undergo a new type of cyclization reaction. The reactions of both the linear and cyclic compounds are described.

A linear N,N′-dinitroguanidine (N-β-nitroxyethyl-N-nitro-N′-nitroguanidine) has been prepared for the first time. It cyclizes on boiling with water splitting off nitric acid. The cyclized product then hydrolyzes to give 1,2-dinitraminoethane.

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[CONTRIBUTION FROM THE RESEARCH LABORATORIES, THE UPJOHN COMPANY, AND THE DEPARTMENT OF BIOCHEMISTRY AND NUTRITION, TUFTS COLLEGE MEDICAL SCHOOL]

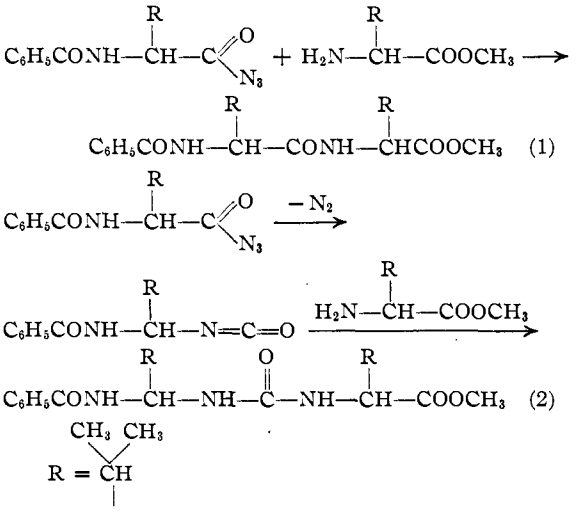
The Isomeric Dipeptides of Valine Including a Correction[1]

BY J. W. HINMAN, E. LOUIS CARON AND HALVOR N. CHRISTENSEN

A dipeptide of astonishing stability was reported to survive the acid hydrolysis of gramicidin and to be present in not one but at least two isomeric forms, namely, D-valyl-D-valine and L-valyl-L-valine.[2,3] The evidence for this view was as follows: low or absent optical activity of the benzoyl derivative; isolation of DL-valine after acid hydrolysis, recovery of half the valine released during hydrolysis as L-valine by microbial assay; comparison of the isolated product with two synthetic mixtures supposed to represent enantiomorphic pairs. Racemization was subsequently observed by the original investigator in the synthetic reaction employed. The product of the reaction of benzoyl-D-valyl chloride and D-valine ethyl ester upon hydrolysis yielded 27 and 30% of the valine as microbiologically available L-valine.[4] Recent studies[5] have pointed out the optical instability of acylamino acid halides. Therefore the problem has been reinvestigated jointly with the conclusion that the dipeptide from gramicidin does indeed consist of a mixture of two enantiomorphic forms, these being D-valyl-L-valine and L-valyl-D-valine, however, rather than the pair originally supposed.

Synthesis

Positive identification of the valylvaline from gramicidin required the synthesis of the isomeric dipeptides of valine by a method which would not permit racemization at any point. Since the benzoyl derivative of the dipeptide from gramicidin was available, the condensation of benzoylvalylazide and valine methyl ether was investigated. However, under conditions normally employed for such a condensation (reaction 1) very little of the desired product was obtained, the major product being the ureide as indicated in reaction 2. Even under the mild conditions employed, the acid azide underwent the Curtius



(1)

(2)

rearrangement before reacting with valine methyl ester. This reaction has been encountered with several other benzamido acid azides and the factors influencing this reaction are now being studied.

The *p*-toluenesulfonyl (tosyl) derivatives of the four optical isomers of valylvaline were prepared by condensing optically active tosylvalyl chloride with optically active valine methyl ester. Preparation of the free dipeptides by removal of the tosyl group through the action of sodium in liquid ammonia did not proceed smoothly; therefore, carbobenzoxy-DL-valyl chloride was condensed in separate reactions with D-valine methyl ester and with L-valine methyl ester to give in each case a mixture of two diastereoisomers of carbobenzoxyvalylvaline methyl ester.[6] The diastereoisomers were readily separated by fractional crystallization. The carbobenzoxy group was removed from one of the pure isomers by catalytic hydrogenation, the valylvaline ester tosylated, and the product identified by com-

(1) An abstract of this paper was presented before the Division of Biological Chemistry at the 116th National meeting of the American Chemical Society in Atlantic City, September, 1949.

(2) Abbreviated, *DvDv* and *LvLv*.

(3) (a) H. N. Christensen, *J. Biol. Chem.*, **151**, 319 (1943); (b) **154**, 427 (1944).

(4) D. M. Hegsted, *ibid.*, **152**, 193 (1944).

(5) H. E. Carter and J. W. Hinman, *ibid.*, **178**, 403 (1949).

(6) It is interesting to note that Polglase and Smith (THIS JOURNAL, **71**, 3081 (1949)) have recently prepared carbobenzoxy-L-leucyl-D-alanine methyl ester and carbobenzoxy-L-leucyl-L-alanine methyl ester from carbobenzoxy-L-leucine and DL-alanine methyl ester. This practice of preparing and separating diastereoisomers of dipeptides, as they point out, has not been widely used, but is particularly useful in some cases.

parison with the previously synthesized tosylvalylvaline methyl ester. The configurations of the remaining three isomers could then be deduced immediately.

The four benzoylvalylvaline methyl esters were prepared from the carbobenzoxyvalylvaline esters of known configuration by reductive decarbobenzoxylation and benzoylation of the resulting dipeptide esters. In each case the free acids were readily prepared by saponification of the esters. The racemic modifications[7] were prepared by mixing equal amounts of the two enantiomorphs and recrystallizing the mixture. The free valylvaline isomers were prepared by the reductive removal of the acyl group of the carbobenzoxyvalylvalines.

Isolation from Gramicidin

Two preparations of gramicidin were used: one studied previously[3b] and a second (L-1199) obtained from The Wallerstein Company through the courtesy of Mr. Leo Wallerstein. The hydrolysis of gramicidin (six hours) and the removal of hydrochloric acid by evaporation and by silver oxide were accomplished as usual.[3a] A portion of this hydrolysate was subjected to paper chromatography. The copper salts soluble in water and absolute ethanol were separated as before, and after removal of copper a portion was examined again by paper chromatography. Other portions of the solution were treated with benzoyl chloride and tosyl chloride, respectively, in alkaline solutions. The benzoyl derivative was separated and recrystallized as previously described.[3b] Portions of the two derivatives were converted to the respective methyl esters by the action of diazomethane.

Comparison of Isomers

Paper Chromatography.—Using a 50% *n*-butanol–25% acetic acid–25% water system in one-dimensional ascending paper strip chromatograms, small but reproducible differences were observed in the R_f values of the synthetic DvDv and LvLv[2] on the one hand and DvLv and LvDv on the other. The diastereoisomers could be separated but the enantiomorphs could not as would be expected in the case of true partition chromatography. When hydrolysates of gramicidin containing valylvaline were subjected to the same procedure, using the synthetic isomers as controls, R_f values of the "natural" valylvaline fell consistently at the same place as for DvLv, LvDv, and the racemic modification of these two. The other two isomers could have been present only in such small quantities as to be indetectable by the procedure.

When the whole hydrolysate was subjected to paper chromatography, spots were observed corresponding to glycine, alanine, valine, leucine, tryptophan, ethanolamine and valylvaline.[8] The fraction obtained by the elimination of copper salts insoluble in water and ethanol contained smaller amounts of free amino acids, although traces of valine, glycine, alanine and ethanolamine were detected in addition to the valylvaline. The results confirm Synge's observation of the presence of ethanolamine.[10]

Other Physical Methods.—The optical inactivity of the benzoyl derivative of the isolated valylvaline along with the chromatographic results suggested that the natural dipeptide might be the racemic modification, DvLv + LvDv. This view was substantiated by the direct comparison of derivatives of synthetic and isolated dipeptides. The melting point of the benzoyl derivative of DvLv + LvDv was the same as that of the natural benzoylvalylvaline, and the melting point was not depressed when the two were mixed. The same melting point behavior was observed for the corresponding methyl esters, the corresponding tosyl derivatives and their methyl esters.

The infrared spectra of chloroform solutions of all the benzoylvalylvaline methyl ester isomers, surprisingly, were too similar for good differentiation. However, the Nujol mull spectra were characteristic (the enantiomorphs giving identical spectra) and the spectrum for the racemic modification DvLv + LvDv was identical to that of the isolated material (Fig. 1). Exactly the same results were obtained in a comparison of the X-ray diffraction patterns of the various benzoyl derivatives. The patterns in Fig. 2 again show the identity of the synthetic benzoyl DvLv + LvDv methyl ester and the corresponding derivative of the isolated dipeptide as well as the non-identity of the latter and synthetic benzoyl DvLv methyl ester.

Stability of the Isomeric Dipeptides to Acid.—With the configuration of the isolated valylvaline rigorously established and the absence of the DvDv and LvLv isomers in the final hydrolysate demonstrated (by paper chromatography) a study was made of the relative stability of the various isomers, for wide differences in the stability of these isomeric dipeptides to acid hydrolysis could be an important factor in determining the configuration of the valylvaline surviving hydrolysis. After thirty hours in 6 *N* hydrochloric acid at 100°, DvDv was split to the extent of 37%; LvLv, 35%; DvLv, 43%; LvDv, 44%. Hence, all four isomers were re-

(7) The term *racemic modification* is used without implication of interaction between the isomers, although evidence was obtained to indicate that the crystal structure of the racemic modifications was different from that of the component enantiomorphs.

(8) No phenylalanine was detected in the gramicidin hydrolysates. This was considered as evidence to show that the gramicidin used in these studies was probably rather pure gramicidin A (J. D. Gregory and L. C. Craig, *J. Biol. Chem.*, **172**, 839 (1948)), for Gregory has reported through Synge[9] that phenylalanine was detected in gramicidin B.

(9) R. L. M. Synge, *Biochem. J.*, **44**, 542 (1949).

(10) R. L. M. Synge, *ibid.*, **39**, 355 (1945).

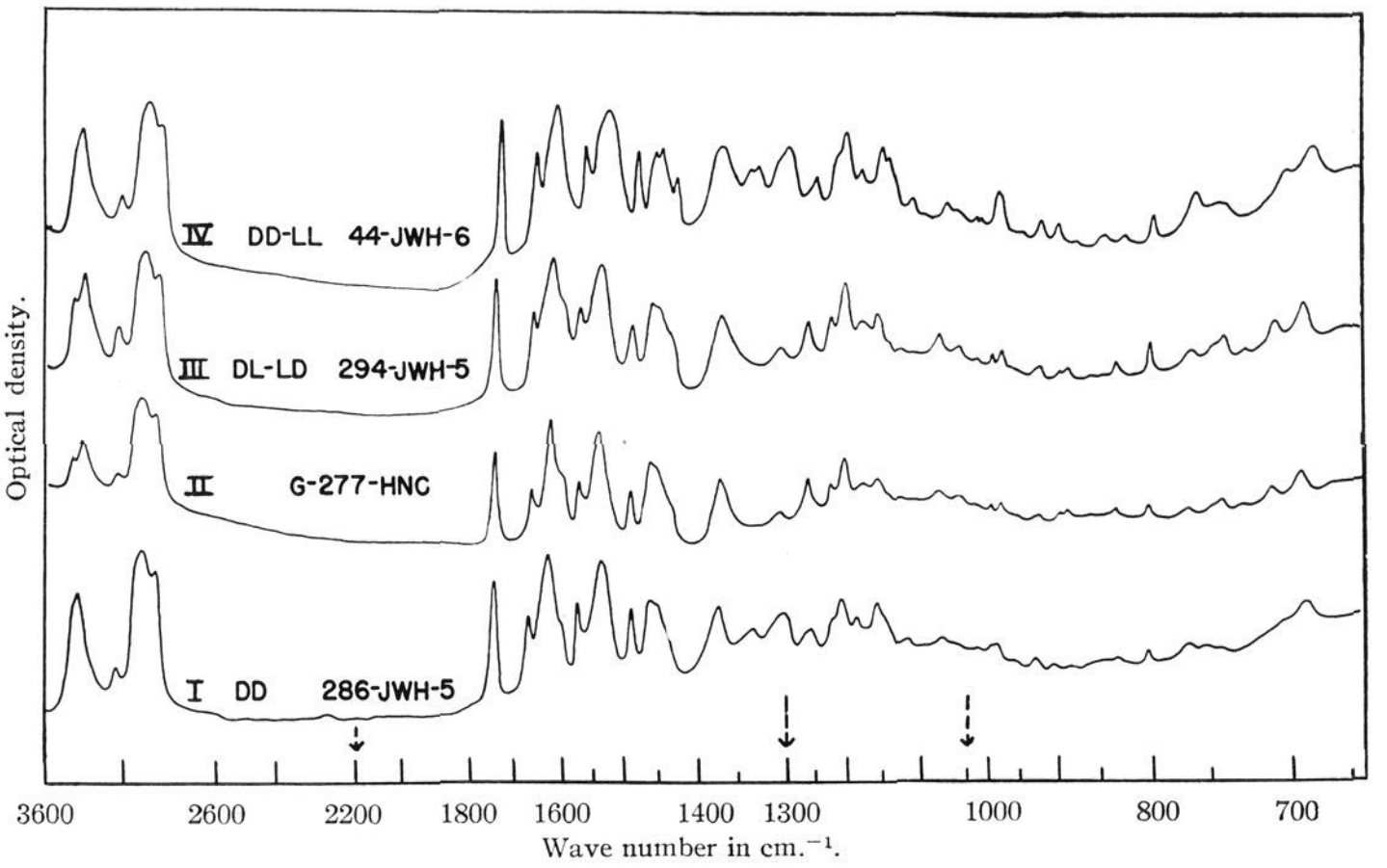


Fig. 1.—Infrared spectra of benzoyl-DvDv methyl ester (Curve I), "natural" benzoylvalylvaline methyl ester (Curve II), benzoyl DvLv + LvDv methyl ester (Curve III), and benzoyl DvDv + LvLv methyl ester (Curve IV); Nujol mulls, Perkin–Elmer infrared spectrometer.

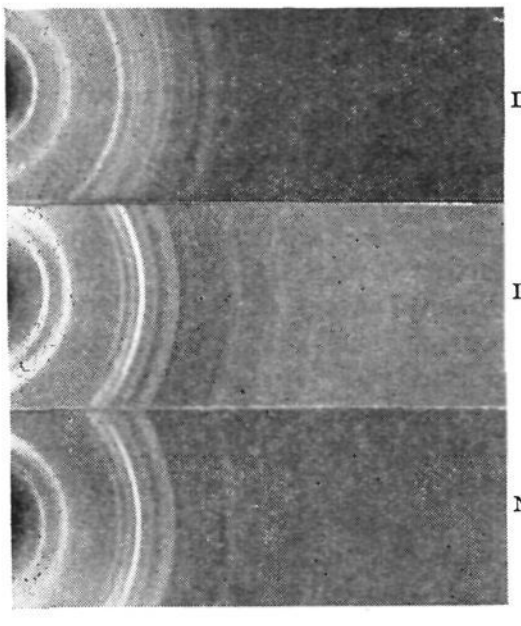


Fig. 2.—X-Ray diffraction patterns of "natural" benzoylvalylvaline methyl ester (natural), benzoyl DvLv–LvDv methyl ester (DL–LD) and benzoyl DvLv methyl ester (DL).

markably resistant to acid,[11] although the isomers containing two valine residues of the same configuration were consistently a little more stable. Therefore, it seems highly unlikely that the DvDv and LvLv isomers were formed in any appreciable quantity at any stage during the hydrolysis or they would have been detected by the methods used.

(11) Abderhalden and Vlassopoulos (*Fermentforschung*, **10**, 365 (1929)) who prepared L-valyl-L-valine in poor yield by amination of α-bromo-isovalerylvaline and by hydrolysis of valine anhydride reported its alkaline hydrolysis to be very slow. Fischer and Scheibler (*Ann.*, **353**, 136 (1908)) prepared D-valyl-L-valine by amination of the bromo derivative and presented data to show that its acid hydrolysis was very slow.

Experimental[12]

Valine.[13]—DL-Valine was resolved through its formyl derivative by the method of Fischer[14] yielding formyl-D-valine, m. p. 155–155.5°, $[\alpha]^{29}D$ +17.2° (5.0% in water); formyl-L-valine, m. p., 153–154.5°, $[\alpha]^{24}D$ −16.6° (1.0% in water); D-valine, $[\alpha]^{28}D$ −5.7° (5.0% in water); and L-valine, $[\alpha]^{25}D$ +5.9° (5.0% in water).

Valine Methyl Ester Hydrochloride.—The enantiomorphs of this compound were prepared from resolved valine by the methanolic hydrogen chloride method according to Synge[15] yielding D-valine methyl ester hydrochloride, m. p. 149–152°, $[\alpha]^{24}D$ −13.8° (1.16% in water) and L-valine methyl ester hydrochloride, m. p. 151–153°, $[\alpha]^{24}D$ +14.3° (1.12% in water).

Benzoyl-D-valine Methyl Ester.—D-Valine was benzoylated according to the procedure of Carter and Stevens.[16] After recrystallization from benzene the crystals melted at 134.5–135.5°; $[\alpha]^{31}D$ −51.0° (1.04% in chloroform).

Anal. Calcd. for $C_{12}H_{15}NO_3$ (221.3): N, 6.33. Found: N, 6.46.

The benzoyl derivative was converted to the methyl ester by the action of diazomethane or by the methanolic hydrogen chloride method according to the procedure of Synge.[15] A nearly quantitative yield was obtained by

(12) All melting points are given corrected as obtained on a Kofler microhotstage with a heating rate of 2–3° per minute at the melting point. Optical rotations were determined in all-glass polarimeter tubes 0.95 dm. long. Concentrations are expressed as g. per 100 ml. of solution. Neutralization equivalent values were obtained by potentiometric titration using *ca.* 0.05 mM. samples.

(13) Gifts of valine or valine derivatives from Dr. Wm. C. Rose, Dr. C. E. Meyer and Dr. H. E. Carter are gratefully acknowledged.

(14) E. Fischer, *Ber.*, **39**, 2320 (1906).

(15) R. L. M. Synge, *Biochem. J.*, **42**, 99 (1948).

(16) H. E. Carter and C. M. Stevens, *J. Biol. Chem.*, **133**, 117 (1940).

either method; m. p. 109.0–109.5°; $[\alpha]^{28.5}D$ −43.0° (1.17% in chloroform).

Anal. Calcd. for $C_{13}H_{17}NO_3$ (235.3): C, 66.36; H, 7.29. Found: C, 66.18; H, 7.11.

Benzoyl-D-valyl Hydrazide.—Benzoyl-D-valine methyl ester (1.19 g., 5.06 mM.) was dissolved in 2.4 ml. of commercial absolute ethanol and 0.5 ml. of 100% hydrazine hydrate. During three hours of heating under reflux the solution gradually filled with a gel. About 10 ml. of ethanol was added to dissolve the gel and after repeated warming, cooling and scratching fine needle crystals were obtained. The product was collected, washed with water, and dried *in vacuo* to yield 0.92 g. (67%); m. p., 209–210°. The analysis indicated a dihydrate.

Anal. Calcd. for $C_{12}H_{17}N_3O_2 \cdot 2H_2O$ (271.3): N, 15.49. Found: N, 15.55.

Benzoyl-L-valyl Hydrazide.—This compound was prepared from benzoyl-L-valine methyl ester according to the above procedure without isolation of the intermediates; m. p. 208–209°.

N-(1-Benzamidoisobutyl)-N'-(1-carbomethoxyisobutyl)-urea.—Benzoyl-D-valyl hydrazide dihydrate (235 mg., 0.867 mM.) was dissolved in 1.2 ml. of glacial acetic acid (by warming) and 1.2 ml. of water. The solution was chilled in an ice–salt-bath and treated with an ice-cold solution of 83 mg. (1.2 mM.) of sodium nitrite in 0.4 ml. of water. An opaque oil separated at once. On stirring the oil crystallized, was collected by filtration, and washed with ice-cold water. The crystalline azide was dissolved in cold ether and the solution was filtered through a pad of anhydrous magnesium sulfate into an ethereal solution containing *ca.* 1.5 mM. of valine methyl ester (liberated from the hydrochloride by the method of Fischer[17]). Crystallization began within a few minutes and soon became quite voluminous. During this time the evolution of gas (presumably nitrogen) was evident. After standing overnight at room temperature the crystals were collected, washed with ether and recrystallized from boiling acetone. The D–D isomer (assuming retention of configuration during the Curtius rearrangement) was obtained in 70% yield as fine colorless rods which melted at 250–251° with decomposition; $[\alpha]^{26}D \cong +9.5°$ (0.35% in chloroform).

Anal. Calcd. for $C_{18}H_{27}N_3O_4$ (349.4): C, 61.87; H, 7.79; N, 12.02. Found: C, 61.98; H, 7.47; N, 11.85.

The D–L isomer was prepared in the same manner from benzoyl-D-valyl hydrazide and L-valine methyl ester. The crystals of this isomer (m. p. 252–252.5° dec.) appeared under the microscope as long rods or elongated plates which tended to mat together in balls; $[\alpha]^{24}D$ +28.2° (0.37% in chloroform).

Anal. Calcd. for $C_{18}H_{27}N_3O_4$ (349.4): C, 61.87; H, 7.79; N, 12.02. Found: C, 61.61; H, 7.45; N, 12.01.

By working up the mother liquors small amounts of benzoylvalylvaline methyl ester could be obtained. Perhaps at a lower reaction temperature this compound would be the major product. The ureide was characterized by its elementary analysis and its infrared absorption spectrum. The L–L and L–D isomers of this ureide compound were prepared also, but were not as fully characterized.

Tosyl-D-valine.—D-Valine (2.37 g., 0.02 mole) was dissolved in 10 ml. of 2 *N* sodium hydroxide and 20 ml. of water, and the solution was cooled in an ice-water-bath. To this solution was added dropwise and with stirring a solution of 3.82 g. (0.02 mole) of tosyl chloride dissolved in 100 ml. of acetone with concurrent dropwise addition of 10 ml. of 2 *N* sodium hydroxide. After all of the reagents were added, another 50 ml. of acetone was added. The reaction mixture was allowed to come to room temperature and after three hours most of the acetone was removed *in vacuo*. The solution was made acid to congo red paper with hydrochloric acid and placed in the refrigerator for several hours. The crystals were collected and recrystallized from ethanol and water. The yield of pure product was about 70%; m. p. 149.5–150.5° with sublimation starting at 147°; $[\alpha]^{22}D$ −27.7° (1% in ethanol).

Anal. Calcd. for $C_{12}H_{17}NO_4S$ (271.3): neut. equiv., 271.3. Found: neut. equiv., 274.

Tosyl-L-valine.—This compound was prepared from L-valine by the method given above for the D-isomer; m. p. 149.5–150.5° with sublimation starting at 147°; $[\alpha]^{22}D$ +27.4° (1% in ethanol). Karrer and van der Slugs Veer[18] reported m. p. 147° and $[\alpha]D$ +25.0° (in ethanol) for tosyl-L-valine.

Anal. Calcd. for $C_{12}H_{17}NO_4S$ (271.3): neut. equiv., 271.3. Found: neut. equiv., 274.

Tosylvalylvaline Methyl Ester.—The four optical isomers of this compound were prepared according to the following procedure. The racemic modifications were prepared by mixing equal amounts of enantiomorphs and recrystallizing the product to constant melting point.

Phosphorus pentachloride (1.75 g., 8.4 mM.) was added to a solution of 1.90 g. (7 mM.) of optically active tosylvaline in 50 ml. of commercial anhydrous ether. The mixture was shaken for approximately one hour at 5–10° during which time most of the phosphorus pentachloride had dissolved. The solution was washed quickly with 2 30-ml. portions of ice-cold water and then dried over magnesium sulfate for about thirty minutes. The solution was filtered into an ethereal solution of 8 mM. of optically active valine methyl ester (prepared from the hydrochloride by suspending the crystals in ether and adding a slight excess of ethereal diazomethane. When the nitrogen evolution ceased the solution was filtered and concentrated *in vacuo* for a few minutes to remove the excess diazomethane.) Crystals of the product began to separate almost immediately. After four to five minutes 10 ml. of saturated potassium bicarbonate solution was added and the reaction mixture was swirled from time to time during the next hour. The crystals were collected and the ether layer evaporated to dryness to obtain a second crop of the product. The two crops of crystals were combined and dissolved in chloroform. This solution was washed with bicarbonate solution, water, *N* hydrochloric acid and finally with water. The washed chloroform solution was then concentrated *in vacuo* until crystals began to form. Skellysolve B was added and after cooling in the refrigerator overnight 75–90% of the theoretical amount of substantially pure product was obtained. Further purification was accomplished by recrystallization from chloroform and Skellysolve B. Physical constants and analytical values for the individual isomers are given in Table I.

TABLE I

PHYSICAL CONSTANTS AND ANALYTICAL DATA FOR THE ISOMERS OF TOSYLVALYLVALINE METHYL ESTER

Isomer	M. p., °C.	1% in $CHCl_3$ °C.	1% in $CHCl_3$ $[\alpha]D$	Analyses, % Carbon	Hydrogen	Nitrogen
D–D	159.0–159.5	22	−12.1	56.41	7.17	7.31
L–L	159.0–159.5	22	+12.9	56.58	6.99	7.64
D–L	167.5–168.0	25	+14.6	56.33	7.26	7.52
L–D	167.5–168.0	25	−14.5	56.01	7.18	7.37
DD–LL	136–139	..	....	56.76	7.14	7.29
DL–LD	149–151	..	....	56.35	7.12	7.48
Calculated for $C_{18}H_{28}N_2O_5S$ (384.5)				56.23	7.34	7.29

Tosylvalylvaline.—The isomers of tosylvalylvaline methyl ester were saponified according to the following procedure. To a solution of 0.50 g. (1.3 mM.) of the ester in 5 ml. of 3A alcohol was added 5.0 ml. of 0.5 *N* sodium hydroxide (prepared by diluting 50% aqueous sodium hydroxide with 3A alcohol). After standing for eighteen to twenty-four hours at room temperature, 100 ml. of water was added and the solution was made acid to congo red paper by addition of 6 *N* hydrochloric acid.

(17) E. Fischer, *Ber.*, **34**, 433 (1901).

(18) P. Karrer and F. C. van der Slugs Veer, *Helv. Chim. Acta*, **15**, 746 (1932).

Crystallization was allowed to proceed overnight in the refrigerator. The crystals were collected, washed with water and dried in a vacuum desiccator to obtain 90–95% of the theoretical yield. Final purification was effected by recrystallization from 3A alcohol and water except with the DL–LD racemic modification which was recrystallized from chloroform and Skellysolve B. The physical properties of the individual isomers are given in Table II.

TABLE II

PHYSICAL CONSTANTS AND ANALYTICAL DATA FOR THE ISOMERS OF TOSYLVALYLVALINE

Isomer	M. p., °C.	1% in acetone °C.	[α]D	% Nitrogen Found
D–D	194.0–194.5	26	−62.1	7.49
L–L	194.0–194.5	26.5	+64.2	7.51
D–L	200.0–200.5	26.5	−47.7	7.54
L–D	200.0–200.5	27	+49.2	7.28
DD–LL	162–165	..	....	7.50
DL–LD	195–197	..	....	7.31

Calculated for $C_{17}H_{26}N_2O_5S$ (370.5): N, 7.56

Carbobenzoxy-DL-valine.—This compound was prepared according to the method described by Carter, *et al.*,[19] for the preparation of carbobenzoxyglycine. When the reaction mixture was acidified a colorless oil separated. Upon cooling and seeding (seed crystals were kindly supplied by Drs. Marguerite Fling and S. W. Fox) the oil crystallized. After recrystallization from chloroform and Skellysolve B the crystals melted at 72–73°; yield 75–80%.

Anal. Calcd. for $C_{13}H_{17}NO_4$ (251.3): N, 5.58. Found: N, 5.66.

Carbobenzoxyvalylvaline Methyl Ester.—The four optical isomers of this compound were prepared in pairs: carbobenzoxy-DL-valyl chloride was condensed with D-valine methyl ester to yield the D–D and the L–D isomers; the condensation of the DL-acid chloride with L-valine methyl ester yielded the D–L and L–L isomers.

A mixture of 5.00 g. (0.02 mole) of carbobenzoxy-DL-valine and 4.20 g. (0.02 mole) of phosphorus pentachloride in 100 ml. of commercial absolute ether was shaken at 5–10° for approximately one hour. The resulting solution was washed quickly with two 75-ml. portions of ice-cold water and dried briefly over anhydrous magnesium sulfate. The solution was then filtered into a solution of *ca.* 0.025 mole of D-valine methyl ester (liberated from the hydrochloride by the action of diazomethane). At the same time 50 ml. of cold saturated aqueous potassium bicarbonate solution was added to the reaction mixture. The mixture was swirled occasionally and more ether was added (to keep the product from separating) to a final volume of about 350 ml. After thirty minutes at room temperature the bicarbonate layer was diluted with *ca.* 100 ml. of water, and the ether layer was washed successively with water, *N* hydrochloric acid, bicarbonate solution and water, dried for an hour over magnesium sulfate and concentrated *in vacuo*. From time to time the distillation was interrupted and chloroform was added to keep the product from crystallizing. When the solvent became predominantly chloroform, the volume was concentrated to *ca.* 25 ml. and 100 ml. of Skellysolve B was added. Crystallization was allowed to proceed for twenty to thirty minutes at room temperature and then at 3° in the refrigerator overnight. The crystals (long needles) were collected, washed with Skellysolve B, and dried to yield 1.34 g. of what was later shown to be carbobenzoxy-L-valyl-D-valine methyl ester. One recrystallization from chloroform and Skellysolve B provided 1.31 g. of analytically and optically pure material.

The mother liquor from the L–D isomer was concentrated *in vacuo* to a volume of 5 ml. and the rather dense granular crystals which formed were collected, washed with Skellysolve B and dried, yielding 1.72 g. of the crude D–D isomer. Two or three recrystallizations of this material from ethyl acetate and Skellysolve B gave 1.3 g. of substantially pure D–D compound. In this way the crystals were obtained as spherical bundles of very fine needles.

The other two isomers were obtained in the same manner from carbobenzoxy-DL-valine and L-valine methyl ester. The over-all yields based on the carbobenzoxy-DL-valine were between 40 and 50%. Additional material could be obtained by working up the mother liquors. The D–L and L–D isomers were very readily crystallized and purified and their melting points were sharp. On the other hand, the more soluble D–D and L–L isomers were not so readily crystallized and melted over rather wide ranges even after repeated recrystallizations, and when other criteria of purity (*e. g.*, paper chromatography of the valylvaline derived from them) indicated them to be substantially free from contamination. The physical constants and analytical data for these isomers are recorded in Table III.

TABLE III

PHYSICAL CONSTANTS AND ANALYTICAL DATA FOR THE ISOMERS OF CARBOBENZOXYVALYLVALINE METHYL ESTER

Isomer	M. p., °C.	Specific rotation °C.	[α]D	1%	Analyses, % Carbon	Hydrogen	Nitrogen
D–D	100–104	22	+28.5	EtOH	62.92	7.52	7.85
L–L	100–103	22	−21.0	EtOH	62.77	7.49	7.61
D–L	163.0–163.5	25	+12.0	$CHCl_3$	62.89	7.72	7.89
L–D	163.0–163.5	25	−12.0	$CHCl_3$	62.98	7.54	7.73
	Calculated for $C_{19}H_{28}N_2O_5$ (364.4)				62.64	7.74	7.69

Determination of the Configuration of the Carbobenzoxyvalylvaline Methyl Ester Isomers.—The higher melting isomer (250 mg., 0.687 mM.) from the condensation of carbobenzoxy-DL-valyl chloride and D-valine methyl ester was dissolved in 10 ml. of methanol, 1 ml. of glacial acetic acid and 2 ml. of water and hydrogenated for one hour at 15 lb. pressure in the presence of 0.3 g. of 5% palladium-on-charcoal.[20] The catalyst was removed by filtration and washed several times with methanol. Removal of the solvent *in vacuo* left a colorless gum which was dissolved in *ca.* 10 ml. of saturated sodium bicarbonate solution. A portion (0.5 ml.) of this solution was removed for paper chromatography studies and a solution of 0.13 g. (0.68 mM.) of tosyl chloride dissolved in 20 ml. of acetone was added. On stirring, carbon dioxide was evolved and finely divided solid separated. After *ca.* one hour the acetone was removed *in vacuo;* the crystals were collected and recrystallized from ethanol and water to yield 98 mg. (37%) of crystals[21] which melted at 165–168°. A second recrystallization from the same solvents yielded 90 mg. of silky needles which melted at 167–168°. On admixture with a sample of authentic tosyl-L-valyl-D-valine methyl ester (m. p. 167.5–168°) there was no depression in melting point. Thus the configuration of the original carbobenzoxy derivative was established and the configurations of the remaining three isomers could be deduced.

Carbobenzoxyvalylvaline.—The isomers of carbobenzoxyvalylvaline methyl ester were saponified by allowing a solution of 500 mg. (1.37 mM.) of the ester in 5 ml. of 0.5 *N* sodium hydroxide solution (prepared by diluting 9 *N* sodium hydroxide with 3A alcohol) to stand for eighteen to twenty hours at room temperature. The solution was diluted with 35 ml. of water, filtered and acidified with *N* hydrochloric acid. The white precipitate was collected, washed with water, and dried yielding 80–95% of the theoretical amount of carbobenzoxyvalylvaline. The D–L and L–D isomers were obtained as sharp-melting glossy needles by recrystallization from ethanol and water, while recrystallization of the D–D and L–L isomers first from ethanol

(19) H. E. Carter, R. L. Frank and H. W. Johnston, "Org. Synthesis," Vol. 23, John Wiley and Sons, Inc., New York, N. Y., p. 14.

(20) Supplied by The American Platinum Works, Newark, N. J.

(21) The low yield in this experiment appeared to be due to adsorption of the valylvaline methyl ester on the charcoal in the catalyst, for in later experiments of this type the catalyst was washed thoroughly with dilute methanolic hydrogen chloride and good yields were obtained.

and water and then from ethyl acetate and Skellysolve B yielded dense prismatic crystals which melted poorly and appeared to be hydrated (neut. equiv. calcd. for $C_{18}H_{26}N_2O_5 \cdot 2H_2O$, 386.4; found, 385). Drying at 60° in high vacuum gave the anhydrous material. The physical constants and analytical data for the individual isomers are given in Table IV.

TABLE IV

PHYSICAL CONSTANTS AND ANALYTICAL DATA FOR THE ISOMERS OF CARBOBENZOXYVALYLVALINE

Isomer	M. p., °C.	°C.	Specific rotation $[\alpha]_D$	1%	Analyses, % N
D–D	136–139	23	− 7.2	Acetone	7.78
L–L	135–139	24	+ 7.4	Acetone	8.03
D–L	186.5–187	22	+10.5	EtOH	7.86
L–D	187.5–188	21	−11.5	EtOH	7.87
	Calculated for $C_{18}H_{26}N_2O_5$ (350.4)				8.00

Valylvaline.—The four optical isomers of this compound were prepared as the hydrochlorides from the corresponding carbobenzoxy derivatives according to the procedure given above for the decarbobenzoxylation of carbobenzoxyvalylvaline methyl ester. However, upon completion of the hydrogenation, the catalyst was washed with 0.2 *N* methanolic hydrogen chloride to remove any of the dipeptide which was adsorbed on the carbon of the catalyst. The solutions were taken to dryness *in vacuo* and the residues used in the paper chromatography studies without further purification.

Paper Chromatography.—The general techniques of Williams and Kirby[22] were employed in the paper chromatography studies. A number of solvent systems were investigated, but the only one which gave satisfactory results was the 50% *n*-butanol–25% acetic acid–25% water system. Several solvents which are satisfactory for separating amino acids (*e. g.*, phenol–water) failed to separate the diastereoisomers of valylvaline.

In general about 3 λ of the solutions of the synthetic isomers of valylvaline hydrochloride containing approximately 10 mg. per ml. were applied to sheets of Whatman no. 1 paper. The chromatograms were developed for seven and one-half hours at room temperature, then processed according to the procedure used for amino acids.[22] Considering fifty typical experiments, the average R_f value for DvLv and LvDv was 0.84 ± 0.02. On the basis of 35 experiments, the average R_f value for DvDv and LvLv was 0.88 ± 0.02. In analyzing the unknowns control determinations were made with the synthetic isomers on the same chromatogram.

Three gramicidin hydrolysates were examined: an unfractionated six-hour hydrolysate[3a] and two hydrolysates from which most of the amino acids had been removed by fractionation of the copper salts. In each case the valylvaline present in the hydrolysate had an R_f value in good agreement for those obtained with the control DvLv and LvDv or the racemic modification of the two. No evidence for the presence of the other isomers could be detected. The amino acids present in the hydrolysates (especially the unfractionated one) and their approximate R_f values were as follows: glycine 0.37; alanine 0.49; tryptophan 0.61; valine 0.67; leucine 0.78; and ethanolamine 0.57. No phenylalanine could be detected.[9] In making an accurate determination of the amino acids, a smaller quantity of the hydrolysate was applied to the paper, for the color reaction with ninhydrin was much more sensitive for amino acids than for the dipeptides. Traces of faster moving components (presumably other dipeptides) than valylvaline were detected in the hydrolysates but no attempt was made to identify them.

Benzoylvalylvaline Methyl Ester.—The isomers of this compound were prepared as follows: carbobenzoxyvalylvaline methyl ester (250 mg., 0.687 mM.) was decarbobenzoxylated as previously described. The resulting valylvaline methyl ester hydrochloride was dissolved in 10 ml. of water containing 0.4 g. of sodium bicarbonate. Benzoyl chloride (0.20 ml.) was added in two portions with shaking and the benzoyl derivative separated as oily globules which soon crystallized. After about one hour the product was collected, washed with water and recrystallized from ethanol and water yielding 70–75% of the theoretical amount of benzoyl derivative. The racemic modifications were prepared by mixing equal amounts of enantiomorphs and recrystallizing the mixtures from ethanol and water to constant melting point. Further data on the individual isomers are given in Table V.

TABLE V

PHYSICAL CONSTANTS AND ANALYTICAL DATA FOR THE ISOMERS OF BENZOYLVALYLVALINE METHYL ESTER

Isomer	M. p., °C.	°C.	Specific rotation $[\alpha]_D$	c, Solvent	Analyses, % Carbon	Hydrogen	Nitrogen
D–D	171–172	20.5	+11.3	0.93% in acetone	64.45	7.78	8.44
L–L	171.5–172	21	−11.4	0.92% in acetone	...	..	8.42
D–L	187–187.5	27	+ 2.0	1.0% in $CHCl_3$	64.64	7.53	8.43
L–D	187–188	27	− 2.0	1.0% in $CHCl_3$	64.81	7.66	8.19
DD–LL	168–168.5	..	....	.....	64.44	7.81	8.59
DL–LD	186–187.5	..	....	.....	64.81	7.75	8.17
			Calculated for $C_{18}H_{26}N_2O_4$ (334.4)		64.65	7.84	8.38

Benzoylvalylvaline.—Only the DL–LD isomer of this compound was prepared. A solution of 50 mg. (0.15 mM.) of benzoyl DvLv + LvDv methyl ester in 0.4 ml. of 0.5 *N* sodium hydroxide (ethanolic) and 1.0 ml. of 3A alcohol was allowed to stand at room temperature for forty hours. The solution was diluted with 30 ml. of water, filtered and acidified with 6 *N* hydrochloric acid. After cooling the mixture overnight in the refrigerator, 42.5 mg. (88%) of the acid was obtained. Recrystallization from boiling acetone yielded small diamond-shaped crystals which melted at 231–232°.

Anal. Calcd. for $C_{17}H_{24}N_2O_4$ (320.4): neut. equiv., 320.4. Found: neut. equiv., 322.

"Natural" Tosylvalylvaline.—A valylvaline-rich fraction from a six-hour gramicidin hydrolysate[3] was chilled, made alkaline with sodium hydroxide and treated with tosyl chloride. After acidification the resulting crude product was crystallized and recrystallized from ethanol and water yielding 5 mg. of nearly colorless needles, m. p. 194–197°. The infrared absorption of this preparation established its identity as tosylvalylvaline, although both the spectrum and the melting point, as well as the neutralization equivalent, indicated that the crystals were not of analytical purity. However, a mixture of this material and the synthetic tosyl DvLv–LvDv (m. p. 195–197°) melted at 194–197° while admixture of this natural derivative with any of the other synthetic isomers produced a marked depression in the melting point.

"Natural" Tosylvalylvaline Methyl Ester.—A portion (1.6 mg.) of the "natural" tosylvalylvaline was suspended in 0.2 ml. of ether and treated with a few drops of ethereal diazomethane. When the evolution of nitrogen subsided, the solution was filtered and concentrated to *ca.* 0.2 ml. A drop of chloroform and 0.4 ml. of Skellysolve B were added and upon scratching fine needle crystals began to

(22) R. J. Williams and H. Kirby, *Science*, **107**, 481 (1948).

form. After refrigerating overnight a nearly quantitative yield of ester was obtained; m. p. 143–150°. A mixture of this ester and the synthetic tosyl DvLv–LvDv methyl ester (m. p. 149–151°) melted at 148–151°. The melting points of other mixtures were depressed, *e. g.*, a mixture of the natural ester and synthetic tosyl DvDv–LvLv methyl ester melted over the range 125–145°.

"Natural" Benzoylvalylvaline.—This compound was prepared by benzoylation of the valylvaline from gramicidin according to the method reported previously.[3a] Recrystallization from acetone yielded tiny diamond-shaped crystals which melted at 231.5–232° alone and on admixture with synthetic benzoyl DvLv–LvDv.

"Natural" Benzoylvalylvaline Methyl Ester.—Methylation of a few mg. of the above acid was accomplished using ethereal diazomethane and a chloroform solution of the benzoyl compound. Recrystallization of the methyl ester from ethanol and water yielded fine glossy needles; m. p. 186–187°. When mixed with synthetic benzoyl DvLv–LvDv methyl ester the melting point was not depressed, but admixture with synthetic benzoyl DvLv methyl ester (m. p. 187–187.5°) depressed the melting point to 172–176°. Comparison of the infrared spectra and the X-ray diffraction patterns (Figs. 1 and 2) confirmed the identity of the natural benzoylvalylvaline methyl ester as the DL–LD racemic modification.

Acid Hydrolysis of Isomeric Dipeptides.—The four free, isomeric, valylvalines were subjected, in solutions of 0.021 to 0.024 *M* concentration (by weighing) to the action of 6 *N* hydrochloric acid at 100° in sealed tubes for thirty hours. After removal of the hydrochloric acid *in vacuo*, duplicate aliquots of the hydrolysates were analyzed for α-amino nitrogen by the manometric ninhydrin method at *p*H 2.5[23,24] and the degree of hydrolysis calculated.

Discussion

The dilemma presented by the appearance of similar quantities of two enantiomorphic dipeptides upon the hydrolysis of gramicidin still remains. Racemization, unless it occurred as an epimerization, as suggested by Neuberger,[25] would not appear to be an adequate explanation. The differences in stability to hydrolysis of the isomers are in the wrong direction to account for the isomers found. The incomplete homogeneity of gramicidin[8] does not provide any obvious explanation. The racemic dipeptide isolated might conceivably arise by the hydrolysis of a structure of the type R-D-valyl-L-valyl-D-valyl-R'. Synge has isolated two peptides, alanylvaline and valylglycine, containing excesses of D- and L-valine, respectively.[9,26] It is probably significant that valylvaline, despite its great stability, apparently is not formed when the acid hydrolysis is performed under at least two other sets of conditions.

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Summary

1. Paper chromatography of the unfractionated gramicidin hydrolysate revealed the presence of valylvaline of R_f corresponding to that of the racemic modification D-valyl-L-valine plus L-valyl-D-valine or its component isomers. The other two isomers could not be detected.

2. Comparison of several derivatives of the valine dipeptide separated from gramicidin hydrolysates with corresponding synthetic products as to melting point, infrared absorption and X-ray diffraction studies indicated that the isolated dipeptide consisted of the above racemic modification.

3. The isomeric dipeptides of valine and a number of their derivatives have been synthesized.

(23) P. B. Hamilton and D. D. Van Slyke, *J. Biol. Chem.*, **150**, 231 (1943).

(24) P. B. Hamilton and D. D. Van Slyke, *ibid.*, **164**, 249 (1946).

(25) A. Neuberger, *Adv. Protein Chem.*, **4**, 297 (1948).

(26) R. L. M. Synge, *Biochem. J.*, **38**, 285 (1944).

KALAMAZOO 99, MICHIGAN RECEIVED OCTOBER 27, 1949

[CONTRIBUTION FROM THE VENABLE CHEMICAL LABORATORY OF THE UNIVERSITY OF NORTH CAROLINA]

The Pyridine Acylation of Sarcosine and Esters of Alpha-Acylamino Acids[1]

BY RICHARD H. WILEY[1b] AND OLIN H. BORUM[1a]

Previous communications[2,3] have described the conversion of α-amino acids to acylamido ketones on refluxing with acid anhydrides in pyridine.

$$RCH(NH_2)CO_2H + 2(R'CO)_2O \xrightarrow{\text{pyr.}} RCH(NHCOR')COR' + 2RCO_2H + CO_2$$

The present paper describes the course of the reaction when applied to the α-acylamino acid esters and to the N-acetyl derivative of sarcosine.

Acylation of Acetylsarcosine.—Acetylsarcosine reacts on refluxing with acetic anhydride in the presence of pyridine to form a mixture of N-methylacetamidoacetone and its acetyl derivative.

$$CH_3NHCH_2CO_2H + (CH_3CO)_2O \longrightarrow CH_3CON(CH_3)CH_2COCH_3 + CH_3CON(CH_3)CH{=}C(OCOCH_3)CH_3\ (?)$$

The two products, which are obtained as a mixture boiling at 99–109° (3–4 mm.), are separated

(1) Taken from the thesis submitted by Olin H. Borum to the Graduate School of the University of North Carolina in partial fulfillment of the requirements for the Ph.D. degree. Presented before the Division of Organic Chemistry of the American Chemical Society, Atlantic City, September, 1949. (a) du Pont Company, Philadelphia, Pa. (b) University of Louisville, Louisville, Ky.

(2) Wiley, *J. Org. Chem.*, **12**, 43 (1947).

(3) Wiley and Borum, THIS JOURNAL, **70**, 2005 (1948).