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ISOLATION AND STRUCTURE ELUCIDATION OF HYMATOXINS B - E AND OTHER PHYTOTOXINS FROM *HYPOXYLON MAMMATUM* FUNGAL PATHOGEN OF LEUCE POPLARS

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Abstract: *Two groups of phytotoxic substances were isolated from a culture filtrate of the plant pathogenic fungus* Hypoxylon mammatum *(Wahl.) Miller, responsible of the canker formation in aspen. The main group consists of a mixture of original diterpenes, hymatoxins, from which have been isolated hymatoxins A, B, C, D and E that belong to the pimarane class. The minor group of toxins is composed of isomeric 3,4,5- and 3,4,8-trihydroxynapthalenones. The structures of the isolated compounds were determined by spectroscopic methods.*

INTRODUCTION

The fungus *Hypoxylon mammatum* (Wahl.) Miller (Xylariaceae) causes a stem canker in certain poplars of the Leuce section, especially in aspens from North America (*Populus tremuloides*) and Europe (*P. tremula*) and also in some poplars of the Tacamahaca section (*P. trichocarpa*). The disease in nature is characterized by a flattened sunken surface with a yellow-orange margin and an inhibition of host callusing. Hubbes [1] suggested that the fungus produced diffusible substances toxic to the living tissues. Later, Schipper [2], Manion [3] and Stermer [4] showed that the culture filtrate of *H. mammatum* induced necrosis of bark and leaves of sensitive aspens and contained toxic substances responsible for canker development. In addition, it was observed that necrosis of leaves induced by the culture filtrate occurred more easily as the poplar clones were more susceptible to cankering under natural conditions, thus suggesting the involvement of host-specific toxins. However the high correlation of disease incidence in wild clones with sensitivity to toxic culture filtrate was not reproducible in a subsequent investigation [5]. In order to test clonal reaction, Pinon has established culture conditions for the fungus to give optimum of toxin production and has developed two toxicity bioassays representative of the disease in nature. The first one, a leaf bioassay, shows the necrosis of

the leaves leading to a blackening, induced by the toxins [6]; the bioassay, a cambium bioassay, measures the inhibition of cambium development in the presence of toxins [7].

By using these two bioassays we undertook the isolation of the bioactive compounds from a phytotoxic culture of *H. mammatum*. We have already reported the isolation and structural determination, based on spectroscopic evidence, of hymatoxin A, one of the main toxins, which is a diterpene of the pimarane class [8]. We now report on the other phytotoxins that we have characterized from the broth of *H. mammatum*..

RESULTS AND DISCUSSION

Isolation:

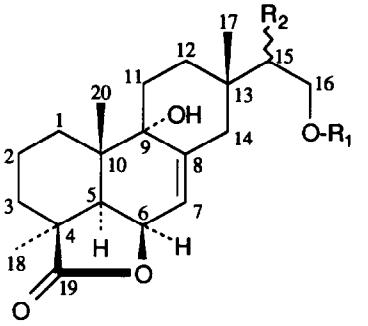
H. mammatum was cultivated on a wort medium at 27°C for 5 weeks in Erlenmeyer flasks. Two procedures were used successively for the extraction and isolation of the toxins from the toxic culture filtrate. In the first, the filtrate was concentrated under reduced pressure at 40°C to 10% of its original volume and then extracted several times with *n*-butanol. The toxic extract was fractionated by repetitive chomatography over reversed and direct silica gel phases, and each fraction controlled with the foliar and cambium bioassays, leading to pure hymatoxins A (**1**), B (**2**), C (**3**), D (**4**) and E (**5**). A second procedure involved XAD amberlite resins for adsorbing the toxins from the culture filtrate. The toxins were desorbed from the collected resins with a methanol/water gradient from water to pure methanol. The last fractions contained the hymatoxins that were purified in the usual way. The first fractions were extracted with EtOAc and from this extract, were isolated two other toxic compounds, the 3,4,8-naphthalenone, **6**, and the 3,4,5-naphthalenone, **7**.

Structural analysis:

Hymatoxin A ($C_{20}H_{29}O_7S^-$) was the major component of the hymatoxin mixture. Its structure, **1**, has been previously determined from a spectroscopic study involving ^1H and ^{13}C NMR analysis. The connectivities of carbon atoms was established from INADEQUATE experiments and the relative stereochemistry from NOE measurements [8].

Hymatoxin B was obtained as a colourless amorphous solid, soluble in water and methanol, and having optical activity: $[\alpha]_D^{20}$ -46.6 (c = 1.0, CH_3OH). Its negative ion FAB mass spectrum showed the pseudomolecular ion $[M]^-$ at *m/z* 429 in agreement with the molecular formula $C_{20}H_{29}O_8S^-$: hymatoxin B has an oxygen atom more than hymatoxin A. Its IR spectrum is similar to that of hymatoxin A, indicating that the molecule contains a carbonyl group of a γ-lactone (ν : 1766 cm^{-1}), but suggests the presence of an additional hydroxyl group. In the ^{13}C nmr spectrum, all the 20 individual resonances are exhibited and a ^{13}C J-modulated experiment shows 3 methyl, 7 methylene, and 4 methine groups in addition to six quaternary carbon atoms; the main difference, as compared to the spectrum of hymatoxin A, is the replacement of a methylene group (δ_C= 44.64 ppm) by a methine (δ_C= 79.00 ppm), suggesting that the additional oxygen atom is involved in a secondary alcohol function. This function was easily located at carbon atom -15, from analysis of the ^1H-^1H and ^1H-^{13}C COSY spectra: the deshielded methine proton at δ_H = 3.504 ppm shows vicinal coupling to the methylene (δ_H = 4.237 and 3.922 ppm) which is linked to the sulphate group. The total assignment of ^1H and ^{13}C NMR spectra is accomplished through ^1H-^1H COSY and ^1H-^{13}C COSY

experiments (Tables 1 and 2). The results led to the assignment of hymatoxin B as structure **2**, showing that this compound is the 15-hydroxy derivative of hymatoxin A.



1 $R_1 = SO_3^-$, $R_2 = H$
2 $R_1 = SO_3^-$, $R_2 = OH$
5 $R_1 = R_2 = H$

Cn°	1		2		3		4		5	
1	28.42	*t*	28.39	*t*	41.22	*t*	35.30	*t*	28.39	*t*
2	19.15	*t*	19.14	*t*	20.93	*t*	20.75	*t*	19.14	*t*
3	29.60	*t*	29.59	*t*	39.62	*t*	38.71	*t*	29.56	*t*
4	43.92	*s*	43.91	*s*	48.0	*s*	48.0	*s*	43.86	*s*
5	45.10	*d*	45.14	*d*	52.99	*d*	56.54	*d*	45.10	*d*
6	75.60	*d*	75.62	*d*	25.66	*t*	129.20	*d*	75.48	*d*
7	120.64	*d*	120.91	*d*	122.54	*d*	128.78	*d*	120.54	*d*
8	146.82	*s*	146.58	*s*	136.11	*s*	138.59	*s*	146.83	*s*
9	73.75	*s*	73.68	*s*	52.99	*d*	50.67	*d*	73.81	*s*
10	39.37	*s*	39.25	*s*	36.96	*s*	44.36	*s*	39.26	*s*
11	27.44	*t*	27.07	*t*	22.10	*t*	20.00	*t*	27.40	*t*
12	33.41	*t*	29.81	*t*	38.28	*t*	38.66	*t*	33.48	*t*
13	34.46	*s*	38.97	*s*	34.06	*s*	35.17	*s*	34.31	*s*
14	45.14	*t*	41.42	*t*	48.36	*t*	134.28	*d*	45.28	*t*
15	44.64	*t*	79.00	*d*	44.87	*t*	43.53	*t*	48.23	*t*
16	65.85	*t*	70.46	*t*	66.01	*t*	66.52	*t*	59.03	*t*
17	22.22	*q*	17.80	*q*	22.10	*q*	28.01	*q*	22.30	*q*
18	25.11	*q*	25.13	*q*	29.91	*q*	28.81	*q*	25.12	*q*
19	185.69	*s*	185.69	*s*	181.60	*s*	181.27	*s*	185.58	*s*
20	22.71	*q*	22.70	*q*	14.81	*q*	12.35	*q*	22.70	*q*

Table 1: ^{13}C NMR chemical shift values of hymatoxins A, **1**, B, **2**, C, **3**, D, **4** and E, **5**.
(δ ppm, *m* : multiplicity, *s* singlet, *d* doublet, *t* triplet, *q* quartet; CD_3OD, TMS, 100 MHz)

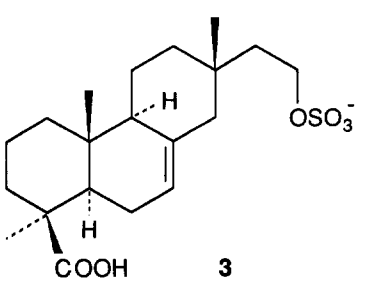
Hymatoxin C, **3**, is a colourless amorphous solid, optically active $[\alpha]_D^{20}$ +10.1. The negative ion FAB mass spectrum shows a molecular ion peak at *m/z* 399 $[M]^-$ corresponding to the molecular formula $C_{20}H_{31}O_6S^-$. The IR spectral band at ν 1691 cm^{-1} indicates the presence of a carboxylic acid function. The twenty signals of the ^{13}C NMR spectrum were assigned as follows: three CH_3, nine CH_2, three CH and five quaternary carbon atoms. A comparison of ^1H and ^{13}C NMR spectra with those of hymatoxin A indicated similar chemical shifts of the chain carrying the sulfate group but differed mainly in rings *A* and *B*. The structure of this moiety was elucidated on the basis of ^1H-^1H and ^1H-^{13}C COSY spectra. The ethylenic proton H-7 (δ_H = 5.360 ppm) showed a vicinal coupling (5.9 Hz) with the proton at δ_H = 2.467 ppm belonging to a methylene group (C-6) and showed cross-peaks not only with this methylene but also with an other methylene (C-14) and a methine (C-9).

	A, 1			B, 2			C, 3		
Cn°	δ	*m*	J (Hz)	δ	*m*	J (Hz)	δ	*m*	J (Hz)
1*ax*	1.703	*m*		1.70	*m*		1.084	*ddd*	13.0, 12.6, 3.7
eq	1.301	*m*		1.30	*m*		1.891	brd	12.6
2*ax*	1.688	*m*		1.69	*m*		1.958	*ddddd*	13.8,13.7,13.0,3.3,3.3
eq	1.556	*m*		1.55	*m*		1.436	*ddddd*	13.8, 3.7, 3.7, 3.3, 3.3
3*ax*	1.450	*ddd*	14.4, 9.0, 5.6	1.454	*ddd*	14.5,8.5,6.0	1.051	brddd	13.7, 13.2, 3.7
eq	2.080	*ddd*	14.4, 7.5, 5.6	2.084	*ddd*	14.5,7.3,4.9	2.135	brd	13.2
5*ax*	2.385	*d*	5.0	2.394	*d*	5.0	1.335	*dd*	12.1, 4.3
6*ax*							2.467	brddd	14.0, 12.1, 5.9
eq	4.853	*dd*	5.0, 5.0, 3.0	4.860	*dd*	5.0,4.9,3.0	2.135	brdd	14.0, 4.3
7	5.743	*dd*	5.0, 2.5	5.738	*dd*	4.9, 2.1	5.360	brd	5.9
9*ax*							1.675	brd	#10
11*ax*	1.862	*ddd*	14.5, 14.0, 4.1	1.86	*m*		1.31	*m*	
eq	1.535	*ddd*	14.5, 3.0, 3.0	1.55	*m*		1.56	*m*	
12*ax*	1.731	*ddd*	14.0, 13.0, 3.0	1.84	*m*		1.28	*m*	
eq	1.413	*dddd*	13.0,4.1,3.0,3.0	1.36	*m*		1.55	*m*	
14*ax*	2.373	*ddd*	14.7, 3.0, 2.5	2.397	*ddd*	15.0, 3.0, 2.1	1.917	brs	
eq	2.060	*dd*	14.7, 3.0	2.214	*dd*	15.0, 2.8	1.917	brs	
15	1.654	*t*	7.3	3.504	*dd*	8.5, 2.6	1.584	*t*	7.4
16	4.129	*t*	7.3	4.237	*dd*	10.5, 2.6	4.094	*t*	7.4
				3.922	*dd*	10.5, 8.5			
17	0.896	*s*		0.857	*s*		0.820	*s*	
18	1.289	*s*		1.293	*s*		1.227	*s*	
20	0.961	*s*		0.961	*s*		0.834	*s*	

Table 2: ^{1}H NMR spectral data of hymatoxins A (**1**), B (**2**), C (**3**), D (**4**) and E (**5**)

The proton at δ_H = 2.467 ppm has a geminal partner at δ_H = 2.135 ppm ($^2J_{H\text{-}H}$ = 14.0 Hz) which was in a *trans*- pseudoaxial disposition with methine H-5 (δ_H = 1.335 ppm) in a six-membered ring, as indicated by their large vicinal coupling constant value ($^3J_{H\text{-}H}$ = 12.1 Hz). The signal at δ_H = 1.675 ppm in the ^{1}H NMR spectrum was assigned to the methine proton H-9. The large value of its coupling constant (^3J = 10 Hz) with one proton from the C-11 methylene group indicates that it is axial. Long-range couplings were observed in the ^{1}H-^{1}H COSY spectrum between methyl H_3-20 and both protons H-1*ax* and H-5, and between methyl H_3-17 and both protons H-12*ax* and H-14*ax* indicative of *trans* diaxial dispositions [9].

As no cross-peak was detected between methyl H_3-18 and proton H-5*ax*, this methyl was in an equatorial position, as in the hymatoxin A structure. A *W* $^4J_{H\text{-}H}$ coupling was also detected between protons H-12*eq* and H-14*eq*, in agreement with their di-equatorial disposition. In sum, the data are only compatible with structure **3** for hymatoxin C.



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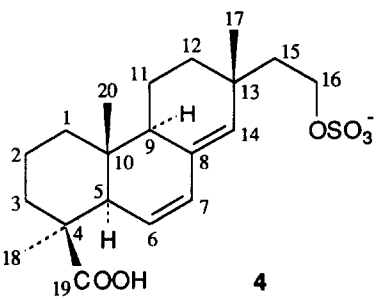


4

D, 4			E, 5		
δ	*m*	J (Hz)	δ	*m*	J (Hz)
1.129	*ddd*	13.9, 13.4, 4.0	1.70	*m*	
1.743	*ddd*	13.4, 3.5, 3.5	1.29	*m*	
1.888	*ddddd*	13.9,13.8,13.8,3.5,3.5	1.69	*m*	
1.52	*ddddd*	13.8, 4.2, 4.0, 3.5,3.5	1.56	*m*	
1.082	*ddd*	13.8, 13.7, 4.2	1.451	*ddd*	14.3, 8.5, 5.8
2.177	*ddd*	13.7, 3.5, 3.5	2.083	*ddd*	14.3, 7.2, 4.8
2.155	*dd*	3.1, 2.0	2.382	*d*	5.0
6.089	*dd*	10.1, 2.0	4.849	*ddd*	5.0, 4.9, 3.0
5.895	*dd*	10.1, 3.1	5.732	*dd*	4.9, 2.5
1.956	br*dd*	11.0, 5.0			
1.40	*m*		1.854	*ddd*	14.0, 13.9, 4.2
1.65	*m*		1.527	*ddd*	14.0, 3.6, 2.7
1.49	*m*		1.715	*ddd*	13.9, 12.8, 3.6
1.53	*m*		1.372	*dddd*	12.8, 4.2, 2.8, 2.7
			2.360	*ddd*	14.9, 3.0, 2.5
5.445	br*s*		2.006	*dd*	14.9, 2.8
1.687	*t*	7.4	1.530	*t*	7.7
4.077	*dt*	10.0, 7.4	3.673	*t*	7.7
4.022	*dt*	10.0, 7.4			
			0.864	*s*	
1.268	*s*		1.290	*s*	
0.650	*s*		0.961	*s*	

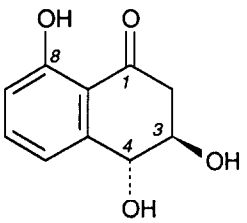
(δ ppm; *m* : multiplicity; br: broad; CD_3OD, TMS as int. standard, 400 MHz).

The HR negative ion FAB MS of hymatoxin D revealed the molecular ion at *m/z* 397.1703 corresponding to $C_{20}H_{31}O_6S^-$. Hymatoxin D thus contains two hydrogen atoms less than hymatoxin C. Their IR spectra are similar especially for the carbonyl absorption at 1691 cm^{-1} assigned to a carboxylic acid group. The UV spectrum of hymatoxin D is characteristic of a conjugated diene showing absorptions at λ 240.0 and 247.8 nm. The ^1H and ^{13}C NMR spectra of hymatoxin D, **4**, differ mainly from those of hymatoxin C in the ethylenic part of the molecule. The ^{13}C NMR spectrum of hymatoxin D displays four sp2 carbon atoms between δ_C 128 and 139 ppm, three of which were protonated (Table 1). The three ethylenic protons detected in the ^1H NMR spectrum form a >CH-CH=CH-C=CH- system as shown by coupling analysis. The ethylenic proton at δ_H 5.895 ppm (H-7) shows a vicinal coupling ($^3J_{H\text{-}H}$ = 10.1 Hz) with the proton at δ_H 6.089 ppm (H-6) and both (H-6 and H-7) show long range couplings with proton H-5 (δ_H = 2.155 ppm). The remaining ethylenic proton at δ_H 5.445 ppm, which gives an allylic coupling with H-9 and a *W* long-range coupling with H-12*eq* , as shown by cross-peaks on the ^1H-^1H LR COSY, was assigned to H-14. Reciprocal assignments of H-6 and H-7 were accomplished by NOE difference measurements, as strong NOEs were observed between methyl-18 and both H-5 and H-6, between H-6 and H-5, and between H-7 and H-14. Weaker NOEs were detected between H-14 and both methylene-15 and methyl-17. The double bonds were thus located as indicated in structure **4**, which was thus assigned to hymatoxin D.

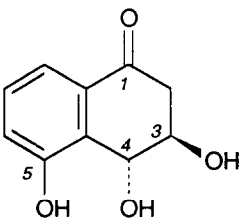
Hymatoxin E, **5**, was also obtained as a non crystalline, optically active compound ($[\alpha]_D^{20}$ -82.9). The HR EI MS (positive mode) shows the molecular ion $[M]^{+\bullet}$ at *m/z* 334.2076 corresponding to $C_{20}H_{30}O_4$. The IR absorption at 1750 cm^{-1} indicates this compound to have the same lactone moiety as

hymatoxin A, but the two strong absorption bands between 1210 and 1260 cm-^{1}, characteristic of the sulphate group, were not observed. The ^{1}H and ^{13}C NMR spectra are almost identical with those of hymatoxin A and the ^{1}H-^{1}H COSY spectrum leads to the same substructures. They only differ in the chemical shift of methylenes -15 and -16 which are weakly shielded in hymatoxin E. As this compound contains no sulphate group but a secondary alcohol group located at C-16 (δ_C = 59.03 ppm), structure **5** was assigned to hymatoxin E.

The EI MS of 3,4,8-trihydroxy-1-tetralone, **6**, shows the molecular ion $[M]^{+\bullet}$ at *m/z* 194 in agreement with the molecular formula $C_{10}H_{10}O_4$. The ^{1}H and ^{13}C NMR spectra were indicative of a trisubstituted aromatic ring, a carbonyl carbon (δ_C = 204.3 ppm) and an ABXX' proton spin system analysed as -CH_2-CH(OH)-CH(OH)-, which close the second ring to form a naphthalenone nucleus. The methinoxy proton at δ_H = 4.651 ppm (doublet, J = 6.8 Hz) was assigned the C-4 position, as a NOE effect is observed between this proton and the aromatic proton at δ_H =7.167 ppm (H-5). The C-4 proton is axial as it gave a large coupling constant with the proton at C-3 and a NOE effect with one proton at C-2 (H-2*ax*) compatible with structure **6**. This compound has been previously isolated from a *Pyricularia oryzae* culture [11,12].



6



7

The IR spectrum of 3,4,5-trihydroxy-1-tetralone, **7**, shows strong absorptions of a conjugated carbonyl (ν: 1685 cm-^{1}) and of hydroxyl groups (ν: 3350 cm-^{1}). The EI mass spectrum has a molecular ion $[M]^{+\bullet}$ at *m/z* 194 ($C_{10}H_{10}O_4$). These data and the NMR spectra suggest that it is an isomer of tetralone **6**: the ^{1}H NMR spectrum exhibits the signals of a methylene and two methinoxy protons forming a -CH_2-CH(OH)-CH(OH)- system, and three aromatic protons. The relative stereochemistry of this compound was determined from a X-Ray crystallographic study of a single crystal. The aromatic hydroxyl was clearly at C-8 and the aliphatic one's at C-3 and C-4 in a *trans* relative disposition (Figure 1). A 3,4,5-trihydroxy-1-tetralone with the same relative stereochemistry has been reported in a *P. diversum* var. *aureum* culture [10].

CONCLUSION

Several toxins belonging to two different chemical groups have been isolated from the culture filtrate of *H. mammatum*, and their chemical structure (relative configuration) determined. The first group consists of diterpenes from the pimarane class, having either a sulphate group (hymatoxins A to D) or a hydroxyl group (hymatoxin E) at C-16. They are structurally related to momilactones A and B, which belong to the same diterpene class and are growth and germination plant inhibitors [13,14]. It should be noted that callus formation inhibition observed with hymatoxins may be compared to the growth inhibition by momilactones.

	X/A	Y/B	Z/C	UEQ
C (1)	-0.0061 (2)	-0.0777 (2)	-1.2975 (3)	0.036 (0)
O (1)	-0.0447 (2)	-0.0634 (2)	-1.4467 (2)	0.062 (0)
C (2)	-0.1207 (3)	-0.0114 (2)	-1.1091 (3)	0.031 (0)
C (3)	-0.0194 (3)	-0.0240 (2)	-0.9815 (3)	0.027 (0)
O (3)	-0.1826 (2)	-0.1146 (2)	-1.0821 (2)	0.033 (0)
C (4)	-0.1400 (2)	-0.0921 (2)	-0.9404 (2)	0.027 (0)
O (4)	-0.0131 (2)	-0.1751 (2)	-0.8051 (2)	0.034 (0)
C (5)	-0.4275 (3)	-0.2473 (2)	-1.1261 (3)	0.033 (0)
O (5)	-0.4824 (2)	-0.2512 (0)	-0.9580 (2)	0.040 (0)
C (6)	-0.5333 (3)	-0.3210 (3)	-1.2933 (3)	0.045 (0)
C (7)	-0.4655 (4)	-0.3154 (3)	-1.4576 (3)	0.054 (1)
C (8)	-0.2950 (4)	-0.2369 (3)	-1.4603 (3)	0.048 (1)
C (9)	-0.1897 (3)	-0.1622 (2)	-1.2941 (2)	0.035 (0)
C (10)	-0.2539 (2)	-0.1679 (2)	-1.1252 (2)	0.028 (0)

Table 3: Carbon and oxygen atoms: positional parameters and isotropic temperature factors.

	X/A	Y/B	Z/C	UEQ
H (21)	0.200 (4)	0.064 (3)	-1.136 (4)	0.051 (7)
H (22)	0.235 (4)	-0.072 (2)	-1.041 (3)	0.036 (5)
H (3)	0.069 (4)	0.062 (2)	-0.856 (3)	0.036 (5)
H (31)	-0.120 (4)	0.177 (2)	-1.121 (4)	0.041 (6)
H (4)	-0.254 (4)	-0.063 (2)	-0.886 (3)	0.037 (5)
H (41)	0.044 (5)	-0.144 (3)	-0.673 (3)	0.065 (8)
H (5)	-0.587 (4)	-0.304 (3)	-0.968 (3)	0.038 (6)
H (6)	-0.663 (4)	-0.367 (3)	-1.289 (3)	0.040 (6)
H (7)	-0.537 (5)	-0.375 (3)	-1.569 (4)	0.064 (8)
H (8)	-0.260 (4)	-0.238 (3)	-1.571 (4)	0.055 (7)

Table 4: Hydrogen atoms: positional parameters and isotropic temperature factors.

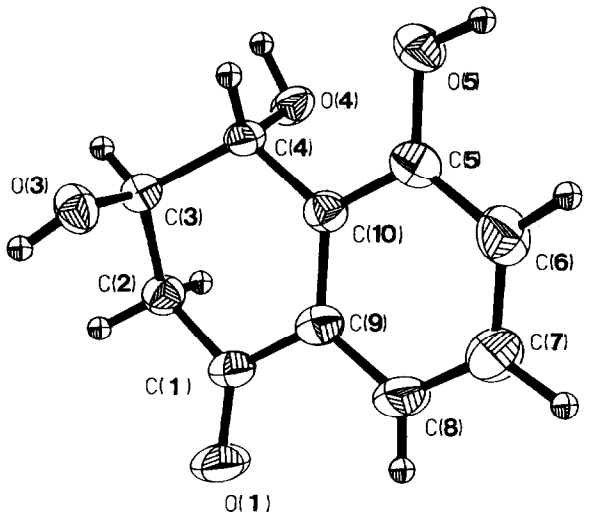


Figure1: Perspective view of the crystal structure of 3,4,5-trihydroxy 1-naphthalenone, 7.

The second group of isolated toxins is composed of naphthalenone derivatives. Such toxins have been isolated from other phytopathogenic fungi (*Penicillium* , *Pyricularia* and *Verticillium* species) [10-12]. The relative toxicity of each of these toxins will be studied in order to determine their structure/toxicity relationship. Their involvement in the disease process will be further examined, as phytotoxin production appears to be an important component of plant pathogenesis.

EXPERIMENTAL

General methods. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra were recorded on a Bruker AM 400 spectrometer. Chemical shifts and coupling constant values of proton coupled systems have been determined after simulation of the calculated values with the PANIC program (Bruker). EI and CI MS were obtained with a Nermag Sidar V 3.0 mass spectrometer.

Isolation The isolate of *H. mammatum* used in this study was collected in the canker margin of an infected quaking aspen (*Populus tremula*). Growth of the fungus for toxin production was achieved within five weeks at 27°C, with unshaken cultures on liquid wort medium complemented with unbleached kraft pulp. The culture filtrate was then harvested.

Two main procedures were used for isolation of the toxins. In the first one, the filtrate was concentrated to a tenth of its volume and then extracted three times with *n*-butanol. The crude extract was purified by column chromatography. The second procedure used neutral adsorbing resin (Amberlite type, XAD 1180, Rohm and Haas): 2% (vol) of the well washed and hydrated resin was added to the culture filtrate with intermittent stirring for 48 h, the resin was filtered off and new resin was added to the filtrate for 48 h. The whole resin was collected, put in a column and washed with a water/methanol (w/m) step gradient from pure water to pure methanol (w/m = 100/0; 80/20; 50/50; 20/80; 0/100). The water fraction was not toxic when tested using the foliar bioassay, whereas the w/m = 80/20 fraction was weakly toxic. Most of the toxicity was concentrated in the last fractions (w/m =20/80). The 80/20 and 50/50 w/m fractions were dissolved in water, the solution extracted with EtOAc and the remaining aqueous solution evaporated to dryness to give respectively residues a and b that contained hymatoxins.

Purification of hymatoxins from XAD resin: Residue a was a mixture consisting mainly of hymatoxins A and B that were separated by reversed-phase column chromatography over RP-8 silica gel, using a w/m gradient for elution. The minor compound hymatoxin B was further purified by repetitive RP-8 column chromatography with w/m: 7/3 as eluent. Purification of the major compound, hymatoxin A, was achieved successively by 1) adsorption on a DEAE column, 2) desorption with a NaCl solution and 3) chromatography on XAD-2 resin with a w/m gradient from 1/1 to pure methanol.

Residue b, chromatographed over RP-8 silica gel column (w/m gradient) gave four main fractions, each of which contained a main compound, respectively hymatoxins A, E, D and C. Purification of hymatoxin C was accomplished by repetitive RP-8 column chromatography (w/m: 1/1) and then gel permeation on Sephadex LH 20 (MeOH). The same procedure was applied for hymatoxin D purification. Purification of hymatoxin E was done in two steps: 1) chromatography over RP-8 silica gel (w/m: 4/6) and then column chromatography over silica gel (CH_2Cl_2/ *iso*-PrOH: 9/1).

Purification of naphthalenones: The EtOAc extract from the m/w:1/1 fraction, (250mg) was chromatographed 1) over Sephadex LH 20 (MeOH), 2) over silica gel (toluene/EtOH: 9/1) to give **6**, (10 mg).

The EtOAc extract from the m/w : 2/8 fraction (250mg) was chromatographed 1) over silica gel (CH_2Cl_2/MeOH: 1/9), 2) over RP-8 silica gel (w/m: 9/1) to give **7**, (6.5 mg).

Chromatographical characteristics of hymatoxins: CCM (silica gel Merck 5554, EtOAc/*n*-ButOH/AcOH: 6/3/1), (Rf): hymatoxins A (0.45), B (0.33), C (0.61), D (0.62), E (0.57). Purity of isolated hymatoxins was controlled by C-18 ODS 2 reversed-phase HPLC (methanol/water gradient from MeOH 10% to 100%; UV detection 220 nm). Retention times (min): hymatoxins A (26), B (14), C (37), D (34), E (39).

Hymatoxin A, **1**, $C_{20}H_{29}O_7S^-$, (mw 413), colourless amorphous solid, $[\alpha]_D^{20}$ -49.0 (c = 0.5, CH_3OH). UV λ^{max}_{MeOH} (log ε): 214.0 (3.3), 238.1 (2.8). IR (KBr, ν cm^{-1}): 3465, 1753, 1640, 1461, 1385, 1240, 1215, 1064, 992, 977. FAB(-) MS: *m/z* 413 (10, $[M]^-$), 321 (13), 251 (11), 215 (28), 143 (18), 107 (100); EI-MS (70 eV, 200 °C) *m/z* (%): 316 (20, $C_{20}H_{28}O_3$), 298 (21), 296 (11), 271 (100, $C_{19}H_{27}O$), 270 (48), 253 (67, $C_{19}H_{25}$), 225 (70, $C_{17}H_{21}$), 211 (38), 209 (40), 197 (44, $C_{15}H_{44}$), 195 (37), 169 (54, $C_{13}H_{13}$), 155 (44), 128 (34), 109 (33).

Hymatoxin B, **2**, $C_{20}H_{29}O_8S^-$, (mw 429), colourless amorphous solid, $[\alpha]_D^{20}$ -46.6 (c = 1.0, CH_3OH). UV λ^{max}_{MeOH} (log ε): 204.6 (3.8), 245.4 (1.9). IR (KBr, ν cm^{-1}): 3450, 2935, 2877, 1766, 1453, 1386, 1259, 1206, 1073, 992, 917, 832. FAB(-) MS: *m/z* 429 $[M]^-$.

Hymatoxin C, **3**, $C_{20}H_{31}O_6S^-$, (mw 399), colourless amorphous solid, $[\alpha]_D^{20}$ +10.1 (c = 1.0, CH_3OH). UV λ^{max}_{MeOH} (log ε): 206.6 (3.6), 242.0 (2.5). IR (KBr, ν cm^{-1}): 2931, 2868, 2850, 2821, 1691, 1465, 1451, 1384, 1255, 1220, 1071, 981, 834. FAB(-) MS: *m/z* 399 $[M]^-$.

Hymatoxin D, **4**, $C_{20}H_{29}O_6S^-$ (mw = 397), colourless amorphous solid, $[\alpha]_D^{20}$ -29.4 (c = 1.0, CH_3OH). UV λ^{max}_{MeOH} (log ε): 225.4 (4.0), 233.2 (4.2), 240.0 (4.2), 247.8 (4.0). IR (KBr, ν cm^{-1}): 2941, 2871, 2856, 1691, 1481, 1382, 1257, 1214, 1083, 977, 859. HR FAB(-) MS: *m/z* 397.1703 $[M]^-$; calc. $C_{20}H_{29}O_6S^-$: 397.1685

Hymatoxin E, **5**, $C_{20}H_{30}O_4$, (mw 334), colourless amorphous solid, $[\alpha]_D^{20}$ -82.9 (c = 1.3, CH_3OH). UV λ^{max}_{MeOH} (log ε): 205.6 (3.7), 241.8 (2.6), 253.4 (2.5). IR (KBr, ν cm^{-1}): 3420, 2933, 2877, 1750, 1457, 1382, 1204, 1156, 1122, 1056, 1021, 996, 917. EI MS: *m/z* 334 (3, $[M]^{+\bullet}$), 317 (26), 307 (17), 299 (6), 289 (16), 271 (9), 154 (100), 136 (77), 107 (34).

3,4-Dihydro-3,4,8-trihydroxy-1(2H)-naphthalenone, **6**, $C_{10}H_{10}O_4$. EI MS: *m/z* 194, $[M]^{+\bullet}$. ^1H NMR (CD_3OD, 300.13 MHz, TMS), δ (ppm) J (Hz): 2.744 (1H, dd, 17.2, 8.1, H-2ax), 3.123 (1H, dd, 17.2, 4.0, H-2eq), 4.116 (1H, ddd, 8.1,6.8, 4.0, H-3ax), 4.651 (1H, d, 6.8, H-4ax), 7.167 (1H, d, 7.6, H-5), 7.576 (1H, dd, 8.4, 7.6, H-6), 6.905 (1H, d_b, 8.4, H-2ax). ^{13}C NMR (CD_3OD, 75.47 MHz, TMS), δ

(ppm): 204.3 (s, C-1), 163.22 (s, C-8), 145.85 (s, C-8a), 140.0 (s, C-4a), 137.9 (d, C-7), 120.0 (d) and 117.7 (d) (C-5 and C-6), 73.3 (d, C-4), 71.7 (d, C-3), 44.4 (t, C-2).

3,4-Dihydro-3,4,5-trihydroxy-1(2H)-naphthalenone, **7**, $C_{10}H_{10}O_4$. EI MS: *m/z* 194, $[M]^{+\bullet}$. IR (KBr, ν cm^{-1}): 3350, 2925, 2858, 1685, 1590, 1465, 1378, 1293, 1166, 1108, 1069, 1023, 803, 697. ^1H NMR (CD_3OD, 300.13 MHz, TMS), δ (ppm), J (Hz): 7.504 (1H, ddd, 7.8, 1.1, 0.5, H-8), 7.303 (1H, dd, 7.8, 7.8, H-7), 7.131 (1H, dd, 7.8, 1.1, H-6), 5.154 (1H, dd, 4.2, 0.5, H-4), 4.323 (1H, ddd, 4.8, 4.2, 3.1, H-3), 3.169 (1H, dd, 16.8, 3.1, H-2a), 2.673 dd (1H, dd, 16.8, 4.8, 1.1, H-2b).

Crystal data: $C_{10}H_{10}O_4$, molecular weight 194.19. Crystals were obtained by slow crystallization of **7** from methanol; crystals belong to the monoclinic system, space group $P2_1$, Z = 2. Cell parameters: a = 6.480 (7), b = 10.476 (11), c = 7.134 (8) Å, β = 108.90 (9)°; V = 458.18 (9) $Å^3$; Synthex-$P2_1$-diffractometer, F(000) = 204, λ (Cu Kα) = 1.5418 Å, μ = 8.27 cm-1.

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