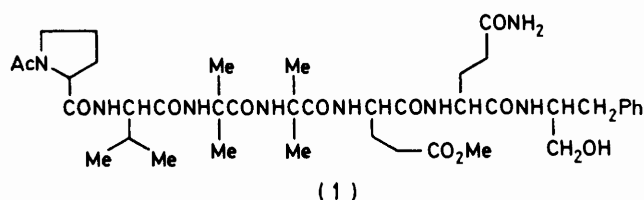
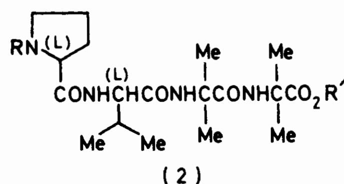
The Chemistry of Peptides Related to Metabolites of *Trichoderma* spp. Synthesis of L-Prolyl-L-valyl-2-methylalaninyl-2-methylalanine

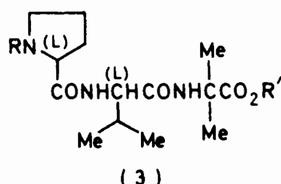
By **I. M. Shaw** and **A. Taylor,*** National Research Council of Canada, Atlantic Regional Laboratory, Halifax, Nova Scotia, Canada

L-Prolyl-L-valyl-2-methylalaninyl-2-methylalanine has been synthesised in 33% overall yield on the 100 g scale, from benzyloxycarbonyl-L-proline.

THE genus *Trichoderma* produces acidic metabolites [1] that affect the physiological properties of membranes [2] and inhibit growth and cellulolytic activity of rumen bacteria.[3] Reusser [1] showed that glycine, 2-methylalanine, L-alanine, L-glutamic acid, L-leucine, L-proline, and L-valine were hydrolysis products of U-22324, and that some of these residues were probably linked by peptide bonds. The isolation of the peptide (1),[4] after







treatment of alamethicin with trifluoroacetic acid, supports this conclusion. Studies of the biology of the *Trichoderma* metabolites [5] requires their characterisation, but this is difficult because knowledge of the likely physical and chemical properties of such complex mixtures is lacking.[6] This ignorance may be dissipated by a study of suitable model compounds, and to this end a synthesis of L-prolyl-L-valyl-2-methylalaninyl-2-methylalanine, regarded as a starting material for the elaboration of such models, is reported.

RESULTS AND DISCUSSION

Benzyloxycarbonyl-L-prolyl-L-valine [7] treated with pivaloyl chloride,[8] and the resulting mixed anhydride with methyl 2-methylalaninate gave the tripeptide (3, R = $PhCH_2OCO$, R′ = Me) in 73% yield having $[\alpha]_D^{21}$ −69°. A fourfold increase in scale (5→20 g) reduced the yield to 45% and this result, together with difficulties of using pivaloyl chloride on a large scale led us to investigate other routes to (3, R = $PhCH_2OCO$, R′ = Me). Excellent yields of this tripeptide were obtained using 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline [9] as the condensing agent when it was found that the yield was solvent-dependent. After purification of the tripeptide (3, R = $PhCH_2OCO$, R′ = Me), prepared in toluene the yield was *ca.* 90% (82% overall from proline) and it had $[\alpha]_D^{21}$ −82° (the yield in methanol was 55%). A similar optical rotation was obtained on the tripeptide (3, R = $PhCH_2OCO$, R′ = Me, 25% overall from *N*-t-butyloxy-L-valine) prepared by condensation of benzyloxycarbonyl-L-proline with methyl L-valyl-2-methylalaninate, a reaction unlikely to be attended by racemisation.[10] When the product with $[\alpha]_D^{21}$ −82° was saponified, reversed-phase partition chromatography of the acid gave a single peak of retention volume 54.6 ± 0.1 ml. Similarly, benzyloxycarbonyl-L-prolyl-D-valyl-2-methylalanine gave a single peak of retention volume 58.2 ml. However, chromatography of the crude saponification products after condensation of benzyloxycarbonyl-L-prolyl-L-valine or benzyloxycarbonyl-L-prolyl-D-valine with methyl 2-methylalaninate, indicated 3% of the D-valine isomer in the former, and 7% of the L-valine isomer in the latter. Benzyloxycarbonyl-L-prolyl-L-and -D-valines were also separated, having retention volumes of 42.4 and 39.8 ml respectively. The tetrapeptide derivative (2, R = $PhCH_2OCO$, R′ = H) had a retention volume of 61.2 ml under the same conditions, hence all five compounds could be detected in the same chromatographic run. The limit of detection of the tetrapeptide (2, R = $PhCH_2OCO$, R′ = H) was about 10 μg, and 1—2% of a contaminating diastereoisomer could be determined. Similar procedures have been reported.[11]

Benzyloxycarbonyl-L-prolyl-L-valyl-2-methylalanine condensed with methyl 2-methylalaninate to give the tetrapeptide (2, R = $PhCH_2OCO$, R′ = Me) but despite about 20 exploratory experiments we were unable to achieve yields greater than 50% on the 30 g scale. About 20 g of the starting tripeptide could be recovered. This protected tetrapeptide was then converted into its derivatives (2, R = $PhCH_2OCO$, R′ = H; 2, R = H, R′ = Me) by appropriate hydrolysis and hydrogenolysis reactions, and similarly to the tetrapeptide (2, R = R′ = H).

Signals for 19 carbon atoms were found in the ^{1}H-broadband-decoupled ^{13}C n.m.r. spectrum of the ester (2, R = H, R′ = Me) in [$^{2}H_4$]methanol, and included four carbonyl carbon resonances (δ_C 177.1, 176.3, 175.8, 173.0) and four α-carbon signals, two of which (δ_C 57.6, 57.0) were quaternary, and two were α-CH (δ_C 61.3, 60.3). Resonances due to seven methyl carbon atoms [δ_C 19.0, 19.6 (valine); 24.1, 24.6 (?), 25.5, 26.3 (2-methylalanine); 52.6 (ester)] were observed, and the remaining CH signal at δ_C 31.4 was therefore assigned to the β-CH group of valine. The CH_2 triplets in the off-resonance-decoupled spectrum centred at δ_C 48.0 and 31.8 were assigned to the δ- and β-CH_2 groups of proline.[12] The remaining signal at δ_C 27.0 is tentatively assigned to the γ-CH_2 proline carbon atom. In the ^{1}H-broadband-decoupled ^{13}C n.m.r. spectrum of the ester (2, R = $PhCH_2OCO$, R′ = Me) in [$^{2}H_4$]methanol at 35 °C, the carbonyl carbon atom (δ_C 156.7, 156.1) of the benzyoxycarbonyl group and the proline carbon atoms (CO, δ_C 175.1 and 174.3; α-CH, δ_C 61.3 and 60.5; β-CH_2, δ_C 32.3 and 31.0; γ-CH_2, δ_C 24.4 and 24.0; δ-CH_2, δ_C 48.4 and 48.1) appeared as doublets, the components of which were of equal intensity. Assignments for the signals in the ^{1}H n.m.r. spectrum of (2, R = $PhCH_2OCO$, R′ = Me) are given in the Table and are

Assigned ^{1}H chemical shifts (p.p.m. downfield from $SiMe_4$) for methyl benzyloxycarbonyl-L-prolyl-L-valyl-2-methylalaninyl-2-methylalaninate

δ_H (C^2HCl_3)	δ_H ($C^2H_3O^2H$)	Intensity	J_{HH} [a]	Assignment
7.36(s)	6.91(m)	5		Ph
7.12(e) [b]		1		NH (2Me-Ala)
6.91(e) [b]		1	7.5	NH (Val)
6.83(e) [b]		1		NH (2Me-Ala)
5.23(d)		1	12.5	$PhCH_2$
5.12(d)		1	12.5	$PhCH_2$
	5.14, 5.08	2		$PhCH_2$
4.36(m)	4.39(m), 4.37(m)	1		α-H (Pro)
3.99(d)	3.90(d), 3.85(d)	1	6	α-H (Val)
3.71(s)	3.61(s)	3		ester-Me
3.55(m)	3.43(t)	2	*ca.* 6	δ-CH_2 (Pro)
	2.25(m)	1	*ca.* 7	β-H (Val)
ca. 2.2(m)		3		β-H (Val) + β-CH_2
1.95(m)		2		γ(?)-CH_2 (Pro)
	1.93(m)	4		β + γ-CH_2 (Pro)
1.43(s)		12		Me (2Me-Ala)
	1.45, 1.43	12		Me (2Me-Ala)
	1.01(d), 0.98(d)	3	6.5, 6.5	Me (Val)
0.90(d)		3	7	Me (Val)
	0.91(d), 0.89(d)	3	6.5, 6.5	Me (Val)
0.81(d)		3	7	Me (Val)

[a] For C^2HCl_3 solutions. [b] e = Exchangeable.

based on decoupling experiments, deuterium exchange, and comparisons with published coupling constants and chemical shifts for analogous compounds.[13] All spectra of *N*-acylproline derivatives were complex, and were temperature- and solvent-dependent. The data in the Table for the [$^{2}H_4$]methanol solution suggests the presence of two conformers in this solvent, in agreement with the ^{13}C n.m.r. data, and previous work.[12,13] However, by contrast to the ^{13}C n.m.r. data, the most noticeable duplication of signals occurs in those assigned to the benzylic-CH_2 protons, and the 2-methylalaninyl-CH_3 and valine-CH_3 groups. Thus signals, apparently singlets, in the ^{1}H-broadband-decoupled ^{13}C n.m.r. spectra are presumably unresolved doublets.

Neither the tetrapeptide (2, R = R′ = H), nor its simple derivatives, nor their products of permethylation [14] gave molecular ions or sequence information on mass spectroscopy. Despite this anomaly, the data are in accord with the proposed structure for the tetrapeptide, which is therefore regarded as an intermediate, available in quantity, for the elaboration of model peptides resembling the *Trichoderma* metabolites.

EXPERIMENTAL

^{1}H N.m.r. spectra were measured on Varian HA 100 (for di- and tri-peptides) and HR 220 instruments. ^{13}C N.m.r. spectra were measured using a Varian XL 100 instrument operated as previously described;[15] ^{13}C n.m.r. data separated by semicolons refer to single carbon atoms. Positive chemical shifts are quoted in p.p.m. downfield from tetramethylsilane. Mass spectra were measured after direct introduction of samples into the source of a Dupont 21-110B mass spectrometer. Peptide derivatives were chromatographed on a 'μ-Bondapack-C_{18}' (Waters) column (50 × 0.95 cm) using a mixture of methanol and pH 4, 0.01M ammonium acetate buffer (11 : 9). It was found that the efficiency of separation varied slightly from one column to another, and could be adjusted by making small changes in the proportion of the two liquids pumped on to the column. The proportion of the two liquids was controlled by a solvent programmer (Waters, model 660) which regulated the pumping speed of pumps (Waters), one pumping methanol and the other buffer. The buffer was prepared with water which had 100% transmission of 210 nm light through 1 cm; all buffer solutions were filtered through glass fibre filters (Millipore, $<3\mu$) before use. The retention volumes were independent of flow through the column in the range 2—6 ml min^{-1}; slightly better separations were obtained at slower flow rates. The effluent from the column was led through 0.09 mm (i.d. diameter) stainless steel tubing to a u.v. detector (Dupont, model 837 spectrophotometer) which measured the absorbance at 209 nm. In suitable cases the effluent from the detector was collected and the solute isolated.

Methyl N-*t-butyloxycarbonyl*-L-*valyl-2-methylalaninate*.—*N*-t-Butyloxycarbonyl-L-valine (Sigma, 19 g), toluene (30 ml), methyl 2-methylalaninate[16] (10.2 g), and 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) (24 g) were stirred together at room temperature for 7 d. The toluene was evaporated, the residue taken up in ethyl acetate and the solution washed with citric acid solution (2N), water, sodium bicarbonate solution (5%), water, and then dried (Na_2SO_4). The solution was filtered, the filtrate evaporated, and the residue (20 g, 72%) crystallised by tituration with di-isopropyl ether. The *ester* separated from isopropyl ether as needles, m.p. 151 °C (sublimes above 143 °C) (Found: C, 57.0; H, 9.0; N, 8.9; O, 25.2. $C_{15}H_{28}N_2O_5$ requires C, 57.0; H, 9.0; N, 9.0; O, 25.3%); $[\alpha]_D^{20}$ −28° (*c* 1.1, MeOH); δ_H (C^2HCl_3) 6.88 (1 H), 5.33 (1 H,

J 9 Hz), 3.91 (1 H, m), 3.73 (3 H, 2.13 (1 H, m), 1.53 (6 H, 1.46 (9 H), 0.98 (3 H, d, *J* 7 Hz), and 0.95 (3 H, d, *J* 7 Hz).

Methyl Benzyloxycarbonyl-L-*prolyl*-L-*valyl*-2-*methylalaninate*.—(*a*) Benzyloxycarbonyl-L-prolyl-L-valine [7] (20 g), toluene (200 ml), methyl 2-methylalaninate (6.7 g), and EEDQ (16.3 g) were stirred together at 40 °C for 70 h. The solution was evaporated, the residue dissolved in ethyl acetate (75 ml), and the solution washed with hydrochloric acid (2N, 2×), water, sodium bicarbonate solution (5%, 2×), and finally water. The dried (Na_2SO_4) ethyl acetate solution was evaporated and the residue was precipitated from di-isopropyl ether as a colourless glass (24 g, 94%) (Found: C, 61.6; H, 7.1; N, 9.2. $C_{23}H_{33}N_3O_6$ requires C, 61.7; H, 7.4; N, 9.4%). This ester (1 g) in di-isopropyl ether (1 ml) was applied to a silica gel column (60 × 5 cm, 400 g, Woelm 'for dry column chromatography'). The column was developed with isopropyl alcohol–di-isopropyl ether (1 : 9), fractions (10 ml) were collected and analysed by high-pressure chromatography as described, the fractions containing the component of greatest retention time being collected. These fractions were combined, evaporated, and the residue precipitated from di-isopropyl ether; $[\alpha]_D^{22}$ −82.6° (*c* 1.0, MeOH).

(*b*) Methyl *N*-t-butyloxycarbonyl-L-valyl-2-methylalaninate (1 g) in [^{2}H]chloroform (0.4 ml) was treated with a solution (5 ml) of trifluoroacetic acid (2.5 ml) in [^{2}H]chloroform. The reaction was followed by the decrease of the t-butyl proton resonance at δ_H 1.46. After 1 h, when the signal was undetectable, the solution was evaporated at 0 °C, benzyloxycarbonyl-L-proline (0.8 g), toluene (80 ml), and EEDQ (0.9 g) added, and the mixture set aside for 3 d at room temperature. The reaction mixture was worked-up as described in (*a*) above, giving the tripeptide ester as needles (0.5 g), m.p. 122—123 °C; $[\alpha]_D^{22}$ −82.5° (*c* 0.6, MeOH); δ_H (C^2HCl_3) 7.33 (5 H), 6.76 (2 H, exchangeable, m), 5.23 (1 H, d, *J* 12.5 Hz), 5.07 (1 H, br d, *J* 12.5 Hz), 4.33 (1 H, m), 4.20 (1 H, m), 3.70 (3 H), 3.55 (2 H, m), 2.06 (5 H, m), 1.51 (6 H, br s), 0.90 (3 H, d, *J* 6 Hz), and 0.82 (3 H, d, *J* 6 Hz); *m/e* 447, 416, 388, 331, 303, 232, 204, 160, and 91.

(*c*) *N*-Benzyloxycarbonyl-L-prolyl-L-valine (4.5 g), triethylamine (1.8 ml), and toluene (40 ml) were treated at −10 °C with pivaloyl chloride (1.6 ml). Methyl 2-methylalaninate (1.5 g) was added, the slurry set aside at 0 °C for 2 h, and then shaken during 72 h at room temperature. Ethyl acetate (100 ml) was added and the solution washed with dilute hydrochloric acid (2N), sodium bicarbonate solution, and water. The dried (Na_2SO_4) solution was filtered, evaporated to dryness, and the residue {4.3 g, 73%, $[\alpha]_D^{20}$ −69° (*c* 1.0, MeOH)} was purified as described in the previous paragraphs.

(*d*) *N*-Benzyloxycarbonyl-L-prolyl-L-valyl-2-methylalanine (1 g) was dissolved in tetrahydrofuran (20 ml) and the solution treated with a solution of diazomethane (0.15 g) in diethyl ether. The solution was set aside for 1 h, evaporated to dryness, and the residue recrystallised from di-isopropyl ether as needles, m.p. 123 °C, $[\alpha]_D^{20}$ −83° (*c* 1.0, MeOH).

L-*Prolyl*-L-*valyl*-2-*methylalanine*.— Methyl *N*-benzyloxycarbonyl-L-prolyl-L-valyl-2-methylalaninate (24 g) in methanol (100 ml) was treated with a solution (25 ml) of sodium hydroxide (6 g) in water. After 1 h at 20 °C the solution was concentrated, diluted with water (100 ml), extracted with ethyl acetate, and the raffinate acidified; the acid (18.3 g, m.p. 185—188 °C) was collected and washed with ether. The N-*benzyloxycarbonyltripeptide* separated from acetonitrile as colourless rosettes, m.p. 187—189 °C (Found: C, 60.7; H, 7.3; N, 9.7; O, 22.4. $C_{22}H_{31}N_3O_6$ requires C, 61.0; H, 7.2; N, 9.7; O, 22.1%); $[\alpha]_D^{22}$ −95° (*c* 1.7, MeOH): δ_C ($C^2H_3O^2H$) 177.4; 174.9, 174.8; 172.4; 156.7, 156.3; 137.9; 129.4; 128.9; 128.7; 68.1; 61.6, 61.1; 59.8; 56.8; 48.5, 48.1; 32.6, 31.2; 32.0; 25.3; 25.1; 25.3, 24.4; 19.6; and 18.8. This acid (10 g) in methanol (30 ml) was shaken with triethylamine (1 ml), palladium–charcoal (5%, 0.5 g), and hydrogen at 3.5 kg cm^{-2} for 24 h. The reaction mixture was filtered and the filtrate evaporated. L-*Prolyl*-L-*valyl*-2-*methylalanine* separated from ethyl acetate–light petroleum (10 : 1) as colourless rods, sharp at one end, m.p. 166 °C (Found: C, 56.2; H, 8.4; N, 14.0; O, 21.4. $C_{14}H_{25}N_3O_4$ requires C, 56.2; H, 8.4; N, 14.0; O, 21.4%); $[\alpha]_D^{23}$ −63°, (*c* 1.0, MeOH).

N-*Benzyloxycarbonyl*-L-*prolyl*-D-*valine*.— *N*-Benzyloxycarbonyl-L-proline [7] (20 g), methyl D-valinate ($[\alpha]_D^{20}$ −18.3°, *c* 3.0 in 0.4N-HCl) (10.5 g), EEDQ (22 g), and toluene (600 ml) were stirred together at room temperature for 3 d; the solution was then concentrated, the residue dissolved in ethyl acetate (250 ml) and the solution washed with acid, base, and then water. The dried (Na_2SO_4) filtered solution was evaporated and the residue was recrystallised from n-heptane to give *methyl* N-*benzyloxycarbonyl*-L-*prolyl*-D-*valinate* as needles, m.p. 57—58 °C (Found: C, 60.0; H, 7.4; N, 7.4; O, 25.0. $C_{19}H_{26}N_2O_5$ requires C, 60.0; H, 7.4; N, 7.4; O, 25.2%); $[\alpha]_D^{22}$ −19.3° (*c* 1.5, MeOH): δ_C ($C^2H_3O^2H$) 174.9, 174.8; 173.1; 156.4 (br s); 137.9; 129.4; 128.8; 128.6; 68.0; 61.2 (br s); 58.8; 52.4; 48.1 (br s); 32.7, 31.3; 32.0; 24.5 (br s); 19.5; and 18.4. This ester (20 g) in methanol (150 ml) was treated with sodium hydroxide solution (4N, 80 ml) and the solution set aside for 90 min at room temperature; the methanol was then evaporated, water added, the solution washed with ethyl acetate (2×), and the aqueous layer acidified with hydrochloric acid (6N). The ethyl acetate remaining in solution was blown off with a stream of nitrogen, the residual solution set aside at 4 °C for 18 h and the crystalline solid that separated was collected (in some experiments the product separated as a gum, which usually crystallised after trituration with ether). The *acid* (13.6 g, 70%) crystallised from xylene as needles, m.p. 89—91 °C (Found: C, 62.0; H, 6.9; N, 8.0; O, 23.0. $C_{18}H_{24}N_2O_5$ requires C, 62.0; H, 7.0; N, 8.0; O, 23.0%); $[\alpha]_D^{22}$ −38.6° (*c* 2.0 MeOH).

L-*Prolyl*-D-*valyl*-2-*methylalanine*.—*N*-Benzyloxycarbonyl-L-prolyl-D-valine (9 g), methyl 2-methylalaninate (3 g), EEDQ (7 g), and toluene (200 ml) were stirred together at room temperature for 3 d. The reaction mixture was worked-up as described for the L-valyl stereoisomer and gave a glass (10 g) which was re-precipitated when a hot di-isopropyl ether solution was allowed to cool. *Methyl* N-*benzyloxycarbonyl*-L-*prolyl*-D-*valyl*-2-*methylalaninate* (7.0 g) {$[\alpha]_D^{22}$ −10° (*c* 1.0, MeOH) (Found: C, 61.3; H, 7.4; N, 9.3; O, 22.0. $C_{23}H_{33}N_3O_6$ requires C, 61.7; H, 7.4; N, 9.4; O, 21.5%); δ_H (C^2HCl_3) 7.32 (5 H), 7.04 (1 H, exchangeable), 6.86 (1 H, exchangeable), 5.14 (2 H), 4.32 (1 H, m), 4.26 (1 H), 3.69 (3 H), 3.53 (2 H, m), *ca.* 2.1 (5 H, m), 1.49 (6 H), 0.92 (3 H, d, *J* 6.5 Hz), and 0.86 (3 H, d, *J* 6.5 Hz)} was dissolved in methanol (100 ml) and the solution treated with sodium hydroxide solution (2N, 50 ml). After 1 h at room temperature the reaction mixture was processed as described for the L,L-tripeptide and gave N-*benzyloxycarbonyl*-L-*prolyl*-D-*valyl*-2-*methylalanine* (5.7 g, 59%) as needles (from acetonitrile), m.p. 161—162 °C (Found: C, 60.9; H, 7.2; N, 9.7; O, 22.3. $C_{22}H_{31}N_3O_6$

requires C, 61.0; H, 7.2; N, 9.7; O, 22.1%); $[\alpha]_D^{22}$ +3.6° (*c* 0.72, MeOH). This acid (0.5 g) in methanol (20 ml) was shaken with triethylamine (0.5 ml), palladium–carbon (5%, 0.1 g), and hydrogen at 3.5 kg cm^{-2} for 18 h. The reaction mixture was filtered, evaporated, and the residue (0.35 g) was taken up in methanol (20 ml) and ether added. The precipitate (0.3 g) was recrystallised from methanol (1 ml per 10 mg product) to give the *tripeptide* (0.25 g) as needles, m.p. 249—250 °C (Found: C, 54.6; H, 8.3; N, 13.4; O, 23.4. $C_{14}H_{25}N_3O_4,0.5H_2O$ requires C, 54.5; H, 8.4; N, 13.6; O, 23.4%); $[\alpha]_D^{22}$ −11.3° (*c* 0.34, MeOH).

L-Prolyl-L-valyl-2-methylalaninyl-2-methylalanine.— *N*-Benzyloxycarbonyl-L-prolyl-L-valyl-2-methylalanine (30.2 g), toluene (100 ml), EEDQ (19 g), and methyl 2-methylalaninate (8.2 g) were stirred together at 40 °C for 144 h. The reaction mixture was evaporated, the residue dissolved in ethyl acetate (200 ml), and the solution worked up as described for the tripeptides by washing with acid and base. The gum (31 g) could be induced to crystallise by seeding. Otherwise, it (2 g) was dissolved in isopropyl alcohol (10 ml), adsorbed onto silica gel (5 g), the solvent evaporated and the silica packed on the top of a silica gel column [Woelm, 'for dry column chromatography', 400 g, equilibrated with isopropyl alcohol–di-isopropyl ether (3 : 20) 40 ml]. The column was developed with isopropyl alcohol–di-isopropyl ether (3 : 20), the first 650 ml of eluate was discarded, and the next 900 ml collected. The latter fraction was evaporated and the residue (1.25 g, m.p. 131—133 °C) crystallised when set aside at −18 °C for 48 h. *Methyl N-benzyloxycarbonyl-L-prolyl-L-valyl-2-methylalaninyl-2-methylalaninate* was recrystallised from ethyl acetate–light petroleum (1 : 20) as rosettes, m.p. 135—136 °C (Found: C, 61.0; H, 7.4; N, 10.4; O, 21.1. $C_{27}H_{40}N_4O_7$ requires C, 60.9; H, 7.6; N, 10.5; O, 21.0%); $[\alpha]_D^{20}$ −57° (*c* 1.0, MeOH): δ_C ($C^2H_3O^2H$) 176.3; 175.8; 175.1, 174.8; 172.8; 156.7, 156.1; 137.8; 137.7; 129.3; 128.9; 128.6; 68.0; 61.3, 60.5; 61.1; 57.6; 56.9; 52.6; 48.4, 48.1; 32.3, 31.0; 26.3; 25.4; 24.7; 24.3, 24.0; 24.4; 19.5; and 19.3. This ester (40 g) in methyl alcohol (200 ml) was treated with a solution (130 ml) of sodium hydroxide (10.4 g) in water. The solution was set aside at 20 °C for 1 h, concentrated, diluted with water (200 ml), and extracted with ethyl acetate. The raffinate was acidified, extracted with ethyl acetate, and the dried (Na_2SO_4) extract evaporated. The crude acid (34 g) was taken up in methanol and precipitated with ether. The colourless, amorphous *N-benzyloxycarbonyltetrapeptide* was recrystallised from methyl alcohol–ether (1 : 10), m.p. 145—147 °C (Found: C, 60.2; H, 7.4; N, 10.6; O, 21.6. $C_{26}H_{38}N_4O_7$ requires C, 60.2; H, 7.4; N, 10.8; O, 21.6%); $[\alpha]_D^{21}$ −38° (*c* 1.2, MeOH): δ_C ($C^2H_3O^2H$) 177.7; 175.6; 175.1, 174.9; 172.9; 156.6, 156.1; 137.7; 137.6; 129.3; 128.8; 128.5; 68.0; 61.3, 60.6; 60.9; 57.7; 56.9; 48.4, 48.0; 32.3; 31.1; 26.1; 25.3; 24.7; 24.5; 24.2; 19.5; and 19.2. This acid (18 g) in methyl alcohol (100 ml) and triethylamine (20 ml) was shaken at room temperature with palladium–carbon (10%, 1 g) and hydrogen at 3.5 kg cm^{-2} for 72 h. The filtered reaction mixture was evaporated and the residue (13.8 g) was recrystallised from methanol–ether (2 : 1) to give *L-prolyl-L-valyl-2-methylalaninyl-2-methylalanine*, m.p. 258—259 °C (Found: C, 56.0; H, 8.4; N, 14.4; O, 20.8. $C_{18}H_{32}N_4O_5$ requires C, 56.2; H, 8.4; N, 14.6; O, 20.8%); $[\alpha]_D^{20}$ −38° (*c* 0.74, MeOH).

Deuteriation of Methyl N-Benzyloxycarbonyl-L-prolyl-L-valyl-2-methylalaninyl-2-methylalaninate.— Palladium chloride (40 mg) in hydrochloric acid (11N, 99% ^{2}HCl, 0.2 ml) was treated with a solution of sodium acetate (1 g) in ^2H_2O (5 ml), carbon (Darco G-60, 0.5 g) added, and the mixture shaken with $^2H_2$ until gas absorption ceased. The mixture was centrifuged, the sediment washed with [$^2H_4$]-methanol (4 × 2 ml) and then treated with a solution of the tetrapeptide (2, R = $PhCH_2OCO$, R′ = Me, 0.7 g) in [$^2H_4$]methanol (5 ml). The mixture was heated under reflux for 24 h, filtered, and the filtrate evaporated. Hyflo-supercel (0.2 g) was suspended in [$^2H_4$]methanol (2 ml) and the mixture filtered on to a plug of fibre-glass and cotton wool. The filter bed was washed with [$^2H_4$]methanol (4 × 1 ml) and then with [^{2}H]chloroform (2 × 1 ml). The reaction product in [^{2}H]chloroform (0.5 ml) was filtered through the prepared filter plug to give a colourless crystal-clear filtrate which was treated with di-isopropyl ether (*ca.* 3 ml) until the solution was opalescent. The trideuterio-ester {0.48 g, m.p. 135—136 °C; $[\alpha]_D^{22}$ −60° (*c* 1.2, MeOH)} separated as needles after 18 h at 4 °C.

N-Chloroacetyl-L-prolyl-L-valyl-2-methylalaninyl-2-methylalanine.—The tetrapeptide (2, R = R′ = H) (8 g) was dissolved in sodium hydroxide solution (2N, 10.4 ml) at 0 °C and chloroacetyl chloride (2.8 g) added at the same rate as sodium hydroxide solution (2N, 12.8 ml) during 45 min. The alkaline solution was allowed to warm to room temperature and after 3 h was washed with ether, acidified, extracted with ether (3×), the extract washed with water, dried (Na_2SO_4), and evaporated. The *chloroacetyltetrapeptide* (2, R = $ClCH_2CO$, R′ = H) was recrystallised from ethyl acetate–light petroleum (2 : 1) as needles, m.p. 179—180 °C (Found: C, 52.0; H, 7.3; Cl, 7.5; N, 12.2; O, 21.3. $C_{20}H_{33}ClN_4O_6$ requires C, 52.1; H, 7.2; Cl, 7.7; N, 12.2; O, 20.9%); $[\alpha]_D^{22}$ −83° (*c* 0.7, MeOH).

Methyl L-Prolyl-L-valyl-2-methylalaninyl-2-methylalaninate.—(*a*) The tetrapeptide (2, R = R′ = H) (0.7 g) suspended in tetrahydrofuran (40 ml) was stirred with a solution (1 ml) of diazomethane (20 mg) in ether for 24 h. The solution was evaporated and the residue (0.75 g, m.p. 106 °C) was recrystallised from cyclohexane to give needles of the *methyl ester* (2, R = H, R′ = Me), m.p. 110—115 °C (Found: C, 57.2; H, 8.5; N, 13.4; O, 20.3. $C_{19}H_{34}N_4O_5$ requires C, 57.3; H, 8.5; N, 14.1; O, 20.1%); $[\alpha]_D^{21}$ −15° (*c* 0.4, MeOH); $\nu_{max.}$ (KBr) 1 740, 1 675, and 1 520 cm^{-1}.

(*b*) The tetrapeptide methyl ester (2, R = $PhCH_2OCO$, R′ = Me) (5 g) in methyl alcohol (120 ml) was shaken with palladium–carbon (5% 1 g) and hydrogen at 4 kg cm^{-2} for 20 h at room temperature. The reaction mixture was filtered through a pad of Celite 535 (2.5 g) and the filtrate evaporated. The residue (3.7 g), on recrystallisation from cyclohexane, gave needles, m.p. 113—115 °C, identical with material as prepared in the previous paragraph.

We thank Dr. A. G. McInnes for helpful discussions.

[8/1530 *Received, 21st August,* 1978]

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