Structure elucidation of two differentiation inducing factors (DIF-2 and DIF-3) from the cellular slime mould *Dictyostelium discoideum*

Howard R. MORRIS,* Mark S. MASENTO,*|| Graham W. TAYLOR,†¶ Keith A. JERMYN‡ and Robert R. KAY§

*Department of Biochemistry, Imperial College of Science and Technology, South Kensington, London SW7 2AZ, †Department of Clinical Pharmacology, Royal Postgraduate Medical School, Hammersmith Hospital, DuCane Road, London W12 0HS, ‡Imperial Cancer Research Fund, Clare Hall Laboratories, South Mimms, Herts. EN6 3LD, and §MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, U.K.

Two endogenous differentiation-inducing factors (DIF-2 and DIF-3), which induce stalk-cell differentiation in the cellular slime mould *Dictyostelium discoideum*, have been identified as the pentan-1-one and monochloro analogues respectively of DIF-1 {1-[(3,5-dichloro-2,6-dihydroxy-4-methoxy)phenyl]hexan-1-one}. These compounds represent a new chemical class of effector molecules.

INTRODUCTION

Starvation of amoebae of the cellular slime mould *Dictyostelium discoideum* results in their aggregation by chemotaxis to relayed cyclic AMP signals to form a multicellular mass. Shortly thereafter, a pattern becomes apparent in the cell mass, with differentiation occurring toward stalk cells in the anterior region and toward spore cells in the posterior. During this process, a number of low M_r substances are released which can induce cell differentiation among isolated amoebae (Town *et al.*, 1976; Town & Stanford, 1979; Kay *et al.*, 1983). The major biologically active differentiation-inducing factor, DIF-1, is active at 10^{-10} M and has recently been identified as 1-[(3,5-dichloro-2,6-dihydroxy-4-methoxy)phenyl]hexan-1-one (Morris *et al.*, 1987). Two further biologically active species (DIF-2 and DIF-3) have been purified from developing *Dictyostelium discoideum* and have been identified and synthesized.

EXPERIMENTAL

Chemicals were of AnalaR grade from BDH Chemicals, Poole, Dorset, U.K. H.p.l.c. solvents were from Rathburn Chemicals, Walkerburn, Peeblesshire, Scotland, U.K.; water was of Milli Q grade (Waters Associates, Harrow, Middx., U.K.).

DIFs were obtained from the medium of developing *Dictyostelium discoideum*, and purified by solvent extraction and two or three steps of reverse-phase h.p.l.c. as previously reported (Kay *et al.*, 1983). Biological activity was determined using the stalk-cell-induction bioassay (Brookman *et al.*, 1982).

Probe-electron-impact mass spectra were recorded at 70 eV on a Kratos MS50 instrument, using a temperature-programmed ion source to fractionally volatilize the sample. Accurate mass measurement was undertaken at

10000 resolving power using perfluorokerosene as a reference. G.c. was carried out on an SE 54 capillary column (30 m × 0.2 mm), eluting with helium as the carrier gas over a $10\text{ }^{\circ}\text{C}\cdot\text{min}^{-1}$ temperature gradient between 100 and 280 $^{\circ}\text{C}$. Samples were loaded in octane at 200 $^{\circ}\text{C}$ with a Grob injector in the splitless mode. The g.c. column was routed into a Finnigan 4500 quadrupole mass spectrometer, which was operated in the electron-impact mode. Desorption chemical ionization was carried out on a Finnigan 4500 mass spectrometer in both positive- and negative-ion modes. Methane was used as the reagent gas, and the sample was desorbed at $10\text{ mA}\cdot\text{s}^{-1}$.

U.v. spectra were obtained in methanol or 50% methanol/aq. 0.2 M-HCl on either a Cary 210 or a Perkin-Elmer 555 spectrophotometer. N.m.r. spectra were obtained on a Bruker 250 MHz instrument in (^2H)chloroform.

Additional h.p.l.c. was undertaken on a Waters instrument; samples were chromatographed on a μ Bondapak C_{18} column (30 cm × 0.8 cm), eluting at 1 ml/min isocratically with propan-2-ol/acetic acid/water (140:13:247, by vol.). The h.p.l.c. eluate was monitored at 280 and 254 nm and by bioassay.

Synthetic DIF-2 and DIF-3 were prepared by the Hoesch reactions, followed by chlorination; essentially the synthesis of DIF-2 involved allowing 1,3-dihydroxy-5-methoxybenzene (1 mmol) to react with valeronitrile (1 mmol) at 0 $^{\circ}\text{C}$ for 48 h in the presence of ZnCl_2 and anhydrous HCl. The resulting imino-zinc salt was hydrolysed, and the two isomeric 1-[dihydroxymethoxyphenyl]-1-ketones were extracted into chloroform, treated with chlorine water and finally purified by h.p.l.c. to obtain the dichlorinated butyl ketone (DIF-2). DIF-3 was prepared in an analogous manner, using hexanonitrile; chlorination was limited to generate mainly the monochlorinated species, which were purified by h.p.l.c. to give DIF-3. Synthetic materials were characterized by m.s. and by u.v. and n.m.r. spectroscopy.

Abbreviation used: DIF, differentiation-inducing factor.

|| Present address: Chester Beatty Laboratories, Institute of Cancer Research, Fulham Road, London SW3 6JB, U.K.

¶ To whom correspondence and reprint requests should be sent.

Fig. 1. Electron-impact mass spectra of DIF-2 (a) and DIF-3 (b)

The spectrum is homologous with that of DIF-1 (Morris *et al.*, 1987). The 9:6:1 isotope ratio of the major ions arises from the presence of two chlorine atoms in the molecule. The molecular ion (M^{+} , m/z 292) loses $C_2H_5^+$ (m/z 263) and $C_4H_9^+$ (m/z 235). The McLafferty-rearrangement ion (m/z 250) defines the presence of a butyl ketone side chain. (b) This is a monochlorinated species (3:1 isotope ratio). Losses of $C_3H_7^+$, $C_5H_{11}^+$ and C_4H_8 (McLafferty rearrangement) define the $CO \cdot C_5H_{11}$ substitution.

RESULTS

The medium surrounding developing *Dictyostelium* cells yields a number of substances which induce stalk-cell differentiation among isolated amoebae in the DIF bioassay. These activities may be resolved by reverse-phase h.p.l.c. The major biologically active species, DIF-1, has been identified as 1-[(3,5-dichloro-2,6-dihydroxy-4-

methoxy)phenyl]hexan-1-one (Morris *et al.*, 1987); substantial amounts of biological activity were also associated with two other u.v.-absorbing peaks, namely DIF-2 and DIF-3.

Previous work had shown that the biological activity of DIF-2 is destroyed by reduction with $NaBH_4$ (Kay *et al.*, 1983), indicating that, like DIF-1, this substance possesses a carbonyl group. Purified DIF-2 possessed a

Table 1. High-resolution mass measurement of DIF-2

Mass measurements were obtained at a resolution of 10000 against a perfluorokerosene reference. The mass of the molecular ion defines the atomic composition of DIF-2. The butyl side chain is confirmed by the measured mass of m/z 235. The $^{37}Cl^{35}Cl$ isotope was also measured to increase confidence in the structural assignments.

Nominal mass ion		Accurate mass	Molecular formula
m/z	Assignment		
292	M^{+}	292.027	$C_{12}H_{14}O_4^{35}Cl_2$
294	$[^{37}Cl^{35}Cl] M^{+}$	294.024	$C_{12}H_{14}O_4^{37}Cl^{35}Cl$
235	$M^{+} - C_4H_9^+$	234.957	$C_8H_5O_4^{35}Cl_2$
237	$[^{37}Cl^{35}Cl] M^{+} - C_4H_9^+$	236.954	$C_8H_5O_4^{37}Cl^{35}Cl$
220	$M^{+} - C_5H_{12}$	219.933	$C_7H_2O_4^{35}Cl_2$

Fig. 2. Chemical structures of DIF-2 (a) and DIF-3 (b)

The structure of the major biologically active species released by *Dictyostelium discoideum*, DIF-1, is shown (c) for comparison (from Morris *et al.*, 1987).

u.v.-absorbance spectrum similar to that of DIF I, characteristic of a highly conjugated, probably aromatic, species ($\lambda_{\max}$ 274 nm).

The M_r of DIF-2 was defined as 292 by positive- and negative-ion desorption chemical ionization ($M+H^+$, m/z 293; M^- , m/z 292). DIF-2 was eluted on g.c. with a retention time of 12.5 min and generated the electron-impact mass spectrum shown in Fig. 1(a). A series of polyisotopic ion clusters were observed between m/z 200 and m/z 296; each cluster has an isotope ratio of 9:6:1, defining the presence of two chlorine atoms. The mass spectrum is homologous with that of DIF-1 (Morris *et al.*, 1987) and essentially indistinguishable in the region m/z 200–240. Accurate mass measurement of the major ions was undertaken (Table 1); the molecular ion (M^{+}) at m/z 292.027 defined the atomic composition of DIF-2 as $C_{12}H_{14}O_4Cl_2$. Losses of $C_2H_5^+$ (m/z 263), $C_4H_9^+$ (m/z 235), together with the rearrangement ions at m/z 250 and 220, established the presence of a $CO \cdot C_4H_9$ functional group. These data indicated that DIF-2 is 1-[(3,5-dichloro-2,6-dihydroxy-4-methoxy)phenyl]pentan-1-one (Fig. 2a), a homologue of DIF-1. This compound was synthesized and characterized by its n.m.r. spectrum: signals were observed at δ (chemical shift, p.p.m.) 10.2 (singlet, 2H), 3.96 (singlet, 3H), 3.11 (triplet, 2H) 3.05 (triplet, 2H) 1.68 (multiplet, 4H) and 0.90 (multiplet, 3H). Natural and synthetic DIF-2 were co-eluted on h.p.l.c. and g.c.; their electron-impact mass spectra were indistinguishable. The synthetic material was active in the bioassay with a specific activity of $69.5 \pm 30.2 \times 10^6$

units $\cdot \mu\text{mol}^{-1}$ ($n = 3$). (A unit of activity is defined as the amount that will induce 1% stalk-cell differentiation in a 2 ml bioassay; Brookman *et al.*, 1982.) The 1-(3-methylbutanone) isomer was also synthesized; although it possessed biological activity, it differed in terms of h.p.l.c. and g.c. retention times and its mass spectrum from the natural substance.

DIF-3 was eluted before DIF-2 on h.p.l.c. as a u.v.-absorbing substance; it also contains an essential carbonyl group (Kay *et al.*, 1983). After g.c. (retention time 11.5 min), isotope clusters were again observed in the electron-impact mass spectrum (Fig. 1b). The isotope ratio (3:1) for each of the more intense ions was consistent with the presence of a monochlorinated species. The M_r was defined by the molecular ion (M^{+} , m/z 272); insufficient sample was available for accurate mass measurement. Fragmentation of M^{+} led to the formation of ions at m/z 229 ($M^{+} - C_3H_7^+$), 201 ($M^{+} - C_5H_{11}^+$), and 216 ($M^{+} - C_4H_8^+$; McLafferty rearrangement). The spectrum was analogous to that of DIF-1, indicating that DIF-3 is a monochloro analogue of DIF-1 [1-(3-chloro-2,6-dihydroxy-4-methoxyphenyl)hexan-1-one; Fig. 2b]. The monochloro derivative was formed as a by-product in the synthesis of DIF-1, with synthetic yields increased by a decreased chlorination time. Synthetic DIF-3 was indistinguishable from the natural material by h.p.l.c. retention time and by its electron-impact mass spectrum. Synthetic DIF-3 was active in the bioassay, but insufficient pure material was available to give a precise biological activity.

Two further weakly bioactive species (DIF-4 and DIF-5) (Kay *et al.*, 1983) have also been examined by electron-impact mass spectrometry; neither contains chlorine. Further investigation will be required to determine their full structure.

DISCUSSION

The major stalk-cell differentiation-inducing activity released by developing *Dictyostelium* cells has recently been identified as 1-[(2,6-dihydroxy-3,5-dichloro-4-methoxy)phenyl]hexan-1-one (Morris *et al.*, 1987). It is accompanied by a number of related species of lower specific activity. DIF-2 and DIF-3 represent about 3 and 1% respectively of the recovered bioactivity (Kay *et al.*, 1983; Brookman *et al.*, 1987). DIF-2 is a lower homologue of DIF-1, having a C_4 rather than a C_5 alkyl side chain, whereas DIF-3 lacks one of the chlorine atoms. Although a number of naturally occurring chlorinated phenols are known (Suida & DeBernardis, 1973; Niedleman & Geigert, 1986), we are not aware of any in which there is an additional alkyl ketone substitution on the benzene ring. DIFs 1–3 therefore represent a new class of biological effector molecules.

The biosynthetic pathway for DIF-1 is not yet known, but the alternating oxy substitution on the ring suggests that it is made by the polyketide pathway, in common with many other natural products (Luckner, 1972). Assembly of the carbon skeleton would be followed by *O*-methylation and chlorination by chloroperoxidase (Niedleman & Geigert, 1986). DIF-2 and DIF-3 could easily arise from variants of this pathway. Incomplete chlorination is the obvious source of DIF-3, whereas DIF-2 could be produced by the incorporation of a C_3 propionyl-CoA unit into the polyketide in place of two molecules of acetyl-CoA. The close chemical relationship

of DIF-2 and DIF-3 to DIF-1 does not preclude them from having distinct functions in *Dictyostelium* development. The identification and synthesis of DIF-2 and DIF-3 should assist in determining whether such distinct functions exist.

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