β-(3-Bromo-4-hydroxy-5-methoxyphenyl)alanine (RIT 1412), an Anti-hypertensive Agent

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β-(3-Bromo-4-hydroxy-5-methoxyphenyl)alanine (I) exerts a long-acting antihypertensive effect in rats and dogs.[1] This unnatural amino-acid was synthesized by two different methods, *i.e.*, *via* the azlactone (VI) and the corresponding phenylpyruvic acid (VIII), and also *via* the acetamidomalonic ester (V). Some derivatives of (I) [(IX)—(XII)], and the optical resolution of (I) into its enantiomers, are described. The results of the determination of the configuration of (−)-(I) and (+)-(I) by the enzymic path are in agreement with those obtained by the application of the Lutz–Jirgensons rule.

THE interest in unnatural phenylalanine derivatives has been enhanced this last decade by the discovery of α-methyldopa, a dopa decarboxylase inhibitor [2] with antihypertensive properties.[3] This amino-acid is catabolised *in vivo* into a 'false transmitter' which is supposed to take the place of norepinephrine in the adrenergic nerve terminals. Other unnatural amino-acids have been tested for antiviral and antineoplastic activity,[4] inhibition of bacterial growth,[5] and for their action on the thyroid gland [6] and on the central nervous system.

The purpose of our study was to determine the influence of a halogen substitution *ortho* to the hydroxy-group in 4-hydroxy-3-methoxyphenylalanine in a screening for antihypertensive compounds.

Furthermore, if α-methyl-norepinephrine, a catabolite of α-methyl-dopa, cannot be attacked by monoamine oxidase because of the presence in the molecule of a methyl α to the amino-group,[7] RIT 1412 (I) and its metabolites are left unmodified in the presence of catechol *O*-methyl transferase, which is the second enzyme responsible for the destruction of the hypertensive catecholamines.

As shown in the Scheme, the amino-acid (I) was obtained by two different routes. The common starting material is 5-bromovanillin (II), obtained in quantitative yield by bromination of vanillin in acetic acid.[8] Sodium borohydride reduction of (II) led to vanillyl alcohol (III) which, in a benzene solution saturated with hydrogen chloride, is converted into (IV). This benzyl chloride can then be condensed with acetamidomalonic esters in the usual way (using sodium hydride or sodium ethoxide as condensing agent and dimethylformamide or anhydrous ethanol as solvent).

The best way of hydrolysing (V) to give (Ia) is to use concentrated hydrochloric acid in acetic acid. This system leaves the methoxy-group and the bromine atom unattacked on the benzene ring. Another way of obtaining (I) consists in the reduction of an ammonium hydroxide solution of the phenylpyruvic acid derivative (VIII).[9] We obtained this α-keto-acid, in one or two steps, from the corresponding azlactone (VI). The overall yield amounts to 56·1% *via* the malonic derivative compared to 42% *via* the azlactone. Nevertheless, this second route is easier, more rapid, and cheaper than the first one.

Several derivatives of (Ia), namely, the ethyl ester (IX), the free base (Ib), the *ON*-diacetyl derivative (X), the *N*-acetyl (XI), and *N*-palmitoyl (XII) derivatives, were synthesized to explore the degree of resorption of such compounds.

The purity of all our derivatives was more particularly assessed by potentiometric titration in non-aqueous medium. By this technique, we determined the hydrochloride of the amine hydrochloride moiety, using the mercuric acetate–acetic acid–perchloric acid method.

A benzene–methanol–tetrabutylammonium hydroxide solution is employed as a titrant for quantitative determination of the ammonium, carboxylic, and phenolic groups in phenylalanine derivatives.

Compound (I) can be separated into its enantiomers on Whatman chromatographic paper with n-butanol saturated with 3N-hydrochloric acid as a developing system. Such a resolution is not rare in the case of phenylalanine derivatives. For instance, (±)-2,3-dihydroxy-β-phenylalanine was resolved on No. 4 Whatman filter paper by Dalgliesh [10] using the n-butanol–acetic acid–water (4 : 1 : 5) system. A separation also occurred with (±)-2,5-dihydroxy- and with (±)-3,4-dihydroxy-2-methyl-β-phenylalanine but not with (±)-3,4-dihydroxy-5-methyl-β-phenylalanine.

If gram quantities of (+)-(Ia) and (−)-(Ia) are needed, (I) can be resolved by formation of diastereoisomers.

The diastereoisomeric salts formed by reaction of the *N*-acetyl derivative (XI) with L(−)-ephedrine are resolvable in acetone. A third method which gives good results is the enzymic resolution with L-amino-acid oxidase. When DL-(Ia) is treated with L-amino-acid oxidase in a buffered solution at 37°, a mixture of D-(Ia) and of the corresponding phenylpyruvic acid is obtained. A separation of these two compounds in acidic medium leaves pure D-(Ia).

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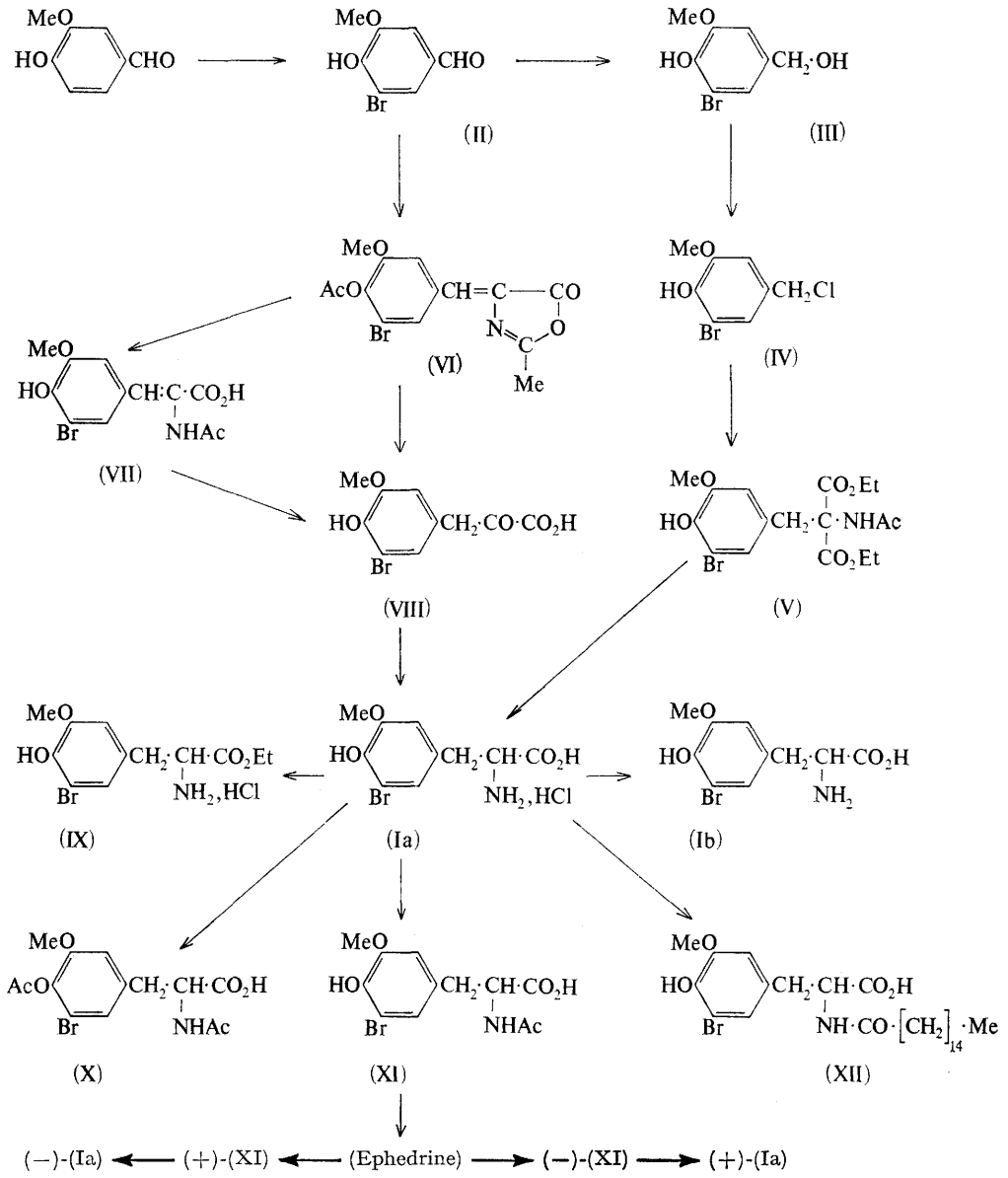
[8] (*a*) O. L. Brady and F. P. Dunn, *J. Chem. Soc.*, 1915, **107**, 1858; (*b*) R. A. McIvor and J. M. Pepper, *Canad. J. Chem.*, 1953, **31**, 298.
[9] ASAHI Chem. Ind. Co. Ltd., Jap.P. 6684/1963.
[10] C. E. Dalgliesh, *J. Chem. Soc.*, 1952, 3940.



The establishment of the configuration of the two enantiomers of (Ia) was done by two independent methods which gave concordant results. The results obtained by studying the variation of the rotatory power in neutral and acid medium (Lutz–Jirgensons rule [11]) were in agreement with those obtained by submitting the two enantiomers to the action of L- and D-amino-acid oxidase.

EXPERIMENTAL

The potentiometric determinations were carried out with a pH-meter Radiometer 22, using a combined glass calomel electrode.

5-Bromovanillin (II) *and 5-Bromovanillyl Alcohol* (III).—These two compounds were synthesized according to McIvor.[8b]

3-Bromo-4-hydroxy-5-methoxybenzyl Chloride (IV).—5-Bromovanillyl alcohol (21 g.) is suspended in benzene (150 ml.). A stream of hydrogen chloride is allowed to pass through the medium which is maintained at 7—8°. The stream of hydrogen chloride is maintained for a further 15 min. after complete dissolution of the reactant. The medium is dried over anhydrous sodium sulphate, filtered, and evaporated. The dry residue is treated twice with anhydrous benzene (150 ml.) which is evaporated to eliminate residual hydrogen chloride, then poured into hexane (1·5 l.) previously dried over calcium chloride. The precipitate is filtered off and dried to yield the desired *benzyl chloride* (21·17 g., 96%), m.p. 84—86°. Purity by titration (HCl), 98% (Found: C, 38·25; H, 3·3; Br, 31·7; Cl, 14·1. $C_8H_8BrClO_2$ requires C, 38·2; H, 3·2; Br, 31·8; Cl, 14·1%).

Diethyl (3-Bromo-4-hydroxy-5-methoxy)benzylacetamidomalonate (V).—Diethyl acetamidomalonate (6·95 g.) is suspended in anhydrous ethanol (12 ml.) and an ethanolic solution of sodium ethoxide (0·735 g. of sodium in 24 ml. of ethanol) is added with stirring. A yellowish solution is

[11] O. Lutz and Br. Jirgensons, *Ber.*, 1930, **63**, 448; 1931, **64**, 1221.

obtained which is stirred for 30 min. at 65°. The benzyl chloride obtained above (4 g.) is dissolved in anhydrous ethanol (15 ml.). A precipitate of sodium chloride immediately appears. The medium is refluxed for 3½ hr. and then evaporated to dryness. The residue is taken up with 500 ml. of water. After vigorous stirring, the white *precipitate* obtained is filtered off, washed with water, and dried to yield (V) (6·1 g., 89%), m.p. 161—162°, purity by phenol-group titration, 96·5% (Found: C, 47·1; H, 5·05; Br, 18·4; N, 3·1. $C_{17}H_{22}BrNO_7$ requires C, 47·6; H, 5·15; Br, 18·6; N, 3·25%).

β-(3-*Bromo*-4-*hydroxy*-5-*methoxyphenyl*)*alanine* HCl (Ia).—Diethyl α-(3-bromo-4-hydroxy-5-methoxybenzyl) α-acetamidomalonate (5 g.) is dissolved at 70° in a mixture of glacial acetic acid (12 ml.) and concentrated hydrochloric acid (20 ml.) and the solution is saturated with sulphur dioxide. After a 6 hr. period of heating at 95°, the solvent is evaporated and the residue is taken up with anhydrous ether. After filtration, the precipitate is dried under reduced pressure to yield DL-(Ia) (2·75 g., 73%), m.p. 220—223° (decomp.), purity by titration, 99·6% (Found: C, 36·95; H, 4·15; Br, 24·6; Cl, 10·9; N, 4·45. $C_{10}H_{13}BrClNO_4$ requires C, 36·8; H, 4·1; Br, 24·45; Cl, 10·85; N, 4·3%).

Only one spot, of R_F 0·33, can be observed by t.l.c. on silica gel. Solvent: $CHCl_3$–MeOH–NH_4OH 17% (20 : 20 : 5).

The ultraviolet spectrum of (Ia) was taken on a Beckman DB spectrophotometer at a concentration of $3·75 \times 10^{-4}$M, $\lambda_{max.}$ 284 mμ (ε 2319) with a shoulder at 280 mμ (ε 2282), $\lambda_{min.}$ 255 mμ (ε 440).

The n.m.r. spectrum was taken on a Varian A 60 at 60 Mc., with an internal standard of acetonitrile. The integrated n.m.r. spectrum was in good agreement with the proposed structure: δ (p.p.m.) 3·03—3·23t (2H), 2·82s (3H), 4·08—4·38t (1H), 6·77—6·9d (1H) (*J* 2 c./sec.), 6·95—7·06d (1H) (*J* 2 c./sec.).

4-(4-*Acetoxy*-3-*bromo*-5-*methoxybenzylidene*)-2-*methyl*-5-*oxazolone* (VI).—This product [12] was obtained in 73% yield, m.p. 208—209° (lit., 206—207°).

By t.l.c. on silica gel, one spot, of R_F 0·67, can be detected by the sulphuric acid–formaldehyde reagent. Solvent: benzene–dioxan–acetic acid (90 : 25 : 4).

α-*Acetamido*-3-*bromo*-4-*hydroxy*-5-*methoxycinnamic Acid* [12] (VII).—Yield, 92·5%, m.p. 208—209° (lit., 203—204°) (Found: C, 43·3; H, 4·1; Br, 24·3; N, 4·1. Calc. for $C_{12}H_{12}BrNO_5$: C, 43·7; H, 3·65; Br, 24·2; N, 4·25%). Purity by titration: CO_2H, 99·7; OH, 100%. Only one spot can be detected by ferric chloride in t.l.c. on silica gel, R_F 0·21, in benzene–methanol–acetic acid (45 : 8 : 4).

3-*Bromo*-4-*hydroxy*-5-*methoxyphenylpyruvic Acid* (VIII).—This acid [12] was obtained by the following methods.

(*a*) The above cinnamic acid (VII) (25 g.) is suspended in acetic acid (60 ml.) and hydrochloric acid (140 ml.) and heated for 6 hr. at the reflux temperature under vigorous stirring. The medium is then cooled, and poured under stirring into water (2 l.). The crystals obtained are filtered off, washed with water, and dried to yield 17·9 g. (82%) of (VIII), m.p. 238—240° (decomp.) (Found: C, 41·7; H, 3·3; Br, 27·7. Calc. for $C_{10}H_9BrO_5$: C, 41·55; H, 3·15; Br, 27·6%). Purity by titration: CO_2H, 98·7; OH, 97%. One spot, R_F 0·25, can be detected by ferric chloride in the solvent benzene–methanol–acetic acid (45 : 8 : 4).

(*b*) The azlactone (VI) (2·5 g.) is dissolved in a 25% solution of sodium hydroxide (25 ml.) and heated for 3½ hr. at reflux. After treatment with activated charcoal, the pH of the solution is brought to 1·5 with hydrochloric acid. The crystals obtained are filtered off, washed with water, and dried at 40° under a vacuum to yield 1·7 g. (83·4%) of (VIII), m.p. 236—237°.

3-*Bromo*-4-*hydroxy*-5-*methoxyphenylalanine Hydrochloride* (Ia).—The above phenylpyruvic acid (VIII) (10 g.) is dissolved in 28% ammonium hydroxide (100 ml.), and after 10 min. stirring, treated with water (10 ml.) containing sodium borohydride (0·35 g.). After 2 hr. stirring, the solution is concentrated under a vacuum. The residue is taken up twice with water and concentrated to dryness. The final residue is taken up with water and brought to pH 1·5 with concentrated hydrochloric acid. After 1 hr. of stirring, the acid solution is concentrated to dryness. The ethanolic solution of the residue is filtered. The filtrate is concentrated under a vacuum to a syrup which is precipitated in ether to yield 7·8 g. (69%) of (Ia), m.p. 218—220° (decomp.), giving a resolution in its enantiomers by paper chromatography on Whatman No. 1. R_F values of 0·645 and 0·827 were obtained in n-butanol saturated with 3N-HCl.

3-*Bromo*-4-*hydroxy*-5-*methoxyphenylalanine Ethyl Ester Hydrochloride* (IX).—A solution of DL-(Ia) (10 g.) in anhydrous ethanol (250 ml.) is saturated at 0° with hydrogen chloride. The solution is then heated for 1 hr. at 80°. The solvent is evaporated and the residue is taken up twice with anhydrous ethanol and precipitated by pouring the solution into anhydrous ether to yield (IX) (10 g., 92%), m.p. 194—197°, purity by titration (NH_2), 101·3% (Found: C, 40·75; H, 4·95; Br, 22·55; Cl, 10·0; N, 4·1. $C_{12}H_{17}BrClNO_4$ requires C, 40·6; H, 4·85; Br, 22·5; Cl, 10·0; N, 3·95%).

3-*Bromo*-4-*hydroxy*-5-*methoxyphenylalanine* (Ib).—(*a*) A solution of DL-3-bromo-4-hydroxy-5-methoxyphenylalanine hydrochloride (1 g.) in water (50 ml.) is batch treated by Amberlite LA-2 (2·5 ml.) dissolved in benzene (60 ml.). After stirring for 24 hr., the aqueous layer is washed twice with benzene and the organic layer is washed twice with water.

The aqueous portions are collected and the solvent is evaporated to yield (Ib) (0·8 g., 92%), m.p. 177°, purity by titration (NH_2), 96·4% (Found: C, 41·35; H, 4·3; Br, 27·45; N, 4·7. $C_{10}H_{12}BrNO_4$ requires C, 41·45; H, 4·15; Br, 27·55; N, 4·8%).

(*b*) DL-(Ia) (16·33 g.) dissolved in water (50 ml.) is treated with a solution of triethylamine in ethyl alcohol, until pH 5·1 is obtained. The solution is then filtered and the filtrate is evaporated to dryness. The residue is treated with two portions of ethanol which are evaporated.

The residue is triturated twice with chloroform, in order to eliminate the triethylamine hydrochloride, and then with acetone. The solution is filtered and the filtrate is evaporated to dryness.

The residue is crystallised from hot water to yield (Ib) (9·6 g., 66%), m.p. 177° (decomp.). Purity by titration (NH_2), 95·2%; water content (Karl Fischer), 2·4%.

DL-4-*Acetoxy*-3-*bromo*-5-*methoxy*-N-*acetylphenylalanine* (X).—(Ia) (1 g.) dissolved in N-sodium hydroxide (33·6 ml.) is treated under stirring with acetic anhydride (2·4 ml.). The reaction is exothermic. The medium is stirred at room temperature for 17 hr. and filtered. The pH of the

[12] L. C. Raiford and C. H. Buurman, *J. Org. Chem.*, 1943, **8**, 466.

filtrate is brought to 2·3 with hydrochloric acid and the solution is then evaporated to dryness. The residue is taken up with methylene chloride. After filtration, the solution is treated with charcoal, filtered, concentrated to small volume, and hexane is added to yield 1·05 g. (91·7%) of (X) which is filtered off and dried under reduced pressure, m.p. 165—167° (Found: C, 44·8; H, 4·2; Br, 21·5; N, 3·8. $C_{14}H_{16}BrNO_6$ requires C, 44·9; H, 4·3; Br, 21·4; N, 3·75%).

By t.l.c. this product is no longer detectable, either by ninhydrine or by $FeCl_3$. Bromocresol Purple must be used. R_F 0·45 ± 0·02 in propanol–water (75 : 25).

3-*Bromo*-4-*hydroxy*-5-*methoxy*-N-*palmitoylphenylalanine* (XII).—(Ia) (15 g.) dissolved in N-sodium hydroxide (100 ml.) is treated dropwise and under stirring with a solution of palmitoyl chloride (15 ml.) in freshly distilled tetrahydrofuran (30 ml.), the pH of the medium being adjusted between 6·5 and 7·5 with N-sodium hydroxide during the addition. When the addition is completed, the pH is brought to 8. The stirring is maintained during the next 5 hr., while the pH of the medium is slowly brought to 9 by addition of N-sodium hydroxide. The medium is then allowed to stand overnight.

The solution is concentrated under reduced pressure in the presence of a small amount of sodium hydrosulphite to 1/3 of its initial volume and extracted with two portions of ether, the pH being brought to 2 by addition of N-hydrochloric acid.

The organic layers are collected, treated with charcoal, filtered, dried over anhydrous sodium sulphate, and concentrated under reduced pressure. By addition of hexane, a precipitate is obtained which is filtered off on sintered glass and dried under reduced pressure at 40° to yield (XII) (14·5 g., 60%), m p. 104—107° (Found: C, 59·25; H, 7·9; Br, 15·2; N, 2·75. $C_{26}H_{42}BrNO_5$ requires C, 59·1; H, 8·05; Br, 15·1; N, 2·65%). Purity by titration of the phenolic group, 102·6%. This product gave no colouration with ninhydrin in t.l.c. (XII) was detected (one spot) by $FeCl_3$ and Bromocresol Purple.

3-*Bromo*-4-*hydroxy*-5-*methoxy*-N-*acetylphenylalanine* (XI).—A solution of (Ia) (50 g.) in N-sodium hydroxide (500 ml.) is treated successively with sodium hydrosulphite (0·5 g.) and by acetic anhydride until pH 7 is reached. The addition of acetic anhydride is then continued while maintaining the pH at about 8 with N-sodium hydroxide. After complete addition of the acetic anhydride (36·25 ml.), the pH is brought to 10·8 with N-sodium hydroxide (total amount, 602·5 ml.). The temperature is maintained around 35° during the whole reaction time. The medium is kept at room temperature for 4 hr. and then acidified with 6N-hydrochloric acid.

Crystallization of the *N*-acetylated compound begins at pH *ca.* 4 but acidification is continued to pH 1·4 and crystallization is allowed to take place at that pH for 15 hr. at 4°. The crystals are filtered off, washed with cold water, and dried to yield (XI) (47·25 g., 93·3%), m.p. 222—223° (Found: C, 43·6; H, 4·4; Br, 24·25; N, 4·4. $C_{12}H_{14}BrNO_5$ requires C, 43·4; H, 4·25; Br, 24·05; N, 4·2%). Purity by titration (CO_2H), 98·25%. In t.l.c., one spot, R_F 0·4 ± 0·02, is obtained in the ammonium hydroxide–methanol–chloroform (5 : 20 : 20) system. This spot was detected by $FeCl_3$ and by Bromocresol Purple.

Resolution of (Ia) *into its Enantiomers by Formation of Diastereoisomers with Ephedrine.* (−)-3-*Bromo*-4-*hydroxy*-5-*methoxy*-N-*acetylphenylalanine*.—DL-(XI) (45 g.) is added to an ethanolic solution (31·6 ml.) of 4·3M-L-(−)-ephedrine, diluted with acetone (174·5 ml.). The solution obtained is kept for 5 days at room temperature. The crystals are then filtered off, washed with acetone, dried, powdered, and taken up in acetone (400 ml.). The suspension is stirred for 1 hr. and then filtered. The crystals collected are dissolved under stirring in hot anhydrous ethanol (80 ml.). By slow addition of ether (360 ml.) under stirring and with cooling, a precipitate is obtained which is allowed to stand for 4 hr. at room temperature. The crystals are filtered off, washed with ether, and dried to yield 25·4 g. (75·4%) of the L-(−)-ephedrine salt of (−)-(XI), m.p. 128—131°, $[\alpha]_D$ −66° (*c* 1 in ethanol). The above salt (21·5 g.) is dissolved in ethanol–water (75 : 25) (1075 ml.) and poured onto a 5-cm. diameter column of Amberlite IR 124 (H^+), the flow rate being adjusted to 5 ml./min. Elution is performed with the same solvent mixture. The eluate is concentrated to a small volume which is taken up with ethanol, treated with charcoal, and filtered. The solvent is evaporated under reduced pressure at 40° to yield (−)-(XI) (13·2 g., 92%), $[\alpha]_D$ −40° (*c* 1 in ethanol). Purity by phenolic group titration, 93·4%.

(+)-3-*Bromo*-4-*hydroxy*-5-*methoxyphenylalanine Hydrochloride*.—A suspension of (−)-(XI) (11·5 g.) in 1·2 N-hydrochloric acid (230 ml.) is saturated with sulphur dioxide. A solution is obtained after 30 min. heating under stirring at 110° (bath temperature), and heating and stirring are maintained for a further 3½ hr. to reach complete hydrolysis.

After this reaction time, the solution is discoloured with charcoal and filtered. The solution is concentrated to a small volume, taken up with water, and evaporated to dryness by azeotropic distillation with ethanol. The residue is taken up with anhydrous ether. After filtration and drying under reduced pressure at 40°, (+)-(Ia) (10·1 g.) (90·3%) is obtained with a 98·5% titration purity (NH_2), m.p. 207—210°, $[\alpha]_D$ +10° (*c* 1 in water).

(+)-3-*Bromo*-4-*hydroxy*-5-*methoxy*-N-*acetylphenylalanine*.—(a) *Isolation of the partially resolved* (+)-(XI). After crystallization and filtration of the L(−)-ephedrine salt of (−)-(XI), the mother-liquor is concentrated under a vacuum to yield impure (+)-(XI). The L(−)-ephedrine salt, $[\alpha]_D$ +16·5° (*c* 1 in ethanol) (43 g.), is dissolved in ethanol–water (80 : 20) (2·151), passed through a column of Amberlite IR/124 (H^+ form), and eluted with the same solvent mixture.

The eluate (9 l.) is concentrated under a vacuum until disappearance of the ethanol. A white powder is then obtained, which is filtered off, washed with water, and dried under a vacuum to yield partially resolved (+)-(XI) (9·1 g., 31·7%), $[\alpha]_D$ +4° (*c* 1 in ethanol).

The mother-liquor is discoloured with charcoal, concentrated to dryness, taken up with ethanol, again concentrated to dryness, and dried under a vacuum to yield impure (+)-(XI) (18 g., 62·75%), $[\alpha]_D$ +38·5° (*c* 1 in ethanol). Purity by titration: CO_2H, 92; OH (phenol), 97%. Total yield, 94·45%. By t.l.c. on silica gel, only one spot, R_F 0·61 ± 0·2, in the methanol–chloroform–ammonium hydroxide (20 : 20 : 4) system may be observed by detection with $FeCl_3$. No detection occurred with ninhydrin.

(b) *Salt formation of partially resolved* (+)-(XI) *with* D(+)-*ephedrine*. Impure (+)-(XI) (12·7 g.), $[\alpha]_D$ +38·5° (*c* 1 in ethanol), are dissolved in acetone (49 ml.) and treated with a 3·4M-ethanolic solution (11·25 ml.) of D(+)-ephedrine. After 2½ hr. at room temperature and the addition of

acetone (150 ml.), the crystals obtained are filtered off, washed with acetone and ether, and dried under a vacuum. These crystals are dissolved in hot ethanol (40 ml.). The solution is cooled and ether (70 ml.) is added to yield, after washing with ether and drying, 11·15 g. (58·7%) of (+)-(XI), D(+)-ephedrine. $[\alpha]_D$ +60° (*c* 1 in ethanol).

(c) (+)-(XI). The above diastereoisomeric salt (10·1 g.) dissolved in ethanol–water (80 : 20) (610 ml.) is poured on a column of Amberlite IR-124 (H^+) and eluted with the same solvent. The acid eluate is collected and concentrated under a vacuum until free from ethanol. The aqueous solution is then diluted with water, treated with charcoal, filtered, concentrated, and dried under a vacuum. The residue is taken up with ethanol and concentrated to dryness to yield 6·4 g. (95·7%) of (+)-(XI). Purity by titration: CO_2H, 93%, $[\alpha]_D$ +40·5° (*c* 1 in ethanol). By t.l.c., one spot, R_F 0·605, was detected with $FeCl_3$, but not with ninhydrin. The solvent was methanol–chloroform–ammonium hydroxide (20 : 20 : 4).

(d) (−)-(Ia). (+)-(XI) (5·5 g.) is dissolved in 1·2N-hydrochloric acid (110 ml.) and hydrolysed at 110° during 4½ hr., the reaction being followed by t.l.c. The solution is then discoloured with charcoal, filtered, and concentrated to dryness. The residue is taken up with ethanol, concentrated to a small volume, and diluted with one volume of ether. (−)-(Ia) crystallized on cooling (5 g., 92·5%), m.p. 204—210°, $[\alpha]_D$ −9° (*c* 2 in water). Purity by titration: NH_2, 92%.

By paper chromatography on Whatman No. 1 (n-butanol saturated with 3N-hydrochloric acid) DL-(Ia) is resolved in its enantiomers, R_F 0·645 and 0·827. For the enantiomers of (Ia), we obtained the following values: (+)-(Ia), R_F 0·645 (single spot); (−)-(Ia), R_F 0·827 (single spot).

Determination of the Configuration of (+)-(Ia) *and* (−)-(Ia). —(1) *Determination of the configuration with amino-acid oxidase.* (a) *Experimental conditions.* Buffer pH 7·4 prepared according to Sörensen,[13] and used with L-amino-acid oxidase, grade C, extract from *Crotaleus adamanteus* (Calbiochem No. 12.993). Buffer pH 8·8: sodium pyrophosphate (M/15) buffer, used with D-amino-acid oxidase of hog kidney (Mann Research Lab. No. 191) (0·06 enzymic units/mg. enzyme). Methylene Blue solution (0·033% Methylene Blue, HCl, Merck, in water). Chromatography on Whatman paper No. 1; n-butanol saturated with 3N-HCl; paper saturation time, 4·5 hr.; migration time, 24 hr.; detection by ninhydrin.

(b) *Description of the methods. Thunberg's method* [14] (Experiments 1 and 2). The following preparations are introduced into a 50-ml. Erlenmeyer flask: D- or L-amino-acid oxidase dissolved in its buffer; the buffered solution of (±)-(Ia) or of one of its enantiomers, and the solution of Methylene Blue. The reaction flask is placed under a vacuum, maintained at 37°, and stirred with a little magnet. Eventual changes in colour in the medium are noted. At the end of the experiment, the medium is chromatographed on Whatman paper No. 1.

Oxidation in the presence of air (Experiments 3 to 8). The buffered solution of the enzyme and of (Ia) is stirred and left in contact with air at 37°. After a reaction period varying from 2 to 13 hr., the medium is chromatographed on Whatman paper.

(c) *Results.* All the results obtained are summarized in Table 1.

TABLE 1

Experiment	Mg. (Ia)/ml. buffer	Enzyme/ml. buffer	Methylene Blue (ml.)	Method, time	R_F
1	10 mg. (+)/2 ml.	3 mg. L/1 ml.	0·2	Vacuum, 30 min.	0·645
2	10 mg. (−)/2 ml.	3 mg. L/1 ml.	0·2	Vacuum, 20 min.	No spot
3	10 mg. (±)/2 ml.	30 mg. D/3 ml.	—	Air, 13 hr.	0·827
4	5 mg. (+)/1 ml.	30 mg. D/4 ml.	—	Air, 13 hr.	No spot
5	5 mg. (−)/1 ml.	30 mg. D/4 ml.	—	Air, 13 hr.	0·827
6	15 mg. (±)/1 ml.	10 mg. L/2 ml.	—	Air, 2 hr.	0·645
7	10 mg. (+)/2 ml.	5·5 mg. L/1 ml.	—	Air, 2¼ hr.	0·645
8	10 mg. (−)/2 ml.	5·5 mg. L/1 ml.	—	Air, 2¼ hr.	No spot

(d) *Conclusions.* (*i*) The (−)-(Ia) enantiomer discolours Methylene Blue in presence of L-amino-acid oxidase (expt. No. 2) and no longer gives a spot in chromatography. This fact is confirmed by experiment No. 8. The enantiomer (−)-(Ia) is not destroyed by D-amino-acid oxidase, as shown in expt. No. 5. (−)-(Ia) belongs then to the L(or *S*)-configuration.

(*ii*) The (+)-(Ia) enantiomer does not discolour Methylene Blue in presence of L-amino-acid oxidase (expt. No. 1) but gives a spot in chromatography. This experiment is confirmed by the experiment No. 7.

The (−)-(Ia) enantiomer is completely destroyed by L-amino-acid oxidase (expt. No. 2) and is unattacked by D-amino-acid oxidase (expt. No. 5).

(+)-(Ia) belongs to the D(or *R*)-configuration.

(2) *Determination of the configuration by the method of Lutz and Jirgensons.*[11] The rotatory power values of the two enantiomers of (Ia) in different media are given in Table 2. As seen in Table 2, the L(or *S*)-configuration must be attributed to (−)-(Ia), and the D(or *R*)-configuration to (+)-(Ia). These results are in perfect agreement with those obtained by the enzymic method.

TABLE 2

	$[\alpha]_D$				
HCl	4N	N	N/2	N/33	0
(+)-(Ia)	+0·5°	+0·5°	+3·8°	+10°	+14°
(−)-(Ia).....	−0·01	−0·6	−2	−9	−18

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[14] See E. Balwin, 'Dynamic Aspects in Biochemistry,' 2nd edn., Cambridge Univ. Press, 1953, p. 151.