Cyto-active Amino-acid and Peptide Derivatives. Part I. Substituted Phenylalanines.

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In a search for more selective tumour inhibitors we have prepared, as a start, some derivatives of phenylalanine. The syntheses of DL-, L-, and D-*p*-di-(2-chloroethyl)aminophenylalanine and of *p*-thioformamido-DL-phenylalanine are described. For the synthesis of the corresponding *p-iso*thiocyanato-compound diethyl α-*p-iso*thiocyanatobenzyl-α-formamidomalonate has been prepared. In biological tests so far carried out, the L-form of the chloroethyl derivative proved the most active.

PRESENT developments in the field of tumour- or leukæmia-inhibiting agents reveal a growing interest in α-amino-acid derivatives. This is, to a considerable extent, due to the occurrence of such units in the molecules of antibiotics and to the discovery of antimetabolite effects with analogues of essential amino-acids. Thus, recent reports disclose tests against neoplastic conditions of a microbiologically produced serine derivative, *O*-diazoacetyl-L-serine (azaserine) (Stock, Reilly, Buckley, Clarke, and Rhoads, *Nature*, 1954, **173**, 71), of a synthetic phenylalanine isostere, thienylalanine (Jacquez, Stock, and Barclay, *Cancer*, 1953, **6**, 828), and of a number of other substances of an amino-acid or peptide nature (cf. Reilly, Stock, Buckley, and Clarke, *Cancer Res.*, 1953, **13**, 684; Brockmann, *Angew. Chem.*, 1954, **66**, 1). Before this trend became noticeable, it had occurred to us that certain natural amino-acids or peptides (either synthetic or isolated from proteins) might,

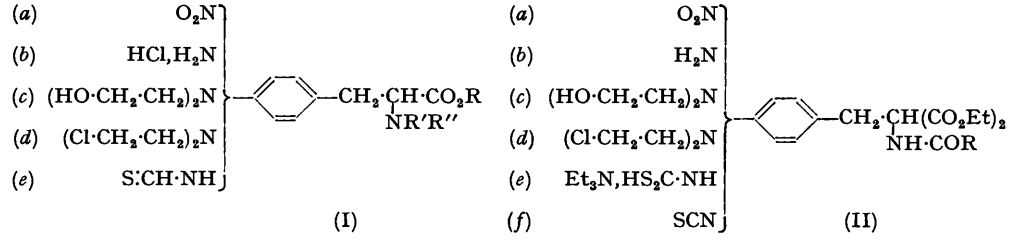
when modified by appropriate groups, show anti-tumour activity, possibly by interaction with cellular constituents in such a manner as to interfere specifically with the nucleic acid or protein metabolism of malignant cells.

Our first approach was greatly stimulated by the systematic work of Ross and his co-workers (see Ross, *Adv. Cancer Res.*, 1953, **1**, 397) on aryldi-(2-chloroethyl)amines. This refers particularly to those carrying additional substituents by which these workers hoped to obtain more pronounced biological specificity. In fact, certain members of a homologous series, $M{\cdot}C_6H_4{\cdot}[CH_2]_n{\cdot}CO_2H$ [where $M = (Cl{\cdot}CH_2{\cdot}CH_2)_2N$; $n = 0$—4] (Everett, Roberts, and Ross, *J.*, 1953, 2386) showed greater activity in their inhibition of experimental tumours than did others (Haddow, personal communication).

Although the " nitrogen mustard " group on aromatic systems, evidently acting as an alkylating agent, proved to be a very active cytotoxic radical, it is not unique. Other cyto-active types include diepoxides, ethyleneimines, and methanesulphonates (cf. Ross, *loc. cit.*) and more will no doubt be discovered. In our first set of compounds derived from phenylalanine we made use mainly of the di-(2-chloroethyl)amino-group in the *para*-position. This was done also with the purpose of adding a new variant to the series studied by Everett, Roberts, and Ross (*loc. cit.*). However, with thioformamido- and *iso*thiocyanato-derivatives, we have made a start in modifying the carrier molecule with alternative groupings. It is hoped that these may interfere with abnormal growth by different mechanisms, such as acylation of, or addition to, cellular constituents.

Our syntheses of the *p*-di-(2-chloroethyl)aminophenylalanines followed two routes. One was *via* *p*-nitro-*N*-phthaloylphenylalanine, an intermediate lending itself readily to the preparation of the three optical isomers, and prepared also for subsequent use in peptide synthesis. The second procedure, which is more suitable for the larger-scale preparation of substituted racemic phenylalanine, involved the intermediate preparation of α-acetamido- or α-formamido-α-*p*-nitrobenzylmalonic ester.

The racemic and optically active forms of *p*-nitrophenylalanine were prepared by the method of Erlenmeyer and Lipp (*Annalen*, 1883, **219**, 213), with modifications in the isolation procedure in most experiments. Oxidation with nitric acid confirmed their observation that production of nitro-phenylalanines other than the *p*-substituted one was insignificant.



In the synthesis of the racemic compound the intermediate ethyl *p*-nitro-*N*-phthaloyl-DL-phenylalaninate [I*a*; R = Et, R'R'' = *o*-$C_6H_4(CO)_2$] was prepared by heating *p*-nitro-DL-phenylalanine with phthalic anhydride alone (cf. Billman and Harting, *J. Amer. Chem. Soc.*, 1948, **70**, 1473) or in pyridine (cf. King and Kidd, *J.*, 1949, 3315) and esterifying the product in ethanolic hydrogen chloride. With the optically active isomers, however, in order to avoid racemisation, another method due to King and Kidd (*loc. cit.*), used in their case to prepare ethyl phthaloyl-L-glutamate, was followed. After a successful trial with the DL-compound, phthalic anhydride was allowed to react with the ethyl esters of *p*-nitro-L- and -D-phenylalaninate severally in benzene solution at room temperature. The resulting *N*-*o*'-carboxybenzoyl-*p*-nitro-L- and -D-phenylalanine esters were converted into the corresponding *N*-phthaloyl derivatives [I*a*; R = Et, R'R'' = *o*-$C_6H_4(CO)_2$] by refluxing ethanolic hydrogen chloride. The three isomeric phthaloyl esters were reduced catalytically and the *p*-amino-compounds obtained as their hydrochlorides [I*b*; R = Et, R'R'' = *o*-$C_6H_4(CO)_2$]. In the case of the DL-isomer the free base was characterised. Hydroxy-

ethylation and conversion into the di-(2-chloroethyl)amino-derivatives (I*d*; R = R′ = R″ = H) followed conventional methods except that in the final hydrolysis of the chlorinated optically active products, 6N-hydrochloric acid was used instead of the concentrated acid in order to minimise the risk of racemisation. The specific rotation of *p*-di-(2-chloroethyl)amino-D-phenylalanine was not significantly altered when the compound was subjected to the hydrolysis conditions used in the final stage of preparation, a result in line with the known stability of optically active amino-acids in acid media (cf. Neuberger, *Adv. Protein Chem.*, 1948, **4**, 297). This fact did not, however, eliminate the possibility of partial racemisation during the hydrolysis of the phthaloyl and ester groups. As a check, therefore, L-phenylalanine was taken through the same cycle of reactions *via* the ethyl ester of *N*-*o*′-carboxybenzoyl-L-phenylalanine and the *N*-phthaloyl ester, back again to the L-amino-acid. The starting material had $[\alpha]_D^{21}$ −6·9° ± 0·3° and the final product $[\alpha]_D^{21}$ −7·0° ± 0·3° (both in N-hydrochloric acid); so no detectable racemisation had occurred. In this experiment, the hydrolysis of the *N*-phthaloyl-L-phenylalanine ester was much slower than that of the corresponding *p*-di-(2-chloroethyl)amino-compound.

The same cycle of reactions was not tried with the optically active " nitrogen mustards " themselves since these were available only in relatively small amounts which were reserved for biological testing.

No attempt was made to ensure that the L- and D-phenylalanines employed as starting materials were optically pure. This was justified by the fact that the intermediate *p*-nitro-*N*-phthaloyl-L- and -D-phenylalanine esters [I*a*; R = Et, R′R″ = *o*-$C_6H_4(CO)_2$] were readily obtained in their optically pure forms of $[\alpha]_D^{20}$ −207° and +207° respectively, showing that contaminating isomer had been removed during the preceding stages.

The free di-(2-chloroethyl)amino-substituted amino-acids (I*d*; R = R′ = R″ = H) were obtained by precipitation with sodium acetate from the aqueous solutions of their hydrochlorides. The L- and the D-acid each crystallised from methanol with one molecule of solvent of crystallisation, had m. p. 182° and $[\alpha]_D^{22}$ +7·5°, $[\alpha]_D^{21}$ −7·5° respectively in N-hydrochloric acid.

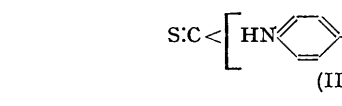
As indicatedabove, the DL-derivative (I*d*; R = R′ = R″ = H) was also synthesised by preparing first α-acetamido- or α-formamido-α-*p*-nitrobenzylmalonic ester (II*a*; R = Me or H). Catalytic reduction gave the corresponding amino-compounds (II*b*; R = Me or H) and treatment with ethylene oxide the di-(2-hydroxyethyl)amino-derivatives (II*c*; R = Me or H). While chlorination with phosphorus oxychloride did not work well here, the use of thionyl chloride with short refluxing in chloroform led, after hydrolysis and decarboxylation with hydrochloric acid, to a relatively good yield of (I*d*; R = R′ = R″ = H). The formamido-intermediate (II*d*; R = H) showed less tendency to darken during chlorination and gave a cleaner hydrolysate than did the acetamido-compound (II*d*; R = Me).

In order to ascertain the reactivity of *p*-di-(2-chloroethyl)amino-DL-phenylalanine as compared with other aromatic " nitrogen mustards," the compound was hydrolysed by Ross's method (*J.*, 1949, 183). Titration of hydrogen ion was carried out electrometrically since the usual method using phenolphthalein as indicator was unsatisfactory. The result indicated a 21% decomposition of the 2-chloroethyl group. Chloride-ion determination showed that 22% of the halogen was liberated, a result in accord with the acidity determination. The hydrolysis rate is of the same order as that of other aryl-*p*-di-(2-chloroethyl)-amines of known biological activity (cf. Ross, *loc. cit.*, 1953).

p-Thioformamido-DL-phenylalanine was prepared by the action of sodium dithioformate on *p*-amino-DL-phenylalanine in aqueous solution (cf. Todd, Bergel, Karimullah, and Keller, *J.*, 1937, 361). The crystalline product, analysis of which indicated a monothioformamido-derivative, had no definite melting point. In order to ascertain which of the two amino-groups had been thioformylated we converted the thio-compound into the corresponding formamido-compound by refluxing aqueous formic acid. The product melted at 227—229° (decomp.), and was clearly different from *p*-amino-N^{α}-formyl-DL-phenylalanine, m. p. 168—169°, prepared by hydrogenation of *N*-formyl-*p*-nitro-DL-phenylalanine. The thioformamido-compound was therefore assigned structure (I*e*; R = R′ = R″ = H).

In our attempt to prepare *p-iso*thiocyanato-DL-phenylalanine, we again made use of the malonic acid synthesis. The reaction of α-*p*-aminobenzyl-α-formamidomalonic ester (II*b*;

R = H) with carbon disulphide and triethylamine in ethanol led to the expected triethylammonium dithiocarbamate (II*e*; R = H) (cf., *e.g.*, Dains, Brewster, and Olander, *Org. Synth.*, Coll. Vol. I, 1932, p. 437). A by-product of this reaction was the symmetrically disubstituted thiourea (III), m. p. 182—183°. Its structure was confirmed by the identity of the compound with the product obtained by condensing diethyl *p*-aminobenzylformamidomalonate (II*b*; R = H) with diethylformamido-*p*-*iso*-thiocyanatobenzylmalonate (II*f*; R = H; see below).



(III)

When the dithiocarbamate was allowed to react with ethyl chloroformate in dioxan solution at room temperature (cf. Andreasch, *Monatsh.*, 1906, **27**, 1211) diethyl formamido-*p*-*iso*thiocyanatobenzylmalonate (II*f*; R = H) was obtained directly, the presumed intermediate carbonic–dithiocarbamic mixed anhydride decomposing spontaneously under our conditions (cf. Johnson and Ticknor, *Proc. Nat. Acad. Sci.*, 1917, **3**, 303). Attempts to hydrolyse the *iso*thiocyanatomalonate to the *iso*thiocyanatophenylalanine have so far been unsuccessful.

The three optically isomeric *p*-di-(2-chloroethyl)phenylalanines, carrying the experimental numbers CB 3007, 3025, and 3026 respectively, have been submitted to tests on rats carrying the Walker carcinoma 256. Details will be published elsewhere by Professor A. Haddow to whom thanks are due for his interest in this work and for his permission to refer to his biological results. Under his experimental conditions the L-isomer has shown the greatest effect, 1 mg./kg. producing complete inhibition of the tumours. This is in conformity with the results of Stock *et al.* (*loc. cit.*) who found that of the three azaserines the L-isomer showed maximum activity against mouse sarcoma 180.

Experimental

p-*Nitro*-DL-, -L-, *and* -D-*phenylalanine* (I*a*; R = R′ = R″ = H).—(*a*) Erlenmeyer and Lipp's method (*loc. cit.*) was employed several times for the DL-compound but the yields were lower than claimed by them and were in general about 80%.

(*b*) A satisfactory simplification of method (*a*) avoided the use of lead carbonate and hydrogen sulphide. In one experiment DL-phenylalanine (12·5 g.) was nitrated in the usual way and the viscous mixture poured into cold water (500 ml.) with stirring. A slight excess of diluted aqueous ammonia was added and the solution evaporated until solid began to appear. The cooled mixture gave *p*-nitro-DL-phenylalanine in 90% yield, the first crop having m. p. 240—243° (decomp.) and the second m. p. 235—240° (decomp.) after recrystallisation from water.

L-Phenylalanine {3·9 g.; $[\alpha]_D^{25}$ −33·5° ± 0·5° (*c*, 1·94 in H_2O)} was nitrated and the product (84%) isolated in the same way. Recrystallisation from water gave colourless needles of p-*nitro*-L-*phenylalanine monohydrate*, m. p. 238—241° (decomp.; softening at 230°), $[\alpha]_D^{25}$ +9·8° ± 0·3° (*c*, 1·77 in N-HCl) (Found: C, 47·3; H, 5·3; N, 12·8. $C_9H_{10}O_4N_2,H_2O$ requires C, 47·4; H, 5·3; N, 12·3%).

Similarly, D-phenylalanine {2·95 g.; $[\alpha]_D^{22}$ +31·5° ± 0·5° (*c*, 1·18 in H_2O); prepared by resolution of DL-phenylalanine by Fischer and Schoeller's method (*Annalen*, 1907, **357**, 4, 7)} was nitrated in 88% yield. Recrystallisation from water (charcoal) gave almost colourless needles of p-*nitro*-D-*phenylalanine monohydrate*, m. p. 238—240° (decomp.), $[\alpha]_D^{20}$ −8·9° ± 0·3° (*c*, 2·41 in N-HCl) (Found: C, 47·2; H, 5·4; N, 12·4%).

(*c*) An ion-exchange resin was employed in another method. The acid mixture from the nitration of DL-phenylalanine (11·05 g.) was poured into water (800 ml.), and the solution added to a mechanically stirred slurry of Amberlite IR-4B resin (wet wt. 300 g.) and water (500 ml.). Stirring was continued until the pH had risen to about 5 (approx. 15 min.). Evaporation of the combined filtrate and washings gave *p*-nitro-DL-phenylalanine in 80% yield.

Oxidation of Nitro-DL-*phenylalanine*.—The nitration product (0·485 g.), m. p. 240—243° (decomp.), prepared as in (*a*) above, was heated with concentrated nitric acid (4·5 ml.) and water (10·5 ml.) for 4 hr. at 180°. The tube was cooled in an acetone–solid carbon dioxide bath before being opened. Yellow plates of *p*-nitrobenzoic acid, m. p. 240—243°, were obtained in 83% yield.

p-*Nitro*-N-*phthaloyl*-DL-*phenylalanine* [I*a*; R = H, R′R″ = *o*-$C_6H_4(CO)_2$].—(*a*) In one experiment, *p*-nitro-DL-phenylalanine (10·0 g.) was refluxed with phthalic anhydride (7·05 g., 1·00 mol.) in dry pyridine (75 ml.) for 1·5 hr. (cf. King and Kidd, *loc. cit.*). The solution was concentrated to small bulk (vacuum; steam-bath), and the residue refluxed for 5 min. with acetic anhydride (25 ml.). The cooled liquid yielded a sticky solid when poured into water (250

ml.), and further precipitation occurred when the mixture was acidified to Congo-red with hydrochloric acid. The washed product (96%) became granular when rubbed. Two successive crystallisations from chloroform–light petroleum yielded the N-*phthaloyl*-DL-compound as colourless needles, m. p. 180—181° (Found: C, 59·6; H, 3·8; N, 8·2. $C_{17}H_{12}O_6N_2$ requires C, 60·0; H, 3·55; N, 8·2%).

(*b*) *p*-Nitro-DL-phenylalanine (2·10 g.) and powdered phthalic anhydride (1·48 g., 1·00 mol.) were mixed and heated in a Pyrex tube at 170—190° for 15 min. (cf. Billman and Harting, *loc. cit.*). The brown mass crystallised from aqueous ethanol (charcoal) in colourless needles (86%), m. p. 177—178° rising to 179—180° when recrystallised from chloroform–light petroleum; the product had a mixed m. p. of 180—181° with the analytical specimen of *p*-nitro-*N*-phthaloyl-DL-phenylalanine described under (*a*).

Ethyl Esters of p-*Nitro*-N-*phthaloyl*-DL-, -L-, *and* -D-*phenylalanine* [Ia; R = Et, R'R'' = o-$C_6H_4(CO)_2$].—(*a*) Crude *p*-nitro-*N*-phthaloyl-DL-phenylalanine [16·7 g.; prepared as in (*a*) above] was esterified by refluxing it for 1·5 hr. in saturated ethanolic hydrogen chloride. The product, crystallised from *n*-propanol, had m. p. 91—93°. Two successive crystallisations from propanol (charcoal) gave colourless prisms of the DL-*ester*, m. p. 96·5—97° (Found: C, 61·7; H, 4·4; N, 7·6. $C_{19}H_{16}O_6N_2$ requires C, 61·95; H, 4·4; N, 7·6%).

(*b*) (i) p-*Nitro*-DL-, -L-, *and* -D-*phenylalanine ethyl ester hydrochlorides*. The DL-ester hydrochloride was prepared in 82% yield by refluxing a solution of the nitro-amino-acid in 3·5N-ethanolic hydrogen chloride, evaporating the product, and recrystallising the residue from acetone–methanol. Recrystallisation gave almost colourless needles of the hydrochloride, m. p. 185—186° (Found: C, 48·6; H, 5·8; N, 10·0. Calc. for $C_{11}H_{15}O_4N_2Cl$: C, 48·1; H, 5·5; N, 10·2%). Dornow and Winter (*Chem. Ber.*, 1951, **84**, 307) prepared the same compound by hydrolysis and decarboxylation of diethyl formamido-*p*-nitrobenzylmalonate and subsequent esterification. They recorded m. p. 179—180°. The optical isomers were prepared similarly. From *p*-nitro-L-phenylalanine (2·50 g.) was obtained p-*nitro*-L-*phenylalanine ethyl ester hydrochloride* (83%) in colourless rods (from dioxan–ethyl acetate), m. p. 207—208° (decomp.), $[\alpha]_D^{25}$ +12·7° ± 0·3° (*c*, 2·24 in H_2O) (Found: C, 47·95; H, 5·55; N, 10·3. $C_{11}H_{15}O_4N_2Cl$ requires C, 48·1; H, 5·5; N, 10·2%). Esterification of the D-isomer (3·0 g.) gave, in comparable yield, colourless rods (from dioxan–ethyl acetate) of p-*nitro*-D-*phenylalanine ethyl ester hydrochloride*, m. p. 204—205°, $[\alpha]_D^{20}$ −12·0° ± 0·3° (*c*, 2·06 in H_2O) (Found: C, 48·2; H, 5·7; N, 10·1%).

(ii) *Ethyl esters of* N-*o*'-*carboxybenzoyl*-p-*nitro*-DL-, -L-, *and* -D-*phenylalanine* (Ia; R = Et, R' = H; R'' = o-CO·C_6H_4·CO_2H) (cf. King and Kidd, *loc. cit.*). *p*-Nitro-DL-phenylalanine ethyl ester hydrochloride (1·00 g.) was suspended in dry benzene (8 ml.), and diethylamine (0·27 g., 1·00 mol.) was added. The mixture was shaken until all the ester hydrochloride crystals had dissolved. Dry ether was added to the turbid liquid until precipitation was complete, the diethylamine hydrochloride filtered off, and the filtrate evaporated almost to dryness under vacuum. The oily residue was taken up in dry benzene (15 ml.) and powdered phthalic anhydride (0·54 g., 1·00 mol.) added with shaking. In less than a minute, the mixture suddenly thickened to a white pasty mass and its temperature rose. The mixture was evaporated under vacuum. The residue crystallised from acetone–light petroleum in colourless crystals of N-*o*'-*carboxybenzoyl*-p-*nitro*-DL-*phenylalanine ethyl ester* (93%), m. p. 181° (Found: C, 59·1; H, 4·9; N, 7·4. $C_{19}H_{18}O_7N_2$ requires C, 59·1; H, 4·7; N, 7·25%).

Ethyl *p*-nitro-L-phenylalaninate hydrochloride (1·90 g.) gave, after similar treatment, a 94% yield of colourless needles (from acetone–light petroleum) of the L-*carboxybenzoyl* compound m. p. 180—182°, $[\alpha]_D^{23}$ +19·4° ± 0·3° (*c*, 2·94 in dioxan) (Found: C, 59·1; H, 4·7; N, 7·3%).

From *p*-nitro-D-phenylalanine ethyl ester hydrochloride (2·15 g.) were obtained colourless needles (93%) of N-*o*'-*carboxybenzoyl*-p-*nitro*-D-*phenylalanine ethyl ester*, m. p. 180—181°, $[\alpha]_D^{21}$ −19·7° ± 0·3° (*c*, 3·91 in dioxan) (Found: C, 58·9; H, 4·9; N, 7·2%).

(iii) *Ring closure of the* DL-, L-, *and* D-*carboxybenzoyl compounds*. The DL-compound (1·10 g.) was refluxed for 1·5 hr. with ethanolic 3·5N-hydrogen chloride (25 ml.). Removal of the solvent gave a pale yellow gum (1·08 g.) which crystallised from *n*-propanol in colourless prisms of *p*-nitro-*N*-phthaloyl-DL-phenylalanine ethyl ester (86%), m. p. 96·5—97·5°, undepressed on admixture with the compound of m. p. 96·5—97° prepared as under (*a*) above.

The corresponding L-*isomer* was prepared similarly from the L-carboxybenzoyl compound (1·85 g.), except that ethanolic 2N-hydrogen chloride was used. The product crystallised readily in 85% yield from amyl alcohol in colourless prisms, m. p. 80—81°, $[\alpha]_D^{23}$ −207° ± 1° (*c*, 1·15 in dioxan) (Found: C, 61·7; H, 4·5; N, 7·5%).

In the same way, the ethyl ester of *N*-*o*'-carboxybenzoyl-*p*-nitro-D-phenylalanine (2·18 g.) yielded the corresponding D-*isomer* (88%), m. p. 78—80° (from amyl alcohol). This recrystal-

lised from methanol in colourless prisms, m. p. 79—80°, $[\alpha]_D^{20}$ +207° ± 1° (*c*, 1·72 in dioxan). After a further recrystallisation from amyl alcohol, the m. p. and specific rotation of the product were unchanged (Found: C, 61·4; H, 4·7; N, 7·8%).

p-*Amino*-N$^{\alpha}$-*phthaloyl*-DL-, -L-, *and* -D-*phenylalanine Ethyl Ester Hydrochlorides* [*Ib*; R = Et; R′R″ = *o*-$C_6H_4(CO)_2$].—The DL-nitro-compound (9·0 g.) was hydrogenated at atmospheric pressure in ethyl acetate–methanol over palladium–calcium carbonate. The orange gum obtained on evaporation of the filtrate was converted into the hydrochloride (93%) in ether. Two successive crystallisations from ethyl acetate–acetone gave slightly tinted needles of the DL-*amine hydrochloride*, m. p. 205—207° (decomp.) (Found: N, 7·4; Cl, 9·3. $C_{19}H_{19}O_4N_2Cl$ requires N, 7·5; Cl, 9·5%).

The corresponding L-nitro-compound (1·30 g.) was converted into the amine salt (96%) in the same way, and was recrystallised from dioxan–ethyl acetate, giving colourless needles of the L-*amine hydrochloride*, m. p. 234—235° (decomp.; slight softening at 215°), $[\alpha]_D^{22}$ −153° ± 1° [*c*, 0·945 in 3 : 1 (by vol.) H_2O–EtOH] (Found: N, 7·3%).

The D-*amine hydrochloride* was prepared in an analogous manner in 88% yield as needles (from dioxan–ethyl acetate), m. p. 233—234° (decomp.), $[\alpha]_D^{22}$ +157° ± 1° [*c*, 1·01 in 3 : 1 (by vol.) H_2O–EtOH] (Found: N, 7·1%).

Isolation of the free DL-*base*. Excess of dilute aqueous ammonia was added to a warm, opalescent, filtered aqueous solution of the DL-amine hydrochloride (1·83 g.). The precipitated gum was washed with water and crystallised from aqueous methanol, giving large pale yellow needles, m. p. 109—111°. Recrystallisation yielded faintly tinted needles of p-*amino*-N$^{\alpha}$-*phthaloyl*-DL-*phenylalanine ethyl ester*, m. p. 110—112° (Found: N, 8·3. $C_{19}H_{18}O_4N_2$ requires N, 8·3%).

p-*Di*-(2-*chloroethylamino*)-DL-, -L-, *and* -D-*phenylalanine* (*Id*; R = R′ = R″ = H) (cf. Everett *et al.*, *loc. cit.*).—(*a*) *p*-Amino-*N*-phthaloyl-DL-phenylalanine ethyl ester (1·00 g.) was suspended in water (12 ml.), and glacial acetic acid was added with stirring until dissolution was complete (about 8 ml. of acid were required). Ethylene oxide (2 ml.) was added with shaking and the mixture kept for 24 hr. at room temperature. The clear yellow solution was poured into water (100 ml.), a slight excess of sodium hydrogen carbonate carefully added with stirring, the gummy precipitate extracted with ethyl acetate, and the dried ($MgSO_4$) solution evaporated to dryness on a steam-bath. The clear yellow gummy *p*-di-(2-hydroxyethyl)-amino-N$^{\alpha}$-phthaloyl-DL-phenylalanine ethyl ester did not crystallise.

The DL-di(hydroxyethyl)amino-compound (6·0 g.), prepared as above, was dissolved in benzene (100 ml.), and the solution was dried by distilling off part (25 ml.) of the solvent. Freshly distilled phosphorus oxychloride (15 ml.) was added and the mixture refluxed for 25 min. The solution was evaporated to dryness under vacuum and the clear gummy residue refluxed with concentrated hydrochloric acid for 6 hr. The pink solution was filtered from the red-tinted phthalic acid (whose identity was checked through the toluidide, m. p. and mixed m. p. 199—201°) and evaporated to small bulk under vacuum. Addition of concentrated sodium acetate solution precipitated a white granular solid which crystallised from methanol in tiny colourless needles of p-*di*-(2-*chloroethyl*)*amino*-DL-*phenylalanine*, m. p. 180—181° (46%, calculated on the free amino-compound) (Found: C, 51·1; H, 6·0; N, 8·9; Cl, 23·2. $C_{13}H_{18}O_2N_2Cl_2$ requires C, 51·1; H, 5·9; N, 8·5; Cl, 23·3%). The L-*isomer* was obtained similarly, starting from the unpurified free base obtained by ammonia treatment of *p*-amino-N$^{\alpha}$-phthaloyl-L-phenylalanine ethyl ester hydrochloride (1·06 g.). The chlorination product was refluxed for 3·5 hr. with 6N-hydrochloric acid. Precipitation with sodium acetate and crystallisation from methanol gave small colourless needles of monosolvated amine (37·5%, calc. on the amine hydrochloride), m. p. 182—183° (decomp.), $[\alpha]_D^{22}$ +7·5° ± 0·5° (*c*, 1·33 in N-HCl), $[\alpha]_D^{22}$ −31·5° ± 0·5° (*c*, 0·67 in MeOH) (Found: N, 8·3; Cl, 21·1. $C_{13}H_8O_2N_2Cl_2$,MeOH requires N, 8·3; Cl, 21·3%).

In the same way, the D-hydrochloride (1·15 g.) yielded colourless needles of monosolvated D-*base* (34%), m. p. 181·5—182° (decomp.), $[\alpha]_D^{21}$ −7·5° ± 0·5° (*c*, 1·26 in N-HCl) (Found: N, 8·6; Cl, 21·2%).

(*b*) Thionyl chloride (2 ml.) was added to a solution of diethyl α-acetamido-α-*p*-di-(2-hydroxyethyl)aminobenzylmalonate (1·38 g.) (see below) in dry alcohol-free chloroform (25 ml.). The clear solution was refluxed for 10 min. by which time it had begun to darken appreciably. It was evaporated under vacuum, and the residual gum refluxed with concentrated hydrochloric acid (20 ml.) for 3 hr. The method of isolation was as described above, and crystallisation of the product from methanol gave the di(chloroethyl)amine, m. p. 179—180° (decomp.), undepressed on admixture with material prepared from DL-phenylalanine, in 63% yield.

The corresponding hydroxyethylated formamido-compound (1·0 g.) was chlorinated as

described in (*a*). In this case, however, there was no tendency to darken during the thionyl chloride treatment. Evaporation of the chloroform solution yielded a pale yellow granular solid which was refluxed with hydrochloric acid for 40 min. only. The chloro-compound (74%), m. p. 182° (decomp.), had a mixed m. p. of 180° (decomp.) with the material of m. p. 179—180° obtained as under (*a*).

Diethyl α-Formamido-α-p-nitrobenzylmalonate (IIa; R = H)*.—Condensation of *p*-nitrobenzyl chloride (9·75 g.) and diethyl formamidomalonate (13·0 g., 1·12 mol.) was carried out as described by Dornow and Winter (*Chem. Ber.*, 1951, **84**, 307) except that the mixture was heated under reflux for 2 hr. The residue obtained on filtration of the hot reaction mixture crystallised from aqueous ethanol in colourless rods (13·6 g.) of *diethyl formamido-p-nitrobenzylmalonate*, m. p. 190°. The filtrate from the reaction mixture deposited the same compound (0·83 g., total yield, 75%), m. p. 189—190° (Found: C, 53·6; H, 5·5; N, 8·3. $C_{15}H_{18}O_7N_2$ requires C, 53·25; H, 5·4; N, 8·3%).

Diethyl α-p-Aminobenzyl-α-formamidomalonate (IIb; R = H).—Diethyl formamido-*p*-nitrobenzylmalonate (4·0 g.) was hydrogenated over palladium–calcium carbonate in suspension in ethyl acetate–methanol. The nitro-compound went into solution as reduction proceeded and the p-*aminobenzyl-formamidomalonate* crystallised in colourless needles (94%), m. p. 131—132° from ethyl acetate–light petroleum (Found: C, 58·3; H, 6·6; N, 9·2. $C_{15}H_{20}O_5N_2$ requires C, 58·4; H, 6·5; N, 9·1%).

Diethyl α-Acetamido- and α-Formamido-p-di-(2-hydroxyethyl)aminobenzylmalonate (IIc; R = Me or H).—Diethyl acetamido-*p*-aminobenzylmalonate (Elliott and Harington, *J.*, 1949, 1374) (3·84 g.) was hydroxyethylated with ethylene oxide (6 ml.) in 25% aqueous acetic acid (40 ml.) at room temperature (17 hr.). The product was worked up in the usual way. Crystallisation from ethyl acetate gave colourless needles of *diethyl acetamido-p-di-(2-hydroxyethyl)-aminobenzylmalonate* (82%), m. p. 100° (Found: C, 58·3; H, 7·5; N, 6·7. $C_{20}H_{30}O_7N_2$ requires C, 58·5; H, 7·4; N, 6·8%).

Hydroxyethylation of the formamido-compound (2·80 g.) led similarly to *diethyl α-p-di-(2-hydroxyethyl)aminobenzyl-α-formamidomalonate* (83%), m. p. 118°, rising to 120·5° on recrystallisation from ethyl acetate–light petroleum (Found: C, 57·4; H, 7·3; N, 7·1. $C_{19}H_{28}O_7N_2$ requires C, 57·6; H, 7·1; N, 7·1%).

Racemisation Experiments.—(*a*) *p*-Di-(2-chloroethyl)amino-D-phenylalanine (33 mg.) in 6N-hydrochloric acid (1·5 ml.) had $[\alpha]_D^{22}$ −4·75° ± 0·25° (*c*, 2·2). The solution was heated in a sealed tube for 3·5 hr. in refluxing 6N-hydrochloric acid, after which $[\alpha]_D^{22}$ was −4·5° ± 0·25°.

(*b*) L-Phenylalanine {0·600 g.; $[\alpha]_D^{21}$ −6·9° ± 0·3° (*c*, 1·68 in N-HCl)} was converted into the ester hydrochloride by refluxing ethanolic 2N-hydrogen chloride for 2 hr. The free ester, obtained from the hydrochloride (0·500 g.; acetone-crystallised) by diethylamine treatment, was allowed to react in benzene solution with phthalic anhydride (0·33 g., 1·00 mol.). After 2 days, the clear solution was evaporated, and the gummy residue taken up in ether. Crystallisation set in on cooling, and recrystallisation of the product from acetone–light petroleum gave colourless needles of N-o′-*carboxybenzoyl-L-phenylalanine ethyl ester*, m. p. 119—120°, $[\alpha]_D^{20}$ +31·0° ± 0·5° (*c*, 1·56 in dioxan) (Found: C, 66·8; H, 5·5; N, 4·3. $C_{19}H_{19}O_5N$ requires C, 66·85; H, 5·6; N, 4·1%).

The carboxybenzoyl compound (0·50 g.) was refluxed with ethanolic 2N-hydrogen chloride for 1·75 hr. The gummy residue obtained by evaporation of the solution was refluxed with 6N-hydrochloric acid (10 ml.) for 2·5 hr. A considerable quantity of oil was present at this stage. The mixture was evaporated to dryness under vacuum, concentrated hydrochloric acid (7 ml.) was added to the residue, and refluxing continued for a further hour. Much oil remained at the end of this period. The mixture was cooled and kept overnight in the ice box. Water (10 ml.) was added and the mixture shaken to take up any phenylalanine hydrochloride which may have crystallised. The filtrate was evaporated to dryness (vacuum), and the residue taken up in a little water and filtered. The crystalline residue from the evaporated filtrate was recrystallised from acetone–methanol and gave colourless rhombic crystals of L-phenylalanine hydrochloride (0·025 g.), $[\alpha]_D^{21}$ −7·0° ± 0·3° (*c*, 1·37, calc. as free amino-acid, in N-HCl).

Hydrolysis in 1 : 1 Acetone–Water at 66° (cf. Ross, *J.*, 1949, 183).—*p*-Di-(2-chloroethyl)-phenylalanine (0·076 g., 0·25 mmole) was refluxed in " AnalaR " acetone (12·5 ml.)–water (12·5 ml.) for 30 min. The cooled solution was titrated electrometrically (Pye pH meter, model 605) against 0·1N-sodium hydroxide. An unhydrolysed solution in the same medium was titrated similarly. The shift in the titration curve corresponded to 21% hydrolysis.

* [*Added in Proof.*]—This compound has recently been characterised by Schlögl, Wessely, and Korger (*Monatsh.*, 1952, **83**, 845).

Liberation of chloride ion was determined as follows. A hydrolysate obtained simultaneously with and exactly as the above was cooled and set aside for 0·5 hr. (during the titration of the other solution). 0·1N-Silver nitrate (5·0 ml.) and a few drops of 2N-nitric acid were added. The mixture was shaken for 1—2 min., the silver chloride was filtered off, and the filtrate and washings were titrated against 0·1N-potassium thiocyanate in presence of ferric ammonium sulphate. This indicated 22% hydrolysis.

p-*Thioformamido*-DL-*phenylalanine* (I*e*; R = R′ = R″ = H).—A filtered solution of sodium dithioformate (0·35 g.) in water (2 ml.) was added to a solution of *p*-amino-DL-phenylalanine (0·180 g.) in water (10 ml.). After 20 min. crystals began to separate from the yellow solution. The mixture was kept at room temperature for 2 hr. and was then placed in the ice-box for a further 2 hr. The product (81%) recrystallised from water as pale yellow rods of the monohydrated *thioformamido*-compound (indefinite decomp. temp.; began to char at 210°) (Found: C, 49·6; H, 5·9; N, 11·5; S, 13·4. $C_{10}H_{12}O_2N_2S,H_2O$ requires C, 49·6; H, 5·8; N, 11·6; S, 13·2%).

p-*Formamido*-DL-*phenylalanine*.—The thioformamido-derivative (0·155 g.) was refluxed in 98% formic acid (3 ml.) and water (6 ml.) until evolution of hydrogen sulphide ceased (about 1·5 hr.). The solution was evaporated to dryness under reduced pressure. A little water was added to the colourless residue, and the solution again evaporated to dryness to remove traces of formic acid. The residue was twice crystallised from aqueous ethanol, yielding very small needles of p-*formamido*-DL-*phenylalanine* (75%), m. p. 227—229° (decomp.) (Found: C, 57·3; H, 6·0; N, 13·2. $C_{10}H_{12}O_3N_2$ requires C, 57·7; H, 5·8; N, 13·5%).

N-*Formyl*-p-*nitro*-DL-*phenylalanine* (cf. Waley, *Chem. and Ind.*, 1953, 107).—*p*-Nitro-DL-phenylalanine (1·90 g.) was dissolved in 98% formic acid (15 ml.), and acetic anhydride (5 ml.) was added. The solution was kept at 45—55° for 30 min., then water (50 ml.) was added slowly with stirring and warming. Crystallisation began on cooling. The mixture was left in the ice-box overnight and yielded pale yellow prisms (75%) of the N-*formyl* compound, m. p. 185—186°, raised to 187° on recrystallisation from water (Found: C, 50·3; H, 4·2; N, 11·5. $C_{10}H_{10}O_5N_2$ requires C, 50·4; H, 4·2; N, 11·8%).

p-*Amino*-N-*formyl*-DL-*phenylalanine*.—The *p*-nitro-compound (0·60 g.) was hydrogenated at room temperature and pressure in water (30 ml.) over a 5% palladium–charcoal catalyst. Reduction was complete in about an hour. Evaporation of the filtrate gave a gum which crystallised from ethanol and in a 77% yield of the reduction product. Recrystallisation from ethanol (charcoal) gave colourless prisms of p-*amino*-N-*formyl*-DL-*phenylalanine*, m. p. 168—169° (decomp.) (Found: C, 57·4; H, 6·1; N, 13·2. $C_{10}H_{12}O_3N_2$ requires C, 57·7; H, 5·8; N, 13·5%).

Reduction of the nitro-compound over palladium–calcium carbonate gave a granular product which was twice precipitated from its aqueous solution by addition of ethanol. Analysis of the compound, which gave an ash on ignition, showed it to be the *calcium salt* of *p*-amino-N-formyl-DL-phenylalanine, m. p. 316—318° (decomp.) (Found: N, 12·3. $C_{20}H_{22}O_6N_4Ca$ requires N, 12·3%).

Reaction of Diethyl p-*Aminobenzylformamidomalonate with Carbon Disulphide and Triethylamine*.—A sealed tube containing the malonate (6·0 g.), carbon disulphide (2·0 ml., 2·0 mol.), and triethylamine (4·0 ml., 2·9 mol.) in ethanol (40 ml.) was heated in a water bath at 70° for 10 min. and then allowed to cool slowly (30 min.) to room temperature. The filtered solution was left in the ice-box overnight. The pale yellow crystalline product (6·60 g.), m. p. 114—115° (decomp.), was recrystallised from *n*-propanol and gave yellow prisms (2·63 g.) of *triethylammonium* N-p-(*di*-2-*ethoxycarbonyl*-2-*formamidoethyl*)*phenyldithiocarbamate* (II*e*; R = H), m. p. 106° (decomp.), unchanged on recrystallisation from propanol. The fall in m. p. on the first recrystallisation was noticed also in a second experiment (Found: C, 54·4; H, 6·9; N, 8·4; S, 13·1. $C_{22}H_{35}O_5N_3S_2$ requires C, 54·4; H, 7·3; N, 8·7; S, 13·2%).

The ethanolic mother-liquor from the crude dithiocarbamate was evaporated to smaller bulk and yielded needles, m. p. 179—181°, of NN′-*bis*-[p-(*di*-2-*ethoxycarbonyl*-2-*formamidoethyl*)-*phenyl*]*thiourea* (III) (1·40 g.). Recrystallisation from ethanol rendered the product colourless and raised the m. p. to 182—183° (Found: C, 56·8; H, 6·0; N, 8·4; S, 4·9. $C_{31}H_{38}O_{10}N_4S$ requires C, 56·5; H, 5·8; N, 8·5; S, 4·9%).

Reaction between Diethyl p-*Aminobenzylformamidomalonate and Diethyl Formamido*-p-iso*thiocyanatobenzylmalonate*.—The amine (31 mg.) and the *iso*thiocyanato-compound (see below) (35 mg., 1·0 mol.) were refluxed for 1 hr. in ethanol (1·5 ml.). The cooled solution deposited colourless needles (35 mg.), m. p. 181·5—182·5°, undepressed on admixture with the disubstituted thiourea (III).

Diethyl Formamido-p-iso*thiocyanatobenzylmalonate* (II*f*; R = H).—The above triethyl-

ammonium dithiocarbamate (0·88 g.), dissolved in dry dioxan (7 ml.), was cooled somewhat in ice-water. Ethyl chloroformate (0·215 g.; 0·19 ml.; 1·10 mol.) was added with shaking, the mixture was kept at room temperature for 30 min., then filtered from the crystalline triethylamine hydrochloride, and the filtrate was evaporated under reduced pressure. Recrystallisation of the colourless crystalline residue from pentanol gave prisms of the iso*thiocyanato*-compound, m. p. 142° (73%) (Found: C, 55·1; H, 5·2; N, 7·9; S, 8·7. $C_{16}H_{18}O_5N_2S$ requires C, 54·85; H, 5·2; N, 8·0; S, 9·0%).

N-p-(*Di*-2-*ethoxycarbonyl*-2-*formamidoethyl*)*phenyl*-N′-*phenylthiourea*.—The above *iso*thiocyanate (63 mg.) and aniline (19 mg., 1·1 mol.) were warmed over a small flame for about a minute. The mixture solidified to a white mass on cooling slightly. Crystallisation from ethanol gave colourless needles of the *thiourea* derivative, m. p. 174° (Found: C, 59·4; H, 5·8; S, 7·0. $C_{22}H_{25}O_5N_3S$ requires C, 59·6; H, 5·7; S, 7·2%).

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