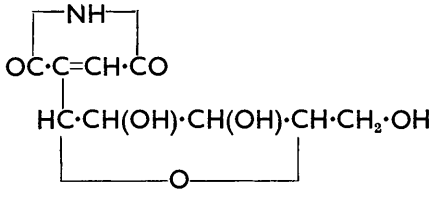
Natural Acetylenes. Part XXXVIII.[1] Biosyntheses of Acetylenedicarboxamide (Cellocidin) in *Streptomyces SF-536* Cultures

By **Sir Ewart R. H. Jones,* J. W. Keeping, M. G. Pellatt,** and **V. Thaller,** The Dyson Perrins Laboratory, Oxford University, Oxford OX1 3QY

Biosynthetic experiments with a number of differently ^{14}C-labelled hypothetical precursors have shown that cellocidin (acetylenedicarboxamide) is an offshoot of the Krebs cycle and arises in *Streptomyces SF*-536 cultures directly from carbon atoms 2—5 of 2-oxoglutarate.

THE reactions leading to carbon–carbon triple bond formation in nature are not known; biosynthetic experiments on the production of simple acetylenic metabolites could help to elucidate them. Several micro-organisms have been reported to produce monoacetylenes and we have made attempts to find favourable culture growth conditions and to isolate the metabolites. In this we succeeded only with cellocidin; our efforts to produce propiolic acid [2] and undecynoic acid [3] were fruitless. Cellocidin (I) (also known as lenamycin [4] and aquamycin [5]) was first isolated from culture fluids of *Streptomyces chibaensis* [6] and was identified as acetylenedicarboxamide.[7]

$$H_2N{\cdot}OC{\cdot}C{\equiv}C{\cdot}CO{\cdot}NH_2 \qquad (I)$$



(II)

Streptomyces SF-536 † has been used in the biosynthetic experiments described in this paper. It produces both cellocidin and chloramphenicol and proved to be highly susceptible to growth conditions; consistent cellocidin production was eventually achieved in a medium consisting of corn steep liquor, yeast extract, and glucose. Its presence was detected in cultures 24 h after inoculation (a chloramphenicol-resistant *E. coli* strain † was used in the bioassay); maximum levels were reached after about 55 h and these fell to zero after *ca.* 120 h.

Attempts to isolate cellocidin by continuous extraction with chloroform or ethyl acetate [it is slightly soluble in these (hot) solvents] failed to yield a crystalline product. The original activated charcoal extraction [6] on the other hand suffers from the disadvantage that not all the cellocidin is recovered from the culture fluid. This was overcome in our biosynthetic work by using charcoal extraction in conjunction with dilution analysis. The latter allowed an estimation of the weight of labelled cellocidin which remained in the culture fluid and thus gave the total weight of cellocidin produced.

The percentage of radioactivity residing on the amide carbon atoms was determined as follows: cellocidin was hydrogenated and the resulting succinamide hydrolysed to succinic acid, which was subjected to a Schmidt [8] degradation. The combined efficiencies of degradation and carbon dioxide recovery and counting were determined on [1,4-^{14}C]succinic acid (nominal label specificity 98%) and amounted to 87·5%; the label distributions for cellocidin quoted in the Table have been corrected accordingly (all degradations were repeated up to three times).

Compound added [a]			Cellocidin isolated			
				% Incorporation		
	Sp. act. (mCi mmol^{-1})	μCi per flask	Sp. act. (μCi mmol^{-1})	Isolated	Estimated [b]	% ^{14}C on $CO{\cdot}NH_2$
Sodium [1-^{14}C]acetate	29	3·0	0·052	0·1		100
Sodium [2-^{14}C]acetate (90%)	56	3·05	0·24	0·2	0·4	40
[1,4-^{14}C]Succinic acid	20·4	5·0	<0·005	<0·005		
[2,3-^{14}C]Succinic acid	23	2·05	0·35	0·4	0·8	51
[1,4-^{14}C]Fumaric acid	20·4	5·0	<0·005	<0·005		
[2,3-^{14}C]Fumaric acid	19·4	2·45	0·44	0·95	1·75	47
[1,4-^{14}C]Maleic acid	20·4	2·5	0·02	<0·02		
Sodium 2-oxo[1-^{14}C]glutarate	17·0	2·75	<0·005	<0·001		
Sodium 2-oxo[5-^{14}C]glutarate	21·0	2·55	0·11	0·25	0·4	97
DL-[2-^{14}C]Glutamic acid	4·26	3·1	0·33	0·5	1·1	76

[a] The labelled compounds were added to ten flasks after 24 h. Cellocidin was isolated 15—17 h later. Yields of cellocidin were usually 3—7 mg per flask. These incubations were all repeated at least once; some two and three times. [b] These figures were arrived at by dilution analysis (see Experimental section).

† Meiji Seika Kaisha Ltd., Yokohama, Japan.

[1] Part XXXVII, Sh. O. Badanyan, R. K. Bentley, J. K. Jenkins, Sir Ewart R. H. Jones, and V. Thaller, preceding paper.

[2] W. F. Lange, *Proc. Soc. Exp. Biol. Med.*, 1931—2, **29**, 1134.

[3] L. Nogueira Prista, *Anais. Fac. Farm. Porto*, 1954, **14**, 19.

[4] (*a*) Y. Sekizawa, *Meiji Seika Kenkyu Nempo*, 1960, 42 (*Chem. Abs.*, 1962, **56**, 14,609b); (*b*) Y. Sekizawa, *J. Biochem.* (*Japan*), 1958, **45**, 159 (*Chem. Abs.*, 1958, **52**, 12,981h).

[5] M. Funaki, K. Kageyama, S. Takemura, and H. Taniyama, *Yakugaku Zasshi*, 1959, **79**, 1510 (*Chem. Abs.*, 1960, **54**, 21,314e); Jap. P. 18,443/1960 (*Chem. Abs.*, 1961, **55**, 21,473g).

[6] G. Nakamura, K. Okuma, S. Suzuki, and Y. Tomiyama, *J. Antibiotics* (*Japan*), *Ser. A*, 1958, **11**, 81.

[7] K. Okuma and S. Suzuki, *J. Antibiotics* (*Japan*), *Ser. A*, 1958, **11**, 84.

[8] M. V. Long and E. F. Phares, *J. Amer. Chem. Soc.*, 1955, **77**, 2556.

The conditions and the results of the biosynthetic experiments are summarised in the Table. The incorporation of [1-^{14}C]acetate was less than that of [2-^{14}C]acetate and the carbon-14 derived from these two sources was very differently distributed over the cellocidin molecule. Both of these differences, *i.e.* incorporation and label distribution, can be explained in the light of computations which have been made for the incorporation of [1-^{14}C]- and [2-^{14}C]-acetates into 2-oxoglutarate *via* the tricarboxylic acid (TCA) cycle [9] (see Figure). It is therefore strongly indicated that cellocidin originates directly from carbon atoms 2—5 of 2-oxoglutarate.

Carbon atoms 2 and 3 of fumarate and succinate were incorporated uniformly into all four carbon atoms of cellocidin, whilst C(1) and C(4) were not incorporated at all. This type of labelling pattern is consistent with the expected [9] distribution of label in 2-oxoglutarate derived from fumarate or succinate after they have entered the TCA cycle, and excludes them as direct C_4 precursors of cellocidin. This is also the case for maleic acid, since the 1,4-labelled acid was not incorporated.

The incorporation results obtained with differently labelled 2-oxoglutaric and glutamic acids clearly point to the direct derivation of cellocidin from C(2)—C(5) of 2-oxoglutarate. 2-Oxo[1-^{14}C]glutaric acid was, as expected, not incorporated, whilst all the label from 2-oxo[5-^{14}C]glutaric acid resided on the amide groups of cellocidin. There was some label randomisation (the reasons for this are not understood) in cellocidin derived from DL-[2-^{14}C]glutamic acid, but most of the carbon-14 resided, again as expected, on the amide groups. The incorporation of 2-oxo[5-^{14}C]glutaric and DL-[2-^{14}C]-glutamic acids also proves that these acids cannot make a complete passage round the TCA cycle before they are incorporated into cellocidin; if this were to happen, all the label would be lost as $^{14}CO_2$ and the resulting cellocidin would be inactive. No evidence is available to indicate whether the triple bond is formed before or after decarboxylation.

Experiments are in progress to learn more about the biosynthesis of cellocidin from 2-oxoglutarate with the emphasis on the mechanism of triple bond formation. The obvious alternatives involve elimination, *e.g.* from an enol phosphate (this has been suggested [10] already in connection with polyacetylene biosynthesis), or stepwise dehydrogenation, but the stages at which these might be effected cannot be envisaged at present.

The maleimide ring of the nucleoside antibiotic showdomycin (II), produced by *Streptomyces showdoensis*, has been proved [11] to originate from carbon atoms 2—5 of 2-oxoglutarate, but no information is available about the transformations involved. The great similarity between cellocidin and the C_4 unit of showdomycin and the possible relationship between the organisms which produce them makes similar biosynthetic pathways for the two compounds likely.

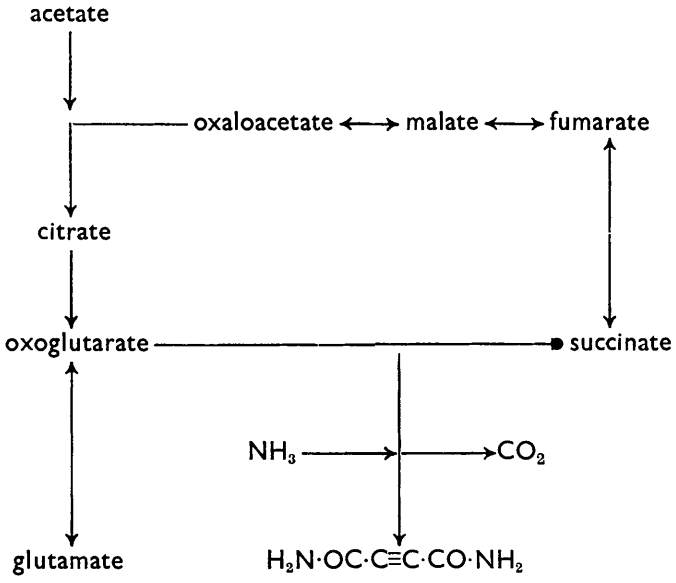


FIGURE Krebs cycle and derivation of cellocidin

EXPERIMENTAL

M.p.s were determined with a Kofler hot-stage apparatus. Activated charcoal (decolorising powder) was obtained from B.D.H. Ltd. Dimethylformamide (AnalaR) was dried over molecular sieve (B.D.H., type 4A).

DL-[2-^{14}C]Glutamic acid was obtained from International Chemical and Nuclear Corp, and 2-oxo[1-^{14}C]glutaric acid from New England Nuclear Corp; all other radiochemicals were obtained from the Radiochemical Centre, Amersham.

[9] I. D. Spenser in 'Comprehensive Biochemistry,' vol. 20, eds. M. Florkin and E. H. Stotz, Elsevier, Amsterdam, 1968, p. 364.

[10] E. R. H. Jones, *Chem. Eng. News*, 1961, **39**, 46; J. Cymerman Craig, M. D. Bergenthal, I. Fleming, and J. Harley-Mason, *Angew. Chem. Internat. Edn.*, 1969, **8**, 429.

[11] E. F. Elstner and R. J. Suhadolnik, *Biochemistry*, 1971, **10**, 3608.

Radioactive samples were counted on a Liquid Scintillation System (Beckman Instruments Inc., type LS 100) fitted with a Direct Data Readout Module. A solution (12 ml) of 5-(biphenyl-4-yl)-2-(4-t-butylphenyl)-1,3,4-oxadiazole (6·00 g) in AnalaR toluene (1 l) containing naphthalene (60 g) and POPOP (0·25 g) was used as scintillator. At least 10,000 counts were taken, so that the standard deviation was within 2%. ' Constant activity ' was taken to be within 2%. Cellocidin and succinic acid solutions for counting were prepared as follows: scintillator solution was added to the solution of a known weight (2—3 mg) of the specimen in warm dimethylformamide (2 ml) and counting was carried out immediately. Active barium carbonate samples were counted as suspensions with Cab-O-sil (Koch–Light Ltd.).

Bioassays for cellocidin were carried out as follows. Assay plates (Peptone 0·5%; Lab Lemco 0·3%; agar powder 1·5% w/w, in distilled water) were seeded with *E. coli* (chloramphenicol-resistant strain). Four holes (1 cm diameter) were bored in each plate: two holes were filled with culture filtrates (0·25 ml) and the other two with standard cellocidin solutions (0·25 ml). The plates were incubated at 37° for 24 h, and the area of spore inhibition in each case was calculated. All assays were performed in triplicate.

Growth of Streptomyces SF-536 *with Precursors and Extraction of Cellocidin.*—Cultures of *Streptomyces SF-536* were obtained from Meiji Seika Kaisha Ltd., Yokohama, Japan, and maintained on agar slopes (composition 1% w/v soluble starch, 0·2% yeast extract, 2% agar in distilled water, adjusted to pH 7·0).

Inoculum development medium. The inoculum was developed in a medium containing 0·5% peptone (Oxoid) and 0·3% Lab Lemco in distilled water. The medium was autoclaved at 15 lb in^{-2} for 15 min, and placed in a reciprocating tube shaker (150 rev. min^{-1}; eccentric throw 4 cm; 10 ml per tube). After inoculation the cells were incubated for 24 h at 25°.

Cellocidin production medium. The composition of the medium for the production of cellocidin was as follows: 0·28% cornsteep liquor, 0·25% yeast extract, 1·25% glucose, 0·30% ammonium dihydrogen phosphate, 0·25% calcium carbonate in Oxford tap water. The medium (100 ml per 500 ml conical flask) was autoclaved at 15 lb in^{-2} for 15 min. The cells from three tubes of the developed inoculum were distributed between 10 flasks, and incubated on a gyrotary shaker (190 rev. min^{-1}; eccentric throw 6 cm) at 25°. After 24 h incubation the precursor, dissolved in 70% ethanol (1 ml per flask), was distributed among the flasks (total and specific activities are given in the Table). After a further 15—17 h growth the culture fluid (1 l) was separated from the cells by filtration (Whatman No. 4), and the cells were washed with warm (40°) water (200 ml). The combined culture fluid and washings were adjusted to pH 6·4—6·8 with 2M-sodium hydroxide and activated charcoal (20 g) was added. After stirring for 1 h at 20°, the charcoal was removed by vacuum filtration through a sintered glass disc (porosity 2) and the filtrate was set aside for dilution analysis. Cellocidin was eluted from the activated charcoal by the addition of warm (50°) aqueous methanol (80%; 100 ml). This procedure was repeated 3 times and was followed by elution with hot methanol (100 ml) and hot water (100 ml). The eluates were combined, cooled to 20°, filtered, and concentrated *in vacuo* to *ca.* 3 ml. The oily concentrate was then cooled to 0°; cellocidin was filtered off and washed successively with water (1 ml), ethanol (1 ml), and ether (5 ml), dried in air, and weighed (3—7 mg per flask). The filtrate and washings were again set aside for dilution analysis. Cellocidin was dissolved in water (1 ml per mg cellocidin), boiled for 2 min with activated charcoal (25 mg), and filtered hot. The filtrate was concentrated in vacuum, and the residual cellocidin was recrystallised to constant specific activity from hot water in Craig tubes. Usually 2—3 crystallisations were necessary; pure cellocidin (long needles) decomposed between 218 and 221° (lit.,[6] m.p. 216—218°). The specific activities obtained for the various compounds administered to the micro-organism and the % incorporations based on pure cellocidin isolated are given in the Table.

Dilution analysis. A known weight of inactive synthetic cellocidin (30—40 mg) in water (60 ml) was added to the combined filtrates which had been set aside for dilution analysis. Cellocidin was recovered, purified, and recrystallised to constant specific activity as already described. The weight of the cellocidin not extracted by the charcoal followed then from the expression $WB/(A - B)$ (W represents the weight of inactive cellocidin added, A the specific activity of cellocidin from the original extraction, and B the specific activity of the cellocidin obtained by dilution with the inactive compound). It was added to the weight isolated and gave weights on the basis of which the estimated incorporations quoted in the Table were calculated.

Degradation of Cellocidin.—Succinamide. Cellocidin (15 mg) in 60% aqueous methanol (30 ml) was hydrogenated at atmospheric pressure for 3 h over PtO_2. The crude reduction product was recrystallised from water giving needles of succinamide, m.p. 269° (lit.,[4a] 265°).

Succinic acid. Succinamide (15 mg) was hydrolysed in sulphuric acid (3M; 2 ml) at 100° for 6 h. The product was isolated by continuous extraction with ether for 20 h, and recrystallised from water giving plates of succinic acid, m.p. 185° (lit.,[12] 185°). In a typical experiment, active cellocidin (0·189 μCi $mmol^{-1}$) gave succinic acid (0·187 μCi $mmol^{-1}$) in 85% overall yield.

Isolation of carbon dioxide from the carbonyl groups of cellocidin. A 25 ml pear-shaped flask was connected *via* a stirred, degassed acid permanganate trap to a Dreschel bottle in which barium hydroxide solution (12 g l^{-1}) was protected from the atmosphere by a potassium hydroxide guard tube. Succinic acid (50 mg) was dissolved in fuming sulphuric acid (19M; 0·5 ml) in the flask, and cooled to 0°; sodium azide (100 mg) was added and the mixture was shaken and warmed while purified nitrogen was passed slowly through the system. When formation of barium carbonate ceased, the precipitate was filtered off, washed thoroughly with degassed water, ethanol, and ether, and dried at 100° and 1 mmHg (yield 23 mg).

We thank Professor H. Umezawa for helpful counsel, Dr. T. Niida, Meiji Seika Kaisha Ltd., for provision of cultures and advice on the production and estimation of cellocidin, and Dr. J. K. Jenkins for his contribution to the preliminary investigations with the *Streptomyces* cultures. We also thank the S.R.C. for support.

[2/1717 *Received*, 20*th July*, 1972]

[12] R. Adams and M. T. Leffler, *J. Amer. Chem. Soc.*, 1936, **58**, 1553.