Communication

Synthesis of N-Heterocyclic Analogues of 28-*O*-Methyl Betulinate, and Their Antibacterial and Antifungal Properties

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Abstract: The paper presents the results on the one-pot pyridine quaternization using betulinic 28-*O*-methyl ester (**1**) and Tempo$^+$Br$_3^-$ cation followed by reduction of the resulting salt (**2**) to 1,2,5,6-tetrahydropyridine derivative (**3**). The structures of new compounds are confirmed by means of 1D and 2D-NMR spectroscopy, as well as MALDI TOF/TOF spectrometry. The derivatives **2** and **3** are active against *S. aureus* at the minimum inhibitory concentration (MIC) of 4 μg/mL and 16 μg/mL, correspondingly.

Keywords: C28-methyl ester of betulinic acid; Tempo$^+$Br$_3^-$ cation; pyridinium salts; antibacterial activity; antifungal activity
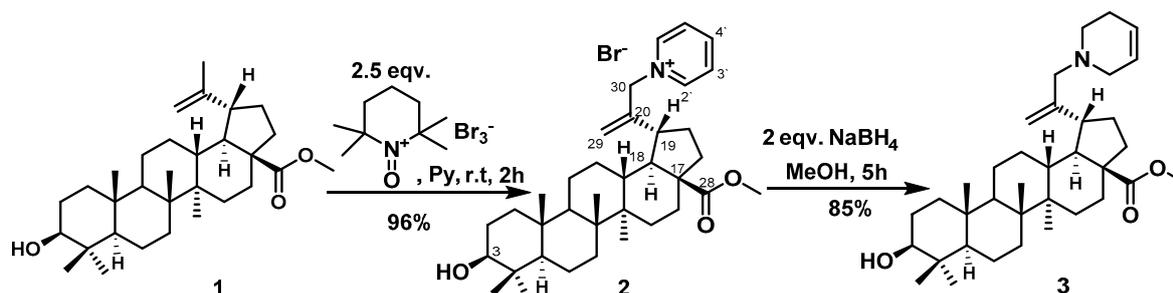
1. Introduction

N-Heterocyclic compounds represent a large class of organic molecules, many of which are biologically active substances, thus widely applied in medicine [1]. Various nitrogen heterocycles are involved in the numerous biochemical processes occurring in living cells. A perfect example is group B vitamins (B1, B2, B6, folic acid, etc.) and some coenzymes. In studies of N-heterocycles, quaternary ammonium compounds and their reduction products—1,2,5,6-tetrahydropyridines—are of particular interest as they can act as membrane-active substances exhibiting antitumor and antimicrobial properties [2,3].

Currently, the synthesis of compounds that selectively act upon tumor cells or microorganisms is of significant interest. One approach to solve this problem is the creation of molecules that can cause a general loss of structural organization and integrity of the cytoplasmic membrane in bacteria. Moreover, an important factor is the ability of the synthesized compounds to selectively bind to nucleic acids and cleave them, as well as to accumulate in the mitochondria of tumor cells and cause their death by cell apoptosis. In world practice, a direction in which natural compounds are used as the initial synthetic matrix owing to their low toxicity and high biocompatibility is widely developed. Among them, lupane triterpenoids are considered to be one of the most promising compounds, as they contain a hydrophobic carbocyclic backbone, which possesses a high affinity to lipid membranes [4,5]. The synthesis of their ammonium derivatives is a promising task for the development of targeted agents against bacterial infections and malignant neoplasms. Usually, the reactions proceed in several stages, involving preparation and isolation of intermediate products—halogen or amino derivatives, the corresponding modification of which provides quaternary ammonium salts [6,7]. The development of new one-pot methods and approaches to the synthesis of target derivatives would not only create effective and quite simple methods for their preparation, but also significantly enrich the range of quaternized ammonium analogues of natural compounds.

2. Results and Discussion

Recently, we reported the first case of pyridine quaternization using Tempo$^+$Br$_3^-$ cation (2,2,6,6-tetramethyl-1-oxo-piperidine tribromide) and betulin triterpenoids as a lipophilic substrate [8,9].

In continuation of our research on the synthesis of pyridinium analogue of natural compounds, we prepared a quaternized pyridine derivative of betulinic acid C28-methyl ester (**1**) via the one-pot method developed by us [8,9], which avoids the additional stage of halogenation and increases the yield of the target product. Thus, in the presence of Tempo$^+$Br$_3^-$ cation (Scheme 1), in situ allylic bromination of substrate **1** at the C-30 position of the triterpenoid takes place. Subsequent quaternization provides a pyridine derivative **2** with a yield of 96%. The reaction proceeds under mild conditions, and the method is distinguished by tolerance to the functional groups of the initial natural molecule.

Scheme 1. Synthesis of pyridine derivative **2** of 28-*O*-methyl betulinate (**1**) and its reduction product **3**.

It should be noted that the reaction pathway significantly depends on the position of the double bond in the molecule (Scheme 2). Thus, the reaction with an internal double bond goes via electrophilic addition (Path B) [10]. In the case of compound **1**, the reaction can go via an electrophilic addition or substitution, but if this path were realized, a brominated product with a pyridine moiety attached to the C-20 quaternary atom would be observed. However, this product was not found. Therefore, we suggest that for lupane-type triterpenes with the isopropenyl group, the radical allylic bromination is realized on the first steps of the reaction (path A). Further, terpene bromide, obtained, in situ, the N-alkylate pyridine molecule. Probably, in our reaction, Tempo$^+$Br$_3^-$ acts as a carrier of Br$_2$, although in some cases, it can also work as an oxidizing agent [10]. The radical nature of the process may also be indicated by the use of azobisisobutyronitrile (AIBN) as the initiator in the lupane triterpene bromination by the N-bromosuccinimide (NBS) [6].
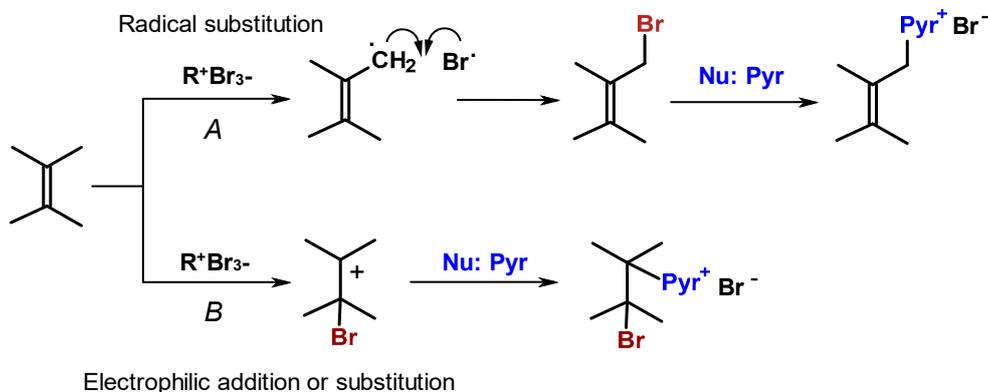
Scheme 2. Tentative reaction mechanisms.

Furthermore, to show the possibility of further transformations of pyridine analogue, we synthesized tetrahydropyridine derivative **3** via the reduction of **2** by NaBH$_4$ [11]. The formation of N-heterocycle **3** proceeded in dry methanol for five hours at room temperature with a yield of 85%.

The structures of the synthesized compounds were identified by means of MALDI TOF/TOF mass spectrometry (MS), as well as one- (^{1}H and ^{13}C) and two-dimensional (COSY HH, NOESY, HSQC, HMBC) NMR spectroscopy. Owing to the presence of a pyridinium fragment in the triterpene molecule at the C-30 position, methylene AB protons resonate as two doublets at 5.57 and 5.69 ppm, whereas the signal of the corresponding carbon atom is shifted to 66.4 ppm. Furthermore, the HMBC spectra exhibit cross-peaks of the C-30 atom with the pyridine ring protons in the *ortho*-position. Moreover, the NOESY spectra show the cross-peaks of the same protons with protons at C-30 and C-29. In compound **3**, the C-30 atom resonates at 63.00 ppm. The signals of the double-bond protons H-3′ and H-4′ are shifted to the upfield to 5.68 ppm and 5.74 ppm, respectively. They correlate with resonance lines of carbon atoms at 125.7 and 125.2 ppm, respectively, in the HSQC spectrum and with signals of protons at C-2′ and C-5′ atoms in the COSY HH spectrum.

The results of primary screening on the antibacterial and antifungal activity of the initial methyl ester **1**, its quaternized salt **2**, and the 1,2,5,6-tetrahydropyridine derivative **3** are presented in Table 1. The initial compound **1** showed weak antimicrobial activity against all tested bacterial strains and fungi. The compounds **2** and **3** exhibited antimicrobial activity, inhibiting growth and reproduction of gram-positive *Staphylococcus aureus* bacteria in the range of 81.7–88.0%. The compounds **2** and **3** showed moderate inhibitory activity towards the gram-negative bacteria and proved to be inactive against fungi.

The derivatives **2** and **3** were selected for further testing. Following the CLSI [12] recommendations, the minimum inhibitory concentration (MIC) was examined, calculating the lowest concentration at which the complete inhibition of bacteria or fungi was registered (Table 2). The complete inhibition of growth was revealed at ≤20% of growth (or >80% inhibition), while the exposures were chosen only if the next maximum concentration exhibited complete inhibition of 80–100%. Colistin and vancomycin were used as positive standards for the bacterial inhibition of gram-negative and gram-positive bacteria, respectively. Fluconazole was used as a positive standard for the inhibition of *C. albicans* and *C. neoformans* fungi. The compounds **2** and **3** were active against *S. aureus* at the MIC of 4 μg/mL and 16 μg/mL, correspondingly. It can be concluded that the introduction of the pyridine fragment while preserving the ester group at the C-28 position and the hydroxyl function at the C-3 atom of betulinic acid provides an increase in the antibacterial effect towards the *S. aureus* bacteria, which is comparable with the level of inhibitory activity of the comparison drug, Vancomycin (Table 2).

Table 1. Percentage growth inhibition of bacterial strains and fungi by compounds **1**–**3** at a concentration of 32 μg/mL.

Comp.	Gram-Positive Bacteria	Gram-Negative Bacteria				Fungi	
	Staphylococcus aureus	*Escherichia coli*	*Klebsiella pneumoniae*	*Acinetobacter baumannii*	*Pseudomonas aeruginosa*	*Candida albicans*	*Cryptococcus neoformans*
1	3.51	−5.37	10.43	15.17	1.63	10.10	−28.74
2	88.00	16.90	23.10	34.70	5.70	8.80	−52.50
3	81.70	12.60	−0.70	−7.30	−9.30	7.8	−64.60

Table 2. Values of the minimal inhibitory concentration (MIC, μg/mL) of compounds **2** and **3** against previously studied strains of bacteria and fungi.

Comp.	Gram-Positive Bacteria	Gram-Negative Bacteria				Fungi	
	Staphylococcus aureus	*Escherichia coli*	*Klebsiella pneumoniae*	*Acinetobacter baumannii*	*Pseudomonas aeruginosa*	*Candida albicans*	*Cryptococcus neoformans*
2	4	>32	>32	>32	>32	32	32
3	16	>32	>32	>32	>32	32	32
Fluconazole						0.125	8
Colistin–sulfate		0.125	0.25	0.25	0.25		
Vancomycin–HCl	1						

3. Materials and Methods

The starting compounds and reagents were purchased from standard commercial suppliers and used without any further purification. ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance 500 HD spectrometer (Karlsruhe, Germany) (500.17 MHz for ^1H and 125.78 MHz for ^{13}C) in CDCl$_3$ or CD$_3$OD. All the experiments were set up with standard Bruker pulse sequences. Chemical shifts are given in ppm relative to tetramethylsilane as the internal standard. IR spectra (thin films) were obtained with the use of a Bruker Vertex 70v spectrometer (Karlsruhe, Germany). Optical rotation was determined on a Perkin–Elmer 141 polarimeter (Beaconsfield, UK). Specific rotation $[\alpha]_D$ is expressed in $(deg \cdot mL)/(g \cdot dm)^{-1}$; the concentration of the solution *c* is expressed in g/100 mL. Elemental analysis was carried out on a Carlo Erba 1106 analyzer (Milan, Italy). The UV/visible spectra were recorded by Perkin Elmer Lambda 750 spectrometer (*l* = 1; 0.1 cm) in C$_2$H$_5$OH. Mass-spectra were measured by the MALDI TOF/TOF method on a Bruker Autoflex III spectrometer with the registration of positive ions; 3,5-dimethoxy-4-hydroxycinnamic (sinapic) acid was used as a matrix. Thin-layer chromatography was carried out on Sorbfil plates (Sorbpolimer, Krasnodar, Russia) in CHCl$_3$–MeOH (20:1); spots were visualized with anisaldehyde. Silica gel L (KSKG grade, 50–160 μm) was employed for column chromatography. 2,2,6,6-Tetramethyl-1-oxopiperidinium tribromide was obtained as dark-red crystals, which were formed in the reaction of 0.05 mol of TEMPO radical with 0.08 mol of Br$_2$ in dry CCl$_4$. Physico-chemical characteristics of the obtained compound Tempo$^+$Br$_3^-$ correspond to those described in the literature [13–15]. Starting triterpenoid **1** was prepared as previously reported [16].

3.1. 1-{2-[(1R, 3aS, 5aR, 5bR, 7aR, 9S, 11aR, 13aR, 13bS)-9-hydroxy-3a-(methoxycarbonyl)-5a, 5b, 8, 8, 11a-pentamethyl-icosahydro-1H-cyclopenta [a] chrysen-1-yl] prop-2-en-1-yl} pyridin-1-ium bromide (**2**)

Tempo$^+$Br$_3^-$ (2.5 eq.) was added to a solution of the initial triterpenoid **1** (0.1 mmol) in pyridine (2 mL). The reaction mixture was stirred for 2 h, when TLC indicated complete consumption of the starting triterpene. The mixture was evaporated repeatedly by diluting EtOAc. The residue was separated by column chromatography on SiO$_2$, sequentially eluting with a solvent system in order of polarity increasing (hexane/EtOAc, 30:1→1:1; CHCl$_3$; MeOH). Compound **2** was obtained as beige crystalline powder (recrystallized from EtOAc) (150 mg, 95%): $[\alpha]_D^{21}$ = −10° (c 0.43, CHCl$_3$), m.p. 207–209 °C. UV (CH$_3$OH) λ_{max} (log ε): 256 nm (2.21). IR (film, v cm^{-1}) 3390 (m), 2924 (m), 1718 (s), 1631 (s), 1461 (s), 1377 (s). ^1H-NMR (δ, ppm, CDCl$_3$, 500 MHz): 9.29 (2H, d, ^3J = 4.3 Hz, Py), 8.61 (1H, t, ^3J = 7.1 Hz, Py), 8.14–8.19 (2H, m, Py), 5.57 and 5.69 (2H, both d, ^2J = 14.3 Hz, H-30), 5.18 and 4.89 (2H, both br.s, H-29), 3.64 (3H, s, OCH$_3$), 3.18 (1H, dd, ^3J = 3.1 Hz, ^3J = 10.4 Hz, H-3), 2.86–2.75 (1H, m, H-19), 2.28–0.65 (23H, m, CH, CH$_2$), 1.71 (1H, t, ^3J = 10.9 Hz, H-18), 0.95, 0.90, 0.86, 0.79, 0.74 (15H, all s, 5CH$_3$). ^{13}C-NMR (δ, ppm, CDCl$_3$, 125.5 MHz): 176.2 (C=O), 149.1 (C), 145.8, 145.6, 128.1 (CH, Py), 113.7 (CH$_2$), 78.7 (CH), 66.4 (CH$_2$), 56.4 (C), 55.2 (CH), 51.5 (OCH$_3$), 50.6 (CH), 50.3 (CH), 42.6 (CH), 42.3 (C), 40.6 (C), 38.8 (C), 38.7 (CH$_2$), 38.1 (CH), 37.1 (C), 36.3 (CH$_2$), 34.2 (CH$_2$), 32.2 (CH$_2$), 31.7 (CH$_2$), 29.5 (CH$_2$), 28.0 (CH$_3$), 27.4 (CH$_2$), 27.3 (CH$_2$), 20.9 (CH$_2$), 18.2 (CH$_2$), 16.1 (CH$_3$), 15.9 (CH$_3$), 15.4 (CH$_3$), 14.7 (CH$_3$). MS (MALDI TOF/TOF), *m/z* [*M*-Br]$^+$ 548.446, calcd. for C$_{36}$H$_{54}$BrNO$_3$: 628.736. Anal. calcd. for C$_{36}$H$_{54}$BrNO$_3$, %: C 68.77; H 8.66; Br 12.71; N 2.23. Found, %: C 68.75; H 8.59; Br 12.78; N 2.24.

3.2. Methyl (1R, 3aS, 5aR, 5bR, 7aR, 9S, 11aR, 13aR, 13bS)-9-hydroxy-5a, 5b, 8, 8, 11a-pentamethyl-1-[3-(1,2,5,6-tetrahydropyridin-1-yl)prop-1-en-2-yl]-icosahydro-1H-cyclopenta [a] chrysene 3a-carboxylate (**3**)

Compound **2** (100 mg, 0.16 mmol) was dissolved in 5 mL of MeOH, then 10 mg of sodium borohydride (0.26 mmol) was added portionwise at room temperature. The mixture was stirred for 5 h. The solvent was evaporated under reduced pressure, then 5 mL of water was added, and the mixture was extracted with CH$_2$Cl$_2$ (3 × 10 mL). The combined organic phases were dried over Na$_2$SO$_4$ and evaporated. The residue was purified by column chromatography on SiO$_2$, sequentially eluting with a solvent system in order of polarity increasing (hexane/EtOAc, 30:1→1:1; CHCl$_3$). Compound **3** was

obtained as beige crystalline powder (recrystallized from EtOAc) (75 mg, 85%): $[\alpha]_D^{19} = -6.3°$ (c 0.23, CHCl$_3$), m.p. 210–212 °C. UV (C$_2$H$_5$OH): 256 nm (log ε 1.79). IR (film, v cm^{-1}) 3441 (m), 2945 (m), 2869 (s), 1722 (s), 1454 (s), 1388 (s). ^{1}H-NMR (δ, ppm, CDCl$_3$, 500 MHz): 5.74–5.76 (1H, m, H-4'), 5.6–5.69 (1H, m, H-3'), 4.91–4.95 (2H, m, H-30), 5.32 and 4.93 (2H, both br.s, H-29), 3.68 (3H, s, OCH$_3$), 3.18–3.21 (1H, m, H-3), 2.98–0.89 (30H, m, CH, CH$_2$), 0.98, 0.98, 0.92, 0.83, 0.77 (15H, all s, 5CH$_3$). ^{13}C-NMR (δ, ppm, CDCl$_3$, 125.5 MHz): 176.6 (C=O), 151.2 (C), 125.7 (CH), 125.2 (CH), 109.4 (CH$_2$), 78.9 (CH), 62.9 (CH$_2$), 56.6 (C), 55.3 (CH), 53.5 (CH$_2$), 51.2 (OCH$_3$), 50.5 (CH$_2$), 50.06 (CH), 49.7 (CH), 44.3 (CH), 42.3 (C), 40.6 (C), 38.8 (C), 38.7 (CH$_2$), 38.2 (CH), 37.2 (C), 36.8 (CH$_2$), 34.3 (CH$_2$), 32.1 (CH$_2$), 32.0 (CH$_2$), 29.7 (CH$_2$), 27.9 (CH$_3$), 27.4 (CH$_2$), 26.5 (CH$_2$), 26.2 (CH$_2$), 21.0 (CH$_2$), 18.3 (CH$_2$), 16.1 (CH$_3$), 15.9 (CH$_3$), 15.3 (CH$_3$), 14.7 (CH$_3$). MS (MALDI TOF/TOF), m/z [M − H]$^+$ 550.243, calcd. for C$_{36}$H$_{57}$NO$_3$: 551.431. Anal. calcd. for C$_{36}$H$_{57}$NO$_3$, %: C 78.35; H 10.41; N 2.54. Found, %: C 78.38; H 10.49; N 2.50.

3.3. Biological Screening

The antimicrobial screening was performed by CO-ADD (The Community for Antimicrobial Drug Discovery), funded by the Wellcome Trust (United Kingdom) and The University of Queensland (Australia).

3.3.1. Antimicrobial Assay

The bacteria were cultured in cation-adjusted Mueller–Hinton broth (CAMHB) at 37 °C overnight followed by a series of dilutions, and then added to each well of the compound-containing plates, giving a cell density of 5×10^5 CFU/mL and a total volume of 50 mL. All the plates were covered and incubated at 37 °C for 18 h without shaking.

3.3.2. Antifungal Assay

Fungi strains were cultured for three days on yeast extract-peptone dextrose (YPD) agar at 30 °C. A yeast suspension of 1×10^6 to 5×10^6 CFU/mL (as determined by OD530) was prepared from five colonies. The suspension was subsequently diluted and added to each well of the compound-containing plates, giving a final cell density of fungi suspension of 2.5×10^3 CFU/mL and a total volume of 50 mL. All plates were covered and incubated at 35 °C for 24 h without shaking.

The inhibition of bacterial growth was determined by the measuring of absorbance at 600 nm (OD$_{600}$), using a Tecan M1000 Pro monochromator plate reader. The percentage of growth inhibition was calculated for each well, using negative control (media only) and positive control (bacteria without inhibitors) on the same plate as references. The significance of the inhibition values was determined by modified Z-scores, calculated using the median and MAD of the samples (no controls) on the same plate. Samples with an inhibition value above 80% and a Z-score above 2.5 for either replicate (n = 2 on different plates) were classed as active. Samples with inhibition values between 50% and 80% and a Z-score above 2.5 for either replicate (n = 2 on different plates) were classed as partially active.

3.3.3. Hit Confirmation

The percentage of growth inhibition was calculated for each well, using negative control (media only) and positive control (bacteria without inhibitors) on the same plate. The MIC was determined as the lowest concentration at which the growth was fully inhibited, defined by inhibition of ≥80%. Also, the maximal percentage of growth inhibition is reported as DMax, indicating any compounds with partial activity. Hits were classified by MIC ≤16 μg/mL in either replicate (n = 2 on different plates).

4. Conclusions

Thus, we demonstrated an example of pyridine quaternization using betulinic acid 28-*O*-methyl ester and Tempo$^+$Br$_3{}^-$ cation, followed by reduction of the resulting salt to 1,2,5,6-tetrahydropyridine derivative. The prepared quaternized and reduced pyridine derivatives of betulinic acid exhibited

high antibacterial activity against gram-positive *Staphylococcus aureus* bacteria compared with the initial compound.

Supplementary Materials: NMR, IR, UV, and mass-spectra for compounds are available online.

Author Contributions: E.R.S. carried out the synthesis, analyzed the data, and prepared the manuscript; L.V.P. managed the research, and edited and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Asif, M. Biological significances of nitrogen hetero atom containing heterocyclic compounds. *Int. J. Bioorg. Chem.* **2017**, *2*, 146–152. [CrossRef]
2. Sundararaman, M.; Kumar, R.R.; Venkatesan, P.; Ilangovan, A. 1-Alkyl-(*N*,*N*-dimethylamino)pyridinium bromides: inhibitory effect on virulence factors of Candida albicans and on the growth of bacterial pathogens. *J. Med. Microbiol.* **2013**, *62*, 241–248. [CrossRef] [PubMed]
3. Serafim, T.L.; Carvalho, F.S.; Bernardo, T.C.; Pereira, G.C.; Perkins, E.; Holy, J.; Krasutsky, D.A.; Kolomitsyna, O.N.; Krasutsky, P.A.; Oliveira, P.J. New derivatives of lupane triterpenoids disturb breast cancer mitochondria and induce cell death. *Bioorg. Med. Chem.* **2014**, *22*, 6270–6287. [CrossRef]
4. Alakurtti, S.; Mäkelä, T.; Koskimies, S.; Yli-Kauhaluoma, J. Pharmacological properties of the ubiquitous natural product betulin. *Eur. J. Pharm. Sci.* **2006**, *29*, 1–13. [CrossRef] [PubMed]
5. Furtado, N.A.J.C.; Pirson, L.; Edelberg, H.; Miranda, L.M.; Loira-Pastoriza, C.; Preat, V.; Larondelle, Y.; André, C.M. Pentacyclic triterpene bioavailability: An overview of in vitro and in vivo studies. *Molecules* **2017**, *22*, 400–424. [CrossRef] [PubMed]
6. Holy, J.; Kolomitsyna, O.; Krasutsky, D.; Oliveira, P.J.; Perkins, E.; Krasutsky, P.A. Dimethylaminopyridine derivatives of lupane triterpenoids are potent disruptors of mitochondrial structure and function. *Bioorg. Med. Chem.* **2010**, *18*, 6080–6088. [CrossRef] [PubMed]
7. Bernardo, T.C.; Cunha-Oliveira, T.; Serafim, T.L.; Holy, J.; Krasutsky, D.; Kolomitsyna, O.; Krasutsky, P.; Moreno, A.M.; Oliveira, P.J. Dimethylaminopyridine derivatives of lupane triterpenoids cause mitochondrial disruption and induce the permeability transition. *Bioorg. Med. Chem.* **2013**, *21*, 7239–7249. [CrossRef] [PubMed]
8. Shakurova, E.R.; Pozdnyakova, D.A.; Tretyakova, E.V.; Parfenova, L.V. One-Pot Synthesis of betulin triterpenoid quaternized pyridine derivatives and their antimicrobial activity. *Lett. Drug. Des. Discov.* **2020**. [CrossRef]
9. Parfenova, L.V.; Shakurova, E.R.; Ponedel'kina, I.Y. The method of synthesis of 1-(3-hydroxy,28-acetoxylup-20(29)-en-30-yl)–pyridinium bromide and its use as an antibacterial and antifungal agent. Patent application RU 2018 130 200 A, 20 August 2018.
10. Shakurova, E.R.; Salimova, E.V.; Mescheryakova, E.S.; Parfenova, L.V. One-pot synthesis of quaternary pyridinium salts and tetrahydropyridine derivatives of fusidane triterpenoids. *Chem. Heterocycl. Comp.* **2019**, *55*, in press.
11. Tang, Z.; Mayrargue, J.; Alami, M. Synthesis of piperidine derivatives by reduction of pyridinium salts. *Synth. Commun.* **2007**, *37*, 3367–3379. [CrossRef]
12. Cockerill, F.R.; Wikler, M.A.; Alder, J.; Dudley, M.N.; Eliopoulos, G.M.; Ferraro, M.J.; Hardy, D.J.; Hecht, D.; Hindler, J.; Patel, J.; et al. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard*, 9th ed.; Clinical and Laboratory Standards Institute: Wayne, NJ, USA, 2012.
13. Zhadanov, R.I.; Golubev, V.A.; Rozantsev, E.G. Synthesis and structure of certain 1-oxopiperidinium tribromides. *Izv. Akad. nauk SSSR. Ser. Khim.* **1970**, *1*, 186–187. [CrossRef]

14. Shibuya, M.; Tomizawa, M.; Iwabuchi, Y. Oxidative rearrangement of tertiary allylic alcohols employing oxoammonium salts. *J. Org. Chem.* **2008**, *73*, 4750–4752. [CrossRef] [PubMed]

15. Percino, M.J.; Cerón, M.; Soriano-Moro, G.; Pacheco, J.A.; Castro, M.E.; Chapela, V.M.; Bonilla-Cruz, J.; Saldivar-Guerra, E. 2,2,6,6-Tetramethyl-1-oxopiperidinetribromide and two forms of 1-hydroxy-2,2,6,6-tetramethylpiperidinium bromide salt: syntheses, crystal structures and theoretical calculations. *J. Mol. Struct.* **2015**, *1103*, 254–264. [CrossRef]

16. Urban, M.; Sarek, J.; Klinot, J.; Kornikova, G.; Hajduch, M. Synthesis of A-seco derivatives of betulinic acid with cytotoxic activity. *J. Nat. Prod.* **2004**, *67*, 1100–1105. [CrossRef] [PubMed]