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SYNTHESIS AND ANTIMICROBIAL ACTIVITY OF SEVERAL BIS-QUATERNIZED AMMONIUM DERIVATIVES OF THE DITERPENOID ISOSTEVIOL

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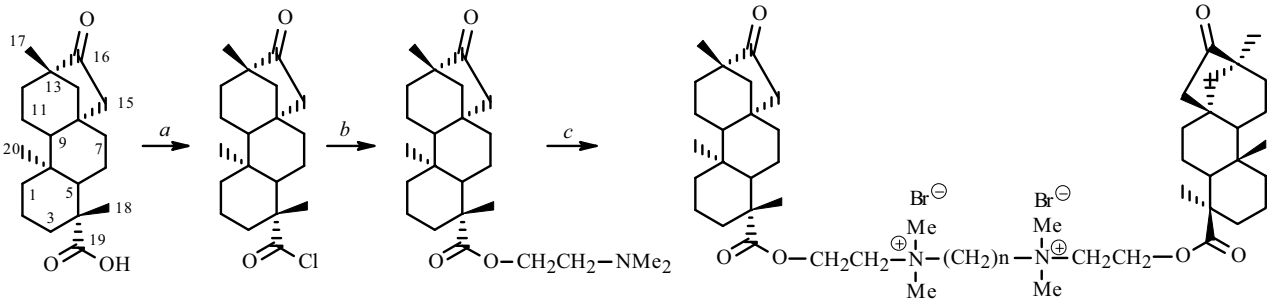
*Previously unreported bis-quaternized ammonium derivatives of the diterpenoid isosteviol (16-oxo-*ent*-beyeran-19-oic acid) were synthesized. The compound in which the two quaternized N atoms were joined by a dodecamethylene spacer had the greatest antimicrobial activity and was comparable in activity with ciprofloxacin and clotrimazole.*

Keywords: terpenoids, isosteviol, ammonium compounds, antimicrobial activity.

We synthesized previously monoammonium esters of the diterpenoid isosteviol that exhibited moderate bacteriostatic and fungistatic activity [1]. Keeping in mind that increasing the number of quaternized N atoms in ammonium compounds enhances their biological activity [2–5], herein we report on the synthesis and antimicrobial activity of previously unknown semi-synthetic ammonium diterpenoids in which two molecules of isosteviol (**1**) (16-oxo-*ent*-beyeran-19-oic acid) are covalently linked by a spacer with two quaternized N atoms that are joined in turn by a polymethylene chain.

Target compounds **4**–**7** were prepared by the published method [1] in which 1,ω-dibromoalkanes were used instead of alkylhalides in the quaternization step of amine **3**.



1 **2** **3** **4 - 7**

4: n = 5; **5:** n = 9; **6:** n = 10; **7:** n = 12

a. $SOCl_2$, *b*. $Me_2N(CH_2)_2OH$, *c*. $Br(CH_2)_nBr$

The formation of ammonium compounds **4**–**7** was proved by PMR spectroscopy and mass spectrometry. PMR spectra of the salts had characteristic resonances for protons of the isosteviol hydrocarbon framework, namely C17, C18, and C20 methyl protons, H-15$_\alpha$ and H-3$_{eq}$ protons, and protons for the methyls on the N atoms and in the –O–CH_2CH_2–N(+)–CH_2– spacer. Weak-field shifts of the singlet for the methyl protons (by 0.7 ppm) and multiplets for methylene protons of the –O–CH_2CH_2–N(+)– group (by 0.8 ppm) indicated that the N atoms were quaternized on going from amine **3** to **4**–**7**. Compounds **4**–**7** were yellow sticky compounds that crystallized with time and were soluble in DMSO, MeOH, and $CHCl_3$ but poorly soluble in H_2O.

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TABLE 1. Bacteriostatic, Fungistatic, Bactericidal, and Fungicidal Activity of **4**–**7**

Bacterium	Minimum inhibiting concentration, μg/mL						
	4	**5**	**6**	**7**	Ciprofloxacin	Amphotericin B	Clotrimazole
				Bacteriostatic and fungistatic			
St. aureus 209p	7.8	3.0	0.78	0.5	0.25	–	–
B.cereus 8035	31.25	3.9	1.5	1.5	0.25	–	–
E.coli F-50	62.5	31.25	31.25	15.6	0.5	–	–
Ps. aeruginosa 9027	250	125	125	62.5	0.5	–	–
Aspergillus niger	500	500	500	500	–	20	–
Trich. gypseum	15.6	7.8	12.5	7.8	–	–	3.13
Candida albicans	12.5	1.56	1.9	0.78	–	–	0.39
				Bactericidal and fungicidal			
St. aureus 209p	300	30	5	0.5	0.25	–	–
B.cereus 8035	500	500	50	5	0.25	–	–
E.coli F-50	> 500	> 500	> 500	> 500	0.5	–	–
Ps. aeruginosa 9027	> 500	> 500	> 500	> 500	0.5	–	–
Aspergillus niger	> 500	> 500	> 500	> 500	–	20	–
Trich. gypseum	> 500	> 500	> 500	62.5	–	–	3.13
Candida albicans	> 500	> 500	300	30	–	–	0.39

Compounds **4**–**7** were tested *in vitro* for bacteriostatic, fungistatic, bactericidal, and fungicidal activity (Table 1). The results showed that compound **7** had the most clearly pronounced antimicrobial activity. The bacteriostatic and bactericidal properties of this compound against Gram-positive bacteria (*Staphylococcus aureus* 209p, *Bacillus cereus* 8035) were comparable with those of the antibacterial drug ciprofloxacin; the fungistatic properties, with those of the antifungal drug clotrimazole. The activity of compound **7** also surpassed those of all aforementioned compounds with respect to Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*). In general, the antimicrobial activity of bis-quaternized isosteviol derivatives **4**–**7** was greater than that of the mono-quaternized derivatives [1]. This agreed with the known tendency mentioned above [2–5].

It can be concluded from the results that the antimicrobial activity of bis-quaternized isosteviol derivatives **4**–**7** depended on the length of the polymethylene chain between the N atoms. Furthermore, salts **4**–**7** represented one of those rare instances where a single compound exhibited simultaneously high antibacterial (in this instance against *S. aureus* 209p and *B. cereus* 8035) and high fungistatic (in this instance against *Trichophyton gypseum* and *Candida albicans*) activities.

EXPERIMENTAL

IR spectra were recorded from emulsions in mineral oil on a Bruker Vector 22 Fourier-spectrometer (Germany) in the range 400–4000 cm^{-1}. PMR spectra were obtained on a Bruker MSL-400 instrument (Germany). MALDI mass spectra were measured on a Bruker Ultraflex III instrument (Germany) equipped with a solid-state laser and time-of-flight mass analyzer. The accelerating potential was 25 kV. Samples were placed on an Anchor Chip target. Spectra were recorded in positive-ion mode. The resulting spectrum was the sum of 300 spectra obtained from various points of the sample. The matrix was 2,5-dihydroxybenzoic acid or *p*-nitroaniline (Acros). Methanol (Merck) was used to prepare the matrices. Samples were deposited on the target by drying drops. Electrospray mass spectra were measured on a DFS Thermo Electron Corporation instrument (USA). The sprayer capillary potential was 4.5 kV. The completeness of the reaction and the purity of the compounds were monitored by TLC on Silufol UV-254 plates with elution by $CHCl_3$:MeOH (5:1). Spots were detected using I_2 vapor. Column chromatography was performed over silica gel 60 (0.06–0.2 mm, Alfa Aesar).

Isosteviol (**1**) was obtained by the literature method [6] from the sweetener Sweta (Steivan Biotechnology Corp.). Acid chloride **2** and oxoamine **3** were synthesized as before [1]. The physical constants of **1**–**3** agreed with those in the literature.

General Method for Preparing Quaternized Derivatives of Oxoamine 3. A solution of **3** (0.9 mmol) and anhydrous CH_3CN (5 mL) was treated with dibromoalkane (0.3 mmol) and heated on a bath at 70–75°C for 20–25 h. The solvent was removed at reduced pressure. The resulting precipitate was chromatographed over silica gel ($CHCl_3$:MeOH eluent, 40:1).

1,5-Bis(19-*nor*-16-oxo-*ent*-beyeran-4α-ylcarbonyloxyethyl-2-dimethylammonio)pentane Dibromide (4). Yield 45%, yellow paste. IR spectrum (ν, cm^{-1}): 1130, 1147 (C–O), 1732 (C19=O, C16=O).

PMR spectrum (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 0.69 (6H, s, H-20), 0.95 (6H, s, H-17), 1.22 (6H, s, H-18), 0.70–2.10 (48H, m, *ent*-beyerane framework and -$CH_2(C\underline{H}_2)_3CH_2$- spacer), 2.13 (2H, d, J = 13.6, H-3_{eq}), 2.62 (2H, d, J = 18.5, H-15_α), 3.41 [12H, s, $N^+(C\underline{H}_3)_2$], 3.70–3.76 [4H, m, -$N^+C\underline{H}_2(CH_2)_n$], 3.92–4.00 (4H, m, -$OCH_2C\underline{H}_2N^+$), 4.48–4.63 (4H, m, -$OC\underline{H}_2$-).

Mass spectrum (ESI, *m*/*z*): 1008.66 $[M + H]^+$. Mass spectrum (MALDI-TOF, *m*/*z*): 928 $[M – Br]^+$ (exp.); 1008.50 $[M]^+$ (theor.) ($C_{53}H_{88}Br_2N_2O_6$).

1,9-Bis(19-*nor*-16-oxo-*ent*-beyeran-4α-ylcarbonyloxyethyl-2-dimethylammonio)nonane Dibromide (5). Yield 48%, yellow paste. IR spectrum (ν, cm^{-1}): 1131, 1147 (C–O), 1732 (C19=O, C16=O).

PMR spectrum (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 0.67 (6H, s, H-20), 0.95 (6H, s, H-17), 1.21 (6H, s, H-18), 0.68–2.10 (56H, m, *ent*-beyerane framework and -$CH_2(C\underline{H}_2)_7CH_2$- spacer), 2.13 (2H, d, J = 13.9, H-3_{eq}), 2.59 (2H, d, J = 18.7, H-15_α), 3.44 [12H, s, $2(N^+(CH_3)_2)$], 3.73–3.80 [4H, m, -$N^+C\underline{H}_2(CH_2)_n$], 3.96–4.05 (4H, m, -$OCH_2C\underline{H}_2N^+$), 4.45–4.61 (4H, m, -$OC\underline{H}_2$-).

Mass spectrum (MALDI-TOF, *m*/*z*): 984.16 $[M – Br]^+$ (exp.); 1064.56 $[M]^+$ (theor.) ($C_{57}H_{96}Br_2N_2O_6$).

1,10-Bis(19-*nor*-16-oxo-*ent*-beyeran-4α-ylcarbonyloxyethyl-2-dimethylammonio)decane Dibromide (6). Yield 26%, yellow paste. IR spectrum (ν, cm^{-1}): 1131, 1147 (C–O), 1732 (C19=O, C16=O).

PMR spectrum (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 0.67 (6H, s, H-20), 0.95 (6H, s, H-17), 1.21 (6H, s, H-18), 0.68–2.10 [58H, m, *ent*-beyerane framework and -$CH_2(C\underline{H}_2)_8CH_2$- spacer), 2.12 (2H, d, J = 13.9, H-3_{eq}), 2.60 (2H, d, J = 18.7, H-15_α), 3.43 [12H, s, $2(N^+(CH_3)_2)$], 3.71–3.83 [4H, m, -$N^+C\underline{H}_2(CH_2)_n$], 3.92–4.04 (4H, m, -$OCH_2C\underline{H}_2N^+$), 4.45–4.62 (4H, m, -$C\underline{H}_2$-).

Mass spectrum (MALDI-TOF, *m*/*z*): 997.90 $[M – Br]^+$ (exp.); 1078.58 $[M]^+$ (theor.) ($C_{58}H_{98}Br_2N_2O_6$).

1,12-Bis(19-*nor*-16-oxo-*ent*-beyeran-4α-ylcarbonyloxyethyl-2-dimethylammonio)dodecane Dibromide (7). Yield 46.2%, yellow paste. IR spectrum (ν, cm^{-1}): 1130, 1147 (C–O), 1727 (C19=O), 1728 (C16=O).

PMR spectrum (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 0.68 (6H, s, H-20), 0.96 (6H, s, H-17), 1.22 (6H, s, H-18), 0.68–2.10 [62H, m, *ent*-beyerane framework and -$CH_2(C\underline{H}_2)_{10}CH_2$- spacer], 2.12 (2H, d, J = 13.9, H-3_{eq}), 2.60 (2H, d, J = 18.7, H-15_α), 3.46 [12H, s, $2(N^+(CH_3)_2)$], 3.75–3.81 [4H, m, -$N^+C\underline{H}_2(CH_2)_n$], 3.99–4.04 (4H, m, -$OCH_2C\underline{H}_2N^+$), 4.45–4.61 (4H, m, -$OC\underline{H}_2$-).

Mass spectrum (MALDI-TOF, *m*/*z*): 1026.11 $[M – Br]^+$ (exp.); 1106.61 $[M]^+$ (theor.) ($C_{60}H_{102}Br_2N_2O_6$).

The bacteriostatic and fungistatic properties were studied using serial dilutions in liquid growth medium [7]; bactericidal and fungicidal, by the literature methods [8, 9]. The test subjects were bacterial cultures of *S. aureus* 209-P, *E. coli* F-50, *B. cereus* 8035, *P. aeruginosa* 9027 and fungal cultures of *A. niger* BKMF-1119, *Trichophyton mentagrophytes* var. gypseum-1773, *C. albicans* 855-653. The standards were the antibacterial drug ciprofloxacin, which is widely used in medicine, and the antifungal drugs amphotericin B and clotrimazole.

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