

A New Ursane-Type Triterpene from the Fermented Shallot *Allium Ascalonicum*

Nguyen Van Chuyen[#], Nguyen Hong Son[#], Pham Van Hien, Dang Truong Giang, Ho Ba Ngoc Minh, Ngo Thi Tuyet Mai, Chu Van Men, Ho Anh Son^{*}, Vu Binh Duong^{*}

Nguyen Van Chuyen[#], Nguyen Hong Son[#], Pham Van Hien, Dang Truong Giang, Ho Ba Ngoc Minh, Ngo Thi Tuyet Mai, Chu Van Men, Ho Anh Son^{*}, Vu Binh Duong^{*}

Vietnam Military Medical University, 160 Phung Hung, Ha Dong District, Hanoi, VIETNAM.

[#]These authors contributed equally to this work.

Correspondence

Vu Binh Duong, Ph.D

The Drug R&D Center, Vietnam Military Medical University, Hadong District, Hanoi, VIETNAM.

Phone no: +84-98251310

E-mail: vbduong2978@gmail.com

Ho Anh Son, Ph.D

Institute of Biomedicine and Pharmacy, Vietnam Military Medical University, 160 Phung Hung, Ha Dong District, Hanoi, VIETNAM.

Phone no: +84-35-321-2500

E-mail: hoanhsonghp@gmail.com

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ABSTRACT

3,24-acetonideclethric acid (**1**), a new ursane-type triterpene, and four known compounds including ursolic acid (**2**), randiasaponin IV (**3**), ilekudinoside W (**4**) and (25S)-1 β ,3 β ,24 β -trihydroxyspirost-5-en 1-O- α -L-rhamnopyranosyl-(1 \rightarrow 2)- α -L-arabinopyranoside (**5**), and were isolated from the fermented shallot *Allium ascalonicum*. Their structures were determined by analysis of HR-ESI-MS, NMR spectral data, as well as comparison with those reported in the literature. All of the saponins (**3-5**) exhibited antimicrobial activity against three strains *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* with IC₅₀ values in the range from 89.49 \pm 2.24 to 95.71 \pm 3.86 μ M.

Key Words: 3,24-Acetonideclethric acid, Saponin, *Allium ascalonicum*, Antimicrobial activity.

INTRODUCTION

Genus *Allium* is one of the largest genera of the Amaryllidaceae family. It was reported comprising of more than 800 species and widely distributed throughout temperate regions in the world including Europe, Asia, North America and Africa.¹ *Allium* species were also highly regarded worldwide for both therapeutic and culinary values including *A. cepa* (onion), *A. sativum* (garlic), *A. porrum* (leek), *A. schoenoprasum* (chive), and *A. ascalonicum* (shallot). They have been used for thousands of years as flavor-enhancing foods and folk medicines having hypocholesteremic, hypotensive, hypoglycemic, anti-thrombotic, anti-allergic, anti-inflammatory, anti-microbial, anti-fungal, and anti-oxidant activity.² Phytochemical studies suggested that *Allium* species are rich of sulfur-containing volatile compounds, phenolics, steroids, triterpenes, as well as saponins. The saponins from *Allium* species were highlighted for their cytotoxic and antimicrobial activity.^{3,4} However, the sulfur compounds responsible features and its distinctive smell. In addition, fermented shallot is delicious. In our research to find new antimicrobial agents from *Allium* species, herein, we report the isolation, structural elucidation of a new ursane-type triterpene and four known compounds from the fermented shallot *A. ascalonicum*. The anti-microbial activity of isolated triterpenoids and saponins were also evaluated by liquid-dilution method using resazurin as a redox indicator of microbial viability.

RESULTS AND DISCUSSION

The fermented shallot of *A. ascalonicum* was extracted in methanol, partitioned successively in dichloromethane and ethyl acetate. The crude extracts were separated and purified by combination of various chromatographic technique to obtain 5 compounds (1-5, Figure 1)

Compound **1** was isolated as a white amorphous powder. Its molecular formula was determined to be C₃₃H₅₂O₆ by a quasi-molecular ion peak at *m/z* 543.3691 [M-H]⁻ (calcd for C₃₃H₅₁O₆, 543.3686) in the HR-ESI-MS and in conjunction with the ¹³C-NMR spectral data. The ¹H-NMR spectrum of **1** contained signals for 7 methyl groups [δ_{H} 1.40, 1.37, 1.26, 1.19, 0.91, 0.69 (each 3H, s) and δ_{H} 0.93 (3H, d, *J* = 6.8 Hz)], five carbinol protons [δ_{H} 3.95 (1H, br s) and δ_{H} 3.87, 3.73, 3.68, 3.59 (each 1H, d, *J* = 6.8 Hz)], an olefinic proton [δ_{H} 5.33 (1H, br s)], and a lot of shielded signals in the range δ_{H} 1.02~2.51. The ¹³C-NMR and HSQC spectra of **1** revealed signal of 33 carbons. Of these, a deshielded signal at δ_{C} 183.4 was assigned for a carbonyl carbon. Two olefinic carbon signals at δ_{C} 137.8 and 129.3 indicated for the presence of a C-C double bond. An acetal and others four oxygenated carbons were characterized by signals at δ_{C} 98.7, 73.1, 67.2, 65.0, 64.4. Aforementioned spectral data indicated that compound **1** to be a triterpene. Moreover, the NMR data of **1** were recognized in close similarity with those of clethric acid⁵ except for the additional signals of an acetonide group (δ_{C} 98.7, δ_{C} 27.9/ δ_{H} 1.37, and δ_{C} 20.5/ δ_{H} 1.40). The presence of acetonide group was also confirmed by HMBC correlations between H₃-2' (δ_{H} 1.40)/ H₃-3' (δ_{H} 1.37) and C-1' (δ_{C} 98.7) (Figure 2). Others HMBC correlations between H-3 (δ_{H} 3.95)/ H₂-24 (δ_{H} 3.87, 3.73) and acetal carbon C-1' indicated location of acetonide group binding between C-3 and C-24. Remaining hydroxy methylene group was assigned to be C-23 by HMBC correlations between H₂-23 (δ_{H} 3.68, 3.59) and C-3 (δ_{C} 67.2)/ C-24 (δ_{C} 65.0)/ C-5 (δ_{C} 44.4)/ C-4 (δ_{C} 40.0). Position of C-C double bond at C-12/C-13 and carbonyl group C-28 were confirmed by HMBC correlations between H-18 (δ_{H} 2.51) and C-12 (δ_{C} 129.3)/ C-13 (δ_{C} 137.8)/ C-28 (δ_{C} 183.4). And the HMBC correlations between H₃-29 (δ_{H} 1.19) and C-18 (δ_{C} 52.8)/ C-19 (δ_{C} 73.1)/ C-20 (δ_{C} 41.0) suggested the last oxygenated carbon to be C-19. The presence of hydroxy group at C-19 was also well

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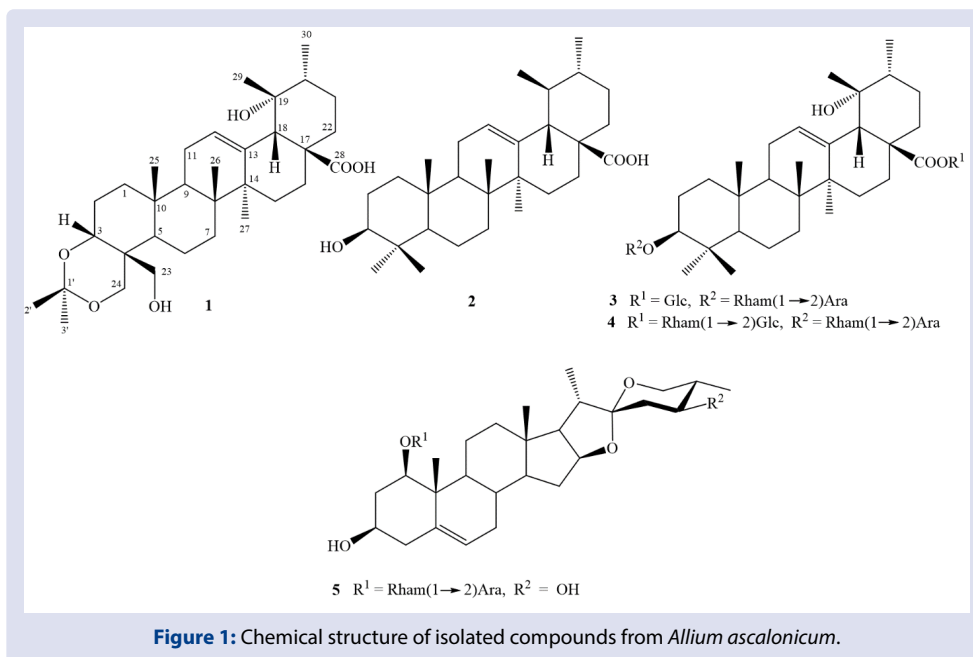


Figure 1: Chemical structure of isolated compounds from *Allium ascalonicum*.

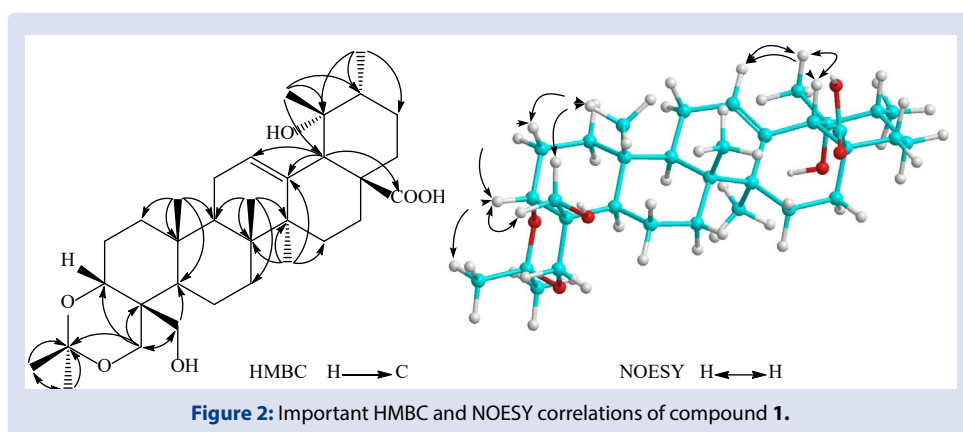


Figure 2: Important HMBC and NOESY correlations of compound **1**.

agreed with singlet multiplicity of H-18 (δ_{H} 2.51) and H₃-29 (δ_{H} 1.19). Finally, relative stereochemistry of **1** was established by NOESY analysis (Figure 2). NOESY correlations between H₃-25 (δ_{H} 3.68) and H-23 (δ_{H} 3.68) / H_{axial}-2 (δ_{H} 1.80), H_{axial}-2 and H-3 (δ_{H} 3.95) indicated that H₃-25, H_{axial}-2, H-3, and H-23 were close proximity and hence C-25, H_{axial}-2, H-3, C-23 were all β -orientations. The β -orientation (equatorial position) of H-3 was also supported by its broad singlet signal (δ_{H} 3.95) in the ¹H-NMR spectrum. On the other hand, the NOESY correlations between H₃-29 (δ_{H} 1.19) and H-18 (δ_{H} 2.51) / H-12 (δ_{H} 5.33) suggested for β -orientations of H-18 and methyl group C-29, showing α -orientation of hydroxy group at C-19. Consequently, structure of compound **1** was determined to be 3,24-acetonideclethric acid.

Other compounds were determined to be ursolic acid (**2**),⁶randiasaponin IV (**3**),⁷ilekudinoside W (**4**),⁸ (25S)-1 β ,3 β ,24 β -trihydroxy Spirost-5-en 1-O- α -L-rhamnopyranosyl-(1→2)- α -L-arabinopyranoside (**5**),⁹ by the good agreement of their NMR spectral data with those reported in the literature.

The steroids, triterpenoids, and saponins from *Allium* species have been reviewed as potential antimicrobial activity.^{3, 10} Therefore, compounds **1-5** were evaluated their antimicrobial activity against three pathogenic microbial strains including Gram-positive bacteria (*Staphylococcus aureus*), Gram-negative bacteria (*Escherichia coli*), and yeast (*Candida albicans*). As shown in the Table 2, except compound **2**, the tested compounds exhibited antibacterial activity against both Gram-

positive (*S. aureus*) and Gram-negative (*E. coli*) with IC₅₀ values in the range from 42.73±2.24 to 117.12±4.93 μ M. Saponins (**3-5**) displayed cytotoxic to all tested microbial strains with IC₅₀ values in the range from 60.18 ± 4.10 to 95.71 ± 3.86 μ M. Among ursane-type triterpene (**1-4**), saponins exhibited better anti-microbial activity in comparison with the aglycone triterpene. The standard drugs chloramphenicol and fluconazole were used as positive control for antibacterial and antifungal test. Once again, these results confirmed that triterpenoid, steroid, especially the saponins are anti-microbial components from *A. ascalonicum*. Compound **1**, a new derivative of ursolic acid presented better anti-bacterial activity than ursolic acid (**2**).

MATERIALS AND METHOD

General experimental procedures

Optical rotation was recorded on a Jasco P2000 polarimeter. NMR spectra were measured on a Bruker 500 MHz spectrometer using TMS as an internal standard. HR-ESI-MS was acquired on an Agilent 6530 Accurate Mass Q-TOF LC/MS system. Column chromatography was performed using silica gel, reverse phase C-18, and diaion HP-20 resins. Thin layer chromatography was carried out using pre-coated silica gel 60 F₂₅₄ and RP-18 F_{254S} plates. The plates were visualized under UV radiation (254 and 365 nm) and by spraying with aqueous H₂SO₄ solution (5%) followed by heating with a heat gun.

Plant materials

The bulbs of *A. ascalonicum* were collected in An Hai Commune, Ly Son island district, Quang Ngai Province, Vietnam in 2019. Its scientific name was identified by Dr. Do Thi Xuyen, Department of Biology, VNU University of Science, Vietnam National University, Hanoi. A voucher specimen (VNU181019) was deposited at the Herbarium of the VNU University of Science, Vietnam National University, Hanoi. The fresh bulbs were fermented under suitable temperature and humidity for a certain period of time to get the fermented ones.

Isolation and extraction

The dried powdered of fermented shallot *A. ascalonicum* (5.0 kg) was ultrasonically extracted with methanol for three times in room temperature (each 10 L, 30 min). After removal of the solvent, methanol extract (400 g) was suspended in 3 L of water and then successively partitioned with dichloromethane, ethyl acetate to give corresponding extracts dichloromethane (ASD, 73 g), ethyl acetate (ASE, 12 g), and water layer (ASW). The ASD and ASE extract were combined and separated on a silica gel column, eluting with gradient system of n-hexane/acetone (0-100% acetone) to give four fractions ASD1-ASD4. The ASD3 fraction was loaded on a RP-18 column and eluted with methanol/water (3/1, v/v) to give compounds 2 (61 mg).

The ASD4 fraction was purified on a silica gel column chromatography, eluting with dichloromethane/acetone (7/1, v/v) to give compound 1 (14 mg). The water layer (ASW) was poured on a diaion HP-20 column chromatography, washed with water (2L), and then eluted with methanol/water (stepwise, 25%, 50%, 75%, 100% methanol) to give four fractions ASW1-ASW4. Fraction ASW2 was separated on a silica gel column, eluting with dichloromethane/methanol (0-100% methanol) to give six fractions ASW2A-ASW2F. Fraction ASW2D was also purified on a RP-18 column, eluting with acetone/water (5/2, v/v) to give compounds 3 (27 mg) and 4 (16 mg). Fraction ASW2C was separated on a silica gel column, eluting with dichloromethane/methanol (0-100% methanol) to give five fractions ASW3A-ASW3E. Fraction ASW3C was purified on a RP-18 column, eluting with methanol/water (3/2, v/v) to obtain compound 5 (24 mg).

3,24-acetonideclethric acid (1)

White amorphous powder; $[\alpha]_D^{25}$: -43.1 (c 0.1, MeOH); HR-ESI-MS m/z : 543.3691 [M-H]⁻ (calcd for C₃₃H₅₁O₆, 543.3686); ¹H- and ¹³C-NMR spectral data are given in the Table 1.

Antimicrobial assay

Three microbial strains from American Type Culture Collection including *Candida albicans* ATCC 10231 (yeast), *Escherichia coli* ATCC

Table 1: ¹H and ¹³C NMR spectral data for compound 1.

No.	$\delta_c^{a,b}$	$\delta_H^{a,c}$ (mult, J in Hz)
1	33.0	1.33 (m)/1.52 (m)
2	23.5	1.51 (m)/1.80 (m)
3	67.2	3.95 (br s)
4	40.0	-
5	44.4	1.83 (m)
6	18.8	1.35 (m)/1.43 (m)
7	32.8	1.65 (m)/1.79 (m)
8	40.7	-
9	46.9	1.78 (m)
10	36.4	-
11	23.6	1.90 (m)/2.03 (m)
12	129.3	5.33 (br s)
13	137.8	-
14	41.0	-
15	27.9	1.02 (m)/1.69 (m)
16	25.9	1.26 (m)/1.65 (m)
17	47.7	-
18	52.8	2.51 (s)
19	73.1	-
20	41.0	1.38 (m)
21	25.3	1.56 (m)/2.50 (m)
22	37.5	1.64 (m)/1.79 (m)
23	64.4	3.59 (d, 12.0)/3.68 (d, 12.0)
24	65.0	3.73 (d, 12.0)/3.87 (d, 12.0)
25	15.5	0.91 (s)
26	17.0	0.69 (s)
27	24.4	1.26 (s)
28	183.4	-
29	27.4	1.19 (s)
30	16.1	0.93 (d, 6.8)
1'	98.7	-
2'	20.5	1.40 (s)
3'	27.9	1.37 (s)

Measured in ^aCDCl₃, ^b500 MHz, ^c125 MHz. Assignments were done by HMQC, HMQC, COSY, and NOESY experiments

Table 2: Antimicrobial activity of compounds 1-5.

Comp.	IC ₅₀ (μM)		
	<i>S. aureus</i>	<i>E. coli</i>	<i>C. albicans</i>
1	102.62 ± 2.07	117.12 ± 4.93	>128
2	>128	>128	>128
3	88.21 ± 4.84	73.02 ± 3.48	89.49 ± 2.24
4	72.04 ± 2.71	60.18 ± 4.10	95.71 ± 3.86
5	67.98 ± 3.64	78.31 ± 2.42	93.81 ± 4.52
Chlo	7.11 ± 0.24	4.47 ± 0.16	-
Fluc	-	-	1.93 ± 0.21

Chloramphenicol (Chlo) and fluconazole (Fluc) were used as positive control, (-): not tested.

25922 (Gram-negative bacterium), and *Staphylococcus aureus* ATCC 13709 (Gram-positive bacterium) were used to evaluate antimicrobial activity. Assay was performed in 96 well plates by liquid-dilution method using resazurin as a redox indicator of microbial viability.¹¹ In brief, each well containing 10 μL of compounds and 190 μL of microorganism suspension (bacteria inoculum 5 × 10⁵ CFU/mL and yeast inoculum 5 × 10³ CFU/mL) was incubated at 37°C in 18h for yeast and 20h for bacteria. After addition of 10 μL of resazurin (0.1 mg/mL), the microbial viability was assessed fluorimetrically (λ_{ex} = 550 nm, λ_{em} 590 nm) on a microplate reader (TecanGenios). The results were expressed in term of percent reduction of microorganism viability compared with control well and IC₅₀ values were determined from dose-response curve.

CONCLUSIONS

Five compounds were isolated from the bulbs of *A. ascalonicum*. Their chemical structures were determined to be 3,24-acetonideclethric acid (1), ursolic acid (2), randiasaponin IV (3), ilekudinoside W (4), (2S)-1β,3β,24β-trihydroxyspirost-5-en 1-O-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside (5). Compound 1 was a new ursane-type triterpene and exhibited weak anti-bacterial activity against *E. coli* and *S. aureus* strains. Saponins (3-5) displayed cytotoxic to *E. coli*, *S. aureus*, *C. albicans* microbial strains with IC₅₀ values in the range from 36.62 ± 1.88 to 101.99 ± 3.17 μM.

CONFLICTS OF INTEREST

The authors declare that there is no potential conflicts of interest

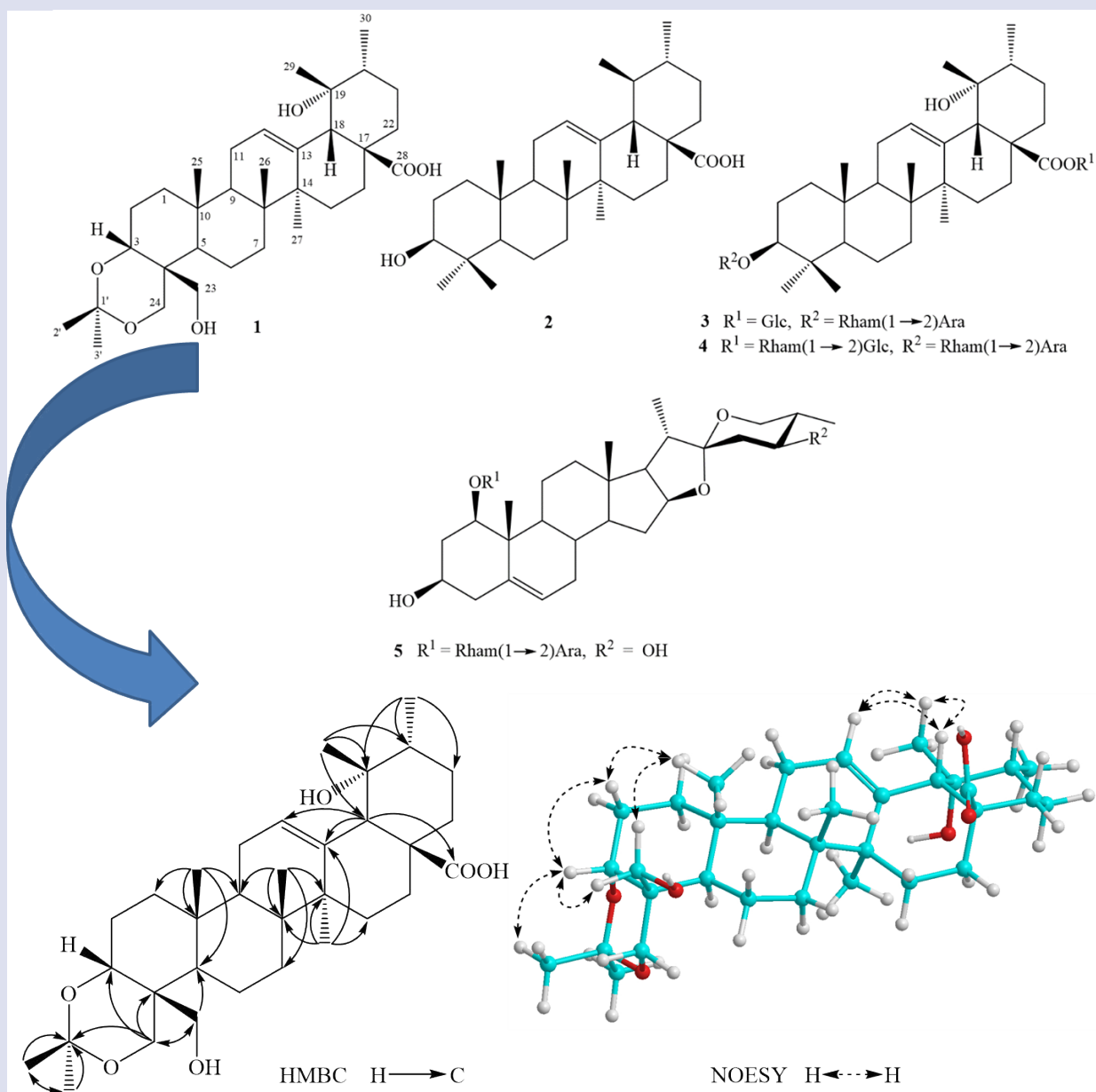
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GRAPHICAL ABSTRACT



SUMMARY

3,24-acetonideclethric acid (1), a new ursane-type triterpene, and four known compounds including ursolic acid (2), randiasaponin IV (3), ilekudinoside W (4) and (25S)-1 β ,3 β ,24 β -trihydroxyspirost-5-en 1-O- α -L-rhamnopyranosyl-(1 \rightarrow 2)- α -L-arabinopyranoside (5), and were isolated from the fermented shallot *Allium ascalonicum*. Their structures were determined by analysis of HR-ESI-MS, NMR spectral data, as well as comparison with those reported in the literature. All of the saponins (3-5) exhibited antimicrobial activity against three strains *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* with IC₅₀ values in the range from 89.49 \pm 2.24 to 95.71 \pm 3.86 μM .

ABOUT AUTHORS



Nguyen Van Chuyen: is an Assistant Professor, Vice head, Department of Military Hygiene, VMMU.



Nguyen Hong Son: A Researcher of The Drug R&D Center, VMMU.



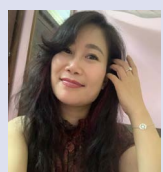
Nguyen Van Hien: A Researcher of The Drug R&D Center, VMMU.



Ho Ba Ngoc Minh: A Researcher of the Research Center for Drug Manufacturing Applications, VMMU.



Dang Truong Giang: A Researcher of The Drug R&D Center, VMMU.



Ngo Thi Tuyet Mai: A Researcher of The Drug R&D Center, VMMU.



Chu Van Men: Assistant Professor, Director, Clinical Trial and Bioequivalent Testing Centre, Institute of Biomedicine and Pharmacy, VMMU.



Ho Anh Son: Associate Professor, Vice Director of the Institute of Biomedicine and Pharmacy, VMMU.



Vu Binh Duong: Associate Professor, Director of The Drug R&D Center, VMMU.

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