Table II. Effect of 24-h Treatment on Uptake of [$^3$H]Thymidine by Secondary Mouse Embryo Fibroblast Cultures[a]

| compd | concn, mg/mL | change, % |
|---|---|---|
| $PtCl_4$-ICRF-159 | 10.0 | −71 |
| | 1.0 | −16 |
| | 0.1 | −8 |
| $PtCl_2$-ICRF-159 | 10.0 | −79 |
| | 1.0 | −30 |
| | 0.1 | −7 |
| $PdCl_2$-ICRF-159 | 10.0 | −17 |
| | 1.0 | −2 |
| | 0.1 | +21 |
| ICRF-159 | 10.0 | −73 |
| | 1.0 | −57 |
| | 0.1 | −20 |

[a] A. M. Creighton, Imperial Cancer Research Fund, private communication; for method, see A. M. Creighton and G. D. Birnie, *Int. J. Cancer*, 5, 47–54 (1970).

and washing the oily yellow residue with THF: yield 75%; mp 280–290 °C dec. The visible absorption spectrum indicated that a chemical change did take place, but the product was not purified further.

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# Synthesis of All the Stereoisomers of Statine (4-Amino-3-hydroxy-6-methylheptanoic Acid). Inhibition of Pepsin Activity by *N*-Carbobenzoxy-L-valyl-L-valyl-statine Derived from the Four Stereoisomers

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Synthesis of all four stereoisomers of the novel amino acid statine, 4-amino-3-hydroxy-6-methylheptanoic acid, found in pepstatin, a potent acid protease inhibitor, has been accomplished. Carbobenzoxy-L-valyl-L-valyl-statine tripeptides derived from all four stereoisomers have been prepared and their effect on pepsin activity is compared to that of pepstatin.

Pepstatin, isovaleryl-L-valyl-L-valyl-(3*S*,4*S*)-statyl-L-alanyl-(3*S*,4*S*)-statine,[1] is one of several peptide antibiotics isolated in a deliberate attempt to find low-molecular-weight protease inhibitors that were amenable to chemical synthesis.[2] This peptide inhibits renin, pepsin, and cathepsin D activities and shows promise in the treatment of ulcers, inflammation and hypertension,[2] and, recently, muscular dystrophy.[3,4] While pepstatin is a very effective inhibitor, it is not very selective, which may be a contributing factor as to why it is not in clinical use. Thus, synthesis of analogues and derivatives that might be more selective is warranted. The development of a convenient synthetic route to the novel amino acid statine, (3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid (**3**), and analogues was the initial objective of the research. While several syntheses of statine have been reported,[5-9] a more convenient method for separation of the (3*S*,4*S*) and (3*R*,4*S*) diastereomers is outlined in this paper. The difficulty of separating the diastereomers was solved by chromatographing them on commercial Lobar silica gel columns as did Rich et al.[9] In this note, the preparation of all four stereoisomers of statine, the synthesis of the four statine *tert*-butyl esters and their use in the preparation of Cbz-L-valyl-L-valyl-statine tripeptides, and a comparison of the tripeptides and pepstatin as inhibitors of pepsin activity are reported.

**Chemistry.** Synthesis of statine (**3**) is outline in Scheme I. The mixtures of diastereomers, **2a,b** and **2c,d**, were prepared by condensation of the appropriate phthalyl-leucinal with the zinc enolate of *tert*-butyl acetate.[8] Each diastereomeric mixture was resolved into its pure components by preparative liquid chromatography for a combined yield of 57%. The use of Boc and ethyl ester blocking groups appears to make the separation easier;[9] the increased bulk of the blocking groups used in the work reported here offers greater steric hindrance to the interaction of the polar groups with the silica gel. The free amino acids, **3**, were obtained in 94% yield from **2** by removal of the *tert*-butyl ester with $CF_3COOH$ and the phthalyl group by hydrazinolysis. Physical data for statine stereoisomers are given in Table I. The melting points of **3a** and **3c** differed from those of **3b** and **3d** as expected of diastereomers, but all of our melting points were higher than those reported.[5-7] The differences could in part be due to variations in thermometers, heating rates, or the temperature at which the samples were introduced during the melting point determinations and to the greater purity achieved by high-pressure liquid chromatography than by methods used in previous work. The optical rotations of **3a–d** are in reasonable agreement with reported[6] values. The tripeptide *tert*-butyl esters were prepared (Scheme II) from *tert*-butyl esters, **4**, which were obtained in 68%

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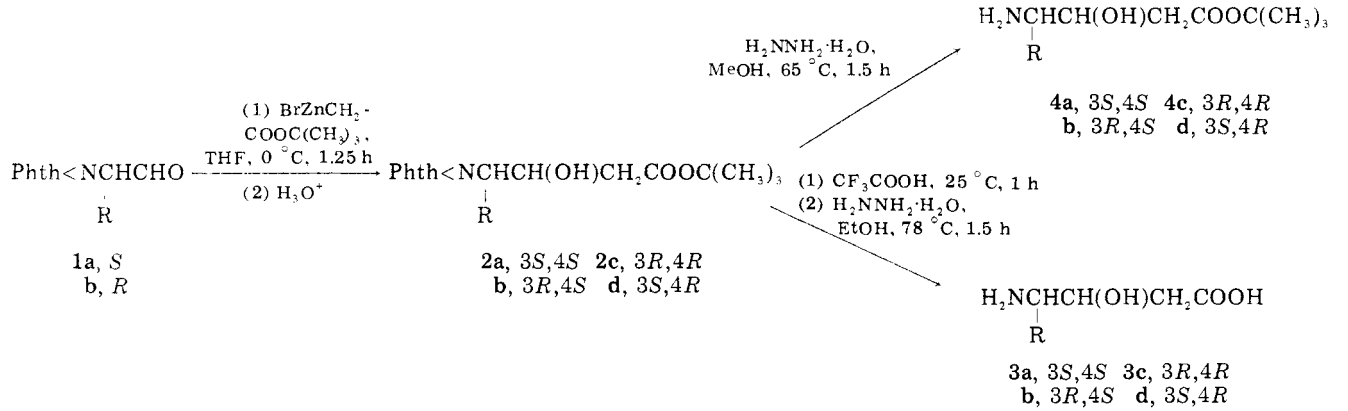
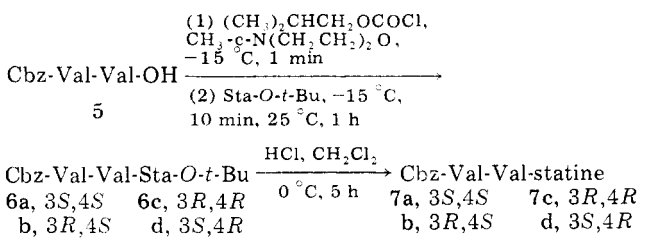
Table I. Properties of the Stereoisomers of Statine (3)

| compd | melting points, °C: this work | melting points, °C: reported[5,6] | optical rotations: $[\alpha]^{24}_D$, deg, this work | optical rotations: $[\alpha]^{21}_D$, deg, reported[6] |
|---|---|---|---|---|
| 3a | 235.5-237 | 201-203 | −20 (*c* 1, $H_2O$) | −20 (*c* 0.64, $H_2O$) |
| 3b | 217-218 | 202-203 | −19 (*c* 0.73, $H_2O$) | −20 (*c* 0.66, $H_2O$) |
| 3c | 234-236 | 200-202 | +20 (*c* 1.0, $H_2O$) | +19 (*c* 0.55, $H_2O$) |
| 3d | 216-217 | 202.5-203.5 | +18 (*c* 0.88, $H_2O$) | +20 (*c* 0.55, $H_2O$) |

Scheme I




Scheme II




yield by hydrazinolysis of the phthalyl blocking group of **2**. Cbz-L-valyl-L-valine (**5**)[10] was coupled to **4** by the mixed anhydride method,[11] giving 65% yields of the tripeptides, **6**. The homogeneity of each blocked tripeptide, **6a–d**, was established by analytical high-pressure liquid chromatography. The tripeptides, **7**, were obtained in quantitative yield by removal of the *tert*-butyl ester with HCl in $CH_2Cl_2$. Physical data for **7a–d** are found in Table II. The melting point of **7a** is in good agreement with that reported,[12] but the optical rotations are not very similar. Since the coupling methodology employed has been shown to give no racemization in coupling dipeptides to amino acids,[11] the discrepancy probably does not result from racemization occurring during the coupling steps.

**Inhibition of Pepsin Activity.** The *N*-Cbz tripeptides were prepared as analogues of pepstatin rather than the *N*-valeryl tripeptides because it has been reported that Cbz-L-valyl-L-valyl-(3*S*,4*S*)-statine was slightly more inhibitory against pepsin activity than was the corresponding *N*-valeryl derivative[12] and the Cbz group offers greater flexibility in the preparation of other *N*-acyl derivatives. Table III summarizes the data for inhibition of the peptic hydrolysis of hemoglobin and Cbz-Phe-Tyr by the tripeptides **7a–d** and by pepstatin. The data show that the additional residue of statine in pepstatin is quite important to the inhibition of hemoglobin hydrolysis but is not significant in the dipeptide hydrolysis. It is also of interest to note that, while the (3*S*,4*S*) tripeptide and pepstatin differ significantly in their inhibition of dipeptide and protein hydrolysis, the other tripeptides are similar in their ability to inhibit either hydrolysis. The stereochemistry

Table II. Properties of Cbz-L-valyl-L-valyl-statine (7)

| compd | mp, °C | $[\alpha]^{30}_D$ (*c* 1.3, $CH_3COOH$), deg |
|---|---|---|
| 7a | 200-202[a] | −75.5[b] |
| 7b | 215.5-218 | −50.0 |
| 7c | 201-203 | −10.2 |
| 7d | 216-218 | −14.2 |

[a] Reported[12] mp 199.5-200 °C. [b] Reported[12] $[\alpha]^{22}_D$ (*c* 1.3, $CH_3COOH$) −47.9°.

Table III. Inhibition of Pepsin Activity by Synthetic Tripeptides and Pepstatin

| inhibitor | $ID_{50}$, μg/mL[a]; hydrolysis of: Cbz-Phe-Tyr | $ID_{50}$, μg/mL[a]; hydrolysis of: Hb |
|---|---|---|
| pepstatin | 2 | 0.0052 |
| 7a | 1 | 0.10 |
| 7b | 12 | 21.0 |
| 7c | 42 | 18.0 |
| 7d | 219 | 224.0 |

[a] $ID_{50}$ is an estimation of the concentration of inhibitor giving 50% inhibition of pepsin activity under the experimental conditions.[13] Briefly, percent inhibition = $[(A - B)/A]100$, where $A$ is the absorbance at 280 nm observed in the hemoglobin assay or the rate of decrease in absorbance at 237 nm observed in the dipeptide assay in the absence of inhibitor and $B$ is the value obtained in the presence of inhibitor. The concentration giving 50% inhibition was obtained by plotting the percent inhibition on a probability graph (National Blank Book Co., no. 12-083) on the ordinate and the log of the inhibitor concentration on the abcissa. In all cases, the plot was linear between a minimum of four different concentrations encompassing the 50% inhibition point.

of statine is important; i.e., the change at position 4 is more significant. This position corresponds to the α carbon of the amino acid at the carboxyl side of the peptide bond which is to be cleaved, so that the change of 4*S* to 4*R* is equivalent to changing an L-amino acid to a D-amino acid. Even though **7a** is 20-fold less effective than pepstatin, it is effective at the low concentration of 0.1 μg/mL, indicating that synthesis of tripeptides as analogues of pep-

statin appears to be a reasonable approach to finding protease inhibitors that are more selective than pepstatin.

## Experimental Section

Melting points were determined on a Mel-Temp apparatus in capillaries and are uncorrected. NMR spectra were consistent with the structures of compounds reported and were recorded on Varian T-60 or HA-100 spectrometers. Elemental analyses were within 0.4% of theoretical values and were performed by Galbraith Laboratories. Lobar columns prepacked with silica gel 60 (EM Reagents) were used for preparative separations. Analytical high-pressure liquid chromatography was performed on a 4.1 × 300 mm column of Partisil-10 silica gel (Whatman). Amino acids and proteins were obtained from Sigma. *N*-Cbz-L-phenylalanyl-L-tyrosine was purchased from Chemical Dynamics Corp. Pepstatin was purchased from the Peptide Institute, Inc. (Osaka, Japan).

***tert*-Butyl 4-(Phthalylamino)-3-hydroxy-6-methylheptanoate (2).** *N*-Phthalyl-L-leucinal (**1a**) was condensed with the zinc enolate prepared from *tert*-butyl α-bromoacetate as described previously.[8] The two diastereomers **2a** and **2b** were separated in yields of 25 and 32%, respectively, by preparative chromatography eluting with 1% *i*-PrOH in $CH_2Cl_2$. The other two stereoisomers **2c** and **2d** were prepared similarly. The compounds were recrystallized from $Et_2O$–petroleum ether, giving crystalline **2a**, mp 79.5–82 °C; **2b**, mp 90–91 °C; **2c**, mp 79–82 °C; and **2d**, mp 90–91 °C. Anal. ($C_{20}H_{26}NO_5$) C, H, N. The purity of each stereoisomer was confirmed by analytical high-presure liquid chromatography, eluting with 1% *i*-PrOH in $CH_2Cl_2$.

**4-Amino-3-hydroxy-6-methylheptanoic Acid (3).** Deblocking of **2a** was effected by treatment of 0.7 g (1.9 mmol) at 25 °C in 10 mL of $CF_3COOH$ for 1 h, evaporation of the solvent in vacuo, and refluxing the residue with 82 mg (2.2 mmol) of 95% hydrazine hydrate in 10 mL of ethanol. The phthalhydrazide salt of **3a**, obtained by evaporation of the ethanol and excess hydrazine, was treated with an excess of 2 N HCl. The phthalhydrazide was removed by suction filtration and the filtrate concentrated to a gummy residue of **3a**·HCl. Crude **3a** was purified by ion-exchange chromatography on Dowex 50, eluting with 0.1 M pyridine adjusted to pH 5 with acetic acid. The pure amino acid (0.32 g) was obtained in 94% yield. The other stereoisomers **3b–d** were obtained similarly. Anal. ($C_8H_{17}NO_3$) C, H, N. Physical data are given in Table I.

***tert*-Butyl 4-Amino-3-hydroxy-6-methylheptanoate (4).** Compound **2a** (2 g, 5.4 mmol) and 95% hydrazine hydrate (0.25 g, 6.7 mmol) were refluxed in 10 mL of MeOH for 1.5 h. The solvent and excess hydrazine were removed in vacuo and the phthalhydrazide salt of **4** was decomposed by shaking the residue with 34 mL of 0.2 N HCl at 25 °C for 1.5 h. The mixture was chilled in an ice bath, the phthalhydrazide was filtered off, and the filtrate was poured into a cold mixture of 70 mL of 2 N NaOH and 150 mL of $Et_2O$. The aqueous phase was washed with $Et_2O$ (2 × 100 mL), and the combined organic extracts were dried ($Na_2SO_4$) and evaporated, yielding 0.85 g (68%) of an oil that crystallized on storage at −20 °C. The other stereoisomers were prepared similarly, and all were used without further purification.

***N*-Carbobenzoxy-L-valyl-L-valine (5).** Carbobenzoxy-L-valine was coupled to L-valine ethyl ester by the mixed anhydride method[11] using isobutyl chloroformate and *N*-methylmorpholine in THF, yielding the ethyl ester of **5**, mp 89–90 °C (lit.[10] mp 90–92 °C). Saponification of the ester in alcoholic sodium hydroxide and recrystallization from EtOAc–petroleum ether yielded **5**, mp 133–135 °C (lit.[10] mp 132–134 °C).

***N*-Carbobenzoxy-L-valyl-L-valyl-statine *tert*-Butyl Ester (6).** A solution of 1.28 g (3.7 mmol) of **5** and 0.37 g (3.7 mmol) of *N*-methylmorpholine in 10 mL of dry THF was cooled to −15 °C and 0.51 g (3.7 mmol) of isobutyl chloroformate was added. After 1 min, a solution of 0.85 g (3.7 mmol) of **4a** in 5 mL of THF was added and the reaction was maintained at −15 °C for 10 min and then allowed to warm to room temperature. The THF was removed in vacuo, and the residue was taken up in EtOAc and extracted with 0.2 N HCl and 5% $NaHCO_3$. The organic solution was dried ($Na_2SO_4$) and evaporated to the crude tripeptide, which was purified by preparative chromatography in 3% *i*-PrOH in $CH_2Cl_2$. Recrystallization with EtOAc–petroleum ether yielded 1.35 g (65%) of **6a**. Compounds **6b–d** were obtained similarly. The homogeneity of the tripeptides was confirmed by high-pressure liquid chromatography: **6a**, mp 150–152 °C; **6b**, mp 168.5–170 °C; **6c**, mp 149.5–152 °C; **6d**, mp 168–169 °C.

***N*-Carbobenzoxy-L-valyl-L-valyl-statine (7).** The tripeptides were obtained in quantitative yield by treatment of **6** with $CH_2Cl_2$ saturated with HCl at 0 °C for 5 h, evaporation of the solvent, dissolving the residue in a minimal amount of $CH_3OH$, and precipitating the pure tripeptides with a large volume of EtOAc. The physical properties of **7a–d** are listed in Table II. Anal. ($C_{26}H_{41}N_3O_7$) C, H, N.

**Inhibition of Pepsin Activity by 7a–d and Pepstatin. Hemoglobin Assay.**[13] One milliliter of 0.5% hemoglobin solution, 0.8 mL of 0.02 M KCl–HCl buffer (pH 2.0), and 100 μL of the same buffer containing appropriate concentrations of the inhibitors were mixed and incubated for 3 min at 37 °C. Porcine pepsin, 100 μL of a 10 μg/mL solution, was added, and incubation was continued for 25 min at 37 °C. The reaction was terminated by the addition of 2.0 mL of 1.7 M perchloric acid. After 1 h at room temperature, the precipitated proteins were centrifuged and the absorbance of the supernatant at 280 nm was measured. The absorbance of a blank containing no enzyme was subtracted from each reading.

**Dipeptide Assay.**[14] Enzyme stock solutions were prepared immediately before use by dissolving 17 mg of porcine pepsin in 1 mL of water and centrifuging for 30 min at 10000*g*. A mixture of 3.0 mL of buffer and 100 μL of enzyme solution was equilibrated to 35 °C in a Beckman Acta III UV–visible spectrophotometer equipped with a thermostatted cell holder. Methanolic solutions of the inhibitors were prepared so that 100 μL could be added to give the desired final concentrations. Hydrolysis was initiated by adding 100 μL of a methanolic solution of *N*-Cbz-L-phenylalanyl-L-tyrosine, the cell was loosely stoppered, and the decrease in absorbance at 237 nm vs. time was recorded for 20–40 min. The slopes of the lines obtained were taken as the measured rates of hydrolysis.

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