New steroid haptens for radioimmunoassay: synthesis of steroids substituted with thioether or ester linkages at the 2α-position

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Haptens with bridge at the 2-position have not yet been explored. Radioimmunoassays with antibodies directed against 2α-alkyl bridged steroid haptens are expected to be highly specific due to greater topographical exposure and similarity in conformation to the native steroid. The 2α-alkyl bridged haptens were synthesized by first adding a cyclopropane ring to 2-methylene-4-en-3-one. Selective opening of the three-membered ring with trimethyl silyl iodide and transformation of the iodo group gave a carbocyclic acid, the desired analog for conjugation with protein. (*Steroids* **56**:185–188, 1991)

Keywords: steroids; synthesis; haptens; 7-(3-oxocholest-4-en-2α-yl)-5-oxa-4-oxoheptanoic acid; 6-(3-oxocholest-4-en-2α-yl)-4-thiahexanoate acid; spiro[4-cholestene-2,1′-cyclopropane]-3-one, radioimmunoassay; 2α-(2′-hydroxyethyl)cholest-4-en-3-one; 2β-(2′-iodoethyl)-cholest-4-en-3-one; methyl 6-(3-oxocholest-4-en-2α-yl)-4-thiahexanoate

Introduction

Since Erlanger et al.[1] successfully produced antisteroid antiserum with steroid-protein conjugates, many radioimmunoassays for steroid hormones have been reported with advances in the areas of specificity, selectivity, and sensitivity. Midgley and Niswender[2] pointed out that specificity of an antibody is increased when the "bridge" between steroid and protein antigen does not utilize the functional groups in the native steroid. Although quantitation of binding has not been carried out with regard to number of intervening "spacer" atoms, it is generally believed that as the number of spacer atoms increases, the specificity of the corresponding antibody is enhanced due to increase in "topographical exposure."[3] The concept of bridging to proteins from the α- or β-face of steroids has also been explored.[4,5] Rao and Moore[5] have reported a highly specific antiserum from steroid-protein conjugates that utilizes the 15β-position of testosterone. Indeed, the 15-position appears to be better than other positions for a variety of conjugates of testosterone. Accordingly, it was reasoned that the 2-position offers advantages similar to the 15-position. However, the 2β-linkage is expected to distort the "normal" A ring conformation due to 1,3-diaxial interaction with the C-10 methyl group, producing an antibody with lower specificity. An alkyl substituent with a 2α-configuration is expected to retain the native conformation,[6] and introducing additional methylenes would increase the specificity. This report describes methods for synthesis of thioether or ester "bridged" hapten on a model steroid.

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Experimental

Melting points (mp) are reported uncorrected. Nuclear magnetic resonance (NMR) spectra were recorded with Varian VXR300S or Bruker AM500 spectrometers with trimethylsilyl (TMS) as internal standard at ambient temperature. Infrared (IR) spectra were obtained in a Perkin-Elmer 688 spectrometer, mass spectra (MS) were recorded in a Shimadzu QP1000 spectrometer, and ultraviolet (UV) spectra were recorded with a Shimadzu UV-VIS spectrophotometer. Elemental analyses were carried out on a CEST MOD.110 analyzer. All laboratory solvents were dried prior to their use. Sodium iodide was oven dried at 150 C for 5 hours and vacuum dried at 110 C for 2 hours before use. TMSCl was purchased from Fluka AG and used as such.

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2-(Ethoxycarbonyl hydroxymethylene)cholest-4-en-3-one **(2)**

Sodium hydride (55% in petroleum wax, 657 mg, 12.53 mmol) was freed of petroleum wax with hexane under nitrogen. Cholestenone (960 mg, 2.48 mmol) in benzene (10 ml) was added to the NaH dropwise. After 15 minutes, diethyl oxalate (666 mg, 4.97 mmol) in benzene (2 ml) was added all at once. After stirring for 5 hours, the solution was acidified and extracted with ether. Evaporation of the solvents followed by column chromatography yielded a yellow oil (939 mg, 78%): IR (neat) $\tilde{\nu}$, 1,730 cm^{-1} (COOEt), 1,630 (C=O), 1,590 (C=C); ^1H NMR ($CDCl_3$) δ, 15.06 (broad, enolic OH), 5.87 (d, 1H, J = 1.2 Hz, 4-H), 4.35 (q, 2H, J = 7.14 Hz, OCH_2CH_3), 3.38 (d, 1H, J = 15.38 Hz, 1β-H), 2.29 (d, 1H, J = 15.38 Hz, 1α-H), 1.39 (t, 3H, J = 7.14 Hz, OCH_2CH_3), 1.06 (s, 3H, 19-H_3), 0.91 (d, 3H, J = 6.41 Hz, 21-H_3), 0.87 (d, 3H, J = 6.59 Hz, 26-H_3), 0.86 (d, 3H, J = 6.59 Hz, 27-H_3), 0.70 (s, 3H, 18-H_3).

2-Methylenecholest-4-en-3-one **(3)**

To a stirred solution of the steroid **2** (50.5 mg, 0.1 mmol) in MeOH (14 ml) and benzene (1 ml), 40% aqueous formaldehyde solution in water (400 μl) was added dropwise during 15 minutes. After 30 minutes, a solution of K_2CO_3 (40.5 mg) in water (620 μl) was added slowly, then stirring was continued for 90 minutes. The precipitated product was collected by filtration, then crystallized from petroleum ether (boiling point [bp], 60 to 80 C)/ethyl acetate. The filtrate was extracted with four portions of CH_2Cl_2 (20 ml). After evaporation of the CH_2Cl_2 under reduced pressure, chromatography provided a combined yield of 35 mg (87.5%) of the pure product: MS, 396 (M^+); IR (KBr) $\tilde{\nu}$, 3,100 cm^{-1} (OH), 1,680 (C=O), 1,620 (C=C), 935, 885; UV (MeOH) λ_{max} ($\log_e$), 257.8 nm (4.301); ^1H NMR ($CDCl_3$) δ, 5.94 (t, 1H, J = 2.19 Hz, 2'-HC=), 5.83 (s, 1H, 4-H), 5.22 (t, 1H, J = 0.8 Hz, 2'-HC=), 2.69 (d, 1H, J = 14.07 Hz, 1β-H), 1.09 (s, 3H, 19-H_3), 0.92 (d, 3H, J = 6.59 Hz, 21-H_3), 0.87 (d, 3H, J = 6.59 Hz, 26-H_3), 0.86 (d, 3H, J = 6.59 Hz, 27-H_3), 0.71 (s, 3H, 18-H_3). Analysis calculated: C, 84.78; H, 11.18. Found: C, 84.54; H, 11.20.

Spiro[2,1'-cyclopropane]cholest-4-en-3-one **(4)**

Sodium hydride (55% in petroleum wax, 68.4 mg, 1.57 mmol) was freed of petroleum wax with hexane under nitrogen. Addition of trimethyl sulfoxonium iodide (243.9 mg, 1.2 mmol) in dimethyl sulfoxide (DMSO) (4 ml) gave an effervescent mixture. After stirring the mixture for 45 minutes, a solution of **3** (475.2 mg, 1.2 mmol) in DMSO and tetrahydrofuran (THF) (1 ml) was added dropwise, and the resulting mixture was allowed to stir for 5 hours. Work-up of the reaction mixture with CH_2Cl_2 gave, after recrystallization from petroleum ether (bp, 60 to 80 C)/ethyl acetate, a colorless solid (254 mg, 52%): mp, 80 C; MS, 410 (M^+); IR (KBr) $\tilde{\nu}$, 1,670 cm^{-1} (C=O), 1,620 (C=C); UV (MeOH) λ_{max} ($\log_e$), 241.8 nm (4.239); ^1H NMR ($CDCl_3$) δ, 5.79 (d, 1H, J = 1.59 Hz, 4-H), 2.27 (d, 1H, J = 13.92 Hz, 1α-H), 1.22 (s, 3H, 19-H_3), 0.91 (d, 3H, J = 6.4 Hz, 21-H_3), 0.87 (d, 3H, J = 6.59 Hz, 26-H_3), 0.86 (d, J = 6.59 Hz, 27-H_3), 0.79 to 0.71 (m, 1H, cyclopropyl), 0.70 (s, 3H, 18-H_3), 0.51 to 0.44 (m, 1H, cyclopropyl). Analysis calculated: C, 84.81; H, 11.29. Found: C, 85.22; H, 11.47.

2β-(2'-Iodoethyl)cholest-4-en-3-one **(5)**

Compound **4** (50 mg, 0.122 mmol) was dissolved in CH_3CN (5 ml) under nitrogen, and solid NaI (37 mg, 0.24 mmol) was added. Freshly distilled TMSCl (30 μl, 0.24 mmol) was added dropwise, resulting in an immediate precipitation of product. After stirring the reaction mixture in the dark for 2 hours, the precipitated product was filtered and sequentially washed with distilled water, 10% sodium thiosulfate, and distilled water, then vacuum dried in the dark to yield 50 mg of a colorless solid (76%). The combined aqueous solutions were further extracted to yield an additional 5 mg of product: mp, 102 C (dec.); MS, 538 (M^+); IR (KBr) $\tilde{\nu}$, 1,660 cm^{-1} (C=O), 1,620 (C=C); UV ($CHCl_3$) λ_{max} ($\log_e$), 241.8 nm (4.012); ^1H NMR ($CDCl_3$) δ, 5.74 (s, 1H, 4-H), 3.44 (m, 1H, CH_2I), 3.37 (m, 1H, CH_2I), 1.29 (s, 3H, 19-H_3), 0.95 (d, 3H, J = 6.4 Hz, 21-H_3), 0.92 (d, 6H, J = 6.30 Hz, 26,27-H_3), 0.76 (s, 3H, 18-H_3).

Methyl 6-(3-oxocholest-4-en-2α-yl)-4-thiahexanoate **(8)**

The 2β-iodoethyl steroid **5** (20 mg, 0.037 mmol) in DMSO (1 ml) was added to a solution of methyl thiopropionate (8.8 mg, 0.074 mmol) in DMSO (0.5 ml). The resulting mixture was slowly added with stirring to a chilled solution of n-BuLi in hexane (32 μl, 15%, 0.074 mmol) in DMSO (0.5 ml) at 5 C under nitrogen. After stirring the reaction mixture for 0.5 hours at 5 C, the work-up was performed by pouring the mixture into water and extracting with four portions of ether (15 ml). Chromatography on silica gel yielded a yellow oil (7.1 mg, 42%) based on recovery of starting material: MS, 530 (M^+); IR (KBr) $\tilde{\nu}$, 1,738.6 cm^{-1} (COOMe), 1,666.4 (C=O), 1,620.0 (C=C); UV (MeOH) λ_{max} ($\log_e$), 246.0 nm (4.235); ^1H NMR ($CDCl_3$) δ, 5.69 (s, 1H, 4-H), 3.70 (s, 3H, OCH_3), 2.80 (t, 2H, J = 7.0 Hz, $O{=}CCH_2CH_2$), 2.61 to 2.67 (m, 2H, CH_2S), 1.21 (s, 3H, 19-H_3), 0.91 (d, 3H, J = 6.4 Hz, 21-H_3), 0.86 (d, 3H, J = 6.56 Hz, 26-H_3), 0.87 (d, 3H, J = 6.56 Hz, 27-H_3), 0.71 (s, 3H, 18-H_3).

6-(3-Oxocholest-4-en-2α-yl)-4-thiahexanoic acid **(9)**

Sodium ethoxide (15.64 mg, 0.23 mmol) in ethanol (5 ml) and thiopropionic acid (20.4 mg, 0.19 mmol) were heated under reflux for 30 minutes under nitrogen. A solution of the 2β-iodoethyl steroid **5** (51.8 mg, 0.096 mmol) in EtOH (2 ml) was added dropwise to the thiol solution. After reflux for 3 hours, the solution was neutralized with 10% HCl and then reduced to half of the original volume under reduced pressure. Extraction

of the residual mixture with four portions of $CHCl_3$ (25 ml), evaporation, then concentration of the extract followed by chromatography of the crude product on silica gel yielded a colorless solid (31 mg, 70%). The compound was recrystallized from petroleum ether (bp, 60 to 80 C)/ethyl acetate for analytic purposes: mp, 119 C (dec.); MS, 516 (M^+); IR (KBr) $\bar{\nu}$, 3,200 cm^{-1} (OH), 1,741.6 (COOH), 1,648.4 (C=O), 1,612.1 (C=C); UV ($CHCl_3$) λ_{max} ($\log_e$), 244.0 nm (4.274); ^1H NMR ($CDCl_3$) δ, 5.70 (d, 1H, J = 1.28 Hz, 4-H), 2.81 (t, 2H, J = 6.96 Hz, O=C$CH_2$$CH_2$), 2.67 (m, 4H, CH_2S), 1.21 (s, 3H, 19-H_3), 0.91 (d, 3H, J = 6.29 Hz, 21-H_3), 0.87 (d, 3H, J = 6.59 Hz, 27-H_3), 0.86 (d, 3H, J = 6.59 Hz, 26-H_3), 0.71 (s, 3H, 18-H_3). Analysis calculated: C, 74.37; H, 10.14. Found: C, 74.14; H, 10.02.

2α-(2'-Hydroxyethyl)cholest-4-en-3-one ***(10)***

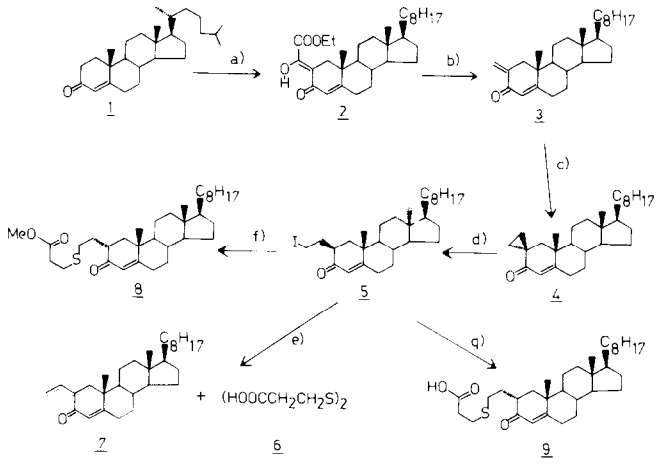
A mixture of the 2β-iodoethyl steroid **5** (63 mg, 0.012 mmol) and NaOH (17 mg, 0.047 mmol) in THF (10 ml) and H_2O (1 ml) was stirred at room temperature under nitrogen for 9 hours. Tetrahydrofuran was evaporated with a stream of nitrogen, then the resulting mixture was extracted with three portions of CH_2Cl_2 (25 ml), followed by the usual work-up and chromatography to yield a colorless solid (45 mg, 88%). The compound was crystallized from petroleum ether (bp, 60 to 80 C)/ethyl acetate mixture for analytic purposes: mp, 89 C; MS, 428 (M^+); IR (KBr) $\bar{\nu}$, 3,600 to 3,200 cm^{-1} (broad, OH), 1,665 (C=O), 1,620 (C=C); UV ($CHCl_3$) λ_{max} ($\log_e$), 246.8 nm (4.097); ^1H NMR ($CDCl_3$) δ, 5.74 (s, 1H, 4-H), 3.83 to 3.79 (m, 1H, OCH_2), 3.69 to 3.64 (m, 1H, OCH_2), 2.53 to 2.50 (broad, D_2O exchangeable, OH), 1.22 (s, 3H, 19-H_3), 0.91 (d, 3H, J = 6.7 Hz, 21-H_3), 0.85 (d, 6H, J = 6.4 Hz, 26,27-H_3), 0.71 (s, 3H, 18-H_3). Analysis calculated: C, 78.45; H, 11.28. Found: C, 79.01; H, 11.15.

7-(3-Oxocholest-4-en-2α-yl)-5-oxa-4-oxoheptanoic acid ***(11)***

A mixture of **10** (30 mg, 0.07 mmol), imidazole (9 mg, 0.13 mmol), and succinic anhydride (30 mg, 0.3 mmol) in pyridine (3 ml) was heated under reflux under nitrogen for 5 hours. Pyridine was removed with a stream of nitrogen, and the residue was taken up in $CHCl_3$. The usual work-up followed by chromatography yielded a colorless oil (16 mg, 43%): MS, 528 (M^+); IR ($CHCl_3$) $\bar{\nu}$, 2,600 to 2,653 cm^{-1} (OH), 1,737.5 (COOH), 1,674.8 (C=O), 1,620.2 (C=C); UV ($CHCl_3$) λ_{max} ($\log_e$), 240 nm (4.003); ^1H NMR ($CDCl_3$) δ, 5.71 (d, 1H, J = 1.46 Hz, 4-H), 4.20 to 4.25 (m, 2H, OCH_2), 2.59 to 2.69 (m, 4H, OC$CH_2$$CH_2$CO), 1.20 (s, 3H, 19-$H_3$), 0.91 (d, 3H, J = 5.93 Hz, 21-H_3), 0.86 (d, 6H, J = 6.78 Hz, 26,27-H_3), 0.71 (s, 3H, 18-H_3).

Results and Discussion

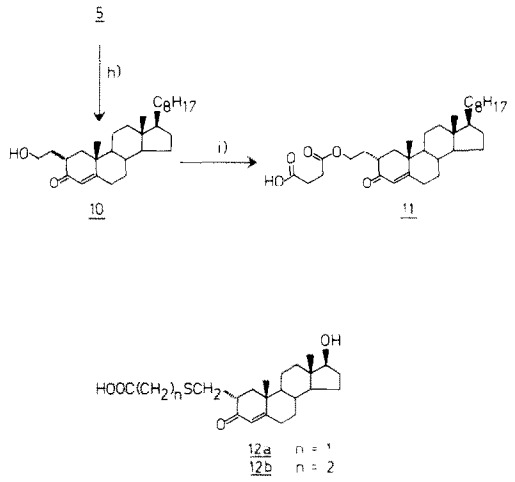
Cholest-4-en-3-one **(1)** was converted to 2-methylene 4-en-3-one substrate **3** in a yield of 68% (Scheme 1) by modifying the method of Evans et al.[7] The cyclopropane ring in **4** was produced in 59% yield with dimethyl sulfoxonium methylide at room temperature. Although Danishefsky[8] reports the nucleophilic opening of bisactivated cyclopropanes in good yields, the same is not true for monoactivated cyclopropanes. Sulfur and selenium nucleophiles[9] also do not open monoactivated cyclopropane rings in good yields.



Scheme 1 (a) $(COOEt)_2$/NaH/PhH/RT, (b) HCHO/K_2CO_3/MeOH/RT, (c) $CH_2SO(CH_3)_2$/DMSO/RT, (d) TMSCl/NaI/CH_3CN/RT, (e) $HSCH_2CH_2COOH$/base/solvent, (f) $MeOOCCH_2CH_2SH$/n-BuLi/DMSO/5 C, (g) $HSCH_2CH_2COOH$/EtONa/EtOH/reflux.

Mioskowski et al.[10] have used the alkyl cuprates complexed with CuCN to effect monoactivated cyclopropane cleavage. Thiolate anions from thiopropionic acid failed to open the cyclopropane ring in spirocyclopropane **4**, even when the strong Lewis acids $BF_3 \cdot Et_2O$ or $AlCl_3$ were used. Free radical ring opening with benzoyl peroxide did not yield any fission products, contrary to previous reports.[11] Trimethyl silyl iodide generated in situ[12] opened the cyclopropyl ring in **4** to give the desired product in high yield.

The ^1H NMR spectrum indicated formation of a single stereoisomer with the 10-methyl signal observed at 1.29 δ. It is also known that 6β-substituents deshield the 10-methyl relative to 6α-substituents; this has been the basis of assignment of configuration at the 6-position. Although this cannot be used in strict sense for the substitution at the 2-position, we have assigned the 2β-iodoethyl substitution on the basis of 10-methyl occurring at 1.29 δ and 2α-substitution on the basis of 10-methyl occurring at 1.20 to 12.1 δ. Two 1H multiplets at 3.44 δ and 3.37 δ could be assigned to two protons α to the iodo group. Thus, the product was inferred to be 2β-(2'-iodoethyl)cholest-4-en-3-one **(5)**. On repeated trials of displacement of the iodo group with thiolate anions from thiopropionic acid, a variety of combinations of bases NaH and n-BuLi and solvents THF, DMSO, and dioxane did not produce the desired product. Instead, the dimer **6** and a mixture of dehydroiodinated compound **7** were obtained as products. Although the mechanism of thiolate displacement of



Scheme 2 (h) TMSCl/NaI/CH_3CN/RT/2 hours, then KOH/THF/H_2O; (i) $(CH_2CO)_2O$/pyridine/imidazole/reflux.

halides postulated by Bunnett[13] proceeds through a radical anion species, displacement is known to occur. Therefore, it was surprising that oxidative dimerization took place. Displacement of the iodo group with thiolate anion from methyl thiopropionate proceeded smoothly to yield 42% of **8**. The 10-methyl signal in the ^{1}H NMR spectrum occurs at 1.21 δ, suggesting a 2α-configuration. Thus, the reaction produces epimerization at the 2-position. Such epimerizations are well documented for 2β-methyl substituted 4-en-3-one steroids[14] in basic medium, and it is quite plausible for the more stable 2α-isomer to be formed in this case. Although the hydrolysis of esters of these types has been reported,[5,15] the overall yield for the synthesis of hapten would be less heartening. Hence, the displacement of the iodo group in **5** with thiolate anions was tried in ethanol/sodium ethoxide medium. The resulting product exhibits IR bands at 1,741.1 cm^{-1} for the carbonyl group of the carboxyclic acid and 1,643.5 and 1,615.2 cm^{-1} for the carbonyl and double bond of α,β-unsaturated ketone moieties. The ^{1}H NMR spectrum indicates a two-proton triplet at 2.81 δ, a four-proton multiplet at 2.67 δ, and a 10-methyl signal at 1.21 δ that imply an α-orientation at the 2-position. The hapten represented as **9** with six spacer atoms was obtained in an overall yield of 22% from cholestenone.

The iodide **5** was treated with KOH in THF to give good yield of **10** (Scheme 2) with a 2α-configuration. The hydroxy group was then esterified with succinic anhydride,[2,3] giving the hemisuccinate **11** in excellent yields. Thus, the ester "bridged" hapten **11** with seven spacer atoms was synthesized in an overall yield of 39% from 2β-iodoethyl steroid **5**.

A study of the binding characteristics of the antibodies made from steroids described here will be reported elsewhere. However, preliminary results for testosterone show that the cross-reactivity of the hapten with four spacer atoms is more than that of the hapten with five spacer atoms. The antisera generated from steroid-protein conjugates of haptens[16] **12a** and **12b** showed 41.6% and 29.4% cross-reactivity with 5α-dihydrotestosterone (at 50% concentration of 5α-dihydrotestosterone), indicating the applicability of the above concept. The cross-reactivity of antisera generated from the 3-(O-carboxymethyl oxime) hapten of testosterone with 5α-dihydrotestosterone was 41.0%.

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