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Synthesis of 5α-androstane-3α,17β-diol 17-*O*-glucuronide histaminyl conjugate for immunoassays

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ABSTRACT

Simple method of preparation of 5α-androstane-3α,17β-diol 17-*O*-glucuronide *N*-histaminyl amide was developed for the construction of immunoanalytical kit. Improved method of glucuronide derivative synthesis was used, followed by hydroxybenzotriazole–dicyclohexylcarbodiimide coupling with histamine.

1. Introduction

Immunoassays, especially in the field of biological steroids, retain its importance even in time of progress of sophisticated instrumental analyses mainly for its simplicity and costs [1]. Syntheses of components for construction of a kit may bring some challenging tasks due to the structural complexity of products. Similar studies from our laboratory targeted to preparation of haptens for endocrinologists and clinical analysts may serve as an example [2,3].

Current project is aimed on the simple synthesis of histaminyl amide 17-*O*-glucuronide conjugate of 5α-androstane-3α,17β-diol. Suitably protected starting compound, acetate **1**, was accessible from commercial 3α-acetoxy-5α-androstan-17-one. For a glycosylation into hindered 17β-position of androstane skeleton, the modified Koenigs–Knorr reaction [4] was selected. The mentioned paper [4] is aimed on the mass spectrometry and gives only limited information on the synthesis and its results (esp. yields), so we present our procedure which may be considered in part as experimental specification of the published troublesome general procedure. Several methods were tested for the deprotection of glucuronide **2**, and also for the preparation of *N*-histaminyl amide **4**.

2. Experimental

2.1. General

NMR spectra were measured at Varian Gemini 300 HC instrument (300 MHz, FT mode) in solvents indicated below and were referenced to solvent signals. Spectra of final compound **4** were taken on Bruker Avance 600 III (600 MHz; 150 MHz). Chemical shifts are given in ppm, coupling constants (*J*) and ranges of multiplets in Hz. Beside ^{1}H-NMR and ^{13}C-NMR (APT), homonuclear 2D-spectrum (COSY) and heteronuclear 2D-spectra (HSQC, HMBC) were taken for complete structural assignment of compound **4**. High resolution mass spectra were recorded using LTQ Orbitrap Velos (Thermo Scientific) instrument with electrospray ionisation in positive mode. Analytical TLC was performed on Merck TLC Silica gel 60 F_{254} plates using visualization by solution of H_2SO_4 in MeOH (1/1) and consecutive heating. For column chromatography, silica gel 60 (100–160 μm, Fluka) was used. For reverse-phase flash column chromatography, C-18 modified silica gel (Sigma-Aldrich) was filled into glass columns. Starting 3α-acetoxy-5α-androstan-17-one was purchased from Steraloids, so as a reference sample of deprotected acid **3**. Other chemicals and solvents were purchased from commercial sources in reagent grade and were not further purified before use if not stated otherwise below. The solutions were dried over anhydrous $MgSO_4$ and the solvents were removed on rotary evaporator under reduced pressure.

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2.2. Chemical synthesis

*2.2.1. Methyl 2,3,4-tri-O-acetyl-1-O-(3α-acetyloxy-5α-androstan-17β-yl)-β-D-glucopyranosiduronate (**2**)*

17β-Hydroxy-5α-androstan-3α-yl acetate [4–6] (**1**, 100 mg, 1 eq, 0.299 mmol) was dissolved in dry freshly distilled benzene (8 ml) and treated by methyl 2,3,4-tri-O-acetyl-1α-bromo-1-deoxy-β-D-glucuronate (**6**) (300 mg, 2.5 eq, 0.75 mmol) in presence of silver carbonate (200 mg, 2.4 eq, 0.725 mmol) and ground molecular sieves (250 mg). Reaction mixture was stirred in dark, under argon at room temperature for 3 days. Then, the mixture was diluted by dichloromethane, filtered, and the solvent was evaporated. The crude product was chromatographed on silica gel column using gradient of diethyl ether (1–10% v/v) in dichloromethane. Glycoside **2** (36 mg, 18%) was obtained as white amorphous solid. ^{1}H NMR ($CDCl_3$, 300 MHz): 5.24–5.15 m, 2 H; 5.01 m, 2 H; 4.58 d, 1 H, J = 7.9; 3.99 d, 1 H, J = 9.7; 3.75 s, 3 H; 3.57 t, 1 H, J = 8.8; 2.05 s, 3 H; 2.04 s, 3 H; 2.01 m, 6 H; 0.79 s, 3 H; 0.70 s, 3 H. The title compound was previously prepared by another method [7] and was found to have identical structure.

*2.2.2. 3α-Hydroxy-5α-androstan-17β-yl β-D-glucopyranosiduronic acid (**3**)*

Protected glycoside **2** (165 mg, 1 eq, 0.254 mmol) was dissolved in methanol (2 ml) and the solution was diluted by 10 ml of water. Solution of lithium hydroxide in methanol (0.5 M, 10 ml) was then added. Reaction mixture was stirred in dark at room temperature for 3 days. After dilution by another 10 ml of methanol, pH was adjusted to neutral by a solution of acetic acid (10% in methanol). Resulting solution was passed through a column of catex (Amberlite, IR-120, H-cycle, 20–50 mesh, 15 ml) using the methanol/water (2/1) mixture as eluent. After evaporation, the crude product was purified by column chromatography on reverse phase (C-18 modified silica gel), using gradient of MeOH in water. Deprotected glucuronide **3** (63 mg, 53%) was obtained as amorphous white solid. This commercial product was described previously [4,7]. ^{1}H NMR (CD_3OD, 300 MHz): 4.36 d, 1 H, J = 7.91 (H-1′); 3.95 br t, 1 H (H-3); 3.77–3.64 m, 2 H (H-5′, H-17); 3.50 t, 1 H, J = 9.1 (H-4′); 3.34 t, 1 H, J = 9.1 (H-3′); 3.20 t, 1 H, J = 7.91 (H-2′); 0.82–0.81 2 × s, 6 H (3 × H-18, 3 × H-19). HRMS ESI + (m/z): [M+Na]$^+$ calcd. for $C_{25}H_{40}O_8$: 491.262087. Found: 491.26158.

*2.2.3. 3α-Hydroxy-5α-androstan-17β-yl N-(4-imidazolyl)ethyl-β-D-glucopyranosiduronamide (**4**)*

To a solution of glucuronide **3** (9 mg, 1 eq, 19.2 nmol) in DMF (200 μl), hydroxybenzotriazole (hydrate, 90%, 6 mg, 3.5 eq, 6.7 nmol) was added. DCC (14 mg, 3.5 eq, 6.7 nmol) was being added in 3 portions. 6 mg were added at the beginning of the reaction, another 6 mg were added after 2 days and at last, 2 mg were added after 2 more days. The reaction was completed after 6 days of slow stirring at room temperature. The reaction mixture was filtered from crystals of dicyclohexylurea, which were washed by another 100 μl of DMF. Solution of histamine (7.5 mg, 3.5 eq, 6.7 nmol) in DMF (100 μl) and DIPEA (20 μl) were added. After stirring in dark at room temperature for 3 days, the reaction was completed. Reaction mixture was evaporated to dryness and residue was submitted to the column chromatography on reverse phase, using gradient of MeOH in water as eluent. Fractions containing target compound were combined and evaporated, yielding 7 mg of compound **4** which was further purified by column chromatography on silica gel (CH_2Cl_2/MeOH). Target compound **4** (3.7 mg, 34%) was obtained as amorphous white solid. Prior to measurements of NMR spectra, the compound was further purified from trace impurities by semi-preparative HPLC (gradient of MeOH in water). ^{1}H NMR (CD_3OD, 600 MHz): 7.60 s, 1 H (H-2″); 6.89 br s, 1 H (H-5″); 4.38 d, 1 H, J = 7.7 (H-1′); 3.97 br s, 1 H (H-3β); 3.68–3.63 m, 2 H (H-5′, H-17α); 3.57–3.43 m, 3 H (2 × H-7″, 1 × H-4′); 3.39–3.36 m, 1 H (H-3′); 3.21 t, 1 H, J = 8.8 (H-2′); 2.82 br t, 2 H, J = 6.6 (2 × H-6″); 1.99–1.90 m, 2 H (H-12a, H-16a); 1.73–1.66 m, 2 H (H-7a, H-2a); 1.64–1.58 m, 5 H (H-5, H-8, H-11a, H-15a, H-16b); 1.52 dt, 1 H, J_1 = 13.2, J_2 = 2.2 (H-4a); 1.48–1.40 m, 2 H (H-1a, H-2b); 1.39–1.35 dd, 1 H, J_1 = 13.7, J_2 = 3.3 (H-11b); 1.29–1.21 m, 3 H (H-6a, H-6b, H-15b); 1.15 dt, 1 H, J_1 = 12.6, J_2 = 3.8 (H-12b); 1.03–0.91 m, 2 H (H-14, H-7b); 0.84 s, 6 H (3 × H-18, 3 × H-19); 0.77 dt, 1 H, J_1 = 12.1, J_2 = 3.8 (H-9). ^{13}C NMR (CD_3OD, 150 MHz): 170.49 (C-6′); 134.74, 2 C (C-2″, C-4″); 117.99 (br.[8], C-5″); 103.58 (C-1′); 89.22 (C-17); 76.17 (C-3′); 74.80 (C-5′); 73.50 (C-2′); 72.15 (C-4′); 65.78 (C-3); 54.62 (C-9); 50.85 (C-14); 42.93 (C-13); 38.93 (C-5); 38.59 (C-7″); 37.31 (C-12); 35.81 (C-10); 35.33 (C-4); 35.31 (C-8); 32.12 (C-1); 31.46 (C-7); 28.56 (C-16); 28.28 (C-2); 28.19 (C-6″); 28.18 (C-6); 22.97 (C-15); 20.08 (C-11); 10.69 (C-18); 10.30 (C-19). HRMS ESI + (m/z): [M+H]$^+$ calcd. for $C_{30}H_{47}N_3O_7$: 562.349341. Found: 562.34900.

3. Results and discussion

For the synthesis of complex target amide **4** from commercial building blocks of reasonable price, pathway starting with 3α-acetoxy-5α-androstan-17-one was selected. Protected androstanolone was reduced to secondary alcohol (Scheme 1), glycosylated by acetylated halogenose, and after hydrolysis of protecting ester groups subjected to the conjugation with histamine. Glycosylation and amide formation with subsequent purification were critical steps.

Ketone group of starting material was readily reduced to obtain corresponding alcohol **1** in very good yield by common procedure using sodium borohydride in mixture of methanol and ethyl acetate. As reported previously [4–6], only 17β isomer was isolated.

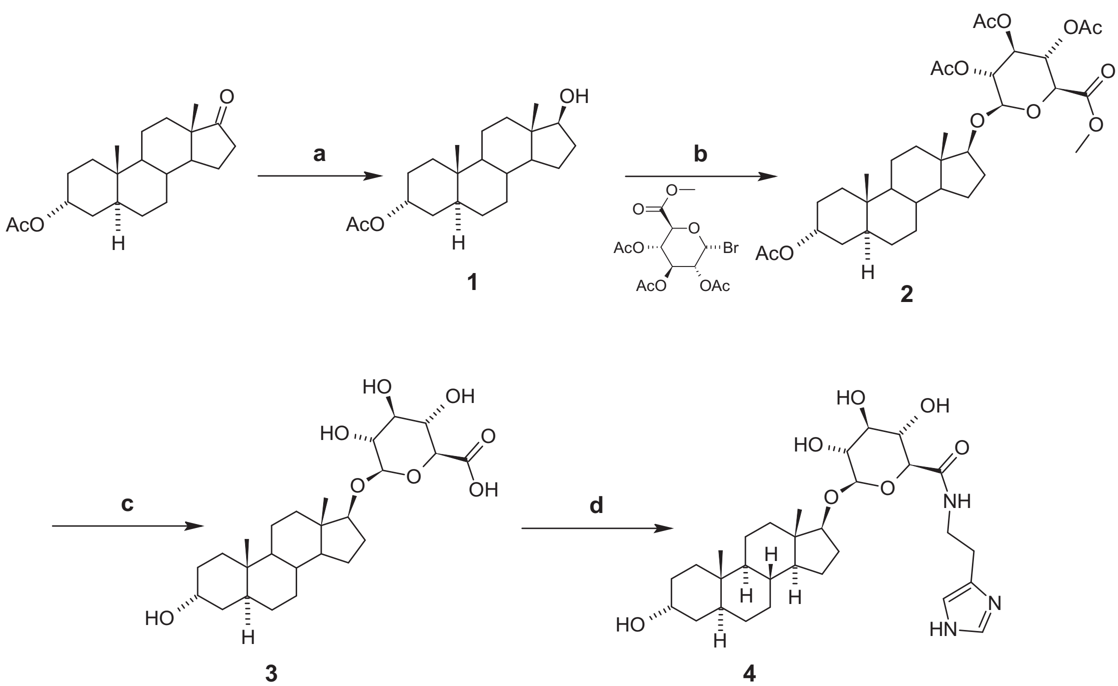
The suitable halogenose **6** was prepared (see Scheme 2) with the method of Bowering and Timell [9] from β-D-glucuronolactone.

From obtained building blocks, glycoside **2** was prepared by modified Koenigs–Knorr reaction [10], following to method of Thevis et al. [11] using silver carbonate as catalyst (even though in stoichiometric excess).

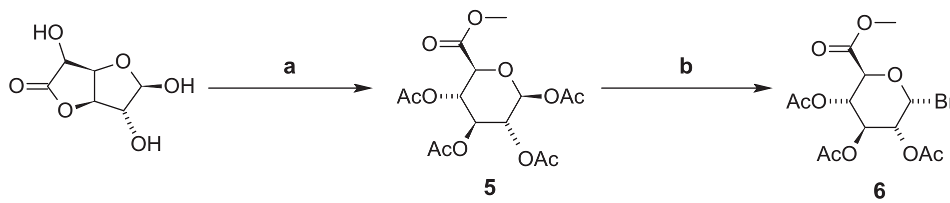
It is noteworthy that this reaction is very sensitive and requires absolutely dry solvents, giving increasing ratio of corresponding orthoester **7** (Fig. 1) hand in hand with rising moisture content. During our early experiments with this method, the orthoester was even isolated in good yield as main product, using "dry" reagent-grade toluene. After such experience, toluene was substituted by freshly dried and distilled benzene over grinded activated molecular sieves. Nevertheless, the orthoester remained troublesome side product and thorough purification via column chromatography was necessary. This caused additional losses and the overall yield was low (about 20% in total). Orthoester content can be detected by characteristic signal of its methyl group in ^{1}H NMR spectrum (1.71 ppm in $CDCl_3$).

The deprotection of acetyl groups was achieved by treatment by solution of LiOH in water/methanol over two days [12]. Despite the general swiftness and ease of deacetylation reactions, ordinary protocols using MeONa or NaOH with reaction times of several hours did not lead to complete deacetylation of the substrate.

Several methods of amide formation were tried out for the final step. First, T3P® [13] (propylphosphonic anhydride, 50% solution in EtOAc) was employed in DMF/pyridine. Even with an excess (4–6 mol. eq.) of the reagent, only starting steroid **3** was detected in reaction mixture. Then, experiments with DEPC (diethyl phosphoryl cyanide) were made [14]. This reagent readily promoted amide group formation in DMF with DIPEA, but only phosphate of target structure **4** was retrieved (single product was obtained, showing MW = 698 in MS spectrum and two excessive ethyl groups in ^{1}H



Scheme 1. Proposed synthetic route towards amide **4**. (a) $NaBH_4$, MeOH, EtOAc; (b) Ag_2CO_3, 6, benzene, mol. sieves; (c) LiOH, MeOH, H_2O; (d) 1. HOBT, DCC, DMF; 2. Histamine, DIPEA, DMF.



Scheme 2. Preparation of bromosugar for glycosylation reaction. (a) 1. NaOH, MeOH; 2. Ac_2O, Py; (b) HBr, AcOH.

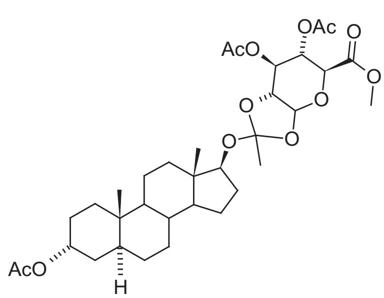


Fig. 1. Structure of orthoester impurity detected after glycosylation reaction.

NMR). Finally, method using hydroxybenzotriazole with dicyclohexylcarbodiimide [15] and consecutive active-ester addition to histamine in DMF/DIPEA solution was employed to obtain amide **4**. Followed by reverse-phase and subsequent normal-phase chromatography, this method provided desired conjugate in sufficient purity.

It should be stated that all efforts to get the compounds prepared in decent crystalline form were not successful.

4. Conclusion

Synthetic pathway towards target androstanyl glucuronate marked by histamine starting from basic building blocks was developed. It consists of conventional reactions, which were verified for given reagents, affording new reproducible synthetic route. Very low overall yield of 3–4% offers possibilities for further optimization, however it is strongly dependent on desired purity of target product. Small quantity of conjugate prepared was enough to be successfully used in immunoanalytical sets both in performance and stability by the industrial partner.

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