Antimycobacterial Eudesmanolides from *Inula helenium* and *Rudbeckia subtomentosa*

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Abstract: **In a bioassay guided search for antimycobacterial compounds from higher plants, the root extracts of Elecampane (*Inula helenium* L.; Asteraceae) and Sweet Coneflower (*Rudbeckia subtomentosa* Pursh.; Asteraceae) were chemically investigated for their active constituents. Chromatographic fractions of root extracts of *I. helenium*, which exhibited significant activity against *Mycobacterium tuberculosis*, provided the known eudesmanolides alantolactone, isoalantolactone, and 11α*H*,13-dihydroisoalantolactone. Peracid epoxidation of alantolactone and isoalantolactone provided 5α-epoxyalantolactone and 4(15)α-epoxyisoalantolactone, respectively and oxidation of alantolactone with OsO_4 gave 11,13-dihydroxyalantolactone. Active fractions from *R. subtomentosa* contained the known alloalantolactone and 3-oxoalloalantolactone. The structures of the above compounds were established by spectroscopic methods including 1D and 2D NMR techniques as well as spectral comparison with previously reported data. The molecular structure of 5α-epoxyalantolactone was determined by single crystal X-ray diffraction. Eleven natural and semisynthetic eudesmanolides were tested in a radiorespirometric bioassay for activity against *M. tuberculosis*. 5α-Epoxyalantolactone and encelin from *Montanoa speciosa* showed minimum inhibitory concentrations (MICs) of 8 and 16 μg ml^{-1}, respectively. Alantolactone, isoalantolactone and its 4α,15-epoxide, 1,2-dehydro-3-epi-isotelekin and alloalantolactone gave MICs of 32 μg ml^{-1}. All other compounds showed MIC values of 128 μg ml^{-1} or higher.**

Key words: *Inula helenium*, *Rudbeckia subtomentosa*, *R. mollis*, *Montanoa speciosa*, Asteraceae, sesquiterpene lactones, eudesmanolides, *Mycobacterium tuberculosis*, antituberculosis activity.

Introduction

Worldwide, the number of tuberculosis cases is currently on the rise and it is estimated that the number of new infections by *Mycobacterium tuberculosis* is greater than 8 million annually and more than 3 million people die of this disease each year (1), creating a need for the discovery and development of new and more effective antituberculosis drugs (2). Our previous research efforts focused on a search for crude plant extracts with significant *in vitro* antimycobacterial activity (3), followed by a bioassay-guided isolation of active constituents (3 – 6). For instance, crude flower extracts of the Sea Daisy (*Borrichia frutescens*) exhibited significant activity, which from active chromatographic fractions provided antimycobacterial cycloartane-type triterpenes with minimum inhibitory concentrations (MICs) of $< 10\,\mu$g ml^{-1} (6).

In continuation of our search for new structural types of antimycobacterial natural products, active root extracts of *Inula helenium* and *Rudbeckia subtomentosa* were chemically investigated. Native Americans (Iroquois, Cherokees, and Mohegans) used infusions and decoctions of *I. helenium* roots for the treatment of lung disorders and against tuberculosis (7). Since at 100 μg ml^{-1}, the crude root extracts of *I. helenium* and *R. subtomentosa* exhibited 100 and 99 percent inhibition, respectively against *M. tuberculosis*, the active fractions were chemically investigated for their active constituents.

Materials and Methods

^{1}H- and ^{13}C-NMR spectra were recorded in $CDCl_3$ on a Bruker ARX 300 spectrometer at 300 MHz (^{1}H-NMR) and 75 MHz (^{13}C-NMR). Mass spectra were obtained on a Hewlett-Packard 5971A GC-MS instrument. IR spectra were run on a Perkin-Elmer 1760X spectrometer as a film on KBr plates. Vacuum-liquid chromatographic (VLC) separations (8) were carried out on silica gel (MN Kieselgel).

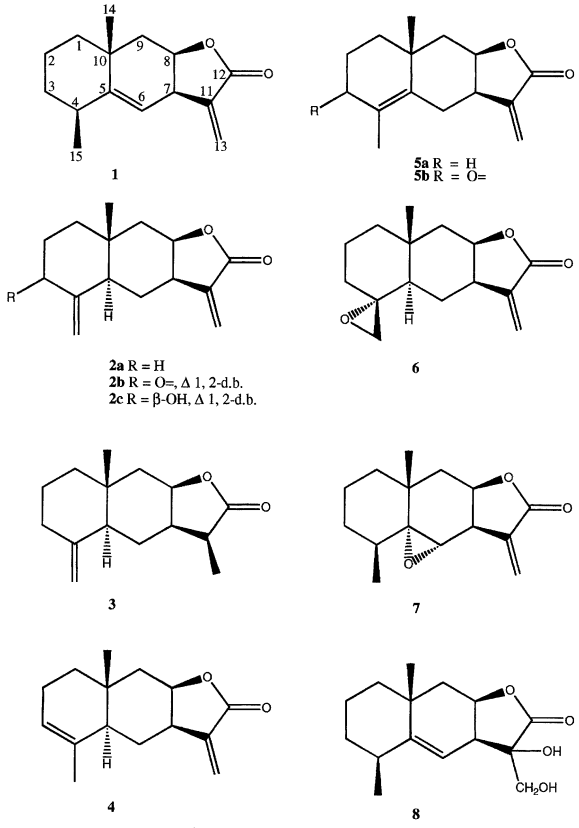
Plant material

Roots of *I. helenium* L. were collected on June 25, 1995 and obtained from Mr. George Sturtz of Aromagen, 31787 Peoria Road, Albany, Oregon 97321, U.S.A. A voucher (Sturtz-Fischer No 570) is deposited at the Louisiana State University Herbarium. The crude plant extract of *R. subtomentosa* Pursh. from a previous chemical study (9) was obtained from our repository. The voucher (Cox No 4923) is deposited at Louisiana State University Herbarium, U.S.A.

Extraction and isolation

Small pieces of fresh roots (4.5 kg) of *I. helenium* were dried at room temperature for two weeks and then soaked in hexane (8.5 l) for 24 hours. The solvent was decanted from the plant residue and evaporated in vacuo to yield 15.2 g of crude

Planta Medica 65 (1999) 351 – 355



extract. The residual plant material was resoaked for 24 hours in CH_2Cl_2 (7.8 l) and subsequently extracted for 24 hours in MeOH (7.8 l), yielding 23.3 g and 85.2 g, respectively. The CH_2Cl_2 extract (23.3 g) was adsorbed onto silica gel (15.2 g) and separated by VLC (8) into 11 fractions using solvents of increasing polarity (hexane, ethyl acetate, and MeOH) as summarized in Table **2**. Fractions 2 – 4 were further separated by repeated VLC procedures on silica gel, as described previously (10, 11), to yield pure compounds **1**, **2a**, and **3**.

Previously obtained CH_2Cl_2 root extract (1.75 g) of *R. subtomentosa* (9) was adsorbed on 1.6 g silica gel and placed onto a VLC column (2.3 cm in diameter) packed with 25 g of silica gel. The extract was separated into 8 fractions of increasing polarity using hexane, EtOAc, and MeOH and mixtures thereof, as listed in Table **2**. Fraction 2 (120 mg) was adsorbed on 200 mg of silica gel and placed onto a VLC column (1.3 cm in diameter) containing 5 g of silica gel. Subsequent elution using 10 ml fractions of hexane-EtOAc of increasing polarity afforded 17 mg of alloalantolactone (**5a**), which eluted with hexane-EtOAc (95 : 5). Fraction 3 (88 mg) was separated as described above for fraction 2 to afford 7 mg of compound **5a**. Fraction 4 (167 mg) was adsorbed on 240 mg of silica gel and placed onto a VLC column (1.3 cm in diameter) packed with 6 g of silica gel. Elution of the column with 10 ml fractions of hexane-EtOAc of increasing polarity afforded 19 mg of pure **5b**, which eluted with hexane-EtOAc (3 : 1).

Epoxidations of **1** *and* **2a**

Lactone **1** (101 mg) in 10 ml of CH_2Cl_2, was added to a solution of 10 ml of CH_2Cl_2 containing 1.2 equivalents *m*-chloroperbenzoic acid (*m*-CPBA), and stirred at 0 °C until TLC indicated that all of **1** had reacted (1.5 hours). The solution was washed with 10 ml of 10 % aqueous $NaHCO_3$ followed by 10 ml of H_2O. The organic phase was dried with anhydrous $MgSO_4$, evaporated and the residue was separated by preparative TLC (SiO_2, 1 mm) using hexane-EtOAc (5 : 2) as the mobile phase to yield 103 mg of **7**. Lactone **2a** was converted to epoxide **6** by a previously described method (13).

5α-Epoxyalantolactone (**7**): $C_{15}H_{20}O_3$ (M_r: 248) colorless crystals (EtOAc); m.p. 164 – 166 °C; $[\alpha]_D^{25}$: + 84.2° (*c*, 0.0057, $CHCl_3$); IR (KBr): ν_{max} = 1741 (C=O) cm^{-1}; EI-MS (70 eV): *m/z* (% rel. int.) = 248 (M^+, 9), 233 (M^+ − Me, 7), 204 (14), 192 (6), 159 (6), 149, (28), 133 (11), 126 (30), 123 (34), 109 (86), 95 (44), 81 (100), 67 (74), 55 (73); ^{1}H-NMR and ^{13}C-NMR see Table **3**.

OsO_4 oxidation of **1**

To an ice-cooled solution of 135 mg of a 50 % aqueous solution of 4-methylmorpholine *N*-oxide was added a solution of 0.33 mg of OsO_4 in 0.33 ml *t*-BuOH (14). Lactone **1** (126 mg), dissolved in 3 ml of acetone, was added and the reaction mixture stirred for 18 hours. A solution of aqueous 10 % $NaHSO_3$ (3 ml) was added and all solvents evaporated on a rotary evaporator. The crude mixture (136 mg) was absorbed on silica gel (208 mg) and separated on a VLC column (2.3 cm in diameter) packed with 5 g of silica gel. Elution of the column with 50 ml fractions of hexane and hexane-EtOAc mixtures of increasing polarity resulted in pure **8** (102 mg), which eluted with hexane-EtOAc (94 : 6).

11,13-Dihydroxyalantolactone (**8**): $C_{15}H_{22}O_4$ (M_r: 266) colorless oil; $[\alpha]_D^{25}$: − 27.9° (*c*, 0.0068, $CHCl_3$); IR (KBr): ν_{max} = 3388 (OH) cm^{-1}; EI-MS (70 eV): *m/z* (% rel. int.) = 266 (M^+, 16), 251 (M^+ − Me, 1), 235 (10), 221 (6), 215 (19), 207 (3), 191 (12), 162 (100), 147 (33), 133 (15), 119 (25), 105 (96), 91 (89), 77 (30), 67 (18), 55 (37); ^{1}H-NMR and ^{13}C-NMR see Table **3**.

Compounds **2b**, **2c**, *and* **4**

Isoalloalantolactone (**4**) had been previously obtained from *Rudbeckia mollis* Ell. (15). Encelin (**2b**) and 1,2-dehydro-3-epi-isotelekin (**2c**) were isolated from *Montanoa speciosa* DC., Asteraceae (16).

X-Ray crystallographic analysis of 5a-epoxyalantolactone (**7**)

A colorless fragment of dimensions 0.62 × 0.35 × 0.35 mm was used for data collection at T = 100 K on an Enraf-Nonius CAD4 diffractometer equipped with MoK_α radiation (λ = 0.71073 Å) and a graphite monochromator. The temperature of the sample was maintained by an Oxford Cryostream device. Crystal data are: $C_{15}H_{20}O_3$, M_r = 248.3, monoclinic space group $P2_1$, *a* = 7.968 (6), *b* = 6.181 (2), *c* = 13.132 (4) Å, β = 105.58 (6)°, V = 623.0 (8) Å^3, Z = 2, d_c = 1.324 g cm^{-3}. Intensity data were measured by ϖ-2θ scans of variable rate. Two quadrants of data were collected within the limits $2 < \theta < 30°$. Data reduction included corrections for background, Lorentz, and polarization effects. 3938 Intensities were averaged to yield 1974 unique data (Rint = 0.019), of which 1932 had $I > 0$ and were used in the refinement. The structure was solved by direct methods using SIR92 (17) and refined by full-matrix least squares, treating nonhydrogen atoms anisotropi-

cally. Hydrogen atoms were located in difference maps and refined isotropically. Convergence was achieved with R = 0.031, R_w = 0.036, and GOF = 1.687. The Enraf-Nonius MolEN programs (18) were used for all computations. The structure of **7** is illustrated in Figure **1**. A full list of crystallographic data and parameters including fractional coordinates is deposited at the Cambridge Crystallographic Data Center, University Chemical Laboratory, 12 Union Road, Cambridge, CB2, 1EZ, UK.

Radiorespirometric bioassays

Bioassays were performed essentially as described previously (6, 12). Experiments for *M. tuberculosis* were usually completed within ten days; rifampin was used as a positive control with an MIC of 0.25 – 0.125 μg ml^{-1}. The MIC values (μg ml^{-1}) for lactones **1 – 8** are **1**: 32; **2a**: 32; **2b**: 16; **2c**: 32; **3**: > 128; **4**: 128; **5a**: 32; **5b**: 128; **6**: 32; **7**: 8; **8**: > 128.

Table 1 Percent inhibition of crude plant extracts of *Inula helenium* and *Rudbeckia subtomentosa* against *Mycobacterium tuberculosis* (H_{37}Rv) at 1000 and 100 μg ml^{-1}.

Species	plant part	extract. solv.*	1000 μg ml^{-1}	100 μg ml^{-1}
I. helenium	roots	H	100	100
	roots	D	100	100
	roots	M	100	83
R. subtomentosa	roots	D	100	99
	leaves	D	96	43
	stems	D	70	–
	flowers	D	95	30

* H = hexane; D = dichloromethane; M = methanol.

Results and Discussion

The results of radiorespiratory antituberculosis bioassays of crude extracts of *I. helenium* and *R. subtomentosa* are presented in Table **1**. The hexane and CH_2Cl_2 root extracts of *I. helenium* demonstrated 100 percent inhibition at 100 μg ml^{-1}. VLC procedures of the CH_2Cl_2 root extract of *I. helenium* and subsequent bioassay of the fractions against *M. tuberculosis* indicated that the nonpolar fractions 2 – 4 were the most active, all three fractions giving 99 percent inhibitions at 33 μg ml^{-1} (Table **2**). Chemical investigation of these fractions resulted in the isolation of the known eudesmanolides alantolactone (**1**) (19, 20), isoalantolactone (**2a**) (19, 20), and 11,13-dihydroisoalantolactone (**3**) (20), as shown by spectroscopic comparison with data previously reported for these three sesquiterpene lactones.

Bioassays of CH_2Cl_2 extracts of various plant parts of *R. subtomentosa* indicated that root extracts were most active against *M. tuberculosis* with a 99 percent inhibition at 100 μg ml^{-1}, which after VLC fractionations gave eight fractions, their activities being summarized in Table **2**. Further VLC separation of the most active fractions 2 and 3 provided the known alloalantolactone (**5a**) and fraction 4 afforded the known 3-oxoalloalantolactone (**5b**) as the major component and **5a** as a minor constituent. The structures of **5a** and **5b** were determined by spectral comparison with previously reported data (11, 21). Both **5a** and **5b** had been previously isolated from *Eupatorium quadrangularae* (21), but this is the first report of their isolation from *R. subtomentosa*.

In our previous study of cycloartane-type triterpenes from *Borrichia frutescens* (6), epoxides were more active against *M. tuberculosis* than their corresponding alkene analogs, suggesting that epoxidation of the C-5 double bond of **1** or the C-4 (15) double bond of **2a** may result in derivatives of higher activity.

Epoxidation of **1** gave epoxide **7** which was identified by MS, ^{1}H-, ^{13}C-NMR, including 90° and 135° DEPT methods as well as comparison of spectral data with values previously reported for structurally related lactones (11, 20). The molecular structure of **7** was confirmed by single crystal X-ray diffraction (Fig. **1**).

Epoxidation of **2a** provided epoxide **6**, which had been previously isolated (13) and synthesized (22). Its structure was determined by spectral comparison, mainly of ^{1}H-NMR with previously reported values (13), and was shown to be essentially identical.

Table 2 Percent inhibition of *Inula helenium* and *Rudbeckia subtomentosa* fractions against *M. tuberculosis* (H_{37}Rv).

Fraction	solvent percentages (hexane : EtOAc : MeOH)		*I. helenium*		*R. subtomentosa*	
	I. helenium[a]	*R. subtomentosa*[b]	100 μg ml^{-1}	33 μg ml^{-1}	100 μg ml^{-1}	33 μg ml^{-1}
1	100 : 0 : 0	100 : 0 : 0	100	94	79	43
2	95 : 5 : 0	95 : 5 : 0	100	99	100	97
3	90 : 10 : 0	75 : 25 : 0	100	99	99	91
4	85 : 15 : 0	50 : 50 : 0	99	99	99	67
5	80 : 20 : 0	25 : 75 : 0	99	96	53	26
6	70 : 30 : 0	0 : 100 : 0	92	84	26	24
7	50 : 50 : 0	0 : 50 : 50	90	80	28	23
8	0 : 100 : 0	0 : 0 : 100	−20	−9	70	44
9	0 : 50 : 50	–	−14	−10	–	–
10	0 : 50 : 50	–	−3	10	–	–
11	0 : 0 : 100	–	−19	−12	–	–

[a] Using a column (6.5 cm in diameter) with 200 ml of solvent for each fraction.
[b] Using a column (2.3 cm in diameter) with 100 ml of solvent for each fraction.

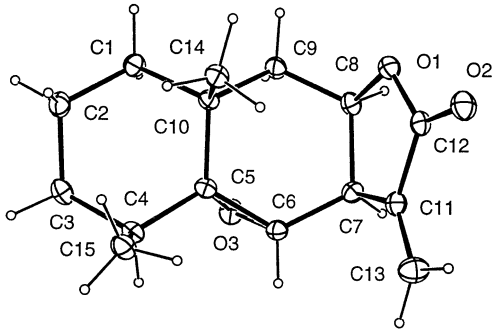


Fig. 1 Molecular structure of **7** at 100 K.

In an attempt to synthesize the 5,6-diol derivative of lactone **1**, it was reacted with OsO_4 (14). The ^{1}H-NMR spectrum of product **8** indicated that the olefinic H-6 doublet of **1** at δ 5.11 was nearly unchanged (δ 5.03) in **8**. Instead, the two diagnostic exocyclic methylene lactone signals (H-13) were missing in **8**, suggesting that glycol formation had occurred exclusively at the 11,13-rather than at the 5,6-position. ^{1}H-NMR values of **8** are summarized in Table **3** with peak assignments based on spectral comparison with data of structurally related analogs (10, 11, 19, 20). Attempts to determine the relative configuration of C-11 in **8** by NMR experiments were inconclusive.

The eudesmanolides isolated from *I. helenium*, *R. subtomentosa*, lactone **4** from *R. mollis* (15), and lactones **2b**, **2c** previously obtained from *M. speciosa* (16), as well as their semisynthetic derivatives, **6**, **7**, and **8**, were tested for their biological activities against *M. tuberculosis*. The *M. speciosa* constituents, encelin (**2b**) and 1,2-dehydro-3-epi-isotelekin (**2c**) showed MICs of 16 μg ml^{-1} and 32 μg ml^{-1}, respectively. Lactones **1**, **2a**, and **5a** gave values of 32 μg ml^{-1} while isoalloalantolactone (**4**) from *R. mollis* (15) and compound **5b** exhibited MICs of 128 μg ml^{-1}.

Previous structure-activity studies within a series of natural and semisynthetic germacranolides suggest that the α-methylene-γ-lactone moiety is an essential, but not sufficient, structural requirement for significant *in vitro* activity against *M. tuberculosis* (23). The necessity of the presence of an α-methylene-γ-lactone group is supported by the activity of compound **2a** with a MIC of 32 μg ml^{-1}, when compared with the inactive 11α*H*,13-dihydroderivative **3** with a value of $>$128 μg ml^{-1}. The presence of a second alkylating site, such as an α,β-unsaturated carbonyl group or an epoxide function together with a moderate to high lipophilicity (24), seems to enhance the *in vitro* antimycobacterial activity of sesquiterpene lactones. For instance, encelin (**2b**) is more active than **2a** and 5α-epoxyalantolactone (**7**), the most active lactone within this eudesmanolide series with a MIC of 8 μg ml^{-1}, is significantly more active than its precursor **1** with an MIC of 32 μg ml^{-1}. Both the 4α,15-epoxide **6** and its precursor **2a** gave MICs of 32 μg ml^{-1}, which suggests that the distance between the two alkylating sites and possibly the stereochemical relationship of the methylene lactone moiety and the epoxide, seems to influence the antimycobacterial activity. In contrast to **1** with an MIC of 32 μg ml^{-1}, its more polar 11,13-diol derivative (**8**), showed no biological activity with a MIC of $>$128 μg ml^{-1}. This is most likely due to the increased polarity and/or the loss of the lactonic alkylating site.

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Table 3 ^{1}H-NMR (300 MHz) and ^{13}C-NMR (75.4 MHz) spectral data of compounds **7** and **8** ($CDCl_3$).[a]

Atom	**7**		**8**	
	^{1}H-NMR	^{13}C-NMR	^{1}H-NMR	^{13}C-NMR
1	–	37.7 t*	–	42.3 t*
2	–	16.5 t	–	16.9 t
3α	–	29.5 t	1.54 m	32.9 t
3β	–	–	1.54 m	–
4	1.34 m	37.1 d**	2.47 m	38.6 d
5	–	67.5 s	–	152.9 s
6β	2.89 d (2.6)	61.2 d	5.03 d (3.5)	112.5 d
7α	3.66 dddd (2.5, 2.6, 2.9, 8.8)	37.4 d**	2.98 dd (3.5, 5.6)	44.9 d
8α	4.66 ddd (1.9, 4.5, 8.8)	75.2 d	5.12 ddd (3.0, 3.0, 5.6)	78.0 d
9α	1.55 dd (1.9, 15.0)	39.6 t*	1.54 dd (3.0, 15.0)	42.6 t*
9β	1.87 dd (4.5, 15.0)	–	2.14 dd (3.0, 15.0)	–
10	–	32.6 s	–	33.0 s
11	–	136.7 s	–	77.4 s
12	–	169.7 s	–	177.9 s
13a	5.77 d (2.5)	123.8 t	3.72 br d (12.0)	64.2 t
13b	6.39 d (2.9)	–	3.93 d (12.0)	–
14	1.10 s	24.0 q	1.22 s	28.8 q
15	1.03 d (2.7)	18.1 q	1.12 d (7.6)	23.1 q
11-OH	–	–	3.77 br s	–
13-OH	–	–	2.93 br d (9.9)	–

[a] Expressed as δ values in ppm, with *J* values in Hz in parentheses.
* Assignments in the same column are interchangeable.

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