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Structure elucidation of xanthone derivatives with CD4-binding activity from *Penicillium glabrum* (Wehmer) Westling

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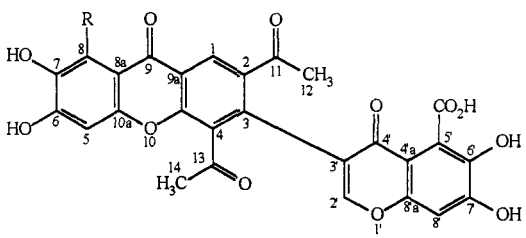
Abstract

Fermentations of *Penicillium glabrum* (Wehmer) Westling produce a complex mixture of metabolites with potent CD4-binding activity in an ELISA based on the binding of the monoclonal antibody anti-Leu 3a to soluble recombinant CD4. On purification this mixture was found to include the known polyketide metabolites citromycetin and anhydrofulvic acid and a series of novel xanthone derivatives with CD4-binding activity. The structure elucidation of these xanthone derivatives was achieved by a combination of mass spectrometric and NMR spectroscopic techniques including HMQC, HMBC and ROESY. These new C_{27}/C_{28} polyketide metabolites probably arise from a dimerisation of two C_{14} polyketides such as the citromycetin precursor polivione.

INTRODUCTION

Extracts of aqueous culture filtrates of fermentations of *Penicillium glabrum* (Wehmer) Westling contained a complex mixture of metabolites with potent CD4-binding activity in an ELISA based on the binding of the monoclonal antibody anti-Leu 3a to soluble recombinant CD4.[1] CD4 is a glycoprotein expressed on the surface of mature helper/inducer T lymphocytes and has an essential role in many immune responses.[2] It is also the cellular receptor for the human immunodeficiency virus (HIV).[2] Anti-Leu 3a blocks both CD4-dependant T cell responses and the binding of HIV to T cells. Compounds with activity in the anti-Leu 3a / CD4 ELISA therefore have potential as selective immunusuppressive agents and also as anti-HIV agents. The *P. glabrum* fermentation extracts were purified by preparative reversed phase HPLC and were found to contain the known compounds citromycetin, polivione and anhydrofulvic acid, which had low activity in the CD4-binding assay, and a series of novel xanthone derivatives with potent CD4-binding activity. Citromycetin, and polivione, are known metabolites of *P frequentans*.[3,4] Polivione exists as a mixture of two slowly interconverting tautomers.[4] Anhydrofulvic acid, has not previously been reported as a fermentation product. It has been reported as a dehydration product of the *Penicillium* sp. metabolite fulvic acid[5].

This paper presents the structure elucidation of the xanthone derivatives 411F, **1**, 411P, **2**, and 411J, **3**, by a combination of mass spectrometric and NMR spectroscopic techniques. Solutions of 411J also contained the cyclised form **4**. After this work was completed the first of these new xanthone derivatives, 411F, was reported independantly as vinaxanthone, a phospholipase C inhibitor produced by *P vinaceum*.[6] The biological properties of the compounds described here will be reported elsewhere.[7]



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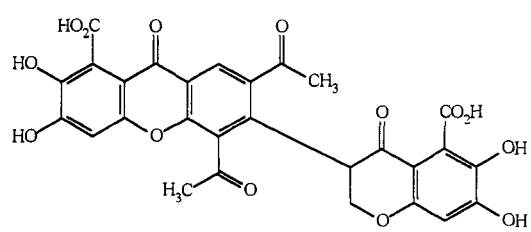
2 R = H



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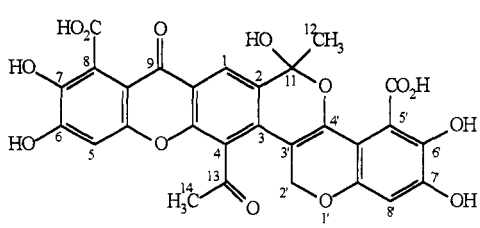

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MATERIALS AND METHODS

The compounds reported here were purified from fermentations of Xenova organism 8063, *Penicillium glabrum* (Wehmer) Westling, by solvent extraction and preparative reversed phase HPLC. Details of the purifications and of the fermentation and identification of the organism will be reported elsewhere. UV/visible spectra were measured on a Perkin-Elmer Lambda 17 UV/visible spectrophotometer. IR spectra were recorded on a Nicolet 5PC FTIR spectrometer using a Spectra Tech "Collector" diffuse reflectance accessory. Low resolution mass spectra (MS) including fast atom bombardment mass spectra (FAB-MS) were obtained on a VG Trio 3 triple quadrupole mass spectrometer using *m*-nitrobenzyl alcohol (MNBA) as the matrix. High resolution FAB-MS were obtained by the SERC Mass Spectrometry Service at the University of Swansea (MNBA was used as the reference compound and as the matrix). ^{1}H and ^{13}C NMR spectra were recorded in d_6-DMSO 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, COLOC, HMQC, HMBC and ROESY spectra. The COLOC experiment was optimised for a J_{CH} of 10 H_Z; 61 increments were collected in t_1 and 4k points in t_2. In HMQC experiments for 411J the J_{CH} was optimised for 145 Hz. In HMBC experiments the long range coupling constant $^{3\text{-}5}J_{CH}$ was optimised for 5 Hz and 256 increments in t_1 and 2k points in t_2 were collected. Sine-bell window functions were applied to the data in both dimensions for all the heteronuclear shift correlation experiments. Experiments were run for 40 or 16 hours depending on concentration. The concentrations of 411F, 411P and 411J used for NMR spectroscopy were 80, 15 and 80 mM respectively. Mixing times from 200 - 800 ms were used in the ROESY experiments for 411J. 256 increments were collected in t_1, 2k points in t_2 and Q-sine window functions applied to the data in both dimensions.

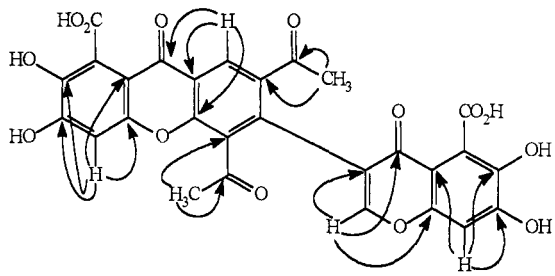
RESULTS AND DISCUSSION

The physico-chemical properties of 411F, 411P and 411J are summarised in Table 1. High resolution FAB-MS indicated that compound 411F had a molecular weight of 576 and a molecular formula of $C_{28}H_{16}O_{14}$. On treatment with diazomethane, a derivative of 411F with a molecular weight of 660, as indicated by MS (desorption chemical ionisation (DCI), NH_3), was formed. On treatment of 411F with *N*-methyl-*N*-(trimethylsily)trifluoroacetamide (MSTFA), a derivative with molecular weight 1008, as indicated by MS (DCI, NH_3), was formed. These micro-derivatisation suggested that 411F possessed six exchangeable protons.

TABLE 1 PHYSICO-CHEMICAL PROPERTIES OF 411F, 411P AND 411J

	411F	411P	411J
Appearance	Yellow-brown powder	Yellow-brown powder	Yellow powder
Molecular Formula	$C_{28}H_{16}O_{14}$	$C_{27}H_{16}O_{12}$	$C_{28}H_{18}O_{14}$
MW	576	532	578
FAB-MS (m/z)	577 $(M+H)^+$, 559, 541	533 $(M+H)^+$, 515, 489	579 $(M+H)^+$, 562, 543
UV: λ_{max} (MeOH) /nm (ε)	226 (28,100), 275 (10,800), 315 (10,800), 383 (12,900)	230 (25,200), 285 (13,100), 381 (11,200)	225 (23,700), 319 (12,400), 402 (14,700)
UV: λ_{max} (MeOH-0.1M NaOH) /nm (ε)	225 (19,200), 284 (9,300), 356 (11,900), 400sh	230 (22,900), 285 (15,000), 300sh, 345 (16,200), 420sh	240 (23,300), 345sh, 400 (16,300)
UV: λ_{max} (MeOH-0.1M HCl) /nm (ε)	237 (23,100), 286 (17,500), 322 (15,300), 365sh	230 (22,900), 285 (15,000) 321 (12,800)	240sh (20,000), 316 (16,100) 365 (10,900)
IR: υ_{max} (KBr) /cm^{-1}	3600-3200 (br), 1696, 1660, 1620, 1570, 1464, 1290, 1220, 1170	3600-3200 (br), 1692, 1659, 1621, 1566, 1518, 1456, 1291, 1228, 1196, 1163	3600-3200 (br), 1705, 1662, 1603, 1560, 1470, 1281, 1200

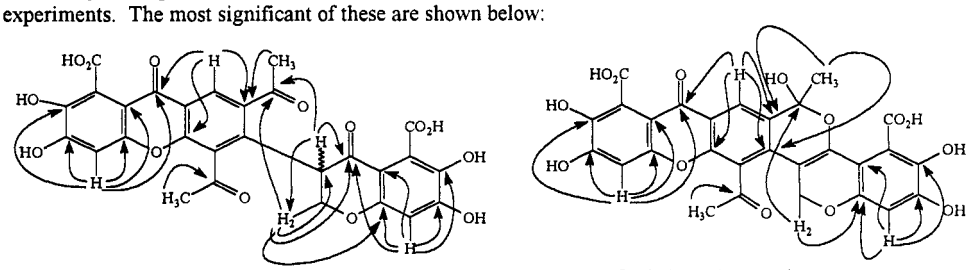
Close comparison of the ^{1}H and ^{13}C NMR data for 411F with literature data for citromycetin, polivione, anhydrofulvic acid and for two recently reported *P. italicum* metabolites[8] suggested the presence of two 3-substituted 6,7-dihydroxychromone-5-carboxylic acid-like moieties in the structure of 411F. Consideration of the possible origin of a C_{28} entity with these characteristics suggested that it might have arisen from the combination of two C_{14} polyketides such as polivione or one of the recent *P. italicum* chromone metabolites to generate structure 1. This structure accounts for the physico-chemical properties and NMR characterisics of 411F Further circumstantial evidence, for this structure was obtained from correlations observed in the COLOC NMR experiment; these correlations are shown below:



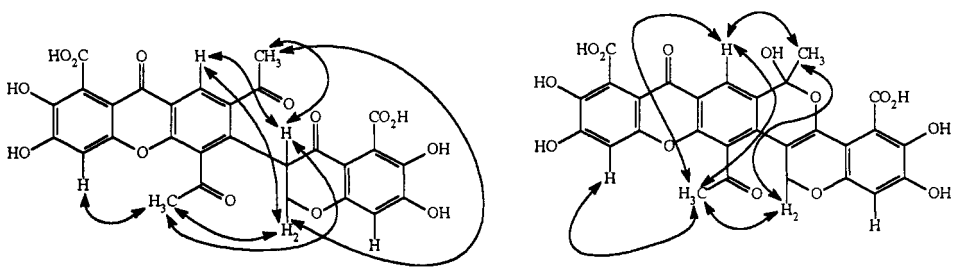
After this work was completed structure **1** was reported for the phospholipase C inhibitor vinaxanthone from *P. vinaceum*[6]. The structure of vinaxanthone was assigned on the basis of HMBC, COLOC, 2D- and selective 1D-INADEQUATE experiments[6]. The reported physico-chemical properties and NMR spectra for vinaxanthone are the same as those for 411F.

MS experiments (FAB, DCI) indicated that 411P had a molecular weight of 532, suggesting a molecular formula of $C_{27}H_{16}O_{12}$. Treatment with diazomethane formed a derivative with a molecular weight of 602 as indicated by MS (DCI, NH_3), suggesting the presence of five exchangeable protons. DEPT analysis showed the presence of a new methine carbon at δ_C 108.4. Otherwise the ^{13}C NMR spectrum of 411P was very similar to that of 411F, with some signals shifted slightly. The ^1H NMR spectrum was almost identical to that of 411F except for an additional singlet at δ_H 7.48. This proton was shown to be attached to the carbon at δ_C 108.4. Consideration of this MS and NMR data suggested that 411P was best represented by structural formula **2**.

High resolution FAB-MS indicated that compound 411J had a molecular weight of 578 and a molecular formula of $C_{28}H_{18}O_{14}$. On treatment with diazomethane a derivative was formed with molecular weight of 676, indicating the formation of a heptamethylated derivative. The ^1H and ^{13}C NMR spectra of 411J in d_6-DMSO consisted of two sets of overlapping signals from two different, but very closely-related, chemical species, which were present in an approximately 4:1 ratio. Both components were very closely related to 411F. Consideration of the NMR data for 411J suggested that it was best accounted for by the presence of two slowly interconverting ring-chain tautomers of structural formulae **3**, **4**. The minor component is the 2', 3'-dihydro form of 411F and the major component is its cyclised hemiacetal tautomer. Correlations supporting these conclusions were obtained by HMBC experiments. The most significant of these are shown below:



Initial attempts to probe through-space proton-proton interactions by the NOESY NMR experiment were unsuccessful. No cross-peaks were observed, probably because of unfavourable tumbling rates. An alternative approach to probe these through-space interactions using the ROESY NMR experiment was successful. The correlations observed are shown below.



Other possible structures for the major and minor components of 411J based on different dimerisations of possible C_{14} precusors and different cyclisation possibilities for the tautomerisation were considered and rejected on the grounds that they fitted the NMR data less well than did structural formulae **3** and **4**.

The ^1H and ^{13}C NMR data for 411F, 411P and the two tautomers of 411J, and their assignments in terms of structures **1**, **2**, **3** and **4**, are given in Table 2.

CONCLUSIONS

Three of the metabolites reponsible for the activity of fermentation samples from *P. glabrum* in the anti-Leu 3a/CD4 binding assay have been studied and structures proposed. They are highly substituted polyketides which are possibly formed from the dimerisation of a C_{14} polyketide related to the known *P. glabrum* metabolite polivione. Their structure elucidation was made especially difficult by their highly substituted nature. Heteronuclear shift correlation experiments such as COLOC, HMQC and HMBC were used extensively. The data from these experiments can be equivocal and in these cases, although providing good evidence for the proposed structures, did not provide a full confirmation for them. For 411F confirmatory evidence has been obtained from published data for vinaxanthone, where INADEQUATE NMR experiments were used to define the carbon skeleton. INADEQUATE NMR experiments would be very useful for 411J but they are not sensitive experiments and the amounts of this compound produced in *P. glabrum* fermentations falls a long way short of the amount required. Further circumstantial evidence for the structures proposed for 411J was obtained by observing through-space proton-proton interactions in the ROESY NMR experiment. This demonstrates the

value of ROESY as an alternative approach to the NOESY NMR experiment when parameters such as the tumbling rate are unfavourable for NOESY.

TABLE 2 ^{1}H and ^{13}C NMR data in $(CD_3)_2SO$ for 4, 5, 6, and 7

ATOM	1[a]		2[a]		3[b]		4[b]	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	126.5	8.52,s	126.1	8.56,s	127.5	8.53,s	125.8	7.97,s
2	136.4		136.0		139.1		138.2	
3	132.7		132.3		134.9		129.8	
4	133.5		133.5		132.4		131.8	
4a	152.2		152.5		152.1		151.6	
5	102.3	6.94,s	103.0	6.96,s	102.3	6.93,s	102.3	6.94,s
6	152.7		152.6		153.8		153.8	
7	141.1		144.5		140.8		140.7	
8	119.7		108.4	7.48,s	120.6		120.6	
8a	110.2		113.4		110.1		110.0	
9	172.8		173.3		172.5		172.7	
9a	120.7		119.7		118.2		118.6	
10a	150.4		150.6		150.1		150.2	
11	199.3		c		199.0		104.4	
12	29.2	2.52,s	28.8	2.52,s	16.9	2.31,s	16.4	2.33,s
13	201.3		c		202.5		202.4	
14	32.3	2.53,s	31.7	2.53,s	32.4	2.68,s	32.2	2.71,s
2′	152.9	8.14,s	152.4	8.13,s	66.9	4.73,dd (11,5), 4.64,dd (11,9)	65.8	4.69, br s
3′	121.0		120.8		56.2	5.04, dd (9,5)	e	
4′	173.0		172.8		186.1		182.8	
4′a	112.6		112.4		108.7		109.2	
5′	119.9		119.5		122.2		121.6	
6′	141.8		141.7		137.5		138.6	
7′	154.1		154.3		154.5		154.5	
8′	102.4	6.95,s	102.3	6.94,s	102.4	6.43,s	102.8	6.40,s
8′a	150.8		150.7		156.2		155.6	
8-CO_2H	167.5		-		167.4		167.4	
5′-CO_2H	167.5		167.2		167.4		167.5	

a Chemical shifts are given in ppm referenced to $(CH_3)_2SO$ at 2.50 ppm for ^{1}H and $(CD_3)_2SO$ at 39.51 ppm for ^{13}C.
b Chemical shifts are given in ppm referenced to internal TMS for ^{1}H and ^{13}C spectra. Coupling constants in parentheses are quoted to the nearest Hz.
c Signals for these carbons were not observed.
e No signal which could be assigned to this carbon was observed in the ^{13}C NMR spectrum.
s Singlet
dd Doublet of doublets

ACKNOWLEDGMENT

The authors would like to acknowledge the considerable contributions of Peter J Hylands to the work described in this paper.

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