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SYNTHESIS OF S-RIBOSYL-L-HOMOCYSTEINE SPECIFICALLY TRITIATED AT THE 5-C POSITION

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SUMMARY

S-Ribosyl-L-homocysteine, tritiated specifically at the 5-C position, has been synthesized in order to assay S-Ribosyl-L-homocysteine hydrolase (EC. 3.3.1.3).

Key Words : Tritium labelling, S-Ribosyl-L-homocysteine.

INTRODUCTION

The main pathway for regeneration of free homocysteine in the activated methyl cycle of various prokaryote cells is initiated by cleavage of the glycosyl linkage of S-adenosyl homocysteine (Ado Hcy) by Ado Hcy nucleosidase (1,2,3,4) yielding adenine and S-ribosyl-L-homocysteine. S-ribosyl-L-homocysteine is then hydrolysed to free homocysteine and ribose by S-ribosyl-L-homocysteine hydrolase (5,6).

For the purification of S-ribosyl homocysteine hydrolase and evaluation of inhibitors of this enzyme we developed a more simple and reliable enzymatic assay than those previously described (5,6,7). It uses S-ribosyl homocysteine tritiated on the ribose moiety, which is split during the enzymatic reaction into homocysteine and ribose. Chromatographic separation of ribose from S-ribosyl homocysteine and homocysteine is easily performed on a microcolumn of cellex P (cation exchanger) and allows direct determination of the amont of labelled ribose formed in the enzymatic reaction.

This assay procedure required specifically labelled S-ribosyl-L-homocysteine with the highest possible specific activity.

We describe here the preparation of chemically and radiochemically pure [5-^{3}H]-S-ribosyl-L-homocysteine.

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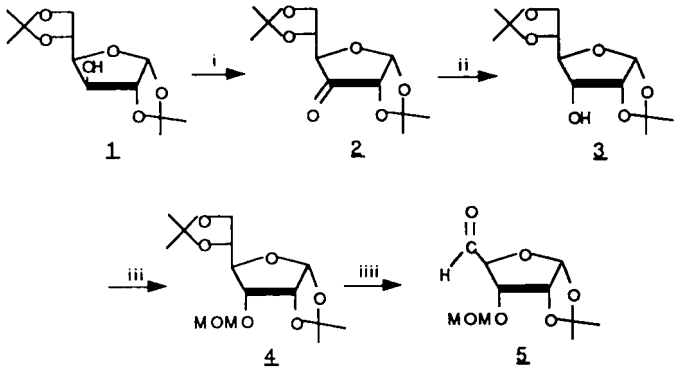
DISCUSSION

The 1,2-isopropylidene-3-O-methoxymethyl-α-D-ribopentodialdo-1,4-furanose 5 has been prepared in our laboratory (8) starting from the diacetonide 1 of D(+)-glucose using classical methods (9,10,11) (Scheme 1).



1 2 3 4 5

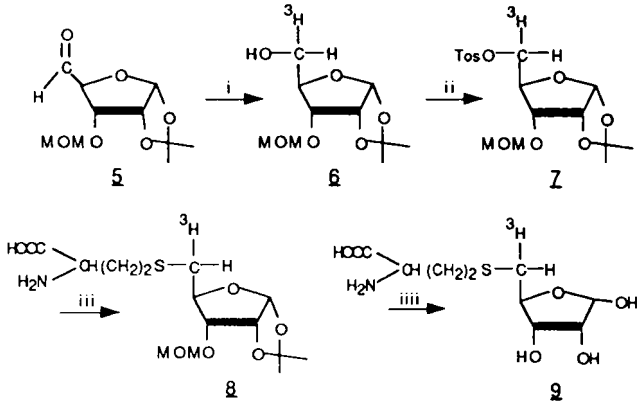
Scheme 1

Reagents : i : PDC , CH_2Cl_2 ; ii : $NaBH_4$, EtOH ; iii : MOMCl , CH_2Cl_2 , $(iPr)_2NEt$;

iiii : H_2SO_4 0,8% , H_2O/MeOH (36 h) , $NaHCO_3$ 10% , $NaIO_4$, MeOH

This aldehydosugar 5 was a useful precursor to the synthesis of [5-^{3}H] ribosylhomocysteine 9.

The general route for synthesis of 9 is illustrated on the following scheme (Scheme 2).



5 6 7 8 9

Scheme 2

Reagents : i : NaB^3H_4 (4000 MBq , 0.67 TBq/mmole) , EtOH ; ii : TsCl , pyridine ;

iii : sodium L-homocysteinate , DMF , 80°C , iiii : 0 25 M HCl , AG11A8 resin

The procedure for each step, and the method of purification of each intermediate was adapted for secure manipulation of radioactive material.

Reduction of 5 (0,04 mmole) with sodium [³H] borohydride (4000 MBq, 0.67 TBq/mmole) in ethanol for one hour, followed by treatement with excess of $NaBH_4$ gave chemically pure labelled ribose deritative 6 with 47% isotopic dilution.

Tosylation of 6 in pyridine with a large excess of tosyl chloride (10 eq.) afforded 7 in 69% yield. Condensation of 7 in DMF with the disodium salt of L-homocysteine generated by the classical method (12,13) gave 8 in 22% yield after purification on HP 20 SS resin. Deprotection of the 1,2 and 3 hydroxyl groups of 8 was achieved by treatment of 8 with 0.25 M HCl. Purification of 9 on cellulose precoated plates afforded chemically and radiochemically pure [5-³H]-S-ribosyl-L-homocysteine with a specific activity of 75 GBq/mmole.

Unlabelled S-ribosyl-L-homocysteine was also synthesized by using the same reaction sequence. The structure of the unlabelled intermediates as well as the corresponding target tritiated derivatives were established by spectroscopic techniques (^1H, ^3H NMR and mass spectroscopy).

EXPERIMENTAL

All chemicals were of the highest purity available. [³H] $NaBH_4$ (4000 MBq, at 0.67 TBq/mmole) was purchased from CEA (Saclay - France). ^1H NMR spectra were recorded on a BRUCKER 300 MHz spectrometer, ³H NMR on a BRUCKER 106 MHz spectrometer, chemical shifts are reported in ppm (δ), using TMS as internal standard and J values in Hz. The mass spectra were obtained using a NERMAG (DCI/NH_3) mass spectrometer. UV spectra were recorded using a Beckman DU-8 model spectrophotometer. HP 20 SS Dianion resin was from Mitsubishi fine chemicals. Counting was carried out with a β 5 Kontron Liquid scintillation Counter in Beckman scintillation cocktail HP/B and NT. All the results were corrected for quenching by the external standard method.

For all the prepared radioactive compounds the chemical homogenety was controlled by TLC (silica gel precoated plates N°5545J Merck) and radiochemical purity by radiochromatography using a LB 285 BERTHOLD Scanner equipped for CHROMA 2D.

1,2-O-isopropylidene-3-O-methoxymethyl-[5-^3H]-α-D-ribopentofuranose 6

In a 5 ml long-necked flask containing the delivered lyopholized powder NaB^3H_4 (4000 MBq, 0.67 TBq/mmole) was added 1 ml of an ethanolic solution of 5 (9.6 mg, 41.6 µmole). The solution was magnetically stirred at 0°C for 2 hours. The reduction was completed by a further addition of 5 mg of unlabelled $NaBH_4$ dissolved in 1 ml of ethanol. After neutralisation of the mixture with a few drops of acetic acid, the solution was evaporated under vacuo and the crude labelled 6

extracted by trituration of the residue with 4 x 10 ml of chloroform. After washing the chloroformic solution with brine the organic phase was dried on $MgSO_4$ and evaporated, yielding pure 6 (3227 MBq) in 81 % yield, the chemical purity of which was checked by ^1H NMR and the radiochemical purity by ^3H NMR and TLC radiochromatography.

300 MHZ-^1H NMR : ($CDCl_3$) external TMS. δ 1.37 (s) and 1.59 (s) (CMe_2), 3.45 (s, CH_3-O), 3.68 (dd, J_{gem} = 12, $J_{4,5a}$ = 2.7, H_{5a}), 3.97 (dd, J_{gem} = 12, $J_{4,5b}$ = 2.7, H_{5b}), 4.06 (dd, $J_{3,4}$ = 9, $J_{2,3}$ = 4, H_3), 4.13 (dt, $J_{3,4}$ = 9, $J_{4,5a}$ = $J_{4,5b}$ = 2.7, H_4), 4.66 (t, $J_{1,2}$ = $J_{2,3}$ = 4, H_2), 4.73 (d, J = 6.7) and 4.78 (d,J = 6.7) (-O-CH_2-O), 5.76 (d, $J_{1,2}$ = 4, H_1).

106 MHz ^3H RMN : ($CDCl_3$) : with irradiation of ^1H δ 3.4 (s) 3.90 (s), $^3H_5$.

1,2-O-isopropylidene-3-O-methoxymethyl-5-deoxy-5-O-p.toluenesulfonyl-[5-^3H]-α-D ribopentofuranose 7

In a 5 ml stoppered test tube, 3227 MBq of 6, prepared as described above and without any further purification, was dissolved in dry pyridine (1 ml) and freshly cristallyzed tosyl chloride (33.4 mg, 175 μmole) was added in two portions. The mixture was stirred at room temperature for 18 hours under argon pressure before evaporation to dryness under vacuo. The residue was reevaporated twice with 3 ml of toluene and the resulting solid was dissolved in water (8 ml) and 7 extracted with 4 x 8 ml of $CHCl_3$. The organic phase was dried on $MgSO_4$ and evaporated. The crude residue was purified on preparative silicagel plates using ethyl acetate/chloroform (1V/9V) as eluant. The product was located by bidimentionnal autoradiography of the plates which showed that a good separation of 7 and remaining starting material was achieved. The product 7 (1184 MBq, 75 GBq/mmole) was obtained in a 36 % yield.

300 MHz ^1H NMR : ($CDCl_3$)

δ 1.34 (s) and 1.54 (s) (CMe_2), 2.45 (s, CH_3-Ar), 3.41 (s, CH_3-O), 3.91 (dd, $J_{3,4}$ = 9, $J_{2,3}$ = 4, H_3), 4.16 (ddd, J = 9, $J_{4,5a}$ = 4, $J_{4,5b}$ = 2, H_4), 4.21 (dd, J_{gem} = 11, $J_{4,5a}$ = 4, H_{5a}), 4.31 (dd, J_{gem} = 11, $J_{4,5b}$ = 2, H_{5b}), 4.61 (t, $J_{1,2}$ = $J_{2,3}$ = 4, H_2), 4.68 (d, J = 6.7) and 4.72 (d, J = 6.7) (-O-CH_2-O), 5.65 (d, $J_{1,2}$ = 4, H_1).

106 MHz - ^3H NMR ($CDCl_3$) : with irradiation of ^1H. δ 4.20 (s), 4.30 (s) $^3H_5$

UV (MeOH) λ_{max} = 220 nm (10000)

1,2-O-isopropylidene-3-O-methoxymethyl-5-deoxy [5-^{3}H]-L-homocysteinyl α-D-ribopentofuranose 8

An excess of the dry disodium derivative of L-homocysteine (20 mg), prepared from L-homocystine (13), was suspended in 0.5 ml of dry DMF in a stoppered test tube flushed with argon. The product 7 (1184 MBq, 75 GBq/mmole) from above was dissolved in 0.5 ml of DMF and the solution stirred for 16 hours at 80°C.

After evaporation of the solvent under vacuo, the residue was dissolved in water (10 ml) and the remaining tosylate extracted with $CHCl_3$ (3 x 5 ml). After neutralisation of the aqueous solution with a few drops of 0.1N HCl, desalting and purification of 8 was carried out using a column containing HP 20 SS hydrophobic resin (100 ml). Elution was performed successively with 200 ml of water and 200 ml of methanol-water (4v/6v). Fractions containing the desired product 8 were combined, the methanol was removed and the residual solution lyophilized to afford 248 MBq of pure 8 (yield 22 %, sp. act. 75 GBq/mmole) the chemical homogeneity of which was checked by TLC (silicagel) using ethanol/water (4v/1v).

300 MHz ^{1}H NMR (D_2O, external TMS)
δ 1.44 (s) and 1.63 (s) (CMe_2), 2.10 (m, H_{7a}, H_{7b}), 2.76 (t, $J_{6,7}$ = 7.6, H_{6a}, H_{6b}), 2.86 (dd, J_{gem} = 13, $J_{4,5a}$ = 6.5, H_{5a}), 3.10 (dd, J_{gem} = 13, $J_{4,5b}$ = 4.3, H_{5b}), 3.50 (s, CH_3-O), 3.71 (dd, $J_{7a,8}$ = 8.6, $J_{7b,8}$ = 6.5, H_8), 4.06 (ddd, $J_{3,4}$ = 8.6, $J_{4,5a}$ = 6.5, $J_{4,5b}$ = 4.3, H_4), 4.82 (d, J = 6.7) and 4.85 (d, J = 6.7) (O-CH_2-O), 4.92 (t, $J_{1,2}$ = $J_{2,3}$ = 4.3, H_2), 5.93 (d, $J_{1,2}$ = 4.3, H_1).

106 MHz ^{3}H NMR with irradiation of ^{1}H. δ 2.90 (s) 3.15 $^3H_5$.

[5-^{3}H]-S-Ribosyl-L-homocystéine 9

200 MBq of 8 (sp. act. 75 GBq/mmole) was dissolved in 1 ml of 0.25 M HCl and the solution was heated at 50°C. The removal of 1,2-O-isopropylidene and 1-MOM protecting groups was completed in 2 hours (checked on TLC plates RP-18, eluant MeOH/H_2O, 1v/4v). Excess of HCl was removed by AG 11 A8 resin (2 ml) and the resulting neutral solution was lyophilized. Purification of 9 was achieved on 1 mm cellulose precoated plates (elution with Ethanol/Water, 3v/1v) which were autoradiographied using TLC radio CHROMA 2D method. 160 MBq (sp. act. 75 GBq/mmole) of 9 was obtained in 80 % yield, with a radio chemical purity > 99,5 % by the radio TLC method.

300 MHz ^{1}H NMR (D_2O, external TMS)
δ 2.15 (m, H_{7a}, H_{7b}), 2.75 (m, H_{6a}, H_{6b}), 2.85 (dd, J_{gem} = 14.1, $J_{4,5a}$ = 4.8, H_{5a}), 2.94 (dd, J_{gem} = 14.1, $J_{4,5b}$ = 4.6, H_{5b}), 3.84 (m, H_8), 4.01 (dd, $J_{2,3}$ = 4.8, $J_{1,7}$ = 1.7, H_2 β form), 4.06 (m, H_4), 4.14 (dd, $J_{2,3}$ = 5.7, $J_{1,2}$ = 4.1, H_2 α form), 4.20 (dd, $J_{3,4}$ = 6.5, $J_{2,3}$ = 4.8, H_3), 5.22 (d, $J_{1,2}$ = 1.7, H_1 β form), 5.38 (d, $J_{1,2}$ = 4.1, H_1 α form).

106 MHz ^{3}H NMR with irradiation of ^{1}H. δ 2.8 (s), 2.95 (s) H_5.

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8. 5 is obtained as a mixture of free aldehyde and its hydrated form. ^{1}H NMR (300 MHz) : δ 1.39 (s) and 1.62 (s) (CMe_2), 3.42 (s, CH_3-O), 4.05 (dd, $J_{2,3}$ = 4.5, $J_{3,4}$ = 9.0, H_3), 4.44 (dd, $J_{3,4}$ = 9, $J_{4,5}$ = 2.3, H_4) 4.72 (t, $J_{1,2}$ = $J_{2,3}$ = 4.5, H_2), 5.89 (d, $J_{1,2}$ = 4.5, H_1), 9.70 (d, $J_{4,5}$ = 2.3, H_5). MS (DCI/NH_3) : m/z 233 $(MH)^+$.

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