BIOSYNTHESIS OF THE ANTITUMOR ANTIBIOTIC CC-1065 BY *STREPTOMYCES ZELENSIS*

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The biosynthesis of the antitumor antibiotic, CC-1065, has been investigated by radioactive isotope techniques, in combination with chemical degradation of CC-1065. Tyrosine, dopa, serine and methionine (S–CH_3 group) have been shown to be precursors of CC-1065. Tyrosine is proposed to be a precursor of all three benzodipyrrole subunits, while dopa is only apparently incorporated into subunits B and C. Serine is postulated to contribute three 2C units, with loss of C-1, to all three subunits of CC-1065. The S–CH_3 group of methionine probably contributes four C-1 units to CC-1065 of which one is incorporated with considerable loss of tritium, most probably into the cyclopropane ring of subunit A.

CC-1065[1] is a potent antitumor antibiotic produced by *Streptomyces zelensis*[1,2,3] which interacts with double stranded DNA[4,5]. Mechanism of action studies suggest CC-1065 forms a covalent adduct in which the drug is bound in the minor groove of DNA. Although CC-1065 is highly active against a variety of mouse tumors[2] its potential as a clinical agent has yet to be proven.

Materials and Methods

Fermentations

S. zelensis Dietz and Li sp. m. was maintained on Hickney Tresner agar at 4°C. In a typical fermentation, spores were inoculated into 100 ml of seed medium [Bacto-tryptone (Difco) 5.0, Bacto-yeast extract (Difco) 3.0, dextrin 1.0, $MgSO_4 \cdot 7H_2O$ 1.0, $FeSO_4 \cdot 7H_2O$ 0.02, $MnCl_2$ 0.002, $CoCl_2 \cdot 7H_2O$ 0.02, $ZnSO_4 \cdot 7H_2O$ 0.01 (grams/liter) distilled H_2O to 1 liter], contained in a 500-ml Erlenmeyer flask and incubated for 48 hours at 28°C at 250 r.p.m. on a rotary incubator shaker. For production 5 ml of the 48 hours seed culture was transferred to 100 ml of a fermentation medium [Dextrose 3.0, dextrin 10.0, cornmeal 10.0, torula yeast 4.0, $CaCO_3$ 5.0, $MgSO_4 \cdot 7H_2O$ 1.0, Na citrate 2.5, fish meal 10.0, KCl 2.0, Na_2HPO_4 2.0 (grams/liter) distilled H_2O to 1 liter] which is incubated for up to 90 hours, under the same conditions as the seed flask. Production starts at about 66 hours reaching a maximum of between $2 \sim 4$ μg/ml at 90 hours.

Radioactive precursors were added at 66 hours and the incubation continued for 24 hours before harvesting.

Radioactively Labelled Compounds

L-[1′-^{14}C]Dopa, L-[3′-^{14}C]dopa, L-[3-^{14}C]serine, L-[Ala-2 or 3-^{3}H]dopa, L-[$^{14}CH_3$]methionine, L-[U-C^{14}]threonine and L-[3-^{3}H]serine were purchased from Amersham. L-[$C^{3}H_3$]Methionine, L-[U-^{14}C]serine, L-[U-^{14}C]tyrosine, L-[1′-^{14}C]tyrosine, L-[2- or 6-^{3}H]tyrosine, L-[3- or 5-^{3}H]tyrosine, L-[8-^{14}C]dopamine and [2-^{14}C]acetate were purchased from New England Nuclear and DL-[7a-^{14}C]tryptophan from International Chemical and Nuclear.

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Nonlabelled Compounds

CC-1065 (U-56,314, lot No. 15168-DGM-33.17) was obtained from Dr. DAVID MARTIN at the Upjohn Company and dissolved in 5% DMF in water prior to use. The methylpyrrole compound (4-methylpyrrole-2,3-dicarboxylic acid) used in the Kuhn-Roth oxidation was obtained from Dr. KOSTENBAUDER at the University of Kentucky.

Isotope Analysis

Radioactivity measurements were carried out on Packard Model 2425 liquid scintillation counter. Radioactive samples were added to 8 ml of Aquasure and counted to at least 2% statistical error and counting efficiencies were determined using an external standardization method. Radioactivity on chromatograms was detected using a Packard Model 7201 radiochromatogram scanner.

Chromatography

Thin-layer chromatography on 5×20 cm precoated Silica gel 60 F-254 was used throughout this study. Visualization of CC-1065 was by short UV light in which CC-1065 produced a blue fluorescence. Chromatography systems consisted of methylethylketone - acetone - water, 80: 17: 3; CC-1065 Rf 0.66 (TLC-1) and chloroform - methanol - ammonium hydroxide, 80: 20: 0.5; CC-1065 Rf 0.56 (TLC-2).

Sephadex G-100 column chromatography on the CC-1065-DNA adduct was carried out in 5 ml syringes (about $1 \sim 2$ ml of Sephadex) and eluted with water. Although adduct formation with DNA and then Sephadex chromatography will eliminate non-DNA reactive compounds, other CC-1065 degradation products still containing the DNA alkylating moiety will not be removed. CC-1065-DNA adduct were prepared by incubation of the isolated CC-1065 dissolved in 450 μl of 0.1 SSC, with an excess of calf thymus DNA (62 μg in 50 μl of 0.1 SSC) at 4°C overnight. 1 ml fractions were collected and 200 μl used for liquid scintillation counting in 8 ml of Aquasure and the remaining 800 μl used for O.D. measurements at 260 nm (DNA) and 363 nm (CC-1065). The CC-1065-DNA eluted in fractions $2 \sim 4$ and fraction 3 was used for radioactivity determinations.

Isolation of CC-1065 from Cultures and Determination of Percentage Incorporation of Precursors

At the end of the incubation period after adjusting the pH of the broth to 10 with concentrated NH_4OH, the whole fermentation broth is extracted twice with 200 ml of methylene chloride. The organic layers are pooled, dried over anhydrous sodium sulfate and taken to dryness on a rotary evaporator at 40°C. The residue is redissolved in acetone (about 3 ml) and 10 μl taken for radioactivity determination. An aliquot equivalent to about 2×10^4 dpm (^{14}C) or 2×10^5 dpm (^3H) is spotted on a TLC plate alongside reference CC-1065 and developed in TLC-1. The CC-1065 is located on the developed plate by exposure to UV light. The plate was then scanned for radioactivity using a radiochromogram scanner and the percentage incorporation determined from the combined radioactivity and scanning results as previously described[6]. Those samples found to be significantly labelled in TLC-1 were further purified by scraping off the band corresponding to CC-1065 rechromatographing in TLC-2 and Sephadex G-100 system to check for radiochemical purity.

Degradation of CC-1065 by Kuhn-Roth Oxidation

Radiochemically pure samples of CC-1065 labelled from methionine and serine were degraded by Kuhn-Roth oxidation[7]. Due to the unavailability of significant amounts of carrier CC-1065, a methylpyrrol compound was used as a carrier material (100 mg) since it carries an equivalent C–CH_3 group in a pyrrole ring. The acetic acid obtained from the Kuhn-Roth oxidation was isolated and counted for radioactivity.

Results

Determination of the Biosynthetic Precursors for CC-1065

CC-1065 consists of three very similar benzodipyrrole subunits (A, B, and C in Fig. 1) of which B and C are identical and subunit A is distinguished by having an extra carbon atom incorporated into a cyclopropane ring and also containing an indole quinone in place of a dihydroxyindole moiety. Based upon biosynthetic considerations it seemed most likely that the indole ring of tryptophan or cycli-

Table 1. Incorporation of labelled substrates in CC-1065 by *S. zelensis*.

Experiment No.	Substrate fed	Radioactivity fed (μCi)	% Incorporation into CC-1065
1	L-[$^{14}CH_3$]Methionine	10	0.5
2	L-[U-^{14}C]Tyrosine	20	0.5
3	L-[1′-^{14}C]Tyrosine	10	0.8
4	L-[1′-^{14}C]Dopa	10	0.3
5	L-[U-^{14}C]Serine	10	0.3
6	L-[U-^{14}C]Threonine	10	<0.03
7	DL-[7a-^{14}C]Tryptophan	10	<0.01
8	DL-[8-^{14}C]Dopamine	10	<0.01
9	L-[G-^{3}H]Tyramine	50	<0.1
10	[2-^{14}C]Acetate	10	<0.01

zation of tyrosine or dopa would give rise to two of the three rings in each of the benzodipyrrole subunits. Assuming this to be the case, then the remaining C_2–N unit in subunits B and C and C_3–N unit in subunit A could reasonably be derived from serine (B and C), serine and a C-1 unit (A) or threonine (A).

The results of radioisotope tracer experiments are shown in Table 1 and demonstrate that of the suspected precursors fed, only tyrosine, dopa, serine and methionine were detectable incorporated into CC-1065. Scarcity of cold carrier material precluded recrystallization to constant specific activity, however, the isolated biosynthetically labelled CC-1065 was shown to be radiochemically pure by both chromatography in two TLC systems and then binding to DNA which was then purified by Sephadex chromatography. The relative low levels of incorporation (*i.e.* 0.3~0.8%) of precursors into CC-1065 is most likely due to dilution by the relatively large amounts of amino acids (approximately 20 mg of each per 100 ml of media) contained in the fish meal which was found to be essential for antibiotic production. The incorporation of tyrosine, dopa, serine and methionine into CC-1065 leaves open several options how these precursors could be incorporated into subunits A or B and C. A series of double-isotope (^{3}H/^{14}C) experiments and chemical degradation of biosynthetically labelled CC-1065 molecules were designed and performed to answer these questions.

Fig. 1. Structure of CC-1065.

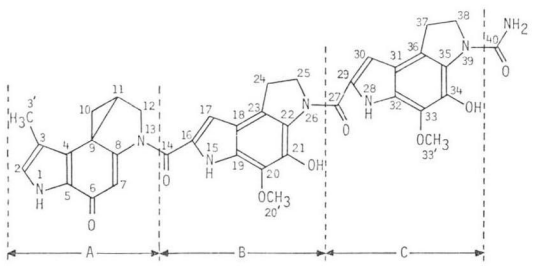
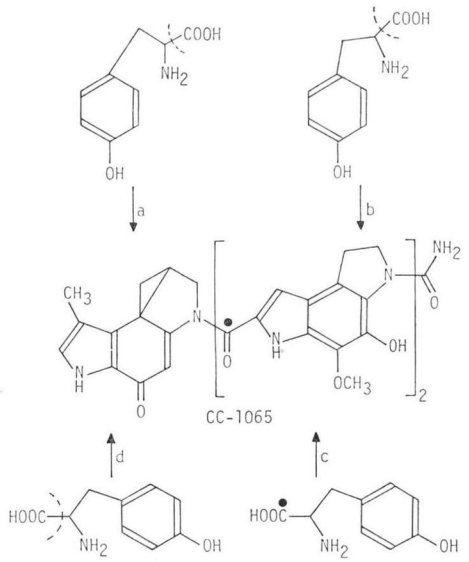


Fig. 2. Possible modes of incorporation of tyrosine into subunit A (pathways a and d) and subunits B and C (pathways b and c) showing metabolic fate of the 1′ carbon atom of tyrosine.



Incorporation of Tyrosine into CC-1065

The possible modes in which tyrosine can be incorporated into the A or B and C subunits of CC-1065 are shown in Fig. 2. Of these alternatives only pathway "c" leading to subunits B and C results in retention of the carboxyl carbon of tyrosine. Parallel feeding experiments in which tyrosine doubly labelled with carbon-14 either at C-1′ or uniformly labelled and carrying tritium in the side chain as a reference label were carried out, and the CC-1065 isolated and purified by successive chromatography and DNA binding. The results (experiments 1 and 2 in Table 2) demonstrate that while L-[1′-^{14}C]-tyrosine is incorporated into CC-1065, the L-[U-^{14}C]tyrosine is one third more efficiently incorporated into the antibiotic than the carboxyl labelled tyrosine. These results eliminate pathway "b" as an alternative and are in accord with both B and C subunits being derived by pathway "c". While these double labelling experiments do not distinguish between pathways "a" and "d" in Fig. 2, they are in accord with the incorporation of tyrosine with loss of the carboxyl carbon in the case of subunit A.

Dual labelled feeding experiments with both L-[3- or 5-^{3}H] and L-[2- or 6-^{3}H]tyrosine and L-[U-^{14}C]-tyrosine as a reference label were carried out to shed some light on the fate of the aromatic hydrogens in CC-1065. Since the six membered rings of subunits B and C are fully substituted, complete loss of tritium from both tyrosine species was expected. However, in the case of subunit A the operation of pathway "a" might allow a 16% retention of tritium from L-[3- or 5-^{3}H] tyrosine barring other chemical or enzymatic events which might eliminate this aromatic proton. The results shown in Table 2 (experiments 3 and 4) indicate a virtual complete loss of tritium from all aromatic positions of tyrosine during conversion into subunits A, B and C. In fact, the 98~99% loss of tritium from these substrates is further proof that our purification of CC-1065 using thin-layer and Sephadex chromatography leads to radiochemically pure material. While the virtual complete loss of tritium from L-[2- or 6-^{3}H]tyrosine was expected and is in accord with all four pathways in Fig. 2, the complete loss of tritium from L-[3- or 5-^{3}H]tyrosine does not necessarily distinguish between pathways "a" and "d" in Fig. 2. If pathway "a" is indeed operative the loss of tritium from L-[3- or 5-^{3}H]tyrosine would be attributed to some event directly or indirectly associated with the biosynthetic pathway to subunit A.

Incorporation of Dopa into CC-1065

The incorporation of dopa into CC-1065 (see Table 1) could be explained by its sole incorpora-

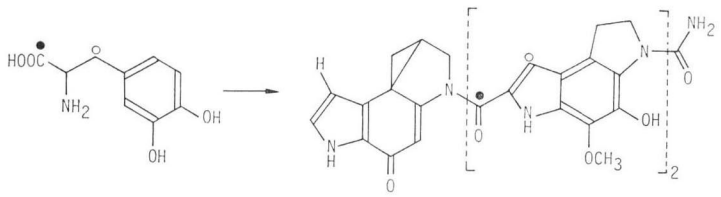
Table 2. Results of purification of CC-1065 from feeding experiments with various double-labelled precursors.

Experiment No.	Species fed	^{3}H/^{14}C ratio of precursor	^{3}H/^{14}C ratio of CC-1065			Final tritium[a] retention (%)
			TLC-1	TLC-2	Sephadex	
1	L-[1′-^{14}C, Ala-2′,3′-^{3}H]tyrosine	9.92	5.05	—	4.76	47.9
2	L-[U-^{14}C, Ala-2′,3′-^{3}H]tyrosine	9.06	3.21	4.50	2.95	32.6
3	L-[1′-^{14}C, Ala-2′,3′-^{3}H]dopa	9.26	2.48	2.80	2.59	28.0
4	L-[3′-^{14}C, Ala-2′,3′-^{3}H]dopa	10.30	4.12	2.83	3.03	29.4
5	L-[U-^{14}C, 2- or 6-^{3}H]Tyrosine	8.25	0.11	—	0.01	0.1
6	L-[U-^{14}C, 3- or 5-^{3}H]Tyrosine	9.63	0.12	—	0.06	0.6
7	L-[3-^{14}C, 3-^{3}H]Serine	8.90	5.70	—	5.70	64.0
8	DL-[1-^{14}C], L-[3-^{3}H]Serine	7.01	454.0	—	—	(98.5)[b]
9	L-[^{14}C^{3}H$_3$]Methionine	7.80	6.0	—	5.38	69.0

[a] Tritium retention = (^{3}H/^{14}C ratio of CC-1065 after Sephadex chromatography/^{3}H/^{14}C ratio of precursor) × 100.

[b] Relative loss of carbon-14 with respect to tritium based upon TLC-1.

Fig. 3. Possible mode of incorporation of dopa into subunits B and C, showing metabolic fate of the 1′ and 3′ carbon atoms of dopa.



tion into subunits B and C (see Fig. 3), which would rationalize the hydroxylation pattern in the six membered rings. If tyrosine, but not dopa, is a precursor of subunit A this would strongly support pathway "a" in Fig. 2, since the quinone oxygen would be in the correct position relative to the phenolic hydroxy of tyrosine. Incorporation *via* pathway "d" would require a dopa intermediate and then loss of the tyrosine phenolic hydroxyl group. To distinguish between these possibilities dopa labelled either in the 1′ or 3′ position was fed in parallel experiments with a common tritium reference label [[L-Ala-2′- or 3-^{3}H]dopa]. The results (experiments 5 and 6) in Table 2 demonstrate that both carbon-14 labels are incorporated to almost the same extent. This result is consistent with dopa not being incorporated into subunit A. Consequently pathway "a" in which tyrosine but not dopa is an intermediate is supported by these experiments.

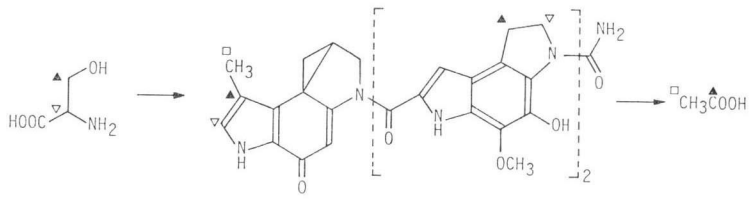
The retention of between 28 and 30% of tritium from L-[Ala-2′ or 3′-^{3}H]dopa in CC-1065 may have some significance in terms of the stereospecificity of elimination of one of the hydrogen atoms originally at 3′ of dopa (and by inference tyrosine). The C-3′ carbon of dopa should be located exclusively at C-17 and C-30 of subunit B and C respectively of CC-1065, both of which are methine carbons. Since C-2′ of tyrosine is predicted to label C-16 and C-29 of CC-1065, which is a quaternary carbon in both cases the tritium must be lost from this position. The tritium labelling pattern in the side chain of phenylalanine and tyrosine prepared in a similar manner to the dopa used in this experiment has been shown by KIRBY[8] to be as follows: First, the tritium is distributed equally between positions 2′ and 3′ of the side chain. Second, the tritium at C-3′ of the side chain is predominantly in the pro-*S* position (41.5±0.5%) relative to 8.5±0.5% in the pro-*R* position. The 28~30% retention of tritium is in between that expected for loss of the pro-*S* hydrogen (8.5% retention) or the pro-*R* hydrogen (41.5% retention). However, previous investigations[9,10] have also revealed excess tritium and deuterium retentions in sibiromycin and gliotoxin respectively. By analogy with sibiromycin (19% retention of tritium) we would predict the stereospecific loss of the 3′-(*R*) hydrogen of dopa during its conversion of subunits B and C, however, until experiments with stereospecific labelled substrates and chemical degradation are carried out, this conclusion should only be very tentatively offered.

Incorporation of Serine into CC-1065

Based upon the results of double-labelling experiments with tyrosine and dopa we can predict that serine should label CC-1065 as shown in Fig. 4. In this figure we proposed C-2 and 3 serine give rise to C-2 and 3 of subunit A and C-24 and 25 plus C-37 and 38 of subunits B and C respectively. A number of single, and double labelling experiments using serine in conjunction with chemical degradation of CC-1065 were designed and executed to test this postulate.

Fig. 4 predicts that while the tritium at C-3 of serine should be retained at C-24 and C-37 of subunits B and C of CC-1065 respectively, the tritium should be lost in subunit A and furthermore C-1

Fig. 4. Postulated labelling pattern of serine in CC-1065 and origin of the acetic acid derived from Kuhn-Roth oxidation of CC-1065.



of serine should not be incorporated into CC-1065. The results (experiments 7 and 8) in Table 2 demonstrate that as expected one third of the tritium is lost from L-[3-^{3}H]serine and C-1 of serine is negligibly incorporated into CC-1065. A sample of CC-1065 biosynthetically labelled from L-[U-^{14}C]-serine and degraded by Kuhn-Roth oxidation to remove C-3 and 3′ as acetic acid showed 91% of the calculated amount of carbon-14 in the acetic acid. (Table 3). These results taken together provide strong evidence that serine contributes three 2C units to CC-1065, as shown in Fig. 4.

Incorporation of Methionine into CC-1065

Our previously described results with tyrosine, dopa and serine account for all the carbon atoms of CC-1065, except the *C*-methyl group in subunit A (C-3′) the cyclopropane carbon in subunit B (C-10), the two *O*-methyl groups in subunits B and C (C-20′ and C-33′) and the terminal amide carbonyl (C-40). The *C*- and *O*-methyl groups seemed likely to be derived from the *S*-methyl group of methionine, while the extra cyclopropane carbon by analogy with cyclopropane fatty acids[11] seemed likely also to be derived from the C-1 pool. Experiments using double labelled L-[^{14}C^{3}H$_3$]methionine and chemical degradation of CC-1065 biosynthetically labelled from L-[^{14}CH$_3$]methionine were designed to investigate this postulate.

The results of double labelling experiments using L-[^{14}C^{3}H$_3$]methionine (experiment 9) in Table 2 indicate an 69% retention of tritium in CC-1065. The less than 100% retention of tritium in CC-1065 is in accord with an additional C-1 unit, other than the three intact methyl groups found in subunits A (C-3′), B (C-20′) and C (C-33′), which is incorporated with significant loss of tritium. Assuming that the C-1 units contribute equally to each of the 4 carbons in CC-1065 derived from methionine,

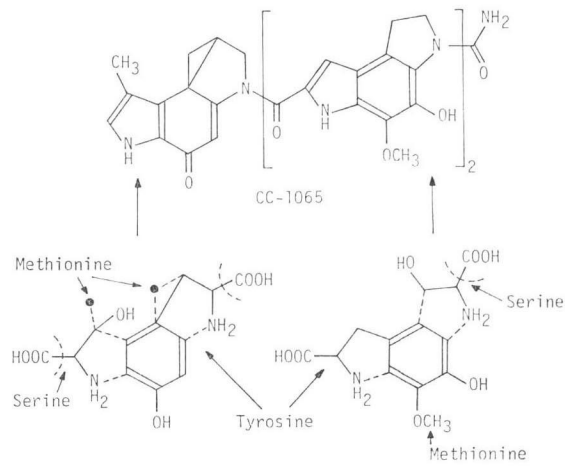
Table 3. Kuhn-Roth oxidation of CC-1065 biosynthetically labelled from L-[U-^{14}C]serine and L-[^{14}CH$_3$]methionine.

Precursor	Relative specific CC-1065	Radioactivities acetic acid
L-[^{14}CH$_3$]Methionine	100	22.5 (25)[a]
L-[U-^{14}C]Serine	100	15.2 (16.7)[b]

[a] Figure in parenthesis is the theoretical expected assuming L-[^{14}CH$_3$]methionine contributes 4 C-1 units equally to CC-1065.

[b] Figure in parenthesis is the theoretical expected assuming L-[U-^{14}C]serine contributes 3 C-2 units equally to CC-1065.

Fig. 5. Precursors of CC-1065 and their postulated labelling pattern in CC-1065.



then transfer of the C-1 unit to the cyclopropane ring with 0, 1 or 2 associated hydrogens would give rise to theoretical tritium retentions of 75, 83 and 92% respectively. [*e.g.* $(3\times CH_3)+(1\times CH_1)\div 12=$ 83.3%]. Therefore the tritium retention of 69% is suggestive of a transfer of a C-1 unit to the cyclopropane ring without any associated hydrogen. This latter result should, however, be treated with some caution since not only is this dependant upon the assumption that methionine labels each C-1 unit equally but that isotope effects are not operating during this transfer.

Kuhn-Roth oxidation of CC-1065 (see Fig. 4), biosynthetically labelled from L-[$^{14}CH_3$]methionine yielded acetic acid possessing 22.5% of the total radioactivity of CC-1065. This is in reasonable agreement of the calculated 25% that would be expected if methionine contributed four C-1 units all of which approximately equally labelled the CC-1065.

Discussion

The results of this study demonstrate that tyrosine, dopa, serine and methionine (C-1 units) are biosynthetic precursors of CC-1065. Our double-labelling experiments with these precursors provide a reasonable basis for the postulated biosynthetic labelling pattern of these amino acids in CC-1065 which is shown in Fig. 5. This study also provides an example of how thoughtful experimental design can reveal much useful information on probably biosynthetic labelling patterns in a secondary metabolite, where stable isotope techniques have proven to be difficult to apply.

CC-1065 is produced in relatively low amounts (2~4 mg/liter) in a complex media containing large amounts of amino acids in a fish meal extract. Experiments using radioactively labelled tyrosine and methionine reveal at best, dilution values of in excess of 500 which rules out the possibility of stable-isotope experiments. Persistent efforts to develop a synthetic or semisynthetic media which will support production of more than trace amounts of CC-1065 have also so far failed. Likewise we have been unsuccessful in developing a washed cell suspension that will support antibiotic production, although possibly a resting cell system in which amino acids were limited but antibiotic synthetases were already formed prior to cell collection might be successful. Therefore, more definitive experiments with stable isotopes will have to await the isolation of CC-1065 producing strains with improved properties or fermentation conditions.

Acknowledgments

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