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THE NOVEL SYNTHESIS OF THE PROTEASE INHIBITOR (S)-1-CHLORO-3-[(p-TOLYLSULFONYL)AMINO]-7-AMINO-2-[5,5,6,6-^{3}H]HEPTANONE ([^{3}H]TLCK) LABELED TO HIGH SPECIFIC ACTIVITY WITH TRITIUM

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ABSTRACT

The protease inhibitor (S)-1-chloro-3-[(p-tolylsulfonyl)amino]-7-amino-2-[5,5,6,6-^{3}H]heptanone ([^{3}H]TLCK) was prepared in an overall 41% radiochemical yield with a specific activity of 1.6 Ci/mmol in a 'one-pot' 3 step sequence beginning with (S)-6-[[(1,1-dimethylethyl)oxy]carbonyl]-2-(p-tolylsulfonyl)amino[4,4,5,5-^{3}H]hexanoic acid. The latter was prepared with a specific activity of 123 Ci/mmol in a 3 step sequence beginning with the stereospecific enzymatic hydrolysis of the commercially available racemic 2-acetylamino-6-[[(1,1-dimethylethyl)-oxy]carbonyl]aminohexan-4-ynoic acid, followed by tosylation and then palladium catalyzed reduction of the triple bond under tritium.

Keywords: Protease inhibitor, tritiated TLCK, high specific activity, 'one-pot' synthesis

INTRODUCTION

The elevated levels of the immunoglobulin IgE in persons subject to allergic reactions have been known for some time (1,3). Hence IgE production and control would appear to be an important factor in allergic diseases (4). It is also well known that the protein CD23, a low affinity receptor for IgE, has a regulatory effect on IgE production. Intact CD23 is known to down-regulate IgE synthesis when IgE binds to it. By contrast, some of the soluble products (sCD23) formed when the intact molecule is proteolytically cleaved from the cell surface appear to stimulate IgE synthesis (5,6).

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Hence the feasibility of inhibiting the cleavage of the intact molecule and thus reducing circulating IgE levels, became the central focus of study by our Immunotherapeutics group based in the UK. To probe the cleavage mechanism, tritium labeled (S)-1-chloro-3-[(p-tolylsulfonyl)-amino]-7-amino-2-[5,5,6,6-^{3}H]heptanone ([^{3}H]TLCK) was required, since TLCK itself has been shown to inhibit a portion of the cleavage process (7-9).

Since the protease responsible for that particular cleavage could be labeled with tritiated TLCK *in vitro,* it was envisaged that the cleavage process could be followed by autoradiography. A specific activity greater than 1 Ci/mmol for [^{3}H]TLCK was necessary in order to achieve the required sensitivity. Described herein is the synthesis of high specific activity tritium labeled [^{3}H]TLCK in a three step consecutive process beginning with the appropriately labeled tritium lysine derivative.

RESULTS AND DISCUSSION

The chemical literature reports several ways to synthesize TLCK. The method of Shaw (Figure 1), starting with N$^\alpha$-tosyl-N$^\varepsilon$-carbobenzyloxy-L-lysine (1), appeared the most relevant from a radiosynthetic point of view, since 1 can be readily labeled with tritium.

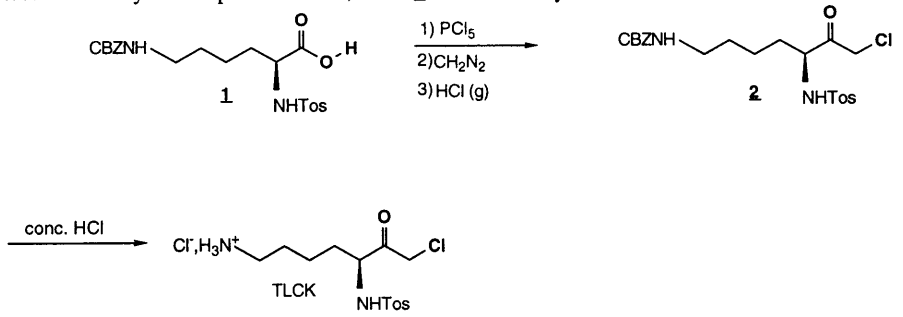


Figure 1. Synthesis of TLCK by the method of Shaw (10).

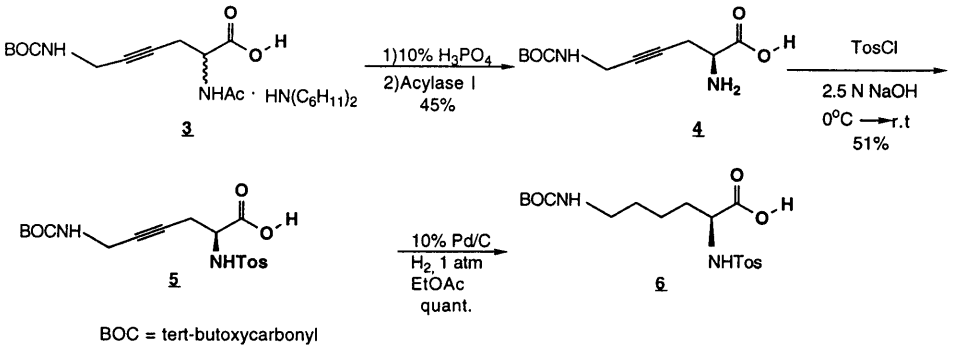
However, a low yield in the carbobenzoxy deprotection step makes it an unattractive route to follow. Since the usual palladium catalyzed deprotection could not be performed on this particular substrate due to concomitant dechlorination, a hydrolytic procedure was employed by Shaw. This, however, led to an extremely low yield (14 %) of TLCK. The hydrolytic procedure employed by Shaw (10) used concentrated hydrochloric acid in ethanol at 75°C. Attempts to improve the yield by heating 2 with hydrogen bromide in acetic acid caused extensive decomposition. Subsequently, Shaw (11,12) and other authors have improved on his deprotection step by employing trifluoroacetic acid.

For example, Sebestyen and Samu (13) have obtained a 50% yield of TLCK when 2 was heated at 40°C for 4 h in a sealed tube in the presence of trifluoroacetic and concentrated hydrochloric acid. However, the use of such drastic conditions on a compound labeled with tritium to high specific activity seemed to us untenable from a stability point of view.

We therefore decided to design our synthesis using a t-butoxycarbonyl protecting group in the N $^\varepsilon$ position, and take advantage of the facility with which this group can be removed. We

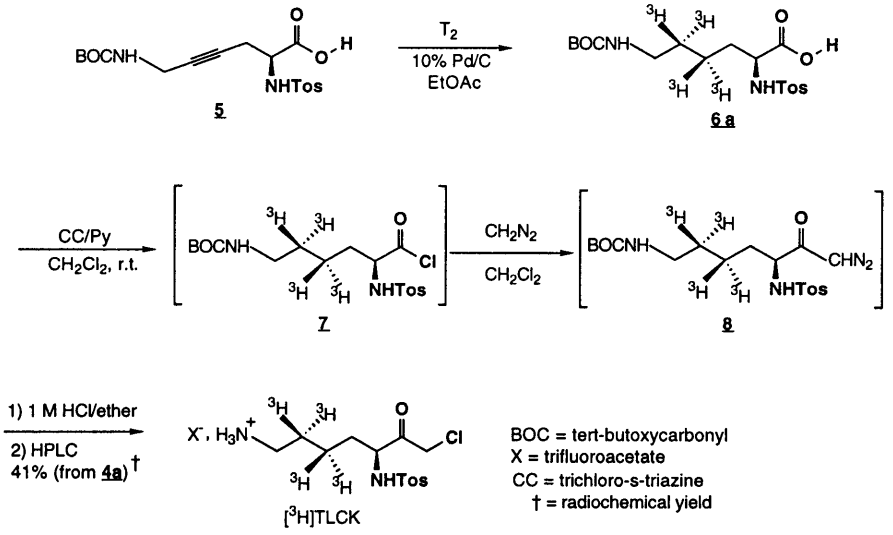
envisaged that the conversion of the diazo ketone to the chloromethyl ketone would occur concurrently with the removal of the Boc group in HCl (g) at room temperature. To implement this strategy, we needed first to find a method which would allow the formation of an acid chloride in the presence of an acid labile group such as t-butoxycarbonyl.
Venkaturanan and Wagle (14) have shown that Boc-L-valine, when treated with trichloro-s-triazine (CC), formed the acid chloride, and the resultant solution could be used directly for further reactions. Similarly, we have shown that **6** (Scheme 1) , when treated with CC followed by a methanol quench, gave a 100% conversion to the methyl ester by reverse phase HPLC analysis. In addition, we found that the one pot/3 step conversion of **6** to TLCK (Scheme 2) in practice runs was equally successful; affording TLCK in an overall chemical yield of 62%.

The next extension of the synthetic strategy involved the synthesis of the appropriately substituted, tritium labeled L- lysine precursor (**6a**). Here we relied on the work of Sasaki and Bricas (15), who developed a method for the stereospecific enzymatic hydrolysis of the acetylamino group of **3** (Scheme 1) using porcine kidney acylase I. Due to the absence of sufficient experimental detail, it was difficult at first repeating this work. However, we found the grade of porcine kidney acylase I used was crucial to the success of the enzymatic hydrolysis; and grade III obtained from Sigma being the most effective. Although it was evident from reverse phase HPLC that enzymatic hydrolysis was successful, we nonetheless had difficulty in isolating the product, since the desired amino acid (**4**) remained in solution. We found the best way to isolate **4** was to lyophilize the reaction solution to dryness, then separate the mixture via a silica gravity column. By this procedure, **4** was obtained in 45 % yield having a specific rotation of -24° (c 2.0, H_2O). This compared most favorably with the literature value (15) of - 17.5°. Chiral chromatography provided a method of confirming the specific rotation results. However, since the separation of enantiomers of **4** and lysine derived from **4** (via hydrogenation and removal of the BOC protecting group with trifluoroacetic acid) were not successful, the determination had to be done on **6** instead. The result of 97.4% e.e. for **6** was consistent with the specific rotation data, and unequivocally established the optical purity of compound **4.**



Scheme 1. Preparation of the chiral tritium precursor (**5**)

In the hot run, the reduction of **5** under 4.7 Ci of tritium gas (Scheme 2) gave **6a** with a specific activity of 123 Ci/mmol by mass spectrometric analysis. A 106 mCi quantity of this was diluted with carrier (**6**) to a specific activity of 1.6 Ci/mmol, and then carried through to [^{3}H]TLCKon a 53 micromole scale as depicted in Scheme 2. We feel confident that the micro-chemical techniques developed here can be scaled to the 5-10 micromole range thus producing [^{3}H]TLCK with a specific activity of ≥ 20 Ci/mmol.



Scheme 2. Synthesis of [^{3}H]TLCK

EXPERIMENTAL SECTION

GENERAL

DL-2-acetylamino-6-[[(1,1-dimethylethyl)-oxy]carbonyl]aminohexan-4-ynoic acid dicyclohexylamine salt, N$^{\varepsilon}$-BOC-L-lysine, and N$^{\varepsilon}$-BOC-D-lysine were obtained from Bachem Feinchemikalien AG. Porcine kidney acylase I (grade III, 7000 units/mg) was obtained from Sigma. Analytical and preparative HPLC conditions are given in the individual preparations. All radiochemical purities were determined using a Ramona-D radioactivity flow detector equipped with a 750 μL flow cell and Tru-Count scintillation cocktail. All radioactivity counting was done on a TM Analytic model 6881 scintillation counter using the internal quench curve to calculate DPM. All HPLC retention times of labeled compounds matched those of authentic samples of the respective unlabeled compounds. Tritium gas was obtained from DuPont-NEN. Proton, and carbon-13 nuclear magnetic resonance spectra were obtained on both Bruker WM-360 and 400 instruments. The solvents and chromatography conditions are indicated in the individual preparations. Chemical shifts (δ) are reported downfield from tetramethylsilane.

(S)-2-Amino-6-[[(1,1-dimethylethyl)oxy]carbonyl]aminohexan-4-ynoic acid (**4**)

A 10% aqueous solution of phosphoric acid (10 mL) was added to a suspension of racemic 2-acetylamino-6-[[(1,1-dimethylethyl)-oxy]carbonyl]aminohexan-4-ynoic acid, dicycloyhexylamine salt **3** (1.6 g, 3.5 mmol) in ethyl acetate (50 mL). The organic layer was separated, washed with ice water (50 mL), transferred to a 125 mL round bottom flask, and solvent removed in vacuo. The resulting white foam was dissolved in 0.1 M phosphate buffer (91 mL, pH 7.4) and a trace of $CoCl_2 \cdot 6\ H_2O$, acylase I (150 mg) was added. The flask was stoppered and the mixture heated at 37° C for 23 h. The reaction mixture was filtered through a layer of Celite™ then lyophilized to dryness. HPLC analysis (Rainin Dynamax C 18; 0.1% aqueous trifluoroacetic acid:acetonitrile, 70:30, wt/wt, 220 nm) of the resulting white solid revealed an approximate 1:1 mixture of **4** and (R)-2-acetylamino-6-[[(1,1-dimethylethyl)-oxy]carbonyl]aminohexan-4-ynoic acid. The solid was dissolved in 95% ethanol (50 mL), filtered, and placed atop a 30 mm I.D X 300 mm Silica Gel (Merck Silica 60, 230-400 mesh) column. The mixture was separated by elution with 95% ethanol to give 183 mg (22%, theoretical yield 50%) of the desired product as a white solid, mp 195-200° C.

360 MHz ^{1}H-NMR Spectrum (DMSO-d_6/TFA): δH 1.45 (s, 9H), 2.52 (m, 2H), 3.29 (m, 1H), 3.73 (m, 2H), 7.19 (s, 1H)
^{13}C-NMR Spectrum: δC 22.01 ($CH_1\underline{C}H_2\underline{C}{\equiv}\underline{C}$), 28.96 (($\underline{C}H_3)_3\underline{C}$), 30.54 ($C{\equiv}\underline{C}\underline{C}H_2NH$), 79.05 ($CHCH_2\underline{C}{\equiv}\underline{C}$), 80.29 ($C{\equiv}\underline{C}CH_2NH$)
$[\alpha]21^oC, D) = -24.0^o$ (c 2, H_2O)

(S)-6-[[(1,1-Dimethylethyl)oxy]carbonyl]-2-(p-tolylsulfonyl)aminohexan-4-ynoic acid (**5**)

Compound **4** (99.22 mg, 0.41 mmol) was dissolved in 2.5 N aqueous sodium hydroxide(3.5 mL) and placed in a 10 mL screw cap vial containing a magnetic stirrer. The solution was cooled in ice, and of p-tolylsulfonyl chloride (781.7 mg, 4.1 mmol) was added in portions. The vial was removed from the ice bath, capped then stirred at room temperature for 22.5 h. The clear basic solution was diluted with water and extracted with diethyl ether. The aqueous layer was chilled in ice and acidified to pH = 2 with 10% aqueous phosphoric acid which produced a white oil. This was extracted out with diethyl ether. The diethyl ether phase was washed with water and dried (sodium sulfate), filtered, and concentrated to dryness in vacuo giving 83 mg (51%) of a white solid, mp 80-85° C.

Mass Spectrum (CI, ammonia, m/e (% base)): 189 (100), 297 (14), 358 (68), $(M + H)^+$ 397 (2), $(M + NH_4)^+$ 414 (70)
400 MHz ^{1}H-NMR Spectrum ($CDCl_3$): δH 1.45 (s, 9H), 2.41 (s, 3H), 2.76 (m, 2H), 3.78 (broad s, 2H), 4.06 (broad s, 1H), 7.28 (m, 2H), 7.81 (broad s, 2H)
Calcd. for $C_{18}H_{24}N_2O_6S$: C, 54.53; H, 6.10; N, 7.07
Found: C. 54.24; H, 6.24; N, 6.73

(S)-6-[[(1,1-Dimethylethyl)oxy]carbonyl]-2-(p-tolylsulfonyl)aminohexanoic acid (**6**)

Compound **5** (7.5 mg, 18.9 μmol) was dissolved in ethyl acetate (5 mL), and then treated with 10% Pd/C (3.1 mg). The suspension was stirred under of hydrogen gas (1 atm) at room temperature for 21 h. The mixture was filtered and concentrated in vacuo to a white solid. The solid was identical by reverse phase HPLC (Beckman Ultrasphere ODS C18; 0.1% aq. trifluoroacetic acid/acetonitrile, 1:1, v/v, 220 nm) to that obtained from the tosylation of N^{ε}-Boc-L-lysine using literature procedures (16-18). The product (**6**) was compared to authentic N^{ε}–Boc-α-tosyl-L and D-lysine [each prepared via the tosylation (16-18) of commercially obtained L, D-N^{ε}-Boc lysines] by chiral chromatography on a Diacel 4.6 mm I.D. Chiracel AS column using a mobile phase of hexane/ethanol/trifluoroacetic acid (80:20:0.1, v/v/v), 1 mL/min, 254 nm. Admixtures of the authentic D, and L enantiomers showed retention times of 11.13 and 12.19 min, respectively. Compound **6** eluted at 12.13 min with <1.3% D enantiomer being detected.

(S)-6-[[(1,1-Dimethylethyl)oxy]carbonyl]-2-(p-tolylsulfonyl)aminohexan-4,4,5,5-^{3}H-oic acid (**6a**)

Compound **5** (2.73 mg, 7 μmol) in a 5.2 mL spherical O-ring flask equipped with a magnetic stirrer was dissolved in ethyl acetate (1 mL) and10% Pd/C (1.58 mg, 58 wt%) was added. The flask was attached to the tritium manifold and the mixture subjected to three cycles of freeze (liquid nitrogen)-pump-thaw . Tritium gas (7.6 Ci) was introduced to a partial pressure of 355 mm. The mixture was stirred at room temperature for 23 h, then frozen with a liquid nitrogen cooling bath, and the unreacted tritium gas removed. The frozen mixture was removed from the tritium manifold and attached to a glass bridge equipped with a receiver flask. This assembly was then attached to a glass vacuum manifold and the ethyl acetate removed by static vacuum transfer. Any labile tritons were exchange by static vacuum transfer with methanol. The resulting residue was taken up in ethanol and then passed through a Acrodisc™ PTFE, 13 mm (0.2 μm) nylon filter, removing the spent catalyst. The resulting solution was diluted to a final volume of 10 mL with absolute ethanol giving 735 mCi of the desired product. An 184 mCi portion of the above solution was purified by semi-preparative reverse phase HPLC (Rainin Microsorb C18 10 mm I. D. X 250 mm column, 0.05 M aqueous ammonium acetate (pH 7)/acetonitrile, 74:26, 7.5 mL/min, UV detection at 254 nm). The desired fraction was collected, and then the mobile phase was removed by lyophilization over a 24 h period. The resulting residue was dissolved in absolute ethanol (10 mL) to give 111 mCi of **6a**, having a radiochemical purity of 77% by HPLC (Ultrasphere Octyl C18, 0.005 M octanesulfonic acid, sodium salt (pH 2.9)/acetonitrile, 55:45, 1 mL/min), and a specific activity of 123 Ci/mmol as determined by mass spectrometry. The radiochemical purity was 92% before lyophilization.

Mass Spectrum [DCI, ammonia, negative ion, m/e (% base)]: 155 (100), 253 (32), $[M-H]^-$ 407 (1.5)
Tritium Distribution: t_0 (1%), t_1 (1%), t_2 (5%), t_3 (21%), t_4 (41%), t_5 (10%), t_6 (11%), t_7 (10%) for an average incorporation of 4.24 tritons/molecule.

(S)-6-[[(1,1-Dimethylethyl)oxy]carbonyl]-2-(p-tolylsulfonyl)aminohexan-4,4,5,5-^{3}H-oyl chloride (**7**)

To the above ethanol solution of **6a** (106 mCi) was added carrier (**6** , 20.6 mg, 51.5 μmol) . The ethanol was removed in vacuo and the resulting residue recrystallized from ethyl acetate/hexane. The solid was filitered off then dissolved in 3 mL of methylene chloride and placed in a 24 mL screw cap vial equipped

with a magnetic stirrer. The radiochemical purity of the solution was 94.7%. To the above solution was sequentially added: 4.1 mg (51.5 µmol) pyridine (freshly distilled over KOH), and 11.9 mg (64.4 µmol) of trichloro-s-triazine (Aldrich, 99%, lot #05022EY). The vial was capped and the reaction solution stirred at room temperature. After a few hours stirring, a white precipitate formed. After 25.5 h stirring at room temperature, the reaction mixture was filtered and the filtrate used directly in the next step.

(S)-6-[[(1,1-Dimethylethyl)oxy]carbonyl]-2-(p-tolylsulfonyl)aminohex-4,4,5,5-^{3}H-oyl diazomethane (**8**)

The inner chamber of an Aldrich MNNG diazomethane apparatus was charged with of 1-methyl-3-nitro-1-nitrosguanidine (209.5 mg,1.42 mmol) (Aldrich, 97%, lot # 00330MY) suspended in water (0.5 mL). The outer chamber was charged with methylene chloride (5 mL) and a magnetic stirrer.. The apparatus was immersed in a dry ice/acetone bath so that only the methylene chloride was cooled. With stirring of the methylene chloride layer, 5N aq sodium hydroxide (1.2 mL) was added dropwise by syringe (23 gauge needle) through the septum inlet of the inner chamber. After stirring in the dry ice/acetone bath for 1 h, the inner chamber was removed. To the methylene chloride solution of diazomethane was added the solution of **7** in portions. The color of the solution changed from dark to light yellow. The cooling bath was removed, and the reaction vessal was capped and stirred at room temperature for 23 h. Any gas that formed was allowed to exit through a bubbler. Some gas evolution was noted. The solution was used directly in the next step.

(S)-1-Chloro-3-[(p-tolylsulfonyl)amino]-7-amino-2-heptan-5,5,6,6-^{3}H-one ([^{3}H]TLCK)

To the above solution chilled in ice, was added 1M ethereal hydrogen chloride solution (5 mL) in portions. The reaction vessel was capped (bubbler) and stirred at room temperature for 24 h. A brownish gum , adhering to the walls of the vessel, precipitated out of solution. The solvent was removed under a stream of nitrogen, and the brownish gum dissolved in mobile phase (0.125% aqueous trifluoroacetic acid/acetonitrile, 70 : 30, v/v, 5 mL) giving a solution amounting to 85 mCi of activity. Reverse phase HPLC analysis on a Beckman Octyl C18, 4.6 mm ID (5 µm) column (220 nm UV detection, 1 mL/min) eluting with above mobile phase, showed that 50.8% of the radioactivity was associated with product [^{3}H] TLCKfor an overall radiochemical yield of 41%. The crude material was purified by semi-preparative reverse phase HPLC (5 X 1 mL injections, 10 mm I.D. Ultrasphere Octyl C18, 5 micron particle size, 5 mL/min, UV detection at 240 nm). The desired fractions were collected and combined to give a volume of 45 mL having a radioactivity concentration of 0.3 mCi/mL for a total activity of 13 mCi. The mobile phase solution was stored at -80^o C. Reverse phase HPLC assay as before showed a radiochemical purity of 94% (some decomposition occurs on the column), and a specific activity determination (derived from mass and radioactivity concentration) gave a value of 1.6 Ci/mmol.

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