy-3′-sulfamoyloxy-estra-1′,3′,5′(10′)-trien-11′α-yl]oxypropyl ethanethioate (**19**)*

To a solution of compound **18** (1.1 g, 2.12 mmol) in a mixture of dichloromethane (30 ml) and 2,6-di-tert-butyl-pyridine (2.3 ml, 10.43 mmol) was added sulfamoyl chloride (1.3 g, 11.3 mmol). After stirring for 1 h at 22°C, the solution was diluted with water (30 ml) and extracted with ethyl acetate. The combined organic phases were washed with saturated aqueous sodium hydrogen carbonate solution and water and dried. After evaporation in vacuo, the residue was purified by column chromatography (eluent:toluene: ethyl acetate 9:1) to give compound **19** (1.03 g, 84%) as an amorphous powder. $[\alpha]_D$-49° (pyridine). ^{1}H nmr (d_6-DMSO): 7.89 (s, -NH_2), 7.53 (d, 8.9, H-1), 7.02 (dd, 8.9, 2.5, H-2), 6.98 (d, 2.5, H-4), 2.91 (t, 7.4, H-3′), 2.77 (t, 6.8, H-6), 2.32 (s, -Ac), 1.05 (t, 11.5, H-12), 0.89 (s, -$C(CH_3)_3$), 0.65 (s, H-18), 0.06 (s, Si-CH_3), 0.04 (s, Si-CH_3). ms: (EI) m/z 597.25873 $(M)^+$. $C_{29}H_{47}NO_6S_2Si$ (597.90) calculated C 58.26 H 7.92 N 2.34 S 10.72 found C 58.37 H 7.95 N 2.57 S 10.86.

*2.17. S-3-(17′β-Hydroxy-3′-sulfamoyloxy-estra-1′,3′,5′(10′)-trien-11′α-yl)oxypropyl ethanethioate (**20**)*

Following the procedure described for the formation of compound **16**, compound **19** (1.0 g, 1.67 mmol) was treated with a mixture of acetic acid, tetrahydrofuran, and water (3:2:1, 115 ml) for 6 days at room temperature to give compound **20** (0.69 g, 85%) as a foam. $[\alpha]_D$-69° (pyridine). ^{1}H nmr (d_6-DMSO): 7.90 (s, -NH_2), 7.52 (d, 8.2, H-1), 7.02 (dd, 8.2, 2.7, H-2), 6.98 (d, 2.7, H-4), 4.62 (d, 4.7, -OH), 2.90 (t, 7.1, H-3′), 2.77 (t, 7.0, H-6), 2.42 (dd, 12.0, 4.9, H-12), 2.34 (s, -Ac), 1.00 (t, 10.9, H-12), 0.62 (s, H-18). ms: $(ESI)^-$ m/z 482.5 $(M\text{-}H)^-$, 965.9 $(2\ M\text{-}H)^-$, 1448.8 $(3\ M\text{-}H)^-$; $(ESI)^+$ m/z 484.8 $(M+H)^+$, 506.5 $(M+Na)^+$, 989.9 $(2M+Na)^+$. $C_{23}H_{33}NO_6S_2$ (483.64) calculated C 57.12 H 6.88 N 2.9 S 13.26 found C 57.46 H 7.31 N 2.87 S 13.13.

*2.18. 17β-Hydroxy-11α-(3′-sulfanylpropyl)oxy-estra-1,3,5(10)-trien-3-yl sulfamate bovine γ- globulin conjugate (**21**)*

Hapten **17** was coupled to bovine γ-globulin (BGG) in a two-step procedure. BGG (25 mg) was dissolved in 0.1 M sodium phosphate (0.9 ml, pH 7.2) containing 0.9% sodium chloride. β-Maleimido propionic acid N-hydroxysuccinimide ester (Sigma) (13 mg, 48.9 μmol) was dissolved in dimethylformamide (0.3 ml) and immediately added to the protein solution with stirring. The mixture was stirred for 1 h at room temperature followed by centrifugation (~10 000 *g*, 5 min). The clear supernatant was passed through a Sephadex G 50 column (15 × 90 mm), equilibrated in phosphate buffer (vide supra) and the absorbance of the effluent was monitored at 280 nm. The protein peak (~4 ml) was collected and after dilution with dioxane (1.4 ml), compound **17** (10 mg, 22.6 μmol) in dioxane (150 μl) was slowly added with fast stirring. The cloudy mixture was stirred for 5 h at room temperature and then dialyzed once against 25% dioxane in phosphate buffer (1 l) and twice against phosphate-buffered saline (PBS) (1 l). As control, cysteine was coupled to BGG in a similar procedure (5 mg were added in 150 μl pf buffer), followed by identical treatment. The amount of steroid coupled to the BGG was determined by comparing the UV spectra of the conjugate, the control and of a defined concentration of compound **17**. The mole ratio calculated for the coupling was 32 mol steroid to 1 mol BGG.

2.19. Production of antisera

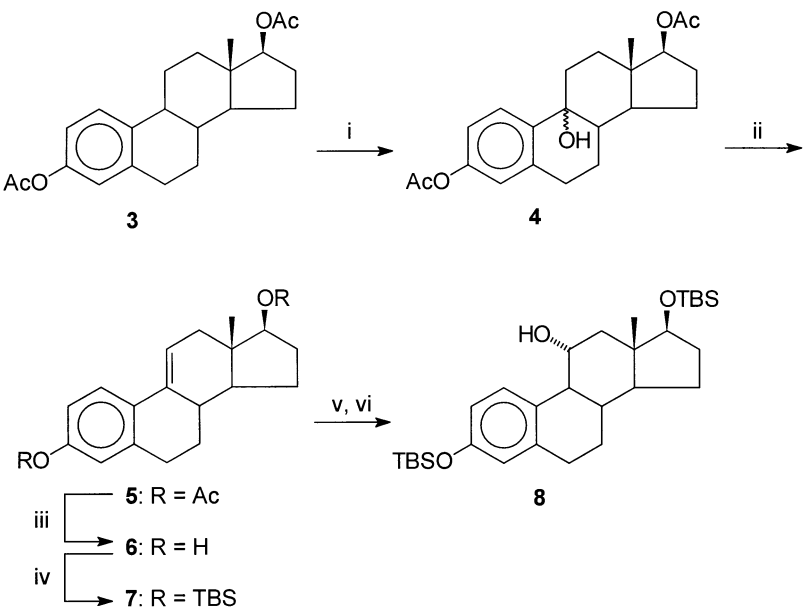
Six male rabbits (~3 kg) were subcutaneously (s.c.) immunized with conjugate **21** (1 mg) in emulgate (1.5 ml) containing complete Freund's Adjuvant and boosted thereafter with conjugate (0.5 mg) in incomplete adjuvant at 6 to 8 weekly intervals. Animals were bled 2 weeks after the third boost.

*2.20. Biotinylated compound **22***

Compound **17** (1 μmol) dissolved in dimethylformamide (20 μl) was added to a solution of N-biotinoyl-N′-[6-maleidohexanoyl]-hydrazide (Sigma) (1 μmol) in dimethylformamide (150 μl). The mixture was incubated for 2 h at room temperature with stirring. The biotinylated compound **22** was purified by thin-layer chromatography using silica G 60 coated aluminium sheets and the solvent mixture ethyl acetate/ methanol/acetic acid (70/30/2) as mobile phase. The coupling product (Rf = 0.77) was localized under UV light, cut off and extracted with aqueous methanol (80%). On the basis of UV absorption, a yield of about 50% of compound **22** was estimated.

2.21. Enzyme immunoassay protocol

Microtiter plates (NUNC 460348) were coated with affinity purified goat-anti-rabbit γ-globulin (Scantibodies, USA) by pipetting 150 μl antibody solution (6.6 μg/ml 50 mM sodium carbonate pH 9.6) into each well, followed by an overnight incubation at room temperature. Then, 250 μl blocking buffer (0.16 M sodium phosphate pH 7.0, 1.44% sodium chloride, 0.8% bovine serum albumin (BSA), 0.08% Tween 20, 0.02% Thimerosal) were added to each well and the incubation was continued until next day. The plates were emptied by decantation and patted dry onto absorbent paper. They were washed once with 375 μl wash solution (0.02%



Scheme 2. 11α-Oxyfunctionalization of estradiol diacetate. Reaction conditions: (i) Oxone, acetone, CH_2Cl_2, H_2O, $NaHCO_3$, TBAHS, 15°C, 80%. (ii) H_2SO_4, CH_2Cl_2, −30°C; 95%. (iii) MeOH, KOH, 45°C, 65%. (iv) TBSCl, DMF, Py, 60°C, 92%. (v) $BH_3.Me_2S$, THF, 50°C. (vi) H_2O_2, NaOH, 40°C, (v)+(vi) 74%.

Tween 20, 0.5% sodium chloride) and then stored at −20°C for up to 6 months. Prior to assay, the frozen plates were brought to room temperature and washed once with assay buffer (0.1 M sodium phosphate pH 7.0 containing 0.15 M sodium chloride, 0.005 M EDTA, 0.2% BSA, 0.01% Thimerosal, 0.005% Tween 20). Estrogen sulfamate standards or test compounds for cross-reaction studies were added in assay buffer (100 μl) followed by the addition of the biotinylated tracer (~1 fmol) and the antiserum (K4; dilution 1:80 000) in assay buffer (50 μl each). The covered plates were incubated overnight in a humidity chamber at 4°C and then emptied and patted dry. Two hundred μl of streptavidine-horse radish peroxidase conjugate (Camon; 0.15 μg/ml assay buffer, 4°C) were added, and after incubation for 30 min at 4°C, the wells were emptied and washed four times with 375 μl cold wash solution. Finally, the emptied wells were brought to room temperature and filled with 250 μl freshly prepared substrate solution (90 mM sodium acetate, 4.5 mM citric acid, 0.004% H_2O_2, 0.01% 3,3′,5,5′-tetramethylbenzidine, 2% DMSO) adjusted to room temperature. The enzyme reaction was run for 40 min at room temperature and stopped by the addition of 50 μl 2 M sulfuric acid. The absorption was measured at 450 nm in a microplate photometer.

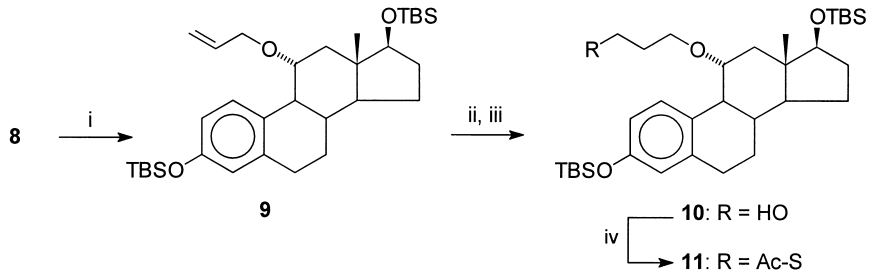
3. Results and discussion

*3.1. Synthesis of hapten 17β-hydroxy-11α-(3′-sulfanylpropyl)oxy-estra-1,3,5 (10)-trien-3-yl sulfamate (**17**)*

Effective oxyfunctionalization of estradiol at 11α-position was an essential prerequisite to the synthesis of title compound **17**. In previous work on the large-scale synthesis of the progestin desogestrel we presented a novel efficient approach to the synthesis of an 11α-hydroxylated A-ring aromatic 18a-homo steroid involving as key steps (i) a C-9 hydroxylation of the corresponding aromatic steroid by in situ formed dimethyldioxirane (DMD) and (ii) a very effective hydroboration/alkaline hydrogen peroxide oxidation of the 9(11)-dehydro compound formed upon regioselective water elimination of the 9-hydroxy group [15]. Adapting our protocol to estradiol diacetate (**3**), 9-hydroxy steroid **4** was obtained in 80% yield using DMD generation in a phase transfer catalyzed two-phase system (Scheme 2).

Water elimination by sulfuric acid transformed hydroxy compound **4** into the 9(11)-olefin **5**. By HPLC, formation of the undesired 8-dehydro isomer was detected in less than 3%. The diol **6**, prepared from diacetate **5**, was converted into the bis-silyl ether **7**. Hydroboration/alkaline hydrogen peroxide oxidation of compound **7** then furnished the bis-protected 11α-hydroxy estradiol **8**.

Initially, alkylation of the 11-hydroxy group in compound **8** was attempted by reaction of compound **8** with 2-trimethylsilyloxy bromoethane [16] and 3-trimethylsilyloxy bromopropane [17] using various bases and solvents. A trimethylsilyl (TMS) ether group at the ω-position of the spacer was thought to undergo chemoselective cleavage in the presence of the 3- and 17-tert-butyl-dimethylsilyl (TBS) ethers, thus opening a route for a subsequent thiofunctionalization of the side chain. However, in all cases studied, only marginal alkylation of the 11-hydroxy group was observed. The reactions suffered from cleavage of the phenolic 3-TBS ether by the bases used and resilylation by the C_2- and C_3-building blocks to give compounds with a TMS group in position 3 or 11 or 3 and 11 as



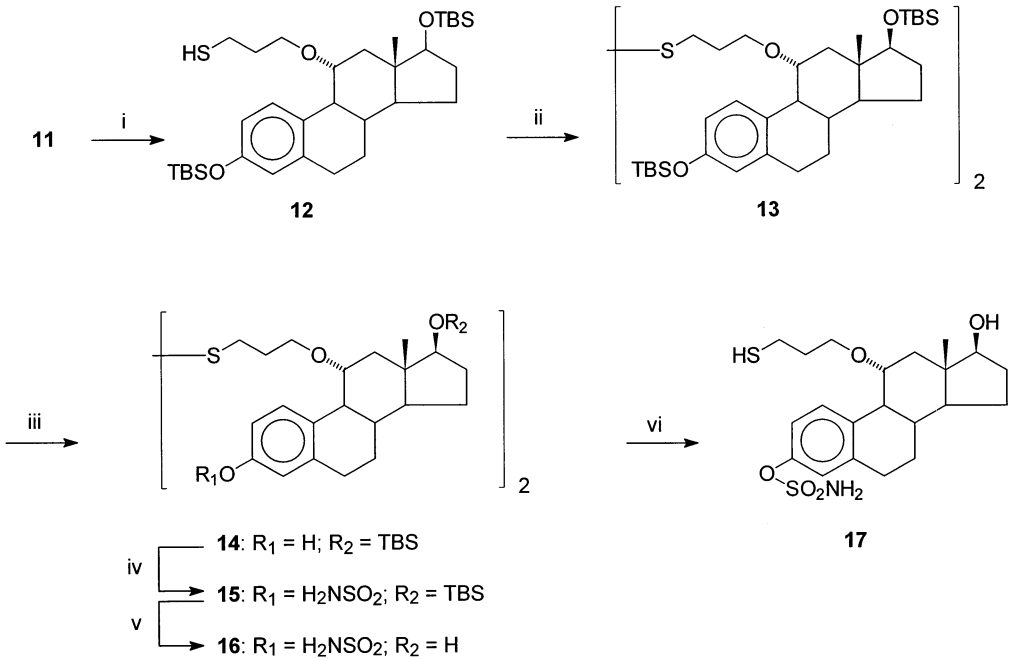
Scheme 3. Introduction of the 11α-(ω-functionalized) side chain. Reaction conditions: (i) Allylbromide, KO*t*Bu, THF, 0°C, 74%. (ii) 9-BBN, THF, 65°C. (iii) H_2O_2, NaOH, (ii)+(iii) 96%. (iv) PPh_3, DIAD, AcSH, THF, 0°C, 90%.

well. C_2- or C_3-alkylation at position 3 instead of position 11 was also observed.

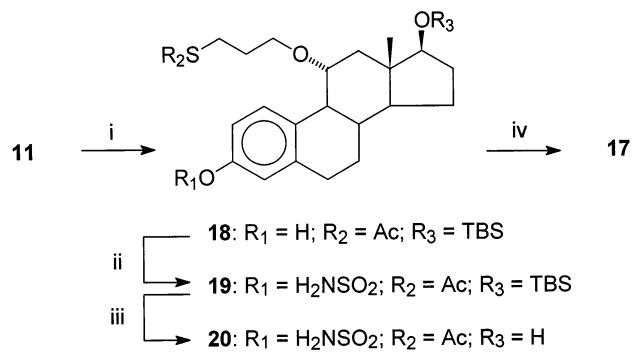
As outlined in Scheme 3, switching to allyl bromide as a C_3-building block was more successful, after the reaction had been carefully optimized. When, at 0°C, a solution of potassium tert-butoxide (3 equiv.) in tetrahydrofuran was added to a mixture of compound **8** and allyl bromide (6 equiv.) in tetrahydrofuran, and, thereupon, the mixture was allowed to react for a strictly limited time of 2 min, allyl ether **9** was isolated in 63% yield after work-up. Subsequent allyl ether functionalization was realized by hydroboration/alkaline hydrogen peroxide oxidation of compound **9** with 9-BBN to give alcohol **10,** which was transformed into compound **11** by thioacetic S-acid using Mitsunobu conditions [18]. Attempted hydroboration of compound **9** using the borane–THF complex or the borane–dimethyl sulfide complex suffered from formation of various by-products.

The synthesis was continued as depicted in Scheme 4: Thioate **11**, when subjected to lithium aluminum hydride, afforded thiol **12**. Subsequent iodine oxidation of the thiol **12** achieved the disulfide **13**. The disulfide bridging provided a valuable thiol group protection with regard to the following sulfamate formation. Treatment of disulfide **13** with tetrabutylammonium fluoride (2 equiv., 30 min) chemo- and regioselectively removed the 3-TBS ether and gave phenol **14**. Sulfamoylation of the exposed phenolic hydroxy group in compound **14** with sulfamoyl chloride/2,6-di-tert-butyl 4-methylpyridine/dichloromethane [10] gave a modest yield of ester **15** which was deprotected at C-17 by acetic acid in THF/water (6 days, room temperature), thus providing compound **16**. As described before [10], this protocol, though requiring a rather long reaction time, succeeded in a clean silyl ether cleavage rigorously avoiding a concomitant hydrolysis of the phenolic sulfamate group.

Finally, title compound **17** was obtained by reductive cleavage of the disulfide bridge in compound **16**. Sodium



Scheme 4. Synthesis of title compound **17** *via* disulfide formation. Reaction conditions: (i) LAH, THF, 82%. (ii) I_2, EtOH, CH_2Cl_2, nearly quantitative yield. (iii) TBAF (1 equiv.), THF, 53%. (iv) H_2N-SO_2Cl, CH_2Cl_2, DTBP (26%). (v) AcOH, H_2O, THF (3:1:1), 58%. (vi) $NaBH_4$, $AlCl_3$, diglyme, nearly quantitative yield.



Scheme 5. Synthesis of title compound **17** *via* ethane thioate **18** and **19**. Reaction conditions: (i) TBAF (1 equiv.), THF, 62%. (ii) $H_2N\text{-}SO_2Cl$, CH_2Cl_2, DTBP, 84%. (iii) AcOH, H_2O, THF (3:1:1), 85%. (iv) $NaBH_4$, $AlCl_3$, diglyme, 48% or $BH_3.Me_2S$, THF, 56%.

borohydride which was reported to reduce disulfides to the corresponding thiols [19], failed completely. On the other hand, lithium aluminum hydride [19] and diisobutyl aluminum hydride (tetrahydrofuran, 0°C) led to disulfide and sulfamate reduction as well. Finally, we found that in the presence of aluminum chloride a diethylene glycol dimethyl ether solution of sodium borohydride [20,21] resulted in disulfide cleavage without attacking the phenolic sulfamate group of compound **16** (0°C, then 2 h at room temperature). Sodium borohydride and aluminum chloride apparently form aluminum borohydride to some extent [20,22]. However, we speculated that, additionally, borane should be a player. Thereupon, various boranes have been studied for being capable of reducing disulfide **16**. Among them, the borane–dimethyl sulfide complex (0°C, then 2 h at room temperature) was found to successfully reduce disulfide **16** as well.

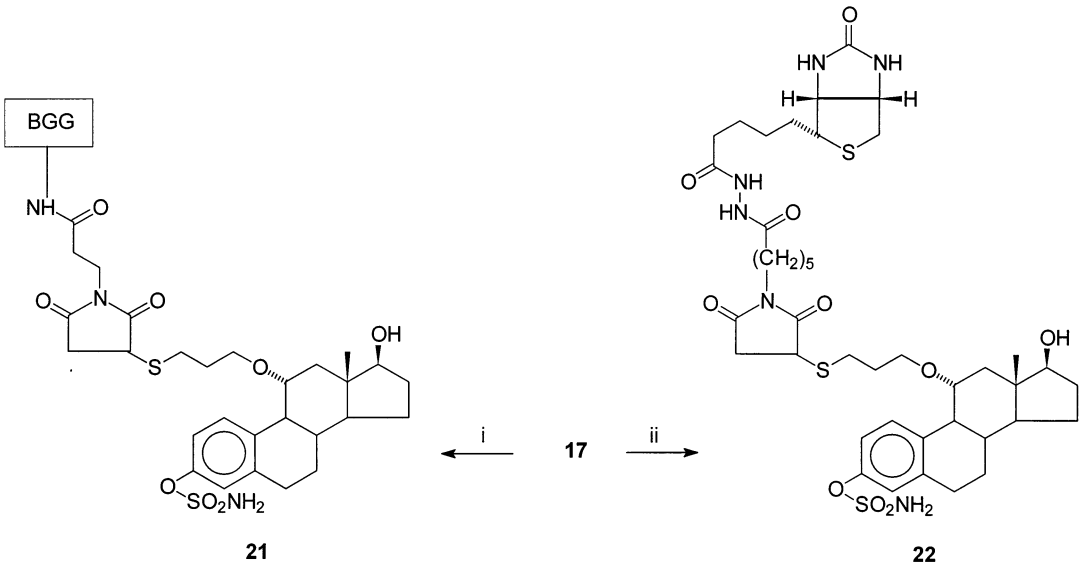
The low yield of sulfamate **15** from the phenolic disulfide **14** and the moderate purity of title compound **17** obtained from compound **15** prompted us to look for an alternative approach to hapten **17**. As outlined in Scheme 5, this was realized from thioate **11** via the intermediates **18**, **19**, and **20**. Analogously to the disulfide bridge reduction of compound **16,** the thioate moiety of compound **20** was readily removed by sodium borohydride/aluminum chloride to yield title compound **17** in very high purity as demonstrated by HPLC measurement. Borane–dimethyl sulfide reduction of thioate **20** was also effective, however, gave some less pure compound **17**.

*3.2. Synthesis of 17β-hydroxy-11α-(3′-sulfanylpropyl)oxy-estra-1,3,5 (10)-trien-3-yl sulfamate bovine γ-globulin conjugate (**21**)*

To exclude a possible coupling between the carrier protein and hapten **17** through its sulfamate moiety, 3-maleido propionic acid N-hydroxysuccinimide ester, a heterobifunctional cross-linker carrying a thiol-reactive maleimide group at the one end and an amino group-specific N-hydroxysuccinimide ester at the other, has been used as coupling reagent (see Scheme 6). In a two-step procedure the cross-linker was first reacted with the amino groups of bovine γ-globulin (which lacks any thiol groups), and second, after removal of unbound cross-linker, with the ω-thiol group of the hapten **17**.

3.3. Immunoassay

All 6 rabbits responded to the immunization against conjugate **21** with the development of antibodies against estradiol sulfamate (**1**). Using a tracer concentration of ~5 pM (1 fmol/well) it was possible to dilute the antisera



Scheme 6. Synthesis of BGG conjugate **21** and biotinylated compound **22**. Reaction conditions: (i) *a)* BGG, 3-maleido propionic acid N-hydroxy-succinimide ester, buffer, DMF *b)* **17**, dioxane *c)* dialysis. (ii) **17**, DMF, N-biotinoyl-N′-(6-maleidohexanoyl) hydrazide, 50%.

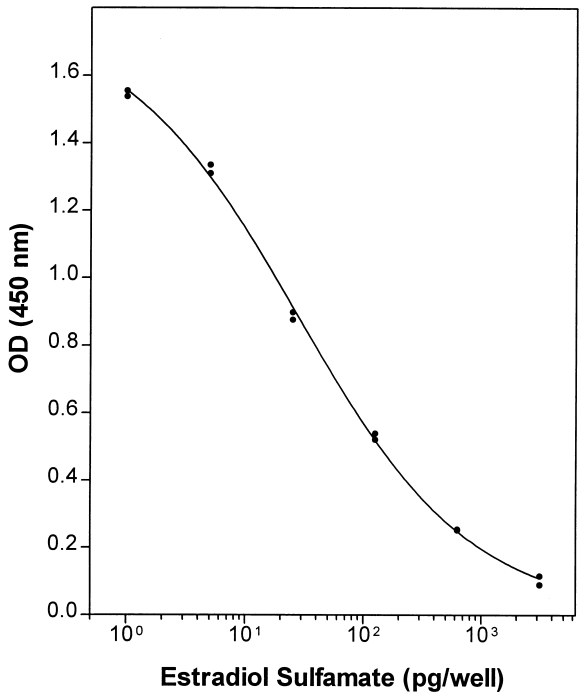


Fig. 1. Typical standard curve for estradiol sulfamate (**1**) obtained with antiserum No. 4. The assay was performed in duplicate as described under Experimental. Antiserum dilution (final): 1:320 000; Tracer concentration: ~1 fmol/well. Non-Specific Binding (NSB): OD = 0.068. Zero standard binding (Bo): OD = 1.748.

between 1:140 000 and 1:1 000 000 (final dilutions) to produce an optical density (OD) of about 1.5.

The most sensitive standard curve was obtained with antiserum No. 4. As illustrated in Fig. 1, the curve covers a concentration range from about 1 to 1000 pg/well (ED_{50} = 27 pg/well), which appears to satisfy future demands with respect to sensitivity.

Concerning the specificity of antiserum No. 4, the cross-reactions of various sulfamoylated and non-sulfamoylated estrogens listed in Table 1 indicate that this antiserum is very specific to the intact sulfamate moiety, and does not tolerate any changes at the C-3 position. On the other hand, the specificity to the D-ring appears to be low, since the sulfamates of estrone, estriol and 17α-estradiol show high cross-reactions (100%, 33%, and 100%, respectively). Another antiserum (No. 5) showed a higher specificity for the D-ring with cross-reactions of 10%, 1.5%, and 10%, respectively; however, its sensitivity was about five times lower in comparison to antiserum No. 4, which makes it unsuitable for future measurements of low plasma levels.

The low specificity at the D-ring appears not to be a great disadvantage, since only estrone sulfamate will be formed from estradiol sulfamate in substantial amounts in the blood circulation. Of course, it is desirable to differentiate between these two estrogen sulfamates, and therefore we have started to establish a simple routine derivatization step, which selectively modifies estrone sulfamate at the 17-keto group, thereby decreasing drastically its cross-reactivity.

Table 1
Cross reactivities of antiserum No. 4

Compound	Cross-reaction (%)
Estradiol sulfamate	100.0
Estrone sulfamate	100.0
17α-Estradiol sulfamate	100.0
Estriol sulfamate	33.3
Estriol 3-(N-acetyl)sulfamate	1.7
Estra-1,3,5(10)-triene-3,17β-diyl 17-Pentanoate, 3-sulfamate	1.0
Estrone (N-acetyl)sulfamate	0.1
Estradiol 3-(N-acetyl)sulfamate	0.1
Estradiol 3-pentanoate	0.01
Estradiol	0.01
17α-Estradiol	<0.005
Ethinyl-estradiol	<0.005
Estradiol-3-sulfate	<0.005
Estradiol-3-glucuronide	<0.005

Preliminary results have shown that estrone sulfamate is easily converted into the 2,4-dinitrophenylhydrazone derivative according to Knapp [23], and that this modification decreases its cross-reaction to a value below 0.2%. Under the reaction conditions used, estradiol sulfamate (**1**) apparently remains unchanged as indicated by the retention of its immunoreactivity (data not shown).

In a previous work by Webb et al. [24], a specific radioimmunoassay for estradiol was developed showing very little cross-reaction with estrone and estriol. For the preparation of the immunogen the authors coupled 11β-hemisuccinyl-estradiol to BSA, and as tracer they used estradiol-11α-succinyl-tyrosinemethylester-^{125}I. The high specificity of their antiserum for the D-ring suggests that the 11β-configuration of the hapten in the immunogen might be advantageous over the 11α-configuration, perhaps, by leaving the D-ring of the steroid molecule more unmasked.

Although our estradiol sulfamate assay shows a very low detection limit of ~1 pg/well, the large assay range up to 1000 pg/well is disadvantageous in terms of precision. The large assay range is presumably due to the homologous design of the assay, in which the immunogen and the biotinylated tracer possess the same steroid structure and bridge configuration (11α). As a consequence, the tracer is more tightly bound by the polyclonal antibodies than the ligand to be measured. In this case relatively large amounts of ligand would be required to displace the tracer from the antibody binding sites, resulting in flat standard curves. Webb et al. [24] circumvented this problem of 'bridge recognition' by using the 11β-configuration for the preparation of the immunogen and the 11α-configuration for the synthesis of the tracer. The advantage of this heterologous assay design became significant in a steeper slope of the standard curve and an increased sensitivity in comparison to the homologous system (both configurations 11β).

The disadvantage of homologous hapten immunoassays with respect to sensitivity is also well recognized in other competitive immunoassay systems. In this context, the kind

of labeling (e.g. ^{125}I-iodotyrosine, biotin, enzyme) appears to play a minor role. There are in general two ways to circumvent this problem:

i) The ligand to be measured is modified by a chemical derivatization step prior to assay in a way that makes it more similar to the hapten structure, as it is shown for the cAMP and cGMP RIAs (Harper and Brooker [25]) or for the serotonin RIA (Gow et al. [26]). Such an assay design, however, cannot be adapted to the assay of estradiol sulfamate described here, since the latter is not easily derivatized at 11α-position.

ii) A tracer molecule with a heterologous structure in comparison to the hapten structure in the immunogen is used, which still binds to the antibody, but is more easily displaced by the ligand thereby increasing the sensitivity. Besides Webb et al. [24], numerous other authors have followed such assay designs, some of which being exemplified below. Franec et al. [27] drastically increased the sensitivity of an estradiol RIA by using an anti-estradiol-2 (4)-azo-serum in combination with estradiol-6-O-(CMO)-[^{125}I]iodohistamine as tracer. Tiefenauer et al. [28] used antibodies against estradiol-6-CMO-albumin in combination with a tracer, in which 6α-aminoestradiol was labeled with [^{125}I]iodophenyl propionic acid N-hydroxysuccinimide ester. Meyer et al. [7] developed an ELISA using an antiserum directed against estradiol-17β-hemisuccinate-albumin, and a tracer in which a biotin derivative was coupled to estradiol-17β-D-glucuronic acid. Allen and Redshaw [29] compared eight homologous and heterologous ^{125}I-tracers in a progesterone RIA using an antiserum raised to progesterone-11α-hemisuccinate-albumin. They found that all tracers with the 11α-hemisuccinate structure or similar structures produced very unsensitive standard curves compared to the tritiated tracer (reference tracer), whereas 3- or 12-(O-carboxymethyl)oxime tracer derivatives resulted in standard curves equal to, or even more sensitive than, that obtained with the tritiated tracer.

At present, our efforts are ongoing to synthesize a suitable heterologous tracer derivative which is more easily displaced by the unlabeled ligand than the one described here.

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