Studies of Peptide Antibiotics. I. Dipeptide Anhydrides as Models of Cyclic Peptide Antibiotics

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Some peptide antibiotics,[1] such as gramicidin S, tyrocidin A and polymixin B, possess several structural features in common. These include a cyclic structure, a basic character due to the presence of one or more diamino acid residues, and the possession of at least one D-amino acid residue. The effectiveness of a cyclic structure for an antibiotic activity is indicated by the finding that a synthetic open-chain decapeptide, with the same sequence of amino acid residues as is found in gramicidin S, shows a weaker antibacterial activity than that of gramicidin S.[2]

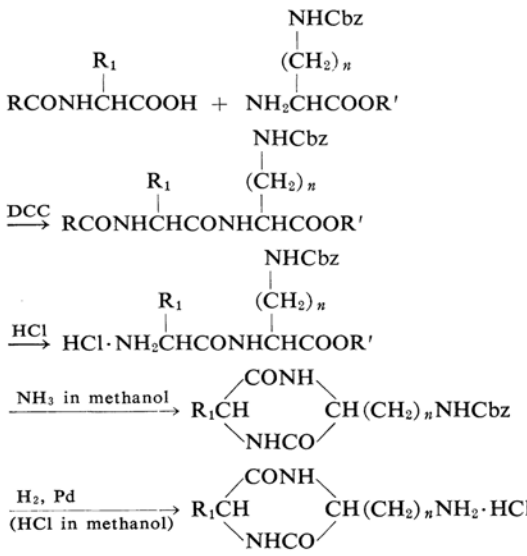
Since dipeptide anhydrides, wherein one (or

1) R. O. Studer and K. Vogler, *Helv. Chim. Acta*, **45**, 819 (1962).

2) B. F. Erlanger and L. Goode, *Nature*, **174**, 840 (1954); *Science*, **131**, 669 (1960).

both) of the component amino acid residues possesses a D-configuration and a basic character, reveal the characteristics mentioned above, it became of interest to ascertain whether these relatively simple compounds would exhibit the antibacterial properties. For that purpose, the L-L, D-D, D-L and L-D stereomers of both valyllysine anhydride and phenylalanyllysine anhydride, and the L-L and D-L stereomers of both valylornithine anhydride and phenylalanylornithine anhydride were selected as model compounds. Lysine was used as one of the diamino acids because Schwyzer and Sieber[3] had reported that no loss in activity occured upon the replacement of an ornithine residue in gramicidin S with a lysine residue. Ornithine, valine and phenylalanine were chosen because of their relatively high frequency of occurrence in cyclic peptide antibiotics of natural origin.[1]

The sequence of reaction employed for the synthesis of the anhydrides is indicated in the following equations. All final dipeptide anhydride hydrochlorides were crystalline materials which showed satisfactory elemental analyses. The L-L and D-D antipodes of the final products exhibited specific rotation values that were equal in absolute value within the limits of experimental error, as did the D-L and L-D antipodes. The products revealed no contamination with other ninhydrin-positive materials when subjected to paper chromatography.



R-: H- or $C(CH_3)_3O$-, R_1: $(CH_3)_2CH$- or $C_6H_5CH_2$-, n: 3 or 4, R'-: $-CH_3$ or $-C_2H_5$, Cbz-: $C_6H_5CH_2OCO$-, DCC: dicyclohexylcarbodiimide.

3) R. Schwyzer and P. Sieber, *Helv. Chim. Acta*, **41**, 1582 (1958).

Since in this laboratory it has been reported that L-valyl-L-lysine amide and L-phenylalanyl-L-lysine amide are susceptible to the hydrolytic action of trypsin at the lysine carbonyl linkage,[4] it became relevant to determine whether this same enzyme would similarly cleave L-valyl-L-lysine anhydride and L-phenylalanyl-L-lysine anhydride at the same linkage with the formation of valyllysine and phenylalanyllysine respectively as the hydrolytic products. In addition, the known susceptibility of phenylalanine-containing peptides to the hydrolytic action of chymotrypsin[5] indicated that the possible conversion of L-phenylalanyl-L-lysine anhydride to lysylphenylalanine by this enzyme should also be studied. For the purpose of chromatographic comparison, valyllysines, phenylalanyllysines and lysylphenylalanine were synthesized via the mixed anhydride method, and their R_f values ascertained. Finally, paper chromatography of the enzymic digests showed only a single ninhydrin-positive spot, which possessed the same R_f values as the unhydrolyzed dipeptide anhydride substrate.

In order to ascertain whether or not the dipeptide anhydrides described herein possessed antibacterial activity, the effect of various levels of the anhydrides on the growth response of *B. subtilis*, *St. aureus*, *E. coli* and *Pr. vulgaris* was examined. No retardation of growth was noted with any one of these microorganisms, at levels for each compound at 100 μg. per ml. of the assay medium. In this connection, it should be noted that Fruton has shown no influence by D-leucyl-L-tryptophan anhydride[6] on the growth of several microorganisms. From these findings, it appears that other structural characteristics in addition to those described previously are necessary, such as a certain minimum ring size, before a peptide molecule can exhibit antibacterial properties. Further experiments on the preparation and properties of larger cyclic peptides are thus in progress in this laboratory.

Experimental

Melting points are uncorrected. Samples are prepared for analysis by drying them at 100°C in vacuo for 2 hr.

Formyl-D-valine (I) and Formyl-D-phenylalanine (II).—Each of these compounds was prepared, according to the procedure of Sheehan and Yang,[7] by the treatment of D-valine or D-phenylalanine

4) N. Izumiya, T. Yamashita, H. Uchio and K. Kitagawa, *Arch. Biochem. Biophys.*, **90**, 170 (1960).
5) H. Neurath and G. W. Schwert, *Chem. Revs.*, **46**, 69 (1950); T. Yamashita and N. Izumiya, *J. Biochem.*, **46**, 991 (1959).
6) J. S. Fruton, *J. Am. Chem. Soc.*, **70**, 1280 (1946).
7) J. C. Sheehan and D-D. H. Yang, ibid., **80**, 1154 (1958).

with 98% formic acid and acetic anhydride; yield, 84% and 80%, m. p. 152~153°C and 167°C, $[\alpha]_D^{20}$ −13.1° and −75.2° (*c* 2, ethanol), for I and II respectively. Each of these compounds had been obtained previously by the resolution of formyl-DL-valine or -phenylalanine with brucine;[8-10] m. p. 153°C[9] and 148~149°C[9], $[\alpha]_D^{20}$ −12.8°[8] and −13°[9] for I, and m. p. 167°C,[10] $[\alpha]_D$ −75.2°[10] for II.

ε-Carbobenzoxy-D-lysine Methyl Ester Hydrochloride.—This compound was prepared as was the L-antipode;[11] m. p. 114~116°C; $[\alpha]_D^{25}$ −16.9° (*c* 2, water). The reported values for the L-antipode[11] are m. p. 117°C and $[\alpha]_D^{25}$ +17.2°.

δ-Carbobenzoxy-L-ornithine Ethyl Ester *p*-Toluene-sulfonate (III).—This compound was prepared according to the general procedure of Kato et al.[12] Thus, a suspension of δ-carbobenzoxy-L-ornithine[13] (53.3 g.) and *p*-toluenesulfonic acid monohydrate (42 g.) in a mixture of ethanol (80 ml.) and carbon tetrachloride (400 ml.) was refluxed, and the water liberated was removed as an azeotropic mixture. The reaction mixture was then concentrated in vacuo, and the residual material treated with ether. The precipitate filtered off was recrystallized from hot acetone (170 ml.)-ether (650 ml.); 87 g. (95%); m. p. 111~112°C; $[\alpha]_D^{20}$ +8.9° (*c* 2, dimethylformamide).

Found: C, 56.19; H, 6.56; N, 6.17. Calcd. for $C_{22}H_{30}O_7N_2S$: C, 56.63; H, 6.48; N, 6.01%.

***t*-Butyloxycarbonyl-D-phenylalanine (IV).**—This compound was prepared as an oily product in the same manner as was the L-antipode;[14] yield, 88%. As a minor modification, sodium bicarbonate was used instead of magnesium oxide.[14] The reported yield of the oily L-antipode is 79%.[14]

Formylvalyl-ε-carbobenzoxylysine Methyl Esters (V—VIII).—The coupling reaction was achieved according to the general procedure of Sheehan et al.[15] To a stirred solution of formyl-L-valine[7] or I (7.26 g., 0.05 mol.), ε-carbobenzoxy-L(or D)-lysine methyl ester hydrochloride (16.55 g., 0.05 mol.), and triethyl amine (7.0 ml.) in a mixture of tetrahydrofuran (150 ml.) and chloroform (150 ml.), at 0°C, was added DCC (10.3 g.) The mixture was stored in the cold overnight, after which time the L-L and D-D stereomers were recovered as described under a), and the D-L and L-D stereomers, as described under b). a) The precipitated dicyclohexylurea was removed, and the filtrate was concentrated in vacuo to an oil. After the latter had been dissolved in chloroform (300 ml.), the solution was washed successively with 4% sodium bicarbonate, 2% hydrochloric acid, and water. The organic layer was dried over sodium sulfate and concentrated in vacuo, and the residual material was treated with ether and petroleum ether. The precipitate filtered off was recrystallized from ethyl acetate (30 ml.) - ether (60 ml.) - petroleum ether (60 ml.). b) The dicyclohexylurea, admixed with a small amount of the desired product, precipitated. After the crystals of the latter had been taken into a solution by warming them at 50°C, the reaction mixture was cooled to room temperature under tap water and the urea filtered off. The remainder of the procedure was identical with that described under a) above. Recrystallization was effected from ethyl acetate (90 ml.) - ether (50 ml.) - petroleum ether (50 ml.). The elemental analyses, yields, and physical constants of the compounds so procured are given in Table I.

Formylphenylalanyl-ε-carbobenzoxylysine Methyl Esters (IX—XII).—These compounds were prepared on a 0.05 mol. scale following the procedure described above. The recrystallization of the crude products was achieved from ethyl acetate - petroleum ether (70 ml.: 50 ml. for IX and X, and 110 ml.: 30 ml. for XI and XII).

Formyl-L-phenylalanyl-ε-carbobenzoxy - L - lysine Ethyl Ester (XIII).—This compound was prepared from formyl-L-phenylalanine and ε-carbobenzoxy-L-lysine ethyl ester *p*-toluenesulfonate[12] in the same manner as that used for V and was recrystallized from ethyl acetate - ether - petroleum ether.

Formylvalyl - δ - carbobenzoxyornithine Ethyl Esters (XIV—XV).—These compounds were prepared following the procedure described for the preparation of V. The L-L stereomer (XIV) was more soluble in ethyl acetate than the D-L stereomer (XV), as was observed in the cases of V and VII.

***t*-Butyloxycarbonyl - L - valyl - δ - carbobenzoxy-L-ornithine Ethyl Ester (XVI).**—This compound was prepared by the treatment of a solution of *t*-butyloxycarbonyl-L-valine[14] (2.18 g.), III (4.66 g.) and triethyl amine (1.4 ml.) in tetrahydrofuran-chloroform with DCC (2.06 g.), following essentially the same procedure as that employed for the preparation of V. As a minor alteration, however, 0.5 M citric acid was used instead of 2% hydrochloric acid to wash the reaction mixture.

Formylphenylalanyl-δ-carbobenzoxyornithine Ethyl Esters.—The L-L stereomer (XVII) was prepared following the procedure described for the preparation of V. The D-L stereomer was obtained as crystals with a m. p. of 145~149°C in a yield of 63%; however, the elemental analyses were not satisfactory.

***t*-Butyloxycarbonyl-D-phenylalanyl - δ - carbobenzoxy-L-ornithine Ethyl Ester (XVIII).**—This compound was obtained from IV and III by the procedure described in the case of XVI. The recrystallization of the crude product was achieved from ethyl acetate - ether - petroleum ether.

Valyl-ε-carbobenzoxylysine Methyl Ester Hydrochlorides (XIX—XXII).—A suspension of 8.43 g. each of V—VIII in 0.5 N methanolic hydrogen chloride (60 ml.) was stored at room temperature for 2 days. The resulting clear solution was then concentrated in vacuo. The treatment of the residual material with acetone and ether led to a

8) E. Fischer, *Ber.*, **39**, 2320 (1906).
9) M. A. Nyman and R. M. Herbst, *J. Org. Chem.*, **15**, 108 (1950).
10) E. Fischer and W. Schoeller, *Ann.*, **375**, 1 (1907).
11) M. Bergmann, L. Zervas and W. F. Ross, *J. Biol. Chem.*, **111**, 245 (1935).
12) T. Kato, S. Makisumi, M. Ohno and N. Izumiya, *J. Chem. Soc. Japan, Pure Chem. Sec.* (*Nippon Kagaku Zasshi*), **83**, 1151 (1962).
13) J. I. Harris and T. S. Work, *Biochem. J.*, **46**, 582 (1950).
14) R. Schwyzer, P. Sieber and H. Kappeler, *Helv. Chim. Acta*, **42**, 2622 (1959).
15) J. C. Sheehan and G. P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955); J. C. Sheehan, M. Goodman and G. P. Hess, ibid., **78**, 1367 (1956).

TABLE I. YIELDS, PHYSICAL CONSTANTS AND ANALYSES OF COMPOUNDS

Compound[a]		Yield %	M. p. °C	$[\alpha]_D^{20}$ [b]	Molecular formula	Calcd., %			Found, %		
						C	H	N	C	H	N
For-Val-LysCbz-OMe	L-L (V)	56	140~143	−11.9	$C_{21}H_{31}O_6N_3$	59.84	7.41	9.97	60.26	7.43	9.84
	D-D (VI)	59	140~143	+12.0	$C_{21}H_{31}O_6N_3$	59.84	7.41	9.97	60.07	7.34	9.82
	D-L (VII)	55	149	−16.0	$C_{21}H_{31}O_6N_3$	59.84	7.41	9.97	60.15	7.41	9.92
	L-D (VIII)	58	148	+14.9	$C_{21}H_{31}O_6N_3$	59.84	7.41	9.97	59.98	7.47	9.83
For-Phe-LysCbz-OMe	L-L (IX)	75	125	− 4.5	$C_{25}H_{31}O_6N_3$	63.94	6.66	8.95	63.84	6.78	9.02
	D-D (X)	78	125	+ 3.9	$C_{25}H_{31}O_6N_3$	63.94	6.66	8.95	64.02	6.77	9.25
	D-L (XI)	68	134~136	−11.8	$C_{25}H_{31}O_6N_3$	63.94	6.66	8.95	64.20	7.01	9.21
	L-D (XII)	67	133~135	+11.0	$C_{25}H_{31}O_6N_3$	63.94	6.66	8.95	64.25	6.73	9.16
For-Phe-LysCbz-OEt	L-L (XIII)	74	118~120	− 7.1	$C_{26}H_{33}O_6N_3$	64.58	6.88	8.69	64.24	6.89	8.72
For-Val-OrnCbz-OEt	L-L (XIV)	54	117~120	−12.1	$C_{21}H_{31}O_6N_3$	59.84	7.41	9.97	59.59	7.53	9.94
	D-L (XV)	55	140~142	−15.8	$C_{21}H_{31}O_6N_3$	59.84	7.41	9.97	59.79	7.83	9.67
BOC-Val-OrnCbz-OEt	L-L (XVI)	57	107~109	−6.5	$C_{25}H_{39}O_7N_3$	60.83	7.96	8.51	60.82	7.71	8.68
For-Phe-OrnCbz-OEt	L-L (XVII)	73	124~126	− 9.0	$C_{25}H_{31}O_6N_3$	63.94	6.66	8.95	64.18	6.79	9.20
BOC-Phe-OrnCbz-OEt	D-L (XVIII)	78	117	− 3.7	$C_{29}H_{39}O_7N_3$	64.30	7.26	7.76	64.41	7.33	7.71
H-Val-LysCbz-OMe·HCl	L-L (XIX)	77	154	0	$C_{20}H_{32}O_5N_3Cl$	55.87	7.50	9.77	55.81	7.52	9.72
	D-D (XX)	73	153~154	0	$C_{20}H_{32}O_5N_3Cl$	55.87	7.50	9.77	55.54	7.61	9.74
	D-L (XXI)	80	161	−43.0	$C_{20}H_{32}O_5N_3Cl$	55.87	7.50	9.77	55.63	7.68	9.69
	L-D (XXII)	75	161	+43.8	$C_{20}H_{32}O_5N_3Cl$	55.87	7.50	9.77	55.82	7.57	9.79
H-Phe-LysCbz-OMe·HCl	L-L (XXIII)	72	153~154	− 9.3	$C_{24}H_{32}O_5N_3Cl$	60.32	6.75	8.79	60.31	6.95	8.83
	D-D (XXIV)	75	154~155	+10.1	$C_{24}H_{32}O_5N_3Cl$	60.32	6.75	8.79	60.14	6.99	8.64
H-Val-OrnCbz-OEt·HCl	L-L (XXVII)	83	175~177	+19.9	$C_{20}H_{32}O_5N_3Cl$	55.87	7.50	9.77	55.66	7.59	9.58
H-Phe-OrnCbz-OEt·HCl	L-L (XXIX)	85	120~125	− 7.7	$C_{24}H_{32}O_5N_3Cl$	60.32	6.75	8.79	59.74	6.92	8.69
Val◇LysCbz	L-L (XXXI)	88	162~164	−46.1	$C_{19}H_{27}O_4N_3$	63.14	7.53	11.63	63.04	7.53	11.62
	D-D (XXXII)	86	161~164	+45.0	$C_{19}H_{27}O_4N_3$	63.14	7.53	11.63	63.11	7.34	11.44
	D-L (XXXIII)	90	198~200	− 4.3	$C_{19}H_{27}O_4N_3$	63.14	7.53	11.63	63.16	7.44	11.64
	L-D (XXXIV)	91	199~201	+ 3.2	$C_{19}H_{27}O_4N_3$	63.14	7.53	11.63	63.12	7.44	11.71
Phe◇LysCbz	L-L (XXXV)	83	206~208	−26.1	$C_{23}H_{27}O_4N_3$	67.46	6.65	10.26	67.51	6.76	10.22
	D-D (XXXVI)	87	205~207	+25.5	$C_{23}H_{27}O_4N_3$	67.46	6.65	10.26	67.34	6.76	10.23
	D-L (XXXVII)	85[c]	171	−10.0	$C_{23}H_{27}O_4N_3$	67.46	6.65	10.26	67.85	6.95	10.31
	L-D (XXXVIII)	75[c]	171	+10.5	$C_{23}H_{27}O_4N_3$	67.46	6.65	10.26	67.87	7.05	10.25
Val◇OrnCbz	L-L (XXXIX)	80	206~208	−47.4	$C_{18}H_{25}O_4N_3$	62.23	7.25	12.10	62.25	7.28	12.24
	D-L (XL)	68[c]	208~210	− 6.8	$C_{18}H_{25}O_4N_3$	62.23	7.25	12.10	62.74	7.54	12.08
Phe◇OrnCbz	L-L (XLI)	72	210~212	−12.9	$C_{22}H_{25}O_4N_3$	66.82	6.37	10.63	66.74	6.41	10.59
	D-L (XLII)	66[c]	175~178	− 3.4	$C_{22}H_{25}O_4N_3$	66.82	6.37	10.63	66.30	6.67	10.40
Val◇Lys·HCl	L-L (XLIII)	77	254~256	−54.1	$C_{11}H_{22}O_2N_3Cl$	50.07	8.41	15.92	50.02	8.24	15.82
	D-D (XLIV)	84	253~255	+53.6	$C_{11}H_{22}O_2N_3Cl$	50.07	8.41	15.92	49.84	8.48	15.73

TABLE I. (Continued)

Compound[a]		Yield %	M. p. °C	$[\alpha]_D^{29}$ [b]	Molecular formula	Calcd., %			Found, %		
						C	H	N	C	H	N
	D-L (XLV)	90	282	− 5.4	$C_{11}H_{22}O_2N_3Cl$	50.07	8.41	15.92	50.26	8.47	15.73
	L-D (XIVI)	90	282～283	+ 5.8	$C_{11}H_{22}O_2O_3Cl$	50.07	8.41	15.92	50.04	8.41	15.71
Phe◇Lys·HCl	L-L (XLVII)	94	>270	+ 2.5	$C_{15}H_{22}O_2N_3Cl$	57.80	7.12	13.48	57.72	7.26	13.53
	D-D (XLVIII)	91	>270	− 2.0	$C_{15}H_{22}O_2N_3Cl$	57.80	7.12	13.48	57.64	7.10	13.51
	D-L (XLIX)	88	>255	−46	$C_{15}H_{22}O_2N_3Cl$	57.80	7.12	13.48	58.14	7.24	13.66
	L-D (L)	90	>255	+48	$C_{15}H_{22}O_2N_3Cl$	57.80	7.12	13.48	57.83	7.26	13.42
Val◇Orn·HCl	L-L (LI)	90	255～257	−54.7	$C_{10}H_{20}O_2N_3Cl$	48.10	8.07	16.83	48.07	8.08	16.32
	D-L (LII)	66	265～268	− 4.0	$C_{10}H_{20}O_2N_3Cl$	48.10	8.07	16.83	48.11	8.28	16.80
Phe◇Orn·HCl	L-L (LIII)	68	245～246	+ 8.7	$C_{14}H_{20}O_2N_3Cl$	56.46	6.77	14.38	56.65	6.86	14.16
	D-L (LIV)	75	249～250	−50.4	$C_{14}H_{20}O_2N_3Cl$	56.46	6.77	14.38	56.57	6.82	14.52
Cbz-Val-LysCbz-OMe	L-L (LV)	76	124～126	0	$C_{28}H_{37}O_7N_3$	63.74	7.07	7.97	63.71	7.10	7.98
	D-D (LVI)	74	124～126	0	$C_{28}H_{37}O_7N_3$	63.74	7.07	7.97	63.34	7.28	7.93
	D-L (LVII)	71	163	−15.4	$C_{28}H_{37}O_7N_3$	63.74	7.07	7.97	63.62	7.18	7.84
	L-D (LVIII)	77	164	+16.1	$C_{28}H_{37}O_7N_3$	63.74	7.07	7.97	63.33	7.08	7.97
Cbz-Phe-LysCbz-OEt	L-L (LIX)	85	150	− 8.7	$C_{33}H_{39}O_7N_3$	67.21	6.67	7.13	66.74	6.53	6.84
	D-L (LX)	82	148	+ 4.0	$C_{33}H_{39}O_7N_3$	67.21	6.67	7.13	67.17	6.72	7.04
Cbz-Val-LysCbz-OH	L-L (LXI)	90	139～140	+ 5.0	$C_{27}H_{35}O_7N_3$	63.14	6.87	8.18	62.84	6.90	8.14
	D-D (LXII)	94	139～140	− 5.9	$C_{27}H_{35}O_7N_3$	63.14	6.87	8.18	62.74	6.73	8.20
	D-L (LXIII)	96	123～125	+13.7	$C_{27}H_{35}O_7N_3$	63.14	6.87	8.18	62.91	6.99	8.17
	L-D (LXIV)	97	124～126	−14.5	$C_{27}H_{35}O_7N_3$	63.14	6.87	8.18	63.08	6.82	8.12
Cbz-Phe-LysCbz-OH	L-L (LXV)	95	108～111	− 3.3	$C_{31}H_{35}O_7N_3$	66.29	6.28	7.48	66.23	6.31	7.48
	D-L (LXVI)	93	147	+ 4.3	$C_{31}H_{35}O_7N_3$	66.29	6.28	7.48	66.31	6.29	7.43
H-Val-Lys-OH·HCl	L-L (LXVII)	85	241～243	+25.4	$C_{11}H_{24}O_3N_3Cl$	46.89	8.59	14.91	46.83	8.86	14.89
	D-D (LXVIII)	84	242～243	−25.3	$C_{11}H_{24}O_3N_3Cl$	46.89	8.59	14.91	46.64	8.76	14.99
	D-L (LXIX)[d]	81	—	−50	$C_{11}H_{24}O_3N_3Cl$	46.89	8.59	14.91	46.42	8.81	14.53
	L-D (LXX)[d]	78	—	+48	$C_{11}H_{24}O_3N_3Cl$	46.89	8.59	14.91	46.71	8.83	14.49
H-Phe-Lys-OH·HCl	L-L (LXXI)[d]	79	—	+24	$C_{15}H_{24}O_3N_3Cl$	57.42	7.70	13.39	56.87	7.91	13.31
	D-L (LXXII)[d]	74	—	−80	$C_{15}H_{24}O_3N_3Cl$	57.42	7.70	13.39	57.21	8.03	13.11
Cbz-LysCbz-Phe-OBz	L-L (LXXIII)	67	141	−10.4	$C_{38}H_{41}O_7N_3$	70.03	6.34	6.45	70.01	6.28	6.48
H-Lys-Phe-OH·HCl	L-L (LXXIV)	76	143～145	+35.0	$C_{15}H_{24}O_3N_3Cl$	54.64	7.33	12.75	54.55	7.42	12.63

a) Abbreviations are as follows; For: formyl, BOC: *t*-butyloxycarbonyl, LysCbz: ε-carbobenzoxylysine residue, OrnCbz: δ-carbobenzoxyornithine residue, Val◇Lys·HCl L-L: L-valyl-L-lysine anhydride monohydrochloride.

b) Optical rotations were determined using a 20 cm. tube. All concentrations were 1% except the compounds, XLIX and L. Concentrations of XLIX and L were 0.3%.

c) Yield was based on the parent formyl or *t*-butyloxycarbonyl carbobenzoxy-substituted dipeptide ester.

d) This was hydroscopic crystal; optical rotation was approximate.

crystalline product which was recrystallized from acetone-ether. Although the crystallization of the L-D and D-L stereomers occurred more readily than that of the L-L and D-D stereomers, the latter nevertheless crystallized within an hour by the procedure described above.

Phenylalanyl-ε-carbobenzoxylysine Methyl Ester Hydrochlorides (XXIII—XXVI). — These compounds were prepared following the procedure described above. Although the L-L and D-D stereomers (XXIII, XXIV) could be recrystallized from methanol-acetone-ether, attempts to solidify the D-L and L-D forms (XXV, XXVI) were unsuccessful.

Valyl-δ-carbobenzoxyornithine Ethyl Ester Hydrochlorides (XXVII,XXVIII).—The L-L stereomer (XXVII) was prepared as follows: To a solution of XVI (4.94 g.) in ethyl acetate (15 ml.), 4 N hydrogen chloride in ethyl acetate (50 ml.) was added. The reaction mixture was stored at room temperature for 3 hr. and then evaporated in vacuo. The residual crystals were filtered off with the aid of ether and recrystallized from methanol-ether. The melting point was 175~177°C, as is shown in Table I. The crystalline product was also obtained from the formyl dipeptide ester (XIV) with methanolic hydrogen chloride. This compound, however, showed the low melting point of 108~115°C and did not give the analytically-pure valyl-δ-carbobenzoxyornithine anhydride.

The oily D-L stereomer (XXVIII) was obtained from the formyl dipeptide ester (XV). Attempts to solidify the oil were unsuccessful, although this oily product gave the analytically-pure dipeptide anhydride (XL).

Phenylalanyl-δ-carbobenzoxyornithine Ethyl Ester Hydrochlorides (XXIX, XXX).—The crystalline L-L stereomer (XXIX) was obtained from the formyl dipeptide ester (XVII) by the procedure described in the case of XIX, while the oily D-L stereomer (XXX) was obtained from the *t*-butyloxycarbonyl dipeptide ester (XVIII) as in the case of XXVII.

Valyl-ε-carbobenzoxylysine Anhydrides (XXXI—XXXIV).—A solution of 4.3 g. each of XIX—XXII in 100 ml. of methanol, which had been previously saturated with dry ammonia at 0°C, was stored in a glass-stoppered bottle at room temperature for 2 days.[16] In the case of the L-D and D-L stereomers (XXXIII and XXXIV), a mass of crystals appeared within about 1 hr. whereas a complete solution was maintained with the L-L and D-D stereomers (XXXI and XXXII) over the entire reaction period. The reaction mixture was then concentrated to dryness with a rotary evaporator, and the crystalline residue was filtered off with the aid of a little water. The recrystallization of XXXI and XXXII was achieved from 30% methanol (85 ml.), whereas 50% ethanol (100 ml.) was used with XXXIII and XXXIV.

Phenylalanyl-ε-carbobenzoxylysine Anhydrides (XXXV—XXXVIII). — The conversion of XXIII—XXVI to XXXV—XXXVIII followed the same procedure as was employed in the preparation of XXXI—XXXIII. Recrystallization was effected from 70% ethanol.

Valyl-δ-carbobenzoxyornithine Anhydrides (XXXIX, XL) and Phenylalanyl-δ-carbobenzoxyornithine Anhydrides (XLI, XLII).—These compounds were obtained from the dipeptide ester hydrochlorides, XXVII, XXVIII, XXIX and XXX, as described in the cases of XXXI and XXXIII. Recrystallization was effected from 40% ethanol for XXXIX and XL, and from 70% ethanol for XLI and XLII.

Valyllysine Anhydride Hydrochlorides (XLIII—XLVI).—A suspension of 3.61 g. (0.01 mol.) each of XXXI—XXXIV in 0.1 N methanolic hydrogen chloride (105 ml.) was subjected to hydrogenolysis with dry hydrogen in the presence of palladium black which had previously been washed with methanol. Upon the completion of the reaction, the catalyst was removed by filtration, with prior warming if necessary to dissolve any product which may have precipitated, and the filtrate was concentrated to dryness in vacuo. The crystalline product was then filtered off, with the aid of a mixture of acetone and ether, and recrystallized from methanol-acetone.

Phenylalanyllysine Anhydride Hydrochlorides (XLVII—L). — A suspension of 2.05 g. each of XXXV—XXXVIII in acetic acid (50 ml.) was subjected to hydrogenolysis as has been described above. The filtrate was then concentrated to dryness, the residual material was dissolved in water, and the solution was treated with 1 N hydrochloric acid (5.3 ml.). After the concentration of the solution, the residual product was recovered by filtration with the aid of acetone. Recrystallization was effected from methanol-acetone.

Valylornithine Anhydride Hydrochlorides (LI, LII).—These compounds were obtained from XXXIX and XL as has been described in the case of XLIII. The D-L stereomer (LII) was less soluble in methanol than the L-L stereomer (LI).

Phenylalanylornithine Anhydride Hydrochlorides (LIII, LIV). — These compounds were obtained from XLI and XLII as has been described in the case of XLIII. The air-dried crystals contained half moles of crystal waters; they lost 3.0% (LIII) and 3.2% (LIV) of their weight upon being dried for 3 hr. in vacuo at 100°C. Calcd. for $C_{14}H_{20}O_2N_3Cl \cdot 1/2H_2O$: H_2O, 2.94%.

Carbobenzoxyvalyl-ε-carbobenzoxylysine Methyl Esters (LV—LVIII).—To a stirred solution of carbobenzoxy-L(or D)-valine[17] (5.03 g.) and triethyl amine (2.8 ml.) in toluene (40 ml.) at 0°C, isobutylchlorocarbonate (2.7 ml.) was added.[18] After 15 min., a mixture of ε-carbobenzoxy-L(or D)-lysine methyl ester hydrochloride (6.61 g.) and triethyl amine (2.8 ml.) in chloroform (40 ml.) was also added. The reaction mixture was then left at room temperature overnight, after which time LV and LVI were recovered as has been described under a), and LVII and LVIII, as has been described under b). a) After being washed successively with 4% sodium bicarbonate, 2% hydrochloric acid, and water, the organic layer was dried over sodium

16) E. Fischer and K. Raske, *Ber.*, 39, 3981 (1906).

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

18) J. R. Vaughan, Jr., and R. L. Osato, *J. Am. Chem. Soc.*, 73, 5553 (1951).

sulfate and concentrated to dryness. The crystalline residue was recrystallized from ethyl acetate-petroleum ether. b) The mass of crystals which precipitated was filtered off after the addition of 80 ml. of petroleum ether and some water. Recrystallization was achieved from acetone-ether-petroleum ether.

Carbobenzoxyphenylalanyl-ε-carbobenzoxylysine Ethyl Esters (LIX, LX).—These compounds were prepared from carbobenzoxy-L-phenylalanine[19] and ε-carbobenzoxy-L-lysine ethyl ester *p*-toluenesulfonate[12] in the manner described above.

Carbobenzoxyvalyl-ε-carbobenzoxylysines (LXI—LXIV).—To a solution of 5.28 g. each of LV—LVIII in dioxane (100 ml.), 1 N sodium hydroxide (11 ml.) was added. The reaction mixture was then stored at room temperature for 6 hr., after which time it was neutralized with 1 N hydrochloric acid (12 ml.) and concentrated to dryness in vacuo. The residual material was filtered off with the aid of water, dried well, and recrystallized from ethyl acetate-petroleum ether.

Carbobenzoxyphenylalanyl-ε-carbobenzoxylysines (LXV, LXVI).—These compounds were prepared from LIX and LX following the procedure described above.

Valyllysine Hydrochlorides (LXVII—LXX).—A solution of 2.57 g. (0.005 mol.) each of LXI—LXIV in a mixture of methanol (60 ml.) and 0.5 N hydrochloric acid (10.3 g.) was subjected to hydrogenolysis in the presence of palladium black. The filtrate was then evaporated to dryness in vacuo, and the residual product was filtered off with the aid of ethanol or a mixture of ethanol and acetone. The L-L and D-D stereomers (LXVII, LXVIII) were recrystallized from water (5 ml.)-ethanol (30 ml.). The L-D and D-L stereomers (LXIX, LXX) were obtained as hygroscopic crystalline products. However, their paper chromatographic behavior in a number of solvent systems indicated that they gave only a single ninhydrin-positive spot and, hence, were suitable as chromatographic reference compounds for the present purposes.

Phenylalanyllysine Hydrochlorides (LXXI, LXXII).—The hydrogenolysis of LXV and LXVI was carried out in the manner described above. The free dipeptide hydrochlorides were obtained as hygroscopic crystals. However, each of them yielded only a single ninhydrin-positive spot when subjected to paper chromatographic criteria.

α,ε-Dicarbobenzoxy-L-lysyl-L-phenylalanine Benzyl Ester (LXXIII).—This compound was prepared from α,ε-dicarbobenzoxy-L-lysine[11] and, L-phenylalanine benzyl ester[20] in the same manner as was used for LV.

L-Lysyl-L-phenylalanine Hydrochloride (LXXIV). This compound was obtained from LXXIII by hydrogenolysis, and was recrystallized from water-ethanol.

***R*f Values.**—The *R*f values of the compounds on ascending paper chromatography, employing *n*-butanol-acetic acid-pyridine-water (15 : 3 : 10 : 12, v/v) and No. 50 paper of the Toyo Roshi Co., are given in Table II. The *R*f values of the L-L and D-D antipodes were the same within the limits of experimental error, as were those of the D-L and L-D antipodes. The *R*f value for a L-L stereomer, however, differed appreciably from that of the diastereomeric L-D stereomer. In this connection, it should be noted that, recently, some results on the paper chromatographic separation of diastereomeric dipeptides have been published.[21]

Table II. *R*f values of dipeptide anhydrides and dipeptides

Compound	Configuration	*R*f volue
Val◇Lys·HCl	L-L or D-D	0.50
	D-L or L-D	0.52
Phe◇Lys·HCl	L-L or D-D	0.54
	D-L or L-D	0.60
Val◇Orn·HCl	L-L	0.47
	D-L	0.51
Phe◇Orn·HCl	L-L	0.53
	D-L	0.58
H-Val-Lys-OH·HCl	L-L or D-D	0.25
	D-L or L-D	0.20
H-Phe-Lys-OH·HCl	L-L	0.32
	D-L	0.27
H-Lys-Phe-OH·HCl	L-L	0.34

The Action of Enzymes on Dipeptide Anhydrides.—To a 1 ml. flask were added 0.01 mmol. each of XLIII—XLVI and XLVII—L, crystalline trypsin (3.0 mg.) and a 0.2 M phosphate buffer (0.5 ml.) at pH 7.5 The solution was made up to 1 ml. by water and incubated at 37°C for 24 hr. Similar experiments with each of XLVII—L and crystalline α-chymotrypsin (3.0 mg.) were also carried out. Paper chromatographic analysis of an aliquot sample of the enzymic digests yielded only a single ninhydrin-positive spot, which, in each instance, possessed the same *R*f values as the unhydrolyzed dipeptide anhydride substrates.

Biological Assays.* — The microorganisms employed were *B. subtilis* (*Bacillus subtilis* PCI 219), *St. aureus* (*Staphylococcus aureus* FDA 2099), *E. coli* (*Escherichia coli* IFO 3044), and *Pr. vulgaris* (*Proteus vulgaris* IFO 3045). The minimum amounts of the compounds necessary for the complete inhibition of growth were determined by a dilution method with a bouillon agar medium at pH 7.0. As is shown in Table III, no antibacterial activity by the dipeptide anhydrides was observed against any of the microorganisms used even at a high concentration of each compound. In contrast with the lack of activity of each dipeptide anhydride, gramicidin S, a cyclic decapeptide, exhibited considerable activity against some of the microorganisms.

Erlanger and Goode have reported that, in a synthetic medium, the minimum amount of gramicidin S for the complete inhibition of the growth of *E. coli* was 5 μg. per ml.[2] As is indicated in

19) W. Grassmann and E. Wünsch, *Chem. Ber.*, **91**, 449, 462 (1958).

20) N. Izumiya and S. Makisumi, *J. Chem. Soc. Japan, Pure Chem. Sec.* (*Nippon Kagaku Zassi*), **78**, 662, 1768 (1957).

21) T. Sokolowska and J. F. Biernat, *J. Chromatogr.*, **13**, 269 (1964).

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TABLE III. INHIBITORY ACTIVITY OF PEPTIDE ANHYDRIDES ON MICROORGANISMS

Compound	Amount of compound necessary for complete inhibition of growth, μg./ml.			
	B. subtilis	*St. aureus*	*E. coli*	*Pr. vulgaris*
Gramicidin S·H_2SO_4[a)]	12.5	12.5	>100	50
Val◇Lys·HCl (XLIII—XLVI)	>100	>100	>100	>100
Phe◇Lys·HCl (XLVII—L)	>100	>100	>100	>100
Val◇Orn·HCl (LI, LII)	>100	>100	>100	>100
Phe◇Orn·HCl (LIII, LIV)	>100	>100	>100	>100

a) A product of Astra Co., U.S.A.

Table III, in a bouillon medium, gramicidin S exhibited no activity at the 100 μg. per ml. level. In this connection, experiments concerning the growth inhibition of *E. coli* in a synthetic medium by the dipeptide anhydrides, XLIII—LIV, are in progress in this laboratory.

Summary

The D-D, L-L, D-L and L-D stereomers of both valyllysine anhydride and phenylalanyllysine anhydride, and the D-L and L-L stereomers of both valylornithine anhydride and phenylalanylornithine anhydride, have been synthesized in order to compare them with the cyclic peptide antibiotics, with which they possess several structural features in common.

Synthesis has been achiveved by the dicyclohexylcarbodiimide-induced condensation of formyl, (or *t*-butyloxycarbonyl) valine (or phenylalanine) with the ω-carbobenzoxylysine (or ornithine) ester, followed by the treatment of the resulting acyl dipeptide ester with hydrogen chloride in methanol or ethyl acetate to remove the formyl- or *t*-butyloxycarbonyl-blocking group. The dipeptide ester hydrochlorides so derived have been converted by the action of ammonia to the corresponding carbobenzoxy-substituted dipeptide anhydrides and these, in turn, transformed to the desired unacylated dipeptide anhydride hydrochlorides by palladium-catalyzed hydrogenolysis.

Enzymic experiments have been carried out to determine whether the dipeptide anhydride hydrochlorides are attacked by trypsin or chymotrypsin. The results indicate that no hydrolysis of any of the anhydrides occurs. In this connection, valyllysines, phenylalanyllysines and lysylphenylalanine have been synthesized for the purposes of chromatographic comparison.

The effects of the dipeptide anhydride hydrochlorides on bacterial growth have been examined. The results indicate that no antibacterial activities are observed against microorganisms in a bouillon agar medium.

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