Peptide Studies V

Synthesis and Racemization of Some Optically Active Tripeptides of L- and D-Leucine and L- and D-Valine

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Seven tripeptides of leucine and valine of the optical configuration L-L-D were synthesized by the mixed anhydride procedure. Configuration of each amino acid in the peptides was established bacterimetrically. Racemization was found on the internal amino acid.

IN PREVIOUS investigations of antibacterial peptides (1, 2), we found that the L-L-D structure of the 8 valine tripeptides was unique in its ability to inhibit growth of lactic acid bacteria. Synthesis of other tripeptides was desirable to determine further the relation of structure to inhibitory activity. We now report that the 7 other possible L-L-D tripeptides of leucine and valine have been synthesized via the mixed anhydride procedure (3) by the sequence Z.A.B. + C. ester → Z.A.B.C. ester. All di- and tripeptide intermediates were obtained in the crystalline state. The phthalyl valyl-valyl esters were previously reported (4) as uncrystallizable oils.

In the synthesis of carbobenzoxy-leucyl-valyl-leucine methyl ester and carbobenzoxy-leucyl-valyl-valine methyl ester, both the desired L-L-D structure and an L-DL-D mixture were isolated. The latter was formed by racemization of the central amino acid. This mixture, which melted sharply, is composed of two compounds, L-L-D and L-D-D, which crystallized together but are not optical enantiomorphs. The L-DL-D mixtures contained approximately equal amounts of L-L-D and L-D-D isomers for both the Z.leu.val.-val.ester and the Z.leu.val.leu.ester.

The optical purity of peptides has been established classically by rotation of the protected peptide after synthesis by two procedures. Several workers (5–10) have evaluated coupling procedure for racemization by using model systems with isolation of the optically active and racemic compounds. The coupling of acylamino acid to the peptide esters has been recommended to avoid racemization (11, 12). Z-blocked dipeptides may racemize during *p*-nitrophenyl ester formation (13). Racemization of an internal amino acid may occur during exposure of the peptide to base (14) during the saponification of peptide esters.

Biologic evaluation of optical purity has proved useful when the peptide can be compared to natural all L isolated material (15, 16). In one case, however, presence of D-amino acid did not destroy or prevent biologic activity (17). Enzymatic methods, employing leucine aminopeptidase (18) and chymotrypsin (19), have been used to establish optical purity after decarbobenzoxylation of peptides. Still another method for proving optical purity of peptides is the bacterimetric determination of the free L-amino acids after hydrolysis (4). Here we report the application of the bacterimetric procedure to determine the configuration of each amino acid in the free tripeptides of leucine and valine and two carbobenzoxy-tripeptide esters.

EXPERIMENTAL

Carbobenzoxy-valyl-valyl-methyl ester.—Z.L-valine (0.138 mole) (m.p. 59–60°) in cold (−10°) ether was condensed via the chlorcarbonate (3) with valine methyl ester (0.153 mole) freshly distilled. The mixture was washed with water, 0.1 N hydrochloric acid, water, 5% potassium carbonate, water, with appropriate backwashes, dried over sodium sulfate, and evaporated to dryness. The compound solidified after the residual solvent was removed *in vacuo*. The peptide was recrystallized from chloroform:petroleum ether. Yield 37.5 Gm., 75%, m.p. 109–110°. The other three protected dipeptides were similarly made. Yields and m. p. are given in Table I.

Carbobenzoxy-valyl-valine.—Z.val.val.OMe (0.088 mole) was dissolved in a minimal amount of methanol at RT and sodium hydroxide (0.096 mole) (2 *N* in water) was added. After 4 hours water was added and the methanol was removed *in vacuo*. The unsaponified residue was removed by filtration. The aqueous filtrate was treated with about 1 Gm. of activated carbon, filtered, and acidified with 1 *N* hydrochloric acid to Congo red. The Z.val.val was extracted into ethyl acetate, washed with water, dried, evaporated to dryness, and recrystallized from chloroform:petroleum ether. Yield 26.9 Gm., 88%, m.p. 140–141.5°. The remaining Z.dipeptides were similarly made.

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TABLE I.—CARBOBENZOXY DIPEPTIDES OF L-LEUCINE AND L-VALINE

Compound	Z. Dipeptide Ester			Z. Dipeptide		
	Yield, %	M.P., °C.[a] Found	Lit.	Yield, %	M.P., °C. Found	Lit.
Leu.leu	70	95–96	97–98.5(20)	80	114–115	114–116(21)
Val.leu	60	103–104	112(22)	89	137–137.5	143(22)
Val.val	75	109–110	100–103(23)	88	140–141.5	139.5–140(24)
Leu.val	70	...	...	80	109–110	109–110(25)

[a] Uncorrected.

Carbobenzoxy-tripeptide Esters.—Z.leu.leu, 2.5 Gm. (6.6 mmole) in cold ether (−5°) was condensed via the chlorcarbonate (3) with 7.3 mmole of D-valine ester freshly distilled. Some precipitation occurred on standing overnight. To the entire mass was added sufficient ethyl acetate to form one phase, and the ethyl acetate was washed with water, 0.1 *N* hydrochloric acid, water, 5% potassium carbonate, and water, with appropriate backwashes. The ethyl acetate-ether phase was dried over sodium sulfate and evaporated to dryness *in vacuo*. The residue was dissolved in chloroform and fractionally precipitated with petroleum ether (30–60°). The first and second crops were recrystallized repeatedly from the same solvent pair to constant melting point before any analysis. The remaining six compounds were similarly prepared unless noted. Yields and analytic data are given in Table II.

On the Z.leu.val.val.OMe and Z.leu.val.leu.OMe, the above procedure gave the sharp melting material which melted intermediate to the compounds in Table III.

Fractionation of Z.leu.val.val.OMe.—The first crop, 1.5 Gm. of product from chloroform:petroleum ether crystallization, had a wide melting range 130–150°. This material was very slowly crystallized five times from ethanol:water to yield 0.5 Gm. of material, m.p. 131–132°, which proved by analysis to be a mixture of L-L-D and L-D-D. The second crop from the chloroform:petroleum ether was obtained by evaporating the filtrate from crop No. 1 to dryness. It was recrystallized three times from ethanol:water to yield 0.4 Gm. of material, m.p. 162–163°, which proved on analysis to be chiefly L-L-D.

Fractionation of Z.leu.val.leu.OMe.—The crude tripeptide ester (3.2 Gm.) was recrystallized from methanol:water to give 2.4 Gm., m.p. 130-153°. After two recrystallizations from chloroform:hexane and one from alcohol:water, 1.0 Gm. of material, m.p. 126–127.5°, was obtained, which was by analysis a mixture of L-L-D plus L-D-D. The second crop from chloroform:hexane was obtained by evaporating the filtrate from crop No. 1 to dryness. It was recrystallized very slowly three times from ethanol:water to give 0.8 Gm., m.p. 162–164°, which proved to be the L-L-D compound.

Carbobenzoxy Tripeptides.—These compounds were saponified as above, but overnight. The methanol was reduced to one-fourth volume *in vacuo*, and water was added to precipitate completely the unsaponified starting compound. The filtrate was stripped of methanol on a rotary evaporator, extracted with ethyl ether, treated with charcoal, and acidified with 0.1 *N* hydrochloric acid to Congo red. The Z.tripeptides were filtered off and recrystallized from chloroform:petroleum ether. Ethanol:water was used only when material with wide melting range was obtained.

Tripeptide Acetates and Tripeptides.—The Z.tripeptide (0.5 Gm.) was dissolved in 30 ml. methanol and 0.1 ml. acetic acid, 2 ml. water, and 0.17 Gm. palladium oxide catalyst (26) was added. Hydrogen was passed in until carbon dioxide was no longer evolved. The catalyst was removed and the filtrate was evaporated to dryness *in vacuo*. The residue was dissolved in water, filtered, and again evaporated to dryness. This procedure gave tripeptide acetates. The tripeptide acetates were put on a Dowex-50-X-8 column and eluted with 4 *N* ammonium hydroxide. The eluate was taken to dryness *in vacuo*.

Analytic Procedures.—*L. plantarum* was used to assay L-valine under conditions in which D-valine was inactive. *P. cerevisiae* parent (ATCC 8081) or *L. casei* was used to assay for L-leucine, since *L. plantarum* can utilize the D-form. Media and test conditions have been reported (27). The growth period was 3 days. Several commercial D-leucines had variable amounts (3–11%) of L-leucine under our conditions. The lot used for synthesis assayed 0.1–0.3% L-leucine in several trials. The D-valine employed assayed about 0.1% L-valine. The L-amino acids from Calbiochem or Mann assayed about 100% of control by bacterimetry. Possible impurities, such as tyrosine in L-leucine, were negligible. D-Amino acid in L-valine or L-leucine was not determined.

Free peptides were hydrolyzed overnight in 4 *N* hydrochloric acid, in sealed tubes, at 116°, which is conveniently maintained by refluxing *n*-butanol. Z.peptides or Z.peptide esters were treated first with hydrogen bromide in dioxane, taken to dryness, then hydrolyzed with hydrochloric acid. Hydrochloric acid treatment alone led to charring of sample and poor assays.

Rotations were measured with the Keston polarimetric attachment to the Beckman DU spectrophotometer, employing Aminco 5.00-cm. cells of 2.6 ml. volume. The technique for inhibition measurements has been described (2). All samples were autoclaved with the test medium.

DISCUSSION

The bacterimetric procedure, applied to any all L-peptide, gives (as a measure of optical purity of a given amino acid) the L-amino acid found compared with theory for that amino acid. In the L-L-D peptides described here, the bacterimetric procedure is applicable to each amino acid residue with maximum accuracy when used to determine the C-terminal amino acid with D-configuration. Any L-amino acid in the C-terminal position is determined

TABLE II.—L-L-D TRIPEPTIDES OF LEUCINE AND VALINE AND DERIVATIVES

Compound	Z.Tripeptide Ester		Z.Tripeptide		Tripeptide							
	Yield, %	M.P., °C.[a]	Yield, %	M.P., °C.	Form	Yield, %	M.P., °C.	% N Found	% N Theory	% L-Leucine Found	% L-Leucine Theory	% L-Valine Found	% L-Valine Theory
Leu.leu.leu	44	122–124	50	163–165	HOAc	85	230–231	10.2	10.1	64.2	62.5	<0.1	0
Leu.leu.val	40	129–130	40	166–167	HOAc	60	219–221	10.6	10.4	61.0	65.0	0.2	0
Leu.val.val													
L-L-D + L-D-D	25	148–9.5	50	187–188	HOAc	90	148–150	10.8	10.8	32.5	33.6	20.7	30.3[b]
Leu.val.val													
L-L-D	...	162–163	...	...	...	...	...	...	...	...	...	...	...
Leu.val.leu													
L-L-D + L-D-D	33	150–152	25	177.5-178.5	...	65	205–207	12.0	12.2	34.8	38.1	31.0	34.2[b]
Leu.val.leu													
L-L-D	...	162–164	...	...	...	...	...	...	...	...	...	...	...
Val.val.leu	28	181–182	25	199–200	...	20	270–300[c]	...	...	1.8	0	69.4	71.1
Val.leu.val	55	176–177	75[d]	193–195	...	55	232–233.5	12.7	12.8	35.9	40.0	35.8	35.1
Val.leu.leu	33	170–171	60	182–183	HOAc	45	222–224	9.85	10.4	32.0	32.4	31.0	29.0

[a] Uncorrected. [b] Theory for the pure L-L-D compound. [c] Decomposition. [d] Saponified twice.

directly, without interference from the existing D-amino acid. The limit of the detection is determined by the extent of racemization under the hydrolytic conditions employed. The determination of the D-amino acid which is present due to racemization of the L is made by subtracting the L found from that which should be theoretically present when no racemization occurs.

Maximal assay values for all peptides were obtained after hydrolysis with 4 *N* hydrochloric acid in sealed tubes, after 12–24 hours at 116°, in contrast to the 60 hours at 126° needed for D-valyl-L-valine (4). Increasing the hydrolysis time to 48 hours or longer did not give increased leucine or valine assay values in the peptides val.leu.val, leu.leu.val, or leu.val.leu. The free amino acids D-leucine and D-valine gave 0.7% and about 0.2%, respectively, of the L-forms when hydrolyzed 12 hours, and 3.5% and 1.0% of the L-forms at 60 hours. Approximately 10–20 γ of L-amino acid per ml. in the sample was sufficient for analysis.

Yields of the Z.dipeptide esters of valyl-valine, valyl-leucine, leucyl-valine, and leucyl-leucine were satisfactory. Alkaline saponification of these Z. dipeptide esters also proceeded smoothly and in good yield (Table I).

In the Z.tripeptide series (Table II), yields of the crude Z.tripeptide esters were from 60–80% of crystalline material. However, after several recrystallizations (needed to obtain constant melting point and analytic purity, indicated by amino acid analysis) the yields of pure Z.tripeptide esters were from 25–35%. Saponification proceeded quite slowly compared with the Z.dipeptide esters. After 16 hours, yields of Z.tripeptides from 25–60% were obtained, with most of the remaining Z.tripeptide ester recoverable. There were distinct differences in the yields of the free tripeptides after hydrogenation. N-Terminal leucine peptides were obtained in 60–90% yields; N-terminal valine peptides in 20–55% yields. The N-terminal valine tripeptides which are reported here were obtained optically pure after recrystallization, but this is not conclusive proof of absence of racemization on synthesis (28).

Two of the four protected N-terminal leucyl peptides were obtained in both optical forms. Previously (4), with the phthaloyl blocking group and carbodiimide coupling, the eight optically active tripeptides of L- and D-valine were obtained pure. This probably was due in large part to the use of valine in the N-terminal position, as has been noted above, since Anderson (6) has reported that the carbodiimide procedure also permits racemization to occur.

The data in Table II indicate that no significant racemization occurred on the N-terminal or C-terminal amino acids during coupling, saponification, or hydrogenation. However, leu.val.val was found to be low in L-valine. A second coupling was fractionally crystallized as the Z.tripeptide ester, and yielded both the desired L-L-D peptide ester and the mixture of L-L-D and L-D-D. The Z.L-L-D peptide esters melted some 30° higher than the mixture of L-L-D and L-D-D (Table III).

Rotations, R_f values, and inhibitory powers of the free peptides are given in Table IV. No resolution of the mixtures of L-L-D and L-D-D occurred in the butanol:acetic acid:water system used. Previously (4), we obtained R_f's of 0.70 and 0.73, respectively, for the L-L-D and L-D-D trivalines.

TABLE III.—L-L-D AND L-DL-D Z. TRIPEPTIDE ESTERS OF LEUCINE AND VALINE

Compound	M.P., °C.	% L-Leucine Found	% L-Leucine Theory	% L-Valine Found	% L-Valine Theory	$[\alpha]_D$[a]
Z.leu.val.val.OMe L-L-D	162–163	27.2	27.6	20.9	24.6	−35.1
Z.leu.val.val.OMe L-L-D + L-D-D	131–132	27.1	27.6	13.2	24.6[b]	−22.8
Z.leu.val.leu.OMe L-L-D	162–164	27.6	26.6	24.0	24.0	−27.6
Z.leu.val.leu.OMe L-L-D + L-D-D	126–128	27.5	26.6	12.4	24.0[b]	−10.1

[a] C = 2, $CHCl_3$; T = 25°. [b] Theory for the pure L-L-D compound.

TABLE IV.—PROPERTIES OF L-L-D TRIPEPTIDES OF LEUCINE AND VALINE

Compound	$[\alpha]_D$	R_f[b]	ID_{50},[a] mg./ml. *P. cerevisiae* Parent	ID_{50},[a] mg./ml. *P. cerevisiae* Mutant
Leu.leu.leu.HOAc	+7.4[c]	0.84	0.4	0.3
Leu.leu.val.HOAc	+22.0[c]	0.81[d]	I[e]	0.4
Leu.val.val.HOAc L-L-D + L-D-D	+23.5[g]	0.75	0.2[f]	0.3[f]
Leu.val.leu L-L-D + L-D-D	+22.0[c]	0.78	I[f]	0.2[f]
Val.val.leu		0.75	0.01	0.06
Val.leu.val	+ 6.6[c]	0.75[d]	I	I
Val.leu.leu.HOAc		0.75	I	I
Val.val.val	−37.0[c,g] −37.3[i]		0.03 ± 0.03	0.03 ± 0.01[h]

[a] Concentration at which growth is reduced 50%, maximum test level 0.25 or 0.50 mg./ml. [b] Solvent butanol:water:acetic acid 10:8:1 upper phase, compounds applied as hydrochlorides. [c] C = 2, 1 *N* HCl; T = 25°. [d] Very small second spot at R_f 0.34. [e] I = Inhibition occurred less than 50%. [f] The L-D-D tripeptide of valine was inert (2). [g] Reported −41.8 (4). [h] This compound also inhibited *L. plantarum*, the other compounds stimulated this growth of *L. plantarum* under the test conditions. [i] C = 2, H_2O.

SUMMARY

Seven possible tripeptides of leucine and valine of the L-L-D configuration were synthesized.

Their optical purity was determined bacterimetrically.

These peptides were not effective as antibacterial agents compared with L-valyl-L-valyl-D-valine.

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