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NATIVE AMERICAN FOOD AND MEDICINAL PLANTS 7.

ANTIMICROBIAL TETRONIC ACIDS FROM *Lomatium dissectum*[1]

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Abstract - A pair of homologous 2-alkenyl-3-hydroxy-penta-2,4-dien-4-olides has been isolated from the umbellifer Lomatium dissectum and found to be the principal antimicrobial metabolites in the crude extracts of the whole plant. These unstable tetronic acids were partially characterized from spectroscopic data, but chemical transformations were required to secure the complete structures. Details of the isolation, identification and bioassay results are provided; a probable biosynthetic pathway to these compounds is discussed.

Of the eighteen species which represent the genus Lomatium in the United States and Canada, only L. dissectum has a reputation as an untrustworthy foodstuff[2a] and is considered toxic by the Okanagan and the Kalispel[2b]. Icthyotoxicity and insecticidal activity[2b] have been attributed to L. dissectum and it has been used in the treatment of trachoma[3] and lung disease[4], as well as for colic and distemper in horses[5]. As part of our investigation of umbelliferous plants used by Native American nations, we report herein the isolation and identification of the antimicrobial constituents of Lomatium dissectum.

Lomatium dissectum is a much larger plant than the species of Lomatium which serve as food sources, such as L. cous and L. macrocarpon. A single, whole plant collected in the summer of 1983 yielded 162 g of dichloromethane soluble extracts (8.7 % of the fresh weight) and 71 g of water soluble material (3.1 % of the fresh weight). In vivo (PS) screening revealed no antineoplastic activity in the water solubles, but toxicity (@ 400 mg/kg) and slight life extension (T/C 110 @ 100 mg/kg) in the organic solubles. Antimicrobial assays indicated promising antifungal activity in the organic solubles and inhibition of bacterial growth in both extracts.

Solvent partitioning of the dichloromethane soluble extracts dispersed the antifungal activity over the hexane, carbon tetrachloride and chloroform solubles, while the antibacterial activity was found in all fractions. Gel permeation (Bio-Beads S-X4, S-X8 and Sephadex LH-20) and low pressure adsorption chromatography of the carbon tetrachloride solubles gave the mixture **1** as a relatively unstable colorless oil. This material tended to decompose, by apparent polymerization, to form an insoluble, colorless film. The two compounds could be separated and analyzed individually by capillary GC-MS, but all attempts at preparative separation ended in large losses to decomposition. Partial resolution of the derived acetates **2** by HPLC (Ultrasphere-Cyano) was achieved, but, again, decomposition claimed most of the mixture. Consequently, the pair was characterized as the mixture; additional quantities of **1** were obtained from the hexane and chloroform solubles. In all, the mixture **1** comprised nearly 15% of the total organic soluble extracts.

Mass spectral analysis provided the molecular formulas $C_{21}H_{34}O_3$ and $C_{19}H_{30}O_3$ for the mixture **1**, suggesting two compounds differing by only two methylene groups and indicating five sites of unsaturation. The ^{1}H-NMR spectrum, then, was something of a surprise in that it resembled that of a monounsaturated fatty acid. Only a pair of doublets near δ5.0, indicative of a 1,1-disubstituted olefin, belied a more complex structure for the antifungal complex.

Table I

^{13}C-NMR Data, sp^2 Carbons in 1,2,4,5[a]

C#	1	2[b]	4[c]	5
1	173.49	167.69	170.21	174.07
2	162.91	153.97	161.19	(48.70)[d]
3	105.19	121.09	105.66	197.65
4	149.75	149.00	149.74	150.59
5	93.49	92.20	91.03	95.59
12[e]	129.96	129.98	129.97	130.08
13[e]	129.56	129.50	129.50	129.45

[a]recorded in $CDCl_3$, data reported as δ units

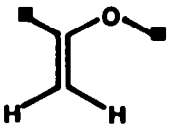
[b]acetate C=O: δ165.69

[c]OCH_3: δ 58.74

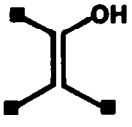
[d]quaternary sp^3

[e]assignments in each column may be reversed

The ^{13}C-NMR spectrum (see Table I) suggested that this 1,1-disubstituted olefin (δ149.75,s, and 93.49,t) was attached directly to oxygen; catalytic hydrogenation of 1 gave a tetrahydro derivative 3 whose chemical shifts and multiplicities (δ4.88, 1H,q; 1.49, 3H,d) confirmed part structure 1a and revealed that the other substituent on the olefin was a fully substituted carbon atom.



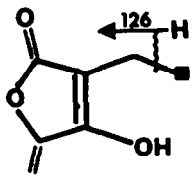
1a



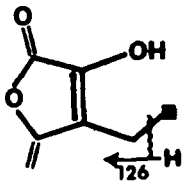
1b

While hydroxyl absorptions in both the IR and ^{1}H-NMR and reaction of 1 with diazomethane to form the methoxy derivative 4 seemed to support the "fatty acid" hypothesis, the chemical shifts of the O-methyl compounds (δ4.09) and O-acetate (δ2.32) groups and the absence of a characteristic carboxylic acid carbonyl group in the IR led to an alternative proposal, an enol. A positive ferric chloride test and appropriate ^{13}C-NMR resonances (δ162.91, 105.19, both singlets) provided part structure 1b and demonstrated that the enolic double bond was fully substituted and unaffected by the catalytic reduction.

Part structures 1a and 1b could be combined and extended to either 1c or 1d after consideration of the UV absorption maximum at 251 nm and the IR absorption at 1777 cm^{-1}. The base peaks in the mass spectra of 1-4 all corresponded to allylic cleavage as shown in 1c. Confirmation of 1c as the correct assemblage was obtained from the alkylation of 1 with CH_3I/Na_2CO_3; a mixture of O-methyl (4) and C-alkyl (5) products was obtained. The final evidence lay in 5, λ_{max} 222 nm; the ^{1}H-NMR signals for the 1,1-disubstituted olefin were separated and shifted downfield (δ5.42, 5.11), a pattern reminiscent of α-methylene cyclopentanones and γ-lactones. The infrared absorptions near 1820 and 1750 cm^{-1} correlated with the γ, δ-unsaturated γ-lactone and α-methylene cyclopentanone moieties, respectively. The dominant mass spectral fragmentation in 1-4 (allylic cleavage) gave way to cleavage at the quaternary sp^3 carbon in 5, as shown below.



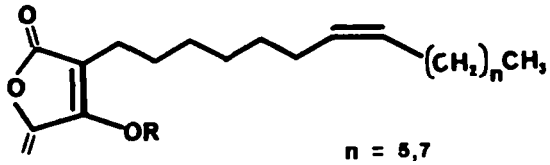
1c



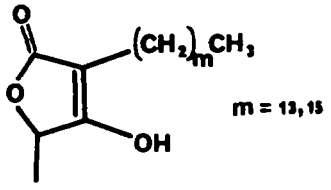
1d

With 1c securely in hand, all that remained was to locate and determine the geometry of the olefin in the alkenyl chain. The absence of an IR absorption around 970 cm^{-1} and calculations of the expected ^{13}C-NMR shifts for the olefinic carbons indicated a cis geometry. Ozonolysis of 1 and reduction and acetylation of the products provided 1-heptyl acetate and 1-nonyl acetate, analyzed by ^{1}H-NMR and GC-MS.

A more polar fraction, also analyzed by GC-MS and ^{1}H-NMR, corresponded to 6, the other fragment from the ozonolyzis of 1. Two acetates were indicated (δ2.02 and 2.13; M^{+}-HOAc and M^{+}-2 HOAc).



1 R = H
2 = Ac
4 = Me



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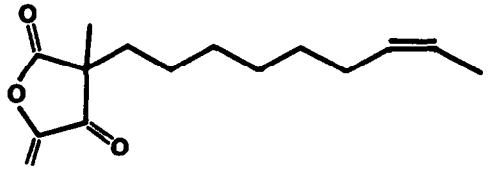

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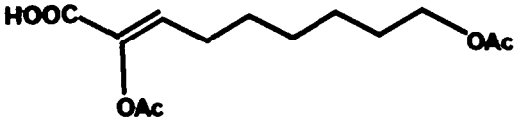
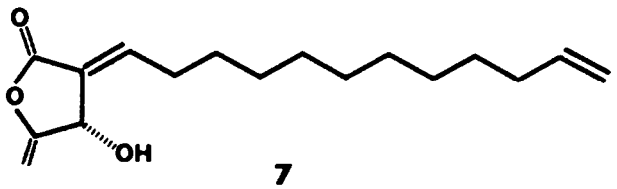


6

The mixture 1, then, was comprised of two homologs whose biogenesis apparently proceeds through a condensation of pyruvate and oleic or palmitoleic acid, enolization and lactone ring closure. The higher homolog has previously been found and characterized as a component of a complex mixture by Bohlmann's group from three umbellifers, Peucedanum veneturn, P. alsaticum and Seseli hippomarathrum[6]. A number of related tetronic acids are known from microbial sources, exemplified by multicolic and multicolosic acids from Penicillium multicolor[7]. Other, more distantly related compounds include the cytotoxic obtusilactone, 7, with both olefinic bonds exocyclic to the γ-lactone[8], and the dihydrotetronic acid 8 from the gorgonian Plexaura flava[9]. The fimbrolides, represented by 9[10,11] from the red alga Delisea fimbriata, would appear to be halogenated analogs of 1, although a different biosynthesis of these compounds has been proposed[12].



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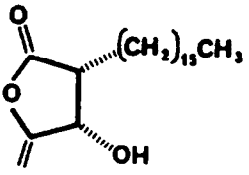

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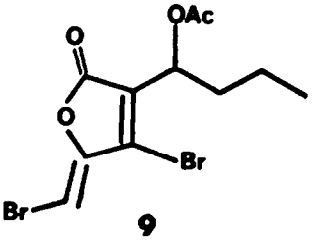

9

The tetronic acids (1) provide the antifungal and at least some of the antibacterial activity observed in the crude extracts. As illustrated in Table II, the mixture 1 and some of its transformation products (2,4,5) exhibited quite respectable zones of inhibition in the impregnated disk assay at relatively low concentrations; the data do reveal that any manipulation of the tetronic acid moiety, except for acetylation, results in a dramatic loss of activity.

Since 7 was reported to be cytotoxic[8], it is possible that 1 is responsible for the slight activity observed by us in the PS screen; the instability of 1 might be the cause of the low T/C value. Despite literature reports on insecticidal activity[2b], neither the crude extracts (organic and aqueous) nor 1 and its derivatives (2,4,5) exhibited any activity in tests with the tobacco hornworm, Manduca sexta.

Table II

Antimicrobial Activity of the Tetronic Acids 1a and Derivatives 2,4 and 5[a]

	Compound[c]					
Microorganism[b]	1(50)	1(12)	2(50)	2(12)	4(200)	5(200)
Staphylococcus aureus	6	4	4	2	-	1
Corynebacterium michiganense	7	5	6	4	NT[d]	NT[d]
Bacillus cereus	6	4	4	3	-	1
Xanthomonas campestris	5	1	5	3	1	2
Phythium ultimum	5	3	5	3	-	2
Rhizoctonia solani	12	7	5	3	-	1

[a]Zone of inhibition on inoculated, incubated plates reported in mm from edge of impregnated disk.

[b]All compounds were inactive against *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, *Candida tropicalis*, *Aspergillus terreus*, *Helminthosporium sativum*.

[c]Dosage, in μg/disk, in parentheses.

[d]Not tested

EXPERIMENTAL

NMR spectra were obtained with a Bruker WM-250 spectrometer with $CDCl_3$ as solvent and internal standard. Mass spectral analyses were performed on VG Instruments MM16F and 7070 EHF mass spectrometers. IR spectra were recorded with a Nicolet 5DX spectrophotometer; UV spectra were determined with a Varian G34 spectrophotometer.

Collection, Extraction and Initial Partitioning of *Lomatium dissectum*.

The plant was collected on a ridge near the Olsen Creek/Skunk Creek divide in the Bridger Mountains, about sixteen miles from Bozeman, Montana, and was frozen until extracted. The entire plant (1.878 kg fresh weight) was chopped into small pieces and ground in a Waring blender with MeOH. The solvent was removed by suction filtration and the marc was steeped overnight in fresh MeOH. Again the MeOH was removed and the plant residue was soaked in CH_2Cl_2 (2X). The MeOH extracts were reduced to an aqueous suspension, which was then equilibrated with the CH_2Cl_2 extracts. Evaporation of the CH_2Cl_2 phase gave 162.39 g of dark green oil; lypophilization of the aqueous phase yielded 71.06 g of tan solid.

A 38.43 g portion of the crude CH_2Cl_2 solubles was dissolved in 500 mL of 9:1 MeOH:H_2O and extracted with 300 mL of hexane (3x) to yield 28.76 g of hexane solubles. The aqueous MeOH phase was increased in polarity to 3:1 MeOH:H_2O and extracted with 300 mL of CCl_4 (3x) to yield 6.1 g of CCl_4 solubles. The remaining aqueous phase was increased in polarity to 13:7 MeOH:H_2O and extracted with 300 mL of $CHCl_3$ (3x) to yield 2.65 g of $CHCl_3$ solubles. The MeOH was then removed from the upper phase, in vacuo, and the residual aqueous suspension was extracted with 300 mL portions of EtOAc (3x) to yield 409 mg of EtOAc solubles. The remaining water solubles were freeze dried to give 511 mg of tan solid.

Isolation of the Tetronic Acids

A portion (2.09 g) of the CCl_4 soluble material was permeated through Bio-Beads S-X4 (column 78 x 4 cm) with 4:4:1 CH_2Cl_2-hexane-EtOAc to yield 10 fractions. Fraction 6 (875 mg) was then permeated through Sephadex LH-20 (column 142 x 2.5 cm) with 1:1 MeOH-CH_2Cl_2 to yield 5 fractions. Fraction 5 (512 mg) was then chromatographed on silica gel (column 38 x 2.5 cm) at low pressure (10 psi N_2); elution with 1:2 MeOtBu-hexane gave 5 fractions. Fraction 4, 454 mg, a clear faint yellow oil, was the mixture 1; λ_{max} (cyclohexane) 251 nm; ^{1}H-NMR: δ5.33 (2H, m), 5.18 (1H,J=3), 5.09 (1H,d,J=3), 5.09 (1H,d,J=3), 2.27 (2H,t,J=7.5), 1.97 (4H,m), 1.49 (2H,m),1.27 (XH, br s), 0.85 (3H,t,J=6.9); ν_{max}(CCl_4) 3138, 3007, 2927, 2858, 1777, 1626, 1270, 1063 cm^{-1}; MS: m/z 334.2525 (M^+, 1%, calc'd for $C_{21}H_{34}O_3$-334.2505), 306.2186 (M^+,41, calc'd for $C_{19}H_{30}O_3$-306.2193), 126.0333 (100, calc'd for $C_6H_6O_3$-126.0316).

Acetylation of 1

A solution of 95 mg of 1 in 2 mL dry pyridine was purged with N_2; 0.5 mL Ac_2O and one crystal of 4-N,N-dimethylaminopyridine were added and the mixture was refluxed for 6 hours. The pyridine was removed, in vacuo, and the residue was passed through a 1 cm silica gel plug with hexane-MeOtBu (2:1). The residue was permeated through Sephadex LH-20 as described earlier; fraction 5 of five fractions was comprised of 58 mg 2; ^{1}H-NMR: δ5.32 (2H,m), 5.06 (1H,d,J=3), 4.77 (1H,d,J=3), 2.33 (3H,s), 2.23 (2H,t,J=7.7), 1.98 (4H,m), 1.50 (2H,m), 1.27 (XH,br s), 0.85 (3H, t,J=6.7); $\nu_{max}$$CCl_4$ 3006, 2933, 2856, 1788, 1651, 1368, 1171, 1060 cm^{-1}; MS: m/z 376/348 (M^+), 334/306, 168, 126.

Catalytic Reduction of 1

To a solution of 92.9 mg of 1 in absolute ethanol were added ~20 mg Pd/C; the system was vented and purged with H_2. The mixture was shaken 6 hours at 10 psi H_2, then filtered through Hyflo Super Cel to yield, after evaporation of solvent, 92 mg of an off-white solid. This material was permeated through Sephadex LH-20 as described above to yield 4 fractions. Fraction 2, 38 mg white waxy solid, was 3; ^{1}H-NMR: δ4.88 (1H,q,J = 6.6). 2.16 (2H,t,J = 7.5), 1.49 (3H,d,J = 6.6), 1.23 (XH,br s), 0.86 (3H,br t); MS: m/z 338/310 (M^+; 3,20%), 253/225 (3,6), 128 (82), 115 (100).

Methylation of 1 with Diazomethane

Diazomethane was bubbled into a solution of 283 mg 1 in 35 mL Et_2O until a faint yellow color persisted. The mixture was evaporated to give a light yellow oil (290 mg), which was subjected to low pressure silica gel chromatography; elution with hexane-MeOtBu-EtOAc (8:3:1) at 10 psi N_2 gave 127 mg unreacted 1 and 102 mg of 4, a colorless oil; λ_{max} (cyclohexane) 253 nm; ^{1}H-NMR: δ5.31 (2H,m),4.93 (1H,d,J = 2.4),4.91 (1H,d,J = 2.4), 4.09 (3H,s), 2.42 (2H, t, J = 7.7), 2.00 (4H, m), 1.27 (XH,br s) 0.98 (3H,t,J = 6.8); ν_{max}(CCl_4) 3007, 2930, 2853, 1779, 1637, 1276, 1060 cm^{-1}; MS: m/z 348/320 (M^+; 8, 0.4%), 140 (100).

Methylation of 1 with Na_2CO_3/MeI

A solution of 227 mg of 1 in 25 mL anhydrous acetone was purged with N_2 prior to addition of 60 mg anhydrous Na_2CO_3 and 1 mL MeI. The resulting mixture was refluxed (10 hrs) until TLC showed that no 1 remained. After cooling the solution was filtered to remove solids; the pH was adjusted to ~6 with 4% HCl and the acetone was removed in vacuo. The remaining aqueous suspension was extracted with CH_2Cl_2 (4 x 20 mL). Evaporation of the combined dichloromethane phase gave 205 mg light orange oil. Low pressure chromatography on silica gel (49 x 3.0 cm column) gave two major fractions, A (71 mg) and B (82 mg), eluted with hexane-MeOtBu (500:1). Both fractions were permeated through Sephadex LH-20; Fraction B gave 73 mg of 4 and Fraction A gave 60 mg of the keto-lactone 5; λ_{max} (cyclohexane) 222 nm; ^{1}H-NMR: δ 5.42 (1H, d, J=2.7), 5.33 (2H,m), 5.11 (1H,d,J=2.7), 1.97 (4H,m), 1.78 (2H,m), 1.32 (3H,s), 1.24 (XH, br s), 0.86 (3H,t,J=6.6); ν_{max}(CCl_4) 3007, 2926, 2856, 1823, 1754, 1648, 1379, 1266, 1094, 1048 cm^{-1}; MS: m/z 348/320 (M+;3,7%), 125(11).

Ozonolysis of 1

A solution of 130 mg of 1 in 30 mL anhydrous CH_2Cl_2 was exposed to ozone for 5 hours at -15°C. The resulting sky blue solution was purged with N_2 and 30 mL anhydrous EtOH and 125 mg of $NaBH_4$ were added. The mixture was refluxed gently for 2 hours. Then 10 mL of acetone were added and the solution was refluxed another 0.5 hours. The solution was evaporated to dryness and then taken up in 50 mL of H_2O. This basic solution was made acidic (pH-3) with 5% HCl and extracted with CH_2Cl_2 (4 x 20 mL); the CH_2Cl_2 phase was filtered through anhydrous Na_2SO_4 and evaporated to leave a thin clear oil (111 mg). This reaction mixture was then acetylated with 1 mL pyridine and 0.5 mL Ac_2O at reflux for 2 hours. After 2 hours 20 mL MeOH were added and the solution was reduced in vacuo to a dark brown oil. This oil was washed through a silica gel plug with CH_2Cl_2-MeOH (9:1) to yield 64 mg of a light yellow oil. GC-MS analysis of the mixture revealed the presence of n-heptyl acetate, n-nonyl acetate, and the acid 6. Relevant spectral data:

n-heptyl acetate: m/z 157 ($M-1^+$, 2%), 97 (15), 43 (100).

n-nonyl acetate: m/z 185 ($M-1^+$, 7%), 143 (29), 43 (100).

6: m/z 272 (M^+,9%) 271 ($M-1^+$,50) 211 (3), 151 (9), 95 (59), 43 (100);

^{1}H-NMR (partial): δ4.99 (1H,t,J=7), 4.10 (2H,t,J=7), 2.28

(2H,dt,J=7), 2.13 (3H,s), 2.02 (3H,s).

Acknowledgements

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