

**NEW STEROID HAPTENS FOR RADIOIMMUNOASSAY PART II.
SYNTHESIS OF THIOETHER BASED HAPTENS FOR TESTOSTERONE.[#]**

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ABSTRACT : Steroid-haptens with thioether bridge at 2-position have been synthesized using the Michael addition approach as well as cyclopropanation and stereospecific opening of the three membered ring. The methodology allows the bridge length to be varied between 3 to 5 spacer atoms.

The utility of Radioimmunoassay as a technique for the quantitation of steroid hormones lies in the development of monoclonal antibodies vis a vis highly specific haptens. Numerous haptens have yielded considerable information as to the constitution of a specific hapten. Midgley et al.¹ pointed out that the bridge which utilizes a position remote from the native functional groups on the steroid provides

[#] For Part I of the series see reference 5.

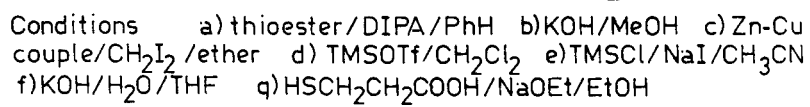
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better specificity. It is generally felt that more the number of spacer atoms, better the specificity². The importance of stereoconfiguration of the bridge has also been highlighted^{3,4}. Literature survey reveals that the potential offered by position-2 on steroid framework remains unexplored. Our earlier forays into the synthesis of steroid haptens at 2-position⁵ reveals a methodology to generate stereospecific haptens on a model steroid. We present here the synthesis of thioether based haptens at 2-position for testosterone. The overall strategy can be used to an advantage due to its manoevrability for the synthesis of haptens with different spacer atoms. Bridges with 3-5 spacer atoms have been synthesized. The methodology could be used for the synthesis of two types of bridges - the thioether and ester bridges.

Testosterone **1** is transformed into 2-Methylene testosterone by the procedure established by Evans et al.⁶ in an overall yield of 82%. On Michael addition of thiopropionic acid, the compound isolated could be analysed for the required Michael adduct **5b**; however, the method required very long reaction time as well as elaborate chromatography for separation from starting thiopropionic acid resulting in lowered yields. Hence Michael addition with methyl ester of thioacid was performed. Methyl thioglycolate yielded a compound homogeneous on tlc and showing bands at 1730, 1670 and

1620 cm^{-1} indicative of addition at 2-position. However the ^1H NMR indicated two signals of the 4-H olefinic proton as doublets at 5.73 and 5.75 δ indicative of both, the α - and β -isomers at 2-position. Although two signals arising from the 19- H_3 protons were clearly observed, the assignment and isomeric distribution was difficult. The ^{13}C NMR spectrum shows two signals for each carbons at 1, 2, 4 and 5 positions. Based upon the resonances of these carbons for major and minor isomers, a configuration of α - at 2-position was assigned to major isomer. The isomeric ratio was found to be 3:1. When the ester **5a** was hydrolyzed the expected butanoic acid **5b** was obtained in overall yield of 37% from **1**. Exposure to alkali yields the α -isomer only. The α -isomer would be expected to retain the conformation of native testosterone and hence stereo-characters of native conformation would be expected to be expressed in the antibodies. Similar treatment yields the pentanoate ester **4a** with Methyl thiopropionate in the isomeric distribution of 70:30 which is hydrolyzed to **4b**. Thus the method yields two steroid-haptens with bridge length of 3 and 4 spacer atoms. The RIA features will be discussed elsewhere.

In order to increase the chain length, the method of cyclopropanation of 2-Methylene testosterone followed by cleavage and transformation to hapten, as adopted in our



earlier approach⁵ for a model steroid, was taken up. Corey's reagent was found to provide a yield of 52%. A simple technique of Simmons Smith reaction with 1:1 equivalent of methylene iodide and substrated 2, as developed in our laboratory⁷, was found to provide much improved yields. Since the hydroxy group is known to be transformed into the iodo group with TMSI⁸, the direct use of TMSI, was not advisable. Trimethyl silyl trifluoroacetate is known to be able to cleave fused activated cyclopropanes⁹; however on trying the cleavage of cyclopropane 3 with TMSOTf at elevated temperature, the product obtained was found to be the trifluoroacetate 6 instead of the opened up cyclopropane. This incidentally forms a very high yielding synthesis of trifluoroacetates in near neutral medium. A trial experiment with TMSI on cyclopropane 3 with 1:1 equivalent of NaI followed by displacement with hydroxide was found to yield the 2 α -(2'-Hydroxyethyl) derivative 8 in good yields. This indicated the higher reactivity of cyclopropane towards TMSI in preference to the hydroxy group. Hence in accordance with our earlier observation of instability of iodo intermediate 7 the cyclopropane 3 was transformed by cleavage with TMSI and displacement of the iodo group in ethoxide-ethanol medium into the hexanoate 9. The ¹H NMR of the product indicated the compound to be a mixture of α - and β -isomers in the ratio of 1:1. The reason for the formation of both isomers,

in contrast to uniform α -isomer observed in our earlier work⁵, could be the room temperature conditions coupled with reduced reaction time. The isomeric mixture could be easily epimerised to more stable α -isomer. Thus steroid hapten with chain length of 5 spacer atoms was synthesized in an overall yield of 42%.

As a part of expanding the applicability of the methodology, the 2-Methylene testosterone **2** was converted into **10** with PCC. Cyclopropanation with Simmons Smith method followed by opening of cyclopropane with TMSI and displacement with hydroxide yielded the 2α -(2'-Hydroxyethyl) derivative **12**. This could easily lead to the synthesis of hemisuccinate steroid haptens⁵ for the steroids like androstenedione, 17-ethynyl testosterone etc.

EXPERIMENTAL

All melting points are uncorrected. Laboratory solvents were predried before use according to standard procedures. TMSCl , TMSOTf and thioacids were purchased from FLUKA AG. and used as such. NaI was dried at 140°C for two hours under a vacuum of 5 mm of Hg. Testosterone was purchased from Sigma. IR spectra were recorded on Perkin Elmer 688 spectrometer. UV spectra were obtained on Shimadzu UV-VIS spectrophotometer. ^1H NMR spectra were recorded on Bruker

AM500 or Varian VXR300S spectrometers. Mass spectra were obtained on Shimadzu QP1000 spectrometer. Elemental Analyses were performed on CEST MOD.110 analyser.

2-Methylene-testosterone 2

Prepared according to the procedure of D.D. Evans et al.⁶ in an overall yield of 82%.

IR (KBr) $\bar{\nu}$ = 3320 cm^{-1} (OH), 1665 (C=O), 1620 (C=C), 945, 890 ; UV (CHCl_3) λ_{max} (log ϵ) = 258.6 nm (4.195); ^1H NMR (CDCl_3) δ (ppm) = 5.95 (t, J = 2.01 Hz, 1H, 2'-C=CH), 5.84 (t, J = 0.32 Hz, 1H, 4-H), 5.23 (dd, J = 2.01, 2.38 Hz, 1H, 2'-C=CH), 3.66 (t, J = 8.24 Hz, 1H, 17-H), 2.71 (d, J = 14.10 Hz, 1H, 1-H), 1.12 (s, 3H, 19-H₃), 0.80 (s, 3H, 18-H₃); Mol. mass = 300 ;

Elemental Analyses Calculated C 79.95 H 9.39

Found C 78.96 H 9.61

Methyl-4-(2'-testosterone)yl-3-thia-butanoate 4a

A mixture of 2-Methylene testosterone (209 mg, 0.66 mmol), Methyl thioglycolate (70 mg, 0.66 mmol) and few drops of diisopropyl amine in dry benzene (10 mL) is stirred under nitrogen until tlc indicates disappearance of all starting material (4-5 days). Benzene is vacuum evaporated and mixture adsorbed on silica for chromatography. Elution with

ethyl acetate - petroleum ether (bp 60 - 80°C) mixture yields a white crystalline solid (201 mg, 75%).

mp = 102°C ; IR (KBr) $\bar{\nu}$ = 3600-3350 cm^{-1} (OH), 1730 (COOR), 1670 (C=O), 1620 (C=C), 1250, 1070 ; UV(CHCl_3) λ_{max} = 244.4nm; ^1H NMR (CDCl_3) δ = 5.73 (d, J = 1.28 Hz, 1H, 4-H), 3.74 (s, 3H, OCH_3), 3.65 (t, J = 8.24 Hz, 1H, 17-H), 3.29 (s, 2H, $\text{O}=\text{CCH}_2\text{S}$), 3.22-3.29 (m, 1H, SCH_2), 1.23 (s, 3H, 19- H_3), 0.80 (s, 3H, 18- H_3); ^{13}C NMR (CDCl_3) major isomer (α) ppm = 41.44 (C1), 42.46 (C2), 198.69 (C3), 123.30 (C4), 170.52 (C5), 39.35 (C10), 17.59 (C19), minor isomer (β) ppm = 37.29 (C1), 42.06 (C2), 198.69 (C3), 121.76 (C4), 172.31 (C5), 39.35 (C10), 17.59 (C19) ; Mol. mass = 407; Elemental Analyses

| | | | | |
|------------|---|-------|---|------|
| Calculated | C | 68.53 | H | 8.62 |
| Found | C | 67.61 | H | 8.49 |

3-Thia-4-(2'-testosterone)yl-butanoic acid 4b

The ester **4a** (54 mg, 0.133 mmol) is dissolved in MeOH (2 mL) at ice-water temperature and KOH (39 mg, 0.66 mmol) in MeOH (2 mL) is added dropwise over 15 minutes. The reaction mixture is stirred at RT for 4 hrs. and extracted with ethyl acetate. The alkaline aqueous extract is acidified and then repeatedly extracted with ethyl acetate (4 * 20 mL). On concentration under reduced pressure and column chromatography an oil is obtained (30 mg, 60%).

UV (CHCl_3) $\lambda_{\text{max}} = 245.2 \text{ nm}$; IR (CHCl_3) $\bar{\nu} = 2500\text{--}2700 \text{ cm}^{-1}$ (broad, OH), 1717.5 (COOH), 1664.0 (C=O), 1616.4 (C=C) ; ^1H NMR (CHCl_3) $\delta = 5.77$ (s, 1H, 4-H), 3.43 (t, $J = 7.54 \text{ Hz}$, 1H, 17-H), 3.35 (dd, $J = 4.06, 13.46 \text{ Hz}$, 1H, SCH_2), 3.01 (s, 2H, $\text{O}=\text{CCH}_2\text{S}$), 2.75 (dd, $J = 7.91, 13.25 \text{ Hz}$, 1H, 6-H), 2.41–2.53 (broad, 1H, OH), 2.02 (dd, $J = 4.49, 13.25 \text{ Hz}$, 1H, SCH_2), 0.80 (s, 3H, 19- H_3), 0.69 (s, 3H, 18- H_3) ; Mol. mass = 392.

Methyl-5-(2'-testosterone)yl-4-thia-pentanoate 5a

Prepared as in the case of **4a** with Methyl thiopropionate.

Yield = 68%.

IR (neat) $\bar{\nu} = 3400\text{--}3500 \text{ cm}^{-1}$ (OH), 1735 (COOR), 1680 (C=O), 1620 (C=C) ; UV (CHCl_3) $\lambda_{\text{max}} = 244.4 \text{ nm}$; ^1H NMR (CDCl_3) $\delta = 5.73$ (d, $J = 1.47 \text{ Hz}$, 1H, 4-H), 3.70 (s, 3H, OCH_3), 3.66 (t, $J = 7.63 \text{ Hz}$, 2H, $\text{O}=\text{CCH}_2$), 2.65 (t, $J = 7.65 \text{ Hz}$, 2H, $\text{O}=\text{CCH}_2\text{CH}_2\text{S}$), 3.19 (d, $J = 9.34 \text{ Hz}$, 1H, SCH_2), 1.23 (s, 3H, 19- H_3), 0.809 (s, 3H, 18- H_3) ; ^{13}C NMR (CDCl_3) major isomer (α) ppm = 41.38 (C1), 42.49 (C2), 198.99 (C3), 123.28 (C4), 170.77 (C5), 39.33 (C10), 17.59 (C19), minor isomer (β) ppm = 37.21 (C1), 42.17 (C2), 198.99 (C3), 121.73 (C4), 172.34 (C5), 39.33 (C10), 17.59 (C19) ; Mol. mass = 420.

4-Thia-5-(testosterone)yl-pentanoic acid 5b

Prepared as in the case of **4b**.

Yield = 64%.

UV (MeOH) λ_{max} = 245.0 nm ; IR (KBr) $\bar{\nu}$ = 3550-3250 cm^{-1} (OH), 2700-2500 (OH), 1722.5 (COOH), 1666.4 (C=O), 1620.3 (C=C) ; ^1H NMR (CDCl_3) δ = 5.79 (s, 1H, 4-H), 3.43 (t, J = 11.4 Hz, 1H, 17-H), 3.30 (dd, J = 3.48, 13.00 Hz, 1H, SCH_2), 2.63 (t, J = 6.59 Hz, 1H, O=CCH_2), 2.41 (t, J = 7.77 Hz, 2H, $\text{O=CCH}_2\text{CH}_2\text{S}$), 2.12 (dd, J = 4.58, 13.00 Hz, 1H, SCH_2), 0.82 (s, 3H, 19- H_3), 0.69 (s, 3H, 18- H_3) ; Mol. mass = 406.

Spiro[cyclopropane-1,2'-testosterone] 3

Cupric acetate dihydrate (60 mg) is dissolved in hot boiling anhydrous acetic acid (3 ml) under nitrogen and zinc dust (500 mg, 325 mesh) added all at once. After stirring for 30 sec. the solution is allowed to cool. Excess acetic acid is syringed off and the reddish grey couple is repeatedly washed with anhydrous Et_2O until it is freed of acetic acid. A drop of methylene iodide is then added to a stirring solution of couple in anhydrous ether (10 mL) with reflux. A solution of **2** (57.4 mg, 0.19 mmol) in anhydrous ether (10 mL) and anhydrous THF (1 mL) containing CH_2I_2 (23 μL , 0.19 mmol) is added dropwise under reflux. After 4 hrs. of reflux the couple is filtered and solution washed with 10% HCl.

Repeated washings with ether followed by usual work up and chromatography yields a white solid (56.0 mg, 92%).

mp 147°C ; IR (KBr) $\bar{\nu}$ = 3440 cm^{-1} (OH), 1650 (C=O), 1615 (C=C) ; UV (MeOH) λ_{max} (log ϵ) = 242.0 nm (4.199) ; ^1H NMR (CDCl_3) δ = 5.80 (d, J = 1.83 Hz, 1H, 4-H), 3.66 (t, J = 8.61 Hz, 1H, 17-H), 2.19 (d, J = 13.37 Hz, 1H, 1-H), 1.24 (s, 3H, 19-H₃), 0.80-0.74 (m, 1H, cyclopropyl), 0.79 (s, 3H, 18-H₃), 0.50-0.43 (m, 1H, cyclopropyl) ; Mol. mass = 314 ;

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|--------------------|------------|---|-------|---|------|
| Elemental Analyses | Calculated | C | 80.21 | H | 9.62 |
| | Found | C | 79.81 | H | 9.47 |

4-Thia-6-(2'-testosterone)yl-hexanoic acid 9

To a stirring solution of cyclopropane **3** (60 mg, 0.19 mmol) and dry NaI (30 mg, 0.20 mmol) in anhydrous acetonitrile (2 mL) is added TMSCl (1 mL) dropwise at room temperature under nitrogen during a period of 10 min. After stirring for 2 hrs. the CH_3CN is evaporated under a flush of nitrogen. The yellow residue is taken up in THF (35 mL) and washed with 1:1 mixture of brine and 10% Sodium thio-sulfate to remove all traces of iodine. The colorless organic layer is put under a stream of nitrogen where upon all THF is removed and the residue is subjected to a high vacuum (0.3 mm of Hg) for drying under dark. After 1.5 hrs., during which the residue picks up yellowish tinge, the residue

is dissolved in anhydrous EtOH (2 mL) and added to a stirring solution of thiopropionic acid (42.4 mg, 0.40 mmol) in anhydrous EtOH containing NaOEt (61.20 mg, 0.90 mmol) under nitrogen at room temperature. Stirring continued for 4 hrs. after which the alkaline solution is acidified with dil. HCl and ethanol evaporated with nitrogen flush. The remaining solution is taken up in CHCl_3 (40 mL) and worked up as usual. Column chromatography yields a yellow oil (44 mg, 55%).

UV (CHCl_3) λ_{max} ($\log \epsilon$) = 245.8 nm (4.193) ; IR (CHCl_3) $\bar{\nu}$ = 3400-3500 cm^{-1} (broad OH), 1713.7 (COOH), 1660.8 (C=O), 1614.3 (C=C) ; ^1H NMR (CDCl_3) δ = 6.08 (s, 1H, 4-H), 5.65 (s, 1H, 4-H), 3.65 (t, J = 8.65 Hz, 1H, 17-H), 3.60 (t, J = 8.63 Hz, 1H, 17-H), 2.79-2.73 (m, 2H), 2.63-2.54 (m, 5H), 1.17 (s, 3H, 19- H_3), 1.16 (s, 3H, 19- H_3), 0.90 (s, 3H, 18- H_3), 0.88 (s, 3H, 18- H_3) ; Mol. mass 420.

2-(2'-Hydroxyethyl)-testosterone 8

The spirocyclopropane 3 (50 mg, 0.16 mmol) was dissolved in anhydrous CH_3CN (2 mL) and dry NaI (33 mg, 0.22 mmol) suspended with nitrogen flush in dark at room temperature. TMSCl (0.5 mL) added dropwise and solution is allowed to stir for 2 hrs. after which CH_3CN is evaporated with stream of nitrogen. The residue is taken up in THF (2 * 20 mL). The THF solution is washed with a solution

of 10% sodium thiosulfate and brine (1:1). The colorless THF solution is concentrated to a volume of 10 mL and a 1 mL, 1.6% solution (w/v) of KOH in water added dropwise. Stirring for 10 hrs. at room temperature followed by usual workup and column chromatography yields a white solid (22 mg, 66%).

IR (KBr) $\bar{\nu}$ = 3400 cm^{-1} (broad OH), 1644.3 (C=O), 1612.8 (C=C); UV (CHCl_3) λ_{max} (log ϵ) = 243.5 nm (4.023); ^1H NMR (CDCl_3) δ = 5.75 (d, J = 1.28 Hz, 4-H), 3.71-3.84 (m, 1H, 17-H), 3.63-3.70 (m, 2H, CH_2OH), 1.24 (s, 3H, 19- H_3), 0.80 (s, 3H, 18- H_3); Mol. mass 332.

2-Methylene-androst-4-en-3, 17-dione 10

Solid PCC (19 mg, 0.09 mmol) is added to a stirring solution of 2-Methylene-testosterone (20 mg, 0.07 mmol) in alcohol free CH_2Cl_2 (2.5 mL) at room temperature. After 30 min. silica gel is added to adsorb compound and short column chromatography resorted to. Elution with 25% ethyl acetate- pet. ether (bp 60-80 C) mixture yields a white solid (19 mg, 100%).

IR (KBr) $\bar{\nu}$ = 1736.9 cm^{-1} (C=O), 1674.4 (C=O), 1620.7 (C=C), 900, 957; UV (CHCl_3) λ_{max} (log ϵ) = 257.8 nm (4.478); ^1H NMR (CDCl_3) δ = 5.96 (t, J = 2.20 Hz, 1H, C=CH), 5.87 (d, J = 0.73 Hz, 1H, 4-H), 5.25 (dd, J = 1.92, 2.38 Hz, 1H, C=CH), 2.72 (d, J = 14.10 Hz, 1H, 1-H), 1.13 (s, 3H, 19- H_3), 0.92 (s, 3H, 18- H_3); Mol. mass = 298.

Spiro[androst-4-en-2.1'-cyclopropane]-3,17-dione 11

The cyclopropanation procedure remains the same as for 3; however co-solvent THF need not be used.

Yield = 95% ;

mp = 144°C ; IR (KBr) $\bar{\nu}$ = 3076 cm^{-1} , 1733.8 (C=O), 1656.4 (C=O), 1613.7 (C=C); UV (CHCl_3) λ_{max} ($\log \epsilon$) = 244.8 (4.225) ; ^1H NMR (CDCl_3) δ = 5.82 (d, J = 1.47 Hz, 1H, 4-H), 1.26 (s, 3H, 19- H_3), 0.92 (s, 3H, 18- H_3), 0.75-0.81 (m, 1H, cyclopropyl), 0.45-0.52 (m, 1H, cyclopropyl) ; Mol. mass = 312

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|--------------------|------------|---|-------|---|------|
| Elemental Analysis | Calculated | C | 80.72 | H | 9.03 |
| | Found | C | 80.60 | H | 9.24 |

2 α -(2'-Hydroxyethyl)-androst-4-en-3,17-dione 12

The procedure remains the same as for 8.

Yield = 64% ;

mp = 107°C ; IR (KBr) $\bar{\nu}$ = 3300 cm^{-1} (broad, OH), 1735.4 (C=O), 1669.1 (C=O), 1625.0 (C=C) ; UV (CHCl_3) λ_{max} ($\log \epsilon$) = 244.6 (4.095) ; ^1H NMR (CDCl_3) δ = 5.78 (d, J = 1.28 Hz, 1H, 4-H), 3.83-3.78 (m, 1H, OCH_2), 3.72-3.65 (m, 1H, OCH_2), 1.26 (s, 3H, 19- H_3), 0.93 (s, 3H, 18- H_3) ; Mol. mass = 330 ;

| | | | | | |
|--------------------|------------|---|-------|---|------|
| Elemental Analyses | Calculated | C | 76.32 | H | 9.15 |
| | Found | C | 75.98 | H | 9.07 |

Spiro[cyclopropane-1,2'-testosterone]-17 β -trifluoroacetate 6

The cyclopropane 3 (34.9 mg, 0.11 mmol) is dissolved in anhydrous CH_2Cl_2 (1 mL) and TMSOTf (1 mL) added with stirring under nitrogen atmosphere. After stirring for 7 hrs. the organic solution is washed with water, 5% bicarbonate, water, and brine and dried on anhydrous MgSO_4 . Vacuum evaporation of CH_2Cl_2 layer followed by chromatography on silica gel yield a white solid (45 mg, 96%).

IR (KBr) $\bar{\nu}$ = 1779.0 cm^{-1} (COOCF_3), 1659.6 (C=O), 1611.0 (C=C), 871.8, 775.7 ; UV (CHCl_3) λ_{max} ($\log \epsilon$) = 244.0 nm (4.163) ; ^1H NMR (CDCl_3) δ = 5.81 (d, J = 1.65 Hz, 1H, 4-H), 4.80 (dd, J = 7.69, 9.16 Hz, 1H, 17-H), 1.24 (s, 3H, 19- H_3), 0.89 (s, 3H, 18- H_3), 0.75-0.81 (m, 1H, cyclopropyl), 0.45-0.52 (m, 1H, cyclopropyl) ; Mol. mass = 410;

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|--------------------|------------|---|-------|---|------|
| Elemental Analyses | Calculated | C | 67.30 | H | 7.12 |
| | Found | C | 67.07 | H | 7.33 |

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(Received in UK 30 May, 1990)