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L-Canavanine Synthesis by Zinc-Mediated Guanidination of L-Canaline with Cyanamide

ALVYDAS J. OZINSKAS AND GERALD A. ROSENTHAL

T H Morgan School of Biological Sciences, University of Kentucky, Lexington, Kentucky 40506

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A novel procedure is described for the synthesis of L-canavanine, 2-amino-4-(guanidinooxy)butyric acid, using cyanamide in the presence of zinc cations for guanidination of the copper salt of L-canaline in nearly quantitative yield. [^{14}C]Cyanamide–zinc(II), obtained from the barium salt, is used to prepare L-[*guanidinooxy*-^{14}C]canavanine in high yield. Application of cyanamide-zinc(II) to the synthesis of isotopically labeled canavanine derivatives is discussed. Improvements are reported in the procedure for guanidination of L-canaline with *O*-methylisourea.

L-Canavanine (**1**)[1], the 5-oxa analog of L-arginine, is found in hundreds of leguminous plants (*1*). Canavanine can be stored in both the plant and its seeds, and there is considerable evidence that such stored canavanine provides an effective protective barrier against predation and disease (*1–3*). In canavanine-susceptible organisms, canavanine is activated by arginyl-tRNA synthetase, and the resulting formation of canavanyl-tRNA^{Arg} culminates in substitution of canavanine for arginine in proteins (*4*). This causes structural and functional defects that can elicit a wide range of deleterious biochemical effects. This potentially toxic nonprotein amino acid also exerts its antimetabolic effects through competition with arginine in virtually all enzymatic reactions for which arginine is a substrate (*3*).

Most procedures for the synthesis of canavanine have relied on guanidination of the Cu^{2+} salt of canaline (**2**) with *O*-methylisourea in modest yields (*5–7*). Canavanine has also been prepared by reaction of canaline α-*N*-benzoyl ethyl ester hydrochloride salt with cyanamide, followed by deprotection (*8*). The value of [^{14}C]canavanine as a biochemical probe prompted us to reevaluate the synthesis of canavanine with the emphasis on high yielding procedures that are suitable for isotopic labeling of the guanidinooxy group. We studied various metal-catalyzed guanidination reactions and found that in the presence of zinc cations, cyanamide reacts quantitatively with a slight excess of the Cu^{2+} salt of L-canaline (**2**) to produce L-canavanine (**1**). Since, similarly, [^{14}C]cyanamide–zinc(II) was applied

[1] Due to the structural similarity between canavanine and arginine, it has been commonly assumed that the guanidinooxy group exists in the imino form, comparable to the guanidino group of arginine. The recent elucidation of the X-ray crystal structure of canavanine demonstrated that contrary to prevalent belief, the guanidinooxy double bond occurs between the carbon and the internal 6-nitrogen, at least in the crystalline state, so that the guanidinooxy group is in the amino form. See Boyar, A., and Marsh, R. E., *J. Amer. Chem. Soc.* (1982) **104**, 1995.

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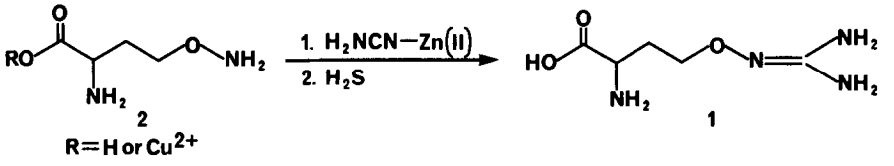
successfully to the high yield synthesis of enantiomerically pure L-[*guanidinooxy*-^{14}C]canavanine, universally labeled [^{14}C]canavanine can now be prepared from [^{14}C]canaline.

RESULTS AND DISCUSSION

Improved conditions were established for protection of the α-amino group of canaline as the cupric salt. Reaction of canaline with CuO for a longer time[2] than used previously (*7*) circumvented potential interference by the aminooxy group, and resulted in complete protection of the α-amino group during canaline guanidination with *O*-methylisourea. This eliminated α-guanidinated side products which we observed in a combined yield of 25%, and which previously attenuated the final canavanine yield of 25%. This and other improvements described in the Experimental section resulted in crystalline canavanine in 50% yield, potentially useful when *O*-methylisourea is the starting material of choice.[3]

Cyanamide in the presence of zinc cations was markedly superior to *O*-methylisourea as a guanidinating agent for the synthesis of L-canavanine (**1**) from L-canaline (**2**). Stoichiometric amounts of Zn^{2+} and various other metal cations were compared for their efficacy in cyanamide–metal-mediated guanidination of canaline–Cu^{2+} to canavanine (**1**) (Table 1). Quantitative canavanine (**1**) formation occurred in the presence of Zn^{2+}, whereas Cu^{2+} and Fe^{3+} catalyzed canavanine synthesis from cyanamide to a lesser extent. In the presence of Ba^{2+} or Mg^{2+}, or in the absence of a metal catalyst, no detectable canavanine formation occurred under the given reaction conditions. Due to the limited amount of unreacted canaline (**2**), the canavanine (**1**) so formed was crystallized directly from the crude reaction mixture following filtration of the insoluble metal sulfides. Shorter reaction times at reduced temperature were possible by increasing the canaline (**2**) concentration (Table 2), but ion-exchange chromatographic purification of the product then became necessary to eliminate unreacted canaline (**2**).



[2] This reaction required vigorous stirring with an excess of CuO for several minutes at 100°C, followed by overnight stirring at reduced temperatures. In comparison, formation of the copper salt of ornithine from CuO is complete within minutes at 100°C.

[3] *O*-Methylisourea is more stable than free cyanamide, and for various reasons, may on occasion be the starting material of choice. Metal salts of cyanamide which contain isotopes of carbon can be prepared by classical routes using carbonate metal salts in reaction with a large excess of N_2 (*7*). Due to the expense of $^{15}N_2$, other synthetic routes are used to prepare [^{15}N]cyanamide, and these result in free [^{15}N]cyanamide. Free cyanamide is less stable than cyanamide in the salt form, and can undergo dimerization and other reactions. For example, samples of [^{15}N]cyanamide which were prepared commercially at our request were void of dectable cyanamide upon receipt, whereas ^{15}N-labeled *O*-methylisourea may have been supplied more advantageously.

TABLE 1

EFFECT OF VARIOUS METAL CATIONS ON L-CANAVANINE (1) YIELD FROM THE REACTION BETWEEN CYANAMIDE AND L-CANALINE–Cu^{2+} (2)[a]

Cation[b]	Yield (%)
—	0
Mg^{2+}	0
Ba^{2+}	0
Co^{2+}	0
Cu^{2+}	50
Fe^{3+}	60
Zn^{2+}	100

[a] Reactions were conducted at 45°C for 72 h with stoichiometric ratios of reactants.

[b] All metals were added as the chloride salts.

Reaction mixtures that contained a nearly stoichiometric ratio of canaline–Cu^{2+} to cyanamide with Zn^{2+} developed an unusual pink precipitate within 1 h at 45°C. This was accompanied by a solution color shift from blue to violet and a severe reduction in color intensity. These phenomena were not observed when either Zn^{2+} or Cu^{2+} were excluded from the reaction mixture,[4] or if the canaline–Cu^{2+} concentration was twofold or greater. This apparent concentration-dependent interaction between zinc(II) and copper(II) facilitated the reaction, because exclusion of Cu^{2+} from the reaction mixture required a significant excess of canaline (**2**) to drive the reaction to completion (Table 2). This result revealed that selective reaction of the aminooxy group was possible under these reaction conditions. Although the 16-h reaction period to form canaline–Cu^{2+} in this case can be eliminated, ion-exchange chromatography must be used to purify the product.

We found that ^{14}C- or ^{13}C-labeled cyanamide–zinc(II) were easily prepared from $Ba^{14}CO_3$ or $Ba^{13}CO_3$, respectively (*8*). These reagents were applied effectively to the synthesis of L-[*guanidinooxy*-^{14}C]canavanine or L-[*guanidinooxy*-^{13}C]canavanine, respectively. The value of this guanidinating agent was further revealed by the radiochemical and enantiomeric purity of the ^{14}C-labeled canavanine, demonstrated by enzymatic assay (see Experimental section).

We further observed that cyanamide–zinc(II) converted hydroxylamine hydrochloride to hydroxyguanidine in 95% yield, higher than with cyanamide alone (*9*). This was determined by pentacyanoammonioferrate assay (*7*) in comparison with authentic hydroxyguanidine, which had a linear absorbance response up to 1000 nmol. Preliminary observations have also indicated that *O*-substituted hydroxylamines may be converted to hydroxyguanidines with this procedure.

The relative ease of producing cyanamide, the ability to incorporate a variety of isotopes into this compound, and its successful application in preparing canava-

[4] Reaction mixtures in which copper was excluded were clear and colorless.

TABLE 2

PARAMETERS OF L-CANAVANINE (1) SYNTHESIS BY GUANIDINATION OF CANALINE (2)

Guanidinating agent, eq		Canaline–Cu^{2+}	Zn^{2+}	Temperature	Time	Yield
Cyanamide	*O*-Methylisourea	(eq)	(eq)	(°C)	(h)	(%)
	1.0	4		45	72	60
	1.0	4	1.3	45	72	40–50
1.0		4	0.5	45	48	84
1.0		4	1.0	45	48	92
1.0		4	1.3	45	48	100
1.0		1.1	1.3	45	48	100
1.0		1.1 (–Cu^{2+})	1.3	45	24	71
1.0		2 (–Cu^{2+})	1.3	25	24	100

nine from canaline derivatives will greatly improve production of a variety of isotopically labeled canavanine derivatives for subsequent chemical, biochemical, biological, and toxicological studies.

EXPERIMENTAL PROCEDURES

Synthesis of L-*canavanine* (1) *from O-methylisourea and* L-*canaline* (2). L-Canaline (2) (*10, 11*) (536 mg, 4.0 mmol) and 500 mg (6.3 mmol) of CuO were swirled in 15 ml of deionized water at 100°C for 3–5 min. The mixture was then stirred vigorously for 2 h at 45°C, and finally for 24 h at room temperature. Reaction of CuO with canaline for a longer time or at an excessive temperature must be avoided since an insoluble copper salt of canaline forms. Unreacted CuO was removed by filtration. *O*-Methylisourea hydrochloride (1.0 mmol) was dissolved in the canaline–Cu^{2+} solution and the resulting clear-blue solution was adjusted to pH 9.1 with 1 N NaOH. The reaction mixture was sealed and stirred at 45°C for 72 h; the pH was adjusted periodically to 9.1 with 1 N NaOH. The reaction mixture pH was reduced to 5.5 with 1 N HCl and treated with H_2S for 10 min, followed by a stream of air for an equal time. Precipitated materials were removed by filtration, the colorless solution was evaporated under reduced pressure to about 5 ml, and applied to a 20 × 40 mm column of Dowex 50-X8 (NH_4^+). After washing the column with 1 liter of deionized water, canavanine was eluted with 300 ml of 50 mM ammonia. The eluant was evaporated to 4–5 ml under reduced pressure, adjusted to ph 5.5 with 1 N HCl, and applied to a second column exactly as described above. The tandem ion-exchange chromatographic procedures removed more than 99% of the unreacted canaline from the canavanine product; the latter was obtained in 60% yield. The column eluant was concentrated under reduced pressure, decolorized with charcoal, and crystalline canavanine (water/ethanol) was obtained in 50% yield. Product analysis is described in the following section.

Synthesis of L-*canavanine* (1) *from cyanamide–zinc(II) and* L-*canaline* (2). A

solution containing 1.1 mmol of canaline–Cu^{2+} (see previous section) in 4 ml of water was adjusted to pH 9.1 with 1 N NaOH. This solution was added to a solution containing 42 mg (1.0 mmol) of cyanamide and 177 mg (1.3 mmol) of $ZnCl_2$ in 2 ml of water; the final reaction mixture pH was 6.4. It is most important to observe the stated addition sequence of reagents to prevent zinc precipitation as the relatively insoluble hydroxide. The reaction mixture, stirred vigorously at 45°C for 48 h, was terminated by bubbling H_2S through the reaction mixture for 10 min, followed by air for an equal time. The metal sulfides were removed by filtration. A faint yellow color in the filtrate was observed occasionally; it was removed with charcoal. The decolorized filtrate was concentrated under reduced pressure and canavanine was obtained by crystallization (*10*) from water/ethanol in 90 to 95% yield. An analytical sample was obtained by recrystallization from the same solvent mixture: mp (corr) 172°C [lit (*11*) mp 172°C]; NMR (D_2O, DSS internal reference) δ2.18 (m, 2H, β-CH_2), 3.88 (m, 3H, α-CH, γ-CH_2). *Anal.* Calcd for $C_5H_{12}N_4O_3$: C, 34.12; H, 6.87; N, 31.98. Found: C, 34.41; H, 6.87; N, 31.91.

The identity and purity of canavanine were further verified by automated amino acid analysis using a Durrum D-300 Amino Acid Analyzer and by the pentacyanoammonioferrate colorimetric assay for canavanine (*10*).

Radiochemical synthesis of L-[*guanidinooxy*-^{14}*C*]*canavanine.* Since barium [^{14}C]cyanamide (*12*) generally contains variable amounts of the unreacted precursor $Ba^{14}CO_3$, a purified solution of [^{14}C]cyanamide was prepared. To 2 ml of water were added 9.28 mg (3.0 mCi, 58 mCi/mmol) of barium [^{14}C]cyanamide. The suspension was stirred vigorously for 1 h at 2°C to fragment the solid prior to the dropwise addition of 0.60 ml of 0.1 M H_2SO_4. The mixture was stirred for a second hour while the temperature was maintained at 2°C. Insoluble materials were removed by filtration through Whatman No. 50 paper. The filtrate was adjusted to pH 6.6 with 1 N NaOH, 9 mg (0.06 mmol) of $ZnCl_2$ in 1 ml H_2O were added to the filtrate, and the solution was filtered again as above. The resultant solution contained 2.1 mCi (0.036 mmol) of [^{14}C]cyanamide–zinc(II), and was evaporated under reduced pressure to a volume of 1 ml. The reaction was initiated by the addition of 0.06 mmol of canaline–Cu^{2+} (pH 9.1, see previous sections); the final volume was 1.5 ml. L-[*Guanidinooxy*-^{14}C]canavanine synthesis was conducted for 72 h at 45°C. The reaction mixture was filtered and titrated to pH 4.6 with 1 N HCl. After applying the filtrate to a 20 × 40-mm column of Dowex 50-X8 (NH_4^+), the resin was washed with 2 liters of water to ensure complete removal of carbon-14-containing impurities prior to elution of L-[*guanidinooxy*-^{14}C]canavanine. The product was eluted with 500 ml of 300 mM ammonia. The eluant was reduced to a final volume of 2 ml by rotary evaporation and adjusted to pH 2 with 2 N HCl. This solution contained 1.8 mCi (0.031 mmol) of L-[*guanidinooxy*-^{14}C]canavanine, 87% yield, based on purified [^{14}C]cyanamide–zinc(II). The radiochemical purity was established by treating the L-[*guanidinooxy*-^{14}C]canavanine with arginase and urease and quantitatively trapping evolved $^{14}CO_2$ (*12*). This revealed that virtually all of the carbon-14 contained in the product was L-[*guanidinooxy*-^{14}C] canavanine. This radiometric assay also established the optical purity of the canavanine since D-canavanine is not a substrate for arginase. The acidified L-[*guanidinooxy*-^{14}C]canavanine solution was stored at −60°C. Our preparations

have retained full radiochemical stability and biological activity for several months under these storage conditions.

Finally, it is important to note that pure barium [^{14}C]cyanamide is difficult to prepare, can polymerize or exchange with atmospheric CO_2, is subject to decomposition on storage, and conversion to urea can occur upon adsorbing water. Moreover, it may contain $Ba^{14}CO_3$ from which it is routinely prepared. *The possibility of generating $^{14}CO_2$ upon contact with acid must not be overlooked. As a result, care must be taken with any preparation to ensure that manipulations described are conducted in a well-ventilated fume hood.*

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