SYNTHESIS OF *p*-HYDROXYUBENIMEX

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p-Hydroxyubenimex, (2*S*,3*R*)-3-amino-2-hydroxy-4-*p*-hydroxyphenylbutyryl-L-leucine, was synthesized starting from D-tyrosine. The structure and stereochemistry of the synthesized product were confirmed by comparison with *p*-hydroxyubenimex that was chemically transformed from ubenimex, an aminopeptidase inhibitor of microbial origin. Compared to ubenimex, *p*-hydroxyubenimex is more active against aminopeptidase B but less active against leucine aminopeptidase. By using the synthetic *p*-hydroxyubenimex as a reference sample, one of the metabolites of ubenimex was identified as *p*-hydroxyubenimex. The (2*R*,3*R*)-stereoisomer of *p*-hydroxyubenimex was also prepared. However, its activity against aminopeptidases was much weaker.

Ubenimex[†††], (2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutyryl-L-leucine, is a dipeptide isolated from culture filtrates of *Streptomyces olivoreticuli* as an inhibitor of aminopeptidase B[1]. We have already reported on the synthesis of ubenimex and its analogues and their inhibitory activities[2]. Ubenimex has been tested as a therapeutic agent against cancer[3], and recently, the clinical use of this product was permitted in Japan. Ubenimex can be administered orally and has an extremely weak toxicity (LD_{50} in mice is 4.0 g/kg *per os*).

In this paper, the synthesis of *p*-hydroxyubenimex, (2*S*,3*R*)-3-amino-2-hydroxy-4-*p*-hydroxyphenylbutyryl-L-leucine, starting from D-tyrosine is reported.

Synthesis

p-Hydroxyubenimex was synthesized from D-tyrosine by a procedure similar to that of preparing ubenimex from D-phenylalanine[2,4] (Scheme 1). D-Tyrosine was benzyloxycarbonylated with benzyl *S*-4,6-dimethylpyrimidin-2-yl-thiolcarbonate (Z-S) by the method of NAGASAWA *et al.*[5]. The benzyloxycarbonyl-D-tyrosine (Z-D-Tyr, **1**) thus obtained was coupled with 3,5-dimethylpyrazole using dicyclohexylcarbodiimide (DCCD) as the coupling agent to yield pyrazolide (**2**). This was reduced with sodium dihydro bismethoxyethoxy aluminate (RD-2) in tetrahydrofuran at $-15\sim-20$°C to give Z-D-tyrosinal (**3**), which was precipitated as the adduct of sodium hydrogen sulfite (**4**). To the vigorously agitated suspension of **4** in a mixed solvent of water and ethyl acetate was added sodium cyanide. After dissolution, the ethyl acetate layer was separated and dried to yield cyanohydrin (**5**). This was then hydrolyzed in a mixed solvent of concentrated hydrochloric acid - dioxane (1 : 1) containing phenol as an anti-oxidant and refluxed under nitrogen atmosphere. Thus a diastereoisomeric mixture of (2*S*, 3*R*)- and (2*R*,3*R*)-3-amino-2-hydroxy-4-*p*-hydroxyphenylbutyric acids, (2*RS*,3*R*)-(*p*-OH)-AHPA (**6**), was prepared. The overall yield from **2** to **6** was 30%.

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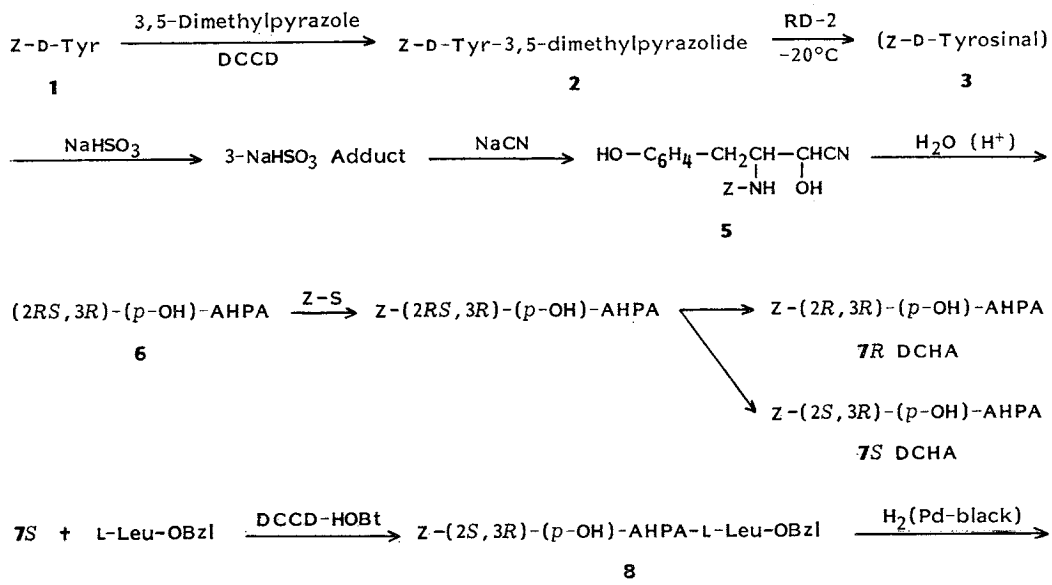
Scheme 1. Synthesis of *p*-hydroxyubenimex from D-tyrosine.



1 **2** **3** **5** **6** **8**

H-(2*S*,3*R*)-(*p*-OH)-AHPA-L-Leu-OH (*p*-Hydroxyubenimex)

Z=Benzyloxycarbonyl, Bzl=benzyl.

Compound **6** was benzyloxycarbonylated with Z-S in a quantitative yield. The Z-derivative (**7**) was then transformed to the dicyclohexylamine (DCHA) salt, and subjected to the fractional crystallization to separate the diastereoisomers.

One diastereoisomer (**7***R* DCHA) was isolated as crystals from a mixed solvent of methanol, ethyl acetate and petroleum ether, and the other (**7***S* DCHA) was recovered from the mother liquor. The former was purified by recrystallization from the mixed solvent described above, and the latter was purified by reprecipitation from ethyl acetate and petroleum ether. The purity of each diastereoisomer was subsequently confirmed by HPLC of the deprotected product, (*p*-OH)-AHPA, on Aminex A-7 developed with citrate buffer. The stereochemistry of **7***S* was determined as (2*S*,3*R*) after achieving the synthesis of *p*-hydroxyubenimex from this isomer as described in the Experimental section. The optical purity of **7***S* was also established in this synthesis.

The free acid yielded from **7***S* DCHA by acidic solvent extraction was coupled with L-leucine benzyl ester by DCCD-*N*-hydroxybenzotriazole (HOBt). The coupling product (**8**) was hydrogenated on palladium black to form *p*-hydroxyubenimex. The (2*R*)-epimer of *p*-hydroxyubenimex was prepared from **7***R* in a similar manner.

In order to determine the stereochemistry of the synthetic *p*-hydroxyubenimex, ubenimex was transformed to *p*-hydroxyubenimex. Ubenimex was first nitrated with fuming nitric acid in concentrated sulfuric acid. The amino function of *p*-nitroubenimex thus obtained was protected with a *tert*-butyloxycarbonyl group[5], and the nitro group was then reduced to the amino group by catalytic hydrogenation on palladium black. The resulting *N*-*tert*-butoxycarbonyl(BOC)-protected *p*-aminoubenimex was diazotized with sodium nitrite in a mixture of hydrochloric acid - acetic acid (1 : 1) at 0°C for 1 hour, followed by heating at 100°C for 2 hours to give *p*-hydroxyubenimex. This was then purified by silica gel column chromatography developed with butyl alcohol - acetic acid - water (4 : 1 : 1). The overall

Table 1. Enzyme inhibitory activities.

Compounds	IC_{50} (μg/ml)	
	AP-B	LAP
p-Hydroxyubenimex (from D-Tyrosine)	0.007	0.02
p-Hydroxyubenimex (from ubenimex)	0.007	0.02
epi-p-Hydroxyubenimex	2.5	6.3
Ubenimex	0.05	0.003

AP-B: Aminopeptidase B.
LAP: Leucine aminopeptidase.

transformation yield was about 2%.

The synthetic and transformed *p*-hydroxyubenimex had the same optical rotation, Rf values on TLC, IR and ^{1}H NMR spectra, and biological activities (Table 1).

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. NMR spectroscopy was carried out on a Jeol-PMX-60 spectrometer. The abbreviations s, d, dd, m and b indicate singlet, doublet, double of doublets, multiplet and broad respectively. 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 UV absorption, or visualized with iodine and ninhydrin reagent.

Aminopeptidase B (E.C.3.4.11.6), purified according to the method of HOPSU *et al.*[6], and L-arginine-β-naphthylamide, purchased from Protein Research Foundation, Japan, were used for the assay of aminopeptidase B activity. Leucine aminopeptidase (E.C.3.4.11.1), purchased from Miles Laboratories, Inc., and L-leucine-β-naphthylamide, purchased from Tokyo Chemical Industry Co., Ltd., Japan, were used for the assay of leucine aminopeptidase activity. For details of the assay methods see ref 1.

Synthesis of *p*-Hydroxyubenimex from D-Tyrosine

Z-D-Tyr-3,5-dimethylpyrazolide (**2**): To 200 ml of $CHCl_3$ solution containing 63.0 g of **1** and 19.2 g of 3,5-dimethylpyrazole was added 41.2 g of DCCD at -10°C and the reaction mixture was stirred at room temp overnight. The resulting dicyclohexylurea was filtered and the filtrate was evaporated to dryness. The residue was purified by recrystallization twice from $CHCl_3$ and *n*-hexane to give 55.4 g of **2** in a 70.4%-yield: MP 134~136°C; $[\alpha]_{578}^{28}$ $-61.7°$ (*c* 1.2, AcOH); Elemental *Anal* calcd for $C_{22}H_{23}N_3O_4$: C 67.14, H 5.90, N 10.69, found: C 67.51, H 6.13, N 10.35.

H-(2*RS*,3*R*)-(*p*-OH)-AHPA-OH (**6**): To a solution of 79.2 g of sodium dihydro bismethoxyethoxy aluminate (70% toluene solution) in 200 ml of THF was added a solution of 36.0 g of **2** in 500 ml of THF over a period of 1 hour at -15~-20°C. After standing for another 1 hour at -15~-20°C, the solution was dropped into 400 ml of 1 N HCl solution at -5°C. The precipitates were removed by centrifugation and the solvent evaporated. The residue was dissolved in EtOAc, washed with water, and evaporated. To the oily residue was added an ice cooled solution of $NaHSO_3$ (9.69 g) and the mixture was stirred overnight at 5°C. To the resulting suspension of $NaHSO_3$ adduct was added 500 ml of EtOAc and aq NaCN solution (4.48 g/100 ml) and the reaction mixture was stirred for 5 hours at room temp. The EtOAc layer was washed with water, then evaporated to give the cyanohydrin **5** as an oil. Oily **5** (30.0 g) was dissolved in 200 ml of conc HCl and 200 ml of dioxane, and after adding 17.2 g of phenol, the mixed solution was refluxed for 4 hours under a nitrogen stream. The solution was washed with ether, and the water layer was evaporated to dryness. Water (300 ml) was then added to the residue to remove insoluble materials. The same volume of acetone was added and pH of the solution adjusted to 5.5 with ammonia water. The solution was refrigerated overnight and the deposited crystals were filtered, providing 12.61 g of **6** in a 29.7% yield from **2**: $[\alpha]_{578}^{25}$ $+20.5°$ (*c* 0.7, 1 N

Table 2. Z-(*p*-OH)-AHPA-L-Leu-OBzl (**8** and **8'**).

Compounds	Configuration	Yield (%)	MP (°C)	$[\alpha]_{578}^{20}$ (AcOH)	*Elemental *Anal*		
					C	H	N
8	2*S*,3*R*, L	98.0	134.5 (sinter)	+22.3° (*c* 2.3)	68.03,	6.88,	5.39.
8'	2*R*,3*R*, L	87.6	138.5 (sinter)	+13.3° (*c* 3.3)	68.11,	6.59,	5.33.

* Calcd for $C_{31}H_{36}N_2O_7$: C 67.85, H 6.62, N 5.11.

HCl); ^{1}H NMR (CF_3COOD, TMS) δ 3.3 (2H, m, C*H*$_2$C$_6$H$_5$), 4.0~4.5 (1H, m, C*H*NH$_2$), 4.7 (1H, d, J=4 Hz, C*H*OH), 6.5~7.8 (8H, m, C$_6$*H*$_4$, N*H*$_2$, O*H*, COO*H*).

Z-(2*R*,3*R*)- and (2*S*,3*R*)-(*p*-OH)-AHPA-OH DCHA (7*R* DCHA and 7*S* DCHA): Compound **6** (7.2 g), 11.2 g of benzyl *S*-4,6-dimethylpyrimidin-2-ylthiolcarbonate and 7.17 ml of triethylamine were allowed to react in 30 ml of dioxane and 30 ml of water overnight at room temp. The reaction mixture was condensed to a half volume and washed with EtOAc. The aqueous layer was adjusted to pH 2 with 1 N HCl. The separated oily material was extracted with EtOAc, washed with water and dried over anhydrous $MgSO_4$. The filtrate was neutralized with DCHA and evaporated. The residue was washed with ether to give 15.2 g of the DCHA salt of the diastereomeric mixture 7*RS*. Crystallization of 15.2 g of 7*RS* DCHA salt from 50 ml of MeOH, 75 ml of EtOAc and 40 ml of petroleum ether gave 3.2 g of crude 7*R* DCHA. From the mother liquor, crude 7*S* DCHA was recovered and precipitated three times from EtOAc and petroleum ether to give 5.0 g of optically pure 7*S* DCHA: MP 121~122°C; $[\alpha]_{578}^{28}$ +55.3° (*c* 4.2, AcOH); ^{1}H NMR (DMSO-d_6+D$_2$O, TMS) δ 0.9~2.2 (20H, m, (C$_6$H$_{10}$)$_2$), 2.4~3.3 (4H, m, C*H*-NHC*H*, C*H*$_2$C$_6$H$_5$), 3.6 (1H, d, J=2 Hz, C*H*OH), 3.7~4.3 (1H, m, C*H*NH), 4.8~5.1 (2H, m, OC*H*$_2$C$_6$H$_5$), 6.7 and 7.0 (each 2H, d, J=9 Hz, C$_6$*H*$_2$), 7.2 (5H, s, C$_6$*H*$_5$).

The crude salt of 7*R* DCHA was recrystallized from MeOH, EtOAc and petroleum ether to give 1.85 g of optically pure salt: MP 195~197°C; $[\alpha]_{578}^{20}$ +3.1° (*c* 0.89, AcOH).

Z-(2*S*,3*R*)- and (2*R*,3*R*)-(*p*-OH)-AHPA-L-Leu-Obenzyl(Bzl) (**8** and **8'**): 7*R* or 7*S* DCHA (1.05 g) was suspended in 100 ml of EtOAc and treated with 1 N H_2SO_4 to remove DCHA. To the ice cooled solution resulting from 7*R* or 7*S*, 866 mg of H-L-Leu-OBzl tosyl (Tos)OH, 0.31 ml of triethylamine and 400 mg of HOBt was added 412 mg of DCCD. The reaction mixture was stirred overnight at the same temp, then evaporated. Dicyclohexylurea was removed from the residue by adding EtOAc. The solution was washed with 1 N HCl, water, 5% $NaHCO_3$ aq solution and water, successively, and then dried over anhydrous $MgSO_4$. The filtrate was evaporated under reduced pressure and the residue re-precipitated with EtOAc and petroleum ether to afford pure **8** or **8'**.

These data are summarized in Table 2.

p-Hydroxyubenimex and *epi-p*-Hydroxyubenimex: Z-(2*S*,3*R*) or (2*R*,3*R*)-(*p*-OH)-AHPA-L-Leu-OBzl (**8** or **8'**) in MeOH and AcOH was hydrogenated on palladium black for 4 hours. The resulting solution was evaporated to dryness and *p*-hydroxyubenimex or *epi-p*-hydroxyubenimex was filtered and washed with acetone.

p-Hydroxyubenimex was obtained in a quantitative yield: Rf 0.48 (BuOH - AcOH - H_2O, 4:1:1); $[\alpha]_{578}^{25}$ −19.7° (*c* 1.2, AcOH), ^{1}H NMR (DMSO-d_6+D$_2$O, TMS) δ 0.9 (6H, m, (CH$_3$)$_2$), 1.3~1.9 (3H, m, C*H*C*H*$_2$), 2.6~3.0 (2H, m, C*H*$_2$C$_6$H$_5$), 3.3~3.7 (1H, m, C*H*NH$_2$), 3.7~4.2 (2H, m, C*H*OH, C*H*COOH), 6.7 and 7.1 (each 2H, d, J=8 Hz, C$_6$*H*$_2$), 7.8~9.1 (0.3H, b, CON*H*). *epi-p*-Hydroxyubenimex was also obtained quantitatively: Rf 0.52 (BuOH - AcOH - H_2O, 4:1:1); $[\alpha]_{578}^{25}$ +29.9° (*c* 1.0 AcOH) and showed the same NMR spectrum except for the signal between 3.7~4.2 ppm because of an overlap with the signal of water.

Chemical Transformation of Ubenimex to *p*-Hydroxyubenimex

BOC-*p*-aminoubenimex: *p*-Nitroubenimex (7.40 g), which was obtained from ubenimex by nitration with fuming HNO_3 in conc H_2SO_4 in a 67%-yield[3], 5.01 g of *tert*-butyl *S*-4,6-dimethylpyrimidin-2-yl-thiolcarbonate[5] and 4.40 ml of triethylamine was allowed to react at room temp in 50 ml of dioxane and 50 ml of water. The reaction mixture was evaporated to a half volume and washed with 300 ml of EtOAc. The water layer was acidified to pH 2 with 1 N H_2SO_4, and the separated oily material was

extracted with 300 ml of EtOAc. The EtOAc layer was washed with water and dried over anhydrous $MgSO_4$. To the filtrate was added 3.62 g of DCHA and the DCHA salt deposited was filtered and washed with EtOAc. Recrystallization from 50 ml of MeOH and 100 ml of ether gave 6.48 g of the DCHA salt of BOC-*p*-nitroubenimex in a 49%-yield: MP 221~222°C; $[\alpha]_{578}^{20}$ +34.5° (*c* 2.4, AcOH).

The oily BOC-*p*-nitroubenimex, which was obtained by extraction with EtOAc from the acidic solution of 6.40 g of the DCHA salt, was hydrogenated on palladium black for 6 hours in 50 ml of MeOH and 50 ml of AcOH. Evaporation of the filtrates gave a solid material. Recrystallization from 100 ml of MeOH and 50 ml of ether gave 2.78 g of BOC-*p*-aminoubenimex in a 64.5%-yield: MP 230°C (dec); $[\alpha]_{578}^{20}$ +28.8° (*c* 1.1, AcOH); Elemental *Anal* calcd for $C_{21}H_{33}N_3O_6$: C 59.53, H 7.86, N 9.93, found: C 60.01, H 7.78, N 9.85.

p-Hydroxyubenimex: To a chilled solution of 421 mg of BOC-*p*-aminoubenimex in 10 ml of AcOH and 1 ml of 1 N HCl was added 12 ml of 0.1 N $NaNO_2$ aq solution at 0~5°C. This reaction mixture was stirred for 1 hour at 0~5°C, and 10 mg of urea was then added.

The reaction mixture was then heated for 2 hours at 100°C. The solution was adjusted to pH 5.0 with 10 ml of conc ammonia water and evaporated to dryness. The residue was dissolved in 100 ml of water and the solution was subjected on a column packed with 150 ml of Dowex 50W-X4 (H^+, 50~100 mesh). Crude *p*-hydroxyubenimex was eluted with 1 N NH_4OH and purified with silica gel column (2.5×35 cm) chromatography using BuOH - AcOH - H_2O (4 : 1 : 1) as a developing solvent. Solid material (212 mg) obtained by evaporation of the eluates from 45 ml to 60 ml was treated on a column packed with 3 ml of Dowex 50W-X4 (H^+, 100~200 mesh) to remove silica gel. *p*-Hydroxyubenimex (84.6 mg) was obtained by lyophilization of the eluates in a 26.1%-yield from BOC-*p*-aminoubenimex: $[\alpha]_{578}^{25}$ −19.5° (*c* 1.3, AcOH); Rf 0.48 (BuOH - AcOH - H_2O, 4 : 1 : 1); ^1H NMR (DMSO-d_6+D_2O, TMS) δ 0.9 (6H, m, $(CH_3)_2$), 1.3~1.9 (3H, m, $CHCH_2$), 2.6~3.0 (2H, m, $CH_2C_6H_5$), 3.3~3.7 (1H, m, $CHNH_2$), 3.7~4.2 (2H, m, CHOH, CHCOOH), 6.7 and 7.1 (each 2H, d, J=8 Hz, C_6H_2), 7.7~9.0 (0.6H, b, CONH).

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