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Synthesis of Deuterated Steroids for GC/MS Quantification of Endogenous Steroids.

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INTRODUCTION

Steroid profiling in human sport has become an additional tool in doping control to detect changes in the normal pattern of excreted steroids. This changes can be caused by the misuse of testosterone, a potent precursor or metabolite of testosterone and/or synthetic anabolic steroids.

To obtain exact quantitative results of endogenous steroids in urine and blood plasma by gas chromatography-mass spectrometry (GC-MS) deuterated standards are used to control the sample preparation, the GC-MS analysis and to calculate urinary steroid concentrations.

Deuterium-labelled steroids have been applied as the most proper internal standards in GC/MS quantification of steroid hormones [1-3].

The most interesting steroids analyzed are testosterone, epitestosterone, the main metabolites of testosterone: androsterone, etiocholanolone, 5α-androstane-3α,17ß-diol and 5ß-androstane-3α,17ß-diol. Further 11ß-hydroxyandrosterone and 11ß-hydroxy-etiocholanolone, metabolites of 11ß-hydroxyandrost-4-ene-3,17-dione, are estimated to obtain information about the activity of the adrenal gland.

The synthesis of [16,16,17-$^2H_3$]-testosterone **1**, [16,16,17-$^2H_3$]-epitestosterone **2**, [2,2,4,4-$^2H_4$]-androsterone **3**, [2,2,4,4-$^2H_4$]-etiocholanolone **4** and [2,2,4,4-$^2H_4$]-11ß-hydroxyandrosterone **5** (Fig.1) is described.

EXPERIMENTAL

[16,16,17-$^2H_3$]-Testosterone 1 (Fig.2)

1. *17ß-Hydroxyandrost-5-en-3-on 3-ethyleneketal* **7**: Testosterone **6** (25 g) was dissolved in 100 ml of benzene, 30 ml of ethylene glycol, 100 mg of p-toluenesulfonic acid and refluxed using a water extractor for 24 hours. The reaction mixture was diluted with 600 ml of diethyl ether and extracted against 200 ml of bidestilled water. The aqueous phase was further extracted twice with 600 ml of diethyl ether. The ether layers were combined and dried yielding 27.5 g of 70% 17ß-hydroxyandrost-5-en-3-on 3-ethyleneketal **7**.

2. *Androst-5-en-3,17-dion 3-ethyleneketal* **8**: The dried reaction products were dissolved in 250 ml of dimethylformamide, 70 ml of dichloromethane and added with 65 g of pyridinium dichromate while stirring. After 1h at room temperature the reaction mixture was added to 1000 ml of diethyl ether and stirred for 5 min. After standing for 5 min the upper layer was separated and extracted against 250 ml of bidestilled water. The organic layer was concentrated to dryness and crystallized from 650 ml of methanol yielding 10.2 g of pure androst-5-en-3,17-dion 3-ethyleneketal **8**.

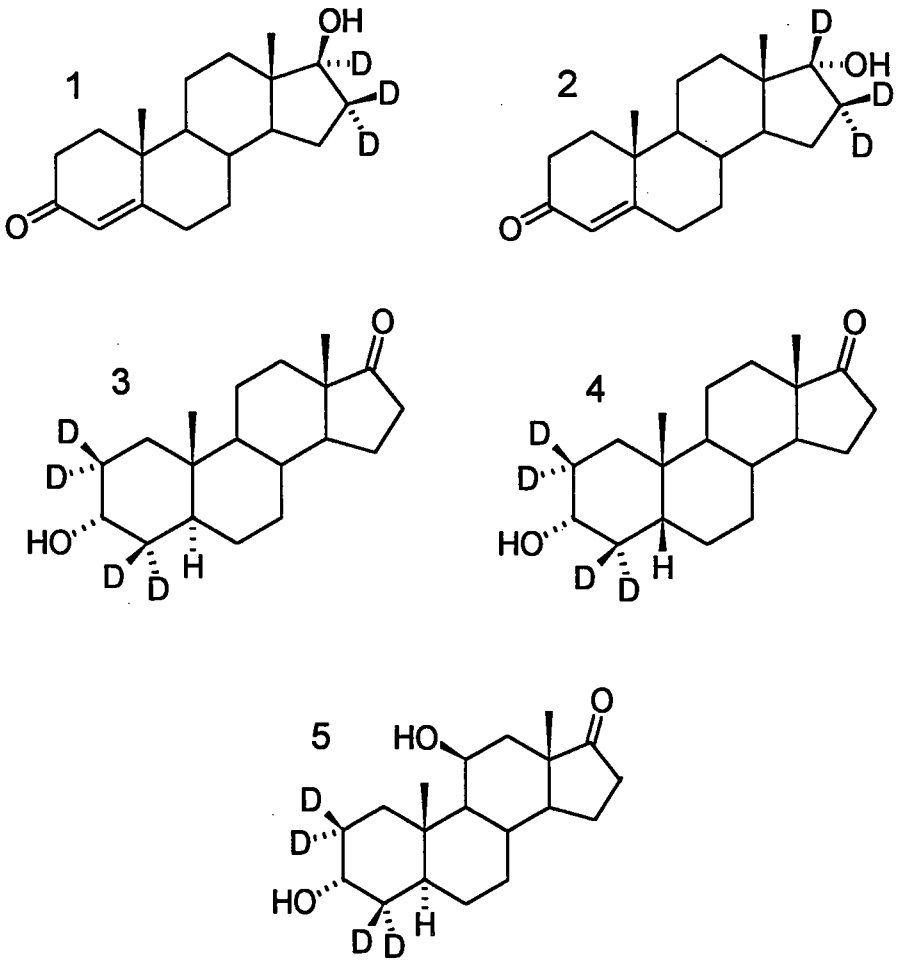


Fig. 1 Synthesized deuterated steroids: **1** [16,16,17-$^2H_3$]-testosterone: **2** [16,16,17-$^2H_3$]-epitestosterone; **3** [2,2,4,4-$^2H_4$]-androsterone: **4** [2,2,4,4-$^2H_4$]-etiocholanolone and **5** [2,2,4,4-$^2H_4$]-11ß-hydroxyandrosterone.

3. *[16,16-$^2H_2$]-Androst-5-en-3,17-dion 3-ethyleneketal* **9**: 10.2g of **8** was dissolved in 50 ml of methyl alcohol-d, 50 ml of dichloromethane, 10 ml of deuterium oxide and 0.2 ml of 40% sodium deuteroxide in D_2O and refluxed over night. The reaction mixture was concentrated to dryness and the deuterium exchange repeated using the same conditions. The reaction yielded [16,16-$^2H_2$]-androst-5-en-3,17-dion 3-ethyleneketal **9**.

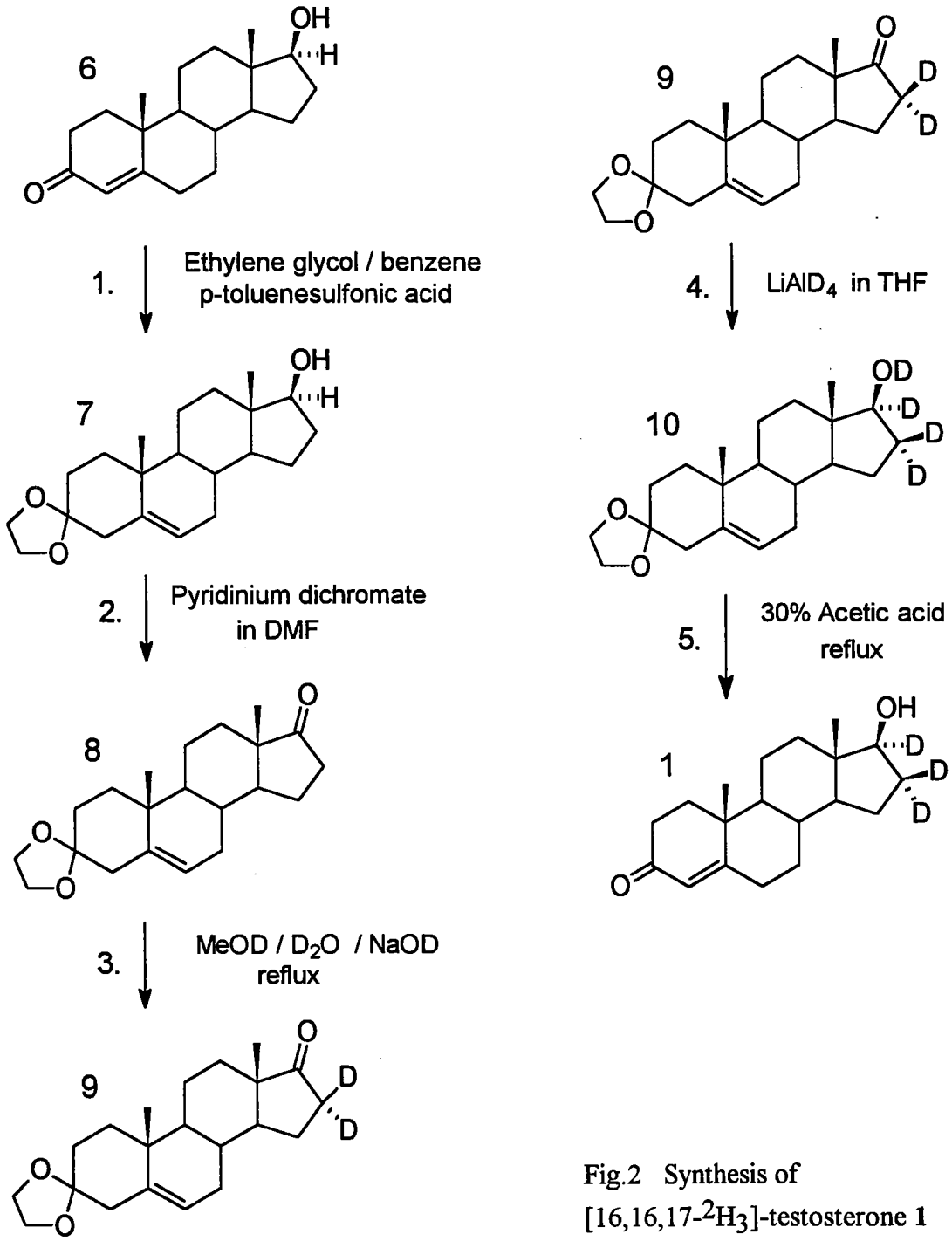


Fig.2 Synthesis of
[16,16,17-$^2H_3$]-testosterone **1**

4. *[16,16,17-$^2H_3$]-17ß-Hydroxyandrost-5-en-3-on 3-ethyleneketal* **10**: The reaction mixture containig **9** was concentrated to dryness, dissolved in 100 ml of dichloromethane, 300 ml of diethyl ether and added with 2 g of lithium aluminium deuteride while stirring. After 1 h the reacion mixture was added to 250 ml of water, the reaction products were extracted 8 times with 600 ml of diethyl ether und concentrated to dryness. The main reaction product was [16,16,17-$^2H_3$]-17ß-hydroxyandrost-5-en-3-on 3-ethyleneketal **10**.

5. *[16,16,17-$^2H_3$]-testosterone* **1**: Without further purification [16,16,17-$^2H_3$]-17ß-hydroxyandrost-5-en-3-on 3-ethyleneketal **10** was dissolved in 100 ml of 30% acetic acid

and refluxed for 30 min to hydrolyze the 3-ethyleneketal completely. The solution was diluted with 200 ml of water and extracted twice with 600 ml of diethyl ether. The combined organic layers were washed with 100 ml of 2% potassium hydroxide, 100 ml of bidestilled water, concentrated to dryness and crystallized from 100 ml of ethyl acetate yielding 3.6 g of [16,16,17-$^2H_3$]-testosterone **1** (yield 14.3%). EI-spectrum of the bis-TMS derivative see Fig.3B and deuteration yield see Table 1.

[16,16,17-$^2H_3$]-Epitestosterone 2 (Fig.4)

The synthesis of [16,16,17-$^2H_3$]-epitestosterone was prepared following the method of Radüchel **[4]**.

1. *[16,16,17-$^2H_3$]-Testosterone 17-tosylate* **11**: 8 g of [16,16,17-$^2H_3$]-testosterone **1** was dissolved in 100 ml of pyridine, added with 18 g of p-toluenesulfonyl chloride and stirred for 24 h at room temperature. The reaction mixture was added to 500 ml of water and the precipitate was filtered, washed two times with 250 ml of water and dried over potassium hydroxide/phosphorus pentoxide under vacuo. The reaction yielded 11.3 g of [16,16,17-$^2H_3$]-testosterone 17-tosylate **11**.

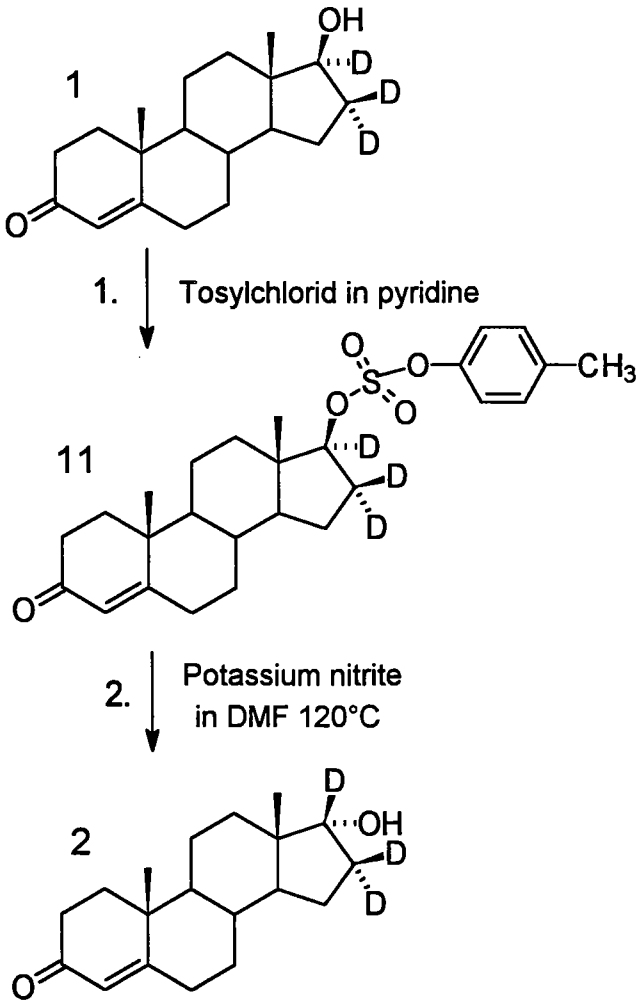


Fig.4 Synthesis of [16,16,17-$^2H_3$]-epitestosterone **2**

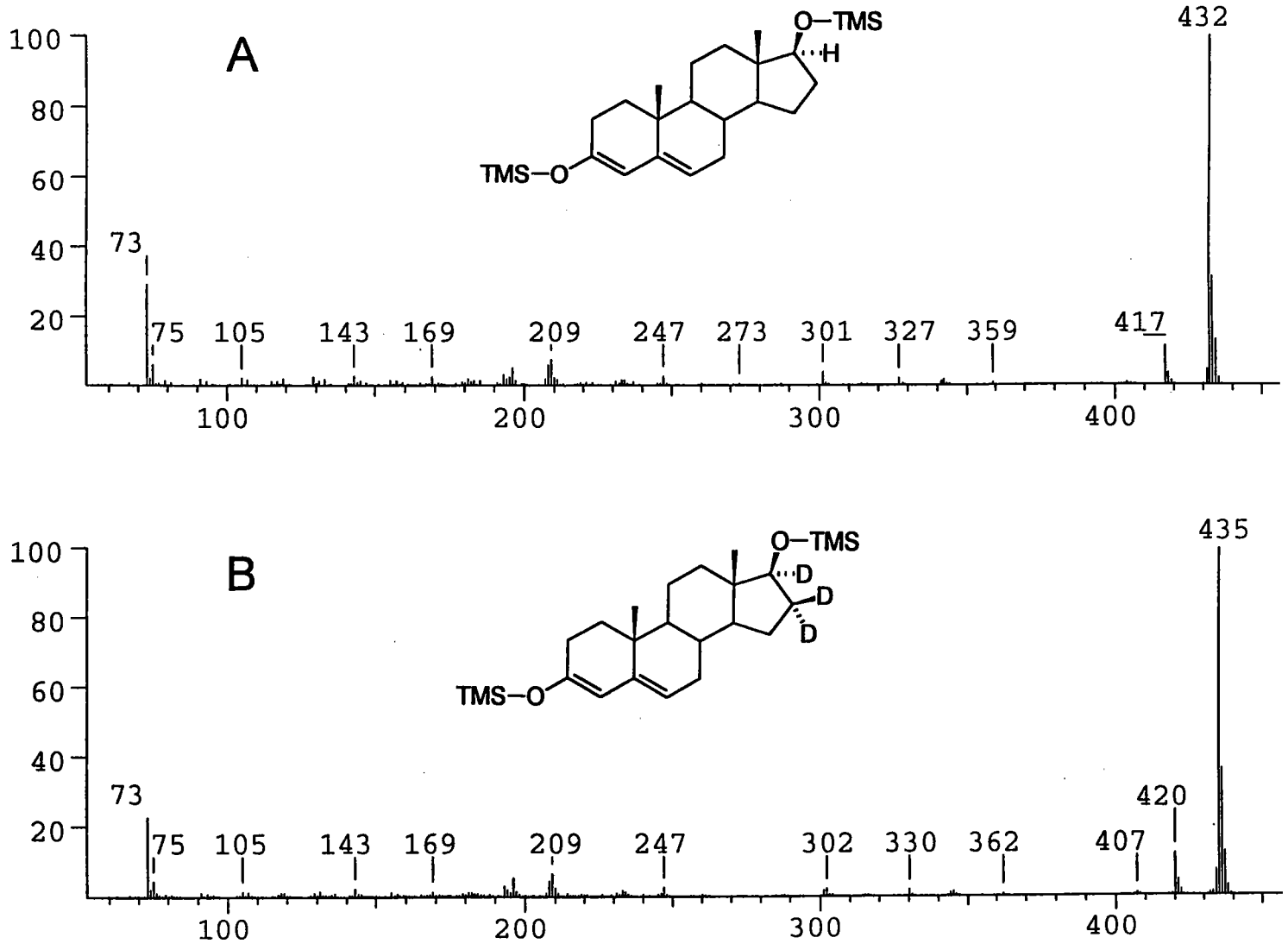


Fig.3 EI mass spectra of A) testosterone bis-TMS and B) [16,16,17-$^{2}H_3$]-testosterone bis-TMS

Table 1 Isotope composition and deuteration yield of deuterium labelled steroids

Steroid	Mass Spec.	Deuterium content in % $^2H_0$	$^2H_1$	$^2H_2$	$^2H_3$	$^2H_4$	Overall yield[a]
[16,16,17-$^2H_3$]-Testosterone	MAT	1.1	0	2.7	96.2		98.0
	MSD	1.2	0.2	2.7	95.7		98.0
[16,16,17-$^2H_3$]-Epitestosterone	MAT	1.3	0.4	2.4	95.9		97.6
	MSD	1.4	0.3	2.5	95.8		97.6
[2,2,4,4-$^2H_4$]-Androsterone	MAT	1.4	0.1	0.6	9.8	88.1	95.7
	MSD	1.5	0.3	0.9	10.3	87.0	95.2
[2,2,4,4-$^2H_4$]-Etiocholanolone	MAT	0	0	0	1.5	98.5	99.6
	MSD	0	0.1	0	1.4	98.5	99.6
[2,2,4,4-$^2H_4$]-11ß-Hydroxy-androsterone	MAT	0	0	0.3	5.7	94.0	98.4
	MSD	0	0.1	0.7	3.6	95.6	98.7

Mass Spec. = Mass spectrometer,

MAT = High Resolution Instrument MAT 95 (Finnigan),

MSD = Mass selective detector (Hewlett-Packard),

a = Ratio of introduced deuterium atoms versus calculated number of maximum exchangable deuterium atoms,

All steroids were measured as their per-TMS derivatives after gas-chromatographic separation, MAT values were registrated in SCAN mode, whereas the MSD values are mean values of three analysis using the selcted ion monitoring mode (SIM).

Extent of deuteration was estimated by correction for $[M-H]^+$, $[M-2H]^+$ and the isotopic contribution of the non labelled steroid.

2. *[16,16,17-$^2H_3$]-Epitestosterone* **2**: 11 g of [16,16,17-$^2H_3$]-testosterone 17-tosylate **11** was dissolved in 150 ml of dimethylformamide, added with 33 g of potassium nitrite and heated exactly at 125°C for 16 h. The reaction mixture was diluted with 200 ml of water and extracted with 1000 ml of diethyl ether. The organic layer was concentrated to dryness and separated via silica gel 60 (Merck, 35-70 mesh, ASTMS, bed 1 x 50 cm) using n-pentan/ethyl acetate (70:30, v:v) as solvent. The fractions containing the deuterated epitestosterone were crystallized from ethyl acetate yielding 550 mg of [16,16,17-$^2H_3$]-epitestosterone **2** (yield 6.8%). EI-spectrum of the bis-TMS derivative see Fig.5B and deuteration yield see Table 1.

[2,2,4,4-$^2H_4$]-Androsterone 3 (Fig.6)

1. *Epiandrosteron 17-ethyleneketal* **13**: 10 g (34.6 mmol) of epiandrosterone **12** was dissolved in 120 ml of benzene, 20 ml of ethylene glycol, 50 mg of p-toluenesulfonic acid and refluxed over night using a water extractor. 250 ml of bidestilled water were added to the mixture and the reaction products were extracted with 1000 ml of diethyl ether. The organic layer was concentrated to dryness with a yield of 13 g of crude crystals, 96% epiandrosteron 17-ethyleneketal **13** (GC-MS result).

2. *5α-Androstane-3,17-dion 17-ethyleneketal* **14**: The crude crystals of 1. were dissolved in 100 ml of dimethylformamide and oxidized with 41 g (110 mmol) of pyridinium dichromate, which was slowly added to the reaction mixture while stirring. After 1h at room temperature the reaction solution was added to 1000 ml of diethyl ether and stirred for 5 min. After standing for 5 min the upper layer was separated, washed with 250 ml of bidestilled water and evaporated to dryness. The oxidation yielded 5α-androstane-3,17-dion 17-ethyleneketal **14**.

3. *[2,2,4,4-$^2H_4$]-5α-Androstane-3,17-dion 17-ethyleneketal* **15**: The 5α-androstane-3,17-dion 17-ethyleneketal **14** was not further purified, dissolved in 50 ml of methyl alcohol-d, 50 ml of dichloromethane, 10 ml of deuterium oxide, 0.1 ml of 40% sodium deuteroxide in D_2O and refluxed over night. The reaction mixture was concentrated to dryness and the deuterium exchange repeated using the same conditions. The reaction solution was evaporated to dryness yielding **15** as main product..

4. *[2,2,4,4-$^2H_4$]-3β-Hydroxy-5α-androstan-17-on 17-ethyleneketal* **16**: [2,2,4,4-$^2H_4$]-5α-Androstane-3,17-dion 17-ethyleneketal **15** obtained from reaction 3. was dissolved in 100 ml of dichloromethane, 300 ml of diethyl ether and added with 1 g of lithium aluminium hydride while stirring. After 1 h at room temperature 250 ml of water was added tothe reation mixture and the reaction products were extracted 2 times with 800 ml of diethyl ether. The organic phases were combinded, and concentrated to dryness yielding [2,2,4,4-$^2H_4$]-3ß-hydroxy-5α-androstan-17-on 17-ethyleneketal **16** as main reduction product.

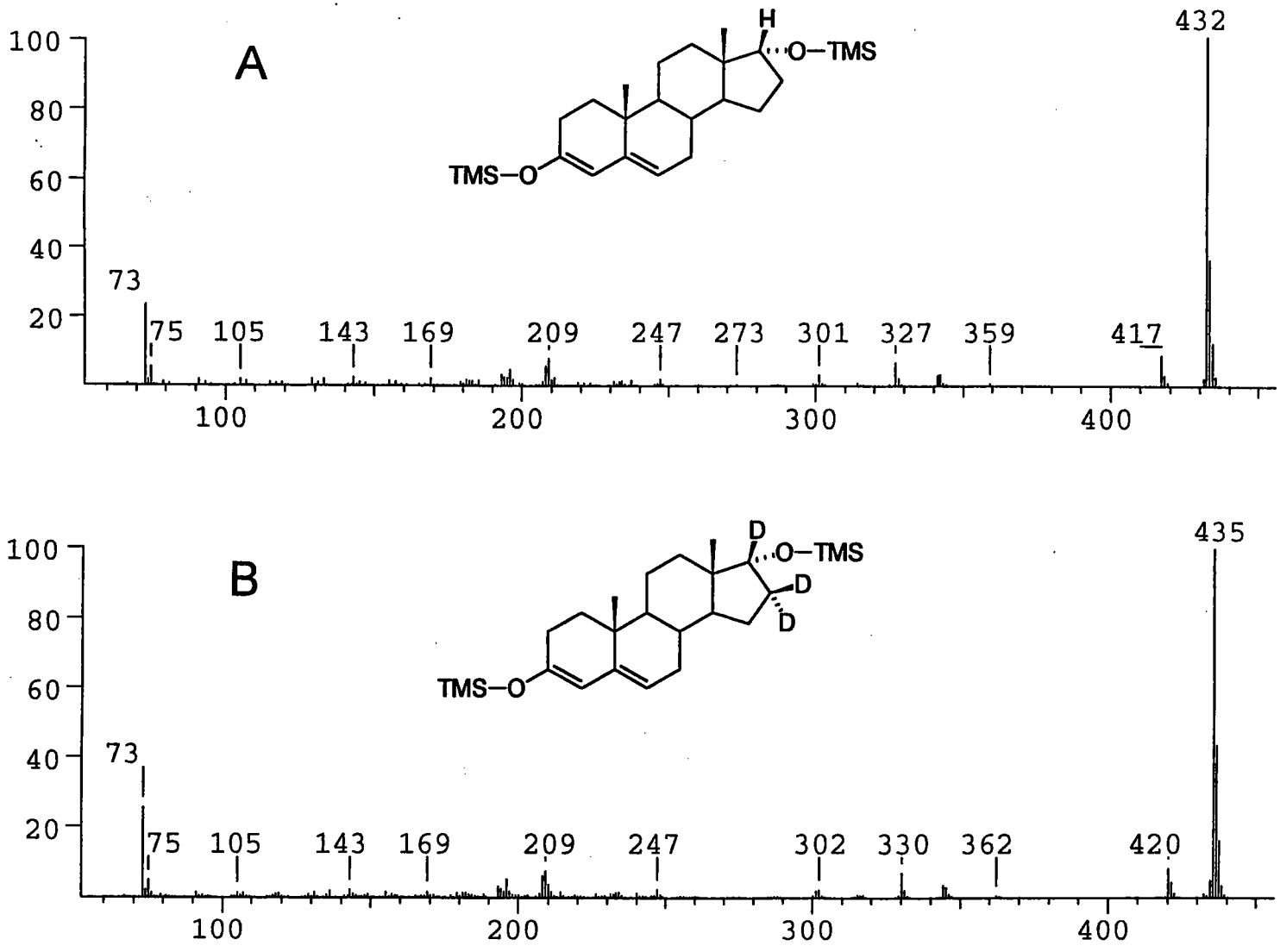


Fig.5 EI mass spectra of A) epitestosterone bis-TMS and B) [16,16,17-$^{2}H_3$]-epitestosterone bis-TMS

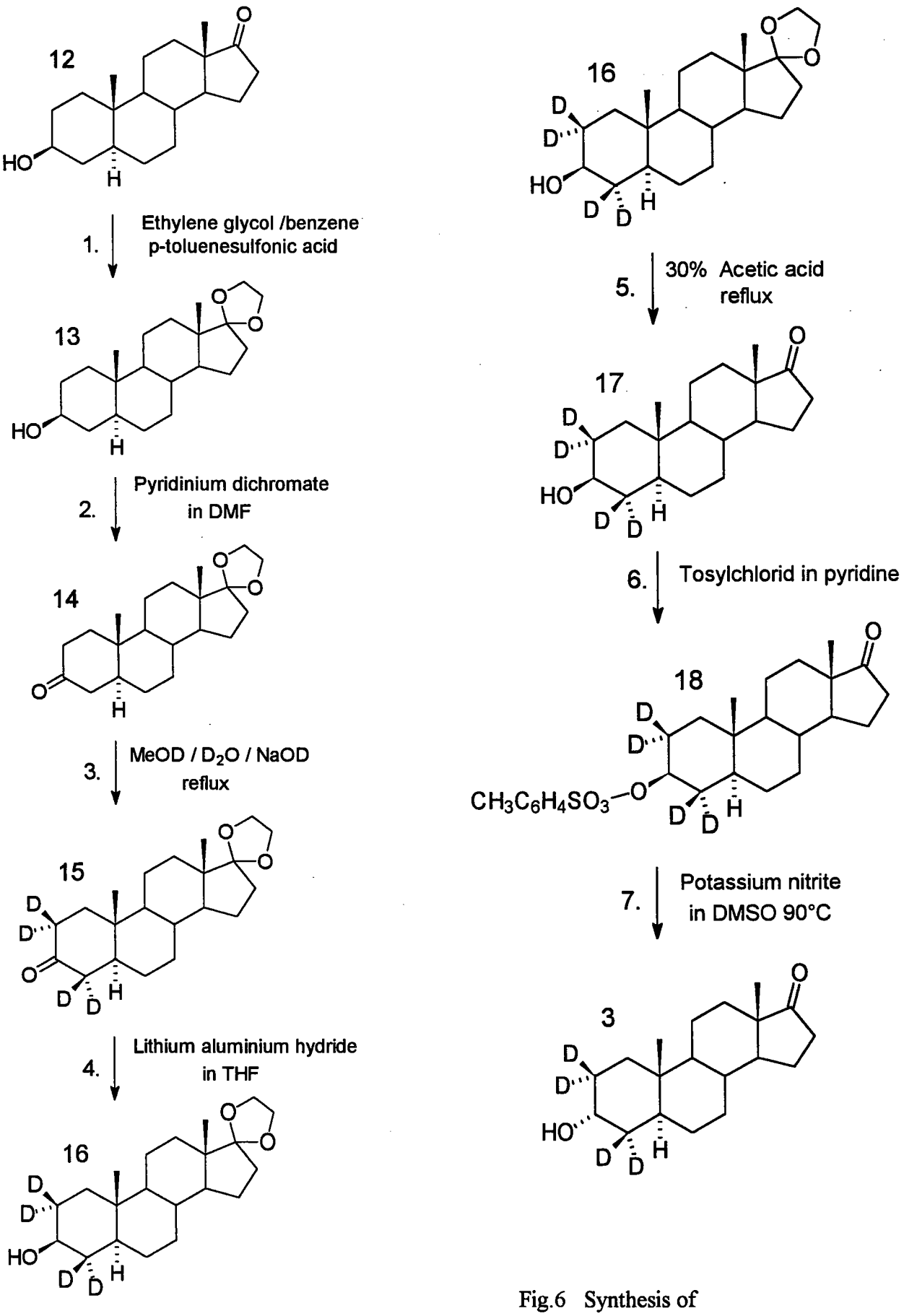


Fig.6 Synthesis of
[2,2,4,4-$^2H_4$]-androsterone **3**

5. *[2,2,4,4-$^2H_4$]-3β-Hydroxy-5α-androstan-17-on* **17**: The crude reaction products of reaction 4. were dissolved in 100 ml of 30% acetic acid and refluxed for 30 min to hydrolize the 17-ethyleneketal completely. The solution was diluted with 200 ml of water and extracted with 1000 ml of diethyl ether. The organic phase was washed with 100 ml of 2% potassium hydroxide, 100 ml of bidestilled water and evaporated to dryness. [2,2,4,4-$^2H_4$]-3ß-Hydroxy-5α-androstan-17-on **17** was obtained and not further purified.

6. *[2,2,4,4-$^2H_4$]-3β-Hydroxy-5α-androstan-17-one 3-tosylate* **18**: The dried residue of reaction 5. containing the [2,2,4,4-$^2H_4$]-3ß-Hydroxy-5α-androstan-17-on **17** was dissolved in 100 ml of pyridine, added with 9 g (60 mmol) of p-tolunesulfonyl chlorid and stirred for 18 h at room temperature. The reaction mixture was diluted with 500 ml of water and extracted with 800 ml and 600 ml of diethyl ether. The organic layers were combined and evaporated to dryness.

7. [2,2,4,4-$^2H_4$]-Androsterone **3**: The crude residue of reaction 6. with [2,2,4,4-$^2H_4$]-3ß-hydroxy-5α-androstan-17-one 3-tosylate **18** was dissolved in 100 ml of dimethylsulfoxide, 59.5 g of potassium nitrite and heated to 90°C for 150 min. 300 ml of bidestilled water was added, the reaction products extracted with 900 ml of diethyl ether and separated after concentration to dryness via silica gel 60 (Merck, 35-70 mesh, ASTMS, bed 3 x 40 cm) using n-pentan/ethyl acetate (60:40, v:v) as solvent. The fractions containing the [2,2,4,4-$^2H_4$]-androsterone **3** were combined and crystallized from ethyl acetate/n-heptan yielding 1.4 g of crystals (yield 14%; 97% pure, GC/FID and GC/MS analysis). EI-spectrum of the bis-TMS derivative of [2,2,4,4-$^2H_4$]-androsterone **3** see Fig.7B and deuteration yield see Table 1.

[2,2,4,4-$^2H_4$]-Etiocholanolone 4 (Fig.8)

1. *Dehydroepiandrosteron 17-ethyleneketal* **20**: 10 g (34 mmol) of dehydroepiandrosterone **19** (DHEA) was dissolved in 120 ml of benzene, 20 ml of ethylene glycol, 50 mg of p-toluenesulfonic acid and refluxed over night using a water extractor. The reaction mixture was diluted with 200 ml of bidestilled water and the reaction product was extracted twice with 700 ml of diethyl ether. The organic layers were combined and evaporated to dryness. DHEA 17-ethyleneketal **20** was formed with a yield of more than 98%.

2. *Androst-4-en-3,17-dion 17-ethyleneketal* **21**: The dried residue with DHEA 17-ethyleneketal **20** was held under argon, dissolved in 145 ml of acetone, 300 ml of benzene, added with 12.8 g (50 mmol) of aluminium t-butoxide **[5]** and refluxed over night. The reaction yielded 85% of androst-4-en-3,17-dion 17-ethyleneketal **21** and 15% of unchanged DHEA 17-ethyleneketal **20**. 300 ml of bidestilled water was added to the reaction solution and the steroids were extracted twice with 500 ml of diethyl ether. The organic layers were combined and evaporated to dryness.

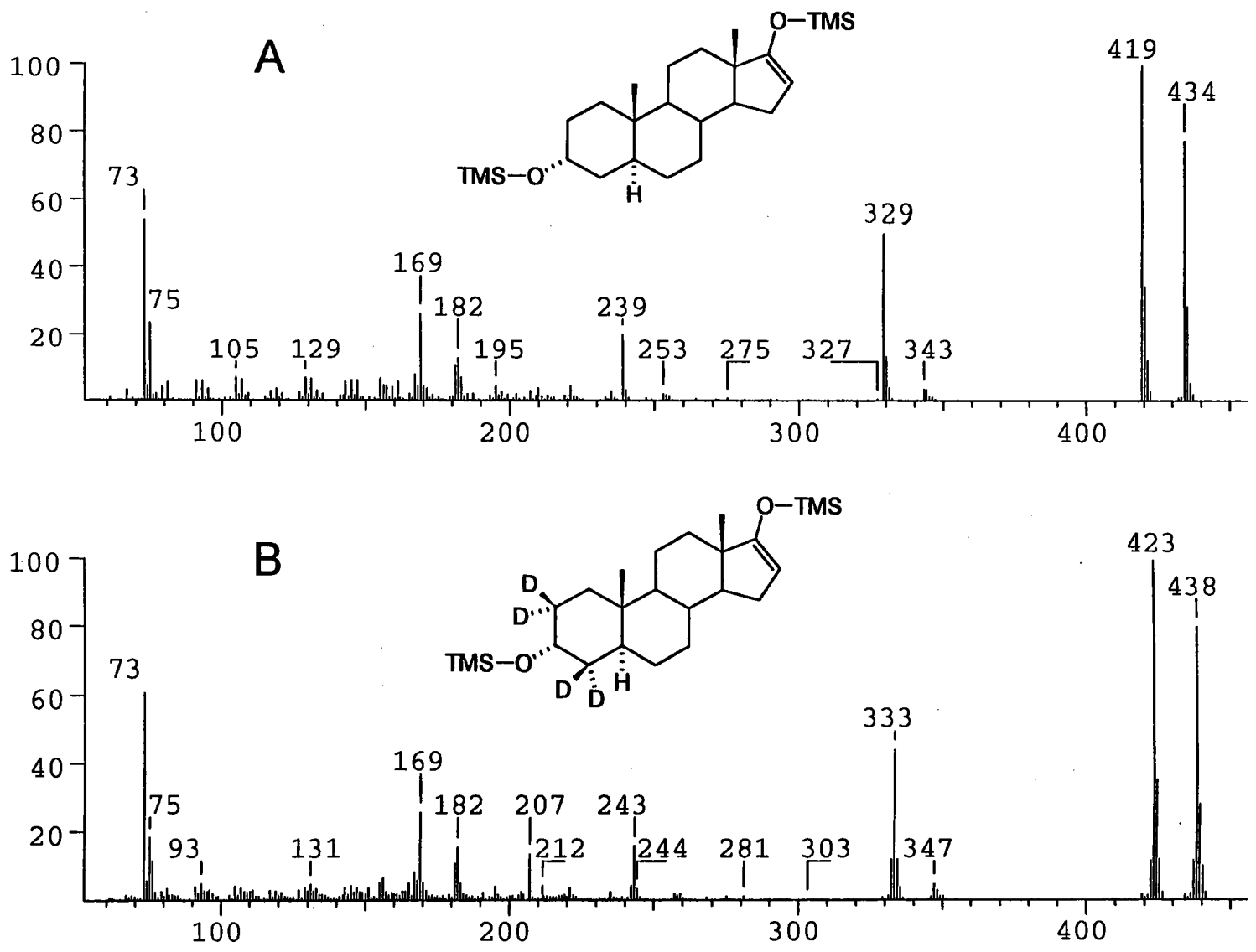


Fig.7 EI mass spectra of A) androsterone bis-TMS and B) [2,2,4,4-$^{2}H_4$]-androsterone bis-TMS

3. *5ß-Androstane-3,17-dione 17-ethyleneketal* **22**: The crude residue of reaction 2 was dissolved in 60 ml of methanol, 5 ml of benzene, added with 1 g of potassium hydroxide and the C-4,5 double bond was reduced with hydrogen using 40 mg of platinum dioxide as catalyst yielding more than 90% of the 5ß-isomer 5ß-androstane-3,17-dion 17-ethyleneketal **22**. The reaction mixture was evaporated to dryness and not further purified.

4. *[2,2,4,4-$^2H_4$]-5ß-androstane-3,17-dion 17-ethyleneketal* **23**: The dried reaction mixture containing the 5ß-androstane-3,17-dion 17-ethyleneketal **22** was dissolved in 60 ml of methyl alcohol-d, 50 ml of dichloromethane, 10 ml of deuterium oxide, 0.1 ml of 40% sodium deuteroxide in D_2O and refluxed over night. The reaction mixture was concentrated to dryness and the deuterium exchange repeated using the same conditions. The obtained [2,2,4,4-$^2H_4$]-5ß-androstane-3,17-dion 17-ethyleneketal **23** was not further purified.

5. *[2,2,4,4-$^2H_4$]-3α-Hydroxy-5ß-androstan-17-on 17-ethyleneketal* **24**: [2,2,4,4-$^2H_4$]-5ß-Androstane-3,17-dion 17-ethyleneketal **23** obtained from reaction 4 was dissolved in 30 ml of dichloromethane, 300 ml of diethyl ether and added slowly with 1.3 g of lithium aluminium hydride while stirring. After 60 min 250 ml of water was added, the steroids were extracted with 750 ml of diethyl ether and the organic phase was evaporated to dryness. The reduction of the 3-keto group yielded the 3α-isomer [2,2,4,4-$^2H_4$]-3α-hydroxy-5ß-androstan-17-on 17-ethyleneketal **24** as main product. The ratio of the formed 3 α-hydroxy:3ß-hydroxy isomer was approximately 10:1.

6. *[2,2,4,4-$^2H_4$]-Etiocholanolone* **4**: To hydrolyze the 17-ethyleneketal the reduced products of reaction 5 were refluxed in 150 ml of 30% acetic acid for 30 min. 300 ml of water was added and the steroids were extracted with 800 ml and 300 ml of diethyl ether. The organic layers were combined and washed with 100 ml of 2% potassium hydroxide and 100 ml of bidestilled water. After evaporation of the organic phase the reaction products were separated via silica gel 60 (Merck, 35-70 mesh, ASTMS, bed 3 x 40 cm) using n-pentane/ethyl acetate (70:30, v:v) as solvent. The fractions containing the [2,2,4,4-$^2H_4$]-etiocholanolone **4** were crystallized from ethyl acetate/n-heptane yielding 1.78 g of pure [2,2,4,4-$^4H_2$]-etiocholanolone **4** (yield 17.8%). EI-spectrum of the bis-TMS derivative are shown in Fig.9B and deuteration yield see Table 1.

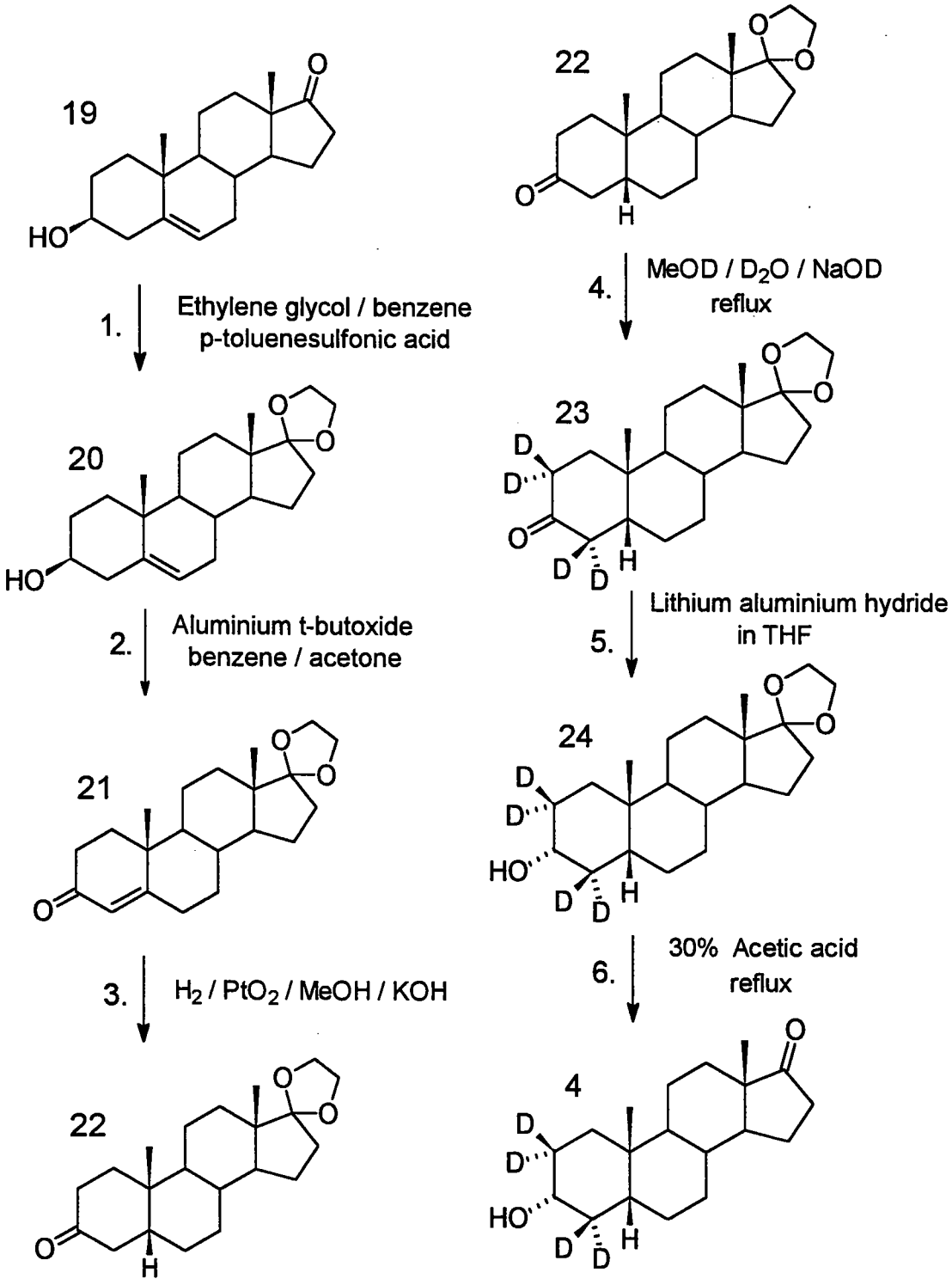


Fig. 8 Synthesis of [2,2,4,4-$^2H_4$]-etiocholanolone 4

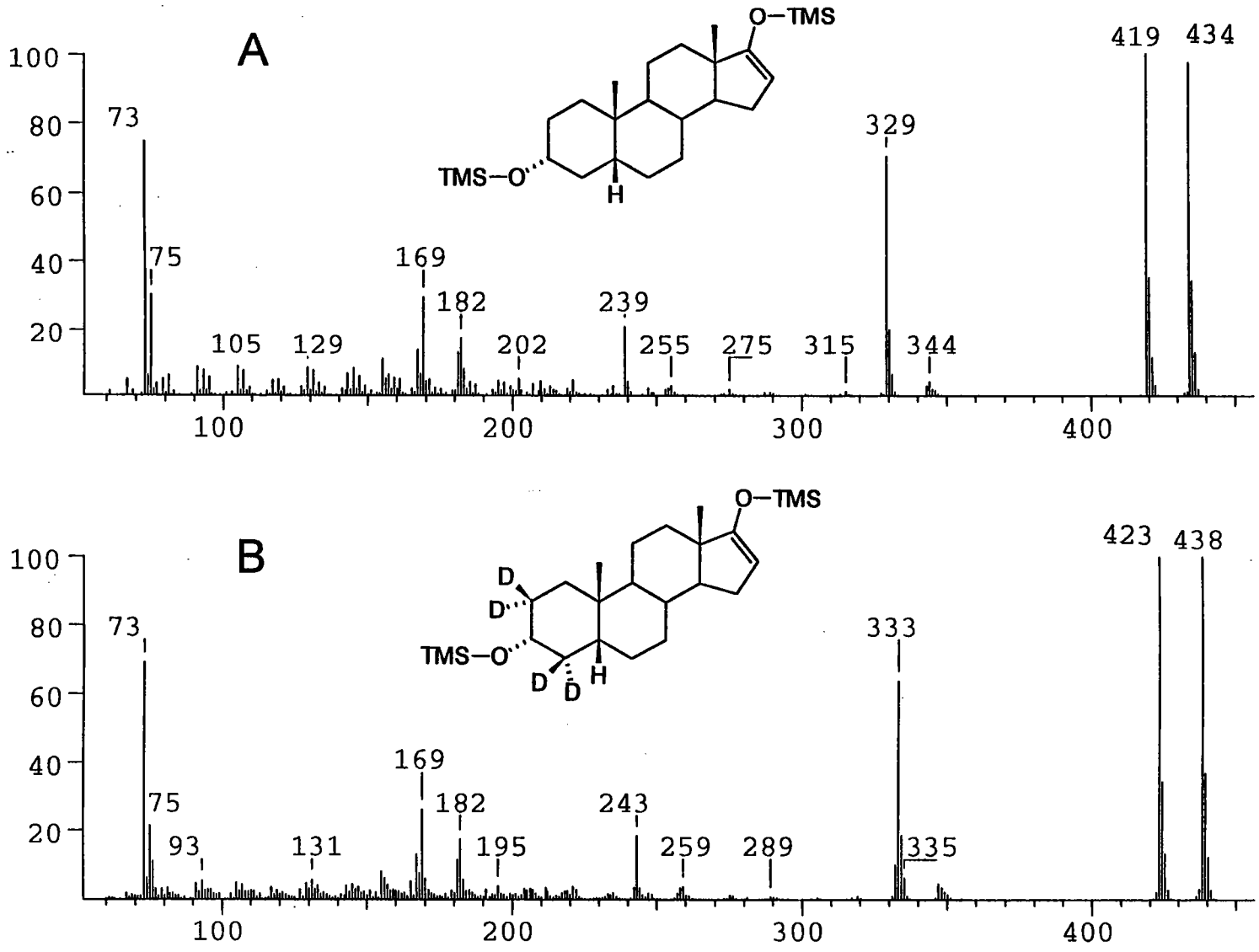


Fig.9 EI mass spectra of A) etiocholanolone bis-TMS and B) [2,2,4,4-$^{2}H_4$]-etiocholanolone bis-TMS

[2,2,4,4-$^2H_4$]-11ß-Hydroxyandrosterone 5 (Fig.10)

1. *5α-Androstane-3,11,17-trione* **26** and *5ß-androstane-3,11,17-trione* **27**: 24 g (80 mmol) of adrenosterone **25** was dissolved in 400 ml of methanol, 150 ml of dichloromethane and reduced with hydrogen using 20 mg of platinum dioxide as catalyst. The reaction was stopped when more than 98% of adrenosterone was reduced. The reaction yielded 40% of 5α-androstane-3,11,17-trione **26**, 42.5% of 5ß-androstane-3,11,17-trione **27**, 1.5% of unchanged adrenosterone **25** and 16% of side products.

2. *5α-Androstane-3,11,17-trione 3,17-diethyleneketal* **28**: The reaction solution of 1. was evaporated to dryness, dissolved in 150 ml of benzene, 30 ml of ethylene glycol, 75 mg of p-toluenesulfonic acid and refluxed over night using a water extractor. The reaction products were concentrated and tried to separate via silica gel (3 x 40 cm column) using ethyl acetate/n-pentane (50:50, v:v) as solvent. The 5α- **28** and 5ß-isomeres **29** were not separated. The fractions containing both isomers were concentrated to dryness and crystallized from methanol.yielding 7.8 g of 5α-androstane-3,11,17-trione 3,17-diethyleneketal **28** (98% purity, GC/MS analysis).

3. *11ß-Hydroxy-5α-androstane-3,17-dione 3,17-diethyleneketal* **30**: 7.8 g of 5α-Androstane-3,11,17-trione 3,17-diethyleneketal **28** was dissolved in 50 ml of dichloromethane, 200 ml of diethyl ether and added slowly with 1 g of lithium aluminium hydride while stirring. After 30 min the reaction mixture was diluted with 300 ml of water and the reduced product was extracted with 1000 ml of diethyl ether. The organic phase was concentrated to dryness. The reduction yielded with over 95% the 11ß-hydroxy-5α-androstane-3,17-dione 3,17-diethyleneketal **30**.

4. *11ß-Hydroxy-5α-androstane-3,17-dione* **31**: The ethyleneketal groups of 11ß-hydroxy-5α-androstane-3,17-dione 3,17-diethyleneketal **30** were hydrolyzed by 30 min refluxing in 150 ml of 30% acetic acid. The reaction mixture was evaporated to dryness, the reaction products were dissolved in 1000 ml of diethyl ether and washed with 200 ml of 2% potassium hydroxide and two times with 200 ml of bidestilled water. The organic phase was evaporated and dried over potassium hydroxide/phosphorus pentoxide under reduced pressure yielding 5.2g of 11ß-hydroxy-5α-androstane-3,17-dione **31** (96.5% purity, GC-MS analysis).

5. *[2,2,4,4,16,16-$^2H_6$]-11ß-Hydroxy-5α-androstane-3,17-dione* **32**: 5 g of 11ß-hydroxy-5α-androstane-3,17-dione **31** was dissolved in 50 ml of methyl alcohol-d, 50 ml of dichloromethane, 10 ml of deuterium oxide, 0.1 ml of 40% sodium deuteroxide in D_2O and refluxed over night. The reaction mixture was concentrated to dryness and the deuterium exchange repeated using the same conditions. The reaction yielded [2,2,4,4,16,16-$^2H_6$]-11ß-hydroxy-5α-androstane-3,17-dione **32** which was not further purified but dried over potassium hydroxide/phosphorus pentoxide under reduced pressure.

6. *[2,2,4,4,16,16-$^2H_6$]-3α,11ß-Dihydroxy-5α-androstan-17-one* **33**: 4.5 g of dried [2,2,4,4,16,16-$^6H_2$]-11ß-hydroxy-5α-androstane-3,17-dione **32** was reduced with K-Selektride in portions to minimize a possible reexchange of deuterium against hydrogen in the time between adding of the reagent and reduction of the 3-keto group: 4.5 g of **32** held under argon was dissolved in 10 ml of dichloromethane, 30 ml of diethyl ether (freshly distilled over lithium hydride). 4 ml portions of this solution were used and added with 1.4 ml of 1M K-Selektride in tetrahydrofurane within 5-10 seconds while stirring. The fractions were combined, added with 200 ml of bidistilled water and the reduced products were extracted with 800 ml of diethyl ether. The organic phase was concentrated to dryness yielding 76% of [2,2,4,4,16,16-$^2H_6$]-3α,11ß-dihydroxy-5α-androstan-17-one **33**, 11% of [2,2,4,4,16,16-$^2H_6$]-3ß,11ß-dhydroxy-5α-androstan-17-one, 3% of [2,2,4,4,16,16-$^2H_6$]-5α-androstan-3α,11ß,17ß-triol and 11% of further side products.

7. *[2,2,4,4-$^2H_4$]-11ß-Hydroxyandrosterone* **5**: The crude residue of reaction 6 containing [2,2,4,4,16,16-$^2H_6$]-3α,11ß-dihydroxy-5α-androstan-17-one **33** was dissolved in 30 ml of dichloromethane, 50 ml of methanol, 10 ml of bidestilled water, 1 ml of 5N potassium hydroxide and refluxed over night. The reaction exchanged the deuterium atoms at C-16. The solution was diluted with 200 ml of water and extracted with 1000 ml of diethyl ether. The ether phase was concentrated to dryness and the reaction products were separated via silica gel (3 x 40 cm column) using n-pentane/ethyl acetate (20:80, v:v) as solvent. The fractions containing the deuterated 11ß-hydroxyandrosterone were combined and crystallized from 50 ml of methanol yielding 1.09 g of 95% [2,2,4,4-$^2H_4$]-11ß-hydroxy-androsterone **5** (yield 4.2%), EI-spectrum of the tris-TMS derivative see Fig.11B and deuteration yield see Table 1.

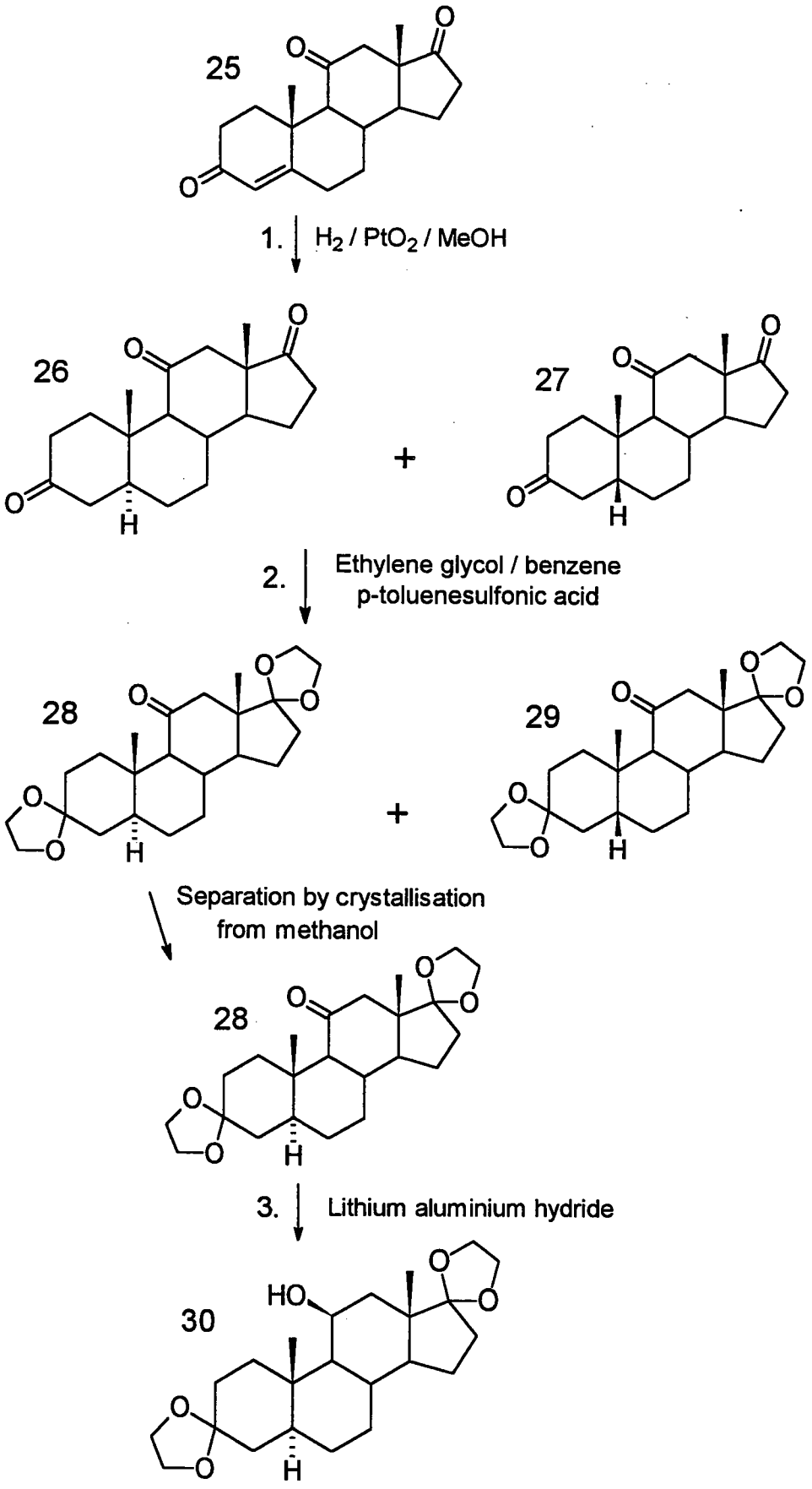
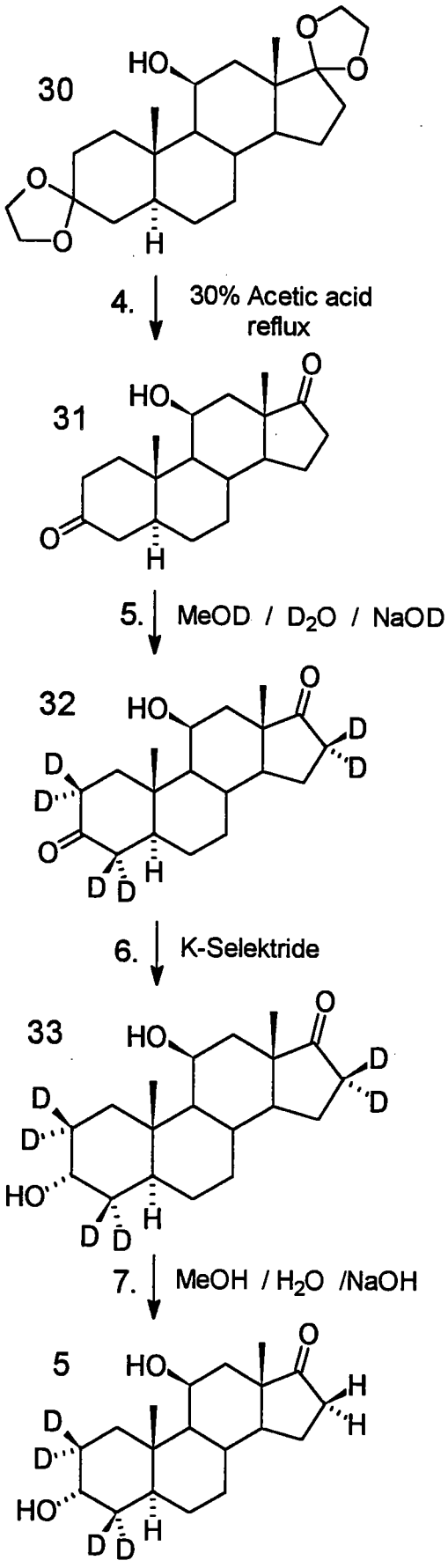


Fig. 10 Synthesis of [2,2,4,4-$^2H_4$]-11ß-hydroxyandrosterone **5**, continue next page



continue Fig. 10 Synthesis of [2,2,4,4-$^{2}H_4$]-11ß-hydroxyandrosterone **5.**

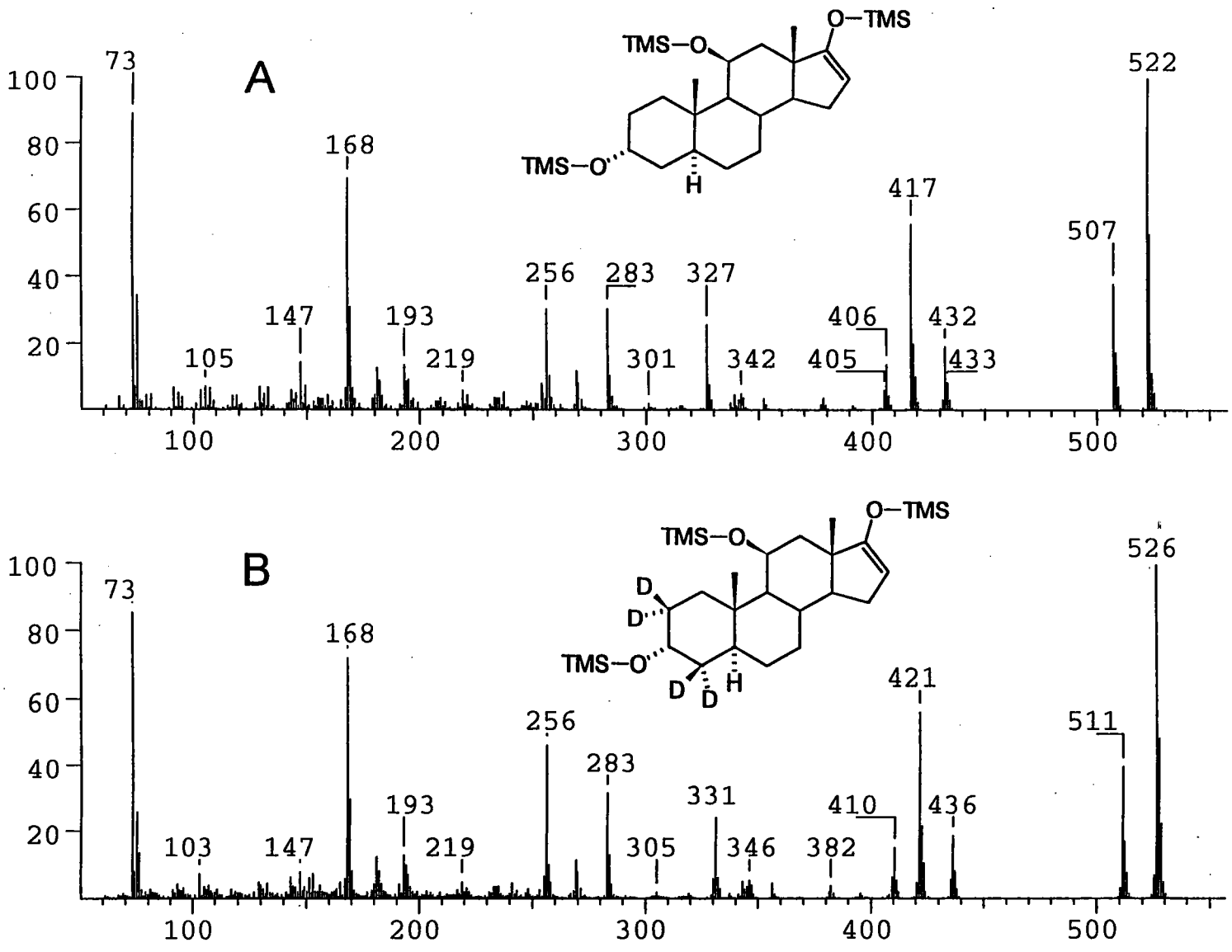


Fig.11 EI mass spectra of A) 11ß-hydroxyandrosterone tris-TMS and B) [2,2,4,4-$^{2}H_4$]-11ß-hydroxyandrosterone tris-TMS

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