PREPARATION OF ^{15}N LABELED TETRANITROMETHANE $C(^{15}NO_2)_4$

William Skawinski, Joseph Flisak, Alex C. Chung, Frank Jordan, Richard Mendelsohn

Department of Chemistry, Rutgers, the State University , Newark, NJ 07102.

SUMMARY

^{15}N labeled tetranitromethane, $C(^{15}NO_2)_4$, was prepared from anhydrous nitric acid and acetic anhydride. A procedure for the preparation of the nitric acid from $Na^{15}NO_3$ is described. Tyrosine was nitrated with $H^{15}NO_3$ and its ^{15}N NMR spectrum compared well with that of tyrosine nitrated with $C(^{15}NO_2)_4$.

Key words: nitrogen isotopes, tetranitromethane, tyrosine nitration

INTRODUCTION

Tetranitromethane, $C(NO_2)_4$ can be used as a reagent for the nitration of tyrosyl residues in peptides and proteins (1,2). The reaction is specific and is carried out under mild conditions. The nitro group is introduced onto the tyrosyl ring ortho to the phenolic group. The resulting nitrotyrosyl group is a chromophore which can be observed with a variety of spectroscopic techniques and thus is suitable to monitor the local environment into which it is inserted. The chromophore has been monitored by UV/VIS absorption (3-5), ^1H NMR (6,7), and Resonance Raman spectroscopy (7,8) to investigate properties of a number of globular proteins. In addition the nitro group can be readily reduced to an amino functionality which itself can be chemically altered to yield a number of different potential probes (9-12).

When more than one nitrotyrosyl group is present it may be difficult to resolve the signals due to the individual groups because of overlap or interference from other signals. The introduction of an ^{15}N label into the nitro group would thus extend the utility of this probe to ^{15}N NMR spectroscopy.

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EXPERIMENTAL

Anhydrous Nitric Acid $H^{15}NO_3$.

A 25 ml. two-necked round bottom flask was equipped with a 10 ml dropping pressure equalizing funnel, a short path distillation head, thermometer, and receiving flask. To the dropping funnel was added 8 ml of concentrated sulfuric acid (30% SO_3). The system was then put under full pump vacuum and evacuated to a pressure of 0.002 tor. and the receiving flask was placed in a dry ice/acetone bath. The excess SO_3 was collected in the receiving flask as a white powder. Once all the excess SO_3 had been removed, the receiving flask was changed, then 6.00 g (69 mmol) of $Na^{15}NO_3$ was added to the round bottom flask and the receiving flask was again placed in the dry ice/acetone bath. The system was again placed under full pump vacuum and the concentrated sulfuric acid was added to the reaction flask over a 10 minute period. Initially the reaction is vigorous, then it subsides. The flask was then gently heated to 50 °C for 15 minutes to insure complete reaction. The anhydrous $H^{15}NO_3$ was then collected as a white crystalline solid which upon removal from the dry ice/acetone bath became a colorless liquid. A total of 3.167 g (49.5 mmol, ca. 70% yield) of $H^{15}NO_3$ was obtained.

Tetranitromethane, $C(^{15}NO_2)_4$

The procedure described in the literature was followed with minor modifications (13). To a 25 ml two-necked round bottom flask fitted with a thermometer, was added 1.58 g (24.7 mmol) of anhydrous nitric acid, $H^{15}NO_3$. A 10 ml pressure equalizing dropping funnel was then attached to the flask. To the funnel was then added 2.34 ml (24.6 mmol) of acetic anhydride. The flask was then placed in an ice water bath. When the temperature reached 4 °C, the acetic anhydride was slowly added (over approximately 20 min) not allowing the temperature to rise above 10 °C. After the addition of acetic anhydride was completed the thermometer was replaced with a glass stopper and the dropping funnel was replaced with a U-tube fitted with a receiving flask and a calcium chloride drying tube attached to the side arm. The entire apparatus, which was still in the ice bath, was placed in a cold room (ca. 3 °C) for 24 hours. The apparatus was then removed and allowed to warm to room temperature. The pale yellow reaction mixture was allowed to stand at room temperature for 14 days. To the resulting dark yellow solution was added 15 ml of distilled water and this was then allowed to stand for 2 hours. The crude light yellow

tetranitromethane lower layer was then cautiously drawn off with a disposable pipet and placed in a micro steam distillation apparatus and cautiously steam distilled. The clear product was then collected, separated from the distillate and dried over anhydrous sodium sulfate. The product was then gently drawn off with a disposable pipet to yield 715 mg (3.57 mmol, 57.9%) of a colorless liquid.

Following this procedure both ^{14}N and ^{15}N labelled tetranitromethane were prepared. Raman spectra of the products were obtained and their infrared spectra were compared with the spectrum of authentic ^{14}N tetranitromethane. The infrared spectrum of the ^{15}N labelled product in CCl_4 contained a strong peak at 1256.8 cm^{-1} and a weaker feature at 592.1 cm^{-1}. The spectrum of an authentic ^{14}N labelled sample of tetranitromethane contained corresponding bands at 1269.5 cm^{-1} and 602.7 cm^{-1}. Assuming these modes to arise mostly from the symmetric stretching and bending of a bent NO_2 moiety, respectively, the direction and magnitude of the isotopic shifts are in good accord (to within 0.5%) with a simple relationship given by Herzberg(14) for a bent triatomic molecule. IR thus provides excellent structure proof for the isotopically labeled material.

m-[^{15}N]-Nitro-L-tyrosine.

L-Tyrosine was nitrated with nitric acid by a modification of a previously reported method (15). L-tyrosine (479 mg) in 1.72 ml of distilled water was treated with 0.51 ml of 45.45% $H^{15}NO_3$ for 30 minutes with stirring at room temperature. The heterogeneous mixture was then cooled to a temperature of 5-8 °C, and treated during 2 hours with an additional 1.236 ml of the $H^{15}NO_3$. The mixture was then stirred for 5 hours at room temperature and refrigerated overnight. The solid yellow brown product was filtered from the dark red solution by suction and dried in air to yield 475 mg of nitrated product *m*-[^{15}N]-nitro-L-tyrosine.

^{15}N NMR Spectroscopy

All chemical shifts are reported in ppm downfield, with the zero ppm set at 25 ppm upfield from external $^{15}NH_4Cl$ dissolved in nitric acid. The *m*-[^{15}N]-nitro-L-tyrosine was dissolved in D_2O (10 mg/0.5 ml) and its ^{15}N NMR spectrum contained a single resonance at 375 ppm. The spectrum was obtained using the INEPT method, which enhances the signal/noise of ^{15}N resonance by transfering polarization to the ^{15}N nucleus from the ortho ring proton. The $C(^{15}NO_2)_4$ was dissolved in ethanol (0.012 ml/1.0 ml) and this was added to a solution containing 2.8 ml of 0.13 M. Tris, pH 8.0, 0.7 ml ethanol, and 1 ml D_2O. The ^{15}N NMR spectrum of this solution was obtained by direct ^{15}N detection using inverse gated decoupling to

suppress the undesirable negative Nuclear Overhauser enhancement of ^{15}N. One large resonance at 347.43 ppm was observed for tetranitromethane, far upfield from the nitrotyrosine resonance.

Nitration of N-Acetyl-L-tyrosine Amide with $C(^{15}NO_2)_4$.

To a solution of 20 mg of N-acetyl-L-tyrosine amide in 2.8 ml of 0.13 M Tris, pH 8.0, 0.7 ml ethanol, and 1 ml D_2O was added a solution of 0.010 ml of $C(^{15}NO_2)_4$ in 0.5 ml of ethanol. This was allowed to react. The solution rapidly changed from clear to dark orange indicating the formation of nitroformate ion and nitrotyrosinate ion. The ^{15}N NMR spectrum was obtained via direct detection using inverse gated decoupling. A large resonance at 349.82 ppm corresponding to nitroformate was observed. The ^{15}N NMR spectrum recorded under INEPT conditions produced a single resonance at 375.59 ppm corresponding to nitrotyrosine. Besides the nitration reaction, a number of side reactions occur producing several cross-linked products lacking the nitro group (16,17). The INEPT method was used since it enhances only those resonances that correspond to nitrogen nuclei coupled to protons by a specific coupling constant value. Thus the signal obtained under INEPT conditions is due solely to the aromatic nitro group coupled to an ortho ring proton, the various dissociation products of tetranitromethane are not observed.

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