Differentiation-inducing factor from the slime mould *Dictyostelium discoideum* and its analogues

Synthesis, structure and biological activity

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Previous work has led to the identification of a novel class of effector molecules [DIFs (differentiation-inducing factors) 1–3] released from the slime mould *Dictyostelium discoideum*. These substances induce stalk-cell differentiation in *Dictyostelium discoideum* and are thought to act as morphogens in the generation of the prestalk/prespore pattern during development. The DIFs are phenylalkan-1-ones, with chloro, hydroxy and methoxy substitution on the benzene ring. DIFs 1–3 and a number of their analogues have been synthesized by using a simple two-step procedure, and each analogue has been characterized by m.s., u.v. and n.m.r. spectroscopy. The crystal structure of synthetic DIF-1 [1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)hexan-1-one, was investigated. The specific biological activity of each analogue was determined in a bioassay, where isolated *Dictyostelium* amoebae are induced to differentiate into stalk cells. The major biologically active substance, DIF-1, caused 50 % stalk-cell differentiation at 1.8×10^{-10} M; the C₄ alkyl homologue (DIF-2) and C₆ homologue possessed 40 and 16 % of the activity of DIF-1 respectively. Further increase or decrease in the alkyl chain length resulted in a marked decrease in specific activity. The pattern of substitution on the benzene ring is a major determinant of bioactivity, since the specific activities of the 2,4-dihydroxy-6-methoxy and trihydroxy analogues were less than 1 % of that of DIF-1. Substitution of bromine in DIF-1 had little effect on bioactivity; in contrast the activity of monochloro-DIF-1 (DIF-3) was diminished. There was no evidence for antagonism or synergy between DIF-1 and any of its analogues. This series of analogues will facilitate further studies in the biological effects and mode of action of DIF-1.

INTRODUCTION

The cells of the slime mould *Dictyostelium discoideum* grow as single amoebae, but when starved they aggregate in response to propagating waves of cyclic AMP to form a multicellular organism which transforms itself into a migrating 'slug' (Loomis, 1982; Gerisch, 1987). Within this slug there is a simple spatial pattern of cell differentiation with prestalk cells in the anterior and prespore cells in the posterior. This pattern reflects the final stalk/spore proportion of the mature fruiting body. Stalk-cell differentiation *in vitro* can be induced by DIF (differentiation-inducing factor)-1 (Town *et al.*, 1976; Brookman *et al.*, 1982), a factor released by developing cells which has recently been defined as 1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)hexan-1-one (Morris *et al.*, 1987). Smaller amounts (in terms of bioactivity) of the C₄ homologue (DIF-2) and monochloro analogue (DIF-3) are also released (Kay *et al.*, 1983; Morris *et al.*, 1988). DIFs 1–3 are of great interest both chemically and biologically. Chemically they represent a new, and possibly more widespread, class of effector molecules, and there is preliminary evidence that they are present

in other dictyostelid species (Brookman *et al.*, 1982). Biologically, DIF-1 is a key regulatory molecule in *Dictyostelium* development and appears to induce the differentiation of a new cell type, the prestalk cell (Kopachik *et al.*, 1983, 1985; Williams *et al.*, 1987). Together with other diffusible signals, such as cyclic AMP, adenosine and NH₃, DIF-1 may be involved in generating the prestalk/prespore pattern in the aggregate (Kay *et al.*, 1983; Weijer & Durston, 1985; Schaap & Wang, 1986; Williams, 1988). The generation of such spatial patterns is a fundamental problem in developmental biology and is thought to involve gradients in the embryo of signal substances such as DIF-1 (Wolpert, 1971; Gross *et al.*, 1981).

DIF-1 is very potent, with measurable biological activity at 10^{-10} M (Kay *et al.*, 1983; the present study) and is able to induce the transcription of the pDd63 prestalk-specific mRNA within 15 min of addition to responsive cells (Williams *et al.*, 1987). It thus seems certain that DIF-1 must have a specific receptor, and it is possible, given the novel nature of DIF-1, that this receptor couples to a response pathway having some novel features (Gross *et al.*, 1983). The study of receptors

Abbreviations used: DIF, differentiation-inducing factor; -OMe, methyl ester.

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has been facilitated by the use of a range of effector analogues. These can be used to define separate classes of related receptors, where such exist (Lefkowitz *et al.*, 1983), to reveal details of receptor–ligand interaction (Van Haastert & Kein, 1983) and to provide information for the design of specific labelled probes and affinity matrices for the purification of the receptor.

In the work described here, we have developed a simple synthetic procedure for DIF-1 and related compounds. A number of analogues have been synthesized and their biological activity in the DIF bioassay has been quantified; a potency series has been established which will be invaluable in characterizing the DIF receptor(s).

EXPERIMENTAL

Chemicals were obtained from either BDH Chemicals (Poole, Dorset, U.K.) or Aldrich (Gillingham, Dorset, U.K.), and were of analytical-reagent grade. H.p.l.c. solvents were obtained from Rathburn (Walkerburn, Peeblesshire, Scotland, U.K.). DIF-1 and its analogues were synthesized by using the Hoesch reaction, followed by aqueous-phase chlorination. HCl gas was bubbled into 25 ml of anhydrous ether containing 1 mmol each of 3,5-dihydroxyanisole and hexanonitrile in the presence of anhydrous ZnCl_2 (2 g). After 72 h in an ice/salt bath, the resulting iminochloride was hydrolysed for 2 h at 100 °C in 200 ml of water; the isomeric hexanones were extracted into chloroform. The hexanones were repeatedly chlorinated with chlorine water (20 min, room temp.) and the yield of dichlorinated product monitored by m.s. When optimum chlorination had been achieved, the products were purified by reverse-phase h.p.l.c. Analogues of DIF-1 were prepared similarly by substituting the nitrile, polyhydric phenol or bromine water as appropriate. H.p.l.c. of the synthetic mixture was undertaken on a Waters dual-pump instrument. Samples were chromatographed on a μ Bondapak C_{18} column, eluting isocratically at 1 ml $\cdot$ min $^{-1}$ in 35% (v/v) propan-1-ol in aq. 5% (v/v) acetic acid. The h.p.l.c. eluate was monitored at 280 nm and 254 nm and by bioassay.

DIF activity was measured as previously described (Brookman *et al.*, 1982; Kay *et al.*, 1983) with minor modifications. Aliquots (2 ml) of washed vegetative cells of strain *V12M2*, at a density of $10^4 \cdot \text{ml}^{-1}$ in 10 mM-potassium Hepes/10 mM-KCl/2 mM-NaCl/1 mM- CaCl_2 , pH 6.2, containing 200 μg of streptomycin sulphate/ml and 0.5 mM-cyclic AMP, were pipetted into 5 cm-diameter tissue-culture dishes (Sterilin 302 V). The test substance, in up to 2 μl of ethanol, was then mixed into the medium and, after 2 days incubation in the dark at 22 °C, cell differentiation was scored by phase-contrast microscopy. Cells which were at least 50% vacuolated were defined as stalk cells, and at least 100 cells were counted per plate. Duplicate assays of four concentrations of the test substance were always performed and compared with a DIF-1 standard assayed at the same time. The sensitivity of the assay measured from the standard varied by up to 5-fold from day to day. However, the relative activities of the analogues were much less variable, except for those with very low activity (such as the 6-methoxy C_8 analogue; Table 2 below), which were assayed at close to their lethal concentration. One unit of DIF activity is defined as the

amount that will induce 1% stalk-cell differentiation in the above 2 ml assay.

M.s. was undertaken in the electron-impact mode on an MS 50 instrument (Kratos U.K.); the electron energy was set at 70 eV and exponential scans (10 s) obtained over the mass range 50–500 units. Samples were fractionally evaporated from a quartz tip over a temperature range of 100–300 °C. G.c.–m.s. was undertaken on a Finnigan 4500 instrument. Samples were injected at 200 °C in octane on to an SE 54 capillary column. Elution was undertaken with helium as the carrier gas over a temperature gradient (10 °C $\cdot$ min $^{-1}$) from 100 °C to 280 °C. The g.c. column was routed directly into the mass spectrometer, which was operated in the electron-impact mode at 70 eV; 1 s scans over the mass range 70–500 mass units were obtained.

N.m.r. spectra were obtained in [^2H]chloroform on a Bruker 250 MHz instrument, calibrated against the proton resonance arising from a trace amount of [^1H]chloroform present. U.v. spectra were obtained in methanol [aq. 50% (v/v) methanol containing 0.1 M-HCl or 0.1 M-NaOH] on a Cary 210 spectrophotometer.

Crystals of synthetic DIF-1 were obtained by crystallization from [^2H]chloroform over 14 days. X-ray-diffraction data were collected on a Nicolet R3m/Eclipse S140 diffractometer system using an ω -scan technique with graphite monochromated Cu- $\text{K}\alpha$ radiation. A total of 2953 independent reflections were measured (to $\theta = 50^\circ$), of which 668 were judged to be ‘unobserved’. The structure was solved by direct methods, and least-squares refinement has reached $R = 0.048$ and $R_w = 0.040$.

RESULTS

The Hoesch reaction between 3,5-dihydroxyanisole and hexanonitrile generated two products, the 4- and 6-methoxy isomeric substituted hexanophenones. Controlled chlorination of the mixture produced the corresponding dichlorinated products (DIF-1 and its 6-*O*-methyl isomer; Scheme 1), further contaminated by mono- and some tri-chlorinated species, as judged by mass-spectrometric analysis. The mixture could be resolved by h.p.l.c. (Fig. 2). Among the u.v.-absorbing peaks in the h.p.l.c. profile were the two isomeric dichlorinated species (**I** and **II**, Fig. 1), which were eluted at 31 and 39.5 min respectively. We had shown previously that natural DIF-1 is identical with isomer **II** by h.p.l.c., g.c., m.s. and n.m.r. analysis (Morris *et al.*, 1987). Here we present in more detail the evidence that isomer **I** is 1-(3,5-dichloro-2,4-dihydroxy-6-methoxyphenyl)hexan-1-one, and that isomer **II** (and hence DIF-1) is the 2,6-dihydroxy-4-methoxyphenyl isomer.

The u.v. spectra of the two DIF-1 isomers in methanol differed markedly, with λ_{max} values of 335 nm and $\epsilon = 14000$ (**I**) and 277 nm and $\epsilon = 10600$ (**II**) suggesting that isomer **I** possesses phenoxide ion characteristics. In the presence of strong acid, however, λ_{max} for isomer **I** shifted to 284 nm (with little change in the spectrum of isomer **II**), indicating that the spectral differences observed for the two isomers were probably related to the pK_a of the hydroxy functions. The isomers were separated by g.c. (14.16 min and 14.33 min) and generated similar electron-impact mass spectra (Table 1), differing mainly in the diminished intensity of the McLafferty rearrangement ion ($M^{++} - \text{C}_4\text{H}_8$, m/z 250)

Scheme 1. Synthetic route to DIF-1

The Hoesch reaction generates two isomers (4-OMe and 6-OMe), which are then chlorinated to generate 1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)hexan-1-one (DIF-1, isomer II) and its 6-methoxy isomer (I). Homologues were prepared by replacing hexanenitrile with the appropriate alkyl cyanide. Partial chlorination results in the formation of monochlorinated species.

Fig. 1. H.p.l.c. separation of the reaction products in DIF-1 synthesis

Five u.v. (A_{280})-absorbing compounds were detected. The three unresolved peaks (21.5–27.3 min) each possessed e.i. mass spectra consistent with monochlorination of the C_5 ketone mixture ($M^{+} m/z$ 272; $M^{+} - C_5H_{11}$ m/z 201). Only one of these isomers was biologically active in the assay (i.e. DIF-3, the monochloro analogue of DIF-1; see Morris *et al.*, 1988). The two later-eluted substances (31 min and 39.5 min, isomers I and II) were dichlorinated C_5 alkyl ketones ($M^{+} m/z$ 306, $M^{+} - C_5H_{11}$ m/z 235). Biological activity was associated with isomer II; the activity of isomer I was below the limit of detection of the assay (see Table 1). Isomer II was co-eluted with natural DIF-1.

for isomer I. This fragmentation involves interaction of a γ -H with the carbonyl function, suggesting that the ability of the carbonyl to accept protons in the rearrangement process is impaired in isomer I. These observations were interpreted as arising through the influence of different H-bonding in the hydroxy groups of the two isomers. Strong H-bonding occurs between the carbonyl group and the single *ortho*-OH for the 6-OMe isomer, resulting in a highly acidic hydroxyl

proton (together with a high proportion of phenoxide ion) and a normal phenolic proton. In the 4-OMe isomer, the effect of H-bonding is spread over the two *ortho*-OH groups, resulting in two equivalent protons of acidity intermediate between a phenol and an H-bonded OH group. This interpretation is borne out by the n.m.r. spectra: the high-field regions of the spectra were similar; however, isomer II generated a 2H broad signal at δ (chemical shift in p.p.m.) 10, whereas in isomer I the signal was split between δ 6 p.p.m. (1 H, phenolic) and δ 14 (1 H, acidic). Thus the earlier-eluted isomer (I) is 6-OMe-substituted, with isomer II (DIF-1) being the 4-OMe species.

The crystal structure of synthetic DIF-1 (isomer II) was determined by X-ray-crystallographic analysis. Crystals of isomer II are monoclinic with $a = 1.3329$ (3), $b = 0.7505$ (1), $c = 2.9490$ (9) nm, $\beta = 103.29$ (2) $^\circ$ (at 19 $^\circ$ C), space group $P2_1/n$ (no. 14) and $Z = 8$ units of $C_{13}H_{16}O_4Cl_2$. In the crystal structure of this isomer there are two crystallographically independent molecules, but there are no chemically significant differences between them. The molecular structure is shown in Fig. 2. In each molecule there is an intramolecular hydrogen bond (shown by the dotted line in Fig. 2a) between a hydroxy group (on the equivalent 2- or 6-position of the ring) and the oxygen of the carbonyl group. In solution, of course, both hydroxy groups within any molecule may form this hydrogen bond with equal probability (cf. n.m.r. and u.v. spectra).

A further five pairs of isomeric analogues differing in the length of their alkyl substituent were also synthesized by the Hoesch/chlorination method, starting with the appropriate nitrile; for example, valeronitrile was used to prepare DIF-2, the naturally occurring C_4 homologue. In addition, the trihydroxy (desmethyl) analogue was

Table 1. Synthetic DIF analogues: effect of alkyl chain length on n.m.r. and mass spectra

The molecular ion in each mass spectrum is italicized, with relative intensities given in parentheses. The mass spectra of the 6-methoxy (I) and 4-methoxy (II) isomers for each analogue are similar, except for the McLafferty rearrangement ion (m/z 250), which is noticeably less intense in the 6-methoxy isomers (I). N.m.r. spectra also distinguish between the isomers. In isomers I, the strongly hydrogen-bonded *ortho*-hydroxy proton appears at δ 14, with the phenolic (*para*) proton at δ 6.5. For isomers II, the two (*ortho*-hydroxy) proton signals are averaged to a broad singlet at δ 10.3. The number of protons and signal type are given in parentheses (s, singlet; t, triplet; m, multiplet).

Alkyl chain	Isomer	Electron-impact mass spectra	N.m.r. spectra
C ₃ H ₇	I	278 (11.5), 263 (3.8), 247 (6.0), 235 (100), 220 (34.5)	13.98 (s, 1H), 6.48 (s, 1H), 3.92 (s, 3H), 3.03 (t, 2H), 1.73 (m, 2H), 0.98 (m, 3H)
C ₃ H ₇	II	278 (15.8), 263 (8.2), 250 (3.2), 245 (4.6), 235 (100), 220 (17.1)	10.31 (s, 2H), 3.96 (s, 3H), 3.10 (t, 2H), 1.72 (m, 2H), 0.98 (m, 3H)
C ₄ H ₉	I	292 (12.1), 263 (18.5), 250 (3.5), 235 (100), 220 (36.4)	14.00 (s, 1H), 6.48 (s, 1H), 3.92 (s, 3H), 3.05 (t, 2H), 1.68 (m, 4H), 0.90 (m, 3H)
C ₄ H ₉ (DIF-2)	II	292 (13.3), 263 (27.7), 250 (13.6), 235 (100), 220 (36.4)	10.32 (s, 2H), 3.96 (s, 3H), 3.11 (t, 2H), 1.67 (m, 4H), 0.90 (m, 3H)
C ₅ H ₁₁	I	306 (10.4), 288 (9.2), 263 (13.6), 250 (4.8), 235 (100), 220 (23.7)	14.01 (s, 1H), 6.49 (s, 1H), 3.90 (s, 3H), 3.05 (t, 2H), 1.66 (m, 4H), 1.30 (m, 3H)
C ₅ H ₁₁ (DIF-1)	II	306 (12.7), 288 (13.3), 263 (25.9), 250 (14.6), 235 (100), 220 (12.9)	10.32 (s, 2H), 3.96 (s, 3H), 3.10 (t, 2H), 1.68 (m, 4H), 1.32 (m, 2H), 0.89 (m, 3H)
C ₆ H ₁₃	I	320 (8.9), 302 (8.2), 263 (19.8), 250 (6.7), 235 (100), 220 (25.6)	14.03 (s, 1H), 6.47 (s, 1H), 3.92 (s, 3H), 3.08 (t, 2H), 1.68 (m, 4H), 1.29 (m, 3H)
C ₆ H ₁₃	II	320 (11.1), 302 (11.4), 273 (8.1), 263 (26.6), 250 (17.4), 235 (100), 220 (29.4)	10.34 (s, 2H), 3.96 (s, 3H), 3.11 (t, 2H), 1.69 (m, 4H), 1.33 (m, 4H), 0.90 (m, 3H)
C ₇ H ₁₅	I	334 (7.0), 316 (7.5), 263 (32.8), 250 (7.6), 235 (100), 220 (36.7)	14.02 (s, 1H), 6.49 (s, 1H), 3.92 (s, 3H), 3.07 (t, 2H), 1.68 (m, 4H), 1.30 (m, 6H), 0.87 (m, 3H)
C ₇ H ₁₅	II	334 (14.7), 316 (12.7), 263 (32.8), 250 (22.6), 235 (100), 220 (10.9)	10.35 (s, 2H), 3.96 (s, 3H), 3.13 (t, 2H), 1.61 (m, 4H), 0.88 (m, 2H)
C ₈ H ₁₇	I	348 (7.0), 330 (7.4), 263 (19.3), 250 (6.9), 235 (100), 220 (27.1)	14.03 (s, 1H), 6.47 (s, 1H), 3.92 (s, 3H), 3.07 (t, 2H), 1.68 (m, 4H), 1.26 (m, 8H), 0.85 (m, 3H)
C ₈ H ₁₇	II	348 (13.2), 330 (8.3), 263 (34.2), 250 (24.8), 235 (100), 220 (10.1)	10.35 (s, 2H), 3.96 (s, 3H), 3.12 (t, 2H), 1.68 (m, 4H), 1.26 (m, 8H), 0.85 (m, 3H)

Fig. 2. (a) Molecular structure of DIF-1 (the broken line shows an intramolecular hydrogen bond) and (b) space-filling representation of DIF-1

synthesized using phloroglucinol instead of 3,5-dihydroxyanisole, and the bromo analogues (of DIF-1 only) were prepared by halogenation with bromine water. After purification by h.p.l.c., each analogue was subjected to g.c. and was characterized in terms of its mass spectra, u.v. spectrum and, in most cases, its n.m.r. spectrum. By analogy with DIF-1, the isomers of each analogue could be defined by their u.v. and n.m.r. spectra and relative intensity of their McLafferty rearrangement ion. The n.m.r. spectra and mass spectra of the analogues are summarized in Table 1. DIF-3 (monochloro DIF-1) was prepared as a by-product of the DIF-1 synthesis, along with two other monochloro isomers derived from the 6-methoxyphenyl ketone. Although the three monochloro isomers could be separated by g.c., they were not well resolved by h.p.l.c., and insufficient pure DIF-3 was available for extensive analysis. The monoiodo analogue of DIF-1 was also prepared; it was not possible to incorporate two iodines using standard aqueous halogenation procedures.

DIF was originally discovered using an assay *in vitro* for stalk-cell differentiation. In the present form of the assay, isolated amoebae are incubated in tissue-culture dishes under a simple salts medium containing cyclic AMP. After 2 days, cell differentiation is scored by phase-contrast microscopy, with stalk cells being those cells that are at least 50% vacuolated and have a refractile cell wall (Brookman *et al.*, 1982; for photographs, see Kay, 1987). Most of the non-stalk cells

Table 2. Relative biological activity of DIF-1 analogues

Each synthetic compound was tested in a number of independent assays (n) against a DIF-1 standard (**bold**, 100 %) to minimize variability in the assay. Results are presented as a relative specific activity on a molar basis ($\pm$ s.d.), normalized to the DIF-1 standard. The molar concentration required to induce 50 % stalk-cell differentiation in the assay (D_{50}) is also shown; where the D_{50} is bracketted, it is a nominal value only, since it is above the lethal concentration for the cells. Pure monochloro-DIF-1 was not available in sufficient quantities to obtain an accurate result in the assay; however, biological activity was always decreased compared with DIF-1 (cf. Fig. 2).

General structure of DIF:

No.	R ¹	R ²	R ³	R ⁴	R ⁵	D_{50} (M)	Bioactivity (%) (mean $\pm$ s.d.)	(n)
1.	n-C ₃ H ₇	Cl	OH	Cl	OCH ₃	3.7×10^{-6}	0.005 ± 0.003	(3)
2.	n-C ₃ H ₇	Cl	OCH ₃	Cl	OH	7.0×10^{-9}	2.64 ± 0.81	(3)
3.	n-C ₄ H ₉	Cl	OH	Cl	OCH ₃	1.8×10^{-6}	0.01 ± 0.005	(3)
4.	n-C ₄ H ₉	Cl	OCH ₃	Cl	OH	4.6×10^{-10}	40.1 ± 9.5	(3)
5.	n-C ₅ H ₁₁	Cl	OH	Cl	OCH ₃	1.2×10^{-7}	0.15 ± 0.04	(4)
6.	n-C ₅ H ₁₁ (DIF-1)	Cl	OCH ₃	Cl	OH	1.8×10^{-10}	100	
7.	n-C ₅ H ₁₁	Br	OH	Br	OCH ₃	5.3×10^{-8}	0.35 ± 0.13	(5)
8.	n-C ₅ H ₁₁	Br	OCH ₃	Br	OH	3.5×10^{-10}	53.2 ± 25.5	(4)
9.	n-C ₅ H ₁₁	Cl	OH	Cl	OH	4.6×10^{-8}	0.40 ± 0.25	(4)
10.	n-C ₅ H ₁₁	H	OH	H	OH	$[1.2 \times 10^{-3}]$	0.00002 ± 0.000007	(4)
11.	n-C ₅ H ₁₁	Cl	OH	Cl	CH ₃	5.1×10^{-7}	0.036	(1)
12.	n-C ₆ H ₁₃	Cl	OH	Cl	OCH ₃	2.6×10^{-8}	0.72 ± 0.71	(4)
13.	n-C ₆ H ₁₃	Cl	OCH ₃	Cl	OH	1.3×10^{-9}	14.3 ± 1.10	(3)
14.	n-C ₇ H ₁₅	Cl	OH	Cl	OCH ₃	1.8×10^{-6}	0.01 ± 0.01	(5)
15.	n-C ₇ H ₁₅	Cl	OCH ₃	Cl	OH	7.4×10^{-9}	2.50 ± 0.71	(5)
16.	n-C ₈ H ₁₇	Cl	OH	Cl	OCH ₃	$[1.8 \times 10^{-5}]$	0.001 ± 0.0005	(5)
17.	n-C ₈ H ₁₇	Cl	OCH ₃	Cl	OH	6.1×10^{-7}	0.030 ± 0.01	(6)

remain amoeboid, though a few are partially vacuolated. Although this assay relies on morphological criteria, these morphological changes reflect an extensive programme of gene expression which is induced by DIF (Kopachik *et al.*, 1985; Williams *et al.*, 1987).

In preliminary experiments the lethal concentration and approximate activity range was established for each compound. They were then titrated over a 10–20-fold concentration range and the results normalized to a DIF-1 standard titrated at the same time. In this way, the effects of day-to-day responsiveness (up to 5-fold variation) of the tester cells was minimized.

The absolute specific activity of DIF-1 was $(136 \pm 75) \times 10^6$ units $\cdot \mu\text{mol}^{-1}$ ($n = 16$). Thus 10^{-10} M-DIF-1 should induce about 27 % of the amoebae to differentiate into stalk cells: DIF-1 is clearly a very potent effector molecule. At much higher concentrations (2×10^{-7} M) DIF-1 became lethal to the low-density cells in the conditions of the stalk-cell-differentiation assay. In other experiments using submerged cells (as in the stalk-cell assay), the lethal concentration of DIF-1 was found to vary with cell density and ionic conditions. It can be as high as 2×10^{-6} M with cells at $10^7/\text{ml}$, whereas cells developing normally on agar can tolerate up to 2×10^{-5} M (results not shown). Stalk-cell induction and cell killing are presumably mediated by different mechanisms, since the other compounds tested are also lethal in the micromolar range, although some of them are 10^6 fold less potent at stalk-cell induction than DIF-1.

It is apparent from Table 2 that the C₅ alkyl tail of DIF-1 (compound 6, Table 2) represents the optimum

chain length for bioactivity, although homologues differing by up to two methylene groups retained substantial activity. Thus DIF-2, the naturally occurring C₄ homologue (compound 4), had 40 % of the specific activity of DIF-1, and the C₆ homologue (compound 13) had 14 % activity. The exact configuration of the alkyl tail does not seem to be critical, since, in preliminary experiments, we found that the C₄ and C₅ isoalkyl compounds (2-methylpropyl and 3-methylbutyl) had a potency similar to that of their straight-chain isomers (results not shown). By contrast, the pattern of ring substitution is critical for bioactivity: with the exception of chlorine $\rightarrow$ bromine exchange (compound 8, Table 2), which may be regarded as a conservative change, all modifications in the substitution of the benzene ring result in at least a 100-fold decrease in potency. Thus the 6-methoxy isomer of DIF-1 and the trihydroxy analogue possessed 0.15 and 0.4 % respectively of DIF-1 bioactivity (compounds 5 and 9 respectively, Table 2). Since these two analogues are of broadly similar activity, it seems that the decrease in activity of the 6-methoxy isomer can be ascribed to a lack of 4-methoxy substitution rather than the presence of the 6-methoxy group. Monochloro DIF-1 (i.e. DIF-3) is much less active than DIF-1; further, there is a greater than 10^4 -fold decrease in activity in going from the trihydroxy compound to its non-chlorinated analogue (compounds 9 and 10, Table 2).

One purpose of the synthetic programme was to obtain DIF antagonists. Compounds were tested at concentrations below those which induce stalk-cell

differentiation, but none were found to antagonize DIF-1 activity. No synergy between DIF-1 and any of its analogues was observed.

DISCUSSION

The identification of DIFs 1–3 (Morris *et al.*, 1987, 1988) has opened a number of new avenues for research. These include a search for the DIF receptor and transduction pathway to gene expression (Gross *et al.*, 1983; Williams *et al.*, 1987) and investigation of the biosynthesis and metabolism of DIF. To pursue these avenues requires a ready source of DIF and related compounds. Since only minute amounts of the natural compound are available, we have developed a simple synthetic route for this class of compounds. This procedure requires little apparatus and can readily be carried out in a biochemical laboratory. A detailed chemical characterization of many of the compounds is also provided to assist identification. The Hoesch method of acylation was chosen to produce the required ring-substitution pattern, followed by a simple room-temperature chlorination step. The reaction mixture can be separated by h.p.l.c. to give essentially homogeneous preparations of the 4- and 6-OMe isomers (isomers I and II). The three monochlorinated isomers DIF-1 require multiple h.p.l.c. purification for complete resolution. Further modification of the synthetic protocol has enabled us to synthesize isotopically labelled DIF-1, which is proving invaluable for metabolic studies (D. Traynor & R. R. Kay, unpublished work).

X-ray-crystallographic analysis of synthetic DIF-1 defined the presence of an intramolecular hydrogen bond between the carbonyl and *ortho*-hydroxy groups in DIF-1, as previously inferred from the n.m.r. and u.v. spectroscopic data. The physicochemical differences between the 2- and 4-methoxy isomers can be largely ascribed to differences in this bonding.

Full interpretation of the activity–structure data is clouded by the uncertainty about the location of the DIF receptor. DIF-1 is soluble in both water and hexane at neutral pH, and it is therefore possible that it could penetrate cellular membranes. The DIF receptor could reside in the membrane (like many peptide-hormone receptors) or be intracellular, as in the case of steroid receptors. If the receptor is intracellular, the activity of these analogues may reflect their ability to penetrate the cell: analogues with short alkyl chains may be insufficiently membrane-soluble to penetrate efficiently, whereas those with long alkyl chains may interact too strongly with the membrane. Alternatively, if the receptor is at the cell surface, the effect of chain length would suggest the presence of a specific hydrophobic site on the surface of the receptor. The effect of changes in the substitution on the ring was investigated with a limited number of analogues, each having C₅ chains. It appears that the 4-methoxy group and two chlorine atoms are important for activity. When a hydroxy group is substituted for the methoxy in DIF-1, there is a 250-fold decrease in activity and a further 2700-fold decrease when the chlorine atoms are replaced by hydrogen atoms. The brominated analogue possesses 53 % of the activity of DIF-1, showing that the increased molecular size *per se* has only a limited effect and that the electron-withdrawing effects of the

halogens, perhaps on the pK_a of the *ortho*-hydroxy groups, is probably more important.

DIF-1 has a number of important biological actions, including induction of stalk-cell differentiation, suppression of spore-cell differentiation and the rapid induction of specific mRNA transcription (see Kay & Jermyn, 1983; Jermyn *et al.*, 1987; Williams *et al.*, 1987). A number of analogues of DIF-1 can now readily be obtained by simple synthetic procedures. For example, by using only three separate Hoesch reactions, followed by chlorination, a potency series of DIF analogues covering a 10⁶-fold range in specific activity is now available. In order of potency these compounds are DIF-1 > DIF-2 ≫ desmethoxyhydroxy-DIF-1, *ortho*-methoxy-DIF-1 ≫ 2,4,6-trihydroxyphenyl-hexan-1-one (compounds 6, 4, 9, 5 and 10 respectively in Table 2). These analogues could be used to determine whether the effects of DIF-1 are mediated by single or multiple pathways and to help identify the DIF receptor.

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