SYNTHESIS OF STABLE ISOTOPE ENRICHED METABOLITES OF THEOPHYLLINE

Edward R. Matjeka*, Richard C. Banks*, Gary D. Mercer*,
Beth Musser**, and Robert E. Vestal**†

* Department of Chemistry, Boise State University, Boise, ID 83725

** Clinical Pharmacology and Gerontology Unit, Veterans Administration Medical Center, Boise, ID 83702 and Divison of Gerontology & Geriatric Medicine, Department of Medicine, University of Washington, Seattle, WA

SUMMARY

Four isotopically labelled metabolites of theophylline were synthesized. The syntheses of 1-(methyl-d_3)uric-2,8-$^{13}C_2$-acid; 1,3-di(methyl-d_3)uric-2,8-$^{13}C_2$ acid; 3-(methyl-d_3)xanthine-2-^{13}C,7-^{15}N and 1-(methyl-d_3)xanthine-2-^{13}C are described. Modifications in published procedures for the synthesis of the corresponding unlabelled compounds were required to achieve the micro scale necessary to accommodate the small starting quantities of expensive stable isotope labelled precursors.

KEY WORDS: theophylline metabolites, 1-methyluric acid, 1,3-dimethyluric acid, 1-methylxanthine, 3-methylxanthine, stable isotope labelling

INTRODUCTION

Theophylline is a drug which is frequently used in the acute and chronic management of obstructive airways disease. Recent studies (1-3) of the disposition of this important, but potentially toxic, therapeutic agent have utilized stable isotope methodology (4) in order to obtain estimates of the pharmacokinetic parameters at steady state under conditions of chronic dose administration.

†Author to whom correspondence should be addressed at the VA Medical Center (151), 500 West Fort Street, Boise, Idaho 83702.

0362-4803/86/090967-12$06.00

Received April 24,1985
Revised April 7, 1986

Although a method for the assay of the metabolites of theophylline in plasma and urine by gas chromatography/mass spectrometry has been reported (5), it utilizes deuterium-labelled caffeine as the internal standard. To ensure accurate quantification, an internal standard for each metabolite would be desirable. In order to use stable isotope labelled compounds as internal standards in the analysis, the syntheses of 1-methyluric acid, 1,3-dimethyluric acid, 3-methylxanthine and 1-methylxanthine were undertaken utilizing starting materials enriched with ^{13}C, ^{15}N and deuterium.

RESULTS AND DISCUSSION

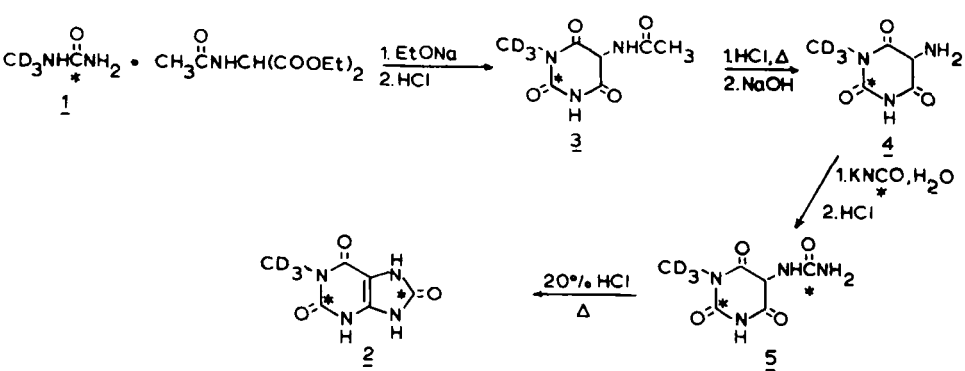
The syntheses of the compounds were accomplished by means of published procedures which required modifications because of the small scale. Each synthesis is described.

The compound N-(methyl-d_3)urea-^{13}C (1) was prepared by the reaction of (methyl-d_3)amine hydrochloride with potassium cyanate-^{13}C (6).

$$CD_3NH_3Cl + K\overset{*}{N}CO \longrightarrow CD_3N\overset{*}{H}\overset{O}{\overset{\|}{C}}NH_2 + KCl$$

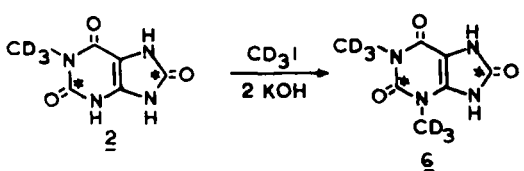
1

The synthesis of 1-(methyl-d_3)uric-2,8-$^{13}C_2$ acid (2) with a mass enhancement of 5 atomic mass units more than natural 1-methyluric acid was based upon the synthesis developed by Stein (7) and Fischer (8) and required the following four steps.



The reaction of 1 and diethyl acetamidomalonate when treated with sodium ethoxide, according to the procedure of Stein (7), produced 3 in good yield with no modifications necessary in the procedure. Compound 4 was prepared from 3 by Fischer's (8) procedure using potassium cyanate in lieu of calcium cyanate. It was found in that procedure, because of the micro scale used, more water was needed to dissolve the reactants and the amount of base used in the isolation of 4 had to be carefully controlled in order to isolate the product. The final steps to produce 2 were carried out without modification (8).

The synthesis of 1,3-di(methyl-d_3)uric-2,8-$^{13}C_2$ acid (6) was accomplished with the further methylation of 2 according to the procedure of Fischer (8). The very micro scale of the reaction required more base to effect solution of 2 than was indicated by the literature (8), and the methyl-d_3 iodide was added in portions and allowed to react for a longer period of time. It was determined by high performance liquid chromatography (9) that, when the reaction was worked-up according to Fisher's procedure (8), the 1,7-dimethyl isomer was the compound that crystallized out of solution during recrystallization and was the major product. The desired 1,3-dimethyl product was isolated by evaporation of the filtrate under nitrogen gas after crystallization of the 1,7-isomer. The purity of 6 isolated by this procedure was quite high as determined by high performance liquid chromatography. The mass difference achieved between natural 1,3-dimethyluric acid and 6 was 8 atomic mass units.



2 6

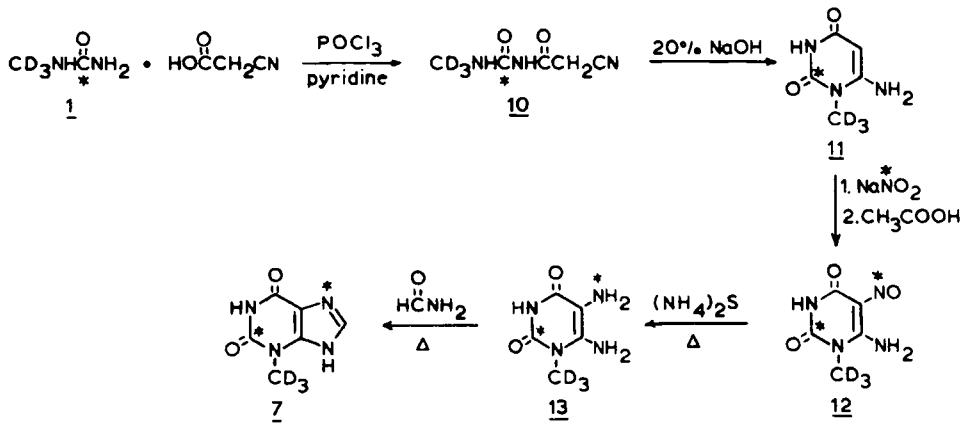
The synthesis of 2-(methyl-d_3)xanthine-2-^{13}C,7-^{15}N (7) was accomplished without modification according the to scheme below (7, 10-12).

Table 1

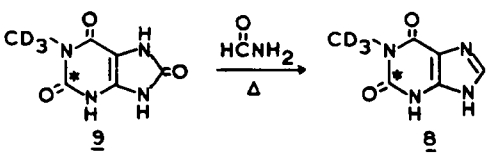
Comparison of Retention Time and Peak Height Ratios*

	1-Methyluric Acid		1,3-Dimethyluric Acid		3-Methylxanthine		1-Methylxanthine	
	Unlabelled	Labelled	Unlabelled	Labelled	Unlabelled	Labelled	Unlabelled	Labelled
R_f (min)	8.3	8.1	11.4	11.0	5.4	5.3	7.0	6.9
280/254	2.3	2.4	2.2	2.2	1.6	1.6	0.9	0.9

*Each value is the mean of five injections.



The final synthesis produced 1-(methyl-d_3)xanthine-2-^{13}C (8) by the reduction of 1-(methyl-d_3)uric-2-^{13}C acid (9) with formamide (12). The mass enhancement for 8 was 4 atomic mass units.



A standard mixture of all four labelled compounds was assayed by high performance liquid chromatogrpahy (9). Absorbance was monitored simultaneously at 280 and 254 nm. A standard mixture of the unlabelled compounds was assayed in the same manner. As shown in Table 1, the 280/254 peak height ratio of each labelled compound was identical to the ratio of its unlabelled analog (13).

EXPERIMENTAL

All labelled compounds used as starting materials were obtained from KOR Isotopes, Cambridge, MA. Mass spectral analyses were performed with a Finnigan 3200F mass spectrometer using a solid sample probe at 300°C. Melting points were taken on an Electro-Thermal apparatus (uncorrected). High performance liquid chromatographic analyses were performed using the method of Muir et al (9) with two Waters Model 510 pumps, Waters 440 and 480 detectors, Waters Intelligent Sample Processor autosampler, Waters Model 752 gradient controller

and Waters Model 730 Data Module. The column used was Spheri-5, RP-18 (4.5 mm x 25 cm) obtained from Brownlee Laboratories.

N-(Methyl-d_3)urea-^{13}C (1)

A solution of 2.6 g (37 mmol) (methyl-d_3)amine hydrochloride (containing 98 mol% CD_3NH_2) and 3.1 g (38 mmol) potassium cyanate-^{13}C (containing 90 mol% ^{13}C) was prepared in 18.6 ml of deionized water. The solution was stirred magnetically and the water slowly distilled out through a short-path distillation head. After 15 ml of water had been recovered, the residue was dried under vacuum. The crude product was then transferred to a Soxhlet extraction thimble and extracted for 3 hr with pentane. The extraction was then continued for 20.5 hr with methylene chloride. Upon evaporation of the methylene chloride, 2.01 g (69.6%) of a white solid was obtained. The compound was used without further purification.

The mass spectrum of N-methylurea indicates major ions at m/e 74 (M^+), m/e 58 (M^+-NH_2) and m/e 44 (M^+-$NHCH_3$). If ions below m/e 40 are not considered, the relative abundances of the ions are 100, 18.5 and 48.2% respectively and there is no significant M-1 ion (14). The mass spectrum of N-(methyl-d_3)urea-^{13}C (1) was consistant, exhibiting ions at m/e 78 (M^+), m/e 62 (M^+-NH_2) and m/e 45 (M^+-$NHCD_3$) with relative abundances of 100, 26 and 54.3% respectively. Based upon the isotope analyses stated by the starting material supplier, (methyl-d_3) amine hydrochloride containing 98 mol% deuterium and potassium cyanate-^{13}C containing 90 mol% ^{13}C, the M^+ to M-1 ions in 1 should have been 100 to 13.4%. We observed M-1 in 1 to be 57.1% of M^+. Since m/e 43 in the unlabelled compound had a relative abundance of 14.8% and m/e 44 in 1 had a relative abundance of 36.7%, the potassium cyanate-^{13}C appears to have been the major source of the difference. The potassium cyanate-^{13}C would have to have been about 65 mol% ^{13}C to account for the results.

5-Acetamido-1-(methyl-d_3)-2,4,6-trioxopyrimidine-2-^{13}C (3)

Sodium (0.324 g, 0.0144 gram atom) was dissolved in 10 ml of absolute ethanol. Compound 1 (0.9 g, 12 mmol) and 2.65 g (12.2 mmol) of diethyl acetamido-

malonate were added and the resulting mixture was heated for 6 hr in an oil bath at 90°C. The reaction mixture was cooled to 0°C and 15 ml deionized water was added. Concentrated hydrochloric acid (2.4 ml) was added and the precipitate was filtered, yielding 2.29 g (98.3%) white solid. This material was used without purification.

5-Amino-1-(methyl-d_3)-2,4,6-trioxopyrimidine-2-^{13}C (4)

Compound 3 (0.80 g, 4 mmol) was suspended in 40 ml of absolute methanol. Concentrated hydrochloric acid (6 ml) was added and the mixture was refluxed for 80 min in an oil bath. The reaction mixture was cooled to 0°C and 4 ml of 20% sodium hydroxide solution was added dropwise in 0.25 ml increments with stirring. The solution was stirred for about 2 min after the addition of each 0.25 ml of base to allow for a delayed precipitation. Excess base was found to dissolve the product irreversibly. The product was filtered, yielding 0.80 g (82%) light pink solid. Trial reactions with the unlabelled starting material yielded the 5-amino-1-methyl-2,4,6-trioxopyrimidine as a light pink solid, m.p. 265-270°C dec. (lit. 253-6) (8). The material was used without further purification. Occasionally the hydrochloride salt was isolated but this was used as is in the next reaction with no adverse effects.

5-(Carbamoyl-^{13}C-amino)-1-(methyl-d_3)-2,4,6-trioxopyrimidine-2-^{13}C (5)

The hydrochloride of 4 (0.40 g, 1.6 mmol) and 0.13 g of potassium cyanate-^{13}C (90 mol% ^{13}C, 1.7 mmol) and 2 ml of deionized water were heated for 80 min at 100°C in an oil bath. The reaction mixture was cooled to 0°C and 0.5 ml of 10% hydrochloric acid solution was added dropwise to precipitate the product. The white solid was filtered, yielding 0.15 g (46%). A trial reaction forming the unlabelled compound yielded a white solid m.p. 230°C dec. (lit. 220°C)(8). This material was used without further purification.

1-(Methyl-d_3)uric-2,8-$^{13}C_2$ acid (2)

Compound 5 (0.30 g, 1.5 mmol) and 2.5 ml of 20% hydrochloric acid solution were heated for 1 hr at 100°C in an oil bath. The reaction mixture was cooled

to 0°C and the solid precipitate was filtered. This solid was dissolved in 2.5 ml of 20% sodium hydroxide solution and brought up to a total volume of 10 ml with deionized water. Hydrochloric acid solution (10%) was added in excess until precipitation was complete. The white solid was filtered and washed with water, yielding 0.05 g (18%). A trial reaction forming the unlabelled 1-methyluric acid yielded a white solid m.p. 400°C dec. (lit. 400°C)(8). Mass spectral analysis showed a molecular ion at m/e 187 which indicated the expected gain of 5 a.m.u. A mass spectrum of an authentic sample of the unlabelled compound showed no M-1 ion but major fragments at m/e 125 for a loss of 57 mass units and at 97 for a loss of 85 mass units. The labelled 2 showed significant ions at m/e 126 and 98 but M-1 at m/e 186 became the base peak. As discussed earlier for 6, the incorporation of a second ^{13}C with the same potassium cyanate-^{13}C would lead to a still more important ion at M-1 for this molecule.

1-(Methyl-d_3)uric-2-^{13}C acid (9)

This compound was prepared from 4 using unlabelled potassium cyanate according to the same procedures used to prepare 5 and 2.

1,3-Di(methyl-d_3)uric-2,8-$^{13}C_2$ acid (6)

Compound 2 (0.10 g, 0.5 mmol) was dissolved in 2 ml of 1N potassium hydroxide. Trideuteromethyl iodide (0.10 ml, 99.5 mol% d_3, 1.6 mmol) was added and the mixture was stirred rapidly and heated to 50°C. Trideuteromethyl iodide (0.02 ml, 0.3 mmol) was added every 30 min for the first 3 hr of the reaction to replace any that was lost past the reflux condenser. The reaction was refluxed for an additonal 3 hr without further addition of alkyl halide. Hydrochloric acid solution (10%, 0.5 ml) was added to precipitate the product. This product was added to a solution of 10 ml of methanol and 5 ml of deionized water. The material that did not dissolve was shown by HPLC analysis (9) to be a mixture of the 1,7-dimethyluric acid and unreacted 1-methyluric acid. The solution was cooled to 0°C and a second crop of crystals was collected and analyzed by HPLC. They were shown to be primarily 1,7-dimethyluric acid with some 1,3-dimethyluric acid. The filtrate was evaporated under a stream of dry nitro-

gen yielding 0.012 g (12%) of white solid that was essentially pure 1,3-dimethyluric acid with a trace of the 1,7-isomer. A trial reaction forming the unlabelled 1,3-dimethyluric acid yielded a white solid, m.p. 345-350°C dec. (lit. 370°C)(8). Mass spectral analysis showed a molecular ion at m/e 204 which indicated the expected gain of 8 a.m.u. Again the M-1 ion at m/e 203 was the base peak because the same potassium cyanate-^{13}C source was used twice during the synthesis.

N-(Cyanoacetyl)-N'-(methyl-d_3)urea-^{13}C (10)

To an oven-dried and nitrogen-flushed 100-ml, three-neck, round bottom flask fitted with a nitrogen inlet tube, septum and drying tube was added 0.70 g (9 mmol) of 1, 0.76 g (9 mmol) of cyanoacetic acid and 1.4 ml of pyridine which had been dried over NaOH. The mixture was stirred magnetically under nitrogen and warmed with a water bath to dissolve the solids. The water bath was brought to room temperature and 0.43 ml (0.70 g, 4.6 mmol) of phosphorous oxychloride was added dropwise by means of a syringe. The reaction was exothermic and produced a thick, dark brown syrup which was allowed to remain at room temperature for one-half hr. To the reaction mixture was then added 5.6 ml of deionized water which produced a mustard yellow slurry. The slurry was chilled in an ice bath and filtered. The collected solid was washed with a small amount of ice cold water. The solid was taken up in about 35 ml of deionized water, activated charcoal was added and the resulting mixture was heated to boiling. Filtration through a hot funnel yielded a slightly yellow filtrate which began to precipitate upon cooling. After chilling the filtrate in ice, 0.58 g of slightly yellow crystals were collected. Evaporation of the filtrate under vacuum eventually yielded two more small crops of crystals for a total of 0.63 g (48%). The melting point of unlabelled N-(cyanoacetyl)-N'-methylurea obtained in a trial run was 217-221°C (lit. 205°C)(15). The product was used without further purfication.

4-Amino-2,6-dihydroxy-3-(methyl-d_3) pyrimidine-2-^{13}C (11)

To 0.63 g (4.5 mmol) of 10 in a crucible was added 1.89 ml of 20% NaOH with stirring. A slightly red solution briefly occurred and then a solid reformed. On continued stirring, the stiff mixture became almost pure white and less viscous. Glacial acetic acid was then added until the pH was less than 7. The resulting white slurry was then chilled in ice and filtered to yield a white solid. After being recrystallized from about 60 ml of deionized water, 0.52 g (83%) of white crystals were obtained. The product was used without further purification.

4-Amino-2,6-dihydroxy-3-(methyl-d_3)-5-nitroso-^{15}N-pyrimidine-2-^{13}C (12)

To 0.52 g (3.6 mmol) of 11 in 40 ml of boiling deionized water was added 0.27 g (3.9 mmol) of sodium nitrite-^{15}N (99 mol% ^{15}N) which effected dissolution of 11. To the solution was then added 0.18 ml (0.19 g, 4.3 mmol) of glacial acetic acid which produced a purple color. Upon cooling, crystallization began to occur. After chilling in ice, purple crystals were collected and washed with ice cold water. After collecting a small second crop of crystals, a total of 0.57 g (83%) was obtained. The product was used without further purification.

4-Amino-5-amino-^{15}N-2,6-dihydroxy-3-(methyl-d_3)pyrimidine-2-^{13}C (13)

To 0.54 g (3 mmol) of 12 in a 50-ml round-bottom flask fitted with a magnetic stirring bar and heated by a boiling water bath was added 22% diammonium sulfide solution which had been previously heated in a boiling water bath. The dropwise addition was continued until the purple color of 12 had been discharged and bubbling ceased. To the resulting yellow solid and orange liquid was added concentrated hydrochloric acid until pH 2 was obtained. The light yellow solid and liquid were then heated in a boiling water bath and filtered. The solid was washed on the filter with several portions of hot deionized water. The light yellow filtrate was brought to pH 8-9 with concentrated ammonium hydroxide whereupon a light yellow precipitate formed. The volume was reduced by warming the mixture. The mixture was then chilled and filtered. The light

yellow solid was washed with cold deionized water. Several more small crops of product were collected to yield a total of 0.425 g (88.5%).

3-(Methyl-d_3)xanthine-2-^{13}C,7-^{15}N (7)

To 0.425 g (2.6 mmol) of 13 in a 10-ml round-bottom flask was added 3 ml (3.39 g, 0.075 mol) of formamide. The mixture was stirred magnetically, maintained under a positive flow of nitrogen and heated by means of a 180°C silicon oil bath. Dissolution occurred rapidly, with slight bubbling, to form a deep black-green solution but after 15 min solid began to form. The mixture was heated a further 15 min, then cooled and 12 ml of deionized water added to produce a yellow-green mixture. The mixture was then chilled and filtered to yield a green-black solid which was dissolved in 25 ml of deionized water and about 4 ml of concentrated ammonium hydroxide. The resulting olive colored solution was decolorized with activated charcoal. After filtration the hot filtrate was brought to pH 2 with concentrated hydrochloric acid, whereupon crystals began to form. After chilling and filtration, 0.32 g (73%) of 7 was obtained as a very pale yellow solid. The overall yield from 1 was 21%. The mass spectrum of 7 showed a molecular ion at m/e 171 and major ions at m/e 127 and 99 corresponding to ions at m/e 166, 123, and 95 for unlabelled 3-methylxanthine (16). There was no M-1 shown for unlabelled-3-methylxanthine but the M-1 ion at 170 for 7 was 54.7% of M^+ as would be expected based upon the analysis of 1.

1-(Methyl-d_3)xanthine-2-^{13}C (8)

A mixture of 0.11 g (0.59 mmol) of 9 and 4 ml of formamide (4.5 g, 0.1 mol) was stirred for 2.5 hr under a nitrogen flow. The mixture was then heated for two hr by a 180°C silicon oil bath while nitrogen flow was maintained. After cooling, 10 ml of deionized water was added and the mixture chilled and filtered to yield a tan-grey solid. Upon evaporation of the filtrate a second crop of crystals was obtained. The combined crystals were taken up in 90 drops of 1N NaOH, activated charcoal was added and the mixture warmed by a boiling water bath before being filtered to yield a light yellow-green filtrate. When the

temperature of the filtrate was 60°C, 2 drops of 30% hydrogen peroxide were added, followed by 6N sulfuric acid dropwise to pH 2 when the solution reached 65°C. The solution was then warmed to 85°C before being chilled. Upon chilling a pale yellow solid formed which was collected by centrifugation to yield 0.042 g (42%). Mass spectral analysis showed a molecular ion at m/e 170 which indicated the expected gain of 4 a.m.u. There was again a significant M-1 ion (55% of M^+) for the labelled compound but none observed for unlabelled 1-methylxanthine. Both labelled and unlabelled compounds exhibited a major fragment at m/e 109.

ACKNOWLEDGEMENTS

This work was supported in part by a Faculty Research Grant from Boise State University, by the Veterans Administration and by a grant (AG 02901) from the National Institutes of Health, U.S. Department of Health and Human Services.

REFERENCES

1. Vestal R.E., Thummel K.E., Musser B. and Mercer G.D. - Br. J. Clin. Pharmacol. 15:411 (1983)
2. Cusack B.J., Dawson G.W., Mercer G.D. and Vestal R.E. - Clin. Pharmacol. Ther. 37:330 (1985)
3. Vestal R.E., Thummel K.E., Mercer G.D. and Koup J.R. - Europ. J. Clin. Pharmacol. (1986, in press)
4. Vestal R.E., Thummel K.E., Musser B., Jue S.G., Mercer G.D. and Howald W.N. - Biomed. Mass Spectrom. 9:340 (1982)
5. Tserng K.-Y. - J. Pharm. Sci. 72:526 (1983)
6. Adams R. and Johnson J.R. - Laboratory Experiments in Organic Chemistry, (Fourth Edition), Macmillan Co., 1949, p. 285
7. Stein A., Gregor H.P. and Spoerri P.E. - J. Amer. Chem. Soc. 78: 6185 (1956)
8. Fischer E. and Clemm H. - Ber. 30:3089 (1897)
9. Muir K.T., Jonkman J., Tang D-S, Kunitani M and Riegelman S. - J. Chromatog. 221:85 (1980)
10. Traube W. - Ber. 33:3035 (1900)
11. Bergmann F. and Dikstein S. - J. Amer. Chem. Soc. 77: 691 (1955)
12. Brederick H., von Schuh H.-G. and Martini A. - Ber. 83:201 (1950)
13. Yost R., Stoveken J., MacLean W. - J. Chrom. 134:73 (1977)
14. Stenhagen E., Abrahamson S. and McLafferty F.W., eds. - Registry of Mass Spectral Data, Vol. 1, Wiley, 1974, p. 30
15. Beilsteins Handbuch der Organischen Chemie, Vol. 29, 1909, p. 83
16. Stenhagen, op. cit., p. 546