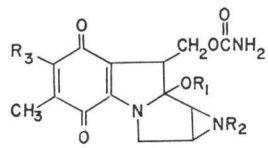
UTILIZATION OF THE INTACT CARBAMOYL GROUP OF L-[NH_2CO-^{13}C, ^{15}N] CITRULLINE IN MITOMYCIN BIOSYNTHESIS BY *STREPTOMYCES VERTICILLATUS*

Sir:

It was shown previously that both L-[guanidino-^{14}C] arginine and L-[ureido-^{14}C] citrulline are efficient mitomycin (**I**) precursors which specifically label the carbamoyl-group of these antibiotics[1,2]. Sodium-[^{14}C] carbonate[1], [^{14}C] urea[1] and potassium-[^{14}C] cyanate[3] are inefficient precursors and this is presumably due to either inefficient uptake or loss of the label as carbon dioxide from the slightly acidic fermentation broth. Other precursors label the carbamoyl group probably after being metabolized to carbon dioxide[4]. The results of a competition feeding experiment between L-citrulline and L-arginine indicated, but did not prove, that the former was the more proximate precursor[2] and they suggested that L-arginine is converted into L-citrulline prior to incorporation into the mitomycins. Thus the assumption can be made that the arginine dihydrolase pathway[5] is operative in *S. verticillatus* and that the carbamoylphosphate generated gives rise to the carbamoyl group of these antibiotics. However, a variety of factors, such as differences in uptake and different rates of metabolic conversions, may have influenced the outcome of the competition feeding experiment, which taken alone, was not completely self consistant, in that the suppression of labeled arginine incorporation in the presence of excess unlabeled citrulline was not as efficient as expected. Therefore, the possibility that L-citrulline was converted into L-arginine, which was serving as the more proximate precursor, could not be completely excluded. In addition, it was conceivable that the carbamoyl group of L-citrulline was undergoing extensive metabolic conversion leading to a separation of its carbon and nitrogen atoms prior to their separate incorporation into the carbamoyl group of the mitomycins. These possibilities were investigated in feeding experiments with intramolecularly double labeled L-[NH_2CO-^{13}C, ^{15}N] citrulline.

The synthesis of the labeled citrulline started with [^{13}C, ^{15}N] urea (92 atom % ^{13}C, 99 atom % ^{15}N, Stohler Isotopes, Waltham, Massachusetts) which was fused with potassium carbonate to give potassium[^{13}C, ^{15}N] cyanate in 57 % yield[6]. The cyanate was heated with an equimolar amount of a copper ornithine complex[7] to give an insoluble copper citrulline complex, which was decomposed in hydrochloric acid solution with hydrogen sulfide to afford crystalline L-[NH_2CO-^{13}C, ^{15}N] citrulline hydrochloride in 11.4 % overall yield (based on urea). Suitable conditions, in particular a low dilution factor*, for the feeding studies with this precursor were established in feeding experiments with L-[ureido-^{14}C] citrulline. Two days before harvest of replaced mycelia, three cultures of *S. verticillatus* received $1.1 \cdot 10^7$ dpm L-[ureido-^{14}C] citrulline and 10 mg, 25 mg and 50 mg of unlabeled L-citrulline, respectively. Approximately 4 mg of mainly mitomycin A (**Ia**) and mitomycin B (**Ib**) were produced and were extracted with ethyl acetate from the filtered fermentation broth, purified by thin-layer chromatography[1], recrystallized from acetone - ligroin (1 : 10), the specific radioactivity determined and the dilution factors calculated as 3.3, 1.7, and 1.4 respectively. These low dilution factors indicate a remarkably efficient utilization of L-citrulline in mitomycin biosynthesis. The incorporation of the label occurred specifically into the carbamoyl group as shown by chemical degradation[2].



Ia : $R_1 = CH_3$, $R_2 = H$, $R_3 = OCH_3$ Mitomycin A
Ib : $R_1 = H$, $R_2 = CH_3$, $R_3 = OCH_3$ Mitomycin B
Ic : $R_1 = CH_3$, $R_2 = H$, $R_3 = NH_2$ Mitomycin C

L-[NH_2CO-^{13}C, ^{15}N] citrulline (15 mg) and

* Specific radioactivity of precursor / specific radioactivity of product, or atom % enrichment of the heavy isotope(s) in precursor / atom % enrichment of heavy isotope(s) in product.

Table 1. Relative abundance of the ions NH_2CO^+ of mitomycin B labeled from L-[NH_2CO-^{13}C, ^{15}N]citrulline.

Ion	NH_2CO^+	$^{15}NH_2CO^+$	$NH_2{}^{13}CO^+$	$^{15}NH_2{}^{13}CO^+$
Relative abundance $\times 100$	60.8	9.9	<3.3	29.0

$1.0 \cdot 10^7$ dpm of L-[ureido-^{14}C]citrulline (admixed as a reference label) each were added 36 hours before harvest to two 100-ml cultures of replaced *S. verticillatus*. Mitomycins A and B were isolated and purified and a total incorporation of 3.8 % and a dilution factor of 2.94 were determined for the carbon-14 label. Mitomycin A was converted into mitomycin C (**Ic**), which was recrystallized with carrier material to constant specific radioactivity (6.35×10^5 dpm/mmol) and converted into a tetraacetyl derivative lacking the $-OCONH_2$ and the $-OCH_3$ group as previously outlined[2]. The derivative was essentially devoid of radioactivity (less than 1.68×10^3 dpm/mmol) indicating that the carbon label resided exclusively in either the $-OCONH_2$ or the $-OCH_3$ group, most likely exclusively in the former. Mitomycin B was analyzed by low and high resolution mass spectrometry (CEC 21 110B direct inlet probe, 200°, 70 eV, mass marker: carbon dioxide at *m/e* 43.98982; accuracy 3 m mass units). All mitomycins show a *m/e*=44 fragment ion of the composition NH_2CO^+ which arises from the carbamoyl group[8]. The ions belonging to the NH_2CO^+ ion cluster of the mitomycin B sample were unexpectedly weak but nonetheless clearly identifiable, and their intensities were determined from an average of 10~20 scans. The results obtained are shown in Table 1.

A dilution factor of 3.12 can be calculated from these data for the incorporation of the carbon-13 label, which is in close agreement with the dilution factor for the carbon-14 label. The results show that essentially the intact carbamoyl group of L-citrulline is utilized in mitomycin biosynthesis and that only a relatively small amount of ammonia and apparently no usable carbon dioxide is generated in the metabolism of L-citrulline and incorporated into the carbamoyl group of mitomycin B as judged from the low intensity of the $^{15}NH_2CO^+$ and $NH_2{}^{13}CO^+$ peaks. It can be concluded that L-citrulline, under the conditions of the feeding experiment, is not converted into either arginine or urea prior to its incorporation. If this conversion had taken place, it would have been expected that 50 % of the nitrogen-15 label would have been lost due to transient equilibration of the labeled nitrogen with an unlabeled nitrogen in either compound and this should have resulted in equal intensities of the $NH_2{}^{13}CO^+$ and $^{15}NH_2{}^{13}CO^+$ ions which is clearly not the case.

The observations of this study are most readily explained by assuming that ornithine transcarbamoylase, which has been detected in cell free extracts of *S. verticillatus* by measuring the formation of citrulline from ornithine and carbamoylphosphate (U. HORNEMANN, *et al.*, unpublished observations), acts in the reverse direction to generate carbamoylphosphate which then serves as a direct precursor in mitomycin biosynthesis. Further studies will be necessary to determine how L-[guanidino-^{14}C]arginine provides its label to the carbamoyl group. The assumption that the arginine dihydrolase pathway is active in *S. verticillatus* is attractive but pathways involving agmatine and carbamoylputrescine[9] or the initial transfer of an amidino group[10] cannot be excluded.

ULFERT HORNEMANN
JAMES H. EGGERT

Dept. of Medicinal Chemistry and Pharmacognosy
School of Pharmacy and Pharmacal Sciences
West Lafayette, Indiana 47907
U.S.A.

(Received July 24, 1975)

References

1) HORNEMANN, U. & J. C. CLOYD: Studies on the biosynthesis of the mitomycin antibiotics by *Streptomyces verticillatus*. J. Chem. Soc. Chem. Comm. 1971: 301~302, 1971
2) HORNEMANN, U.; J. P. KEHRER, C. S. NUNEZ & R. L. RANIERI: D-Glucosamine and L-citrulline, precursors in mitomycin biosynthesis by *Streptomyces verticillatus*. J. Amer. Chem. Soc. 96: 320~322, 1974

3) HORNEMANN, U.; J. P. KEHRER, C. S. NUNEZ, R. L. RANIERI & Y. K. HO: Precursors in mitomycin biosynthesis by *Streptomyces verticillatus*. Developments Ind. Microbiol. 15: 82~92, 1974
4) BEZANSON, G. S. & L. C. VINING: Studies on the biosynthesis of mitomycin C by *Streptomyces verticillatus*. Canad. J. Biochem. 49: 911~918, 1971
5) SCHIMKE, R. T.; C. M. BERLIN, E. W. SWEENEY & W. R. CARROLL: The generation of energy by the arginine dihydrolase pathway in *Mycoplasma hominis* 07. J. Biol. Chem. 241: 2228~2236, 1966
6) SCATTERGOOD, A.: Alkali metal cyanates. Inorg. Synth. 2: 86~90, 1946
7) SMITH, Jr., L. H.: A simple synthesis of isotopic citrulline and two of its homologs. J. Amer. Chem. Soc. 77: 6691~6692, 1955
8) VAN LEAR, G. E.: Mass spectrometric studies of antibiotics. I. Mass spectra of mitomycin antibiotics. Tetrahedron 26: 2587~2597, 1970
9) ROON, R. J. & H. A. BARKER: Fermentation of agmatine in *Streptococcus faecalis*: Occurrence of putrescine transcarbamoylase. J. Bact. 109: 44~50, 1972
10) WALKER, J. B.: Biosynthesis of the monoguanidinated inositol moiety of bluensomycin, a possible evolutionary precursor of streptomycin. J. Biol. Chem. 249: 2397~2404, 1974