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Polyacylated Neohesperidosides From *Geranium caespitosum*: Bacterial Multidrug Resistance Pump Inhibitors

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Abstract—Bioassay-directed fractionation for *Staphylococcus aureus* multidrug resistance efflux pump inhibitors resulted in isolation of novel acylated neohesperidosides from *Geranium caespitosum*. The more highly acylated compounds had no direct activity against *S. aureus,* but potentiated activity of the antibiotics berberine, rhein, ciprofloxacin and norfloxacin. Cellular concentrations of berberine were greatly increased in the presence of active esters.

In a continued search for plant sources of antimicrobial multidrug resistance (MDR) efflux pump inhibitors[1] we found that hexanes extracts of *Geranium caespitosum* Gray and James[2] had both direct and antibiotic-potentiating antibacterial growth inhibitory activity. Thus, a 20 s dip of fresh, sticky-leaved whole plant material into hexanes and evaporation of the hexanes yielded 11.1 g of residue (62.5 μg/mL activity against wild-type strain 8325-4 *Staphylococcus aureus*) and 271 g of plant marc (after drying). Similar activity was present in 1.48 g of a hexanes extract of 68 g of whole above-ground plant material. The basic bioassay[1a–c] involved testing the sample for bacterial growth inhibition and for potentiation in combination with a subinhibitory dose of the weak antibiotic berberine.

Bioactivity-directed chromatography of the extracts yielded the new compounds **1**–**4**.[3] Structures were determined by MS data, detailed 2D NMR experiments and by hydrolysis of **3** to butyl neohesperidoside (**5**).[4,5] The NMR spectra of **5** were compared to those for commercially available neohesperidose (2-*O*-α-L-rhamnopyranosyl-β-D-glucopyranose) and to literature values for steroidal neohesperidosides.[6] Molecular formulas were established by HRMS or MS in combination with ^{13}C and ^{1}H NMR. These analyses were facilitated by the facts that **1**–**4** were all derivatives of butyl neohesperidoside (**5**) and that spectral data could be compared across the series. The presence of the rhamnose methyl, the glucose C-6 methylene, and the known relationship of the two anomeric carbons were keys from which ring C–H assignments could be made from the HMQC-TOCSY spectra. Once the sugar ring protons were assigned, HMBC correlations to the individual acyl side chain carbonyls and from the carbonyls to the side chains allowed for placement of each of the acyl groups.

Details of spectral assignments and structure determinations will be published elsewhere, but exemplary NMR spectral data for **3** are given in Table 1. The molecular formula for **3** was established as $C_{32}H_{52}O_{14}$ by FABMS (M+H found: 661.3440 and calcd 661.3435). The summary data of Table 2 for all isolates are presented to provide an easier focus on comparative relationships. Data for the side chain groups of the other compounds were essentially the same as those for **3** with the exception of the hexadecanoyl moiety of **1** and **2**. The position of that group in **1** and **2** was established by HMBC spectra which correlated the side chain methylene to the C-4″ proton in **1** and the C-6′ proton in **2**. The nature of this side chain was established by a combination of NMR and MS. Thus, **2** showed loss of BuO from the glucose anomeric carbon site to yield fragment **6** ($C_{32}H_{57}O_{11}$; 617.3893 found, 617.3887 calcd) and further fragments $C_{22}H_{41}O_5$ (385.2958 found,

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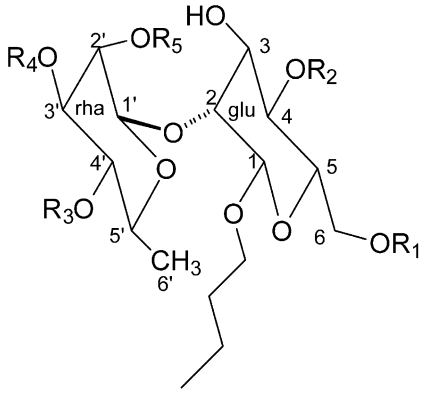
385.2954 calcd) and $C_{10}H_{16}O_6$ (232.0935 found, 232.0933 calcd) as indicated on **6**, where one hydrogen was transferred between fragments. Compound **2** formed a tetraacetate whose HRMS fragments exactly correlated with those of **2**. Congestion of NMR resonances for the carbons and hydrogens of the hexadecanoyl moiety in **2** with those of other groups did not allow for exact proton and carbon count, but the spectra were consistent with the hexadecanoyl (palmitic acid) formulation. The exact mass for the $C_{22}H_{41}O_{11}$ fragment was the key to assignment since the mass did not match that for one less or one more CH_2 moiety. Structures for **1** and **4** were supported by related MS data.



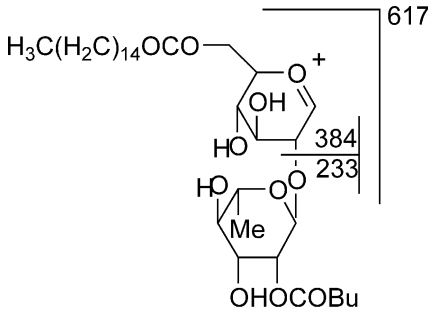
1: $R_1 = R_2 = R_4 = R_5 = H$; $R_3 = -CO(CH_2)_{14}CH_3$

2: $R_1 = -CO(CH_2)_{14}CH_3$; $R_2 = R_4 = R_5 = H$; $R_3 = -COBu$

3: $R_1 = R_2 = R_3 = -COiPr$; $R_4 = -COBu$

4: $R_1 = R_2 = R_3 = -COiPr$; $R_4 = OAc$; $R_5 = -COBu$

5: $R_1 = R_2 = R_3 = R_4 = R_5 = H$



6

The monoester **1** was inactive against *S. aureus* and *B. megaterium* 11561 and did not show potentiation activity with the weak antibiotic berberine. The diester **2** was also inactive against *S. aureus* and was not potentiating, but directly inhibited *B. megaterium* growth (12.5 μg/mL). The tetraester **3** and pentaester **4** were inactive against *S. aureus* but directly inhibited the growth of *B. megaterium* (3.12–6.25 μg/mL). Esters **3** and **4** did, however, potentiate the antigrowth activity of subinhibitory berberine (3.12–6.25 μg/mL) in the presence of 30 μg/mL berberine. More detailed data for **4** are in Table 3. Thus, there was potentiation with the fluoroquinoline antibiotics norfloxacin and ciprofloxacin as well as rhein (1,8-dihydroxyanthraquinone-2-carboxylic acid), an active component of rhubarb These potentiations can be ascribed to inhibition of the NorA MDR efflux pump in *S. aureus* as is indicated by (1) the increased activity of the antibiotics against the pump-lacking mutant (NorA *S. aureus,* strain KLE 8) (Table 1) and (2) by the data of Figure 1. Figure 1 demonstrates the increased uptake and maintainance of berberine in cells of *S. aureus* treated with esters **3** and **4** as compared to the control, inactive **2**, and the standard potent inhibitor INF_{271}.[7] Details of the bioassay and transport experiments have been published.[1b,c] The potentiation by **4** for antibiotics of such different structures (a quaternary alkaloid, an anthraquinone, and furoquinolines) points to the broad activity of this MDR pump inhibitor.

Table 1. NMR spectral data for **3** ($CDCl_3$, 400 MHz)

	δH (mult., *J*, Hz)	δC	HMBC	HSQC-TOCSY
Glucose				
1	4.28 (d, 6.4)	101.7	3, 4, 5, 7	1, 2, 3, 4, 5
2	3.56 (m)	77.1	1, 1′, 3	1, 2, 3, 4, 2′
3	3.67 (t, 8.8)	76.4	2, 4	1, 2′, 3, 4, 5
4	4.76 (m)	70.9	1‴, 3, 5, 6	
5	3.52 (m)	72.0		1, 3, 4, 5, 6
6	4.07 (d, 9.6)	62.6	4, 5, 1‴	3, 4, 5, 6
7	Ha 3.44 (dd, 7.2, 16)	70.0		7, 8, 9, 10
7	Hb 3.83 (dd, 7.2, 16)	70.0	1, 8, 9	7, 8, 9, 10
8	1.51 (m)	31.8		
9	1.31 (q, 7.6, 15.2)	19.3	7, 8, 10	
10	0.87 (m)	13.9		
Rhamnose				
1′	5.25 (s)	99.8	2, 3′, 5′	1′, 2′
2′	4.05 (d, 9.6)	69.3	1′, 3′, 1″	1′, 2′
3′	5.10 (m)	71.5	4′	2′, 3′,5′ 6′
4′	5.05 (m)	71.0	1′ 3′ 5′ 6′, 1‴	
5′	4.23 (q, 6.4, 10)	66.5	1′, 4′, 6′	2′, 3′, 4′, 6′
6′	1.04 (s)	17.3		4′, 5′, 6′
Butanoyl (rha-3′)				
1″		173.6		
2″	2.22 (dd, 4.8, 7.6)	36.1	1′,3″, 4″	
3″	1.54 (m)	18.4	1″, 2′, 2‴, 4″	
4″	0.85 (m)	13.7		
Isobutanoyl (rha-4′)				
1‴		176.2		
2‴	2.42 (m)	34.2	1‴, 3‴, 4‴	
3‴	1.06 (d, 7.2)	18.99	1‴, 2‴, 4‴	
4‴	1.06 (d, 7.2)	18.99	1‴, 2‴, 3‴	
Isobutanoyl (glu-4)				
1⁗		177.1		
2⁗	2.48 (m)	33.9	1⁗, 3⁗, 4⁗	
3⁗	1.09 (d, 6.8)	18.95	1⁗, 2⁗, 4⁗, 4	
4⁗	1.09 (d, 6.8)	18.95	1⁗, 2⁗, 3⁗, 4	
Isobutanoyl (glu-6)				
1⁗′		176.9		
2⁗′	2.51 (m)	34.1	1⁗′, 3⁗′, 4⁗′	
3⁗′	1.10 (d, 7.2)	19.04	1⁗′, 2⁗′, 4⁗′	
4⁗′	1.10 (d, 7.2)	19.04	1⁗′, 2⁗′, 3⁗′	

These compounds are in part related to glucose and sucrose polyacyl exudates from glandular trichomes of species in the family Solanaceae,[8] for some of which weak (250 μg/mL) activity against *Bacillus subtilis* has

Table 2. NMR spectral data for the neohesperidose moieties of **1**–**4** ($CDCl_3$; 400 MH_z)

	1		2		3		4	
glu 1	101.9	4.33[a]	101.5	4.29[a]	101.7	4.28[a]	101.7	4.32[a]
2	77.6	3.49	76.3	3.51	77.1	3.56	77.9	3.58
3	78.0	3.61	77.9	3.60	76.4	3.67	76.7	3.73
4	70.5	3.88	70.5	3.34	70.9	4.76	71.3	4.75
5	75.5	3.28	73.7	3.42	72.0	3.52	72.0	3.56
6	61.7	3.87	63.5	4.35	62.6	4.07	62.6	4.12
rha 1′	100.5	5.23	99.8	5.29	99.8	5.25	99.7	5.32
2′	71.0	4.07	70.8	4.06	69.3	4.05	69.5	4.18
3′	69.6	3.86	69.6	3.83	71.5	5.10	71.8	5.19
4′	74.4	4.86	74.5	4.83	71.0	5.05	71.0	5.07
5′	66.5	4.17	66.1	4.19	66.5	4.23	66.6	4.27
6′	17.4	1.14	17.2	1.13	17.3	1.04	17.3	1.11

[a]The ^1H NMR resonance values in each column are at the center of a multiplet.

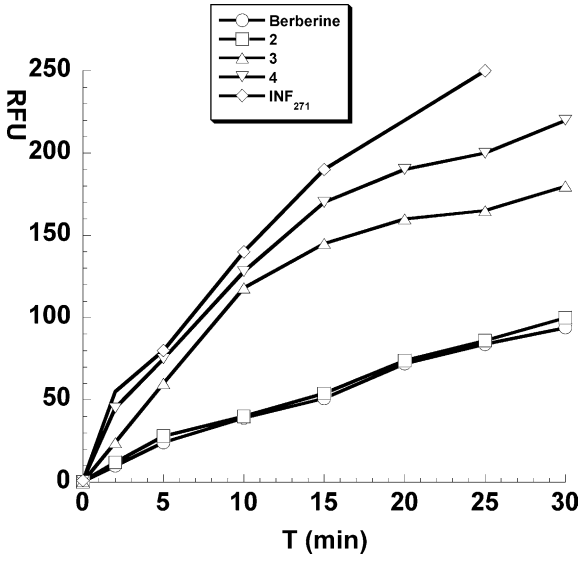


Figure 1. *Staphylococcus aureus* cellular uptake of berberine alone and in the presence of **2**, **3**, **4**, and INF_{271} (a known *S. aureus* MDR efflux pump inhibitor).[7] Relative fluorscence units (RFU) measured are a result of cellular DNA-berberine binding.[1b]

been reported.[9] The Procter & Gamble synthetic fat substitute Olestra[10] is a non-natural member of this class of compound. Some species of the Convolvulaceae contain other resin glycosides, usually tetrasaccharides with small fatty acid acyl groups as well as a hydroxyhexadecanoic acid lactone moiety.[11] These compounds generally have plant growth regulatory activity. Oligoacylated sucrose mixtures from the Japanese herb Atractylodis Lancae Rhizoma were reported to show growth inhibition against P-gp mediated MDR tumor cells.[12] A synthetic pentaisovalerylsucrose was later reported to be a more readily accessible analogue.[13]

Esters **2** and **4** were evaluated for antitumor activity[14] at the National Cancer Institute and both resulted in low or no growth of human cancer cells MCF-7 (mammary adenocarcinoma), NCI-H460 (lung large cell carcinoma), and SF-268 (breast carcinoma) in the standard one dose (10^{-4} M) screen. In the follow-up 60 cell line tests, however, **2** showed no activity, while **4** was slightly active only against three leukemia lines.

Acknowledgements

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2. Identified by J. Rowens and M. K. Simmons, Department of Biology, Colorado State. A voucher specimen has been deposited in the Colorado State University Herbarium.
3. Isolation procedures: Plants of *Geranium caespitosum* James were collected on 29 June and 6 August 2001 off SH 14 at the Tunnel turn-off about 8 miles east of Cameron Pass, Larimer County, Colorado. Plants were in flower at the time of collection and leaves were heavily covered with a sticky exudate.

Table 3. Minimum concentration (μg/mL) of antimicrobials alone and in combination with 10 μg/mL of **4** required to inhibit growth

	S. aureus		NorA *S. aureus*		*B. megaterium*		*B. subtilis*	
	Alone	+4	Alone	+4	Alone	+4	Alone	+4
Berberine	500	3.12	64	1.56	125	6.25	500	125
Rhein	4	1	4	1	4	1	16	4
Ciprofloxacin	0.5	0.1	0.2	0.1	0.8	0.1	0.8	0.1
Norfloxacin	1	0.2	0.4	0.1	1	0.2	1	0.2

A 4.0 g portion of the hexane-dipped leaf extract was fractionated (flash chromatography; Si gel, hexanes/EtOAc gradient). Fractions 22–27 (1:1 hexanes/EtOAc) were combined and evaporated to leave 620 mg of gum. This was further purified by Si gel chromatography ($CHCl_3$/MeOH 3:1) to yield 271 mg of 3 as a gum. Fraction 43 (1.2 g; 1:4 hexanes/EtOAc) yielded 487 mg of gum. This was purified by Si gel chromatography ($CHCl_3$/MeOH 9:1) to yield 179 mg of 1 (frs. 10–14) as a gum.

Another 4.0 g portion of the hexane-dipped extract was fractionated by Si gel vacuum liquid chromatography (VLC) (hexanes/EtOAc/MeOH gradient) to give 833 mg of gum in fr. 64 (100% MeOH). Of this, 400 mg was chromatographed (Si gel; flash; 4:1 hexanes/EtOAc) to yield 105 mg of gum in combined frs. 25–37. This was purified by Si gel MPLC (ISCO, Inc. CombiFlash Retrieve) with 1:1 hexanes/EtOAc. Combined frs. 20–25 yielded 34 mg of gum **4**.

The 1.48 g of whole plant hexanes extract was purified first by VLC (hexanes/EtOAc gradient) to yield 167 mg of gum from frs. 7–9, which was further purified by flash Si gel chromatography ($CHCl_3$/MeOH 9:1) to yield 2 (30 mg; combined frs. 12 and 13) as a gum. Additional **2** was obtained from the hexane-dipped extracts by similar methods.

4. Hydrolysis of **3** to **5**: Ester **3** (150 mg) was treated with MeOH (1 mL) and 1 M NaOH in MeOH (1 mL) for 90 min at 70 °C. The MeOH was removed in vacuo and the residue was partitioned between aqueous acid (HCl, 10 mL, pH 3) and $CHCl_3$ (3×10 mL). The aqueous portion was evaporated to dryness and chromatographed by VLC (RP Si gel; water/acetone 3:1, then water/acetone 1:1) to give 14 fractions. Fractions **3–6** were combined to yield 130 mg of **5** as a gum.

5. Structural data: Butyl 6-*O*-hexadecanoyl-4-*O*-butanoylneohesperidoside (**2**), $C_{42}H_{66}O_{12}$, a colorless clear gum, $[\alpha]_D$ −54° (*c* .016, $CHCl_3$), MS see text, ^{1}H NMR (500 MHz, $CDCl_3$) 0.87 (t, 3H), 0. 92 (t, 3H), 0.94 (t, 3H), 1.13 (d, 3H), 1.16 (dd, 2H), 1.2–1.3 (m, 22H), 1.38 (sextet, 2H), 1.61 (m, 4H), 1.63 (sextet, 2H), 2.34 (m, 4H), 3.35 (m, 1H), 3.46 (m, 2H), 3.52 (m, 1H), 3.61 (m, 2H), 3.85 (m, 2H), 4.06 (m, 1H), 4.29 (d, 1H), 4.35 (m, 2H), 4.84 (t, 1H), 5.29 (s, 1H), ^{13}C NMR Table 2 and 174.6, 174.3, 69.8, 36.1, 31.7, 29.3, 25.0, 19.2, 18.6, 14.2, 13.9, 13.7 and 29.6-29.8 for 12 carbons of the hexadecanoyl group. Butyl 3′-*O*-butanoyl-4, 6, 4′-tri(O-2-methylpropanoyl)neohesperidoside, **3**, a colorless semisolid, $[\alpha]_D$ −43 (*c* .012), HRMS FAB$^+$ 663.3588 (calcd for $C_{32}H_{55}O_{14}$, 663.3592), NMR data Tables 1 and 2. Butyl 3′-*O*-acetyl-2′-*O*-butanoyl-3,4,4′-tri(*O*-2-methylpropanoyl)-neohesperidoside, **4**, a white semisolid, $[\alpha]_D$ −51 (*c* .017), $C_{34}H_{56}O_{15}$ by ^{13}C- and ^{1}H NMR and MS (no molecular ion seen by electrospray or FABMS, but FABMS *m/z* 561 (loss of OBu and BuC=O or *i*PrC=0), ^{1}H NMR (400 MHz, $CDCl_3$) 0.87 (t, 3H), 0.90 (t, 3H), 1.09 (t, 6H), 1.11 (d, 3H), 1.13 (t, 12H), 1.35 (m, 2H), 1.60 (m, 2H), 1.60 (m, 2H), 2.02 (s, 3H), 2.29 (t, 2H), 2.48 (sept, 1H), 2.55 (sept, 1H), 2.57 (sept, 1H), 3.56 (m, 1H), 3.58 (m, 1H), 3.73 (m, 1H), 3.87 (m, 2H), 4.09–4.15 (m, 2H), 4.18 (m, 1H), 4.27 (m, 1H), 4.32 (d, 1H), 4.75 (m, 1H), 5.07 (m, 1H), 5.19 (m, 1H), 5.32 (br s, 1H), ^{13}C NMR Table 2 and 177.6, 177.0, 176.4, 173.6, 170.5, 70.1, 36.1, 34.3, 34.2, 34.0, 31.8, 19.4, 19.2 (2C), 19.1 (2C), 19.0 (2C), 18.5, 14.0, 13.8. Butyl neohesperidoside, **5**, $C_{16}H_{30}O_{10}$, a white semisolid, $[\alpha]_D$ −76° (*c* .0065, MeOH), MSEI$^-$ 381 (M-1), MSEI$^+$ 383 (M + H), 400 (M + NH_4^+), 309 (M$^+$–BuOH). ^{1}H NMR (400 MHz, CH_3OD), 0.94 (t, 3H), 1.22 (d, 3H), 1.40 (m, 2H), 1.60 (m, 2H), 3.24 (1H, m), 3.30 (1H, m), 3.38 (1H, m), 3.40 (1H, m), 3.44 (1H, m), 3.46 (1H, m), 3.52 (1H, m), 3.66 (1H, m), 3.67 (1H, m), 3.88 (1H, m), 3.67 (1H, m), 3.88 (1H, m), 3.91 (1H, m), 3.92 (1H, m), 4.02 (1H, m), 4.31 (1H, d), 5.19 (1H, d), ^{13}C NMR 14.5, 18.2, 20.5, 33.1, 62.9, 69.9, 70.7, 71.9, 72.3 (2C), 71.9, 77.9, 79.3, 79.4, 102.3, 103.2.

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