SYNTHESIS OF TRITIUM-LABELED AZALINE B ACETATE

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SUMMARY

Reaction of azaline B acetate (**1**) with freshly prepared trifluoroacetyl hypoiodite in trifluoroacetic acid - methylene chloride solution under anhydrous conditions produced a complex mixture of products from which compounds **2** and **3**, resulting from iodination at the 1'- and 8'-positions of the Ac-D Nal[1] residue, were isolated by preparative reversed-phase HPLC chromatography. Mass spectral analysis and ^{1}H, 2D DQF-COSY and homonuclear 2D J-resolved NMR experiments confirmed these structures. Reductive tritiation of **2** over 10% Pd/C in EtOH - DMF (1:9, v/v) with 50 Ci T_2 followed by chromatographic purification and salt exchange gave 37.5 mCi of tritiated azaline B acetate (**4**) having >98% chemical and radiochemical purities and a specific radioactivity of 11.2 Ci / mmol. A single peak at 7.59 ppm was observed in the proton decoupled tritium NMR spectrum.

Key words: azaline B, tritium, decapeptide

INTRODUCTION

Azaline B acetate (**1**), a synthetic decapeptide containing both natural L- and several unusual, unnatural D- amino acids, is a potent GnRH (gonadotropin-releasing hormone) antagonist with reduced histamine-releasing properties.[1-3] Our interest in azaline B acetate (**1**) necessitated preparation of tritium-labeled material for use in drug metabolism studies. We report herein the synthesis of tritiated azaline B acetate (**4**) by iodination of **1** followed by reductive tritiation.

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CCC 0362–4803/97/020147–11
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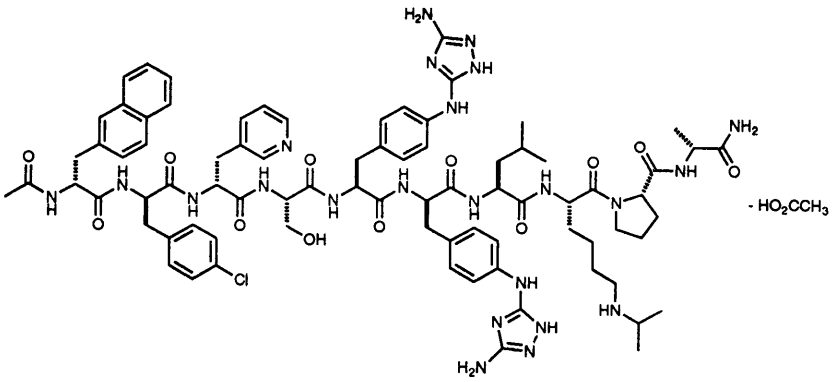
Received 10 September 1996
Revised 30 September 1996



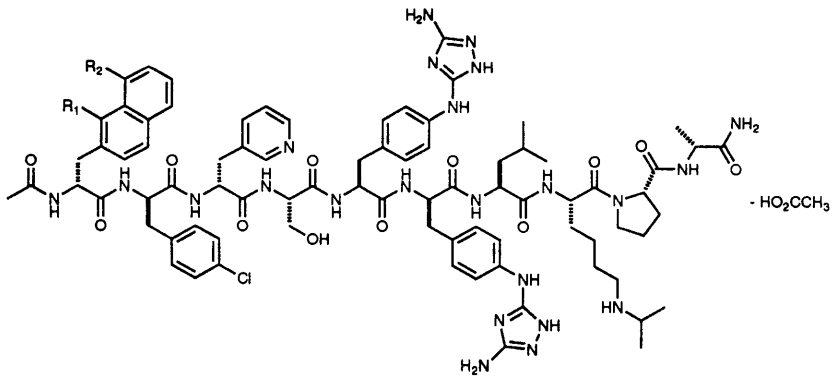
Ac-D-Nal[1],D-Cpa[2],D-Pal[3],Ser[4],Aph[5](atz),D-Aph[6](atz),Leu[7],ILys[8],Pro[9],D-Ala[10]-NH_2-HOAc

Azaline B Acetate (1)

RESULTS AND DISCUSSION

Of several strategies considered for preparation of the tritiated material, it seemed most expedient to proceed by direct iodination of **1** followed by reductive tritiation. Regarding the choice of iodinating agent, prior work in our laboratory[4] suggested the use of freshly prepared trifluoroacetyl hypoiodite[5,6] in trifluoroacetic acid - methylene chloride solution.

Although only methylene chloride was used as the solvent in a model system, the use of trifluoroacetic acid - methylene chloride (1:3, v/v) was necessary for peptide dissolution.[4] Acceptable iodination results were observed as long as the reaction was conducted with short reaction times, under strictly anhydrous conditions with freshly prepared trifluoroacetyl hypoiodite, and with immediate removal of trifluoroacetic acid after reaction completion.[4] Drying of silver trifluoroacetate at 60°C in vacuo and distillation of trifluoroacetic acid immediately prior to the reaction were also required.[4] Thus, addition of 2.8 equiv of freshly prepared trifluoroacetyl hypoiodite into a solution of **1** in anhydrous trifluoroacetic acid - methylene chloride, stirring for 105 min at ambient temperature and workup produced a number of new products in addition to recovered starting material (HPLC). Given the functional diversity present in **1**, it was not surprising to observe that the reaction proceeded with a lack of chemospecificity. The largest area % component in the crude reaction mixture was isolated by preparative reversed-phase HPLC chromatography using an acetonitrile - triethylammonium phosphate mobile phase, converted to an acetate salt[7] and recovered by lyophilization. Mass spectral analysis indicated the material was mono-iodinated while hydrogenation over a palladium catalyst[4] with



2 (R_1 = I, R_2 = H)

3 (R_1 = H, R_2 = I)

4 (R_1 = ^{3}H, R_2 = H)

excess triethylamine regenerated starting **1** (HPLC, NMR). NMR spectroscopy was used to identify the site of iodination. Results from ^{1}H, 2D DQF-COSY and homonuclear 2D J-resolved NMR experiments[10] indicated the sample was a ca. 1:1 mixture of two isomeric iodides, **2** and **3**. A second preparative reversed-phase HPLC method using a tetrahydrofuran - aqueous trifluoroacetic acid mobile phase was developed to obtain pure **2**.

Attention was next turned to improving the chemoselectivity of the iodination and the extent of reaction completion. Lowering the iodination temperature to ≥ -25°C resulted in a reduction of area % levels of early t_R by-products (HPLC). Below this temperature, **1** oiled from solution in 1:3 (v/v) trifluoroacetic acid - methylene chloride at concentrations ≥ 12 mg / mL. Near complete consumption of **1** was achieved by titration with ≤ 2.2 equiv trifluoroacetyl hypoiodite solution. In other experiments, complete reaction of starting **1** resulted by addition of more hypoiodite solution, but increased by-product formation was also observed.

The final iodination conditions involved the slow addition of freshly prepared trifluoroacetyl hypoiodite (2 X 1.04 equiv) in 1:3 (v/v) trifluoroacetic acid - methylene chloride solution into a solution of azaline B acetate **1** at -20°C followed by workup after 90 min. The crude reaction mixture was first purified by preparative reversed-phase HPLC using a tetrahydrofuran - aqueous trifluoroacetic acid mobile phase to separate **2** from **3**. A second chromatography in an acetonitrile - aqueous triethylammonium

phosphate system followed by conversion to the acetate salt[7] and lyophilization afforded very pure iodoazaline B acetate **2** (HPLC, NMR).

The reductive tritiation was performed by reacting a solution of **2** in 1:9 (v/v) absolute ethanol - *N,N*-dimethylformamide with 50 Ci of tritium gas over 10% Pd/C in the presence of excess triethylamine for 5 h. After workup, the product solution containing **4** (radiochemical purity of 58-60% by HPLC/RAM) was purified by preparative reversed-phase HPLC chromatography, first using the gradient acetonitrile - aqueous triethylammonium phosphate system, then by the isocratic tetrahydrofuran - aqueous trifluoroacetic acid system. Following conversion from the trifluoroacetate to the acetate salt, 37.5 mCi (16% chemical yield) of azaline B-*^3H* acetate **4** having a specific radioactivity of 11.2 Ci / mmol was obtained. This material was found to have radiochemical and chemical purities exceeding 98% (HPLC/RAM). The proton decoupled tritium NMR spectrum of **4** showed a single resonance at 7.59 ppm (D_2O).

It is important to note that azaline B acetate is readily deposited from aqueous solution onto plastic and glass surfaces. Reproducible radioactivity measurements were obtained only by sampling a solution of **4** prepared by its addition into a glass volumetric flask containing 2% trifluoroacetic acid in water (v/v).

EXPERIMENTAL

^1H NMR spectra were recorded on either a Bruker AM-400 or a Varian XL-400 spectrometer. Chemical shifts are expressed on the δ scale and reported downfield of 3-(trimethylsilyl)-1-propanesulfonic acid, sodium salt (TSP) internal standard. The proton decoupled tritium NMR spectrum was recorded in D_2O on a Bruker ACE-300 NMR spectrometer operating at 320 MHz. Mass spectral analysis was performed on a Finnigan TSQ-7000 mass spectrometer under electrospray conditions (5 KV). Samples were dissolved into 50:50:0.5 (v/v/v) methanol - water - acetic acid and injected (sample loop) into the instrument. Radioactivity measurements were performed using a Beckman model LS6000LL liquid scintillation counter and Formula-989 liquid scintillation cocktail (DuPont/NEN Research Products, Boston, MA).

The analytical HPLC data were acquired on a Waters Millennium 2010 HPLC system consisting of a model 600E system controller, a model 996 diode array detector and a model 717 WISP injector, on a

Waters HPLC system consisting of a model 600E system controller, a model 486 variable wavelength detector, a model 717 WISP injector and a 746 data module, or on a Hewlett-Packard model 1090M liquid chromatograph. Analyses were performed using a Zorbax SB-C8 column (5 μm packing, 4.6 x 250 mm) purchased from MAC-MOD (Chadds Ford, PA) with monitoring at 227 and 260 nm. Isocratic analyses were performed using 36:64:0.15 (v/v/v) tetrahydrofuran - water - trifluoroacetic acid with a flow rate of 1 mL / min and a column temperature of 40°C. Gradient analyses (Table 1; all gradient changes were linear) were performed at ambient temperature at a flow rate of 1.5 mL / min using mobile phases composed of acetonitrile - pH 2.5 aqueous triethylammonium phosphate (prepared by

Table 1

Time (min)	0	10	14	15	17	18	22
%A	80	65	50	15	15	80	80
%B	20	35	50	85	85	20	20

A: 1:9 (v/v) MeCN - pH 2.5 TEAP

B: 9:1 (v/v) MeCN - pH 2.5 TEAP

acidifying a 1% (v/v) solution of triethylamine in water to pH 2.5 with 85% phosphoric acid). Radiochemical detection was accomplished using a Radiomatic model A250 flow detector equipped with a 0.5 mL flow cell connected to the Hewlett-Packard 1090M liquid chromatograph equipped with Chemstation software. Atomflow scintillation solution was used for radiochemical detection.

Purification of iodoazaline B acetate (**2**) was performed using a Zorbax SB-C8 column (7 μm packing, 21.2 x 250 mm) purchased from MAC-MOD (Chadds Ford, PA) at ambient temperature on a Rainin Dynamax HPLC system consisting of two Rainin model SD-1A pumps, a Rainin model UV-1 detector and a Rheodyne 7125 manual injector equipped with a 0.5 mL sample loop. For the 34:66:0.15 (v/v/v) tetrahydrofuran - water - trifluoroacetic acid isocratic preparative method, absorption was monitored at a wavelength of 285 nm and the run time was 26 min. Sample solutions were prepared in the mobile phase at a concentration of ca. 100 mg / mL. For the acetonitrile - pH 2.5 aqueous triethylammonium phosphate gradient preparative method, absorption was monitored at a wavelength of 280 nm and the run time was 17 min.

Purification of azaline B-^{3}H (**4**) was performed at ambient temperature on a Waters HPLC system consisting of a model 600E system controller, a model 486 variable wavelength detector, a model 746 data module, a Zorbax SB-C8 column (7 μm packing, 21.2 x 250 mm) and a Rheodyne 7125 manual injector equipped with a 2 mL sample loop. For preparative chromatography in the gradient acetonitrile - pH 2.5 triethylammonium phosphate system, absorbance was monitored at 290 nm with

2.0 AUFS, and the run time was 25 min. For the isocratic 36:64:0.15 (v/v/v) tetrahydrofuran - water - trifluoroacetic acid preparative method, absorbance was monitored at 290 nm with 2.0 AUFS, and the run time was 35 min. Removal of the buffer and conversion to the acetate salt was also performed on this Waters HPLC system using a Zorbax SB-C8 column (5 μm packing, 4.6 X 250 mm) with monitoring at 290 nm using 2.0 AUFS and a run time of 55 min.

Anhydrous *N,N*-dimethylformamide (99+%, HPLC grade), anhydrous methylene chloride (CH_2Cl_2), silver trifluoroacetate (98%), triethylamine (99%) and trifluoroacetic acid (TFA, 99+%) were purchased from Aldrich Chemical Co. (Milwaukee, WI). The trifluoroacetic acid was freshly distilled prior to use. The silver trifluoroacetate was dried in vacuo at 60°C prior to use. The anhydrous 10% Pd/C was purchased from Alfa Products (Danvers, MA) and used as received. Azaline B acetate (**1**) was obtained from Bachem Bioscience, Inc. (King of Prussia, PA) and dried at ambient temperature in vacuo over P_2O_5 prior to use. Glacial acetic acid (ACS grade), acetonitrile (HPLC grade), iodine (ACS grade), 85% phosphoric acid (ACS grade) and phosphorous pentoxide (ACS grade) were purchased from Fisher Scientific Co. (Malvern, PA) and used as received. Tetrahydrofuran (THF, HPLC grade) was purchased from Baxter Healthcare Corporation (Muskegon, MI) and used as received. Absolute ethanol was purchased from Pharmco Products, Inc. (Brookfield, CT) and used as received.

Iodoazaline B (2)

An oven-dried 300 mL three-neck flask containing a stir bar was cooled to ambient temperature under a stream of dry nitrogen. After adding AgO_2CCF_3 (1.046 g, 4.64 mmol, 5.25 equiv) and TFA (30 mL) and stirring at ambient temperature for 5-10 min, CH_2Cl_2 (95 mL) and iodine (1.122 g, 4.42 mmol, 5.0 equiv; solid addition) were added. The resulting slurry was stirred 30 min at ambient temperature under an inert atmosphere of nitrogen with protection from light, then cooled 15 min in an ice water bath. The magenta solution of trifluoroacetyl hypoiodite was held at 0-5°C under nitrogen until needed.

An oven-dried 500 mL three-neck flask, stir bar and 60 mL constant pressure addition funnel were assembled hot, then cooled to ambient temperature under a stream of dry nitrogen. The flask was charged with azaline B acetate (**1**, 1.426 g, 0.85 mmol) and CH_2Cl_2 (90 mL). To the resulting slurry was added TFA (30 mL) at ambient temperature. The solution was stirred under an inert atmosphere of nitrogen while cooling 15 min in a dry ice - isopropanol bath maintained at -20 ±2°C.

A 25 mL aliquot (0.88 mmol, 1.04 equiv) of the trifluoroacetyl hypoiodite solution was transferred into the addition funnel and added dropwise into the solution of **1** at -20°C over ca. 13 min. After an additional 40 min,[11] a second 25 mL aliquot (0.88 mmol, 1.04 equiv; total equiv added = 2.08) of trifluoroacetyl hypoiodite solution was added dropwise over 12 min into the reaction at -20°C. After an additional 40 min,[11] the reaction mixture was filtered under anhydrous conditions and the dark red filtrate was concentrated to dryness by rotary evaporation at 20°C. The resulting oil was twice reconcentrated from 1:1 (v/v) acetonitrile - water (46 mL) and dried in vacuo for 16 h at ambient temperature with protection from light to yield 2.46 g (>100%) of crude product as a rusty brown powder. This material was dissolved in 34:66:0.15 (v/v/v) tetrahydrofuran - water - trifluoroacetic acid (ca. 100 mg / mL) and injected onto a Zorbax SB-C8 preparative HPLC column (0.5 mL / injection). The peak eluting from 11.6 - 14.0 min from each injection was collected, combined fractions were concentrated to a volume of ≤ 200 mL by rotary evaporation at 20°C and the solid was recovered by lyophilization. This material was dissolved into 10 mL of the initial mobile phase (Table 2) and injected onto a Zorbax SB-C8 preparative HPLC column (0.5 mL / injection; flow rate = 20 mL / min). The peak eluting from 6.4 - 7.1 min from each injection was collected. This solution containing **2** was reinjected

Table 2

Time (min)	0	10	15	16	17
%A	80	70	15	80	80
%B	20	30	85	20	20

A: 1:9 (v/v) MeCN - pH 2.5 TEAP

B: 9:1 (v/v) MeCN - pH 2.5 TEAP

onto the Zorbax SB-C8 preparative HPLC column (40 mL / run; 5 mL loading loop) by repetitive injections while maintaining the initial mobile phase composition (Table 3; flow = 8 mL / min). A center

Table 3

Time (min)	0	10	15	34	34.5	37
%A	100	100	90	50	100	100
%B	0	0	10	50	0	0
Flow (mL/min)	18	18	18	18	18	8

A: 1% (v/v) HOAc in water

B: MeCN

cut (28.0 - 30.4 min) of the major peak from each injection was collected, and the combined fractions were concentrated by rotary evaporation at 20°C to a volume of <50 mL. This solution was diluted to a volume of 100 mL with water and lyophilized to dryness to yield 60.9 mg (4%) of iodoazaline B acetate (**2**) as a fluffy, pale yellow powder. HPLC analyses indicated purities of >99 area% in both the isocratic

36:64:0.15 (v/v/v) tetrahydrofuran - water - trifluoroacetic acid and gradient acetonitrile - pH 2.5 triethylammonium phosphate systems. The mass spectrum showed ions at m/z 1740.3, 870.2 and 580.7, representing $(M + H)^+$, $[(M + 2H) / 2]^{2+}$ and $[(M + 3H) / 3]^{3+}$, respectively. ^1H NMR[12] (partial spectrum) δ 6.89 (m, 4H), 7.03 (m, 4H), 7.08 (1/2 AB_q, 1H, J = 8.3 Hz), 7.18 (m, 4H), 7.47 (dd, 1H, J = 7.5, 7.5 Hz), 7.54 (dd, 1H, J = 7.5, 7.5 Hz), 7.63 (d, 1H, J = 8.4 Hz), 7.72 (d, 1H, J = 8.0 Hz), 7.98 (dd, 1H, J = 8.0, 6.0 Hz), 8.08 (d, 1H, J = 8.6 Hz), 8.37 (d, 1H, J = 8.0 Hz), 8.60 (s, 1H), 8.66 (d, 1H, J = 6.0 Hz).

Azaline B-*3H* (4)

Into a solution of 38 mg (21.1 μmol) iodoazaline B acetate (**2**) in 1:9 (v/v) abs. ethanol - *N,N*-dimethylformamide (3.8 mL) at ambient temperature was added 233 μL (1.67 mmol, 79 equiv) triethylamine and 38 mg of 10% Pd/C. After degassing the slurry in vacuo, 50 Ci of tritium gas was introduced and the reaction stirred for 5h at ambient temperature. Excess tritium gas was removed and the catalyst was suction filtered through a prewashed Celite® pad (100 mg). The filtercake was washed with 50% aqueous ethanol (2 X 2 mL) and the combined filtrates were concentrated in vacuo at ≤30°C to a low volume.[13] Labile tritium was removed by addition of 50% aqueous ethanol (2.5 mL) followed by stirring for 1-2 min and concentration in vacuo at 30°C to a low volume.[13] A second exchange was performed by adding 50:50:0.1 (v/v/v) abs. EtOH - water - TFA (2.5 mL), stirring 1-2 min and concentrating in vacuo at 30°C to a low volume.[13] The remaining solution was diluted with 50% aqueous ethanol to a volume of 25 mL and returned from the vendor for further purification.

The solution containing **4** was concentrated to a low volume by distillation at 30°C.[13] The remaining light brown oil (ca. 0.8 mL) was diluted with 1:9 (v/v) abs. EtOH - water solution (1 mL) and injected onto a preparative Zorbax SB-C8 column for purification using the conditions shown in Table 4 (all gradient changes were linear). An initial flow 1 mL / min was necessary until the column back pressure, high due to the viscosity of the sample solution, was sufficiently reduced. Fractions containing the major band (10.0 - 12.3 min) were collected and concentrated by rotary evaporation at 15°C to a

Table 4

Time (min)	0	2	14	15	20	21
%A	82	82	72	15	15	82
%B	18	18	28	85	85	18
Flow (mL/min)	1	3	4	4	4	4

A: 1:9 (v/v) MeCN - pH 2.5 TEAP

B: 9:1(v/v) MeCN - pH 2.5 TEAP

volume of ca. 2 mL. This solution containing **4** was further purified by preparative reversed-phase HPLC chromatography over the Zorbax column using the isocratic 36:64:0.15 (v/v/v) THF - water -

Table 5

Time (min)	0	2	4	8
Flow (mL/min)	0.5	1	1.5	2
Curve	*	9	9	6

TFA method (Table 5). Fractions containing pure **4** were combined, concentrated by rotary evaporation at 15°C and diluted with 50% aqueous EtOH (4 mL). Following concentration to ca. 1 mL by rotary evaporation at 15°C, glacial acetic acid (15 µL) was added and the clear, colorless solution was injected onto the analytical Zorbax SB-C8 column (Table 6). Fractions containing pure **4** were collected, combined, concentrated to ca. 3 mL by rotary evaporation at 13°C then diluted to a volume of 15 mL with 1:9 (v/v) abs. EtOH - water to give a clear, colorless solution of azaline B-*3H* acetate(**4**). HPLC/RAM analysis showed the sample to have chemical and radiochemical purities exceeding 98%.

Table 6

Time (min)	0	3	10	11	22	23	33	37	42	45	46
%A	10	10	10	10	10	20	50	50	60	80	10
%B	0	0	0	90	90	80	50	50	40	20	0
%C	90	90	90	0	0	0	0	0	0	0	90
Flow (mL/min)	0.5	1	1	1	1	1	1.2	1.3	1.5	1.5	1.5
Curve	*	9	6	6	6	6	6	6	6	6	6

A: MeCN B: water C: 1% (v/v) HOAc in water

The total activity recovered was 37.5 mCi (16% chemical yield), while the specific activity was determined to be 11.2 Ci / mmol. The proton decoupled tritium NMR spectrum (D_2O) showed a single resonance at 7.59 ppm (DuPont/NEN Research Products).

ACKNOWLEDGEMENTS

The authors are grateful to Dr. Tom Williams for acquisition and interpretation of ^1H and 2D NMR experiments leading to the assignment of structures to **2/3**. We also thank Diane Gauthier and Xinzhen Xiang for NMR and mass spectral analysis work, respectively. Finally, we thank Amersham Corporation of Arlington Heights, IL for tritiation work and Dr. P.R. Srinivasan of DuPont/NEN Research Products, Boston, MA for acquisition of the proton decoupled tritium NMR spectrum of **4**.

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10. NMR experiments (Varian XL-400; probe temperature = 30°C) were conducted on a solution containing 5 mg of **2/3** (acetate salt) in 1 mL of D_2O acidified to pH 2.0 with DCl.

11. HPLC analysis was performed 15 min after addition of the trifluoroacetyl hypoiodite solution by diluting 20 μL of the reaction stream into 1 mL mobile phase (34:66:0.15 (v/v/v) tetrahydrofuran - water - trifluoroacetic acid) and injecting 30-40 μL of the resulting solution onto the column.

12. NMR experiments (Bruker AM-400; probe temperature = 32°C) were conducted on a solution containing 1 mg **2** (acetate salt) dissolved into 0.75 mL of pH 2.0 D_2O/DCl.

13. The solution was not permitted to concentrate to dryness during this operation.