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Semisynthesis of ^{14}C-Labelled Leupeptin[1]

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^{14}C-Labelled leupeptin, Ac-L-[U-^{14}C]-leucyl-L-leucyl-L-argininal, with a specific radioactivity of 20.9 mCi/mmol was prepared by a semisynthetic procedure in which L-leucyl-L-argininal dibutylacetal obtained by an enzymatic cleavage of leupeptin dibutylacetal with thermolysin was employed as a key compound.

The chemical and radiochemical purities of the labelled leupeptin were found to be over 99% by high performance liquid chromatography using radioisotope and ultraviolet detectors.

Keywords—^{14}C-labelled leupeptin; semisynthesis; protease inhibitor; DCCD-HOSu method; HPLC; FD-MS.

Introduction

Leupeptin, Ac–L-Leu–L-Leu–L-Argal,[2] which was isolated from the culture filtrates of *Streptomyces*,[3] inhibits the activities of proteases such as trypsin, papain, kallikrein, *etc.*

In 1978, Libby *et al.*[4] and Stracher *et al.*[5] suggested independently that leupeptin prevents the degradation of protein in diseased muscle due to inhibition of protease activity.

The synthesis of radioisotope-labelled leupeptin was indispensable to investigate its absorption, excretion, distribution, metabolism and bioavailability in preclinical studies.

There are reports on chemical synthesis of leupeptin.[6] However, these methods seem to be unsuitable for the synthesis of radioactive leupeptin, because they require skillful and

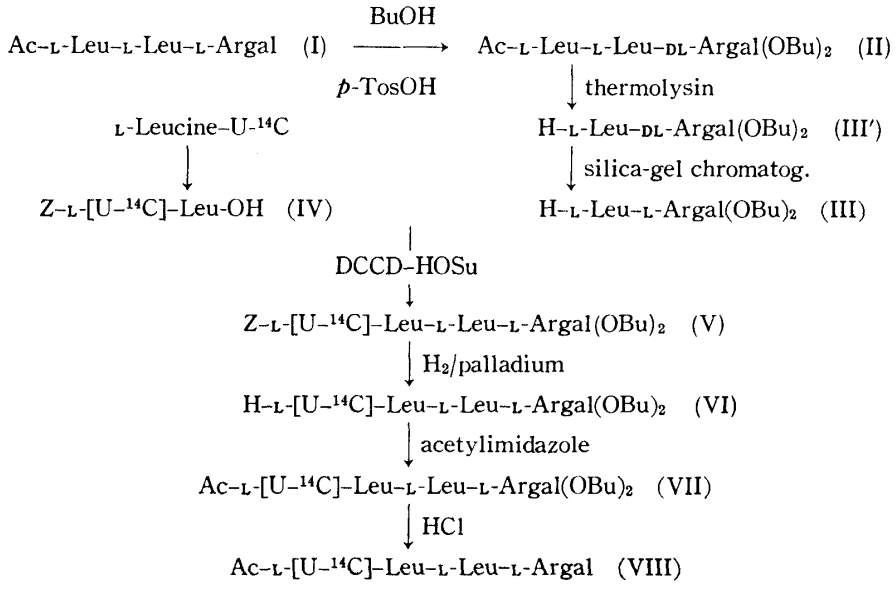

Chart 1. Semisynthetic Scheme for ^{14}C-Labelled Leupeptin

Abbreviations: Argal=Argininal, Argal(OBu)$_2$= Argininal dibutylacetal

tedious procedures due to the instability of the aldehyde in the molecule.

On the other hand, a biosynthetic method using radioactive acetic acid, glycine, leucine or arginine as a precursor has the disadvantages that uptake of radioactivity is low and that the labelling positions are spread over the molecule.[7]

Recently, Ishii *et al.* have reported that thermolysin, an endopeptidase of *Bacillus thermoproteolyticus*, can cleave leupeptin dibutylacetal into acetyl-L-leucine and L-leucyl-L-argininal dibutylacetal in a highly specific manner.[8]

This paper deals with a semisynthetic method for the preparation of ^{14}C-labelled leupeptin using the L-leucyl-L-argininal dibutylacetal as a starting material, and in addition, presents an analytical method for determining the purity of the resulting products by high performance liquid chromatography (HPLC) using radioisotope and ultraviolet detectors.

Synthesis

The scheme for the semisynthesis is shown in Chart 1.

H–L-Leu–L-Argal dibutylacetal (III), which was used as a key compound in this semisynthesis, could be obtained from biosynthetic leupeptin, Ac–L-Leu–L-Leu–L-Argal (I). First, the reactive aldehyde moiety in I was protected with a chemically inert acetal group by treating I with an alcohol in the presence of *p*-toluenesulfonic acid as a catalyst. 1-Butanol was selected as the alcohol, because the resulting dibutylacetal (II) was highly lipophilic and easily extractable from the reaction mixture with organic solvents.

II was a diastereomeric mixture due to inevitable racemization during the acetal formation, but the direct fractionation of the mixture was difficult. Therefore, the thermolysin digest of

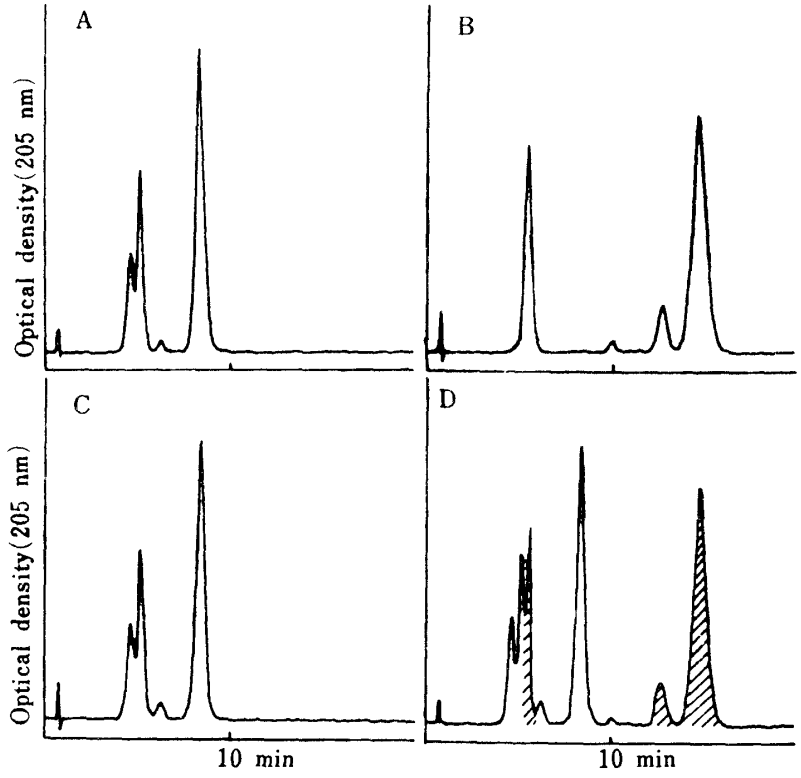

Fig. 1. HPLC of Leupeptin obtained from Fraction A (A), from Fraction B (B), Biosynthetic Ac–L-Leu–L-Leu–L-Argal (C) and Authentic Ac–L-Leu–L-Leu–DL-Argal (D)

Three main peaks and one minor peak in all the chromatograms except D are considered to arise from the equilibrium structures Ia, Ib and Ic having two diastereomers owing to a newly formed asymmetric center as described in Chart 2.

The assignment of these peaks in the chromatogram to the equilibrium structures will be reported elsewhere.

II was subjected to silica-gel column chromatography in order to isolate of III from H–L-Leu–DL-Argal dibutylacetal (III'). Two compounds, fractions A and B, were obtained when the column eluate was monitored by thin layer chromatography (TLC) using solvent system D (see "Experimental"). The *Rf* values of fractions A and B were found to be 0.27 and 0.30, respectively.

Since we could not determine which fraction, A or B, is identical with III, we led each of the compounds to the acetyl-L-leucyl derivative with a regenerated aldehyde group (I or its diastereomer) through four reaction steps similar to those shown in Chart 1, and subjected the derivatives to HPLC analysis to determine which was identical with biosynthetic I. The products derived from fractions A and B gave the chromatograms distinct from each other as shown in Fig. 1-A and 1-B, respectively, and the former chromatogram was in good agreement with that of I (Fig. 1-C). On the other hand, the chromatogram of the product from fraction B (Fig. 1-B) almost coincided with the shaded portions in the chromatogram of Ac–L-Leu–L-Leu–DL-Argal (Fig. 1-D), in which the unshaded portions correspond to the chromatogram of I.

These results indicate that fraction A is III which can be used as the starting material for the synthesis of labelled leupeptin.

In order to minimize the radiation hazards through the process of labelling, a simple synthetic route was required.

If the racemization of Ac–L-[U-^{14}C]-Leu–OH in the coupling process with III could be limited to a few per cent by the use of mild reaction conditions, Ac–L-[U-^{14}C]-Leu–L-Leu–L-Argal dibutylacetal (VII) would be directly prepared. However, approximately 30% racemization at the leucine-U-^{14}C moiety was observed in the coupling product by HPLC, even when the reaction was carried out with diphenylphosphoryl azide,[9] a condensing agent with low racemization effect, under mild conditions at -10°C.

In the HPLC experiments to confirm the above mentioned racemization, Ac–D-Leu–L-Leu–L-Argal was used as an authentic sample, as shown in Fig. 2.

It was concluded, therefore, that the use of a benzyloxycarbonyl group, which can be removed selectively without affecting the acetal group under neutral conditions, is the best choice as a protecting group for leucine-U-^{14}C in this coupling process.

Z–L-[U-^{14}C]-Leu–OH (IV) was coupled with III by a DCCD–HOSu method to yield

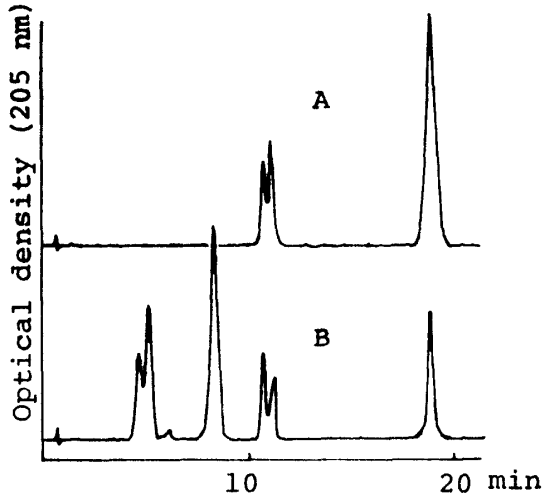

Fig. 2. HPLC of Authentic Ac–D-Leu–L-Leu–L-Argal (A) and Ac–[U-^{14}C]–Leu–L-Leu–L-Argal (B) synthesized from Ac–L-[U-^{14}C]–Leu–OH and H–L-Leu–L-Argal $(OBu)_2$ (III) using Diphenylphosphoryl Azide as a Condensing Agent

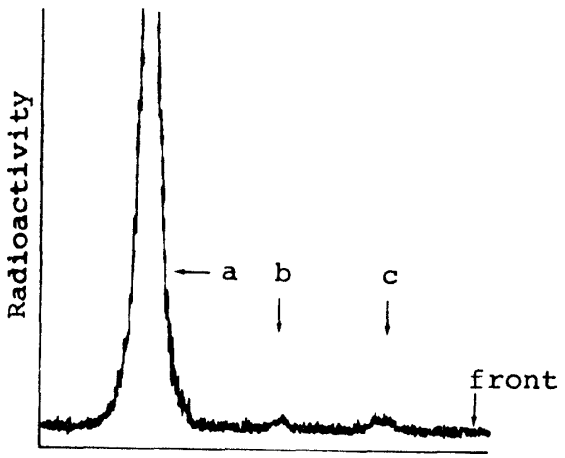

Fig. 3. TLC Radioscannogram of Ac–L-[U-^{14}C]–Leu–L-Leu–L-Argal(VIII) using Solvent System C

a; Ac-L-[U-^{14}C]-Leu-L-Leu-L-Argal(VIII), b; Ac-[U-^{14}C]-Leu-L-Leu-L-Argal$(OBu)_2$(VII), c; Ac-L-[U-^{14}C]-Leu-L-Leu-OH.

Z–L-[U-^{14}C]-Leu–L-Leu–L-Argal dibutylacetal (V).

The benzyloxycarbonyl group in V was eliminated by catalytic hydrogenation to give H–L-[U-^{14}C]-Leu–L-Leu–L-Argal dibutylacetal (VI). VI was acetylated with acetylimidazole under mild conditions to yield crude Ac–L-[U-^{14}C]-Leu–L-Leu–L-Argal dibutylacetal (VII). VII containing a protected aldehyde group has to be purified completely by silica-gel chromatography, because Ac–L-[U-^{14}C]–Leu–L-Leu–L-Argal (VIII) is very prone to racemization upon contact with silica gel and so cannot be purified by chromatography.

VII was then treated with 1 N HCl to afford VIII in a manner similar to that reported for the hydrolysis of semicarbazone.[6a]

When the reaction mixture was analyzed by TLC, the presence of a by-product was detected on the chromatogram using solvent system C as shown in Fig. 3.

The by-product was identified as Ac–L-[U-^{14}C]-Leu–L-Leu–OH by comparing its behavior with that of an authentic Ac–L-Leu–L-Leu–OH in TLC and HPLC. The unexpected side reaction leading to this compound was probably caused by cleavage of the amide linkage due to an acid-catalyzed N→O acyl migration of the intermediate Ie in Chart 2, which had arisen from a six-membered cyclic alkanolamine form of leupeptin Ic[10] produced in acidic media.

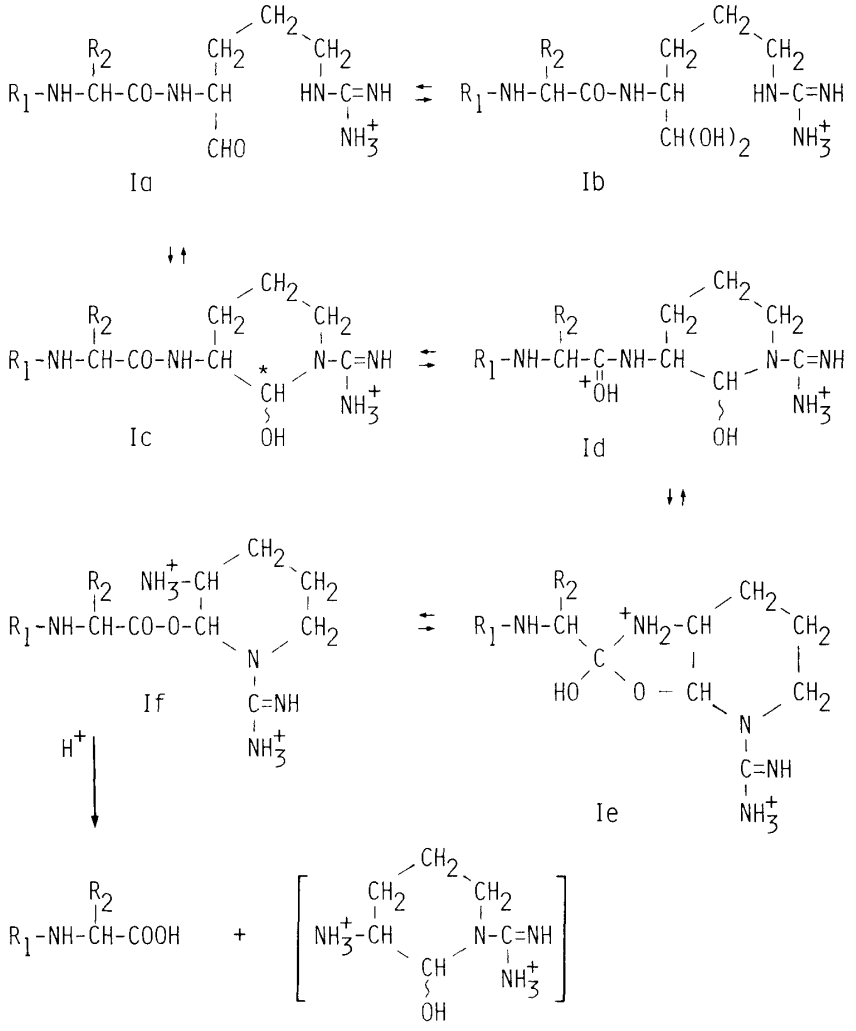

Chart 2. Equilibrium Structures of Leupeptin and the Cleavage Mechanism of the Leucyl-argininal Linkage due to N→O Acyl Migration

R_1=Ac-L-[U-^{14}C]-Leu, R_2=isobutyl.

It is essential to find conditions for hydrolysis of the acetal which minimize the amounts of the by-product and of the remaining VII in the reaction mixture, because silica-gel chromatography is not applicable to the purification of VIII for the reason described above. The best hydrolytic procedure, as described in "Experimental," was selected after comparison by HPLC analysis of the purity of VIII obtained under various conditions.

The resulting reaction mixture was adjusted to pH 4.8 with Dowex WGR (OH^- form) and lyophilized to give VIII.

Chemical and Radiochemical Purities

HPLC chromatograms of VIII (Fig. 4-A) and ^{14}C-labelled leupeptinol (Fig. 4-B) obtained by sodium borohydride reduction without racemization of VIII coincided completely with those of biosynthetic I and leupeptinol, respectively.

Both the chemical and radiochemical purities of VIII were found to be over 99% by HPLC with radioisotope and ultraviolet detectors. The radiochemical yield from L-leucine-U-^{14}C was 22.4% and the specific radioactivity was 20.9 mCi/mmol.

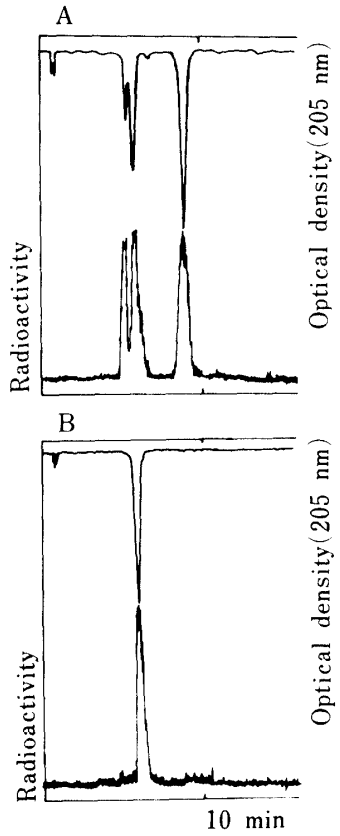

Fig. 4. HPLC of Synthesized Ac-L-[U-^{14}C]-Leu-L-Leu-L-Argal (VIII) (A) and Its Reduced Product with Sodium Borohydride (B)

Experimental

Materials and Methods——Leupeptin hydrochloride was obtained from fermentation broth of *Streptomyces* in our research laboratories. Ac-L-Leu-L-Leu-DL-Argal was obtained by treatment of leupeptin with 0.1 M aqueous sodium carbonate for 20 h at 37°C. Ac-D-Leu-L-Leu-L-Argal was semisynthesized from D-leucine and H-L-Leu-L-Argal dibutylacetal in the manner shown in Chart 1. Thermolysin was purchased from Seikagaku Kogyo Co. Ltd., Tokyo, Japan, and L-leucine -U-^{14}C (344 mCi/mmol) from New England Nuclear, Mass., U.S.A.

The reaction products were purified by silica-gel chromatography, and the purities were examined by TLC on silica-gel plates (Merck precoated silica gel 60 F_{254} plate) using Sakaguchi reagent for color reaction. Solvent systems used in these chromatographies were as follows: A) $CHCl_3$: MeOH: AcOH=95: 5: 3, B) $CHCl_3$: MeOH: AcOH=90: 10: 5, C) $CHCl_3$: MeOH: AcOH=75: 25: 3, D) 1-BuOH: AcOBu: AcOH: H_2O=4: 2: 1: 1 (v/v).

Radiochemical as well as chemical purities were confirmed by TLC with a Packard radiochromatogram scanner, Model 7200, and by HPLC on a Shimadzu LC-2 chromatograph equipped with an Aloka rapid chromatograph detector, Model PSM-RLC-621. The conditions of HPLC were as follows; column, 150 mm × 4 mm i.d. packed with Nucleosil 7C-18; solvent, 1% H_3PO_4: CH_3CN=83: 17 (v/v); flow rate, 1.5 ml/min; monitoring wavelength, 205 nm. Radioactivity was counted with an Aloka liquid scintillation spectrometer, Model LSC-753. Melting points (uncorrected) were measured on a Shibata melting point apparatus, and specific optical rotations on a Perkin Elmer 241 polarimeter. Field desorption mass spectrometry (FD-MS) was carried out on a JEOL JMS-DX300 mass spectrometer equipped with a JEOL JMS-2000S mass data analysis system; emitter current, 15—20 mA; acceleration voltage, 3 kV.

Ac-L-Leu-L-Leu-DL-Argal Dibutylacetal II——Ac-L-Leu-L-Leu-L-Argal I (50 g) and *p*-toluenesulfonic acid (5 g) were refluxed for 6 h in 1-butanol (600 ml) and benzene (1000 ml). The reaction mixture was concentrated under reduced pressure to afford a powder, which was dissolved in ethyl acetate (1000 ml) and the resulting solution was washed with 10% aqueous sodium chloride. The organic layer was dried over anhydrous magnesium sulfate and concentrated to give 27.6 g of powdery II. It gave a single spot on a TLC plate using solvent system D: mp 154°C (dec.), $[\alpha]_{578}^{25}$ −55.5° (c=2.3, AcOH), FD-MS for $C_{28}H_{56}N_6O_5$:

m/z: 557 $(M+H)^+$ (base peak), 558 (32.8%), 559 (6.6%).

H-L-Leu-L-Argal Dibutylacetal III——Digestion of II (36 g) with thermolysin (1.8 g) in 0.1 M *N*-ethylmorpholine buffer solution (1800 ml) containing 0.02 M $CaCl_2$ at pH 8.0 was carried out for 72 h at 38°C on a reciprocating shaker. The resulting solution was extracted with 1-butanol (1200 ml × 2). The combined extract was concentrated and chromatographed on a column (8 × 70 cm) of silica gel using solvent system D (5.5 l). Sakaguchi-positive fractions containing the desired material, which gave *Rf* 0.27 on a TLC plate using solvent system D, were collected and concentrated to afford 12.6 g of oily III: FD-MS for $C_{20}H_{43}N_5O_3$; *m/z*: 402 $(M+H)^+$ (base peak), 403 (24.9%), 404 (21.0%), 328 (5.5%).

Z-L-[U-^{14}C]-Leu-OH IV——L-Leucine-U-^{14}C (5 mCi, 344 mCi/mmol) and L-leucine (29.2 mg) were treated with benzyl S-4,6-dimethylpyrimidin-2-ylthiolcarbonate (92 mg) in water (5 ml), dioxane (5 ml) and triethylamine (90 μl).

The reaction mixture was evaporated to dryness and the residue dissolved in chloroform was charged on a column (1.5 × 30 cm) of silica gel and chromatographed with solvent system A (200 ml).

The effluent fractions with radioactivity were collected and concentrated. IV with a radioactivity of 3.97 mCi was thus obtained in 79.4% yield.

Z-L-[U-^{14}C]-Leu-L-Leu-L-Argal Dibutylacetal V——IV (3.97 mCi, 0.177 mmol), HOSu (40.7 mg, 0.353 mmol) and 0.1 N DCCD in chloroform (2.12 ml, 0.212 mmol) were allowed to react at 0°C for 4 h. The residue (obtained by evaporation of the chloroform) and III (115 mg, 0.262 mmol) were dissolved in dimethylformamide (2 ml). The solution was adjusted to pH 7.5 with triethylamine, stirred for 2 d at room temperature, and then concentrated under reduced pressure. The soluble material in the residue was taken up with chloroform. The resulting solution was charged on a column (1.5 × 30 cm) of silica gel and chromatographed with solvent system B (310 ml). The effluent fractions which gave a radioactive spot with *Rf* 0.40 on a TLC plate were combined and concentrated. V with a radioactivity of 1.664 mCi was obtained in 33.3% yield. For non-labelled V: mp 161°C (dec.), $[\alpha]_{578}^{25}$ −35.5° (*c* = 0.9, AcOH), FD-MS for $C_{34}H_{60}N_6O_6$; *m/z*: 649 $(M+H)^+$ (base peak), 650 (25.0%), 651 (21.0%), 541 (4.3%), 108 (31.4%).

Ac-L-[U-^{14}C]-Leu-L-Leu-L-Argal Dibutylacetal VII——V (1.664 mCi) was hydrogenated for 4.5 h in the presence of palladium in methanol (10 ml) to give H-L-[U-^{14}C]-Leu-L-Leu-L-Argal dibutylacetal VI. Acetylimidazole (300 mg) was added to the solution of VI in dimethylformamide (2 ml) and water (2 ml), and the mixture was adjusted to pH 8.0 by addition of triethylamine.

The reaction mixture was allowed to stand for 3 d at room temperature, then evaporated to dryness. Chloroform was added to the residue in order to remove insoluble material by filtration.

The resulting solution was charged on a column (1.5 × 30 cm) of silica gel and chromatographed with solvent system C (120 ml).

The fractions which showed a radioactive spot with *Rf* 0.60 on a TLC plate were collected. VII with a radioactivity of 1.359 mCi was thus obtained in 81.7% yield: FD-MS for labelled VII; *m/z*: 557 $(M+H)^+$ (base peak), 558 (31.0%), 559 (14.4%), 567 $(M+10+H)^+$ (3.1%), 569 $(M+12+H)^+$ (12.5%). For non-labelled VII: mp 164°C (dec.), $[\alpha]_{578}^{23}$ −52.7° (*c* = 0.9, AcOH), FD-MS for $C_{28}H_{56}N_6O_5$; *m/z*: 557 $(M+H)^+$ (base peak), 558 (41.4%), 559 (10.0%).

Ac-L-[U-^{14}C]-Leu-L-Leu-L-Argal VIII——VII (1.227 mCi) was treated in 1 N HCl (4 ml) and acetonitrile (8 ml) at 37°C for 2 h. The reaction mixture was adjusted to pH 4.8 with Dowex WGR (OH^- form). The filtrate was concentrated and lyophilized to afford 1.12 mCi of VIII in 22.4% yield from L-Leucine-U-^{14}C. It showed high chemical and radiochemical purities and a specific radioactivity of 20.9 mCi/mmol: FD-MS for labelled VIII; *m/z*: 427 $(M+H)^+$ (base peak), 428 (24.6%), 429 (5.7%), 435 $(M+8+H)^+$ (1.8%), 437 $(M+10+H)^+$ (3.6%), 439 $(M+12+H)^+$ (5.5%), 415 (10.3%), 418 (11.0%), 409 (15.8%), FD-MS for I ($C_{20}H_{38}N_6O_4$); *m/z*: 427 $(M+H)^+$ (base peak), 428 (20.9%), 429 (2.7%), 409 (5.0%).

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References and Notes

1) Abbreviations used are those recommended by the IUPAC-ICB Commission on Biochemical Nomenclature: *Biochem.*, **5**, 2458 (1966); *ibid.*, **6**, 362 (1967); *ibid.*, **11**, 1726 (1976). Other abbreviations used in this paper are: Ac = acetyl, Z = benzyloxycarbonyl, DCCD = dicyclohexylcarbodiimide, HOSu = *N*-hydroxysuccinimide, Argal = argininal, Argal(OBu)$_2$ = argininal dibutylacetal, *p*-TosOH = *p*-toluenesulfonic acid.

2) All compounds containing argininal in this paper are mono hydrochlorides.

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