[CONTRIBUTION FROM THE LABORATORY OF BIOCHEMISTRY, NATIONAL CANCER INSTITUTE, NATIONAL INSTITUTES OF HEALTH]

Studies on Carcinogenic Amines. I. L- and D-Amino Acid Derivatives of 2-Aminofluorene, 4-Aminobiphenyl and 4,4′-Diaminobiphenyl, and their Susceptibility to Hydrolysis by Tissue Homogenates

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Fifty amino acid derivatives of three known carcinogenic amines in which the carboxyl group of the amino acid is combined in peptide linkage with the amino group of the amine have been prepared in crystalline form. The amino acids employed for this purpose have included both L- and D-varieties. The compounds were subjected to hydrolytic studies with homogenates of rat liver, kidney, spleen, lung, intestine and pancreas. Compounds containing L-amino acids with straight aliphatic side-chains were the most susceptible to hydrolysis, those with aromatic groups or with branching on the side-chains were much less susceptible, while those compounds containing D-amino acids, with a few weak exceptions, were completely inert.

The relatively unique ability of 2-aminofluorene and of such related compounds as 4-aminobiphenyl and benzidine to induce cancer in a variety of tissues in experimental animals has evoked much interest in this class of compounds. The original report of Wilson, DeEds and Cox in 1941 was based on the N-acetyl derivative of 2-aminofluorene,[1] and was followed by a number of significant studies, particularly by Bielschowsky, Haddow, Morris, J. and E. Weisburger, Ray, Walpole and J. and E. Miller.[2] Although both aminofluorene and N-acetylaminofluorene induced a variety of tumors in rats, the distribution of the tumors was not the same for the two compounds.[3] Tumors were induced at five sites by aminofluorene and at ten sites by acetylaminofluorene. These observations are of some interest in view of the fact that the acetyl group of acetylaminofluorene is known to be hydrolytically removed *in vivo*[4] and by several tissue homogenates *in vitro*.[5,6] That the acetyl group is split from the amino group of the fluorene molecule during the metabolism of the compound appears to be further substantiated by the failure of 2-*p*-toluenesulfonylaminofluorene to be carcinogenic as well as by the very weakly carcinogenic activity of 2-benzoylaminofluorene; the tosyl group is not hydrolyzed at all and the benzoyl group only with difficulty.[7]

Rats fed 2-acetylaminofluorene-9-C^{14} bind C^{14} in the liver,[8,9] and it would appear from recent concepts of the initial stages of carcinogenesis by this and similar compounds that these agents possess the capacity of combining with tissue proteins or protein precursors.[10] The nature of this binding is not known, but in the case of the carcinogenic amines it is not inconceivable that the binding may be of a peptide nature between the amine and a carboxyl group of the protein or protein precursor. Consequently, it was considered of interest to prepare a series of such peptide derivatives, in which the carcinogenic amine would be combined with the carboxyl group of a number of different amino acids and polypeptides. Unlike the acetyl derivatives, these compounds would still possess a free amino group, and unlike the free carcinogenic amine, this amino group would be on an aliphatic rather than on an aromatic residue. The present communication is concerned with the synthesis of a variety of L-and D-amino acid derivatives of 2-aminofluorene, 4-aminobiphenyl and 4,4′-diaminobiphenyl (benzidine), and with the subsequent study of their susceptibility to hydrolysis by different tissue homogenates. Studies now under way conducted by S. M. Birnbaum on the carcinogenicity of these compounds in rats is concerned in part with finding whether a correlation exists between the sites of carcinogenic action and the susceptibility to hydrolysis at these sites.

The general synthetic procedure involved the coupling of the N-carbobenzoxy derivative of the amino acid with the aromatic amine in the presence of isovaleryl chloride or isobutyl chlorocarbonate and triethylamine, followed by treatment of the product in glacial acetic acid with hydrobromic acid gas to remove the carbobenzoxy group. The final product was isolated either as the crystalline hydrobromide salt, or, on subsequent treatment with ammonia, as the free crystalline base. With one exception, no special difficulties were encountered in any of the reactions studied, which may be generalized as

$$\text{CbzoNHRCOOH} + \text{NH}_2\text{R}^1 \longrightarrow \text{CbzoNHRCONHR}^1 \longrightarrow \text{NH}_2\text{RCONHR}^1$$

where R is the amino acid residue and R^1 the nucleus of the aromatic amine, and the yields of the coupling and decarbobenzoxylated products were quite satisfactory. The exception noted concerned the preparation of L-prolylaminofluorene. The decarbobenzoxylation of carbobenzoxy-L-prolylaminofluorene led to the isolation of a sirup which could not be brought to crystallization and from which the benzyl bromide could not be completely removed. On treating the sirup in methanol solution with ammonia water a reaction of the residual benzyl bromide with the imino group of the prolyl residue occurred, leading to the formation of N-ben-

(1) R. H. Wilson, F. DeEds and A. J. Cox, *Cancer Research*, **1**, 595 (1941).

(2) For a brief review of the pertinent literature, *cf.* J. P. Greenstein, "Biochemistry of Cancer," Academic Press, Inc., New York, N. Y., 1954, pp. 105–113.

(3) H. P. Morris, C. S. Dubnik and J. M. Johnson, *J. Natl. Cancer Inst.*, **10**, 1201 (1905).

(4) J. H. Weisburger, E. K. Weisburger and H. P. Morris, *ibid.*, **11**, 797 (1951).

(5) J. H. Weisburger, *Biochem. Biophys. Acta*, **16**, 382 (1955).

(6) H. R. Gutmann and J. H. Peters, *J. Biol. Chem.*, **211**, 63 (1954).

(7) F. E. Ray and M. F. Argus, *Cancer Research*, **11**, 783 (1951).

(8) E. C. Miller and J. A. Miller, *ibid.*, **12**, 547 (1952).

(9) E. K. Weisburger, J. H. Weisburger and H. P. Morris, *Archiv. Biochem. Biophys.*, **43**, 474 (1953).

(10) J. A. Miller and E. C. Miller, *Adv. Cancer Res.*, **1**, 340 (1953).

TABLE I

PHYSICAL CHARACTERISTICS OF CARBOBENZOXY AMINO ACID DERIVATIVES OF CARCINOGENIC AMINES[a]

Compound	Formula	M.p., °C.	Calculated C	Calculated H	Calculated N	Found C	Found H	Found N
Cbzo-Glycyl-AF	$C_{23}H_{20}O_3N_2$	200	74.2	5.4	7.6	73.8	5.5	7.6
Cbzo-L-Alanyl-AF	$C_{24}H_{22}O_3N_2$	221	74.5	5.7	7.3	74.0	5.7	7.3
Cbzo-D-Alanyl-AF	$C_{24}H_{22}O_3N_2$	221	74.5	5.7	7.3	74.4	5.5	7.4
Cbzo-L-Butyrinyl-AF	$C_{25}H_{24}O_3N_2$	220	74.9	5.9	6.9	75.5	5.9	7.0
Cbzo-D-Butyrinyl-AF	$C_{25}H_{24}O_3N_2$	220	74.9	5.9	6.9	75.2	6.1	6.9
Cbzo-L-Norvalyl-AF	$C_{26}H_{26}O_3N_2$	208	75.3	6.3	6.8	75.5	6.3	6.6
Cbzo-D-Norvalyl-AF	$C_{26}H_{26}O_3N_2$	208	75.3	6.3	6.8	75.5	6.5	6.5
Cbzo-L-Norleucyl-AF	$C_{27}H_{28}O_3N_2$	204	75.6	6.6	6.5	75.7	6.6	6.5
Cbzo-D-Norleucyl-AF	$C_{27}H_{28}O_3N_2$	204	75.6	6.6	6.5	75.6	7.0	6.5
Cbzo-L-S-Benzylcysteinyl-AF[b]	$C_{31}H_{28}O_3N_2S$	197	73.2	5.5	5.5	73.2	5.6	5.5
Cbzo-D-S-Benzylcysteinyl-AF[b]	$C_{31}H_{28}O_3N_2S$	197	73.2	5.5	5.5	73.4	5.6	5.5
Cbzo-L-Methionyl-AF[c]	$C_{25}H_{26}O_3N_2S$	198	69.9	5.8	6.3	69.9	6.1	6.3
Cbzo-D-Methionyl-AF[c]	$C_{25}H_{26}O_3N_2S$	198	69.9	5.8	6.3	70.3	5.9	6.3
Cbzo-L-Valyl-AF	$C_{26}H_{26}O_3N_2$	>230	75.3	6.3	6.8	75.4	6.2	6.7
Cbzo-D-Valyl-AF	$C_{26}H_{26}O_3N_2$	>230	75.3	6.3	6.8	75.3	6.2	6.7
Cbzo-L-Leucyl-AF	$C_{27}O_{28}O_3N_2$	208	75.6	6.6	6.5	75.9	6.5	6.3
Cbzo-D-Leucyl-AF	$C_{27}H_{28}O_3N_2$	208	75.6	6.6	6.5	76.1	6.6	6.3
Cbzo-L-Isoleucyl-AF	$C_{27}H_{28}O_3N_2$	>230	75.6	6.6	6.5	76.0	7.0	6.5
Cbzo-D-Alloisoleucyl-AF	$C_{27}H_{28}O_3N_2$	>230	75.6	6.6	6.5	75.6	6.5	6.5
Cbzo-L-Phenylalanyl-AF	$C_{30}H_{26}O_3N_2$	211	77.9	5.6	6.1	78.1	5.7	6.0
Cbzo-D-Phenylalanyl-AF	$C_{30}H_{26}O_3N_2$	211	77.9	5.6	6.1	78.0	5.6	5.9
Cbzo-L-Tryptophyl-AF	$C_{32}H_{27}O_3N_3$	219	76.6	5.4	8.4	76.2	5.6	8.4
Cbzo-D-Tryptophyl-AF	$C_{32}H_{27}O_3N_3$	219	76.6	5.4	8.4	76.3	5.5	8.3
Cbzo-L-Prolyl-AF	$C_{26}H_{24}O_3N_2$	174	75.7	5.8	6.8	75.7	5.9	6.8
Cbzo-Glycyl-AD	$C_{22}H_{20}O_3N_2$	205	73.3	5.5	7.7	73.6	5.7	7.7
Cbzo-L-Alanyl-AD	$C_{23}H_{22}O_3N_2$	219	73.7	5.8	7.5	73.9	5.8	7.3
Cbzo-D-Alanyl-AD	$C_{23}H_{22}O_3N_2$	219	73.7	5.8	7.5	74.1	5.8	7.3
Cbzo-L-Butyrinyl-AD	$C_{24}H_{24}O_3N_2$	204	74.2	6.2	7.2	74.5	6.2	7.2
Cbzo-D-Butyrinyl-AD	$C_{24}H_{24}O_3N_2$	204	74.2	6.2	7.2	75.0	6.5	7.2
Cbzo-L-Norvalyl-AD	$C_{25}H_{26}O_3N_2$	215	74.6	6.5	6.9	74.7	6.5	6.7
Cbzo-D-Norvalyl-AD	$C_{25}H_{26}O_3N_2$	215	74.6	6.5	6.9	74.8	6.4	6.8
Cbzo-L-Norleucyl-AD	$C_{26}H_{28}O_3N_2$	205	74.9	6.7	6.7	75.1	6.8	6.7
Cbzo-D-Norleucyl-AD	$C_{26}H_{28}O_3N_2$	205	74.9	6.7	6.7	75.1	6.7	6.7
Cbzo-L-S-Benzylcysteinyl-AD[d]	$C_{30}H_{28}O_3N_2S$	198	72.5	5.6	5.6	72.8	5.8	5.6
Cbzo-D-S-Benzylcysteinyl-AD[d]	$C_{30}H_{28}O_3N_2S$	198	72.5	5.6	5.6	72.4	5.7	5.6
Cbzo-L-Valyl-AD	$C_{25}H_{26}O_3N_2$	>230	74.6	6.5	6.9	74.4	6.5	6.9
Cbzo-D-Valyl-AD	$C_{25}H_{26}O_3N_2$	>230	74.6	6.5	6.9	74.9	6.6	6.9
Cbzo-L-Isoleucyl-AD	$C_{26}H_{28}O_3N_2$	>230	74.9	6.7	6.7	75.1	6.9	6.7
Cbzo-D-Alloisoleucyl-AD	$C_{26}H_{28}O_3N_2$	>230	74.9	6.7	6.7	75.1	6.9	6.8
Cbzo-L-Leucyl-AD	$C_{26}H_{28}O_3N_2$	205	74.9	6.7	6.7	75.1	6.7	6.6
Cbzo-D-Leucyl-AD	$C_{26}H_{28}O_3N_2$	205	74.9	6.7	6.7	75.0	6.7	6.5
Cbzo-L-Phenylalanyl-AD	$C_{29}H_{26}O_3N_2$	219	77.3	5.8	6.2	78.0	6.1	6.2
Cbzo-D-Phenylalanyl-AD	$C_{29}H_{26}O_3N_2$	219	77.3	5.8	6.2	77.9	6.1	6.2
Cbzo-L-Tryptophyl-AD	$C_{31}H_{27}O_3N_3$	206	76.0	5.5	8.6	76.0	5.7	8.5
Cbzo-D-Tryptophyl-AD	$C_{31}H_{17}O_3N_3$	206	76.0	5.5	8.6	76.0	5.7	8.6
Dicbzo-Diglycyl-B	$C_{32}H_{30}O_6N_4$	>230	67.8	5.3	9.9	67.3	5.5	9.7
Dicbzo-Di-L-Alanyl-B	$C_{34}H_{34}O_6N_4$	>230	68.6	5.7	9.4	68.2	5.8	9.3
Dicbzo-Di-D-Alanyl-B	$C_{34}H_{34}O_6N_4$	>230	68.6	5.7	9.4	68.7	5.8	9.2
Dicbzo-Di-L-Leucyl-B	$C_{40}H_{46}O_6N_4$	224	70.8	6.8	8.3	70.9	7.0	8.3
Dicbzo-Di-D-Leucyl-B	$C_{40}H_{46}O_6N_4$	224	70.8	6.8	8.3	71.2	6.9	8.3

[a] AF refers to 2-aminofluorene, AD to 4-aminobiphenyl, and B to 4,4′-diaminobiphenyl or benzidine. [b] Calcd. S, 6.3. Found: S (for the L-compound), 6.3; (for the D-compound), 6.3. [c] Calcd. S, 7.2. Found: S (for the L-compound), 7.2; (for the D-compound), 7.1. [d] Calcd. S, 6.4. Found: S (for the L-compound), 6.3; (for the D-compound), 6.2.

zylprolylaminofluorene. The final product therefore was a mixture of this compound and of prolylaminofluorene which, on crystallization from ethanol, yielded only the pure N-benzyl derivative.

Incubation of the various compounds with aqueous rat tissue homogenates at *p*H 8.0 revealed appreciable hydrolysis of the L-butyrinyl, L-norvalyl and L-norleucyl derivatives of aminofluorene and of aminobiphenyl in all of the homogenates studied (liver, kidney, spleen, lung, small intestine and pancreas). The glycyl and the L-amino acid derivatives with branched side chains or with aromatic residues, were generally less susceptible. Of the three benzidine compounds, diglycyl, di-L-alanyl and di-L-leucyl, only the di-L-alanyl derivative was appreciably hydrolyzed by homogenates of the six tissues studied. With the exceptions of the D-alanyl derivative of aminofluorene, and of the D-ala-

TABLE II

PHYSICAL CHARACTERISTICS OF FREE AMINO ACID DERIVATIVES OF CARCINOGENIC AMINES[a]

Compound	Formula	$[\alpha]^{25}D$,[f] degree	Calcd. N	Calcd. Br	Found N	Found Br	Crystallization medium
Glycyl-AF·HBr	$C_{15}H_{15}ON_2Br$	.....	8.8	25.1	8.8	25.3	MeOH–ether
L-Alanyl-AF·HBr	$C_{16}H_{17}ON_2Br$	+ 11.0	8.4	24.0	8.4	24.3	MeOH–ether
D-Alanyl-AF·HBr	$C_{16}H_{17}ON_2Br$	− 11.5	8.4	24.0	8.4	24.3	MeOH–ether
L-Butyrinyl-AF·HBr	$C_{17}H_{19}ON_2Br$	+ 32.0	8.1	23.1	8.0	22.8	MeOH–ether
D-Butyrinyl-AF·HBr	$C_{17}H_{19}ON_2Br$	− 32.0	8.1	23.1	7.9	22.9	MeOH–ether
L-Norvalyl-AF·HBr	$C_{18}H_{21}ON_2Br$	+ 45.5	7.7	22.1	7.7	22.5	H_2O
D-Norvalyl-AF·HBr	$C_{18}H_{21}ON_2Br$	− 46.0	7.7	22.1	7.7	21.9	H_2O
L-Norleucyl-AF·HBr	$C_{19}H_{23}ON_2Br$	+ 52.0	7.5	21.3	7.4	21.1	H_2O
D-Norleucyl-AF·HBr	$C_{19}H_{23}ON_2Br$	− 52.0	7.5	21.3	7.4	21.1	H_2O
L-S-Benzylcysteinyl-AF·HBr[b]	$C_{23}H_{23}ON_2SBr$	+117.0	6.1	17.5	6.1	17.1	Ethanol–H_2O
D-S-Benzylcysteinyl-AF·HBr[b]	$C_{23}H_{23}ON_2SBr$	−117.5	6.1	17.5	6.1	17.9	Ethanol–H_2O
L-Methionyl-AF(base)[c]	$C_{18}H_{20}ON_2S$	+ 79.0	9.0	..	9.0	..	Ethanol–H_2O
D-Methionyl-AF(base)[c]	$C_{18}H_{20}ON_2S$	− 79.0	9.0	..	9.0	..	Ethanol-H_2O
L-Valyl-AF(base)	$C_{18}H_{20}ON_2$	+ 75.0	10.0	..	10.0	..	Ethanol–H_2O
D-Valyl-AF(base)	$C_{18}H_{20}ON_2$	− 74.5	10.0	..	10.0	..	Ethanol–H_2O
L-Leucyl-AF·HBr	$C_{19}H_{23}ON_2Br$	+ 53.5	7.5	21.3	7.5	21.2	H_2O
D-Leucyl-AF·HBr	$C_{19}H_{23}ON_2Br$	− 54.0	7.5	21.3	7.5	21.2	H_2O
L-Isoleucyl-AF·HBr	$C_{19}H_{23}ON_2Br$	+ 70.0	7.5	21.3	7.5	21.2	H_2O
D-Alloisoleucyl-AF(base)	$C_{19}H_{22}ON_2$	− 87.0	9.5	..	9.3	..	Ethanol
L-Phenylalanyl-AF·HBr	$C_{22}H_{21}ON_2Br$	+146.0	6.8	19.5	6.8	19.8	H_2O
D-Phenylalanyl-AF·HBr	$C_{22}H_{21}ON_2Br$	−148.0	6.8	19.5	6.8	19.4	H_2O
L-Tryptophyl-AF(base)	$C_{24}H_{21}ON_3$	+194.0	11.4	..	11.3	..	Ethanol
D-Tryptophyl-AF(base)	$C_{24}H_{21}ON_3$	−195.0	11.4	..	11.2	..	Ethanol
L-N-Benzylpropyl-AF (base)[d]	$C_{23}H_{24}ON_2$	− 49.0	7.7	..	7.8	..	Ethanol
Glycyl-AD·HBr	$C_{14}H_{15}ON_2Br$	.....	9.1	26.1	9.1	26.2	H_2O
L-Alanyl-AD·HBr	$C_{15}H_{17}ON_2Br$	+ 12.5	8.7	24.9	8.5	24.6	H_2O
D-Alanyl-AD·HBr	$C_{15}H_{17}ON_2Br$	− 12.3	8.7	24.9	8.5	24.7	MeOH–ether
L-Butyrinyl-AD·HBr	$C_{16}H_{19}ON_2Br$	+ 38.0	8.3	23.8	8.3	23.8	MeOH–ether
D-Butyrinyl-AD·HBr	$C_{16}H_{19}ON_2Br$	− 38.0	8.3	23.8	8.3	24.2	MeOH–ether
L-Norvalyl-AD·HBr	$C_{17}H_{21}ON_2Br$	+ 43.0	8.0	22.9	8.0	22.8	H_2O
D-Norvalyl-AD·HBr	$C_{17}H_{21}ON_2Br$	− 43.0	8.0	22.9	7.7	22.6	H_2O
L-Norleucyl-AD·HBr	$C_{18}H_{23}ON_2Br$	+ 49.5	7.7	22.0	7.6	21.7	H_2O
D-Norleucyl-AD·HBr	$C_{18}H_{23}ON_2Br$	− 49.0	7.7	22.0	7.7	21.8	H_2O
L-S-Benzylcysteinyl-AD·HBr[e]	$C_{22}H_{23}ON_2SBr$	+103.0	6.3	18.1	6.3	18.0	Ethanol–H_2O
D-S-Benzylcysteinyl-AD·HBr[e]	$C_{22}H_{23}ON_2SBr$	−102.0	6.3	18.1	6.4	18.0	Ethanol–H_2O
L-Valyl-AD(base)	$C_{17}H_{20}ON_2$	+ 72.0	10.5	..	10.3	..	Ethanol–H_2O
D-Valyl-AD(base)	$C_{17}H_{20}ON_2$	− 72.0	10.5	..	10.3	..	Ethanol–H_2O
L-Isoleucyl-AD(base)	$C_{18}H_{22}ON_2$	+ 76.0	9.9	..	9.9	..	Ethanol
D-Alloisoleucyl-AD(base)	$C_{18}H_{22}ON_2$	− 81.0	9.9	..	9.8	..	Ethanol–H_2O
L-Leucyl-AD·HBr	$C_{18}H_{23}ON_2Br$	+155.0	7.7	22.0	7.7	21.8	H_2O
D-Leucyl-AD·HBr	$C_{18}H_{23}ON_2Br$	−155.0	7.7	22.0	7.5	22.2	H_2O
L-Phenylalanyl-AD(base)	$C_{21}H_{20}ON_2$	+148.0	8.9	..	8.8	..	Ethanol
D-Phenylalanyl-AD(base)	$C_{21}H_{20}ON_2$	−147.0	8.9	..	8.8	..	Ethanol
L-Tryptophyl-AD·HBr	$C_{23}H_{22}ON_3Br$	+155.0	9.6	18.4	9.6	18.5	H_2O
D-Tryptophyl-AD·HBr	$C_{23}H_{22}ON_3Br$	−155.0	9.6	18.4	9.6	18.3	H_2O
Diglycyl-B·2HBr	$C_{16}H_{20}O_2N_4Br_2$	.....	12.2	34.8	12.0	35.1	MeOH–H_2O
Di-L-Alanyl-B·2HBr	$C_{18}H_{24}O_2N_4Br_2$	+ 18.0	11.5	32.8	11.4	32.8	MeOH–ether
Di-D-Alanyl-B·2HBr	$C_{18}H_{24}O_2N_4Br_2$	− 18.0	11.5	32.8	11.5	32.8	MeOH–ether
Di-L-Leucyl-B(base)	$C_{24}H_{34}O_2N_4$	+130.0	13.8	..	13.7	..	Ethanol
Di-D-Leucyl-B(base)	$C_{24}H_{34}O_2N_4$	−131.0	13.8	..	13.7	..	Ethanol

[a] AF refers to 2-aminofluorene, AD to 4-aminobiphenyl and B to 4,4′-diaminobiphenyl or benzidine. [b] Calcd. S, 7.0. Found: S (for the L-compound), 7.0; (for the D-compound), 7.1. [c] Calcd. S, 10.1. Found: S (for the L-compound), 9.7; (for the D-compound), 9.9. [d] Calcd. C, 81.5; H, 6.8. Found: C, 81.5; H, 6.8. [e] Calcd. S, 7.1. Found: S, 7.1 (for the L-compound); (for the D-compound), 6.9. [f] The rotation values of the hydrobromide salts were determined in methanol solutions, those of the free bases in glacial acetic acid. In all cases $c = 1$.

nyl and D-butyrinyl derivatives of aminobiphenyl, which were slowly hydrolyzed by liver homogenates, all of the D-amino acid derivatives of the aromatic amines were inert toward all of the tissue preparations. It is not improbable that the susceptible substrates are hydrolyzed by different enzyme complexes in the various tissues studied, as suggested by the lack of uniformity in the activity ratios of each substrate in different tissues. The data by Weisburger on the hydrolysis of acetylaminofluorene by liver and by kidney homogenates[5] show the former tissue to be about twice as active as the latter; the present data on the hydrolysis of glycylaminofluorene by the same tissue shows just

the reverse. It is not likely that the acetyl and the amino acid derivatives of the aromatic amines are hydrolyzed by the same enzyme or group of enzymes.[11]

Experimental

Preparation of the Amino Acid Derivatives of the Carcinogenic Amines.—The general mixed anhydride procedure of Vaughan and Osato[12] was employed for the synthesis of all the carbobenzoxy derivatives described herein. In essence, 0.1 mole of the carbobenzoxy amino acid was dissolved in 200 ml. of chloroform which contained 14.0 ml. of triethylamine. The solution was chilled to 0–5° in an ice-bath and subsequently treated with 0.1 mole of isovaleryl chloride or isobutyl chlorocarbonate. After 45 minutes of standing at 4°, a solution of 0.1 mole of the aromatic amine in 200 ml. of chloroform was added to the resultant mixed anhydride. After standing at room temperature overnight, 5 to 10 volumes of petroleum ether was added to effect a more nearly complete precipitation of addition product. The precipitate was filtered over suction, washed copiously with water, and eventually boiled with 1 to 3 liters of absolute ethanol. In some instances complete solution of the addition product was effected. After cooling at −10° for several hours, the precipitate was filtered and the alcohol treatment repeated. Final crystallization was accomplished by precipitation of a solution of the coupling product in a 50:50 mixture of dimethylformamide and chloroform by the addition of petroleum ether. The yields were generally 60 to 80% of the theoretical. The analytical data are given in Table I.

Removal of the carbobenzoxy group from each of the products was effected by the method of Ben-Ishai and Berger.[13] The carbobenzoxylated derivative was dissolved with heating in the 20-fold amount of glacial acetic acid and the solution cooled to 25°. In most cases the compounds crystallized. Dry HBr gas was introduced into the mixture which was maintained at a temperature not exceeding 40°. In the special cases of the tryptophyl and methionyl derivatives the reaction temperature was kept at 20°. With the exception of the diglycyl and dialanyl derivatives of benzidine, all of the compounds went into solution during the HBr gassing procedure which was carried on for a period of 30 minutes to 1 hour. The exceptional compounds noted which did not dissolve appeared to change their crystalline shape during this interval. In all cases the gassed mixtures were allowed to stand for an hour at 25° and were then evaporated to dryness *in vacuo* or blown down to dryness in a stream of dry air. The residues were in the form either of crystals or oils, and in any event were thoroughly washed with ether to remove adherent benzyl bromide. Crystallization of the hydrobromide salts was attempted as such, and where this procedure appeared to fail, the compound was dissolved in the minimum amount of methanol and the solution treated with an excess of ammonia water to convert the compound to the free base. In this form the compound readily precipitated and could be subsequently brought to crystallization. The analytical and other pertinent data on these compounds are described in Table II. The optical rotation values for the optical antipodes are opposite in sign and nearly equal in magnitude, thus excluding the possibility of any appreciable racemization during the synthesis. With the exception of the L-N-benzylprolyl derivative, all of the L-compounds rotated in the positive, and all of the D-compounds rotated in the negative direction. Prior to analysis, the compounds, many of which appeared to contain water of crystallization, were carefully dried at 78° *in vacuo* for several hours to remove all traces of water. The yields were generally about 60% of the theoretical. The removal of the carbobenzoxy group from 17 g. of carbobenzoxy prolylaminofluorene followed by treatment with ammonia water and recrystallization from ethanol–water yielded 8 g. of a mixture of prolylaminofluorene and N-benzylprolylaminofluorene the analysis of which for C, H and N revealed nearly equal amounts of each compound. A single crystallization from absolute ethanol sufficed to yield analytically pure N-benzylprolylaminofluorene.

Most of the optically active amino acids employed in these syntheses were prepared in this Laboratory by the general enzymatic procedure described earlier,[14] and all contained less than 0.1% of the respective optical antipode.[15]

Enzymatic Studies.—Aqueous homogenates in ice-cold distilled water were prepared from the following tissues removed immediately from freshly-sacrificed male rats of the Sprague–Dawley strain: liver, kidney, spleen, lung, small intestine and pancreas. Hydrolytic rates of the substrates were determined by mixing 0.5 ml. of a neutralized suspension containing 10 micromoles of the substrate with 0.5 ml. of 0.2 *M* tris buffer at *p*H 8.0 and 0.5 ml. of the tissue homogenate (approximately 1 to 2 g. of tissue per 5 ml. water). The digests were incubated at 37° in a Dubnoff shaker for periods varying from 1 to 2 hours, and the control reaction prepared by incubating only homogenate with buffer, the substrate being added after termination of the digestion period. These controls were negative in every case, indicating complete freedom of the substrates from free aromatic amines. After standing for 20 minutes at 25° the acetone–digest mixture was filtered through Whatman no. 4 paper, the precipitate was washed with 5 ml. of acetone, and the combined filtrate and washings blown down to a volume of 2 to 3 ml. in a stream of dry air. The concentrate was brought to 5 ml. by addition of glacial acetic acid and an aliquot of this solution used for the determination of diazotiz-

TABLE III

HYDROLYSIS OF AMINO ACID DERIVATIVES OF CARCINOGENIC AMINES BY TISSUE HOMOGENATES[a]

Derivatives of aminofluorene	Liver	Kidney	Spleen	Lung	Intestine[b]	Pancreas
Glycyl	10	28	5	4	0	0
L-Alanyl	22	30	10	17	20	11
D-Alanyl	3	0	0	0	0	0
L-Butyrinyl	107	108	62	49	47	13
L-Norvalyl	87	112	43	23	19	14
L-Norleucyl	19	28	10	6	10	6
L-Valyl	2	1	0	0	0	0
L-Leucyl	27	19	6	4	0	0
L-Isoleucyl	4	1	5	<1	<1	0
L-Methionyl	5	0	6	2	6	0
Aminobiphenyl						
Glycyl	15	53	8	7	15	0
L-Alanyl	75	73	42	55	70	0
D-Alanyl	4	0	0	0	0	0
L-Butyrinyl	69	80	45	42	22	9
D-Butyrinyl	4	0	0	0	1	0
L-Norvalyl	44	53	17	16	19	21
L-Norleucyl	14	24	7	7	11	6
L-Valyl	7	5	0	0	0	0
L-Leucyl	27	34	10	7	10	0
L-Isoleucyl	7	4	0	0	0	0
L-Phenylalanyl	1	<1	<1	<1	<1	0
L-Tryptophyl	4	3	0	0	0	0
Benzidine						
Diglycyl	0	2	0	0	0	0
Di-L-alanyl	53	72	27	25	15	8

[a] Figures represent rates in terms of micromoles of substrate hydrolyzed per gram of wet tissues. [b] Small intestine.

(11) While these experiments were in progress, the paper by L. Rombach and I. R. MacGregor, *J. Org. Chem.*, **19**, 428 (1954), came to our attention in which these authors described the preparation of glycyl-2-aminofluorene and of di-(DL-phenylalanyl)-2-aminofluorene by treating the phthalylamino acid chloride with aminofluorene in the presence of pyridine followed by removal of the phthalyl group from the coupling product by means of hydrazine. No enzyme experiments were reported.

(12) J. R. Vaughan and R. L. Osato, THIS JOURNAL, **73**, 5553 (1951); **74**, 676 (1952).

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(14) S. M. Birnbaum, L. Levintow, R. B. Kingsley and J. P. Greenstein, *J. Biol. Chem.*, **194**, 455 (1952); J. P. Greenstein, S. M. Birnbaum and M. C. Otey, *ibid.*, **204**, 307 (1953); J. P. Greenstein, *Advances in Prot. Chem.*, **9**, 122 (1954).

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able amines by the general procedure of Westfall and Morris.[16] The color developed was measured in a Coleman model 14 spectrophotometer against a standard curve prepared from freshly prepared glacial acetic acid solutions of the corresponding aromatic amines, using the maxima of 525 mμ for aminofluorene, 505 mμ for aminobiphenyl, and 520 mμ for benzidine. Control runs of aromatic amines incubated at 37° with the tissue homogenates up to 4 hours revealed no appreciable destruction of the amines and recoveries of over 90%. The data in terms of micromoles of substrate hydrolyzed per hour per gram of wet tissue are given in Table III.

No evidence of hydrolysis up to 2 hours of incubation was observed by any of the tissue homogenates for the D-butyrinyl, D-norvalyl, D-norleucyl, D-valyl, D-leucyl, D-alloisoleucyl, D-methionyl, L- and D-S-benzylcysteinyl, L- and D-phenylalanyl, L- and D-tryptophyl and the mixed L-prolyl and L-N-benzylprolyl, derivatives of aminofluorene, nor for the D-norvalyl, D-norleucyl, D-valyl, D-leucyl, D-alloisoleucyl, D-tryptophyl, D-phenylalanyl and L- and D-S-benzylcysteinyl derivatives of aminobiphenyl, or for the di-D-alanyl, and di-L- and di-D-leucyl derivatives of benzidine. These data have therefore been omitted from Table III.

(16) B. B. Westfall and H. P. Morris, *J. Natl. Cancer Inst.*, **8**, 17 (1947); *cf.* H. M. Dyer, H. E. Ross and H. P. Morris, *Cancer Research*, **11**, 307 (1951).

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[CONTRIBUTION FROM THE RESEARCH DEPARTMENT OF CIBA PHARMACEUTICAL PRODUCTS, INC.]

Rauwolfia Alkaloids. XX.[1] 11-Methoxyalloyohimbane from Reserpine

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Two reserpones were obtained by hydrolysis and decarboxylation of methyl anhydroreserpate (III). They were transformed *via* their thioketals to two reserpanes. The negatively rotating reserpane was shown to be 11-methoxyalloyohimbane (IV) by an independent synthesis thus providing proof for the *cis* juncture of rings D and E in reserpine. IV was prepared by catalytic hydrogenation of 11-methoxysempervirine which in turn resulted from the reaction of the lithium derivative of harmine with isopropoxymethylenecyclohexanone.

We have demonstrated in a previous paper[1] that the stereochemistry of the pentacyclic ring skeleton of deserpidine is that of 3-epialloyohimbane. It also was reported briefly that reserpine can be degraded to a negatively rotating reserpane which was proved by synthesis to possess a *cis* fusion of rings D and E. The details of this proof are given here. The nature of this ring juncture has been confirmed recently by another group of workers who showed that methyl reserpate tosylate can be converted to a salt derived by intramolecular quaternization.[2] Since both deserpidine and reserpine and their derivatives can be epimerized at C-3 and since the two alkaloids possess virtually equivalent pharmacological activities,[3] reserpine also can be regarded as a derivative of 3-epialloyohimbane. The high degree of dependence of biological properties on steric factors is indicated by the complete inactivity of 3-isoreserpine.[1]

Two reserpones, one (I) with a negative and the other (II), with a positive rotation, are formed by the acid-catalyzed hydrolysis and decarboxylation of methyl anhydroreserpate (III). Both of these ketones have been converted to their thioketals and thence by Raney nickel desulfurization to the two corresponding reserpanes IV and V. There is little change in the magnitude of rotation in proceeding from the ketone to the hydrocarbon. Since alloyohimbane and 3-epialloyohimbane obtained from the naturally occurring alkaloid, 3-epi-α-yohimbine, show a negative and positive rotation, respectively[4] (Table I), it seemed likely that the negatively rotating reserpane was 11-methoxyalloyohimbane (IV). This supposition was proved by the synthesis of the racemic form of IV.

TABLE I

ROTATIONS IN CHLOROFORM OF ALLO- AND 3-EPIALLOYOHIMBANE DERIVATIVES

Compound	$[\alpha]^{25}D$
Methyl anhydroreserpate	−129°
Alloyohimbane	−130
11-Methoxyalloyohimbane	−149
3-Epialloyohimbane[4]	+105
11-Methoxy-3-epialloyohimbane	+ 72

Harmine (VI) was converted to its lithium derivative with phenyllithium and treated with isopropoxymethylenecyclohexanone (VII) according to the sempervirine synthesis of Woodward and McLamore.[5] The resulting 11-methoxysempervirine (VIII) was hydrogenated over platinum oxide in the presence of base to yield *dl*-11-methoxyalloyohimbane (IV). Le Hir and co-workers[6] have shown that the reduction of sempervirine under these conditions leads to *dl*-alloyohimbane. The infrared spectrum of the synthetic *dl*-11-methoxyalloyohimbane in chloroform solution was identical to that of the negatively rotating reserpane (IV) thus proving that rings D and E are *cis* fused in reserpine. The infrared spectra of IV and the positively rotating reserpane (V) are distinctly different.

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