The Biosynthesis of Xanthocillin-X Monomethyl Ether in *Dichotomomyces cejpii*. Experiments on the Origin of the Isocyanide Carbon Atoms

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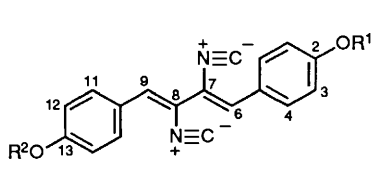
Precursor feeding experiments establish that the isocyanide groups in xanthocillin-X monomethyl ether (XME) **1** do not originate from (i) compounds associated with C_1-tetrahydrofolate metabolism; (ii) the ureido group of citrulline, carbamoyl phosphate, or potassium cyanate; or (iii) cyanide and arguably related compounds. However the results with D-[U-^{13}C]glucose show that the origin of the isocyanide groups is metabolically closer to glucose than is the origin of the backbone; DL-[1-^{13}C]-glyceraldehyde and D-[1-^{13}C]glucose are not specific precursors.

The isocyanide function is a curious structural feature found diversely, but in only a few secondary metabolites,[1] of which xanthocillin-X monomethyl ether, XME **1**, produced by the fungus *Dichotomomyces cejpii*, is an example.[2] Marine sponges elaborate some terpenoid metabolites bearing isocyanide groups[1] and their origin has convincingly been shown to be the cyanide ion.[3,4] It is most probable that biosynthesis, in these examples, involves formal quenching of a carbonium ion by reverse addition of a cyanide ion. Hapalindole **4** is a metabolite of the cyanobacterium *Hapalosiphon fontinalis* and the origin of its isocyanide group is, again, the cyanide ion; glycine and other compounds related through tetrahydrofolate metabolism were also specific precursors.[5] It has been reported[6] that the isocyanide groups in the bacterial hazimycins **5** are labelled specifically by L-[*methyl*-^{13}C]methionine. But the observation that *N*-methyltyrosine, which would seem to be an obligatory intermediate, is not incorporated[6] casts some doubt on the validity of the result with methionine. Apart from this last result the biosynthetic origins of bacterial/fungal isocyanides remains obscure.[1,7-10] Some of our results reported here have been published in preliminary form.[9,10]

Most of the skeleton of the xanthocillins, *e.g.* XME **1**, is accounted for by two molecules of tyrosine[10-13] and it is fairly certain that the isocyanide nitrogen atoms originate primarily in the α-amino group of tyrosine.[10] Inspection of the structure (as **1**) for the xanthocillins immediately suggests that it is constructed through dimerization of either **6** or **7**. The latter would afford **8**, a known metabolite of *Penicillium notatum*; this organism also produces xanthocillin-X **2**.[14] Simple dehydration of **8** would yield **2**. Tuberin **9**, a metabolite of *Streptomyces amakusaensis*, is structurally very similar to **7** and it has been shown to originate in tyrosine with the *O*-methyl and *N*-formyl groups arising *via* C_1-tetrahydrofolate metabolism (incorporation of, *e.g.* glycine and serine).[15] Accordingly we examined the incorporation into XME **1** of various precursors for C_1-tetrahydrofolate metabolism. These, and the other experiments described here, were carried out with the fungus *D. cejpii* which we were able to culture successfully and which produced substantial amounts of **1**. Also the presence of the *O*-methyl group in the metabolite **1** provided an invaluable internal check on the incorporation of various C_1 precursors.

[2-^{13}C, ^{14}C]Glycine yielded radioactive **1** but ^{13}C NMR spectroscopy showed labelling only of the *O*-methyl group (specific incorporation: 4.9% ^{14}C, 5.1% ^{13}C). The hydroxymethyl group of serine is a better source of C_1 units than is the C-2 of glycine and L-[3-^{14}C]serine was well incorporated into **1** (1.6% total incorporation). Degradation[16] to **10** was, however, without loss of radioactivity. Thus the C-3 of serine is not a source of the isocyanide carbon atoms. A similar, negative result was obtained with [^{14}C]formic acid (5% total incorporation). This is in agreement with the results of others who also obtained negative results for **2** with DL-[1-^{14}C]tyrosine, [1-^{14}C]-



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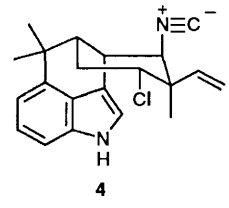
2 R^1 = R^2 = H
3 R^1 = R^2 = Me



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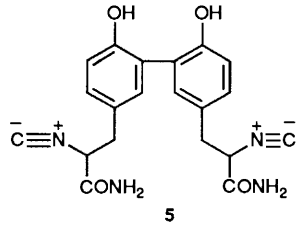

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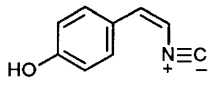

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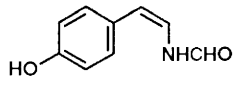

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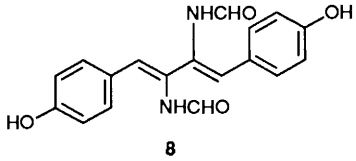

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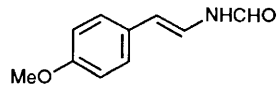

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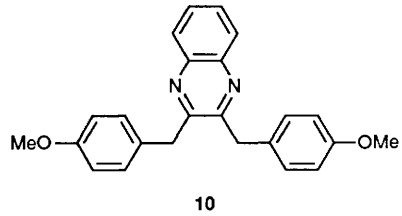

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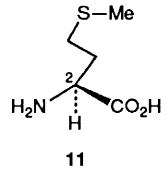

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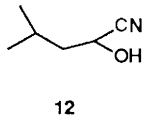

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Table 1 ^{13}C NMR data for xanthocillin-X dimethyl ether **16/17** obtained from XME **1** in a feeding experiment with D-[U-^{13}C]glucose to glucose-depleted cultures of *D. cejpii*[a]

Position	Signal pattern	J/Hz	Total enrichment[b] (%)	Average per carbon (%)
1	s	—	0.8	0.8[d]
2	d + t	65, 65	1.0	0.5
3 + 3′	s + d + t	59, 65	3.2	0.8
4 + 4′	d + d	58, 59	3.3	0.8
5	d	58	1.3	0.65
6	d	86	1.6	0.8
7[c]	d	86	1.8	0.9
8	s	—	3.1	1.55

[a] ^{14}C Incorporation: 1.5% (total), 14.3% (specific); ^{13}C specific enrichment: 15.8%. [b] Total enrichment above natural abundance. [c] The value for this carbon is not completely reliable because of broadening arising from ^{13}C–^{14}N coupling. [d] Only one methyl group in XME *vs.*, *e.g.*, four for C-3/3′ and C-4/4′.

and [2-^{14}C]-acetate, and L-[*methyl*-^{14}C]methionine.[12] As expected, L-[*methyl*-^{13}C]methionine labelled only the *O*-methyl group of **1** (^{13}C NMR, 6.5% specific incorporation). The high level of incorporation of precursors into **1**, particularly with proven labelling of the *O*-methyl group in the case of glycine, establishes that C_1-tetrahydrofolate intermediates were well labelled. That no labelling of the isocyanide carbon atoms occurs excludes C_1-tetrahydrofolate metabolism as a source for the isocyanide groups in **1** (*cf.* the result[6] for the hazimycins above).

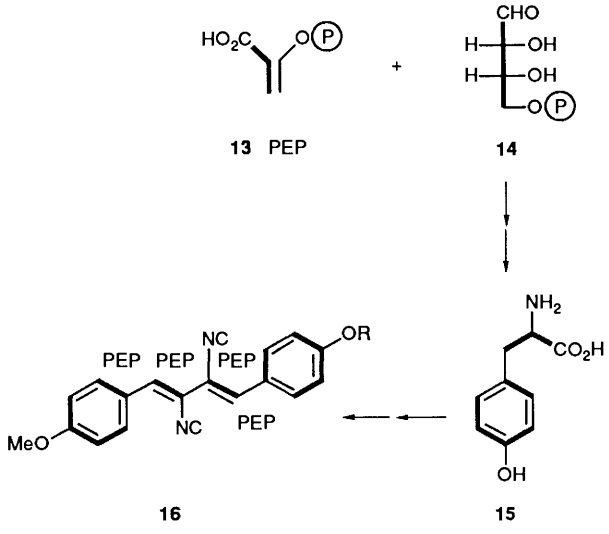
It seemed just possible that cyanide ion could be a precursor for the isocyanide groups of the xanthocillin **1**, as it is for terpenoid isocyanides (see above). It was, *a priori*, possible that any cyanide precursor might be derived either from an amino acid along the route followed in plants affording cyanogenic glycosides[17] or along the path converting methionine **11** into ethylene and hydrogen cyanide, the latter being derived from the nitrogen atom and C-2 of the amino acid.[18] The cyanohydrin **12** (synthesized from isovaleraldehyde[19]) was chosen to be representative of the former pathway; it could alternatively be viewed as a protected form of cyanide ion for safe delivery to the putative site of biosynthesis in the cell.

Sodium [^{14}C]cyanide was utilized well for the biosynthesis of XME **1** (total incorporation, 2.6%) and [1-^{14}C]-2-hydroxy-4-methylvaleronitrile (as **12**) was incorporated at a lower level (0.35%). These observations justified experiments with the two precursors labelled with ^{14}C, ^{13}C and ^{15}N and a similar set of results was obtained. In each case, however, ^{13}C NMR analysis revealed that only the *O*-methyl group of XME **1** was labelled. Mass spectral analysis of the XME which was derived from the experiment with sodium cyanide showed that there was some separate incorporation of ^{15}N (enhancement of the M + 1 peak only, 1% ^{15}N enrichment; by NMR there was 5% ^{13}C enrichment). Presumably the incorporations observed were the result of hydrolysis to formate (which was incorporated into the *O*-methyl group of XME *via* C_1-tetrahydrofolate metabolism) and ammonia. No incorporation of L-[2-^{14}C]methionine (as **11**) into XME **1** was observed. On a similar tack to the experiments with **12**, [2-^{13}C]phenylalanine was examined: it was only incorporated *via* hydroxylation to tyrosine and thence into the backbone of XME. All these results taken with those which show that the nitrogen atoms of XME derive from tyrosine[10] indicate strongly that the isocyanide functions in XME do not arise through cyanide.

We considered that the isocyanide carbon atoms in XME **1** might originate from metabolites at the carbon dioxide level of oxidation, *i.e.* at a higher oxidation level than those of tetrahydrofolate metabolism. Accordingly [^{14}C]carbamoyl phosphate, [*ureido*-^{14}C]citrulline, and also potassium [^{14}C]-cyanate were examined as precursors but only insignificant incorporations were observed (total incorporation, 0.015, <0.001, and 0.006%, respectively, *cf.* [3-^{14}C]serine in a parallel experiment: 4.9%). Label from sodium [^{14}C]carbonate was also not incorporated into **1**. This last result is somewhat ambiguous since, at the time of the experiment, the organism was evolving carbon dioxide.

Some evidence has been obtained[14] that the formyl derivative **8** is a precursor for xanthocillin-X **2**. We tested ^{14}C-labelled **8** as a precursor for XME **1**; a negligible amount of radioactivity was incorporated, indicating that **8** is not an intermediate in the biosynthesis of **1**. This is consistent with the C_1-tetrahydrofolate results above.

A new approach to the problem was clearly needed at this stage and we chose to examine D-[U-^{13}C]glucose as a precursor. This material has found powerful application in a number of problems.[20,21] In the case of XME **1** normal, unexceptional labelling of the tyrosine part **15** of the skeleton **16** by phosphoenol pyruvate (PEP) **13** (4 molecules) and erythrose 4-phosphate **14** (2 molecules) *via* the shikimate pathway was expected (Scheme 1). If labelling of the isocyanide functions did occur then the level of enrichment of these carbons relative to that of other carbons would provide information about how close metabolically to glucose was the source of the isocyanide carbons. In this regard the four PEP units would provide a reference (Scheme 1). For example passage through the tricarboxylic acid cycle prior to incorporation into the isocyanide carbons would result in a lower enrichment into these carbons than into the PEP units.



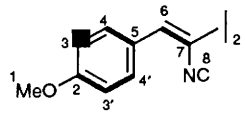
Scheme 1

D-[U-^{13}C, U-^{14}C]Glucose was incorporated into XME in normal cultures of *D. cejpii*. The incorporation (^{14}C, total incorporation = 0.58%, specific incorporation = 1.35%) was not high enough to give interpretable ^{13}C NMR spectra. Success came, however, with a change in experimental procedure.

Cells of *D. cejpii* were cultured normally and then isolated at the stage when XME production had just begun. The cells were resuspended in culture medium which contained no added glucose (the medium still contained other carbon sources). Incubation was continued and the labelled glucose was added over 60 h when XME production had resumed. The XME isolated was converted into the dimethyl ether **3** (to halve the number of NMR signals) which was then examined by ^{13}C NMR spectroscopy. To our delight the isocyanide carbons

were labelled and the level of enrichment was higher than for any other carbon atoms (Table 1). Thus, the isocyanide carbons are closer to glucose (smaller number of reactions, less dilution by endogenous intermediates) than are any of the PEP units (the side-chain PEP units are, as expected, on the same argument more enriched than those in the ring). This excludes biosynthesis for the isocyanide carbons as being through, *e.g.*, the tricarboxylic acid cycle (confirmed by an experiment with [$^{13}C_2$]acetate). Enrichment was also higher than for the *O*-methyl group, thus once again excluding events and intermediates associated with C_1-tetrahydrofolate metabolism.

The ^{13}C-labelling pattern seen in the aromatic rings of **17** = **3** was not entirely C_4 (erythrose 4-phosphate) plus C_2 (PEP); the C_4 pattern was overlaid by a $C_3 + C_1$ pattern, *i.e.* **16** + **17**, which is consistent with cycling *via* the pentose phosphate pathway (*cf.* obafluorin biosynthesis in *Pseudomonas fluorescens*,[21] though here a similar set of observations is most probably accounted for by operation of the Entner–Doudoroff pathway).



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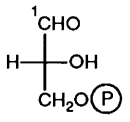

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In considering possible precursors for the isocyanide groups in **1** which could be related to glucose, we thought that C-1 of glyceraldehyde 3-phosphate **18** could provide the isocyanide functions in a mechanistically reasonable way. Labelled glyceraldehyde (as **18**) could be incorporated into **1**. Surprisingly, the incorporation was better with normal cultures than with conditions of glucose-depletion. DL-[1-^{13}C, 1-^{14}C]Glyceraldehyde (as **18**) afforded labelled XME under both experimental conditions. In neither was there significant enhancement of the isocyanide signal relative to the others in the ^{13}C NMR of **3**. This failure of C-1 of glyceraldehyde to label the isocyanide carbons is consistent with our observation that, although [U-^{14}C]glycerol was incorporated into XME (0.85%), no radioactivity was present in the isocyanide groups.

A further attempt to find the vexed origin of the isocyanide groups involved D-[1-^{13}C]glucose as a precursor. It was fed, mixed with D-[U-^{14}C]glucose, under both of the above sets of conditions. No significant incorporation into the isocyanide groups of XME was observed. Further, well directed experiments are needed to solve this problem. So far the apparently secure pieces of evidence relate to the incorporation of D-[U-^{13}C]glucose and the incorporation of nitrogen from tyrosine.[10]

Experimental

M.p.s were determined on a Reichert hot stage and are uncorrected. ^{1}H NMR spectra were recorded at 90 MHz on a JEOL FX90Q or at 400 MHz on a Bruker AM400 spectrometer. ^{13}C NMR spectra were recorded at 100 MHz on the Bruker AM400 spectrometer. *J* Values are given in Hz. IR spectra were recorded on a Perkin-Elmer 1420 ratio-recording spectrophotomer, and UV spectra on a Pye-Unicam PU8800 or SP800 spectrophotometer. Mass spectra were obtained with Kratos MS25 or MS9/50 instruments. Column chromatography was with Kieselgel G type 60 (Merck 7731).

Labelled compounds were purchased from Amersham International plc, Aldrich Chemical Co. and MSD Isotopes, Canada. Radioactivity was assayed by scintillation counting with a Packard 300CD instrument. Total incorporation = total radioactivity in metabolite/total activity in precursor × 100%. Specific incorporation was calculated similarly from molar activities.

Growth of Dichotomomyces cejpii *and Isolation of Xanthocillin-X Monomethyl Ether* (XME).—A culture of *D. cejpii* (SANK 23575) was kindly provided by Dr. M. Arai, Sankyo Co. Ltd., Tokyo. It was cultured essentially as described.[2] The culture was maintained on slants of oat meal (25 mesh or smaller) (10 g), $MgSO_4 \cdot 7H_2O$ (1.0 g), KH_2PO_4 (1.0 g), $NaNO_3$ (1.0 g), agar (20 g), tap water (1 dm^3) (pH not adjusted). Innoculation from the slant was into a 250 cm^3 conical flask containing 50 cm^3 of growth medium: glucose (50 g), Bactopeptone (20 g), corn-steep liquor (3 g), distilled water (1 dm^3) (pH not adjusted). Incubation was at 27 °C and 180 r.p.m. in a Gallenkamp orbital incubator for 72 h. This culture was used at 2.5% to innoculate 200 cm^3 of growth medium per 500 cm^3 conical flask. Incubation was then as before. Cultures were sampled at regular intervals. The samples (each 5 cm^3) were extracted with ethyl acetate, dried and evaporated. The residue was dissolved in ethanol and its UV absorbance at λ = 365 nm was determined.

Typically, XME production began after *ca.* 48 h and reached a maximum after *ca.* 72 h. XME was isolated after *ca.* 96 h. The mycelium was collected by filtration on a bed of Celite and thoroughly dried *in vacuo* over phosphorus pentoxide. The filtrate contained no XME and was discarded. The dried mycelium was ground to a powder and extracted (Me_2CO) in a soxhlet apparatus (4 h). The extract was evaporated to dryness and the residue was chromatographed (2% acetone in benzene) to give XME which was recrystallized from benzene to give yellow plates, m.p. 183–186 °C (decomp.). Typically, 100 mg were obtained from 600 cm^3 of culture medium. λ_{max}(EtOH)/nm 235, 295 and 365; ν_{max}($CHCl_3$)/cm^{-1} 3580 (OH), 2120 (N≡C) and 1605; δ_H(400 MHz, [$^2H_5$]pyridine) 7.81 (2 H, d, *J* 9), 7.77 (2 H, d, *J* 9), 7.11 (2 H, d, *J* 9), 7.07 (1 H, s), 7.02 (1 H, s), 6.91 (2 H, d, *J* 9) and 3.6 (3 H, s); δ_C(100 MHz, [$^2H_5$]pyridine) 55.3 (C-1, CH_3), 114.7 (C-3), 115.3 (C-7), 116.5 (C-8), 116.8 (C-12), 123.5 (C-5), 125.1 (C-10), 127.1 (C-6), 128.3 (C-9), 131.8 (C-4), 132.5 (C-11), 161.0 (C-13), 161.1 (C-2) and 174.4 and 174.5 (isocyanide carbons); *m/z* 302.10514 (M$^+$, calc. for $C_{19}H_{14}N_2O_2$, 302.10552), 209, 170, 151, 108 and 94.

Treatment of an ice-cold solution of XME in dry tetrahydrofuran with ethereal diazomethane for 4 h gave *xanthocillin-X dimethyl ether*. It was purified by chromatography with benzene as eluent and recrystallized from benzene to give green needles, m.p. 186 °C (decomp.) (87%). ν_{max}($CHCl_3$)/cm^{-1} 2120 and 1600; δ_H(90 MHz, $CDCl_3$) 7.78 (4 H, d, *J* 9), 7.0 (2 H, s), 6.97 (4 H, d, *J* 9) and 3.88 (6 H, s); δ_C(100 MHz, $CDCl_3$), 55.23 (C-1), 114.14 (C-3), 115.77 (C-7), 124.43 (C-5), 127.09 (C-6), 131.38 (C-4), 160.73 (C-2) and 172.85 (C-8); *m/z* 316.12176 (M$^+$, calc. for $C_{20}H_{16}N_2O_2$, 316.12117), 229, 209, 170 and 108 (Found: C, 75.5; H, 4.95; N, 8.6. $C_{20}H_{16}N_2O_2$ requires C, 75.92; H, 5.10; N, 8.86%).

2,3-*Bis*(p-*methoxybenzyl*)*quinoxaline* **10**.[16]—Xanthocillin-X dimethyl ether (60 mg) in a mixture of glacial acetic acid (2.25 cm^3) and aqueous sulphuric acid (1 mol dm^{-3}, 0.15 cm^3) was heated at 100 °C for 40 min. Water (5 cm^3) was added. The mixture was stirred and cooled in ice. The precipitate was collected and stirred for 2 h with *o*-phenylenediamine (20.5 mg, 1 equiv.) in ethanol (2 cm^3). Evaporation gave a brown oil which was chromatographed with 10% methanol in chloroform or 2% ethyl acetate in dichloromethane to give 2,3-*bis*(p-*methoxybenzyl*)*quinoxaline* (10 mg, 14%), m.p. 80–81 °C (lit.,[16] 80 °C). λ_{max}(MeOH)/nm 316; ν_{max}(CH_2Cl_2)/cm^{-1} 1600 and 1510; *m/z* 370 (M$^+$) 261, 121 (Found: C, 77.6; H, 6.1; N, 7.7. $C_{24}H_{22}N_2O_2$ requires C, 77.8; H, 5.94; N, 7.57%).

1-(p-*Hydroxyphenyl*)-4-(p-*methoxyphenyl*)-2,3-*diformamidobuta*-1,3-*diene*.—To XME (0.25 g) was added glacial acetic acid (2.5 cm^3) and the mixture was refluxed for 5 min. The white precipitate was collected from the cooled mixture and washed

with water. The solid was recrystallized from methanol–ethanol (10:1) to give 1-(p-*hydroxyphenyl*)-4-(p-*methoxyphenyl*)-2,3-*diformamidobuta*-1,3-*diene* (0.25 g, 90%), m.p. 216–219 °C. λ_{max}(EtOH)/nm 334; ν_{max}(Nujol)/cm^{-1} 3200, 1635 and 1600; *m/z* 338.12675 (M^+, $C_{19}H_{18}N_2O_4$ requires 338.12665), 121 and 94.

1,4-*Bis*(p-*hydroxyphenyl*)-2,3-*diformamidobuta*-1,3-*diene* **8**.—To a stirred suspension of the monomethyl ether (20 mg, 0.06 mmol) in dry dichloromethane (1 cm^3), under nitrogen at −25 °C, was added a solution of boron tribromide (0.012 cm^3), 0.12 mmol) in dichloromethane. The mixture was stirred at this temperature for 15 min and then for 2 h at room temperature. Ice was added and after 30 min the mixture was evaporated to dryness. The pale yellow solid was dissolved in aqueous sodium hydroxide (0.2 mol dm^{-3}) and then precipitated by the dropwise addition of glacial acetic acid. The white solid was collected, washed with water and dried *in vacuo* to give 1,4-*bis*(p-*hydroxyphenyl*)-2,3-*diformamidobuta*-1,3-*diene* **8** (17 mg, 87%), m.p. >290 °C. λ_{max}(MeOH)/nm 336; ν_{max}(Nujol)/cm^{-1} 3200, 1625 and 1600; *m/z* 324.11071 (M^+, $C_{18}H_{16}N_2O_4$ requires 324.1110).

The ^{14}C-labelled material was obtained *via* a feeding experiment with L-[U-^{14}C]tyrosine (30 μCi; 28.6% incorporation). The XME (26 mg) was converted into ^{14}*C-labelled* **8** in 75.3% yield; 7.86 μCi mmol^{-1}. This material when fed in aqueous solution to *D. cejpii* gave negligible incorporation into XME.

2-*Hydroxy*-4-*methylvaleronitrile* **12** (cf. *ref.* 19).—Isovaleraldehyde (5.0 g, 58.1 mmol) was added to a vigorously stirred, freshly prepared, and ice-cold aqueous sodium metabisulphite (16.6 g in 20 cm^3, 87.2 mmol). After the mixture had been stirred for 30 min with ice-cooling the white precipitate was collected, washed with ethanol and dried *in vacuo* to give an isovaleraldehyde bisulphite addition complex (10 g, 91%); ν_{max}(Nujol)/cm^{-1} 3600–3200, 1220, 1160, 1040 and 635. This was used to prepare both unlabelled material for characterization and labelled material. Experimental detail is given for the latter.

To a solution of the bisulphite addition complex (49.3 mg, 0.26 mmol) in water (5 cm^3) was added sodium [^{14}C]cyanide (25 μCi). After swirling for 2 min a solution of unlabelled sodium cyanide (11.8 mg, 0.24 mmol) in water (10 cm^3) was added. After swirling again for a few minutes this solution was used in feeding experiments. To prepare [1-^{13}C,^{14}C,^{15}N]-2-hydroxy-4-methylvaleronitrile, potassium [^{13}C,^{15}N]cyanide (19.2 mg) was added (instead of unlabelled sodium cyanide) to the reaction mixture (as above) containing radioactive cyanide.

Unlabelled material was isolated from aqueous solution by ether extraction. After drying, the organic solution was evaporated and the residual pale yellow oil was distilled (Büchi Kugelrohr, oven temp. 100–105 °C at 15 mmHg). The product **12** was a colourless oil (67%). ν_{max}/cm^{-1} 3700–3100 and 2225; δ_H(90 MHz, $CDCl_3$) 4.5 (1 H, q, *J* 6), 2.6 (1 H, d, *J* 6, OH), 1.6–2.1 (3 H) and 0.98 (6 H, d, *J* 6); *m/z* 113.08388 (M^+, $C_6H_{11}NO$ requires 113.08413).

DL-[1-^{14}C]- *and* DL-[1-^{13}C]-*Glyceraldehyde*.—These compounds were prepared following a published procedure.[22] DL-[1-^{14}C]Glyceraldehyde (1.45 μCi mmol^{-1}) was obtained from commercial glycolaldehyde dimer and sodium [^{14}C]cyanide (300 μCi) in 25% yield. DL-[1-^{13}C]Glyceraldehyde (0.28 g, 41%) was obtained similarly from potassium [^{13}C]cyanide (99 atom % ^{13}C). TLC (10% ethanol in ethyl acetate; stain: *p*-anisaldehyde), ^{1}H and ^{13}C NMR spectroscopy established purity and identity with authentic glyceraldehyde; TLC was particularly valuable for analysis. Synthetic (and authentic) glyceraldehyde was in the form of the hydrated gem-diol: δ_C(100 MHz, D_2O) 63.3 (C-3), 75.4 (C-2) and 91.1 (C-1, exclusively enriched in the ^{13}C-labelled material).

Feeding Experiments.—These experiments which were carried out with cultures of *D. cejpii* were as follows, with the exception of experiments with glucose-depleted medium: The precursors were dissolved in distilled water (15 cm^3) and the pH was adjusted to 7.0 (where necessary). The solutions were administered, typically, to 3 culture flasks each containing 200 cm^3 of medium in 4 batches over 48 h commencing when XME production began. The cultures were worked up and the XME was isolated as described above.

Analysis of the incorporation of ^{13}C-labelled compounds was by ^{13}C NMR spectroscopy. In all cases $Cr(acac)_3$ (*ca.* 10 mg per NMR sample) was also present as a relaxation agent.

[2-^{14}C,^{13}C]Glycine (50 mg, 6.6 μCi) gave XME (specific incorporation: 4.9% ^{14}C, 5.1% ^{13}C); enhancement only of the signal at δ 55.3. L-[3-^{14}C]Serine (4.9 μCi): 1.6% total incorporation; essentially no loss of activity on degradation to **10**. [^{14}C]Formic acid (22.65 μCi, 56 mCi mmol^{-1}): 5.0% total incoporation; essentially no loss of activity on degradation to **10**. L-[*Methyl*-^{13}C]methionine (60 mg): 6.5% specific incorporation; enhancement only of the signal at δ 55.3. D-[U-^{14}C]-Glucose (10 μCi, 3 mCi mmol^{-1}) and [U-^{14}C]glycerol (10 μCi, 170 mCi mmol^{-1}): 0.7% and 0.85% total incorporation, respectively; essentially no loss of activity on degradation to **10**. L-[2-^{14}C]Methionine (5 μCi): <0.001% total incorporation. In a parallel set of experiments, L-[3-^{14}C]serine (10 μCi, 53 mCi mmol^{-1}), [^{14}C]carbamoyl phosphate (25 μCi, 55 μCi mmol^{-1}), L-[*ureido*-^{14}C]citrulline (10 μCi, 55 mCi mmol^{-1}) and potassium [^{14}C]cyanate (10 μCi, 55 mCi mmol^{-1}) gave total incorporations, respectively, of 4.9, 0.015, <0.001 and 0.006%. The carbamoyl phosphate was prepared from potassium [^{14}C]cyanate.[23] The details for sodium cyanide and 2-hydroxyvaleronitrile are given above.

D-[U-$^{13}C_6$,^{14}C]Glucose (48 mg, 66.1 atom %^{13}C, 10 μCi), ^{14}C incorporation in normal cultures: 0.58% (total), 1.35% (specific). D-[U-$^{13}C_6$,^{14}C]Glucose (100 mg, 98.2 atom %^{13}C, 0.5 μCi) fed to glucose-depleted cultures; ^{14}C incorporation: 1.5% (total), 14.3% (specific); ^{13}C NMR spectroscopic results are given in Table 1. The enrichment was calculated by weighing the peaks in a spectrum of the labelled material and a spectrum, obtained under closely similar conditions, of unlabelled compound. Correlation was following the reasonable assumption that neither C-5 nor C-6 (a secure C_2 PEP unit) in **3** would be singly labelled. Subtraction then gave the enhancement of the natural abundance singlets (C-1, C-3/3′, C-8). The culture conditions were as follows: cultures were grown normally until XME production just commenced. The mycelium from 400 cm^3 (2 flasks) of culture were carefully filtered off and transferred to two flasks each containing 200 cm^3 of growth medium which contained no added glucose. Incubation was continued and the recommencement of the XME production was monitored in one of the flasks for *ca.* 24 h. The glucose was fed to the other flask in 4 batches over 48 h. Similarly, D-[1-^{13}C,U-^{14}C]glucose [50 mg, 99 atom %^{13}C, 13 μCi; ^{14}C incorporation in normal cultures: 0.2% (total), 0.2% (specific) and separately (50 mg, 12 μCi) in glucose-depleted cultures: ^{14}C incorporation: 0.24% (total), 0.41% (specific)]. In neither case was the xanthocillin-X dimethyl ether specifically labelled on the isocyanide carbons (^{13}C NMR).

DL-[1-^{13}C,1-^{14}C]Glyceraldehyde (100 mg, 0.5 μCi) was fed to normal [^{14}C incorporation: 0.2% (total), 0.63% (specific)] and glucose-depleted cultures [^{14}C incorporation: 0.02% (total), 0.12% (specific)]; in each case 200 cm^3 of culture were used. No specific ^{13}C incorporation (NMR) was observed in either case.

Sodium [1,2-$^{13}C_2$,^{14}C]acetate (100 mg, 99 atom %^{13}C, 4.1 μCi) was incorporated in normal cultures; ^{14}C: 0.43% (total), 3.83% (specific). Average enrichment per carbon by ^{13}C NMR on **3**: C-1, 0.31; C-2, 0.03; C-3 + C-3′: 0.09; C-4 + C-4′: 0.10; C-5: 0.34; C-6, 0.25; C-7: 0.49; C-8: 0.10%; all except C-1 and

C-8 showed low intensity satellites due to ^{13}C–^{13}C coupling (numbering is as in **17**).

Acknowledgements

We thank Dr. M. Arai, Sankyo Co. Ltd., Tokyo for a culture of *D. cejpii*, Dr. A. G. Swanson and Dr. B. M. Watson for NMR spectra, Miss A. M. Mattocks for technical assistance, and SERC and the University of Leeds for research studentships (to J. M., K. M. C. and A. R. K.).

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Paper 0/04673F
Received 17*th October* 1990
Accepted 14*th November* 1990