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[^{3}H]-LABELLING OF HYDROXYETHYL GROUPS - SYNTHESIS OF S-2-HYDROXY [2-^{3}H] ETHYL) GLUTATHIONE AND OF [^{3}H]-MELPHALAN

M. VERNY and C. NICOLAS
INSERM U 71 - B.P. 184
Rue Montalembert - 63005 Clermont-Ferrand Cedex

SUMMARY

The easy preparation of 2-bromo [1-^{3}H] ethanol allows the tritium labelling of molecules bearing S- or N- hydroxyethyl groups. Thus S-(2-hydroxy [2-^{3}H] ethyl) glutathione and [^{3}H]-Melphalan were synthesised with specific radioactivities of around 10 mCi/mmol (370 MBq/mmol). These values could be theoretically raised to 10 Ci/mmol (370 GBq/mmol), according to the specific activity of the labelling precursor, sodium [^{3}H] borohydride.

Key words : *2-Hydroxy [2-^{3}H] ethyl groups*
S-(2-hydroxy [2-^{3}H] ethyl) glutathione
[^{3}H] Melphalan

INTRODUCTION

*The study of two oncostatics, namely **RFNCU** = 1-(2-chloroethyl) 3-[1'-(5'-p-nitrobenzoyl 2',3'-isopropylidene) α,β-D-ribofuranosyl] 1-nitrosourea and **RPNCU** = 1-(2-chloroethyl) 3-[2',3',4'-tris O-acetyl α,β-ribopyranosyl] 1-nitrosourea, at INSERM, Unit 71, led to the proposal of a biotransformation scheme in animals (1).*

2-Chloroethanol was identified in plasma and after oxidation into 2-chloroacetaldehyde, appeared to be eliminated mainly as four sulphur containing metabolites (1,2). Two intermediates in this metabolic scheme could be S-(2-chloroethyl) glutathione and S-(2-hydroxyethyl) glutathione (3), the latter being expected to exhibit high concentrations in thymus and pancreas.

In order to study the metabolism and biodisposition of RFNCU and RPNCU, it was useful to synthesize the supposed intermediates as labelled species. The S-alkylation of glutathione by the means of ethylene oxide (available as the [^{14}C]-labelled reagent), according to the method described by Johnson (4), failed to give the expected results. Thus, following the methods of Zilka and Weinstein (5) and Nachtomi (6), we attempted to use bromoethanol as the alkylating reagent (Scheme 1), on the assumption that the [^{3}H]-labelled compound could be easily obtained, by the reduction of ethyl bromoacetate with a tritiated hydride :

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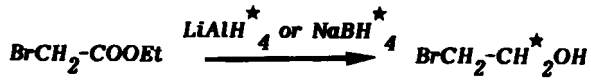
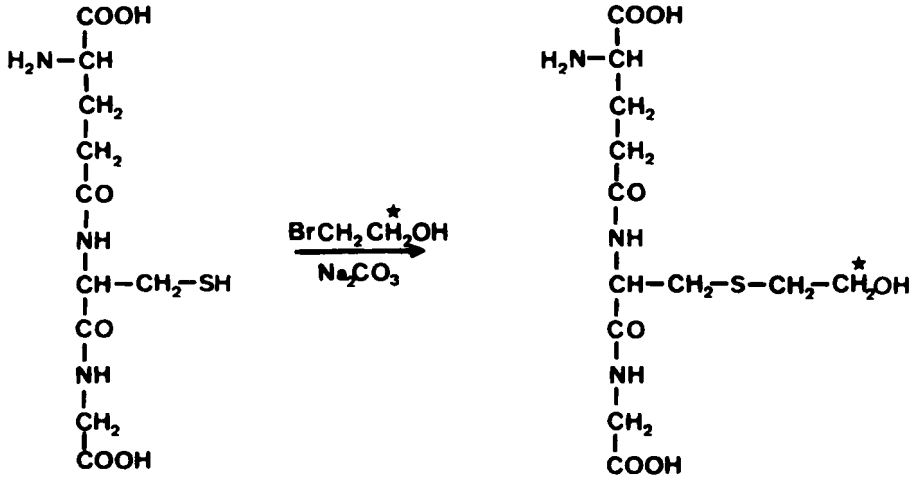
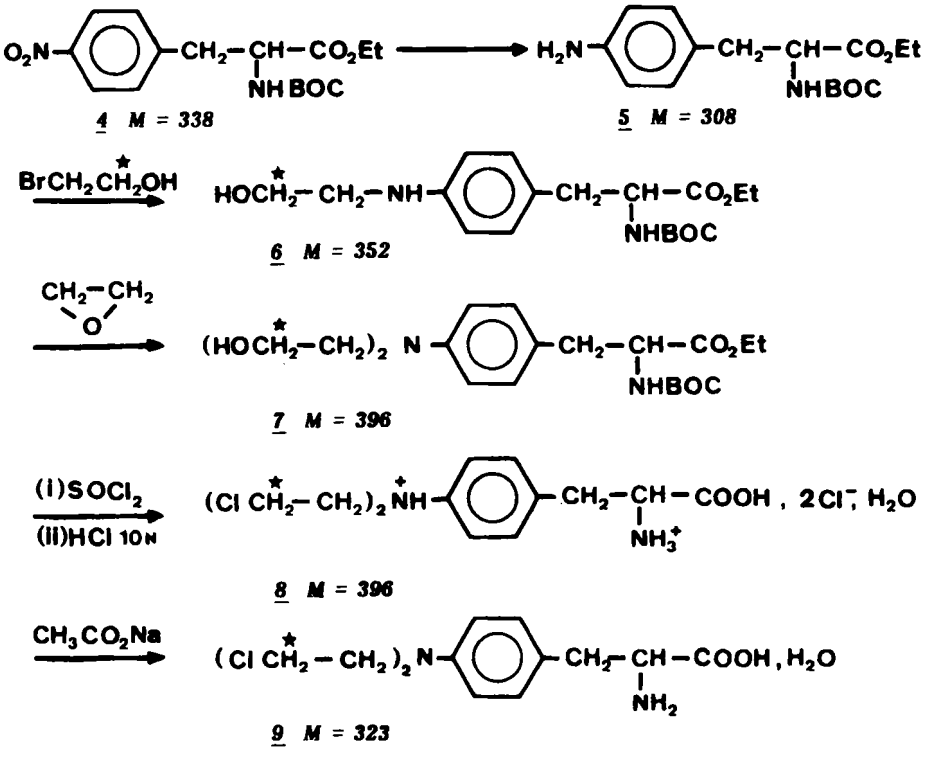


SCHEME 1



G S H, 1 *GSCH$_2$CH$_2$OH,* 2

SCHEME 2



4 *M = 338* 5 *M = 308*

6 *M = 352*

7 *M = 396*

8 *M = 396*

9 *M = 323*

As the synthesis appeared to be carried out without difficulty and with high chemical yields, it was hoped that it could offer a general method for introducing a labelled hydroxyethyl group : thus, the same procedure was applied successfully to the synthesis of [^3H]-labelled Melphalan. The [^{14}C]-labelling of Melphalan was described in a previous paper (7). A method of [^3H]-labelling was prospected to provide highly radioactive samples, proper to study the behaviour of this compound at the cellular level.

The classical synthesis of Melphalan (7,8) consists in reacting ethylene oxide with p-amino phenylalanine, after blocking the COOH and α -NH_2 functions. When ethylene oxide was replaced by 2-bromoethanol, the N-alkylation was restricted to the introduction of only one hydroxyethyl group, and the synthesis was completed with the aid of ethylene oxide, according to scheme 2.

As indicated in the Experimental Part, the reduction of ethyl bromoacetate into bromoethanol, to be quantitative, needs a hydride/ester ratio at least equal to 1. Under such conditions, only two of the four hydrogens of the reagent are incorporated in the product, and the radioactive yield cannot be raised over 50%.

Since sodium [^3H] borohydride is commercially available with activities up to 20 Ci/mmol, this method would be expected to give specific radioactivities up to 10 Ci/mmol.

EXPERIMENTAL

1°) Reduction of ethyl bromoacetate

In preliminary experiments, ethyl bromoacetate was reacted, in parallel, with $LiAlH_4$ and $NaBH_4$, in water at room temperature. The hydride/ester ratios used were 1.25, 1.0 and 0.75 respectively. In the last case, whatever reagent was used, significant amounts of the unreacted ester were recovered.

Though the reaction was faster with $LiAlH_4$, the use of $NaBH_4$ was preferred, as the labelled reagent is more readily available and more stable.

Sodium borohydride (0.2 g, 5.25 mmol) was added with stirring to ethyl bromoacetate (0.85 g, 5.1 mmol) in water (15 ml) at room temperature. After 1 hr stirring, the mixture became homogeneous, and the irritating odour of the bromoester gradually vanished. The aqueous medium was then saturated with NaCl and extracted three times with Et_2O. After drying over $MgSO_4$, the solvent was distilled as slowly as possible through a Crismer column. On analysis by NMR, the residue (0.68 g) appeared to contain 80 % of 2-bromoethanol and 20 % of ether (mole percent), with unsignificant amounts of ethyl bromoacetate and ethanol. This corresponded to 0.59 g of 2-bromoethanol (92 % yield). Any attempts to improve the evaporation of ether resulted in a lower yield of 2-bromoethanol.

It was possible to use either the crude residue obtained as above, or, as in the case of glutathione, directly the aqueous solution.

When ethanol was used instead of water as the solvent, the reaction was much slower, and could not be brought to completion in 24 hrs.

2°) S-(2-hydroxy [2-^{3}H] ethyl) glutathione 2.

Ethyl bromoacetate (167 mg ,1 mmol) and $NaBT_4$ (6.8 mg, 20 mCi, 740 MBq) (CEA France) were stirred in 5 ml H_2O. After 15 min. unlabelled hydride (31.2 mg) was added, and the stirring was continued for 1 hr.

Glutathione hemihydrate 1 (300 mg, 1 mmol) and Na_2CO_3 (320 mg, 3 mmol) were then added, and the clear, homogeneous solution was allowed to stand overnight at room temperature. The reaction was made to go to completion by a further addition of unlabelled 2-bromoethanol (50 mg) and then left to stand for a further 24 hrs.

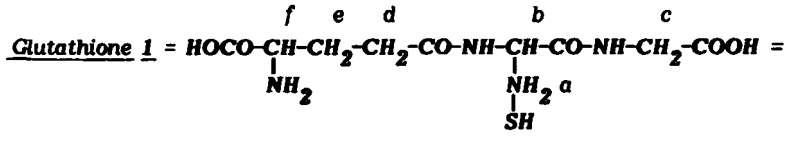
The solution was acidified to pH 4 by dropwise addition of acetic acid, then evaporated to dryness, and the residue was taken up in 1 ml of water. Absolute ethanol (20 ml) was added with vigorous shaking and the precipitate filtered and dried under vacuum, to yield S-(2-hydroxy [2-^{3}H] ethyl) glutathione 2 (380 mg ; radioactive yield : 10.55 mCi, 390 MBq, 53 % ; specific radioactivity : 9.8 mCi/mmol, 360 MBq/mmol). The fine hygroscopic powder so obtained, according to its NMR spectrum, contains per mole about 1/2 EtOH and 2 H_2O, which could not be removed by freeze-drying.

TLC indicates a 100 % radiochemical purity. A minor non-radioactive impurity corresponded by TLC to glutathione itself, and not to S-(ethoxycarbonyl methyl) glutathione $GSCH_2COOEt$ 3. An authentic sample of the latter was prepared from glutathione and ethyl bromoacetate, according to the procedure described above.

R_f (propanol/pyridine/water 1/1/1) :

1 : 0.59 2 : 0.77 3 : 0.85

NMR (solvent : D_2O) :



δ (ppm) : 4.77 (s) = exchangeable protons ; 4.62 (t) = b ; 4.03 (s) = c ; 3.90 (t) = f ; 3.00 (d) = a ; 2.65 (m) and 2.25 (m) = d and e.

S-(2-Hydroxyethyl) glutathione 2 = $GSCH_2CH_2OH$

In comparaison with the previous spectrum : addition of two triplets (J = 6.4 Hz) at 3.80 ppm (partly concealed, OCH_2) and 2.85 ppm (SCH_2).

NOTE : All attempts to prepare S-(2-chloroethyl) glutathione were unsuccessful. The hydroxy-compound 2 failed to react with classical reagents such as $SOCl_2$ or PCl_5, due to its complete insolubility in non-aqueous media. On the other hand, the alkylation of glutathione by $ClCH_2CH_2Br$ or $ClCH_2CH_2OTs$ led to a single product, identified as $GSCH_2CH_2SG$ (6) (NMR : singlet at 2.75 ppm, intensity 4). Reed and coll. (9) claimed that they had prepared S-(2-chloroethyl) glutathione (identified only by a TLC spot), and that this compound was readily converted to S-(2-hydroxyethyl) glutathione when placed in water at room temperature.

3°) Melphalan = p-[N,N-bis(2-chloro [2-^{3}H]ethyl)amino] L-phenylalanine

a) BOC p-amino phenylalanine ethyl ester 5

The nitration of L-phenylalanine, and the subsequent esterification of the product, are carried out following Nicolas and Godenèche (8).

p-Nitro phenylalanine ethyl ester hydrochloride (5.49 g , 20 mmol), dissolved in chloroform (40 ml), was treated with a solution of $NaHCO_3$ (1.68 g , 20 mmol) and NaCl (4 g) in water (30 ml), and finally with di-tert.butyl dicarbonate (4.36 g, 20 mmol) (10). The mixture was refluxed for 1.5 hr. with stirring, then the aqueous layer was extracted twice with $CHCl_3$. The extracts were dried and evaporated, and the residue recrystallized from hexane to yield 4 (5,9 g, 87 %).

NMR δ ($CDCl_3$) : 7.67 (2d, J = 9 Hz, C_6H_4) ; 4.57 and 3.17 (2 m, CH-CH_2) ; 4.15 and 1.25 (q and t, COOEt) ; 1.40 (s, tBu) ; 5.17 (d, NHCO).

The catalytic hydrogenation of compound 4 was achieved as described previously (8). The crude product 5 (yield 92 %) was pure enough to be used as such in the following reaction.

NMR δ($CDCl_3$) : 6.63 (2d, J = 8 Hz, C_6H_4) ; 4.42 and 2.83 (t and d, CH-CH_2) 4.08 and 1.13 (q and t, COOEt); 1.40 (s, tBu) ; 5.43 (d, NHCO) ; 3.75 (s, NH_2).

b) BOC p-[N-(2-hydroxy [2-^{3}H] ethyl) amino] phenylalanine ethyl ester 6

Ethyl bromoacetate (334 mg, 2 mmol) was stirred, as described in 2°), with $NaBT_4$ (10.8 mg, 32.4 mCi, 1200 MBq) for 15 min., and then for 1 hr. after addition of 70 mg of unlabelled hydride. The next steps are carried out as described in 1°), and the crude 2-bromo [2-^{3}H] ethanol (16.2 mCi, 600 MBq, 50 %) was dissolved in ethanol (10 ml) and heated under reflux for 24 hrs in the presence of 5 (616 mg, 2 mmol).

The solvent was evaporated and the residue was treated with a saturated solution of $NaHCO_3$ and extracted with $CHCl_3$. The extracts were dried and evaporated to yield crude 6 (750 mg).

NOTE : in earlier experiments, the reaction was carried out with the aid of triethylamine and 2 equivalents of bromoethanol, without any improvement in the yield.

NMR δ ($CDCl_3$) : 6.73 (2d, J = 8 Hz, C_6H_4) ; 4.47 and 2.93 (t and d, J = 6 Hz, CH-CH_2) ; 3.75 and 3.18 (2t, J = 5.5 Hz, CH_2-CH_2) ; 4.13 and 1.22 (q and t, COOEt) ; 1.43 (s, tBu) ; 5.25 (s, OH) ; 5.15 (d, NHCO)

c)BOC p-[N,N-bis(2-hydroxy[2-^{3}H]ethyl)amino] phenylalanine ethyl ester 7

The crude product of the above reaction was dissolved in anhydrous acetic acid (5 ml) and reacted with ethylene oxide (90 mg, 2 mmol). A slight excess of the latter was added after 1 hr. and then after 2 hrs. After standing 24 hrs., the mixture was evaporated to dryness, aqueous $NaHCO_3$ added and extracted with $CHCl_3$. The extracts are dried and evaporated to yield crude 7 (700 mg).

NMR δ ($CDCl_3$) : 6.80 (2d, J = 8 Hz, C_6H_4) : 4.48 and 2.93 (t and d, J = 6 Hz $CH-CH_2$) ; 3.77 and 3.50 (2t, J = 4 Hz, 2 x CH_2-CH_2) ; 4.20 and 1.23 (q and t, COOEt) ; 1.42 (s, tBu) ; 5.23 (s, OH) ; 5.13 (d, NHCO).

d) [^{3}H] Melphalan dihydrochloride 8 and [^{3}H] Melphalan 9

Crude 7 was dissolved into $CHCl_3$ (6 ml) and treated with a solution of $SOCl_2$ (1.5 ml) in $CHCl_3$ (3 ml), with stirring and cooling in an ice-bath. The mixture was warmed gently up to 60°C, then allowed to cool, and evaporated to dryness. The dark gummy residue was taken up in 10 N HCl (10 ml) and the mixture refluxed overnight. After treatment with charcoal and filtration, the clear solution was evaporated to dryness, and the white, crystalline residue was washed with Et_2O and dried, to yield [^{3}H] dihydrochloride 8 (480 mg, 1.2 mmol, 60 %).

NMR δ (CD_3OD) : 7.35 (s, C_6H_4) ; 4.30 and 3.28 (t and d, J = 6 Hz, $CH-CH_2$) ; 3.97 and 3.67 (J = 5 Hz, 2 x CH_2-CH_2) ; 4.95 (s, mobile protons)

The hydrochloride 8 (120 mg, 0.3 mmol) was dissolved in water (1 ml). Melphalan 9 was precipitated by the progressive addition of an icy saturated solution of sodium acetate, then quickly filtered and washed with isopropyl ether. The purification was achieved by dissolving crude 9 in hot methanol, treatment with charcoal, filtration and finally evaporation to dryness, to yield 82 mg (51 % from 5).

Specific radioactivity : 6.8 mCi/mmol, 252 MBq/mmol, 21 %

TLC (n.BuOH/AcOH/H_2O : 4/1/1) : Rf = 0.45

Optical rotations :

8 : $[\alpha]_J^{20}$ = + 12° (c = 0.01, N HCl)

9 : $[\alpha]_J^{20}$ = + 13,5° (c = 0.0085, N HCl)

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