Applications of high-performance liquid chromatography to quantitation of metabolites and enzymes of the patulin pathway from *Penicillium patulum*

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ABSTRACT

Conditions for extraction and high-performance liquid chromatographic (HPLC) analysis for fourteen of the patulin pathway metabolites from *Penicillium patulum* are described which allow quantitation of the metabolite content of cultures at hourly intervals. The HPLC analysis is more sensitive than gas–liquid chromatographic analysis and is more quantitative than thin-layer chromatographic analysis. Separations on a preparative column allow for the collection and identification of new metabolites. The column elution program can be varied to optimize analysis time for individual metabolites, allowing individual enzymes of the pathway to be assayed by following the conversion of substrate to product. Analysis of product formation in crude enzyme mixtures can be used to assay an enzyme in the presence of subsequent enzymes of the pathway and to establish the pathway reaction sequence.

INTRODUCTION

Fig. 1 shows the polyketide family of products derived from the simple aromatic polyketide 6-methylsalicylic acid (6-MS) in the organism *Penicillium patulum* and in several related fungal species. The metabolites and the enzymes producing them are absent from young cultures undergoing balanced "trophophase" growth, and they are not produced until the culture enters "idiophase" growth where some essential nutrient has become limiting[1] (these terms were suggested by Bu'Lock *et al.*[2]). Patulin is the major end product accumulating under some culture conditions but changes in media composition, pH, temperature, aeration rate, etc. can affect not only the relative amounts of the metabolites but also the timing of their appearance[1,3,4]. Variations in the induction rates and final activities of the several enzymes involved are presumably responsible for the variability in metabolite profile, though limitations on transport in

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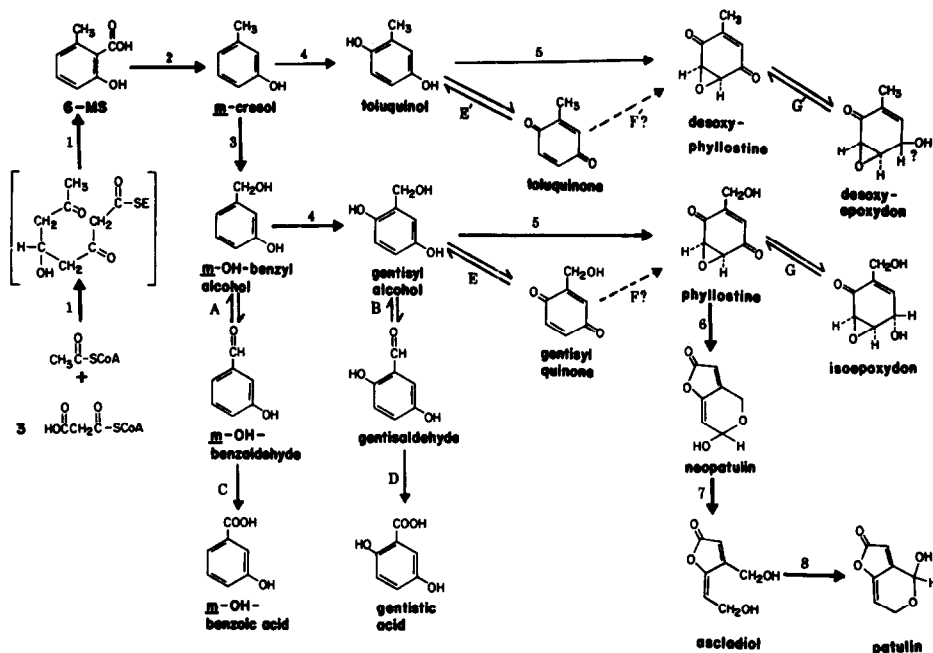
Fig. 1. Products derived from polyketide 6-methylsalicylic acid in the organism *Penicillium patulum* and related species.

and out of the cells may also play a role in what is found in the culture medium at any one time.

While this polyketide "pathway" is one of the most studied examples of secondary metabolism, a thorough study of many of the subtleties involved in induction of the pathway enzymes has been hindered by the lack of a rapid, sensitive, and quantitative assay for the metabolites and a sensitive, general assay for the enzymes. A crude measure such as the color intensity with FeCl$_3$ can quickly quantitate total 6-MS in the absence of significant amounts of the other metabolites[5], but is an inadequate indication of total "phenolic" metabolite production. Thin-layer chromatography (TLC) provides sensitive information[4,6], but spots on thin-layer plates are difficult to quantitate, interpretation can be difficult when compounds comigrate, and the collection of data is time-consuming. Gas–liquid chromatography (GLC) has been used in some laboratories for metabolite quantitation but this method is less sensitive and requires preparation of silylated derivatives[7]. The dehydrogenase enzymes of the pathway which employ NAD$^+$ or NADP$^+$ cofactors are simple to assay spectrophotometrically[8,9] but the other enzymes studied have required a specialized assay development for each enzyme, usually involving the use of isotopically labeled precursors and the chromatographic isolation and counting of a radiolabeled product[5,10,11].

We report here the application of HPLC to the rapid (1 h) simultaneous analysis of metabolites with a sensitivity one to two orders of magnitude higher than GLC, and with only two cases of peak overlap requiring special attention. Minor variations of the

procedure allow a generalized assay of any enzyme in the pathway for which substrate is available.

EXPERIMENTAL

Metabolite standards

Several compounds were obtained from commercial sources and purified by recrystallization or, in the case of *m*-cresol, redistillation. These include *m*-cresol, *m*-hydroxybenzyl alcohol, *m*-hydroxybenzaldehyde, and gentisaldehyde from Aldrich, toluquinol from Matheson, and patulin from Applied Science. Gentisaldehyde was also synthesized from *p*-hydroquinone (Matheson) by the Riemer-Tieman reaction[12]. 6-MS was synthesized by the method of Eliel *et al.*[13]. Toluquinone was synthesized by oxidation of toluquinol with Ag_2O (ref 14). Gentisyl alcohol was prepared by the $NaBH_4$ reduction of gentisaldehyde, and gentisyl quinone by the further oxidation of this product with $NaIO_4$ (ref. 15). Ascladiol was synthesized by the $NaBH_4$ reduction of patulin[16]. Phyllostine was kindly supplied by Dr. G. M. Gaucher, University of Calgary, Canada[17]. Desoxyphyllostine and neopatulin were isolated from preparative scale *in vitro* enzyme incubations[18]. Standard curves were obtained by dissolving a known quantity of standard (10 to 160 μg) to two or more 0.5 ml samples of HPLC buffer (14 mM potassium phosphate, pH 6.0 and 20% methanol) and analyzing 20 μl aliquots by HPLC. The integrated peak areas on the recorder were used to calculate the conversion factor "pmoles/10^3 area units" (Table I).

HPLC conditions

The HPLC system consisted of two Model 114M solvent pumps, a Model 421A controller, a Model 210A sample injection valve, a Model 160 UV detector with 280 nm and 254 nm filters (all Beckman), and a Shimadzu model C-R3A integrator. Reversed-phase columns were packed with Altex Ultrasphere ODS C_{18} for either analytical (25 cm × 4.6 mm I.D., 5 μm packing or 7.5 × 4.6 mm I.D., 3 μm packing) or preparative (25 × 1.0 cm I.D., 5 μm packing) analyses. Samples isolated by ether extraction followed by drying and removal of ether *in vacuo* were dissolved in 14 mM potassium phosphate buffer, pH 6.0, containing 20% methanol [and 0.7 mM tetrabutylammonium phosphate (Sigma, 90%) if this ion-pair reagent was being used in the elution]. Analytical samples were cleared of fine particles by a 30-s high-speed centrifugation in an Eppendorf tube prior to injection of a 20-μl aliquot. Preparative samples were centrifuged for 30 min at 30 000 g prior to injection of 500 μl aliquots. The eluting solvents were HPLC grade and de-aerated prior to use. The elution programs are described in the figure legends.

Metabolite extraction

Fermentor cultures of *P. patulum* were grown as described previously[18]. Aliquots of the culture were harvested at various times and the mycelium separated by filtration. For analytical runs, a 30-ml portion of the filtered culture medium was acidified to pH 2–3 with concentrated HCl and extracted twice with 30-ml portions of anhydrous ether. The ether was dried over 20 g of anhydrous Na_2SO_4 and removed by rotary evaporation under an aspiration vacuum. The residue was redissolved in 10 ml of buffer as described above. For preparative isolations, usually one liter of medium

was acidified and extracted with two 500-ml portions of anhydrous ether. The combined ether extracts were equilibrated with saturated aqueous NaCl solution, dried over anhydrous Na_2SO_4, and the ether was removed by rotary evaporation. The residue was redissolved in 3 ml of buffer as described above.

Enzyme assays

Mycelial samples of *P. patulum* were harvested from cultures grown as previously described[18], washed with 0.5 volumes of 20 mM potassium phosphate, pH 7.5, and suspended in this same buffer for lyophilization. Lyophilized samples were stored at $-70°C$ until utilized for enzyme isolation or analysis. To facilitate cell breakage and to remove phenolic metabolites which might inhibit activity[19] the lyophilized mycelium was frozen in liquid nitrogen, ground to a fine powder with a mortar and pestle, and stirred into 10 ml of $-20°C$ acetone per gram of powder. The acetone was removed by filtration through a sintered glass filter, and the mycelial powder was rinsed with 10 ml of $-20°C$ anhydrous ether and dried thoroughly by suction filtration. A 0.5-g portion of the dried mycelium was mixed in a cold mortar with 5 ml of extraction buffer (100 mM potassium phosphate, pH 7.5, 2.5 mM dithiothreitol, and 15% glycerol) and 0.5 g of sand. The sample was ground with a pestle to a paste and the thick suspension centrifuged at $4°C$ for 10 min at $10\,000\,g$ in a Sorvall RC-2B centrifuge with an SS-34 rotor. The supernatant was transferred to a second tube and centrifuged at $4°C$ for 30 min at $30\,000\,g$. This process yielded approximately 3.5 ml of crude extract with a protein concentration of 18–48 mg/ml. Aliquots of this crude extract were utilized for the enzyme assays. The conditions for each assay are described in the figure legends. The metabolite extraction efficiency for the enzyme assay (Table I) was determined by adding known quantities of metabolite (10 to 160 μg) to two or more 5.0 ml portions of 100 mM potassium phosphate buffer, pH 7.5, acidifying to pH 1–2, and extracting twice with two volumes of ether. The ether was dried over sodium sulfate and evaporated as in the enzyme assay extraction, the residue was dissolved in 0.5 ml of HPLC buffer (14 mM potassium phosphate, pH 6.0 and 20% methanol) and 20 μl aliquots were analyzed by HPLC. Integrated peak areas were compared with those obtained from an equivalent amount of standard added directly to 0.5 ml of HPLC buffer.

RESULTS AND DISCUSSION

Fig. 2 shows an analytical HPLC profile of eight of the more common metabolites from 6-MS (Fig. 1), including the most polar (gentisyl alcohol, peak a) and the least polar (*m*-cresol, peak i). A gradient elution was necessary to achieve the best separation of the early peaks while keeping elution of *m*-cresol within 30 min. An ion-pair reagent, tetrabutylammonium phosphate, was necessary to alter the elution position of 6-MS (peak h), the one carboxylic acid in the set of standards. In the absence of the ion pair the carboxylate ion of 6-MS elutes very early as a broad, polar peak and interferes with detection of the other polar metabolites. At the indicated concentration of 0.7 mM ion pair reagent, 6-MS eluted in a region clear of other metabolites. Instability of the column at low pH precluded attempts to suppress ionization using an acidic elution mixture. For samples not containing 6-MS or another carboxylic acid, the ion-pair reagent can be omitted. Only two pairs of
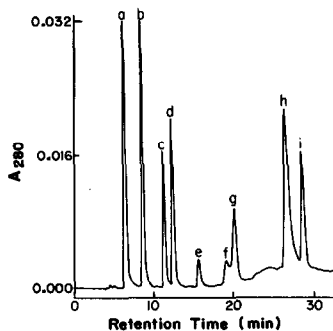
Fig. 2. HPLC profile of metabolite standards. A 20-μl sample of a mixture of standards in a buffer containing 14 mM potassium phosphate at pH 6.0, 0.7 mM tetrabutylammonium phosphate, and 20% (v/v) methanol was injected on a 25 cm × 4.6 mm I.D., 5 μm C$_{18}$ column. The column was eluted at a flow-rate of 0.6 ml/min using a gradient of solvent B [14 mM potassium phosphate at pH 6.0, 0.7 mM tetrabutyl-ammonium phosphate, and 50% (v/v) methanol] in solvent A (14 mM potassium phosphate at pH 6.0 and 0.7 mM tetrabutylammonium phosphate). The elution program was 40% B to 60% B (linear gradient) in 10 min, 60% B to 100% B (linear gradient) in 10 min, and 100% B (isocratic) for 10 min. The metabolite standards are: a = gentisyl alcohol, 0.4 μg; b = patulin, 0.05 μg; c = toluquinol, 0.2 μg; d = m-hydroxy-benzyl alcohol, 0.4 μg; e = impurity; f = gentisaldehyde, 0.5 μg; g = m-hydroxybenzaldehyde, 0.4 μg; h = 6-MS, 1.0 μg; and i = m-cresol, 0.4 μg.

compounds tested did not give satisfactory resolution under these conditions. On a preparative column ascladiol co-eluted with gentisyl alcohol at 10.8 min, and toluquinone co-eluted with m-hydroxybenzaldehyde at 32.8 min. For the culture induction and enzymatic assays reported here, this lack of resolution did not cause ambiguity in interpretation of results. Gentisyl alcohol and ascladiol are not likely to be present in the same enzymatic assays, and in culture analyses gentisyl alcohol should be the first of the pair appearing. The presence of toluquinone would be indicated if toluquinol were also found. Nevertheless, should it be necessary to verify the content of these peaks, one can re-run the sample at a different detector wavelength or collect material from the peaks to analyze by UV–VIS spectroscopy or by TLC.

Ideally, one would like to inject a culture medium or an enzymatic reaction mixture directly on the HPLC. The major components of either the culture medium (glucose, salts, nutrients) or the enzyme solution (proteins, buffers) would likely interfere or drastically shorten the life of the column, so metabolites were first extracted with ether prior to analysis. While ether extraction adds some time to the analysis, it also adds flexibility in manipulating sample size and ultimate sensitivity. One can vary the volume of sample extracted, the volume of elution buffer used to reconstitute the extract, and the volume of the aliquot injected.

The sensitivity, of course, also varies with the compound and the wavelength of the detector. The quantity of standards shown in Fig. 2 ranged from 50 ng (0.32 nmol) for patulin (peak b) to 1.0 μg (6.6 nmol) for 6-MS (peak h) in a 20-μl injection, or 2.5 ng/μl and 50 ng/μl respectively. Discernable peaks could be seen with as little as 2% of this quantity, whereas detection limits reported for GLC by Ehman and Gaucher[7] range from 4–18 ng/μl of extract for a 1-μl injection.

In addition to monitoring the appearance of known metabolites, the HPLC procedure provides the possibility of observing new metabolites. Samples can be
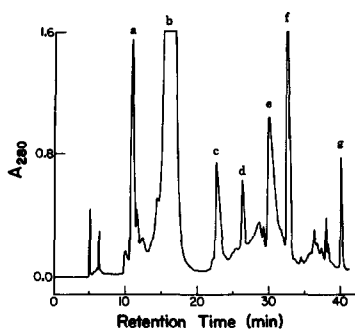
Fig. 3. Preparative HPLC metabolite profile of extract from an induced culture of *Penicillium patulum*. One liter of medium from a 32-h culture was extracted and reconstituted in 3.0 ml of buffer containing 14 m*M* potassium phosphate at pH 6.0 and 20% (v/v) methanol. A 500-μl aliquot was injected on a 25 × 1.0 cm I.D., 5 μm C_{18} column. The column was eluted at a flow-rate of 2.0 ml/min using a methanol–water solvent as follows: 17.5% methanol(isocratic) for 15 min, 17.5% methanol to 100% methanol (linear gradient) in 30 min, and 100% methanol (isocratic) for 5 min. Metabolite identities were determined by comparison with retention times and by examining the UV spectra of collected material as described in the text (the ion-pair reagent was not included because no 6-MS was detected in the culture at that time).

collected from a preparative column for structural verification of identification using other spectral techniques.

Fig. 3 shows a typical preparative HPLC elution profile of a culture extract obtained from a 32-h fermentor culture. The lettered peaks were identified both by comparison of retention times to standards and by collecting the corresponding eluent and comparing its UV spectrum to that of a standard. Peak a contained a mixture of gentisyl alcohol and ascladiol. Peak b contained neopatulin in early fractions and patulin in late fractions (neopatulin can be seen as a distinct shoulder on the patulin peak with a smaller sample). Peak c contained toluquinol with a small unknown contaminant. Peaks d and g contained *m*-hydroxybenzyl alcohol ad *m*-cresol respectively. Material from peak e had four absorption maxima at 210, 242, 285, and 350 nm and that from peak f had a broad absorption band with a maximum at about 270 nm. These latter two substances may turn out to be new compounds related to the pathway, but have yet to be identified.

The HPLC procedure was easily adapted to assay of individual enzymes of the patulin pathway. We modified the extraction method to start with a smaller sample volume and achieve a greater concentration of extract in the elution buffer in order to increase sensitivity. The gradient elution profile was modified for each specific assay to minimize elution time while still achieving separation of substrate and product. The ion-pair reagent was unnecessary for reactions not involving 6-MS, nor was it necessary for 6-MS decarboxylase since the product *m*-cresol elutes much later than 6-MS. Details are given under Experimental.

Figs. 4 and 5 show an example of the assay of 6-MS decarboxylase in crude extract. The production of *m*-cresol is linear with respect to both time up to one hour (Fig. 4) and extract concentration up to 20 μl (Fig. 5). Fig. 6 shows similar results for *m*-hydroxybenzyl alcohol dehydrogenase. A more convenient spectrophotometric assay has been developed for the dehydrogenase[8], but the HPLC method can be used to verify activity in crude extract where competing reactions of NADH could
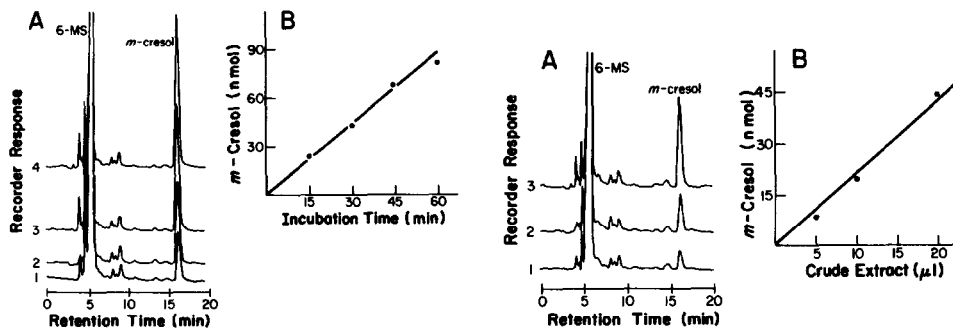
Fig. 4. HPLC assay of 6-MS decarboxylase: effect of incubation time on product formation. Each assay tube contained 0.50 μmol of 6-MS, 7.5 mg of serum albumin, and 20 μl of crude extract in 5.0 ml of 100 mM potassium phosphate buffer, pH 7.5. Tubes were incubated at 28°C for the indicated time, acidified to pH 1–2 with concentrated HCl, and the products were extracted with ether as described under Experimental. Aliquots of the reconstituted extract (20 μl of 500 μl) were injected on a 25 cm × 4.6 mm I.D., 5 μm C$_{18}$ column which was eluted isocratically with 50% methanol at a flow-rate of 0.6 ml/min. (A) Tracing of the recorder response at 280 nm from (1) 15 min, (2) 30 min, (3) 45 min, and (4) 60 min incubations respectively. (B) Plot of the quantity of m-cresol in the extract determined from the integrated peak areas in A. These values were not corrected for extraction efficiency.

Fig. 5. HPLC assay of 6-MS decarboxylase: effect of crude extract concentration on product formation. Assays were carried out as in Fig. 4 except incubations were for 60 min at crude extract concentrations of (1) 5 μl, (2) 10 μl, and (3) 20 μl in the 5.0-ml incubation mixture. (A) Tracing of the recorder response at 280 nm from the HPLC analysis. (B) Plot of the quantity of m-cresol in the extract determined from the integrated peak areas in A. These values were not corrected for extraction efficiency (this extract had been stored longer than that used in Fig. 4, consequently its decarboxylase activity was lower).

complicate the assay with a high background. A similar assay procedure has been reported for toluquinol and gentisyl alcohol epoxidase activity[18].

The data in Figs. 4–6 were not corrected for extraction efficiency. If one desires

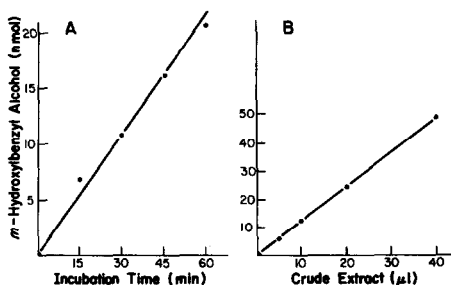

Fig. 6. HPLC assay of m-hydroxybenzyl alcohol dehydrogenase: effect of incubation time and enzyme extract concentration on product formation. Each assay tube contained 0.50 μmol of m-hydroxyben-zaldehyde, 0.50 μmol of NADPH, 50 nmol of ATP, 5 μmol of MgCl$_2$ and the indicated amount of crude extract in 5.0 ml of 100 mM phosphate buffer, pH 7.5. The enzyme extract was a saturated ammonium sulfate precipitate of crude extract resuspended in an equivalent volume of enzyme buffer. Tubes were incubated at 28°C for various times with 40 μl of enzyme extract (A) or for 60 min with varying amounts of enzyme extract (B). After acidification and extraction with ether as described under Experimental, aliquots of the reconstituted extract (20 μl of 500 μl) were injected on a 25 cm × 4.6 mm I.D., 3 μm C$_{18}$ column which was eluted at a flow-rate of 0.6 ml/min with a gradient of 20% methanol to 50% methanol in 4 min followed by 50% methanol for 14 min. The quantity of m-hydroxybenzyl alcohol (retention time, 4.8 min) produced was determined from the integrated peak areas. These values were not corrected for extraction efficiency.

to calculate enzyme activities on an absolute basis for comparison with each other, then the extraction efficiency of the method must be determined. Table I shows the efficiency under conditions we employed for our enzymatic assays as determined for thirteen of the metabolites we have studied. These are clearly lower than those reported by Ehman and Gaucher[7] who were preparing samples for GLC analysis, and who obtained extraction efficiencies above 90% for all but the very polar gentisyl alcohol at 78%. Different sample sizes and volume ratios of extracting solvent to sample probably account for these different results, and our method could be modified to improve efficiency if necessary. Table I also compares the detector sensitivity at 280 nm for each of the compounds. Multiplying the factor in the table by the integrated peak area gives the quantity of material in the injected sample. The retention times for standards are given for both the analytical column with the ion-pair reagent and the preparative column without the ion-pair reagent under the gradient elution programs described in Figs. 2 and 3.

TABLE I

PARAMETERS FOR QUANTITATING METABOLITE CONCENTRATIONS BY HPLC ANALYSIS

The metabolite extraction efficiency for enzyme assays, and the recorder response for each metabolite were determined as described under Experimental. Retention times on an analytical (25 cm × 4.6 mm I.D.) and a preparative (25 cm × 1.0 cm I.D.) column were determined using the elution programs described in Figs. 2 and 3, respectively. They varied by less than 5% in any one day's experiments and less than 15% between two columns over a period of eighteen months. The elution order is constant.

Metabolite	Extraction efficiency[a] (%)	Detector response[b] 280 nm (pmoles/10³ area units)	Retention time (min)	
			Analytical	Preparative
Gentisyl alcohol[c]	32 ± 3	9.6	6.61	10.8
Phyllostine	68 ± 4	16.7	n.d.[e]	12.2
Neopatulin	n.d.[d]	2.7	n.d.[e]	15.1
Patulin	49 ± 9	1.5	8.8	15.6
Gentisyl quinone	66 ± 5	34.7	n.d.[e]	16.8
Toluquinol	76 ± 10	12.1	11.5	22.5
m-Hydroxybenzyl alcohol	68 ± 5	18.2	12.6	25.6
Desoxyphyllostine	60 ± 5	12.8	n.d.[e]	29.1
Gentisaldehyde	77 ± 12	62.5	19.3	31.9
m-hydroxybenzaldehyde	76 ± 8	28.6	20.4	32.8
Toluquinone	43 ± 1	62.6	n.d.[e]	32.8
6-Methylsalicylic acid	80 ± 5	15.1	26.9	n.d.[d]
m-Cresol	74 ± 7	22.0	28.9	39.1

[a] Average of 3–5 extractions.
[b] Arbitrary area units on a Shimadzu model C-R3A integrator at a setting of 0.04 a.u.f.s. This factor converts peak area to pmoles of analyte.
[c] Ascladiol co-elutes with gentisyl alcohol. We did not determine extraction efficiency or a standard peak response for this compound.
[d] Not determined.
[e] Not determined under the specific elution conditions described in Fig. 2.

   We did not systematically study the sensitivity of the method for different assays, but Fig. 5 can give one a feeling for the sensitivity of the decarboxylase assay. The lowest point (5 μl of extract) gives a small but clearly discernable peak when the recorder is set at 0.04 a.u.f.s. After correction for 74% extraction efficiency, this value corresponds to about 10 nmoles or about 1 μg of *m*-cresol in 5 ml of assay mixture. By comparing the 280 nm recorder responses shown in Table I, one can calculate that other compounds would be detected with one-third to ten times the sensitivity of *m*-cresol.

   The HPLC procedure also provides an advantage beyond speed and sensitivity in studying the pathway enzymes. Assays based on incorporation of substrate radioactivity into product involve isolation and counting of product accumulated after a given time of incubation. The presence of other enzymes in the crude extract, especially the next pathway enzyme, can prevent product accumulation by further metabolizing it. Thus assay values in crude extract can be misleading. The HPLC analysis allows one to observe accumulation of other products as well as the one being assayed and to determine whether subsequent reactions are occuring. One also need not go to the trouble and expense of preparing radiolabeled substrate.

   Finally, the ability to observe several products at once has proven useful in determining the reaction sequence of the pathway. As an example, Fig. 7 shows the complexity of products formed after incubation of crude extract with gentisyl alcohol. Four products are observed: gentisyl quinone (d), which might have been produced chemically or enzymatically; gentisaldehyde (e), product of gentisyl alcohol dehydrogenase; phyllostine (b), the epoxidase reaction product on the way to patulin; and neopatulin (c), the product of phyllostine ring opening[20]. Fig. 7 describes a preparative HPLC run used for the purpose of collecting and identifying these metabolites. An analytical column was used under similar conditions [isocratic elution with methanol–water (10:90) at a flow-rate of 0.35 ml/min] to assay different enzyme fractions. These assays played an important role in unraveling the reaction sequence as gentisyl
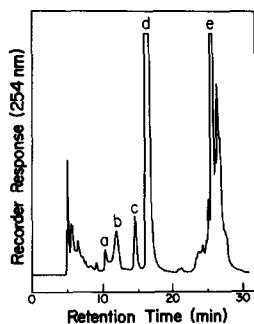

Fig. 7. Preparative-scale HPLC of products from the incubation of crude extract with gentisyl alcohol. A 1.0-l assay containing 30 mg of gentisyl alchol, 100 mM potassium phosphate buffer, pH 7.5, and 6.0 ml of mycelial crude extract from a 32-h culture was incubated for 2 h at 28°C. The product was isolated by ether extraction, the ether removed *in vacuo* and the residue dissolved in 4.0 ml of 17.5% methanol–water. A 0.5-ml aliquot was injected on a 1.0 × 25 cm 5 μm C$_{18}$ column which was eluted isocratically with 17.5% methanol–water at a flow-rate of 2.0 ml/min. The detector was set at 254 nm and the recorder response at 2.0 a.u.f.s. Material in the lettered peaks was collected and identified as: a = gentisyl alcohol; b = phyllostine; c = neopatulin; d = gentisyl quinone; e = gentisaldehyde.

alcohol → phyllostine → neopatulin, with gentisaldehyde and gentisyl quinone as products of side reactions[18].

With the relatively rapid HPLC method it becomes possible to assay metabolite production at hourly intervals while the culture is in progress. Culture experiments from several laboratories have shown similarity in induction patterns, but the time of induction varies considerably[1,2,4,5]. Even in our hands under carefully controlled conditions the induction time may differ by a couple of hours. Timely assay of the induction state of the culture is important if one wants to harvest large samples of mycelia at a given stage of induction. We will report elsewhere the use of these assays to carefully define the condition of culture samples for the purpose of isolating mRNA from uninduced and early induction-stage cells and the use of this mRNA in isolating induction-specific genes from a genomic library.

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