NOTES

XR651, A Novel Naphthacene-5,12-dione

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In our search for microbial inhibitors of interleukin-1 (IL-1) signal transduction we detected promising activity in the absence of cytotoxicity in extracts of fermentations of a naturally occuring actinomycete which was classified as belonging to the genus *Amycolatopsis* (Xenova culture collection number X16735). The active principle was purified and identified as a novel naphthacene-5,12-dione derivative, XR651, **1** (structure shown in Fig. 1). Our screening assay was based on IL-1-induced activation of the KB epidermoid carcinoma cell line measured by the modulation of IL-6 production[1].

Materials and Methods

Source of Organism

The actinomycete designated Xenova culture collection number X16735 was isolated from heather roots collected from lowland heath in the U.K. during 1987. It was deposited at the National Collections of Industrial and Marine Bacteria Ltd., Aberdeen, Scotland on 14 June 1996 under accession number NCIMB 30107.

Fermentation

A cryovial containing 1 ml of vegetative mycelium suspended in a 10% glycerol solution was rapidly thawed from −135°C and used to inoculate a 1 liter conical flask containing 100 ml of seed medium (glucose, glycerol, soya-bean, peptone, malt - extract, yeast - extract, Tween-80 and junlon; pH = 7.0) which was incubated with shaking at 240 rpm and 28°C for four days. This seed culture was used to inoculate a 3-liter fermenter containing 2 liters of production medium (2[-*N*-morpholino] ethanesulfonic acid, proline, glycerol, sucrose, NaCl, K_2HPO_4, Tween-80, $MgSO_4$, $CaCl_2$, trace element mix, vitamin mix and $FeSO_4$; pH = 7.0). The production vessel was stirred at 500 rpm, aerated at 0.5 vvm and incubated at 28°C for nine days after which the culture was harvested and the biomass and liquor separated by centrifugation.

Isolation of XR651

The fermentation liquor was extracted with ethyl acetate (3 × 1.5 liters). The organic layers were combined, concentrated to dryness *in vacuo* and resuspended in methanol (40 ml). Purification of the extract was achieved using flash chromatography on a silica gel column (15 × 9 cm, Merck Kieselgel 60 silica gel (230 ~ 400 mesh)) eluted with dichloromethane. The fraction containing the compound of interest was further purified by chromatography on a column of Sephadex LH20 (12 × 12 cm) eluted with methanol. Fractions containing **1** were concentrated to yield a red solid (10 mg).

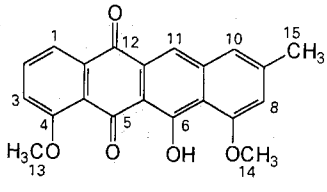
Spectroscopic Studies

UV/Visible spectra were measured on a Perkin-Elmer Lambda 17 UV/Visible spectrophotometer. IR spectra were recorded on a Nicolet 5PC FTIR spectrometer. Low resolution EI-MS were obtained on a VG Trio 3 triple quadrupole mass spectrometer. High resolution EI-MS were obtained on a Finnigan Mat 95 mass spectrometer. ^1H and ^{13}C NMR spectra were recorded at 308 K on a Bruker ACF400 spectrometer at 400 MHz and 100 MHz respectively. All chemical shifts (δ) are quoted in ppm. Standard techniques were used to obtain the DEPT, COSY-45, HMQC, HMBC and NOESY spectra. In HMQC experiments the J_{CH} was optimised for 145 Hz. In HMBC experiments the long range coupling constant $^{3-5}J_{CH}$ was optimised for 7 Hz. A mixing time of 1.8 seconds was used in the NOESY experiments.

IL-1 Signal Transduction Assay

The assay for inhibition of IL-1 signal transduction measured IL-1-induced activation of the KB epidermoid carcinoma cell line by monitoring IL-6 production[1]. Non-specific effects due to cytotoxicity were assessed by concomitant measurement of KB cell proliferation using either an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma Chemical Co.)[2] assay to determine mitochondrial activity or a resazurin[3] assay to examine redox potential.

Fig. 1. Structure of **1**, XR651.



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Results and Discussion

Morphological observations of a fourteen day old culture grown at 28°C on oatmeal agar revealed the presence of a manilla[4] coloured vegetative mycelium and sparse white aerial mycelium with no spore structures or spore pigments being produced. Cultures consisted of a mycelium of fragmenting hyphae (0.5 μm in diameter) which formed non-motile, bacillary elements as shown in Fig. 2.

Chemotaxonomic analysis of the strain revealed the presence of *meso*-diaminopimelic acid in the cell wall, with major amounts of arabinose and galactose detected following whole cell sugar analysis[5]. These results confirm that the strain X16735 has a type IVA cell wall composition of LECHEVALIER and LECHEVALIER[6]. Mycolic acids were not present following saponification of whole cell material and lipid analysis[7]. The strain was also resistant to *Nocardia*-specific phage[8].

The data suggest that the most likely identification for this strain is the genus *Amycolatopsis*.

The IR spectrum of **1** showed an hydroxyl absorption at 3436 cm^{-1}, two carbonyl stretches at 1682, 1642 cm^{-1} and absorptions at 2972, 2932, 1295, 1143, 1042 and 574 cm^{-1}. Its UV/Visible spectrum showed characteristic absorptions in methanol at λ_{max} (ε): 213 nm (22968), 291 nm (12180), 317 nm (15347), 414 nm (6508) and 500 nm (2436).

The ^{13}C NMR spectrum of **1** exhibited 21 carbon signals. DEPT spectra revealed the presence of three CH_3, six CH and twelve quaternary carbons. All of the ^{13}C NMR signals were characteristic of unsaturated carbons with the exception of the methyl signals at 56.5, 55.8 and 22.1 ppm. These were connected to the proton signals at 4.06, 3.93 (characteristic of methoxyl protons) and 2.47 ppm respectively in the HMQC spectrum. The quaternary carbon resonances at 188.5 and 186.3 ppm indicated the presence of two carbonyl groups, confirmed by the IR absorption bands at 1682 and 1642 cm^{-1}.

The ^1H NMR spectrum exhibited six aromatic proton signals and three methyl proton signals. Three of the aromatic signals comprised a spin system consisting of a triplet at δ 7.72 (J=7.9 Hz) and two doublets of doublets at δ 7.66 (J=7.5, 1.4 Hz) and 7.27 (J=8.0, 1.0 Hz) indicating the presence of three protons at adjacent positions on one aromatic ring. The remaining aromatic proton signals were broad singlets at δ 7.34 and 7.06 and a broad doublet at δ 6.64 (J=1.0 Hz). Two of the methyl proton signals at δ 4.06 and 3.93 were singlets characteristic of methoxyl groups while the third methyl signal, also a singlet, was at a higher field, δ 2.47.

The EIMS of XR651 contained prominent ions at m/z: 350 and 348. NMR evidence suggested that the molecular formula was $C_{21}H_{16}O_5$ and the molecular weight 348. Addition of two mass units in mass spectroscopy is characteristic of quinones[9]. HREI-MS confirmed this molecular formula (m/z observed 348.0993; calculated for $C_{21}H_{16}O_5$ 348.0998).

Examination of the COSY-45 spectrum indicated a second aromatic ring containing the two protons at δ 6.64 and 7.06 as *m*- or *p*- coupled protons. The presence of two aromatic rings and a quinone moiety suggested a naphthacenedione skeleton. The structure of **1** was then established using correlations observed in an HMBC NMR experiment. These correlations are shown in Fig. 3(a). This structure was confirmed by the correlations observed in a 2D-NOESY NMR experiment, which are shown in Fig. 3(b). The ^1H and ^{13}C NMR assignments for XR651 are given in Table 1.

The IC_{50} of **1** for the inhibition of IL-1 stimulated IL-6 production by KB cells was 9 μM. This effect initially appeared to be specific and only slight inhibition of KB cell proliferation was observed at concentrations of up to 50 μM as judged by the MTT assay. Further investigations utilising resazurin to assess cell viability did, however, indicate that **1** did cause some cytotoxic effects

Fig. 2. Scanning electron micrograph of *Amycolatopsis* sp. X16735.

Bar represents 5.0 μm.

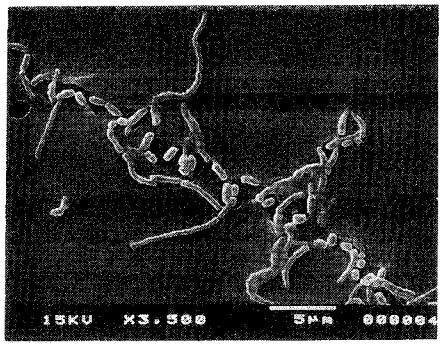
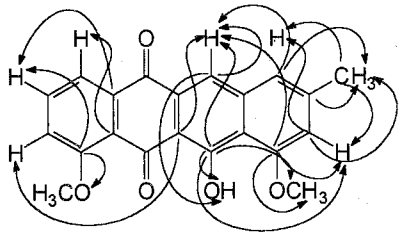


Fig. 3. ^1H-^{13}C correlations observed in the HMBC NMR spectrum of **1** (a) and NOE interactions observed in the 2D-NOESY NMR experiment (b).

(a)



(b)

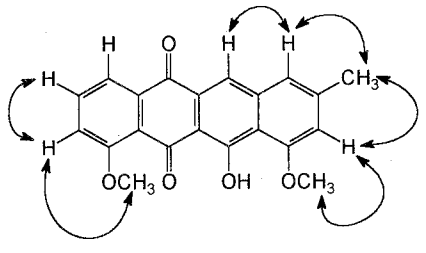


Table 1. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR assignments for **1**.

Position	^{13}C	^{1}H
1	118.5	7.66 (dd, J=7.5, 1.4)
2	135.8	7.72 (t, J=7.9)
3	116.5	7.27 (dd, J=8, 1)
4	159.9	—
4a	120.1	—
5	188.5	—
5a	119.5	—
6	156.8	—
6a	115.6	—
7	156.9	—
8	108.4	6.64 (br d, J=1)
9	141.2	—
10	118.4	7.06 (br s)
10a	140.1	—
11	116.8	7.34 (br s)
11a	136.7	—
12	186.3	—
12a	139.1	—
13	56.5 (s)	4.06 (s)
14	55.8 (s)	3.93 (s)
15	22.1 (s)	2.47 (s)

δ_H and δ_C expressed in ppm and referenced to external TMS (=0 ppm).

at concentrations above 10 μM, as expected for this chemical class and that its effects on the IL-1 signal transduction process were indirect. This illustrates the importance of multiple approaches to determine cytotoxicity.

Literature reports of the biological activities of metabolites related to XR651 also suggest that the IL-1 signal transduction inhibitory effects of XR651 observed by us were indirect. Natural product derivatives of the naphthacenedione class and related anthracyclines and tetracenomycins are produced by a number of actinomycete and fungal species. Members of these classes of compounds possess a range of biological activities including antibacterial[10] and antitumour[11] properties. The streptomycete metabolites maggiemycin and anhydromaggiemycin show antitumour activity against KB, P388 and L1210 murine tumour cell lines[12]. Saintopin was isolated from the mycelium of fermentations of a *Paecilomyces* sp. as an inducer of topoisomerase II-mediated DNA cleavage. It showed weak antimicrobial activity against Gram-positive bacteria[13].

Acknowledgements

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