Selective manipulation of steroid hydroxyl groups with boronate esters: efficient access to antigenic C-3 linked steroid–protein conjugates and steroid sulfate standards for drug detection†‡

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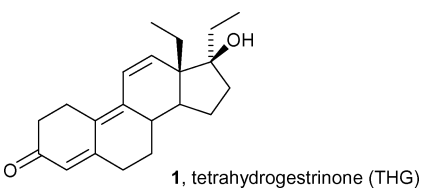
The temporary protection of 17α-alkyl-5α-androstane-3β,16β,17β triols as boronate esters is an efficient method for their regioselective functionalisation. This has been applied to the synthesis of protein–steroid conjugates **7–10** suitable for the development of immunoassays targeting classes of steroids banned from competition in Australian horse racing and other sports. The synthesis of steroids sulfate conjugates **42** and **44** for use as reference standards is also reported.

Introduction

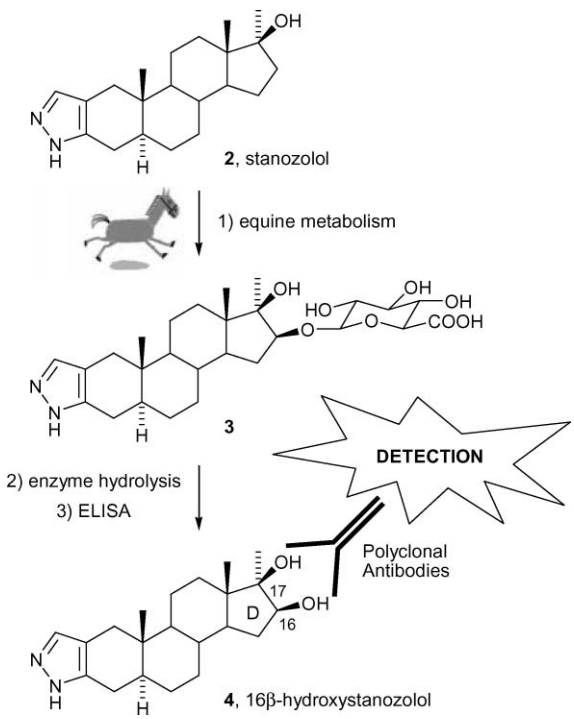
Anabolic androgenic steroids are an important class of performance enhancing drugs with potential for misuse in horse racing and other sport. As a result, the integrity of sporting contests relies on stringent doping control measures targeting these agents. Despite ongoing research, the detection of illicit steroid use presents significant challenges due to a range of complicating factors. Among these, the administration of anabolic steroids frequently results in little or no excretion of the parent steroid in the urine and instead, the steroid is converted into more hydrophilic metabolites. The detection of steroid abuse therefore requires appropriate reference materials and methods for the detection of the metabolites derived from known steroidal agents. Of greater concern, in 2003, the previously unknown anabolic steroid tetrahydrogestrinone **1** (THG, Fig. 1)[1] was uncovered as a doping agent at the highest levels of world sport. This so-called 'designer' steroid was conceived to evade standard methods of selected ion monitoring mass spectrometric detection and highlights the need for broader screening methods for the detection of drugs in sport.



1, tetrahydrogestrinone (THG)

Fig. 1

Recently, we described[2] an enzyme-linked immunosorbent assay (ELISA) based screen for the detection of 17α-methyl steroid metabolites in urine. Steroids with 17α-alkyl substituents are common agents of abuse in horse racing and other sports, owing to the oral bioavailability imparted by the alkyl substituent. In horses, these 17-alkyl anabolic steroids are extensively metabolised prior to excretion[3,4] and our approach was to target the D-ring structures common to many equine metabolites of these steroids. The application of this assay to the detection of equine stanozolol metabolites is depicted in Scheme 1. Stanozolol, **2**, administration leads to excretion of the 16β-hydroxystanozolol glucuronide conjugate **3** as the major phase II metabolite.[3] Enzyme hydrolysis liberates the 16β-hydroxystanozol **4** which is then detected in the raw hydrolysed urine by an ELISA employing polyclonal antibodies targeting the D-ring of the steroid.[2] The assay has been deployed by the Australian Racing Forensic Laboratory as



Scheme 1

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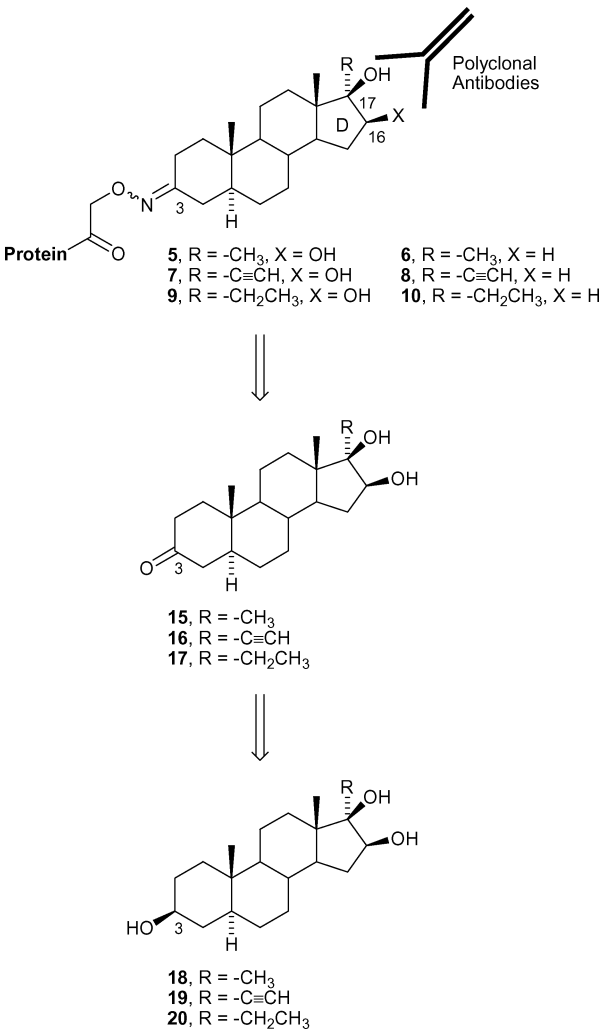
† Electronic supplementary information (ESI) available: General experimental details together with experimental procedures and spectroscopic data for compounds **15**, **19**, **20**, **26**, **30–40**, **44** and **47**. See DOI: 10.1039/b610499a

‡ The HTML version of this article has been enhanced with colour images

a primary screen for the detection of stanozolol metabolites in race day samples.

The ELISA-based methods of drug detection have a number of positive attributes. The assays do not routinely require sample extraction and derivatisation procedures and are readily conducted in array format leading to significant efficiencies. More importantly, ELISA is a broad screen with the potential to detect families of steroid metabolites that contain a common structural motif (such as the hydroxylated D-ring of metabolite **4**). ELISA thus has significant promise as a forensic tool for the early detection of previously unknown steroids or their metabolites.

The success of the reported ELISA targeting 17α-methyl steroids suggested a number of avenues for further research. Our first goal was the development of assays targeting other commonly occurring D-ring substitution patterns associated with World Anti-Doping Agency (WADA) and International Federation of Horseracing Authorities (IFHA) prohibited anabolic steroids such as 17α-ethyl and 17α-ethynyl steroids. This required the development of polyclonal antibodies raised against these D-ring motifs, which in turn required extension of the previously reported methods for the synthesis of protein–steroid antigens **5** and **6**[2] to derivatives **7–10** bearing different D-ring substituents (Scheme 2). It was anticipated that 17α-ethyl antigens **9** and **10** would afford antibodies suitable for the detection of prohibited steroids such as ethylestrenol **11**, norethandrolone **12** (Fig. 2) and their 16β-hydroxylated metabolites.[3] The 17-ethynyl antigens **7** and **8** would afford antibodies suitable for the detection of danazol **13** and its putative metabolites. The antibodies could also show some selectivity for the detection of 18-homo steroids such as THG **1** and gestrinone **14**.



Scheme 2

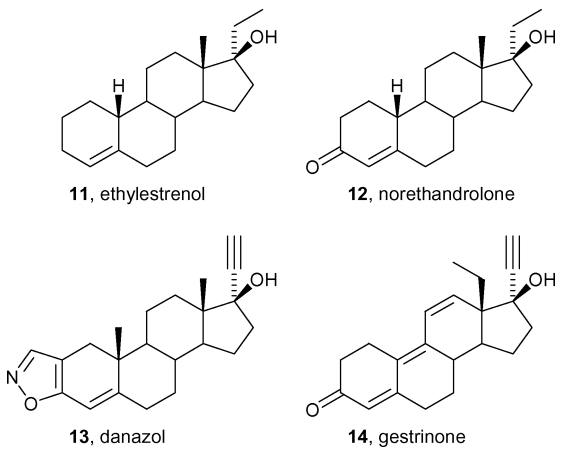


Fig. 2 WADA and IFHA prohibited anabolic steroids.

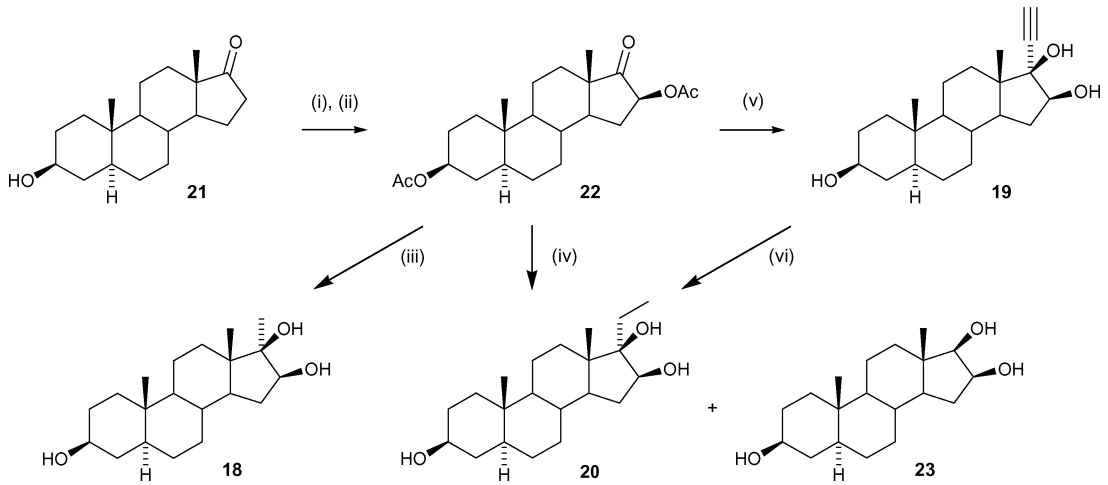
A second and related goal of this study was to improve synthetic access to the antigenic steroid–protein conjugates **5**, **7** and **9**. The 3-keto steroids **15–17** were key intermediates required for conjugate synthesis and could be derived from the steroid triols **18–20** by selective oxidation of the C3-hydroxyl group. In the reported synthesis of **15**, the multi-step oxidation sequence (**18** → **15**) suffered from the formation of isomeric steroids, necessitating HPLC separation to afford pure 3-keto steroid **15**.[2] In addition to the development of antibodies already reported, the conjugate **5** was required as a reagent for the ELISA and this bottleneck to the large-scale synthesis potentially limited the future application of the successful assay. Furthermore, these difficulties also posed problems for the synthesis of the new steroid antigens **7** and **9**.

This paper reports an improved synthesis of protein–steroid conjugates **7–10** suitable for the development of ELISA targeting 17α-ethyl and 17α-ethynyl steroids. The synthesis hinged on the development of effective boronate ester-mediated strategies for the selective functionalisation to afford steroid ketones **15–17**. As a further demonstration of the scope of this chemistry, the boronate ester methodology is also employed for the selective synthesis of steroid sulfate reference materials. Such steroid sulfates are of interest as reference materials for phase II anabolic steroid metabolites commonly observed following steroid administration.

Results and discussion

Synthesis of 16β,17β-dihydroxy-17α-alkyl-3-keto-steroids for ELISA development

Synthesis of these 17α-alkyl-5α-androstane-3β,16β,17β-triols (17α-alkyl-triols, **18–20**) was achieved starting from commercially available epiandrosterone **21**. The diacetoxyketone **22** was synthesised in two steps by enol acetate formation followed by lead tetraacetate oxidation. The addition of excess methylmagnesium bromide afforded 17α-methyl-triol **18** as previously described



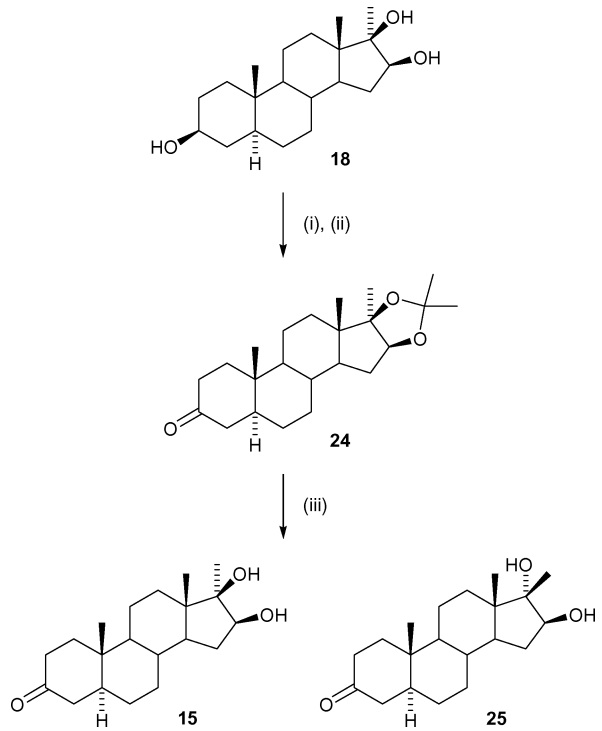
Scheme 3 Reagents and conditions: (i) isopropenyl acetate, cat. H_2SO_4, 80% (ii) $Pb(OAc)_4$, AcOH, Ac_2O, 70% (iii) MeMgBr, Et_2O, then H^+, 97% (iv) EtMgBr, Et_2O, then H^+ (v) HC≡CLi·en, THF, then H^+, 71% (vi) Pd/C, MeOH, H_2, 96%.

(Scheme 3).[5,6] This route was then adapted for the synthesis of ethynyl- and ethyl-substituted steroids **19** and **20**. Attempted treatment of ketone **22** with ethylmagnesium bromide afforded the desired 17α-ethyl-triol **16** together with the reduction product **23**, arising from hydride transfer from the Grignard reagent β-carbon.[7] The triols **20** and **23**, were not readily separable by flash chromatography. A convenient alternative was found in the addition of commercially available lithium acetylide–ethylenediamine complex to give the 17α-ethynyl-triol **19**, as a single diastereomer. Catalytic hydrogenation then gave access to 17α-ethyl-triol **20** in 96% yield, as shown in Scheme 3.

Elaboration of these triols **18–20** to 16β,17β-dihydroxy-5α-androstan-3-ones (17α-alkyl-3-ketones, **15–17**) proved more challenging. This required selective manipulation of the C-3 hydroxyl group in 17α-alkyl-triols **18–20**. Selective protection of the vicinal diol in steroid **18** as the acetonide was followed by oxidation to ketone **24** (Scheme 4). Subsequent access to 17α-methyl-ketone **15** required acetonide deprotection under acidic conditions to furnish the 16β,17β-diol array.[2] Unfortunately, the acid promoted deprotection of acetonide **24** under a wide range of conditions provided mixtures of epimers **15** and **25**. Separation of epimers was only partially achieved by flash chromatography, and HPLC separation was required.

To increase efficiency of the synthesis and to avoid the acidic deprotection conditions and C17 epimerisation, exchange of the acetonide protecting group for a benzylidene acetal protecting group was investigated. The 17α-methyl-triol **18** was treated with benzaldehyde dimethyl acetal in the presence of concentrated sulfuric acid to give the acetal **26**, as a single diastereomer (Scheme 5). NOESY experiments were used to assign the stereochemistry at the newly formed acetal stereocentre. Parikh–Doering[8] oxidation conditions gave higher yields (78%) for the conversion of the 3-hydroxyl group to the corresponding ketone. Benzylidene deprotection was then achieved, without epimerisation, with $Pd(OH)_2$ under a hydrogen atmosphere, to give the desired triol **15** exclusively, which was isolated in 93% yield.

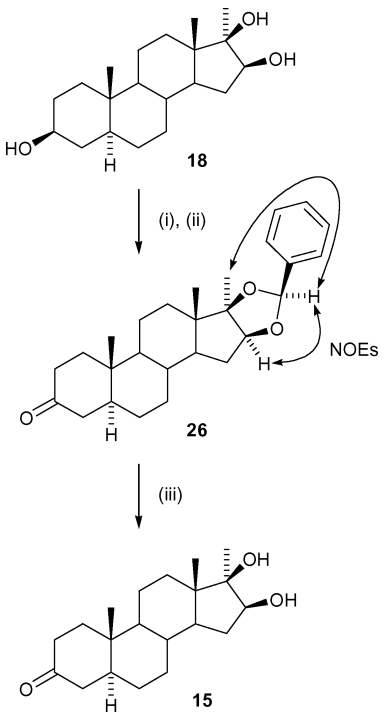
While the benzylidene protecting group conveniently gave access to 17α-methyl-ketone **15**, and would be suitable for the preparation of 17α-ethyl-ketone **17**, the methodology would not



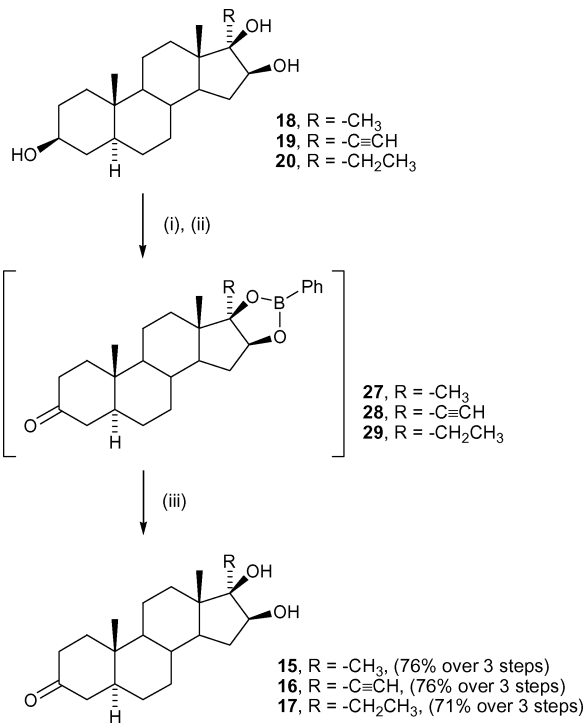
Scheme 4 Reagents and conditions: (i) 2,2-dimethoxypropane, *p*TsOH, CH_2Cl_2, 74% (ii) CrO_3, py, 70% (iii) Dowex 50W-X8, MeOH–H_2O (1 : 1), 70%.

be appropriate for the conversion of 17α-ethynyl-triol **19** to the corresponding 17α-ethynyl-ketone **16**, as the hydrogenolysis would be incompatible with an alkyne. Hence, a more general method was sought.

The use of boronate esters as protecting groups for diols has been reviewed,[9] with many applications to carbohydrate synthesis and the regioselective manipulation of hydroxyl groups. Appealing features include the ease of protection/deprotection and *in situ* derivatisation which minimise the number of reaction and purification steps. Such features would be well suited to steroid hydroxyl group manipulation. Notably, Harmatha and



Scheme 5 Reagents and conditions: (i) $C_6H_5CH(OCH_3)_2$, conc. H_2SO_4, DMF, CH_2Cl_2, 95% (ii) $Py{\cdot}SO_3$, Et_3N, 4 Å mol. sieves, DMSO, CH_2Cl_2, 78% (iii) H_2, $Pd(OH)_2$, THF, 93%.



Scheme 6 Reagents and conditions: (i) $PhB(OH)_2$, DMF, CH_2Cl_2, 4 Å mol. sieves (ii) PCC/Al_2O_3 (iii) H_2O_2, NaOH(aq), THF.

co-workers[10] utilised phenylboronic acid to selectively protect an ecdysteroid side-chain vicinal diol in the presence of a more rigid A-ring 2β,3β-diol. This enabled subsequent derivatisation of the remaining secondary hydroxyl groups as acetate esters.

In the present case, the use of phenylboronic acid to protect the 16β,17β-diol unit in 17-alkyl-triols **18–20** would require boronate ester formation on the rigid D-ring allowing for selective oxidation of the 3-hydroxyl to give ketones **15–17**. The sequential boronate ester formation and *in situ* oxidation followed by oxidative removal of the boronate ester, would provide an expedient alternative to the step-wise strategies, and associated purifications, described above (Schemes 4 and 5) for 17α-methyl-ketone **15**. In the event, treatment of **18** with phenylboronic acid (Scheme 6), in DMF–dichloromethane in the presence of molecular sieves, followed by direct oxidation at C-3, by addition of pyridinium chlorochromate (PCC) on alumina,[11] gave intermediate **27**. Oxidative removal of the boronate ester with sodium hydroxide and hydrogen peroxide, then gave **15** exclusively, in 76% yield over the 3 steps. The basic deprotection conditions avoided any epimerisation problems and 16β-hydroxymestanolone **15** was prepared in an extremely clean, rapid, and efficient sequence that compares favourably to the 69% yield obtained for the three step sequence *via* benzylidene acetal **26** (Scheme 5).

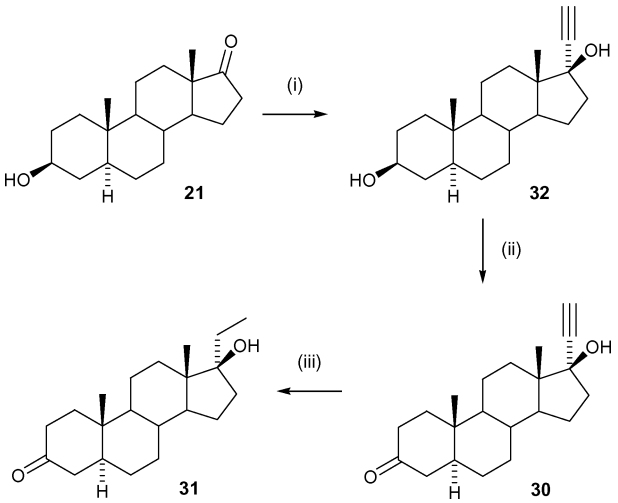
This approach was then applied to the conversion of 17α-ethynyl-triol **19** and 17α-ethyl-triol **20**, to ketones **16** and **17** respectively. In each case, intermediates **28** and **29** were formed by treatment with phenylboronic acid and *in situ* oxidation (PCC/Al_2O_3). Deprotection then ($NaOH–H_2O_2$) afforded ketones **16** and **17**, in 76% and 71% yield (over the 3 steps), respectively (Scheme 6). Again the deprotection conditions caused no epimerisation, particularly in the case of sensitive 17α-ethynyl-ketone **19**.[12]

The application of phenylboronate ester chemistry to trihydroxylated steroids provided regioselective access to ketones **15–17**, ready for elaboration to conjugates **5–9**. Efficient protection, *in situ* and regioselective oxidation, followed by deprotection rapidly afforded the desired dihydroxylated 3-keto-steroids. The chemistry was readily scaleable and did not result in the production of stereoisomeric steroids nor require HPLC to afford pure compound.

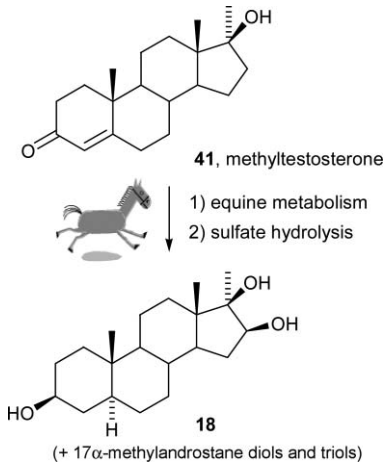
Synthesis of 17β-hydroxy-17α-alkyl-3-keto-steroids for ELISA development

A major finding of previous studies was that antibodies generated against 16β,17β-dihydroxy-17α-alkyl-antigens such as **5** were more selective and sensitive than antibodies generated against the parent 17β-hydroxy-17α-alkyl-antigens such as **6** (Scheme 2).[2] This provides significant advantages in the development of ELISA for drug detection. Antibodies raised against antigen **5** were used for the detection of stanozolol metabolites in raw enzyme hydrolysed urine. In contrast, antibodies raised against antigen **6** are used for the detection of methandriol metabolites but required solid phase extraction to remove interferences presumed to arise from endogenous compounds. To explore the generality of this phenomenon the synthesis of additional mono-hydroxylated 17α-ethynyl and 17α-ethyl-antigens **8** and **10** was required.

Epiandrosterone **21** was treated with excess lithium acetylide–ethylenediamine complex, giving alkyne **32** in 94% yield (Scheme 7). Oxidation of the 3-hydroxyl group was achieved cleanly using Parikh–Doering conditions[8] to give target 17α-ethynyl-ketone **30** in 75% yield. Catalytic hydrogenation (Pd/C, H_2, $NaHCO_3$) afforded corresponding 17α-ethyl-ketone **31**, in 65% yield.



Scheme 7 Reagents and conditions: (i) HC≡CLi·en, THF, then H^+, 94% (ii) Py·SO_3, Et_3N, DMSO, CH_2Cl_2, 4 Å mol. sieves, 75% (iii) H_2, Pd/C, $NaHCO_3$, 65%.



Scheme 9

Synthesis of C-3 linked protein-steroid conjugates

Conversion of ketones **16**, **17**, **30** and **31** to steroid–protein conjugates **7–10** is summarised in Scheme 8.[2] Treatment of the ketones with carboxymethoxylamine hemihydrochloride, gave the corresponding carboxymethyloximes **33–36**. Subsequent reaction with *N*-hydroxysuccinimide and 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) yielded the activated esters **37–40** respectively. Standard techniques then provided access to steroid–protein conjugates **7–10**, with the steroid linked to the lysine residues of human serum albumin to provide the antigenic material for antibody generation.[2] The development of antibodies and their application to the development of ELISA is in progress using previously reported methods and will be reported elsewhere.

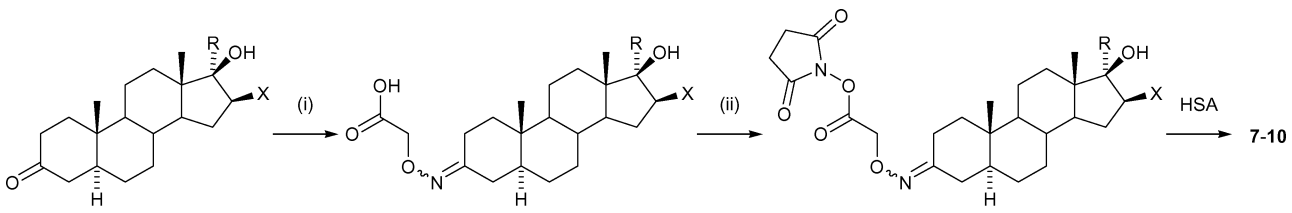
Synthesis of steroid sulfate reference materials

Steroid sulfate esters are commonly observed phase II metabolites that are derived from polyhydroxylated steroids.[13] The charged sulfate residue renders the hydrophobic steroid more soluble in aqueous environments and more readily excreted in the urine. An example of steroidal metabolism is given in Scheme 9. In recent work, the administration of methyltestosterone **41** to a thoroughbred gelding resulted in the excretion of a number of androstane diol and triol metabolites as their sulfate or glucuronide conjugates.[14] These were identified by a process of conjugate hydrolysis to give the free steroid, followed by derivatisation and GCMS analysis against synthetically derived reference standards. Of relevance to this work, the 17α-methyl triol **18** was identified as a major steroidal metabolite occurring as the sulfate conjugate.

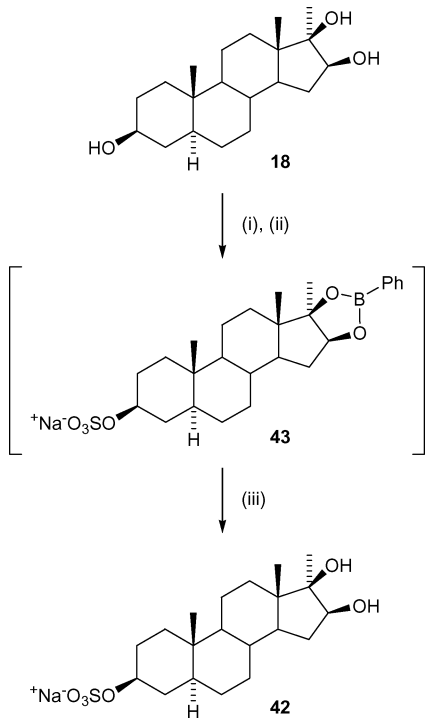
The routine observation of sulfate esters as metabolites makes the synthesis of these compounds highly desirable as reference standards that allow each step of the analytical procedure, including sulfate ester hydrolysis, to be monitored. In the case of 17α-methyl triol **18**, this also raised the question of which hydroxyl group of the steroid carried the sulfate ester residue. Evidence based on the configurational stability of the C17 stereogenic centre suggested the sulfate was not conjugated to the tertiary hydroxyl group.[14]

The ability to regioselectively manipulate the hydroxyl groups in steroid triols, through boronate ester protection of the steroid 16,17-diol, would also enable selective and rapid access to steroid sulfate derivatives. Previously, Vasella and co-workers[15] employed phenylboronic acid to protect the 4,6-diol of a methyl glycoside, with subsequent stannylidene activation of the remaining diol enabling regioselective sulfation.

As shown in Scheme 10, the flexibility of the boronate ester approach, as applied to anabolic steroid metabolites, is demonstrated by the selective conversion of triol **18** to 3-sulfate derivative **42**. Diol protection and sulfation are rapidly achieved in the one-pot, giving intermediate **43**. Oxidative phenylboronate deprotection then provided the desired 3-sulfate **42** in 79% yield, over the 3 steps.



Scheme 8 Reagents and conditions: (i) $(NH_2OCH_2CO_2H)_2$·HCl, py (ii) EDC, 1,4-dioxane, CH_2Cl_2, *N*-hydroxysuccinimide.



Scheme 10 Reagents and conditions: (i) $PhB(OH)_2$, DMF, CH_2Cl_2, 4 Å mol. sieves (ii) SO_3·py, DMF (iii) H_2O_2, aq. $NaHCO_3$, THF, MeOH, 79% (over 3 steps).

The alternative steroid 16-sulfate **44** could also be prepared selectively as shown in Scheme 11. Treatment of triol **18** with phenylboronic acid and *in situ* protection[16] of the 3-hydroxyl group by addition of *tert*-butyldimethylsilyl (TBS) chloride and imidazole gave intermediate **45**. Oxidative removal of the boronate ester then gave diol **46** in 73% yield, over the 3 steps. Treatment of diol **46** with excess sulfur trioxide–pyridine complex, and excess pyridine, for 50 minutes, then resulted in regioselective sulfation of the remaining secondary hydroxyl group at C16 to give protected sulfate **47**. The tetrabutylammonium fluoride (TBAF) mediated silyl ether deprotection of sulfate **47** was unsuccessful, with excess TBAF resulting only in formation of the tetrabutylammonium salt of the sulfate and retention of the TBS protecting group. However, TBS deprotection was effected with 80% acetic acid in water, over 1 hour.[17] These conditions were sufficiently mild to retain the acid labile sulfate group, and 16-sulfate **44** was obtained in 69% yield.

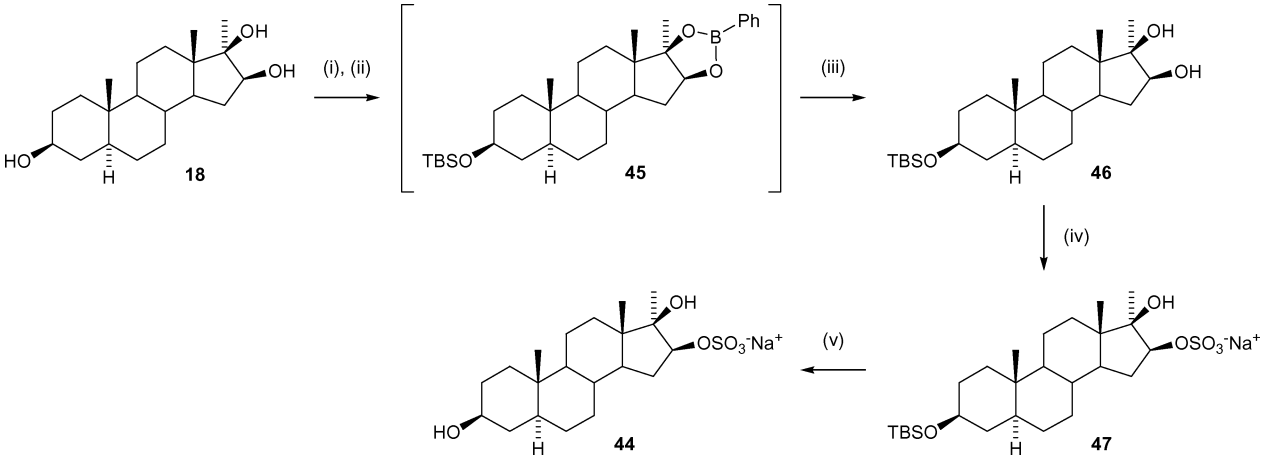
Both sulfate standards were used in a recent study of the phase II metabolites derived from methyltestosterone in a drug administration trial. Both the C3-sulfate **42** and C16-sulfate **44** displayed retention time matches and mass spectrometric behaviour consistent with the presence of significant sulfate metabolites.[18] Unfortunately, due to inadequate chromatographic resolution and the likely presence of a number of isomeric sulfated steroid triols that were not targeted for synthesis by this study, it was not possible to unambiguously confirm the presence of these sulfate compounds in urine. Nevertheless, the chemistry highlights the application of boronate esters in the synthesis of regioisomeric sulfate standards.

Experimental

General experimental details together with experimental procedures and spectroscopic data for compounds **15**, **19**, **20**, **26**, **30–40**, **44** and **47** have been deposited in ESI.†

Boronate ester mediated oxidation

16β,17β-Dihydroxy-17α-methyl-5α-androstan-3-one (β-hydroxymestanolone) (15). Method 1. 17α-Methyl triol **18**[2] (0.410 g, 1.27 mmol) was stirred (**18** was partially insoluble) in a mixture of DMF–CH_2Cl_2 (1 : 1, 2.4 mL). Phenylboronic acid (0.248 g, 2.04 mmol) was added, followed by 4 Å molecular sieves (20). After 15 minutes, no starting material remained insoluble, and after 1 hour, TLC (ethyl acetate–hexane, 1 : 1) showed complete conversion of starting material (R_f 0.2) to the corresponding boronic ester (R_f 0.5). Additional CH_2Cl_2 (2 mL) was added, followed by pyridinium chlorochromate (PCC)/alumina[11] (3.18 g, 25% w/w) and 4 Å molecular sieves (10). The reaction mixture was stirred for 20 hours after which time TLC (ethyl acetate–hexane, 1 : 1) showed complete conversion to intermediate product **27** (R_f 0.7). The reaction mixture was diluted with Et_2O (20 mL) and filtered through a pad of silica topped with celite, with Et_2O



Scheme 11 Reagents and conditions: (i) $PhB(OH)_2$, DMF, CH_2Cl_2, 4 Å mol. sieves (ii) TBSCl, imidazole (iii) H_2O_2, aq. NaOH, THF, 73% (over 3 steps) (iv) SO_3·py, DMF, py, 4 Å mol. sieves, 61% (v) 80% AcOH–H_2O, 69%.

(500 mL) elution. Concentration *in vacuo* gave a colourless foam which was directly dissolved in THF (12 mL) and treated with H_2O_2 (30% aqueous solution, 3.2 mL) and NaOH (12% aqueous solution, 2 mL) for 15 minutes. TLC (ethyl acetate–hexane, 1 : 2) suggested the deprotection to be complete. The reaction mixture was diluted with H_2O (70 mL) and extracted into ethyl acetate (4 × 30 mL). The combined ethyl acetate extracts were washed with saturated Na_2SO_3 solution (20 mL), saturated NaCl solution (20 mL), then dried (Na_2SO_4) and concentrated *in vacuo.* The residue was dissolved in CH_2Cl_2–MeOH and pre-adsorbed onto silica for flash chromatography (ethyl acetate–hexane, 1 : 2 then 1 : 1), which afforded β-hydroxymestanolone (**15**) (0.308 g, 76%). R_f 0.2 (ethyl acetate–hexane, 1 : 1); mp 185–186 °C (lit.[2] 185–186 °C) $[\alpha]_D^{20}$ +8.4 (*c* 1.0, CH_2Cl_2) (lit.[2] $[\alpha]_D^{20}$ +8.4 (*c* 1.0, CH_2Cl_2)); ν_{max}/cm^{-1} (film) 3600–3000 (OH), 2939, 2920, 2854, 1713 (C=O), 1447, 1381, 1356, 1271, 1219, 1175, 1124, 1067, 1040, 1024; δ_H (200 MHz, $CDCl_3$) 3.64 (1H, dd, *J* 7.9, 5.4 Hz, $C_{16}H$), 3.00–2.80 (1H, br s, OH), 2.80–2.60 (1H, br s, OH), 2.48–1.92 (6H, m), 1.78–0.63 (14H, m), 1.11 (3H, s, $C_{20}H$), 1.01 (3H, s, $C_{18}H$), 0.86 (3H, s, $C_{19}H$); δ_C (50 MHz, $CDCl_3$) 212.0, 79.1, 77.6, 53.9, 46.9, 46.7, 44.9, 44.6, 38.5, 38.1, 35.7, 35.6, 34.8, 32.3, 31.5, 28.7, 23.7, 20.8, 13.5, 11.4; *m/z* (EI+) 320.2350 (M^+, $C_{20}H_{32}O_3$ requires 320.2351, 85%), 232 (60), 217 (100), 159 (45).

16β,17β-Dihydroxy-5α,17α-pregn-20-yn-3-one (16). 17α-Ethynyl triol **19** (0.107 g, 0.32 mmol) was stirred (**19** was partially insoluble) in CH_2Cl_2 (1.0 mL). Phenylboronic acid (0.062 g, 0.51 mmol) was added, followed by 4 Å molecular sieves (10). After 5 minutes, the starting material had dissolved, and after 1 hour, TLC (ethyl acetate–hexane, 1 : 1) showed complete conversion of starting material (R_f 0.3) to the corresponding boronic ester (R_f 0.5). Additional CH_2Cl_2 (1 mL) was added, followed by pyridinium chlorochromate (PCC)/alumina[11] (1.2 g, 25% w/w). The reaction mixture was stirred for 3 hours after which time TLC (ethyl acetate–hexane, 1 : 1) showed complete conversion to intermediate product **28** (R_f 0.8). The reaction mixture was diluted with Et_2O (15 mL) and filtered through a pad of silica topped with celite, with Et_2O (80 mL) elution. Concentration *in vacuo* gave a colourless foam which was directly dissolved in THF (3 mL) and treated with H_2O_2 (30% aqueous solution, 0.8 mL) and NaOH (12% aqueous solution, 0.5 mL) for 15 minutes. TLC (ethyl acetate–hexane, 1 : 2) suggested the deprotection to be complete. The reaction mixture was diluted with H_2O (70 mL) and extracted into ethyl acetate (5 × 20 mL). The combined ethyl acetate extracts were washed with saturated Na_2SO_3 solution (20 mL), saturated NaCl solution (20 mL), then dried (Na_2SO_4) and concentrated *in vacuo.* The residue was dissolved in CH_2Cl_2–MeOH and pre-adsorbed onto silica for flash chromatography (ethyl acetate–hexane, 1 : 3 then 1 : 1), which afforded diol **16** (0.081 g, 76%). R_f 0.5 (ethyl acetate–hexane, 1 : 1); mp 253–256 °C; $[\alpha]_D^{22}$ −18.9 (*c* 0.19, CH_2Cl_2–MeOH, 4 : 1); ν_{max}/cm^{-1} (film) 3550–3200 (OH), 3238 (≡C–H), 2918, 2106 (C≡C), 1703 (C=O), 1150; δ_H (300 MHz, $CDCl_3$–MeOD, 3 : 1) 3.93–3.85 (1H, m, H16), 2.39 (1H, s, ≡CH), 2.26–1.94 (4H, m), 1.87–1.76 (2H, m), 1.56–0.49 (14H, m), 0.80 (3H, s, CH_3), 0.60 (3H, s, CH_3); δ_C (75 MHz, $CDCl_3$–MeOD, 3 : 1) 213.5, 85.7, 77.4, 76.5, 74.0, 53.2, 46.3, 45.7, 44.1, 38.1, 37.6, 35.3, 35.0, 34.0, 33.2, 31.0, 28.3, 20.4, 12.2, 10.8; *m/z* (EI+) 330.2187 (M^+, $C_{21}H_{30}O_3$ requires 330.2195. 8%), 312 (15), 297 (38), 231 (54), 217 (62), 173 (65), 159 (56), 119 (55), 105 (63), 91 (100), 79 (82).

16β,17β-Dihydroxy-5α,17α-pregnan-3-one (17). 17α-Ethyl triol **20** (0.350 g, 1.04 mmol) was stirred (**20** was partially insoluble) in CH_2Cl_2 (3.0 mL). Phenylboronic acid (0.203 g, 1.7 mmol) was added, followed by 4 Å molecular sieves (20). After 5 minutes, the starting material had dissolved, and after 1 hour, TLC (ethyl acetate–hexane, 1 : 1) showed complete conversion of starting material (R_f 0.33) to the corresponding boronic ester (R_f 0.6). Additional CH_2Cl_2 (2 mL) was added followed by pyridinium chlorochromate (PCC)/alumina[11] (3.3 g, 25% w/w). The reaction mixture was stirred for 3 hours after which time TLC (ethyl acetate–hexane, 1 : 1) showed complete conversion to intermediate product **29** (R_f 0.7). The reaction mixture was diluted with Et_2O (20 mL) and filtered through a pad of silica topped with celite, with Et_2O (100 mL) elution. Concentration *in vacuo* gave a colourless foam which was directly dissolved in THF (9 mL) and treated with H_2O_2 (30% aqueous solution, 2.4 mL) and NaOH (12% aqueous solution, 1.5 mL) for 1.25 hours. TLC (ethyl acetate–hexane, 1 : 2) suggested the deprotection to be complete. The reaction mixture was diluted with H_2O (210 mL) and extracted into ethyl acetate (4 × 50 mL). The combined ethyl acetate extracts were washed with saturated Na_2SO_3 solution (40 mL), saturated NaCl solution (50 mL), then dried (Na_2SO_4) and concentrated *in vacuo.* The residue was dissolved in CH_2Cl_2–MeOH and pre-adsorbed onto silica for flash chromatography (ethyl acetate–hexane, 1 : 3 then 1 : 2), which afforded diol **17** (0.248 g, 71%). R_f 0.20 (ethyl acetate–hexane, 1 : 2); mp 170–175 °C; $[\alpha]_D^{22}$ +3.4 (*c* 0.71, $CHCl_3$); ν_{max}/cm^{-1} (KBr disk) 3600–3000 (OH), 2927, 1710 (C=O), 1447, 1055, 999; δ_H (200 MHz, $CDCl_3$) 3.86–3.73 (1H, m, H16), 2.99 (1H, br d, *J* 4.1, 16-OH), 2.76 (1H, br s, 17-OH), 2.48–1.93 (6H, m), 1.76–0.60 (16H, m), 1.00 (3H, s, CH_3), 0.89 (3H, t, *J* 7.1, C_{21}–H_3), 0.84 (3H, s, CH_3); δ_C (50 MHz, $CDCl_3$) 212.2, 80.2, 72.8, 53.8, 46.6, 46.4, 45.8, 44.6, 38.5, 38.0, 35.7, 35.5, 35.2, 32.3, 31.6, 28.7, 27.1, 20.9, 14.2, 11.4, 7.2; *m/z* (EI+) 334.2515 (M^+, $C_{21}H_{34}O_3$ requires 334.2508, 25%), 247 (100), 229 (60), 217 (60), 159 (45), 91 (40).

Boronate ester mediated sulfation

Sodium 3β,16β,17β-trihydroxy-17α-methyl-5α-androstane 3-sulfate (42). 17α-Methyl triol **18**[2] (0.030 g, 0.093 mmol) was stirred (**18** was partially insoluble) in a mixture of DMF–CH_2Cl_2 (1 : 1, 0.3 mL). Phenylboronic acid (0.018 g, 0.15 mmol) was added, followed by 4 Å molecular sieves (5). After 10 minutes, additional CH_2Cl_2 (0.05 mL) was added, and after another 10 minutes, TLC (ethyl acetate–hexane, 1 : 1) showed complete conversion of starting material (R_f 0.2) to the corresponding boronic ester (R_f 0.5). Additional DMF (1 mL) was added, followed by sulfur trioxide–pyridine complex (0.044 g, 0.28 mmol). The reaction mixture was stirred for 70 minutes after which time TLC (ethyl acetate–hexane, 1 : 1) showed incomplete conversion. Additional sulfur trioxide–pyridine complex (0.024 g, 0.15 mmol) was added and stirring continued (30 minutes). TLC (CH_2Cl_2–MeOH, 4 : 1) showed a single spot (R_f 0.4) due to intermediate product **43**. The molecular sieves were removed and the reaction mixture was poured into saturated $NaHCO_3$ solution (30 mL), which was extracted with ethyl acetate (1 × 20 mL) and $CHCl_3$–^{i}PrOH (3 : 1, 3 × 20 mL). The organic extracts were dried (Na_2SO_4)

and concentrated. The residue obtained was directly dissolved in a mixture of THF–MeOH (2 : 1, 1.5 mL) and treated with H_2O_2 (30% aqueous solution, 0.23 mL) and saturated $NaHCO_3$ solution (0.17 mL) for 10 minutes. TLC (CH_2Cl_2–MeOH, 4 : 1) suggested the complete conversion to deprotected product (R_f 0.3). The reaction mixture was diluted with saturated Na_2SO_3 solution (30 mL) and extracted into $CHCl_3$–^{i}PrOH (3 : 1, 5 × 15 mL). The combined organic extracts were dried (Na_2SO_4) and concentrated *in vacuo.* The residue was dissolved in CH_2Cl_2–MeOH and pre-adsorbed onto silica for flash chromatography (CH_2Cl_2–MeOH–H_2O, 60 : 15 : 1), which afforded 3-sulfate **42** (0.031 g, 79%). R_f 0.3 (CH_2Cl_2–MeOH–H_2O, 60 : 15 : 1); mp 176–179 °C; $[\alpha]_D^{23}$ −41.8 (*c* 0.22, MeOH); ν_{max}/cm^{-1} (film) 3700–3100 (OH), 2943, 2914, 1641, 1447, 1379, 1223 (O–SO_2), 1061; δ_H (300 MHz, $CDCl_3$–MeOD, 1 : 1) 4.32–4.16 (1H, m, H3), 3.56 (1H, dd, *J* 8.0, 5.6, H16), 2.19–1.94 (2H, m), 1.84–0.78 (17H, m), 1.07 (3H, s, CH_3), 0.80 (3H, s, CH_3), 0.78 (3H, s, CH_3), 0.68–0.56 (1H, m); δ_C (75 MHz, DMSO) 77.8, 76.2, 74.9, 53.8, 46.5, 44.5, 44.5, 36.6, 35.4, 35.2, 35.1, 34.9, 32.2, 31.6, 28.6, 28.3, 24.1, 20.2, 13.8, 12.0; *m/z* (ESI-) 401.1988 ($C_{20}H_{33}O_6S$ requires 401.1998, 100%).

Boronate ester mediated alcohol protection

3β-(*tert*-Butyldimethylsilyloxy)-17α-methyl-5α-androstane-16β, 17β-diol (46). 17α-Methyl triol **18**[2] (0.250 g, 0.78 mmol) was stirred (**18** was partially insoluble) in a mixture of DMF–CH_2Cl_2 (1.3 : 1, 1.6 mL). Phenylboronic acid (0.151 g, 1.24 mmol) was added, followed by 4 Å molecular sieves (20). After 4 hours, TLC (ethyl acetate–hexane, 1 : 1) showed complete conversion of starting material (R_f 0.2) to the corresponding boronic ester (R_f 0.5). Additional DMF (0.7 mL) was added, followed by imidazole (0.423 g, 6.21 mmol) and TBSCl. The reaction mixture was stirred for 40 hours, when TLC (ethyl acetate–hexane, 1 : 4) showed a single spot (R_f 0.7) due to intermediate product **45**. The reaction mixture was diluted with ethyl acetate (30 mL) and filtered through celite. Saturated $NaHCO_3$ solution (50 mL) was added, and the layers separated. The aqueous portion was further extracted with ethyl acetate (2 × 30 mL). The combined organic extracts were washed with saturated NaCl solution (20 mL), dried over Na_2SO_4 and concentrated. The resulting residue was dissolved in THF (7 mL) and treated with H_2O_2 (30% aqueous solution, 1.7 mL) and NaOH (3 M, 1.1 mL) for 4 hours. TLC (ethyl acetate–hexane, 1 : 4) suggested the deprotection to be complete. The reaction mixture was diluted with H_2O (100 mL) and extracted into ethyl acetate (3 × 40 mL). The combined ethyl acetate extracts were washed with saturated Na_2SO_3 solution (30 mL), saturated NaCl solution (30 mL), then dried (Na_2SO_4) and concentrated *in vacuo.* The residue was dissolved in CH_2Cl_2 and pre-adsorbed onto silica for flash chromatography (ethyl acetate–hexane, 1 : 4 to 1 : 2), which afforded diol **46** (0.248 g, 73%). R_f 0.5 (ethyl acetate–hexane, 1 : 1); mp 216–220 °C; $[\alpha]_D^{22}$ −18.0 (*c* 0.51, $CHCl_3$); ν_{max}/cm^{-1} (film) 3600–3000 (OH), 2931, 1377, 1360, 1250, 1091, 1070, 1053; δ_H (200 MHz, $CDCl_3$) 3.64 (1H, dd, *J* 8.0, 5.4, H16), 3.60–3.44 (1H, m, H3), 2.64 (1H, br s, OH), 2.57 (1H, br s, OH), 2.26–2.08 (1H, m), 1.74–0.78 (18H, m), 1.12 (3H, s, CH_3), 0.88 (9H, s, $(CH_3)_3CSi$), 0.83 (3H, s, CH_3), 0.81 (3H, s, CH_3), 0.66–0.50 (1H, m), 0.04 (6H, s, $(CH_3)_2Si$); δ_C (50 MHz, $CDCl_3$) 79.2, 77.8, 72.1, 54.6, 47.1, 45.1, 44.9, 38.6, 37.2, 35.8, 35.6, 34.9, 32.5, 32.0, 31.9, 28.6, 25.9, 23.8, 20.6, 18.2, 13.6, 12.4, −4.6; *m/z* (EI+) 436.3364 (M^+, $C_{26}H_{48}O_3Si$ requires 436.3373, 2%), 379 (M^+-^{t}Bu, 100), 303 (10), 285 (26), 267 (30).

Conclusions

The selective manipulation of steroid hydroxyl groups in steroid triols, using boronate ester protection of the vicinal diol, has enabled the regioselective derivatisation of the remaining hydroxyl group. One-pot phenylboronate protection, followed by regioselective oxidations, sulfations or silyl ether protections were achieved, with subsequent oxidative deprotection of the boronate ester rapidly providing the derivatised steroid. This methodology has been applied to the efficient preparation of steroid antigens **5**, **7** and **9** and sulfates **42** and **44**. In the future, this methodology could be extended to the preparation of further derivatised steroids, *e.g.* glycosylated steroids.

A range of new steroid protein conjugates **7–10** have been prepared. These are currently being applied to develop ELISAs as screening tools for the detection of steroid metabolites. It is anticipated based on previous results that these conjugates will provide assays to detect D-ring structures associated with ethylestrenol **11**, norethandrolone **12** and danazol **13**, their metabolites and structurally related steroids. The generation of antibodies and the development of ELISAs is currently in progress and will be reported elsewhere.

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