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Structure elucidation of clavilactone D: an inhibitor of protein tyrosine kinases ☆

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Abstract

Clavilactones D and E were isolated from an agar culture of the Basidiomycetous fungus *Clitocybe clavipes*, and their structure was elucidated by ^{1}H- and ^{13}C-NMR studies. Clavilactone D is an inhibitor of tyrosine kinases. © 2000 Elsevier Science Ltd. All rights reserved.

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1. Introduction

In a program aimed to isolate new biologically active secondary metabolites from Basidiomycetes, we isolated recently Clavilactones A–C (CA–CC, **2b**–**2c**) from *Clitocybe clavipes* (Arnone, Cardillo, Meille, Nasini & Tolazzi, 1994), grown on MPGA cultures. Metabolites CA and CB showed antibacterial activity, were active in some antifungal tests and inhibited the growth of *Lepidum sativum*.

Further investigations on the biological activity of these compounds showed inhibition of protein tyrosine kinases (Cassinelli, Lanzi, Polizzi, Gambetta, Pensa, Merlini et al., 1998) and prompted us to study the production of similar compounds using different culture conditions. MYGA (malt, yeast, glucose, agar) medium, followed by chromatographic separation led to the isolation, besides CA, of the novel Clavilactone D (CD, **1a**). A second new metabolite, Clavilactone E (CE, **2a**), was also obtained in very poor yield.

2. Results and discussion

Clavilactone D, **1a**, isolated (see Section 3) as a red powder, mp 172–175°C, $[\alpha]_D$ +376° (MeOH, *c* 0.05), analyzed for $C_{16}H_{14}O_6$, showed in the IR spectrum a band at 3400 cm^{-1} (OH) and in addition a strong band at 1780 cm^{-1} attributable to an ester-like function; a large band at 1650 cm^{-1}, which suggested the presence of a quinone carbonyl group (Arnone et al., 1994); the UV spectrum showed absorption at 210, 285 and 330sh nm (ε 14570, 4700 and 1600).

The great similarity of the IR and ^{1}H-NMR spectra (Tables 1 and 2) of **1a** with those of clavilactone B indicated that the two compounds must have the same structure except for the presence of an additional OH in the quinone ring of **1a**. This is consistent with the appearance of only one proton signal at higher field (5.90 δ) than the two ones (7.01 and 7.04) in clavilactone B, and is confirmed by EIMS (M^+ 302). The assignment of the position of the OH was not straightforward, but could be obtained from the long-range

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Table 1
^{1}H-NMR chemical shifts (δ) and long-range ^{1}H–^{13}C correlations for compound **1a** in acetone-d_6

H	δ_H	Long-range correlations
9α	1.36	61.13 (C-8), 172.52 (C-15)
Me-16	1.57	27.21 (C-13), 123.99 (C-11), 136.38 (C-12)
10α	2.27	
10β	2.37	61.13 (C-8), 123.99 (C-11), 136.38 (C-12)
9β	2.62	23.21 (C-10), 61.13 (C-8), 63.58 (C-7), 123.99 (C-11)
13α	2.86	22.59 (Me), 123.99 (C-11), 134.38 (C-14), 136.38 (C-12), 151.33 (C-5), 184.17 (C-1)
13β	3.76	123.99 (C-11), 134.38 (C-14), 136.38 (C-12), 151.33 (C-5), 184.17 (C-1)
7	4.42	73.12 (C-6), 172.52 (C-15)
11	5.38	23.21 (C-10), 27.21 (C-13)
3	5.90	27.21 (C-13), 148.78 (C-2), 151.33 (C-5), 183.23 (C-4)
6	5.99	61.13 (C-8), 63.58 (C-7), 134.38 (C-14), 151.33 (C-5), 183.23 (C-4)

correlations in the HMBC-NMR spectrum (Table 1). The positioning of the OH was derived from the correlation of H-3 with C-4 and C-5 and not with C-1 and C-14, the carbonyl carbons on their turn being assigned on the basis of the correlations with H-6 and with both H_2-13, respectively. The close similarity of the ^{1}H–^{1}H coupling constants observed in CD and CB implies that the stereochemistry of the alicyclic rings is the same in the two compounds.

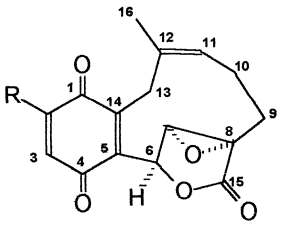
HRMS data and comparison of the ^{1}H- and ^{13}C-NMR spectra of CE **2a** with that of CC **2c** (Arnone et al., 1994) indicated that the structure of CE differs from that of CC only for the presence of an OMe group in position 13. This assignment is confirmed by NOE effect on H-13 and OH-1 on irradiation of the OMe signal in the ^{1}H-NMR spectrum.

The clavilactones form a new class of compounds that may be biosynthesized from geranylhydroquinone via several oxidative steps (Arnone et al., 1994). The recent revision of the structure of flavidulol **3**, isolated from the mushroom *Lactarius flavidulus* (Takahashi, Kusano, Ohta & Nozoe, 1993) confirms this hypothesis.

Clavilactone D was found to be a specific inhibitor of tyrosine kinases, such as Epidermal Growth Factor Receptor (EGFR), and showed selective antiproliferative action against A431 cell line, that overexpressed this receptor (Cassinelli et al., 1998). In particular, Clavilactone D was a potent compound regarding the inhibition of EGFR in kinase assay (IC_{50} 5.5 μM). In view of the fact that several tyrosine kinases are the products of proto-oncogenes, inhibitors of these kinases offer a potential antitumour approach.

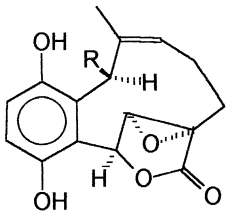
Table 2
^{13}C-NMR chemical shifts (δ) and coupling constants (J) for compound **1a** in acetone-d_6

C	δ_C	$^1J_{C-H}$ (Hz)
1	184.17	
2	148.78	
3	101.67	163.0
4	183.23	
5	151.33	
6	73.12	156.9
7	63.58	201.1
9	25.13	132.3
10	23.21	128.9
11	23.99	151.3
12	136.38	
13	27.21	129.3
14	134.38	
15	172.52	
16 (Me)	22.95	127.4



1a R = OH CD

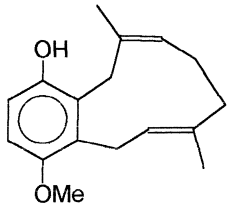
1b R = H CB



2a R = OMe CE

2b R = H CA

2c R = OH CC



3

3. Experimental

Flash column chromatography was performed with Merck silica gel (0.040–0.63 mm) and TLC with Merck HF254 silica gel. Ms were recorded with a Finnigan-Mat TSQ70 spectrometer. NMR spectra were acquired on a Bruker AMX-600 spectrometer operating at 600 MHz.

Owing to the complexity of the purification procedure, we report the R_f values on silica gel plates in hexane–EtOAc (1 : 1) and CH_2Cl_2–MeOH (15 : 1), respectively.

*3.1. Isolation and purification of Clavilactones D **1a** and E **2a***

A strain of *C. clavipes* (CBS 126.44) obtained from Centraal Bureau voor Schimmelcultures (Baarn) was maintained on MPGA (malt, peptone, glucose, agar 20 : 4 : 20 : 15 g l^{-1}) slants and grown in 40 Roux flasks with MYGA (malt, yeast, glucose, agar 10 : 10 : 30 : 15 l^{-1}) medium for one month; the EtOAc extracts (0.8 g) were chromatographed on silica gel with CH_2Cl_2–MeOH (15 : 1) as eluent to obtain, after further purification by PLC in hexane–EtOAc (1 : 1): clavilactone D **1a**, R_f 0.6, 0.6 (20 mg), clavilactone E **2a**, R_f 0.9, 0.7 (2 mg), and clavilactone A **2b**, R_f 0.8, 0.3 (110 mg) (Arnone et al., 1994).

*3.1.1. Clavilactone D **1a***

Found: C, 63.5; H, 4.7. $C_{16}H_{14}O_6$ requires C, 63.6; H, 4.7%; CIMS (CH_4) *m/z* 302 $[M]^+$ (100), 256 (25), 214 (30) and 188 (20). The ^{1}H- and ^{13}C-NMR spectral data are reported in Tables 1 and 2.

*3.1.2. Clavilactone E **2a***

Yellow powder, mp 150–152°C, $[\alpha]_D$ +90° (MeOH; *c* 0.1), λ_{max}^{EtOH} 310 nm (ε 6000), CIMS (CH_4) *m/z* 319 $[MH]^+$ (20), 288 $[MH - 31]^+$ (100), 242 (25) and 189 (26); HRMS *m/z*: found 318.107 $[M^+]$, $C_{17}H_{18}O_6$ requires 318.110; ^{1}H-NMR (600 MHz, acetone-d_6): δ 8.68 (1H, OH-4), 8.66 (OH-1), 6.99 and 6.85 (2H, *AB*, H-2 and H-3, *J* = 8 Hz), 6.42 (1H, *br*s H-6), 5.68 (1H, *m*, H-11), 5.07 (1H, *s*, H-13), 4.17 (1H, *brs*, H-7), 3.42 (3H, *s*, OMe), 2.66 (1H, *m*, H-9β), 2.50 (1H, *m*, H-10β), 2.44 (1H, *m*, H-10α), 1.73 (3H, *s*, Me-16), 1.38 (1H, *m*, H-9α), ^{13}C-NMR (600 MHz, acetone-d_6): 152.31, 121.03, 117.80, 150.02 (C-1 to C-4, interchangeable assignments) 118.67 (C-5), 75.32 (C-6), 64.53 (C-7), 61.87 (C-8), 24.49 (C-9), 23.61 (C-10), 130,01 (C-11), 137.01 (C-12), 79.34 (C-13), 123.69 (C-14), 172.83 (C-15), 18.82 (Me-16), 57.17 (OMe-13).

3.2. Kinase assay

The EGF-R autophosphorylation assay was performed using cell membranes from the EGF receptor overexpressing A431 cell line as a source of enzyme, essentially as described by Levitzki, Gazit, Osherov, Posner and Gilon (1991). Membrane preparations were pre-activated with EGF, then incubated in a reaction mix containing different concentrations of clavilactone D dissolved in DMSO and ethanol (1.7 and 7.5% final concentrations, respectively) and 2 μl of [γ-^{32}P]ATP (2 μM final). After 30 s at 4°C, the reaction was stopped by concentrated Laemmli solution. Samples were then subjected to 7% SDS-PAGE. EGF-R phosphorylation was evaluated by PhosphorImager (Molecular Dynamics, Stunnyvale, CA). Clavilactone D showed an IC_{50} of 5 μM.

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