A FACILE SYNTHESIS OF BESTATIN

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Bestatin, an immunomodulator useful in cancer treatment with the structure: *N*-[(2*S*, 3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl]-L-leucine, has been synthesized from *N*-acyl-α-aminoacetophenone. This novel method using readily available reagents and mild conditions can be applied to a large scale production of this useful agent. The overall yield of bestatin was 10.5% from *N*-acetyl-α-aminoacetophenone and 7.8% from *N*-benzoyl-α-aminoacetophenone.

Bestatin(**1**) is an aminopeptidase B inhibitor discovered in a culture filtrate of Streptomyces[1~3]. Orally administered bestatin augments immunological responses in human and animals[4,5]. Its clinical study has suggested usefulness in the treatment of cancer.

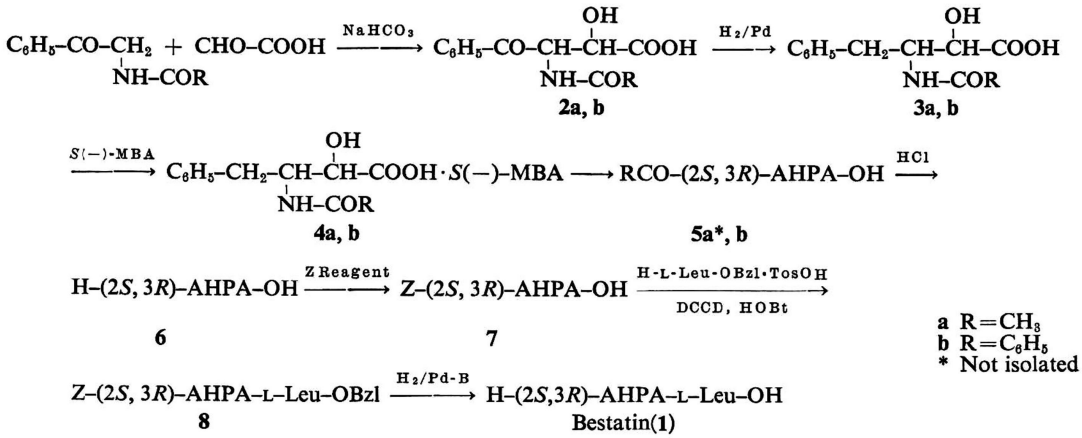
Synthetic studies have been continued to develop a feasible process for the large scale production of bestatin. (2*S*,3*R*)-3-Amino-2-hydroxy-4-phenylbutanoic acid is a key intermediate for synthesis of bestatin and this compound has been synthesized by two different methods. One is a procedure based on the introduction of a C_1 unit into D-phenylalanine[6,7] while the other is based on regio- and stereospecific hydroxyamination of methyl *cis*-benzylglycidate derived from phenylacetaldehyde and Meldrum's acid[8]. Both routes, however, seem to have disadvantages and to be impactical for large scale production. The former procedure needs expensive D-phenylalanine, highly ignitable lithium alminum hydride and low temperatures such as −20°C in the synthesis of D-phenylalaninal and poisonous cyanide in the synthesis of the cyanohydrin intermediate. The latter involves the *cis*-glycidate synthesis requiring explosive pertrifluoroacetic acid as well as expensive thallium nitrate.

In this paper, we report a novel and practical synthesis of bestatin from *N*-acyl-α-aminoacetophenone.

Synthesis

N-Acyl-α-aminoacetophenone and glyoxylic acid were allowed to react overnight in aqueous solution at slight alkalinity to give *N*-acyl-(2*RS*)-3-amino-2-hydroxy-4-oxo-4-phenylbutanoic acid (**2a** and **2b**) in 62% and 65% yield respectively. Compounds **2a** and **2b** were proved to have *threo* configuration as shown in later. *Erythro*-isomers were not detected by TLC, NMR and HPLC as described in detail in the experimental section. Compounds **2a** or **2b** were hydrogenated in an acidic medium at elevated temperature in the presence of a palladium on charcoal catalyst. The benzylic carbonyl group was reduced to afford **3a** or **3b** in 82% and 96% yield respectively. Racemic **3a** and **3b** were resolved by salt

Scheme 1.



AHPA: 3-Amino-2-hydroxy-4-phenylbutanoic acid residue, MBA: α-methylbenzylamine, Z: benzyloxycarbonyl, OBzl: benzyl ester, DCCD: dicyclohexylcarbodiimide, HOBt: 1-hydroxybenzotriazole, Z Reagent: benzyl *S*-4,6-dimethylpyrimidin-2-ylthiolcarbonate.

formation with *S*(−)-α-methylbenzylamine to give a optically pure salt **4a** and **4b** in 43% and 28% yield. After the removal of *S*(−)-α-methylbenzylamine, free *N*-acyl-(2*S*,3*R*)-AHPA-OH (**5a** (not isolated) and **5b**) were hydrolyzed in hydrochloric acid to yield H-(2*S*,3*R*)-AHPA-OH (**6**) in 64% and 73% yield from **4a** and **5b** respectively. Compound **6** was identified with authentic H-(2*S*,3*R*)-AHPA-OH obtained from D-phenylalanine. It was confirmed that the compound **2**~**5** had the *threo* configuration. According to previous reports[6,7], **6** was allowed to react with benzyl *S*-4,6-dimethylpyrimidin-2-ylthiolcarbonate to give Z-(2*S*,3*R*)-AHPA-OH (**7**) in 86% yield, followed by coupling with benzyl L-leucinate *p*-toluenesulfonic acid salt to afford **8** in almost quantitative yield. The protecting groups of **8** were removed by catalytic hydrogenation over palladium black to afford **1** in 85% yield. Bestatin was obtained in 10.5% overall yield from *N*-acetyl-α-aminoacetophenone and 7.8% from *N*-benzoyl-α-aminoacetophenone.

Physicochemical constants and enzyme inhibitory activities of the synthetic sample were the same as those of the authentic sample. The IC_{50} values of **1** thus obtained was 0.05 μg/ml for aminopeptidase B and 0.01 μg/ml for leucine aminopeptidase.

Experimental

Melting points were determined by a Shibata melting point apparatus and were uncorrected. Optical rotations were measured by a Perkin-Elmer 141 automatic polarimeter. Microanalyses were performed on a Yanagimoto MT-2 CHN corder. Nuclear magnetic resonance spectroscopy was carried on a JEOL-PMX-60 spectrometer with tetramethylsilane as internal standard. The abbreviations s, d, dd and multi indicate singlet doublet, double doublets and multiplet respectively. HPLC was carried out on a Hitachi 635 chromatograph under following conditions: column, 150 mm×4 mm ID packed with Nucleosil 5C-18; solvent, 1% H_3PO_4 - CH_3CN, 85: 15 (v/v); flow rate, 1.0 ml/minute; column temperature, 40°C; monitoring wavelength, 254 nm. Thin-layer chromatography (TLC) was used routinely for monitoring the reactions; Merck precoated silica gel plates (Art. 5715) were used and the detections were carried out with ultraviolet absorption, or visualized with iodine and ninhydrin reagent.

Threo-(2*RS*)-3-acetylamino-2-hydroxy-4-oxo-4-phenylbutanoic Acid (**2a**)

N-Acetyl-α-aminoacetophenone (4.43 g, 25 mmole) and sodium hydrogen carbonate (4.20 g, 50 mmole) were added to a mixture of 25% aqueous glyoxylic acid (13.0 g, 44.0 mmole) solution and water

(25 ml). The mixture was allowed to react at 50~60°C overnight and then cooled with an ice bath and adjusted with dilute hydrochloric acid to pH 1~2. The crystals were collected by filtration, washed with water and dried *in vacuo* over P_2O_5 overnight to give 4.70 g (64.9%) of **2a**, which gave a single peak by HPLC, mp 151~152°C (dec.); NMR (DMSO-d_6) δ 2.0 (3H, s, C*H*$_3$), 4.6 (1H, d, J=3 Hz, C*H*–OH), 5.9 (1H, dd, C*H*–NH), 7.7 (5H, multi, phenyl). In addition, broad absorption corresponding to NH and OH was shown at 6.6~8.0 ppm, which disappeared on addition of D_2O.

Threo-(2*RS*)-3-benzoylamino-2-hydroxy-4-oxo-4-phenylbutanoic Acid (**2b**)

N-Benzoyl-α-aminoacetophenone (16.7 g, 70.0 mmole) and sodium hydrogen carbonate (13.0 g, 155 mmole) were dissolved in a mixture of 25% aqueous glyoxylic acid solution (3.75 g, 130 mmole), water (100 ml) and methanol (250 ml). The mixture was allowed to react at 50~60°C overnight. After the removal of insoluble products by filtration, the filtrate was concentrated under reduced pressure to distill off the methanol. Dilute hydrochloric acid was added to the concentrated solution to adjust pH to 1~2. The crystals were collected by filtration, washed with water and dried *in vacuo* over P_2O_5 overnight. The crude material (17.6 g) was recrystallized from ethyl acetate to give 13.5 g (61.8%) of **2b**, mp 175~176°C (dec.); NMR (DMSO-d_6) δ 4.6 (1H, d, J=4 Hz, C*H*-OH), 5.9 (1H, dd, C*H*–NH), 7.7 (10 H, multi, phenyl×2), 8.5 (1H, d, J=9 Hz, NH).

Threo-(2*RS*)-3-acetylamino-2-hydroxy-4-phenylbutanoic Acid (**3a**)

The compound **2a** (3.00 g, 12.0 mmole) was dissolved in acetic acid (25 ml) and hydrogenated in the presence of 0.30 g of 5% palladium carbon at atmospheric pressure and 60°C for 6 hours. After the catalyst was removed by filtration, the filtrate was concentrated under reduced pressure to dryness. The residue was triturated with ethyl acetate (20 ml). The crystalline product was collected by filtration, washed with fresh ethyl acetate and dried *in vacuo* overnight to give 2.33 g (82.3%) of **3a**, mp 174~176°C; NMR (DMSO-d_6) δ 1.8 (3H, s, CH_3), 2.7 and 2.8 (1H, 1H, d, d, J=5 Hz, CH_2), 3.9 (1H, d, J=3Hz, C*H*–OH), 4.3 (1H, multi, C*H*–NH), 7.2 (5H, s, phenyl), 7.6 (1H, d, J=9 Hz, NH). The broad absorption of OH at 7.0~8.0 ppm, disappeared on addition of D_2O.

Threo-(2*RS*)-3-benzoylamino-2-hydroxy-4-phenylbutanoic Acid (**3b**)

Compound **2b** (5.00 g, 16.0 mmole) was hydrogenated in acetic acid (90 ml) for 8 hours at 70°C under atmospheric pressure with 10% palladium carbon (0.50 g) as catalyst. After the removal of the catalyst by filtration, the filtrate was concentrated under reduced pressure to give an oily material. This was triturated with petroleum ether, the resulting crystals were collected, washed and dried *in vacuo* to give 4.61 g (96.4%) of **3b**, mp 144~145°C; NMR (DMSO-d_6) δ 2.9 (2 H, d, J=7 Hz, CH_2), 4.0 (1H, d, J=3 Hz, C*H*–OH), 4.55 (1H, multi, C*H*–NH), 7.25 and 7.5 (10 H, s, multi, phenyl×2), 7.95 (1H, d, J=8 Hz, NH).

S(−)-α-Methylbenzylamine Salt of (2*S*,3*R*)-3-Acetylamino-2-hydroxy-4-phenylbutanoic Acid (**4a**)

Compound **3a** (10.87 g, 46.0 mmole) and *S*(−)-α-methylbenzylamine (5.55 g, 46.0 mmole) were dissolved in ethanol (90 ml) with heating and the solution was allowed to cool to room temperature. The crystals were collected, washed with a small amount of ethanol and dried *in vacuo* to give 6.37 g of optically impure crystals, $[\alpha]_D^{20}$ + 16.8° (*c* 1, MeOH). The salt (6.30 g) was recrystallized from ethanol to give 3.45 g (42.5%) of optically pure **4a**, mp 194~195°C; $[\alpha]_D^{20}$ + 29.0° (*c* 1, MeOH). $[\alpha]_D^{20}$ of the sample prepared from authentic H-(2*S*,3*R*)-AHPA-OH and *S*(−)-α-methylbenzylamine is +29.1°.

S(−)-α-Methylbenzylamine Salt of (2*S*,3*R*)-3-Benzoylamino-2-hydroxy-4-phenylbutanoic Acid (**4b**)

Compound **3b** (6.30 g, 21.1 mmole) and *S*(−)-α-methylbenzylamine (2.57 g, 21.1 mmole) were dissolved in ethanol (18 ml) with heating and then left overnight at room temperature. The crystals were collected and recrystallized from ethanol to give 1.22 g (27.5%) of optically pure **4b**, mp 147~148°C; $[\alpha]_{578}^{29}$ +70.6° (*c* 1.02, AcOH);

Anal. Calcd. for $C_{25}H_{28}N_2O_4$: C 71.39, H 6.72, N 6.67.
Found: C 71.67, H 6.99, N 6.73.

(2*S*,3*R*)-3-Benzoylamino-2-hydroxy-4-phenylbutanoic Acid (**5b**)

The salt **4b** (1.00 g, 2.38 mmole) was added to and shaken with a mixture of 1 N sulfuric acid (20 ml) and ethyl acetate (50 ml). The organic layer was separated and washed with water. The solution was

dried over anhydrous magnesium sulfate. The ethyl acetate was evaporated under reduced pressure and the residue was crystallized from ethyl acetate and petroleum ether to give 0.64 g (89.9%) of **5b**, mp 172~173°C; $[\alpha]_{578}^{25}$ +109.5° (*c* 1.1, AcOH);

Anal. Calcd. for $C_{17}H_{17}NO_4$: C 68.19, H 5.73, N 4.68.
Found: C 68.34, H 5.92, N 4.44.

(2*S*,3*R*)-3-Amino-2-hydroxy-4-phenylbutanoic Acid (**6**)

1) The salt **4a** (4.25 g, 11.9 mmole) and sodium hydrogen carbonate (1.49 g, 17.8 mmole) were dissolved in water (80 ml), and *S*(−)-α-methylbenzylamine was extracted with three 50 ml portions of ethyl acetate. The aqueous phase was acidified with concentrated hydrochloric acid, concentrated to about 40 ml and refluxed for 2 hours after the addition of more concentrated hydrochloric acid (1.7 ml, 20 mmole). The resulting solution was evaporated to dryness. Water (40 ml) was added to the residue, the resulting solution adjusted to pH 5~6 with 2 N sodium hydroxide solution and chilled in an ice bath. The crystals were collected, washed and dried *in vacuo* to give 1.48 g (63.8%) of **6**, $[\alpha]_{578}^{19.5}$ + 32.5° (*c* 0.76, 1 N HCl). The reported value[7] for this compound is $[\alpha]_{578}^{20\sim25}$ +29.5° (*c* 1, 1 N HCl).

2) A suspension of **5b** (0.60 g, 2.0 mmole) in concentrated hydrochloric acid (10 ml) and dioxane (10 ml) was refluxed for 6 hours and evaporated to dryness. Ethyl ether (50 ml) and water (100 ml) were added to the residue to remove benzoic acid by extraction. The water layer was passed through a column (10 ml) packed with Dowex 50 (H^+) and the column was washed with water. Eluates obtained with 2 N ammonia water were evaporated. The residue was triturated with acetone and dried to give 0.29 g (73.2%) of **6**, $[\alpha]_{578}^{20}$ + 31.9° (*c* 0.76, 1 N HCl).

(2*S*,3*R*)-3-Benzyloxycarbonylamino-2-hydroxy-4-phenylbutanoic Acid (**7**)

A mixture of **6** (1.45 g, 7.44 mmole), triethylamine (1.13 g, 11.2 mmole) and benzyl *S*-4,6-dimethylpyrimidin-2-ylthiolcarbonate (2.24 g, 8.20 mmole) in a mixed solvent of water (7 ml) and dioxane (7 ml) was allowed to react under stirring for 3 hours at room temperature. The reaction mixture was diluted with water (20 ml) and washed with two 25 ml portions of ethyl acetate. The aqueous phase was adjusted to pH 1~2 with dilute hydrochloric acid and then the oily product was extracted with two 30 ml portions of ethyl acetate. The combined extracts were washed with water, dried over anhydrous sodium sulfate and concentrated to dryness. The residue was triturated with petroleum ether. The resulting crystals were collected by filtration, washed with petroleum ether and dried *in vacuo* to give 2.10 g (85.7%) of **7**, mp 154~155°C; $[\alpha]_{578}^{20}$ +82.5° (*c* 1, AcOH). The values of an authentic sample[7] are mp 154.5°C and $[\alpha]_{578}^{20\sim25}$ +83.5° (AcOH).

Benzyl *N*-[(2*S*,3*R*)-3-Benzyloxycarbonylamino-2-hydroxy-4-phenylbutanoyl]-L-leucinate (**8**)

To a solution of **7** (2.00 g, 6.00 mmole), the *p*-toluenesulfonic acid salt of benzyl L-leucinate (2.63 g, 6.60 mmole) and 1-hydroxybenzotriazole (0.97 g, 7.20 mmole) in tetrahydrofuran (20 ml), triethylamine (0.67 g, 6.6 mmole) and dicyclohexylcarbodiimide (1.49 g, 7.20 mmole) were added at 0°C. The resulting solution was allowed to stand overnight. The precipitated dicyclohexylurea was filtered out and the filtrate concentrated to dryness. Ethyl acetate (50 ml) was added to the residue. The resulting solution was washed with 0.5 N hydrochloric acid, water, 5% aqueous sodium hydrogen carbonate solution and water and then dried over anhydrous sodium sulfate. The filtrate was concentrated to dryness and the residue triturated with *n*-hexane. The crystals were collected on a filter, washed with *n*-hexane and dried *in vacuo* to afford 3.19 g (99.4%) of **8**, mp 122~123°C; $[\alpha]_{578}^{24}$ +15.2° (*c* 1, AcOH). The reference values of the authentic sample[7] are mp 122°C (dec.) and $[\alpha]_{578}^{20\sim25}$ +15.1° (AcOH).

Bestatin (*N*-[(2*S*,3*R*)-3-Amino-2-hydroxy-4-phenylbutanoyl]-L-leucine, **1**)

The benzyloxycarbonyl and benzyl groups of **8** (3.00 g, 5.60 mmole) were removed by hydrogenation in acetic acid (50 ml) at room temperature using palladium black as catalyst as described in the previous paper[6,7]. After the separation of the catalyst, the filtrate was concentrated to dryness and the residue was triturated with acetone (30 ml). The crystals were separated and dissolved in 1 N hydrochloric acid. The solution was decolorized with activated charcoal and the filtrate adjusted to pH 5~6 with dilute aqueous ammonia. The precipitates was collected, suspended in acetone (20 ml), stirred for 2 hours, filtered and dried *in vacuo* over P_2O_5 to yield 1.47 g (85.0%) of **1**, mp 233~236°C (dec.); $[\alpha]_{578}^{25}$ −21.1° (*c* 1, AcOH). The corresponding value of the authentic sample[7] is $[\alpha]_{578}^{20\sim25}$ −23.5° (AcOH).

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