THE SYNTHESIS OF N-[4-DIMETHYLAMINO-1-BUTYL(1-^{13}C)-N-NITROSOBENZAMIDE, AN INHIBITOR OF TRYSPIN; CYANIDE DOES NOT EXCHANGE WITH ACETONITRILE

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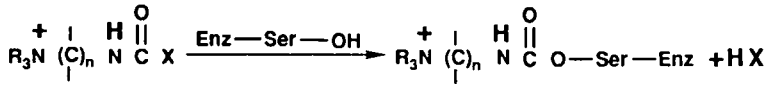
SUMMARY

The synthesis of N-[4-dimethylamino-1-butyl(1-^{13}C)-N-nitrosobenzamide, a powerful active-site-directed inhibitor of trypsin, is reported. An efficient use of ^{13}C-cyanide ion was achieved in the reaction with 1-chloro-3-dimethylaminopropane, the first step of the synthesis. Acetonitrile was used as a co-solvent with water in that step; no evidence was found for cyanide ion exchange with acetonitrile. The earlier report of such an exchange appears to be in error; the formation of hydrocyanic acid under the reaction conditions can account for the observations.

Key Words: carbon-13, trypsin, irreversible inhibitors, cyanide exchange.

INTRODUCTION

Several irreversible inhibitors of trypsin that are 2-aza analogs of the normal lysine-based substrates have been described.(1) Their action is based on the principle of essentially irreversible carbamoylation of a serine hydroxyl group of the enzyme (eq. 1). We report now the synthesis of the ^{13}C-labelled version of a different type of inhibitor,



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R = H or alkyl
X = Good leaving group

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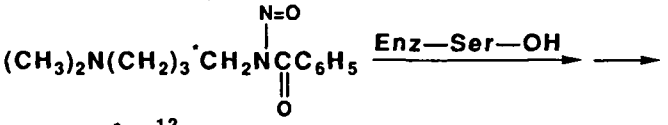
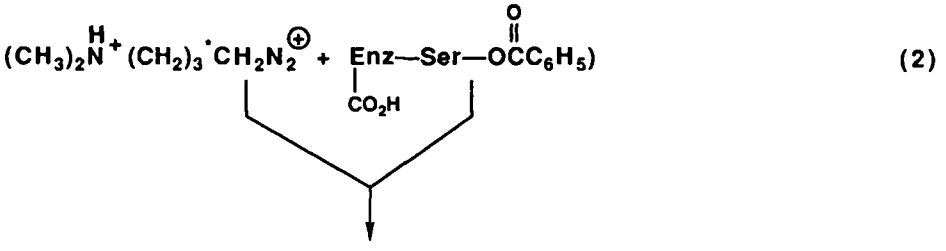
N-(4-dimethylamino-1-butyl)-N-nitrosobenzamide (2), which has the same recognition group as inhibitor 1; nitrosoamide inhibitors of this type inhibit through *alkylation* of the enzyme (2). The ^{13}C-label in analog 2b will be useful in the characterization and location of the



2a,* = ^{12}C
b,* = ^{13}C



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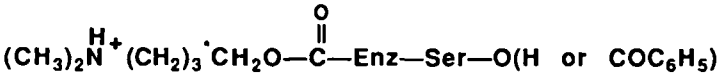
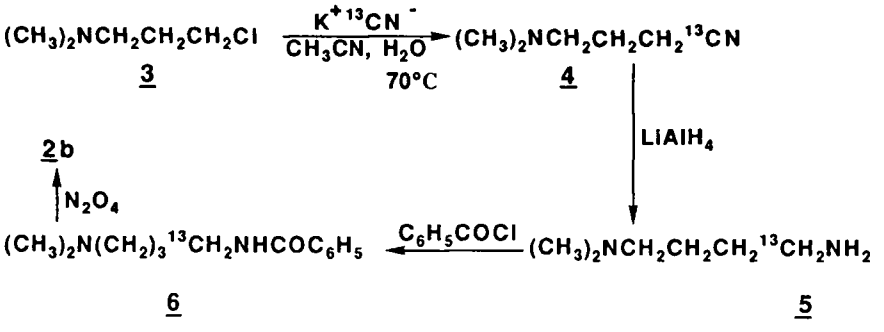


sites of alkylation (3).

The label in inhibitor 2b would appear to be readily introduced by means of ^{13}C-labelled cyanide ion (eq. 3). The synthesis of nitriles by displacement reactions with natural abundance cyanide ion is a common approach; in fact, this reaction has been used in the synthesis of the ^{12}C

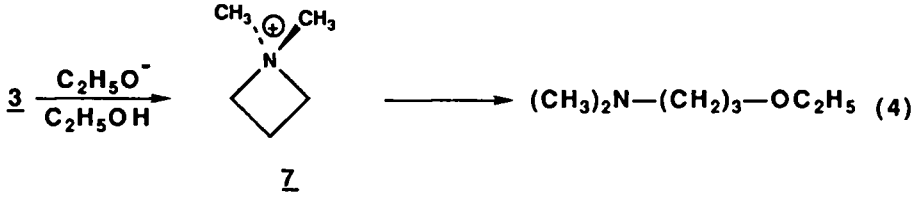


(3)

analog of nitrile 4 (4). Large excesses of cyanide ion are normally employed; the rationale presumably being to increase the speed of the conversion, to efficiently utilize the organic component, and possibly to offset loss of the volatile hydrocyanic acid formed by reaction of the rather basic cyanide ion with proton sources in the medium (see next section). The cost of ^{13}C-labelled potassium cyanide militates against that approach; when labelled nitriles are desired the goal shifts to the efficient use of cyanide ion. Syntheses of labelled nitriles have been reported in which ^{11}C and ^{14}C labelled cyanide ion and water soluble alkylating agents such as chloroacetic acid were used (5). In the case of

^{13}C labelled cyanide ion, however, most of the published uses involve the addition of cyanide ion to the carbonyl groups of aldehydes and ketones (5). As an exception, the reaction of ^{13}C labelled cyanide ion with 1,3-dibromopropane via a displacement reaction has been reported (6), but no literature report of the synthesis of a ^{13}C labelled aminonitrile similar to compound **4** has been found.

The bifunctional starting material, (**3**), for the synthesis of compound **2b** contains a basic amino group, and neighboring group interactions are to be expected. In the reaction of compound **3** with ethoxide ion, the reaction has been shown to proceed via the azetidinium ion **7** (7). Thus, the use of a polar solvent in our case was called for since a solvent for both potassium cyanide and intermediate **7** was desirable . In preliminary runs, the reaction of unlabelled cyanide with 1 molar equivalent of **3** in DMSO, or in water, led to yields of **4** (^{12}C labelled) of about 15-25 and 40-50%,



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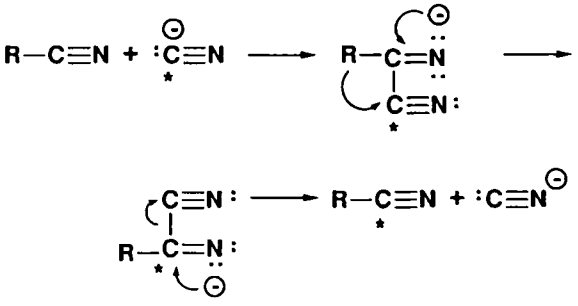
respectively (in the water runs, hydrolysis occurred concurrently to produce the corresponding amide). By changing the reaction conditions, yields of ~ 62% for either the ^{12}C or ^{13}C labelled nitrile **4** were ultimately obtained; the final reaction conditions employed were: **3**/CN^- = 4, solvent = H_2O + acetonitrile (ratio = 1/3, v/v), 70°C heating for 38 h. The four equivalents of the amine function kept the medium basic, obviating the use of external bases and minimizing the formation of hydrocyanic acid.

The subsequent steps, reduction and benzoylation (eq. 3), proceeded smoothly to generate amide **6**. Nitrosation by a modification of the method of White and Aufdermarsh (8) produced inhibitor **2b**.

Non-Exchange Between Cyanide Ion and the Nitrile Function of Acetonitrile. It has been claimed that cyanide ion exchanges with the CN moiety of acetonitrile at 90° (25 min reaction time) (9). The putative exchange was detected and measured via ^{13}C satellites in the ^{1}H-NMR spectra, measurement of the ^{13}CN group in ^{13}C NMR spectra, measurement of the m + 1 peak in mass spectra, and by detection of radioactivity in the distillate when ^{14}C-labelled cyanide ion was used in the experiments. From the data reported, it appears that the extent of exchange was ~ 1%, ~ 0.04%, ~ 4.5%, and 48%, respectively.

To account for these results, the authors proposed the exchange reaction of eq 5. We note, however, that the values measured by NMR and mass spectrometry are small and approximately within normal experimental error ranges. The extent of "exchange" reported for the co-distillation of radioactivity, on the other hand, would appear to be well beyond experimental error. The authors did not analyze the distillate in which radioactivity was found, however, and it is reasonable that the



(5)

distillate contained both acetonitrile and ^{14}C-labelled HCN. The latter compound would arise from proton donation to the relatively basic cyanide ion (pK_A HCN = 9.31) (10) by adventitious water (no indication was given that anhydrous reaction conditions were used). In any case, high incorporation of ^{13}C cyanide ion was observed in our synthesis of nitrile **2b**, even though acetonitrile was used as a co-solvent; had the cyanide ion exchanged with the CN group of acetonitrile, ^{12}C cyanide ion would have been produced, which would have led to ^{12}C-labelled product **2a**. Finally, we heated acetonitrile with potassium cyanide ^{13}C according to the method of Jay, et al. (9), but detected no increase in the NMR signal of the ^{13}C spectrum of the acetonitrile. We conclude that cyanide ion exchange with acetonitrile either does not occur, or it occurs to only a minor extent under the reaction conditions reported (11).

Experimental Section

General. Spectra were measured with Varian XL-400 and Bruker AMX-300 NMR spectrometers, a Perkin-Elmer 1600 Series FTIR spectrometer, and a Beckman Model 25 UV-Vis spectrometer. Potassium cyanide (96.5% pure and 99% ^{13}C labelled) and 1-chloro-3-dimethylaminopropane (3; as the hydrochloride salt) were obtained from the Aldrich Chemical Co.

4-Dimethylaminobutyronitrile-1-^{13}C (4). Acetonitrile (3 ml) and then 1-chloro-3-dimethylaminopropane[12] (3; 2.38 g, 19.4 mmol) were added to a solution of potassium cyanide (0.503 g, 7.34 mmol; 99% ^{13}C) in 1 ml of water (the amine was obtained from the hydrochloride salt by extraction with ether of an aqueous solution containing equimolar amounts of the hydrochloride and sodium hydroxide. The amine was distilled at 25°C and 0.1 Torr). The biphasic mixture was stirred

at 70°C for 38 h. The reaction mixture, on cooling, was diluted with 4 ml of water and extracted with ether (4 x 20 ml). The extracts were pooled and dried over anhydrous sodium sulfate. The organic solvents were removed by distillation at normal atmospheric pressure. The residue (1.70 g) contained, by ^{1}H NMR analysis, acetonitrile and ether and about 50% by weight of compound **4**. This material was heated to 75°C to remove most of the acetonitrile and ether. Heating the product to 80°C at 20 Torr yielded a fraction (0.26 g) containing a small amount (by TLC [silica gel; 1-butanol, acetic acid, water; 40/10/50 (v/v); rf 0.25]) of compound **4**. Distillation was then carried out at 10^{-2} Torr and 25° to yield 0.55 g of an oil containing 94% 4-dimethylaminobutyronitrile (1-^{13}CN) and 6% of acetonitrile (^{1}H NMR integration); the overall yield of nitrile **4** was 63% (4.6 mmol). ^{1}H NMR ($CDCl_3$) δ 2.420 [d of t, J(CH_2 ^{13}CN) = 9.6 Hz and J(CH_2CH_2 CN) = 7.2 Hz, 2H]; 2.380 (t, J = 6.8 Hz, 2H); 2.219 (s, 6H); 1.804 (m, one J value = 6.8 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ 119.8 ppm (relative to $CDCl_3$ at 77.0 ppm (12)). The physical properties are in agreement with natural abundance **4** prepared as described for the ^{13}C-labelled analog and also by a procedure using an excess of cyanide ion (5). This material was used directly in the next step.

N-(4-Dimethylamino-1-butyl)benzamide-1-^{13}C (6). A mixture of lithium aluminum hydride (0.401 g, 10.6 mmol) in 7.5 ml of ether (distilled from lithium aluminum hydride) was added to a solution of 4-dimethylaminobutyronitrile (1-^{13}C)(0.496 g, 4.38 mmol) in 3.0 ml of ether during a period of 10 min, using the general procedure of Zaugg and Horrom (13). The reaction mixture was stirred at O°C under nitrogen for 5 h; then 30 ml of ice-water was added with vigorously stirring, followed by a solution of benzoyl chloride (14) (1.49 g, 10.6 mmol) in 6 ml of ether. The reaction mixture was allowed to warm to room temperature and then it was stirred for an additional 12 h. Water (20 ml) was added, and the mixture was extracted with ether (4 x 40 ml). The remaining aqueous suspension was filtered through Celite and the filter cake was washed with water, ether, and acetonitrile (~ 20 ml @). The filtrate and washings were pooled, then extracted with ether (4 x 40 ml). The organic solvents were dried and removed under vacuum. The residue was dissolved in 30 ml of ether and the solution was extracted with 0.1 *N* HCl (3 x 20 ml). The aqueous phase was taken to pH 14 with 8 *N* NaOH, saturated with sodium chloride, and extracted with methylene chloride (4 x 25 ml). After the organic solvent was removed, the residue was evacuated at 10^{-2} Torr for one h to yield a pale yellow oil (0.97 g); the ^{1}H NMR spectrum showed the presence of ~ 5% of N-ethylbenzamide, which was removed by a second extractive procedure. Most of this material (0.94 g) was dissolved in 20 ml of 0.2 *N* HCl and extracted with ether (4 x 25 ml). The aqueous phase was separated and taken to ~ pH 11 with 2*N* NaOH solution. The basic aqueous mixture was saturated with sodium choride

and extracted with methylene chloride (4 x 40 ml). The solvent was removed and the oily residue was dried *in vacuo* for three h. to produce a pale yellow oil (0.74 g, 3.4 mmol) representing a 77% yield based on the starting nitrile (correcting for the total amount of the crude material (0.97 g), the over-all yield was 80%). ^{1}H NMR ($CDCl_3$) δ 7.783 (d, d, d, J_1 = 6.7 Hz, J_2 = 3.3 Hz, J_3 = 2.0 Hz, 2H); 7.474 (t, t, J_1 = 6.9 Hz, J_2 = 1.6 Hz, 1H); 7.40 (t, d, d, J_1 = 7.1 Hz, J_2 = 2.1 Hz, J_3 = 1.6 Hz, 2H); 3.443 (d, q, J_1 = 138 Hz, J_2 = 6.0 Hz, 2H); 2.351 (t, J = 6.5 Hz, 2H); 2.221 (s, 6H); 1.716 (quintet, J = 6.5 Hz, 2H); 1.618 (quintet, J = 6.5 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ 39.94 ppm ($^{13}CH_2$-) (relative to $CDCl_3$ at 77.0 ppm) (12). In the ^{1}H NMR spectrum, the proton signals for -$^{12}C\underline{H}_2NHCOC_6H_5$ appeared as a quartet at δ 3.443, while those for -$^{13}C\underline{H}_2NHCOC_6H_5$ appeared as quartets on either side of δ 3.443 ($J[^{13}CH_2]$ = 138 Hz); the ratio of the two sets of integrals showed that the 1-position of amide **6** was labelled with ^{13}C to the extent of 99%. IR (^{12}C in $CHCl_3$) 1654, 1604, 1579, 1523, 1487, 1468 cm^{-1}. UV (Ether) 242 nm (ε 2340). TLC (silica gel; CH_3OH, NH_4OH, H_2O, 8:1:1 (v/v); rf 0.52). The physical data are in agreement with values for the natural abundance analog; the elemental analysis for the ^{12}C compound has been reported (15).

N-[4-Dimethylamino-1-butyl(1-^{13}C)]-N-Nitrosobenzamide (2b). (Note: a good fume hood and protective gloves should be used for the following operations.) N-[4-Dimethylamino-1-butyl(1-^{13}C)]benzamide(53 mg, 0.24 mmol) was dissolved in 1.0 ml of chloroform-d) in a tube sealed with a rubber septum and the mixture was cooled to -78°C. Gaseous dinitrogen tetroxide (9) (8.1 ml, 0.29 mmol) was introduced directly over the reaction mixture through a long syringe needle. The solid reaction mixture was then warmed to -20°C and kept at that temperature for 30 min. A ^{1}H NMR spectrum showed that the conversion of the starting material to the product was complete. The solvent and unused dinitrogen tetroxide were removed *in vacuo* at -20°C and the residue was kept under vacuum (10^{-2} Torr) for 5 h at 0°C. The nitrate salt of **2b** was obtained as a yellow oil (55 mg, 0.18 mmol, 75% yield). ^{1}H NMR ($CDCl_3$) δ 9.86 (s, broad, 1H), 7.74 (d, J = 7.7 Hz, 2H), 7.62 (t, J = 6.5 Hz, 1H), 7.50 (t, J = 7.1 Hz, 2H), 3.94 (d, t, J_1 = 143 Hz and J_2 = 7.1 Hz, 2H), 3.15 (m, 2H), 2.91 (d, J = 4.7 Hz, 6H), 1.72 (m, 2H), 1.55 (m, 2H). ^{13}C NMR ($CDCl_3$) δ 38.4 (-$^{13}CH_2$-) (relative to $CDCl_3$ at 77.0 ppm) (12).

To the residue of N-[4-dimethylamino-1-butyl (1-^{13}C)]-N-nitrosobenzamide nitrate salt was added 5.0 ml of CH_2Cl_2 (0°). The yellow solution was transferred to a cold separatory funnel and washed at 0°C with 6.0 ml of an aqueous 3% triethylamine solution saturated with sodium chloride. The organic layer was separated and dried over anhydrous sodium sulphate. The organic solvent was removed *in vacuo* (10^{-2} Torr) at -20°C and the residue was kept under that pressure for 5 h at 0°C. A

yellow oil was obtained (39 mg, 0.16 mmol, 67%). ^{1}H NMR ($CDCl_3$) δ 7.739 (d of t, J_1 = 7.1 Hz and J_2 = 1.7 Hz, 2H), 7.584 (t of t, J = 7.4 Hz and J_2 = 1.7 Hz, 1H), 7.472 (t of t, J_1 = 7.3 Hz and J_2 = 1.4 Hz, 2H), 3.958 (d of t, J_1 = 142 Hz and J_2 = 7.1 Hz, 2H), 2.355 (t, J = 7.2 Hz, 2H), 2.275 (s, 6H), 1.514 (m, 4H). The ratio of the ^{13}C satellites of the 3.958 proton peak to the residual peak at that value indicated an enrichment of ^{13}C of 99%. ^{13}C NMR ($CDCl_3$) δ 39.39 (relative to $CDCl_3$ at 77.0 ppm) (12). IR (CH_2Cl_2) (for ^{12}C analog) 1703, 1602, 1518, 1414 cm^{-1}. UV-VIS (acetonitrile) 425 nm (ε 77), 408 (78), 241 (6890). TLC (silica gel; triethylamine/acetonitrile (3/97, v/v), rf = 0.34).

Heating Potassium Cyanide with Acetonitrile. A mixture of acetonitrile-d_3 (0.50 ml) and potassium cyanide (4 mg, 99% ^{13}C) was prepared in a 5 mm NMR tube and the ^{13}C NMR spectrum was measured. The acetonitrile gave two peaks at 1.93 and 118.9 ppm; the ratio 1.93/118.9 = 1.030 ± 0.028 (7 trials). Acetonitrile-d_3 (0.30 ml) and 18-crown-6 (100 mg) were added and the tube was sealed. It was heated at 90°C for 25 min, producing a clear solution (the conditions used are those reported (10) except that the crown ether concentration was halved to minimize interference of solid in the NMR measurements). Upon cooling, a precipitate formed; the ^{13}C NMR spectrum at this point showed a 1.93/118.9 ratio of 1.053 ± 0.020 (4 trials). Thus, within the experimental error of the method, no change in the ^{13}C content of the acetonitrile occurred.

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