Distribution Coefficients for 5a and 5b.—Ten milliliters each of C_6H_6 and pH 7.4 Krebs–Ringers phosphate were shaken for 1 hr with the tritiated compound (**5a** and **5b**). Each phase (1 ml) was counted in a liquid scintillation spectrometer. The counts per minute found in each phase were corrected to disintegrations per minute and the ratio was calculated.

Hydrolytic Rate Studies on 16a and 16b.—A 10-ml ethanolic 0.25 *N* NaOH solution containing 0.025 g of ester was maintained at 21.5°. Aliquots (1 ml) were drawn at intervals and titrated with 0.04 *N* HCl using phenolphthalein as indicator. A blank experiment was carried out.

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Experimentally Induced Phenylketonuria. II. Potential Inhibitors of Phenylalanine Hydroxylase

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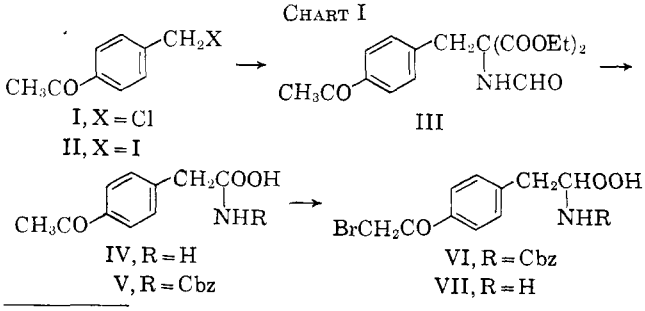
The preparation of a series of alkylating agents derived from phenylalanine is reported. The compounds were found to be ineffective as inhibitors of phenylalanine hydroxylase. Syntheses are described for 4-bromoacetyl-, 4-bromoacetamido-, 3-chloroacetamido-, and 4-fluoro-3-chloroacetamidophenylalanine.

In a previous publication[1] we reported an approach to the creation of a state of phenylketonuria (PKU) by inhibition of the enzyme, phenylalanine hydroxylase. We had confirmed earlier findings that 4-fluorophenylalanine[2] and esculetin (6,7-dihydroxycoumarin)[3] were good inhibitors of the enzyme *in vitro*. Our attempts to find a more potent inhibitor among a series of variously substituted phenylalanine derivatives were unsuccessful. However, we were able to conclude that alteration of the amino acid side chain and substitution of a group larger than fluorine in the 4 position was detrimental to activity. It appeared likely to us that irreversible inhibition of phenylalanine hydroxylase would more closely mimic the genetic defect responsible for PKU.

In the present work we have prepared some potential irreversible inhibitors in the form of alkylating agents derived from phenylalanine. The compounds were 4-bromoacetyl-, 4-bromoacetamido-, 3-chloroacetamido-, and 4-fluoro-3-chloroacetamidophenylalanine. As will be seen in the biological results, no significant degree of inhibition was realized with these compounds. Whether the lack of significant inhibition was due to the bulkiness of the haloalkyl substituents or insufficient reactivity of the alkylating functions is not known.

The synthesis of 4-bromoacetylphenylalanine started with 4-chloromethylacetophenone[4] (I) which was converted to the crude iodide (II), and this in turn was used to alkylate diethyl formamidomalonate (Chart I).

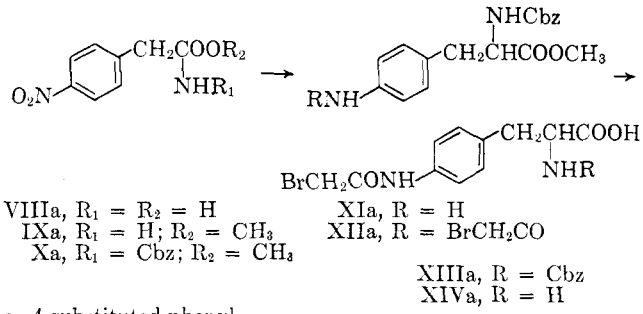
CHART I



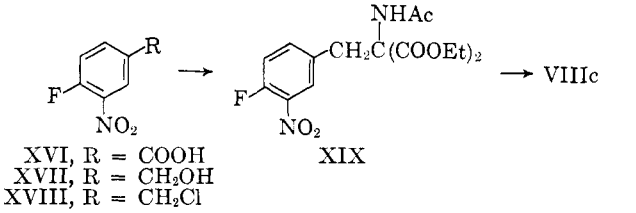
Acid hydrolysis and decarboxylation of the intermediate III yielded 4-acetylphenylalanine (IV). An effort was made to brominate IV directly in acetic acid at 70° but no reaction with bromine was observed. The N-carbobenzoxy derivative V was then prepared and brominated readily with trimethylphenylammonium tribromide[5] in THF to afford the N-carbobenzoxybromoketone (VI) in 44% yield. Treatment of VI with HBr–AcOH smoothly cleaved the blocking group without disturbance of the bromine to yield 4-bromoacetylphenylalanine (VII) as the hydrobromide salt.

4-Nitrophenylalanine was converted to its N-carbobenzoxy methyl ester. The nitro group was reduced and the resulting amine (XIa) was converted to the bromoacetamido compound (XIIa) (Chart II). Mild

CHART II



a, 4-substituted phenyl
b, 3-substituted phenyl
c, 4-fluoro-3-substituted phenyl; chloroacetamides were prepared for the b and c series



exposure of XIIa to HBr–AcOH caused only partial removal of the methyl ester, while more vigorous conditions also caused cleavage of the bromoacetyl moiety.

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Careful saponification of the ester with alkali yielded the N-carbobenzoxy acid (XIIIa) and subsequent treatment with HBr–AcOH afforded 4-bromoacetamidophenylalanine (XIVa).

It was found necessary to prepare 3-chloroacetamidophenylalanine (XIVb) rather than the planned bromo analog since alkaline hydrolysis of the 3-bromoacetamido ester (XV) could not be effected without partial loss of the bromo group. The synthesis was similar to that of XIVa and began with 3-nitrophenylalanine.[6]

3-Chloroacetamido-4-fluorophenylalanine (XIVc) was also prepared from 4-fluoro-3-nitrophenylalanine (VIIIc) in a manner analogous to that described above. The reduction of 4-fluoro-3-nitrobenzoic acid (XVI)[7] with diborane afforded 4-fluoro-3-nitrobenzyl alcohol (XVII). The alcohol was converted to the chloride (XVIII), which was used to alkylate diethyl acetamidomalonate. Hydrolysis of the intermediate amido diester (XIX) with concentrated HCl yielded 4-fluoro-3-nitrophenylalanine (VIIIc).

Biological Evaluation.—Phenylalanine hydroxylase was prepared from rat liver by the method of Kaufman;[8] purification was carried out up to step 2 of his procedure. The incubation mixture consisted of 100 μmoles of sodium phosphate buffer, pH 7.4, 2.0 μmoles of reduced diphosphopyridine nucleotide, 10 μmoles of nicotinamide, approximately 10 mg of enzyme protein, 1.0 μmole of phenylalanine, and appropriate amounts of the test compound in a final volume of 1.74 ml. Incubation was carried out for 20 min at 37° in air. Tyrosine was assayed by the method of Udenfriend and Cooper.[9]

All of the phenylalanine-derived alkylating agents (VII, XIVa–c) showed less than 50% inhibition at a ratio of substrate to inhibitor of 1:1. Prior investigation[1] showed that esculetin and 4-fluorophenylalanine gave 50% inhibition of phenylalanine hydroxylase at ratios of 200:1 and 10:1, respectively.

Experimental Section

Where analyses are indicated only by symbols of the elements, the analytical results obtained for those elements were within ±0.4% of the theoretical values. Melting points were determined on a Fischer-Johns apparatus and are corrected.

Diethyl 4-Acetylbenzylformamidomalonate (III).—To 10.5 g (63.3 mmoles) of *p*-(chloromethyl)acetophenone (I)[4] in 250 ml of Me_2CO was added 34.8 g (0.316 mole) of NaI. The suspension was stirred at reflux for 16 hr and evaporated *in vacuo* to dryness. The crude mixture was partitioned between 200 ml of $CHCl_3$ and 200 ml of H_2O. The $CHCl_3$ layer was washed (two 100-ml portions of H_2O), dried ($MgSO_4$), and evaporated *in vacuo* to yield 15.1 g of II as a light yellow solid (91%). The crude iodide was dissolved in 100 ml of EtOH and added to 1.35 g (58.1 mg-atoms) of Na and 11.82 g (58.1 mmoles) of diethyl formamidomalonate in 200 ml of EtOH. The resulting solution was heated at reflux for 3 hr and evaporated to near dryness, and H_2O (100 ml) was added. The crystalline precipitate that resulted was washed (H_2O), dried in air, and recrystallized (EtOH) to yield 7.5 g (39%) of white crystals, mp 131.5–133.5°. *Anal.* ($C_{17}H_{21}NO_6$) C, H, N.

4-Acetylphenylalanine Hydrochloride (IV).—A solution of 0.59 g (1.7 mmoles) of III in 2.2 ml of concentrated HCl was heated at reflux for 16 hr and evaporated *in vacuo* to dryness. The residue was dissolved in concentrated NH_4OH and filtered. The filtrate was acidified to pH 1, chilled, and filtered. The product was recrystallized from 95% EtOH to yield 0.28 g (63%) of white crystals, mp 182–185° dec. *Anal.* ($C_{11}H_{13}NO_3 \cdot HCl \cdot 0.5H_2O$) C, H, N.

N-Carbobenzoxy-4-acetylphenylalanine (V).—To 1.00 g (4.8 mmoles) of IV in 25 ml of 10% K_2CO_3 at 0° was added 1.65 g (9.6 mmoles) of carbobenzoxy chloride over 10 min with stirring. The mixture was stirred 3 hr, washed (three 20-ml portions of $CHCl_3$), and acidified with 6 *N* HCl to pH 1. The gum was extracted into 50 ml of $CHCl_3$, then washed with two 20-ml portions of H_2O. The $CHCl_3$ extract was dried ($MgSO_4$) and evaporated *in vacuo* to dryness. The resulting crystals were recrystallized twice from EtOAc–cyclohexane (1:5) to yield 0.86 g (52%), mp 119.5–121.5°. *Anal.* ($C_{19}H_{19}NO_5$) C, H, N.

N-Carbobenzoxy-4-bromoacetylphenylalanine (VI).—To a solution of 2.96 g (8.7 mmoles) of V in 60 ml of THF was added 3.17 g (8.6 mmoles) of trimethylphenylammonium tribromide. The orange solution was stirred overnight. The resulting pale yellow mixture was filtered and evaporated *in vacuo* to dryness to yield a light yellow solid. Two recrystallizations (C_6H_6) afforded 1.6 g (44%) of white crystals, mp 144–146°. *Anal.* ($C_{19}H_{18}BrNO_5$) C, H, N, Br.

4-Bromoacetylphenylalanine Hydrobromide (VII).—To 0.50 g (1.2 mmoles) of VI was added 2 ml of 30% HBr in AcOH. The solution was stirred at room temperature for 1 hr and evaporated *in vacuo* to dryness. The white crystalline residue that remained was triturated with Et_2O (20 ml) and filtered. (See Table II for physical data.)

4-Fluoro-3-nitrophenylalanine Hydrochloride (VIIIc).—Following the procedure for 4-acetylphenylalanine hydrochloride (IV), 1.0 g (2.7 mmoles) of diethyl 4-fluoro-3-nitrobenzylacetamidomalonate (XIX) was hydrolyzed with 15 ml of concentrated HCl to yield, after recrystallization from 95% AcOH, 0.57 g (79%) of white crystals, mp 203–205°. *Anal.* ($C_9H_8FN_2O_4 \cdot HCl$) C, H, N.

Methyl 4-Nitrophenylalanate Hydrochloride (IXa).—To 30 ml of MeOH saturated at 0° with dry HCl was added 3.0 g (14.3 mmoles) of 4-nitrophenylalanine (Cyclo Chemical Co.). The resulting solution was stirred at reflux for 16 hr, cooled to room temperature, and evaporated *in vacuo* to dryness. Recrystallization from MeOH gave 2.5 g (77%) of white crystals, mp 213–214°. *Anal.* ($C_{10}H_{12}N_2O_4 \cdot HCl$) C, H, N.

Methyl 3-nitrophenylalanate hydrochloride (IXb) was prepared by the procedure for IXa. The product, mp 182.5–184°, was obtained in an 82% yield after recrystallization from MeOH. *Anal.* ($C_{10}H_{12}N_2O_4 \cdot HCl$) C, H, N.

Methyl 4-fluoro-3-nitrophenylalanate hydrochloride (IXc) was prepared from 3-nitrophenylalanine[6] following the procedure for IXa; mp 192.5–194°, yield 83%, after recrystallization from MeOH. *Anal.* ($C_{10}H_{11}FN_2O_4 \cdot HCl$) C, H, N.

Methyl N-Carbobenzoxyphenylalanates (X).—To an ice-cold mixture of 0.05 mole of the substituted phenylalanine methyl ester hydrochloride in H_2O (400 ml) containing 0.30 mole of K_2CO_3 was added 0.10 mole of carbobenzoxy chloride in 400 ml of $CHCl_3$ at 0°. The mixture was stirred at 0° for 1 hr and at room temperature for 3 hr. The $CHCl_3$ was washed (three 50-ml portions of H_2O, three 50-ml portions of 3 *N* HCl, three 50-ml portions of H_2O), dried ($MgSO_4$), and evaporated *in vacuo*. The resulting products were collected and recrystallized (see Table I).

Methyl N-Carbobenzoxyaminophenylalanates (XI).—To a solution of 1.5 mmoles of the methyl N-carbobenzoxynitrophenylalanate in 15 ml of 90% MeOH at 40° was added 2.5 mmoles of NH_4Cl followed slowly by 15.0 mg-atoms of Zn dust. The mixture was stirred at reflux for 3 hr, filtered while hot, and evaporated *in vacuo* to dryness. The residual mixture was partitioned between ether and water, and the Et_2O was dried and evaporated *in vacuo* to dryness. The resulting gum was used directly for the next reaction. In some cases picrates were prepared for analytical purposes as recorded in Table I.

Methyl N-Carbobenzoxy-α-haloacetamidophenylalanates (XII).—To a suspension of 120 mmoles of powdered, anhydrous K_2CO_3 in 15 ml of C_6H_6 containing 6.0 mmoles of the methyl N-carbobenzoxyaminophenylalanate was slowly added 6.5 mmoles of either chloroacetyl chloride or bromoacetyl bromide keeping the temperature between 15–20°. The mixture was stirred at room temperature for 3 hr and chilled to 5°, and 25 ml of H_2O was added. The benzene was separated, washed (three 10-ml portions of H_2O, three 20-ml portions of 3 *N* HCl, three 10-ml portions of H_2O), dried ($MgSO_4$), and evaporated *in vacuo* to dryness. The product was collected and recrystallized (see Table I).

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TABLE I

SUBSTITUTED N-CARBOBENZOXYPHENYLALANINES

$ArCH_2CHCO_2R$
|
$NH—CBZ$

No.	Ar	R	Yield, %	Mp, °C	Crystn solvent	Formula	Analyses
Xa	$4\text{-}NO_2C_6H_4$	CH_3	72	77.5–78.5	C_6H_6–cyclohexane	$C_{18}H_{18}N_2O_6$	C, H, N
Xb	$3\text{-}NO_2C_6H_4$	CH_3	86	49–52	C_6H_6–hexane	$C_{18}H_{18}N_2O_6$	C, H, N
Xc	$3\text{-}NO_2\text{-}4\text{-}FC_6H_3$	CH_3	83	64.5–67	C_6H_6–cyclohexane	$C_{18}H_{17}FN_2O_6$	C, H, N
XIa	$4\text{-}NH_2C_6H_4$	CH_3	92[a]	128–130	50% EtOH	$C_{24}H_{23}N_5O_{11}$	C, H, N
XIb	$3\text{-}NH_2C_6H_4$	CH_3	90[a]	169–171	50% MeOH	$C_{24}H_{23}N_5O_{11}$	C, H, N
XIc	$3\text{-}NH_2\text{-}4\text{-}FC_6H_3$	CH_3	95[b]				
XIIa	$4\text{-}NHCOCH_2BrC_6H_4$	CH_3	31	100–102	C_6H_6–cyclohexane	$C_{20}H_{21}BrN_2O_5$	C, H, N
XIIb	$3\text{-}NHCOCH_2ClC_6H_4$	CH_3	45	82–84	C_6H_6–cyclohexane	$C_{20}H_{21}ClN_2O_5$	C, H, Cl, N
XIIc	$3\text{-}NHCOCH_2Cl\text{-}4\text{-}FC_6H_3$	CH_3	41	84–85	C_6H_6–cyclohexane	$C_{20}H_{20}ClFN_2O_5$	C, H, N
XIIIa	$4\text{-}NHCOCH_2BrC_6H_4$	H	61	154–155.5	C_6H_6–EtOAc	$C_{19}H_{19}BrN_2O_5$	C, H, Br, N
XIIIb	$3\text{-}NHCOCH_2ClC_6H_4$	H	42	116.5–120	C_6H_6	$C_{19}H_{19}ClN_2O_5$	C, H, Cl, N
XIIIc	$3\text{-}NHCOCH_2Cl\text{-}4\text{-}FC_6H_3$	H	75	145–147	EtOAc	$C_{19}H_{18}ClFN_2O_5$	C, H, Cl, N
XV	$3\text{-}NHCOCH_2BrC_6H_4$	CH_3	25	90–93	C_6H_6	$C_{20}H_{21}BrN_2O_5$	C, H, N

[a] Yield as free base; characterized as picrate. [b] Crude oil used for next reaction without analysis.

TABLE II

SUBSTITUTED PHENYLALANINES

$ArCH_2CHCO_2H$
|
$NH_2 \cdot HBr$

No.	Ar	Yield, %	Mp, °C	Crystn solvent	Formula	Analyses
VII	$4\text{-}COCH_2BrC_6H_4$	68	210.5–212	95% AcOH	$C_{11}H_{12}BrNO_3 \cdot HBr$	C, H, Br, N
XIVa	$4\text{-}NHCOCH_2BrC_6H_4$	57	243–245	95% EtOH	$C_{11}H_{13}BrN_2O_3 \cdot HBr$	C, H, N
XIVb	$3\text{-}NHCOCH_2ClC_6H_4$	51	>300	Me_2CO–Et_2O	$C_{11}H_{13}ClN_2O_3 \cdot HBr$	C, H, N
XIVc	$3\text{-}NHCOCH_2Cl\text{-}4\text{-}FC_6H_3$	78[a]	287–292	Me_2CO–Et_2O	$C_{11}H_{12}ClFN_2O_3 \cdot HBr \cdot HOAc$	C, H, N

[a] Compound quite hygroscopic.

N-Carbobenzoxy-α-haloacetamidophenylalanines (XIII).—To 1.0 mmole of the methyl N-carbobenzoxy-α-haloacetamidophenylalanate in 20 ml of MeOH at 0° was added 1.0 ml of 1.00 *N* KOH. The resulting solution was stirred at room temperature until the pH was approximately 7 (about 5 hr). Tests with acidic, alcoholic $AgNO_3$ were run upon the reaction solution at 1-hr intervals. In the case of the methyl N-carbobenzoxy-3-bromoacetamidophenylalanate (XV) a positive halide test resulted after 1 hr. The reaction mixture was evaporated *in vacuo* to dryness and the resulting gum was partitioned between $CHCl_3$ and saturated $NaHCO_3$. The $NaHCO_3$ extract was acidified to pH 1 with 3 *N* HCl, extracted with $CHCl_3$, dried ($MgSO_4$), and evaporated *in vacuo* to dryness. The products were collected and recrystallized (see Table I).

α-Haloacetamidophenylalanines (XIV) were all prepared from the respective N-carbobenzoxy acids (XIII) by the same procedure used for 4-bromoacetylphenylalanine (VII) (see Table II).

4-Fluoro-3-nitrobenzyl Alcohol (XVII).—To 20 ml (20 mmoles) of 1 *M* borane in THF at 0° was slowly added 1.85 g (10 mmoles) of 4-fluoro-3-nitrobenzoic acid (XVI) in 25 ml of THF. The mixture was stirred at reflux for 1 hr and chilled to 0–5°. Five milliliters of 3 *N* HCl was added slowly and the mixture was evaporated *in vacuo* to dryness. The resulting solid was partitioned between H_2O and Et_2O, and Et_2O extract was washed (three 10-ml portions of saturated $NaHCO_3$, three 10-ml portions of H_2O), dried ($MgSO_4$), and evaporated *in vacuo* to yield 1.5 g (87%) of a yellow oil. A portion of the oil was purified for analysis by glpc on a 16.3 × 0.62 cm column of 20% DC 550 on Chromosorb W 45/60 at 185°. *Anal.* ($C_7H_6FNO_3$) C, H, N.

4-Fluoro-3-nitrobenzyl Chloride (XVIII).—To 7.6 g (44 mmoles) of 4-fluoro-3-nitrobenzyl alcohol (XVII) was added 41.5 g (0.349 mole) of $SOCl_2$. The solution was stirred at room temperature for 1 hr and distilled *in vacuo* through a 10-cm Vigreux head to yield 6.24 g (74%) of colorless oil, bp 111–112° (1.2 mm). *Anal.* ($C_7H_5ClFNO_2$) C, H, N.

Diethyl 4-Fluoro-3-nitrobenzylacetamidomalonate (XIX).—Following the procedure for III, 1.16 g (6.2 mmoles) of XVIII was treated with 4.59 g (30.8 mmoles) of NaI in 50 ml of Me_2CO. The crude iodide was allowed to react with a solution of 0.14 g (6.2 mg-atoms) of Na and 1.34 g (6.2 mmoles) of diethyl acetamidomalonate in 50 ml of EtOH to yield, after recrystallization from EtOH, 1.42 g (62%) of white crystals, mp 121.5–123°. *Anal.* ($C_{16}H_{19}FN_2O_7$) C, H, N.

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