Preparation of Nitroso-^{13}N-Labeled Nitrosoureas

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A method is described for the preparation of ^{13}N-labeled *N*-nitrosoureas, specifically 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea. The ^{13}N is generated as ammonia by the $^{12}C(d,n)^{13}N$ reaction on methane gas. The product is selectively trapped and subsequently oxidized to nitrous acid which reacts with the parent urea in solution to form the ^{13}N-labeled nitrosourea.

Various *N*-nitrosoureas have recently been used in experimental cancer chemotherapy.[1-3] Several workers have studied the mechanistic action of these drugs. Montgomery et al.[4] have examined the modes of decomposition of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and have postulated that intermediates produced during decomposition account for the drug's chemotherapeutic effectiveness. With ^{14}C-labeled BCNU tagged in both ethyl groups and in the carbonyl group, Wheeler et al.[5] have determined tissue distributions of the drug and/or its metabolites in mice and hamsters at intervals of 2, 6, 24, and 48 hr following injection. DeVita[6] and coworkers have shown that drug action probably occurs much more rapidly. With [^{14}C]ethyl-labeled BCNU, equilibration of radioactivity in plasma and cerebral spinal fluid (CSF) occurred in 15 min. Similar results were obtained with man and monkey by De Vita and Oliverio.[6] In the metabolism of *N*-nitrosoureas it is believed that two fragments, an isocyanate and an azohydroxide, result from initial cleavage. These fragments can lead to alkylation or carbamoylation, respectively. Although both fragments could be responsible for the effectiveness of these drugs,[7] Wheeler[8] has postulated that alkylation was predominant in the antileukemia activity of 1-(2-haloethyl)-1-nitrosoureas. Likewise, Oliverio[9] has demonstrated that activity of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) labeled with ^{14}C in the ethyl portion (the alkylating fragment) was found to concentrate in CSF at a level three times greater than in plasma, whereas the activity of CCNU labeled in the cyclohexyl portion (the carbamoylating fragment) was found about equally in CSF and plasma. These data could account for the high activity of CCNU against intracerebral inoculated leukemia. By labeling the alkylating portion of CCNU with a γ-emitting radionuclide, we hope to elucidate further the mechanistic action of these drugs and to gauge their tumor localizing potential and consequent therapeutic effectiveness. Nitrogen-13 was selected for the following reasons: (1) it does not change the chemical nature of the drug; (2) it decays by positron emission with the subsequent production of energetic (511 keV) γ rays which permit facile external scintigraphy for in vivo studies; and (3) its short half-life (10 min) contributes to the reduction of patient body burdens.

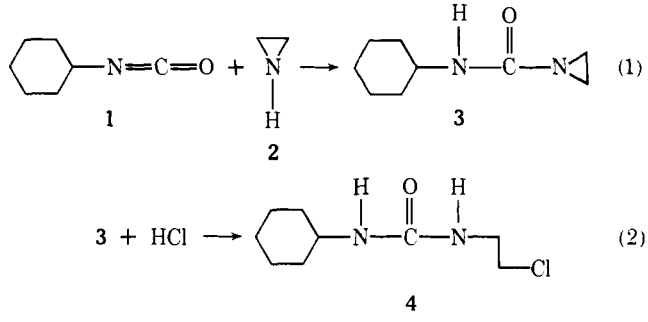
Nitrogen-13 has been prepared as nitrogen gas[10] by irradiation of graphite or carbon dioxide with deuterons and used in lung ventilation and perfusion studies. ^{13}N-Labeled ammonia[11] has been prepared by irradiation of methane with deuterons or by proton irradiation of water with subsequent reduction of NO_3^- to NH_3. Ammonia thus produced has been used in myocardial scanning, liver and kidney function tests, and in the enzymatic synthesis of ^{13}N-labeled glutamine, glutamate, and other amino acids.[11-13] Ammonium chloride with this label also has potential for localizing certain tumors.[14]

Our initial efforts have resulted in the preparation of CCNU with the radionuclide, ^{13}N.

Results and Discussion

Since *N*-nitrosoureas are generally prepared from the reaction of nitrous acid and the corresponding urea, a method was developed for conversion of $^{13}NH_3$ to $HO^{13}NO$. Preparation of CCNU was initially selected because this compound was produced as a more tractable solid in the synthetic procedure than BCNU. The preparation of 1-(2-chloroethyl)-3-cyclohexylurea (CCU), used in this work, is outlined in Scheme I.

Scheme I

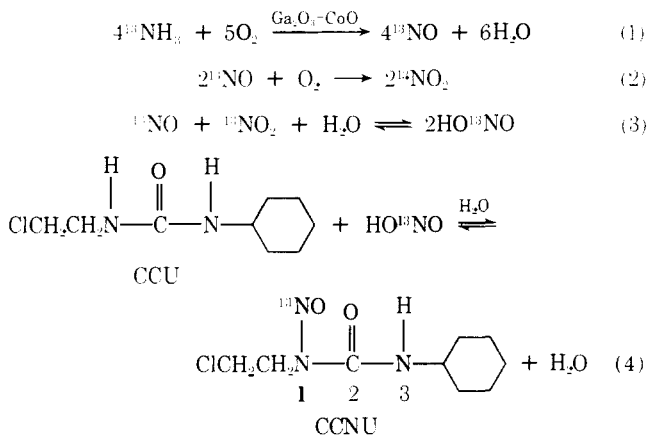


Nitrogen-13 ammonia was produced by deuteron bombardment of methane utilizing the method of Tilbury.[15] Total nitrogen-13 activity could be accounted for as $^{13}NH_3$ and $C^{13}N^-$. Cyanide was a minor product and not considered important to any subsequent chemistry. The $^{13}NH_3$ could be effectively condensed in a trap immersed in a Dry Ice and acetone bath. It was purged from the trap at the end of bombardment with a mixture of carrier ammonia and air. This mixture was passed over a heated catalyst of gallium and cobalt oxides and finally bubbled through cold formate buffer containing CCU. The chemistry involved in this procedure is given in Scheme II.

Following precipitation the nitrosourea was collected by filtration, washed, dried, and counted. In order to ascertain chemical as well as radiochemical purity, the product was dissolved, reprecipitated, and processed again as above. In a series of four determinations [^{13}N]-CCNU was obtained with a specific activity of 0.2 μCi/mg at approximately 1 hr following the end of bombardment. Specific activities before and after reprecipitation were within 10%. A slight increase in specific acitivity was noted following reprecipitation in each determination. This result probably indicates a slight increase in radiochemical purity.

The production of [^{13}N]nitrous acid is significant since [^{13}N]nitrosoureas and a variety of medicinally significant compounds can be prepared from it. Therefore the prob-

Scheme II



lems concerning the conversion of ammonia to nitrous acid merit some additional consideration.

Initial efforts in this investigation were concerned with optimization of the NO and NO_2 ratio (Scheme II, eq 3). By using an air/ammonia ratio of approximately 9:1, György[16] obtained NO in 93% yield. By reducing the concentration of ammonia in the gas stream to approximately 7%, a portion of the NO produced was oxidized to NO_2 (Scheme II, eq 2) by excess oxygen in the gas stream. Peak height of mass spectral analysis of the gas stream from the catalyst showed that both gases were present in the ratio of 1.8:1.0, respectively.

In order to ensure maximum nitrous acid production, aqueous formate buffer was used to shift the equilibrium in eq 3 (Scheme II) to the right. Use of water in the reaction medium introduced an additional problem. Since CCU is an unsymmetrical urea, nitrosation can occur at either of the two nitrogens. In aqueous solution both isomers, the 1- and 3-nitroso compounds, are obtained in 65 and 35% yield, respectively, as demonstrated by Johnston et al.[17] We have found that reprecipitation of the isomeric mixture in water gave the 1-nitroso isomer but only at considerable reduction in yield. The product was analyzed by melting point and TLC and uv spectra and was found to have the same physical properties as those previously reported.[17] Chemical yields based on 50 mg of CCU ranged from 4.6 to 21% following reprecipitation of the nitrosourea. Although determination of both the amount of $^{13}NH_3$ and [^{13}N]-CCNU could not be determined in a single run due to our system design, an approximate radiochemical yield can be derived based on the separate determinations under the same experimental conditions. An average of 10.9 mCi of $^{13}NH_3$ was collected. The radiochemical yield varied with the chemical recovery over a range from 0.3 to 1.5% in a synthesis time of 60–65 min. Presently efforts are underway to increase product specific activity and to determine the applicability of this method to the preparation of other compounds of medical importance.

Experimental Section

A high-voltage Engineering Corp. 6 MeV Van de Graaff accelerator was used to generate the deuteron beam. Methane (UHP grade) was obtained from Matheson Gas Co. Melting points were determined in a Thiele tube containing silicone oil and are uncorrected. Infrared and ultraviolet spectra were obtained from Beckman IR-8 and Beckman-24 uv-visible spectrophotometers, respectively. Mass spectra were performed on a Hitachi Perkin-Elmer RMU-7 spectrometer. Thin-layer chromatograms were carried out on Eastman precoated silica gel plates and were developed in an iodine chamber. Sample radioactivity quantitation was determined on a Squibb dose calibrator Model CRC-6A and a Picker Spectrascaler 4 well counter. Analyses were performed by Micro-Analysis, Inc., Wilmington, Del.

Production of [^{13}N]Ammonia. [^{13}N]Ammonia was produced by the $^{12}C(d,n)^{13}N$ reaction on methane gas in a closed, recirculating system. The amount of [^{13}N]ammonia produced was determined by bubbling the target gas through a bubbler similar to the one previously described.[15] The activity was collected in 5 ml of water which was subsequently tested with Nessler's reagent. For a typical 25-min bombardment at a beam current of 3.5 μA, 10.5 mCi of [^{13}N]ammonia was collected. For [^{13}N]nitrosourea production runs the bubbler was replaced by a glass coil trap cooled in Dry Ice and acetone.

Cyclohexyl-*N*-carbamoylaziridine (3). A solution of 1.25 g (10.0 mmol) of 1 in 10 ml of $CHCl_3$ was added to a solution of 0.50 g (11.6 mmol) of 2 prepared by Wystrach's method[18] in 10 ml of $CHCl_3$ cooled to 0°. The cooled solution was stirred for 10 min. Most of the $CHCl_3$ was evaporated by gentle heating and 15 ml of warm hexane was added. The solution was filtered and on cooling produced 1.26 g (75%), mp 78–79° (lit.[19] 75–76°), of 3 following washing with hexane. Anal. ($C_9H_{16}N_2O$) C, H, N.

1-(2-Chloroethyl)-3-cyclohexylurea (4). 3 (1 g, 5.95 mmol) was added to 10 ml of concentrated HCl in an ice bath. The mixture was stirred for 0.5 hr and diluted with 50 ml of H_2O. The product (0.90 g, 74%), mp 128–129° (lit.[8] 130°), was filtered and washed with water: ir (KBr), uv (MeOH), *m/e* 204.

1-(2-Chloroethyl)-3-cyclohexyl-1-[^{13}N]nitrosourea. General Method. Following collection of $^{13}NH_3$ in the coiled trap, the Dry Ice and acetone bath was replaced by a 50–60° water bath. A mixture of air–NH_3 (93:7) was conducted through the trap at respective flow rates of 125 and 10 ml/min. The gases were passed over 4.1 g of 16% Ga_2O_3 and 84% CoO catalyst heated to 400°. The catalyst was supported by a porous ceramic disk within a 1.7 × 48.6 cm quartz tube. The tube was wrapped with 18-gauge Chromel A wire and covered with asbestos tape. Heating was controlled by a rheostat. The effluent gases from the catalyst were bubbled into 2.0 ml of HCOOH–H_2O (2:1) solution buffered with Na formate (5.3 g/100 ml) and containing 50 mg (0.24 mmol) of 4. The solution was cooled to 0–5° and stirred magnetically. The precipitate was filtered and washed with buffer, followed by 10 ml of H_2O. The product was dried under high vacuum, counted, and weighed to the nearest 0.1 mg. The compound was dissolved in acetone and reprecipitation effected by addition of 10–20 ml of H_2O. Filtration, washing, drying, counting, and weighing were repeated.

Typical Result. Following initial precipitation 8.5 mg (15%) of CCNU was obtained and counted. Subsequent dissolution, reprecipitation, washing, and drying yielded 2.6 mg (4.6%): mp 89–90° (lit.[8] 90°); uv (MeOH); TLC (2:1, hexane–$CHCl_3$) R_f 0.18. The specific activity at 60 min from the end of bombardment was 0.197 μCi/mg.

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3-Hydroxymethyl-*s*-triazolo[3,4-*a*]phthalazine, a Novel Urinary Hydralazine Metabolite in Man

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The elucidation of the structure of a new major metabolic product of hydralazine, 3-hydroxymethyl-*s*-triazolo[3,4-*a*]-phthalazine, is described. The structures of several other previously described metabolites of the drug, phthalazone, *s*-triazolo[3,4-*a*]phthalazine, and 3-methyl-*s*-triazolo[3,4-*a*]phthalazine, are confirmed. A metabolic pathway of hydralazine is also proposed.

The formation of antibodies to hydralazine (1) and/or its metabolites may play an important role in the pathogenesis of drug-induced systemic lupus erythematosus. Furthermore, the occurrence of this syndrome in patients on hydralazine therapy has been correlated with its rate of hepatic acetylation.[1,2] However, the metabolic fate of hydralazine has not been completely described in man or even laboratory animals. Our laboratories[3–5] and others[6] have shown that certain hydralazine metabolites are derived of the *s*-triazolo[3,4-*a*]phthalazine system. While these are unusual metabolites, their chemical structure is not conducive to the ready formation of covalently bound hapten–protein complexes capable of inducing production of antibodies and eventually the lupus syndrome. We wish here to report the discovery and structural elucidation of 3-hydroxymethyl-*s*-triazolo[3,4-*a*]phthalazine (**3**) as a human urinary metabolite of hydralazine. Among the known hydralazine metabolites **3** is the first one which possesses a functional group; the hydroxyl group may provide a handle by which a covalent bond to a protein could be formed. In addition, we confirmed the presence of *s*-triazolo[3,4-*a*]phthalazine (**5**) and phthalazone (**6**) as urinary metabolites of 1.[6]

Possible metabolic pathways giving rise to the production of **3** are shown in Scheme I. It arises very probably from metabolite **2** by enzymatic hydroxylation. An alternate pathway for the formation of **3** may possibly be conjugation of 1 with glycolic acid followed by ring closure. Attempts were made to prepare *s*-triazolo[3,4-*a*]phthalazine-3-carboxylic acid (**4**) by oxidation of **3**. The only product isolated was **5**. This finding suggests that **5** arises from **3** by further oxidation and subsequent decarboxylation. In a separate reaction, phthalazone was obtained by subjecting 1 to air in a slightly alkaline medium. This finding suggests that the appearance of **6** as a urinary metabolite of 1 can be due to its enzymatic and/or chemical oxidation.

The novel metabolite **3** is excreted only as a glucuronic acid conjugate. According to our estimates **3** seems to be a major urinary metabolite of 1 in man.

For this investigation we had 14 hypertensive patients available who were on hydralazine therapy ranging from 100 to 400 mg daily. Their 24-hr urine was collected and routinely checked by paper chromatography[3] for the presence of **2**. Every patient produced **2** which gave rise to a strong spot and could be detected without difficulties.[7]

After incubating the urine of these patients with β-glucuronidase, the combined chloroform extracts of the urine samples were subjected to silica gel preparative layer chromatography resulting in a number of bands. Inspection of the uv spectra of the constituents of these bands revealed the presence of compounds with the triazolo[3,4-*a*]phthalazine system in five of them.

An additional band contained **6**, a previously reported metabolite of 1.[6] A strong band with an R_f value of 0.35–0.40 in chloroform–methanol (15:1) and a blue fluorescence was identified as **3**, as follows. First, its uv spectrum was very similar[3] to that of **2**; in addition the spectrum did not show a red shift upon addition of base. This excludes presence of nuclear hydroxy groups. Second, its mass spectrum showed a molecular ion of 200 and its fragmentation[8] is very similar to that of **2**.[4] Third, its spectroscopic properties are identical with those of an authentic sample synthesized by us via a dehydrative cyclization reaction between 1 and glycolic acid (Figure 1).

A strong band with an intense blue-purple fluorescence and an R_f value of 0.5–0.6 was rechromatographed on silica gel in acetone–cyclohexane (1:1). It separated into three new bands which were identified as **2**, **5**, and **6** in agreement with previous investigations.[6] Several of the other bands according to their uv spectra revealed the presence of additional compounds with the *s*-triazolo[3,4-*a*]phthalazine system. However, they are as yet unidentified because of their occurrence in very small amounts. (In order to attempt to identify them arrangements to get a larger group