Notes

5′-Methoxyhydnocarpin-D and Pheophorbide A: *Berberis* Species Components that Potentiate Berberine Growth Inhibition of Resistant *Staphylococcus aureus*

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A new method of bioactivity-directed fractionation, based on multidrug resistant pump (MDR) inhibition in *Staphylococcus aureus*, was demonstrated. This resulted in the isolation, from berberine-containing *Berberis* species, of two compounds that are themselves devoid of *S. aureus* antibacterial activity, but that form potent synergistic couples with a subinhibitory concentration of berberine. The bacterial MDR pump inhibitors were identified as the flavonolignan **2** and the porphyrin **3**. Another natural flavonolignan, silybin (**8**) from *Silybum marianum*, was also shown to be a bacterial MDR pump inhibitor.

The development of bacterial resistance to antibiotics is a major, and growing, health problem.[1] Resistant strains of *Staphylococcus aureus* have developed in hospitals, but have also recently been discovered in nonhospital situations. Some of these strains even show resistance to the "last resort" antibiotic vancomycin. The expression of multidrug resistant efflux pumps, which extrude antibiotics from the bacterial cell, is one of the most important causes of bacterial resistance.[2] Thus, inhibition of such pumps might present one way to attack the problem of antibiotic resistance.

One of the MDR pumps of *S. aureus*, a protein known as NorA, has been well-characterized, a mutant (KLE 820 nor A) lacking such a pump has been developed, and a screen has been reported for the discovery of MDR pump inhibitory compounds.[3] We have communicated the use of these developments in the discovery of a potent flavonolignan MDR pump inhibitor from three *Berberis* species.[4] Here we wish to present details on the isolation of this flavonolignan as well as to report on a second potent inhibitor from the same plant species. The work demonstrates the validity of a new method[3] for the identification and isolation of bacterial MDR efflux pump inhibitors from plants.

Some amphiphilic cations (for example, benzalkonium chloride) as well as lipophilic agents with multiple protonation sites (chlorhexidine, fluoroquinolines) have had use as sterilization agents or antimicrobial drugs.[3,5] Such compounds have properties that enable them to readily enter bacterial cells and hence exert their antimicrobial action. The multidrug resistance (MDR) efflux pumps of resistant bacteria, however, make such penetration ineffective. Among natural products, the protoberberinium and benzophenanthridinium alkaloids are amphiphilic cations that show a variety of antimicrobial activities, but are also susceptible to extrusion from bacterial cells by the MDR pumps. To search for natural compounds that may inhibit MDR pumps, a bioassay was developed that can be adapted for screening plant extracts and for conducting bioactivity-directed fractionations.[3] This method uses berberine, **1** (Chart 1), as the antibiotic and resistant *S. aureus* as the test organism. The method is particularly useful for finding MDR inhibitors that do not themselves have antibacterial activity. In brief, the extract (or chromatographic fraction) is added to *S. aureus* RN4222 in the absence and in the presence of 30 μg/mL (a subinhibitory dose) of berberine. Berberine chloride inhibits the test organism at 120−240 μg/mL. Lack of bacterial growth inhibition with the extract (or fraction) alone and inhibition of growth when combined with subinhibitory berberine is indicative of a possible MDR pump inhibitor. Once such a potentiator of or synergist with berberine has been isolated, several methods can be used to confirm that the potentiation involves MDR pump inhibition rather than some other mechanism. Details of such methods have been published.[4]

It was hypothesized that plant species that contain the antimicrobial berberine might evolve MDR pump inhibitors to potentiate berberine activity against natural pathogens. Although *S. aureus* is not a natural plant pathogen, inhibitor action evolved against other organisms might also work against the NorA pump. Hence, our first experiments involved screening berberine-containing plant species, rather than a random selection of plant extracts. Since one known, rather poor and nonspecific, natural product inhibitor was an alkaloid (reserpine[6a]), we first tested a number of alkaloids and a lignan previously isolated from *Berberis repens*, *B. fremontii*, and *B. fendleri* or other berberine-containing plant species. Here we describe identification of three potent natural inhibitors, two from *Berberis* and a third from the medicinal herb *Silybum marianum*, milk thistle. Synthetic inhibitors of bacterial MDR pumps were recently reported.[6b]

The alkaloids protopine, allocryptopine, glaucine, isoc-

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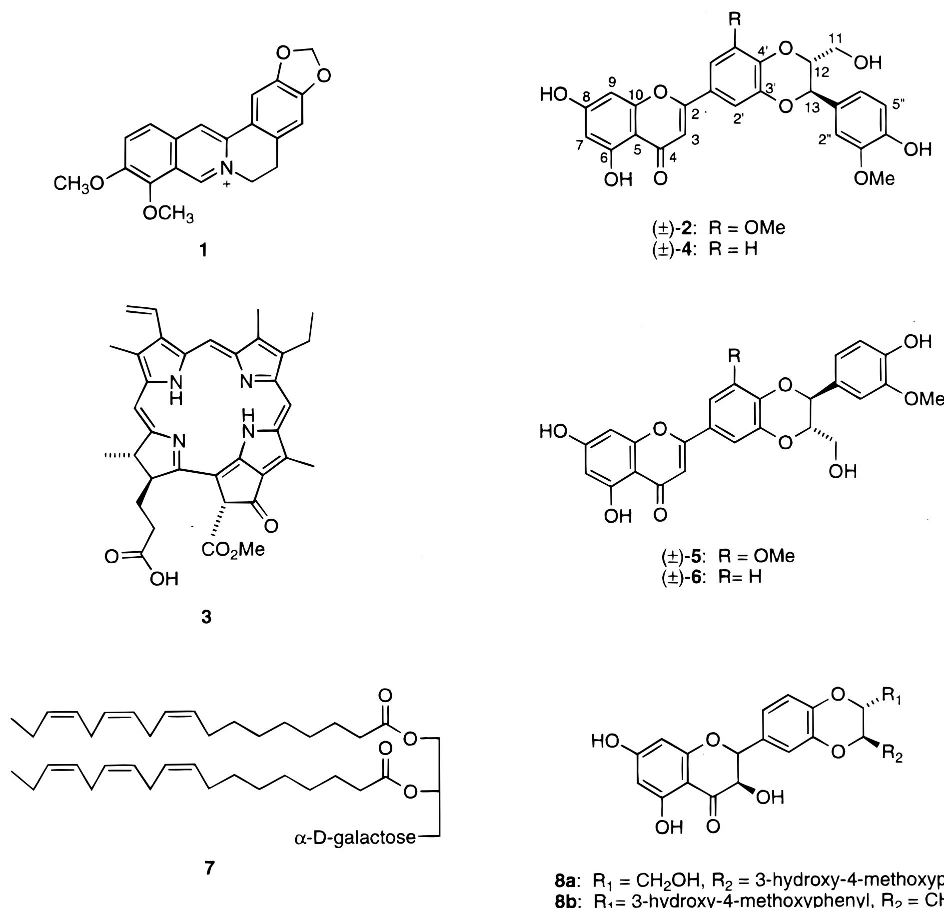
Chart 1



orydine, isocorydine methiodide, laudanosine, argemonine, bisnorargemonine, tetrahydropalmatine, a mixture of bisbenzyltetrahydroisoquinolines, and the lignan asaranin all were inactive against *S. aureus*, and the mixture of each with subinhibitory berberine was also inactive. Chloroform extracts of leaves from *B. fremontii*, *B. repens*, and *B. aquifolium*, when combined with subinhibitory berberine, did, however, inhibit growth of *S. aureus.* Bioactivity-directed fractionation was then undertaken to isolate the MDR inhibitor(s).

Silica gel flash chromatography of a $CHCl_3$ extract of *B. fremontii* leaves yielded several active fractions (Table 1). From these fractions and from the processing of leaves on a large scale (see Experimental Section), we isolated and identified the active components of fraction 5 and combined fractions 8 and 9 as **2** and **3**, respectively.

The UV−visible absorption spectra (in MeOH and with added NaOAc) identified **2** as a phenol, probably a flavone. High-resolution FABMS gave a molecular formula of $C_{26}H_{22}O_{10}$ (495.1299, calc 495.1291 for MH^+), and the ^{13}C NMR spectrum indeed showed 26 carbon resonances. These proved to be identical with those reported for **2**,[7] which had been isolated[8] from *Hydnocarpus wightiana.* The flavonolignan **2** had originally been given the name 5′-methoxyhydnocarpin. This was based on the similarity of its spectra with those of the 5-desmethoxy analogue, which had been isolated[9] from the same plant, given the name hydnocarpin, and assigned structure **4**. There were, however, no data presented that could distinguish regioisomers **2** and **4** from the alternates **5** and **6.** Indeed, hydnocarpin was later proven[10] to actually have structure **6**. We originally adopted[4] the analogous structure **5** for the newly isolated MDR pump inhibitor from *Berberis*, based upon the assumption that what was shown for hydnocarpin would also be true for the methoxy compound isolated from the same plant.[8] To ensure this assignment, we subsequently accomplished a total synthesis of our isolate.[11] Upon the basis of an X-ray diffraction structure determination of an intermediate in the synthesis and extensive HMBC experiments it was determined[11] that the correct structure was **2** and not **5.** Because this is not a hydnocarpin, but its regioisomer, the name 5′-methoxyhydnocarpin[4,8] is not valid for the compound, and we have renamed it 5′-methoxyhydnocarpin-D. The name isohydnocarpin was already in the literature for a structural, not regioisomeric, analogue of hydnocarpin.

Table 1. Bioactivity-Directed Fractionation of a *Berberis fremontii* $CHCl_3$ Extract

fraction no.	MIC (μg/mL)	wt (mg)
1	>450	85
2	>388	664
3	>370	370
4	12.8	109
5	3.5	57
6	6.3	32
7	7.8	21
8	1.25	26
9	1.25	11
10	2.5	19
11	3.9	4
12	3.9	3
13	6.9	2
14	10.9	2
15	16.4	2
16	32.8	2
17	167	2
18	>335	2
19	>825	67

Hydnocarpin was reported to have hypolipidemic, antiinflammatory, and antineoplastic activity,[12] but no biological data were reported[7,8] for **2**.

The visible absorption spectrum identified **3** as a porphyrin, and that spectrum, along with the ^{1}H NMR spectrum, proved identical with those of pheophorbide *a* isolated[13] from *Artemisia capillaris* (Compositae). The ^{13}C NMR spectrum was consistent with that given for methyl pheophorbide *a* from the clam *Ruditapes philippinarum.*[14] The NMR spectra and TLC R_f values were identical with those of a commercial sample of pheophorbide *a*. Pheophorbide *a* is an intermediate in the natural breakdown of chlorophyll in both higher plants and algae,[15] but there are only rare reports of its direct isolation. It was isolated from *Artemisia* as an endothelin receptor antagonist,[13] where it was shown to be a natural component and not an artifact. Pheophorbide *a* has also been studied as a possible inhibition of tumor promotion.[16] Some porphinoid-type compounds from cyanobacteria are known anticancer MDR reversal agents that potentiate P-glycoprotein transported drugs,[17] but none have previously been reported as inhibitors of bacterial MDR pumps.

Many of the fractions of Table 1, including the active ones, contained varying amounts of lipid-like material (by NMR). From these, the monogalatosyldiglyceride **7**, an important lipid of the thylakoid membranes of higher plants,[18] was isolated and identified by its FABMS ($M^+ + Na = 797$) and ^{1}H and ^{13}C NMR spectra in comparison with the literature.[19] It was reported as an antitumor-promoting lipid from the green alga *Chlorella vulgaris*[20] and as one member of a synergistic triad (along with an inositol and aristolochic acid) necessary for a butterfly oviposition on *Aristolochia macrophylla.*[19] The diglyceride **7** was inactive with berberine as an MDR pump inhibitor in *S. aureus* and did not enhance the potency of a pheophorbide *a*/berberine combination.

Crude chloroform extracts of *Berberis repens* and *B. aquifolium* leaves were also active in our bioassay, and fractionations similar to those reported here also resulted in the isolation of 5′-methoxyhydnocarpin-D and pheophorbide *a*. Only two chloroform extracts of several dozen randomly selected plant species were active enough to merit further pursuit, and neither of these was as active as the extracts of *Berberis*.

Neither **2** (the active component of fraction 5) nor **3** (the active component of fractions 8 and 9) had antibiotic activity alone. However, the combination of subinhibitory concentrations of berberine with 1.2 μg/mL of **2**, or 0.5 μg/mL of **3**, completely inhibited growth of *S. aureus***.** In view of the activity of flavonolignan **2**, we prepared for testing a sample of silybin (**8**) by crystallization of the commercial drug silymarin. Silybin (a diastereomeric mixture, **8a** + **8b**) from the widely used herbal plant milk thistle (*Silybum marianum*)[21] also proved active as a synergist with berberine, but was not as active (10 μg/mL) as **2** or **3**. A number of synthetic flavonolignans, some more active and some less active than **2**, have been prepared and will be discussed elsewhere.

This work clearly demonstrates the validity of this method[3] for bioactivity-directed fractionation in the search for naturally occurring inhibitors of bacterial MDR pumps. These inhibitors could be of importance in the control of resistant bacteria. It seems likely that the approach could be extended to antibiotics other than berberine and to bacteria other than *S. aureus*. Both **2** and **3** also potentiated the action of the fluoroquinoline antibiotic norfloxacin. Sensitivity of wild type *S. aureus* cells to norfloxacin increased 4-fold in the presence of either inhibitor and reached the sensitivity of the *norA* mutant.[4] The inhibitors had no effect on the norfloxacin sensitivity of the *norA* mutant, indicating that their action is directed specifically at the NorA pump.

Our original hypothesis that berberine-containing plant species might also contain inhibitors of bacterial pumps that extrude berberine from cells proved to be correct, but the possible ecological meaning of this is unclear. Berberine is found in high concentrations in the roots and bark, but only traces are found in the leaves. Pheophorbide *a*, as a chlorophyll metabolite, is a leaf product. Flavonolignans are thought to be products of antioxidation (radical trapping) processes between *ortho*-dihydroxyphenols and coniferyl alcohol, which presumably also occurs in the leaves. Pheophorbide *a* should also be a leaf component of all green-leaved plants, and yet our random selection of tested plant species extracts showed few with any potency and none as active as the berberine-containing species. Solutions to these complex interactions will require more detailed investigation of the plant processes. Finally, it is perhaps interesting that, although plants of the Berberidaceae and *Berberis* species themselves have been some of the most thoroughly investigated chemically, none of the three metabolites we identified (**2**, **3**, and **7**) were previously isolated from these sources.

Experimental Section

General Experimental Procedures. UV−visible spectra were obtained on a Beckmann DU-600 instrument. NMR spectra were obtained at 400 MHz (^{1}H) and 75 MHz (^{13}C) on a Varian Inova spectrometer in $CDCl_3$ (^{1}H δ 7.24, ^{13}C δ 77.0), acetone-d_6 (^{1}H δ 2.05, ^{13}C δ 24.6), or DMSO-d_6 (^{1}H δ 2.50, ^{13}C δ 39.2). Mass spectra were obtained on Fisons VG Quattro-SQ quadrupole (EI) or Fisons VG AutoSpec double focusing (FAB) spectrometers. For TLC, C_{18} Si gel glass plates were used and visualized with vanillin reagent. For column or vacuum liquid chromatography (VLC), Si gel 60 (0.040−0.063 mm; 230−400 mesh ASTM; EM Science) or a lab-prepared C_{18} Si gel from the same material was used. Silybin was obtained by MeOH recrystallization of commercial silymarin (KADEN Biochemicals GmbH, Hamburg, Germany). Berberine chloride was purchased from Sigma and used directly. Pheophorbide *a* was purchased from ICN Biomedicals, Inc.

Plant Material. *Berberis fremontii* Torrey was collected July 3, 1998 1 km east of Crawford, Delta County, Colorado. *B. repens* Lindley was collected September 26, 1999, 36.3 miles northwest of the junction of Colorado Highway 14 and US Highway 287 (Larimer County, Colorado). *B. aquifolium* Pursh collections (summer 1998, 1999) were from commercial plants (growing on the Colorado State University campus or from Monrovia Nursery, Azusa, California). Vouchers were deposited in the Colorado State University herbarium. Verification of vouchers or living material was by Profs. Dieter Wilken or June Wen, Department of Biology, Colorado State University.

Extraction and Isolation. Dried, ground leaves (188 g) of *B. fremontii* were submerged in 1200 mL of hexanes at room temperature for 24 h and filtered from this inactive extract. The leaves were then treated similarly with 1000 mL of $CHCl_3$ for 24 h, and the $CHCl_3$ was removed in vacuo at 30−40 °C to leave 1.4 g of dark black-green residue. The residue was inactive against *S. aureus*, but inhibited growth at 100 μg/mL in the presence of 30 μg/mL of berberine. To test for complete extraction, a second $CHCl_3$ extraction was made, and this yielded 120 mg of material (48 μg/mL activity). The 1.4 g of primary extract was subjected to flash chromatography over Si gel with 9:l $CHCl_3$/methanol as eluting solvent. Twenty fractions (30−40 mL each) were taken, the solvent was evaporated, and the fractions were weighed and tested (Table 1). TLC (C_{18} Si gel; MeOH/H_2O, 10:1) of fraction 5 showed a major yellow spot at R_f 0.73, while an emerald green spot was

seen at R_f 0.52 of fractions 8 and 9 and some following fractions. The ^{1}H NMR spectra showed the presence of **2** in fraction 5 and **3** in fractions 8 and 9, but both were contaminated by lipid-like material.

Combined fractions **8** and 9 were rechromatographed (flash; 5 g Si gel; elution with hexanes/acetone, 1:1, hexanes/acetone/EtOAc, 1:1:1, hexanes/acetone, 1:2, 1:3, and 1:4, pure acetone, and acetone/$CHCl_3$ mixtures). Fractions eluted with pure acetone were combined (TLC basis) to yield 0.8 mg of pheophorbide *a*, **3**. Extended chromatography and storage of fractions indicated considerable decomposition and loss of **3**. In a somewhat faster, but similar processing, 332 g of dried leaves yielded 14 mg of **3**, but this may still represent a minimum of the concentration in the plant leaves.

To obtain a larger sample of the fraction 5 active component, 3.32 kg of dried leaves was extracted successively with 20 L of hexanes and 19 L of $CHCl_3$. Evaporation of the $CHCl_3$ left 54.8 g of black-green tar (50 μg/mL activity). Of this, 10 g was purified by VLC (Si gel; $CHCl_3$ and $CHCl_3$/MeOH). Fractions (Frs) 7−9 (5:1 to 1:1 $CHCl_3$/MeOH) yielded 8.2 g of residue, which was similarly rechromatographed except eluting with 24:1 $CHCl_3$/MeOH (Frs 1−8), 1:1 $CHCl_3$/MeOH (Fr 9), and pure MeOH (Fr 10): Frs 1−6 (6.7 g; inactive), Fr 7 (202 mg; 12 μg/mL), combined Frs 8 and 9 (339 mg; 3.1 μg/mL), Fr 10 (1.06 g; inactive). Frs 8 and 9 were purified by VLC on C_{18} Si gel (H_2O/MeOH): Frs 1−5 (9:1 to 1:1; inactive), combined Frs 6 and 7 (1:7; 47 mg; 1.2 μg/mL; 5′-methoxyhydnocarpin-D, **2**), Frs 8 and 9 combined (1:9; 15 mg; 6.3 μg/mL), Fr 10 (1:9; 70 mg; 66 μg/mL), Fr 11 (1:9; 148 mg; inactive; 1,2-dilinoleyl-3-galactosyl-*sn*-glycerol, **7**, identified by MS, ^{1}H and ^{13}C NMR spectra[18]). Alternate methods for the isolation and purification of **2** were previously reported.[4]

5′-Methoxyhydnocarpin-D (2): slightly yellow semisolid; $[\alpha]^{25}_{D}$ 0.0° (*c* 0.05, MeOH); MS (see Results and Discussion); UV (MeOH) λ_{max} 272, 345; 279, 369 (NaOAc added); ^{1}H NMR (DMSO-d_6) δ 3.38 (1H, *m*, H-11), 3.62 (1H, *m*, H-11), 3.78 (3H, *s*, C-3″ OMe), 3.92 (3H, *s*, C-5′ OMe), 4.25 (1H, *m*, H-12), 4.98 (1H, *d*, *J* = 9 Hz, H-13), 6.19 (1H, *d*, *J* = 2.1 Hz, H-6), 6.53 (1H, *d*, *J* = 2.3 Hz, H-8), 6.75 (1H, *s*, H-3), 6.82 (1H, *d*, *J* = 8.1 Hz, H-5"), 6.88 (1H, *dd*, *J* = 2, 9 Hz, H-6"), 7.03 (1H, *d*, 2 Hz, H-2″), 7.28 (1H, *d*, *J* = 2.1 Hz, H-2′) 7.32 (1H, *d*, *J* = 2.1 Hz, H-6′), 13.0 (1H, *s*, C-5 OH); ^{13}C (DMSO-d_6) identical with literature values[7] except assignments (based on HMQC and HMBC) have been reversed for C-12 and C-13: δ 56.4 (C−OMe), 56.8 (C−OMe), 61.7 (C-11), 75.9 (C-13), 78.2 (C-12), 94.0 (C-8), 98.7 (C-6), 102.8 (C-6′), 103.8 (C-3), 103.8 (C-10), 108.1 (C-2′), 117.7 (C-2″), 115.2 (C-5″), 120.4 (C-6″), 122.1 (C-1′), 127.1 (C-1″), 136.5 (C-4′), 144.1 (C-3′), 147.5 (C-4″), 147.8 (C-3″), 148.9 (C-5′), 157.2 (C-9), 161.2 (C-5), 162.9 (C-7), 164.1 (C-2), 181.7 (C-4). Acetylation gave a tetraacetate whose ^{1}H NMR spectrum contained peaks identical with those of the partial spectrum that had been reported.[8]

Bioassay. *S. aureus* 4222 parent strain (obtained from the University of Kansas, culture collection unknown) and the *norA* mutant KLE 820(3) were cultured in Mueller-Hinton (MH) broth. 10^5 cells/mL were inoculated into 1 mL of MH broth and dispensed at 0.2 mL/well in microtiter plates. Berberine chloride was added at 1/8 of its 240 μg/mL minimum inhibitor concentration (MIC), and MICs of test fractions or compounds were determined by serial 2-fold dilution in the presence and in the absence of berberine chloride. MIC was defined as the concentration of an antimicrobial 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.

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