914. *Isolation and Synthesis of Balenine, a Dipeptide Occurring in Whale-meat Extract*

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Balenine has been isolated from whale-meat extract and shown by synthesis to have the structure β-alanyl-L-3-methylhistidine. It is isomeric with anserine and ophidine.

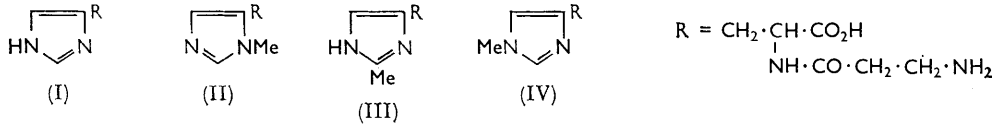
Carnosine (I), β-alanyl-L-histidine, which was first isolated from Liebig's extract of meat, occurs in the muscles of many species of animals.[1-3] Two methylated derivatives of carnosine are known, *viz.* anserine (II), β-alanyl-L-1-methylhistidine,* and ophidine (III), β-alanyl-L-2-methylhistidine, in which the imidazole ring carries a methyl group. Anserine, first isolated from the goose,[4] also occurs in many species,[1-3] often along with carnosine. Ophidine has been isolated from the cobra,[5,6] and the Japanese snake

* Strictly speaking, the trivial names " 1 methylhistidine " and " 3 methylhistidine " are inconsistent with the accepted method of numbering imidazole derivatives (see " Handbook for Chemical Society Authors," 1960, p. 196), the systematic names being α-amino-β-(1-methyl-5-imidazoyl)propionic acid and α-amino-β-(1-methyl-4-imidazoyl)propionic acid, respectively. We have, however, used the trivial names for brevity.

[1] W. A. Wolff and D. W. Wilson, *J. Biol. Chem.*, 1932, **95**, 495.
[2] W. A. Wolff and D. W. Wilson, *J. Biol. Chem.*, 1935, **109**, 565.
[3] J. A. Zapp and D. W. Wilson, *J. Biol. Chem.*, 1938, **126**, 9 and 19.
[4] D. Ackermann, O. Timpe, and K. Poller, *Z. physiol. Chem.*, 1929, **183**, 1.
[5] H. Imamura, *J. Biochem. Japan*, 1939, **30**, 479.
[6] K. Kendo, *J. Biochem. Japan*, 1944, **36**, 265.

Agkistrodon blomhoffi;[7] it has also been reported[8] to occur in the fin whale *Balaenoptera physalus* L., and in the muscle[9] and pancreas[10] of whale (species not reported). The structures of carnosine,[11] anserine,[12] and ophidine[13] have been confirmed by synthesis.

Pocchiari *et al.*[14] and Carisano and Carrà[15] have reported the occurrence in whale-meat extract of a dipeptide of β-alanine and 3-methylhistidine, for which the name balenine and the structure (IV), β-alanyl-3-methylhistidine, have been proposed[14] by analogy with anserine. Our investigations of Norwegian commercial whale-meat extract † (species of whale unknown) led us to the same conclusion as the Italian workers, and we have isolated crystalline balenine from whale-meat extract by the method which Wolff and Wilson[1] used for anserine. The melting point and optical rotation of balenine differ from



those reported for anserine and ophidine (see Table) thus indicating that balenine definitely is a different compound. Balenine may be identical with the unidentified compound, m. p. 261—263°, $[\alpha]_D^{20}$ +30·15° or +31·33° isolated from whale meat by Yazawa,[8,16] although

Properties of bases

	Anserine	Ophidine	Balenine
M. p. of free base	238—239°	249;[6] 246—248;*[13] 238—239	260—262
$[\alpha]_D$	+11·26;[1] +12·25 *[12]	+41·26;[6] +36·5[8]	+34·8; +32·7 *
R_F in solvent B	0·56	—	0·64
M. p. of copper salt ...	228°[1]	210[6]	—
M. p. of picrate	205°[6] (mono)	155[6] (di)	136—139 (di)
Monopicrolonate	—	232°;[6] 220—221[8]	234—236
Dipicrolonate	—	155—156°[8]	164

* Synthetic compound.

this substance was assigned the empirical formula $C_{11}H_{20}N_4O_4$ rather than $C_{10}H_{16}N_4O_3$ which corresponds to structure (IV). Balenine may easily be distinguished from carosine and anserine by paper chromatography in a phenolic solvent (solvent B, see Experimental section) unfortunately, no sample of ophidine could be obtained for comparison. At room temperature balenine reacts with ninhydrin to give a brownish spot which becomes green on heating at 110° for ten minutes. Anserine, 1-methylhistidine, and 3-methylhistidine also give abnormal colours with ninhydrin. Carnosine gives a red colour with Pauly's reagent; balenine and anserine give no colour; ophidine is reported to give a weak red colour. Furthermore, we have found that carnosine, anserine, balenine, histidine, 1-methylhistidine, and 3-methylhistidine all give dark red spots when treated with *p*-nitrobenzenediazonium fluoborate and alkali by the method of Shifrine and Zweig.[17]

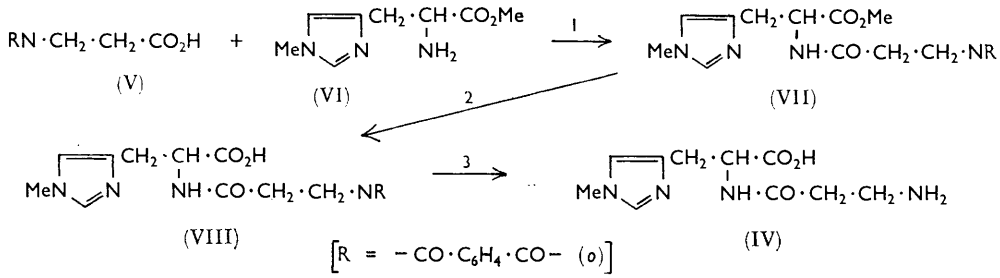
Balenine appears to be unique to whale-meat extract, which appears to contain between

† The manufacture of whale-meat extract on board a factory ship has been described by Ash.[14a] The yield of extract from the meat does not seem to be known exactly, but is probably similar to the yield of extract obtained from beef, *i.e.*, about 3% of the weight of the meat.

[7] M. Tomita, *Z. physiol. Chem.*, 1956, **304**, 72.
[8] T. Nakai, N. Tsujigado, and S. Akiya, *J. Biochem. Japan*, 1963, **54**, (6), 541.
[9] K. Horisaka and A. Mushai, *J. Biochem. Japan*, 1962, **53**, (4), 271.
[10] S. Tsunoo, A. Mushai, and K. Horisaka, *Proc. Japan Acad.*, 1959, **35**, 485.
[11] R. H. Sifferd and V. du Vigneaud, *J. Biol. Chem.*, 1935, **108**, 753.
[12] O. K. Behrens and V. du Vigneaud, *J. Biol. Chem.*, 1937, **120**, 517.
[13] T. Ono and R. Hirohata, *Z. physiol. Chem.*, 1956, **304**, 77.
[14] F. Pocchiari, L. Tentori, and G. Vivaldi, *Sci. Reports Ist. Super. Sanità*, 1962, **2**, 184.
[14a] C. E. Ash, *Chem. and Ind.*, 1964, **38**, 1596.
[15] A. Carisano and F. Carrà, *Ind. Conserve* (*Parma*), 1963, **38**, 28.
[16] T. Yazawa, *Jap. J. Med. Sci.*, II, *Biochemistry*, 1933, **2**, 37; 1935, **3**, 15.
[17] M. Shifrine and G. Zweig, *J. Chromatog.*, 1964, **14**, 297.

10 and 20% of the peptide. We have not found it in extracts of beef, mutton, yeast, herring, chicken, pork, or veal.[18]

A considerable quantity of 3-methylhistidine was isolated from hydrolysed whale-meat extract[19] and used for a synthesis * to confirm the structure (IV), proposed for balenine. Phthaloyl-β-alanine (V) was condensed with L-3-methylhistidine methyl ester (VI), using NN'-dicyclohexylcarbodi-imide as the coupling agent, to give phthaloyl-β-alanyl-L-3-methylhistidine methyl ester (VII) which crystallised well from methanol. Mild acid hydrolysis of compound (VII) gave phthaloyl-β-alanyl-L-3-methylhistidine



Reagents: 1, *NN'*-Dicyclohexylcarbodi-imide; 2, HCl; 3, N_2H_4

(VIII) from which the phthaloyl group was removed by reaction with hydrazine, giving the free dipeptide (IV), which was crystallised from aqueous alcohol. The natural and the synthetic compounds were identical chromatographically in solvent B and they had the same melting point, undepressed on mixing. The specific rotation of the synthetic compound was slightly lower than for the natural compound.

Experimental

Chromatographic separations on Whatman 3MM paper were carried out in the following solvent mixtures: (A) n-butanol–acetic acid–water (60 : 15 : 25 v/v), descending; (B) phenol–water (4 : 1 w/v), ascending, in an atmosphere of hydrochloric acid provided by placing a dish of concentrated hydrochloric acid and water (3 : 2 v/v) in the tank. The locating reagents were: (i) ninhydrin (0·2%) in acetone containing 1% pyridine, for amino-acids; (ii) Pauly's reagent, for histidine derivatives; (iii) iodine (1%) in carbon tetrachloride, which revealed 3-methylhistidine derivatives as faint brown spots. Reagent (iii) was used prior to the discovery of (iv) *p*-nitrobenzenediazonium fluoborate (Eastman Kodak No. P. 7078) (1%) in acetone, and potassium hydroxide, (0·1N) in 95% ethanol. The diazonium reagent was applied to the chromatogram by dipping, and the alkali was sprayed on.[17] All other reagents were applied by dipping.

Chromatography of the Peptides.—Authentic samples of carnosine and anserine were obtained from B. Newton Maine Ltd. and the California Corporation for Biochemical Research, respectively. Balenine (R_F 0·64) was separated from carnosine (R_F 0·38) and anserine (R_F 0·56) by paper chromatography in solvent B. R_F values in this solvent were: 3-methylhistidine 0·51; 1-methylhistidine 0·44; and histidine 0·23.

Isolation of Balenine from Whale-meat Extract.—Ethanol (1600 ml.) was added slowly, with stirring, to a hot solution of whale-meat extract (204 g.) in water (200 ml.) and the mixture was refrigerated overnight, filtered, and then treated with a solution of mercuric sulphate (25% w/v in 10% v/v sulphuric acid) to precipitate the nitrogenous bases as their mercuric salts. After several hours the precipitate was filtered off, resuspended in water (2 l.) and decomposed with

* After the submission of this work, a Paper by H. Rinderknecht *et al.*[19a] came to our attention, in which the synthesis of β-alanyl-3-methylhistidine ("isoanserine") was described. These workers, however, obtained a compound with m. p. 239—243° (decomp.) $[\alpha]_D^{21}$ +33·5° (*c* 1, in water).

[18] D. H. Cocks, P. O. Dennis, and T. H. Nelson, unpublished work; K. Crush, *J. Sci. Food Agric.*, **1964**, **15**, 550.

[19] D. H. Cocks, P. O. Dennis, and T. H. Nelson, *Nature*, 1964, **202**, (4928), 184.

[19a] H. Rinderknecht, T. Rebane, and V. Ma, *J. Org. Chem.*, 1964, **29**, 1968.

hydrogen sulphide. The filtrate and washings from the mercuric sulphide were evaporated under reduced pressure to 1 l. and adjusted to pH 5 with barium hydroxide solution; barium sulphate was filtered off and washed well with water. The filtrate and washings were evaporated to 1 l. and a solution of silver nitrate (50 g. per 100 ml. of water) was added until a drop of the mixture gave a dark brown precipitate with an excess of barium hyroxide (spot test). The brown precipitate at pH 5 (purine fraction) was removed by centrifugation. Similarly the histidine and arginine fractions were precipitated at pH 7 and pH 13, respectively, and removed. The strongly alkaline solution (lysine fraction) was adjusted to pH 7 with sulphuric acid and barium sulphate was filtered off. The lysine fraction was subjected to a further treatment with mercuric sulphate and alcohol and the mercury was removed as before. The solution obtained was adjusted to pH 7, filtered, and evaporated under reduced pressure to 580 ml., it was then heated to boiling and recrystallised picric acid (27·5 g.) was added. The cooled solution rapidly deposited long silky needles of *balenine dipicrate* (37·85 g.) m. p. 135—140° (136—139° after recrystallisation) (Found: picric acid, 65·8. $C_{10}H_{16}N_4O_3,(C_6H_3N_3O_7)_2$ requires picric acid 65·6%). In this way the balenine was completely separated from the other ninhydrin-positive impurities in the lysine fraction. A portion of the picrate was suspended in dilute sulphuric acid and extracted with benzene to remove picric acid. The aqueous phase was treated with charcoal to remove the last traces of picric acid and the sulphuric acid was neutralised with an exactly equivalent amount of barium hydroxide solution; barium sulphate was filtered off and washed with water. The filtrate and washings were evaporated under reduced pressure to a syrup and addition of ethanol caused the peptide to crystallise in clumps of fine needles, m. p. 259—261° (decomp.). A sample of the peptide was recrystallised by dissolving it in the minimum quantity of hot 50% aqueous ethanol and then adding absolute alcohol. The product obtained was stored *in vacuo* over phosphorus pentoxide overnight, it had m. p. 260—262° (decomp.), $[\alpha]_D^{23}$ +34·8° (*c* 1·31, in water). (Found: C, 47·8; H, 6·9; N, 22·2%; *M* (by titration), 254. $C_{10}H_{16}N_4O_3,\frac{1}{2}H_2O$ requires C, 48·2; H, 6·9; N, 22·5%; *M*, 258·3).

Balenine Monopicrolonate and Dipicrolonate.—These were prepared by treating solutions of balenine (10% w/v in 50% ethanol–water) with one and two equivalents, respectively, of picrolonic acid in 80% ethanol–water. Crystallisation was induced by seeding with material obtained by evaporation of small quantities of the solutions. Monopicrolonate, m. p. 234—236° (decomp.); dipicrolonate, m. p. 164° (decomp.).

Hydrolysis of Balenine with Hydrochloric Acid.—A solution of balenine (20 mg.) in 50% hydrochloric acid (1 ml.) was heated on a boiling water bath for 4 hr. The solution was cooled and neutralised by shaking with a solution of tri-n-octylamine in chloroform.[20] Paper chromatograms of the hydrolysate revealed two ninhydrin-positive substances R_F 0·14 and 0·33 in Solvent A and 0·53 and 0·40 in Solvent B. These corresponded to 3-methylhistidine (R_F 0·14 in Solvent A and 0·52 in Solvent B) and β-alanine (R_F 0·31 in Solvent A and 0·40 in Solvent B).

Synthesis of Balenine.—Phthaloyl-β-alanine (V). A mixture of equimolar amounts of phthalic anhydride (resublimed) and β-alanine was heated at 170—180° in an oil-bath for 30 min. The crude material was cooled and recrystallised from water as needles, m. p. 153—156° (lit.,[21] 152—153°) (Found: N, 6·5. Calc. for $C_{11}H_9NO_4$: N, 6·4%).

3-Methylhistidine methyl ester dihydrochloride. 3-Methylhistidine monohydrochloride was dried at 140° and converted into the *ester dihydrochloride*, m. p. 207° (decomp.) by the method of Fischer and Cone [22] (Found: C, 37·8; H, 6·0; N, 16·8. $C_8H_{15}Cl_2N_3O_2$ requires C, 37·5; H, 5·9; N, 16·4%).

Phthaloyl-β-alanyl-3-methylhistidine methyl ester (VII). A solution of 3-methylhistidine methyl ester (free base) (VI) in chloroform, prepared from the dihydrochloride (4·43 g.) by the method of Fischer and Cone,[22] was stirred overnight with phthaloyl-β-alanine (3·78 g.) and DCCl (3·76 g.). The precipitate of dicyclohexylurea was filtered off, the filtrate was evaporated to dryness, and the *ester* was crystallised from methanol (60 ml.) as radiating masses of needles (6·10 g., 76%), m. p. 186—187°, R_F 0·64 in Solvent A (Found: C, 59·7; H, 5·4; N, 14·8. $C_{19}H_{20}N_4O_5$ requires C, 59·4; H, 5·25; N, 14·6%).

Phthaloyl-β-alanyl-3-methylhistidine (VIII). A suspension of the ester (6·1 g.) in 2N-hydrochloric acid (25 ml.) was shaken intermittently for 7 days. An insoluble residue was filtered off,

[20] D. E. Hughes and D. H. Williamson, *Biochem. J.*, 1951, **48**, 487.
[21] R. A. Turner, *J. Amer. Chem. Soc.*, 1953, **75**, 2388.
[22] E. Fischer and L. H. Cone, *Annalen*, 1908, **363**, 107.

the filtrate was diluted with water (150 ml.), and neutralised with tri-n-octylamine in chloroform. The neutral solution was evaporated to a syrup which was dissolved in hot methanol. The methanol solution deposited a white solid on standing overnight, (4·57 g., 76%), m. p. 224—225° (decomp.), R_F 0·48 in Solvent A (Found: C, 56·5; H, 4·95; N, 15·0. $C_{18}H_{18}N_4O_5,\frac{1}{2}H_2O$ requires C, 57·0; H, 5·05; N, 14·8%). Alkaline hydrolysis was not applicable to derivatives containing the phthaloyl group and hot acid hydrolysis caused some fission of the peptide bond.

β-*Alanyl-3-methylhistidine* (*balenine*) (IV). A solution of phthaloyl-β-alanyl-3-methylhistidine (4·99 g.) in water (20 ml.) was treated with ethanolic hydrazine hydrate (4·5 ml.; 25% v/v solution) and stored at room temperature for 4 days. The solution was diluted with water (30 ml.) acidified with glacial acetic acid (1·3 ml.) and the precipitated phthaloyl hydrazide was filtered off and washed well with water. The filtrate and washings were freed from acetic acid by repeated evaporation with water. The residual syrup was dissolved in water (4 ml.), made alkaline with concentrated ammonia, and hot ethanol (30 ml.) was added. The peptide (2·32 g., 71·5%) crystallised from the cooled solution in clumps of needles, m. p. (after recrystallisation) 260—263° (decomp.), undepressed on admixture with the natural peptide; $[\alpha]_D^{20}$ +32·7° (*c* 1·4, in water) (Found: C, 47·8; H, 7·0; N, 21·9%).

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