Antitubercular Natural Products: Berberine from the Roots of Commercial *Hydrastis canadensis* Powder. Isolation of Inactive 8-Oxotetrahydrothalifendine, Canadine, β-Hydrastine, and Two New Quinic Acid Esters, Hycandinic Acid Esters-1 and -2

Elmer J. Gentry, Hanuman B. Jampani, Ali Keshavarz-Shokri, Martha D. Morton, David Vander Velde, Hanumaiah Telikepalli, and Lester A. Mitscher*

Department of Medicinal Chemistry, University of Kansas, Lawrence, Kansas 66045-2506

Ribhi Shawar, Debbi Humble, and William Baker

PathoGenesis Corporation, 201 Elliott Avenue West, Suite 150, Seattle, Washington 98119

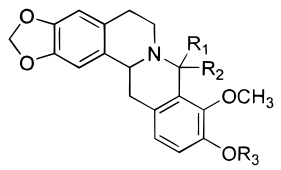
Received March 28, 1997

Berberine **(4)** is responsible for the activity of an extract of a commercial root sample of *Hydrastis canadensis* against multiply drug resistant *Mycobacterium tuberculosis*. Two new quinic acid feruloyl esters, compounds **2** and **3**, have been isolated from the same source along with canadine (**1c**), 8-oxotetrahydrothalifendine (**1**), and β-hydrastine (**5**). These were found to be inactive. The structures of the new compounds were elucidated from spectral (^{1}H, ^{13}C, HMQC, HMBC, and H−H COSY) and chemical evidences.

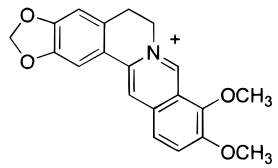
Introduction

The incidence of tuberculosis, also known as "the white plague", has recently been on the upsurge.[1,2] This ancient disease is characterized by comparatively low invasiveness but chronic debility and high mortality. Following decades of decline in developed countries, attributed to improvements in sanitation, public health measures and the development of effective chemotherapy, interest in research on novel agents for this indication declined in governmental and business circles. A general emergence of many kinds of multiply drug-resistant disease-causing microorganisms and the establishment of multiply resistant tubercular strains among highly susceptible recreational drug users, street dwellers, and the AIDS population, along with a corresponding equilibration into the general population, has reawakened interest in screening for novel structures possessing promising activity. Further motivation is supplied by the continuance of tuberculosis as a major health problem in developing countries. Tuberculosis is today one of the most lethal infectious bacterial diseases affecting mankind.

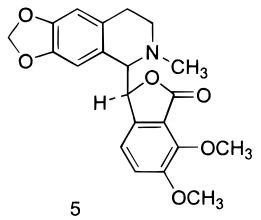
Employing a newly developed screen based upon sequential use of *Mycobacterium smegmatis*, bacille Calmette-Guerin (BCG) (either wild or luminogenic because of luciferase transfection), BACTEC technology with *Mycobacterium tuberculosis*, bioluminescent *Mycobacterium intracellularae* and *Mycobacterium avium* complex, and multiply resistant *M. tuberculosis* strains,[3] we identified several extracts of higher plants as containing promising activity. Those plant extracts chosen for study initially inhibited *M. tuberculosis* in standard BACTEC experiments at 100 mcg/mL. Among these was *Hydrastis canadensis* (Family Ranunculaceae), commonly known as golden seal. This well-known medicinal plant is a perennial herb native to eastern North America, and extracts of its rhizome and roots have seen use for various medicinal purposes among a variety of populations. It is reported[4] that golden seal is among the 25 best selling herbs today. Crude extracts are also widely used in contemporary proprietary preparations. It is generally considered that the activity of these preparations is due to their content of canadine (**1c**), berberine (**4**), and hydrastine (**5**).[4]



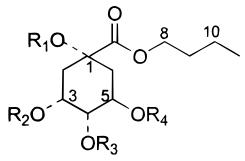
	R1	R2	R3
1		(=O)	H
1a		(=O)	Ac
1b		(=O)	Me
1c	H	H	Me

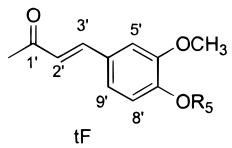


4



5





tF

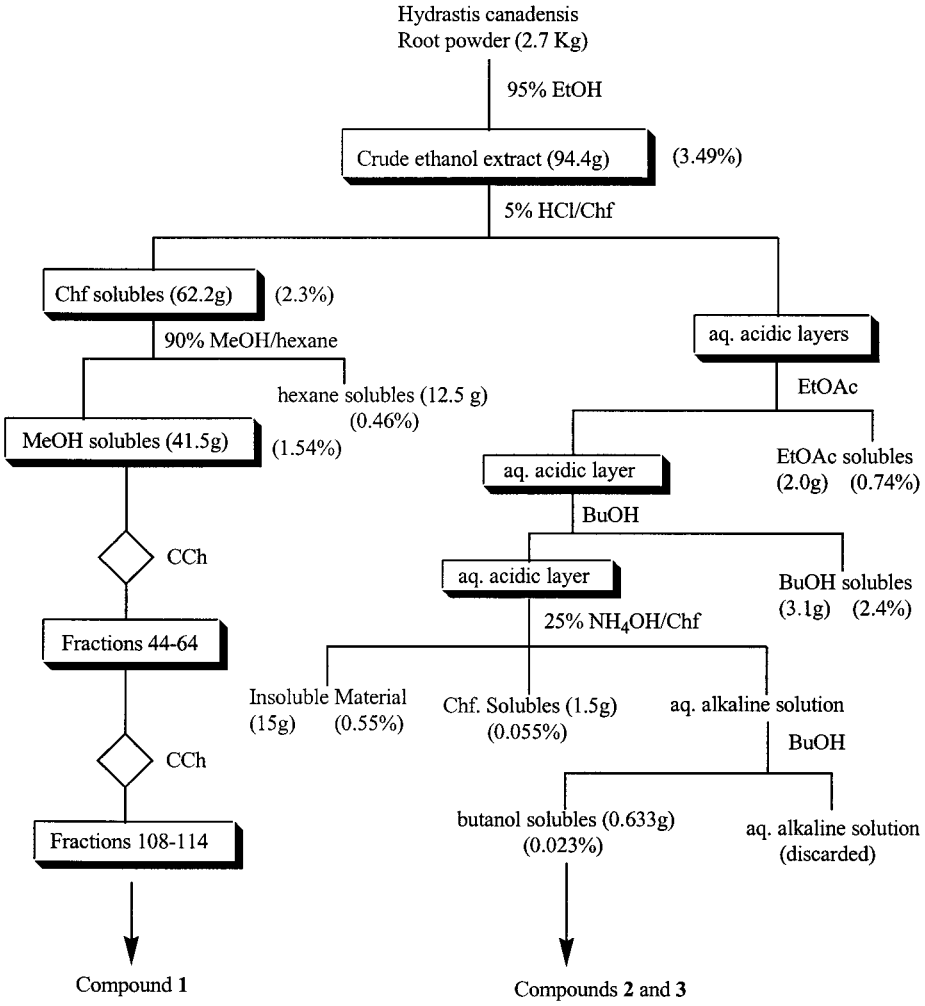
	R1	R2	R3	R4	R5
2	H	H	H	tF	H
2a	H	Ac	Ac	tF	Ac
2b	Ac	Ac	Ac	tF	Ac
3	H	H	tF	H	H
3a	H	Ac	tF	Ac	Ac
3b	Ac	Ac	tF	Ac	Ac

As anticipated, our fractionation led to the identification of berberine (**4**), one of the best known of the higher plant derived antimicrobial agents, as responsible for the antitubercular activity of this plant. Berberine, a membrane

* To whom correspondence should be addressed. Tel.: (913) 864-4562. Fax: (913) 864-5326. E-mail: lmitscher@rx.pharm.ukans.edu.

10.1021/np9701889 CCC: $15.00
Published on Web 09/03/1998

Scheme 1. Bioassay-Directed Fractionation of *Hydrastis canadensis* Roots



poison, has been demonstrated to reduce the infectivity of bacteria, fungi, and protozoa in animals and humans by inhibiting the adherence of microorganisms to the host cells.[5−8] In addition, berberine is known to have immunostimulatory activity by increasing the blood supply to the spleen, thus promoting the protective action of this organ, and by the release of immune-potentiating compounds.[9] Berberine is also known to be a potent activator of macrophages.[9] A number of berberine's biological actions have been attributed to intercalation into DNA.[10] A number of related alkaloids (β-hydrastine, canadine, and 8-oxotetrahydrothalifendine, a lactam-containing alkaloid new to this plant) and unrelated esters (the new compounds, hycandinic acid esters-1 and -2) were uncovered while studying this plant by systematic bioassay-directed experiments as described herein. Only berberine was found to be active when purified.

Results and Discussion

The crude ethanolic extract of *H. canadensis* root powder exhibited reproducible activity at 1000 μg/mL against *Staphylococcus aureus*, *Klebsiella pneumoniae*, *M. smegmatis*, and *Candida albicans* in a primary screen. In secondary screens using *M. tuberculosis* and *M. avium* complex in a BACTEC apparatus, and recombinant strains of BCG and *M. intracellularae* expressing firefly luciferase activity, the antitubercular activity against these clinically more relevant mycobacteria was confirmed.[3] On standing, a crystalline but inactive compound was obtained from the crude extract, which was identified as β-hydrastine (**5**) from its spectral characteristics.[11] The bioassay-directed fractionation of the crude extract by initial bulk solvent partition followed by column chromatography afforded 8-oxotetrahydrothalifendine (**1**), previously isolated from *Coscinium fenestratum*,[12] hycandinic acid ester-1 (**2**), and hycandinic acid ester-2 (**3**) along with canadine (**1c**) and berberine (**4**) (Scheme 1).

The well-known protoberberine anti-infective alkaloid, berberine (**4**), did not partition cleanly in the bulk-transfer experiments. Principally, it was found in the more water-soluble fractions described as chloroform insolubles, as expected. A smaller amount extracted along with the lipophilic components of the crude extract and survived through the various manipulations leading to polar lipids, *n*-butanol-soluble alkaloids, and chloroform-soluble water solubles. The ability of berberine and related alkaloids (e.g., sanguinarine) to add nucleophiles across the C(8)−N(7) olefinic linkage is well established in the literature and leads to a species transiently soluble in lipid solvents. Reversion to berberine readily occurs and accounts for its presence in fractions where one would not normally expect it to be found.[13] The presence of activity in these fractions led us to explore them chemically. As a consequence, we isolated a number of known alkaloids and quinic acid esters, hycandinic acid ester-1 and -2 (whose structures we determined), even though these were ultimately shown to be inactive.

Canadine, berberine, and hydrastine are well-known to be constituents of golden seal. 8-Oxotetrahydrothalifendine is closely related in structure but has not previously been found in this plant. Minor discrepancies from the properties we found for this substance and those published caused us at first to propose an isomeric structure. Ultimately, reexamination of its properties and those of derivatives confirmed identity with the previously known alkaloid.[14]

Acetylation gave a monoacetate (**1a**) whose spectra were in accord with the expectations for the structure. In further confirmation, methylation of **1** with dimethyl sulfate and base in acetone gave monomethylated product **1b**. The ^{1}H and ^{13}C NMR values were identical with those reported for 8-oxocanadine.[12] Furthermore, reduction of **1b** with $LiAlH_4$ afforded canadine identified by its physical and spectral characteristics with those of the isolated sample.[11,15]

Hycandinic acid ester-1 (**2**) and hycandinic acid ester-2 (**3**) were obtained as colorless oils from the *n*-BuOH fraction extractable at pH 1.0. The molecular formulas of **2** and **3**, $C_{21}H_{28}O_9$, were derived from their mass (*m*/*z* 424 $[M]^+$) and ^{13}C NMR spectra (see Experimental Section). Their ir spectra showed characteristic bands for an ester carbonyl group (1710 cm^{-1}) and an aromatic ring system (1620, 1590, and 1510 cm^{-1}). UV absorption at 340 nm is consistent with the presence of an unsaturated carbonyl chromophore conjugated with an aromatic moiety. The ^{1}H NMR spectrum of **2** displayed signals for butyl and ferulic acid ester side chains in addition to corresponding fragment ions at *m*/*z* 57 and 177 in the mass spectrum. The proton-generated signals at δ 6.30, (d, J = 15.9 Hz) and 7.62 (d, J = 15.9 Hz) clearly indicated the presence of trans olefinic protons. In addition, ABX spin system signals were observed for a ferulic side chain at 6.89 (d, J = 8.2 Hz), 7.03 (d, J = 1.6 Hz) and 7.05 (dd, J = 1.6, 8.2 Hz), respectively. A methoxy methyl singlet signal at δ 3.90 and a D_2O exchangeable signal at δ 6.05 were characteristic of a ferulic acid moiety at the 6′- and 7′-positions, respectively. The presence of a butyl ester side chain was evident from the proton signals at 4.18 (H-8), 1.63 (H-9), 1.36 (H-10), and 0.94 ± 0.01 (Me-11), which were in conformance with their H−H connectivities and long-range HMQC and HMBC correlations (Tables 1 and 2).

The basic quinic acid moiety was derived from the proton multiplicities (Table 1), H−H connectivities (Table 2), and ^{1}H, ^{13}C NMR spectral comparisons with reported data.[16] In **2**, the H-4 (axial) signal at δ 3.68 showed connectivities with the H-3 (equitorial) signal at δ 4.20 and the H-5 (axial) signal at δ 5.39. In addition, the $H_{a,e}$-2 and $H_{a,e}$-6 signals exhibited connections with H-3 and H-5, respectively. Further supportive evidence for these assignments and the location of the ferulic acid side chain was obtained from HMQC and HMBC data (Tables 1 and 2) of its acetates. Acetylation (Ac_2O/Py) of **2** yielded a tri- (**2a**) and a tetraacetate (**2b**). In the ^{1}H NMR of **2a** and **2b**, H-3 (equatorial) and H-4 (axial) signals appeared with a downfield shift of 1.27, 1.40 and 1.49, 1.44 ppm, respectively, as compared to their parent (**2**). The shift differences were in good agreement with reported values for similar quinic acid esters.[16,17] The final confirmation of the structure as 5-*O*-feruloylquinic acid butyl ester was obtained from long-range correlations of H-5 and ferulic acid ester carbonyl signals at δ 167.5 in HMBC of **2**.

The spectral characteristics of **3** indicated it to be a positional isomer of **2**. The quinic acid ring proton signals, 3-H (equatorial), 4-H (axial), and 5-H (axial), appeared at δ 4.42, 4.84, and 4.34, respectively. In its H−H COSY spectrum, the 3-H signal showed connectivities with C-2 methylene and C-4 methine protons. Similarly, the H-5 signal exhibited connectivities with the C-6 methylene and C-4 methine protons. These connections gave definitive information on the ferulic acid substitution at the C-4 position. Further, the long-range correlations between H-4 at δ 4.84 and an ester carbonyl signal at δ 167.5 confirmed **3** as 4-*O*-feruloylquinic acid butyl ester and, thus, isomeric with **2**.

Acetylation (Ac_2O/Py) of **3** gave a tri- (**3a**) and a tetraacetate (**3b**). In the ^{1}H NMR spectrum of **3a**, the H-3 and H-5 signals appeared with a downfield shift of 1.06 and 1.29 ppm, whereas 1.20 and 1.23 ppm shifts, respectively, were seen with **3b**. In the ^{13}C NMR spectrum of **2**, C-4 and C-5 produced signals at δ 73.8 and 70.7, while in **3**, the corresponding signals appeared at δ 78.7 and 68.9, thus confirming **3** to be a positional isomer of **2**. On the basis of the above spectral and chemical data **2** and **3** were identified as 5-*O*-feruloylquinic acid butyl ester (**2**) and 4-*O*-feruloylquinic acid butyl ester (**3**), respectively.

Pure samples of these compounds were tested for antimicrobial activity against *S. aureus* and *M. smegmatis* (Table 3). Berberine, surprisingly, showed comparatively weak activity with MIC values at 50 μg/mL against *S. aureus* and 25 μg/mL against *M. smegmatis*, respectively, against the primary screening organisms. The rest of the natural products and their derivatives showed no activity at these concentration levels.

When examined at concentrations ranging from 0.1 to 50 μg/mL in the BCG (rBCG pMV361 lux) and *M. avium* complex (r*M. intracellulare* pMV361 lux) Lux assay systems,[18] none of these compounds were active. On testing berberine at higher concentrations, it exhibited activity at 200 μg/mL against BCG and 50 μg/mL against MAC. These findings were confirmed by conventional broth dilution assays and are likely to be more meaningful with respect to the projected clinical situation than are the findings with indicator organism *M. smegmatis*.

The firefly luciferase assay has been developed comparatively recently[3,18] and has, as yet, not been widely employed in searching for new antitubercular agents. This paper is the first in a series in which it has been employed to find new structures with antitubercular activity. Other papers will relate its efficacy for the purpose in comparison with methods using established surrogate microorganisms. Briefly, a strain of BCG (rBCG lux) was prepared using recombinant technology in which luciferase gene was transfected and expressed. When this strain is grown in a suitable medium containing 1 mM luciferase with the appropriate substrate present, light is emitted as long as cells are viable. Luminescence is measured and compared with the number of relative light units emitted after 30 min, 3 days, and 5 days. The 5 day readings proved to be most accurate and predictive. An agent that inhibits the growth or kills the organism will result in a significant decrease in measured luminosity. This assay is very convenient for screening natural products because it is economical, sensitive, easily quanititated, sample sparing, adaptable to a 96-well format, and comparatively rapid. The presence of materials in the extract that significantly absorb the emitted light give false positive results on occasion. This can be corrected by suitable decrease in concentration. Use of concentrations in the 100−300 μg/mL range minimizes the interference. Clearly, the method is sensitive enough to pick up even weakly bioactive agents, such as berberine.

Table 1. $^{1}H^{a}$ and $^{13}C^{b}$ NMR Spectral Data of Compounds **2**, **2a**, **2b**, **3**, **3a**, and **3b**

	compound															
	2			**2a**			**2b**			**3**			**3a**			**3b**		
position	δH	*J* (Hz)	δC	δH	*J* ((Hz)	δC	δH	*J* (Hz)	δC	δH	*J* (Hz)	δC	δH	*J* (Hz)	δC	δH	*J* (Hz)	δC
1			75.6			73.9			78.8			75.6			73.9			78.8
2	2.08 dd	3.2, 14.7	37.0	2.22 m		35.6	2.4 dd	3.5, 15.7	37.0	2.13 dt		41.5	2.24 m		35.4	2.38 dd	3.4, 16	36.8
2	2.20 dt	2.9, 14.7	37.0	2.22 m		35.6	2.4 dd	3.5, 15.7	37.0	2.17 dd		41.5	2.24 m		35.4	2.38 dd	3.4, 16	36.8
3	4.20 m		70.6	5.47 dd	3.4, 7.0	68.9	5.60 m		67.8	4.34 br s	64.8	5.48 dd	3.5, 7.0	72.1	5.62 m		71.9	
4	3.68 dd	3.2, 9.8	73.8	5.17 dd	3.3, 9.9	71.9	5.12 dd	3.6, 10.0	71.6	4.84 dd	2.9, 9.9	78.7	5.17 dd	3.4, 9.8	69.1	5.13 dd	3.6, 9.8	67.9
5	5.39 ddd	3.2, 9.8, 11.3	70.7	5.63 dt	4.6, 10.2	67.3	5.60 m		66.7	4.42 ddd	4.5, 9.9, 13.0	68.9	5.63 dt	4.4, 10.0	67.1	5.57 m		66.5
6	1.93 dd	11.3, 12.3	38.7	2.10 m		39.1	1.98 m		31.9	1.99 dd	11.6, 13.0	37.5	1.99 m		39.1	1.96 m		32.1
6	2.32 ddd	4.8, 12.3	38.7	2.31 m		39.1	2.68 m		31.9	2.28 dd	4.5, 11.6	37.5	2.30 m		39.1	2.66 m		32.1
7			174.1			174.3			170.2			174.5			174.7			170.2
8	4.18 t	6.6	66.4	4.18 t	6.6	66.4	4.12 t	6.7	65.9	4.20 m		66.6	4.20 t	6.5	66.4	4.14 t	6.5	65.9
9	1.63 quint	7.2	30.5	1.64 dd	6.9, 15.0	30.5	1.60 m		30.3	1.65 quint	7.2	30.4	1.65 dd	6.9, 14.6	30.5	1.6 m		30.4
10	1.36 sext	7.6	19.0	1.37 dd	7.4, 15.0	19.0	1.35 dd	7.1, 14.9	19.0	1.37 sext	7.4	18.9	1.39 dd	7.7, 14.8	19.0	1.37 m		19.0
11	0.94 t	7.4	13.6	0.94 t	7.4	13.6	0.92 t	7.3	13.6	0.95 t	7.3	13.6	0.96 t	7.3	13.6	0.94 t	7.2	13.6
1′			167.5			165.6			165.6			167.5			165.8			165.7
2′	6.30 d	15.9	115.0	6.32 d	16.0	117.6	6.32 d	16.0	117.3	6.37 d	15.8	114.7	6.36 d	16.0	117.2	6.31 d	16.0	117.1
3′	7.62 d	15.9	145.7	7.62 d	16.0 144.9	7.62 d	16.0	145.1	7.67 d	15.8	146.1	7.65 d	16.0	145.3	7.63 d	16.0	145.3	
4′			126.8			133.1			133.0			126.7			133.1			133.1
5′	7.03 d	1.6	109.4	7.10 br s		111.2	7.10 br s		111.2	7.00 d	1.4	109.4	7.12 br s		111.2	7.10 br s		111.3
6′			146.8			151.4			151.5			146.8			151.4			151.4
7′			148.1			141.7			141.7			148.2			141.7			141.7
8′	6.89 d	8.2	114.7	7.08 d	8.0	121.4	7.08 d	8.0	121.5	6.89 d	8.0	114.8	7.06 d	8.4	121.2	7.06 d	8.0	121.5
9′	7.05 dd	1.6, 8.2	123.2	7.11 m		123.3	7.12 m		123.3	7.06 dd	1.4, 8.0	123.4	7.14 m		123.3	7.12 m		123.3
6′-OMe	3.90 s		55.9	3.87 s		56.0	3.88 s		56.0	3.89 s		55.9	3.88 s		56.0	3.88 s		56.0
7′-OAc				2.32 s		168.8	2.33 s		168.7				2.33 s		168.7	2.33 s		168.7
						20.6			20.6						20.6			20.6
1-OAc							2.00 s		170.2							2.07 s		170.0
									21.1									21.1
3-OAc				2.13 s		170.5	2.14 s		169.8				2.11 s		170.4	2.03 s		169.6
						21.1			21.0						21.2			21.0
4-OAc				2.04 s		170.4	2.09 s		169.7									
						20.8			20.7									
5-OAc													2.02 s		170.0	2.13 s		169.6
															21.0			21.0
−OH	1.81 br s									1.76 br s								
−OH	3.14 br s									2.52 br s								
−OH	3.86 br s									4.04 br s								
−OH	6.05 br s									6.15 br s								

[a] Run at 300 and 500 MHz. [b] Run at 125 MHz.

Table 2. HMQC and HMBC Spectral Data of Compounds **1**−**3**[a]

	compound								
	1			**2**			**3**		
position	δH	HMQC	HMBC	δH	HMQC	HMBC	δH	HMQC	HMBC
1	6.69	106.0	C-3, C-4a, C-14						
2				2.08	37.0	C-7	1.99	41.5	C-3, C-4
2				2.20	37.0		2.28	41.5	C-1, C-3, C-4, C-6
3				4.20	70.6		4.42	64.8	C-2, C-4
4	6.67	108.6	C-5	3.68	73.8	C-3, C-5	4.84	78.7	C-1′, C-3
5	2.93	29.8	C-6, C-14a	5.39	70.7	C-4, C-6, C-1′	4.34	68.9	
	2.77	29.8	C-4, C-14a						
6	2.96	38.8		1.93	38.7	C-4, C-5	2.14	37.5	C-1, C-2, C-4, C-5
6	4.96	38.8	C-4a, C-5, C-14	2.32	38.7	C-2, C-5, C-7	2.14	37.5	C-1, C-2, C-4, C-5
8				4.18	66.4	C-7, C-9, C-10	4.20	66.6	C-7, C-9, C-10
9				1.63	30.5	C-8, C-10, C-11	1.65	30.4	C-8, C-10, C-11
10				1.36	19.0	C-8, C-9, C-11	1.37	18.9	C-8, C-9, C-11
11	7.06	118.1	C-9,C-10,C-12,C-12a	0.94	13.6	C-9, C-10	0.95	13.6	C-9, C-10
12	6.91	122.8	C-8a, C-10, C-13						
2′				6.30	115.0	C-1′, C-3′, C-4′	6.37	114.7	C-1′, C-3′, C-4′
3′				7.62	145.7	C-1′, C-2′, C-4′, C-5′, C-9′	7.67	146.1	C-1′,C-2′,C-4′,C-5′,C-9′
5′				7.03	109.4	C-3′, C-7′, C-9′	7.00	109.4	C-3′, C-7′, C-9′
6′					146.8			146.8	
8′				6.89	114.7	C-4′, C-6′, C-7′	6.88	114.8	C-4′, C-6′, C-7′
9′				7.05	123.2	C-3′, C-5′, C-7′	7.02	123.4	C-3′, C-5′, C-7′
6′-OMe				3.90	55.9	C-6′	3.89	55.9	C-6′
13	2.84	39.0	C-12a						
	3.05	39.0	C-8a, C-12, C-12a						
14	4.74	55.3	C-4a						
9-OMe	4.02	62.4	C-9						
10-OH	6.02		C-9, C-10, C-11						
OCH_2O	5.92	101.1	C-2, C-3						

[a] Recorded using a Bruker DRX-400 spectrometer.

Table 3. Antimicrobial Activity of Chemical Constituents Isolated from *Hydrastis canadensis*

	MIC[a] (μg/mL) of microorganism[b]								
	1	2	3	4	5	6	7	8	9
rt ethanol extract	1000	i	i	1000	1000	1000	i	300	300
$CHCl_3$ solubles at pH 1.0	1000	i	i	1000	1000	1000	i		
n-BuOH solubles at pH 1.0	1000	i	i	i	1000	1000	i		
$CHCl_3$ solubles at pH 9.0	1000	i	i	1000	1000	1000	i		
8-oxotetrahydrothalifendine (**1**)	i			i	i	i		>50	>50
9-*O*-acetyl **1** (**1a**)	i			i	i	i		>50	>50
9-*O*-methyl **1** (**1b**)	i			i	i	i		>50	>50
canadine (**1c**)	i			i	i	i		>50	>50
hycandinic acid ester-1 (**2**)	i				i	i			
triacetylhycandinic acid ester-1 (**2a**)	i				i	i			
tetraacetylhycandinic acid ester-1 (**2b**)	i				i	i			
hycandinic acid ester-2 (**3**)	i				i	i			
triacetylhycandinic acid ester-2 (**3a**)	i				i	i			
tetraacetylhycandinic acid ester-2 (**3b**)	i				i	i			
berberine (**4**)	50			+100	25	+100		200	200
β-hydrastine (**5**)	i	i	i	i	i	i	i		
streptomycin sulfate	6.25	6.25	50	1.56	1.56	>100	25		

[a] Minimum inhibitory concentration; i = inactive at 1000 μg/mL. [b] Microorganism: (1) *Staphylococcus aureus* ATCC 13709, (2) *Escherichia coli* ATCC 9637, (3) *Salmonella gallinarium* ATCC 9184, (4) *Klebsiella pneumoniae* ATCC 10031, (5) *Mycobacterium smegmatis* ATCC 607, (6) *Candida albicans* ATCC 10231, (7) *Pseudomonas aeruginosa* ATCC 27853, (8) rBCG pMV361 lux, (9) r*M. intracellularae* pM361 lux.

In sum, the in vitro activity of *H. canadensis* crude extract is attributable to its comparatively high content of the well-known antibacterial alkaloid, berberine, perhaps abetted by artifactual addition of nucleophiles across its C(8)−N(7) olefinic linkage allowing for better cellular penetration and/or by the presence of undetermined auxiliary metabolites, which are themselves individually antibacterially inactive at realistic doses but which may enhance berberine's potency when administered together. This is, of course, rather speculative. Whatever the reason, the potency of the crude extracts in screening experiments led us to hope that more exciting results might attend structure-based screening experiments.

Berberine is known to have immunostimulatory activity that might enhance its antitubercular potential, but this is not likely in experiments such as these in which no functioning immune system is present.

In comparison with established antitubercular agents, the specific potency of pure berberine against resistant tuberculosis strains (data not shown), while interesting, is not sufficient to warrant detailed animal studies. Synthesis and evaluation of a few carefully chosen analogues might prove rewarding as the berberine strucure is clearly different from that of any presently established antitubercular agent.

This work demonstrates again that even thoroughly investigated plants can be shown to contain novel natural products (the hycandinic acid esters) when reexamined by modern methodologies.

Experimental Section

General Experimental Procedures. NMR spectra were recorded with a Varian XL-300, Bruker DRX-400, or a Bruker AM-500 spectrometer in $CDCl_3$, δ, and are reported in ppm downfield to TMS as internal standard and J in Hz. Column chromatography was performed on Merck Si gel 60. TLC was performed on Si gel (Analtech, 0.25 mm, scored plates); preparative TLC was done on Merck Si gel plates; detection: UV lamp, iodine vapors, ferric chloride solution and Dragendorff reagent. IR spectra were recorded with a Perkin-Elmer 1420. Optical rotations were recorded on a Perkin-Elmer polarimeter (Model 241). UV spectra were recorded with a Hewlett-Packard 8450 A instrument; MS were recorded with an Auto Spec-Q mass spectrometer operating at 70 eV in the EIMS mode.

Plant Material. The commerical sample of *H. canadensis* (Golden seal) root powder was purchased from Indiana Botanic Gardens, Hammond, IN. Voucher specimens are deposited in the Kansas Biological Survey herbarium.

Extraction and Isolation. Powdered roots (2.7 kg) of *H. canadensis* were extracted with 95% ethanol (4 L each) at room temperature for 96 h by adding fresh solvent every 24 h and eluting. The combined ethanol extractables were concentrated to a dark brown residue (94.4 g, 3.49%), which showed reproducible antimicrobial activity at 1000 μg/mL (Table 3). After room-temperature extraction, the plant material was extracted with 95% ethanol at boiling temperature by Soxhlet extraction to obtain 117.8 g of additional crude active material. On standing at room temperature, β-hydrastine separated as colorless crystals (MeOH, 500 mg): mp 143−144 °C (132−133 °C, C_6H_6/hexane).[11] The remaining crude extract (93.9 g) was extracted at pH 1.0 with $CHCl_3$, EtOAc, and *n*-BuOH in series. Among these solubles, the $CHCl_3$ and *n*-BuOH extracts exhibited antimicrobial activity. Next, the aqueous solution was basified to pH 9.0 with 25% NH_4OH and extracted with $CHCl_3$ and *n*-BuOH. The insoluble material obtained while partitioning with $CHCl_3$ was filtered and crystallized with MeOH to produce berberine as yellow needles (15.0 g), 208−210 °C dec.[20] The $CHCl_3$ solubles at pH 1.0 were further partitioned with 90% MeOH and hexane to obtain 41.5 g (1.54%) of purified active material. Column chromatography over silica gel with CH_2Cl_2, acetone, and MeOH, using gradient solvent mixtures, gave collected fractions. On the basis of their TLC behavior, fractions were reduced to 36 subfractions, of which 31 were antimicrobially active. The solid obtained on combining fractions 44−64 showed the most activity, and therefore, it was rechromatographed over Si gel with $CHCl_3$, acetone, and MeOH using gradient solvent mixtures to obtain 147 subfractions. 8-Oxotetrahydrothalifendine (**1**) was crystallized with EtOAc from subfractions 108−114 (containing minor amounts of berberine) as colorless crystals, while canadine (**1c**) was isolated from subfractions 128−136 as pale yellow crystals with hexane/acetone (1:0.1), mp 133−134 °C (133−134 °C, C_6H_6/hexane).[11] Similarly, the *n*-BuOH solubles (3.1 g) were also column chromatographed over Si gel with CH_2Cl_2 and MeOH to afford 50 fractions. The total number of fractions was narrowed to 23 subfractions after TLC evaluation, of which, seven fractions showed antibacterial activity. Preparative TLC of subfraction 14 (20 mg, which contained minor amounts of berberine) with $CHCl_3$−acetone−MeOH (4:1:0.075) by multiple development afforded hycandinic acid ester-1 (**2**) (5 mg) and hycandinic acid ester-2 (**3**) (3 mg) as colorless oils.

8-Oxotetrahydrothalifendine (1): white crystals (EtOAc); mp 210−212 °C; $[\alpha]^{24}_D$ −682° (*c* 0.1, $CHCl_3$); UV (EtOH) λ_{max} (log ϵ) 207 (4.79), 217 (4.67), 291 (4.06) nm; (EtOH + NaOH) 211 (4.91), 225 (4.98), 293 (4.04), 344 (3.69) nm; (EtOH + $AlCl_3$) 208 (4.76), 218 (4.64), 293 (4.09) nm; IR (neat) ν_{max} 3220, 2920, 2880, 1620, 1560, 1500, 1480, 1420, 1350, 1270, 1230, 1050, 1030, 920, 850, 800 cm^{-1}; EIMS (70 eV) *m/z* $[M]^+$ 339 (100), 324 (12), 321 (27), 229 (17), 176 (90), 164 (88); the ^{1}H and ^{13}C NMR spectra are essentially identical with those previously published.[12]

Acetyl 8-Oxotetrahydrothalifendine (1a). 8-Oxotetrahydrothalifendine (10 mg) was dissolved in 0.5 mL of pyridine to which 0.5 mL of acetic anhydride was added with stirring. The reaction mixture was kept at room temperature for 12 h. Workup by removing pyridine and unreacted acetic anhydride under vacuum gave a viscous liquid, which on purification by preparative TLC with $CHCl_3$−acetone (5:0.2) yielded amorphous **1a** (12 mg): $[\alpha]^{24}_D$ −319° (*c* 0.2, $CHCl_3$); UV (EtOH) λ_{max} (log ϵ) 211 (5.35), 217 (4.96), 213 (sh, 4.71), 291 (4.52) nm; (EtOH + NaOH) 227 (5.35), 233 (5.07), 238 (5.05), 293 (4.24), 344 (4.11) nm; (EtOH + $AlCl_3$) 207 (4.90), 217 (4.94), 235 (sh, 4.69), 293 (4.50) nm; IR (neat) ν_{max} 2920, 2880, 1750, 1635, 1490, 1470, 1410, 1360, 1320, 1270, 1250, 1200, 1020, 890, 850, 720 cm^{-1}; EIMS (70 eV) *m/z* $[M]^+$ 381-(71), 353 (17), 339 (37), 321 (19), 232 (25), 217 (15), 176 (61), 164 (50), 149 (22), 135 (20), 107 (22), 91 (20), 84 (21), 77 (25), 70 (36), 43 (90); the ^{1}H and ^{13}C NMR data are essentially identical with those previously published.[12]

***O*-Methyl-8-oxotetrahydrothalifendine (8-Oxocanadine) (1b).** 8-Oxotetrahydrothalifendine (12 mg) was dissolved in 5 mL of dry acetone and refluxed with 0.2 mL of dimethyl sulfate over anhydrous K_2CO_3 (100 mg) for 12 h. The reaction mixture was filtered and concentrated to dryness. The residue so obtained was purified by preparative TLC with $CHCl_3$−acetone (4:0.2) to give amorphous **1b** (10 mg): $[\alpha]^{24}_D$ −83.5° (*c* 0.2, $CHCl_3$); UV (EtOH) λ_{max} (log ϵ) 207 (4.89), 234 (4.47), 295 (4.10) nm; (EtOH + NaOH) 212 (5.02), 224 (5.07) 295 (4.17) nm; IR (neat) ν_{max} 2920, 2840, 1635, 1475, 1410, 1320, 1255, 1230, 1070, 1030, 920, 850, 780 cm^{-1}; EIMS (70 eV) *m/z* $[M]^+$ 353 (86), 339 (10), 324 (9), 306 (7), 205 (45), 178 (83), 174 (38), 163 (24), 149 (15), 135 (34), 120 (38), 115 (27), 90 (33), 87 (55), 77 (20); the ^{1}H and ^{13}C NMR data are essentially identical with those previously published.[12]

Canadine (1c). 8-Oxocanadine (**1b**, 5 mg) was dissolved in dry THF (2 mL), and to this solution was added 5 mg of $LiAlH_4$ powder. The reaction mixture was stirred for 5 min at room temperature followed by concentration. The obtained residue was diluted with water and partitioned with $CHCl_3$ (3 × 10 mL each). The combined $CHCl_3$ solution was dried over anhydrous Na_2SO_4, filtered, concentrated, and purified by preparative TLC with $CHCl_3$−acetone (8:0.2) to yield 4 mg of **1c**. Co-TLC, ^{1}H and ^{13}C NMR, and MS spectral data were identical to an isolated canadine sample: mp 133−134 °C (hexane/acetone) (lit.[5] mp 133−134 °C, C_6H_6/hexane); $[\alpha]^{24}_D$ −293° (*c* 0.51, $CHCl_3$); UV (EtOH) λ_{max} (log ϵ) 211 (4.87), 224 (sh, 4.59), 285 (4.18) nm; (EtOH + NaOH) 212 (4.94), 220 (4.94), 289 (4.18) nm; IR (neat) ν_{max} 2920, 2880, 1470, 1450, 1420, 1380, 1320, 1270, 1240, 1210, 1160, 1120, 1080, 1030, 980, 930, 850, 790, 740 cm^{-1}; EIMS (70 eV) *m/z* $[M]^+$ 339 (44), 308 (10), 174 (31), 164 (89), 149 (85), 121 (31), 104 (30), 91 (34), 77 (49); the ^{1}H and ^{13}C NMR data are essentially identical with those previously published.[12]

Hycandinic acid ester-1 (2): colorless oil; $[\alpha]^{24}_D$ −38.6°(*c* 0.145, $CHCl_3$); UV (EtOH) λ_{max} (log ϵ) 223 (4.96), 298 (5.00), 340 (5.02) nm; (EtOH + NaOH) 254 (4.81), 354 (4.99), 412 (5.10) nm; IR (neat) ν_{max} 3420 (br), 2980, 2920, 2860, 1710, 1620, 1590, 1510, 1450, 1420, 1270, 1160, 1120, 1080, 1050, 1030, 980, 910, 850, 810, 730 cm^{-1}; EIMS (70 eV) *m/z* $[M]^+$ 424 (6), 350 (2), 217 (83), 194 (54), 177 (75), 150 (27), 135 (31), 107 (14), 84 (56); for ^{1}H NMR (300 MHz, $CDCl_3$) and ^{13}C NMR data see Tables 1 and 2.

Acetylation of 2. Hycandinic acid ester-1 (5 mg) was dissolved in 0.2 mL of pyridine to which 0.2 mL of acetic anhydride was added dropwise with stirring. The reaction mixture was allowed to stand at room temperature for 12 h. The usual workup by concentrating to dryness under vacuum yielded a pale yellow crude mixture. Acetates **2a** (3 mg) and **2b** (3 mg) were obtained by preparative TLC purification with $CHCl_3$−acetone (20:0.1). For ^{1}H NMR (300 MHz, $CDCl_3$) and ^{13}C NMR data see Tables 1 and 2.

Triacetyl hycandinic acid ester-1 (2a): colorless oil; $[\alpha]^{24}_D$ −18.6° (*c* 0.75, $CHCl_3$); UV (EtOH) λ_{max} (log ϵ) 229 (4.82), 263 (4.76), 311 (4.77) nm; (EtOH + NaOH) 241 (4.71), 280 (4.79), 313 (4.77), 394 (4.07) nm; IR (neat) ν_{max} 2960, 2820, 1730, 1630, 1500, 1410, 1370, 1250, 1150, 1120, 1030, 900, 770

cm^{-1}; EIMS (70 eV) *m/z* [M]$^+$ 508 (43), 466 (7), 315 (10), 219 (57), 194 (20), 177 (40), 146 (20), 111 (20); for ^{1}H and ^{13}C NMR data see Tables 1 and 2.

Tetraacetyl hycandinic acid ester-1 (2b): colorless oil; $[\alpha]^{24}_D$ −5.0° (*c* 0.08, $CHCl_3$); UV (EtOH) λ_{max} (log ϵ) 234 (4.59), 284 (4.58) nm; (EtOH + NaOH) 259 (4.59), 296 (4.57), 395 (3.85) nm; IR (neat) ν_{max} 2960, 2920, 2880, 1740, 1630, 1500, 1420, 1370, 1230, 1150, 1110, 1030, 940, 900, 770 cm^{-1}; EIMS (70 eV) *m/z* [M]$^+$ 550 (57), 508 (7), 406 (10), 357 (7), 194 (17), 177 (33), 146 (20); for ^{1}H and ^{13}C NMR data see Tables 1 and 2.

Hycandinic acid ester-2 (3): colorless oil; $[\alpha]^{24}_D$ −29.6° (*c* 0.25, $CHCl_3$); UV (EtOH) λ_{max} (log ϵ) 239 (4.82), 296 (4.80) nm; (EtOH + NaOH) 252 (4.74), 347 (4.77), 418 (4.88) nm; IR (neat) ν_{max} 3420 (br), 2960, 2920, 2880, 1710, 1620, 1590, 1510, 1450, 1430, 1270, 1180, 1120, 1080, 1030, 980, 910, 850, 820, 730 cm^{-1}; EIMS (70 eV) *m/z* [M]$^+$ 424 (6), 350 (10), 279 (37), 217 (27), 194 (100), 177 (10), 167 (18), 149 (62), 135 (33), 111 (21), 84 (37), 71 (42) cm^{-1}; for ^{1}H and ^{13}C NMR data see Tables 1 and 2.

Acetylation of 3. Hycandinic acid ester-2 (**3**, 3 mg) was dissolved in 0.2 mL of pyridine and reacted with 0.2 mL of acetic anhydride at room temperature for 12 h. The reaction mixture was concentrated to dryness under vacuum. The mixture so obtained was purified by preparative TLC with $CHCl_3$−acetone (20:0.1) to yield **3a** (2 mg) and **3b** (2 mg) as colorless oils.

Triacetyl hycandinic acid ester-2 (3a): colorless oil; $[\alpha]^{24}_D$ −42.0°(*c* 0.1, $CHCl_3$); UV (EtOH) λ_{max} (log ϵ) 233 (4.62), 306 (4.56) nm; (EtOH + NaOH) 250 (4.61), 305 (4.56), 395 (4.32) nm; IR (neat) ν_{max} 2980, 2920, 2880, 1730, 1630, 1500, 1470, 1420, 1370, 1230, 1150, 1120, 1030, 900, 770 cm^{-1}; EIMS (70 eV) *m/z* [M]$^+$ 508 (53), 466 (7), 219 (17), 194 (20), 177 (60), 146 (27) 111 (10); for ^{1}H and ^{13}C NMR see Tables 1 and 2.

Tetraacetyl hycandinic acid ester-2 (3b): colorless oil; $[\alpha]^{24}_D$ −24.0° (*c* 0.075, $CHCl_3$); UV (EtOH) λ_{max} (log ϵ) 231 (5.24), 290 (5.21) nm; (EtOH + NaOH) 234 (5.23), 300 (5.24) 396 (3.68) nm; IR (neat) ν_{max} 2980, 2920, 2880, 1740, 1625, 1600, 1510, 1470, 1410, 1370, 1230, 1150, 1120, 1080, 1030, 940, 900, 700 cm^{-1}; EIMS (70 eV) *m/z* [M]$^+$ 550 (73), 508 (13), 406 (10), 389 (3), 219 (10), 194 (17), 177 (53), 146 (23); ^{1}H and ^{13}C NMR see Tables 1 and 2.

Test Panel Preparation, Inoculation, and Luciferase Assay. The test was performed in 96-place racks containing 1.2 mL Autotubes (Elkay, Shrewsbury, MA). Each test was prepared by transferring extract stock to an Autotube containing Middlebrook 7H9 broth.[18] Autotubes containing no drug and 1% DMSO were included in each test panel and served as growth controls. Twenty-five microliters of the inoculum was added to extract, growth control, and control drug Autotubes, yielding a final inoculum of approximately 5e5 cfu/mL. To perform the luciferase assay, 100 μL of each test was transferred to a 96-well Microlite tray (Dynatech Inc., Chantilly, VA). Using the MicroLumat LB 96P luminometer (Wallac Instruments, Gaithersburg, MD), 100 μL of 1 mM luciferin (R&D Systems, Minneapolis, MN) was automatically dispensed to each well, and luminescence was measured. Luminescence was expressed as the number of relative light units (RLU) detected in the measurement period. The initial, or day 0, RLU was measured within 30 min of inoculation. The Autotubes were capped and incubated at 36 °C in ambient atmosphere. The final RLU output was measured in the same manner after 3 days (rMI) and 5 days (rBCG) of incubation. The Autotubes were recapped and returned to the incubator for later colorimetric end-point determination. Results were expressed as relative change in luminescence. A ≥ 99% inhibition was considered indicative of activity.

Acknowledgments. This work was supported, in part, by Grant Nos. AI-36650 and GM-13155 from the NIH. One of the authors, H.B.J., gratefully acknowledges the additional financial support of the Kansas Health Foundation in the form of a fellowship. We wish to acknowledge Mr. Robert Drake and Ms. Homigol Biesada for the mass spectral measurements. We also acknowledge Dr. S. G. Franzblau, GWL Hansen's Disease Center at Louisiana State University, Baton Rouge, LA, for antitubercular testing of some crude and pure samples in BACTEC experiments.

References and Notes

(1) Fox, J. L. *Am. Soc. Microbiol. News* **1992**, *58*(3), 135−139.
(2) Iseman, M. D.; Cohn, D. L.; Sbarbaro, J. A. *N. Engl. J. Med.* **1993**, *328*, 576−578.
(3) Shawar, R. M.; Humble, D. J.; Van Dalfsen, J. M.; Stover, C. K.; Hickey, M. J.; Steele, S.; Mitscher, L. A.; Baker, W. *Antimicrob. Agents Chemother.* **1997**, *41*, 570−574.
(4) Brevoort, P. *Pharmaceut. News* **1996**, *3*, 26−28.
(5) Amin, A. H.; Subbaiah, T. V.; Abbasi, K. M. *Can. J. Microbiol.* **1969**, *15*, 1067−1076.
(6) Preininger, V. In *The Alkaloids*; Manske, R. H. F., Ed.; Academic Press: New York, 1975; Vol. 15, pp 231, 236.
(7) Subbaiah, T. V.; Amin, *A. H. Nature* **1967**, *215*, 527−528.
(8) Sun, D.; Abraham, S. N.; Beachy, E. H. *Antimicrob. Agents Chemother.* **1988**, *32*, 1274−1277.
(9) Murray, M. T. In *Natural Alternatives to Over-the-Counter and Prescription Drugs*; William Morrow and Company, Inc.: New York, 1994; p 151.
(10) Hahn, F. E.; Ciak, J. In *Antibiotics III: Mechanism of Action of Antimicrobial and Antitumor Agents*; Corcoran, J. W., Hahn, F. E., Springer-Verlag: New York, 1975; p 581.
(11) Messana, I.; Bua, R. L.; Galeffi, C. *Gazz. Chim. Ital.* **1980**, *110*, 539−543.
(12) Pinho, P. M. M.; Pinto, M. M. M.; Kijjoa, A.; Pharadai, K.; Diaz, J. G.; Herz, W. *Phytochemistry* **1992**, *31*, 1403−1407.
(13) Mitscher, L. A.; Park, Y. H.; Clark, D.; Clark, G. W.; Hammersfahr, P. D.; Wu, W. N.; Beal, J. L. *Lloydia* **1978**, *41*, 145−150.
(14) The authors are grateful to a careful reviewer whose report caused us to reopen the question of the appropriate structure for this compound with the result that identity with (−)-8-oxotetrahydrothalifendine was firmly established and we were spared the embarassment of putting forth an incorrect structure.
(15) Malhotra, S.; Taneja, S. C.; Dhar, K. L. *Phytochemistry* **1989**, *28*, 1998−1999.
(16) Rumbero-Sanchez, A.; Vazquez, P. *Phytochemistry* **1991**, *30*, 311−313.
(17) Corse, J.; Sondheimer, E.; Lundin, R. *Tetrahedron* **1962**, *18*, 1207−1210.
(18) Hickey, M. J.; Arain, T. M.; Shawar, R. M.; Humble, D. J.; Langhorne, M. H.; Morgenroth, J. N.; Stover, C. K. *Antimicrob. Agents Chemother.* **1995**, *40*, 400−407.
(19) Gadamer, J. *Arch. Pharm.* **1905**, *243*, 31−43.

NP9701889