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Isoflavones As Potentiators of Antibacterial Activity

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Isoflavones isolated from *Lupinus argenteus* were found to potentiate the antibacterial activity of α-linolenic acid, also found in the same plant. The isoflavones also potentiated the activity of the natural plant antibiotic berberine and the synthetic fluoroquinoline antibiotic norfloxacin. The isoflavones increased the uptake of berberine into *Staphylococcus aureus* cells, indicating that they may be inhibiting a multidrug resistance pump (MDR). Thus, *L. argenteus* contains a weak antibacterial and also MDR pump inhibitors, which increase its potency.

KEYWORDS: ***Lupinus argenteus*; Leguminosae; isoflavones; linolenic acid; antibacterial; potentiation; MDR pump; *Staphylococcus aureus***

INTRODUCTION

Bacterial resistance to antibiotics is a continuing problem, and microorganisms seem able to develop resistance to new drugs as rapidly as they are introduced. One of the major modes by which pathogens develop resistance is through development or enhancement of methods for the removal of antibiotics that have entered cells of the organism. Thus, resistant bacteria possess efficient systems known generically as multidrug resistance (MDR) pumps (*1*). If the action of such pumps can be inhibited, then the cellular content of antibiotics will be increased and the antibiotic treatment will again be effective. We reasoned that plants might develop MDR pump inhibitors to enhance the activity of their own natural antimicrobial compounds, and we have been able to show that this is indeed the case (*2*−*6*). Essentially, we have been looking for plant products that do not themselves possess antibacterial activity but can potentiate known antibiotics by inhibiting microbial MDR pumps.

For this search, a bioassay was developed (*1*) in which a subinhibitory dose of an antibiotic was combined with plant extracts or fractions from a chromatography to test for potentiation. *Staphylococcus aureus* was used as a test organism because of its known resistance and because an *S. aureus* mutant, which lacks the MDR efflux protein (known as NorA), was available (*1*). Berberine was chosen as a model antibiotic since its fluorescent properties when complexed with cellular DNA allowed for measurement of antibiotic uptake and efflux from bacterial cells. The value of this method for bioactivity-directed fractionation was demonstrated by its use in discovery of a potent flavonolignan MDR pump inhibitor from *Berberis* species (*2*−*4*) as well as for structure−activity relationship studies on flavonolignans and flavones as MDR pump inhibitors (*5*, *6*). Screening of randomly chosen plants revealed that extracts of *Lupinus argenteus* Pursh. subspecies *rubricaulis* (Greene) Hess and Dunne had both direct antibiotic activity as well as antibiotic potentiation properties. This species had previously been studied only for its alkaloid content (*7*).

MATERIALS AND METHODS

General Experimental Procedures and Chemicals. Instruments and chromatography procedures used were as previously described (*3*) as were detailed methods for bioassays and berberine uptake into cells (*2*, *3*). Bacterial strains used were *S. aureus* wild-type (8325-4), NorA mutant KLE 8, and *Berberis megaterium* (11561, M. Cannon). Bacteria were cultured in Mueller−Hinton (MH) broth. Amounts of 10^5 cells/mL were inoculated into 1 mL of MH broth and dispensed at 0.2 mL/well in microtiter plates. Berberine chloride (Aldrich Chemical) was added at about one-eighth of its minimum inhibitor concentration (MIC), and MICs of test fractions or compounds were determined by serial 2-fold dilution in the presence and absence of berberine chloride. MIC was defined as the concentration of an antibacterial agent that completely prevented cell growth during an 18 h incubation at 37 °C. Growth was assayed with a microtiter plate reader (Biorad) by reading absorption at 600 nm.

Genistein (**2**), biochanin A (**4**), α-linolenic acid (*Z*,*Z*,*Z*-9,12,15-octadecatrienoic acid, **1**), γ-linolenic acid (*Z*,*Z*,*Z*-6,9,12-octadecatrienoic acid, **5**), and their methyl esters were purchased from Sigma (St. Louis, MO). INF_{271} was a gift from Influx Corp. INF is a designation used for compound numbers by INFlux Corp.

Plant Material. *L. argenteus* Pursh. subsp. *rubricaulis* (Fabaceae) was collected on July 2, 2001 at the same site as previously described (*7*) (Colorado State University voucher GH171, identified by D. B. Dunn, Department of Biology, University of Missouri, Columbia).

Isolation. Dried, ground leaves (164 g) were submerged successively in solvents (two × 1.0 L each), which upon evaporation yielded as follows: hexanes extract (1.2 g; 250 μg/mL direct growth inhibitory activity against *S. aureus*), $CHCl_3$ (5.4 g; 62.5 μg/mL), EtOAc (0.6 g; not tested), and MeOH (12.5 g; inactive). Similarly, dried, ground stems (127 g) were extracted to yield hexanes extract (0.40 g; 125 μg/mL activity), $CHCl_3$ (0.56 g; inactive), EtOAc (0.26 g; 62.5 μg/mL), and MeOH (7.2 g; inactive).

Vacuum liquid chromatography (VLC) of each of the nonpolar extracts followed by bioassay yielded **1** as the directly active component. Typical procedures were as follows.

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Table 1. Growth Inhibition of Bacteria by Antimicrobials (Alone) and in Combination with Isoflavone MDR Inhibitors

	MIC (μg/mL)											
	S. aureus				NorA mutant				*B. megaterium*			
	alone	4[a]	2[b]	3[c]	alone	4[a]	2[b]	3[c]	alone	4[a]	2[b]	3[c]
berberine	500	31.3	31.3	31.3	64	7.81	31.3	16	250	31.3	31.3	31.3
1	62.5	7.81	16	10	10	2	5	5	100	7.81	7.81	16
5	25	7.81	16	5	5	1.5	5	2.5	50	7.81	7.81	16
norfloxacin	1	0.25	0.5	0.25	0.25	0.25	0.5	0.5	1	0.25	1	0.5

[a–c] Compounds **2**–**4** were added at a final concentration of 10 μg/mL.

The dried leaf hexane extract (1.2 g) was separated by VLC (silica gel, 45 g; hexanes followed by hexanes/EtOAc mixtures; 11 fractions, 10−15 mL each). Fraction 3 (9:1 hexanes/EtOAc,101 mg) was **1**. Similarly, 189 mg of **1** was obtained from the leaf $CHCl_3$ extract.

The leaf EtOAc extract was purifed by VLC (silica gel, 45 g; $CHCl_3$, followed by $CHCl_3$ /MeOH mixtures; 15 fractions, 40−50 mL each). Fractions 6 and 7 (9:1 $CHCl_3$/MeOH; 25 μg/mL potentiation activity with subinhibitory berberine) were combined (74 mg) and purified by preparative thin-layer chromatography (PTLC; 9:1 $CHCl_3$/MeOH) to yield **2** (band 2; 7 mg; 25 μg/mL) and orobol (**3**; band 8; 4 mg; 12.5 μg/mL). The stem EtOAc extract also yielded **2** and **3** in similar amounts.

The stem $CHCl_3$ extract was purified by VLC (silica gel, 45 g; gradient of hexanes/EtOAc, EtOAc, MeOH; 15 × 50 mL fractions). Potentiation active fractions (nos. 10−12, 12.5−25 μg/mL) were combined (48 mg) and purified by PTLC (9:1 $CHCl_3$/MeOH) to yield **4** (band 1; 4 mg; 6.25 μg/mL) and **2** (band 2; 2 mg).

Although neither of the MeOH extracts was active, they were also fractionated by VLC. None of the fractions from the leaf extract were active. The stem extract, however, yielded potentiation active fractions, which proved to contain small amounts of **2** and **3**. An inactive fraction yielded the alkaloid 2,9-dihydroxyaphyllidine (*7*).

Identification of **1** was by interpretation of ^{1}H and ^{13}C NMR spectra and by spectral comparison with a commercial sample. Identifications of **2** and **4** were by ^{1}H and ^{13}C NMR spectra and MS in comparison with commercial samples and **3** by analysis of the MS and UV (*12*) spectra and the ^{1}H and ^{13}C NMR spectra in comparison with literature values (*13*).

5,7,4′-Trimethoxyisoflavone (7). A mixture of **4** (92 mg, 0.32 mmol) and 5 mL of a 2.0M solution of trimethylsilyldiazomethane (TMSD) in MeOH was stirred at room temperature overnight. The excess TMSD was decomposed with AcOH, and the mixture was evaporated to dryness. VLC purification (Si gel; 6:4 hexanes/EtOAc) yielded 81 mg (0.26 mmol, 80% yield) of **7** (*14*).

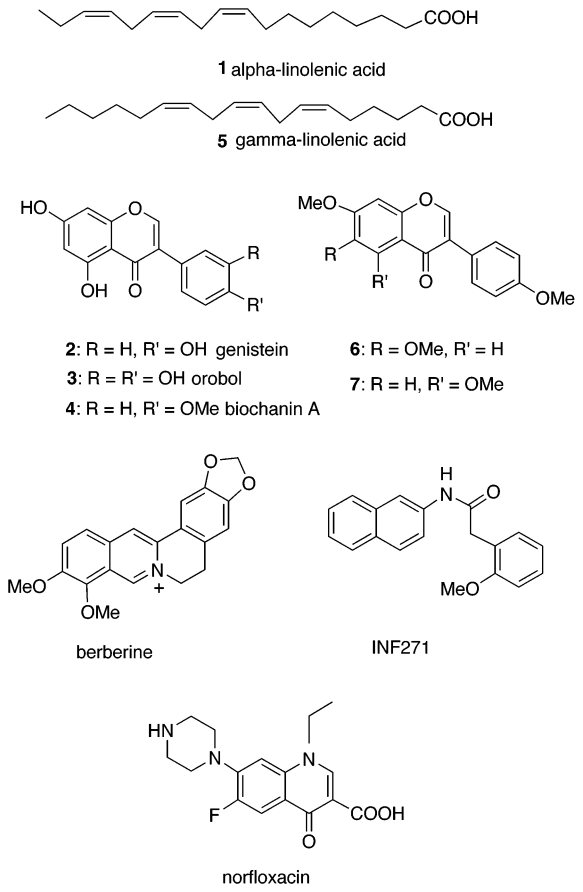

Figure 1. Structures of antibacterial compounds and potentiators.

RESULTS AND DISCUSSION

Dried, ground leaves and stems were extracted successively with hexanes, $CHCl_3$, EtOAc, and MeOH, and the extracts were tested for direct growth inhibition against *S. aureus* and also for potentiation of subinhibitory berberine. The hexanes and $CHCl_3$ extracts yielded directly active fractions after VLC, and these were shown to contain **1** (**Figure 1**). Acid **1** had weak activity (MIC, 62.5 μg/mL). The leaf EtOAc extract, when fractionated, yielded additional **1** and also fractions that were not directly active but that potentiated berberine. PTLC of these fractions yielded **2** and **3** as the active potentiators. The nonpolar stem extracts yielded **1** as well as **2** and **4**. None of the fractions from the leaf MeOH extract were active, but the stem MeOH extract yielded additional small amounts of **2** and **3** as well as the quinolizidine alkaloid 2,9-dihydroxyaphyllidine (*7*). This alkaloid was inactive, either directly or as a potentiator, as was aphyllidine (*7*).

To measure potentiation activities in more detail and to test for structure−activity relationships, we used commercial samples of **1**−**5**. Commercial samples of the methyl esters of **1** and **2** and 6,7,4′-trimethoxyisoflavone (**6**) proved to be inactive in all respects as was **7**, obtained by methylation of **4**. The linolenic acids had weak direct activity against the Gram-positive organisms *S. aureus* and *B. megaterium*, while the isoflavones were inactive at the highest dose tested (100 μg/mL). Compound **4** was, however, a potentiator of berberine and norfloxacin antibiotic activity, as well as in combination with **1** and **5**, against wild-type *S. aureus* and against *B. megaterium*. For example, a combination of 6.25 μg/mL of **4** and subinhibitory (30 μg/mL) berberine or subinhibitory **1** (30 μg/mL) completely inhibited *S. aureus* and *B. megaterium* growth. In **Table 1**, data are given for experiments in which each of the isoflavones was added at 10 μg/mL to berberine, **1**, **5**, and the fluoroquinoline antibiotic norfloxacin and tested against wild-type *S. aureus*, *B. megaterium*, and a mutant *S. aureus* that lacked the NorA efflux pump protein. Potentiation of activity was observed in all cases. Note that the direct activities of the four antibiotics against the NorA mutant *S. aureus* had dropped significantly in comparison with the wild-type. Thus, the linolenic acids (at 5 and 10 μg/mL direct activity against the mutant) can be added to the large group of plant compounds whose inherent activity is hidden by the presence of bacterial efflux pumps (*8*). To

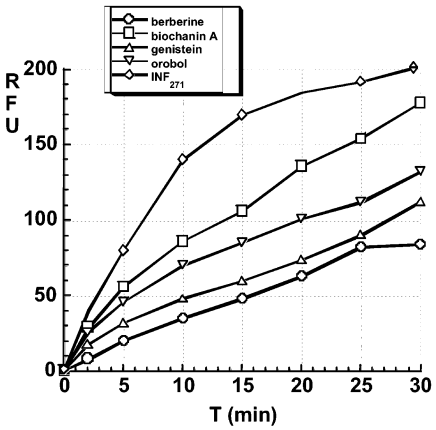

Figure 2. Accumulation of berberine in cells of *S. aureus* alone or with added **2**–**4** or INF_{271}. Berberine uptake was measured by the increase in fluorescence following binding to DNA and expressed as RFU (relative fluorescence units).

provide further evidence that the potentiation of antibiotic activity was due to inhibition of an MDR efflux pump, we examined transport of berberine into cells of *S. aureus* (**Figure 1**). The effects of the isoflavones were compared with those of the known *S. aureus* NorA pump inhibitor INF_{271} (*9*). Although INF_{271} was somewhat more potent than the isoflavones by 30 min, all three isoflavones had increased the content of berberine in the *S. aureus* cells, with **4** being the most effective. If NorA inhibition had been complete or if it was the only efflux pump protein in *S. aureus*, then there should have been no difference in cell berberine content in the NorA mutant in the control as compared to the isoflavone treated case. There is at least one more MDR in *S. aureus* (*11*), and this might explain the presence of residual inhibition of berberine accumulation in the mutant.

There are both similarities and differences among the structure−activity relationships of these isoflavones with some similar flavones previously studied (*5*). Luteolin, the flavone analogue of **3**, was inactive as an MDR pump inhibitor while **3** (present work) was quite active. On the other hand, both tetra-O-methylluteolin and its isoflavone analogue **7** were inactive. In the flavone series, monomethoxy B-ring derivatives were generally more active than the disubstituted B-ring derivatives. Activity was previously reported in the 20−50 μg/mL range for **1** against *S. aureus* and *Berberis cereus*, and a combination of 10 μg/mL **1** with 10 μg/mL of some inactive monoglycerides was more effective than **1** alone (*10*). Linolenic acids are widespread in various seed oils, and isoflavones are common in foods such as soy products. Isoflavone synergists present in the human diet may enhance the effectiveness of standard antibiotic treatments for some bacterial diseases.

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