A Simple Synthesis of ^{3}H-Labelled Ubenimex *

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SUMMARY

^{3}H-Labelled ubenimex, N-[(2S,3R)-3-amino-2-hydroxy-4-(*o*-^{3}H)phenylbutyryl]-S-leucine with a specific radioactivity of 15 mCi/mg was synthesized by a one step catalytic tritiation of N-[(2S,3R)-3-benzyloxycarbonylamino-2-hydroxy-4-(*o*-bromo)phenylbutyryl]-S-leucine benzyl ester previously isolated from a mixture of four optical isomers using preparative high performance liquid chromatography.

Chemical and radiochemical purities were over 99% as determined by thin layer chromatography and high performance liquid chromatography using radioisotope detection. By gas chromatography-mass spectrometric analysis of deuterated ubenimex, the ^{3}H-label was determined to be in the aromatic ring.

Keywords: ^{3}H-Labelled ubenimex, catalytic dehalogenation by ^{3}H-gas, diastreomeric separation, preparative HPLC

INTRODUCTION

The dipeptide ubenimex, N-[(2S,3R)-3-amino-2-hydroxy-4-phenylbutyryl]-S-leucine, isolated from the fermentation broth of *Streptomyces olivoreticuli*, inhibits both aminopeptidase B and leucine aminopeptidase[1]. Oral administration of ubenimex enhances a delayed type hypersensitivity in animals[2], raising the possibility of a new biological response modifier. In preclinical studies, [^{14}C]-ubenimex synthesized from [U-^{14}C]-leucine was investigated for properties such as absorption, excretion, distribution and metabolism. When administered to rats, the dipeptide was partially metabolized to 3-amino-2-hydroxy-4-phenylbutyric acid(AHPA) and [U-^{14}C]-leucine by endogenous peptidase and subsequently excreted into expired air as $^{14}CO_2$.

Consequently, it was desired to synthesize ubenimex radiolabelled in its AHPA moiety to allow a detailed investigation of its metabolism and pharmaco-kinetics. The synthesis of AHPA labelled with ^{14}C, however, is very difficult using the procedures previously reported[3,4,5]. This is because current procedures

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require isolation of each labelled stereoisomer and the handling radioactive intermediates through several steps. In this paper, we report a more simple synthesis of ^{3}H-labelled ubenimex, N-[(2S,3R)-3-amino-2-hydroxy-4-(*o*-^{3}H)phenylbutyryl]-S-leucine.

RESULTS AND DISCUSSION

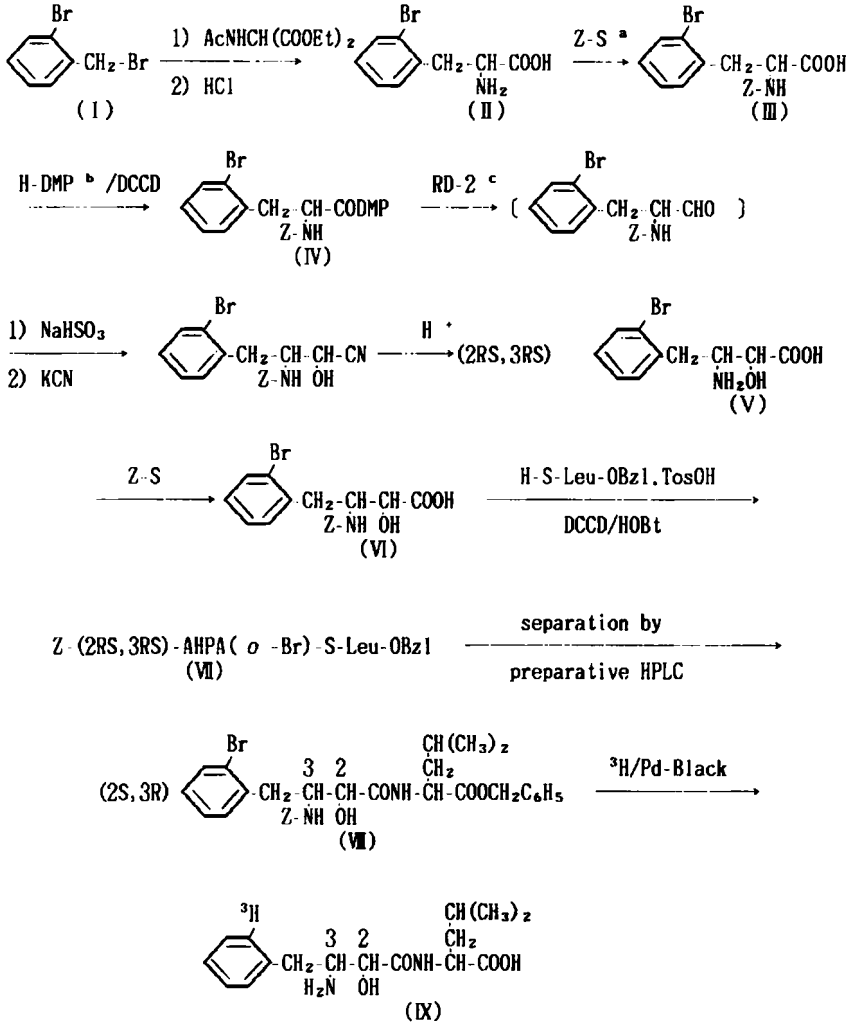
In studies of ubenimex and its analogues, HPLC was found to provide the most efficient analysis of its stereochemistry. Silica gel and reversed phase HPLC were used for analysis of protected or deprotected ubenimex stereoisomers, respectively. Except for one pair of optical enantiomers, four of possible eight optical isomers were successfully separated from each other by the above two HPLC methods. Therefore, if optically active leucine was used in the synthesis of ubenimex analogues, the four possible optical isomers could be separated. Provide that the separated analogue could be converted to ubenimex using mild conditions, the stereochemistry of the separated analogue would be easily determined by using HPLC to compare with standard samples. To achieve this, a catalytic deprotection and simultaneous dehalogenation of N-[(2S,3R)-3-benzyloxycarbonylamino-2-hydroxy-4-(*o*-bromo)phenylbutyryl]-S-leucine benzyl ester by tritium gas in the final step was selected as the strategy for synthesizing of ^{3}H-labelled ubenimex. In the preliminary metabolic study of ubenimex, hydroxylation of an aromatic ring occured in *para* or *meta* position. To avoid problems, the *ortho* position was selected for labelling.

A scheme of the synthesis is shown in Chart 1.

o-Bromobenzylbromide and diethyl acetamidemalonate were allowed to react and then hydrolyzed in 6N hydrochloric acid to give a 77.3% yield of *o*-bromophenylalanine [Phe(*o*-Br)] (II). This II was then treated following the procedures reported by Nishizawa *et al.*[4] to produce four diastereoisomeric mixtures of 3-amino-2-hydroxy-4-(*o*-bromo)phenylbutyric acid [AHPA(*o*-Br)] (V) in a 22% yield. This V was benzyloxycarbonylated using benzyl S-4,6-dimethylpyrimidin-2-ylthiolcarbonate to yield (2S,3R)-3-benzyloxycarbonylamino-2-hydroxy-4-(*o*-bromo)phenylbutyric acid [Z-AHPA(*o*-Br)-OH] (VI).

The VI was coupled with S-leucine benzyl ester *p*-Toluenesulfonic acid salt (S-Leu-OBzl.TosOH) by dicyclohexylcarbodiimide(DCCD)-N-hydroxybenzotriazole(HOBt) method to give Z-AHPA-(*o*-Br)-S-Leu-OBzl(VII) as a syrup in a quantitative yield. Then, VII was subjected to preparative silica-gel HPLC to separate of four optical isomers, (2S,3R-S), (2S,3S-S), (2R,3R-S), (2R,3S-S).

Purity of each fraction were monitored by an analytical silica-gel HPLC and the fractions were confirmed to be pure. Identification of the fractions' stereochemistry was done by a reversed phase HPLC after hydrogenation to ubenimex and its optical isomers using authentic sample as standards[4]. Z-(2S,3R)-AHPA(*o*-Br)-S-Leu-OBzl(VII) was isolated in a 13% yield from VII.



a : Benzyl S-4,6-dimethylpyrimidin-2-ylthiolcarbonate b : 3,5-Dimethylpyrazole,
c : $NaAlH_2(OCH_2OCH_2CH_3)_2$,

Chart 1 Synthetic Scheme of ^{3}H-labelled Ubenimex

Tritiation of VII was performed using palladium as a catalyst and with tritium gas under pressure of not more than 1 atm. The resulting crude ^{3}H-labelled ubenimex was contaminated with 2.88% of unknown radioactive compound. Treatment of the resulting crude IX by silica-gel column chromatography yielded a chemically and radiochemically pure IX.

Figure 1 and 2 show a TLC radiochromatogram and a HPLC radiochromatogram of IX, respectively. The behaviour of optical isomers of ubenimex in HPLC are described in Fig. 2 and each isomer was completely separated under the same conditions. As mentioned above, VII was isolated from a mixture of four optical isomers namely the (2S,3R-S), (2S,3S-S), (2R,3R-S) and (2R,3S-S) derivatives.

Fig 2 shows no contaminating optical isomers and shows IX to be of the 2S,3R-S configuration with high chemical and radiochemical purity.

To confirm the labelled position, VIII was deuterated by use of deuterium gas in the same manner as described here and the resulting deuterated ubenimex was subjected to gas chromatography-mass spectrometry(GC-MS) analysis. The ion, m/z 92 [$(C_6H_4D\text{-}CH_2)^+$, 41.9% in relative intensity] suggested mono-deuteration in the aromatic ring of the AHPA moiety. Considering the above, monotritiation might occured in the same position. Further confirmation of the deutrated position by 400MHz NMR measurement of the deutrated ubenimex was unsuccessful. Consequently, N-[(2S,3R)-3-benzyloxycarbonylamino-2-hydroxy-4-phenylbutyryl]-S-leucine benzyl ester [Z-(2S,3R)-AHPA-S-Leu-OBzl] was treated with deuterium gas under the same conditions as for the compound VIII. When the resulting product was analyzed by GC-MS, the ion(m/z = 92) was not observed. Moreover the NMR spectrum of the product gave excellent agreement with that of authentic ubenimex.

It was considered therefore that the scrambling did not occurred in the tritiation.

Specific radioactivity of IX was 15 mCi/mg.

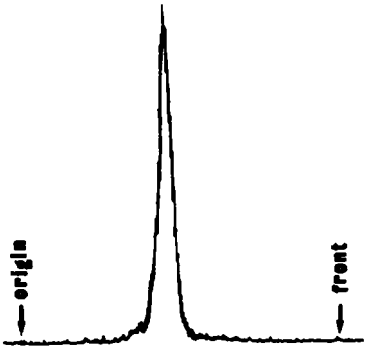


Fig. 1 Radiochromatogram of ^3H-ubenimex

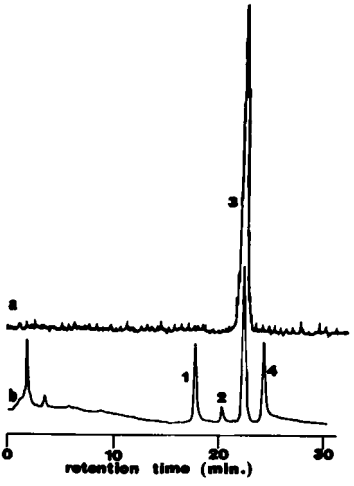


Fig. 2 a) HPLC chromatogram of ^3H-ubenimex using radioisotope detector
b) HPLC chromatogram of authentic ubenimex stereoisomers, 1) (2R,3R-S), 2) (2R,3S-S), 3) (2S,3R-S) and 4) (2S,3S-S) using UV detector

EXPERIMENTAL

Melting points were measured by a Shibata melting point apparatus and were uncorrected. Nuclear magnetic resonance spectroscopy(NMR) was carried out on a JEOL PMX-60 spectrometer with tetramethylsilane as an internal standard. The abbreviations s, d, dd, b and m indicate singlet, doublet, double-doublet, broad and multiplet, respectively. HPLC was carried out for analysis of Ⅶ and Ⅷ using a Hitachi 635 chromatograph under the following conditions : column, 250 x 2.5 mm I.D. packed with Lichrosorb Si-60 ; solvent, chloroform ; flow rate, 1.0ml/min ; column temperature, ambient ; monitoring wavelength, 254 nm. HPLC analyses of ubenimex and its optical isomers was carried out using a Shimadzu LC-3A chromatograph equipped with UV detector and a Berthold LB-503 radioisotope detector under the following conditions : column, 300 x 3.9 mm I.D. packed with μ-Bondapack C_{18} ; solvent system, A = 20mM $(NH_4)_2HPO_4$, pH 6.0, B = methanol ; elution mode, linear gradient of B in a ratio from 2 to 50%(v/v) ; flow rate, 1.0ml/min ; column temperature, ambient ; monitoring wavelength for non-labelled compound, 254nm. Preparative HPLC was performed using a Waters LC System-500 under the following conditions : column, Waters Prepack TM-500 silica-gel column ; solvent, chloroform ; flow rate, 100ml/min ; detector, refractive index detector ; column temperature, ambient. TLC of Ⅸ was done using a Merck precoated silica-gel 60 plate F_{254}. Its purity was confirmed by spraying of ninhydrin reagent and by a Packard 7200 radiochromatogram scanner. Radioactivity was counted with an Aloka liquid scintillation counter Model LSC-753. TLC was used routinely for monitoring the reactions and detections were performed with ultraviolet absorption, or visualized with iodine and ninhydrin reagents. Catalytic tritiation was carried out by New England Nuclear(Mass., USA).

(RS) N-Phe(*o*-Br)-OH Ⅱ

o-Bromobenzylbromide Ⅰ (50g, 0.2mol) and diethyl acetamidemalonate(56g, 0.2mol) were added to a solution of sodium ethoxide(0.2mol) in 350ml of absolute ethanol. The mixture was stirred at room temperature for 2 h, followed by refluxing for 12 h. After filtration of insoluble materials, the filtrate was evaporated to dryness under reduced pressure. The residue was suspended in ice cold water, dried and hydrolyzed in 300ml of 6N HCl for 12 h under reflux. The reaction mixture was evaporated, and the resulting crude material was dissolved in a mixture of water and acetone(1/1,v/v). The solution was adjusted to pH 5.0 by an addition of N NaOH and chilled at 5℃ overnight. The precipitate was filtered, washed and dried to give 37.7g of Ⅱ in a 77.3% yield.

NMR(CF_3COOH) δ 3.16-4.13(2H, m, CH_2), 4.50-5.10(1H, m, CH), 6.80-7.80(6.5H, m, Br-C_6H_4, NH_2, COOH).

Z-(RS)-Phe(*o*-Br)-OH Ⅲ

Compound Ⅱ (35g, 0.143mol), benzyl S-4,6-dimethylpyrimidin-2-ylthiolcarbonate(47.2g, 0.172mol) and triethylamine(17.4g, 0.172mol) were dissolved in a mixture of 150ml of dioxane and 150ml of water and stirred for 12 h. The solution was washed with EtOAc, then the aqueous layer separated was acidified to pH 1 by N HCl. The precipitated syrup was extracted with EtOAc. The layer was washed with water, dried over anhyd. $MgSO_4$ and evaporated to afford 51.3g of Ⅲ in a 94.6% yield. An aliquot of the crude material was recrystallized from EtOAc and petroleum ether to give the analytically pure Ⅲ with mp. 112.5 ℃.

NMR($CDCl_3$) δ 2.90-3.66(2H, m, Br-C_6H_4-CH_2), 4.50-5.60(4H, m, O-CH_2-C_6H_5, CH, NH), 6.87-7.66(9H, m, Br-C_6H_4,-C_6H_5), 9.90(1H, s, COOH).

Z-(RS)-Phe(*o*-Br)-dimethylpyrazolide Ⅳ

To a chilled solution of Ⅲ (50g, 0.132mol) and 3.5-dimethylpyrazole(H-DMP, 13.9g, 0.132mol) was added dicyclohexylcarbodiimide(DCCD, 27.2g, 0.132mol) at -10℃. The resulting solution was stirred at -10℃ for 1 h, followed at room temperature overnight. After removal of precipitates, the resulting solution was evaporated under reduced pressure and the residue was taken up with EtOAc. The layer was washed with N HCl, water, 5% $NaHCO_3$ and water, dried over anhyd. $MgSO_4$. EtOAc was evaporated to give a crude product. Recrystallization from chloroform and *n*-hexane gave 36.8g of Ⅳ in a 61% yield. mp 108.5℃,

NMR($CDCl_3$) δ 2.2(3H, s, CH_3), 2.47(3H, s, CH_3), 3.20-3.60(2H, m, Br-C_6H_4-CH_2), 5.01(2H, s, O-CH_2-C_6H_5), 5.50-6.23(3H, m, NHCO, CH=C x 2), 7.27(5H, s, -C_6H_5), 6.80-7.60(4H, m, Br-C_6H_4).

H-(2RS,3RS)-AHPA(*o*-Br)-OH Ⅴ

To a solution of sodium dihydro-bis-methoxyethoxy aluminate(80% in toluene solution, 11.5g, 57mmol) in 300ml of tetrahydrofuran(THF) was added dropwise a solution of Ⅳ (8.7g, 19mmol) in 100ml of THF at -15--20℃. After allowing to stand for 1 h, 2N HCl was added slowly and the precipitates were removed by filtration. The residue obtained by evaporation of the solvent was redissolved in EtOAc and the EtOAc layer was washed with water and evaporated. To an oily residue was added an ice cold solution of $NaHSO_3$(2.0g, 19mmol), then the mixture was concentrated and allowed to stand at 5 ℃ overnight. To the resulting adduct was added EtOAc and aqueous solution of potassium cyanide(1.2g, 19mmol) and the mixture was stirred for 4 h at room temperature. The EtOAc layer was separated, washed with water and evaporated to give cyanohydrin as a syrup. It was then hydrolyzed under reflux for 12 h in 100ml of dioxane and 100ml of conc. HCl. The solution was washed with ether and concentrated to dryness. The residue obtained was dissolved in 100ml of water and 100ml of acetone, the pH of the solution was then brought to 5.5 with aqueous ammonia.

Crystals deposited after standing at 5℃ overnight were filtered and washed with acetone to provide 8.1g of V in a 38.6% yield.

NMR(CF_3COOH) δ 3.23-3.70(2H, m, CH_2), 4.10-4.93(1H, m, $\underline{CH}$-NH_2), 4.75(1H, d, J=3Hz, $\underline{CH}$-OH), 6.53-7.83(7H, m, Br-C_6H_4, NH_2, OH, COOH).

Z-(2RS,3RS)-AHPA(*o*-Br)-S-Leu-OBzl VII

Compound V (7.1g, 26mmol) was benzyloxycarbonylated in a 88.1% yield under the same procedure described above. To the solution of crude VI (mp 148℃, 8.5g, 20.8mmol), S-Leu-OBzl.TosOH(8.2g, 20.8mmol), N-hydroxybenzotriazole(4.5g, 33.3mmol) and triethylamine(4.6ml, 33mmol) in 200ml of THF was added DCCD(5.36g, 26mmol) and the resulting solution was allowed to react at -5℃ for 1 h and then at room temperature for an additional 20 h. After removal of insoluble materials, the solution was dried. The residue was taken up by EtOAc and the EtOAc layer was washed with N HCl, water, 5% $NaHCO_3$ and water, then dried over anhyd. $MgSO_4$. The filtrate was concentrated to give 12.7g of VII as a syrup.

Isolation of Z-(2S,3R)-AHPA(*o*-Br)-S-Leu-OBzl VII

A solution of VII (2g) in 10ml of chloroform was subjected to preparative high performance liquid chromatography under the conditions described above. Eluates were fractionated in 200ml each and the purity of each fraction examined by analytical HPLC. Fractions eluted from 2400 to 3100ml were collected and evaporated under reduced pressure to give 275mg of VII in a 12.9% yield. mp 114.5 ℃, $[\alpha]_D^{25}$ +19.0 ° (c 1, AcOH).

NMR($CDCl_3$) δ 0.67-1.00(6H, m, CH_3 x 2), 1.23-1.83(3H, m, CH_2-CH), 2.90-3.33(2H, m, Br-C_6H_4-$\underline{CH_2}$), 4.00-4.90(3H, m, CH x 3), 4.97(2H, s, O-$\underline{CH_2}$-C_6H_5), 5.13(2H, s, O-$\underline{CH_2}$-C_6H_5), 5.60(1H, d, J=9Hz, NHCO), 6.90-7.67 (14H, m, Br-C_6H_4, C_6H_5 x 2)

^{3}H-Labelled Ubenimex, H-(2S,3R)-AHPA(*o*-^{3}H)-S-Leu-OH IX

Compound VII (200mg, 0.33mmol) was hydrogenated by tritium gas using palladium black as a catalyst in a mixed solvent(10ml) of AcOH, MeOH and water(4 : 2 : 1, v/v) for 6 h at room temperature. After removal of the catalyst, the solvent was evaporated. This procedure was carried out by New England Nuclear. The crude material was purified by silica-gel column(30 x 1.0cm I.D.) chromatography using a mixed solvent of chloroform, MeOH, AcOH in a ratio of 15 : 5 : 1(v/v) as eluent. Fractions which gave a single radioactive spot with a Rf value of 0.45 on a TLC plate were collected and evaporated to dryness to afford IX with a specific radioactivity of 15 mCi/mg.

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