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ORIGINAL PAPER

Synthesis and characterization of stanozolol *N*-glucuronide metabolites

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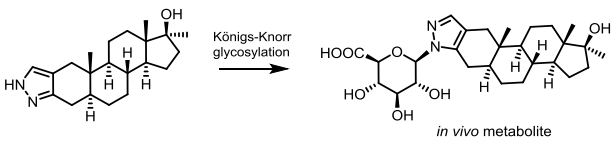
Abstract

The preparation, separation, and analysis of two regioisomeric *N*-glucuronides of the common doping agent stanozolol are described in this manuscript. Glucuronidation was carried out using modified Königs–Knorr conditions developed specifically for pyrazoles. After preparative HPLC separation both isomers could be isolated in a pure form. Global cleavage of protecting groups furnished the putative human phase II metabolites STN1 and STN2 which were compared with human excretion studies.

Graphical abstract



Keywords Carbohydrates · Heterocycles · High-pressure liquid chromatography · Natural products · Steroids

Introduction

Stanozolol (IUPAC: 17α-methyl-5α-androstano[3,2-*c*] pyrazol-17β-ol, **1**) is an exogenous steroid hormone first synthesized in 1959 by Clinton et al. [1] by cyclocondensation of oxymetholone (IUPAC: 2-hydroxymethylene-17α-methyl-17β-hydroxy-5α-androstan-3-one) with hydrazine hydrate. In vivo, it acts weakly androgenic and moderately anabolic and is therefore very popular among amateur and professional athletes. It is a common therapeutic agent in veterinary medicine which only has niche medical applications in humans. The metabolism of stanozolol has been investigated extensively since the late 1980s [2–13]. Hydroxylation can occur enzymatically on C-3′, C-16, C-4, and on C-6/C-7 and conjugate formation on N-1, N-2, C-3′-OH, and C-17-OH.

N-Glucuronides of stanozolol were predicted to be excreted already in 1990 [3], but the analytical methodology for steroid glucuronide conjugates and phase II metabolites in general, was developed mainly in the last decade [12–15]. A synthesis of the two *N*-glucuronidated isomers STN1 and STN2 was reported by Schänzer et al. [13] but was found to be non-reproducible in our hands, is reportedly low-yielding and giving a mixture which was not separated until deprotection.

The two target compounds as well as the parent drug **1** are depicted in Fig. 1. The goal of this project was the development of a concise, reproducible synthesis of these valuable molecules as well as full characterization and analysis of the protected intermediates.

Dedicated to Heinz Falk on the occasion of the 80th anniversary of his birthday.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00706-019-02424-4) contains supplementary material, which is available to authorized users.

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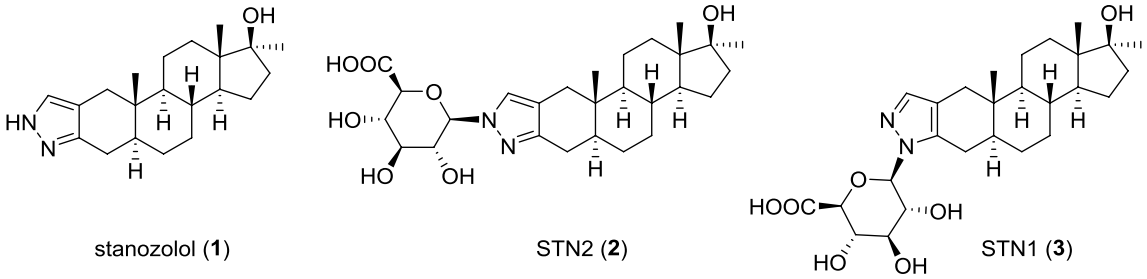


Fig. 1 Relevant structures

Results and discussion

A number of strategies were evaluated before arriving at an efficient synthesis for compounds **2** and **3**. For instance, the silyl-Hilbert-Johnson reaction, for which there are published examples with pyrazole or similar heterocyclic systems was unsuccessful under a plethora of tested reaction conditions [16–20] and with different protecting groups on the C-17 hydroxyl.

The possibility of forming the pyrazole directly during glycosylation of the steroid by cyclocondensation as reported by Schmidt et al. [21, 22] was also examined, but was found to be incompatible with commonly used glucuronic acid-protecting groups (acetate and benzoyl).

Eventually, a modified Königs–Knorr procedure using a glycosyl bromide **4** (synthesis carried out according to Ref. [23]), and activation by mercury salts (see Scheme 1) developed for the *N*-glycosylation of pyrazoles [24] was successful. Presumably, the mercury(II) pyrazolides are formed from stanozolol, which then activate and attack donor **4**. Intriguingly, no glycosylation of the C-17 alcohol was observed under any of the conditions tested. In fact, the inert character of the tertiary alcohol made protection wholly unnecessary, thus the parent drug **1** was used directly in this metabolite synthesis.

The conversion of this reaction stops at 25% and could not be increased by change of temperature, solvent, or stoichiometry. Nevertheless, after column chromatography and preparative HPLC fractionation (C_{18} column, MeOH/H_2O) of the product mixture, the pure protected *N*-2- and *N*-1-glucuronides **5** and **6** were isolated in 5% and 17% yield, respectively, together with 62% recovered stanozolol **1**. The β-anomers were isolated exclusively with both regioisomers.

With the glycosides in hand, global deprotection was achieved by treatment with lithium hydroxide. The final products **2** and **3** were obtained in good yields after preparative HPLC purification and were extensively characterized via 2D-NMR.

The two isomers were compared with excretion studies of stanozolol and compound **3** was found to correspond to the metabolite formed in vivo, while the N-2-isomer **2** was found not to be present.

Conclusion

By synthesis of two *N*-glucuronides of stanazolol and full characterization of the two compounds **2** and **3** the structure of the two compounds was unambiguously determined and it was proved that **3** is formed as phase II metabolite of stanozolol (**1**) in vivo, whereas **2** could not be detected.

Experimental

HPLC-grade solvents (acetonitrile, methanol, isopropanol, water) were from VWR. All other non-specified chemicals were from Sigma-Aldrich. Deuterated chloroform was passed through a column filled with basic alumina.

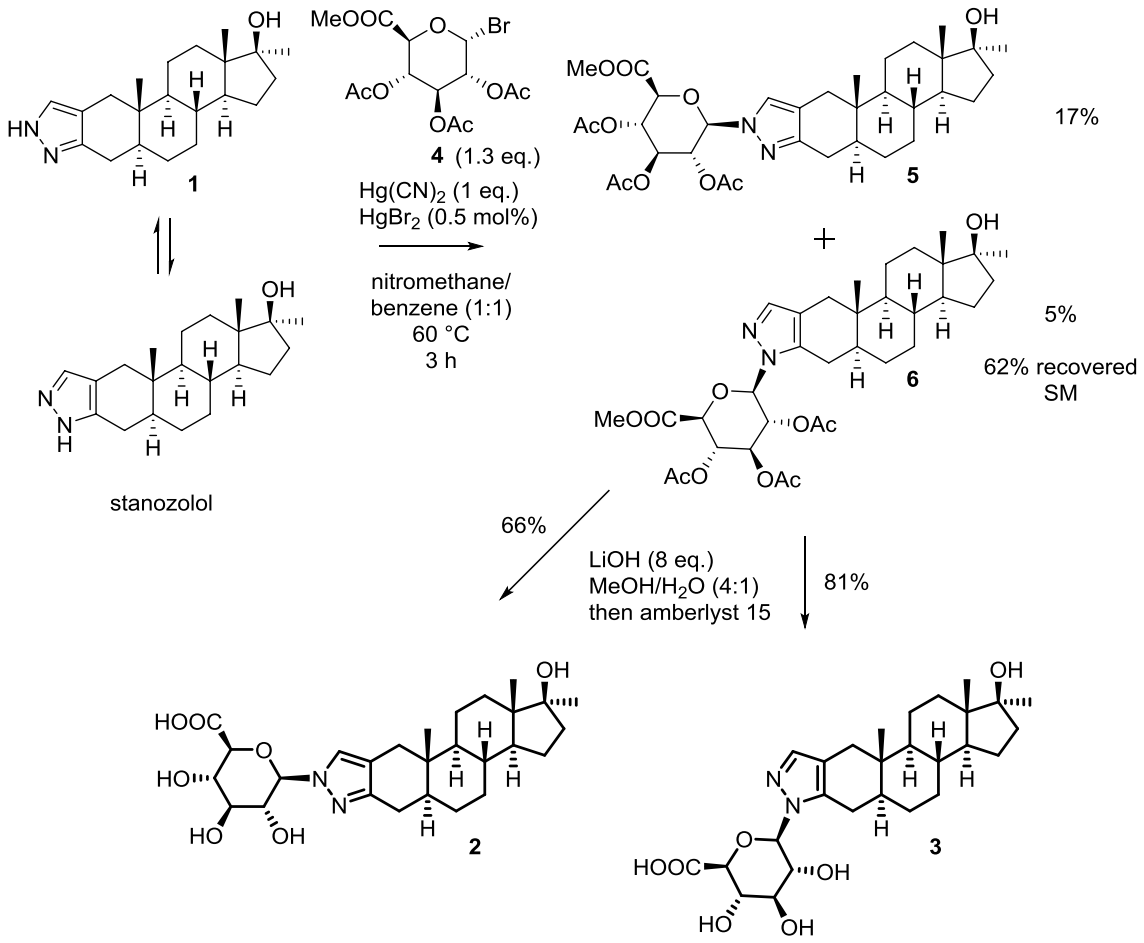
Moisture- and air-sensitive reactions were carried out in flame-dried glass vessels under an argon atmosphere using Schlenk techniques. All reactions were stirred magnetically unless otherwise stated.

Chromatography was performed with glass columns and Merck silica gel 43–60 µm. TLC-analysis was performed with pre-coated aluminium-backed plates (silica gel 60 F_{254}, Merck). Compounds were visualized by submerging in an acidic phosphomolybdic acid/cerium sulfate solution, vanillin/sulfuric acid, or *p*-anisealdehyde/ethanol and drying with a hot air gun.

NMR spectra were recorded on a Bruker Avance 400 at 400 MHz (100 MHz) and Avance 600 at 600 MHz (150 MHz). Chemical shifts are given in ppm and were referenced to the solvent residual peaks [25]. Coupling constants are given in Hertz. IR spectra were recorded on a PerkinElmer Spectrum 65 using thin films (ATR FT-IR).

Melting points were determined with a Leica Galen III Kofler hot-stage apparatus. Specific rotations were measured on an Anton Parr MCP 500 polarimeter at 20 °C and 589 nm.

Scheme 1



HPLC was carried out using an Autopurification system from Waters with an ACQUITY QDa detector, combined with a 2998 Photodiode array detector. Separation was conducted with a XSELECT CSH C18 5 µm 4.6 × 150 mm column for analytical samples and a XSELECT CSH Prep C18 5 µm OBD 30 × 150 column for preparative runs. HPLC-grade methanol and water, containing 0.1% formic acid were used as eluent system.

HRMS analysis was carried out on a Q Exactive orbitrap mass spectrometer coupled to a Vanquish Horizon UHPLC System (Thermo Fisher, Austin, TX, USA). The LC was equipped with a Phenomenex Kinetex C18 Evo column (2.1 × 100 mm, particle size 2.6 µm), and the separation was carried out at constant temperature (25 °C). The mobile phase was composed of water containing 0.2% formic acid (solvent A) and acetonitrile (solvent B). A gradient elution was employed starting at 0% B kept for 2 min; 2–13 min, 0–100% B; 13–17 min, 100% B. The column was finally re-equilibrated at 0% B for 3 min. The flow rate was set at 0.4 cm^3/min and the injection volume was 5 mm^3. Mass detection was carried out in positive electrospray ionization (ESI+) mode. The settings of the mass spectrometer were as follows: spray voltage 3.8 kV, capillary temperature 320 °C. Nitrogen was used as sheath gas (pressure 25 units) and auxiliary gas (pressure 8 units). High-resolution MS full scans were acquired from $m/z = 250$–1000 and a resolution set at 70000, respectively. In case of product ion scan acquisition the resolution was set at 35000, isolation width at 1 Da. The normalized collision energy of 55 was employed for collision-induced dissociation. The inclusion list contained the precursor ion $m/z = 505.2908$, corresponding to the $[M+H]^+$ ion of stanozolol-*N*-glucuronide. All LC–HRMS analyses were performed with an internal mass calibration in each run using a lock-mass. The calibrator ion was the protonated species of diisooctyl phthalate with $m/z = 391.2842$.

1-Deoxy-1-(17*β*-hydroxy-17*α*-methyl-5*α*-androstano[3,2-*c*]-pyrazol-2′-yl)-*β*-D-glucopyranuronic acid (2, $C_{27}H_{40}N_2O_7$) In a 10 cm^3 round-bottom flask 26 mg starting material **5** (0.04 mmol) was dissolved in 3 cm^3 MeOH:water (3:1) and stirred at room temperature. Lithium hydroxide hydrate (13.2 mg, 0.32 mmol, 8 eq.) was added as a solid in two

portions and the mixture was stirred for 6 h. Then, freshly washed amberlite 120 ion resin (H^+-form) was introduced and the mixture vigorously stirred for 15 min. After dilution with ca. 5 cm^3 MeOH the reaction mixture was filtered over a small silica gel plug (0.4 g) and the solvent evaporated in vacuo. Final purification on HPLC (30 cm^3/min, 70 75% MeOH in water + 0.1% formic acid, detection: $m/z = 505$) gave 13.5 mg of product **2** (66% yield).$[\alpha]_D^{20} = 12.6$ ($c = 0.68$, MeOH); ^{1}H NMR (600 MHz, CD_3OD): $\delta = 7.50$ (1H, s), 5.20 (1H, d, $J = 9.28$ Hz), 3.94–4.03 (2H, m), 3.67 (1H, dd, $J = 9.38$ Hz, 9.38 Hz), 3.57 (1H, dd, $J = 9.38$ Hz, 9.38 Hz), 2.69 (1H, d, $J = 16.23$ Hz), 2.60 (1H, dd, $J = 16.23$ Hz, 5.19 Hz), 2.27 (1H, dd, $J = 11.8$ Hz, 16.23 Hz), 2.13 (1H, d, $J = 14.28$ Hz), 1.88 (1H, m), 1.77 (1H, m), 1.54–1.72 (6H, m), 1.24–1.49 (6H, m), 1.21 (3H, s), 0.97 (1H, m), 0.88 (3H, s), 0.86 (1H, m), 0.79 (3H, s) ppm; ^{13}C NMR (150 MHz, CD_3OD): $\delta = 172.97$, 151.01, 129.77, 117.75, 90.39, 82.64, 78.92, 78.48, 73.22, 73.11, 55.51, 52.28, 46.93, 44.07, 39.47, 38.24, 37.63, 36.04, 33.17, 33.01, 30.73, 28.65, 26.37, 24.60, 22.20, 14.88, 12.12 ppm; IR: $\overline{\nu} = 3298$, 2912, 1727, 1368, 1019/cm; HRMS (ESI −: m/z calcd. for $C_{27}H_{39}N_2O_7$ ($[M\text{-}H]^-$) 503.2763, found 503.2908.

1-Deoxy-1-(17*β*-hydroxy-17*α*-methyl-5*α*-androstano[3,2-*c*]-pyrazol-1′-yl)-*β*-D-glucopyranuronic acid (3, $C_{27}H_{40}N_2O_7$) In a 10 cm^3 round-bottom flask 30 mg starting material **6** (0.047 mmol) was dissolved in 3 cm^3 MeOH:water (5:1) and stirred at room temperature. Lithium hydroxide hydrate (15.2 mg, 0.37 mmol, 8 eq.) was added as a solid in two portions and the mixture was stirred for 7 h. Then, freshly washed amberlite 120 ion resin (H^+-form) was introduced and the mixture vigorously stirred for 15 min. After dilution with 5 cm^3 MeOH the reaction mixture was filtered over a small silica gel plug (0.5 g) and the solvent evaporated in vacuo. Final purification on HPLC (30 cm^3/min, 70 75% MeOH in water + 0.1% formic acid, detection: $m/z = 505$) gave 19.5 mg of product **3** (83% yield).$[\alpha]_D^{20} = 5.8$ ($c = 0.95$, CH_2Cl_2); ^{1}H NMR (600 MHz, CD_3OD): $\delta = 7.34$ (1H, s), 5.21 (1H, d, $J = 9.08$ Hz), 4.11 (1H, dd, $J = 9.46$ Hz, 9.46 Hz), 4.05 (1H, d, $J = 9.69$ Hz), 3.70 (1H, dd, $J = 9.30$ Hz, 9.30 Hz), 3.56 (1H, dd, $J = 9.30$ Hz, 9.30 Hz), 2.66 (1H, dd, $J = 16.53$ Hz, 4.57 Hz), 2.60 (1H, d, $J = 15.48$ Hz), 2.31 (1H, dd, $J = 11.92$ Hz, 15.89 Hz), 2.14 (1H, d, $J = 14.78$ Hz), 1.88 (1H, m), 1.78 (1H, m), 1.55–1.73 (6H, m), 1.24–1.49 (6H, m), 1.22 (3H, s), 0.98 (1H, m), 0.85–0.93 (1H, m), 0.89 (3H, s), 0.78 (3H, s) ppm; ^{13}C NMR (150 MHz, CD_3OD): $\delta = 172.28$, 141.16, 140.45, 117.47, 87.27, 82.57, 79.10, 78.46, 72.99, 72.97, 55.47, 52.30, 46.98, 43.74, 39.51, 38.24, 37.83, 36.28, 33.21, 33.00, 30.51, 26.86, 26.40, 24.64, 22.25, 14.90, 12.06 ppm; IR: $\overline{\nu} = 3393$, 2922, 1608, 1368, 1080 /cm; HRMS (ESI +) m/z calcd. for $C_{27}H_{41}N_2O_7$ ($[M + H]^+$) 505.2914, found 505.2889.

ESI + fragmentation MS (55 eV): the by far most abundant fragment obtained by electrospray ionization is the aglycon stanozolol ($[M + H]^+$) with $m/z = 329.2575$ (Fig. 2).

ESI + MS: elemental composition of the target compound was obtained by high-resolution MS analysis, yielding the mass of the molecular ion as 505.2889 ($[M + H]^+$) with a mass accuracy < 5 ppm (theoretical mass 505.2914, error = 4.8 ppm) as well as the elemental composition ($C_{27}H_{40}N_2O_7$) (Fig. 3).

2′-[*N*-(Methyl 2,3,4-tri-*O*-acetyl-1-deoxy-*β*-D-glucuronosyl-uronate)]-17*α*-methyl-5*α*-androstano[3,2-*c*]pyrazol-17*β*-ol (5, $C_{34}H_{48}N_2O_{10}$) and 1′-[*N*-(methyl 2,3,4-tri-*O*-acetyl-1-deoxy-*β*-D-glucuronosyluronate)]-17*α*-methyl-5*α*-androstano[3,2-*c*]pyrazol-17*β*-ol (6, $C_{34}H_{48}N_2O_{10}$) A 250 cm^3 round-bottom flask was charged with 1.0 g stanozolol (**1**, 3.04 mmol, 1 eq.) and 50 cm^3 of a nitromethane/benzene mixture (1:1) followed by addition of 770 mg $Hg(CN)_2$ (3.04 mmol, 1 eq.) and 5 mg $HgBr_2$ (0.015 mmol, 0.005 eq.). 1-Bromo-2,3,4-tri-*O*-acetyl-*α*-D-glucuronic acid methyl ester (**4**, 1.55 g, 3.9 mmol, 1.3 eq.) was dissolved in 10 cm^3 of the same mixture and added dropwise at 40 °C. The mixture was heated to 70 °C. After 3 h the

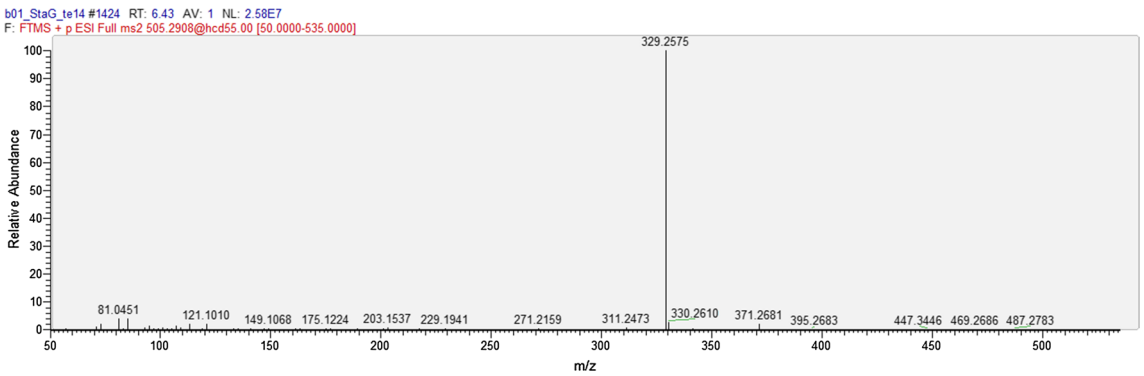


Fig. 2 ESI + product ion spectrum of STN1 at 55 eV collision energy

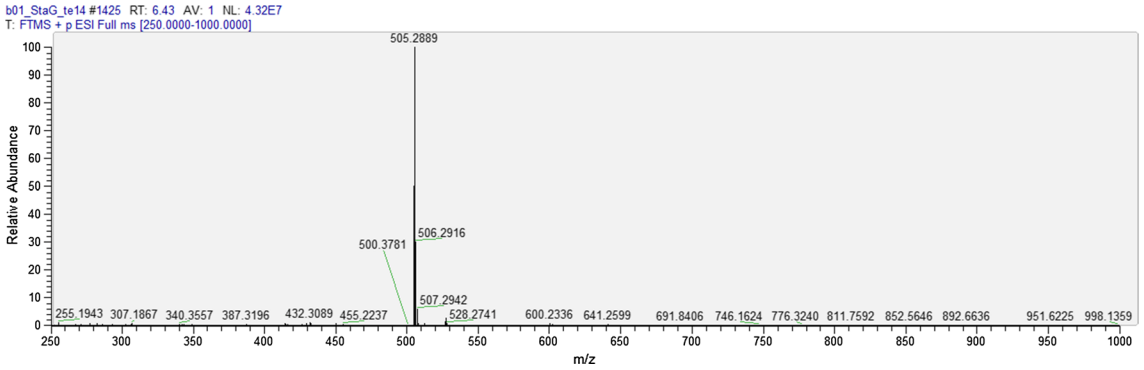


Fig. 3 ESI + high-resolution mass spectrum of STN1

reaction mixture was evaporated, taken up in 100 cm^3 $CHCl_3$ and shaken three times with 30 cm^3 KI solution and once with 30 cm^3 water. Combined organic phases were dried over $MgSO_4$, evaporated, and purified via column chromatography on silica gel (80 g) with PE:Et_2O = 3:1 to give 244 mg of mixed fractions containing almost exclusively **5**, 230 mg containing a 1:1 mixture of **5** and **6**, and 620 mg of starting material **1** (62% recovery). The mixed fractions were further purified on HPLC (30 cm^3/min, 75 80% MeOH in water + 0.1% formic acid, detection: *m/z* = 645). Product fractions were pooled and methanol was stripped at the rotary evaporator. The resulting aqueous solutions were lyophilized to provide in total 335 mg of **5** (17% yield) as well as 102 mg of **6** (5% yield).

2′-[*N*-(Methyl 2,3,4-tri-*O*-acetyl-1-deoxy-*β*-D-glucuronosyl-uronate)]-17*α*-methyl-5*α*-androstano[3,2-*c*]pyrazol-17*β*-ol (5, $C_{34}H_{48}N_2O_{10}$) M.p.: 124–125 °C; $[\alpha]_D^{20}$ = 1.8 (*c* = 0.95, CH_2Cl_2); R_f: 0.47 (PE/Et_2O 2:1); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.28 (1H, s), 5.56 (1H, dd, *J* = 9.30 Hz, 9.30 Hz), 5.48 (1H, d, *J* = 9.50 Hz), 5.39 (1H, dd, *J* = 9.28 Hz, 9.28 Hz), 5.32 (1H, dd, *J* = 9.50 Hz, 9.50 Hz), 4.21 (1H, d, *J* = 9.70 Hz), 3.70 (3H, s), 2.60 (2H, m), 2.23 (1H, dd, *J* = 16.80 Hz, 12.0 Hz), 2.03 (6H, s), 1.86 (3H, s), 1.68–1.80 (4H, m), 1.47–1.66 (8H, m), 1.36–1.45 (2H, m), 1.27–1.35 (2H, m), 1.16–1.25 (1H, m), 1.22 (3H, s), 0.75–0.95 (2H, m), 0.86 (3H, s) 0.71 (3H, s) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 170.15, 169.48, 169.17, 166.76, 150.40, 125.89, 117.97, 87.11, 81.87, 74.79, 72.87, 69.68, 69.42, 53.89, 53.08, 50.72, 45.50, 42.51, 39.10, 36.77, 36.38, 34.79, 31.77, 31.60, 29.42, 27.57, 25.91, 23.44, 20.92, 20.72, 20.61, 20.46, 14.01, 11.55 ppm; IR: $\overline{\nu}$ = 3380, 2922, 1750, 1374, 1215, 1032/cm; HRMS (ESI): *m/z* calcd. for $C_{34}H_{48}N_2O_{10}$ ($[M+H]^+$) 645.3382, found 645.3400.

1′-[*N*-(Methyl 2,3,4-tri-*O*-acetyl-1-deoxy-*β*-D-glucuronosyl-uronate)]-17*α*-methyl-5*α*-androstano[3,2-*c*]pyrazol-17*β*-ol (6, $C_{34}H_{48}N_2O_{10}$) M.p.: 142–143 °C; $[\alpha]_D^{20}$ = 15.8 (*c* = 0.75, CH_2Cl_2); R_f: 0.43 (PE/Et_2O 2:1); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.31 (1H, s), 5.75 (1H, dd, *J* = 9.29 Hz, 9.29 Hz), 5.32–5.44 (3H, m), 4.21 (1H, d, *J* = 10.04 Hz), 3.72 (3H, s), 2.60 (1H, dd, *J* = 15.2 Hz, 4.64 Hz), 2.51 (1H, d, *J* = 15.2 Hz), 2.21 (1H, dd, *J* = 11.9 Hz, 16.98 Hz), 2.05 (3H, s), 2.04 (3H, s), 1.83 (3H, s), 1.71–1.81 (2H, m), 1.50–1.70 (7H, m), 1.28–1.48 (5H, m), 1.16–1.27 (2H, m), 1.22 (3H, s), 0.78–0.96 (2H, m), 0.87 (3H, s), 0.69 (3H, s) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 170.37, 169.43, 168.77, 166.75, 140.17, 138.40, 116.99, 84.58, 81.83, 75.08, 72.80, 69.97, 69.30, 53.75, 53.08, 50.65, 45.51, 42.09, 39.07, 36.70, 36.51, 34.98, 31.73, 31.51, 29.23, 25.98, 25.93, 23.44, 20.89, 20.75, 20.63, 20.43, 14.02 ppm; IR: $\overline{\nu}$ = 3515, 2919, 1750, 1371, 734/cm; HRMS (ESI): *m/z* calcd. for $C_{34}H_{48}N_2O_{10}$ ($[M+H]^+$) 645.3382, found 645.3393.

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