Bioorganic & Medicinal Chemistry Letters 19 (2009) 6637–6639

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis of 3β, 7α, 11α-trihydroxy-pregn-21-benzylidene-5-en-20-one derivatives and their cytotoxic activities

Li-Hong Shan [a], Hong-Min Liu [a,*], Ke-Xue Huang [b], Gui-Fu Dai [c], Chen Cao [a], Rui-Jing Dong [c]

[a] New Drug Research and Development Center of Zhengzhou University, Zhengzhou 450001, China
[b] College of Life Science, Hunan Normal University, Changsha 410081, China
[c] Bioengineering Department of Zhengzhou University, Zhengzhou 450001, China

ARTICLE INFO

Article history:
Received 18 February 2009
Revised 18 September 2009
Accepted 5 October 2009
Available online 14 October 2009

Keywords:
Biotransformation
Pregnenolone
Hydroxylation
Mucor circinelloides lusitanicus
Claisen–Schimidt condensation
Cytotoxicity

ABSTRACT

A series of novel 3β, 7α, 11α-trihydroxy-pregn-21-benzylidene-5-en-20-one derivatives were synthesized and characterized by NMR, HRMS. The pregnenolone (**1**) was first biotransformed by *Mucor circinelloides var lusitanicus* to 3β, 7α, 11α-trihydroxy-pregn-5-en-20-one (**3**), then **3** was treated with various benzaldehydes to produce 3β, 7α, 11α-trihydroxy-pregn-21-benzylidene-5-en-20-one derivatives. These derivatives showed remarkable activity against EC109 cells. The absolute configuration of **3** was also confirmed by signal-crystal X-ray analysis.

It is well known that a variety of steroids are widely used as anti-inflammatory, diuretic, anabolic, contraceptive, antiandrogenic, progestational, anticancer agents as well as in other applications.[1] The distinct structural steroids have aroused great interest, and extensive research have been carried out to develop the methods to synthesize and characterize steroids.[2] Since Peterson and Murray reported the 11α-hydroxylation of progesterone by *Rhizopus arrhizus* in 1952,[3] microbial reactions for the transformation of steroids have proliferated, and specific microbial transformation steps have been incorporated into numerous partial syntheses of new steroids for evaluation as drugs and hormones.[4,5]

Pregnenolone (**1**) is a major hormone mainly present in human nerve tissues,[6] and its therapeutic role in repairing neurons has been well documented.[7] Recent studies indicated that 7α-hydroxylated metabolite of **1** has many profound activities, such as augmenting immune response in mouse,[8] possessing antiglucorticoid potencies,[9] improving spatial memory,[10] and acting as a neuronal activator to stimulate locomotor activity.[11] Compound **1** was also biotransformed to its 7α-hydroxylated, 11α-hydroxylated or 7α, 11α-dihydroxylated derivatives with low yield,[12–15] and these metabolites are not commercially available. This hinders further studies on their structure modification and activities evaluation.[16] Although introduction of new steroids into market is now limited, our interests focus on the improvement of the yields for important metabolites as well as preparation of new steroids that are difficult to synthesize by chemical approaches.

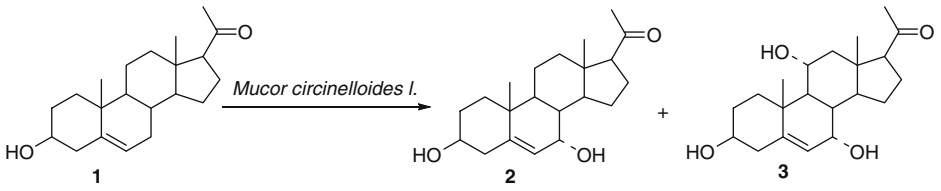
The fungus, *Mucor circinelloides var lusitanicus*, was isolated from local soil and identified by Microbial ID (Newark, USA). The family *Mucor* are widely distributed in nature. *Mucor circinelloides* was used to hydrolyze carboxymethyl cellulose, insoluble cellulose substrates to produce valuable soluble cellodextrins.[17–20] But no report on using it to biotransform steroids was previously reported.

Our studies showed that *Mucor circinelloides var lusitanicus* can transform various 5-en-3β-ol steroids to their 7α-hydroxy derivatives. We were interested in the biotransformation of compound **1** (Scheme 1), mainly because compound **3** could be easily crystallized from the mixture with high yield (46.38%). This made it possible to synthesize 3β, 7α, 11α-trihydroxy-pregn-21-benzylidene-5-en-20-one derivatives **5a**–**k** (Table 1) from **3** in large quantities through Claisen–Schimidt reaction. In the current studies, the cytotoxic effects of **3** and **5a**–**k** on EC109 cells were evaluated.

In order to obtain compound **3**, the fermentation media was optimized,[21] which resulting in an improvement of the transformation efficiency of **1** from 70% to 94%. Furthermore, the recovery yield of **3** reached 46.38% by crystallizing from the extract mixture directly with methanol and chloroform (1:3). The recovery yield of **3** was higher than that of the reported yield 19.6%[22] and 35.0%.[23] Product **2** was purified by silica gel chromatography with a yield of 24.38%. The optimization process showed that the concentration

* Corresponding author. Tel./fax: +86 371 67781739.
E-mail address: liuhm@zzu.edu.cn (H.-M. Liu).

0960-894X/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2009.10.019



Scheme 1. Biotransformation of pregnenolone **1** to compound **2** and **3** by *Mucor circinelloides var lusitanicus*.

Table 1
Structures of compound **5a–k** and the yields

Entry	R	Reaction time (h)	Product	Yield (%)
1	H	8	**5a**	87
2	3-NO_2	8	**5b**	88
3	3-Cl	8	**5c**	85
4	4-Cl	8	**5d**	85
5	4-F	8	**5e**	89
6	4-OCH_3	12	**5f**	74
7	2-OCH_3	12	**5g**	68
8	4-OH	24	**5h**	63
9	4-NH_2	24	**5i**	58
10	4-$N(CH_3)_2$	24	**5j**	51
11	2,4,6-$(OCH_3)_3$	24	**5k**	50

of Mg^{2+} and the initial pH (pH 6.0) in the fermentation media were critical for the transformation of **1** to **3**.

The ^{1}H NMR spectrum[24] of **3** showed three hydroxyl-bearing methine proton signals at δ 3.80 (m, 1H), 3.57 (m, 1H), 3.34 (m, 1H). These values corresponded to the chemical shift of proton attached to a carbon atom bearing a hydroxyl group. Also the ^{1}H NMR spectrum showed a downfield shift of the C-19 methyl protons (δ 1.12) when compared to the spectrum of the substrate **1** (δ 1.00). This is due to the presence of a hydroxyl group at the 11α-position. So δ 3.80 was attributed to 11β proton. The downfield shift (δ 5.44) and split (J = 5.3 Hz) of 6 proton implied a hydroxyl group at 7α-position and assignment of multiple δ 3.57 to 7β proton. Finally, δ 3.34 was attributed to the 3α proton. Based on the NMR, HRMS results, together with the literature data,[23] compound **3** was assigned the structure of 7α, 11α-dihydroxy-pregn-5-en-20-one. The absolute configuration of **3** was established by X-ray crystallographic analysis (Fig. 1), which confirmed the hydroxyl group at 7 and 11 position was in the axial (α) orientation.

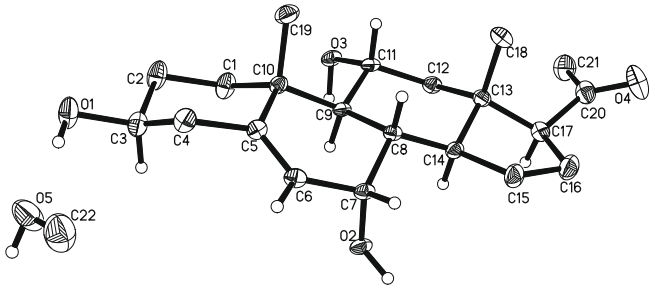
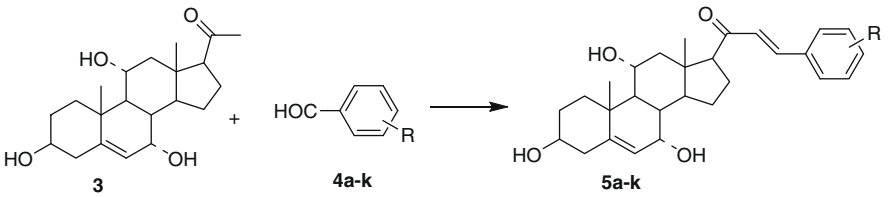


Figure 1. The X-ray diffraction analysis diagram of compound **3**.

Introduction of aromatic group into steroids have led to new physiological activity.[25] Hydroximino derivatives of 16E-arylidenosteroids showed good antineoplastic activity,[26] and 16-(4-substituted benzylidene) derivatives of androst-5-ene also had antiproliferative activity against cancer cells.[27] So we introduced benzylidene groups into **3** and evaluated their cytotoxic activity. Through the Claisen–Schimidt condensation reaction, compound **3** was reacted with benzaldehyde **4a–k** and compounds **5a–k** were produced (Scheme 2). Compared to Na_2CO_3, K_2CO_3 and 1 mol/L of NaOH solution, solid NaOH catalyzed the reaction more efficiently. The effect of solvents in the reaction was also investigated. Only in strong polar protolytic solvent, such as methanol and ethanol, was the reaction performed successfully. When ethanol (95%) was used as the reaction solvent, the reaction was completed under room temperature in short period. The structures of compounds **5a–k** and the reaction conditions are presented in Table 1. The reaction time and yield revealed that the electron-withdrawing group on the benzene ring was favorable to the reaction. Taking **5a** as an example to illustrate how the structures of **5a–k** were determined: From the ^{1}H NMR spectrum,[28] the signal of 21-methyl (δ 2.07) of **3** disappeared, instead of that, two proton signals at δ 6.92 (d), 7.54 (d) were found, and the two relevant protons shared a same J constant (16.07 Hz). Thus they were assigned to the 21 and 22 proton, respectively. Through the J Constant, we concluded that the substitutes on the C21 and C22 were E-type. The chemical shifts at δ 130.5 and 141.1 in ^{13}C NMR spectrum of **5a** confirmed that the desired carbonyl group C=C had been formed. The HRMS spectrum of **5a** also confirmed the formula of **5a** was $C_{28}H_{36}O_4$. Together with other signals in the ^{1}H, ^{13}C NMR spectrum, **5a** was defined as 3β, 7α, 11α-trihydroxy-pregn-21-benzylidene-5-en-20-one. To our knowledge, this is the first report of the synthesis of compounds **5a–k**.

The in vitro cytotoxicity[29] of this novel series of compounds against the human esophageal cancer cell line, EC109, was evaluated. Table 2 shows the inhibitory effects on cell proliferation. Oridonin, an efficacious component of antitumour extracted from Chinese herb Rabdisia rubeseas (hemsl) Hara, was used to treat tumor of digestive tract on clinical.[30] We selected oridonin as the positive contral. From the results, we can find that compound **1** only show faintish activity to EC109. Compared to **1**, both compound **3** and **5a–k** show stronger inhibition activity. This indicated that the 7α- and 11α-hydroxyl group and the structure of benzylidene are essential for the cytotoxic activity we examined. Moreover, the majority of **5a–k** produced a stronger suppressive activity than their parent compound (**3**), and the cytotoxicity response was sen-



Scheme 2. Synthesis of compound **5a–k**. Universal reagent and conditions: solid NaOH, 95% ethanol, rt.

Table 2
Inhibitory effects of compounds against EC109 cells at concentration of 50 μg/mL

Compd	EC109
1	13.6
3	21.72
5a	59.79
5b	72.37
5c	76.43
5d	80.25
5e	76.82
5f	55.29
5g	47.42
5h	45.66
5i	50.21
5j	38.97
5k	35.26
Oridonin	94.65
NC[a]	7.5E-5

[a] Negative control.

sitive to a variety of functional groups and positions on the benzene ring. Furthermore, the results indicate that when there was an electron-withdrawing group on the benzene ring, cytotoxic activity was much higher than when compared to the presence of an electron-donating group on the benzene ring.

In conclusion, we have synthesized a series of new 3β, 7α, 11α-trihydroxy-pregn-21-benzylidene-5-en-20-one derivatives through biotransformation and chemical synthesis, and evaluated their cytotoxicity against EC109 cells. The synthesis procedure was simple and efficient, and the results indicated that *Mucor circinelloides var lusitanicus* can be used to biotransform other steroids to produce novel active steroids. Additionally, other activities of these compounds and further structure modification to improve the bioactivity are under investigation.

Acknowledgment

This research work was supported by NSFC of China (No. 20572103).

Supplementary data

Supplementary data (CCDC-668534 (compound **3**) contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via www. ccdc. com. ac.uk/data_request/cif, by emailing data_request@ccdc.cam.ac.uk, or by contacting the Cambridge Crystallographic Data Center, 12 Union Road, Cambridge CB2 1EZ, UK [fax: +44 1223 336033]) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.10.019.

References and notes

1. Mahato, S. B.; Garai, S. *Steroids* **1997**, *62*, 332.
2. Reese, P. B. *Steroids* **2001**, *66*, 481.
3. Peterson, D. H.; Murry, H. C.; Eppstein, S. H.; Reineke, L. M.; Weintraub, A.; Meister, P. D.; Leigh, H. M. *J. Am. Chem. Soc.* **1952**, *74*, 5933.
4. Fernandes, P.; Cruz, A.; Angelova, B.; Pinheiro, H. M.; Cabral, J. M. S. *Enzyme Microb. Technol.* **2003**, *32*, 688.
5. Herbert, L. H. *Steroids* **1999**, *64*, 178.
6. Morfin, R.; Young, J.; Corpechot, C. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 6790.
7. Baulieu, E. E.; Robel, P.; Fellous, A.; Duchossoy, Y.; Fontaine-Lenoir, V.; David, S. *J. Mol. Neurosci.* **2004**, *24*, 63.
8. Morfin, R.; Courchay, G. *J. Steroid Biochem. Mol. Biol.* **1994**, *50*, 91.
9. Morfin, R.; Calvez, D.; Malewiak, M. I. *Inl. Congr. Hormonal Steroids* (Dallas, Texas, USA), 1994, poster 103.
10. Yau, J. L. W.; Noble, J.; Graham, M.; Seckl, J. R. *J. Neurosci.* **2006**, *26*, 11034.
11. Matsunaga, M.; Ukena, K.; Baulieu, E.; Tsutsui, K. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 17282.
12. Andrew, S. C.; William, A. D.; Sir Ewart, R. H. J.; Denis Meakins, G.; John, T. P. *J. C. S Perkin I.* **1973**, 2137.
13. Madyastha, K. M. *Pro. India Acad. Sci. (Chem. Sci.)* **1994**, *106*, 1203.
14. Schaaf, O.; Dettner, K. *J. Steroid Biochem. Mol. Biol.* **2000**, *75*, 187.
15. Muhammad, I. C.; Iffat, B.; Syed, A. A. S.; Sarfraz, A. N.; Atta, U. R. *Chem. Pharm. Bull.* **2005**, *53*, 1455.
16. Anne-Cécile, C.; Robert, M. *J. Steroid Biochem. Mol. Biol.* **1999**, *68*, 229.
17. Saha, B. C. *Process Biochem.* **2004**, *39*, 1871.
18. Szczesna-Antczak, M.; Antczak, T.; Piotrowicz-Wasiak, M.; Rzyska, M.; Binkowska, N.; Bielecki, S. *Enzyme Microb. Technol.* **2006**, *39*, 1214.
19. Antczak, T.; Graczyk, J.; Szczesna-Antczak, M.; Bielecki, S. *J. Mol. Catal. B: Enzym.* **2002**, *19–20*, 287.
20. Navarro, E.; Sandmann, G.; Torres-Martinez, S. *Exp. Mycology.* **1995**, *19*, 186.
21. The optimized medium consisted of maltose 4.3%, peptone 2.3%, yeast 0.1%, K_2HPO_4 0.9%, $MgSO_4{\cdot}7H_2O$ 0.9 % and pH 6.0. Flasks (500 ml) containing 100 ml medium were inoculated with spore suspension (1.0 ml, 2.2×10^8 spores) from a two-day-old culture grown on the above slants, and incubated at 28 °C on a rotary shaker (220 rpm) for 48 h. After this growth period, 100 mg substrate in 1 ml acetone or DMSO was added to each flask and the incubation was continued for four days. Biotransformation of the substrates was monitored by TLC.
22. Ge, W. Z.; Wang, S. M.; Shan, L. H.; Li, N.; Liu, H. M. *J. Mol. Catal. B: Enzym.* **2008**, *55*, 37.
23. Madyastha, K. M.; Joseph, T. *Appl. Microbiol. Biotechnol.* **1995**, *44*, 339.
24. *Compound* **3**: ^{1}H NMR (400.1 MHz, DMSO-d_6, 25 °C, TMS): δ 0.50 (s, 3H, C(18)-CH_3), 1.12 (s, 3H, C(19)-CH_3), 2.07 (s, 3H, C(21)-CH_3), 3.34 (m, 1H, C(3)-H), 3.57 (m, 1H, C(7)-H), 3.80 (m, 1H, C(11)-H), 5.44 (d, 1H, J = 5.3 Hz, C(6)-H); ^{13}C NMR (100.6 MHz, DMSO-d_6, 25 °C, TMS): δ 14.1, 17.7, 22.6, 23.7, 31.3, 31.6, 37.1, 38.4, 38.5, 42.8, 43.6, 47.8, 49.1, 49.5, 62.8, 63.5, 67.2, 70.2, 124.3, 144.6, 208.7; HRMS: calcd for $C_{21}H_{32}O_4$ $[M+Na]^+$ requires 371.2198, and 371.2201 was found.
25. Dubey, S.; Piplani, P.; Jindal, D. P. *Lett. Drug Des. Discovery* **2005**, *2*, 537.
26. Chattopadhaya, R.; Jindal, D. P.; Minu, M.; Gupta, R. *Arzneim.-Forsch.* **2004**, *54*, 551.
27. Dubey, S.; Piplani, P.; Jindal, D. P. *Chem. Biodivers.* **2004**, *1*, 1529.
28. *Compound* **5a**: ^{1}H NMR (400.1 MHz, DMSO-d_6, 25 °C, TMS): δ 0.51 (s, 3H, C(18)-CH_3), 0.99 (s, 3H, C(19)-CH_3), 3.29 (m, 1H, C(3)-H), 3.61 (d, 1H, J = 2.2 Hz, C(7)-H), 3.79 (m, 1H, C(11)-H), 5.45 (d, 1H, J = 5.4 Hz, C(6)-H), 6.92 (d, 1H, J = 16.07 Hz, C(21)-H), 7.44 (t, 2H, ^2J = 3.6, C(3′)-H and C(5′)-H), 7.45 (s, 1H, C(4′)-H), 7.54 (d, J = 16.07 Hz, 1H, C(22)-H), 7.71 (t, 2H, ^2J = 3.7, C(2′)-H and C(6′)-H); ^{13}C NMR (100.6 MHz, DMSO-d_6, 25 °C, TMS): δ 14.4, 17.6, 22.6, 23.9, 31.6, 37.2, 38.3, 38.5, 42.9, 44.6, 47.8, 49.2, 49.6, 60.4, 63.5, 67.2, 70.1, 124.2, 127.4, 128.5(2C), 129.1(2C), 130.5, 134.8, 141.1, 144.6, 199.9; HRMS: calcd for $C_{28}H_{36}O_4$ $[M+H]^+$ requires 437.2692, and 437.2675 was found.
29. In vitro cytotoxicity studies: EC109 cell lines were obtained from National Laboratory of Molecular Oncology, Cancer Institute, Chinese Academy of Medical Science & Peking Union Medical College. Culture medium was RPMI-1640 (GIBCO Co., Grand Island, NY) supplemented with 10% (v/v) fetal calf serum, 100 IU/mL penicillin and 100 μg/mL streptomycin(Sigma Chemical Co., St. Louis, MO) at 37 °C in humidified air atmosphere of 5% CO_2 (Binder, CB150, Germany). Cell cytotoxicity was assessed by MTT assay. Briefly, cells were plated into 96-well-plate (7000 cells/well). The next day compound at 100 μg/mL diluted in culture medium was added (200 μL/well) to the wells. 48 h later, 20 μL of MTT (Sigma Chemical Co., St. Louis, MO) (0.5 mg/mL MTT in PBS) was added and cells were incubated for a further 4 h. 200 μL DMSO was added to each culture to dissolve the reduced MTT crystals. The MTT-formazan product dissolved in DMSO was estimated by measuring absorbance at 570 nm with a microplate reader (Biotech, Power Wave, CA). Then the inhibitory percentage of each compound was calculated.
30. Li, D.; Wu, L. J.; Tashiro, S. I. *Biol. Pharm. Bull.* **2007**, *30*, 254.