Polyacylated Oligosaccharides from Medicinal Mexican Morning Glory Species as Antibacterials and Inhibitors of Multidrug Resistance in *Staphylococcus aureus*[⊥]

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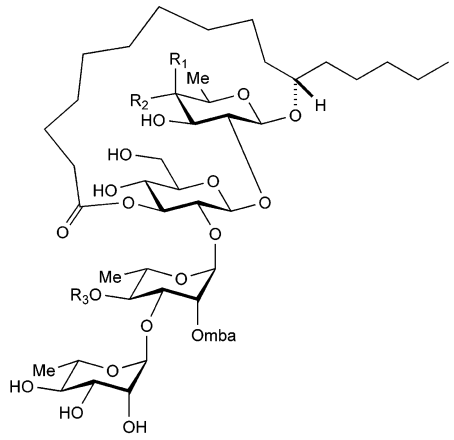
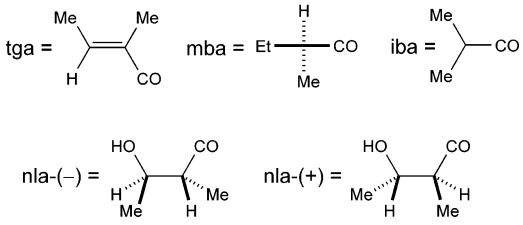
Twenty-two convolvulaceous oligosaccharides selected from the tricolorin (**1**−**7**), scammonin (**8**, **9**), and orizabin (**10**−**22**) series were evaluated for activity against a panel of *Staphylococcus aureus* strains possessing or lacking specific efflux pumps. The minimum inhibitory concentrations (MIC values) for most of the amphipatic compounds ranged from 4 to 32 μg/mL against XU-212 (possessing the TetK multidrug efflux pump) and SA-1199B (overexpressing the NorA multidrug efflux pump), compared with 64 and 0.25 μg/mL, respectively, for tetracycline. This activity was shown to be bactericidal. Two microbiologically inactive members of the orizabin series (**10**, **20**) increased norfloxacin susceptibility of strain SA-1199B. At low concentrations, compound **10** was a more potent inhibitor of multidrug pump-mediated EtBr efflux than reserpine. The wide range of antimicrobial activity displayed by these compounds is an example of synergy between related components occurring in the same medicinal crude drug extract, i.e., microbiologically inactive components disabling a resistance mechanism, potentiating the antibiotic properties of the active substances. These results provide an insight into the antimicrobial potential of these complex macrocyclic lactones and open the possibility of using these compounds as starting points for the development of potent inhibitors of *S. aureus* multidrug efflux pumps.

There are few available antibiotics that can be used to treat life-threatening infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) strains.[1,2] Unfortunately, resistance to the main antibiotic used in its treatment, the glycopeptide vancomycin, has become more frequent[3] and is cause for considerable concern in hospitals and in community life. While the newest oxazolidinone and streptogramin-type antibiotics have been heralded as a solution to therapeutic difficulties associated with MRSA infections, resistance to linezolid (an oxazolidinone) has been reported for vancomycin-resistant *Enterococcus faecium*.[4] Staphylococcal resistance to this antibiotic[5] and to other recently developed agents has also emerged.[6] Clearly, there is an urgent need to identify new compounds that display a broad spectrum of antibiotic activity and develop these leads into new drugs to treat multidrug-resistant (MDR) and methicillin-resistant variants of *S. aureus*. This would alleviate the present situation where few back-up leads are available to complement glycopeptides, oxazolidones, or daptomycin.[7]

Facing this need, we have evaluated the inhibitory activity of a series of oligosaccharides[8,9] from the convolvulaceous resin glycosides on four *S. aureus* strains as well as the effect of the interaction of two inactive natural products when tested combined with norfloxacin on an MDR strain that effluxes this antibiotic. Previously, tricolorins A−E (**1**−**5**) have displayed antimicrobial properties with MIC values in the range 1−40 μg/mL against a standard *S. aureus* strain (ATCC 6538) and toward *Mycobacterium tuberculosis* (MIC 16−32 μg/mL),[10] suggesting the potential of this class of natural bioactive polyacylated oligosaccharides[11] as new antibacterial agents. The current investigation also acknowledges the growing interest in plants historically used in medical treatments as a possible source of antimicrobial agents.[12]



1 : R_1 = OH, R_2 = H, R_3 = mba
2 : R_1 = OH, R_2 = H, R_3 = iba
3 : R_1 = OH, R_2 = H, R_3 = nla-(−)
5 : R_1 = H, R_2 = OH, R_3 = mba



The MIC of all tested compounds was determined against two effluxing strains (SA-1199B and XU-212), an epidemic methicillin-resistant strain (EMRSA-15), and a standard *S. aureus* strain (ATCC 25923), and the data are presented in Table 1. All the amphipathic tetrasaccharides from the tricolorin series (**1**−**5**) exhibited an inhibitory activity against *S. aureus* ATCC 25923, with MIC values

[⊥] Dedicated to Dr. Norman R. Farnsworth of the University of Illinois at Chicago for his pioneering work on bioactive natural products.

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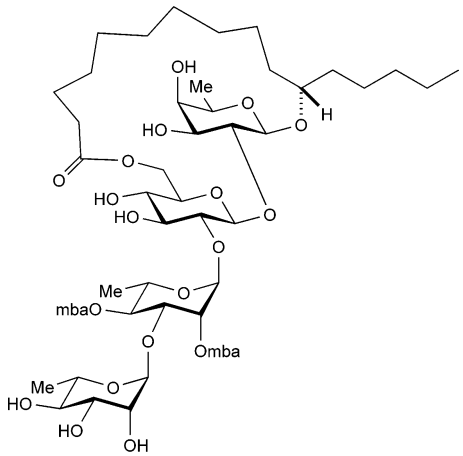
Table 1. Susceptibility of *Staphylococcus aureus* to Selected Convolvulaceous Oligosaccharides

code	compound	MIC (μg/mL)			
		ATCC 25923	XU-212	SA-1199B	EMRSA-15
1	tricolorin A	16	8	8	4
2	tricolorin B	32	16	16	16
3	tricolorin C	32	16	32	32
4	tricolorin D	16	32	32	32
5	tricolorin E	16	8	8	8
6	tricolorin F	>512	>512	>512	>512
7	tricolorin J	>256	>256	>256	>256
8	scammonin I	32	128	32	32
9	scammonin II	256	512	512	128
10	orizabin IX	>512	>512	512	256
11	orizabin X	16	32	4	4
12	orizabin XI	16	32	4	4
13	orizabin XII	>512	>512	256	512
14	orizabin XIII	8	32	4	8
15	orizabin XIV	16	64	8	8
16	orizabin XV	128	512	32	16
17	orizabin XVI	8	128	16	8
18	orizabin XVII	8	32	4	8
19	orizabin XVIII	32	64	64	64
20	orizabin XIX	128	512	512	256
21	orizabin XX	64	>512	8	32
22	orizabin XXI	8	64	8	16
tetracycline		0.08	64	0.25	0.15
norfloxacin		2	16	32	0.5

of 16−32 μg/mL, and toward the effluxing strains with MIC values of 4−32 μg/mL. Not surprisingly, the polar compounds tricolorins F (**6**) and J (**7**) exhibited no antibacterial activity at the concentrations employed. This correlation between lipophilicity and antibacterial activity where the more lipophilic compounds are significantly more active than their polar analogues has been found in other natural products.[10,13] Where growth inhibition by tested compounds was noted, an aliquot of the culture was plated out on nutrient agar and incubated for 24 h. No growth was detected for any of the active compounds, indicating a bactericidal mode of action.



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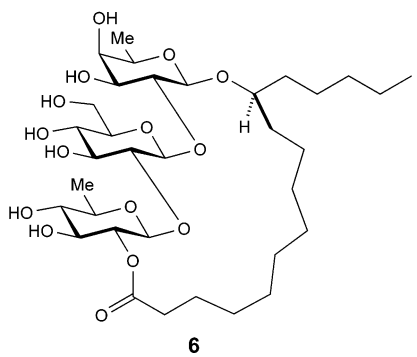

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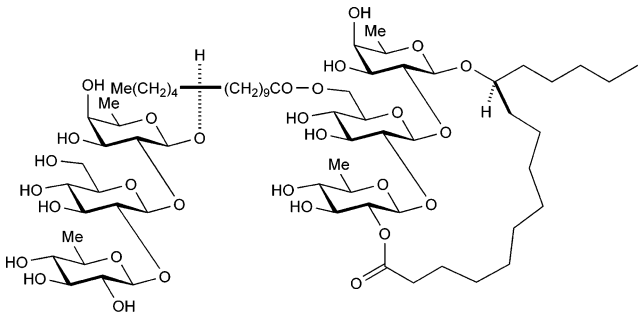

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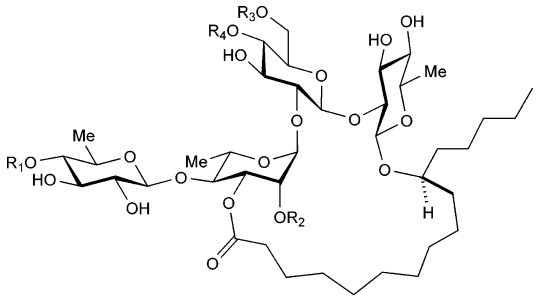
The size of the lactone ring was not crucial for antibacterial activity because the potency of the larger macrocyclic structure of the orizabin series was similar to that of the tricolorin series. It would be interesting to evaluate a higher number of related compounds so as to elaborate conclusions related to the influence of the degree, type, and position of acylation on the activity of these oligosaccharides. However, the following observations can be drawn from our results: (a) the more highly acylated compounds had no direct effect against *S. aureus* (MIC > 512 μg/mL), as indicated by the displayed inactivity of peracetylated derivatives of tricolorin A (**1**), scammonin I (**8**), and orizabin XI (**12**); (b) the activity against *S. aureus* produced by the diesters (**2**−**5**, **8**) was similar to that of tricolorin A (**1**); (c) the monoester compound **9** (scammonin II) was inactive and did not show a potentiation of activity in combination with a subinhibitory dose of the antibiotic norfloxacin; (d) the substitution of (2*S*,3*S*)-nilic acid (nla) by its enantiomer (2*R*,3*R*) in the orizabin series (**11**−**22**), as the esterifying moiety at position C-2 of the inner rhamnose unit, causes, in most of the cases, a 4-fold reduction in the inhibition, e.g., orizabin XII (**13**) versus orizabin XIII (**14**); this effect could be a consequence of a slight difference in the conformations adopted by the macrocyclic structure in each of the diasteroisomeric pairs, modifying their interactions with the microorganism target membranes; (e) the interchange of the other esterifying residues, i.e., tiglic (tga), methylbutyric (mba), and isobutyric (iba) acids, at the remaining



	R_1	R_2	R_3	R_4
8	tga	mba	H	H
9	H	mba	H	H
10	tga	mba	H	nla-(–)
11	tga	nla-(–)	iba	H
12	tga	nla-(+)	iba	H
13	tga	iba	nla-(–)	H
14	tga	iba	nla-(+)	H
15	tga	nla-(–)	mba	H
16	tga	nla-(+)	mba	H
17	tga	mba	nla-(–)	H
18	tga	mba	nla-(+)	H
19	mba	nla-(–)	mba	H
20	mba	nla-(+)	mba	H
21	mba	mba	nla-(–)	H
22	mba	mba	nla-(+)	H

esterifying positions on the oligosaccharide core does not produce any major effect in the inhibitory activity.

The amphipatic orizabins IX (**10**) and XIX (**20**) displayed a strong synergistic effect in combination with norfloxacin. Alone, these compounds had no antimicrobial activity at the concentration tested (MIC > 256 μg/mL), but they strongly potentiated the action of norfloxacin in experiments using a subinhibitory concentration of these oligosaccharides. Orizabin XIX (**20**) at 25 μg/mL reversed norfloxacin resistance 4-fold (8 versus 32 μg/mL) for SA-1199B, while orizabin IX (**10**) at 1 μg/mL completely inhibited SA-1199B growth in the presence of 2 μg/mL of norfloxacin.

Ethidium bromide (EtBr) is a substrate for many multidrug efflux pumps, including NorA of *S. aureus*. The efficiency of any pump for which EtBr is a substrate can be assessed fluorometrically by the loss of fluorescence over time from cells loaded with EtBr.[14] Orizabins IX (**10**) and XV (**16**) were nearly equipotent with respect to the inhibition of EtBr efflux by SA-1199B, with a slight advantage observed for compound **16** (Figure 1). At lower concentrations (less than 10 μM) both test compounds were more efficacious than reserpine. However, at 10 μM or higher their effects reached a plateau and became inferior to that of reserpine. This falloff in activity was due to solubility issues, since above 30 μM the broth solution became cloudy as a result of test compound precipitation. These data suggest that both compounds hold promise as leads in the search for more potent inhibitors of *S. aureus* multidrug efflux pumps.

From the standpoint of their potential use as therapeutic agents, the most important result is that these convolvulaceous oligosaccharides exert their action through inhibition of the multidrug resistance pumps, as previously reported for the structurally related acylated disaccharides found in the leaf exudates of *Geranium* species (Geraniaceae).[15] Therefore, combining these plant products with antibiotics that are substrates for these MDR pumps (e.g., orizabin IX (**10**)/ciprofloxacin) could improve the treatment of refractive infections caused by effluxing staphylococci.

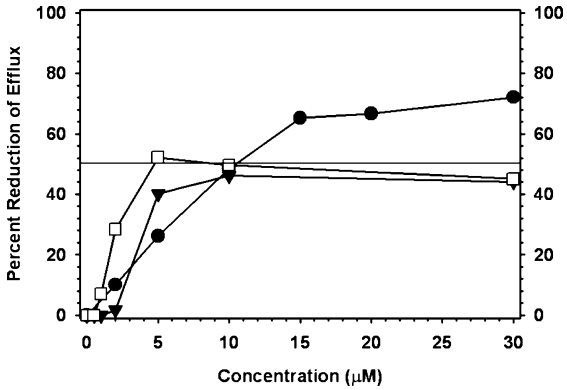


Figure 1. Ethidium efflux inhibition assay from SA-1199B strain cells: (●) reserpine; (▼) compound **10**; (□) compound **16**. The horizontal line indicates 50% efflux inhibition.

The cross activity displayed between members of the tricolorin and orizabin series could represent an example of synergy[16] between related components in medicinal crude drug extracts, with microbiologically inactive compounds disabling a resistance mechanism (e.g., compounds **10**, **16**, and **20**), therefore potentiating the antimicrobial activity of the antibiotic substances.[17] Our results suggest that convolvulaceous plants may elaborate an array of amphipatic oligosaccharides, many of which have evolved to confer selective advantage against microbial attack to plants.[18] This evolutionary process may have potential in the discovery of new antibacterial leads.

Experimental Section

Bacterial Strains and Media. *Staphyloccocus aureus* EMRSA-15 containing the *mecA* gene was provided by Dr. Paul Stapleton, The School of Pharmacy, University of London. Strain XU-212, a methicillin-resistant strain possessing the TetK tetracycline efflux protein, was provided by E. Udo.[19] SA-1199B, which overexpresses the NorA MDR efflux protein,[20] and *S. aureus* ATCC 25923 were also used. All strains were cultured on nutrient agar (Oxoid, Basingstoke, UK) before determination of MIC values. Cation-adjusted Mueller-Hinton broth (MHB; Oxoid) containing 20 and 10 mg/L of Ca^{2+} and Mg^{2+} was used for susceptibility tests.

Antibiotics and Chemicals. Tetracycline and norfloxacin were obtained from Sigma (Poole, UK). Individual glycolipids from the $CHCl_3$ extracts of *Ipomoea tricolor* Cav. and *I. orizabensis* (Pelletan) Ledebour ex Steudel were purified as previously described.[8,9] Glycolipids **1**−**22** were isolated from their respective crude resin mixtures by preparative recycling HPLC using a Waters 600 E multisolvent delivery system equipped with a Waters 410 differential refractometer detector (Waters, Milford, MA). For purification of the tricolorins (**1**−**7**), elution was isocratic (CH_3CN-H_2O, 4:1; flow rate = 3.5 mL/min) on a reversed-phase C_{18} column (20 × 250 mm; 15 μm; ISCO) and a sample injection of 500 μL (100 mg/mL). To perform the separations of compounds **8**−**22**, an HPLC system equipped with an NH_2 column (19 × 150 mm; 10 μm, μBondapak, Waters) was used, and elution was also conducted isocratically (CH_3CN-H_2O, 95:5; flow rate = 4 mL/min).

Susceptibility Testing. Minimum inhibitory concentration values (MIC) were determined at least in duplicate by standard microdilution procedures, as recommended by the National Committee for Clinical Laboratory Standards guidelines.[21] An inoculum density of 5×10^5 cfu of each of the test strains was prepared in 0.9% saline by comparison with a MacFarland standard. MHB (125 μL) was dispensed into 10 wells of a 96-well microtiter plate (Nunc, 0.3 mL volume per well). Glycolipids **1**−**22** were tested at final concentrations ranging from 1 to 512 μg/mL prepared by serial 2-fold dilutions. All test compounds were dissolved in DMSO before dilution into MHB for use in MIC determinations. The highest concentration of DMSO remaining after dilution (3.125% v/v) caused no inhibition of bacterial growth. The MIC was defined as the lowest concentration that yielded no visible growth.

Ethidium Efflux Assay. SA-1199B, which overexpresses NorA, was loaded with EtBr as previously described, and the effect of varying concentrations of compounds **10** and **16** on EtBr efflux efficiency was determined to generate a dose−response profile for each oligosaccharide.[20,22] The effect of reserpine was also determined for comparative purposes. Assays were performed in duplicate, and mean results were expressed as the percentage reduction of total efflux observed for SA-1199B in the absence of inhibitors.

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