BIOSYNTHETIC STUDIES OF ARPHAMENINES A AND B

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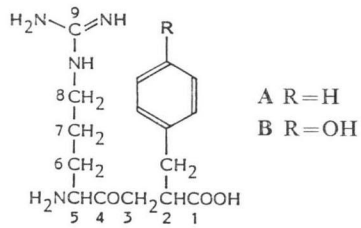
The biosynthetic pathways of arphamenines A and B were studied. Arphamenine A was derived from acetic acid, L-arginine and L-phenylalanine, and arphamenine B from acetic acid, L-arginine and L-tyrosine.

As reported in our previous papers[1,2], two potent aminopeptidase B inhibitors, arphamenines A and B were found in the culture filtrates of *Chromobacterium violaceum* BMG361-CF4. Their structures were determined to be 5-amino-8-guanidino-4-oxo-2-phenylmethyl octanoic acid (**A**) and 5-amino-8-guanidino-2-(4-hydroxyphenylmethyl)-4-oxo-octanoic acid (**B**), respectively.



To study the biosynthetic precursors of these compounds, ^{14}C-labeled acetate, L-arginine, L-phenylalanine and L-tyrosine were chosen for the radiolabeling experiments as they promoted production of arphamenines[3]. The role of acetate as the source of the methylene ketone moiety ($-\overset{*}{C}H_2-\overset{**}{C}O-$) in arphamenines was examined, using sodium [1-^{13}C]**acetate, sodium [2-^{13}C]*acetate or sodium [1,2-^{13}C]acetate.

Materials and Methods

Isotope-labeled Compounds

L-[*U*-^{14}C]Arginine (327.0 mCi/mmol), L-[*methyl*-^{14}C]methionine (48.7 mCi/mmol), L-[*U*-^{14}C]-phenylalanine (510.0 mCi/mmol), L-[*U*-^{14}C]tyrosine (497 mCi/mmol), sodium [1-^{14}C]acetate (56.0 mCi/mmol) and sodium [2-^{14}C]acetate (51.0 mCi/mmol) were purchased from New England Nuclear, U.S.A. Sodium [2-^{13}C]acetate (92.2% enrichment) was purchased from the British Oxygen Co. Ltd., England. DL-[1-^{14}C]Arginine (35~45 mCi/mmol), sodium [1-^{13}C]acetate (90% enrichment) and sodium [1,2-^{13}C]-acetate (90% enrichment) were purchased from Commissarat a L'Energie Atomique, France.

Assay of Anti-aminopeptidase B Activity

Inhibition of aminopeptidase B activity of rat liver by arphamenines was determined as reported previously[4].

Incorporation of Radioactivity into Arphamenines

Cells of *C. violaceum* BMG361-CF4 previously grown on KRAINSKY's asparagine glucose agar slants were incubated in 110 ml of a medium consisting of 3% soluble starch, 0.5% Prorich (Ajinomoto Co.), 1.2% corn gluten meal and 0.2% $CaCO_3$, in a 500-ml Erlenmeyer flask for 20 hours at 27°C on a rotary shaker (180 rpm). After inoculation of 0.5 ml of the seed culture thus prepared into the same medium (110 ml, ±1% L-phenylalanine), the incubation was carried out as before. ^{14}C-Labeled compounds at 10 μCi (except DL-[1-^{14}C]arginine, 20 μCi) were added at the times indicated in Table 1 and the biosynthetic experiments were continued for 4 hours.

Fig. 1. Production of arphamenine A by *C. violaceum* BMG361-CF4.

Medium: Soluble starch 3%, Prorich 0.5%, corn gluten meal 1.2%, L-Phe 1.0%, $CaCO_3$ 0.2%.

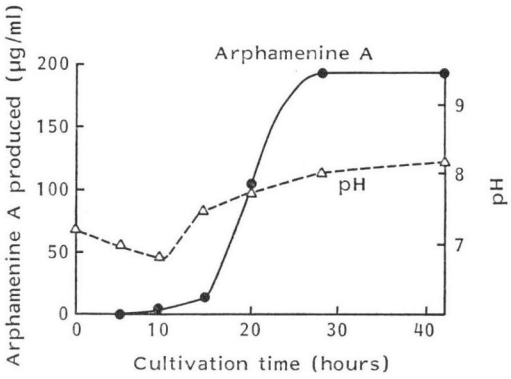


Table 1. Incorporation of ^{14}C-labeled compounds into arphamenines.

Labeled compounds	Medium	Incorporation (%)
Sodium [1-^{14}C]acetate	Med-II	1.6
Sodium [2-^{14}C]acetate	Med-II	2.1
L-[*U*-^{14}C]Arg	Med-II	28.0
DL-[1-^{14}C]Arg	Med-II	0.03
L-[*U*-^{14}C]Phe	Med-I	11.4*1
L-[*U*-^{14}C]Tyr	Med-I	4.7*2
L-[*Methyl*-^{14}C]Met	Med-II	0.05

Med- I: Soluble starch 3%, Prorich 0.5%, corn gluten meal 1.2%, $CaCO_3$ 0.2%.
Med-II: Med-I+L-Phe (1%).
Time of addition of ^{14}C-labeled compounds: Med-I 8 hours, Med-II 15 hours.
*1 Incorporated ratio arphamenine A/B was 99: 1.
*2 Incorporated ratio arphamenine A/B was 0.3: 99.7.

Twenty ml of culture filtrate were chromatographed on a column of CM-Sephadex C-25 (20 ml) which was developed with 0.5 M NaCl. Fractions containing arphamenines were combined (25 ml). Five-tenth ml of the combined solution was applied to a TLC plate of silica gel (E. Merck, Art5721) and developed with the solvent system: butanol - acetic acid - water (4: 1: 1) or chloroform - methanol - acetic acid - water (10: 4: 1: 1). Arphamenines were visualized with 0.4% solution of ninhydrin in acetone. The area containing the arphamenines was cut out from the plate and transferred to vials for counting. Eight ml of scintillation cocktail (Aquasol-2, New England Nuclear, U.S.A.) were added and the radioactivity of each sample was measured in a Beckman LS9800 liquid scintillation counter.

Preparation of ^{13}C-Labeled Arphamenine A

After strain BMG361-CF4 was cultured in 110 ml of the L-phenylalanine-containing medium for 15 hours, 50 mg of sodium [1-^{13}C]acetate, sodium [2-^{13}C]acetate or sodium [1,2-^{13}C]acetate plus 100 mg of unlabeled sodium acetate and 110 mg of L-arginine monohydrochloride were added. The incubation was continued for 4 hours.

One hundred ml of culture filtrate was chromatographed on a column of CM-Sephadex C-25 (100 ml) which was developed with a linear gradient of NaCl from 0 to 0.5 M. Arphamenine A-containing fractions were combined and desalted on an Amberlite XAD-4 adsorption column; elution was carried out with 50% aqueous acetone. The crude arphamenine A thus obtained was rechromatographed on CM-Sephadex C-25 using 0.2 M NaCl as eluant. The purified arphamenine A-containing fractions were subsequently desalted on a column of Sephadex LH-20: elution of ^{13}C-labeled arphamenine A (10~20 mg) was attained with water.

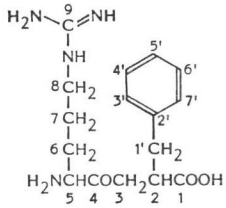
^{13}C NMR Spectrometry

Fourier-transform ^{13}C NMR spectra were obtained on a Jeol FX-200 NMR spectrometer operating at 50.1 MHz. Each sample was dissolved in 0.25 ml of D_2O and was run in a tube with 5 mm diameter at 25°C. The running conditions were as follows: spectral width: 15 KHz, pulse width: 2.5 μseconds and repetition time: 1.73 seconds. Accumulations were as follows: 5,000 for arphamenine A (10 mg) labeled with [1-^{13}C]acetate, 10,000 for arphamenine A (10 mg) labeled with [2-^{13}C]acetate and 31,888 for arphamenine A (4.8 mg) labeled with [1,2-^{13}C]acetate.

Results and Discussion

C. violaceum BMG361-CF4 produced only arphamenine A in a L-Phe-containing medium; production occurred at 15 hours as shown in Fig. 1. By contrast, in the same medium without L-phenylalanine,

Table 2. ^{13}C NMR spectral data for arphamenine A, including enrichments from labeled precursors.



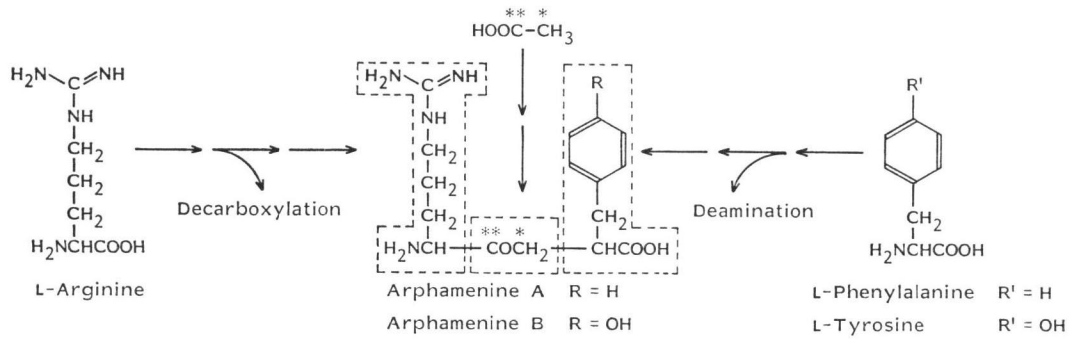
Carbon No.	δC*1	Multiplicity*2	Enrichment factor*3	
			[1-^{13}C]Acetate	[2-^{13}C]Acetate
1	182.6	s	0.48	0.64
2	45.6	d	0.50	0.74
3	41.8	t	1.0	10.8
4	207.2	s	10.4	0.66
5	58.9	d	0.56	0.64
6	26.8	t	0.59	0.72
7	24.1	t	0.73	0.80
8	41.1	t	0.74	1.20
9	157.7	s	0.78	0.85
1′	38.6	t	0.68	0.78
2′	140.3	s	1.00	1.00
3′,7′	129.4	d×2	0.75	0.84
4′,6′	129.9	d×2	0.84	0.94
5′	127.3	d	0.66	0.76

*1 In D_2O (internal dioxane δ 67.4).

*2 Multiplicities in the off-resonance decoupling: s, singlet; d, doublet; t, triplet.

*3 Intensity of each peak in the labeled arphamenine A devided by that of the corresponding peak in the unlabeled arphamenine A, normalized to give a ratio of 1.00 for the peak of 2′-C.

Fig. 2. Building blocks for arphamenines A and B.



both arphamenines A and B production occurred at 8 hours. Thus, ^{14}C-labeled compounds were added to the respective media at 8 and 15 hours.

As shown in Table 1, L-[*U*-^{14}C]arginine, sodium [1-^{14}C]acetate and sodium [2-^{14}C]acetate were efficiently incorporated into arphamenine A, whereas DL-[1-^{14}C]arginine and L-[*methyl*-^{14}C]methionine were not incorporated to any significant extent. In addition, L-[*U*-^{14}C]phenylalanine was incorporated into arphamenine A, but not appreciably into arphamenine B. On the other hand, L-[*U*-^{14}C]tyrosine was incorporated into arphamenine B, but not into arphamenine A. In both cases the ratio of incorpora-

tion of radiolabel into the arphamenines A and B did not change during a further 16 hours' incubation after the addition of ^{14}C-labeled compounds.

These experimental results indicated that the *N*-terminal moiety of arphamenines A and B was derived from L-arginine, the *C*-terminal moiety was derived from L-phenylalanine (arphamenine A) and L-tyrosine (arphamenine B) and the methylene ketone ($-\overset{4}{C}O-\overset{3}{C}H_2-$) moiety was derived from acetate.

In order to establish the distribution of radiocarbon from acetate, ^{13}C-labeled arphamenine A was prepared with sodium [1-^{13}C]acetate, sodium [2-^{13}C]acetate or sodium [1,2-^{13}C]acetate. As shown in Table 2, ^{13}C NMR spectra of ^{13}C-labeled arphamenine A enriched with sodium [1-^{13}C]acetate and sodium [2-^{13}C]acetate indicated that the enrichment of ^{13}C occurred only at C-4 or C-3, respectively. ^{13}C NMR spectra of ^{13}C-labeled arphamenine A enriched with sodium [1,2-^{13}C]acetate also indicated the spin coupling between C-3 (J_{c-c}= 44 Hz) and C-4*.

From the results of our experiments, the building blocks for arphamenines are L-arginine, L-phenylalanine or L-tyrosine and acetic acid, as shown in Fig. 2. The fact that L-[*U*-^{14}C]phenylalanine was only incorporated into arphamenine A whereas L-[*U*-^{14}C]tyrosine was only incorporated into arphamenine B suggests that arphamenine A is not converted to arphamenine B.

In a subsequent paper, we will report on the complete biosynthetic pathway to arphamenines[5].

Acknowledgment

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References

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* J_{c-c} was not determined by overlapping of other signal (C-8).