[CONTRIBUTION FROM THE WESTERN REGIONAL RESEARCH LABORATORY[1]]

A New Sulfur-containing Amino Acid from Subtilin[2]

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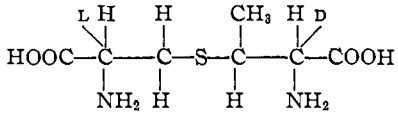
The isolation and some properties of a new naturally occurring thioether amino acid from the antibiotic peptide subtilin are described. The structure has been proposed as one of the α-amino-β-(2-amino-2-carboxyethylmercapto)-butyric acids. The configurations at the two α-carbon atoms have been determined.

In a previous communication[3] part of the sulfur of the antibiotic peptide subtilin was shown to be present as lanthionine. The remainder has been found to be present as a second diamino, dicarboxylic sulfide acid, $C_7H_{14}O_4N_2S$, which forms the subject of this paper. The amino acid was isolated from the mother liquors resulting from the isolation of lanthionine from HCl hydrolyzates of subtilin. Raney nickel desulfurization of the amino acid followed by reaction with dinitrofluorobenzene and chromatography of the DNP product on silica gel gave DNP-L-alanine[4] and DNP D-α-amino-*n*-butyric acid. Since optically active desulfurization products were obtained and since the $C_7H_{14}O_4N_2S$ compound is distinguished from the cystathionines by higher optical rotation and by biological properties,[5] the following structure is proposed



The configuration of the third asymmetric center in this naturally occurring α-amino-β-(2-amino-2-carboxyethylmercapto)-butyric acid has not yet been determined.

Work on degradation and on synthesis of stereoisomers of the above structure *via* the enzymatically resolved thiothreonines will be reported later.

DNP-L-Alanine and DNP-L-α-amino-*n*-butyric acid for comparison with the DNP derivatives of the Raney nickel desulfurization products of the $C_7H_{14}O_4N_2S$ compound were prepared by Porter and Sanger's method[6] from L-alanine and L-α-amino-*n*-butyric acid, which were in turn obtained from L-cysteine·HCl and L-methionine, respectively, by Raney nickel desulfurization. Comparisons on the basis of R_f values on buffered silica gel, X-ray powder diagrams, optical rotation, and ultraviolet and visible spectra established the DNP desulfurization products of the amino acid as DNP-L-alanine and DNP-D-α-amino-*n*-butyric acid. DNP-α-Amino-*n*-butyric acid appears to be a new compound. The specific rotation of the DNP amino acids was found to undergo such a large shift (147° for DNP-alanine) between the free acid and sodium salt as to be of potential utility in establishing optical form of the small amounts of DNP amino acids obtained in protein structure studies by Sanger's method.[7]

Samples of the $C_7H_{14}O_4N_2S$ amino acid from the second preparation described below were supplied by J. C. Lewis and Neva Snell of this Laboratory, who have reported[5] paper chromatographic analysis of the ratio of lanthionine to the $C_7H_{14}O_4N_2S$ compound in subtilin and a Neurospora test of the absence of cystathionine, and to J. F. Carson, who has described[8] the properties of the N,N′-bis-DNP-derivative. Recently a sample of the same material was furnished to E. P. Abraham of Oxford University. He has reported that X-ray powder photographs, taken in the Department of Crystallography, have shown it to be identical with an amino acid occurring in the antibiotic nisin, which Berridge, Newton and Abraham[9] had tentatively assumed was one of the cystathionines. Benedict, *et al.*,[10] reported that an amino acid with similar behavior in paper chromatographic analysis occurs in hydrolyzates of the antibiotic cinnamycin.

The formulation of a name for this amino acid is rendered difficult by the presence of three asymmetric centers for which rules have not yet been adopted. After helpful correspondence on the question with Dr. H. B. Vickery we have deferred naming the substance pending future decisions of the nomenclature committees.

Experimental

Isolation.—The new amino acid is relatively water soluble, as compared to lanthionine, and thus it can be separated from lanthionine fairly readily by fractional crystallization. When the Horn, Jones and Ringel[11] method of lanthionine isolation is applied to subtilin (without prior alkali treatment) the new amino acid follows the lanthionine into the pyridine–alcohol precipitate. On repeated recrystallization of this fraction from water, the $C_7H_{14}O_4N_2S$ compound is found in the mother liquors, from which it can be recovered by addition of alcohol to 30% by volume followed by standing in the cold. Recrystallization is carried out by dissolving the crystals in water, adding alcohol to a concentration of 20–30%, and chilling. By this method 320 mg. of 3 times recrystallized product were obtained from the hydrolyzate of 10 g. of highly active subtilin.

A larger preparation was made from 120 g. of subtilin of lower activity by a variation of the above method as follows: The vacuum-concentrated, carbon-decolorized, 6 *N* HCl hydrolyzate as a thick sirup (95 ml.), was dissolved in 800 ml. of absolute alcohol, neutralized with 50 ml. of pyridine and the resulting suspension was allowed to stand in the cold overnight. After separation and washing with absolute alcohol, the precipitate was mixed with sufficient water

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(2) Presented in part before the Division of Biological Chemistry at the American Chemical Society Meeting, Portland, Oregon, September, 1948, and at the 123rd National Meeting in Los Angeles, March, 1953.

(3) Gordon Alderton and H. L. Fevold, THIS JOURNAL, **73**, 463 (1951).

(4) DNP will be used as an abbreviation for 2,4-dinitrophenyl.

(5) J. C. Lewis and Neva Snell, THIS JOURNAL, **73**, 4812 (1951).

(6) R. R. Porter and F. Sanger, *Biochem. J.*, **42**, 287 (1948).

(7) F. Sanger, *ibid.*, **39**, 507 (1945); **40**, 261 (1946); **42**, 287 (1948).

(8) J. F. Carson, THIS JOURNAL, **74**, 1480 (1952).

(9) N. J. Berridge, G. G. F. Newton and E. P. Abraham, *Biochem. J.*, **52**, 529 (1952).

(10) R. G. Benedict, W. Dvonch, O. L. Shotwell, T. G. Pridham and L. A. Lindenfelser, *Antibiotics and Chemotherapy*, **2**, 591 (1952).

(11) M. J. Horn, D. B. Jones and S. J. Ringel, *J. Biol. Chem.*, **144**, 87 (1942).

to give an alcohol concentration of approximately 40% and then centrifuged, yielding 420 ml. of extract. The precipitate was similarly extracted successively with 20% alcohol (110 ml. of extract) and 10% alcohol (64 ml. of extract), and then dissolved in 1 *N* HCl. This solution was neutralized to *p*H 5 with NaOH, made to 30% in alcohol, chilled for 3 days, and filtered. The precipitate consisted of lanthionine, which was purified further by recrystallization from water. The filtrate plus the above 40, 20 and 10% alcohol extracts, which contained the new amino acid, were vacuum-concentrated to 100 ml. and allowed to crystallize in the cold. The resulting precipitate (15 g.) was recrystallized by solution in water, addition of alcohol to 30%, and chilling to yield 4 g. of product, which was extracted with 50 ml. of hot water. The insoluble portion (71 mg.) was discarded. From the water extract 2.9 g. of the $C_7H_{14}O_4N_2S$ amino acid were recovered in two fractions, one (1.85 g.) by chilling overnight in the refrigerator, the second (1.05 g.) by adding alcohol to 20% at room temperature to the filtrate. Each had essentially the same elementary analysis and rotation.

Anal. Calcd. for $C_7H_{14}O_4N_2S$: N, 12.60; S, 14.42; C, 37.8; H, 6.35; N (amino), 12.6. Found: N, 12.4; S, 14.4; C, 37.4; H, 6.43; N (amino), 12.9; ash, 0.8; $[\alpha]^{24}D$ −34.7° (*l* 1, *c* 5.40 in 1.01 *N* HCl).

The yields mentioned above (approx. 10% as judged by paper chromatographic analysis)[5] could be improved by reworking mother liquors but probably more profitably by ion exchange chromatography.

DNP-L-α-Amino-*n*-butyric Acid (Synthetic).—L-α-Amino-*n*-butyric acid was prepared by a modification of the method of Fonken and Mozingo[12] by Raney nickel desulfurization of L-methionine. The modification, which resulted in a considerably improved yield, consisted of removing dissolved nickel from the bluish-green Raney nickel filtrate by shaking with 2-thenoyltrifluoroacetone (Dow TTA) in ether. After removal of nickel the aqueous phase was evaporated to a sirup and the L-α-amino-*n*-butyric acid precipitated with alcohol in a yield of 57%. No methionine could be detected by paper chromatography.

Anal. Calcd. for $C_4H_9O_2N$: C, 46.6; H, 8.80; N, 13.6. Found: C, 46.7; H, 8.71; N, 13.5; $[\alpha]^{24}D$ 19.6° (*l* 2, *c* 5.00 in 6.0 *N* HCl).

DNP-L-α-Amino-*n*-butyric acid was prepared from this material by the method of Porter and Sanger,[6] and recrystallized from water by acidification of the sodium salt and from acetic acid–water.

Anal. Calcd. for $C_{10}H_{11}O_6N_3$: C, 44.6; H, 4.12; N, 15.6. Found: C, 44.9; H, 4.30; N, 15.5; $[\alpha]^{26}D$ −38.0° (*l* 1, *c* 0.991 in ethyl acetate), $[\alpha]^{27}D$ 98.7° (*l* 1, *c* 0.62 in 0.62% $NaHCO_3$), $[\alpha]^{27}$ 95° (*l* 1, *c* 0.62 in 0.62% $NaHCO_3$, white light). The rotation of the latter solution remained unchanged after 24 hours at room temperature. DNP-α-Amino-*n*-butyric acid (R_f 0.04) can be separated readily from DNP-alanine (R_f 0.01) on *p*H 7.8 buffered silica gel. X-Ray powder data are shown in Table I.

DNP-L-Alanine (Synthetic).—L-Alanine was prepared from L-cysteine·HCl by Raney nickel desulfurization as above.

Anal. Calcd. for $C_3H_7O_2N$: C, 40.4; H, 7.92; N, 15.7. Found: C, 40.1; H, 7.88; N, 15.5; $[\alpha]^{24}D$ 13.6° (*l* 2, *c* 5.00 in 0.999 *N* HCl).

DNP-L-Alanine was prepared from this sample by the method of Porter and Sanger.[6] The rotation of this compound appears not to have been reported.

Anal. Calcd. for $C_9H_9O_6N_3$: C, 42.3; H, 3.55; N, 16.5. Found: C, 42.1; H, 3.83; N, 16.1; $[\alpha]^{27}D$ −11° (*l* 1, *c* 0.99 in ethyl acetate), $[\alpha]^{27}D$ 136° (*l* 1, *c* 1.02 in 1.02% NaHCO), $[\alpha]^{27}$ 133.4° (*l* 1, *c* 1.02 in 1.02% $NaHCO_3$, white light). X-Ray diffraction data are shown in Table I.

Raney Nickel Desulfurization.—Fifty mg. of the $C_7H_{14}N_2O_4S$ amino acid in 25 ml. of 20% alcohol was refluxed for 2 hr. with about 1 g. of Raney nickel (prepared by the method of Mozingo[13] with a digestion period of 1 hr. at 70°). The nickel was extracted 3 times with 10% aqueous ammonia and the extracts were evaporated to dryness in vacuum. Paper chromatograms of the resulting material with butanol–acetic acid and with *o*-cresol showed only two spots which corresponded in position with known DL-alanine and DL-α-amino-*n*-butyric acid run singly and mixed on the same sheets. The light green water solution of the Raney nickel products was freed of nickel by repeated shaking with 2-thenoyltrifluoroacetone (Dow TTA) in ether. The aqueous phase was then treated with dinitrofluorobenzene by the method of Sanger.[6] The resulting DNP amino acids were separated by chromatography on buffered silica gel (500 g. of G. F. Smith silica gel plus 400 ml. of 1 *M* phosphate buffer *p*H 7.80) with water-saturated chloroform as the developing solvent. Two intense bands with R_f values of 0.01 (DNP-alanine) and 0.04 (DNP-α-amino-*n*-butyric acid) were obtained. When the bands were well separated on the column, each was dug out and eluted from the silica gel with water-saturated ethyl ether. Each eluted fraction was rechromatographed in the same way to yield 19 mg. of DNP-α-amino-*n*-butyric acid and 25 mg. of DNP-alanine, which were identified as described below.

TABLE I

X-RAY POWDER DATA

CuK_α = 1.5418 Å.

DNP-L-Alanine		DNP-L-α-Amino-*n*-butyric acid	
d	*I*[a]	*d*	*I*[a]
18.9	10	12.2	10
6.35	10	8.88	25
6.00	15	6.82	8
5.26	18	5.73	47
4.73	12	5.02	7
4.48	12	4.70	7
4.23	40	4.44	27
3.70	100	3.98	39
3.52	25	3.78	28
2.95	30	3.58	31
2.82	10	3.42	100
2.60	12	3.22	18
2.54	50	3.11	40
2.20	50	2.99	47
2.08	90	2.84	22

[a] Intensities were estimated visually by comparison with a calibrated intensity scale.

Identification of DNP Raney Nickel Desulfurization Products.—The fraction of slow mobility on buffered silica gel was established as a resolved DNP-α-alanine by an X-ray powder diagram which was identical with that of known DNP-L-alanine (Table I). Ultraviolet and visible spectra on the Cary recording spectrophotometer likewise checked that of the known. The configuration was shown to be of the L-series by a positive rotation, $[\alpha]^{25}W$ 116° (*l* 2, *c* 0.519 in 1% $NaHCO_3$).

The faster (R_f 0.04) fraction was identified as one of the DNP-α-amino-*n*-butyric acids by identity of the X-ray powder diagram with that of DNP-L-α-amino-*n*-butyric acid. Ultraviolet and visible spectra were similar. A negative rotation $[\alpha]^{25}W$ −81° (*l* 1, *c* 0.725 in 1% $NaHCO_3$), established the configuration as D.

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(12) G. S. Fonken and Ralph Mozingo, THIS JOURNAL, **69**, 1212 (1947).

(13) R. Mozingo, *Org. Syntheses*, **21**, 15 (1941).