Structures of Kelletinins I and II, Antibacterial Metabolites of the Marine Mollusk *Kelletia kelletii*

Adrienne A. Tymiak and Kenneth L. Rinehart, Jr.*

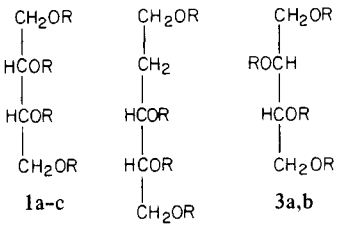
Contribution from the Roger Adams Laboratory, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801. *Received January 14, 1983*

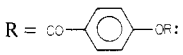
Abstract: Two novel tetraesters, kelletinins I and II [erythrityl tetrakis(*p*-hydroxybenzoate) and 2-deoxy-D-ribityl tetrakis-(*p*-hydroxybenzoate), **1a** and **2a** respectively], were isolated from the marine mollusk *Kelletia kelletii* and were found to inhibit the growth of *Bacillus subtilis* and to inhibit L1210 leukemia cells at 0.4 μg/mL. Structure assignments were based on high-field NMR and high-resolution mass spectrometric [including fast atom bombardment (FAB)] data and were confirmed by spectral comparisons of derivatized natural products and synthetic compounds. Kelletinins I and II represent a new class of antibacterial marine natural products and are rare examples of bioactive compounds from hard-shelled mollusks.

The phylum Mollusca encompasses octopuses, squids, chitons, nudibranchs, bivalves, and shelled snails. Numerous examples of bioactive compounds in mollusks have been noted,[1] mainly in opisthobranchs, whose metabolites have been studied extensively, both for potential sources of drugs and for organic compounds as trail markers.[2] However, bioactive materials isolated from opisthobranchs have often been traced to dietary sources.[3] Similarly, hard-shelled mollusks have been shown to store ingested toxins[4] and to concentrate metals from their aqueous environment.[5] Some of the few bioactive compounds isolated from hard-shelled mollusks, including snails, are quaternary ammonium salts, especially esters of choline,[6] which function as neuromuscular blocking agents. Some antimicrobial and antiviral extracts have also been reported from hard-shelled mollusks, notably abalone.[7] The compounds involved, paolins, appear to be proteinaceous. Similarly, antitumor activity has been reported from some mollusks, notably the edible clam.[8] Finally, there are the cone toxins, which, again, are peptides.[9]

During the Alpha Helix Baja Expedition 1974 (AHBE 1974),[10] which assayed over 190 species of Mollusca, several examples of antibacterial activity were found among the snails (Gastropoda), though nowhere else in the Mollusca. The opisthobranchs were notably active (15 of 27 species), but the Prosobranchia (hard-shelled snails) also showed antimicrobial activity (12 of 96 species).

One hard-shelled snail, *Kelletia kelletii* (AHBE 13-II-74-4-3), collected on Santa Catalina Island, California, during AHBE 1974, displayed modest activity against *Bacillus subtilis*, a Gram-positive bacterium. In subsequent recollections near the Catalina Marine Science Center, Avalon, CA, in 1980 and 1982, extracts of *K. kelletii* were found to be consistently active against *B. subtilis*. We have now investigated *K. kelletii* extracts for their antibacterial compounds and report here our results. Two bioactive components, which we have named kelletinins I and II, have been isolated and assigned structures **1a** and **2a**. Other compounds structurally



R =  : **1a**, **2a**, **3a**, R′ = H; **1b**, **2b**, **3b**, R′ = CH_3;

1c, **2c**, R′ = Ac

related to the kelletinins were also isolated from the mollusk but found to be less bioactive. The structure elucidation of kelletinins I and II and their possible origins will be discussed in this report.

K. kelletii specimens were collected by Scuba techniques on Santa Catalina Island. Frozen mollusks were first deshelled and then dissected into three tissue portions: the orange foot, light-hued viscera, and the black digestive gland. *B. subtilis* inhibition was detected only in the visceral extracts. Solvent partitioning (cf. Experimental Section) afforded a diethyl ether layer from which bioactivity was traced by bioautography to a strongly UV-absorbing thin-layer chromatography (TLC) spot. A silicic acid column or TLC (petroleum ether–diethyl ether mixtures) separated three fractions active against *B. subtilis*, A, B, and C; fraction C, the most polar and most antibacterial contained a mixture of kelletinins I and II. This fraction was also active against *Penicillium atrovenetum*, as well as cytotoxic to monkey kidney (CV-1) cells and L1210 leukemia cells, the latter at a rather low concentration (ID_{50} 0.4 μg/mL). On the basis of characteristic pH-dependent UV absorption, electron ionization (EI) MS fragmentation, aromatic proton resonances, and IR absorptions, bioactivity was correlated with the presence of *p*-hydroxybenzoic acid esters (Table I).

Kelletinin I (**1a**, 2.5 mg) was obtained in excess of kelletinin II (**2a**) by reversed-phase chromatography (C_{18} Sep-PAK) of the mixture eluted from the silica column immediately following fraction B. Its purity was estimated by analytical C_{18} high-performance liquid chromatography (HPLC) (Figure 1, top). A comparison of positive and negative ion fast atom bombardment (FAB) mass spectra (Figure 2) allowed the assignment of 602 as its nominal mass molecular weight. The lower intensity ions corresponding to a neutral species of 616 daltons indicated that the coeluting kelletinin II could be a homologue. All attempts to isolate the homologue from mixtures containing kelletinin I were unsuccessful (Figure 1, bottom). The problem, however, was

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Table I. Spectral Characteristics of *p*-Hydroxybenzoic Acid Derivatives

method	details	*p*-hydroxybenzoic acid	fraction A	fraction B	kelletinins I + II
EIMS, *m/z* (I)[a]	70 eV: HOC_6H_4COOH	138 (66)	138 (2)	138 (13.3)	138 (36)
	HOC_6H_4CO	121 (100)	121 (100)	121 (100)	121 (100)
	HOC_6H_4	93 (30)	93 (3)		93 (20)
	C_4H_9CO[b]		85 (48)[b]	85 (54)[b]	
	C_5H_5	65 (29)	65 (3)		65 (25)
	C_4H_9[b]		57 (34)[b]	57 (5)[b]	
	C_3H_3	39 (27)	39 (2)		39 (20)
^{1}H NMR, ppm	Me_2SO-d_6: Ar-OH	3.8 br s			
	H-3, H-5	6.90 d	6.8[c,d]	6.8[d,e]	6.8[d]
	H-2, H-6	7.86 d	7.8	7.8	7.7
	COOH	10.4 br s			
^{13}C NMR, ppm	Me_2SO-d_6: COOH	167.4	165.3[c]		165[f]
	C-1	121.6	120.9		120
	C-2, C-6	131.7	132.2		131
	C-3, C-5	115.2	115.5		115
	C-4	161.7	161.3		162
UV, nm (log ϵ)	EtOH	250 (4.1)	259 (4.3)	258 (4.8)	258 (4.7)
	0.1 N HCl (EtOH)	252 (4.2)	258 (4.4)	257 (4.8)	258 (4.8)
	0.1 N NaOH (EtOH)	273 (4.3)	298 (4.7)	301 (5.1)	301 (5.0)
IR, cm^{-1}	Et_2O solution	3400–3200	3400–3200	~3200	~3200
		1730	1730	1730	
		1700	1710		1710
		1620	1610	1605	1600
		1600	1600	1595	1590

[a] Intensity relative to base peak = 100. [b] Peaks due to isovaleryl residue. [c] Spectrum was run in $CDCl_3$. [d] Spectrum showed overlapping doublets. [e] Spectrum was run in CD_3CN. [f] Spectrum showed several overlapping signals.

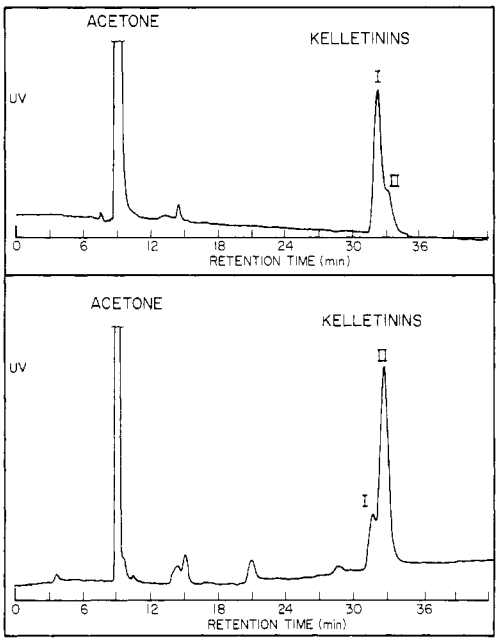


Figure 1. HPLC analysis of silica column fractions enriched in kelletinin I (top) or II (bottom). Conditions: Waters Associates HPLC; 260-nm UV detector; 25 cm × 10 mm Altex Ultrasphere-ODS column; mobile phase methanol–water (65:35); 0.1 N NaOAc/HOAc to pH 4.6; flow rate 1.5 mL/min.

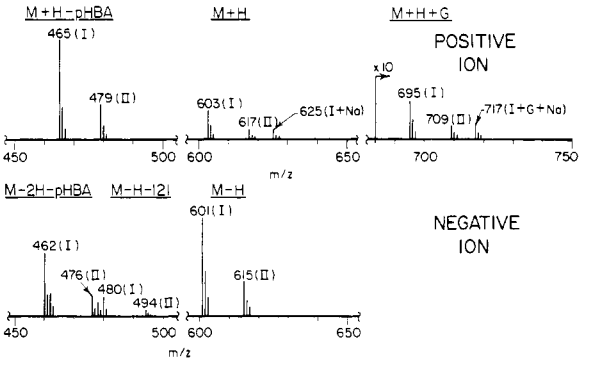


Figure 2. Fast atom bombardment (FAB) mass spectra (partial) of kelletinins I and II (mixture).

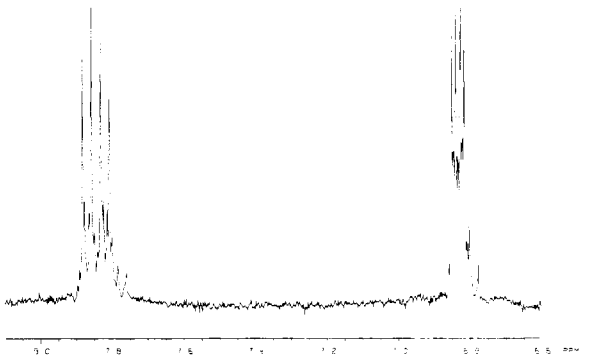
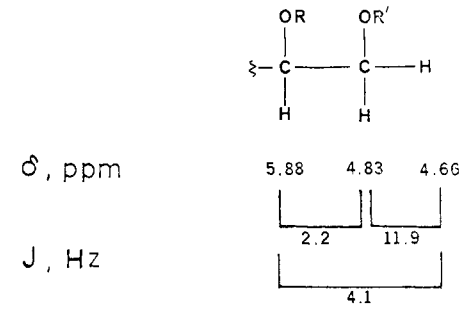


Figure 3. ^{1}H NMR spectrum (CD_3CN) of kelletinin I in the aromatic region.

circumvented by derivatization. Separation of the tetramethyl ethers (**1b** and **2b**) or of the tetraacetates (**1c** and **2c**) of kelletinins I and II was readily achieved by using preparative silica HPLC.

Kelletinin I. The structure of kelletinin I was inferred from spectroscopic data and confirmed by synthesis of the tetramethyl ether **1b**. Molecular ions for **1a** were observed by field desorption (FD) MS or FABMS but not by direct-probe EIMS. High-resolution mass measurement (FAB) of the M + H ion at *m/z* 603 (603.1445) as well as the M + H + glycerol ion at *m/z* 695 (695.1898) suggested the formula $C_{32}H_{27}O_{12}$ (Δ5.7 and 7.7 mmu, respectively). The pattern of aromatic resonances (two overlapping AB quartets) in the 360-MHz ^{1}H NMR spectrum of **1a** (Figure 3) argued the presence of two types of nearly equivalent para-disubstituted benzene rings, in a ratio of 1:1 (or eight protons each). This was verified by the collapse of the downfield or upfield doublet near 6.8 ppm (CD_3OD) when the respective downfield or upfield doublet near 7.9 ppm was irradiated. Four symmetrically arranged *p*-hydroxybenzoate groups would account for these 16 aromatic protons as well as the 20 units of unsaturation and the 12 oxygen atoms required by the molecular formula.

The remaining, aliphatic protons were assigned as in structure a on the basis of homonuclear decoupling experiments (CD_3CN)

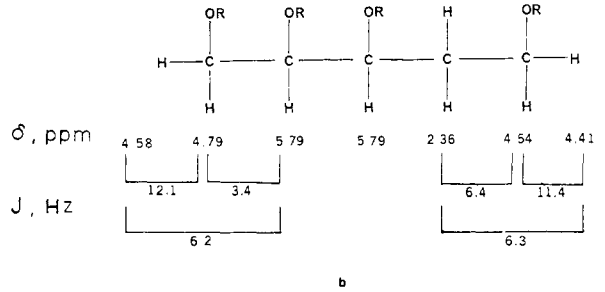


a

conducted on a sample of purified **1c**, leaving only the questions of stereochemistry and positions of esterification in the C_4 chain of unit a (R and R′ = H or acyl). There are only three ways of symmetrically attaching four esters to structure a, by forming either a tetrakis(*p*-hydroxybenzoate) with four free phenols or one of two symmetrical *p*-[(*p*-hydroxybenzoyl)oxy]benzoates with two phenolic and two aliphatic hydroxyl groups. The former possibility is favored by the observed chemical shifts. When kelletinin I was treated with excess diazomethane (Scheme I), a tetramethyl compound (FDMS, *m/z* 658) was isolated, ruling out the two diol arrangements of esters.

The stereochemical assignment of kelletinin I as **1a** or **3a** was resolved by synthesis. Erythritol and DL-threitol were treated with *p*-anisoyl chloride to afford authentic samples of **1b** and **3b**, respectively (Scheme I), which were separable by either silica or reversed-phase HPLC. Coelution then identified the tetramethylated kelletinin I as **1b** (Figure 4a). The ^{1}H NMR data for synthetic and natural samples of **1b** were also in agreement (Table II), thus establishing kelletinin I as the erythritol tetraester **1a**. It is interesting to note that although **1a** gave molecular ions by FD or FAB ionization, the less polar tetramethyl or tetraacetyl derivatives were not suitable for FABMS. Molecular ions for the derivatives could, however, be observed by direct-probe EIMS.

Kelletinin II. Kelletinin II appeared to be a simple homologue of kelletinin I, as judged by its FAB mass spectrum (M + H, *m/z* 617; Figure 2) and the FD mass spectrum (M + H, *m/z* 785) of its tetraacetate, **2c**. In addition to the resonances expected for kelletinin I, the ^{13}C NMR spectrum of a mixture of **1a** and **2a** included a weak signal (off-resonance triplet) at ca. 30 ppm (CD_3OD). The proposed structure **2a** for kelletinin II followed from the partial structure b deduced from irradiation experiments



b

and from the observed formation of a tetramethyl derivative when **2a** was treated with excess diazomethane (FDMS, *m/z* 672) (Scheme I).

Of the four possible stereoisomers of **2a**, only one could be readily prepared as a pure enantiomer. Commercial 2-deoxy-D-ribose was reduced by sodium borohydride to 2-deoxy-D-ribitol, which was esterified with *p*-anisoyl chloride to afford **2b** (Scheme I). The synthetic tetrakis(*p*-anisoate) was found to coelute with kelletinin II tetramethyl ether on both normal and reversed-phase HPLC columns by using conditions known to separate the erythro isomer **1b** from the threo isomer **3b** (Figure 4b). A comparison of ^{1}H NMR chemical shifts and coupling constants of the synthetic **2b** and natural derivatives **2b** and **2c** led to the assignment of an erythro configuration for kelletinin II (Table III). Furthermore, the specific rotations of the derivatized natural product **2c** and

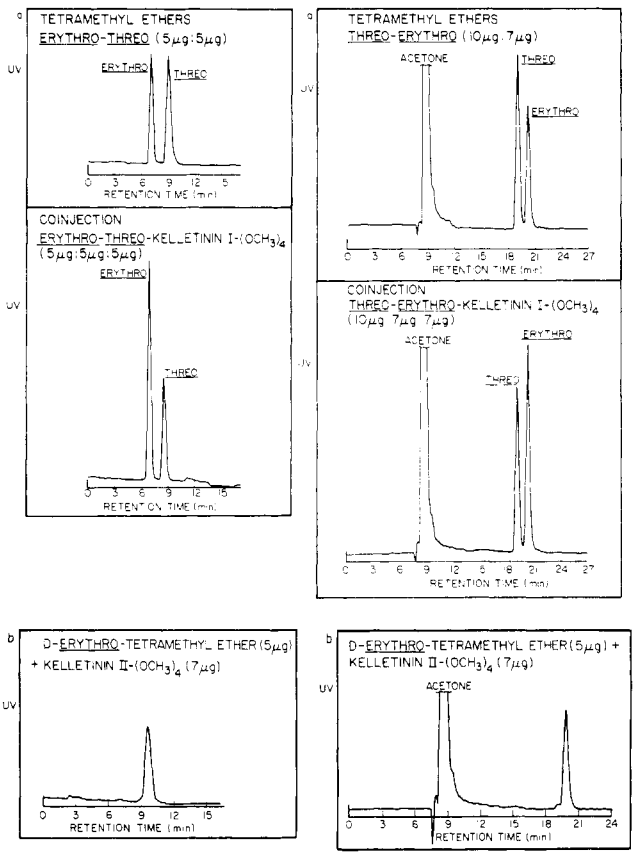
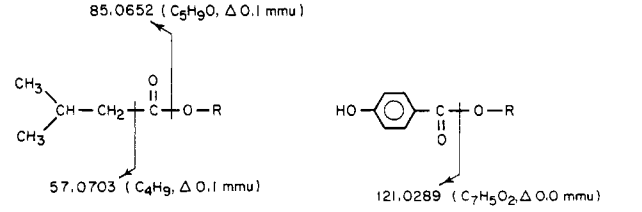


Figure 4. HPLC coelution of (a) kelletinin I tetramethyl ether and synthetic **1b** (erythro) and (b) kelletinin II tetramethyl ether and synthetic **2b**. Conditions: Waters Associates HPLC; 260-nm UV detector; flow rate 1.5 mL/min. Left: 25 cm × 4.6 mm Chromanetics LiChrosorb SI-60 column; mobile phase methanol–dichloromethane (0.25:99.75). Right: 25 cm × 10 mm Altex Ultrasphere-ODS column; mobile phase methanol–water (90:10).

synthetic **2b** were identical within limits of detection of the polarimeter, $[\alpha]^{25}_D$ −9° (*c* 0.11). Thus, the data argue that kelletinin II has the 3*S*,4*R* configuration.

Fractions A and B. Limited attention was paid to the remaining fractions obtained from silicic acid chromatography, since they were less abundant and less bioactive. Fractions A and B were shown by HPLC analysis to be mixtures of compounds absorbing at 260 nm. Both mixtures provided evidence for alditol *p*-hydroxybenzoates similar to kelletinin II (Table I). They differed, however, in the number of phenolic hydroxyl groups and in having aromatic esters replaced by aliphatic esters, as shown in the fragmentations below. Specifically, fraction A contained one or



more tris(isovalerates) and fraction B one or more mono(isovalerates) as derivatives of a deoxypentitol (presumably 2-D-deoxyribitol), with the remaining hydroxyls esterified by *p*-hydroxybenzoic acid. No attempt was made to assign sequences of esters along the alcohol backbones since ^{13}C and ^{1}H NMR spectra suggested complex mixtures. Thus, each of the less polar metabolites was found to be structurally related to kelletinin II.

Discussion

There is ample precedent for mollusks' storing ingested toxins,[4,5] yet kelletinins I and II appear to be examples of modestly bioactive compounds produced de novo by a mollusk. Isolation from the

Scheme I. Synthesis of Tetramethyl Ethers (**1b** and **2b**) of Kelletinins I and II

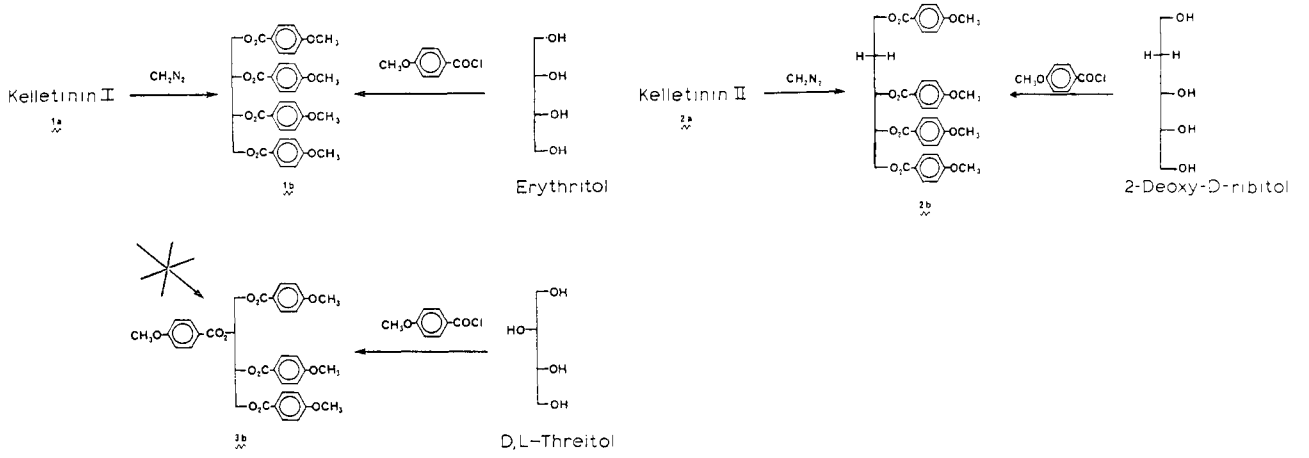
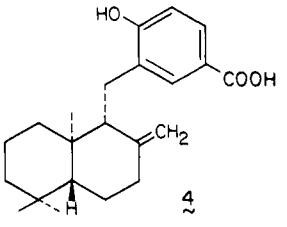


Table II. ^{1}H NMR Data for H_A–OR, H_B–OR, H_C Spin Systems

compound	H_A δ	H_A J, Hz	H_B δ	H_B J, Hz	H_C δ	H_C J, Hz
kelletinin I-$(OCH_3)_4$ (**1b**)[a]	5.86	m	4.79	1.7, 12.0	4.57	3.9, 12.0
synthetic **1b**[a]	5.85	m	4.79	1.6, 12.0	4.57	4.1, 12.2
synthetic **3b**[a]	5.87	m	4.68	3.4, 12.0	4.59	5.6, 12.0
kelletinin I-$(OAc)_4$ (**1c**)[b]	5.88	m	4.83	2.2, 11.9	4.66	4.1, 11.9
kelletinin I (**1a**)[b]	5.78	m	4.74	2.3, 12.1	4.56	5.2, 12.1
synthetic **1b**[b]	5.82	m	4.78	2.4, 12.2	4.61	5.4, 12.4
synthetic **3b**[b]	5.85	m	4.70	3.3, 12.1	4.59	5.5, 12.0

[a] CD_2Cl_2. [b] CD_3CN.

kidney and the hypobranchial gland[11] and detection in *Kelletia* specimens over a long time span support the hypothesis that these are intrinsic metabolites. Since most animals cannot biosynthesize aromatic amino acids,[12] lacking the shikimate pathway, the ultimate source of the *p*-hydroxybenzoic acid is presumably a plant species, although this connection is indirect, since *K. kelletii* is mainly carnivorous.[13] *Dictyopteris undulata* provides a reasonable dietary source for *p*-hydroxybenzoic acid since it incorporates it into zonaroic acid (**4**), a bioactive metabolite.[14] *D. undulata* and



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a *Zonaria* species, two of the dominant brown algae from the Santa Catalina collection sites, were analyzed for kelletinins but no trace of them could be detected in either. Nevertheless, biogenesis involving esterification of *p*-hydroxybenzoic acid from an alga by the mollusk appears attractive.

Experimental Section

IR spectra were recorded on a Perkin-Elmer spectrophotometer, Model 137, and UV spectra on a Perkin-Elmer Lambda 3 double-beam spectrophotometer. Optical rotations were measured with a Rudolph Research Autopol III polarimeter by using a 10-cm cell. NMR spectra were obtained, in part by D. G. Vander Velde or L. P. Johnson, with a Nicolet NTC 360-MHz spectrometer (^{1}H and ^{13}C) or with a JEOL FX60 spectrometer (^{13}C); all chemical shifts (δ) are reported as ppm downfield from the internal tetramethylsilane standard. Low-resolution EI mass spectra were measured by D. W. Phillipson, M. K. Cochran, or Dr. R. M. Milberg with a Finnigan MAT CH-5 DF spectrometer and high-resolution EI, as well as FD, and FAB mass spectra by J. C. Cook with a Finnigan MAT 731 instrument equipped with a multichannel signal analyzer.[15] Melting points were determined on a Thomas-Hoover capillary melting point apparatus and were not corrected. Elemental analyses were performed by J. Nemeth and his associates.

Preparative TLC and bioautography were carried out on commercial plates (Analtech, 20 cm × 20 cm × 2 mm or 10 cm × 2 cm × 0.25 mm, respectively). Gravity columns were prepared with commercial-grade silica gel (Brinkmann, 0.05–0.2 mm), Sephadex LH-20 (Pharmacia, 25–100 μm), or C_{18}-bonded phase (Waters Associates, Sep-PAKS) and reagent-grade solvents. A Waters Associates pump and variable-wavelength detector were used for HPLC on normal-phase (Chromanetics, LiChrosorb Si-60) or reversed-phase (Altex, Ultrasphere-ODS) columns eluted with glass-distilled solvents. Antimicrobial and CV-1 cytotoxicity data were measured at 100 μg/disk; L1210 inhibition was measured by Dr. L. H. Li and J. W. Culp at The Upjohn Co.

Collection and Extraction. *K. kelletii* mollusks (30) were collected by using Scuba techniques at −6- to −10-m depth on sandy flats surrounding Bird Rock near the Catalina Marine Science Center (118°29′ W latitude, 33°26′ N longitude). Specimens were immediately frozen, deshelled, and dissected into three portions, separating the orange foot muscle (191 g), the light-hued viscera (237 g), and the blackish digestive gland (109 g). Each portion was extracted with methanol–toluene (3:1) for an antimicrobial screen. The viscera were then homogenized in a Waring blender by using 2 L of methanol–toluene (3:1). The extract was filtered through Celite and partitioned into toluene and aqueous fractions by addition of 0.1 N sodium nitrate (0.5 L). The resulting aqueous solution was extracted sequentially with toluene (0.5 L), petroleum ether (2 × 0.5 L), diethyl ether (3 × 1 L) and 1-butanol (3 × 0.8 L). Each solvent extract was concentrated and tested for antimicrobial activity. Bioactivity was

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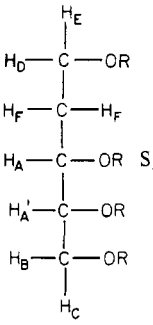

Table III. ^{1}H NMR Data for H_A—C—OR Spin Systems ($CDCl_3$)[a]

compound	H_A, H_A' δ	H_A, H_A' J	H_B δ	H_B J	H_C δ	H_C J	H_D δ	H_D J	H_E δ	H_E J	H_F δ	H_F J
kelletinin II-$(OCH_3)_4$	5.77	m	4.74	~12.0	4.56	5.8, 11.8	4.51	5.3, 11.3	4.38	6.5, 12.0	2.35	m
kelletinin II-$(OAc)_4$	5.79	m	4.79	3.4, 12.1	4.58	6.2, 12.1	4.54	6.4, 11.4	4.41	6.3, 11.4	2.36	m
synthetic **2b**	5.77	m	4.74	3.3, 11.9	4.56	6.0, 12.1	4.52	5.5, 11.2	4.38	6.7, 11.2	2.35	m

[a] Chemical shift (δ) in ppm; coupling constant (J) in Hz; m = unresolved multiplet.

detected mainly in the diethyl ether residue (487.1 mg)—15-mm zone of inhibition against *B. subtilis* and 28-mm zone of cytotoxicity vs. CV-1 cells.

Chromatographic Isolation of Kelletinins. The diethyl ether extract (487.1 mg) was subjected to preparative silica TLC (Et_2O) to provide three bioactive fractions: A (R_f = 0.56, 72.5 mg); B (R_f = 0.35, 8.4 mg); C (R_f = 0.25, 177 mg).

The bioactive components from the least polar band (A, 94.8 mg) were purified by Sephadex LH-20 column chromatography (2.1 × 36 cm) using methanol–dichloromethane (1:1) to afford fraction A-1 (50.7 mg): 13.5-mm zone vs. *B. subtilis* and 17-mm zone vs. CV-1 cells; UV (see Table I); IR ($CHCl_3$) 3500, 2900, 1730, 1710, 1610, 1260, 1170, 1110 cm^{-1}; ^{1}H NMR (C_6D_6) δ 8.14 (d, J = 8.7 Hz), 8.12 (d, J = 10.3 Hz), 8.03 (d, J = 8.6 Hz), 6.66 (d, J = 8.5 Hz), 6.64 (d, J = 7.2 Hz), 6.61 (m), 5.95 (m), 5.75 (dd, J = 4.1, 6.7 Hz), 5.67 (m), 5.61 (dd, J = 3.8, 7.4 Hz), 5.47 (m), 4.75 (dd, J = 2.7, 12.1 Hz), 4.67 (m), 4.46 (dd, J = 7.5, 12.1 Hz), 4.35 (dd, J = 5.8, 12.1 Hz), 4.26 (m), 4.20 (dd, J = 5.4, 11.7 Hz), 4.14 (m), 2.1 (m), 0.83 (m); ^{13}C NMR ($CDCl_3$) δ ca. 173 (several signals), 165.3 (s), 161.3 (s), 132.2 (d), 120.9 (d), 72.2, 71.8, 70.4, 69.3, 65.1, 62.2, 43.2 (t), 25.7 (d), 22.3 (q); EIMS (70 eV) *m/z* 271 (3.8), 200 (3.1), 183 (5.2), 182 (4.2), 155 (7.6), 121 (100), 98 (5.7), 85 (48.7), 57 (3.8); FDMS (10 mA) *m/z* 525, 524; FABMS (+ion) *m/z* 547, 525, 507, 423, 387; FABMS (–ion) 523, 439, 421; HRFABMS (+ion) *m/z* 547.2510 ($C_{27}H_{40}O_{10}Na$ requires 547.2501).

Fraction B was analyzed without further purification: 14-mm zone vs. *B. subtilis*; UV (see Table I); IR (Et_2O) 3400, 1710, 1605, 1595, 1500, 1305, 1250, 885, 760 cm^{-1}; ^{1}H NMR (CD_3CN) δ 7.86 (d, J = 8.4 Hz), 7.81 (d, J = 8.7 Hz), 5.6 (m), 4.66 (dd, J = 2.9, 11.9 Hz), 4.52 (dd, J = 5.9, 12.0 Hz), 4.42 (m), 4.15 (m), 2.17 (m), 1.27 (m), 0.90 (m), 0.84 (d); FABMS (+ion) *m/z* 603, 581, 479, 463, 443, 441, 423, 349, 322, 303, 121; FABMS (–ion) *m/z* 601, 579, 461, 459, 441, 439, 323, 151, 140, 139, 138, 122, 121; HRFABMS (+ion) 581.2011 ($C_{31}H_{33}O_{11}$ requires 581.2000).

The most polar material (C, 156.5 mg) was rechromatographed on a silica gel column (2.1 × 36 cm) by using a step gradient from petroleum ether to diethyl ether to give kelletinin-rich fractions C-1 through C-3 totalling 57.0 mg, where fraction C-3 (37.4 mg) consisted of a 1:3 mixture of **1a** and **2a**, respectively (as estimated by FABMS ion intensities): 15-mm zone vs. *B. subtilis*, 15-mm zone vs. *P. atrovenetum*, and 14-mm zone vs. CV-1 cells; ID_{50} 0.40 μg/mL ID_{90} > 1.0 μg/mL vs. L1210 cells; UV (see Table I); IR (Et_2O) 3400, 1710, 1600, 1590, 1260, 1230, 1010, 850, 770, 760 cm^{-1}; ^{1}H NMR (CD_3CN) δ 7.95–7.80 (m), 6.90–6.80 (m), 5.83 (m), 5.72 (m), 4.79 (dd, J = 2.1, 12.2 Hz), 4.74 (dd, J = 3.6, 12.1 Hz), 4.59 (dd, J = 6.2, 12.1 Hz), 4.44 (dt, J = 5.7, 11.4 Hz), 4.38 (dt, J = 4.8, 7.8 Hz), 2.37 (m); ^{13}C NMR Me_2SO-d_6 δ 71.7, 69.7, 69.6, 61.8, 60.7, 30.6 (t); FDMS (24–25 mA) *m/z* 616, 479; FABMS (+ion) *m/z* 617, 603; HRFABMS (+ion) 617.1639 ($C_{33}H_{29}O_{12}$ requires 617.1651).

Further chromatography of the least polar mixture (C-1, 5.3 mg) on C_{18}–Sep-PAK (2:1 methanol–water) led to the separation of **1a** (1.6 mg, in at least 80% excess over **2a**) as fraction C-1A: 15-mm zone vs. *B. subtilis* and 14-mm zone vs. *P. atrovenetum*; UV (EtOH) 259 nm (ε 24 700); (0.1 N HCl) 257 (ε 33 900), (0.1 N NaOH) 302 (ε 45 600); IR (Et_2O) 3400, 1710, 1600, 1350, 1260, 1010, 790, 775 cm^{-1}; ^{1}H NMR (CD_3CN) δ 7.87 (4 H, d, J = 8.7 Hz), 7.82 (4 H, d, J = 8.7 Hz), 6.84 (4 H, d, J = 8.7 Hz), 6.83 (4 H, d, J = 8.6 Hz), 5.78 (2 H, m), 4.74 (2 H, dd, J = 2.3, 12.1 Hz), 4.56 (2 H, dd, J = 5.2, 12.1 Hz); EIMS (70 eV) *m/z* 138 (47), 121 (100), 94 (67), 66 (20), 65 (21), 44 (22), 40 (6.8), 39 (9.4); HREIMS *m/z* 138.0314 ($C_7H_6O_3$ requires 138.0317), 121.0287 ($C_7H_5O_2$ requires 121.0290), 94.0415 (C_6H_6O requires 94.0419), 93.0338 (C_6H_5O requires 93.0390); FABMS (+ion and –ion), Figure 2; HRFABMS (+ion) 603.1445 ($C_{32}H_{27}O_{12}$ requires 603.1502), 695.1898 ($C_{35}H_{35}O_{15}$ requires 695.1975).

Methylation of Kelletinins I and II. Treatment of fraction C-1A (1.0 mg) with an excess of ethereal diazomethane followed by reversed-phase semipreparative HPLC provided kelletinin I tetramethyl ether (**1b**, 0.3 mg): UV (EtOH) 258 nm (ε 36 900); IR ($CHCl_3$) 1710, 1610, 1250, 1210, 1100, 1005, cm^{-1}; ^{1}H NMR (CD_2Cl_2) δ 7.98 (d, J = 8.8 Hz), 7.94 (d, J = 8.7 Hz), 6.92 (d, J = 8.4 Hz), 6.90 (d, J = 8.3 Hz), 3.85 (s) (see also Table II); FDMS (19–20 mA) *m/z* 658.

Treatment of fraction C-3 (2.0 mg) as above afforded a mixture of the tetramethyl ethers of kelletinins I (**1b**) and II (**2b**). Preparative silica HPLC (97:3 dichloromethane–diethyl ether) then separated **1b** (0.1 mg, cf. above for properties) from **2b** (0.5 mg): UV (EtOH) 258 nm (ε 79 900); IR ($CHCl_3$) 1710, 1610, 1250, 1210, 1090, 1005, 850 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.99 (d, J = 8.8 Hz), 7.92 (d, J = 8.8 Hz), 6.89 (d, J = 8.8 Hz), 6.87 (d, J = 8.7 Hz), 3.85 (s) (see Table III); FDMS (18–19 mA) *m/z* 672.

Acetylation of Kelletinins I and II. Acetic anhydride–pyridine (1:3) was added in excess to a mixture of kelletinins (13.6 mg), and the mixture was stirred at 80 °C overnight. Evaporation and trituration with methanol induced the crystallization of an off-white product that was purified by preparative silica HPLC (97:3 dichloromethane–diethyl ether), yielding **1c** (0.9 mg) and **2c** (3.1 mg). Kelletinin I tetraacetate (**1c**) had the following characteristics: UV (EtOH) 233 nm (ε 29 600); IR ($CHCl_3$) 1750, 1725, 1600, 1370, 1260, 1200, 1160, 1100, 1020, 940, 860 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 8.05 (d, J = 8.7 Hz), 8.02 (d, J = 8.6 Hz), 7.22 (d, J = 8.7 Hz), 7.15 (d, J = 8.5 Hz), 2.32 (s) (see also Table II); FDMS (20 mA) *m/z* 771 (M + H).

Kelletinin II tetraacetate (**2c**) had the following characteristics: $[\alpha]^{25}_D$ –9.1° (*c* 0.11, CH_2Cl_2); UV (EtOH) 234 nm (ε 36 000); IR ($CHCl_3$) 1750, 1725, 1600, 1365, 1260, 1190, 1160, 1100, 1010, 930, 860 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 8.0 (m), 7.1 (m), 2.38 (m), 2.32 (s) (see also Table III); EIMS (70 eV) *m/z* 756, 742 (<0.12), 713, 700 (<0.1), 562 (2.8), 520 (4.2), 163 (18.0), 138 (21.7), 121 (100), 43 (15.2); FDMS (15 mA) *m/z* 785 (M + H); HREIMS *m/z* 742.1863 ($C_{39}H_{34}O_{15}$, M – C_2H_2O, requires 742.1897).

Synthesis of Erythrityl Tetrakis(*p*-anisoate), 1b. Polyacylation[16] was achieved by dissolving erythritol (Aldrich, 120 mg, 1 mmol) in pyridine (3 mL) and adding *p*-anisoyl chloride (0.6 mL, 5.6 mmol) in 3 mL of chloroform. After being stirred for 4 h at room temperature, the mixture was diluted with 0.1 N hydrochloric acid (100 mL) and the product was extracted into diethyl ether. Following base and brine washes, the solution was dried with magnesium sulfate, evaporated, and triturated with methanol to precipitate **1b** as a white solid (228 mg, 35%). Analytically pure **1b** was obtained by recrystallization from absolute ethanol: mp 142–143 °C; UV (EtOH) 256 nm (ε 27 000); IR ($CHCl_3$) 1710, 1600, 1250, 1210, 1170, 1100, 850 cm^{-1}; ^{1}H NMR (CD_2Cl_2) δ 7.98 (*d*, J = 8.7 Hz), 7.94 (d, J = 8.8 Hz), 6.92 (d, J = 8.7 Hz), 6.90 (d, J = 8.7 Hz), 3.85 (s).

Anal. Calcd for $C_{36}H_{34}O_{12}$: C, 65.65; H, 5.20. Found: C, 65.14; H, 5.12.

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Synthesis of DL-Threityl Tetrakis(*p*-anisoate), 3b. DL-Threitol (Sigma, 120 mg, 1 mmol) was esterified with *p*-anisoyl chloride as described above, affording 331 mg (49%) of the ester (**3b**) that was recrystallized from absolute ethanol: mp 120–121 °C; UV (EtOH) 257 nm (ϵ 48 000); IR ($CHCl_3$) 1710, 1600, 1250, 1210, 1170, 1100, 850 cm^{-1}; ^1H NMR (CD_2Cl_2) δ 8.00 (d, J = 8.8 Hz), 7.92 (d, J = 8.7 Hz), 6.91 (d, J = 8.8 Hz), 6.88 (d, J = 8.8 Hz), 3.85 (s); EIMS (70 eV) m/z 658 (M, 0.1), 152 (76.7), 135 (100), 107 (7.6), 92 (15.7), 77 (22.8), 64 (14.9).

Synthesis of 2-Deoxy-D-ribityl Tetrakis(*p*-anisoate), 2b. 2-Deoxy-D-ribose (750 mg) was stirred with sodium borohydride (300 mg) in ethanol (20 mL) for 1 h at room temperature. The solution was acidified with glacial acetic acid and decationized with IR-120 ion-exchange resin (2.1 × 3.4 cm) packed in ethanol. The eluate was evaporated, reevaporated from methanol (3 × 15 mL), and vacuum-dried to remove all borate ester.[17] The residual yellow oil was dissolved in pyridine (2 mL) and added slowly, with shaking, to *p*-anisoyl chloride (0.7 mL) in benzene (3 mL). The reaction mixture was stoppered and left to stand at room temperature overnight and then diluted with water and extracted with diethyl ether. Workup of the organic extract, as described for **1b**, provided 380 mg of **2b**, which was recrystallized from absolute ethanol: $[\alpha]^{25}_D$ −8.4° (c 0.11, CH_2Cl_2); mp 134–135 °C; UV (EtOH) 260 nm (ϵ 51 700); IR ($CHCl_3$) 1710, 1610, 1250, 1200, 1170, 1100, 850 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.95 (m), 6.86 (m), 3.85 (s); FDMS (0 mA) m/z 672.

Anal. Calcd for $C_{37}H_{36}O_{12}$: C, 66.06; H, 5.39. Found: C, 65.64; H, 5.28.

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Deuterium Nuclear Magnetic Resonance Spectroscopy as a Probe of the Stereochemistry of Biosynthetic Reactions: The Biosynthesis of Retronecine

Gunnar Grue-Sørensen and Ian D. Spenser*

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Abstract: The mode of incorporation of ^2H from (*R*)- and from (*S*)-(1-^2H)putrescine into retronecine in *Senecio vulgaris* was determined by ^2H NMR spectroscopy. Retronecine, derived from (*R*)-(1-^2H)putrescine, was labeled with ^2H equally at positions 3-*re*, 5-*re*, 8, and 9-*si*. Retronecine from (*S*)-(1-^2H)putrescine was labeled with ^2H equally at positions 3-*si* and 5-*si*. These results establish the stereochemistry of five of the steps in the biosynthetic conversion of putrescine into retronecine.

The carbon skeleton of retronecine (**8**), the most abundant of the necine bases of the *Senecio* alkaloids,[1] is derived from two C_4 units related to ornithine.[2] Label from ornithine and from putrescine (**1**), its decarboxylation product, is incorporated nonrandomly into retronecine (**8**).[3] A molecule with a C_4–N–C_4 skeleton and C_{2v} symmetry, generated from two putrescine units, is a further intermediate.[3-5] There is some evidence that this nondissymmetric "dimeric" intermediate may be homospermidine (**5**).[5,6] The two routes from putrescine to the pyrrolizidine skeleton, shown in Scheme I, are consistent with the tracer evidence. Beyond the finding that retronecine is derived from L-ornithine[7,8] or L-arginine,[8] rather than from the D enantiomers, stereochemical aspects of retronecine biosynthesis have not hitherto received attention.

We have employed ^2H NMR spectroscopy to determine the stereochemical course of five of the steps of retronecine biosynthesis (Scheme I), involving transformations at the carbon atoms derived from C-1 of putrescine.

Results and Discussion

In two experiments, each with 120 plants of *Senecio vulgaris*, (*R*)-(1-^2H)putrescine dihydrochloride (**9**)[9] (98 atom % ^2H) in admixture with [1,4-^{14}C]putrescine dihydrochloride (experiment 1) and (*S*)-(1-^2H)putrescine dihydrochloride (**10**)[9] (87 atom % ^2H), together with [1,4-^{14}C]putrescine dihydrochloride (experiment 2), were administered by the wick method over a period of 12 days (June 1982). From each experiment a mixture of three alkaloids, senecionine (**11**), seneciphylline (**12**), and retrorsine (**13**), each containing retronecine as the necine base, was isolated.[3] The alkaloid mixture that was obtained contained senecionine, seneciphylline, and retrorsine in a molar ratio of ca. 5:4:1, as determined by ^1H NMR.[10]

The ^2H NMR spectra of the alkaloid mixture (in $CHCl_3$) obtained from each of the two experiments are shown in Figure 1. Chemical shifts were assigned by comparison with the corresponding ^1H NMR chemical shifts of the retronecine moiety of the alkaloids **11**, **12**, and **13** (Table I). Correlation of ^1H NMR spectra of retronecine[11,12] with spectra of 12-membered pyrrol-

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