საქართველოს მეცნიერებათა ეროვნული აკადემიის მოამბე, ტ. 19(193), №2, 2025
BULLETIN OF THE GEORGIAN NATIONAL ACADEMY OF SCIENCES, vol. 19(193), no. 2, 2025

Bioorganic Chemistry

Chemical Characterization and Biological Activity of a Novel Ursolic Acid Derivative

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Abstract. Ursolic acid (UA) demonstrates various biological activities and is considered an important representative of prototypical natural antibiotic molecules and a leading compound with pharmacological and medical significance in the development of new drugs. In the present work, the synthesis of a novel UA derivative and determination of its *in vitro* antimicrobial activity against several bacterial and fungal strains a proposed. Additionally, the effect of the exogenous action of UA and its synthesized derivative on the permeability and sorption capacity of the erythrocyte membrane of cancer patients was studied. The synthesized UA derivative was characterized using IR spectrometry, 1H and 13C NMR spectroscopic methods. The UA derivative showed higher and more selective antibacterial activity against Gram-positive and Gram-negative microorganisms compared to the "parent" compound, UA. However, no significant changes were observed in the sorption capacity and permeability of the erythrocyte membrane. A tendency to decrease the sorption capacity of the erythrocyte membrane and increase the membrane permeability as a result of the UA effect was observed. © *2025 Bull. Georg. Natl. Acad. Sci.*

Keywords: ursolic acid, biological activity, antimicrobial activity, derivative

Introduction

Ursolic acid (UA) demonstrates various biological activities such as anticancer, chemopreventive, hepatoprotective, antiviral, antiinflammatory, anticardiovascular, antiatherosclerotic, antidiabetic, antioxidant, immunomodulatory, neuroprotective, and gastroprotective effects. Additionally, this natural compound enhancing the bacterial susceptibility to other antibiotics has been paid increasing more attention [1-3]. The occurrence of UA and its derivatives as major metabolites in medicinal plants is associated with their microbiocidal activities. This compound is considered an important representative of prototypical natural antibiotic molecules and a leading compound with pharmacological and medical importance in the development of new drugs [4]. UA and some of its derivatives have revealed potent antimicrobial activity against *S. aureus*, *E. coli*, *S. pneumonia, B. subtilis, B. cereus, E. faecalis, E. faecium, P. aeruginosa* and *M. tuberculosis* and some cases even on methi-

cillin- or vancomycin-resistant strains, and a synergistic effect with ampicillin and tetracycline against both *B. cereus* and *S. aureus*. The potentiality of UA in synergistic effects with antibiotics to enhance the antibacterial activity of -lactams can constitute a valuable agent for therapeutic application [3-8]. The bioavailability of this compound is limited by poor water solubility, leading to low efficiency, which is worsened by its nonspecific distribution in the body. Very low and/or no toxicity and structure – activity relationship studies of this compound suggest that certain substituents on the lipophilic 5-ring backbone can increase the selectivity and potency of a desired action and are identified as the potent natural lead compound for drug development [9]. The C3-OH of UA can be exploited to synthesize novel lipophilic or hydrophilic derivates of UA, which is highly essential for enhancing pharmacological activities.

In the present work, the synthesis of a novel derivative containing a hydrazine unit as a side-chain attached to the C3 position of a molecule of UA and the determination of its *in vitro* antimicrobial activity against several bacterial and fungal strains were proposed. Additionally, the effect of the exogenous action of UA and its synthesized derivative on the functional state of red blood cells of cancer patients, precisely, on the permeability and sorption capacity of the erythrocyte membrane was studied.

Materials and Methods

Chemically pure and dry powdered UA (>93%) was extracted from apple pomace and then purified by the laboratory standard method [1,2]. The course of the reaction and the purity of the compounds were monitored using Silufol UV-254 and thin-layer chromatography (TLC) on silica gel Gel 60 F_{254} plates, utilizing a hexane/ethyl acetate eluent in a 3:2 ratio. The developed plates were then visualized using a UV viewing cabinet at 254/365 nm. Infrared (IR) spectra were recorden on a Nicolet TM iS50 FTIR spectrometer (Thermo Fischer Scientific), covering the infrared region of 4000 to 400 cm^{-1} (32 scans, resolution of 4 cm^{-1}). Additionally, ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a NMR-400 spectroscopy (Bruker) at 400 MHz, in DMSO-d_6 as a solvent, with tetramethylsilane as an internal standard. The derivative of UA (compound 3) was synthesized by the following way: at the first stage, 0.624 g (1.37 mmol) of UA dissolved in acetone (24 mL) was cooled, and 2 mL of Jones reagent was added dropwise during 5 min at 0°C under stirring until the reaction turned dark orange. Then isopropanol (5 mL) was added dropwise, and the mixture was stirred for 20 min, then filtered and evaporated under reduced pressure. Crude product was purified by a dry column vacuum chromatography using *n*-hexane: ethyl acetate as an eluent. 3-oxo-ursolic acid (compound 1) was obtained as white powder (0.422 g, yield 67.7%). At the next stage, 0.1 g (0.22 mmol) of the obtained compound 1 dissolved in ethanol was placed in a 50 mL flask, heated under stirring at 60°C. Then two drops of acetic acid and 3 mL of 4-nitrophenylhydrazine solution (compound 2) (0.034 g, 0.22 mmol in ethanol) were added, and the mixture was stirred for 8 hrs. Water (20 mL) was added, and a mixture was cooled and filtered. The crude product was crystallized in ethanol, and the UA derivative – 3-[(4-nitrophenyl)hydrazono]-urs-12-ene-28-oic acid (compound 3) as a yellow crystal was obtained (0.071 g, yield 68%).

The bacterial and fungal Gram-negative and Gram-positive rod, cocci, yeast and spore former microorganisms – *Staphylococcus aureus ATCC 6538, Bacillus cereus ATCC 11778, Pseudomonas aeruginosa ATCC 9027, Candida albicans ATCC 2091* and *Escherichia coli ATCC 8739* were tested for *in vitro* determination of antimicrobial assay of UA and UA derivative (compound 3) by the standard agar disc diffusion method [10]. The test compounds (UA and compound 3) and analytical standards of antimicrobial agents (levofloxacin, LEV – fluoroquinolone antibiotic and fluconazole,

FLU – antifungal agent) were dissolved and diluted with methanol to obtain test solutions at 100 µg/mL concentration. A loopful from each test microorganism (the turbidity of 0.5 McFarland standard for bacterial suspension and 1.0 McFarland standard for fungal suspension equivalent to 1.5×10^8 CFU/mL) was swabbed onto a Mueller-Hinton (MH) agar plate. Sterile paper discs (6 mm in diameter) were impregnated with 100 µL of each test solution (UA and compound 3), then dried and dispensed onto the surface of the inoculated agar plate in triplicates (n=3). A parallel analysis with antimicrobial agents as positive control samples in triplicates (n=3), as well as a blank and negative control samples in duplicate (n=2) was performed. All the plates were incubated in a thermostat-incubator (Thermo Fischer Scientific) at 37°C for 48 hours. The antimicrobial assay was evaluated by measuring the inhibition zone (average value (n=3)±standard deviation (SD) $P<0.05$ at 95% confidence) in mm (including the 6 mm disk) on the plates. The antimicrobial effect (AE) is expressed as a percentage, indicating the percentage difference in the degree of inhibitions in microbial growth between the control antimicrobial agents and test compounds calculated using the following equation: AE,%=(dc-dt)×100/dc, where, d_c – the growth diameter in the positive control sample; d_t – growth diameter in the test sample.

Blood erythrocytes of patients with benign hyperplasia (BH) and adenocarcinoma were taken as a test material for the study of changes in cell membrane properties caused by the effect of test compounds, in particular, the influence of sorption capacity and permeability of membrane. The sorption capacity of erythrocytes was determined by a methyl blue absorption method, the membrane permeability (using different concentrations of urea) was determined by a spectrophotometric method [11-12].

Results and Discussion

The chemical characterization of the synthesized compound was based on changes in the IR, ^{1}H and ^{13}C spectra to confirm the synthesis of (1S,2R,4aS, 6aS,6bR,12aR,12bR,14bS,E)-1,2,6a,6b,9,9,12a-heptamethyl-10-(2-(4-nitro-phenyl) hydrazono)-1,3,4,5,6,6a,6b,7,8,8a,9,10,11,12,12a, 12b,13,14b-octade-cahydro picene-4a(2H)-carboxylic acid) (compound 3). The synthesis pathway of compound 3 is demonstrated in the Figure. The IR spectra of the compounds exhibits characteristic absorption bands of NH groups at 3309 cm^{-1}, as well as the characteristic bands of CH groups from the aromatic ring and aliphatic group at 3095 cm^{-1} and 2951 cm^{-1}, respectively. Additionally, absorption bands corresponding to the functional groups C=O, CH=N, and C–NO_2 are observed in their characteristic regions of the spectra. IR (KBr, νmax, cm^{-1}): 3309.87 (NH), 3095.18 (Ar CH), 2951.42 (CH, CH2) 1612.18 (C=O), 1588, 1327 (NO_2), 1537 (N=C). In the ^{1}H NMR spectra of compound 3, the signals for NH and OH groups appear as singlets at

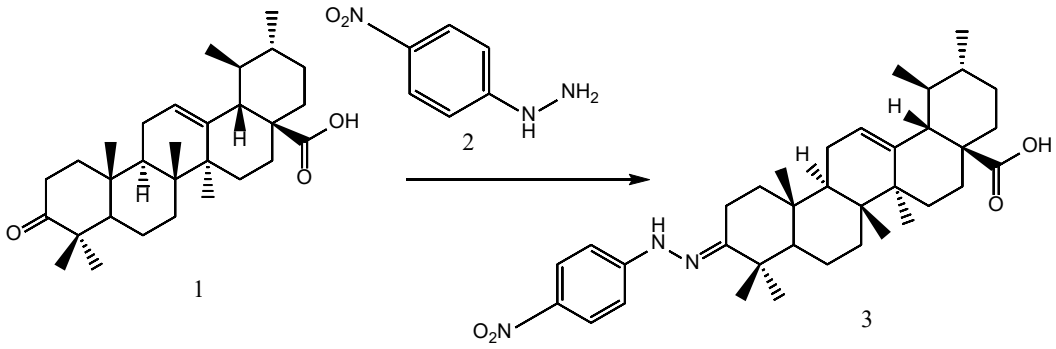

Figure. Synthesis of 3-[(4-nitrophenyl)hydrazono]-urs-12-ene-28-oic acid (compound 3).

12.03 and 10.81 ppm in the downfield region. The aromatic proton signals are observed as doublets of doublets in the 8.87 and 7.85 ppm range. The proton signals of the aliphatic fragment appear as a doublet and multiplets in the upfield region. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.03 (s, 1H), 10.81 (s, 1H), 8.87 (d, *J* = 2.6 Hz, 2H), 7.85 (d, *J* = 9.6 Hz, 2H), 5.17 (t, *J* = 12.2, 3.7 Hz, 1H), 2.29-2.19 (m, 2H), 1.96 – 1.87 (m, 2H), 1.81 (q, *J* = 8.0, 6.2 Hz, 3H), 1.64 – 1.50 (m, 5H), 1.34-3.30 (m, 6H), 1.27-1.23 (m, 4H), 1.20 – 1.12 (m, 3H), 0.97-0.94 (m, 10H), 0.88 – 0.78 (m, 8H). In the ^{13}C NMR spectra of compound 3, the carbon signals of the aliphatic fragment appear in the upfield region between 15.38 and 53.15 ppm. The resonance signals for the functional groups C=O and C=N are observed in the regions of 180.63 ppm and 156.0 ppm, respectively. The carbon signals of the aromatic ring appear in the range of 145.99 to 118.58 ppm. ^{13}C NMR (100 MHz, DMSO-d6) δ: 180.63, 164.04, 145.99, 143.32, 138.67, 125.96, 125.78, 118.58, 53.15, 49.65, 48.10, 47.69, 42.17, 39.83, 39.32, 39.21, 37.79, 37.17, 36.92, 36. 32, 33.17, 31.03, 28.15, 25.90, 24.96, 24.15, 23.16, 20.30, 19.75, 18.43, 17.27, 15.89.

The UA and its derivative were tested against *S. aureus, B. cereus, P. aeruginosa, C. albicans* and *E. coli.* The calculated values of AE, % show quantitatively how different the antimicrobial activity of the test compound is with the comparable antimicrobial agent. The lower a value of the AE, %, the closer the antimicrobial activity of the test compounds to the positive control ones. A value of <35% is considered good antimicrobial activity. It has been revealed that UA is more active against Gram-positive bacterial strains (*S. aureus, B. cereus)*; the inhibitory activity is greater against *S. aureus*, but slightly less compared to LEV(AE>52%). UA does not exhibit antifungal activity at all (Table). The synthesized derivative is more effective against both Gram-negative and Gram-positive bacterial strains than UA; There is no observed a significant difference in relation to the activity of LEV. This derivative has an antifungal effect, but it is significantly different compared to FLU (63%). Based on the results, it is concluded that good antibacterial effect of the derivative is observed against *S. aureus* and *E. coli*.

Based on the study of the effect of the exogenous action of UA and compound 3 on the

Table. The evaluation of the antimicrobial activity and the effect on erythrocyte membrane sorption capacity and permeability

The antimicrobial activity					
Microorganism	**Average value of the inhibition zones ± SD, mm (n=3)**			**AE, %**	
	UA	**Compound 3**	**LEV/FLU**	**UA**	**Compound 3**
S. aureus	13.25±0.82	18.25±1.25	27.32±1.10	52	33
B. cereus	11.05±0.73	17.05±0.87	25.63±1.01	57	37
P. aeruginosa	10.71±0.91	13.71±1.06	26.21±0.87	59	48
C. albicans	-	9.96±0.83	26.85±0.65	-	63
E. coli	8.25±0.94	16.25±0.81	23.65±0.93	65	31

The effect on erythrocyte membrane sorption capacity and permeability				
Sample	**Sorption capacity ± SD, % (n=3)**		**Permeability (relative unit) ± SD, % (n=3)**	
	UA	**Compound 3**	**UA**	**Compound 3**
Control	37.12±1.43	34.68±20	27.32±1.15	17.52±0.10
Benign hyperplasia	38.53±1.72	35.00±1.10	25.63±1.05	14.56±0.22
Adenocarcinoma	67.60±2.83	62.00±2.10	26.21±0.85	14.87±0.32

permeability and sorption capacity of the erythrocyte membrane, it was revealed that there is a tendency to decrease the sorption capacity of the erythrocyte membrane, giving a sharper picture as a result of the UA effect (Table). As for the influence of the mentioned compounds on the membrane permeability, in this case it is observed that the membrane permeability slightly increases under the influence of UA, while the membrane permeability decreases slightly as a result of compound 3. The effect of compound 3 on the sorption capacity and permeability of the erythrocyte membrane shows a slight change. We assume that compound 3 does not affect the mentioned processes. However, it is possible to increase its concentration or time of action to cause significant changes, which is the goal of future research. The decrease in sorption capacity due to the UA effect should be caused by both the antioxidant activity and its ability to reduce the number of toxic compounds in the area. As for the increase in the membrane permeability as a result of the UA exposure, UA affects the permeability and the lipid composition of the membrane. As a result of its influence, the lipid spectrum of the membrane changes and permeability increases, which was also confirmed in our case. This, in turn, increases its role as an auxiliary pharmacological agent, since by increasing the permeability of the membrane, it contributes to the entry of pharmaceutical drugs into the cell, as well as to the apoptosis of tumor cells.

Hence, the influence of the C3 substituent of the ursan-12-ene skeleton on the antimicrobial activity was tested, revealing a preliminary structure-activity relationship. The antimicrobial effect of UA is concentrated in the polar groups present in the non-polar pentacyclic skeleton, since polar substituents increase water solubility and consequently, the bioavailability. It is observed that the substitution of the OH- in C3 by a polar 4-nitrophenylhydrazine usually leads to increased antimicrobial activity and slightly improved water solubility; therefore, it is expected to improve the bioavailability. The novel synthesized UA derivative showed a higher and more selective antibacterial activity against Gram-positive and Gram-negative microorganisms compared to the "parent" compound – UA. The presence of a nitro group in the aromatic ring increases the polarity of the molecule and consequently its bioavailability and water solubility as well, making it more active against bacterial strains. Based on the obtained data, on the one hand, it is concluded that the use of UA in antitumor therapy, by increasing the permeability of the erythrocyte membrane, will contribute to the transport of antitumor drugs in the blood, since erythrocytes are one of the targets for the transport of pharmaceutical drugs in the blood. On the other hand, it will weaken the degree of membrane damage caused by oxidative stress and reduce the amount of toxic compounds that significantly damage the cell membrane. Slightly change in the sorption capacity and permeability of the erythrocyte membrane is observed with the synthesized UA derivative.

Acknowledgements

The research is supported by the Shota Rustaveli National Science Foundation of Georgia within the grant FR-21-4196.

ბიოორგანული ქიმია

ურსოლის მჟავას ახალი წარმოებულის ქიმიური დახასიათება და ბიოლოგიური აქტივობა

ი. რუბაშვილი[*], მ. ციცაგი[*], მ. ჩხაიძე[*], თ. ბუკია[**], მ. ალიბეგაშვილი[**], ა. წიკლაური[§], ქ. ებრალიძე[*], ვ. ციციშვილი[*,#]

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ურსოლის მჟავა (UA) ავლენს სხვადასხვა ტიპის ბიოლოგიურ აქტივობას და ითვლება პროტოტიპური ბუნებრივი ანტიბიოტიკების მოლეკულების მნიშვნელოვან წარმომადგენლად და წამყვან ნივთიერებად, რომელსაც აქვს ფარმაკოლოგიური და სამედიცინო მნიშვნელობა ახალი მედიკამენტების შემუშავებაში. წინამდებარე ნაშრომის ფარგლებში შემოთავაზებულია ახალი UA წარმოებულის სინთეზი და მისი ***in vitro*** ანტიმიკრობული აქტივობის განსაზღვრა რამდენიმე ბაქტერიული და სოკოვანი შტამის მიმართ. გარდა ამისა, შესწავლილია UA-ს და მისი სინთეზირებული წარმოებულის ეგზოგენური მოქმედების ეფექტი კიბოთი დაავადებული პაციენტების ერითროციტების მემბრანის გამტარიანობასა და სორბციულ შესაძლებლობებზე. სინთეზირებული UA წარმოებული დახასიათებულია IR სპექტრომეტრიული, ^{1}H და ^{13}C NMR სპექტროსკოპიული მეთოდების გამოყენებით. UA წარმოებულმა აჩვენა უფრო მაღალი და შერჩევითი ანტიბაქტერიული აქტივობა გრამ-დადებითი და გრამ-უარყოფითი მიკროორგანიზმების მიმართ მისი ე.წ. „მშობელ“ ნივთიერებასთან – UA შედარებით, მაგრამ ერითროციტების მემბრანის სორბციის უნარიანობასა და გამტარიანობაზე მნიშვნელოვანი ცვლილება არ გამოვლენილა. გამოვლენილია UA-ის შემთხვევაში ერითროციტების მემბრანის სორბციის უნარიანობის შემცირებისა და მემბრანის გამტარიანობის ზრდის ტენდენცია.

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Received November, 2024