The average of five replicate determinations for percent intact melphalan in a single vial of dosage formulation was 88.7% with a standard deviation of 1 (range = 2.5%).

Variations of less than 3% were found in the assay of several vials from the same production lot. The samples and reference solutions may be used for quantitative work up to 24 hr. after preparation.

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Synthesis of 4-{*p*-[(2-Chloroethyl)-(2-hydroxyethyl)amino]phenyl}butyric Acid and Its Behavior in the 4-(4-Nitrobenzyl)pyridine Assay Procedure

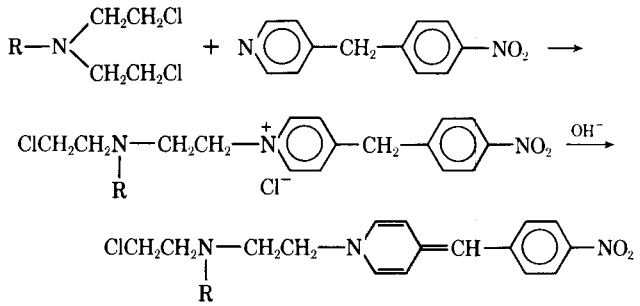
M. K. BALAZS*, C. A. ANDERSON, R. H. IWAMOTO, and P. LIM†

Abstract □ The synthesis of 4-{*p*-[(2-chloroethyl)-(2-hydroxyethyl)-amino]phenyl}butyric acid is reported. Investigation of the behavior of this compound in the NBP assay procedure showed that it alkylates NBP in a manner similar to chlorambucil. Therefore the results obtained from the assay of chlorambucil and similar aryl nitrogen mustards by the NBP method must be interpreted with due caution.

Keyphrases □ 4-{*p*-[(2-Chloroethyl)-(2-hydroxyethyl)amino]-phenyl}butyric acid—synthesis □ 4-(4-Nitrobenzyl)pyridine alkylation—4-{*p*-[(2-chloroethyl)-(2-hydroxyethyl)amino]phenyl}-butyric acid □ Chlorambucil analysis—4-{*p*-[(2-chloroethyl)-(2-hydroxyethyl)amino]phenyl}butyric acid interference □ Colorimetric analysis—spectrophotometer □ TLC—separation, identification □ IR spectrophotometry—structure □ NMR spectroscopy—structure

The compound 4-(4-nitrobenzyl)pyridine (NBP) has been used as an analytical reagent for alkylating agents, among which are the nitrogen mustards such as melphalan,[1] chlorambucil,[2] and uracil mustard.[3] The general method involves the alkylation of NBP; subsequent basification results in the formation of a chromophore whose intensity can be measured photometrically.

The following reaction sequence has been suggested by Petering and Van Giessen (1) (Scheme I).



Scheme I

Holtzman (2) reportedly was able to isolate the product obtained from the reaction of mustard gas with NBP; he identified it as the mono-NBP product.

The NBP procedure has been considered in the authors' laboratories for the assay of chlorambucil. One

[1] 4-{*p*-[Bis(2-chloroethyl)amino]}phenyl-L-alanine.

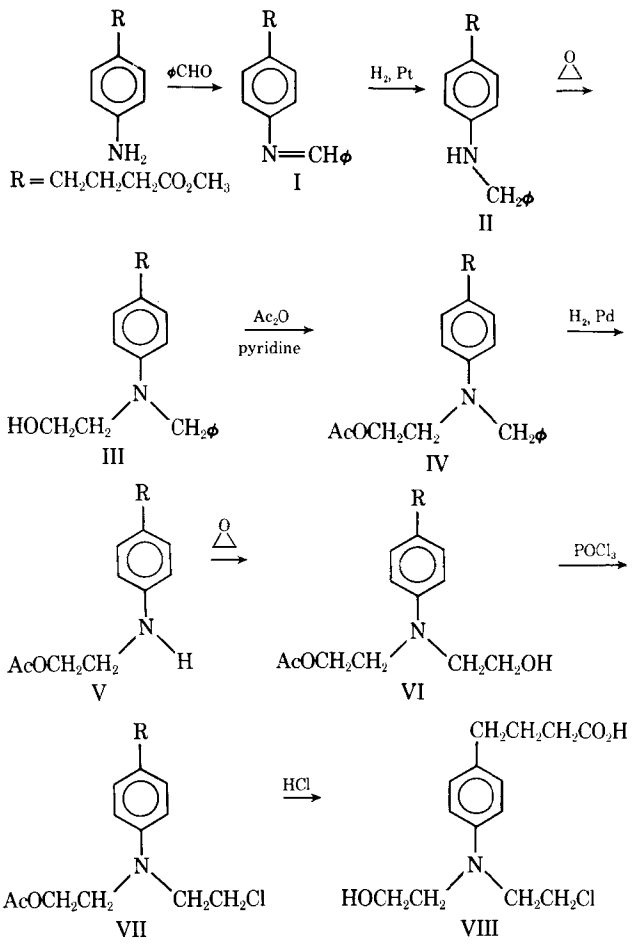
[2] 4-{*p*-[Bis(2-chloroethyl)amino]}phenyl butyric acid was furnished by Cancer Chemotherapy National Service Center.

[3] 5-[Bis(2-chloroethyl)amino]uracil.

possible contaminant is 4-{*p*-[(2-chloroethyl)-(2-hydroxyethyl)amino]phenyl}butyric acid, the chlorambucil hemimustard, VIII. This can result from hydrolysis of chlorambucil or from incomplete chlorination during the preparation of the mustard. Since VIII is still an alkylating agent, it may react with NBP to yield a chromophore characteristically similar to that resulting from the reaction between NBP and chlorambucil. If this happens, the usefulness of the NBP assay of aryl bis-mustards may be questionable. To clarify this situation, the authors have prepared the chlorambucil hemimustard and studied its behavior in the NBP procedure.

RESULTS AND DISCUSSION

The synthesis of VIII was carried out as described in Scheme II; the details are given in the *Experimental* section.



Scheme II

Chlorambucil and its hemimustard were treated with NBP under identical, optimal conditions, which were experimentally determined by varying the substrate–NBP ratio and the period of heating. The results, expressed in molar absorptivities, are given in Tables I and II.

The data show that the hemimustard, VIII, does react with NBP to yield a product whose visible absorption characteristics are similar to those of the chlorambucil–NBP chromophore. Therefore, it is felt that the data obtained from the NBP assay procedure of aryl nitrogen mustards must be interpreted with due caution.

A comparison of the respective molar absorptivities indicates that the intensity of the hemimustard NBP product is only 0.8 that of the chlorambucil–NBP value. The hemimustard appears to reach its maximum color faster than does chlorambucil. At this time, no attempt has been made to seek an explanation for either of these observations because such information is beyond the scope of this communication.

Table I—Color Intensity Developed when the Ratio of Alkylating Compound to NBP was Varied

Moles Compd./ Moles NBP	$\epsilon \times 10^{-4}$, 563 mμ	
	Chlorambucil	Hemimustard, VIII
1:1.2	2.38	1.76
1:2.3	3.14	2.70
1:4.6	3.20	2.84
1:9.2	3.00	2.80

EXPERIMENTAL[4]

NBP Assay Procedure—Chlorambucil (1.2 mg.) or VIII (1.1 mg.) was dissolved in 25.0 ml. of 95% EtOH. A 1.0-ml. aliquot of this solution was placed in a 5.0-ml. volumetric flask together with 1.0 ml. of an NBP solution (3.55 g./50 ml. of acetone) and 1.0 ml. of buffer solution (1.0 g. of potassium hydrogen phthalate/100 ml. of H_2O).

The flask was kept at 85° in a water bath for 30 min. The solution was cooled immediately for 2–3 min. in an ice bath, and 0.1 ml. of a KOH solution (1.4 g./25 ml. of 95% EtOH) was added.[5] The solution was diluted to 5.0 ml. with 95% EtOH and shaken. After 2 min. the absorbance from 560–570 mμ was recorded. This procedure was used by varying the heating time to obtain the data tabulated in Table II. The molar ratios were varied by changing the concentration of NBP solutions so that the data in Table I could be obtained.

Materials—4-Nitrobenzylpyridine (NBP) (Aldrich Chemical Co.) was used without further purification.

The chlorambucil used was chromatographically homogeneous on Whatman No. 1 paper, solvent system *n*-BuOH–HOAc–H_2O, 5:2:3, and by GLC as its silyl derivative on a 1.52-m. (5-ft.) 5% SE-54-diatomaceous earth (Gas Chrom W, Applied Science Laboratories, Inc.) column at 210°. Elemental analysis for the chlorambucil sample was satisfactory.

Anal.—Calcd. for $C_{14}H_{19}Cl_2NO_2$: C, 55.27; H, 6.30; Cl, 23.31; N, 4.60. Found: C, 55.57; H, 6.25; Cl, 23.05; N, 4.60. The IR spectrum of this material was identical to that of an authentic sample. The NMR spectrum is consistent with that expected for the compound; 1.90 δ (m, 2H, $\underline{CH_2}$—CO_2H), 2.40 δ (m, 4H, φ—CH_2—CH_2—), 3.65 δ [s (broad), 8H, $ClCH_2\underline{CH_2}$—], 6.80 δ (q, 4H, aromatic), and 11.0 δ [s (broad), 1H, $CO_2\underline{H}$].

Methyl 4-[(*p*-Benzylidineamino)phenyl]butyrate (I)—A mixture of 6.15 g. (0.03 mole) of methyl 4-(*p*-aminophenyl)butyrate, 0.50 g. of fused potassium acetate, and 3.71 g. of benzaldehyde was refluxed in 50 ml. of absolute EtOH for 4 hr. The cooled solution was diluted with 75 ml. of H_2O and extracted with ether. The ether extracts were combined, dried ($MgSO_4$), filtered, and evaporated at reduced pressure (first at 20 mm. and then at 0.6 mm.) for 7 hr. The product, 8.45 g. (94%), was characterized by spectral and chromatographic evidence. The IR spectrum showed the expected absorption at 6.24 μ, indicative of a benzal imino system. The NMR spectrum, showing expected increase in aryl protons, was consistent with the proposed structure. TLC on silica gel in Solvent System 1 showed only a minor contaminant in addition to a main spot.

Methyl 4-[*p*-(*N*-Benzylamino)phenyl]butyrate (II)—A solution of 8.45 g. (0.03 mole) of crude benzylidene compound in 90 ml. of 95% EtOH was stirred in an atmosphere of hydrogen gas in the presence of 1.0 g. of 5% Pd-C catalyst for 85 min., during which time the theoretical amount of hydrogen was consumed. The catalyst was removed by filtration; the reaction mixture was concentrated to a tan oil, 7.56 g. (89%), which was of sufficient purity to use in the

[4] The IR spectra were recorded as neat liquid films, as mineral oil mulls, or as $CHCl_3$ solutions. The NMR spectra were taken as $CDCl_3$ solutions with internal TMS (δ = 0.0) using a Varian A60-A spectrometer. Where multiplets are involved the chemical shift is measured from TMS to the center of the multiplet. In TLC, Solvent System 1 is benzene–ether, 1:1, and System 2 is $CHCl_3$–MeOH, 2:1.

[5] The quantity of added base has been found to be quite critical, since the addition of insufficient or excess base in this experiment causes a decrease of the absorbance at 560–570 mμ.

Table II—Color Intensity Developed when the Time of the Alkylation Reaction was Varied[a]

Time, min.	$\epsilon \times 10^{-4}$, 563 mμ	
	Chlorambucil	Hemimustard, VIII
10	2.54	2.76
20	3.06	2.75
30	3.32	2.75
40	3.36	2.76
50	3.36	2.78

[a] The ratio of alkylating agent to NBP was 1:2.3.

next step. The IR spectrum showed an absorption at 2.90 μ attributed to N—H. The NMR spectrum exhibited to a two-proton singlet at 4.3 δ and a five-proton singlet at 7.3 δ that were assigned to the benzyl group.

SiO_2 TLC (Solvent System 1) of the reaction mixture shows two major spots. One of these is the desired *N*-benzyl compound, II, while the slower moving spot travels identically as methyl 4-(*p*-aminophenyl)butyrate. In smaller scale preparation, column chromatography on SiO_2 (Solvent System 1) was employed to remove the primary amine. In the subsequent hydroxyethylation reaction, it was found that the bis-hydroxyethylated compound, methyl 4-{*p*-[bis-hydroxyethyl)amino]phenyl}butyrate could be easily separated from the desired compound, III, by chromatography so that extensive purification at this reduction step is not necessary.

Methyl 4-{[*p*-(*N*-Benzyl)-(2-hydroxyethyl)amino]phenyl}butyrate (III)—A 100-ml. round-bottom flask containing 7.6 g. (0.028 mole) of the *N*-benzyl compound, II, 40 ml. of 50% HOAc, and 7.7 ml. of ethylene oxide at 0° was stoppered and kept at room temperature for 16 hr. The reaction mixture was then poured into 75 ml. of H_2O and neutralized with solid $NaHCO_3$. This solution was extracted with several portions of EtOAc. The combined EtOAc extracts were washed thoroughly with H_2O, dried ($MgSO_4$), filtered, and evaporated to a brown oil (7.89 g.).

The crude hydroxyethyl compound was purified by chromatography on a 40 × 2.2-cm. SiO_2 column. The column was developed first with φH-Et_2O, 9:1, followed by φH-Et_2O, 4:1, which eluted 5.22 g. (66%) of Compound III as an oil. The IR spectrum exhibited the expected —OH bands at 2.9 and 9.55 μ. The NMR spectrum exhibited, in addition to those resonances observed for Compound II, a four-proton multiplet centered at 3.65 δ which was assigned to the methylene groups of the newly added 2-hydroxyethyl moiety.

Methyl 4-{*p*-[(*N*-Benzyl)-(2-acetoxyethyl)amino]phenyl}butyrate (IV)—A solution of 5.22 g. (0.016 mole) of the hydroxyethyl Compound III, 15 ml. of dry pyridine, and 15 ml. of acetic anhydride was kept overnight in a 100-ml. round-bottom flask. The reaction mixture was poured into 100 ml. of H_2O, neutralized with $NaHCO_3$, and extracted with EtOAc. The combined EtOAc extracts were washed with *N* HCl (until the aqueous wash remained at pH 1), H_2O, saturated $NaHCO_3$, and again with H_2O. The EtOAc solution was dried ($MgSO_4$), filtered, and evaporated to an oil, 4.97 g. (84%). The material was homogeneous on SiO_2 TLC (Solvent System 1) and its IR showed the typical acetoxy band at 8.10 μ. The NMR spectrum exhibited a three-proton singlet at 1.99 δ which was assigned to the acyl methyl group. In addition, there was a two-proton triplet at 4.25 δ (J = 6 c.p.s.) that was assigned to the AcO—$\underline{CH_2}$— protons.

Methyl 4-{[*p*-(2-Acetoxyethyl)amino]phenyl}butyrate (V)—A solution of 4.95 g. (0.013 mole) of the *N*-benzyl-acetoxyethylamino Compound IV in 35 ml. of 95% EtOH was stirred with 0.56 g. of 5% Pd–C catalyst in the presence of hydrogen gas at atmospheric pressure until sligh ly more than the theoretical amount of gas was taken up (16 hr.). The catalyst was removed by filtration and the ethanol was evaporated under reduced pressure to yield 3.49 g. (93%) of an oil which was homogeneous on SiO_2 TLC (Solvent System 1). The NMR spectrum showed that the benzyl group was removed since the two singlets attributed to the protons of the benzyl group were absent in the spectrum. The IR spectrum showed the expected N—H band at 2.90 μ.

Methyl 4-{*p*-[(2-Hydroxyethyl)-(2-acetoxyethyl)amino]phenyl}butyrate (VI)—Hydroxyethylation of V with ethylene oxide was carried out in a manner similar to that used for the preparation of III. A 2.69-g. (0.009-mole) portion of V gave 2.18 g. (70%) of an oil whose NMR spectrum was consistent with that expected for the desired compound. The additional methylene groups of the hydroxyethyl group were observed as a nine-proton multiplet at 3.6 δ (the integration includes the three protons of the O—CH_3 singlet). At 4.2 δ a two-proton triplet was assigned to the methylene group adjacent to the acetoxy moiety. The IR spectrum showed absorptions at 5.70 and 5.80 μ, the former being assigned to the acetate, and at 2.90 and 9.55 μ, both of which were assigned to the OH.

On SiO_2 TLC (Solvent System 1) the compound was nearly homogeneous and showed three minor contaminants in addition to a large major spot.

Methyl 4-{*p*-[(2-Chloroethyl)-(2-acetoxyethyl)amino]phenyl}butyrate (VII)—Freshly distilled $POCl_3$ (20 ml.) and 2.61 g. (8.0 mmoles) of the hydroxyethyl compound, (VI), were heated at reflux for 1 hr. and then poured into 200 ml. of crushed ice. The mixture was stirred for 20 min., with ice being added periodically, and then extracted with several portions of chloroform. The combined $CHCl_3$ extracts were dried ($MgSO_4$), filtered, and evaporated to give 2.45 g. (88%) of a yellow oil. The oil was essentially homogeneous on silica gel TLC in Solvent System 1. The NMR spectrum exhibited the following resonances: 2.00 δ (s, 3H, $C\underline{CH_3}$); 3.62 δ (m, 6H, —$\underline{CH_2}$—$\underline{CH_2}$— and —$\underline{CH_2}$—CH_2 of *N* substituents); 3.67 δ (s, 3H, O—$\underline{CH_3}$); 4.21 δ (t, J = 6 c.p.s., assigned to $\underline{CH_2}$ adjacent to Cl or OAc); and 6.85 δ (q, 4H, J = 8.5 c.p.s., aromatic). The IR spectrum (neat) showed no absorption at 2.90 μ and an absorption at 13.3 μ which was assigned to C—Cl. The crude material was purified by preparative silica gel plates (Solvent System 1) to yield 1.29 g. (68%) of an analytically pure oil.

Anal.—Calcd. for $C_{17}H_{24}ClNO_4$: C, 59.73; H, 7.08; N, 4.10. Found: C, 59.76; H, 7.23; N, 4.15.

A smaller scale reaction, carried out prior to the above experiment, gave material that had identical IR, NMR, and TLC to the material prepared above. A satisfactory chlorine analysis was obtained.

Anal.—Calcd.: 10.37. Found: 10.3 for this material.

4-{*p*-[(2-Chloroethyl)-(hydroxyethyl)amino]phenyl}butyric Acid "Hemimustard" (VIII)—The hydrolysis of the blocking groups was performed in refluxing HCl. A solution of 1.405 g. (4.1 mmoles) of Compound VII and 3.0 ml. of concentrated HCl was heated at reflux for 4 hr. The reaction mixture was cooled, diluted with H_2O, buffered at pH 5 by the addition of NaOAc, and extracted with several portions of CH_2Cl_2. The CH_2Cl_2 extracts were combined, dried ($MgSO_4$), filtered, and concentrated to a colorless oil, 1.20 g. (84%).

A portion of the crude material was dissolved in Et_2O–petroleum ether (b. p. 30–60°). After 3 days in the cold, there was deposited crystalline VIII; recrystallization from the same solvent yielded an analytically pure product, m.p. 51–53° (uncorrected). The material was homogeneous on silica gel TLC (Solvent System 2).

Anal.—Calcd. for $C_{14}H_{20}ClNO_3$: C, 58.84; H, 7.05; Cl, 12.40; Cl^-, 0.0; N, 4.90. Found: C, 58.72; H, 6.93; Cl, 12.54; Cl^-, 0.0, N, 4.86.

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