BIOSYNTHESIS OF THE SPERMIDINE AND GUANIDINO UNITS IN THE GLYCOCINNAMOYLSPERMIDINE ANTIBIOTIC CINODINE

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The biosynthesis of the spermidine and guanidino groups has been studied with carbon-14 and carbon-13 labeled intermediates. Arginine, citrulline and ornithine are incorporated in good efficiency. The guanidino group of arginine and the ureido group of citrulline both label the guanidino group on the hexose sugar. None of the ureido groups in the antibiotic was enriched. It is likely that citrulline is converted to arginine before use in the biosynthesis. Arginine, citrulline and ornithine are incorporated as the four carbon unit of spermidine. All of the labeled 5 carbon from ornithine or from citrulline appears adjacent to the secondary amine in the four carbon unit of spermidine. This would indicate that unbound putrescine is not an immediate precursor of spermidine.

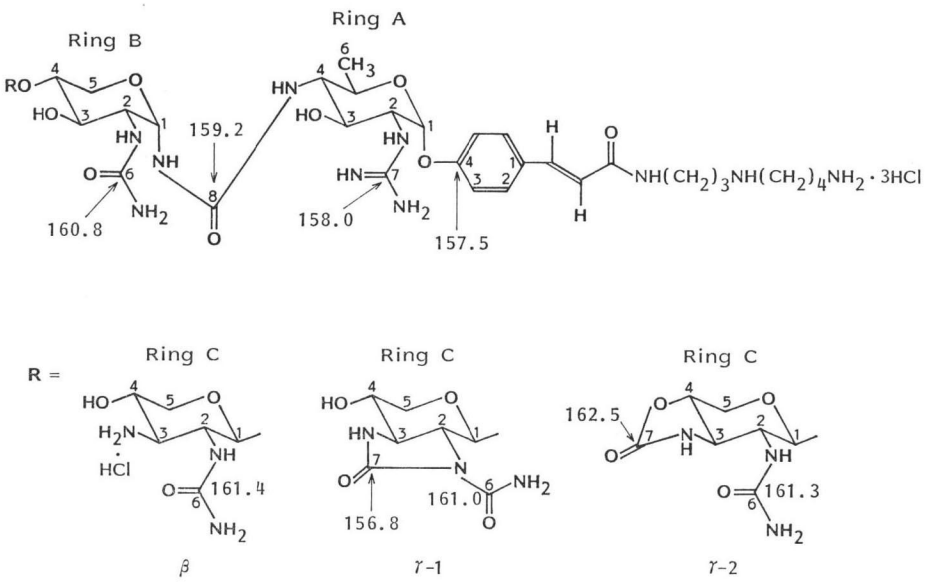
In earlier publications we reported the biogenesis of the *p*-coumaric acid and carbohydrate moieties of cinodine and that certain amino acids in the urea cycle are incorporated in good yields[1,2]. Now we wish to report in more detail the biogenesis of the spermidine and guanidine moieties. In this paper we studied the antibiotic labeling patterns available from arginine, citrulline and ornithine. The structure of the cinodine compounds is given in Fig. 1.

Materials and Methods

Fermentation

Improved strains (LK 5229, LK 1034 and KL 5228) of *Nocardia* culture BM 123 (NRRL 5646) were obtained by mutation and selection by Lederle Laboratories and were supplied to us as frozen suspensions or as agar slants. The cultures were propagated and shaker flask fermentation was done as previously described[1]. The larger scale fermentations designed to yield a gram or more of purified isomers were conducted in a Chemostat (New Brunswick Scientific Model No. C 32) which was fully instrumented for control of temperature, aeration, agitation, pH and transfer of media. In a typical fermentation, the 2,000 ml fermentor was charged with H_2O 800 ml, K_2HPO_4 2.4 g, $CaCl_2$ 0.8 g, $(NH_4)_2SO_4$ 5.6 g, $MgSO_4 \cdot 7H_2O$ 0.8 g, Tween 80 surfactant 1.0 g, $FeSO_4 \cdot 7H_2O$ 0.1 g, Mazur DF40P polypropylene glycol antifoam 0.5 g, monosodium glutamate 8.0 g and 4 ml of a trace metal solution containing $CuSO_4 \cdot 5H_2O$ 33 mg, $ZnSO_4 \cdot 7H_2O$ 500 mg, $MnSO_4 \cdot 4H_2O$ 40 mg, $CoCl_2 \cdot 6H_2O$ 20 mg and $Na_2MoO_4 \cdot 2H_2O$ 10 mg in 50 ml of H_2O. The pH before autoclaving was 6.8 and afterwards was 6.2. Approximately 5 ml of 50% glucose solution were pumped in and the pH adjusted to 6.8 with 20% Na_2CO_3 solution. The fermentor was seeded with 40~45 ml of culture grown as previously described[1,2]. The temperature was set at 26°C. Feed pumps (Masterflex peristaltic pump with No. 7014 pumphead, Cole-Palmer Instrument Co.) driven from Tork timers (Model 18061, Tork Co.) were used to supply 50% glucose, 25% monosodium glutamate (MSG) and antifoam through a silicone rubber tubing as required. The initial feed rate for glucose was approximately 0.5 g/hour and was started at seeding. The MSG feed was started after about 50 hours. The feed rates were adjusted daily so that the glucose concentrations remained at 10~30 g/liter and the MSG concentration was at 10~20 g/liter. During a 160~190-hour run, approximately 150 g of glucose and approximately 70 g of MSG were needed. After 30 hours the pH was gradually lowered 0.25 pH unit every 30 minutes, until the set point of 5.5 was reached

Fig. 1. Cinodine structure and ^{13}C NMR assignments of resonances from 155 to 163 ppm.



where the antibiotic was produced efficiently and was stable in the media. The pH was controlled with 3 N H_2SO_4 which was added with the pH controller through a Viton tubing. When it was desirable to incorporate, a labeled substrate (100~300 mg) was mixed with the first 125 ml of MSG feed and metered in between 50 and 150 hours. The dissolved oxygen was maintained above 50% of free-air saturation during the entire run by varying the stirring rate as cell growth proliferated. We have found empirically that the consumption of one millimole of acid corresponds very well to the consumption of one millimole of MSG. The amount of acid used can be used to calculate the MSG consumed in the fermentor. The levels of MSG in the fermentor were checked by TLC on silica gel plates which were developed with EtOH - H_2O - 12 N HCl (70: 30: 0.3). After spraying with ninhydrin spray and heating at 110°C, the color intensity was compared with standards. Glucose levels were estimated with the glucose oxidase-peroxidase assay[3,4] or by the anthrone colorimetric method[5]. At the termination of the run, 7 g of NaF was added along with some diatomaceous earth filtering aid and the suspension was filtered. The filtrate was diluted until the concentration of Na^+ ion was about 0.4%. The antibiotic was purified by ion exchange chromatography on CM-Sephadex C-25 (Na salt form). The γ fraction was eluted with 3% NaCl, and the β fraction was eluted with 6% NaCl. The salt was removed with an Amicon UM05 (molecular weight cut off 500) membrane filter and the solution containing the antibiotic was lyophilized.

Preparation of Carbon-13 Labeled Substrates

L-[*Guanidino*-^{13}C]arginine Hydrochloride: Thiourea (90 atom % carbon-13, 1.54 g, 20 mmol), was oxidized with yellow mercuric oxide to cyanamide by the method of WALTHER[6], and the cyanamide was converted to 2-methylpseudourea hydrochloride with MeOH and HCl as described by KURZER and LAWSON[7]. This labeled intermediate was reacted with L-ornithine cupric chloride complex[7,8] in H_2O at pH 11 essentially as described by TURBA and LEISMANN[8] for their preparation of carbon-14 labeled arginine. After acidifying to pH 3 and precipitation of the cupric ion as the sulfide, the arginine was precipitated as the monoflavinate (5.64 g, 11.60 mmol). The yield was 58% from thiourea. The infrared spectum was identical to that of an authentic sample prepared by the method of KRUSE[9] who also gave an excellent procedure for further conversion to the hydrochloride. The labeled flavinate was converted to the hydrochloride in 86% yield by this procedure. The product (2.115 g) was recrystallized by dissolving in 1 ml of hot 0.4 N HCl and adding 5 ml of EtOH to give 1.746 g of L-[*guanidino*-^{13}C]arginine hydrochloride.

L-[*Ureido*-^{13}C]citrulline: Carbon-13 labeled KCNO was prepared from KCN (90 atom % carbon-13) by oxidation with $KMnO_4$ in the presence of $Cu(OH)_2$ as described by GALL and LEHMANN[10]. From 4.93 g of ^{13}C-KCN we obtained 4.65 g (75%) of ^{13}C-KCNO. A solution of 5.77 g of 69.1% (10 mmol) L-ornithine cupric chloride complex and 1.62 g (20 mmol) labeled KCNO in 20 ml of H_2O was stirred at reflux for 1 hour. The insoluble citrulline copper complex was filtered from the cooled solution and washed consecutively with H_2O, absolute EtOH and with ether. The yield was 3.544 g (8.603 mmol) 86%: mp 256~257°C. The complex was suspended in 100 ml of H_2O and the cupric ion was precipitated with H_2S. The suspension was filtered and the filtrate was evaporated. The solid residue (2.9 g) was dissolved in 5 ml of H_2O and 20 ml of EtOH were added in portions. The recrystallized product weighed 2.638 g (15.057 mmol), 75% based on ^{13}C-KCNO: mp 220~222°C (ref, mp 221~222°C[11]).

DL-[5-^{13}C]Ornithine and DL-[5, *Ureido*-$^{13}C_2$]citrulline: [1-^{13}C]-3-Hydroxypropionitrile (5.25 g) was prepared from labeled KCN 5.95 g, $MgSO_4 \cdot 7H_2O$ 11.4 g, H_2O 20 ml and ethylene oxide 6 ml by the procedure of TERENT'EV and VINOGRADOVA[12]. A tetrahydrofuran solution (80 ml) of the distilled product (bp 115~118°C/15 mm Hg) was reduced to the amine with 16.2 ml of borane methyl sulfide complex. The reaction mixture was refluxed for 1/2~1 hour to complete the reaction. The excess BH_3 was decomposed with MeOH and the resulting trimethyl borate was distilled. The residue was treated in MeOH with anhydrous HCl. Evaporation of the solvent left 10 g of crude [1-^{13}C]-3-hydroxypropylamine hydrochloride. The salt was suspended in toluene and treated with 20 ml triethylamine followed by 11.16 g of phthalic anhydride. After heating at 110°C overnight, the insoluble triethylamine hydrochloride was removed by filtration. The filtrate was washed with $NaHCO_3$ solution and evaporated leaving 15 g of crude hydroxypropyl-[1-^{13}C]phthalimide as a white solid. The crude product was suspended in toluene (75 ml) and PBr_3 (7 ml) was added dropwise at 0°C. The reaction mixture was heated at 105°C for 3 hours, a small amount of absolute EtOH was added and the mixture poured into ice H_2O. This gave 16.95 g of 3-bromopropyl-[1-^{13}C]-phthalimide: mp 72~73.5°C (ref, mp 72~73°C[13]).

Ethyl acetamidocyanoacetate (16.08 g) was dissolved in dimethyl sulfoxide (160 ml) and sodium methoxide (5.1 g) was added. The labeled phthalimide in 140 ml dimethyl sulfoxide was added and the mixture was heated at 80°C for 4 hours. The mixture was partitioned between ice H_2O and CH_2Cl_2. The organic phase was washed with H_2O, dried and the solvent was evaporated to yield 19.09 g of 2-acetamido-2-carboethoxy-5-phthalimido[5-^{13}C]valeronitrile: mp 209~212°C (ref, mp 210°C[14]). This product was hydrolyzed by refluxing with 6 N HCl for 6 hours as described by GAUDRY[14]. After work up, 7.74 g of DL-[5-^{13}C]ornithine monohydrochloride was obtained: mp 226~230°C (ref, mp 225°C[15]). A portion of this material was converted to DL-[5, *ureido*-$^{13}C_2$]citrulline with ^{13}C-KCNO by the method described above for L-[*ureido*-^{13}C]citrulline: mp 244~246°C.

Analysis of Cinodine for Carbon-13 Enrichment

In the incorporation runs a small amount, usually 50 μCi, of the carbon-14 labeled substrate was added along with 100~300 mg of carbon-13 labeled material. The gross incorporation was calculated from the carbon-14 incorporated into the antibiotic. The carbon-14 content was measured by counting the HPLC eluent containing the antibiotic fraction or in some cases by liquid scintillation counting of the purified antibiotic. The ^{13}C NMR spectra were obtained in D_2O on a Varian FT 80 spectrometer and enrichments were estimated by comparing the enriched peak by integration or by height with the reference natural abundance compound run under the same conditions. A summary of the enrichments obtained are in Table 1.

During the study of these compounds, we were able to obtain good quality ^{13}C NMR spectra of cinodines at 50 and 62.9 MHz, from which we were able to assign the enrichment sites with no ambiguity. ELLESTAD *et al.*[16] reported the complete ^{13}C NMR structural assignment of the three forms of cinodine. The resonances between 155~163 ppm appear to be misassigned, however, most probably because of poor chemical shift dispersion at the 25 MHz field-strength used. They assigned the resonance at 162.1 ppm to the 4-position of the benzene ring. Spectra of γ-2 cinodine taken on a Bruker CXP 200 instrument operating at 50 MHz indicate that this 4-position resonates at 157.5 ppm. The coupled spectrum

Table 1. Incorporation of carbon-13 labeled substrates into cinodine.

Substrate	Amount (g)	Cinodine form	^{14}C Incorporation (%)	Enrichment (×natural enrichment abundance)	^{13}C NMR (ppm) TMS=0
L-[*Guanidino*-^{13}C]arginine	0.3012	γ-2	10.2	9.8	158.0
L-[*Ureido*-^{13}C]citrulline	0.4058	β	8.2	6.2	158.0
		γ-2	8.2	6.3	158.0
DL-[5-^{13}C]Ornithine	0.4056	γ-2	6.2[a]	8.2	47.8
DL-[5, *Ureido*-$^{13}C_2$]citrulline	0.4058	β	—[b]	8.0	47.8
		β	10.5[b]	5.0	158.0

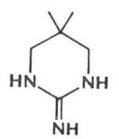
[a] L-[5-^{14}C]Ornithine was used for the tracer.
[b] [*Ureido*-^{14}C]citrulline was used for the tracer.

Table 2. Assignments of ^{13}C NMR chemical shifts at 156~164 ppm for cinodine.

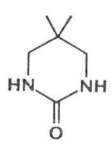
Ring	Carbon number	Ellestad, *et al.*			High field spectra		
		β	γ-1	γ-2	β	γ-1	γ-2
Cinnamoyl	4		162.1		157.4	157.5	157.5
A	7	158.3	158.3	158.3	158.0	158.0	158.0
A	8	157.9	157.9	157.9	159.2	159.1	159.2
B	6	159.7	159.7	159.7	160.9	160.8	160.7
C	6	161.7	160.8	160.5	161.4	161.0	161.3
C	7	—	156.5	163.0	—	156.8	162.5

provided the analyses of 3-bond C–H coupling constants and showed that this resonance is a doublet ($^3J_{CH}$=5 Hz) of triplets ($^3J_{CH}$=3 Hz) arising from coupling to C_1-H of sugar Ring A of Fig. 1 and C_2-H and C_6-H of benzene. No other carbon would produce this pattern.

The H-decoupled spectra of 62.9 MHz (taken on a Bruker WM 250), together with the coupled spectrum of the γ-2 isomer, allowed the remaining resonances to be assigned with little ambiguity, primarily because the high magnetic fields allowed a greater separation of chemical shifts. They are summarized in Table 2 and the numbering scheme is shown in Fig. 1. The two resonances near 161 ppm are clearly attributed from the ureido groups of the amino sugars forming Rings B and C of Fig. 1. The equal peak intensities in the spectrum of the β form suggest that they arise from groups with similar spin relaxation mechanisms, as one might expect for 2-aminoxylose derivatives. Furthermore, they are doublets in the coupled spectrum, as one would predict. The C2 ureido groups in Rings B and C can be differentiated because one at 160.8 ppm is nearly invariant with the cinodine analog studied, whereas those at 161.4 and 161.3 ppm in the β and γ-2 forms move to 161.0 ppm in γ-1. This is consistent with the latter resonance being associated with the C ring. The line at 159.2 ppm is assigned to the ureido group connecting Rings A and B because it is broad and appears to be a doublet of doublets in the coupled spectrum. The resonance at 158.0 ppm, which is the same for three forms of cinodine, is a doublet in the coupled spectrum and, in the β form, its peak height is the same as those of the C2 ureido groups of the aminoxylose rings. Consequently, it is assigned to the C2 amidino group in Ring A. To further support this assignment, one would predict that a guanidino carbon should be more shielded than a ureido carbon. For example, the chemical shift of the guanidino carbon in D is 157.0 ppm, whereas the ureido carbon in E is at 166 ppm.



D



E

Results and Discussion

In a previous study[1] we found that the efficiency of tyrosine incorporation decreases with increased concentration of tyrosine in the media and that high efficiencies would be obtained at low doses. It was also observed that it was important to add the tyrosine at the beginning of antibiotic production. Furthermore, it was observed that greater than 80% of the radioactivity incorporated was incorporated into the antibiotic in the 24 hours following the addition of the labeled substrates and the balance was incorporated during the following 24 hours, and that longer periods did not yield any more labeled antibiotic, although much more cinodine was produced. The same pattern was shown to be true for labeled glucosamine, although the results were less dramatic. The logical explanation is that the glucosamine metabolic pool is much larger than the tyrosine pool, and more labeled substrate can be added without making a large change in the metabolic pool. It would appear that substrates present in excess of what can be utilized in a short time are utilized for purposes other than antibiotic production. By adding the labeled substrate over a period of time that coincides with antibiotic production, we have been able to significantly improve the incorporation of the labels. This procedure keeps the concentration of the substrate low at all times and at the same time allows the overall addition of relatively large amounts that are needed for the preparation of larger amounts of labeled antibiotic. We were able to obtain significantly better yields with a chemically defined media having only glutamate and glucose as carbon sources. The increased yields are likely due to less dilution of the labeled substrates by components in the media. A secondary advantage was that it was easier to control the fermentation because of less foaming and the quantity of acid used in controlling the pH could be correlated well with the progress of the fermentation.

We hope to learn the biogenesis of the four carbonyl and the single guanidino groups in the antibiotic by feeding labeled arginine and citrulline. L-[*Ureido*-^{14}C]citrulline, L-[*guanidino*-^{14}C]arginine and L-[*U*-^{14}C]arginine all are efficiently incorporated[2] and results from the two labeled arginine compounds indicate that both the ornithine and guanido moieties are incorporated. When the antibiotics produced from the ureido labeled citrulline and the guanidino labeled arginine were compared by ^{13}C NMR spectroscopy (Varian FT 80), the labeling patterns were found to be identical. In both cases only the guanidino carbon at 158 ppm[16] showed enrichment and no ureido groups were labeled. Urea was utilized very inefficiently[2]. A single attempt to incorporate potassium [^{13}C]cyanate, a possible precursor of carbamoyl phosphate did not exhibit enrichment of any carbons in the antibiotic. From the results obtained, it would appear that the urea cycle is not directly important in the biosynthesis of cinodine. The guanidino group is derived from arginine by an amidinotransferase. This enzyme has been well established in *Streptomyces*[17,18]. Citrulline may be converted to arginine before use. This could account for the slightly decreased efficiency of citrulline as compared to arginine.

Tracer experiments have established that spermidine is produced from methionine and ornithine[19,20]. Recently BILLINGTON and coworkers[21] have shown that methionine is incorporated exclusively with the No. 4 carbon from methionine attached to the secondary amino group of spermidine. We have prepared DL-[5-^{13}C]ornithine and have found that this substrate is incorporated with good efficiency and exclusively into spermidine with the labeled carbon attached to the secondary amino group. The carbon-13 enriched carbon appeared at 47.8 ppm which has been assigned by ELLESTAD *et al.*[16]. It is interesting that putrescine could also be incorporated at lower yields even though the results obtained here would indicate that free putrescine is not in the biosynthetic pathway to the antibiotic. The results obtained from 5- and ureido carbon-13 doubly labeled citrulline showed an 8-fold enrichment in the

spermidine moiety and 5-fold in the guanidino group. This is consistent with the citrulline being converted to arginine which then serves as the immediate precursor of the guanidino group.

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