Cyto-active Amino-acids and Peptides. Part XV.[1] The Synthesis of *p*-Di-(2-chloroethyl)aminophenyl-L-alanine (Melphalan) Labelled with Tritium

By **Roy Wade,***† and **T. Srinivasa Murthy,** Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, Fulham Road, London S.W.3

Experiments aimed at the preparation of ring-tritiated melphalan by the catalytic replacement of iodine are described. Owing to the lability of tritium *ortho* to the amino ring-substituent much of the incorporated radioactivity was subsequently lost. Nevertheless products of high specific activity were obtained, suitable for most metabolic and mechanism of action studies.

THE synthesis of melphalan labelled with carbon-14 in the aliphatic side chain formed part of a previous paper in this series;[2] material made in this way has been used for distribution studies by Cohn[3] and by Novikova.[4] Since then melphalan with a carbon-14 label in the chloroethyl groups has been prepared[5] and used for similar studies.[6] These preparations were of specific activity 0·2—1·3 mc/mmole, a level too low to detect the binding of drug to nuclear deoxyribonucleic acid *in vivo*. As such binding, currently considered to be a key step in the anti-tumour action of this class of compound,[7] is only of the order of one alkylation for every 10^6 nucleotides in deoxyribonucleic acid from HeLa cells[8] and from Yoshida ascites tumour cells in rats,[9] material of a higher order of activity was needed.

Preliminary experiments (in collaboration with Dr. J. A. Stock) aimed at incorporating tritium by use of the phosphoric acid–boron trifluoride complex method of

† *Present address:* CIBA Laboratories Limited, Horsham, Sussex.

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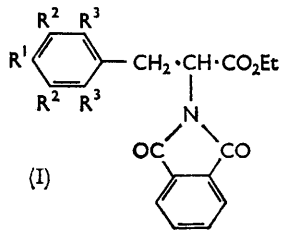
[3] P. Cohn, *Brit. J. Cancer*, 1967, **11**, 258.
[4] M. A. Novikova, *Voprosy Onkol.*, 1961, **7** (No. 3), 48.
[5] A. H. Solaway and E. Nyilas, *J. Org. Chem.*, 1961, **26**, 1091.
[6] A. H. Solaway, E. Nyilas, R. N. Kjellberg, and V. H. Mark, *J. Medicin. Pharm. Chem.*, 1962, **5**, 1371.
[7] J. A. Stock, in 'Biology of Cancer,' ed. E. J. Ambrose and F. J. C. Roe, Van Nostrand, London, 1966, p. 176, and references cited therein.
[8] A. R. Crathorn and J. J. Roberts, *Nature*, 1966, **211**, 150.
[9] C. R. Ball and T. A. Connors, personal communication.

Yavorsky and Gorin [10] were unsuccessful. Melphalan treated with the reagent for 5 hr. showed no incorporation of radioactivity.

We considered that the catalytic replacement of iodine offered a sure means of introducing tritium into the molecule in large amounts and at specific positions. The other commonly used method, that of Wilzbach, gives a relatively low level of incorporation in molecules of this size, and a small but significant degree of racemisation of optically active centres.[11] However, while our work was in progress, the preparation of melphalan tritiated by the exchange method was reported.[12] The specific activity of the purified product (20μc/mg., 6 mc/mmole) was insufficient to detect any binding to deoxyribonucleic acid. The authors state that less than three molecules of drug for every 10,000 nucleotides would not have been detected under their conditions. In view of the more recent data [8,9] quoted above this lack of detection of binding is understandable.

Initially, we attempted to adapt the synthesis of melphalan [13] by the formation of a suitable iodo derivative. The iodine would then be exchanged for tritium and the radioactive product converted into melphalan. 4-Amino-*N*(α)-phthaloylphenyl-L-alanine ethyl ester (I; $R^1 = NH_2$, $R^2 = R^3 = H$) reacted readily with iodine chloride in dilute hydrochloric acid to give the di-iodo-derivative (I; $R^1 = NH_2$, $R^2 = I$, $R^3 = H$), which was then reductively dehalogenated, in the presence of base, back to the amine (I; $R^1 = NH_2$, $R^2 = R^3 = H$) in high yield. The reaction was repeated with the use of tritium (just under 0·1 mM) (tritiation A) and the product, after dilution with non-radioactive material, was converted into melphalan as described previously.[13] The specific activity of the product (293 mc/mmole) was lower than expected. When the dilution factor (13) is allowed for, this implies that the initial tritiation product had a specific activity of 3·8 c/mmole, [*ca.* 7% of theoretical (58 c/mmole)]. This loss of activity was considered to be due to either or both of two factors. First, the iodo-compound contained an unprotected amino-group which would be expected to exchange rapidly with tritium; if this hydrogen was then used in preference to tritium for the dehalogenation stage, a comparatively large deficit of ringbound tritium in the final product would result. Alternatively, exchange at positions *ortho* to the amine substituent may occur during the final stage of acid hydrolysis. The latter reason was largely discounted at this time because the product itself was stable to further acid hydrolysis under conditions (6N-hydrochloric acid, reflux 4—5 hr.) comparable to those used in the preparation. This inference was subsequently shown to be fallacious but at the time we concentrated on removing the first mentioned possible cause of tritium loss.



(I)

We chose the formyl group to protect the aromatic amino-group because it could readily be removed under mild conditions. The di-iodo-amine could be formylated with formic–acetic mixed anhydride. The product (I; $R^1 = HCO{\cdot}NH$, $R^2 = I$, $R^3 = H$) was deiodinated under conditions similar to those used above with the exception that triethylamine was used as the base. A blank run had shown that pyridine was reduced to piperidine to the extent of about 3% under the conditions of the tritiation experiment (room temperature, 48 hr.). Consequently, triethylamine was used to remove this possible source of tritium loss. Tritiation B proceeded smoothly on a scale involving the use of 50 c of tritium. Immediately after the tritiation step, non-radioactive formyl derivative (I; $R^1 = HCO{\cdot}NH$, $R^2 = R^3 = H$) was added, the formyl group was removed with ethanolic hydrogen chloride, and the free amine was converted as before into melphalan (50 mc/mole). When the dilution factor (4·8) was allowed for, this implied that in this preparation again the bulk (99·5%) of the radioactivity had been lost. As the residual activity was stable to acid hydrolysis and steps had been taken to prevent exchange with labile hydrogen in the molecule, we considered that one of the earlier synthetic stages was responsible for this effect. The one most likely to cause exchange of ring hydrogen was the chlorination by phosphoryl chloride of the dihydroxyethyl intermediate [I; $R^1 = (HOCH_2{\cdot}CH_2)_2N$, $R^2 = R^3 = H$]. A similar chlorination of generally ring-tritiated di(2-hydroxyethyl)aniline gave a loss of radioactivity of *ca.* 45%. This could well be accounted for by losses from the two positions *ortho* to the nitrogen substituent.

In order to obviate treatment with phosphoryl chloride after the introduction of tritium, we attempted to convert the amino-group into a nitrogen mustard function before exchange of ring iodo-residues. Such an intermediate would merely require acid hydrolysis after tritiation to convert it into melphalan. The 4-di-(2-chloroethyl)amino-3,5-di-iodo-derivative [I; $R^1 = (ClCH_2{\cdot}CH_2)_2N$, $R^2 = I$, $R^3 = H$], prepared albeit in poor yield, was suitable for this purpose. It was readily dehalogenated under the usual conditions and hydrolysed with acid as before. Only a small first crop of melphalan was obtained from this preparation, so non-radioactive drug was added to the mother-liquor and a second crop of lower specific activity was obtained.

[10] P. M. Yavorsky and E. Gorin, *J. Amer. Chem. Soc.*, 1962, **84**, 1071.
[11] P. Riesy and K. E. Wilzbach, Abstracts of Papers, 134th Meeting of the American Chemical Society, 1958, 27P.
[12] A. N. Milner, O. Klatt, S. E. Young, and J. S. Stehlin, *Cancer Res.*, 1965, **25**, 259.
[13] F. Bergel and J. A. Stock, *J. Chem. Soc.*, 1954, 2409.

The radioactivity of these preparations (106 and 47 mc/mmole) showed that much of the tritium had again been lost. The cause of this, in view of information [14] not originally available to us, is now considered to be exchange at positions *ortho* to the amino-group during strong acid treatment. The proven stability of the residual radioactivity which had led us to reject this possibility is explicable if tritium exchange into the 2- and 6-positions of the aromatic ring can occur under the influence of palladium catalyst.[15] Tritium in these positions would be expected to be much more stable to acid hydrolysis than tritium in the 3- and 5-positions, and would consequently not be lost. The radioactive drug obtained is therefore probably not labelled in the positions intended, and the same material could perhaps be made directly by catalytic gas exchange followed by acid hydrolysis to remove labile tritium without the need to use iodinated intermediates.

Though the specific activity obtained was much lower than had been hoped originally, it was sufficient for most metabolic experiments and for some mechanism of action studies.[9,16] Some of this work has been described.[17]

EXPERIMENTAL

M.p.s. were determined with a Kofler hot-stage apparatus. Assays for radioactivity were carried out with a Packard Tricarb 3375 scintillation counter, with naphthalene, POP and POPOP in ethanol, dioxan, and toluene as scintillation fluid.

Attempted Tritiation with [$^3H_1$]Phosphoric Acid–Boron Trifluoride.—Tritiated water (1 ml.; 4 c) was stirred with phosphorus pentoxide (2·6 g.) with water cooling until a clear solution was obtained, and boron trifluoride was bubbled in until the solution was saturated. Melphalan (1·5 g.) was then added with stirring and the mixture was stirred for 5 hr. at room temperature. Water (15 ml.) was added, then saturated aqueous sodium acetate to pH 6. The precipitated amino-acid was filtered off, washed with water, sucked dry, and recrystallised from methanol. The product had no detectable radioactivity.

*4-Amino-3,5-di-iodo-*N(α)*-phthaloylphenyl-*L*-alanine Ethyl Ester.*—Iodine chloride (4·4 ml.) in 6N-hydrochloric acid (64 ml.) was added to a warm solution of 4-amino-*N*(α)-phthaloylphenyl-L-alanine ethyl ester [13] (12 g.) in warm 2N-hydrochloric acid (200 ml.) and heating (60—70°) was continued for 2 hr. When the mixture was cooled, the dark oil which had separated solidified and the supernatant was decanted. The solid mass was broken up under water containing sodium hydrogen sulphite to remove the excess of iodine chloride, and the solid was filtered off and washed well with water. It was dissolved in benzene and the solution was passed through a column of alumina with benzene as eluant. Evaporation of the eluate gave a pale off-white gum which crystallised under light petroleum. The solid gave prisms (12 g., 58%), m.p. 90—91° [from ethyl acetate–light petroleum (b.p. 60—80°)], of the *di-iodo-derivative*, $[\alpha]_D^{20}$ −154° (*c* 0·74 in AcOH) (Found: C, 39·0; H, 3·0; I, 42·8; N, 4·6. $C_{19}H_{16}I_2N_2O_4$ requires C, 38·6; H, 2·7; I, 43·0; N, 4·8%).

Reductive Tritiation (A) and Conversion of the Product into Radioactive Melphalan.—The iodo-compound (I; R^1 = NH_2, R^2 = I, R^3 = H) (51 mg.) was dissolved in dry dioxan (2 ml.) containing pyridine (0·015 ml.). Palladium–charcoal (5%) was added, and the mixture was tritiated (tritium gas 98% T_2/H_2; 10 c, *ca.* 4 ml.) at room temperature and pressure. Uptake was smooth and stirring was continued overnight. T.l.c. on silica gel in benzene showed that the di-iodo-compound (R_F 0·4) had disappeared but a faint intermediate spot (R_F 0·25) was present in addition to that of the product (R_F 0·05). Fresh catalyst was added and the mixture was hydrogenated until uptake had ceased (0·4 ml.). T.l.c. in benzene now showed only one spot, R_F 0·05, corresponding to deiodinated product.

At this stage 'cold' amino-compound (350 mg.) was added, the catalyst was filtered off, and the solvent was evaporated off. The residue was dissolved in ethyl acetate and the solution was washed with aqueous sodium hydrogen carbonate solution and water and dried (Na_2SO_4). The solvent was removed again and the residue was dissolved in benzene and put on a column of alumina. Elution with benzene removed only a trace of material, which was discarded. Elution with ether–ethyl acetate (1 : 1) removed the required product (checked by t.l.c.) with some coloured impurity. The latter was removed with charcoal and, after filtration, the solution was evaporated to dryness.

The amino-compound (360 mg.) was dissolved in glacial acetic acid (3 ml.) and water (2 ml.), and ethylene oxide (1·5 ml.) was added. Next day the mixture was worked up, chlorinated, and hydrolysed under the conditions used for the preparation of melphalan.[13] One recrystallisation from methanol gave melphalan (62 mg.), R_F 0·75 on silica gel in ethanol–water (2 : 1) and 0·80 on paper (Whatman no. 1) in butanol–ethanol–propionic acid–water (20 : 10 : 4 : 10), identical to those of an authentic specimen. Radiochemically, 98% of the activity was concentrated in the ninhydrin-positive peak; the remainder was located at the origin. Elution and rechromatography of the main peak again showed about 2% activity at the origin.

The specific activity, measured by counting a solution of known concentration with the liquid scintillation counter, with calibration by an internal standard, was 974 μ c/mg. (293 mc/mmole).

*4-Formamido-*N(α)*-phthaloylphenyl-*L*-alanine Ethyl Ester.*—A solution of 4-amino-*N*(α)-phthaloylphenyl-L-alanine ethyl ester (1·3 g.) in formic acid (10 ml.) was treated with acetic anhydride (2 ml.) and set aside for 1 hr. at room temperature. The mixture was poured into water and the solid which separated was filtered off; it gave colourless needles (1·2 g.) of the *product*, m.p. 151—152° (from ethyl acetate–light petroleum), $[\alpha]_D^{20}$ −190° (*c* 1·1 in MeOH) (Found: C, 65·3; H, 4·8; N, 7·7. $C_{20}H_{18}N_2O_5$ requires C, 65·6; H, 4·9; N, 7·7%).

*Deformylation of 4-Formamido-*N(α)*-phthaloylphenyl-*L*-alanine Ethyl Ester.*—The above formyl derivative (36 mg.) was dissolved in 4N-ethanolic hydrogen chloride (2 ml.). The solution was set aside overnight at room temperature and dry ether (20 ml.) was then added. The amine hydrochloride was filtered off and was identical (m.p., specific rotation, and i.r. spectrum) with an authentic specimen.[13]

[14] K. Hempel, in 'Proceedings of Conference on Methods of Preparing and Storing Marked Molecules,' Euratom, 1964, p. 1009.

[15] E. A. Evans, in 'Tritium and Its Compounds,' Butterworths, London, 1966, p. 104, and references cited therein.

[16] S. Eridani, personal communication.

[17] C. R. Ball, T. A. Connors, J. A. Double, V. Ujhazy, and M. E. Whisson, *Internat. J. Cancer*, 1966, **1**, 319; C. R. Ball and T. A. Connors, *Biochem. Pharmacol.*, 1967, **16**, 509.

*4-Formamido-3,5-di-iodo-*N(α)*-phthaloylphenyl-*L*-alanine Ethyl Ester.*—The amino-di-iodo-compound (1·13 g.) was formylated as described above in formic acid (7·5 ml.) and acetic anhydride (2 ml.). The *product* gave colourless needles, m.p. 162—163° in almost quantitative yield (1·1 g.), $[\alpha]_D^{20}$ − 124° (*c* 1·24 in MeOH) (Found: C, 39·6; H, 2·7; I, 40·5; N, 4·2. $C_{20}H_{16}I_2N_2O_5$ requires C, 39·5; H, 2·7; I, 40·4, N, 4·5%).

Reductive Tritiation (B) and Conversion of the Product into Radioactive Melphalan.—The formylated di-iodo-derivative (266 mg.) was dissolved in dry dioxan (2 ml.) containing triethylamine (0·12 ml.). Palladium–charcoal (5%) was added and the mixture was tritiated (tritium gas 98% T_2/H_2; 50 c, *ca.* 20 ml.) as before. The reaction was completed with hydrogen and before working up 4-formamido-*N*(α)-phthaloylphenyl-L-alanine ethyl ester (600 mg.) was added as ' cold ' carrier.

The product was deformylated in ethanolic hydrogen chloride as before and the product was recrystallised from ethanol–ether. Trituration with aqueous ammonia solution was followed by extraction into ethyl acetate, removal of the solvent, and dissolution of the residue in benzene. The benzene solution was put on a column of alumina (2 × 10 cm.) and eluted with benzene (100 ml.). Elution with ethyl acetate removed the required base which ran as a single spot with the same R_F value as authentic material [13] [t.l.c. on silica gel in benzene–ether (3 : 1)]. The amino-compound was then hydroxyethylated, chlorinated, and hydrolysed as described previously to give melphalan (114 mg.), identified by t.l.c. and paper chromatography as in (A). Radiochemically the material contained no significant contaminants, and had specific activity 161 μc/mg. (49 mc/mmole).

*4-Di-(2-chloroethyl)amino-3,5-di-iodo-*N(α)*-phthaloyl-phenyl-*L*-alanine Ethyl Ester.*—4-Amino-3,5-di-iodo-*N*(α)-phthaloylphenyl-L-alanine ethyl ester (1 g.) was dissolved in glacial acetic acid (20 ml.); water (3 ml.) and ethylene oxide (4 ml.) were then added and the flask was stoppered and left overnight at room temperature. The mixture was poured into water (150 ml.) and the solution was neutralised with sodium hydrogen carbonate. The precipitated gum was extracted with ethyl acetate and dried (Na_2SO_4), and the solvent was evaporated off. Attempts at crystallisation were unsuccessful, although no starting material remained and the product appeared homogeneous on t.l.c. [silica gel, benzene–ether (9 : 1); product R_F 0·05, starting material 0·7]. The hydroxyethyl derivative was dissolved in benzene and dried azeotropically three times. The residue was dissolved in dry chloroform (4 ml.), thionyl chloride (1 ml.) was added, and the solution was heated under reflux for 1 hr. The mixture was evaporated to dryness, methanol (3 × 10 ml.) was added and evaporated off, and the residue was dissolved in benzene, put on a column of alumina (2 × 15 cm.), and washed with benzene. Elution with ether removed a substance which gave a positive test for halogen. Evaporation of the solvent left a gum which rapidly crystallised and gave colourless needles (370 mg.), m.p. 126—127° (from methanol), of the required *di-iodo nitrogen mustard*, $[\alpha]_D^{20}$ −100° (*c* 1 in $CHCl_3$) (Found: C, 39·0; H, 3·1; Cl, 9·7; I, 35·2; N, 4·3. $C_{23}H_{22}Cl_2I_2N_2O_4$ requires C, 38·6; H, 3·1; Cl, 9·9; I, 35·5; N, 3·9%).

Reductive Tritiation (C) and Hydrolysis of the Product to Radioactive Melphalan.—The foregoing di-iodo nitrogen mustard (332 mg.) was dissolved in dry dioxan (6 ml.), triethylamine (0·13 ml.) was added, and the mixture was tritiated (tritium gas 97% T_2/H_2; 20 ml., *ca.* 50 c) over palladium–charcoal (5%), to which a trace of Adams platinum oxide had been added, overnight at room temperature and pressure as before. A hydrogen boost (10 ml.) was then introduced and reduction was continued until uptake ceased (further uptake 2 ml.). The catalyst was filtered off and the solvent evaporated off. The residue was dissolved in ethyl acetate and the solution was washed with dilute acid and water, dried (Na_2SO_4), and evaporated. The residue was treated with concentrated hydrochloric acid (2 ml.) and boiled under reflux for 4 hr. The product was worked up as usual and recrystallisation from methanol gave a first crop (55 mg., 40%). Because of the low yield, non-radioactive melphalan (40 mg.) was added to the mother liquor and washings combined, the solution was concentrated, and a second crop (34 mg.) was obtained. Each crop was homogeneous on t.l.c. and paper chromatography as before (ninhydrin test and radiochemically).

The specific activity of first crop was 324 μ c/mg. (106 mc/mmole) and of the second 143 μ c/mg. (47 mc/mmole).

*Action of 6*N*-Hydrochloric Acid under Reflux on Tritiated Melphalan.*—Radioactive melphalan (1 mg.; 160 μc) was dissolved in 6N-hydrochloric acid (2 ml.) and the solution was boiled under reflux. Samples (0·01 ml.) were withdrawn after 0, 5, 10, 20, 30, 60, 120, 240, and 300 min. and chromatographed on Whatman no. 1 paper in butanol–ethanol–propionic acid–water (10 : 5 : 2 : 5). The paper was then cut into strips and scanned for radioactivity with an Actigraph III (Nuclear–Chicago). Integration of the peaks in the melphalan position gave values of 287, 331, 351, 303, 294, 304, 249, 317, and 293 units respectively. The only additional radioactivity was situated at the origin and did not register on the integrator (2% cut-off).

Chlorination of Generally ^{3}H*-Ring-labelled-Di-(2-hydroxy-ethyl)aniline.*—Generally labelled [^{3}H]aniline (Radiochemical Centre, Amersham, TRA20; 135 mc/mmole) was hydroxyethylated with ethylene oxide in aqueous acetic acid. The [^{3}H]-di-(2-hydroxyethyl)aniline (985 μc) was dried by azeotropic distillation with benzene and treated with phosphoryl chloride (0·2 ml.), and the mixture was heated on a steam bath for 30 min. Benzene (5 ml.) was added, and the solution was poured into ice–water, shaken, and quickly separated. The benzene layer was dried (Na_2SO_4) and aliquot portions of it and of the aqueous layer were counted for radioactivity. The benzene layer contained 504 μc and the aqueous layer 443 μc. The benzene layer was subjected to t.l.c. (silica gel–benzene) and 1 cm. bands were scraped off into phosphor and counted; 3% of the activity stayed at the origin, 2% was at R_F 0·7 and 85% at R_F 0·9; the remainder was distributed evenly along the plate.

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