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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF δ-(L-α-AMINOADIPYL)-L-CYSTEINYL-D-VALINE IN COMPLEX MEDIA BY PRECOLUMN DERIVATISATION WITH DANSYLAZIRIDINE

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SUMMARY

A novel method is described for the trace level quantitation of the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) in complex fermentation media, using a high-performance liquid chromatographic, pre-column derivatisation technique. The procedure is based upon the reaction of the ACV monomer with 5-dimethylaminonaphthalene-1-sulphonylaziridine (dansylaziridine) and produces a highly fluorescent product. Reaction conditions between the reagent and tripeptide were investigated and optimal conditions established. Linear calibration graphs were obtained over the ranges 227–0.56 μg/ml and 227–5.6 ng/ml. The extracellular ACV levels produced in fermentation broths of several different fungal strains and species were determined using this technique. The method was compared using ACV standards in buffer solutions for ease of use, sensitivity and selectivity with two other pre-column derivatisation procedures, using dithionitrobenzoic acid and monobromobimane, which also exploit the reaction with the sulphydryl group of the ACV monomer.

INTRODUCTION

The nature of the intermediates involved in the biosynthetic pathways of both penicillins and cephalosporins has been well established[1], but only recently have attempts been made to quantitate levels of these compounds in fermentation broths by the application of high-performance liquid chromatography (HPLC)[2,3]. The tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) undergoes an unusual cyclisation reaction, catalysed by the enzyme isopenicillin N synthetase, to produce the first bioactive intermediate in the pathway, isopenicillin N[4,5]. The enzyme only accepts the sulphydryl form of the tripeptide as substrate and not the disulphide[5]. When excreted into aerated fermentation broths the ACV readily dimerises into the disulphide, but the monomeric form can be produced by reduction of the disulphide bond by mild reducing agents like dithiothreitol (DTT) or one of the alkali metal borohydrides[6,7].

The lack of a suitable chromophore in this tripeptide makes trace level detec-

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tion in fermentation broths difficult. Reversed-phase analytical HPLC has been employed by several workers to assay levels of either the monomeric or the dimeric form of ACV[2,3,8,9] along with other β-lactam intermediates. The methods employed for this purpose have involved the covalent derivatisation of ACV to introduce a chromophore or fluorophore into the molecule. The resultant product may then be monitored at a wavelength at which very few other broth components absorb and also may have an extremely high extinction coefficient making analysis both more convenient and more sensitive.

Several well established methods for the pre-column reactions currently exist for the assay of small peptides and amino acids. Fluorescent products are regarded as preferable because of their greater detection sensitivity and relative freedom from interference. The assay of ACV has been carried out by pre-column derivatization to yield fluorescent products by reaction of its primary amino group[2,3]; using these methods it is possible to monitor both the monomeric and dimeric forms of the tripeptide. More recently ACV has been derivatised via the sulphydryl group[10] and hence greater selectivity has been achieved. This approach has the added advantage of allowing assay of ACV in a form suitable as a substrate for isopenicillin N synthetase. We now report the new use of a specific thiol derivatising reagent, dansylaziridine, which produces stable fluorescent products[11] and is particularly suited to the trace level analysis of this important biosynthetic intermediate by HPLC. We have also compared this procedure with two existing methods for the quantitation of ACV under similar conditions.

EXPERIMENTAL

Materials and methods

Dansylaziridine (5-dimethylaminonaphthalene-1-sulphonylaziridine), glutathione (reduced form), L-cysteine, sodium borohydride and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) were supplied by Sigma (Poole, U.K.). Glacial acetic acid, ethylenediaminetetraacetic acid (EDTA) and potassium hydroxide were obtained from BDH (Poole, U.K.) and were all analytical reagent quality (AnalaR). Analytical reagent grade sodium acetate and potassium dihydrogenphosphate were purchased from Fisons (Loughborough, U.K.). HPLC grade acetonitrile and methanol were acquired from Rathburn Chemicals (Walkerburn, U.K.). Monobromobimane (3,7-dimethyl-4-bromo-
ethyl-6-methyl-1,5-diazobicyclo[3.3.0]-octa-3,6-diene-2,8-dione) was supplied by Calbiochem-Behring (Cambridge, U.K.) and the ACV was kindly donated by Glaxo Group Research (Greenford, U.K.). All aqueous buffers were prepared in deionised water obtained using a Nanopure II system (Fisons).

Equipment

HPLC was performed using a single 2150 HPLC pump connected to a 2152LC controller and a 11300 Ultragrad mixer (LKB, Milton Keynes, U.K.) to produce the gradient. Integration was performed on a 4290 integrator (Spectra Physics, St. Albans, U.K.) and detection was carried out using a RF-530 fluorescence spectrophotometer (Shimadzu, Columbia, MD, U.S.A.). Ultra-violet detection was conducted using a Shimadzu SPD-2AM detector.

Chromatographic separations were carried out on a 5-μm, reversed-phase, C_{18}, 25 cm × 0.46 cm column obtained from Hichrom (Reading, U.K.). A 5 cm × 0.46 cm guard column (cartridge system) containing the same stationary phase was used to protect the analytical column and was supplied by Chrompack (Middleburg, The Netherlands). Fermentations were carried out in a MK X, LH incubator shaker (LH Fermentations, Stoke Poges, U.K.).

Fungal strains and growth media

Penicillium chrysogenum strain numbers P2 and NRRL1951 (Pan Laboratories) and SC6140 (kindly donated by Professor Sir E. P. Abraham and originally from Squibb Institute for Medical Research, New Jersey, U.S.A.) were grown in Jarvis and Johnson[12] defined medium. A mutant strain of *Cephalosporium acremonium*, N2 (a gift from Glaxo Group Research) was grown as described by Shirafuji *et al.*[13]. *Aspergillus niger* IMI17454 and strains G3 and GH52 of *Aspergillus nidulans* were grown in Aspergillus Complete Medium[14] for 5 days. The broth from the former organism was used as a blank (no ACV could be detected; *A. niger* has not been reported as a β-lactam producer).

Sample preparation

Broths were harvested by centrifugation (5600 *g*, 20 min, 4°C), treated with a two-fold excess of acetone and left for 20 min to allow protein precipitation. The resultant suspension was centrifuged and the supernatant passed through a 0.45-μm filter (Millipore, Bedford, MA, U.S.A.). The sample was treated with an excess of solid sodium borohydride (approximately 3.5 mg borohydride per ml of broth) and incubated at 60°C for 30 min. The excess reducing agent was subsequently destroyed by the addition of 20 μl of glacial acetic acid until the pH of the solution reached a value of 4 (usually 20 μl of acid per ml of broth). Degradation of the borohydride was judged to be complete when the effervesence ceased (approximately 5 min). The pH of the broth was readjusted to that required for the reaction by the addition of 2 *M* potassium hydroxide. Buffer samples containing ACV were treated identically, except no protein precipitation step was needed. The reaction involving monobromobimane was conducted with ACV dimer which had been reduced by dithiothreitol. The dimer was dissolved in phosphate buffer at pH 7.4, in contrast to the other two derivatising procedures where the ACV was prepared in water.

Preparation of derivatising reagents and mobile phases

DTNB solution (1 m*M*) was prepared by dissolving the solid in a phosphate buffer (0.1 *M*, pH 8.0). The reagent was prepared daily and kept in the dark when not in use. The mobile phase for HPLC separations consisted of acetate buffer (0.02 *M*, pH 5.8) and acetonitrile run as a linear gradient at a flow-rate of 1 ml/min.

Monobromobimane (2 m*M*) was prepared as a stock solution in HPLC grade acetonitrile. A similar mobile phase to that described by Newton *et al.*[15] was used. Buffer A consisted of 90% (v/v) water containing EDTA (0.5 m*M*), acetic acid (0.04 *M*) and 10% (v/v) acetonitrile. The buffer was then adjusted to a pH of 3.6 with 10 *M*

potassium hydroxide. Buffer B was composed of 90% acetonitrile, 10% water and was brought to a pH of 3.6 with glacial acetic acid. The two buffers were mixed in a step gradient over 30 min using a flow-rate of 1.5 ml/min.

Dansylaziridine (3 m*M*) was prepared in HPLC grade methanol. Once again a gradient elution programme was employed. This time the mobile phase consisted of acetate buffer (0.02 *M*, pH 4.0) containing EDTA (0.5 m*M*) and acetonitrile (Fig. 2a) and separations were effected at a flow-rate of 1 ml/min.

Derivatisation methods

ACV Standards

Fresh stock solutions of ACV (1 m*M*) were produced on a daily basis as described under Sample preparation. These stock solutions were then serially diluted prior to use in the following procedures.

DTNB procedure. A sample of the ACV monomer (10 μl, 1 m*M*) was reacted with DTNB (30 μl, 1 m*M*) at pH 8.0 for 10 min at room temperature. The resultant solution containing the derivatised peptide was injected onto the column (injection volume 20 μl). Detection was by UV absorbance at 310 nm. (This wavelength had previously been determined to correspond to the maximum absorbance of the mixed disulphide.)

Monobromobimane procedure. ACV dimer (20 μl, 0.9 m*M*) was reacted with DTT (20 μl, 2 m*M*) for 5 min. The resultant solution was treated with monobromobimane stock solution (20 μl, 2 m*M*) and after shaking was left to stand for 20 min in the dark at 20°C. HPLC detection was carried out by spectrofluorimetry using the excitation and emission wavelengths of 380 and 477 nm respectively. HPLC was effected using an injection volume of 20 μl.

Dansylaziridine procedure. A solution of ACV (20 μl, 1 m*M* at the pH stated) was reacted with an equal volume of dansylaziridine stock solution. The mixture was incubated at 60°C for 30 min and then cooled to 20°C. A 20-μl sample was injected onto the HPLC column.

The aziridine derivatisation procedure was also carried out on other thiols known to be present in fermentation media, namely glutathione and cysteine. A 1 ml solution containing glutathione (0.21 m*M*), L-cysteine (0.3 m*M*) and reduced ACV (0.1 m*M*) was assayed similarly.

Biological samples

Filtered, deproteinised fermentation broths were obtained and treated with sodium borohydride as described previously to produce the monomeric form of ACV. The pH of each sample was adjusted to 8.8 and an aliquot (50 μl) was reacted with an equal volume of dansylaziridine stock solution. The reaction mixtures were incubated at 60°C for 30 min followed by rapid cooling to 20°C. As in previous experiments an injection volume of 20 μl was employed for HPLC.

RESULTS AND DISCUSSION

Optimisation of the derivatisation procedure

The analytical procedure based upon derivatisation with dansylaziridine was

optimised with respect to excitation and emission wavelengths, reaction time with ACV, molar ratio of reagent to analyte and temperature of the reaction.

Excitation and emission wavelengths were selected by manual scanning of the monochromator, under stopped flow conditions. The maxima for absorption and emission were found to be at wavelengths of 339 and 540 nm respectively.

The pH dependency of the reaction of the aziridine with ACV is shown in Fig. 1a (using a 60-min incubation at 60°C). A working pH of 8.8 was adopted for all subsequent analyses. This pH represented a compromise between product yield and signal-to-noise ratio which decreased with increasing pH. Furthermore the use of samples at a pH greater than 8.9 was incompatible with the buffering capacity of the mobile phase. At these pH's variable retention times, loss of resolution and increased reagent hydrolysis peak were all noted.

The incubation temperature used for the derivatisation was varied between 20 and 70°C (using a 60-min incubation at pH 8.8). As shown in Fig. 1b the yield of fluorescent product increased with temperature. A working temperature of 60°C was adopted in order to maximise the sensitivity since above this temperature the signal-to-noise ratio decreased dramatically.

Reaction times at 60°C were studied and it was found that the product peak area increased rapidly with time up to 30 min but thereafter increased only slowly, reaching a maximum at approximately 400 min. Since the reaction after 30 min was 95% of that after 60 min the former reaction time period was adopted for the incubation in the standard procedure.

The optimal molar ratio of reagent to analyte was investigated for an ACV concentration of 1 m*M*. It was found that it was necessary to use at least a three-fold reagent excess to ensure complete reaction at the time and temperature specified. When working with broth samples, the standard derivatisation employed a concen-

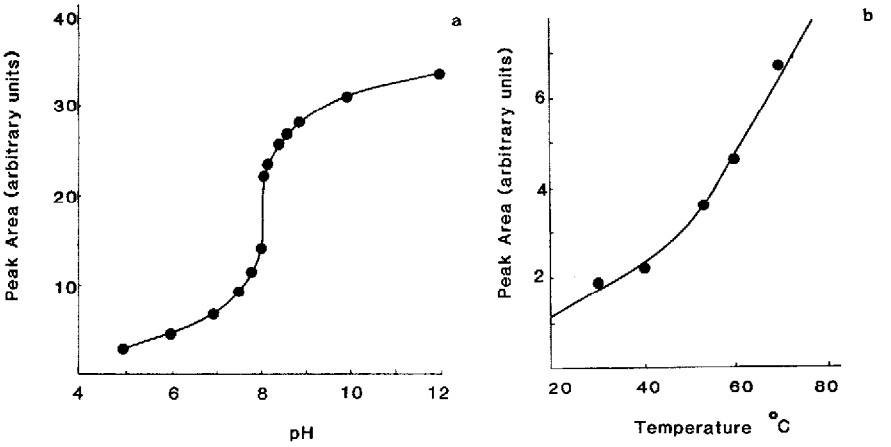

Fig. 1. Optimisation of pH and temperature for the dansylaziridine derivatisation procedure. Chromatographic conditions as described in the Experimental section; detector sensitivity 32; injection volume 20 μl. (a) The effect of reaction pH on peak area was examined using phosphate buffers (50 m*M*) at the appropriate pH (60°C, 60 min) incorporating reagent and ACV at 1.5 and 0.14 m*M* (100 μg/ml) concentrations respectively. (b) The influence of temperature on peak area was examined in phosphate buffer (50 m*M*) at pH 8.8 containing ACV and dansylaziridine at concentrations of 0.14 and 1.5 m*M* respectively.

tration of dansylaziridine (1.5 m*M*) which represented a large molar excess compared to the total thiol concentration in all the complex broths we have studied.

Calibration and detection limits

Two calibration graphs of ACV concentrations were constructed using optimised conditions for the derivatisation as previously described. Regression analysis showed that both plots were linear for ACV concentrations in acetate buffer between 227 and 0.56 μg/ml and 227 and 5.6 ng/ml respectively, and passed through the origin. However, a detection limit of 120 pg/ml for a signal-to-noise ratio of 2:1 was achievable. The regression equations for the two plots were:

Range 1 (227–0.56 μg/ml)
Peak area (arbitrary units) = 3.58 + 0.63 × concentration (μg/ml)

Standard deviation = 2.9
R-squared = 98%

Range 2 (227–5.6 ng/ml)
Peak area (arbitrary units) = 0.52 + 55.43 × concentration (ng/ml)

Standard deviation = 0.223
R-squared = 99.4%

A study of the stability of the product was made. After derivatisation, the reaction mixture was cooled to 20°C and allowed to stand at this temperature for various periods of time prior to HPLC analysis using an injection volume of 20 μl. No variation in peak area was detected after stand-times up to 1250 min (20.8 h). Reproducibility was determined by repeat injections (n = 10) of the same derivatised ACV solution (0.25 m*M*). The standard deviation was found to be 2%.

Comparison with other derivatising reagents

For comparative purposes, assays involving the alternative derivatising reagents DTNB and monobromobimane were carried out using solutions of ACV prepared in acetate buffer. Using the former derivatising reagent good chromatographic resolution was easily obtainable employing a simple gradient and detection at 310 nm. However, the low extinction coefficient of the mixed disulphide did not permit satisfactory detection of ACV below 10 μg/ml in buffer solutions even when maximum detector sensitivity was employed. For this reason, further work in fermentation media was not attempted. The advantages of derivatisation with DTNB are the rapid reaction rates under ambient conditions, and good overall reproducibility in the HPLC analysis. Consequently this method is useful for the assay of thiols in non-complex media.

The use of monobromobimane in the quantitation of thiols from biological sources has been well documented[15–17]. The reagent reacts rapidly with ACV at room temperature and yields highly fluorescent products. Although other workers have reported its use in the study of β-lactam biosynthesis using cell-free extracts[10], when applied to the determination of ACV in fermentation broths problems were encountered in the development of suitable HPLC elution programmes. Additional

problems included the appearance of a very intense peak in the chromatogram attributable to hydrolysed reagent. Other peaks were also obtained which may arise from the reaction of monobromobimane with non-thiol containing broth components. For solutions in buffer, ACV was detectable at concentrations down to 2.5 μg/ml but reproducibility was poor due to poor resolution and the detection limit in broths was subsequently an order of magnitude lower. However, we have developed a procedure for the qualitative visualisation of ACV on paper and thin-layer plates based upon the reaction with monobromobimane and irradiation with UV light (unpublished work).

In conclusion dansylaziridine is the favoured derivatising reagent for the trace analysis of ACV in fermentation samples by an HPLC procedure, on the basis of its selectivity and superior sensitivity when spectrofluorometric detection is employed (Fig. 2a). Detection may also be achieved by the absorption of UV light at 245 nm but this method is considerably less sensitive. Further work was conducted with the dansylaziridine to investigate its applicability to ACV assay in the presence of high levels of other potentially interfering compounds (Fig. 2b). Good resolution between ACV and the likely principle sulphydryl containing metabolites, glutathione and cysteine, was achieved. When equi-molar concentrations of these three metabolites were analysed by the dansylaziridine procedure, a greater signal sensitivity was obtained for cysteine than the two peptides.

Applications of the analytical method to crude fermentation broths

The method has been applied to the measurement of the levels of ACV produced extracellularly in fermentation broths by various fungal species grown under different fermentation conditions. These data are presented in Table I. Broth samples

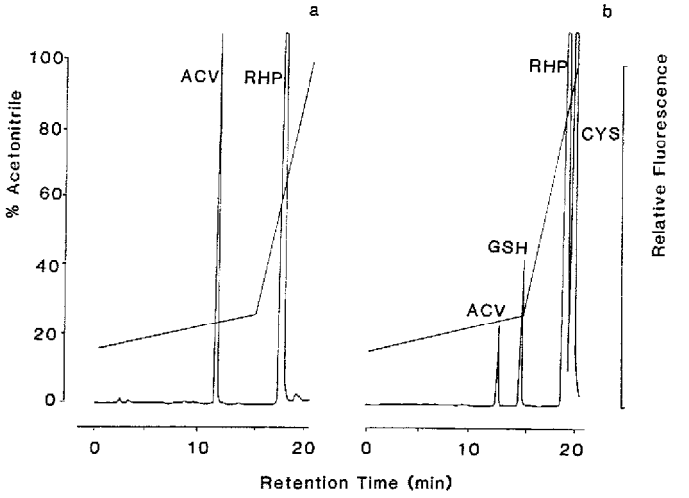

Fig. 2. Reversed-phase HPLC chromatograms of dansylaziridine derivatives. Chromatographic and derivatisation conditions as described in the Experimental section: detector sensitivity 16; injection volume 20 μl. (a) ACV standard at a concentration of 0.07 m*M* (50 μg/ml), showing the reagent peak (RHP). (b) A mixture of glutathione (GSH), cysteine (CYS) and ACV (0.014 m*M*) in a molar ratio 2:3:1 at derivatisation.

TABLE I

DETERMINATION OF ACV CONCENTRATIONS IN CULTURE BROTHS OF FILAMENTOUS FUNGI

After growth of the fungi in the stated complex media for a fixed time, ACV concentrations were determined using the appropriate regression line given in the Results section. Values are the average of duplicate determinations. Media abbreviations used: ACM = *Aspergillus* complete medium; PLFM = *Penicillium* fermentation medium from Pan Labs.; J&J = Jarvis and Johnson defined medium; CSM = *Cephalosporium* sporulation medium.

Species	*Strain*	*Time of assay (h)*	*Media*	*ACV (μg/ml)*
A. nidulans	G3	120	ACM	0.16
A. nidulans	Wild type	120	ACM	0.08
P. chrysogenum	SC6140	0	PLFM	0.13
P. chrysogenum	SC6140	60	PLFM	4.1
P. chrysogenum	NRRL1951	72	J&J	0.00036
P. chrysogenum	P2	120	J&J	1
C. acremonium	N2	120	CSM	4.3

from the fermentation of wild-type *Aspergillus niger* were used as blanks because these were shown to contain no detectable ACV (Fig. 3a). For each fungal species tested several fermentations were carried out and HPLC analyses were made after harvesting broth at fixed times. A typical chromatogram of a given species and fermentation medium is shown (Fig. 3c). ACV concentrations were found to vary according to species, type of liquid culture medium and duration of fermentation prior to assay. To confirm identity of the ACV peaks in the chromatograms from fermentation broths a series of experiments was conducted as follows.

(i) Spiking with standard ACV stock solutions was performed using standard additions calculated to increase the ACV concentration in broth from *P. chrysogenum* SC6140 fermentation by two-, three- and four-fold over the original concentration. This process was also used to confirm the calibration plots described previously.

(ii) Standard additions into spent fermentation broths of *A. niger* (which originally gave no peak at the position corresponding to the ACV derivative) gave an ACV peak which increased in proportion to concentration (Fig. 3a and b).

(iii) When broths treated with and without sodium borohydride were compared, the HPLC of the latter showed no peak corresponding to the ACV derivative. These procedures confirmed the identity of the ACV-dansylaziridine peak in complex media.

Aspergillus nidulans species produced significantly lower ACV levels than the other species, which is consistent with the low penicillin titres obtained from these organisms. This appears to be an intrinsic species trait.

When *P. chrysogenum* strain P2 was grown in defined media with and without the penicillin precursor phenoxyacetic acid (POA) it was observed that addition of POA resulted in reduction of ACV levels by over 50%. There was a concomitant increase in the penicillin V (phenoxymethylpenicillin) titre (unpublished work).

In conclusion, the use of dansylaziridine as a pre-column derivatisation reagent for the HPLC analysis of ACV in fermentation broths has been demonstrated. The method has been shown to be selective and sensitive, with calibrations which are

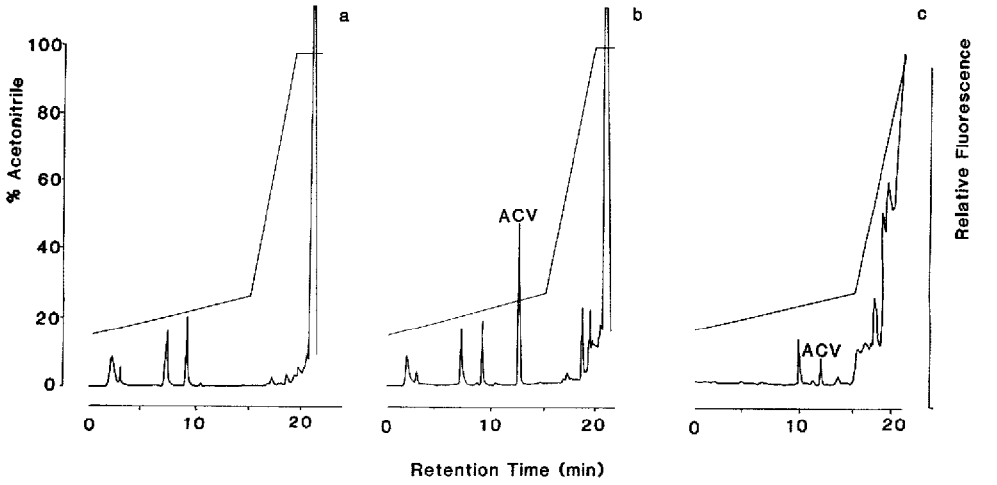

Fig. 3. Reversed-phase HPLC chromatograms of fermentation broth samples after derivatisation with dansylaziridine. Chromatographic procedure, culture conditions and sample derivatisation as described in the Experimental section; detector sensitivity 8; injection volume 20 μl. (a) *A. niger* wild type (IMI17454), 120 h into the fermentation. (b) *A. niger* wild type (IMI17454), 120 h into the fermentation containing a standard addition of ACV (5 μl, 220 μg/ml into 105 μl broth to give a final concentration of 10 μg/ml). (c) *P. chrysogenum* strain SC6140, 60 h into the fermentation.

linear over a wide dynamic range. Dansylaziridine as a derivatising reagent was found to have several advantages over both dithionitrobenzoic acid and monobromobimane, which also react specifically with the sulphydryl group of the ACV monomer.

We believe this is the first direct comparison of broth concentrations of ACV produced extracellularly by the fermentation of different fungal species. In on-going work the procedure is being used to study the influence of media constituents on ACV production in time course studies of fermentations of *A. nidulans*, *P. chrysogenum* and *C. acremonium*. It is intended to monitor both intra- and extracellular levels of the key biosynthetic intermediate, ACV.

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