Journal of Labelled Compounds and Radiopharmaceuticals—Vol. XXXVI, No. 8

SYNTHESIS AND PURIFICATION OF ^{13}C LABELLED XANTHINE DERIVATIVES

M.S. BOUKRAA, D. DERUAZ* , A. BANNIER, M. DESAGE, J.L. BRAZIER

Laboratoire d'Etudes Analytiques et Cinétiques du Médicament
Institut des Sciences Pharmaceutiques et Biologiques.
8 Avenue Rockefeller - 69373 Lyon Cedex 08 - France

SUMMARY

3-[Methyl-^{13}C]xanthine, 7-[Methyl-^{13}C]xanthine, 1,3-[Dimethyl-$^{13}C_2$]xanthine (theophylline-1,3-[$^{13}CH_3]_2$), 1,7-[Dimethyl-$^{13}C_2$]xanthine (paraxanthine-1,7-[$^{13}CH_3]_2$), and 3,7-[Dimethyl-$^{13}C_2$]xanthine (theobromine-3,7-[$^{13}CH_3]_2$) were synthesized by nucleophilic substitution reaction (SN_2) from xanthine (X) and iodomethane-[^{13}C]. The 3-isobutylparaxanthine-7-[$^{13}CH_3$] was prepared from 3-isobutyl-1-methylxanthine (IBMX). The compounds were purified by reverse phase semipreparative liquid chromatography and their chemical structure and purity verified by GC-MS.

INTRODUCTION

The use of stable isotopes as analytical tools in biology and chemistry has gained in importance in the last few years. This development has gone hand-in-hand with the development of new analytical instrumentation, particularly those allowing the detection of compounds labelled with stable isotopes : GC-MS, GC-IRMS and GC-AED.

The use of stable isotopes in the study of metabolisms provides invaluable information on the in vivo evolution of compounds of biological interest, in particular, on the metabolites formed as well as the involved mechanisms. 1,3,7-[Trimethyl-$^{13}C_3$]xanthine (caffeine-1,3,7-[$^{13}CH_3]_3$) is one of the isotopomers used in the study of caffeine metabolism (1-7). It is metabolised by the liver under the influence of the microsomal oxidizing system using different

* to whom correspondence should be sent.

CCC 0362–4803/95/080773–15

Received 27 January 1995
Revised 27 February 1995

forms of cytochrome P-450. One of these forms is involved in the competitive N-demethylations between the three positions leading to the following metabolites:

- labelled dimethylxanthines (DMX*):
  1,3-[Dimethyl-$^{13}C_2$]xanthine; 1,7-[Dimethyl-$^{13}C_2$]xanthine; 3,7-[Dimethyl-$^{13}C_2$]xanthine.
- labelled monomethylxanthines (MMX*):
  1-[Methyl-^{13}C]xanthine; 3-[Methyl-^{13}C]xanthine; 7-[Methyl-^{13}C]xanthine.

Xanthine oxidase by a C-8 hydroxylation leads to their methyl uric acid analogues.

In addition to the properties that make the specificity of methylxanthines (1), the family has become, thanks to the different competitive metabolic pathways it offers, one of the best models for the validation of analytical methods using stable isotopes and these methods are known to be easy to be extrapolated to other molecules of therapeutic interest. The physico-chemical behaviour of 3-isobutylparaxanthine-7-[$^{13}CH_3$] is, in many respects, similar to that of methylxanthines (MX) so this compound can constitute an ideal internal standard for their quantitative determination after derivatization (2, 4).

To synthesize [^{13}C]-labelled methylxanthines (MX*), the reactions of N-methylation described for xanthine, in the same physico-chemical environment as caffeine and its labelled metabolites, were chosen.

The results of the synthesis of caffeine and theophylline isotopomers carried out using xanthine or MX as precursors, and $^{13}CH_3I$ or C^2H_3I as alkylation agent are given in Table 1.

Greeley proposed a method for the derivatization of fatty acids and barbiturates (11, 12). This method can also be applied to MX (2, 4, 13-15) before gas chromatographic analysis.

The reaction yields of the syntheses reported above vary from author to author depending on the number of alkylation sites per precursor with slow kinetics (generally 24 hours). These reactions have been applied for the total alkylation of all the free sites in order to obtain either caffeine and its isotopomers or other derivatives used for gas chromatographic analysis. In addition, Berlioz was able to selectively obtain deuterated theophylline after blocking the (N_7) site with chloromethylpivalate (10).

Two types of methodologies are found in the literature for MX purification:

- chemical purification (3, 8) for caffeine
- thin layer chromatography for caffeine (9) and for theophylline isolation (10).

Although semipreparative liquid chromatographic purification has not been described in the literature, this technique was however chosen because, it not only permits the separation of a complex mixture of compounds (just like analytical liquid chromatography) but also allows isolation of more important amounts of isolated compounds.

Precursors	Base used mmol	Alkylation reagent	Final product	Reference
DMX 0.5g 2.78 mmol	NaOH 12.5	3x250µl C^2H_3I 12.05mmol	1-C^2H_3, 3-C^2H_3 and 7-C^2H_3-Caffeine	(9)
DMX 0.1g 0.56 mmol	NaOH 2.5	3x50µl C^2H_3I 2.41mmol	1-C^2H_3, 3-C^2H_3 and 7-C^2H_3-Caffeine	(3)
MMX 0.5g 3.01 mmol	NaOH 12.5	3x500µl C^2H_3I 24.09mmol	1,3-$(C^2H_3)_2$, 1,7-$(C^2H_3)_2$ and 3,7-$(C^2H_3)_2$-Caffeine	(9)
X 0.5g 3.29 mmol	NaOH 12.5	3x750µl C^2H_3I 36.14mmol	Caffeine-1,3,7-$(C^2H_3)_3$	(9)
X 0.1g 0.66 mmol	NaOH 2.5	3x50µl C^2H_3I 2.41mmol	Caffeine-1,3,7-$(C^2H_3)_3$	(3)
MMX 0.33g 2.0 mmol	KOH	40 mmol $^{13}CH_3I$	Caffeine-1,7-$[^{13}CH_3]_2$	(8)
MMX 1g 6.02 mmol	Na_2CO_3	3x115µl C^2H_3I 5.4 mmol	Theophylline-1-(C^2H_3) Theophylline-3-(C^2H_3)	(10)

(3x): Addition in three steps, at t=0, and after one or two hours.

Table 1: Condition used for the total Alkylation of xanthine and methylxanthines

The major difficulty in the separation of MX by analytical HPLC (16-21) lies in the poor resolution of dimethylxanthines, especially theophylline and paraxanthine. These two isomers play a very important role in the metabolism and there have been many attempts for separating them using a gradient mode (18) or by the addition of modifiers into the mobile phase (21).

The most commonly used stationary phase for the separation of MX is an hexadecylsilane phase such as C18 µBondapack.

The mobile phase is a ternary mixture composed of a buffered aqueous solution (in general, acetate or phosphate) with a pH value between 3 and 6, and two organic components chosen among the following solvents: methanol, tetrahydrofuran, acetonitrile, isopropanol.

EXPERIMENTAL

Two techniques, the Greeley's technique (11, 12) and the Horning's one (3) were chosen, because of their good yield and the ease with which they can be applied to xanthines using methyliodide as alkylation reagent. The Horning's technique was used for the synthesis of MX* and that of Greeley for the synthesis of 3-isobutylparaxanthine-7-$[^{13}CH_3]$.

I - MATERIALS AND REAGENTS

1-MATERIALS

Liquid chromatography

- an isocratic pump, an UV/Visible detector and an integrator (Spectraphysics).
- an injector (500 µl).
- a C18 µBondapack column, L=25 cm, i.d.=8 mm, granulometry=10 µm (Waters), in association with a C18 precolumn, were used.

GC-MS

A series II 5890 gas chromatograph coupled to an HP 5972 Hewlett Packard mass spectrometer, working in the electron impact mode (70 eV) were used . The chromatographic column was an HP 1: L=25 m, i.d.=0.32 mm, film thickness: 0.17 µm.

2 - CHEMICALS

- Xanthine (Fluka, Mulhouse, France) and methylxanthines, iodomethane, iodomethane[^{13}C] 99% atom (Sigma-Aldrich, Saint Quentin, France),
- Acetone, chloroform, isopropanol, hydrochloric acid (Carlo Erba, Milan, Italy),
- NaOH analysis grade,(Merck, Darmstadt, Germany).

II - METHYLXANTHINES SYNTHESIS

1 - METHOD

The synthetic methods gathered in Table I were used to prepare the caffeine isotopomers, with an amount of methyliodide used in molar excess with respect to the precursor. In order to stop the reaction at the steps corresponding to the first or the second alkylation, the amount of iodomethane was varied in the reaction mixture (Table II) while maintaining a constant amount of xanthine : 0.5g (3.29 mmol).

Beyond a certain amount of CH_3I, caffeine becomes the main product formed. With a smaller amount of CH_3I, MMX are the main products formed and DMX is synthesized for intermediate amounts of CH_3I (Fig.1). So two different mixtures were used, one for the synthesis of DMX and the other for MMX, corresponding respectively to 3.21 mmol.(3x1.07) and 1.62 mmol (3x0.54) of $^{13}CH_3I$ (Table II).

NaOH in mmol per alkylation site	CH_3I in mmol per alkylation site	% of Products formed
4.17	3.33	Caffeine 90%
4.17	2.68	caf 28%, DMX 69%, MMX 3%
4.17	2.14	caf 22%, DMX 71%, MMX 7%
4.17	1.61	caf 12%, DMX 63%, MMX 24% *
4.17	**1.07**	**caf 6%, DMX 90%, MMX<5%**
4.17	0.80	caf 6%, DMX 85%, MMX 8%
4.17	**0.54**	**caf<5%, DMX 22%, MMX77%**

(*) Reaction stopped after 4 hours.

Table II: Partial methylation of the xanthine: percentage of each compound formed in the reaction mixture with respect to the of CH_3I amount.

2 - PROCEDURE

0.5 G (3.29 mmol) of xanthine was dissolved in 25 ml of an acetone-water mixture 1:1 in a 100 ml flask under continuous stirring. Then, 25 ml of a 0.5 mol/l (12.50 mmol) of NaOH solution was added. After stirring for two minutes, 200 µl of $^{13}CH_3I$ (3.21 mmol) for the synthesis of the DMX and 100 µl (1.61 mmol) only for the synthesis of MMX were added. The flask was quickly sealed and the mixture stirred for 24 hours at room temperature.

Thereafter acetone was removed using a rotary evaporator. The pH was adjusted to 8 with hydrochloric acid solution (0.5 mol/l) and then 5g of ammonium sulphate was added to the mixture. The reaction products were extracted with 3 x 50 ml of an isopropanol chloroform mixture (85:15, v/v). The solvents were, then, removed with a vacuum rotary evaporator (at 55 °C).

III - PURIFICATION OF THE REACTION PRODUCTS

1 - IMPROVEMENT OF THE CHROMATOGRAPHIC SEPARATION

The starting conditions for the separation of methylxanthines were the use of a C18 µBondapack semi-prep column, with the mobile phases described in (16-18). Under these conditions the separation of all the MX was imperfect so proportions of the various components of the mobile phase had to be modified in order to improve resolution. The use of ammonium or sodium acetate in the acid medium resulted in poor resolution of theobromine and MMX. It was observed that the resolution and retention times were very sensitive to the amount of THF in the mobile phase because THF is less polar than water and methanol, resulting in a significantly modified eluting force for the system. Due to its high polarizability and powerful solvating effect with respect to methylxanthines, THF improved the selectivity and resolution of the chromatographic system.

2 - WORKING CONDITIONS

For improving the chromatographic resolution the following mobile phase was used: water-methanol-THF (93.8 : 5 : 1.2 v/v).

Flow rate: 3 ml/min, injection volume: 500 µl, detection : 270 nm.

After dissolving the reaction products into the mobile phase, 20 injections were made in order to collect a sufficient amount of each component. Methanol and THF were removed from each fraction using a rotary evaporator at 55 °C. Each compound was then extracted from the chromatographic fraction with 3 x 50 ml of a chloroform-isopropanol mixture (85:15, v/v).

IV - 3-ISOBUTYLPARAXANTHINE-7-($^{13}CH_3$) SYNTHESIS

In order to avoid slow kinetics, the Greeley's reaction takes place in an anhydrous medium. N,N-dimethylacetamide (NDA) was replaced by N-dimethylformamide (NDF) which is easier to remove by evaporation at low temperature (70 °C) than NDA at the end of the reaction. Tetramethylammonium hydroxide (TMAH) was used as the base.

1 - PREPARATION

0.5G (2.25 mmol) of IBMX (3-isobutyl-1-methylxanthine) was dissolved in 30 ml of an NDF-methanol (5:1, v/v) mixture in a 100 ml flask under magnetic stirring. Thereafter, 1 ml of 25 % TMAH in methanol (2.6 mmol) was added to the mixture. After stirring for 5 min, a further addition of 190 µl of $^{13}CH_3I$ (3 mmol) to the mixture was performed and the flask was sealed and stirred for two hours, at room temperature. The reaction resulted in the formation of tetramethylammonium iodide, which was eliminated by filtration. The solvents were first evaporated under vacuum (at 55 °C) and then in a water-bath at 70 °C under a stream of nitrogen .

2 - PURIFICATION

According to Greeley, the conditions of synthesis should, yield relatively pure compounds because the reaction is a total one. In addition, there are no parasitic or competitive synthetic pathways since the precursor has only one alkylation site.

The semi prep liquid chromatographic purification was carried out with the same column as previously described and using the following conditions:

-Mobile phase: water-methanol (60:40, v/v) - Flow rate: 1.5 ml/min;-Volume of injection: 400 µl, detection at 274 nm.

After dissolving the product in the mobile phase, 10 injections were necessary to collect a sufficient amount of 3-isobutylparaxanthine 7-$^{13}CH_3$.

RESULTS AND DISCUSSION

The chromatograms obtained after synthesis and semipreparative HPLC separation are shown in Fig. 1. The injected amount of each component was 50 μg (1a). Fig. 1b and 1c show the separation of the mixtures resulting from the synthesis of DMX$_i$* and MMX* respectively.

Spectra obtained by GC-MS (fig. 2a, 2b, 2c and Fig. 3a, 3b, 3c) were compared to the reference obtained from pure unlabelled compounds. This comparison shows similar fragmentation patterns and allows one to verify the authentication and purity of the prepared compounds.

Fig. 2 shows the mass spectra of two MMX and of 3-isobutylparaxanthine as well as those of their [^{13}C]-labelled analogues (MMX* and 3-isobutylparaxanthine-7-[$^{13}CH_3$]).

Pierce (8) studied the two intermediates formed during the synthesis of the various isotopomers of caffeine-1,7-[$^{13}CH_3]_2$ from 3MX and found that theophylline is transformed into caffeine more quickly than theobromine. This provides support for the distinct reactivity between the various alkylation sites of the xanthine moiety.

The results obtained during the synthesis of MMX* from xanthine and $^{13}CH_3I$ (at lower amounts than those given by the reaction stoichiometry) clearly shows this inequality.

3-MX and 7-MX were obtained but only a very small amount of 1-MX was formed. Thus this compound can not be transformed as demonstrated by the absence of DMX* and caffeine in the reaction medium of the MMX*. It can also be observed that the (N_3) site is much more reactive because a greater amount of 3 MX*, is formed during the reaction.

The reactivity of the three sites of xanthine can therefore be given the following order : (N_1)<(N_7)<(N_3) for a first alkylation step of xanthine.

For the second alkylation step from 3-MX* with a greater amount of $^{13}CH_3I$, but still lower than that given by the stoichiometry of the reaction, it can be said that the order of reactivities is the opposite one: ((N_7)<(N_1)) because the quantity of 1,3-DMX* formed in the mixture is higher than that of the 3,7-DMX*. Hence, a methylation on the (N_3) site mostly induces a methylation at the (N_1) position.

The pKa values of the protons (21) of 3-MX (pKa(N-H)$_7$=8.32, pKa(N-H)$_1$=11.92), show that the (N_7) site is more acidic than the (N_1) site although the methylation from 3-MX is more easily obtained on the (N_1) site. So it appears that acidity is not a determining factor in the location of the second alkylation reactions.

Furthermore, the N-methylation reactions of xanthine can be compared to the opposite N-demethylation reactions of caffeine since the (N_3) site is the most involved in the competitive

metabolic demethylations. This may be explained by steric hindrance. The (N_1) site is located between two carbonyl groups and compared to (N_3) and (N_7) sites appears to be the most hindered site for the alkylating reagents.

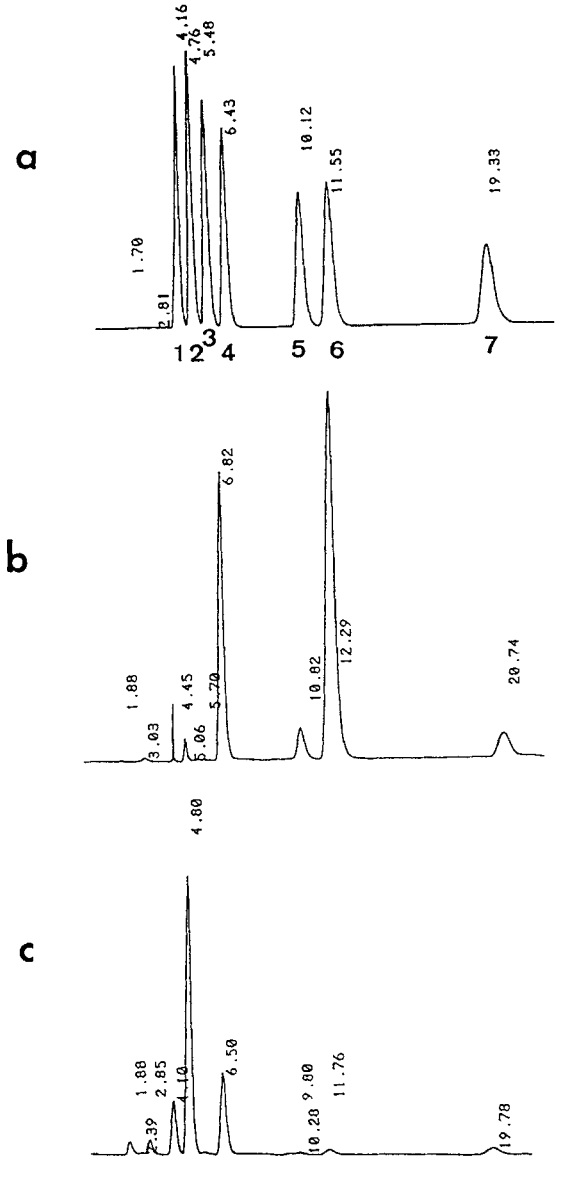
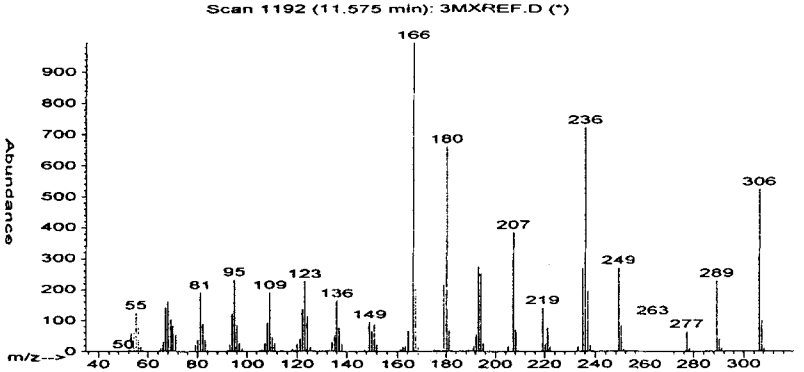
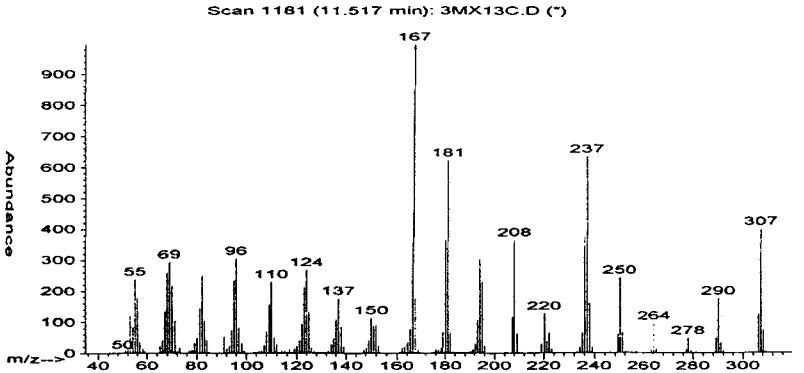


Fig.1: Semipreparative liquid chromatography separation of methylxanthines:

Standard mixture (50 μg of each product) (a), synthesis products (DMX major compounds formed) (b), synthesis products (MMX major compounds formed) (c). 7-MX (1) 3-MX (2) 1-MX (3) 3,7-MX (4) 1,7-MX (5) 1,3-MX (6) caffeine (7).

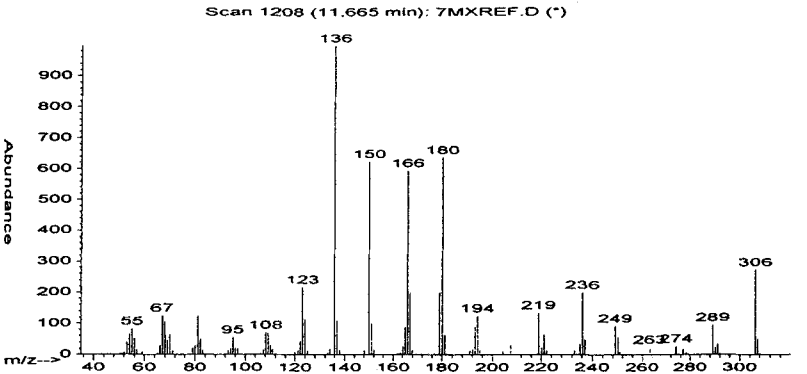
Fig. 2a: electron impact GC-MS analysis

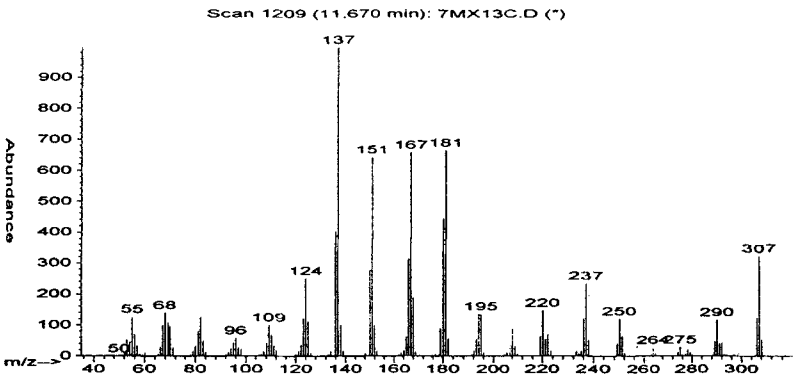




Mass spectra of pentylated compounds: 3-MX m/z = 306 (reference,top) and 3-MX* m/z = 307 (synthesised, bottom).

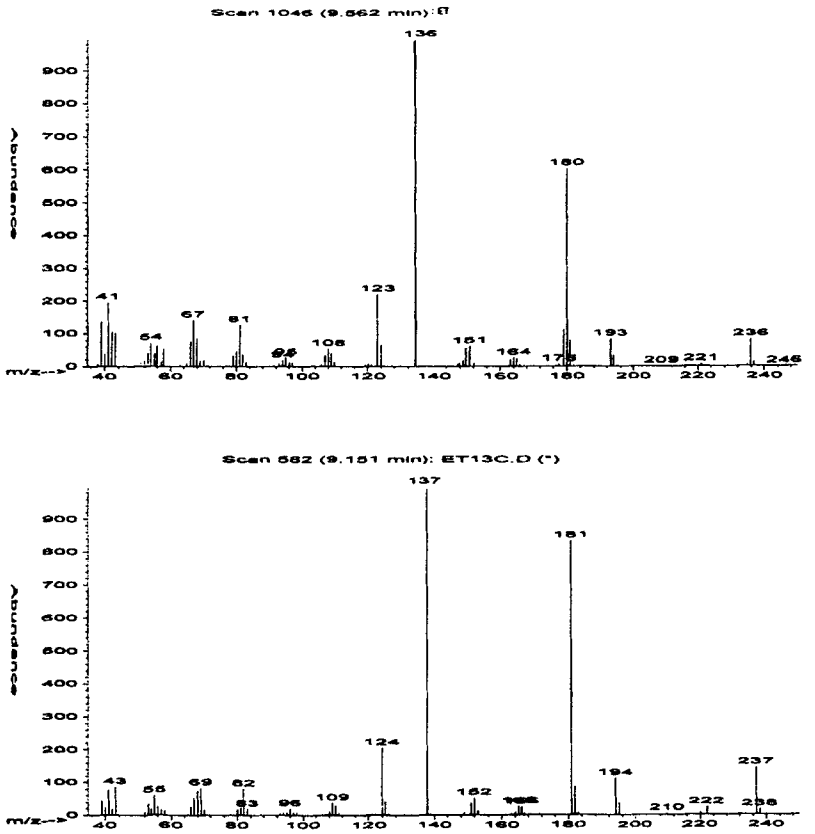
Fig. 2b: electron impact GC-MS analysis





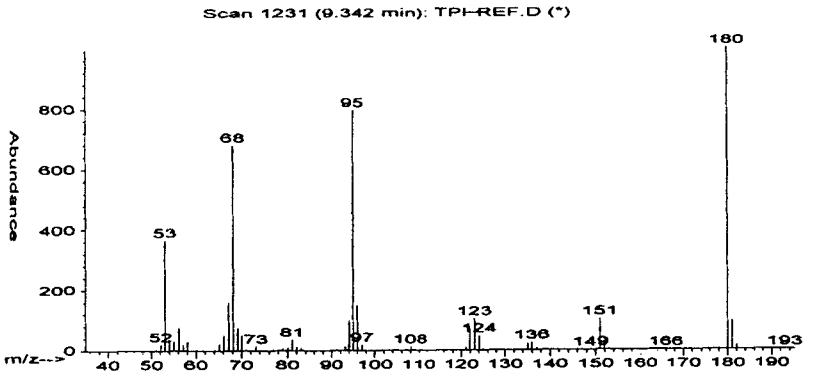
Mass spectra of pentylated compounds: 7-MX m/z = 306 (reference,top) and 7-MX* m/z = 307 (synthesised, bottom).

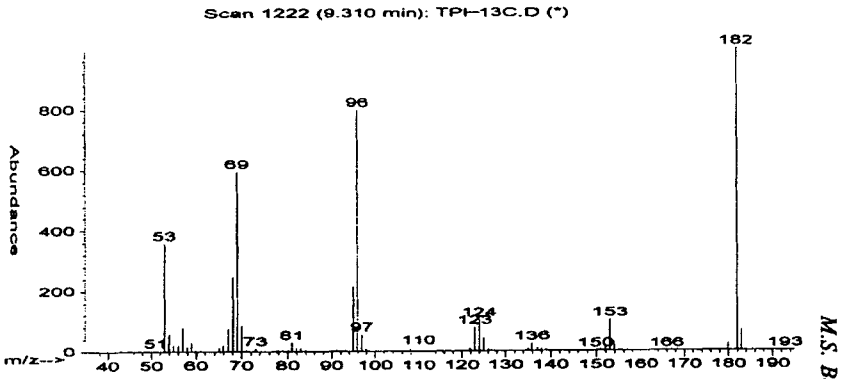
Fig. 2c: electron impact GC-MS analysis



Mass spectra of pentylated compounds:
3-isobutyl-1-methylxanthine m/z = 236 (reference, top) and
3-isobutyl-1-methylxanthine-7-($^{13}CH_3$) m/z = 237 (bottom)

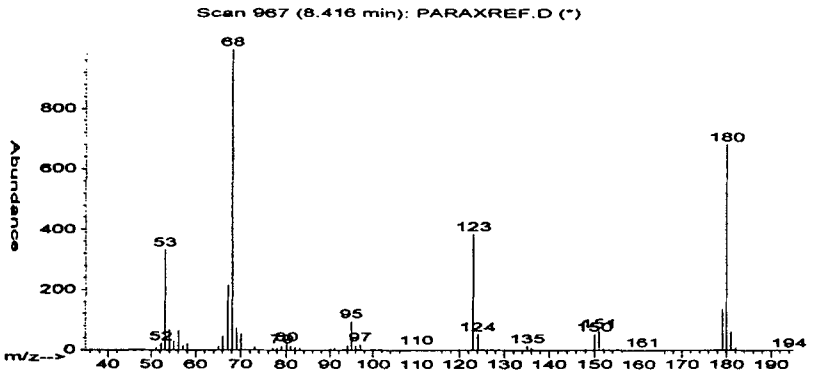
Fig. 3a: GC-MS analysis of DMX

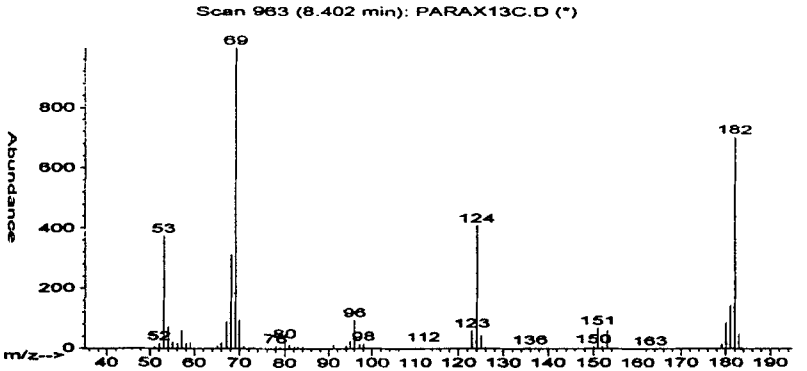




Mass spectra of 1,3-MX m/z = 180 (reference, top) and
1,3-MX* m/z = 182 (synthesised, bottom).

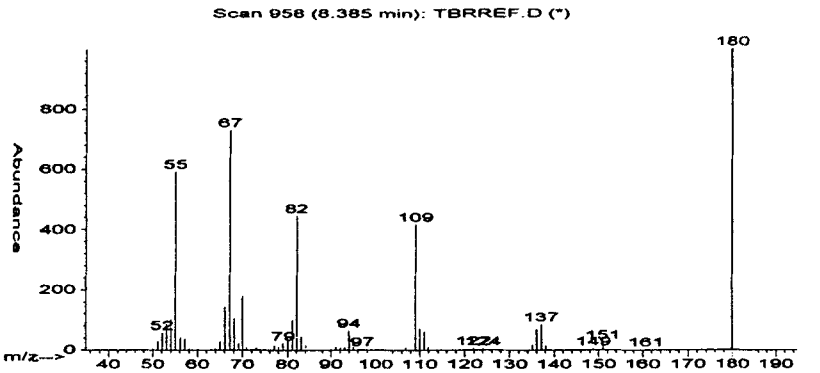
Fig. 3b: GC-MS analysis of DMX

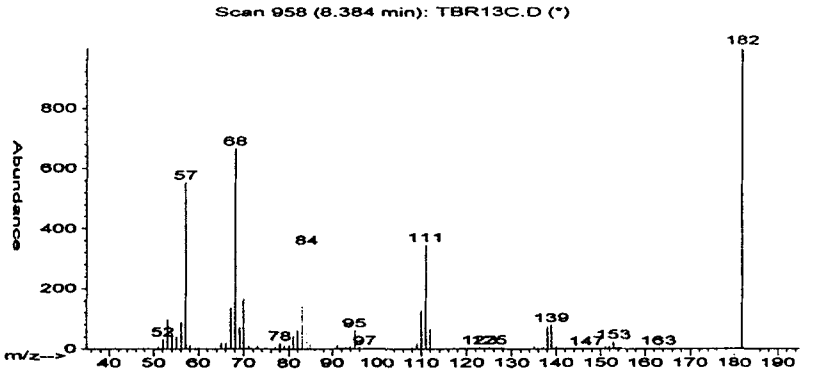




Mass spectra of 1,7-MX m/z = 180 (reference, top) and 1,7-MX* m/z = 182 (synthesised, bottom).

Fig. 3c: GC-MS analysis of DMX





Mass spectra of 3,7-MX m/z = 180 (reference, top) and 3,7-MX* m/z = 182 (synthesised, bottom).

Steric hindrance does not appear to be important in the second methylation step because theophylline is the compound formed (3,7-DMX<1,3-DMX) and, in this case, the reactivity of the (N_1) site is increased as compared to that of the (N_7) site.

Acidity and steric hindrance can not completely explain the unequal reactivity between the three (N-H) sites, with respect to an SN_2-type nucleophilic reaction. This reaction is also sensitive to the nucleophilic character of the different alkylation sites. This effect perhaps explains the low reactivity of the (N_1) site of xanthine, which is located between two electron withdrawing groups, whereas in the case of (N_3) and (N_7) sites the nucleophilic charge can be stabilised by a mesomeric effect (Fig. 4a, 4b, 4c).

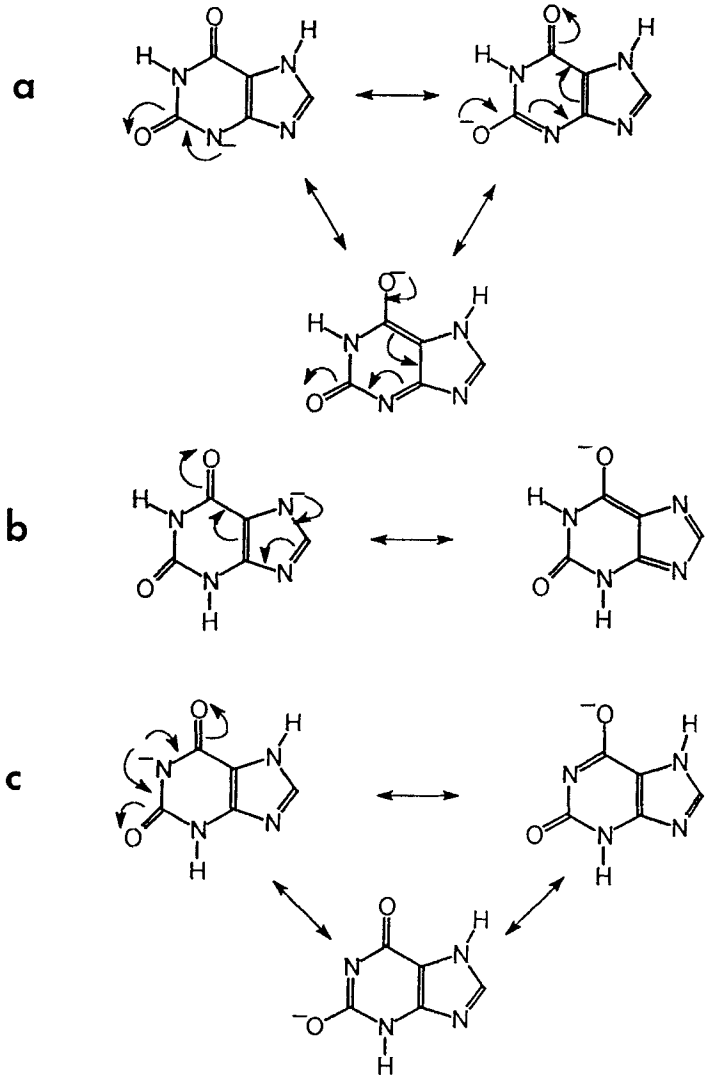


Fig.4: Limited mesomeric forms of the deprotonated xanthine:
at N(3) (a) N(7) (b) and N(1) (c).

From 3-MX, the reactivity of the (N_1) site increases because of the presence of the methyl group at (N_3), which seems to increase the nucleophilicity at (N_1). Methylation on the (N_3) site therefore induces methylation on the (N_1) site.

Fig. 5 shows the compounds resulting from this synthesis.

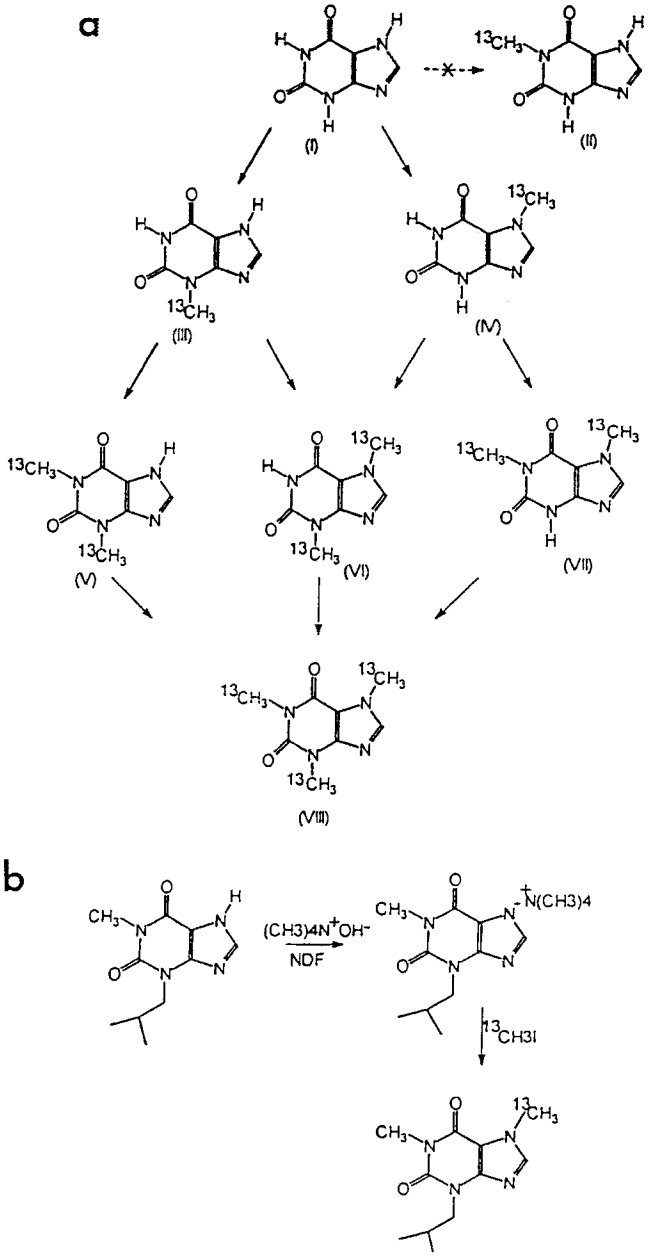


Fig.5: Synthesis of xanthine derivatives: a- from xanthine (I). b- from 3-isobutyl-1-methylxanthine. 1-MX* (II), 3-MX* (III), 7-MX* (IV), 1,3-MX* (V), 3,7-MX* (VI), 1,7-MX* (VII), Caffeine-1,3,7-($^{13}CH_3$)$_3$ (VIII).

CONCLUSION

Synthesis of theophylline-1,3-$[^{13}CH_3]_2$, theobromine-1,3-$[^{13}CH_3]_2$, paraxanthine-1,3-$[^{13}CH_3]_2$, 3-$[^{13}CH_3]$-xanthine and 7-$[^{13}CH_3]$-xanthine starting from xanthine is now possible. This synthesis uses smaller quantities of alkylating reagents than those needed in the stoichiometric conditions used for caffeine synthesis. 3-Isobutylparaxanthine-7-$[^{13}CH_3]$ is prepared from IBMX according to the same process. For the first alkylation step the reactivity of the three sites of xanthine increased as follows : $(N_1) < (N_7) < (N_3)$.

For the synthesis of DMX* from xanthine, 3-MX* was the main intermediate. This compound undergoes a second methylation step, mainly on the (N_1) site, giving rise to the formation of the following compounds: 1,7-DMX*<3,7-DMX*<1,3-DMX*.

Purification was carried out by semi-preparative high performance liquid chromatography on a C18 μBondapack column with water-methanol-THF mixture as mobile phases for methylxanthines and water-methanol mixture for isobutylparaxanthine-7-$[^{13}CH_3]$. The products obtained were analysed by GC-MS. The comparison of the reference chromatograms and spectra shows the authenticity and the high purity of the synthesized compounds.

References

1. Brazier J.L., Ritter J., Berland M., Khenfer D. and Faucon G. - Develop. Pharmacol. Therap. 6: 315 (1983).

2. Boukraa M.S., Deruaz D., Bannier A., Desage M. and Brazier J.L. - J. Pharm. Biomed. Anal. 12: No.2, 185 (1994).

3. Horning M.G., Nowlin J., Thenot J.P. and Bouwsma O.J. - Stable Isotopes: Proceedings of the international conference, E.R. Klein and P.D. Kein, Academic Press, N.Y., 379 (1979).

4. Benchekroun Y., Desage M., Ribon B. and Brazier J.L. -J. Chromatogr. 532: 261 (1990).

5. Cherrah Y., Zini R., Falconnnet J.B., Desage M. and Brazier J.L. - J. Biochem. Pharmacol. 37: 1311 (1988).

6. Cherrah Y., Falconnnet J.B., Desage M. Brazier J.L., Zini R. and Tillement J.P. - Biomed. Environ. Mass Spectrom. 17: 245 (1988).

7. Bechalany A., El Tayar N., Carrupt P.A., Testa B., Falconnet J.B. , Cherrah Y., Benchekroun Y. and Brazier J.L. - Helvetica Chimica Acta 72: 472 (1989).

8. Pierce W.M., Schlager J.J., Madden R.G., and Hurst H.E. - J. Labelled Compounds Radiopharm. 21: No.2, 187 (1984).

9. Falconnnet J.B., Brazier J.L. and Desage M. - J. Labelled Compounds Radiopharm. 23: No.3, 267 (1983).

10. Berlioz C., Falconnet J.B., Desage M. and Brazier J.L. - J. Labelled Compounds Radiopharm. 24: No.3, 275 (1987).

11. Greeley R.H. - J. Chromatogr. 88: 229 (1974).

12. Greeley R.H. - Clin. Chem. 20: 192 (1974).

13. Shah V.P. and Riegelman S. - J. Pharm. Sci. 63: 1283 (1974).

14. Johnson G.F., Dechtiaruk W.A. and Solomon H.M. - Clin. Chem. 21: 144 (1975).

15. Kowblansky M., Scheinthal B.M., Cravello G.D. and Chafetz L. - J. Chromatogr. 76: 467 (1973).

16. Miksic J.R. and Hodes B. - J. Pharm. Sci. 68: 1200 (1979).

17. Hartley R., Cookman J.R. and Smith I.J. - J. Chromatogr. 306: 191 (1984).

18. Ullrich D., Compagnone D., Munch B., Brandes A., Hille H. and Bircher J. - Eur. J. Clin. Pharmacol. 43: 167 (1992).

19. Hartley R., Smith I.J. and Cookman J.R. - J. Chromatogr. 342: 105 (1985).

20. Tanaka E., - J. Chromatogr. 575: 311 (1992).

21. Naline E., Flouvat B., Advennier C. and Pays M. - J. Chromatogr. 419: 177 (1987).