OVINE ILL-THRIFT IN NOVA SCOTIA. 8. ANALYSIS OF ISOCYANIDE ANTIBIOTICS PRODUCED BY *TRICHODERMA HAMATUM* *

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The isocyanide antibiotics, trichoviridin (I), dermadin (II) and 3-(3-isocyanocyclopent-2-enylidene-)propionic acid (III) can be separated on silica and 1 μg can be detected on chromatograms by spraying them with a dilute solution of nickelous chloride. The metabolites II and III can be determined, with poor precision (±8%) by making use of their ultraviolet absorption at 254 nm. These two metabolites (II and III) can be analysed in crude extracts only after a preliminary purification step. This chromatographic step results in losses of the isocyanide II. Hence, periodically, the chromatographic equipment has to be calibrated and always after a change of column. The optimum weights of each of the isocyanides analysed was in the range 0.2 - 12 μg in about 5 μl of solution. About 0.5% of III in dermadin and about 1% of dermadin in III could be determined with the precision of the method.

Les antibiotiques isocyaniques suivants: trichoviridine (I), dermadin (II) et 3-(isocyano-3 cyclopent-enyldene-2) acide propionic-3 (III) feuvent être séparés sur silice et 1 μg est détectable sur les chromatogrammes après vaporisation d'une solution de chlorure de nickel diluie. Les métabolites II et III peuvent être déterminés par leur absorption ultraviolette à 254 nm, mais avec une faible précision (±8%). L' analyse des métabolites II et III dans les extraits, bruts, nécessite une étape de purification préliminaire. Cette étape chromatographique amène des pertes d'isocyanide II.

C'est pour cette raison que l'equipment chromatographique doit être calibré periodiquement, et spécialement après un changement de colonne. Les poids optimum de chacun des isocyanides analysés variaient de 0.2 a 12 μg dans 5 μl de solution. La precision de la méthode perment de déterminer approximativement 0.5% de III dans la dermadiner et 1% de dermadine dans II.

Introduction

Trichoderma hamatum is an important component of the fungal flora of soils of permanent pasture in Nova Scotia (Brewer and Taylor, 1980). Many isolates of this fungus produce unstable metabolites that inhibit the growth of several genera of bacteria (Brewer and Taylor, 1981). Since these metabolites are soluble in water and are of low molecular weight (Brewer et al., 1979) it is possible that they are translocated by pasture plants and thus ingested by ruminants. They may, therefore play a role in the fermentation changes that occur in the rumen, that are thought to be related to ill-thrift (Brewer et al., 1971). Hitherto only biological assays of these compounds have been available, but with some elucidation of their chemistry (Brewer et al., 1979) it seemed possible that an analytical method based on their physical and chemical properties could be developed. Such a procedure might complement and confirm the results of biological assays. An investigation along these lines is reported in this paper.

Methods

All chemicals were of analytical grade. Petroleum ether had b.p. 30 - 60° and diethyl ether was distilled from ferrous sulphate before use. The isocyanides,

trichoviridin (I), dermadin (II), and 3-(3-isocyano*cyclo*pent-2-enylidene-) propionic acid (III) were isolated from fermentations of *Trichoderma hamatum* isolates HLX* 1383, HLX 1388 and HLX 1379-11 as has been described (Brewer et al., 1982). Glass plates coated with silica gel (0.25 mm thick, 60 F-254, Merck) containing a lanthanide fluorescent indicator were used for thin layer chromatography (t.l.c.). Isocyanides were detected on the plates by reflectance short wave (ca. 254 nm) ultraviolet radiation in the cases of II and III, and by spraying the plates with a solution (5 ml) of nickelous chloride (0.01 g) in water (100 ml).

Chromatographic Apparatus for Quantitative Analysis
   The apparatus consisted of a pump (Waters 6000A) fed with premixed solvent through a 5 μm filter and a teflon line. The solvent was kept at room temperature in contact with the filter for 24 h before use. The teflon line passed through a tight - fitting hole in the screw cap of the solvent reservoir to prevent evaporation of the solvent. During chromatography the cap was partially unscrewed to allow air into the reservoir. The pump delivered solvent to an injection block (Waters, model U6K) and thence to 2 silica gel columns (Merck, either LiChrosorb Si 60 (7 μm), 250 x 3 mm, or LiChrosorb Si 60 (5 μm), 250 x 4.5 mm, or intermediate diameters and particle sizes) in series. The effluent from the second column was led to an ultraviolet detector (Waters, model 440, cell light path 1 cm, λ = 254 nm) and then to a second detector (DuPont 837) set at 270 nm and finally to a valve which permitted collection of fractions or disposal to waste. The pump, injection block, columns, detectors and collection valve, were connected by stainless steel tubing of 0.023 cm bore. The signal from the 254 nm detector (0-2 v) was used as input on a digital integrator (Waters data module 700) which calculated the area of the envelope above the base line of the changing signal with time.
   All weights were measured on an analytical balance (P. Bunge, Hamburg 23, W. Germany) with a precision of ±1 μg. Ultraviolet spectra were measured on a Cary 14 spectrometer.

Procedures
   (a) *Qualitative analysis*—A solution (5 μl) of isocyanide (1-5 μg) in diethyl ether was applied to a t.l.c. plate. The plate was developed with a solution (100 ml) of acetic acid (0.2 ml) in petroleum ether (24.8 ml) and diethyl ether until the solvent front had risen to the top of the silica layer. Standard solutions (2 μl, 10% w/v) of the isocyanides I, II, and III were chromatographed at the same time. The plate was examined under ultraviolet light (254 nm), the absorbing spots were marked, and the plate was then sprayed with nickelous chloride solution. After 5 min at room temperature any brown or black spots were marked and the plates were then heated at 100° for 5 min.
   (b) *Quantitative analysis of isocyanides II and III*—A solution (5 μl) containing 0.5-5 μg of III and/or 1-10 μg of II was injected into the sample loop of the injection block. The loop was swept out by the eluting solvent (acetic acid-petroleum ether-diethyl ether: 1:149:350) pumped at 2 ml min^{-1}. The columns were maintained at 24 ±1°. Each chromatogram required about 11 min; III was eluted at an elution volume of about 10 ml and dermadin at 15 ml.
   Replicate chromatograms of all analytical samples were run until the standard deviation of the integrated areas for each isocyanide was less than 10%.
   (c) *Preparation of samples for chromatography*—Samples in aqueous solutions were acidified with phosphoric acid (2N) to pH 4.5. The acidified solution was extracted with diethyl ether (x 4) and the combined ether extracts dried (Na$_2$SO$_4$) at

−15°. After 1 h the extracts were filtered at −15°, were allowed to warm to room temperature and diluted to give a final isocyanide concentration of about 2 mg ml⁻¹. The concentration was judged by the size of the spots on the thin layer chromatograms. Samples in organic solvents were diluted with ether to the same concentration judged in the same way.

Samples of pure trichoviridin (I), and dermadin (II) were weighed as standards as has been described (Brewer et al., 1982). Samples of the isocyanide III were too unstable to be weighed. Thus measurements were made on solutions of known volume whose concentration was then determined by evaporation of an aliquot and weighing the residue. It was assumed that no weight gain or loss of the solute occurred during evaporation.

Results

*Qualitative analysis of isocyanide metabolites by thin layer chromatography*

Separation of the 3 principal isocyanide metabolites of *T. hamatum* by chromatography requires a trace of acetic acid in the developing solvent. The minimum concentration was 0.2% since lower quantities resulted in tailing spots. The maximum concentration was about 0.5% because higher levels induced polymerisation of the isocyanides and hence long streaks of brown polymer on the plates. Most commercially prepared plates were satisfactory and lanthanide fluorescent agents did not appear to catalyse polymerisation. However some polymerisation always occurred at the point of application unless the sample was applied to a 2 cm strip of hyflosupercel along one edge of a composite silica plate.
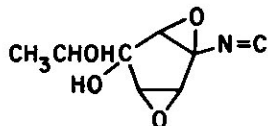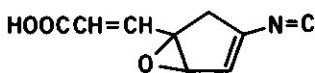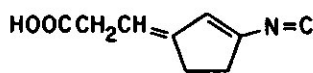
I   II   III

Those metabolites that have the chromophore $-\overset{|}{C}=\overset{|}{C}-N=C$ appear as blue spots on lanthanide-containing plates when the plates are irradiated with short-wave ultraviolet light. The limit of detection of III is about 0.1 μg and of II, about 0.05 μg. The 3 isocyanide metabolites, and indeed the simple isocyanides, cyclohexylisocyanide and benzylisocyanide, polymerise under the catalytic effect of nickelous salts (Nolte et al., 1973) to give brown polymers. Hence they can be detected on t.l.c. plates by spraying the plates with aqueous solutions of nickelous salts. The unsaturated isocyanides (II & III) polymerise instantaneously at room temperature while the other 3 isocyanides polymerise slowly unless the plates are heated to 100°. The lower limit of detection in all cases is about 0.1 μg.

Ultraviolet absorption spectra of isocyanides II and III

The ultraviolet absorption spectra of II and III are shown in Fig 1. The absorption of the latter compound at 270 nm had ε 13630 ± 70 (determinations on 5 samples) but, although the measurement is precise, it is not suitable for analytical purposes because the decomposition/polymerisation products of III also absorb in this region of the spectrum. The value of $\varepsilon_{223\,nm}$ for dermadin was found to be 15200 ± 750 (determinations on 6 samples) but again this was not suitable for direct analysis because of interfering components in mixtures that also absorbed in this region. It was therefore necessary to introduce a purification step into the analytical
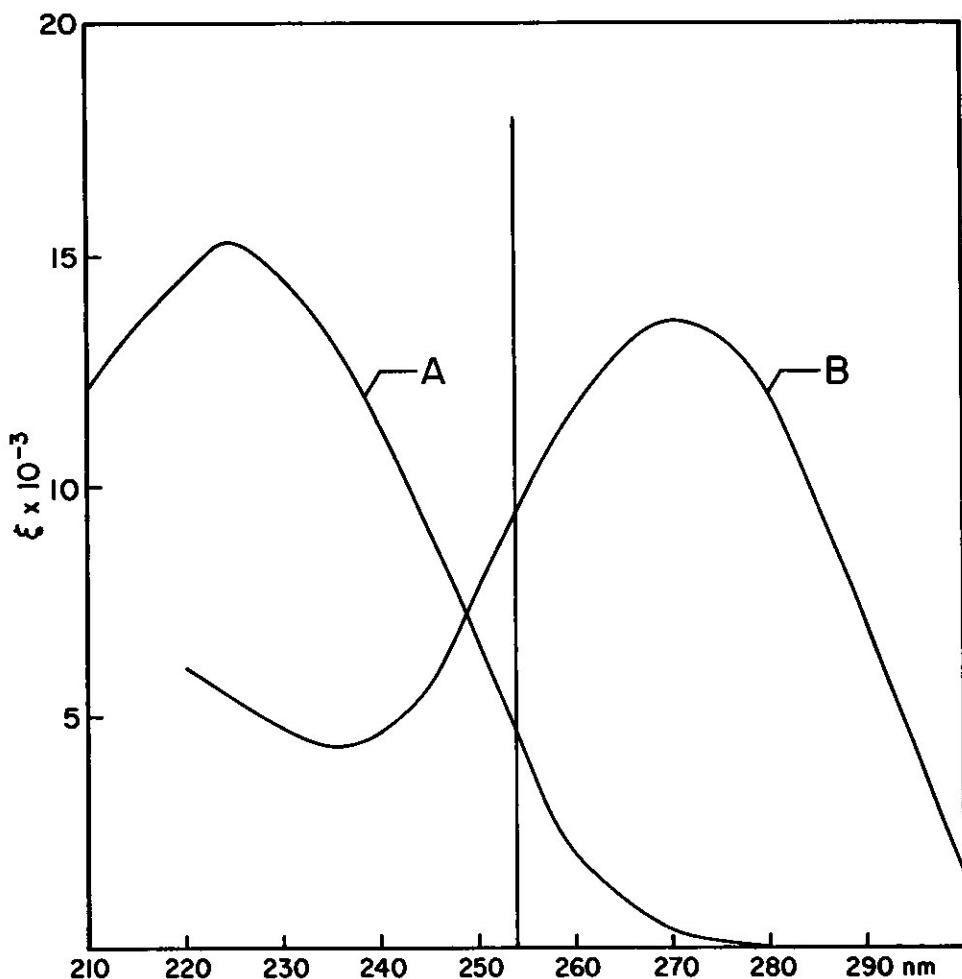
Fig 1   Ultraviolet spectra of A = dermadin (II) and B = 3-(3-isocyanocyclopent-
2-enylidene-) propionic acid (III).

procedure. The 2 metabolites could be separated on slicia gel and could be
detected in the effluent from a chromatography column using a suitable flow cell.
If it is assumed that the detected isocyanide is pure, then its concentration in the
cell at an instant of time, can be calculated from its absorption at that time, with the
precision indicated. Similarly, the absorption detected during the time when the
solute is eluted is proportional to the changing concentration and hence to the
weight of solute present. The proportionality constants can be determined by chro-
matography of known weights of solute. We were unable to determine the absorp-
tion of the effluent at 223 nm and at 270 nm when mixtures of the isocyanides were
analysed on the same chromatogram because of switching difficulties and atten-
dant base line instabilities. The analyses were therefore carried out by measuring
the absorption at 254 nm by the 2 metabolites. The values for $\varepsilon_{254 \text{ nm}}$ for II and III
were found to be, respectively, 4900 ± 240 and 9430 ± 80 (6 determinations on 4
different samples of each metabolite). The larger error for dermadin is due to the
fact that its absorption changes very rapidly with respect to wavelength in this re-
gion of the spectrum (Fig 1; e.g. $\varepsilon_{252 \text{ nm}}$ 5850, $\varepsilon_{256 \text{ nm}}$ 3850), hence accuracy is de-
pendent on the resolution of the spectrometer.

Analysis of Dermadin (II)

(a) *Reproducibility of retention volume*—The R^v of dermadin, i.e. the volume of solvent pumped through the column between injection of the sample, and the point of maximum absorption in the eluate was, as expected, dependent on the temperature of the column and the weight of silica. Under the experimental conditions used the variation in R^v was less than 2%. Most of the variability was due to minute vapour bubbles in the solvent which affected pumping pressure and hence flow rate. For this reason it was essential to allow freshly prepared solvent to stand at the same temperature as the pump for at least 24 h before use.

(b) *Reproducibility of detector response*—A column load of dermadin between 0.1 and 10 μg was easily detected in the eluant. For reasons that will be discussed, loads of about 0.5 μg were used to determine the reproducibility of the system. A series of 27 runs gave a mean value for the integrated areas corresponding to unit injection volume of 1510±126 (range 1123-1762), i.e. an error of ± 8%. Better results were obtained with injection volumes of 4 and 5 μl (Table 1). The F value for the variances of 4 μl and 5 μl injections was significant ($P < 0.05$). Thus a small decrease in the volume injected increased the variability. Nonetheless, the difference in the mean integrated areas was significant ($P < 0.001$) and they were in the proportion 4:5.

Table I Comparison of the reproducibility of integrator response in chromatographic analysis of dermadin solutions in diethyl ether.

Wt. of dermadin on column (μg)	Injected Volume (μl)	No. of Samples	Integrated areas of signal with respect to time in arbitary units			
			Mean	SD	Maximum	Minimum
2.185	5	7	7537	313	7776	6970
1.748	4	8	6093	855	7048	4491
0.435	5	8	1511	74	1612	1417

(c) *Effect of load of dermadin on the column*—The minimum weight of dermadin that could be detected in the effluent was 0.008 μg. At loads ten times greater the integrated areas were 229±60. By increasing the load by a further factor of 5 much better reproducibility was obtained (Table 1). Similar reproductibilities were achieved by using column loads up to and including 11 μg, but higher concentrations, still well within the range of the detector, gave results whose variance increased with load.

(d) *Recovery in column effluents*—The weight of dermadin in the effluent from the column was determined by collecting the fraction, and determining the absorption of the solution of known volume at 223 nm. Losses of dermadin occurred on all columns and depended on the size of the column and the dimensions of the silica particles. Thus on a column of dimensions 25 x 0.3 cm a recovery of 98% was obtained, but when the size of the column was increased to 25 x 0.45 cm (which greatly improved separation—see below) only 76% was recovered. The effect of particle size was smaller, recoveries being about 2% lower when the particle size was ∼5 μm, as compared to ∼ 10 μm. Thus all columns had to be calibrated at regular intervals and especially when new.

(e) Relationship between integrated areas and weight of dermadin in a sample—Eight samples of dermadin, isolated from different *T. hamatum* fermentations (Brewer *et al.*, 1982) and purified by chromatography to constant specific optical rotation ($[\alpha_D^{24} + 138°$) and absorption at 223 nm (see above) were weighed in the quantities shown in Table 2. The weights injected onto the column and the mean values for the areas obtained in quadruplicated runs are also given in Table 2. These data were then used to calculate the least squares regression:

$$\text{Wt. of dermadin in } \mu g = 0.21 + 2.543 \times 10^{-4} \times \text{integrated area,}$$

with $R^2 = 0.987$ and SE_{est}, $0.044\mu g$. The percentage error of predicted weight is smaller for the mid-range of weights (Table II).

Table II Relationship of integrated areas of detector response to known weights of dermadin injected on a silica gel column (20 x 0.45 cm) of particle size ca. 5 μm

Weight of Dermadin		Integrated area (arbitrary units)	Calculated weight of dermadin	Weight – Calc. weight	% Diff.
sample (mg)	on column (μg)		(μg)	(μg)	
0.017	0.085	239	0.27	−0.19	−69
0.087	0.437	1511	0.595	−0.16	−26
0.907	0.907	3025	0.980	−0.073	−7.4
0.437	2.185	7531	2.126	0.059	2.7
1.309	2.618	9539	2.636	−0.018	−0.7
2.100	4.200	16582	4.428	−0.228	−5.1
3.186	6.372	20450	5.410	0.961	17.8
2.212	11.060	44055	11.410	−0.35	−3.1

Analysis of 3-(3-isocyanocyclopent-2-enylidene-) propionic acid (III)

(a) Reproducibility of retention volume—Under the conditions used for chromatography of dermadin, the isocyanide III had a smaller R_v. It was therefore eluted in a band that was less dispersed and hence the variability of the R_v from one chromatogram to another was smaller—$\pm 1.5\%$.

(b) Determination of optimum conditions for analysis—The value of ε at 254 nm for the isocyanide III is 1.9 times greater than that of dermadin at this wave-length. In addition the accuracy of the determination is greater ($\pm 1\%$ compared to $\pm 5\%$). Since the retention volume was also lower, and hence the time of contact of the antibiotic with the silica shorter, polymerisation and/or decomposition on the column might be expected to be less than that of dermadin. However it was found that the mean integrated area for 26 analyses of 1 μg of the pure antibiotic in 5 μl of ether was 7360 ± 624 (range 8731-6143); the same error (8%) as found in the analysis of dermadin. Analysis of data obtained by injection of 3, 4 and 5 μl of the same solution of the isocyanide III showed, as with dermadin, that there was a statistically significant difference in response to each volume injected, but there was no significant differences in the F values of the variances. Recoveries of III from the column were independent of column dimensions and the particle size of the silica. Within the accuracy of the measurements recoveries were 100%.

(c) Relationship between integrated areas and weight of III in a sample—Five samples of III isolated from different fermentations of *T. hamatum* were purified by chromatography to constant ε at 270 nm. The five solutions were adjusted to 50 ml. Samples (2 ml) were then evaporated to dryness to determine the weight of the residue and other samples (5 μl) were analyzed chromatographically. All chromatograms were run in quadruplicate and the data obtained were used to

calculate a least-squares regression:

wt. of isocyanide III in μg = 0.013 + 1.33 x 10^{-4} x integrated area,

with R^2 = 0.994 and SE_{est} = 0.041 μg.

Analysis of mixture of isocyanides II and III

(a) *Factors determining efficiency of separation*—In the early stages of this work stainless steel columns 20 x 0.3 cm, packed with silica suitable for thin layer chromatography, were used. These columns completely separated mixtures of the two isocyanides except for mixtures of ~5% dermadin in III, where the dermadin peak was obscured in the tail of III. Subsequently, better separations were obtained on commercially packed columns and it appeared (no measurements were done) that columns with the smallest particle size (3 μm) were most efficient. Separation also depends on the size of the column, with 20 x 0.45 cm being optimal. Such columns give base-line separations of 1% of III in dermadin and *vice versa*. Larger columns are undesirable because of loss of dermadin. Separation also depends on the ratio of diethyl ether and petroleum ether. The best ratio had to be found by trial and error for each column, balancing complete separation against minimum residence time of the isocyanides on the column. In general decreasing the proportion of ether improves the separation.

(b) *Limits of detection in mixtures of II and III*—The limit of detection of III in dermadin was 0.3%. Thus in a mixture of 0.016 μg of III and 5.5 μg of II the peak of III was detected and integrated in 70% of the chromatograms. At twice the column load the chance of detection and integration of III increased to 90%, whilst at half the column load it declined to 25%. It was advantageous to confirm the presence of III in samples of dermadin by using a second detector set at 270 nm downstream from the 254 nm detector. The 270 nm peak of III (Fig 1) is quite sharp and the absorption of dermadin at 270 nm (ϵ 420) low and thus it is possible to detect about 0.01 μg of III in a mixture containing about 7 μg of dermadin.

The limit of detection of dermadin in samples of III was 1%. In a mixture of 0.05 μg of II and 5μg of III the dermadin peak was detected in 100% of the chromatograms and integrated in 95%. Reduction of the column load by 50% did not change the detection efficiency but reduced the number of times the peak was integrated to 65%.

Discussion

A simple spot test using a nickel salt detects isocyanides in a solution. This test was used to detect isocyanides on chromatograms and gave a rough indication of the quantity present. Unfortunately, we were unable to use the polymerisation reaction for quantitative colorimetric determination of isocyanides because the absorption of the brown polymers formed did not obey Beer's Law.

The concentration of dermadin (II) and the unsaturated isocyanide (III) in solution could be accurately determined by their ultraviolet absorption, but only when they were pure. The chromatography required for their separation and the need to use their absorption at a common wavelength, reduced the precision of the measurements to $\pm$ 8%. It is probable that this imprecision could be reduced by using dual detectors and integrators, but such a development would be expensive. Recent reports (Ott and Formcek, 1981; Schmidt and Dietsche, 1981) of the reactions of isocyanides with specific reagents to give derivatives having characteristic absorption, suggest that such an alternative approach might be more profitable than elaborate instrumentation.

The biological activity of these isocyanides varies greatly and it is therefore difficult to compare the sensitivity of the bioassay with chromatographic analyses. About 5 μg of the isocyanide III can be detected by a disc assay using *Micrococcus luteus* (HLX 701) as the test organism (Brewer et al., 1982). Although

chromatographic procedures are at least an order of magnitude more sensitive, they are no substitute for bioassay, because isocyanides of low biological activity with chromatographic properties similar to III may be present.

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