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Organic Chemistry

Elixir Org. Chem. 164 (2022) 56108-56110

ISSN: 2229-712X

Commercial Quantities of Cytochalasin D and 5-Carboxymellein from Static Cultures of Endophytic Fungi

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ARTICLE INFO

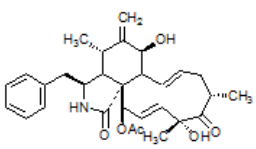
Article history:
Received: 14 January 2022;
Received in revised form:
28 February 2022;
Accepted: 10 March 2022;

Keywords
Cytochalasin,
Cytotoxic,
Molds,
Spectroscopic Analysis,
5-carboxymellein.

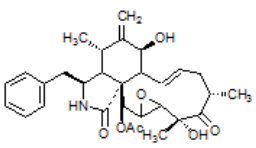
ABSTRACT
Cytochalasin D is a cytotoxic compound used in cellular research and drug development. Cytochalasin D is produced from molds, often in mixtures which are difficult to purify. Yields from the synthesis of the compound are poor, hence the high cost of the cytotoxic compound. Static cultures of a group of endophytic fungi: were sub-cultured in 250 ml conical flask each for three weeks. Each of the endophyte culture was transferred into ten 2.0 dm^3 Thompson bottles and allowed to grow for 8 weeks. TLC studies of the individual crude extracts indicated that the four mangrove endophytes produced the same compounds. The crude extract obtained from RAR 5-6 was dissolved in warm ethyl acetate and left overnight. A white solid (0.90 g) precipitated from the solution. The solid was removed by filtration and recrystallized from the same solvent to give pure cytochalasin D. The resulting filtrate was chromatographed on silica gel. Fraction 5 yielded a yellow viscous liquid (270 mg). This was triturated with alcohol and left to stand for 48 h to give white crystals, Spectroscopic analysis indicated that the compound was 5-carboxymellein. The production of pure cytochalasin D from these endophytic fungi could be a source of commercial production of this important cytotoxic compound.

1.0 Introduction

Cytochalasins are a group of cytotoxic compounds used in cellular research and for drug production. Cytochalasin D (**1**), was first isolated independently at the ICI laboratories in Britain by Aldridge *et al* [1] and by Tamm in Zurich [2]. Since then, it has been isolated by several other workers. Edwards *et al* [3], isolated this metabolite alongside several new cytochalasins from the Fungus *Hypoxylon terricola* in the University of Bradford. Recently, Oppong *et al* [4], reported the isolation of a new Cytochalasin, Englomycin acetate (**2**), a derivative of Cytochalasin D. These fungal metabolites show marked cytotoxic effects on mammalian cells in tissue culture. The mycotoxin, had also been found to be active against HIV-1 protease. Dombrowski *et al*[5] indicated that a cytochalasin L-690-474 isolated from *H. fragiforme* inhibits HIV-1 protease and could be formulated into a retroviral drug. Cytochalasin D is a cell permeable and potent inhibitor of actin polymerization. It is therefore used in drug formulation and cellular research.



[1]



[2]

Cytochalasin D, the most common member of the cytochalasins is very expensive. The high cost of the metabolite is due to difficulties in production. Molds produce the cytochalasin in mixtures, which are structurally very similar. The similarities in physical properties reduces the yield using chromatographic techniques. Attempts to synthesis the compound had not been commercially successful. Merifield et al [6] reported the total synthesis of cytochalasin D, though with low yield. Several other workers had attempted to produce the cytotoxic secondary metabolite by different synthetic routes. It was therefore welcoming that a group of endophytic molds produced pure Cytochalasin D from static cultures. The filtrate that was obtained when the solid that afforded the cytochalasin D was removed gave a yellow viscous liquid (270 mg). Fraction 5, of the column chromatography of this liquid gave white crystals (10 mg) when it was triturated with alcohol and left for 48 h. Spectroscopic analysis identified the compound as 5-carboxymellein.

2.0 Materials and Methods

2.1 Cytochalasin D

Cultures of a group of endophytes RAR 5-6, XMR 12-17, RAJ and XGR 12-5 collected from Thailand were sub-cultured in different conical flasks containing 3% malt and 6% glucose for 3 weeks. All four endophytes developed fruiting bodies after 7 days The dark brown fruiting bodies 0.5 mm base diameter and 1.5-2.0 cm high were visible on the white upper cover of the mycelium. Each of the endophytic mold culture was transferred into ten 2.0 dm^3 Thompson bottles and allowed to grow for 8 weeks. Master cultures of these endophytes were prepared for future reference (figure 1).

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Figure 1. Master cultures of endophytic fungi.

The matured cultures were harvested and the mycelium removed with a muslin cloth. The aqueous filtrates were extracted with neat ethyl acetate in a 5.0 dm^3 separating funnel and the ethyl acetate fraction dried over anhydrous sodium sulphate. The yields obtained were 6.51, 6.30, 3.65 and 5.32 g for RAR 5-6, XMR 12-71, RAJ 17-32 and XGR 12-5 respectively. TLC studies of the individual crude extracts indicated that the groups of endophytes produced the same compounds.

The crude extract obtained from RAR 5-6 was dissolved in warm ethyl acetate and left overnight. A white solid (0.90 g) precipitated from the solution. The solid was removed by filtration and recrystallized from the same solvent to give white needles of cytochalasin D (720 mg) mp (266-267 ^oC) (lit.[9], 267°C) m/z 507 (M^+) $[\alpha]_D^{23}$ -13.5^o (c =1.0, in dioxane). δ_C (C_5D_5N) gave resonance positions at 13.56 (11-CH_3), 19.31 (22-CH_3), 20.48 (25-CH_3), 24.58 (23-CH_3), 33.11 (5-CH), 38.51 (15-CH_2), 42.38 (16-CH), 45.49 (10-CH_2), 47.91 (8-CH), 50.34 (4-CH), 53.96 (9-C), 54.33 3-CH), 71.23 (7-CH), 77.95 (21CH), 78.36 (18-C), 112.13 (12 =CH_2), 126.76 (13-CH), 126.95 (29-Ar-CH), 128.76 (28, 30-ArCH), 129.86 (27, 31-ArCH), 132.23 (20-CH), 132.72 (14-CH), 133.59 (19-CH), 138.41 (26-C), 151.80 (6-C=), 170.44 (24-C=O), 175.08 (1-C=O) and 210.84 (17-C=O).

2.2 *5-Carboxymellein*

The resulting filtrate after the cytochalasin D was removed, was a yellow viscous liquid. This was chromatographed in a 70 cm x 2,5 cm column, packed with silica gel. The column was eluted with 1.5 dm^3 of chloroform-methanol (95:5) and the eluent collected in volumes of 5.0 ml.

Tubes (**136-160**), fraction 5 was a yellow liquid (270 mg). The liquid was dissolved in warm alcohol (1.5 ml) and set aside for 48 h, yielding white needles of 5-carboxymellein (10.0 mg) mp 246 ^{o} C (lit.[4], 247 ^{o}C), m/z 222 (M^+), ν_{max} (KBr) cm^{-1} 3200, 2950, 1695, 1650 and 1590, δ_H (C_5D_5N) 1.27 (3H, d, *J* 6.2 Hz), 3.02 (1H, m), 4.10 (1H, m), 4.55 (1H, m), 7.02 (1H, d, *J* 8.8 Hz) and 8.43 (1H, d, *J* 8.8). δ_C (C_5D_5N) 20.43 (11-CH_3), 32.93 (4-CH_2), 75.68 (3-CH), 109.30 (9-C), 115.85 (7-CH), 139.11 (6-CH), 143.65 (10-C), 165.15 (8-C), 168.47 (12-C=O) and 170.35 (1-C=O).

3.0 Results and Discussion

3.1 Cytochalasin D

3.1.1 Yields from cultures

The four different endophytes gave the following crude yields when they were cultured in 10.0 dm^3 aqueous medium, Table 1

Table 1.Yields of culture extracts from the endophytic fungi.

Endophyte	Yield (g)
RAR 5-6	6.51
XMR12-71	6.30
RAJ 17-32	3.65
XGR 12-5	5.32

3.1.2 TLC studies

TLC studies of the four crude extracts showed that the four different endophytes produced the same compounds.

The crude extract from RAR 5-6 was dissolved in warm ethyl acetate and set aside for 24 hours. A white solid (0.90 g) precipitated from the solution.

The solid was recrystallized from the same solvent to give white needles (720 mg) mp (266-267 ^0C) MW 507 ($C_{30}H_{37}NO_6$), % yield 0.11.

3.1.3 NMR analysis

Both the ^{1}H-NMR and the ^{13}C NMR showed that the metabolite was pure cytochalasin D. (Figure 2 and 3).

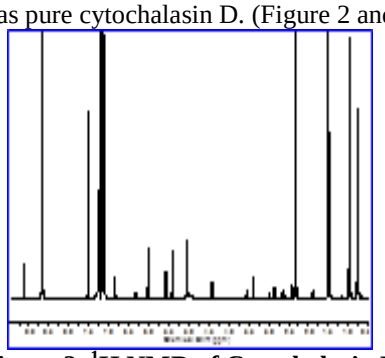


Figure 2. ^{1}H NMR of Cytochalasin D

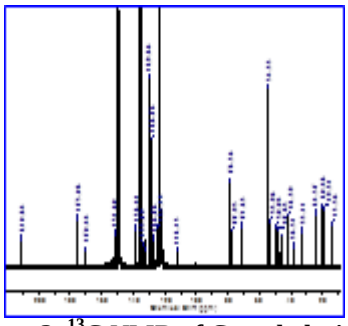


Figure3. ^{13}C NMR of Cytochalasin D

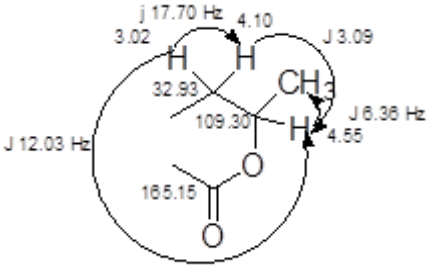
3.2 5-Carboxymellein

Tubes **(136-160),** fraction 5, gave a yellowish oil (270 mg) which was dissolved in warm alcohol (2.0 ml) and set aside for 48 h. White crystals (10.0 mg) were obtained, mp. 246 ^oC, $[\alpha]_D^{23}$ + 198.1^o (c = 0.75, MeOH), $[\alpha]_D^{23}$ + 203.03° c=0.66 EtOH), m/z 222 (100 %), ν_{max} (KBr) 3200, 2950, 1695, 1650, 1590 cm^{-1}. Table 2, gives a summary of the NMR data.

Table 2. NMR data of 5-carboxymellein in C_5D_5N.

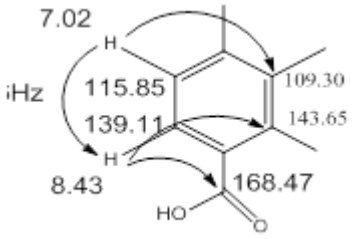
$C_{\#}$	δ_C	Type	δ_H	$^nJ_{C-H}$
1	20.43	CH_3	1.27 (3H, d, *J* 6.2 Hz)	32.93,75.68
2	32.93	CH_2	3.02 (1H, dd, *J* 17.7, 12.0 Hz)	20.43,75.68,143.65
2	32.93	CH_2	4.10 (1H, dd, *J* 17.7, 3.1 Hz)	109.30,120.97,143.65
3	75.68	CH	4.55 (1H, m)	
4	109.30	C	-	
5	115.85	CH	7.02 (1H, d, *J* 8.8Hz)	109.30,120.97,168.47
6	120.97	C	-	
7	139.11	CH	8.43 (1H, d, *J* 8.8 Hz)	143.65,168.47,170.35
8	143.65	C	-	
9	165.15	C	-	
10	168.47	C	-	
11	170.35	C	-	
12	168.47	COOH	6.10 (1H, s(br))	

In the IR spectrum, two strong absorption bands at ν_{max} (KBr) 1695 and1650 cm^{-1} suggested the presence of two carbonyl groups in this compound. The resonance position of the methine proton at δ_H 4.55, indicates that it is attached to a C-O group. This proton must have at least five neighbors due to its multiplicity. In the ^1H-^1H COSY spectrum of this compound, the methine proton correlates with the three methyl protons as well as the two non-equivalent methylene protons. A most likely subunit in this compound is (**A).**



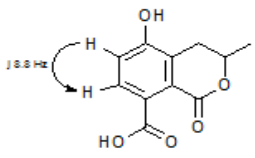
A

The ^1H-^1H COSY spectrum shows coupling between the aromatic protons at δ_H 7.02 and 8.43 with a *J* value of 8.8 Hz, indicating an ortho coupling. A strong absorption in the IR at 1590 cm^{-1} supports the presence of an aromatic ring. The compound gives a yellow coloration with diazotized *p*-nitroaniline and bromocresol green spray reagents (TLC). The former indicates that the compound is a phenol or an enol and the latter shows that it is an acid. In the ^1H NMR, there is a broad peak at δ_H 6.10 which supports the presence of an acid group. The HMBC spectrum shows correlations between the aromatic proton at δ_H 8.43 and 7.02 with carbons at δ_C 143.65 and 109.30 respectively. The other portion of the compound is likely subunit (**B)**.

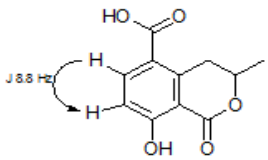


B

Subunits **A** and **B** may be linked in two possible ways **(C1)** and **(C2)**

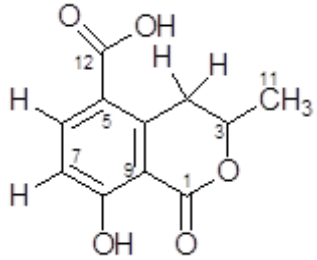


C1



C2

When the COOH functional group occurs at the C-5 position on mellein, it results in a split of the adjacent CH_2 protons. This is the case with this compound, suggesting the structure as **5-carboxymellein (3).**



(3)

5-Carboxymellein is a common fungi secondary metabolite. It was reported in low yield from some species of *Hypoxylon*, *H. illitum* and *H. mammatum* and also from *Nummularia discreta* [8]. Several other fungi cultures have since then produced 5-carboxymellein. One of such fungus is the fungus responsible for the Dutch elm disease, *Phomopsis obloga* [9]. 5-Carboxymellein was also isolated from an unknown fungus that infects wood and in the shake culture of the pathogenic fungus, *Valsa ceratospoma*, responsible for the canker in apple [10]. It is suggested that the compound has anti-boring and anti-feeding deterrent activities.

4.0 Conclusion

Cytochalasin D is cytotoxic and mainly used in cellular research. As mentioned above a member of the cytochalasins had been reported to be anti-viral and could be used in the fight against the covid-19 menace. Cytochalasin D is produced in a mixture with other cytochalasins by molds. Similarity in structure and physical properties amongst the cytochalasins makes them difficult to purify at high yields. Thus, the secondary metabolite is very expensive. It is therefore welcoming that a group of endophytic fungi produce the cytotoxic compound pure and in commercial quantities. This is the first report of the production of pure cytochalasin D from a group of mangrove endophytes. The filtrate obtained after the removal of the pure cytochalasin D gave 5-carboxymellein, a metabolite that had been suggested to have anti-boring and antifeeding deterrent activities.

5.0 References

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