Biotransformation of Vermitaline by *Cunninghamella echinulata*

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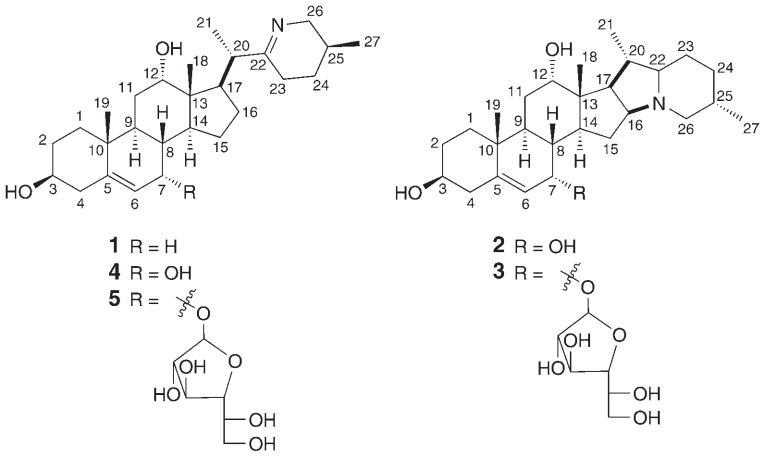
Biotransformation of vermitaline (**1**) by *Cunninghamella echinulata* (ACCC 30369) was carried out. Four biotransformation products were obtained and three of them were characterized as new compounds. On the basis of their NMR and mass-spectral data, their structures were characterized as 7α-hydroxyrubijervine (**2**), 7α-hydroxyrubijervine-7-O-β-D-galactofuranoside (**3**), 7α-hydroxyvermitaline (**4**), and 7α-hydroxyvermitaline-7-O-β-D-galactofuranoside (**5**).

Introduction. – Microbial transformation is an effective tool for the structural modification of bioactive natural compounds. Its application in asymmetric synthesis is increasing due to its versatility and ease [1]. A variety of transformations on natural products such as oxidation, reduction, hydrolysis, isomerization, epimerization, rearrangement, D-homoannulation, *Michael* addition, and reverse aldol reaction can be performed [2]. *Cunninghamella echinulata*, a filamentous fungus, is recognized for its potential for steroid hydroxylation and has been noted for its ability to mimic mammalian hepatic metabolism with other substrates [3][4].

Veratrum alkaloids are a group of potent hypotensive agents that act by reflex suppression of the cardiovascular system [5][6]. Vermitaline[1]) (**1**), a verazine type steroidal alkaloid, is one of the most extensively studied. The interest in *Veratrum* alkaloids has recently been renewed as they were patented several times during the past few years [7–9]. The potent regulatory effects of jervane *Veratrum* alkaloids on hedgehog signaling, modulation of cholesterol biosynthesis and transport, and control of cell proliferation during mandibular arch morphogenesis have been reported recently [10]. Biotransformation of *Veratrum* alkaloids, such as rubijervine, jervine and veratramine by various microorganisms has been reported previously [11–13].

In continuation of our studies on the phytochemistry of *Veratrum* alkaloids [14][15], we now report the characterization of four metabolites of vermitaline (**1**) in cell suspension of *C. echinulata* (ACCC 30369). The metabolites, which were more polar than **1**, were identified as 7α-hydroxyrubijervine (**2**)[1]), 7α-hydroxyrubijervine-7-O-β-D-galactoside (**3**)[1]), 7α-hydroxyvermitaline (**4**)[1]) and 7α-hydroxyvermitaline-7-O-β-D-galactoside (**5**)[1]) by spectroscopic methods.

[1]) For systematic names, see *Exper. Part.*



1 R = H
4 R = OH
5 R =

2 R = OH
3 R =

Figure. *Structures of Compounds* **1**–**5**

Results and Discussion. – Compound **2** was obtained as a white amorphous powder, showing a positive reaction with *Dragendorff*'s reagent. The HR-ESI-MS (positive mode) gave the molecular formula $C_{27}H_{43}NO_3$ from the $[M+H]^+$ signal at *m/z* 430.3314. The ESI-MS of **2** showed a significant fragment ion peak at *m/z* 412 ($[M+H-H_2O]^+$). The IR spectrum exhibited absorptions for OH groups (3370 and 1070 cm^{-1}). The ^{13}C-NMR spectrum (*Table 1*) of **2** showed signals due to four Me, eight CH_2, and twelve CH groups, and three quaternary C-atoms, of which included one C=C bond (δ(C) 141.9 C(5), 122.3 C(6)) and three oxygenated sp^3-C-atoms. The ^{1}H-NMR spectrum (*Table 2*) indicated H-atom resonances for four Me groups at δ 0.69 (*s*, Me(18)), 0.90 (*d*, $J = 6.0$, Me(21)), 0.96 (*s*, Me(19)), 0.97 (*d*, $J = 6.6$, Me(27)), and the ^{13}C-NMR exhibited the signals of three C-atoms attached to an N-atom at δ(C) 73.7 (C(16)), 75.8 (C(22)), and 61.6 (C(26)), which are characteristic for a solanidine type steroid alkaloid. Considering the steroidal alkaloids previously reported from the genus *Veratrum*, **2** was determined to be the known compound 7α-hydroxyrubijervine[1]) [16].

Compound **3** was obtained as a white amorphous powder, showing a positive reaction with *Dragendorff*'s reagent. The HR-ESI-MS (positive mode) gave the molecular formula $C_{33}H_{53}NO_8$ from the $[M+H]^+$ signal at *m/z* 592.3842. The ESI-MS of **3** gave a significant fragment ion peak at *m/z* 430 ($[M+H-162]^+$). The IR spectrum exhibited absorptions for OH groups (3361 and 1073 cm^{-1}). The ^{13}C-NMR data of **3** were similar to those of 7α-hydroxyrubijervine (**2**), except for the presence of six signals at δ(C) 60.8, 68.7, 75.8, 77.0, 80.3 and 101.9. From the above analysis, and considering the elimination of the 162 Da neutral fragment in the ESI-MS, we concluded that there is a β-galactose moiety (D-configured) in **3** by comparison of chemical shifts and coupling constants of the sugar moiety with those of galactofuranose [17], but the absolute configuration of the sugar units have not been proved. Therefore, **3** was determined as an analog of 7α-hydroxyrubijervine (**2**). The galactofuranose moiety must be attached to C(7) due to the HMBC correlations

Table 1. *[13]C-NMR Spectral Data of* **1**–**5**. At 125 MHz in MeOH; δ in ppm.

	1	**2**	**3**	**4**	**5**
CH_2(1)	38.2	38.1	38.1	38.2	38.4
CH_2(2)	32.6	32.5	32.5	32.6	32.4
H–C(3)	71.9	72.1	70.2	71.9	72.1
CH_2(4)	43.0	42.8	42.3	43.0	43.1
C(5)	142.1	141.9	143.2	142.1	143.2
H–C(6)	122.4	122.3	124.5	122.4	122.7
CH_2(7) or H–C(7)	30.2	69.9	73.8	70.1	73.9
H–C(8)	37.0	36.8	38.2	37.0	39.4
H–C(9)	47.6	47.9	47.7	47.6	47.9
C(10)	34.8	34.9	34.8	34.8	35.0
CH_2(11)	33.3	32.9	31.6	33.3	32.9
H–C(12)	72.5	72.4	72.4	72.5	74.3
C(13)	48.3	48.5	48.3	48.3	48.6
H–C(14)	48.2	48.1	48.2	48.2	48.4
CH_2(15)	24.3	24.1	24.0	24.3	23.9
CH_2(16) or H–C(16)	27.4	73.7	72.8	27.5	27.3
H–C(17)	47.3	47.9	47.8	47.3	47.3
Me(18)	13.1	12.5	13.0	13.1	12.7
Me(19)	19.6	19.6	19.7	19.6	19.8
H–C(20)	47.8	47.8	47.6	47.8	47.9
Me(21)	17.6	17.6	17.6	17.6	17.6
C(22) or H–C(22)	179.1	75.8	75.9	179.1	179.1
CH_2(23)	28.0	28.2	28.4	28.0	28.1
CH_2(24)	28.2	28.5	28.5	28.2	28.2
H–C(25)	27.3	27.3	27.5	27.3	27.1
CH_2(26)	56.7	61.6	61.8	56.7	56.8
Me(27)	19.5	19.9	20.1	19.5	19.6
H–C(1′)			101.9		102.0
H–C(2′)			77.0		77.1
H–C(3′)			75.8		76.0
H–C(4′)			80.3		81.9
H–C(5′)			68.7		69.9
CH_2(6′)			60.8		61.9

between H–C(1′) (5.09, *d*, $J = 7.0$) and C(7) (δ(C) 73.8). Therefore, the structure of **3** was identified as 7α-hydroxyrubijervine-7-O-β-D-galactofuranoside[1]).

Compound **4** was obtained as a white amorphous powder, showing a positive reaction with *Dragendorff*'s reagent. The HR-ESI-MS (positive mode) gave the molecular formula $C_{27}H_{44}NO_3$ from the $[M+H]^+$ signal at *m*/*z* 442.3304). The ESI-MS of **4** showed significant fragment ion peak at *m*/*z* 424 ($[M+H-H_2O]^+$). The IR spectrum exhibited absorptions for OH groups (3361 and 1068 cm^{-1}) and a C=C bond (1624 cm^{-1}). The ^{13}C-NMR (*Table 1*), ^{1}H-NMR (*Table 2*) and ESI-MS data suggested that **4** is a monohydroxylated vermitaline derivative [4]. Three OH groups were located at C(3) (δ(C) 71.9), C(7) (δ(C) 70.1), and C(12) (δ(C) 72.5) respectively, due to the HMBC correlations between δ(H) 3.20–3.24 (*m*, H–C(3)) and C(2) (δ(C) 32.6), and C(4) (δ(C) 43.0), δ(H) 3.68 (*dd*, $J = 2.0, 2.0$, H–C(7)) and C(6) (δ(C) 122.4), and C(8) (δ(C) 37.0), δ(H) 3.37 (br. *s*, H–C(12)) and C(11) (δ(C) 33.3), and C(13) (δ(C)

Table 2. *^{1}H-NMR Spectral Data of* **1**–**5**. At 500 MHz in MeOH; δ in ppm, J in Hz.

	1	**2**	**3**	**4**	**5**
CH_2(1)	1.53–1.55 (*m*), 1.48–1.52 (*m*)	1.54–1.57 (*m*), 1.48–1.53 (*m*)	1.53–1.56 (*m*), 1.47–1.52 (*m*)	1.53–1.56 (*m*), 1.49–1.52 (*m*)	1.53–1.57 (*m*), 1.48–1.59 (*m*)
CH_2(2)	1.60–1.64 (*m*), 1.30–1.34 (*m*)	1.60–1.63 (*m*), 1.30–1.35 (*m*)	1.59–1.64 (*m*), 1.32–1.36 (*m*)	1.62–1.64 (*m*), 1.31–1.35 (*m*)	1.58–1.62 (*m*), 1.30–1.34 (*m*)
H–C(3)	3.22–3.26 (*m*)	3.21–3.26 (*m*)	3.22–3.25 (*m*)	3.20–3.24 (*m*)	3.21–3.25 (*m*)
CH_2(4)	2.12–2.15 (*m*), 2.08–2.11 (*m*)	2.11–2.15 (*m*), 2.07–2.10 (*m*)	2.13–2.16 (*m*), 2.09–2.11 (*m*)	2.13–2.16 (*m*), 2.08–2.13 (*m*)	2.13–2.16 (*m*), 2.08–2.12 (*m*)
H–C(6)	5.35 (*d*, J = 2.0)	5.35 (*d*, J = 2.0)	5.32 (*d*, J = 2.0)	5.32 (*d*, J = 2.0)	5.32 (*d*, J = 2.0)
CH_2(7) or H–C(7)	3.68 (*dd*, J = 2.0, 2.0)	3.68 (*dd*, J = 2.0, 2.0)	3.30 (*dd*, J = 2.0, 2.0)	3.68 (*dd*, J = 2.0, 2.0)	3.30 (*dd*, J = 2.0, 2.0)
H–C(8)	1.43–1.47 (*m*)	1.43–1.47 (*m*)	1.75–1.79 (*m*)	1.45–1.49 (*m*)	1.65–1.69 (*m*)
H–C(9)	2.10–2.12 (*m*)	2.08–2.12 (*m*)	2.08–2.13 (*m*)	2.09–2.12 (*m*)	2.07–2.10 (*m*)
CH_2(11)	1.66–1.70 (*m*), 1.43–1.47 (*m*)	1.65–1.69 (*m*), 1.44–1.47 (*m*)	1.67–1.70 (*m*), 1.43–1.46 (*m*)	1.65–1.08 (*m*), 1.43–1.47 (*m*)	1.66–1.70 (*m*), 1.45–1.48 (*m*)
H–C(12)	3.50 (br. *s*)	3.50 (br. *s*)	3.37 (br. *s*)	3.37 (br. *s*)	3.37 (br. *s*)
H–C(14)	2.08–2.12 (*m*)	2.07–2.12 (*m*)	2.09–2.13 (*m*)	2.08–2.11 (*m*)	2.08–2.12 (*m*)
CH_2(15)	1.85–1.87 (*m*), 1.09–1.13 (*m*)	1.85–1.89 (*m*), 1.09–1.12 (*m*)	1.84–1.87 (*m*), 1.08–1.11 (*m*)	1.83–1.87 (*m*), 1.09–1.13 (*m*)	1.85–1.88 (*m*), 1.10–1.13 (*m*)
CH_2(16) or H–C(16)	1.33–1.36 (*m*), 1.58–1.61 (*m*)	2.21–2.24 (*m*)	2.21–2.24 (*m*)	1.34–1.36 (*m*), 1.58–1.62 (*m*)	1.33–1.37 (*m*), 1.57–1.61 (*m*)
H–C(17)	2.24 (*dd*, J = 10.0, 10.0)	2.24 (*dd*, J = 10.0, 10.0)	2.24 (*dd*, J = 10.0, 10.0)	1.50–1.53 (*m*)	1.50–1.53 (*m*)
Me(18)	0.69 (*s*)	0.69 (*s*)	0.69 (*s*)	0.76 (*s*)	0.76 (*s*)
Me(19)	0.96 (*s*)	0.96 (*s*)	0.96 (*s*)	1.01 (*s*)	1.01 (*s*)
H–C(20)	1.63–1.67 (*m*)	1.63–1.66 (*m*)	1.64–1.67 (*m*)	1.88–1.91 (*m*)	1.88–1.92 (*m*)
Me(21)	0.90 (*d*, J = 6.0)	0.90 (*d*, J = 6.0)	0.90 (*d*, J = 6.0)	1.05 (*d*, J = 5.0)	1.05 (*d*, J = 5.0)
H–C(22)	1.48–1.52 (*m*)	1.49–1.52 (*m*)	1.48–1.53 (*m*)	–	–
CH_2(23)	1.64–1.68 (*m*), 1.20–1.23 (*m*)	1.63–1.66 (*m*), 1.21–1.25 (*m*)	1.62–1.66 (*m*), 1.20–1.24 (*m*)	2.33–2.37 (*m*), 2.23–2.27 (*m*)	2.34–2.37 (*m*), 2.23–2.26 (*m*)
CH_2(24)	1.62–1.66 (*m*), 0.94–0.98 (*m*)	1.63–1.66 (*m*), 0.93–0.97 (*m*)	1.64–1.67 (*m*), 0.95–0.98 (*m*)	1.63–1.68 (*m*), 0.97–1.00 (*m*)	1.62–1.65 (*m*), 0.94–0.99 (*m*)
H–C(25)	1.47–1.51 (*m*)	1.49–1.52 (*m*)	1.47–1.50 (*m*)	1.48–1.52 (*m*)	1.47–1.52 (*m*)
CH_2(26)	2.84 (*dd*, J = 11.0, 3.5), 1.34 (*dd*, J = 11.0, 3.5)	2.84 (*dd*, J = 11.0, 3.5), 1.34 (*dd*, J = 11.0, 3.5)	1.45 (*dd*, J = 12.0, 6.0), 1.19 (*dd*, J = 12.0, 5.8)	1.45 (*dd*, J = 12.0, 6.0), 1.19 (*dd*, J = 12.0, 5.8)	1.45 (*dd*, J = 12.0, 6.0), 1.19 (*dd*, J = 12.0, 5.8)
Me(27)	0.95 (*d*, J = 6.6)	0.97 (*d*, J = 6.6)	0.95 (*d*, J = 6.0)	0.94 (*d*, J = 6.0)	0.97 (*d*, J = 6.0)
H–C(1′)			5.09 (*d*, J = 7.0)		5.09 (*d*, J = 7.0)
H–C(2′)			3.96–3.98 (*m*)		3.96–3.98 (*m*)
H–C(3′)			4.51–4.53 (*m*)		4.51–5.54 (*m*)
H–C(4′)			3.91–3.93 (*m*)		3.90–3.93 (*m*)
H–C(5′)			3.62–3.65 (*m*)		3.62–3.65 (*m*)
CH_2(6′)			3.81 (*dd*, J = 2.0, 3.0), 3.79 (*dd*, J = 2.0, 3.0)		3.81 (*dd*, J = 2.0, 3.0), 3.79 (*dd*, J = 2.0, 3.0)

48.3). The configuration of the positions C(3), C(12), C(13), C(10), C(20), and C(25), respectively, was established to be analogous to **1** by analysis of NOESY spectra, and also by comparison of chemical shifts and coupling constants of **4** with those of **1**. The relative configuration of C(7) was deduced to be α, by the observation of the NOESY correlation between H–C(7) with δ(H) 1.45–1.49 (*m*, H_β–C(8)). Therefore, the structure of **4** was identified as 7α-hydroxyvermitaline[1]).

Compound **5** was obtained as a white amorphous powder, showing a positive reaction with *Dragendorff*'s reagent. The HR-ESI-MS (positive mode) of **5** displayed a peak at *m*/*z* 592.3853 ($[M+H]^+$), suggesting the molecular formula $C_{33}H_{53}NO_8$. The IR spectrum exhibited absorptions for OH groups (3352 and 1068 cm^{-1}) and C=C bonds (1618 cm^{-1}). The ^{13}C-NMR (*Table 1*), and ^{1}H-NMR (*Table 2*) spectra of **5** suggested the structure of a vermitaline derivative. Similar to compound **3**, the ^{13}C-NMR spectrum of **5** showed six signals in the region at δ(C) 61.9, 69.9, 76.0, 77.1, 81.9, and 102.0. These data indicated the presence of a β-D-galactose moiety in **5**. H–C(7) (δ(H) 3.30) also showed HMBC coupling with C(1′) (δ(C) 102.0). Metabolite **5** was thus confirmed to be 7α-hydroxyvermitaline-7-O-β-D-galactofuranoside[1]), a new metabolic product reported for the first time in fermentation studies of steroidal alkaloids.

Experimental Part

General. Vermitaline (=*(3β,12α,17β)-17-{1-[(5*S*)-3,4,5,6-tetrahydro-5-methylpyridin-2-yl]ethyl}androst-5-ene-3,12-diol*; **1**) was isolated from the roots of *Veratrum dahuricum* with a purity of 99.1% by HPLC analysis. The compound was authenticated by detailed NMR analysis and comparing with data from the literature [18]. Column chromatography (CC): *MCI* gel *CHP20P* (high-porous polymer 75–150μ; *Mitsubishi Chemicals*, Japan), reversed-phase silica gel *RP-18* (40–63 μm, *Merck*, Germany), silica gel (SiO_2) (200–300 mesh, *Yantai*, P. R.China) and *Sephadex LH-20* (*Pharmacia Co. Ltd.*). TLC: SiO_2 plates; visualization by spraying with 10% H_2SO_4 in EtOH, followed by heating. Optical rotations: *Perkin-Elmer-341* polarimeter. IR Spectra: *Nicolet-Compact-40-Bruker-Vector-22* spectrophotometer; ν_{max} in cm^{-1}. NMR Spectra: *Bruker AVANCE^{II} 500 NMR*, for ^{1}H-NMR at 500 MHz, and ^{13}C-NMR at 125 MHz; δ in ppm with Me_4Si as an internal standard, *J* in Hz. MS: *Varian MAT-212* mass spectrometer (for ESI)and *Q-Tof* micro mass spectrometer (for HR-ESI); in *m*/*z*.

Plant Material. The plants were collected in Yanbian, Jilin Province, P. R. China, in September 2005, and authenticated as *V. dahuricum* by Prof. *Yong-Zhen Liu*, School of Pharmacy, Yanbian University, China. A voucher specimen (No. 211) was deposited at the Herbarium of the School of Pharmacy, Shanghai Jiao Tong University, Shanghai, China.

Organisms. The stock culture of *C. echinulata* (ACCC 30369) was maintained on a potato dextrose agar slant. Four *Erlenmeyer* flasks (1000 ml), each containing 300 ml of liquid medium consisting of 0.1% peptone, 0.1% yeast extract, 0.1% beef extract, and 0.5% glucose were inoculated with freshly obtained *C. echinulata* cultured from the agar slant on a rotary shaker at 180 rpm. After cultivation at 28° for 72 h, the vermitaline (**1**) soln. (50 mg of vermitaline dissolved in 250 μl EtOH) was added to each flask, and the incubation continued for 12 d.

Microbial Metabolism of Vermitaline. After 12 d of incubation, the incubation mixtures were pooled and filtered. The filtrate was applied to a *MCI-gel CHP20P* column, and washed with H_2O to remove sugars, then it was eluted with 20% aq. MeOH, 60% aq. MeOH, and MeOH to give the fractions *A*, *B*, and *C* resp. Each fraction was evaporated to dryness *in vacuo* and analyzed by TLC, which was developed by $CHCl_3$/MeOH (10:1) and visualized by spraying with 10% H_2SO_4 soln. *Frs. B* and *C* were combined and chromatographed repeatedly through a SiO_2 column ($CHCl_3$) and *Sephadex LH-20* (70% MeOH) to furnish **2** (4.9 mg) and **4** (5.6 mg). *Fr. A* was dissolved in 10% aq. MeOH and subjected to RP SiO_2 (*ODS*) CC, eluting with the gradient H_2O/MeOH (100:10–0:100) to give **3** (5.9 mg), and **5** (3.8 mg).

7α-Hydroxyrubijervine (*=(3β,7α,12α,22ξ)-Solanid-5-ene-3,7,12-triol*; **2**). White amorphous powder. IR (KBr): 3370, 2951, 2177, 1623, 1070. ^{1}H - and ^{13}C-NMR: *Tables 1* and *2*. 2D-NMR (HMBC; 500 MHz, $CDCl_3$): H−C(3)/C(4), H−C(7)/C(5), H−C(7)/C(8), H−C(15)/C(14). ESI-MS: 430 ($[M+H]^+$), 412 ($[M+H-H_2O]^+$). HR-ESI-MS: 430.3314 ($[M+H]^+$, $C_{27}H_{44}NO_3^+$; calc. 430.3321).

*7α-Hydroxyrubijervine-7-*O*-β-*D*-galactofuranoside* (*=(3β,7α,12α,22ξ)-3,12-Dihydroxysolanid-5-en-7-yl Hexofuranoside*; **3**). White amorphous powder. IR (KBr): 3361, 2947, 2170, 1617, 1073. ^{1}H- and ^{13}C-NMR: *Tables 1* and *2*. 2D-NMR (HMBC; 500 MHz, $CDCl_3$): H−C(3)/C(4), H−C(7)/C(5), H−C(7)/C(8), H−C(15)/C(14), H−C(1′)/C(7). ESI-MS: 592 ($[M+H]^+$), 430 ($[M+H-162]^+$). HR-ESI-MS: 592.3842 ($[M+H]^+$, $C_{33}H_{54}NO_8^+$; calc. 592.3849).

7α-Hydroxyvermitaline (*=(3β,7α,12α,17β)-17-[1-[(5*S*)-3,4,5,6-Tetrahydro-5-methyl-2-pyridinyl]-ethyl]androst-5-ene-3,7,12-triol*; **4**). White amorphous powder. IR(KBr): 3361, 2937, 2155, 1624, 1068. ^{1}H- and ^{13}C-NMR: *Tables 1* and *2*. 2D-NMR (HMBC; 500 MHz, $CDCl_3$): H−C(3)/C(4), H−C(7)/C(5), H−C(7)/C(8), H−C(15)/C(14). ESI-MS: 430 ($[M+H]^+$), 658 ($[M+H-H_2O]^+$). HR-ESI-TOF-MS: 430.3304 ($[M+H]^+$, $C_{27}H_{44}NO_3^+$; calc. 430.3321).

*7α-Hydroxyvermitaline-7-*O*-β-*D*-galactofuranoside* (*=(3β,7α,12α,17β)-3,12-Dihydroxy-17-[1-[(5*S*)-3,4,5,6-tetrahydro-5-methyl-2-pyridinyl]ethyl]androst-5-en-7-yl Hexofuranoside*; **5**). White amorphous powder. IR(KBr): 3352, 2944, 2162, 1618, 1068. ^{1}H- and ^{13}C-NMR *Tables 1* and *2*. 2D-NMR (HMBC; 500 MHz, $CDCl_3$): H−C(3)/C(4), H−C(7)/C(5), H−C(7)/C(8), H−C(15)/C(14), H−C(1′)/C(7). ESI-MS: 592 ($[M+H]^+$), 430 $[M+H-162]^+$. HR-ESI-MS: 592.3853 ($[M+H]^+$, $C_{33}H_{54}NO_8^+$; calc. 592.3849).

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