PROOF FOR THE BIOSYNTHETIC CONVERSION OF L-[INDOLE-^{15}N]TRYPTOPHAN TO [10-^{15}N]ANTHRAMYCIN USING (^{13}C, ^{15}N) LABELLING IN CONJUNCTION WITH ^{13}C-NMR AND MASS SPECTRAL ANALYSIS*

JOHN M. OSTRANDER and LAURENCE H. HURLEY**

Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Kentucky, Lexington, KY 40506, U.S.A.

A. GAVIN MCINNES, DONALD G. SMITH, JOHN A. WALTER and JEFFREY L. C. WRIGHT

Atlantic Regional Laboratory, National Research Council of Canada***
1411 Oxford Street, Halifax, N.S., Canada, B3H 3Z1

(Received for publication June 10, 1980)

Using ^{13}C-NMR and mass spectral analysis we have demonstrated that the N-10 nitrogen of anthramycin is biosynthetically derived from the indole-nitrogen of tryptophan. Our experimental approach was to bring a ^{15}N atom, which is derived from L-[indole-^{15}N]tryptophan, and a ^{13}C atom which is derived from DL-[1-^{13}C]tyrosine, into adjacent positions of anthramycin. From resonance intensities and ^{13}C-^{15}N spin-spin coupling in the ^{13}C-NMR spectrum of didehydroanhydroanthramycin, a derivative of anthramycin, we could then determine the ^{13}C enrichment at C-11 and the proportion of ^{13}C bonded to ^{15}N at N-10. These results when combined with mass spectral analysis and isotopic dilution measurements proved that the indole nitrogen of tryptophan was completely retained at N-10 of anthramycin.

Anthramycin (**I**) is an antitumor agent belonging to the group of pyrrolo(1,4)benzodiazepine antibiotics[1]. In addition to their biosynthetic origin, these compounds are of interest because of their ability to react with DNA in a unique manner[2,3]. Previously reported biosynthetic studies[4,5] on anthramycin from *Streptomyces refuineus* have established that tyrosine, tryptophan and methionine supply the complete carbon skeleton (Scheme 1) and that the α-amino group of tyrosine is partially retained at N-4 of anthramycin[5]. Recently, the biosynthesis of anthramycin and the related compounds sibiromycin, tomaymycin and the neothramycins has been reviewed[6,7].

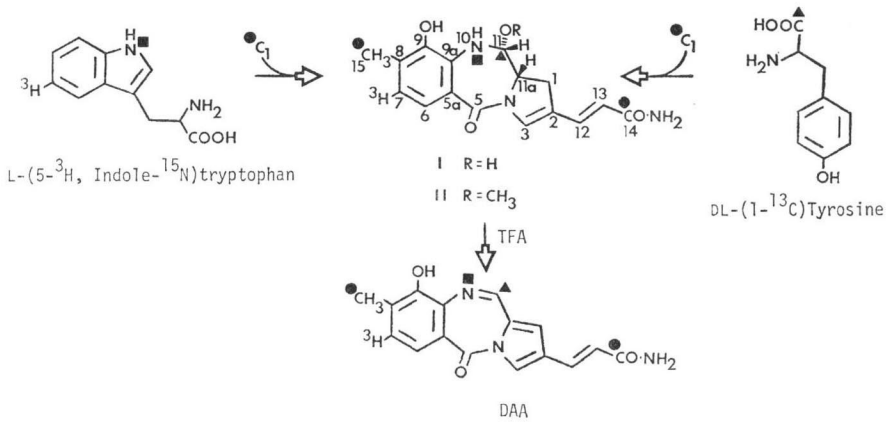
Precursors labelled with contiguous ^{13}C and ^{15}N atoms have been used in recent biosynthetic studies of uroporphyrinogen III[8], streptonigrin[9], nicotine and the tropane alkaloids[10]. A ^{15}N nucleus in a position adjacent to a ^{13}C nucleus produces a doublet in the ^{13}C-NMR spectrum, the fractional intensity of which is a direct measure of the proportion of ^{15}N at the adjacent position. Our present experimental approach differs from those previously described in that the ^{15}N and ^{13}C labels arise from separate precursors brought into contiguous positions during biosynthesis of the antibiotic (see Scheme 1). The adjacent incorporation of the ^{13}C and ^{15}N labels was shown directly by ^{13}C-NMR, and when these results were combined with mass spectral analysis, the degree of dilution of ^{15}N from L-[indole-^{15}N]tryptophan

* This work was presented at the American Society for Pharmacognosy Meeting held in W. Lafayette, Indiana on July 29~August 3, 1979.

** To whom inquiries should be addressed.

*** Issued as NRCC No. 18612.

Scheme 1. Biosynthetic labelling pattern of L-[5-^{3}H, indole-^{15}N]tryptophan and DL-[1-^{13}C]tyrosine in anthramycin and its chemical conversion to DAA.



was calculable. This dilution was then compared with that of ^{3}H from L-[5-^{3}H]tryptophan, incorporated simultaneously*, to determine whether the ^{15}N label was partially or completely retained.

Materials and Methods

Fermentations

Conditions for production and isolation of crystalline anthramycin methyl ether (AME) (**II**) from cultures of *S. refuineus* have been previously described[4]. Precursors were added when the anthramycin titer reached 20 μg/ml and the anthramycin was harvested at peak titer (160 μg/ml) which occurred about 6 hours after addition of precursors.

Chemicals

Media ingredients for fermentations and extractions were as previously described[4]. Trifluoroacetic acid was obtained from Sigma Chemicals. L-[5-^{3}H]Tryptophan was purchased from Amersham Searle; L-[indole-^{15}N]tryptophan (95% ^{15}N) and DL-[1-^{13}C]tyrosine (90% ^{13}C) were purchased from Kor Isotopes and Merck, Sharp and Dohme, Canada, respectively.

Chemical conversion of AME to didehydroanhydroanthramycin (DAA)

Since anthramycin and AME are unsuitable for NMR and MS analysis we routinely converted AME to DAA as previously described[12] before analysis.

Isotope labelling experiment

The stable isotope experiment was carried out by feeding a mixture of 100 μM of L-[5-^{3}H]tryptophan (2 μCi) and L-[indole-^{15}N]tryptophan together with 100 μM of DL-[1-^{13}C]tyrosine to each of nine separate 500 ml baffled shake culture flasks each containing 50 ml of production medium inoculated with *S. refuineus* as described before[4]. After extraction, purification and recrystallization[4], radiochemically pure AME (6 mg) was obtained. A portion (2 mg) of this material was diluted with unlabelled AME (20 mg), converted to DAA using trifluoroacetic acid (TFA)[12] and recrystallized from TFA yielding 14 mg for ^{13}C-NMR and MS analysis.

Instrumental analysis

Mass spectral analysis was carried out on a Hitachi RMU-7 Spectrophotometer. The sample was introduced by direct probe, at a source temperature of 180°C, and ionized by a 10 eV electron beam. The sample sublimed from the probe between 160°C and 190°C. Accelerator voltage was held constant at 2,600 V. For quantitative isotope analysis, several slow scans from m/z 295 to m/z 299 were recorded

* We have previously demonstrated that the tritium from this position of tryptophan is completely retained in anthramycin[11].

each in a 26.7 second period. All data was processed as described by BIEMANN[14].

^{13}C-NMR spectra were recorded on a Varian XL-100/15 NMR spectrometer having a Varian 620L computer and Diablo disk accessory. Conditions, for both natural-abundance and [^{13}C, ^{15}N]-labelled DAA, were: ^{13}C frequency 25.16 MHz, spectral width 5120 Hz, acquisition time 1.6s, pulse width 20 μs (90° pulse=44 μs), ^{1}H broadband decoupling with $\gamma H_2/2\pi$ *ca.* 3800 Hz, internal ^{2}H lock to $[C^2H_3]_2SO$ solvent, sample concentration 10 mg in 0.23 ml solution, reference to internal $[CH_3]_4$ Si, temperature 29°C.

Results

To assess the feasibility of the experimental approach, which required bringing together the ^{15}N- and ^{13}C-enriched positions of tryptophan and tyrosine respectively (Scheme 1), dilution experiments using L-[5-^{3}H]tryptophan and DL-[1-^{14}C]tyrosine were first conducted. The results of Table 1 and reference 4 show that addition of about 100 μM of either tryptophan or tyrosine produces dilutions of 1.86 and 4.6 respectively.

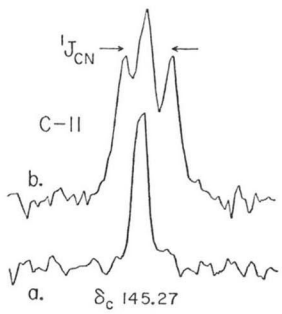
Table 1. Dilution of L-[5-^{3}H]tryptophan in anthramycin obtained by feeding varying amounts of the amino acid to the *S. refuineus*.

L-(5-^{3}H)-Tryptophan μM fed	Sp. act. of tryptophan μCi/μM	Anthramycin		Dilution*
		μM	μCi/μM	
60	0.65	3.6	0.25	2.60
100	0.39	3.9	0.21	1.86
180	0.22	3.6	0.14	1.57

* Specific activity of L-tryptophan/Specific activity of anthramycin.

Fig. 1. Expansion of ^{13}C-11 resonance of DAA.

a at natural isotopic abundance; *b* from culture supplemented with L-[indole-^{15}N]tryptophan and DL-[1-^{13}C]tyrosine, showing ^{13}C-^{15}N coupling ($^{1}J_{CN}$ 8.7 Hz).



[^{13}C, ^{15}N]-Labelled DAA was isolated as described in the methods section, and the results of the isotopic analyses are given in Table 2. Comparison of integrated resonance intensities in ^{13}C-NMR

Table 2. Isotopic analysis of the precursors, AME and DAA, utilized or isolated in the [^{13}C, ^{15}N] labelling experiment.

	Specific activity (μCi/μM)	Dilution[a]	Mass spectral analysis		
L-[5-^{3}H] and L-[Indole-^{15}N]-tryptophan	0.205	NA[b]	95 % ^{15}N		
DL-[1-^{13}C] Tyrosine	NA[b]	NA[b]	90 % ^{13}C		
AME (before dilution)	0.13	1.56	NE[c]		
AME (after dilution)	0.014	15.0	NE[c]		
DAA	0.004[d]	52.5		rel. int.[e]	probability
			$M^{+\cdot}$	100 %±0.56	0.934
			$M+1^{+\cdot}$	5.0 %±0.10	0.047
			$M+2^{+\cdot}$	2.1 %±0.04	0.020
			$M+3^{+\cdot}$	0.0 %	0.0

[a] Specific activity of L-[5-^{3}H] tryptophan/Specific activity of product

[b] Not applicable

[c] Not examined

[d] See footnote p. 1170

[e] Calculated using procedure described by BIEMANN[14].

spectra of this sample with corresponding intensities for natural-abundance DAA[13] showed that C-11 (δ_c 145.27 ppm) was the only position labelled: it contained an average $4.2\pm0.8\%$ ^{13}C ($3.1\pm0.8\%$ above natural ^{13}C-abundance). Spin-spin coupling to ^{15}N at N-10 of DAA was demonstrated by the isotopically-shifted doublet ($^1J_{CN}=8.7\pm0.6$ Hz, intensity I_d, isotope shift $0.02_3\pm0.01$ ppm upfield) about the uncoupled ^{12}C resonance (intensity I_s; Fig. 1). The resonance of C-9a was similarly split into a doublet, for which $^1J_{CN}$ and I_d could not be measured due to overlap with adjoining peaks. The proportion of ^{13}C at C-11 bonded to ^{15}N at N-10 is $I_d/(I_s+I_d)=0.60\pm0.04$. Thus $0.6\times4.2\%=2.5\pm0.5\%$ of the *total* carbon atoms at C-11 are ^{13}C bonded to ^{15}N, and $0.40\times4.2\%=1.7\pm0.4\%$ are ^{13}C bonded to ^{14}N.

The NMR, mass spectral and isotopic dilution results may be reconciled on the basis of the following model: other schemes assuming different sequences of dilution of the labelled precursors and DAA by natural-abundance material are incompatible with the combined observations. Assume that a mole fraction x of 90% [^{13}C]-enriched tyrosine is mixed with $(1-x)$ of natural ^{13}C-abundance tyrosine to form metabolic pool A and, similarly, z of 95% [^{15}N]-enriched tryptophan with $(1-z)$ of natural ^{15}N-abundance tryptophan to form pool B. After combination of molecules from pools A and B to form [^{13}C, ^{15}N]-enriched anthramycin (pool AB), a mole fraction y of AB is diluted further with $(1-y)$ of natural-abundance anthramycin. The probabilities p (N, C) of DAA molecules with various isotopic combinations at N-10 and C-11 (the only positions enriched) are

$$p(^{15}N, ^{12}C)=y[0.95z+0.0037(1-z)]\,[0.1x+0.989(1-x)] \quad (1)$$

$$p(^{14}N, ^{13}C)=y[0.05z+0.9963(1-z)]\,[0.9x+0.011(1-x)] \quad (2)$$

$$p(^{15}N, ^{13}C)=y[0.95z+0.0037(1-z)]\,[0.9x+0.011(1-x)] \quad (3)$$

As $I_d/I_s=p(^{15}N, ^{13}C)/p(^{14}N, ^{13}C)=3/2$, (1) and (3) give $z=0.63_3\pm0.04$.

The mole fractions x and y may then be determined from the probabilities $p(M+1)$ and $p(M+2)$ of excess $M+1^{+\cdot}$ and $M+2^{+\cdot}$ ions, found by mass spectral analysis (Table 2):

$$p(M+1)=0.047=p(^{15}N, ^{12}C)+p(^{14}N, ^{13}C) \quad (4)$$

$$p(M+2)=0.020=p(^{15}N, ^{13}C) \quad (5)$$

Substitution of (1), (2), (3) and $z=0.633$ in (4) and (5), and solution of the resulting simultaneous equations yields $x=0.40\pm0.06$ and $y=0.089\pm0.014$. It is noteworthy that the value of y corresponds exactly to the 1: 10 dilution of labelled AME with natural abundance AME (see Materials and Methods), for which $y=1/11=0.091$. Thus there was no detectable dilution of labelled anthramycin with natural abundance material in the organism. The above results, based solely on the relative doublet intensity from the ^{13}C NMR experiment and the fractional excess $M+1^{+\cdot}$ and $M+2^{+\cdot}$ ions from mass spectral analysis, then predict that the average $\%$ ^{13}C at C-11 $=100y\,[0.9x+0.011(1-x)]+1.1(1-y)=4.3\pm0.4\%$, in good agreement with the independent measurement ($4.2\pm0.8\%$) from the absolute intensity of the C-11 resonance. The fraction of excess ^{15}N is also determinable; it is $0.6y=0.053\pm0.008$. Thus the 95% [^{15}N]-enriched precursor has been diluted by a factor $95/5.3=17.8\pm3.3$, which again compares well with the independent result of 15.0 for the dilution of L-[5-^{3}H]tryptophan* incorporated in the same culture. Therefore the indole-nitrogen of tryptophan is incorporated exclusively at N-10 of anthramycin with complete retention.

* The dilution value was taken from AME rather than DAA since chemical conversion of AME to DAA with TFA is predicted to result in considerable exchange of aromatic protons[15]. This occurred in our hands, as shown by the loss of 72% of the tritium during conversion of AME to DAA (Table 2).

Acknowledgements

This study was supported, in part, by funds from research grant CA-17407, National Institutes of Health, U.S. Department of Health, Education and Welfare.

References

1) HURLEY, L.: Pyrrolo(1,4)benzodiazepine antitumor antibiotics. Comparative aspects of anthramycin, tomaymycin and sibiromycin. J. Antibiotics 30: 349~370, 1977
2) HURLEY, L. H. & R. PETRUSEK: Proposed structure of the anthramycin-DNA adduct. Nature (London) 282: 529~531, 1979
3) HURLEY, L. H.; J. S. ROKEM & R. L. PETRUSEK: Proposed structures of the pyrrolo(1,4)benzodiazepine antibiotic-DNA adducts. Biochem. Pharmacol. 29: 1307~1310, 1980
4) HURLEY, L. H.; M. ZMIJEWSKI & C.-J. CHANG: Biosynthesis of anthramycin. Determination of the labelling pattern by the use of radioactive and stable-isotope techniques. J. Amer. Chem. Soc. 97: 4372~4378, 1975
5) HURLEY, L.; W. L. LASSWELL, J. OSTRANDER & R. PARRY: Pyrrolo(1,4)benzodiazepine antibiotics. Biosynthetic conversion of tyrosine to the C_2 and C_3-proline units of anthramycin, tomaymycin and sibiromycin. Biochemistry 18: 4230~4237, 1979
6) HURLEY, L. H.: Elucidation and formulation of novel biosynthetic pathways leading to the pyrrolo(1,4) benzodiazepine antibiotics anthramycin, tomaymycin and sibiromycin. Acta Chem. Res. 13: 263~269, 1980
7) HURLEY, L. & M. K. SPEEDIE: Pyrrolo(1,4)benzodiazepine antibiotics. Biosynthesis of anthramycin, tomaymycin and sibiromycin. *In* Antibiotics, IV, *Ed.* J. W. CORCORAN, in press
8) BURTON, G.; H. NORDLOV, S. HOSOZAWA, H. MATSUMOTO, P. M. JORDAN, P. E. FAGERNESS, L. M. PRYDE & A. I. SCOTT: Structures of preuroporphrinogen. Exploration of an enzyme mechanism by ^{13}C and ^{15}N NMR spectroscopy. J. Amer. Chem. Soc. 101: 3114~3116, 1979
9) GOULD, S. J. & C. C. CHANG: Studies of nitrogen metabolism using ^{13}C NMR spectroscopy. I. Streptonigrin biosynthesis. J. Amer. Chem. Soc. 100: 1624~1626, 1978
10) LEETE, E.: Biosynthesis of natural products using isotopes detectable by means of NMR. *In* VIII Seminario Latinoamericano de Quimica, pp. 63~83, Buenos Aires, Argentina, November 19~23, 1979
11) HURLEY, L. H.; C. GAIROLA, N. DAS & M. ZMIJEWSKI: Biosynthetic incorporation of DL-(5-^{3}H)tryptophan into anthramycin, sibiromycin and tomaymycin. NIH shift produced by actinomycetes. Tetrahedron Lett. 1976: 1419~1423, 1976
12) MALHOTRA, R. K.; J. M. OSTRANDER, L. H. HURLEY, A. G. MCINNES, D. G. SMITH, J. A. WALTER & J. L. C. WRIGHT: Chemical conversion of anthramycin 11-methyl ether to didehydroanhydroanthramycin and its utilization to studies of the biosynthesis and mechanism of action of anthramycin. J. Nat. Prod., in press
13) WRIGHT, J. L. C.; L. C. VINING, A. G. MCINNES, D. G. SMITH & J.A. WALTER: Use of ^{13}C in biosynthetic studies. The labelling pattern of tenellin enriched from isotope labelled acetate, methionine, and phenylalanine. Canad. J. Biochem. 55: 678~685, 1977
14) BIEMANN, K.: Mass Spectrometry, Organic Chemical Applications. pp. 224~227, McGraw-Hill, N. Y., 1962
15) EVANS, E. A.: Isotope exchange reactions. *in* Tritium and Its Compounds. pp. 282~283, John Wiley & Sons, N. Y., 1974