Tetrahedron Vol. 41, No. 10, pp. 1931 to 1938, 1985
Printed in Great Britain.

0040-4020/85 $3.00 + .00

BIOSYNTHESIS OF 3-(3'-ISOCYANOCYCLOPENT-2-ENYLIDENE)PROPIONIC ACID BY TRICHODERMA HAMATUM (BON.) BAIN. AGGR.+,++

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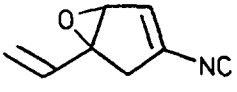
(*Received in UK* 15 *February* 1985)

Abstract - L-Tyrosine is degraded by Trichoderma hamatum to 3-(3'-isocyanocyclopent-2-enylidene)propionic acid having Z-configuration about the exocyclic double bond, by fission of the aromatic ring and cyclization of the side chain of the amino acid.

Isocyanides are known to be produced by bacteria[1,2], fungi[3-6], and marine organisms[7]. In the field of plant pathology, the homothallins[8] (e.g. 1) stimulate oospore formation by the A2 mating type of Phytophthora cinnamomi. The diene-isocyanide (2; R=H)[9,10] inhibits cellulose digestion by Bacterioides succinogenes[11], an important component of the rumen flora; hence it is possible that this metabolite plays a role in animal production[12].



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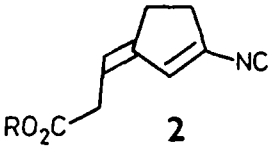

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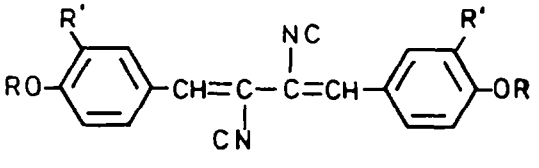
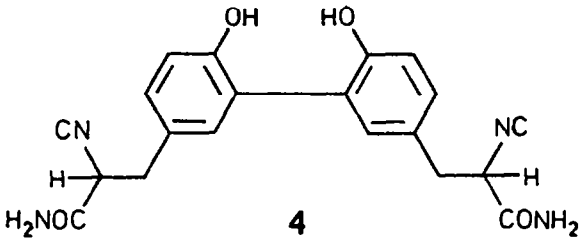
The structures of some of the microbial isocyanides strongly suggest their relationship with amino acids: the xanthocillins (3)[3,4] and hazimycin factors (4)[1] with tyrosine, the indolylacryloisocyanide (5)[2] with tryptophan, and the mannitol diester (6)[5] with valine. For xanthocillin (3; R=R'=H) this relationship has been established[13] through biosynthetic experiments. In the marine compounds, the isocyano group is attached to terpenoid skeletons, e.g. (7)[14], and have thus no obvious relationship with any known naturally occurring amino acid.

The cyclopentyl isocyanides[6] represent one of the two large groups of microbial isocyanides [the xanthocillins (3)[3,4] being the other]. They are biogenetically interesting because they might



3 (R=Me, H;
R'=H, OH, OMe)

be envisaged either as polyketides which are formed by ring contraction of aromatic polyphenols, a ring contraction product of an aromatic amino acid, or a modified monoterpene.



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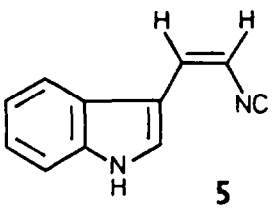

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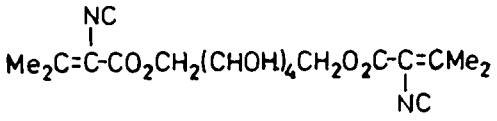

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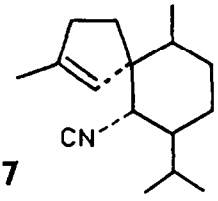

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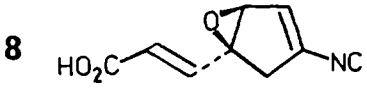

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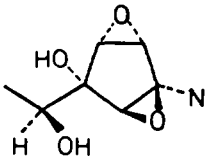

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Wild isolates of T. hamatum were found[9] to produce the isocyanides (2; R=H), (8), and (9) as major metabolites. A strain of the fungus was obtained which formed the diene-isocyanide (2; R=H) consistently in good yields (ca. 40 mgl^{-1} culture fluid). This had also a high activity in the rumen cellulose digestion test[10] and was therefore chosen for our biosynthetic experiments. Since there were some uncertainties concerning the proposed structure and the stereochemistry around the exocyclic double bond was not known, a detailed ^1H and ^{13}C n.m.r. study of the diene-isocyanide (2; R=Me) was carried out simultaneously with the biosynthetic studies. The n.m.r. studies confirmed the proposed structure and are fully discussed in the communication[15]. The results of the ^1H - ^1H nuclear Overhauser effect experiments leading to the allocation of the Z stereochemistry to the exocyclic double bond on the other hand were only mentioned and need illustration. This is given in Figure 1. Attempts at fitting either alternative double bond arrangements or the E stereo-

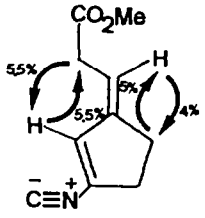


Figure 1. Results of the n.O.e. experiments on the diene-isocyanide (2; R=Me)

chemistry fail to satisfy the requirements of the n.O.e. experiments. These not only determine the stereochemistry involved but also confirm the structure allocated to the metabolite. The final verification of the structure was carried out by synthesis[16] and X-ray crystallography[17].

The incorporation of several potential ^{14}C and ^{3}H labelled precursors of (2; R=H) by T. hamatum is given in Table 1 cf.[15]. These data show that acetate is poorly incorporated but L-tyrosine is converted into the metabolite in yields of about 7%. Thus it is very likely that the biogenesis of (2; R=H) involves the biodegradation of tyrosine with the loss of one ring carbon and both hydrogens flanking the phenolic group. The lower incorporation of specific radioactivity of [U-^{14}C]tyrosine than [1-^{14}C]tyrosine supports this view.

Table 1. Incorporation of radioactive precursors into the diene-isocyanide (2; R=Me) by T. hamatum

Substrate	% Incorporation
$CH_3{}^{14}CO_2Na$	0.05
$^{14}CH_3CO_2Na$	0.035
L-[U-^{14}C]tyrosine	6.5
L-[7-^{14}C]tyrosine^{+}	7.6
L-[7-^{14}C][2a,2a'-$^{3}H_2$]tyrosine^{++}	7.4 (^{14}C), 0 (^{3}H)
L-[1-^{14}C]phenylalanine	0.14
L-3',4'-dihydroxy-[1-^{14}C]phenylalanine	0.55

$^{+}$Numbering of tyrosine is given in fig.2; conventional numbering is used for phenylalanine derivatives.
$^{++}$ ^{3}H : ^{14}C = 3.4

Further details of the biogenesis of the diene-isocyanide (2; R=H), in the first instance the origin of its carbon skeleton, were sought by studying the incorporation of specifically ^{13}C-labelled D,L-tyrosines synthesised[18] for this purpose. The ^{13}C n.m.r. spectrum of the metabolite (2; R=Me) has been fully assigned (cf. Figure 2) and with the high incorporations expected (>3.5%) on the basis of the ^{14}C experiments, routine spectra involving no refinements were recorded for the ^{13}C enriched metabolites. The modified signals in these spectra were easily identified and hence the sites of ^{13}C enrichment; the locations were confirmed by the appearance of satellites in the

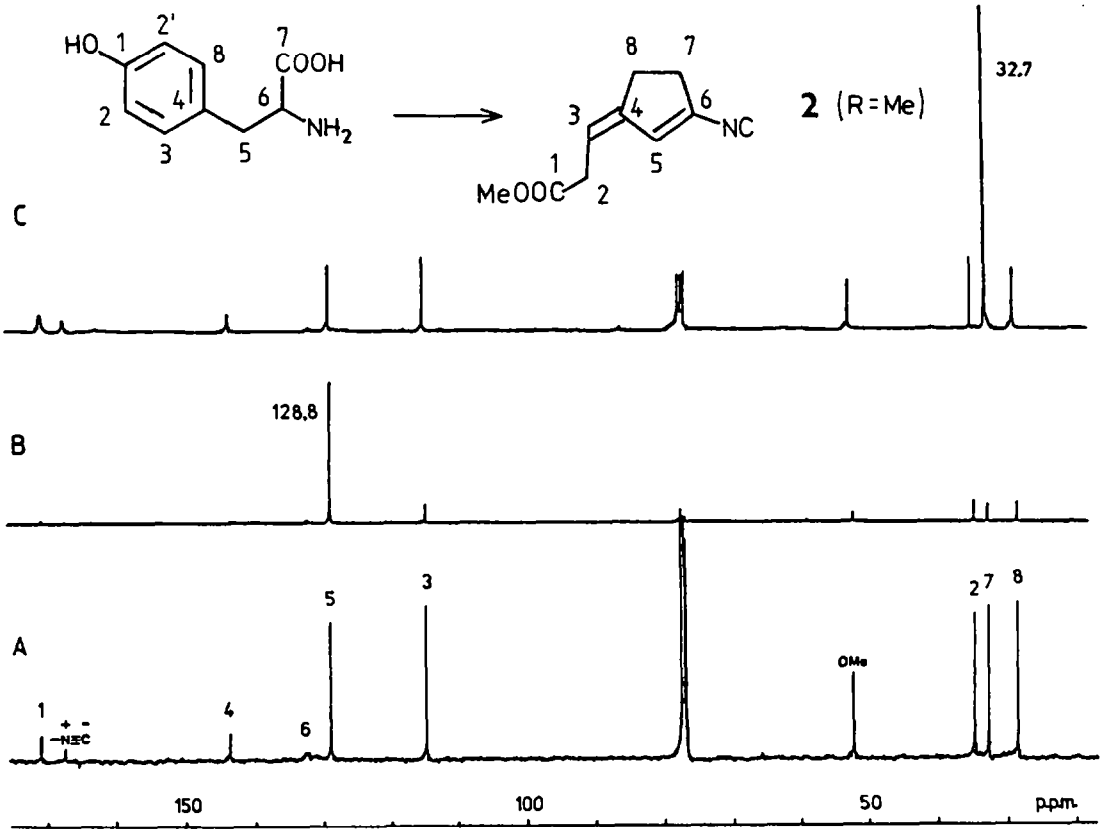
Table 2. T. hamatum culture incubations with [^{13}C]tyrosines. Affected signals in the n.m.r. spectra of the diene-isocyanide (2; R=Me) isolated; enhancements for singlets and coupling constants ($^{J}13_{C-13C}$/Hz) of doublets in the ^{1}H broadband decoupled ^{13}C spectra and satellite appearance ($^{J}1_{H-13C}$/Hz) in the ^{1}H spectra.

D,L-tyrosine substrate^{+}	(2; R=Me)				
	signal allocation	^{13}C n. m. r.		^{1}H n. m. r.	
		δ	$^{J}13_{C-13C}$/Hz	δ	$^{J}1_{H-13C}$/Hz
[7-^{13}C]	7	32.7	s	2.67	135
[5-^{13}C]	5	128.8	s	6.51	172
[3,4-$^{13}C_2$]	3	28.4	d, 39	5.44	160
	8	114.8	d, 77	2.67	132
	4	144	two d, 39 & 77	---	---
[1,2,2'-$^{13}C_3$]	2	34.6	d, 58	3.13	130
	1	171.7	d, 58	---	---

$^{+}$For numbering see figure 2.

corresponding ^{1}H n.m.r. spectra. The relevant signal data from the ^{13}C and ^{1}H n.m.r. spectra of the ^{13}C enriched metabolites are listed in table 2. The ^{13}C n.m.r. spectra recorded for the diene-isocyanide (2; R=Me) biosynthesised from the side-chain labelled tyrosines are given in Figure 2; they demonstrate an increase of intensity of the signals at δ128.8 and 32.7 respectively. The enrichment sites generated in the metabolite by the two multiply labelled tyrosines as precursors

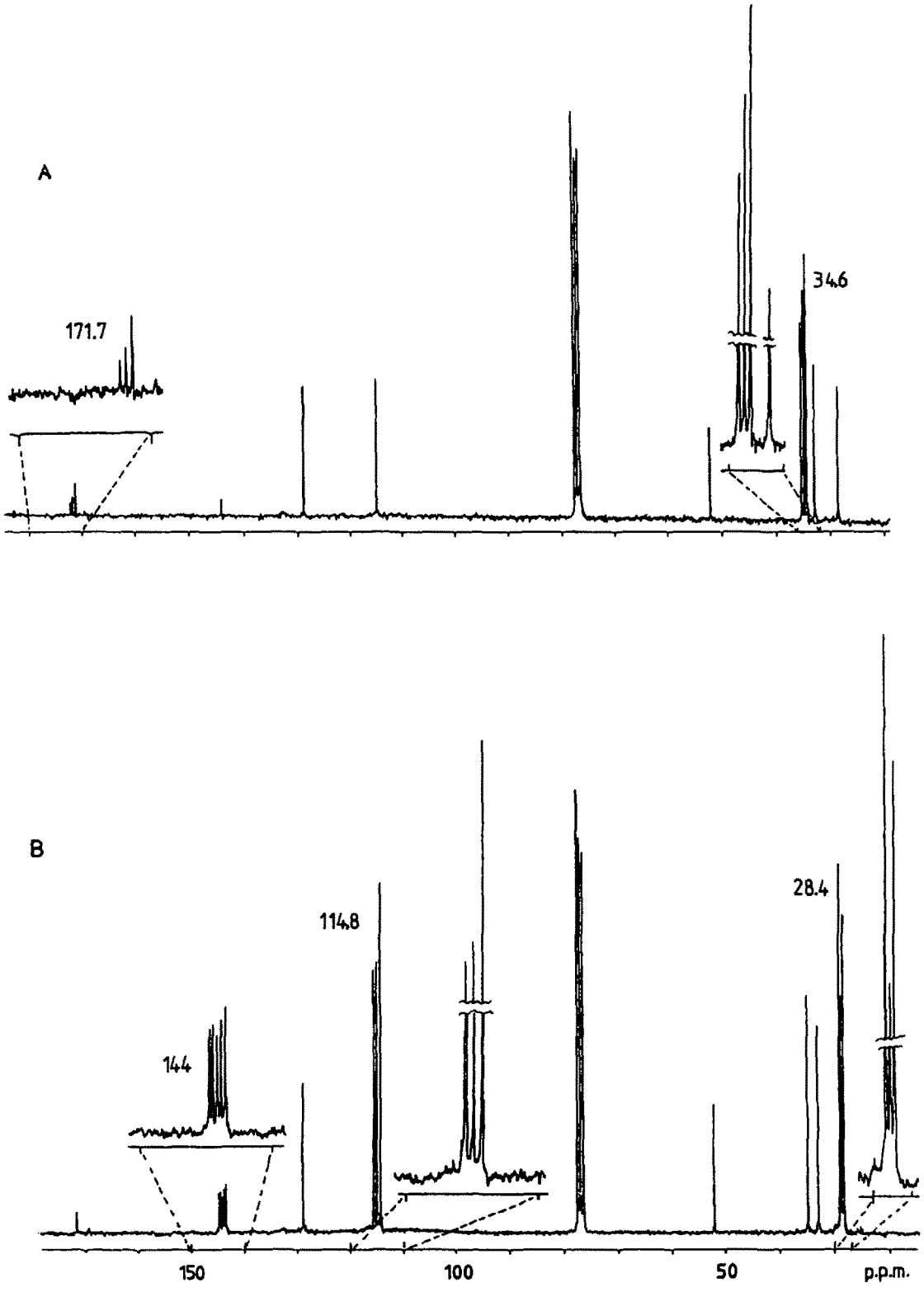
Figure 2. ^{1}H broadband decoupled ^{13}C n.m.r. spectra of the diene-isocyanide (2; R=Me) produced by T. hamatum: A, natural abundance; B, D,L-[5-^{13}C]tyrosine as precursor; C, D,L-[7-^{13}C]tyrosine as precursor.



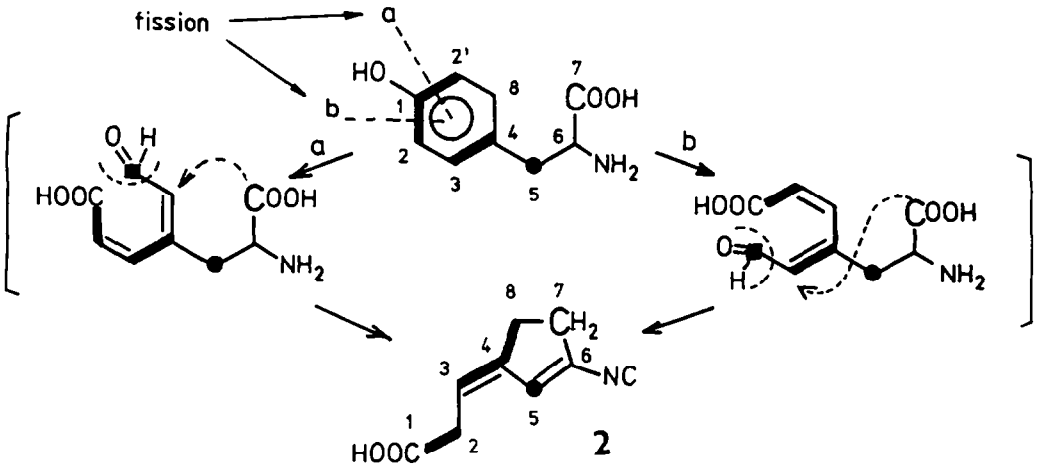
were distinguished in the ^{13}C n.m.r. spectra by ^{13}C-^{13}C nuclear coupling (Figure 3). The coupling of C(4) with the ring methylene C(8) (39 Hz) and, independently, with the olefinic C(3) (77 Hz) suggests that the relative intensities of C(8):C(4):C(3) are 1:2:1.

The incorporation experiments carried out correlate all but C(6) (for numbering see Fig.2) of the diene-isocyanide (2; R=H) to the corresponding tyrosine carbons. Considering the high incorporation of tyrosine and the established link of the neighbouring C(5) and C(7) with the side chain of tyrosine, the derivation of C(6) of the metabolite from the corresponding tyrosine C(6) can be assumed. The observed changes in the ^{13}C and ^{1}H n.m.r. spectra strongly indicate that no significant randomisation of the tyrosine carbon label occurred during biosynthesis and that an intact retention of two contiguous ^{13}C enriched carbons of tyrosine must have occurred with multiply ring-labelled tyrosines as precursors.

Figure 3. ^{1}H broadband decoupled ^{13}C n.m.r. spectra of the diene-isocyanide (2; R=Me) produced by *T. hamatum* with [ring-^{13}C]tyrosines as precursors: A, [1,2,2'-$^{13}C_3$]tyrosine; B, [3,4-$^{13}C_2$]tyrosine (for numbering see Fig. 2).



Scheme



The following conclusions, illustrated in the Scheme, may be drawn from the outcome of the incorporation experiments. The side-chain of tyrosine is incorporated intact by a cyclization process involving the carboxylic carbon atom and an aromatic carbon atom [C(3) or C(8)] that was originally meta to the phenolic group in tyrosine. Thus the cyclopentenyl ring of (2) is formed from the tyrosine side-chain and two carbon atoms of the aromatic ring. (Ring fission occurs at "a" and "b" respectively and, as a consequence, the tyrosine C(3)-C(4) appear in both, the ring and side-chain, of the metabolite.) Secondly, two of the three contiguous enriched carbon atoms of [1,2,2'-$^{13}C_3$] tyrosine are retained intact as C(1) and C(2) in the diene-isocyanide (2) since they are coupled in the metabolite ($^1J_{C(1)-C(2)}$58Hz), the other enriched carbon atom [C(2) or C(2')] being lost during biosynthesis. Hence all of the carbon atoms of tyrosine are retained in the metabolite (2), with the exception of an aromatic carbon atom adjacent to that bearing the hydroxyl Similar conclusions have been drawn by other workers[19] based on a different experimental design.

A small number of natural products is known which are derived from tyrosine by oxidative degradation of the aromatic ring and cyclization of its side-chain into heterocyclic systems[20]. Whilst the oxidative aromatic ring degradation almost certainly applies also to the genesis of the isocyanide (2) and is assumed in the Scheme, the required cyclization of the tyrosine side-chain into a carbocyclic ring appears to have no precedent and thus represents a novel feature of fungal chemistry.

Experimental

Ultraviolet spectra were determined on a Unicam SP800A spectrometer. All n.m.r. spectra were determined in $CDCl_3$ in 5 mm tubes for ^1H and 10 mm tubes for ^{13}C and chemical shifts are reported in p.p.m. downfield from the signal of Me_4Si. ^1H n.m.r. spectra were measured on a Bruker WH 300 spectrometer. ^{13}C n.m.r. spectra were measured on the latter instrument at 75.5 MHz and on a Bruker AM250 spectrometer at 62.8 MHz; the sweep width was 240 p.p.m. accumulated into 16 K data points. Radioactive samples were counted (to ±2%) on a LKB-Walac 1215 RackBeta liquid scintillation counter fitted with DPM software (1215-123). Quench calibration curves, plotted using the hat-trick method were stored in the computer and d.p.m. values were calculated therein. For measurement of specific activity, the sample was dissolved in Et_2O, the solution was made-up to volume (25-50 ml), the concentration was determined[21], and an aliquot (50-100 μl) was dispersed in the scintillation cocktail (LUMAGEL, 10 ml). Compounds were considered to have constant specific activity when the change in spec. act. was<2% after rechromatography. The sign "<" written before an incorporation indicates that specif. act. was not achieved. Solutions of radioactive substrates were made by dissolving the substances in 70% aqueous EtOH. [^{13}C]tyrosine (90% ^{13}C in labelled atoms[18]) solutions (*ca.* 0.5 mg ml^{-1}) were made by dissolving the compounds in water at 40° and allowing the solutions to cool at 25° before administration.

Aluminium oxide (60 PF_{254}, Merck) was used in 1 mm layers for preparative layer chromatography (p.l.c.); spots and bands were detected by the quenching of fluorescence under light of λ 254 nm. Petrol refers to the light petroleum fraction b.p.30-40°; it was purified by shaking with H_2SO_4 (conc.) and then with brine, drying ($MgSO_4$), and distilling. Et_2O was peroxide-free and was dried over sodium.

Growth of T. hamatum cultures, administration of substrates, and isolation of the isocyanide (2; R=Me)

Inocula and production cultures were grown as described[10]. After 15-48 h growth, production cultures were selected on the basis of maximum growth and aliquot samples of the radioactive substrate were added aseptically. Data for the incubation with radioactive precursors are given in Table 3. ^{13}C-precursors were added after 15 h growth and data for these experiments are given in Table 4. All cultures were harvested after 73 h cultivation. All cultures were filtered and extracted as described[10] except that acidifications were with acetic acid and the final extraction was with cold Et_2O. The Et_2O-extract was concentrated at -20° to 10-100 ml and the concentrate was

Table 3. Experimental conditions for the conversion of ^{14}C and ^{3}H labelled substrates into 3-(3'-isocyanocyclopent-2-enylidene)propionic acid (2) by Trichoderma hamatum

Substrate		Yield of (2; R=Me)	
	mci mmol^{-1}	mg	µci mmol^{-1}
L-[U-^{14}C]tyrosine	483 483	18.5 18.3	15.6 15.9
L-[7-^{14}C]tyrosine+	57 57	22.8 17.0	14.7 21.2
L-[7-^{14}C][2,2'-$^{3}H_2$]tyrosine	50 (^{3}H) 57 (^{14}C) 50 (^{3}H) 57 (^{14}C)	11.0 13.3 	0.0 30.7 0.0 23.8
L-[1-^{14}C]phenylalanine	57 57	19.0 24.0	0.32 0.36
L-3',4'-dihydroxy-[1-^{14}C]phenylalanine	7.9 7.9	15.3 18.0	1.6 1.6
Sodium [1-^{14}C]acetate	29	14.0	< 0.05
Sodium [2-^{14}C]acetate	58	11.0	< 0.04

Except for ^{14}C labelled acetates, substrates (25 µci) were added to five 500 ml culture vessels each containing 100 ml of culture 15 h old.

Sodium [1-^{14}C]acetate (25 µci) was added after 48 h growth and sodium [2-^{14}C]acetate (187.5 µci) after 24 h growth.

+The numbering of tyrosine is that used in Figure 2 in the discussion; conventional numbers are used for the phenylalanine derivatives.

Table 4. Experimental conditions for the incorporation of [^{13}C]tyrosines into 3-(3'-isocyanocyclopent-2-enylidene)propionic acid by T. hamatum (for numbering see Fig.2).

Tyrosine		Fermentation fluid volume in l	No. of flasks	(2; R=Me) mg isolated
Label position	mg incubated			
5	500	10	100	200
7	500	10	100	250
1,2,2'	150	4	40	80
3,4	200	4	40	80

All samples of D,L-tyrosine were added to cultures that were 15 h old.

treated with a slight excess of a solution of CH_2N_2 in Et_2O. The reaction mixture was concentrated to ca. 1 ml, applied to an alumina p.l.c. plate, and the chromatogram was developed with petrol-Et_2O (1:1) to give two main bands that reacted with $NiCl_2$ in the cold[21]. The less polar band gave after further purification on alumina layers using petrol-Et_2O (3:1) as the continuous developing solvent, the diene-isocyanide (2; R=Me).

We thank the S.R.C. for a studentship (to H.S.B.) and research grant support, the British Council and the University of Gezira, Sudan, for a studentship (to A.A.T.) and Mr. J. W. Keeping for the mycological work.

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† NRCC No. 24122

†† Part of the work discussed in this paper was published in the communication, reference 15.

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