SYNTHESIS OF THE ANGIOTENSIN CONVERTING ENZYME INHIBITOR ^{3}H-RAC-X-65

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SUMMARY

We prepared the angiotensin converting enzyme (ACE) inhibitor N-[1(S)-carboxy-3-(4′-^{3}H)carboxanilidopropyl]-L-Ala-L-Pro (^{3}H-RAC-X-65). The triflate of D-(+)-lactic acid benzyl ester was reacted with N′-(4-iodophenyl)-L-glutamine methyl ester. The benzyl ester was removed with HF, and the free carboxyl group was coupled to L-proline methyl ester via diphenylphosphorylazide. Methyl ester groups were removed by saponification. The product was dehalogenated in 10 Ci of $^{3}H_2$ to yield ^{3}H-RAC at 24.3 Ci/mmole (89.7% yield). ^{3}H-RAC-X-65 was indistinguishable from its unlabeled counterpart in its ability to inhibit ACE and enter into a tightly bound enzyme:inhibitor complex.

Key words: angiotensin converting enzyme inhibitor, tritium, dehalogenation, tight binding inhibitor.

INTRODUCTION

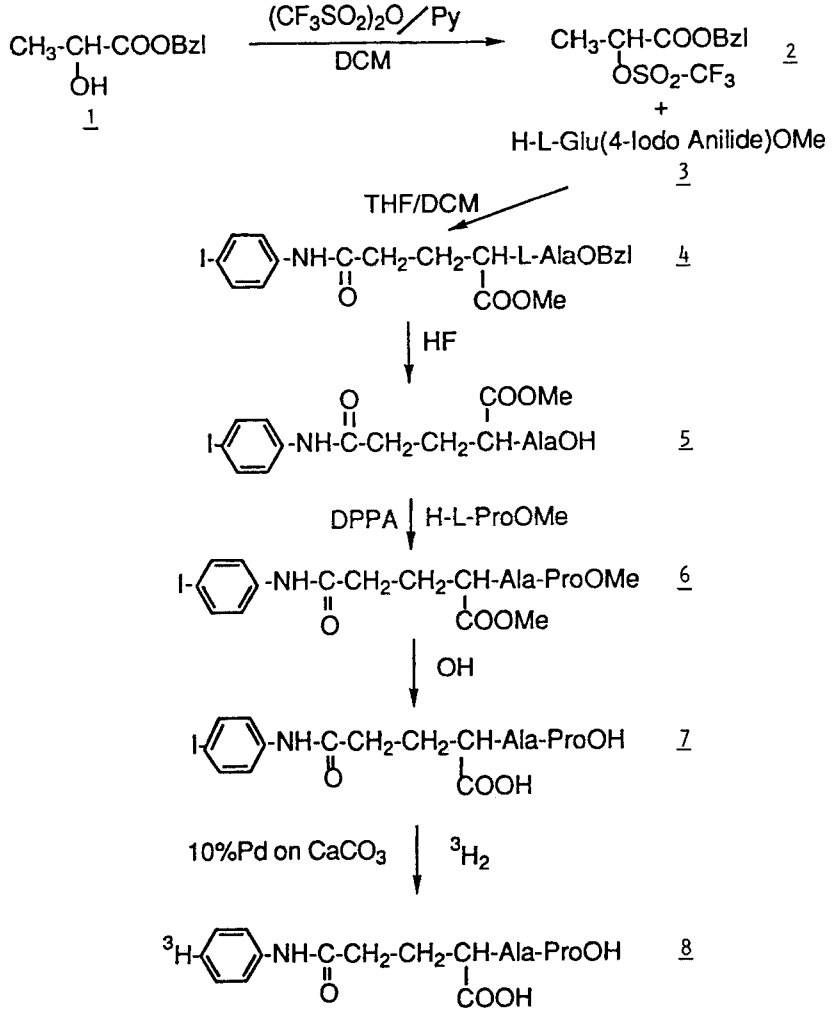
A radiolabeled, chemically stable, high affinity inhibitor of angiotensin converting enzyme (ACE) could be used for a number of purposes, among which are autoradiographic localization of ACE in tissue sections, some forms of radioimmunoassay of ACE inhibitors and of ACE itself, and the assay of ACE in vivo (1, 2). Radiolabeled forms of the ACE inhibitor captopril are available commercially. However, captopril is chemically unstable and does not have a great capacity for tight-binding to ACE. Further, captopril is not as specific for ACE nor as potent as the carboxyalkyl-dipeptide ACE inhibitor RAC-X-65 (N-[l(S)-carboxy-3-carboxanilidopropyl]-L-Ala-L-Pro), an inhibitor with a striking capacity for tight binding (Ki* 10 pM) (3). Thus, we have prepared a ^{3}H-labeled form of RAC-X-65 in which an atom of ^{3}H is introduced into the 4-position of its aniline phenyl group.

0362–4803/93/060483–09$09.50

Received 23 November, 1992
Revised 12 February, 1993

RESULTS AND DISCUSSION

The synthetic scheme is shown in Fig. 1. D-(+)-Lactic acid benzyl ester (1) and N-benzyloxycarbonyl-N′-(4-iodophenyl)-L-glutamine methyl ester were prepared as described in **EXPERIMENTAL**. All other compounds were from commercial sources. By using the triflate of D-(+)-lactic acid benzyl ester, N-[1-carbomethoxy-3-(4-iodocarboxanilido)propyl]-alanine (5) was obtained as the S, S isomer (4). The addition of L-proline methyl ester gave the S, S, S isomer.



Scheme

Tritiation by dehalogenation of 9.75 mg of 7 in $^3H_2$ gas provided the product (8) at an apparent specific radioactivity of 21.8 Ci/mmole, assuming quantitative recovery from the catalyst. Radiochemical purity as judged by co-chromatography of ^{3}H with unlabeled RAC-X-65 was 97.5%.

^{3}H-RAC-X-65 (8) was tested for its ability to inhibit pure guinea pig serum ACE (5). A single concentration of ACE in 50 mM HEPES/NaOH buffer, pH 8.0, containing 0.15 M NaCl and 0.6 M Na_2SO_4, was reacted with several known concentrations of unlabeled RAC-X-65 or several dilutions of ^{3}H-RAC-X-65 of known dpm. In the first study, ACE activity was measured immediately by adding a synthetic substrate, [^{3}H]benzoyl-Phe-Gly-Pro, at a concentration (50 nM) within the range of first order enzyme kinetics. ACE activities were computed as the first order rate constant (6), Vmax/Km, in min^{-1}. In a second study, ^{3}H-RAC, or unlabeled RAC, was preincubated with ACE for 15 min at 37°C, and then residual ACE activity was measured. Results were graphed according to the Henderson equation (7),

$$[I]_T/(1-(v_i/v_o)) = Ki(v_o/v_i) + [E]_T$$

where $[I]_T$ is total inhibitor concentration, $[E]_T$ is total enzyme concentration, Ki is the dissociation constant, v_o is Vmax/Km in the absence of inhibitor, and v_i is Vmax/Km in the presence of inhibitor. For experiments in which ^{3}H-RAC-X-65 was used (Fig. 2b), $[I]_T$ is expressed as Ci/liter.

As illustrated in Fig. 2, inhibition conferred by either unlabeled RAC-X-65 or ^{3}H-RAC-X-65 was progressive. A 15 min preincubation of unlabeled RAC-X-65 with ACE caused apparent Ki to decrease from 4E-10 M to 2.8E-11 M. By dividing slopes of the lines of Fig. 2a into the respective slopes in Fig. 2b, specific radioactivity was computed to be 25.5 and 24.3 Ci/mmole (respectively, for no preincubation and for 15 min preincubation).

Stored at 0.5 mCi/ml of 70% ethanol at -20°C, ^{3}H-RAC-X-65 has been found to be stable for at least 18 mo.

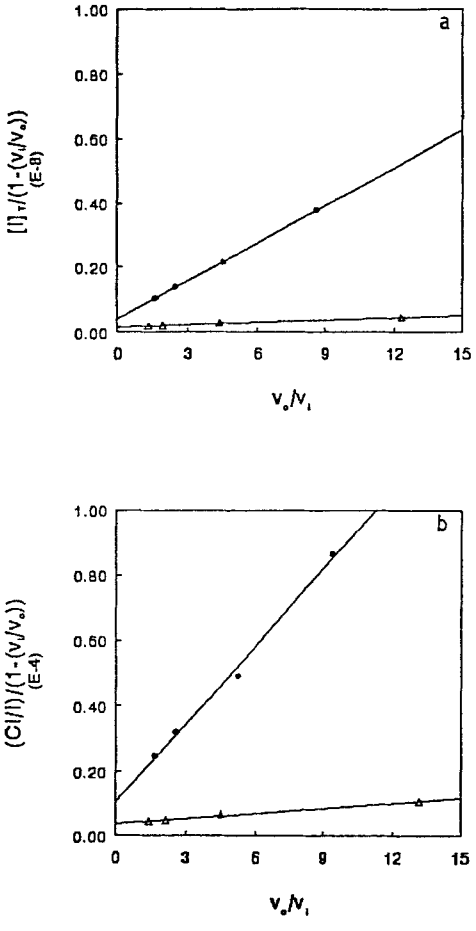


Figure 2. The upper panel (a) shows inhibitory effects of unlabeled RAC-X-65 on guinea pig serum ACE; •, no pre-incubation; ▵, 15 min preincubation. The lower panel (b) shows effects of ^{3}H-RAC-X-65, in Ci/liter, on ACE; •, no preincubation; ▵, 15 min preincubation.

EXPERIMENTAL

Methods. ^{1}H NMR spectra were recorded on a Varian VXR-400 spectrometer or on a Varian R-600 spectrometer. Infrared spectra were recorded on a Perkin-Elmer 267 infrared spectrophotometer. Optical rotations were measured with a Perkin-Elmer 241 polarimeter. Elemental analysis was performed by either Micro-Tech Laboratories, Inc., Skokie, IL or Midwest Microlab, Indianapolis, IN. Analytical thin layer chromatography (TLC) was performed using Analtech silica gel HLF uniplates in the following solvent systems: A, ethyl acetate/pyridine/acetic acid/water; 200:2:6:1; B, methanol/chloroform; 1:1; C, benzene/water/acetic acid; 9:1:9; D, n-butanol/ethyl acetate/acetic acid/water; 1:1:1:1; and E, n-butanol/pyridine/acetic acid/water; 15:10:3:12. R_f subscript letters (e.g. R_{fA}) denote the solvent system used.

Preparation of D-(+)-lactic acid benzyl ester (1). Lithium D-(-)-Lactate (5.0 g, 52 mmol) previously dried in a vacuum desiccator over phosphorus pentoxide was mixed with 30 ml of benzyl alcohol, and the resulting solution was saturated with dry hydrogen chloride at 0 to 5°C for 3.5h. The reaction mixture was then stored at 4°C overnight. Excess hydrogen chloride was removed by rotary evaporation under reduced pressure at 35°C, and the remaining mixture was diluted with ethyl ether. The etheric solution was washed with cold 1M aqueous sodium bicarbonate until slightly alkaline and then with a saturated sodium chloride solution. The mixture was dried over anhydrous magnesium sulfate, filtered and concentrated in vacuo yielding a yellowish liquid. Distillation under vacuum (about 1 mmHg) gave benzyl D-(+)lactate (4.6 g, bp 113-123°C). IR ($CHC1_3$) 3400-3600, 1732, 1190-1280 cm^{-1}. ^{1}H-NMR (60 MHz, $CDC1_3$) (TMS) 7.3 (S, 5H, arom), 5.15 (S, 2H, benzyl CH_2), 4.3 (M, 1H, CH_3-CH-0), 3.75 (S, 1H, OH), 1.38 δ (d, J = 7.2, 3H, CH_3-CH-O). $[\alpha]_D^{25}$ = + 15.3° (C= 3.5, ethanol).

D-1-(1-Carbobenzyloxy)ethyl trifluoromethanesulfonate (Triflate) (2). Dry pyridine (6.3 mmol) in 18 ml of dry dichoromethane was cooled to -22°C. The resulting solution was admixed with trifluoromethane sulfonic anhydride (6.0 mmol) in 1.5 ml of dichloromethane at -22°C, and the resulting mixture was maintained at -22°C for 5 minutes. Benzyl D-(+)-lactate (6 mmol) in 2 ml of dichloromethane was added at -22°C; then the temperature of the reaction mixture was quickly raised to + 22°C on a water bath and was maintained at that temperature for 10 minutes. Pyridinyl salt was removed by filtration, and the filtrate was concentrated in vacuo. The mixture was poured through a silica gel plug (1.1 x 5 cm), and the plug was washed with 40 ml of n-hexane. The combined filtrates were concentrated under reduced pressure yielding 0.95 g of a clear oily residue and was used without further purification. IR (film) 1760 cm^{-1}, ^{1}H-NMR (60 MHz, $CDC1_3$) (TMS) 7.34 (S, 5H, arom.), 5.23 (S,2H, CH_2 benzyl), 4.545 (m, 1H, CH_3-CH-O), 1.66 δ (d, J = 7.0, 3H, CH_3-CH-O).

N′-(4-Iodophenyl)-L-glutamine methyl ester (3). N-Benzyloxycarbonyl-N′-(4-iodophenyl)-L-glutamine methyl ester was prepared as follows: A solution of 6.386 g (31 mmol) of N,N′ dicyclohexylcarbodimide in 15 ml of dry dimethylformamide was added dropwise into a solution of 9.2 g (31 mmol) N$^{\alpha}$-benzyloxycarbonyl-L-glutamic acid methyl ester and 4.19 g

(31 mmol) of 1-hydroxybenzatriazole hydrate in 20 ml of dry dimethylformamide at -5°C with stirring. After 10 minute at -5°C, a solution of 7.23g (33 mmol) of 4-iodoaniline (freshly recrystallized from ethyl acetate and light petroleum ether) in 12 ml of dimethylformamide was added. The reaction mixture was stirred at -5°C for 3/4 hr and then at 4°C overnight. Insoluble dicyclohexylurea was removed by filtration, and the filtrate was concentrated in vacuo. After diluting the residue with ethyl acetate, the organic phase was washed until neutral, dried over anhydrous sodium sulfate, filtered and concentrated in vacuo yielding a white residue. The residue was recrystallized from tetrahydrofuran and isopropyl alcohol resulting in 10.75 g of white crystalline product, mp. 179-180°C: R_{fA} 0.89; R_{fB} 0.82; R_{fC} 0.85. IR(KBr) 3295, 1740 & 1640-1710 cm^{-1}); $[\alpha]_D^{25}$ = - 16.7° (C=2.1; dimethylformamide).

Elemental analysis calculated for C_{20} H_{21} N_2IO_5 : C = 48.40; H = 4.26; N = 5.64; I = 25.57

Found: C = 48.26; H = 4.42; N = 5.62; I = 25.72

The above preparation (4.65g) was treated with 10 ml of 30% HBr in glacial acetic acid at room temperature for 45 minutes. The hydrobromide salt was precipitated with the addition of 50 ml of anhydrous ethyl ether and cooling in an ice bath, resulting in 4.45 g of solid. The crude material was suspended in 40 ml of 20% methanol in diethyl ether with stirring. It was then collected on a filter and washed thoroughly with anhydrous ether resulting in 4.0 g of white solid, d.p. 199-200°C: R_{fA} 0.50; R_{fB} 0.67; R_{fC} 0.46; $[\alpha]_D^{25}$ = + 11.45° (C=2; dimethylformamide): IR(KBr) 3300, 2300-3200, 1720 and 1667 cm^{-1}; Elemental Analysis calculated for $C_{12}H_{16}N_2BrIO_3$ cal: C = 32.53; H = 3.64; N = 6.32; I = 28.64

Found: C = 32.64; H = 3.55; N = 6.28; I = 28.60

The free amino base was obtained by treating the above hydrobromide salt with 1.5 g of $NaHCO_3$ and 0.8 g of NaOH in 5 ml of H_2O admixed with 10 ml of tetrahydrofuran and 40 ml of dichloromethane. The organic phase was dried over anhydrous magnesium sulfate and filtered. The filtrate was concentrated in vacuo yielding 3.09 g of white solid.

1-N-[1-(S)-Carbomethoxy-3-(4′-I)carboxanilidopropyl]-L-alanine (5). A solution of 2.03 g (6.5 mmol) of D-1-(1-carboxbenzyloxy)ethyl triflate in 10 ml of dry dichloromethane was added dropwise over 30 min into a solution of N′-(4-iodophenyl)-L-glutamine methyl ester (1.81 g; 5.0 mmol) and triethylamine (0.9 ml) in 15 ml of dichloromethane with stirring at room

temperature. After stirring at room temperature for an additional 3 hours, the solvent was removed with an evaporator. The residue was diluted with 45 ml of ethyl acetate/diethyl ether (5:1 by vol). The organic solution was washed 2x with saturated NaCl solution; 10x with H_2O and finally 2x with saturated NaCl solution. The organic phase was dried over anhydrous magnesium sulfate and filtered. The filtrate was concentrated in vacuo resulting in 2.73 g of clear liquid. R_{fC} 0.54; R_{fD} 0.77

The benzyl ester protecting group was removed from the above crude material (4) with 20 ml of anhydrous hydrogen fluoride in the presence of 1.5 ml of anisole at 0°C for one hour. After hydrogen fluoride was removed under reduced pressure, the material was treated with a mixture of diethyl ether (20 ml) and H_2O (10 ml) with stirring and cooling in an ice bath for 15 minutes. The resulting white crystalline product was collected by filtration and was washed thoroughly with ice water and then with anhydrous ether. The white crystals were dried in an vacuum desiccator over P_2O_5: weight 1.17 g, d.p. 176-176.5°C (second crop from the filtrate yielded an additional 51 mg of the product d.p. 170-170.5°C): R_{fC} 0.42; R_{fD} 0.58. $[\alpha]_D^{25}$ = - 5.5° (C=2.0; dimethylformamide); IR (KBr) 3340, 1748, 1656 and 1540-1590 Cm^{-1}; ^{1}HNMR (400 MHz, $(CD_3)_2SO$), (TMS) 10.0 (s, 1/2H, COOH), 7.62 (d, J=8.6, 2H, arom); 7.43 (d, J=8.6, 2H, arom), 3.0-4.1 (br; NH), 3.63 (S, OCH_3), 3.33 (t, J=6.4, CH_2-CH_2-CH), 3.22 (q, J=6.2, CH_3-CH) (a total of 7H from 3.0 - 4.1), 2.42 (t, J=7.6, 2H, CH_2), 1.80 - 1.89 (two sets of multiplets, 2H; CH_2-CH_2-CH), 1.18δ (d, 5=6.2cps, 3H, CH_3-CH).

Elemental analysis calculated for C_{15} $H_{19}N_2IO_5$: C, 41.49; H, 4.41; N, 6.45; I, 29.22.

Found: C, 41.37; H,4.71, N, 6.35; I, 29.21.

1-N-[1-(S)-Carboxy-3-(4′-I)carboxanilidopropyl]-L-alanyl-L-proline (7). Diphenylphosphorylazide (0.24 ml; 108 mmol) in 1 ml of dry dimethylformamide was added dropwise with stirring at 0°C into a solution of 0.435 gm (1 mmol) of 1-N-[1-(S)-carbomethoxy-3-(4′-I)carboxanilidopropyl]-L-alanine and 0.179 gm (1.08 mmol) of L-proline methyl ester hydrochloride in 7.0 ml of dry dimethylformamide. After stirring at 0°C for 5 minutes, a solution of 0.29 ml of triethylamine in 2 ml of dry dimethylformamide was added over a period of 15 minutes. The reaction mixture was further stirred at 0°C for 3 hours and then at room temperature overnight. Triethylamine

hydrochloride was removed by filtration, and the filtrate was concentrated in a rotatory evaporator at 35°C. Ethyl acetate (~30 ml) was added to the resulting residue, which then was washed with a succession of half saturated NaCl solution (1x); 1M $NaHCO_3$ (3x); and saturated NaCl solution (2x). The solution was dried over anhydrous magnesium sulfate, filtered and then concentrated in vacuo yielding 0.54 g of oily residue. IR ($CHCl_3$) 3200-3500; 1737, 1673 and 1630 cm^{-1}.

The crude material (6) in 6 ml of acetone was saponified by stirring with 6 ml of 1M NaOH solution at room temperature for 1 hour. After concentration in vacuo, acetic acid (4 ml) was added, and the mixture was concentrated in vacuo. The semi-solid product was purified on an Amberlite XAD-2 (210-350 microns) column (2.0 x 59 cm) and was eluted with 0.1 M NH_4OH/methanol (95:5). Fractions containing the desired product were pooled and concentrated under reduced pressure yielding 0.275 g of white solid. Recrystallization from absolute ethanol and anhydrous ether resulted in 0.223 g of white crystals, m.p. 147-148.5°C. R_{fE} 0.53; R_{fD} 0.49; $[\alpha]_D^{25}$ = -12.1° (C=2.1; EtOH).

Elemental analysis calculated for $C_{19}H_{24}N_3IO_6 \cdot 2H_2O$: C, 41.24; H, 5.10; I, 22.93.

Found: C, 41.57; H, 5.06; I, 23.02

N-[1-(S)-Carboxy-3-(4′-^{3}H)carboxanilidopropyl]-L-alanyl-L-proline (8). The above iodinated precursor (9.75 mg) was subjected to catalytic dehalogenation in 10 Ci of $^3H_2$ gas, with 10% Pd on calcium carbonate in dimethylformamide/water (1:1 by volume) by Amersham Corporation (Arlington Heights, Illinois). Assuming quantitative recovery from the catalyst, the final product had a specific radioactivity of 21.8 Ci/mmol. The radiochemical purity was found to be 97.5% by running with authentic compound in TLC: R_{fE} 0.49.

Acknowledgments

This work was supported in part by EY 07135 and HL 46689, grants from the U.S. Public Health Service.

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