SYNTHESIS OF S-ADENOSYL-L-HOMOCYSTEINE AND 5'-METHYLTHIOADENOSINE SPECIFICALLY TRITIATED AT THE 5'C POSITION

G. Guillerm, M.C. Galas, F. Le Goffic

Laboratoire de Bioorganique et Biotechnologies

ENSCP 11, rue P. & M. Curie 75231 Paris Cédex 05, France

J.P. Beaucourt, L. Sergent

Service des Molécules Marquées, CEN-Saclay 91191 Gif sur Yvette, France

SUMMARY

S-adenosyl-L-homocysteine and 5'-methylthioadenosine were bitritiated specifically at C'-5 for enzymatic assay of S-adenosylhomocysteine nucleosidase (EC.3.2.2.9).

Key Words : Tritium labelling, S-AdoHcy, 5'-methylthioadenosine.

S-adenosyl-L-homocysteine (AdoHcy) is an important metabolic product in a variety of processes (1.2). This compound as well as 5'-methylthioadenosine are substrates for AdoHcy nucleosidase, an enzyme required in various prokaryotes cells (3.4.5.6) for the recovery of the enzymatic activity of methylases through the splitting of S-adenosylhomocysteine.

For the evaluation of inhibitors of AdoHcy nucleosidase we developped (7) a more simple and reliable enzymatic assay than those previously described (3-8). It uses specifically tritiated AdoHcy or 5'-methylthioadenosine which are split during the enzymatic reaction into adenine and ^{3}H S-ribosyl L-homocysteine or ^{3}H 5'-methylthioribose respectively (3).

0362-4803/86/080845-08$05.00

Received December 6, 1985
Revised January 22, 1986

Therefore the assay required specifically labelled AdoHcy or 5'-methylthioadenosine with the highest possible specific activity.

We describe here the preparation of 5'-($^3H_2$)-AdoHcy and 5'-($^3H_2$)-5'methylthioadenosine. The general route (9.10.11) used for synthesis of non radioactive AdoHcy and its analogs was adopted. The procedure for each step and the methods of purification of each intermediate were adapted for secure manipulations of radioactive material. Thus, following the scheme outlined in figure 1, chemically and radiochemically pure 5'-($^3H_2$)methylthioadenosine (6) and 5'-($^3H_2$) AdoHcy 7 were obtained.

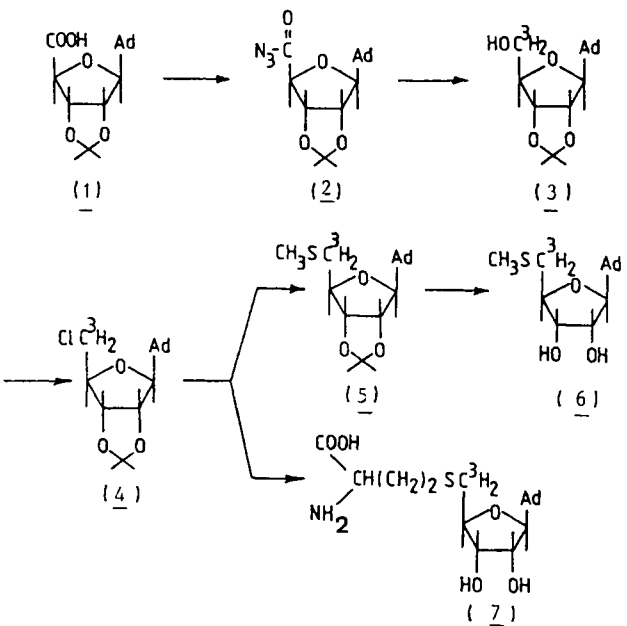

Figure 1

The synthesis of 5'-(^3H)-2',3'-isopropylidene adenosine (3) (specific activity 55 mCi/mMole) was achieved by reduction with (^3H) sodium borohydride of the 5'-ketoazidoadenosine (2) which was prepared from derivative (1) according to the method described by R.R. Schmidt and al. (12).

Chlorination (13) of the labelled-2',3'-isopropylidene adenosine with $SOCl_2$ gave (4) (98 % yield) which was converted into isopropylidene 5'-methylthioadenosine (5) and AdoHcy (7) by substitution of the chlorine with sodium

methylsulfide or sodium L-homocysteinate in liquid ammonia as described in an earlier work (9.11).

Hydrolysis of isopropylidene moiety was achieved with limited removal of adenine by treatment of O-2',3' protected derivative with 0.1 N HCl. In this way 6 mCi of (6) and 2,5 mCi of (7) were obtained with a specific activity of 55 mCi/mMole.

EXPERIMENTAL

All chemicals were of the highest purity available (^3H) $NaBH_4$ (≠100 mCi/mMole) was purchased from C.E.A. (Saclay-France).

^1H NMR spectra were recorded on a CAMECA 250 MHz spectrometer and ^3H NMR on a Bruker 106 MHz spectrometer. Chemical shifts are reported in ppm (δ) with TMS as internal standard. The mass spectra were obtained using a VARIAN CH 7A (E.I.) and NERMAG (DCI/NH_3) mass spectrometers. U.V. spectra were recorded with a Beckman DU-8 model spectrophotometer. Flash column chromatography was conducted with silica gel (No9385, (230-400 mesh), E. Merck, Darmstadt, Germany).

Counting was carried out with a L.K.B. 1211 liquid scintillation counter in Beckman scintillation Coctail HP/Band NA. All the results were corrected for quenching by external standard method.

For all the prepared radioactive compounds, the chemical homogeneity was checked by TLC (silica gel precoated plates N°5554-Merck) and the radiochemical purity by autoradiography using a Berthold β camera.

5'-($^3H_2$) -2,3'-O-isopropylidene adenosine (3)

To a suspension of 320 mg (1.0 mMole) of (1) in 200 ml of acetone, was added 180 µl (1.3 mMole) of triethylamine and 130 µl (1.35 mMole) of ethylchloroformate. The mixture was stirred for 3 hours at 0°C. To the resulting clear solution, 98 mg (1.5 mMole) of sodium azide dissolved in 2 ml of water was added. The mixture was stirred for a further 2 hours. The solution was then evaporated to dryness and the resulting powder was placed on a fritted

glass and washed successively with 20 ml of ether, (2x5 ml) of cold water and quickly dried in vacuo.

The resulting ketoazide (2) (300 mg) was used without further purification. (2) was then suspended in 40 ml of absolute ethanol and 100 mCi (sp. act. 100 mCi/mMole) of (^{3}H) sodium borohydride was added and the solution stirred at room temperature overnight.

After neutralization of the mixture with a few drops of glacial acetic acid, the solution was evaporated under vacuo and the crude labelled adenosine extracted by trituration of the residue with (2x40 ml) of ethylacetate. The ethylacetate solution was concentrated in vacuo and the tritiated adenosine purified on a column of silica gel (flash chromatography) using ethylacetate-methanol (10 v/1.25 v) as eluent. The overall yield of pure (3) was 46 % (138 mg, sp. act. 55.7 mCi/mMole).

250 MHz ^{1}H NMR (Me_2SO-d_6) δ 1.39 (s) and 1.60 (s), (CMe_2), 3.40 (dd, J_{gem}=12, $J_{4',5'}$=5, $H_{5'}$), 3.30 (dd, J_{gem}=12, $J_{4',5'}$=7, $H_{5'}$), 4.30 (ddd, $J_{3',4'}$=3, $J_{4',5'}$=5, $H_{4'}$), 5.02 (dd, $J_{3',4'}$=3, $J_{2',3'}$=6, $H_{3'}$), 4.39 (dd, $J_{2',3'}$=6, $J_{1',2'}$=2.5, $H_{2'}$), 6.20 (d, $J_{1',2'}$=2.5, $H_{1'}$), 8.40 (s) and 8.28 (s) (H_2 and H_8).

MS (low -resolution, EI), m/e 307 ($M^{+\cdot}$), 292 $(M-CH_3)^+$, 277 $(M-H_2CO)^{+\cdot}$, 249 $(M-CH_3COCH_3)^+$, 218 $(C_9H_8N_5O_2)^+$, 173 (sugar moiety)$^+$, 164 (base-$\overset{+}{C}$H=OH), 135 (base, H)$^+$, 59 $(CH_3-C(OH)CH_3)$.

UV (ethanol) λ_{max}=262 nm (14 300).

5'-Chloro-5'-($^3H_2$)-5'deoxy-2',3'-O-isopropylidene adenosine (4)

130 mg (0.42 mMole) of (3) (sp. act. 55.7 mCi/mMole) was dissolved in 1 ml of freshly purified $SOCl_2$. The resulting yellow solution was allowed to stand 24 hours in a stoppered flask before evaporation to dryness under vacuo. The residue was then reevaporated twice with 10 ml of benzene and the resulting solid was taken with 5 ml of H_2O and 1 ml of triethylamine. The labelled (4) was extracted with (5x15 ml) of chloroform and the organic phase washed with

10 ml of water and dried over $MgSO_4$.

The purification of (4) was achieved on a column of silica gel (flash chromatography) using ethylacetate-MeOH (12 V/1V) as eluant. 117 mg (86 %) of pure (4) was obtained (foam), specific activity 56 mCi/mMole.

250 MHz-^{1}H NMR : ($CDCl_3$) δ 1.35 (s), 1.58 (s), (CMe_2), 3.58 (dd, J_{gem}=12, $J_{4',5'}$=5.5, $H_{5'}$), 3.82 (dd, J_{gem}=12, $J_{4',5'}$=7.5, $H_{5'}$), 4.44 (d,d,d, $J_{3',4'}$=2.5, $J_{4',5'}$=7.5, $J_{4',5'}$=5.5, $H_{4'}$), 5.11 (dd, $J_{2',3'}$=5, $J_{3',4'}$=3, $H_{3'}$), 5.44 (dd, $J_{2',1'}$=2.5, $J_{2',3'}$=5, $H_{2'}$), 6.08 (d, $J_{1',2'}$=2.5, $H_{1'}$), 6.44 (NH_2), 7.88 (s) and 8.29 (s), H_2 and H_8.

106 MHz-^{3}H NMR ($CDCl_3$) : with irradiation of ^{1}H. δ 3.58 (s) and 3.76 (s), $^3H_{5'}$. Without irradiation of ^{1}H, 3.48 (dd, J_{gem}=11.9, $J^1H_{4'}{}^3H_{5'}$=5.6, $H_{5'}$) 3.76 (dd, J_{gem}=11.9, $J^1H_4{}^3H_{5'}$=7.4, $H_{5'}$).

MS (low resolution ; EI), m/e 325, 327 ($M^{+\cdot}$) 310 $(M-CH_3)^+$, 267 $(M-CH_3COCH_3)^+$, 218 $(C_9H_8N_5O_2)^+$, 136 (base, 2H)$^+$, 135 (base, H)$^+$, 59 $(CH_3\overset{+}{C}OHCH_3)$.

UV (ethanol) λ_{max} 261 nm (14 000).

5'-($^3H_2$)-5'-deoxy-5'-methylthio-2',3'-O-isopropylidene adenosine (5)

To a solution of 50 mg (0.153 mM, sp. act. 56 mCi/mMole) of (4) in anhydrous DMF, was added 40 mg (0.57 mM) of dry sodium methylsulphide. The solution was stirred at 90°C for 12 hours, then, evaporated to dryness under vacuo. After adding 10 ml of water, the tritiated methylthioadenosine was extracted with (4x10 ml) of chloroform. The organic phase was dried on $MgSO_4$ and evaporated. The crude residue was purified by flash chromatography (silica gel) using ethylacetate-methanol (16 V/1 V) as eluant. 48 mg (96 %) of pure (5) was obtained (sp. act. 55 mCi/mMole).

250 MHz ^{1}H NMR : ($CDCl_3$) δ 1.40 (s), 1.64 (s), (CMe_2), 2.10 (s, CH_3S), 2.76 (dd, J_{gem}=13.5, $J_{4',5'}$=6, $H_{5'}$) 2.86 (dd, J_{gem}=13.5, $J_{4',5'}$=7.5, $H_{5'}$) 4.46 (ddd, $J_{3',4'}$=3, $J_{4',5'}$=6, $J_{4',5'}$=7.5, $H_{4'}$), 5.12 (dd, $J_{2',3'}$=6.5, $J_{3',4'}$=3, $H_{3'}$), 5.6 (dd, $J_{2',3'}$=6.5, $J_{H1',2'}$=2, $H_{2'}$), 6.18 (d, $J_{1',2'}$=2, $H_{1'}$), 7.2 (broad s, NH_2), 8.02 (s), 8.36 (s) (H_2 and H_8).

106 MHz ^{3}H NMR ($CDCl_3$) with irradiation of ^{1}H. 2.70 (s) and 2.78 (s), $^3H_{5'}$.

UV (CH_3OH) λ_{max}=261 nm ε=1400.

5'-($^3H_2$)-5'-deoxy-5'-methylthioadenosine (6)

45 mg (0.133 mM) of (5) was dissolved in 5 ml 0.1 N HCl and heated to 80°C 20 minutes. After cooling the solution was diluted with 5 ml H_2O and neutralized with Dowex AG 2x4 A (OH^-) resin. The resin was filtered and washed with 50 ml of aqueous methanol (MeOH-H_2O, 30 V/70 V). The effluent was evaporated to dryness and the crude mixture purified by flash chromatography on a column of silica gel using ethylacetate-methanol, 7.5 V/2.5 V as eluant. 31 mg (78 %) of pure (6) was obtained (sp. act. 55 mCi/mMole) and separated from small quantities of 5'-($^3H_2$)-5'-deoxy methylthioribose and adenine formed during of the acidic treatment of (5).

250 MHz ^{1}H NMR : ($(CH_3)_2SO$-d_6) δ 2.1 (s, CH_3S), 2.80 (dd, $J_{gem}H_{5'}$=13, $J_{4',5'}$=6, $H_{5'}$), 2.92 (dd, $J_{gem}H_{5'}$=13, $J_{4',5'}$=7, $H_{5'}$), 4.80 (ddd, badly resolved, $H_{4'}$), 5.44 (dd, badly resolved, $J_{2',3'}$=6, $H_{3'}$), 5.64 (dd, badly resolved, $J_{2',3'}$=6, $H_{2'}$), 5.86 (d, $J_{1',2'}$=5, $H_{1'}$), 7.36 (s broad, NH_2), 8.24 (s), 8.44 (s) (H_2 and H_8).

MS : (DCI/NH_3) 298 $(MH)^+$, 136 (base, 2H)$^+$.

UV (MeOH) λ_{max}=261 nm (11 500).

5'-($^3H_2$)-S-adenosyl-L-homocysteine (7)

50 mg (excess) of dry disodium derivative of L-homocysteine (prepared from

L-homocysteine) was dissolved in 15 ml of bidistilled ammonia. 40 mg (0.12 mM, sp. act. 56 mCi/mMole) of (4) in 1.5 ml of dry THF was added in the solution which was stirred for 4 hours at -33°C under argon. Ammonia was allowed to evaporate and the solid residue dissolved in 10 ml of water and the basic solution extracted with 3x10 ml of chloroform to remove the unreacted chloroderivative. The pH of the aqueous solution was brought to 7 with 1N HCl and this solution was lyophilized.

The 5'-($^3H_2$)-O-2',3' isopropylidene- S -adenosyl-L-homocysteine was not isolated and the removal of the isopropylidene protective group achieved by treatment of the amorphous powder, obtained after lyophilization, with 10 ml of 0.1 N HCl at 80°C, 20 mn. After cooling the pH of the solution was adjusted to 6 with 1 N NaOH and the mixtured concentrated in vacuo. The purification of the crude (7) was carried out using a column of 100 ml of Dowex 50 WX4 (100-200 mesh) in NH_4^+ form. Elution was performed successively with 200 ml of water and 200 ml of 1 N NH_4OH solution.

Fractions containing the desired substance were combined and this solution was lyophilized to afford 17 mg (35 %) of pure (7) (sp. act. 55 mCi/mMole), the chemical homogeneity of which was checked by TLC (silica gel) using ethanol/water (4 V/1 V).

250 MHz ^1H RMN : (D_2O +ε Na_2CO_3) External TMS

δ 1.62 (m, $H_{7'}$), 2.4 (t, $J_{6',7'}$=7, $H_{6'}$), 2.74 (dd, $J_{gem}H_{5'}$=15, $J_{4',5'}$=7, $H_{5'}$), 2.84 (dd, $J_{gem}H_{5'}$=15, $J_{4',5'}$=5, $H_{5'}$), 4.14 (dd, $H_{3',4'}$=3, $H_{4',5'}$=7, $J_{4',5'}$=5, $H_{4'}$), 4.24 (t, $J_{7',8'}$=5, $H_{8'}$), 8.0 (s) and 8.14 (s) (H_2 and H_8).

106 MHz ^3H RMN : with irradiation of ^1H.

δ 2.75 (s) and 2.84 (s), $^3H_{5'}$.

MS (DCI/NH_3) 385 $(M, H)^+$.

UV (H_2O +ε Na_2CO_3) λ_{max}=260 nm (12 700).

REFERENCES

1. Shapiro S.K., Schlenk F. - Advances in Enzymology, vol. 22, Interscience Publishers, Inc, New-York, 237 (1960).
2. Ueland P.M. - Pharmacol. Rev. 34 : 223 (1982).
3. Duerre J.A. - J. Biol. Chem. 237 : 3737 (1962).
4. Miller C.H., Duerre J.A. - J. Biol. Chem. 243 : 92 (1968).
5. Walker R.D. and Duerre J.A. - Can. J. Biochem. 53 : 312 (1975).
6. Shimizu S., Shiozaki S., Ohshiro T., Yamamada H. - Eur. J. Biochem. 141 : 385 (1984).
7. Guillerm G., Galas M.C., Le Goffic F. - unpublished results.
8. Duerre J.A. - Methods in Enzymology. Academic Press-NY, 411, vol. XVIIB.
9. Baddiley J. and Jamieson G.A. - J. Am. Chem. Soc. 1085 (1955).
10. Baddiley J. - J. Am. Chem. Soc. 1348 (1951).
11. Borchardt R.T., Huber J.A., Yihschiong Wu. - J. Am. Chem. Soc. 41 : 565 (1976).
12. Schmidt R.R., Schloz U. and Schwille D. - Chem. Ber. 101 : 590 (1968).
13. Gibbs D.E. and Verkade S.G. - Synth. Comm. 6 : 563 (1976).