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Synthesis and *in vitro* activity of oleanane type derivatives against *Chlamydia trachomatis*

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Abstract: Modified synthesis of 3β-nicotinoyloxy-olean-12(13)-en-28-oic acid and 3-deoxy-3a-homo-3a-aza-28-hydroxy-olean-12(13)-ene from natural occurring oleanolic acid is suggested. These compounds and two others of ursane and lupane type triterpenoids (3-oximino-urs-12-en-28-oic acid and 3-deoxy-3a-homo-3a-aza-28-hydroxy-lup-12(13)-ene) were screened *in vitro* against *Chlamydia trachomatis* strain F-3271/Belarus/2015. Oleanane triterpenoids became the leading compounds with chemotherapeutic index > 8 and were chosen for further research.

Keywords: Synthesis; triterpenoids; oleanane; ursane; *Chlamydia trachomatis*.

1. Introduction

Triterpenoids are distributed widely in higher plants and are of interest because of their different types of biological activities.[1] For example, betulinic acid had a great effect against dysplastic nevus,[2] betulin is currently in phase III clinical trials for the treatment of burns and for its impregnation into medicinal bandages.[3] Triterpenoids also exhibit a wide spectrum of antimicrobial activity. Different types of pentacyclic triterpenoids have significant antistaphylococcal activity on at least one strain, and it is postulated that the primary target for their antimicrobial activity is the cell membrane which could also explain one of their other effects.[4]

Chlamydia trachomatis is a common sexually transmitted pathogen that can cause infertility.[5] This infection is treated with broad-spectrum antibiotics which affect pathogens as well as the

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normal endogenous microflora. With conventional antibiotic treatments, there is difficulty in achieving the complete eradication of chronic chlamydial infections, the possibility of developing drug resistance, and the unintended creation of other antibiotic-resistant pathogens. For this reason, it is important to consider new compounds exhibiting various means of antimicrobial activity and finding novel non-toxic compounds for the treatment of chlamydial infections remains an important goal.[6]

2. Background

The properties of different terpenoids as inhibitors of the bacteria family Chlamydiaceae, particularly *C. trachomatis*, are poorly studied. For diterpene lactone Andrographolide IC_{50} values were found to be 46 and 50 µM for two different strains of *C. trachomatis*.[7] Some betulin derivatives showed high (>70% growth inhibition) antichlamydial activity against *C. pneumoniae* at 1 µM.[8] To the best of our knowledge, there is no data about the inhibition of *Chlamydia trachomatis* by triterpenoids and their derivatives.

3. Experimental

General: The spectra recorded at the Center for the Collective Use 'Chemistry' of the Ufa Institute of Chemistry of the UFRC RAS. ^{1}H and ^{13}C-NMR spectra were recorded on a "Bruker AM-500" (Bruker, Billerica, MA, USA, 500 and 125.5 MHz, δ, ppm, Hz) in $CDCl_3$, internal standard tetramethylsilane. Mass spectra were obtained on a liquid chromatograph–mass spectrometer LCMS-2010 EV (Shimadzu, Kyoto, Japan). Melting points were detected on a micro table "Rapido PHMK05" (Nagema, Dresden, Germany). Optical rotations were measured on a polarimeter "Perkin-Elmer 241 MC" (PerkinElmer, Waltham, MA, USA) in a tube length of 1 dm. Elemental analysis was performed on a Euro EA-3000 CHNS analyzer (Eurovector, Milan, Italy); the main standard is acetanilide. Thin-layer chromatography analyses were performed on Sorbfil plates (Sorbpolimer, Krasnodar, Russian Federation), using the solvent system chloroform-ethyl acetate, 40:1. Substances were detected by a 10% solution of a sulfuric acid solution with subsequent heating at 100–120 ºC for 2–3 min. Oleanolic and nicotinic acids were purchased from Sigma – Aldrich.

*Synthesis of 3β-nicotinoyloxy-olean-12(13)-en-28-oic acid **1***: A mixture of oleanolic acid (229 mg, 0.5 mmol), N,N'-dicyclohexylcarbodiimide (103 mg, 1 mmol), DMAP (cat.) and nicotinic acid (160 mg, 1.3 mmol) in CH_2Cl_2 (15 mL) was stirred for 6 h at 5 ºC, then poured into 5% HCl solution (50 mL) and the precipitate was filtered off, washed with H_2O. The product was isolated by crystallization from ethanol with yield of 95% (267 mg) as beige powder. R_f 0.15. M.p. 221 °C. $[\alpha]_D^{20}$ + 97 (c 0.5, $CHCl_3$). [Lit.[9]: M.p. 220-222 °C. $[\alpha]_D^{20}$ + 98 (c 0.5, $CHCl_3$)]. ^{1}H NMR (δ, ppm, 500 MHz, $CDCl_3$): 9.20 (s, 1H, H-6'), 8.75 (d, *J* = 4 Hz, 1H, H-5'), 8.30 (d, *J* = 10, 1H, H-3'), 7.39 (t, *J* = 5 Hz, 1H, H-4'), 5.30 (s, 1H, H-12), 4.75 (t, *J* = 3.8 Hz, 1H, H-3), 2.85 (t, *J* = 3.8 Hz, 1H, H-18), 2.00–1.20 (m, 22H), 1.15 (s, 3H), 1.05 (s, 3H), 1.00 (s, 3H), 0.98 (s, 3H), 0.90 (s, 3H), 0.88 (s, 3H), 0.80 (s, 3H). ^{13}C NMR (δ, ppm, 125.5 MHz, $CDCl_3$): 183.6 (C-28), 164.9 (C-1'), 152.9 (C-6'), 150.6 (C-5'), 143.8 (C-13), 137.3 (C-3'), 126.9 (C-2'), 123.4 (C-4'), 122.4 (C-12), 82.4 (C-3), 55.4, 54.8, 47.6, 46.5, 45.9, 41.6, 40.9, 39.3, 38.1, 37.0, 34.6, 33.8, 33.1, 32.6, 32.5, 30.7, 28.2, 27.7, 25.9, 23.6, 23.4, 22.9, 18.2, 17.2, 16.9, 15.4 Anal. calc. for $C_{36}H_{51}NO_4$: C, 76.97; H, 9.15; N, 2.49. Found: C, 76.89; H, 9.13; N, 2.52. MS(APCI): *m/z* 562.78 $[M + H]^+$, (calcd. 561.81).

*Synthesis of 3-deoxy-3a-homo-3a-aza-28-hydroxy-olean-12(13)-ene **3***. A mixture of compound **2**[10] (235 mg, 0.5 mmol) and $LiAlH_4$ (19 mg, 0.5 mmol) in anhydrous THF was refluxed for 30 min and then poured into a 5% HCl solution (70 mL). The crude product was extracted with $CHCl_3$ (3×50), the organic layer was washed with H_2O, dried under $CaCl_2$ and evaporated *in vacuo*. The product was isolated by crystallization from ethanol with yield of 88% (194 mg) R_f 0.11. M.p. 174 °C. $[\alpha]_D^{20}$ + 2 (c 0.5, $CHCl_3$). [Lit.[11]: M.p. 175 °C. $[\alpha]_D^{20}$ + 1.7 (c 0.5, $CHCl_3$)]. ^{1}H NMR (δ, ppm, 500

MHz, $CDCl_3$): 5.20 (s, 1H, H-12), 3.50 and 3.28 (both d, J = 10.8 Hz 2H, H-28), 3.00–2.92 (m, 2H, H-3), 2.05–1.52 (m, 25H), 1.50 (s, 3H), 1.41 (s, 3H), 1.20 (s, 3H), 1.15 (s, 3H), 1.00 (s, 3H), 0.88 (s, 3H), 0.85 (s, 3H). ^{13}C NMR (δ, ppm, 125.5 MHz, $CDCl_3$): 143.8 (C-13), 122.7 (C-12), 69.5 (C-28), 62.3 (C-3), 54.6 (C-5), 46.2, 44.2, 42.5, 42.4, 41.1, 40.9, 40.1, 39.6, 37.0, 34.1, 33.2, 32.3, 31.0, 30.9, 28.3, 25.4, 25.3, 24.3, 23.7, 23.6, 22.3, 22.1, 22.0, 17.2, 16.6. Anal. calc. for $C_{30}H_{51}NO$: C, 81.57; H, 11.64; N, 3.17. Found: C, 81.49; H, 11.51; N, 3.11. MS(APCI): *m/z* 442.64 $[M + H]^+$, (calcd. 441.74).

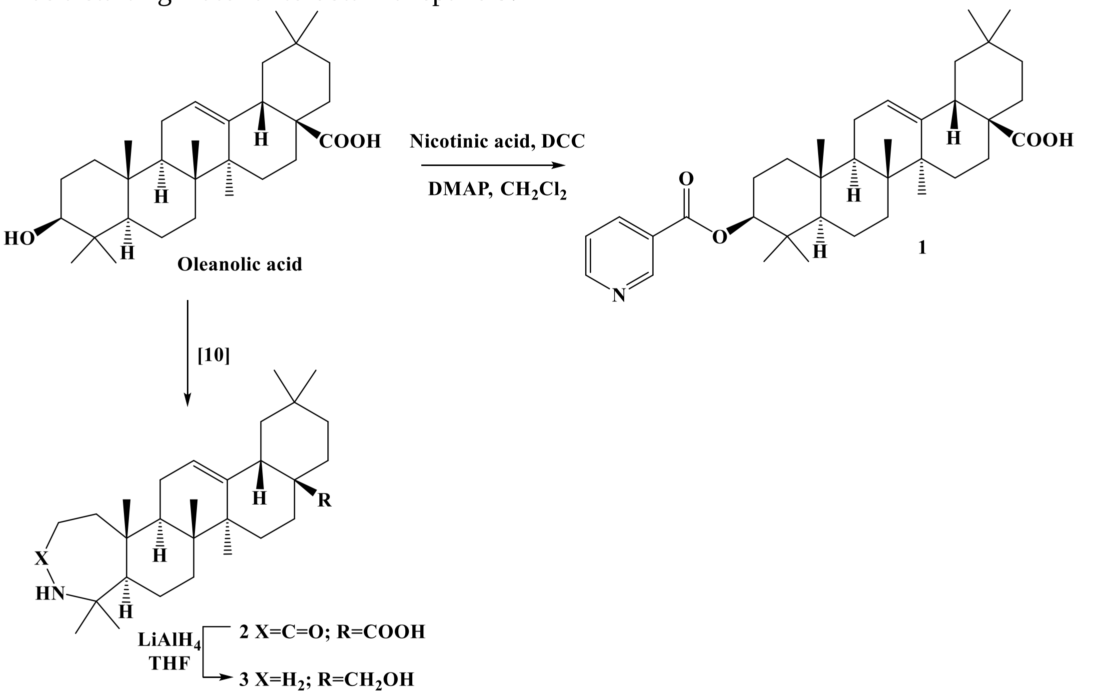
Reagents and organisms: Strain *C. trachomatis* F-3271/Belarus/2015 specialized collection of viruses and bacteria pathogenic for humans http://www.belriem.by/about/collection (Research Center for Epidemiology and Microbiology, Minsk, Belarus) were used. Sequencing of the ompA gene using primers P1/OMP2, NL-F/NL-R, CT6F/OMP2, P1/CT6R[12] showed that this strain corresponds to the serovar F and has 100% homology with the sequence of the strain F/IC-CAL3 https://www.ncbi.nlm.nih.gov/nuccore/DQ064287.1. The nucleotide sequence of the ompA gene of strain *C. trachomatis* F-3271/Belarus/2015 is deposited in the GenBank database https://www.ncbi.nlm.nih.gov/nuccore/MG733343.

Cell Culture: *C. trachomatis* were propagated in McCoy cells grown in DMEM with 10% heat-inactivated fetal bovine serum (HyClone, USA) supplemented with 2 mM L-glutamine, 10 µg/mL gentamicin, 5 mg/mL amphotericin B and 1 µg/mL cycloheximide (AppliChem, Germany). McCoy cells were cultured in glass flasks for 72 h. The cells were suspended after the addition of 0.02% ethylenediamine tetraacetate (Lonza, USA). 1 mL of the cell suspension, containing 10^5 cells, was transferred to flat–bottomed plastic tubes containing a coverslip (13 mm in diameter). The cells were incubated at 37 °C for 24 h to obtain a confluent cell layer. Then 80-90% confluent McCoy cell monolayer was infected stock suspension of *C. trachomatis* at a multiplicity rate of 1 : 1. The plate was centrifuged for 1 h at 1500 x g (relative centrifugal force) to synchronize the infection. After centrifugation, the tubes were incubated for 2 h at 37 °C, and thereafter a wide range of concentrations of compounds **1, 3-5** additions to the culture medium was performed. Tested compounds were preliminarily dissolved in 10% ethanol. Each test was performed in triplicate. All experiments were supplemented with negative (intact McCoy cells) and positive controls (McCoy cells infected by strain *C. trachomatis*). As quantitative criteria of the observable antichlamydial action, a decrease in the titer *C. trachomatis* was calculated in comparison with the control, $TCID_{50}$ (Median Tissue Culture Infectious Dose) of the compounds and chemotherapeutic index (CTI) were determined.

Assessment of Infective Progeny: The infection rate in the McCoy cells and infective progeny formation were estimated 72 h after pathogen inoculation. Titers were determined by infecting cell monolayers with 10-fold dilutions of the thawed stock suspension. All monolayers were stained using FITC-conjugated monoclonal antibody against chlamydial lipopolysaccharide (NearMedic Plus, Russian Federation). Inclusion-containing cells were visualized using the Nikon Eclipse 50i microscope at x1000 magnification.

4. Present Study

The aim of this study was to suggest the most convenient synthesis of two oleanane triterpenoids **1** and **3** from natural occurring oleanolic acid. It was shown that 3β-nicotinoyloxy-olean-12(13)-en-28-oic acid **1** inhibited the influenza type A H1N1 and the papillomavirus HPV-11[9] and 3-deoxy-3a-homo-3a-aza-28-hydroxy-olean-12(13)-en **3** showed activity against *M. tuberculosis*.[11] Environmentally friendly and economical synthesis of drugs and biologically active agents is one of the important aspects for the pharmaceutical industry, which is achieved by reducing the number of stages, using non-toxic solvents, etc. For this purpose, we acylated oleanolic acid by nicotinic acid in CH_2Cl_2 using as a dehydrating agent DCC and a catalytic amount DMAP with the formation of acylate **1** with a yield of 95% after crystallization from ethanol (Scheme 1). Previously this compound was synthesized by a two-step way using preliminary obtained nicotinic acid chloride with the following reaction with oleanolic acid in pyridine.[9] Thus, we reduced the number of stages from two to one, explored nontoxic reagents and increased the yield of target compound **1** by 10%. The synthesis of compound **3** was modified in comparison with the earlier described method[11] by reducing of the number of stages (from 5 to 4) and, respectively, by increasing the total yield. We have excluded the stage of protecting the COOH-group of oleanolic acid by methylation as described in[11] and used A-azepanono-oleanolic acid **2**[10] as a starting material to obtain azepane **3**.



Scheme 1. Synthesis of oleanane type triterpenoid derivatives

The second aim of this research was to evaluate the potential of triterpenoid derivatives against *C. trachomatis*. We decided to take the compounds, which have already shown any anti-infective activity (antiviral[9] for compound **1**, antitubercular[11] for compounds **3**, **5** and antimalarial[13] for compound **4**) (Figure 1).

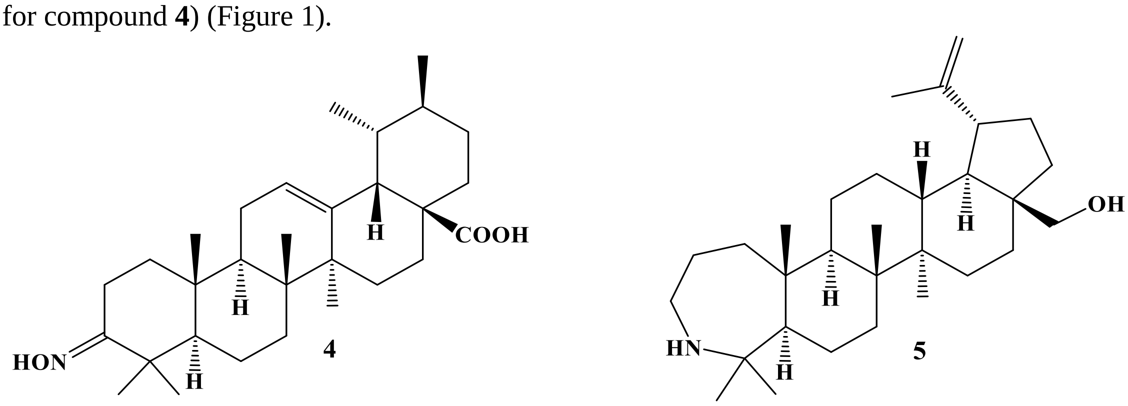


Figure 1. Synthetic derivatives of ursane **4** and lupane **5** type

Compounds **1**, **3–5** were screened *in vitro* toward *Chlamydia trachomatis* strain F-3271/Belarus/2015. $TCID_{50}$ assay method to determine the viability of Chlamydia under compounds action was used. The degree of the pathogen sensitivity to tested compounds was determined on the basis of the chemotherapeutic index (CTI) (Table 1). It was revealed that triterpenoids showed different potential. Compounds **1** and **3** demonstrated a high *Chlamydia* inhibitory activity (chemotherapeutic index was > 8). The range of active non-toxic concentrations for compounds **1** and **3** is quite wide (the lower limit is not reached). The minimum active concentration of compound **4** that reduces the *Chlamydia* titer by at least 1.25l g was less than 100 µg/mL. Azepanobetulin **5** has a chemotherapeutic index of 2 and was classified as low active.

5. Conclusion

As a result of this screening study oleanane type derivatives **1** and **3** were found to be promising for further research and we have developed the most convenient synthesis of these biologically active triterpenoids.

Table 1. An activity of compounds **1, 3-5** against *Chlamydia trachomatis* in McCoy cells test

Compound	Concentration, µg/mL	Titer strain, lg $TCID_{50}$	Difference with positive control, lg $TCID_{50}$	Chemotherapeutic index (CTI)
1	800	<2	>3.8	>8
	400	<2	>3.8	
	200	<2	>3.8	
	100	<2	>3.8	
	50	<2	>3.8	
	25	<2	>3.8	
	12.5	<2	>3.8	
	6.25	<2	>3.8	
3	800	<2	>3.8	>8
	400	<2	>3.8	
	200	<2	>3.8	
	100	<2	>3.8	
	50	<2	>3.8	
	25	<2	>3.8	
	12.5	<2	>3.8	
	6.25	2.20±0.07	3.6	
4	800	<2	>3.8	8
	400	<2	>3.8	
	200	<2	>3.8	
	100	<2	>3.8	
	50	4.70±0.05	1.1	
	25	5.60±0.08	0.2	
	12.5	5.8±0.1	0	
	6.25	5.8±0.1	0	
5	800	<2	>3.8	2
	400	3.6±0.2	2.2	
	200	5.8±0.1	0	
	100	5.8±0.1	0	
	50	4.90±0.15	0.9	
	25	5.8±0.1	0	
	12.5	5.10±0.07	0.7	
	6.25	5.8±0.1	0	
Positive control	Culture cells infected with strain *C. trachomatis* CT-3271/Belarus/2015	5.8±0.1	–	

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Supporting Information

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