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Synthesis of Tritium-Labeled (+)Loxistatin (E-64d)

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SUMMARY

The enantioselective synthesis of tritium-labeled (+)loxistatin is described. N-Boc-[U-^{3}H]L-leucine was the starting material for the three-step synthesis. Boc-leucine was amidated with isoamylamine. The Boc-group was removed with 4N HCl in dioxane. Transesterification with ethyl *p*-nitrophenyl-L-*trans*-epoxysuccinate led to (+)loxistatin with a specific radioactivity of 4.4 mCi/mmole and a radiochemical purity of >97%.

Key words: Synthesis of [^{3}H](+)Loxistatin, E-64d, ethyl *p*-nitrophenyl-L-*trans*-epoxysuccinate.

INTRODUCTION

E-64, a derivative of *trans*-epoxysuccinate isolated from the extract of a solid culture of *Aspergillus japonicus* TPR-64, is a specific inhibitor of thiol proteases (1). Derivatives of E-64 vary in their inhibitory potency and in specificity (2). E-64c and its ethyl ester E-64d (IV), or loxistatin (ethyl-(+)-(2S,3S)-3-[(S)-3-methyl-1-(3-methylbutylcarbamoyl) butylcarbamoyl]-2-oxiranecarboxylate), have been

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studied for their ability to inhibit thiol proteases *in situ*. E-64d, the ethyl ester, is able to penetrate cell membranes and inhibit intracellular thiol proteases, whereas E-64c, the free acid, is unable to enter cells (3). Loxistatin (E-64d) is thus a potentially valuable agent for investigation of the physiological role of thiol proteases. For pharmacokinetic studies and for localization of thiol proteases within cells, radioactive loxistatin is required.

There are four diastereomers of loxistatin. The L,L-isomer possesses the most potent activity against thiol proteases. In this paper we report the synthesis of the L,L-isomer of [^{3}H](+)loxistatin using enantioselective methods of synthesis from the optically active N-*t*-butyloxycarbonyl-[^{3}H]L-leucine (*Boc*-leucine).

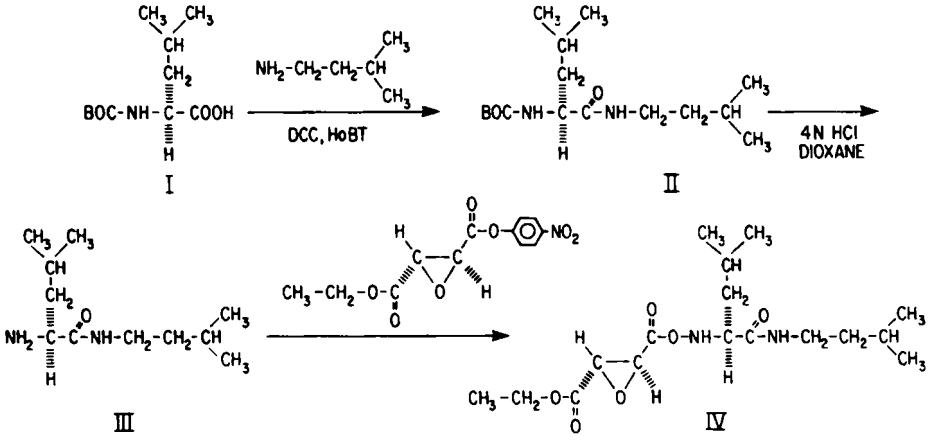
MATERIALS AND METHODS

Infrared spectra were measured with a Model 21 Perkin-Elmer IR spectrophotometer. Proton and ^{13}C-nuclear magnetic resonance spectra were obtained with a AF superconducting magnet 250 MHz IBM/Brucker Fourier transform NMR spectrometer using tetramethylsilane as an internal standard. Chemical shifts are given on the δ scale. [^{3}H]N-Boc-L-leucine (125 Ci/mmole) was supplied by DuPont/NEN Research Products. Radiochemical purity was measured by thin-layer chromatography using Analtech, Inc., Silica Gel HL plates. Ten fractions from the plate were collected in vials containing Hydrofluor scintillation fluid (Manville) and the radioactivity was measured with an LKB 1211 Rackbeta liquid scintillation counter. In ethyl ether:hexane (9:1) the R_f was 0.45, and in chloroform:methanol (98:2) it was 0.55. All physical data for compound IV were compared with those of authentic unlabeled sample obtained from Dr. K. Hanada, Taisho Pharmaceutical, Omiya, Japan.

EXPERIMENTAL

The steps of the synthesis are shown in scheme 1. *t*-Boc-[^{3}H]-L-leucine (I) was amidated by isoamylamine in the presence of N,N'-dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HoBT) to give [^{3}H]-N-Boc-L-leucine isoamylamide (II). The Boc-group of II was removed by treatment with 4N HCl in dioxane at room temperature for one hour to obtain [^{3}H]L-leucine isoamylamide (III). III was transesterified with ethyl *p*-nitrophenyl-L-trans-epoxysuccinate, which was prepared from diethyl

tartrate by the method of enantioselective synthesis (4-6). The product was the configurationally accurate L,L-isomer of (+)loxistatin (IV), identical to an authentic non-radioactive sample by thin-layer chromatography and by NMR and IR spectra. It had a radiochemical purity >97% determined by thin-layer chromatography and a specific radioactivity of 4.4 mCi/mmole. The overall chemical yield of the three steps was 84.7%.



Scheme 1. Synthesis of labeled loxistatin.

Boc-[^{3}H]-L-Leucine Isoamylamide (II). N,N′-Dicyclohexylcarbodiimide (178.4 mg, 0.86 mmole) in ethyl acetate (0.4 mL) was added dropwise to a stirred solution of Boc-[^{3}H]-L-leucine (3.8 μg) plus unlabeled Boc-L-leucine (196.2 mg, 0.84 mmole), isoamylamine (75.4 mg, 0.86 mmole) and 1-hydroxybenzotriazole (116.8 mg, 0.86 mmole) in 2 mL of ethyl acetate at 0°C. The reaction mixture was stirred for 1.5 h at 0°C and then for 2.5 h at room temperature. The precipitate of N,N′-dicyclohexylurea was removed by filtration and washed with ethyl acetate (1.2 mL). The ethyl acetate solution was washed successively with 5% HCl (1 mL), saturated brine (1 mL), saturated aqueous $NaHCO_3$ (1 mL) and saturated brine (1 mL). The ethyl acetate solution was dried over $MgSO_4$ and filtered. The filtrate was evaporated *in vacuo* to dryness. n-Hexane (1.5 mL) was addded to the residue and the insoluble materials were removed by filtration and washed with a small amount of n-hexane. The filtrate was evaporated *in vacuo* to give 236.4 mg (92.9%) of Boc-[^{3}H]-L-leucine isoamylamide

as a colorless solid. This product was used for the next step without further purification.

[^{3}H]-L-Leucine Isoamylamide (III). II (236.4 mg, 0.79 mmole) was dissolved in 4N HCl in dioxane (1 mL), and the reaction mixture was stirred at room temperature for 1 h. After evaporation of the solvent, water (3 mL) was added to the residue and the solution was extracted with dichloromethylene (2 mL). The aqueous layer was adjusted to pH 10 with 20% aqueous NaOH and extracted with ethyl acetate (3 mL once and 1 mL twice). The combined extract was washed with saturated brine, dried over $MgSO_4$ and filtered. The filtrate was evaporated *in vacuo* to dryness to give 148.3 mg (91.1%) of [^{3}H]-L-leucine isoamylamide as a pale yellow oil. This product was used for the next step without further purification.

[^{3}H]-Loxistatin (IV). III (148.3 mg, 0.74 mmole) in 1 mL ethyl acetate was added dropwise to a stirred solution of ethyl-*p*-nitrophenyl-L-*trans*-epoxysuccinate (199.4 mg, 0.74 mmole) in 2 mL ethyl acetate at room temperature. The mixture was stirred at the same temperature for 2 h. A precipitate was removed by filtration, and the filtrate was washed with 2% aqueous NaOH (1 mL x 18) until the washings were colorless. It was then washed with 5% HCl (1 mL) and saturated brine (1 mL x 2), and it was dried over $MgSO_4$. The solvent was evaporated *in vacuo* to give 199 mg (99%) of a white crystalline powder that required no further purification. Radiochemical purity was >97.5% as judged by TLC analysis. Specific radioactivity was 4.4 mCi/mmole. The ^{1}H-NMR spectrum was identical to the published spectrum (7).

Products with higher specific radioactivity can be prepared easily by starting with more radioactive material or by decreasing the total amount synthesized. Products with higher specific radioactivities can be prepared more economically by beginning with [^{3}H]-L-leucine for preparation of labeled (I).

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