Cochliobolic Acid, a Novel Metabolite Produced by *Cochliobolus lunatus*, Inhibits Binding of TGF-α to the EGF Receptor in a SPA Assay

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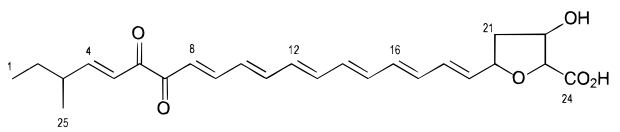
Cochliobolic acid (**1**), a novel biologically active natural product, is produced by submerged fermentation of *Cochliobolus lunatus*. Compound **1** was determined to be a novel polyketide possessing a substituted tetrahydrofuran ring, a conjugated polyene chain and a 1,2-diketone moiety, by interpretation of NMR, MS, and UV/vis spectroscopic data. Compound **1** inhibits the binding of TGF-α to the EGF receptor of the human epidermal cell line A431 in a SPA assay with an IC_{50} of 1.6 μM.

The epidermal growth factor (EGF) receptor, a transmembrane glycoprotein with tyrosine kinase activity, is frequently overexpressed in a wide variety of human malignancies.[1−4] The receptor is activated by the binding of members of a family of structurally related ligands, which includes transforming growth factor alpha (TGF-α), EGF, amphiregulin, and the pox virus growth factors.[5] TGF-α, a 50 amino acid single-chain peptide, is coexpressed with the EGF receptor in some tumors and transformed cell lines and is implicated in tumorigenesis.[6−9]

During a screening program to find inhibitors of the binding of TGF-α to the EGF receptor, we discovered a novel compound produced by a strain of the fungus *Cochliobolus lunatus* Nelson and Haasis (Ascomycete). In this paper, we report the fermentation, isolation, structure elucidation, and biological activity of this novel compound that we have named cochliobolic acid (**1**).



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Results and Discussion

The MeOH extract of the mycelium from submerged liquid fermentation of a strain of *C. lunatus* was found to be active in a ligand-binding assay based on binding of TGF-α to the EGF receptor of the human epidermal cell line A431.[10,11] Bioassay-guided fractionation led to the isolation of **1** as an orange-red powder with an IC_{50} of 1.6 μM in the above assay. Compound **1** exhibited no activity in an EGF receptor tyrosine kinase assay, nor in 11 other primary assays in operation at Xenova. Experiments with NR6 fibroblasts transfected with human EGF receptor, TGF-α dependent cell lines (SiHa carcinoma line; A431), and cells dependent on other growth factors (e.g., Swiss 3T3) demonstrated that **1** does not have a clear window between growth inhibition by selective TGF-α antagonistic activity and non-specific toxicity.

The presence of a highly extended chromophore was indicated by a broad λ_{max} centered at 430 nm; the lack of any fine structure suggested the presence of an oxopolyene moiety.[12] Extensive MS analyses revealed the molecular weight to be 426. Although an accurate mass was not obtained for the molecular ion, HREIMS of a prominent fragment ion at m/z 364 established its elemental formula as $C_{24}H_{28}O_3$. Rationalisation of this fragment as arising by loss of CO_2 and H_2O from the molecular ion suggested the molecular formula to be $C_{25}H_{30}O_6$.

The ^{13}C-NMR spectrum exhibited 25 resolved signals at 100 MHz, comprising 2 × CH_3, 2 × CH_2, 18 × CH, and 3 × quaternary carbons, as revealed by DEPT spectra and consideration of chemical shifts. Signals due to 28 protons were present in the ^{1}H-NMR spectrum, indicating that **1** contains two exchangeable protons. This was confirmed by microderivatization and MS experiments, which revealed that **1** added one methyl group when treated with CH_2N_2 or MeOH−HCL and one acetyl group when acetylated with Ac_2O−pyridine.

The structure elucidation of **1** was hampered by the extensive overlap of the 10 olefinic protons resonating between δ 6.4 and 6.7. The difficulty was exacerbated further by the relatively low solubility of **1** (< 3 mg mL^{-1} in MeOH) and its inherent lability. Nevertheless, interpretation of the ^{1}H, ^{13}C, DEPT, HMQC, and ^{1}H−^{1}H COSY NMR spectra led to the proposal of two spin systems, each terminating in a ketone carbonyl, which correspond to the left and right side of **1**, with only an alcohol hydroxyl and a carboxylic acid group left unaccounted for. The assignment of the relative positions of the hydroxyl and carboxylic acid groups was made on the basis of the chemical shifts of C-22 and C-23. Connection of **2** and **3** through a 1,2-diketone remained the only possibility. Evidence confirming the proposed

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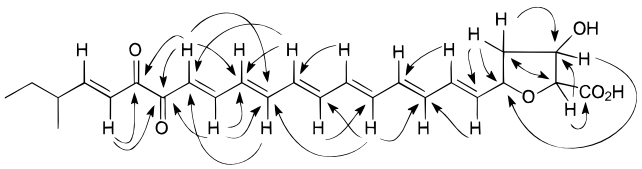

Figure 1. Selected long-range $^1H-^{13}C$ couplings observed for **1** in an HMBC (5 Hz) experiment.

Table 1. ^{13}C- and ^1H-NMR Data of **1** in CD_3OD (30 °C)

position	δC^a	δH^b
1	11.86	1.00 (3H, t, 7.5)
2	29.77	1.55 (2H, quintet, 7.2)
3	40.19	2.42 (2H, septet, 6.7)
4	160.24	7.05 (1H, dd, 16.0, 7.8)
5	124.58	6.66 (1H, dd, 16.0, 1.0)
6	192.68[c]	
7	192.42[c]	
8	140.73	6.71 (1H, d, 15.0)
9	149.41	7.51 (1H, dd, 15.2, 11.4)
10	131.70	6.61 (1H, dd, ~14, ~12)
11	145.93	6.96 (1H, dd, 14.4, 11.4)
12	133.09	6.53 (1H, dd, ~15, ~11)
13	124.06	6.72 (1H, dd, 14.4, 10.2)
14	133.60[c]	6.49 (1H, dd, ~15, ~11)
15	138.10	6.58 (1H, dd, ~15, ~11)
16	133.65[c]	6.44 (1H, d, ~15)
17	136.16	6.46 (1H, s)
18	130.69	6.45 (1H, d, ~15)
19	139.98	5.96 (1H, dd, 14.7, 6.1)
20	69.83	4.48, (1H, m)
21	41.95	1.82 (2H, m)
22	70.49	4.22 (1H, ddd, 9.1, 3.2, 3.0)
23	75.70	4.00 (1H, d, 2.9)
24	178.00	
25	19.03	1.18 (3H, d, 6.7)

[a] Chemical shifts are shown referenced to CD_3OD as 49.00 ppm. [b] Chemical shifts are shown referenced to CD_3OD as 3.40 ppm. The *J* values are in parentheses (Hz). [c] Assignments may be interchanged.

structure of **1** was provided by HMBC (5 Hz) spectral data (Figure 1). Although their chemical shifts are very close, C-6 was assigned tentatively to δ 192.68 and C-7 to δ 192.42, as H-9 appears to exhibit an HMBC cross peak to δ 192.68 only, whereas H-8 and H-5 probably correlate to both. One five-bond coupling was observed in the HMBC experiment between H-15 and C-11; although this is unusual, we have seen such long-range couplings previously in HMBC spectra, optimized for 5 Hz, for other highly conjugated systems. The ^1H- and ^{13}C-NMR assignments for **1** are summarized in Table 1.

The presence of a significant fragment ion of formula $C_7H_{11}O$ in the EIMS is proposed to arise by cleavage of the diketone bond, while the formation of $C_6H_6^{\bullet+}$ is likely to occur by a mechanism similar to the elimination of in-chain units from the conjugated polyene chains of carotenoids.[13]

Compound **1** has four chiral centers (C-3, C-20, C-22, and C-23). Although some insight into the stereochemistry of the tetrahydrofuran ring might be expected from the coupling constants of the protons, the situation is complex. The polyether antibiotic X-14766A contains a terminal tetrahydrofuran ring with the same oxygenation pattern as in **1**. The stereochemistry of X-14766A was determined by X-ray analysis of its thallium and rubidium salts;[14] unfortunately, no information was published on the ^1H chemical shifts or coupling constants for the tetrahydrofuran ring. Data have been published for other compounds containing substituted tetrahydrofuran rings, although the oxygenation pattern does not match exactly that present in **1**. For instance, the stereochemistry present in A83016F was determined by a combination of comparison of coupling constants to model compounds, NOESY, and molecular modeling.[15] Information derived from the model compounds referred to indicated that coupling constants between vicinal *cis* protons in such a ring ranged from 3 to 6 Hz, while *trans* protons had *J* values of < 1 Hz. In acetylated A83016F, however, coupling constants between a proton equivalent to H-23 of **1** and methylene protons equivalent to H-22 were 6.1 Hz (*cis*) and 10.4 Hz (*trans*); whereas, in **1** $J_{22,23}$ was 2.9 Hz. Similarly, there is no basis for comparing $J_{21,22}$ = 9.1 and 3.2 Hz in **1** with 4.9 (*cis*) and 1.2 (*trans*) Hz in acetylated A83016F. Modeling studies with **1** suggest that the observed coupling constant best fits a *trans* orientation for H-22 and H-23.

In an attempt to determine the stereochemistry of the ring, we performed a NOESY experiment. No correlation was seen between H-22 and H-23, supporting a *trans* orientation. Correlations were observed between H_2-21 and protons H-20 and H-22, and between H-20 and olefinic protons H-19 and H-18, as expected. Also consistent with the structure of **1**, weak correlations were observed between H_2-21 and protons H-19 and H-23. Unfortunately, no direct evidence was obtained for the relative stereochemistry of position 20, as neither H-22 nor H-23 exhibited a cross peak with H-20. The conformational flexibility of the ring can result in protons at positions 22 and 23 being too far from H-20 for NOE interactions, even if they are on the same face.

Nonisoprenoid polyene pigments have been reported in a few macromycetes,[16–19] but are relatively rare. Likewise, natural products containing 1,2-diketones are not common; other examples of fungal metabolites possessing such a moiety include podoscyphic acid[20] and grahamimycin A_1.[21]

The biosynthetic origin of **1** is most probably polyketide, with the main points of interest being the origin of the methyl branch (CH_3-25), the formation of the tetrahydrofuran ring, and the unusual dione system. A standard polyketide assembly phase can be envisaged, with CH_3-25 derived from SAM. The dione system is proposed to be formed by oxidation, followed by double-bond migration and tautomeric rearrangement, as suggested for grahamimycin A_1.[22] The formation of the tetrahydrofuran ring is probably via an epoxide-mediated cyclization, analogous to the biosynthesis of polyethers.[23] Assuming a *trans* stereochemistry for the double bond that is oxidized to the epoxide, as in the rest of the molecule, such a mechanism should result in H-22 and H-23 adopting a *trans* configuration, *viz.* 20*S*,22*R*,23*R* or 20*R*,22*S*,23*S*, consistent with the stereochemistry deduced from NMR.

Experimental Section

General Experimental Procedures. UV spectra were recorded on a Perkin-Elmer Lambda 17 UV/vis spectrometer. IR spectra were acquired by diffuse reflectance (KBr) on a Nicolet 5PC FT-IR spectrometer. MS were obtained with a Finnigan Mat 95 or a VG Trio 3 spectrometer. A desorption probe was used for both EIMS and CIMS spectra and NH_3 was used as CI gas. NMR spectra were recorded on a Bruker ACF400 NMR spectrometer.

Organism Details. The microfungus designated Xenova culture collection number X20416 was isolated from a foam sample collected from a tropical stream at Gong Ghiao, Thailand, during 1989. It was deposited at the International Mycological Institute, Egham, UK, on 1 October 1991, under accession number IMI 350304. Colonies grown on potato-dextrose agar were identified as *Curvularia lunata* (Wakker) Boedijn, the anamorph of *C. lunatus* Nelson and Haasis, on the basis of comparison with the published type description.[24]

Fermentation. A suspension from a mature slant culture, grown on PDA (2% dextrose, 15% agar, 0.4% potato extract) was transferred into a 250-mL Erlenmeyer flask containing 20-mL seed medium (1.5% glycerol, 1.5% soya bean peptone, 1% glucose, 0.5% malt extract, 0.3% NaCl, 0.1% $CaCO_3$, 0.1% Tween 80, 0.1% Junlon PW110, adjusted to pH 6 with H_2SO_4 before sterilization). The flask was incubated at 25 °C on an orbital shaker at 240 rpm for 3 days. After this time, another 20-mL seed medium was added to the seed culture and incubated further for 3 days under the same conditions. The seed culture was inoculated into a 2-L Erlenmeyer flask containing 300 mL of seed medium and incubated at 25 °C and 240 rpm for 3 days. The intermediate culture was transferred into a 14-L fermenter containing 10-L production medium (2.4% D-trehalose, 1.0% MES, 0.7% yeast extract, 0.1% Tween 80, 0.1% carboxymethyl−cellulose, pH 6). The fermentation was conducted at 25 °C for 5 days, under 5-L min^{-1} aeration and 350 rpm agitation.

Isolation. The broth (10 L) was centrifuged and the mycelium extracted with MeOH (6 × 1.5 L; ultrasonication). The extract was filtered and evaporated under reduced pressure to dryness (15 g). The extract was redissolved in MeOH (∼1 L) and separated by reversed-phase preparative HPLC on a C_{18} column (Waters Delta-Pak C_{18}, 100 Å, 15 μm, 47 × 300 mm) eluting (50 mL min^{-1}) with a linear gradient program commencing at 90% H_2O − 10% MeCN and terminating at 100% MeCN after 20 min. The eluent was monitored at 430 nm, and the major peak, eluting after about 12 min, was collected and evaporated under reduced pressure to yield a crude fraction containing **1** (∼200 mg). Further purification was achieved by semi-preparative reversed-phase HPLC (Waters Nova-Pak HR C_{18}, 100 Å, 6 μm, 25 × 200 mm) using a solvent program similar to that above, but at a flow rate of 20 mL min^{-1}. A final purification was performed by column chromatography on Sephadex LH-20 (1 × 10 cm; MeOH eluent) to yield **1** as an orange-red powder (∼50 mg) after evaporation under reduced pressure.

Physicochemical Characterization of 1: CIMS *m/z* 444 ($[M + NH_4]^+$, 10%), 427 ($[M + H]^+$, 100), 397 (15), 365 (40); EIMS *m/z* 426 ($M^{\cdot+}$, < 1), 396 (< 1), 364 (5), 111 (30), 78 (70), 44 (100); HREIMS calcd for $C_{24}H_{28}O_3$, 364.2038; observed, 364.2000; calcd for $C_7H_{11}O$, 111.0807; observed, 111.0810; calcd for C_6H_6, 78.0469; observed, 78.0460; UV λ_{max} (MeOH) nm, 420, 320 (sh); UV λ_{max} (MeOH + HCl) nm: 424, 317 (sh); UV λ_{max} (MeOH + NaOH) nm, 407, 387 (sh), 356 (sh), 338 (sh), 321 (sh); UV λ_{max} (H_2O + MeCN) nm: 430, 262 (sh), 226; IR ν_{max} (KBr) cm^{-1} 3300 (br), 3015, 2965, 2930, 2875, 1670, 1600, 1545, 1010, 725; ^{1}H- and ^{13}C-NMR data are listed in Table 1.

TGF-α Binding Assay. The assay used in the screening program utilized scintillation proximity assay (SPA) technology and involved measuring the binding of ^{125}I-TGF-α to SPA beads coated with membranes from A431 cells. Full details of the assay have been published elsewhere.[10,11] Controls for possible quenching by colored compounds were performed with standards covering a range of colors. In the case of **1**, an additional experiment was performed that involved washing the beads free of unbound TGF-α, transferring them to a tube, and determining directly the level of ^{125}I-TGF-α bound by counting in a γ-counter. The activity of **1** determined in this way was the same as in the SPA assay, within the limits of experimental error.

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