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Isolation, trace enrichment and liquid chromatographic analysis of diacetylphloroglucinol in culture and soil samples using UV and amperometric detection

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ABSTRACT

A reversed-phase approach is described for the preparative-scale isolation of the biotechnologically important antibiotic compound 2,4-diacetylphloroglucinol (DAPG), using solid-phase extraction and medium-pressure liquid chromatography. Using the purified sample, a high-performance liquid chromatographic assay was developed for the determination of DAPG in complex matrices such as growth media and soil. A sample pretreatment procedure involving solid-phase extraction on octadecyl silica prior to analytical high-performance liquid chromatography, removed endogenous peaks from the chromatogram when UV (254 nm) and amperometric detection (1.1 V *vs.* Ag/AgCl) were used. The reversed-phase chromatographic method is used to monitor the production of DAPG by a strain of *Pseudomonas* in culture media. The proven higher sensitivity and selectivity of amperometric detection for phenols and catechols are continued here in the analysis of phloroglucinols. Demonstrated selectivity improvements using amperometric detection for the analysis of DAPG in soil samples are however dependent on soil type.

INTRODUCTION

The quest for new and improved antibiotics from microbial sources is an important area of modern biotechnological research. Antibiotics are generally considered to be organic compounds of low molecular mass produced by microbes and are capable of killing bacteria or preventing their growth at low concentrations [1]. Approximately fifty such antibiotic substances have been isolated from pseudomonads [2,3], the largest percentage of these supplied by the sub-species *Pseudomonas fluorescens*. The majority of these antibiotic compounds isolated from *Pseudomonas* culture filtrates are N-containing heterocycles. They include such compounds as phenazines [4–6], pyrrole-type antibiotics [7–9], pyocompounds [10] and indole derivatives [11].

However, there are a small number of non-nitrogen-containing antibiotic compounds produced by *Pseudomonas fluorescens*. 2,4-Diacetylphloroglucinol (DAPG) (2,4-diacetyl-1,3,5-trihydroxybenzene) is one such compound and is one of just three phloroglucinols which have been isolated from the *Pseu-*

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domonas species [12]. This compound has been characterised previously in this laboratory [13] and by a number of other workers [14–16]. However, literature contains no method of harvesting large quantities of DAPG chromatographically nor are there any reported chromatographic methods of analysis for microbially derived phloroglucinols.

A large number of naturally occurring phloroglucinols from the plant genera *Dryopteris, Hagenia* and *Malotus* have already been studied chromatographically by reversed-phase high-performance liquid chromatography (HPLC) using absorption detection at 254 nm [17] and also by gas–liquid chromatography (GLC) [18]. However, these phloroglucinols of plant origin are structurally more complex than those of bacterial origin, existing mostly as large polycyclic molecules.

Phloroglucinols are classified according to the presence of a 1,3,5-trihydroxybenzene group which places them in the structural sequence; phenols, catechols and phloroglucinols (Fig. 1).

Much work has appeared in the literature on phenols and catechols and on how well they lend themselves towards electrochemical detection [19–23]. In many cases electrochemical detection of the above molecules has proven to be the favoured method of analysis, demonstrating higher sensitivity and increased selectivity over UV methods of detection. Results from this laboratory have also demonstrated the usefulness of amperometric detection to the analysis of microbial siderophores, many of which can contain catecholate groups [24].

Recent efforts to define the role of antibiotics in soil have been greatly impeded by a lack of direct evidence that antibiotics are present in soil. The physical and biological factors influencing antibiotic production and detection in soil have been reviewed [25,26] and although some success has been achieved in soil with amended nutrient sources, soil still proves to be a very complex matrix for the detection of antibiotics which are by definition produced in trace amounts [1]. Recently Thomashow *et al.* [6] successfully developed a HPLC method for the detection of the antibiotic phenazine 1-carboxylic acid in the rhizosphere of wheat. This paper reports on the development of a HPLC assay for the detection of a naturally occurring phloroglucinol and demonstrates how electrochemical detection shows enhanced sensitivity and selectivity over UV detection of DAPG. Advantage is taken of a sample pretreatment method involving solid-phase extraction of the antibiotic on octadecylsilica (ODS) prior to chromatographic analysis. The paper also outlines a method for the harvesting of large quantities of DAPG from a *Pseudomonas fluorescens* strain grown on sucrose–asparagine (SA) minimal media [27]. The technique involves preparative chromatography at medium pressures where the precolumn (guard column) is adapted to permit large-scale trace enrichment of DAPG from culture supernate.

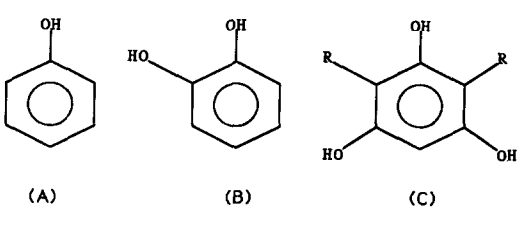

Fig. 1. Chemical structures of electroactive analytes. (A) Phenol; (B) catechol; (C) phloroglucinol (R = H), 2,4-diacetylphloroglucinol (R = CH_3CO-).

EXPERIMENTAL

Medium-pressure liquid chromatography

The preparative medium-pressure liquid chromatography (MPLC) system consisted of a Buchi 681 MPLC pump which can operate up to 40 bar backpressure. The sample was injected using a 10-ml syringe (Segma) directly through a septum into the top of Buchi B-685 chromatographic column (230 mm × 26 mm I.D.) operating at a flow-rate of 6 ml/min. The mobile phase consisted of methanol–water (25:75, v/v). The column was previously packed with LiChroprep RP-18, particle size 5–20 μm, using a standardised dry-packing method with vacuum and nitrogen over-pressure [28]. UV detection was carried out using a Buchi UV-Vis filter photometer at 254 nm equipped with a 2-mm superpreparative flow-through cell. The recorder output was recorded on a Perkin-Elmer 023 strip chart recorder and the fractions were collected manually.

Isolation of diacetylphloroglucinol using off-line solid-phase extraction prior to MPLC

A *Pseudomonas fluorescens* strain (code named

F113) was cultured on minimal SA medium [27] incorporating 0.35% agar and incubated at 28°C for 8 days. Prior to inoculation the medium was sterilized by autoclaving and *ca.* 25-ml aliquots were poured into sterile petri dishes. The bacterial cells and agar gel were sedimented by centrifugation at 16 000 *g* on a Beckmann JA-10 rotor for 20 min at 10°C creating a pellet of the agar and cells. A 1-l volume of supernate was collected, filtered through a 0.45-μm filter and concentrated five-fold by rotary evaporation at 35°C. The concentrated supernate was again filtered through a 0.45-μm filter before commencing solid-phase extraction. The guard column (100 mm × 10 mm I.D.) of the MPLC system was fitted with a porous PTFE frit and packed under gravity using a wet slurry technique. The packing material consisted of LiChroprep RP-18 (Merck) of particle size 25–40 μm. The column was then conditioned by passing methanol and water through it at a flow-rate of 6 ml/min.

By switching the pump inlet, 100 ml of the concentrated supernate was pumped through the guard column. It was then washed with 200 ml of water, followed by 300 ml of methanol, to elute all retained material. The latter washings were collected separately, taken to dryness *in vacuo* and reconstituted in 2 ml of methanol. The sample was then applied to the preparative column for final purification, employing a mobile phase of methanol–water (25:75, v/v). The chromatographic peak corresponding to the antibiotic eluted at a retention time of 32 min. Following solvent evaporation the purity of the isolated product was checked using analytical HPLC where it gave a single peak. An elemental analysis was also performed and the experimental values of C = 57.0%, H = 4.8% and N = 0% compared favourably with the theoretical values of 57.13%, 4.76% and 0% for C, H and N respectively.

HPLC apparatus

The HPLC system consisted of a Waters pump Model 510 attached to a Rheodyne injection valve with a 20-μl loop. The column used was an analytical column (150 mm × 6 mm O.D. × 4.5 mm I.D.) packed with Hypersil ODS (particle size 5 μm). The detector used was a Waters Lambda-Max Model 481 variable-wavelength spectrophotometer. The amperometric detector was an LC4B model from Bioanalytical Systems (BAS, West Lafayette, IN, USA). This was coupled with a BAS column adaptor housing model CC4. The electrode cell was of thin film design with a working electrode of glassy carbon, a platinum auxiliary electrode and a Ag/AgCl reference electrode. The detector was attached to a Philips 8251 strip chart recorder.

Mobile phase composition

Reagents for mobile phase preparation were of AnalaR grade and all mobile phases were filtered and degassed on a Millipore HPLC filtration system using 0.45-μm filters. Unless otherwise stated the HPLC mobile phase consisted of (water–methanol–tetrahydrofuran (THF) (40:45:15) at a flow-rate of 1 ml/min. For electrochemical analysis, 0.05 *M* sodium chloride was incorporated as a background electrolyte.

Preparation of standard solutions and soil samples

Purified antibiotic 2.010 mg was accurately weighed into 25 ml of methanol using a Mettler Me22 electronic balance coupled to a Mettler control unit BA 25. Standard solutions of DAPG were prepared by dilution with methanol to give concentrations in the range of $3.8 \cdot 10^{-4}$ to $1.0 \cdot 10^{-8}$ *M*. Experiments were carried out on a sandy soil sample (pH 6.9) (chemical composition: Ca = 532, K = 203, P = 78, N = 20, Mg = 31, Mn = 0.84, mg/kg soil) taken from a nearby location in Ovens, County Cork, Ireland. The soil was collected from the upper 5 cm of the soil profile, sieved through a 0.5 cm mesh screen and air dried prior to use. Purified DAPG dissolved in diethyl ether (10 ml) was mixed into the soil to yield concentrations of 100, 50 and 25 μg/g of soil. The ether was removed *in vacuo* at 35°C to ensure a uniform dispersion of DAPG throughout the soil.

Pretreatment of growth culture liquid media

A second medium commonly used for the growth of pseudomonads is the complex Luria–Bertani (LB) medium [29]. The production of a range of secondary metabolites by the growing microorganism, as well as the large number of constituents in the media necessitates the use of an efficient sample clean-up step when analysing for DAPG. The following procedure was developed. A Waters Sep-Pak C_{18} cartridge was first wetted with 10 ml of methanol followed by 10 ml water. The antibitiotic

was retained on the cartridge when applied in water. Filtered supernate samples (5 ml) were injected onto the cartridge. The cartridge was then washed with 30 ml of water followed by 20 ml of methanol to elute the retained constituents. The methanol eluate was taken to dryness and the residue reconstituted in 5 ml of mobile phase. The sample was again filtered and 20-μl aliquots injected into the HPLC system. Sample pretreatment of growth cultures on SA minimal media is carried out in the same way; however, the clean-up effect is not as pronounced as that observed in LB media.

Monitoring in vitro DAPG production

To monitor *in vitro* DAPG production over a period of eight days, sixteen 100-ml Erlenmeyer flasks containing 5 ml of SA liquid medium were inoculated to give *ca.* 10^3 colony-forming units (cfu[a])/ml of strain F113 and incubated without shaking at 28°C. At 12-h intervals, a flask was removed from the incubator and a cfu value recorded. The cells were pelleted by centrifugation at 3000 *g* for 10 min at room temperature. The supernate was decanted off and assayed for DAPG production by HPLC. All samples analysed for antibiotic production were pretreated using the outlined solid-phase extraction procedure.

Analysis of spiked soil samples

Spiked soil samples (1 g) were washed three times with 100-ml quantities of diethyl ether. The washings were pooled and rotary evaporated to dryness at 30°C. The residual material was taken up in mobile phase (5 ml) and filtered through 0.45-μm filters. Aliquots (20 μl) were injected into the HPLC system and analysed with UV and amperometric detection. Non-spiked soil samples were washed in the same way and their chromatographic profiles recorded as blanks.

RESULTS AND DISCUSSION

The obvious advantage in employing microbes to produce specific compounds, some of which can be very exotic and chemically difficult to synthesise, lies in the speed and ease of microbial production as opposed to what may involve a multi-step chemical synthesis of the same compound. However this is dependent on whether it is possible to isolate the desired product from what can often be a complex microbial growth matrix. The antibiotic DAPG has been previously synthesised [30], but with the new method of isolation proposed in this paper, from a species of *Pseudomonas* a ready and easily accessible source of DAPG is provided. The purified antibiotic supplied a parent molecular ion in the mass spectrum at *m/z* 210.053 (calculated for $C_{10}H_{10}O_5$:210.0528), a melting point at 167–170°C (Lit. [30] 168–170°C); IR bands (KBr) (*inter alia*) at 3650–2100, 1645–1575, 1435, 1365 and 1200 cm^{-1} and ^1H NMR ($C^2H_3O^2H$) chemical shifts at δ2.63 (S 6H), 5.76 (S 1H) (hydroxyls exchanged).

A sensitive and selective assay for the monitoring of DAPG levels in culture media and in soil samples is required to assist with the optimisation of microbial production of the antibiotic and with its detection in complex matrices such as soil for biotechnological applications including biocontrol. The sensitivity requirements vary with the nature of the application as well as with environmental and time dependent factors. For example, when the microbe is grown under nutritionally stressed conditions such as created by a particular choice of temperature or growth medium [13], only trace amounts of DAPG are present. Selectivity requirements are greatly determined by the microbial sample matrix. These studies examine these attributes of UV and amperometric detection when HPLC is used for the analysis of DAPG in culture media (*i.e. in vitro*) and soil samples.

Optimisation of HPLC assay

Standard solutions of DAPG were used to study the effect of the nature and percentage organic modifier concentration on the capacity factors and peak shapes. The chosen mobile phase containing 15% THF and 45% methanol in water improved the peak shape for DAPG. Hydrodynamic voltammetry was carried out to determine the optimum applied potential for DAPG detection (Fig. 2). A value of 1.10 V *vs.* Ag/AgCl was chosen for amperometric and 254 nm for UV detection.

[a] cfu is the cell count unit providing a representation of the number of viable bacterial cells present in a culture, normally expressed per ml of that culture.

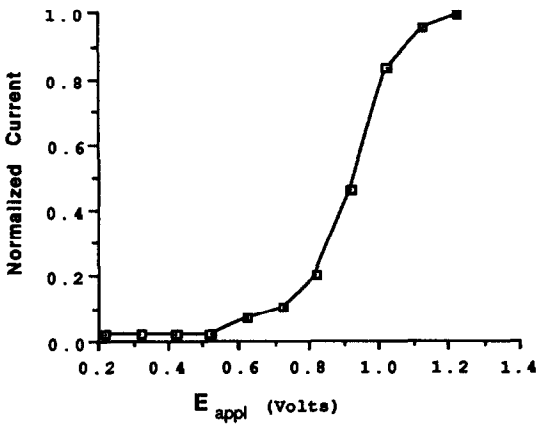

Fig. 2. Hydrodynamic voltammogram of 2,4-diacetylphloroglucinol.

Linearity and precision

The amperometric and UV responses were studied in the ranges $8.86 \cdot 10^{-5}$ to $5.32 \cdot 10^{-6}$ *M* DAPG, each standard being injected five times. Table I summarises a statistical evaluation of the results. Overall, UV detection emerges as being slightly more precise. The mean relative standard deviations were taken as a measure of the precision of the methods with intra-assay values of 2.96 and 3.01% for UV and amperometric detection respectively. Correlation coefficients of 0.9997 and 0.9993 were obtained for amperometric and UV detection respectively.

Limits of detection

The limit of detection (LOD) is defined as the concentration of analyte that will produce a signal-to-noise ratio of 2 and is considered to be the minimum concentration that can be detected [31]. The limit of detection was calculated from a series of measurements made at concentrations close to the blank level and was determined to be $2.309 \cdot 10^{-9}$ *M* at an applied amperometric potential of 1.1 V and $9.767 \cdot 10^{-7}$ *M* at a wavelength of 254 nm. The limit of detection using amperometry is over 400 times lower than with UV detection, and with trace enrichment using the solid-phase extraction procedure described in the experimental section limits of detection in the region of 10^{-12} *M* can be achieved.

Analysis of DAPG in microbial culture media

The monitoring of *in vitro* DAPG production by the *Pseudomonas* bacteria when cultivated on the complex LB growth media clearly benefits from improved chromatographic resolution when the sample pretreatment step is employed. On passing the sample through a cartridge containing octadecylsilica, the poorly retained constituents are washed off, thus eliminating the problem of column overloading caused by the high concentration of media substituents in the analyte sample and allowing for excellent resolution of DAPG (Fig. 3). Similar improvements in chromatographic profile were obtained for UV detection at 254 nm. Furthermore, the recovery of DAPG from culture media was found to be quantitative.

TABLE I

INTRA-ASSAY PRECISION OF THE HPLC ASSAY OF DAPG USING UV AND AMPEROMETRIC DETECTION

HPLC conditions were the same as in Fig. 3. Injection volume, 20 μl; $n = 5$; R.S.D. = relative standard deviation.

Concentration added (10^{-6} *M*)	Concentration found			
	UV		Amperometric	
	Mean ± S.D. (10^{-6} *M*)	R.S.D. (%)	Mean ± S.D. (10^{-6} *M*)	R.S.D. (%)
0.88	0.88 ± 0.04	3.98	0.89 ± 0.02	2.74
1.33	1.18 ± 0.05	3.89	1.41 ± 0.06	4.26
1.77	1.70 ± 0.06	3.58	1.76 ± 0.05	2.84
2.66	3.01 ± 0.06	1.93	2.59 ± 0.06	2.31
5.32	5.22 ± 0.08	1.43	5.39 ± 0.16	2.97

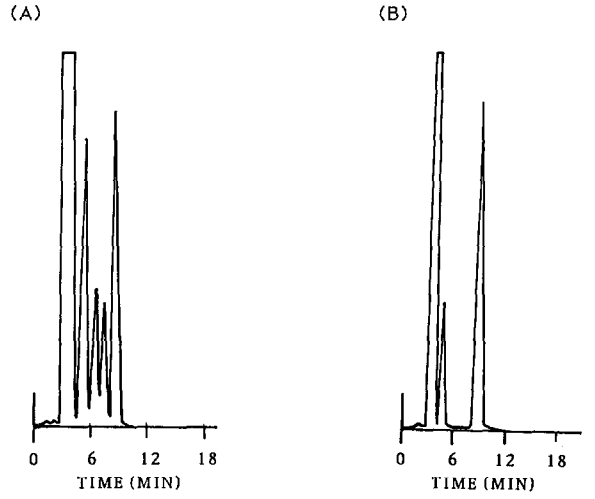

Fig. 3. Chromatograms obtained using amperometric detection from supernates of the pseudomonad grown on complex LB medium (A) without and (B) with sample pre-treatment. Retention time DAPG = 9.15 min. HPLC conditions: mobile phase, water–methanol–THF (40:45:15); flow-rate 1-0 ml/min; detection at 254 nm.

Microbial growth is measured as the change in optical density of the growth media with time and the growth curve obtained shows the characteristic lag, exponential and stationary phases (Fig. 4A). The outlined HPLC assay was employed to investigate the onset and rate of microbial production of DAPG in SA medium (Fig. 4B). DAPG is first detected in the growing culture after 36 h, when the growing microbe has reached its stationary growth phase. In measuring DAPG production, the cell count remained constant in the stationary phase at approximately 10^9 cfu/ml. Thus changes in DAPG production are attributed to an increase in the metabolism of DAPG rather than an increase in cell number. DAPG production increases linearly with time; however, this effect did not continue indefinitely, suggesting that there is a minimum time of approximately four days growth required for optimum DAPG production.

The selectivity of analysis of DAPG in sandy soil samples is enhanced by the use of electrochemical detection. As shown in Fig. 5A the injected sample contained a number of long-retained components together with a component that overlapped the DAPG peak (retention time 8.6 min) when analysed

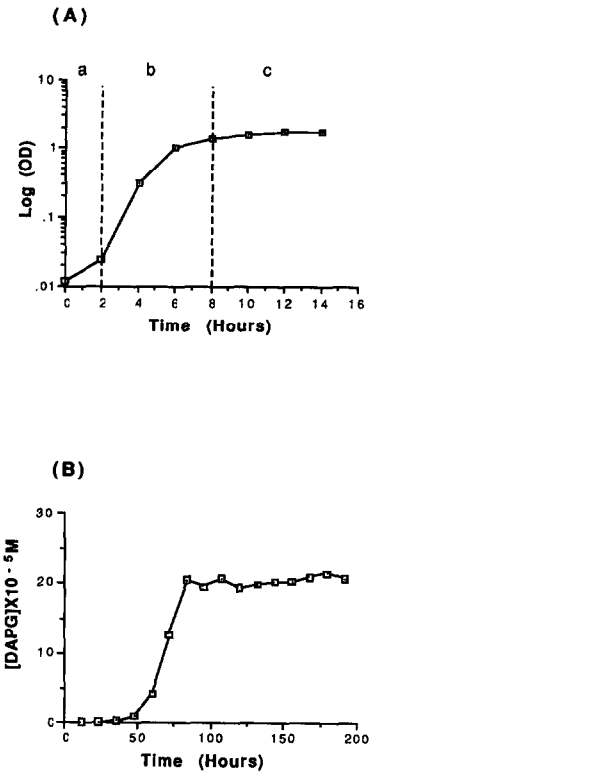

Fig. 4. (A) Growth curve of *Pseudomonas* strain in SA culture medium obtained from optical density measurements at 600 nm showing (a) lag, (b) exponential and (c) stationary phases of growth and (B) DAPG production in SA culture medium monitored by HPLC.

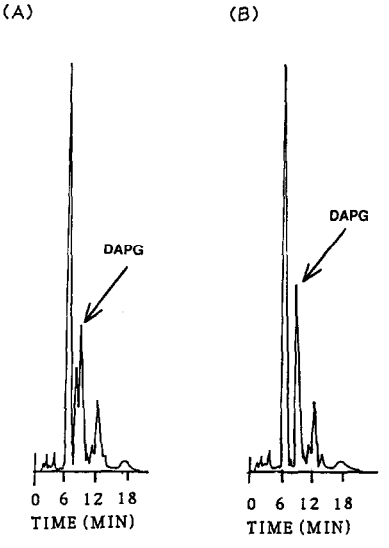

Fig. 5. Chromatograms obtained for extracts of sandy soil samples using (A) UV detection at 254 nm and (B) amperometric detection at + 1.1 V.

with UV detection. When studied with amperometric detection the latter contaminating peak is conveniently absent (Fig. 5B). This improved selectivity was not, however, observed in DAPG analysis of peat soils, highlighting the problem posed by the variation in chemical composition of different soils. Recoveries of DAPG from soil samples were also low, in the range of 60–70%. Further studies are required to optimize selectivity and recovery for detection of microbial antibiotics in these more complex soil matrices.

CONCLUSIONS

The new reversed-phase approach described above for the isolation of DAPG on a preparative scale provides easy access to appreciable amounts of a compound which is already known to be inhibitory to many plant deleterious bacteria. Biological assays for the detection of this compound involve observing the capacity of the DAPG producing strain to inhibit growth of susceptible bacteria or fungi. However, this method presents problems as typical bioassay plates or tubes must be incubated for about 18 h before results can be obtained. There is also the further disadvantage that the technique has no ability to quantify the inhibitory compound produced. The developed HPLC assay employing solid-phase extraction for sample clean-up in conjunction with amperometric detection provides an efficient means of sensitively and selectively monitoring the antibiotic production in complex matrices such as culture media and soil samples.

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