APPLICATION OF SPIN LABELING TO DRUG ASSAYS. III. [^{15}N,$^{2}H_{13}$]2,2,5,5-TETRAMETHYLPYRROLINE-1-OXYL-3-CARBOXYLIC ACID COUPLED TO PHENYTOIN

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SUMMARY

Phorone and [$^{2}H_{14}$]phorone were cyclized with $^{15}NH_3$ or $^{15}N^{2}H_3$ to [^{15}N]- or [^{15}N,$^{2}H_{17}$]-4-oxo-2,2,6,6-tetramethylpiperidine which were then brominated at C-3 and C-5. Ring contraction then gave 2,2,5,5-tetramethylpyrroline-3-carboxamide, which with H_2O_2 gave the corresponding nitroxide. Hydrolysis in base of the doubly labeled nitroxide then gave [^{15}N,^{2}H]2,2,5,5-tetramethylpyrroline-1-oxyl-3-carboxylic acid. The latter, when coupled to phenytoin, afforded a spin-labeled drug of high sensitivity for detection by ESR technique. If the synthesis was started with [$^{2}H_{14}$]phorone, negligible loss of deuterium was noted in the final product even when protiated reagents were used.

Key Words: [^{15}N,$^{2}H_{17}$]Triacetonamine, Pyrroline Nitroxides, Spin-labeled Phenytoin

INTRODUCTION

Over the past few years we have been investigating the application of spin-labeled drugs for the determination of the concentration of drugs in serum by the electron spin resonance (ESR) technique. In this technique, the spin-labeled drug in serum which is not bound to serum protein (free drug) gives an ESR spectrum characteristic of a rapidly rotating molecule (1). The concentration of free drug can be estimated by the height of the peaks of the ESR spectrum. The sensitivity of the technique is determined largely by the width of the peaks of the ESR spectrum. The line width in nonconjugated aliphatic nitroxides is due primarily to the unresolved hyperfine splittings from the protons which are within 3 bonds of the oxyl-N-atom.

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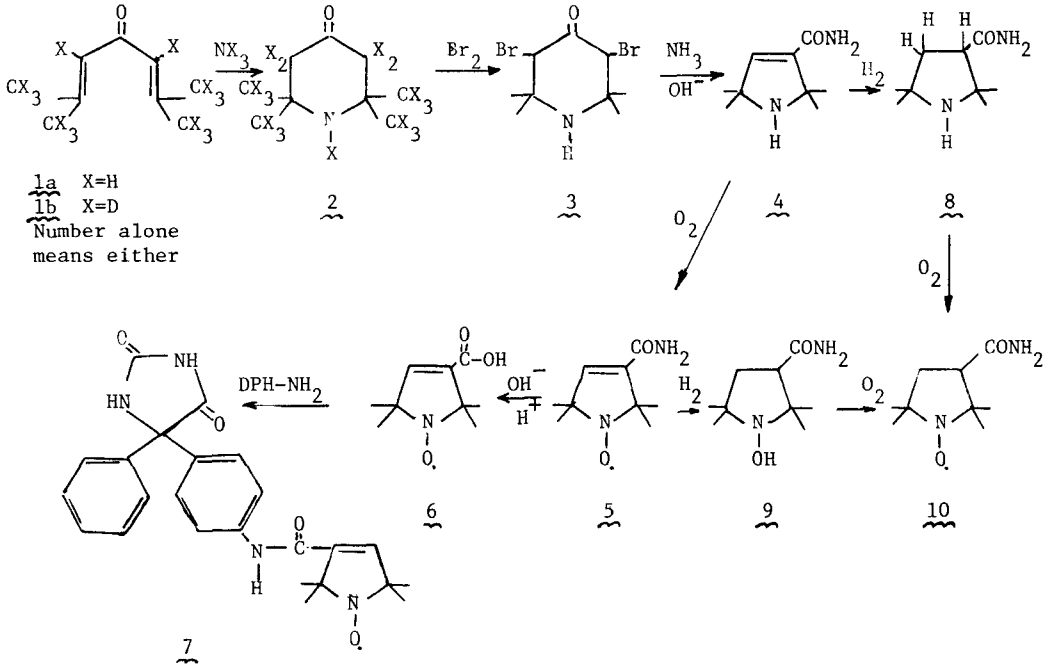
As an extension of our previous report (2) we describe here the preparation of an isotropically-substituted tetramethylpyrroline nitroxide, which is more amenable than a piperidine nitroxide to the introduction of the various functional groups needed for coupling. In the present study, we have prepared [^{15}N]-2,2,5,5-tetramethylpyrroline-1-oxyl-3-carboxylic acid, 6, from $^{15}NH_3$ and phorone. The substitution of ^{15}N for ^{14}N reduces the number of absorption peaks in the ESR spectrum from 3 to 2, thereby increasing the sensitivity by 50%.

Since the gyromagnetic constant of protium is six times that of deuterium, substitution of protium by deuterium reduces the hyperfine splittings and thus the linewidth. Since the area of the ESR absorption spectrum is identical with either set of isotopes, these substitutions should theoretically increase the sensitivity of detection of the free spin labels about seven-fold (3). Therefore, the deuterated isomer of the acid was also prepared by starting with [$^{2}H_{14}$] phorone and [^{15}N]ammonia. The six reaction sequence (Scheme I) gives a 22% yield, based on $^{15}NH_4Cl$. The acids, 6b, 6b-^{15}N, were then coupled to 5-(4-aminophenyl)-5-phenyl hydantoin (DPH-NH_2), thus yielding spin-labeled analog, 7, of the drug phenytoin (1).

Secondly, we have modified the synthesis so that the products were isolated and purified only when deemed crucial, thus streamlining the steps used previously with standard reagents (4). Thirdly, we have examined the extent of protium-deuterium exchange at each step in the reaction sequence in order to ascertain which non-deuterated reagents or solvents could have been used. Finally, we have compared the relative sensitivities of doubly isotopically substituted compounds to those of the standard spin labels.

A primary building block of numerous spin labels is 2a which contains methyl and methylene groups. Chiarelli and Rassat have already shown that the methylene protons of 2a and of Tempone are labile (5). The stability of C-H(D) bonds during the reaction in Scheme I thus remained to be determined.

Scheme I



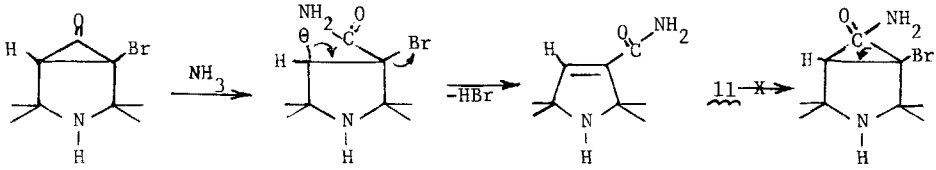
METHODS AND RESULTS

NMR experiments on the enolization of 2a in $CDCl_3$ in the presence of 5% DCl in D_2O showed that the ratio of methyl to methylene H of 2a increased from 3:1 to 3.5:1 in 48 hr. However, using a more polar solvent, as AcOD, it became 3.5:1 in 12 hr and after addition of a strong acid, e.g., 48% aq. HBr, the ratio increased to 7.3:1 in 30 min. This rapid rate of exchange of the methylene hydrogens of 2a in the presence of HBr is expected also to occur under the conditions needed for subsequent bromination, where HBr is generated. In this study 2a·HCl (10 mmoles) was brominated in AcOD (125 mmoles). The NMR spectrum (in DMSO-d_6) of the resulting 3a showed that 67% of the original methine protons was retained. Then, half of the product was treated with NH_4OH followed by NaOH to give 4a. Its NMR spectrum showed 70% of vinyl sites with the protium. The other half was treated with ND_4OD and NaH to give 4a with a similar percent of vinyl H. Subsequently, 3a was treated with ND_4OD followed by NaH to give 4a with methyl to vinyl proton

in a ratio of 12:1, much like when NH_4OH had been used with 3a.

These experiments show that limited exchange occurs during bromination which is typically seen as a fast addition of Br_2 to the enol of 2. Once 3 is formed it precipitates as the hydrohalide salt. (Apparently for this reason the product from bromination of 2a·HCl did not show the labile H and D in a ration of 60:125 present in the reaction mixture.). Thus the limited exchange apparently occurs after bromination at C-3 and C-5 and before the resulting 3·hydrohalide precipitates.

Scheme II



11 12 4 13

The bond-forming steps of subsequent ring contraction seem to be concerted, or faster than any exchanges, thus accounting for lack of exchange when 3a was treated with ND_4OD. The mechanism of this reaction (Favorski) is generally thought to proceed via a cyclopropanone intermediate, 11 (Scheme II). The double bond, in the present case, is thought to form via alpha-bromocarboxamide anion, 12, by a very fast dehalogenation. This results in the carboxamide 4. The formation of a double bond via 13 would entail among the products a monobromocarboxamide which was not found by us using TLC followed by MS.

To obtain 6, 5 was hydrolyzed at 100°C for 24 hr in D_2O to which BaO had been added. This reaction gave no exchange at either the methyl or the vinyl carbon . This was confirmed by treating 5b in H_2O with $Ba(OH)_2$. The resulting acid gave by IR and MS, no evidence of new C-H bonds.

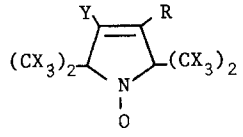
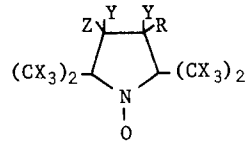
The degree of exchange during the entire synthesis was further examined by starting with 2b. In these studies, 2b was brominated in AcOH; the product was reacted with NH_4OH; the resulting 4 was oxidized with H_2O_2 to give 5b which was then hydrolyzed in H_2O for one day with $Ba(OH)_2$ at 100°C to give eventually 6b. The MS showed a negligible loss of D at the completion of the sequence.

We have further examined whether there is exchange during the reduction of 4 or 5 in the presence of 10% Pd-C with D_2. [This reduction must precede the introduction of an amino group at C-3 by Hofmann degradation. The amino label can then be coupled as in Scheme I to a molecule with a -COOH group (1)]. Reduction of 5 yielded hydroxylamine 9 which was then oxidized by air (O_2) to the respective nitroxide, 10, while still in the presence of the catalyst. Inexplicably, if the catalyst had been filtered, the oxidation was very slow (6) as readily observed by the lack of coloration (from colorless to yellow). Attempts were then made to measure the incorporation of deuterium by MS. Apparently due to the presence of labile hydrogens on many compounds, MS of partly substituted nitroxides were often found to have limited quantitative diagnostic value. Pyrrolines and pyrrolidines gave satisfactory MS spectra (M^+) only after having been oxidized to nitroxides.

The linewidths of the ESR spectra of the resulting nitroxides are compared in Table 1. Noticeably, the pyrroline nitroxides have narrower linewidths

Table 1

The peak-to-peak ESR linewidths, ΔH_{pp}, of five-membered ring nitroxides in aqueous buffer as a function of deuteration. The buffer was deoxygenated with N_2.

	Compound	X	Y	Z	ΔH_{pp} (G)
	5a	H	H	-	1.006 ± 0.008*
	5b	D	D	-	0.387 ± 0.011
	5	D	H	-	0.804 ± 0.008*
	10a	H	H	H	1.096 ± 0.010
	10	H	D	H	1.032 ± 0.006
	10	D	H	D	0.557 ± 0.012
	10	D	D	H	0.556 ± 0.005
R = C(O)-NH_2	10b	D	D	D	0.423 ± 0.015

*Proton superhyperfine splittings resolved.

than the pyrrolidine nitroxides. This is because of the larger proton (deuteron) splittings of the latter. Windle (7) has reported hyperfine splittings for 5a of 0.20 G and 0.35 G for the methyl and vinyl protons, respectively, in water. Using the method of Bales (8) and our spectra we have calculated an effective hyperfine splitting (hfs) of 0.25 G for all 15 protons of 10a. Our simulations also show that if the vinyl deuteron of 5b is replaced by a proton, the observed line would exhibit a splitting. The absence of such a splitting in 7b shows that the vinyl deuteron has been retained during the steps in Scheme I. This was then confirmed as follows: [$^{2}H_{14}$]phorone was cyclized in excess NH_4OH as previously described (2). The resulting 2-tetramethyl-d_{12} was treated with protiated reagents as per Scheme I to afford 5-d_{12}, which showed a vinyl hfs of 0.45 G in the ESR spectrum. Upon reduction of 5-d_{12} with D_2 to 9-d_x and conversion to 10-d_{14}, there was no evidence of resolved hfs from the single proton at C-4. In fact, the linewidth was the same as of the compound resulting from the reduction of 5b by H_2. From these results we inferred that for the pyrrolidine nitroxide, the proton splitting at C-3 is small and the protons at C-4 are nearly equivalent.

For the spin-label drug assay, the peak amplitudes were determined at the modulation amplitude which maximized the amplitude (I) of the low field peak. The sensitivity was given by: I/(receiver gain x [spin-label]). The relative sensitivity of 7b-^{15}N versus 7a was found to be 2.80 $\pm$ 0.05. Yet, when the spectra of both compounds were recorded at the identical non-distorting modulation amplitude, i.e., at 0.1 of the peak-to-peak width for 7b-^{15}N, then the sensitivity of the latter was 6.6 $\pm$ 0.1 times that of 7a. Furthermore, using the hyperfine splittings and intrinsic linewidth of Windle (7), the predicted I(^{15}N,^{2}H)/I(^{14}N,^{1}H) ratio is 6.2:1, in good agreement with our experimental value of 6.6. This is then also in agreement with the relative sensitivity reported while this manuscript was in preparation (3).

Recently, the synthesis of a [^{15}N,^{2}H]piperidine spin-label attached to maleic anhydride has been reported (9). The resulting spin-labeled maleimide was then compared to that made with standard isotopes. These authors reported an increase in sensitivity similar to ours (10). However, when the [^{15}N,^{2}H]piperidine-labeled

maleimide and [^{15}N,^{2}H]pyrroline-labeled phenytoin are compared by using the known splittings (3,7)*, we find that the phenytoin analog has a narrower calculated linewidth than that of the maleimide analog. Thus, the pyrroline label appears to give greater absolute sensitivity than the piperidine label. This may be due to the greater rigidity of the pyrroline.

In summary, this and our previous report describe a method for synthesizing ^{15}N,^{2}H-substituted pyrroline and pyrrolidine spin-labels. The results indicate that non-deuterated reagents can be used, except in the cyclization of 1b where ND_4OD should be used. The reduction of the double bond, of course, requires D_2.

EXPERIMENTAL

IR spectra were recorded on a Beckman Model 11210 spectrophotometer. ESR spectra were obtained on either a Varian Model E-4 or a Model E-109 ESR spectrometer. The latter was interfaced with a mini-computer and the spin label concentration determined by double integration of the ESR spectra. The relative sensitivity of ordinary versus isotopically substituted spin labels was recorded in dilute solutions (less than 10^{-4} M) in 50 mM phosphate buffer and 150 mM NaCl containing 5% ethanol. Ammonium-^{15}N chloride was obtained from Merck and Co., Inc. (Rahway, NJ). For TLC, GS-254 silica gel coated plates were obtained from Analtech, POB 7558, Newark, DE 19711. NMR spectra were recorded on Varian T-60 and HA-80 NMR spectrometers. For gas-liquid chromatography, a Hewlett-Packard Model 5750B chromatograph was used. The synthesis of triacetonamine was monitored by GC with the injection port at 280°C. Retention times for each compound are given in seconds.

The low resolution MS were recorded on an AEI Model MS-30. All measurements were performed using an ionization voltage of 70 eV and an accelerating voltage of 4 KeV and a source temperature of 200°C. Samples were introduced via a direct insertion probe and special care was taken to ensure constant sample evaporation during acquisition of data. Spectra of standard and enriched samples were recorded

*Note that in Reference 3, the proton hyperfine splittings are actually one-half of the correct values, whereas the deuteron splittings are correct.

sequentially under identical conditions. Isotopic enrichment calculations were carried out on data that were the weighted average of a number of similar scans (or of sequential spectra).

[^{15}N]Triacetonamine (2a-^{15}N). [^{15}N]Ammonia was liberated from NH_4Cl (1.0 g, 18.5 mmol) in water (3 ml) and NaOH (1.0 g) and then condensed over phorone (2.76 g, 20.0 mmole) in a vessel* as described previously (2). The reaction mixture was dissolved in ether and the product was extracted with 10% HCl. The aqueous phase was made basic and the product was extracted with ether to give 2a-^{15}N (1.70 g, 59%).

[^{15}N]-2,2,5,5-Tetramethylpyrroline-1-oxyl-3-carboxylic acid (6a-^{15}N). To 2a-^{15}N (1.30 g, 8.30 mmole) in AcOH (4 ml) was added dropwise Br_2 (2.70 g, 17 mmole) in AcOH (20 ml). After 24 hr at room temperature the solvent was removed at reduced pressure and the solid residue, 3a-^{15}N, was washed with ether. Concentrated NH_4OH (18 ml) was added and the solution was stirred for 2 hr. Sodium hyroxide (5 g) was then added, causing formation of white precipitate. The suspension was washed with $CHCl_3$ (4 x 5 ml). The organic phase was dried ($MgSO_4$) and the solvent was evaporated yielding 4a-^{15}N, as shown by GC and MS: where 154 (M^+-CH_3) was the largest ion observed. This material was dissolved in H_2O (12 ml), MeOH (3 ml) and 30% H_2O_2 (12 ml). While keeping the soln. in dark, $NaWO_4$ (10 mg) and EDTA (10 mg) were added and the mixture was stirred at room temperature for two days. The mixture was acidified and washed with $CHCl_3$ (3 x 4 ml). The organic phase was dried ($MgSO_4$) and the solvent evaporated to yield 5a-^{15}N, 630 mg, 24% based on $^{15}NH_4Cl$, IR: ν_{max} (KBr): 3360, 3180, (N-H); 1680 C=O, 1620 C=C cm^{-1}.

Compound 5a-^{15}N (291 mg, 1.57 mmole) in H_2O (5 ml) which contained $Ba(OH)_2 \cdot 5H_2O$ (630 mg) was sealed in an ampule and stirred at 100°C for 24 hr (by means of a

*The vessel was modified by omitting the glass bead. In its stead, as a guard against a sudden surge of liquid from ampule A to ampule B, two staggered opposing indentations were built into glass tubing. The procedure to prepare 1 in Reference 2 calls for use of trichloroacetyl chloride as the dehydrating agent. Since its publication, acetyl chloride with acetone-d_6 was used in an experiment. The MS of the resulting phorone-d_{14} (1-d_{14}) had molecular ion of 152 (8%); M^+-1 (12%), M^+-2 (19%), M^+-3 (17%). It thus appears that the methyl group of acetyl chloride may be mechanistically involved, causing limited scrambling of H and D.

sealed-in microspinning-bar). After cooling, water (5 ml) was added and the mixture was treated with small pellets of Dry Ice while stirred. The suspension was filtered. The filtrate was adjusted with H_2SO_4 to pH of 3 and the resulting mixture was dried with heat and N_2 gas. The solid residue was treated with acetone and the suspension was filtered. Acetone was evaporated, to give 6a-^{15}N as a yellow residue (271 mg, 93%); IR: ν_{max} in KBr: 1720 C=O and 1632 C=C cm^{-1}; MS for $C_9H_{14}{}^{15}NO_3$ calc. 185.0940, found 185.0945. The experiment was repeated by hydrolyzing 5a in D_2O and BaO; the product, 6a, displayed no C-D absorption in its IR spectrum.

[^{15}N]2,2,5,5-Tetramethyl-d_{12}-4-d-pyrroline-3-carboxamide. Bromine (1.70 g, 10.6 mmole) in acetic acid-D (2 ml) was added dropwise to a solution of [^{15}N,d_{17}]-triacetonamine (1.0 g 5.8 mmole) in AcOD (3 ml). The mixture was stirred overnight and the solvent was then removed at reduced pressure. The residue was 2b-^{15}N-deuterio bromide (2.50 g). It was treated with 26% ND_4OD (15 ml) and the mixture was stirred under nitrogen. The next day, 1.5 g of a 50% suspension of NaH in mineral oil was slowly added. The resulting mixture was washed with $CHCl_3$ (4 x 4 ml) and the organic phase was dried ($MgSO_4$). The residue was [^{15}N] 4b (800 mg), which by GC showed a retention time of 140 sec, column temp at 175°C; IR: 2220 (CD_3) and 1680 C=O cm^{-1} in KBr.

2,2,5,5-Tetramethyl-d_{12}-pyrroline-3-carboxamide-1-N-oxyl (5-d_{12}). Phorone-d_{14} was cyclized with NH_4OH as reported previously (2)*. The NMR spectrum ($CDCl_3$) of the resulting tetramethyl-d_{12}-2 showed $-CH_2-$ (2.25 ppm) and a trace of absorption at 1.25 ppm (CH_3). The product was then converted to 5-d_{12} as per Scheme I. In the last step, 4-d_{12} and an equal weight of m-chloroperbenzoic acid were stirred for 2 hr in $CHCl_3$ (11). The reaction mixture was chromatographed by TLC (silica gel, EtAc). The band, R_f 0.50, gave the product (the chlorobenzoic acid moves faster with extended tailing). Its ESR spectrum showed a vinyl hyperfine splitting of 0.45 G.

*In Reference 6, phorone was reacted with hydroxylamine, oxidation with "air" then gave Tempone in 0.3% yield. In our experience, phorone reacted with hydroxylamine to yield preferentially the oxime of phorone, although in low yield. Ammonia, on the other hand, does add preferentially to the double bonds of 1 to give 2.

Isolation and assays for the above pyrroline and its derivatives is conveniently done by TLC with silica gel GF 254. The R_f in $CHCL_3$:acetone = 3:1, are for 4 = 0.38, 5 = 0.76, 10 = 0.68 and 8 = 0.08. Compound 8 is not seen under UV light but can be visualized with I_2 vapor.

Reduction of 4a. The pyrroline 4a (108 mg, 0.64 mmole) in EtOD (8 ml) was allowed to react with D_2 in the presence of 10% Pd-C (30 mg) at ambient conditions until 0.62 mmole of D_2 had been consumed (∿ 40 min). The reaction mixture was filtered and the solvent removed to give product. The IR spectrum (in KBr) showed no C=C bond at 1600, cm^{-1} nor C-D at 2220 cm^{-1} (CD_3) and a weak absorption at 2070 cm^{-1} (methylene or methine C-D). The NMR spectrum (in $CDCl_3$) showed 4 non-equivalent CH_3; (1.10, 1.18, 1.25, 1.35 ppm); MS (rel. heights of the parent peak, P+) 173 (5%), 172 (4%) and 101 (P+, 100%).

Reduction of 5a to 9a-d_x and reoxidation to 10a-d_{12}. The nitroxide 5a (45 mg) in MeOD (10 ml) in the presence of 10% Pd-C (15 mg) was hydrogenated with D_2 at ambient temperature and pressure for 2 hr. Reduction was monitored by TLC. The solvent from an aliquot was removed. The white residue was apparently partly-deuterated 2,2,5,5-tetramethyl-N-hydroxypyrrolidine-3-carboxamide (MS,IR). The rest of the reaction mixture was treated with a stream of air for 30 min, causing the solution to turn yellow. The catalyst was then removed by filtration and the solvent by evaporation. The residue was partly deuterated 10. This material was dissolved in EtOH thereby removing labile D from the amide N. The ethanol was removed, leaving 10a with one D each at C-3 and C-4.

Alternatively, on small scale, pyrrolines were reduced as follows: 5b-^{15}N, (1 mg) in anhydrous EtOH (1 ml) in the presence of 10% Pd-C (1 mg) in a 4 ml vial was first flushed with H_2 for 1 hr and then with air for 15 min. The mixture was filtered, the solvent was removed, the residue was 10-^{15}N-d_{13}.

Compound 7. [^{15}N]2,2,5,5-Tetramethylpyrroline-d_{13}-1-oxyl-3-carboxylic acid, 6b-^{15}N, (18 mg), ethyl chloroformate (10 µl) and N-methylmorpholine (20 µl) in anhydrous tetrahydrofuran (2 ml) were stirred for 3 hr. 4'-Aminodiphenylhydantoin (24 mg) in THF (2 ml) was added. The next day the mixture was filtered. The filtrate was chromatographed by TLC in $CHCl_3$:EtOH:acetone, 8:1:1. The center band

of the three major ones, R_f 0.44, gave pure product (6 mg, 15%); UV: $\nu_{max}(\varepsilon)$ in EtOH: 271 (13000) nm; MS 7-$^{15}N,d_{13}$ (447 = M^+, 40%), 7-$^{15}N,d_{12}$ (446, 25%) and 7-$^{15}N,d_{11}$ (445, 35%) (where % are relative heights of the peaks in the M^+ envelope).

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