Target-Directed Self-Assembly of Homodimeric Drugs Against β-Tryptase

Sarah F. Giardina[†,*], Douglas S. Werner[‡,§], Maneesh Pingle[†,‡,§], Kenneth W. Foreman[‡,‖], Donald E. Bergstrom[⊥], Lee D. Arnold[‡,∇] and Francis Barany[†]

† Department of Microbiology and Immunology, Weill Cornell Medicine, 1300 York Ave, Box 62. New York, NY, 10065

‡ Coferon, Inc., 25 Health Sciences Drive, Mailbox 123, Stony Brook, NY, 11790.

§ Present Address: BlinkBio, Inc., The Scripps Research Institute, 130 Scripps Way #2B2, Jupiter, FL, 33458.

⊥ Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, 575 Stadium Mall, West Lafayette, IN, 47907.

‖ Present Address: Ars Exemplar, LLC, Syosset, NY.

∇ Present Address: Fount Therapeutics, LLC, Wilmington, New Castle, DE, 19801.

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Materials and Methods

Determination of IC_{50}s with Recombinant Human β-Tryptase. Stock solutions of recombinant human β-tryptase from lung (Promega) were diluted to 30 µM, with 50 µM heparin sulfate and 1 M NaCl. Tryptase inhibitors stock solutions were prepared at 50mM in DMSO. Drug plates were prepared at 1.2 X the final concentration in assay buffer (50 mM HEPES, 150mM NaCl, 100 µM EDTA, pH 7.4, 0.02% Tween-20) [1]. Final concentrations of tryptase ranged from 10pM to 1nM. After 3h at RT the coferon-tryptase solution was diluted into assay buffer containing a final concentration of 200µM (2x Km) N-tert-butoxycarbonyl-Gln-Ala-Arg-AMC (Enzo Life Sciences). The release of AMC was immediately measured every 30 seconds for 15 minutes at Ex.: 367nm, Em.: 468nm on a Spectramax M5 (Molecular Devices) microplate reader. The Softmax Pro (Molecular Devices) and GraphPad Prism 6 software were used to determine VMax and IC_{50}s, respectively.

Cell lines. HMC1 cells were a kind gift from Dr. J. H. Butterfield (Mayo Clinic, Rochester, MN [2]). Cultures of HMC1 cells were grown at 37°C, 5% CO_2, in Iscove's modified Dulbecco's medium (IMDM; Life Technologies), supplemented with 36mM sodium bicarbonate, 1.2mM monothioglycerol (Sigma-Aldrich), 10% Normal Calf Serum (NCS; Life Technologies), 100 U/mL penicillin and 100 µg/mL streptomycin.

Inhibition of Cellular Tryptase Activity assessed through assay of Lysates or Degranulation. HMC1 cells were plated on gelatin-coated 96 well plates in IMDM containing 2% NCS. Cells were treated with inhibitors (10nM-100µM) in 100µL for 2h and subsequently washed in PBS. Degranulation was induced with 1 µM of the calcium ionophore, A23187 in PBS. After 1h the supernatant was assayed for tryptase activity as described above. Alternatively, inhibitor-treated cells were lysed in buffer (50mM HEPES, 150mM NaCl, pH 7.4, 0.1% TX-100, with 1mM EDTA, 20µg/ml heparin, 0.5mg/ml soybean trypsin inhibitor, 100nM aprotinin, 0.5 µg /ml pepstatin, and 100 µM N-Ethylmaleimide) and assayed for tryptase activity.

Tryptase Reversibility studies. Bound inhibitors (10 µM) to tryptase (100nM) were separated from unbound using 7 kD cut-off Zeba desalting columns (Pierce), which had been equilibrated with 1M NaCl immediately prior to use. The subsequent eluant was diluted 1:100 in assay buffer and monitored over 216h for tryptase activity as described above. Tryptase activity in controls was consistent with ≥ 90% recovery of the enzyme.

Crystallography. The protein complexes with inhibitors were formed by the addition of 1mM compound to recombinant human β-Tryptase (1.95mg/ml, Promega). The protein-compound mixtures were incubated on ice for 30 minutes and spun down to remove the precipitate. The co-crystallizations were setup using the vapor diffusion method, in brief equal volumes of the protein complex and a reservoir solution (30% PEG 1500, 100mM sodium acetate pH 4.6 and 200mM ammonium sulfate) were mixed and subsequently incubated at 25°C. Monocrystals grew to usable size in 3-5 days. Once formed, a monocrystal was soaked for 20 hours in a solution containing 30% PEG 1500, 200mM ammonium sulfate, 100mM MES pH 5.5 and 1mM compound and flash-frozen in liquid nitrogen until the x-ray diffraction.

Synthesis of compounds:

General procedures. Preparative purification of the compounds were performed on Shimadzu preparative HPLC system composed of the following: CBM-20A system controller, LC-8A binary gradient pump, SPD-M20A photodiode array detector, FRC-10A fraction collector, YMC ODS A 500 X 30mm X 10µm preparative column using 0.05% (v/v) trifluoroacetic acid (TFA) in HPLC grade water (A) and 0.05% (v/v) TFA in HPLC grade acetonitrile (B) at a flow rate of 30mL/min and a run time of 40min. For basic medium purification, the same instrument was utilized with YMC triart C18, 500 X 30mm X 10µm preparative column using 10mM ammonium formate and 0.1% (v/v) ammonia in HPLC grade water (A) and HPLC grade acetonitrile adding 5% (v/v) of mobile phase (A) and 0.1% (v/v) ammonia (B). For both the methods, linear gradient profiles were used depending upon the chromatographic retention and separation of different compounds.

LCMS data was collected on Shimadzu LCMS system equipped with CBM-20A system controller, LC-20AD binary gradient pump, SPD-M20A photodiode array detector, SIL-20AC autosampler, CTO-20AC column oven, LCMS-2010EV single

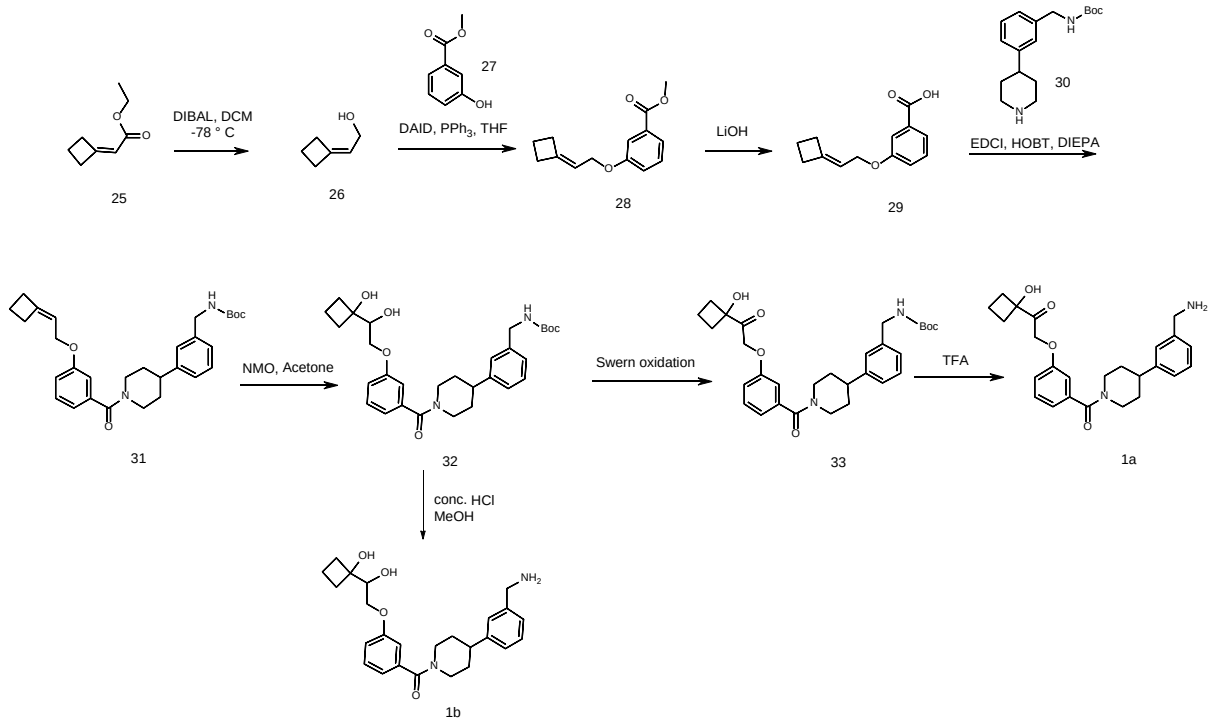
quadrapole mass spectrometer, YMC ODS A 50 X 4.6mm X 3.0µm column using 0.05% (v/v) TFA in HPLC grade water (A) and 0.05% (v/v) TFA in HPLC grade acetonitrile (B) at a flow rate of 1.2mL/min and a run time of 5.0min. The gradient profiles are 20% B to 100% B in 3 minutes, Hold for 0.5min, at 3.51min 20% B, Hold until 5.0min. Maxplot Conditions: wavelength range is 210-400nm.

A) Ammonium Formate & Formic Acid Method for LC-MS. Phase A: 10mM Ammonium Formate in Water + 0.1% Formic Acid. Phase B: acetonitrile + 5% Phase A+ 0.1% Formic Acid. **B) TFA Method for HPLC**. Phase A: 0.05% TFA in water Phase B: 0.05% TFA in Acetonitrile.

All Shimadzu LCMS-2010EV instruments utilized electrospray ionization in positive (ES+) or negative (ES-) ionization mode. The Shimadzu LCMS-2010EV instruments can also be utilized with atmospheric pressure chemical ionization in positive (AP+) or negative (AP-) ionization mode. High resolution mass spec data was obtained using a Waters Synapt G2 (HRMS); Conditions: ESI+ve Mode; Analyzer: TOF.

Nuclear Magnetic Resonance (NMR) spectra were recorded on a Varian spectrometer at 400MHz for proton (^{1}H NMR) and 125MHz for carbon (^{13}C NMR); chemical shifts are reported in ppm (δ) relative to residual protons in deuterated solvent peaks.

Supplementary Scheme 1. Synthesis of 1a and 1b



2-Cyclobutylideneethanol (26): A solution of ethyl 2-cyclobutylideneacetate (0.85g, 6.07mmol) in anhydrous dichloromethane (DCM) (40mL) was cool to -78°C under a N_2 atmosphere. DIBAL-H (1M in toluene) (12.1mL, 12.1mmol) was added dropwise and the solution monitored until starting material was consumed. The reaction mixture was quenched with MeOH/H_2O (1:1) and the DCM layer was separated and dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. Purification by chromatography on silica gel (60-120 mesh) [eluting with 0→20% ethyl acetate (EtOAc) in *n*-hexane] afforded 0.5g, 84% yield of compound **26** as a colorless oil. ^{1}H NMR (400MHz, $CDCl_3$): δ=1.61 (br,1H), 1.91-2.05 (m, 2H), 2.65-2.74 (m, 4H), 4.02 (d, *J*=7.20Hz, 2H), 5.30-5.36 (m, 1H).

Methyl 3-(2-cyclobutylideneethoxy)benzoate (28): A solution of triphenylphosphine (0.56g, 2.25mmol) in anhydrous THF (10mL) was cooled to -20°C and charged with DIAD (0.44 mL, 2.25 mmol). After addition a yellow precipitate was observed in the reaction mixture. Methyl 3-hydroxybenzoate (**27**) (0.26 g, 1.73 mmol) in THF (3 mL) was added dropwise and the

reaction mixture stirred for 10-15min. A solution of compound of **26** (0.17 g, 1.73 mmol) in anhydrous THF (3mL) was added dropwise and the reaction mixture stirred at RT overnight. The reaction was quenched with water (5mL) and the aqueous layer was extracted with diethyl ether (3 x 20 mL) and the combined organic layers were dried over Na_2SO_4, filtered, and concentrated *in vacuo* resulting in crude material which was purified by chromatography on silica gel (60-120 mesh) [eluting with 20% EtOAc in *n*-hexane] resulting in 0.2g, 50% yield of compound **28** as a light yellow oil. ^{1}H NMR (400MHz, $CDCl_3$): δ=1.95-2.06 (m, 2H), 2.70-2.81 (m, 4H), 3.91 (s, 3H), 4.44 (d, *J*=7.20Hz, 2H), 5.38-5.46 (m, 1H), 7.06-7.14 (dd, *J*=2.40, 8.40Hz, 1H), 7.32 (t, *J*=8.0Hz, 1H), 7.57 (t, *J*=2.40Hz, 1H), 7.62 (d, *J*=7.60Hz, 1H); MS (ES+): m/z=233.20 [M+H]+; LCMS calcd. for $C_{14}H_{16}O_3$: 232.28, (M+1) found 232.11; HPLC: t_R=3.22 min.

3-(2-Cyclobutylideneethoxy) benzoic acid (29): A solution of **28** (0.20g, 0.86mmol) in 1:1 THF/water (10mL) was charged with lithium hydroxide monohydrate (0.10g, 2.58mmol) and stirred at RT for 2h. Additional lithium hydroxide monohydrate (0.1g, 2.58mmol) was added and the mixture stirred for 2h. The solvent was concentrated *in vacuo* and the aqueous layer was acidified with citric acid and extracted with EtOAc (3 x 15mL). The combined organic layers were dried over anhydrous Na_2SO_4, filtered, and concentrated *in vacuo*. The crude product was purified by chromatography on silica gel (60-120 mesh) [eluting with 50% EtOAc in *n*-hexane] resulting in 0.14g, 77% yield of compound **29** as a colorless oil. ^{1}H NMR (400MHz, $CDCl_3$): δ=1.96-2.07 (m, 2H), 2.72-2.82 (m, 4H), 4.46 (d, *J*=6.8Hz, 2H), 5.38-5.47 (m, 1H), 7.12-7.18 (dd, *J*=2.4, 8.0Hz, 1H), 7.37 (t, *J*=8.0Hz, 1H), 7.62 (s, 1H), 7.70 (d, *J*=7.6Hz, 1H).

***tert*-Butyl *N*-[[3-[1-[3-(2-cyclobutylideneethoxy)benzoyl]-4-piperidyl]phenyl] methyl]carbamate (31):** A solution of **29** (0.14g, 0.64mmol) in anhydrous DCM (10mL) was charged with **30** (0.18g, 0.64mmol), EDCI (0.14g, 0.70mmol), HOB*t* (0.17g, 1.28mmol) and DIPEA (0.27mL, 1.6mmol) and stirred at RT for 15h under N_2 atmosphere. The reaction mixture was washed with sat. $NaHCO_3$ (20mL) and the organic layer was separated, dried over anhyd. Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by chromatography on silica gel (60-120 mesh) [eluting with 0→40% EtOAc in hexanes] resulting in 0.230g, 73% yield of compound **31** as a colorless oil. ^{1}H NMR (400MHz, $CDCl_3$): δ=1.46 (s, 11H), 1.95-2.0 (m, 2H), 2.71-2.84 (m, 7H), 3.09 (br, 1H), 3.91 (br, 1H), 4.30 (br, 2H), 4.41 (d, *J*=6.8Hz, 2H), 4.82 (br, 2H), 5.40-5.45 (m, 1H), 6.90-7.00 (m, 3H), 7.10-7.20 (m, 3H), 7.26-7.33 (m, 2H); MS (ES+): m/z=491.20 [M+H]+; LCMS calcd. for $C_{30}H_{38}N_2O_4$: 490.64, (M+1) observed 491; HPLC: t_R=3.28min.

***tert*-Butyl *N*-[[3-[1-[3-[2-hydroxy-2-(1-hydroxycyclobutyl)ethoxy]benzoyl]-4-piperidyl]phenyl]methyl]-carbamate (32):** A solution of **31** (0.23g, 0.47mmol) in acetone (7mL) and H_2O (1.5mL) was charged with 4% solution of OsO_4 (0.012mL, 0.0185mmol) and stirred at RT for 10min then charged with a 50% aqueous solution of NMO (0.13mL, 0.56mmol) and stirred at RT for an additional 15h. The reaction mixture was quenched with 10% aqueous sodium bisulphite and stirred for 1h at RT, then the aqueous layer was extracted with EtOAc (3 x 20mL). The combined organic layers were dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by chromatography on silica gel (60-120 mesh) [eluting with 50% EtOAc in *n*-hexane] to resulting in 0.18g, 73% yield of **32** as a colorless oil. ^{1}H NMR (400MHz, $CDCl_3$): δ=1.47 (s, 9H), 1.61-1.76 (m, 4H), 2.05–2.16 (m, 4H), 2.35-2.40 (m, 1H), 2.70-2.90 (m, 4H), 3.11 (br, 1H), 3.86 (br, 1H), 4.05-4.20 (m, 3H), 4.30 (br, 2H), 4.85 (s, 2H), 6.93-7.06 (m, 3H), 7.11-7.17 (m, 3H), 7.26-7.35 (m, 2H); MS (ES+): m/z=525.10 [M+H]+; LCMS calcd. for $C_{30}H_{40}N_2O_6$: 524.66, (M+1) found 525; HPLC: t_R=2.47min.

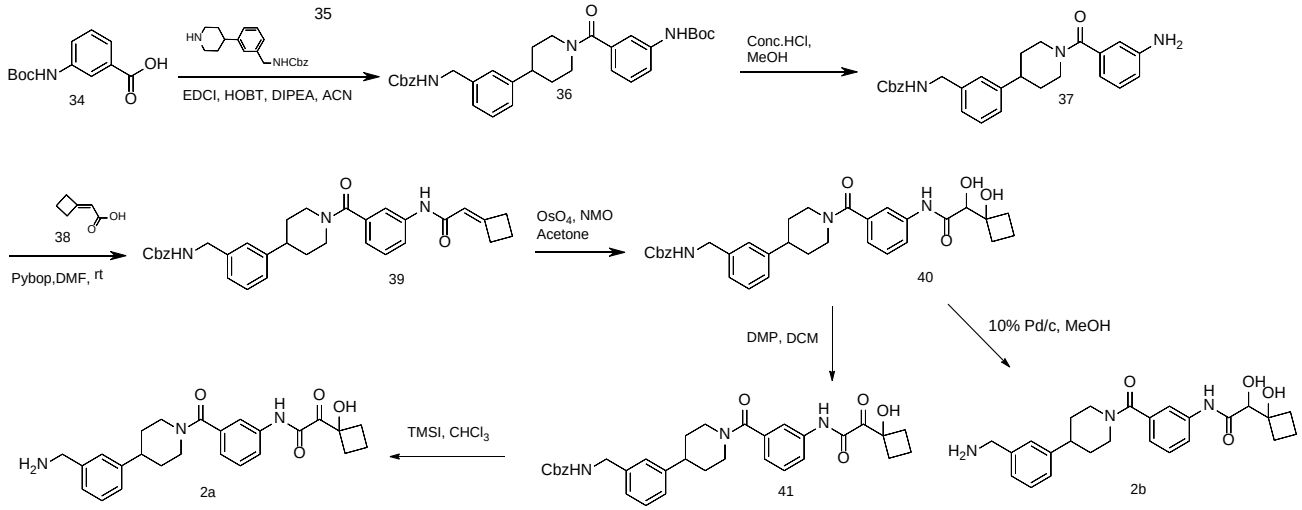
***tert*-Butyl *N*-[[3-[1-[3-[2-(1-hydroxycyclobutyl)-2-oxo-ethoxy]benzoyl]-4-piperidyl] phenyl] methyl]carbamate (33):** A solution of DMSO (0.028mL, 0.4mmol) in DCM (5mL) was cooled to –78°C then dropwise charged with oxalyl chloride (0.032mL, 0.38mmol) in DCM (1mL). Compound **32** (0.10g, 0.19mmol) in DCM (2mL) was added and reaction mixture stirred at -78°C for 1h under a N_2 atmosphere. Triethylamine (0.2mL, 1.52mmol) was added and reaction mixture was allowed to warm to RT and stirred for 15h. The reaction mixture was quenched with sat. NH_4Cl and the aqueous layer was extracted with DCM (3 x 2mL), dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo.* The crude product was

purified by chromatography on silica gel (60-120 mesh) [eluting with 10% EtOAc in *n*-hexane] resulting in 0.028g, 28% yield of **33** as a colorless oil. ^{1}H NMR (400MHz, $CDCl_3$): δ=1.47 (s, 9H), 1.60-1.80 (br, 4H), 1.90-2.10 (m, 4H), 2.30-2.50 (m, 2H), 2.70-3.20 (m, 4H), 3.85 (br, 1H), 4.03 (s, 2H), 4.30 (d, *J*=4.8Hz, 2H), 4.84 (br, 1H), 6.90-7.04 (m, 3H), 7.10-7.20 (m, 3H), 7.28-7.34 (m, 2H); MS (ES+): m/z=545.13 [M+Na]+; LCMS calcd. for $C_{25}H_{30}N_2O_4$: 522.64, m/z (M+1) observed 525; HPLC: t_R=2.58min.

2-[3-[4-[3-(Aminomethyl)phenyl]piperidine-1-carbonyl]phenoxy]-1-(1-hydroxycyclobutyl) ethanone (1a): A solution of **33** (0.02g, 0.038mmol) in TFA:H_2O (9:1)(3mL:0.3mL) was allowed to stir at RT for 2h. The reaction mixture was concentrated *in vacuo* and purified by preparative HPLC resulting in 8.31mg, 41.5% yield of **1a** as a TFA salt. ^{1}H NMR (400MHz, CD_3OD): δ=1.60-1.90 (m, 3H), 1.90-2.10 (m, 4H), 2.25-2.50 (m, 3H), 2.85-3.00 (m, 2H), 3.20-3.30 (m, 1H), 3.84 (br, 1H), 3.95-4.15 (m, 4H), 4.80 (br, 1H), 6.90-7.10 (m, 3H), 7.22-7.44 (m, 5H); MS (ES+): *m/z*=423.10 $[M+H]^+$; LCMS calcd. for $C_{25}H_{30}N_2O_4$: 422.22, (M+1) found 422.52; HPLC: t_R=1.60min.

(4-(3-(Aminomethyl)phenyl)piperidin-1-yl)(3-(2-hydroxy-2-(1-hydroxycyclobutyl)ethoxy)phenyl) methanone (1b): A solution of **32** (0.01g, 0.019mmol) in MeOH (2mL) was charged with conc. HCl (0.1mL) and stirred at RT for 5h. The reaction was charged again with conc. HCl (0.1mL) and stirred overnight. The reaction mixture was concentrated in vacuo and the reaction mixture was washed with diethyl ether then washed with pentane. The crude reaction mixture was purified by preparatory HPLC giving 6.5mg of compound **1b** in 74% yield. ^{1}H NMR (400MHz, CD_3OD): δ=1.60-1.71 (m, 2H), 1.72-2.10 (m, 9H), 2.23-2.46 (m, 2H), 2.83-3.31 (m, 2H), 3.81-4.10 (m, 4H), 4.15-4.21 (m, ^{1}H), 4.60 (brs, 2H), 6.90-7.10 (m, 3H), 7.22-7.44 (m, 5H); MS (ES+): *m/z*=425.15 $[M+H]^+$, 447.20 $[M+Na]^+$; LCMS calcd. for $C_{25}H_{32}N_2O_4$: 424.24, (M+1) observed 424.53; HPLC: t_R=1.56min.

Supplementary Scheme 2. Synthesis of 2a and 2b:



Benzyl N-[[3-(1-[3-[(tert-butoxycarbonyl)amino]benzoyl]piperidin-4-yl)phenyl]methyl]carbamate (36): A solution of 3-((*tert*-butoxycarbonyl)amino)benzoic acid (2.19g, 9.24mmol) (commercial sources) in MeCN (30mL) was charged with benzyl *N*-[[3-(4-piperidyl)phenyl]methyl]carbamate **35** (3g, 9.24mmol), EDCI (1.9g, 10.2mmol), HOB*t* (2.5g, 18.5mmol), and DIPEA (4mL, 23.1mmol) and stirred at RT for 15h. The solvent was concentrated *in vacuo* then partitioned between DCM (50mL) and H_2O (20mL) and separated. The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated *in vacuo*. The resulting crude material was purified by chromatography on silica gel (100-200 mesh) [eluting with 0→80% EtOAc in hexanes] resulting in 1.6 g, 32% yield of compound **36** as a brown semi solid. ^{1}H NMR (400MHz, $CDCl_3$) : δ=1.51 (s, 9H), 1.60-2.00 (br, 4H), 2.70-2.80 (m,. 1H), 2.90-3.30 (br, 2H), 3.80-4.00 (br, 1H), 4.38 (d, *J*= 5.6Hz, 2H), 4.84 (br, 1H), 5.14 (s, 2H), 6.64 (s, 1H), 7.00-7.50 (m, 12H). MS (ES+): *m/z*=566.20 $[M+Na]^+$; LCMS calcd. for $C_{32}H_{37}N_3O_5$: 543.66, (M+Na) observed 566; HPLC: t_R=3.12 min.

Benzyl 3-(1-(3-aminobenzoyl) piperidin-4-yl) benzylcarbamate (37): A solution of compound **36** (1.6g, 2.94mmol) in 16mL of methanol and 6.4mL of conc. HCl was allowed to stir at RT for 16h. The solvent was removed under reduced pressure and water (20mL) was added to the reaction mixture followed by basified to pH 9-10 with 2 N NaOH solution. The aqueous layer was extracted with EtOAc (3 x 30mL). The combined organic fractions were washed with water (20mL), brine (10mL) and dried over anhydrous Na_2SO_4. The organic layer was filtered, concentrated and evaporated under reduced pressure to isolate crude compound. The compound was purified by column chromatography on silica gel (60-120 mesh) eluting with 0→80% EtOAc in *n*-hexane giving 1.10g of **37** in 84% yield as white solid. ^{1}H NMR (400MHz, $CDCl_3$): δ 1.70-2.00 (br, 4H), 2.70-2.81 (m, 1H), 2.82-3.20 (br, 1H), 3.92 (br, 1H), 4.38 (d, *J*=5.6Hz, 2H), 4.70-5.10 (br, 2H), 5.15 (s, 2H), 6.70-6.82 (m, 3H), 7.00-7.50 (m, 11H); MS (ES+): *m/z*=466.60 $[M+Na]^+$; LCMS calcd. for $C_{27}H_{29}N_3O_3$: 443.55, (M+23) found 466; HPLC: t_R=2.03min.

2-Cyclobutylidene-acetic acid (38): A solution of ethyl 2-cyclobutylideneacetate (1.2g, 8.57mmol) in THF/water/MeOH (10:10:5mL each) was charged with lithium hydroxide monohydrate (2.15g, 51.4mmol) and stirred at RT for 16h. The solvent was concentrated *in vacuo* and the aqueous layer was acidified with citric acid and extracted with EtOAc (3 x 20mL). The crude product was purified by chromatography on silica gel (60-120 mesh) [eluting with 30% EtOAc in *n*-hexane] to afford 0.6g (62% yield) of compound **38** as a white solid. ^{1}H NMR (400MHz, $CDCl_3$): δ=2.02-2.20 (m, 2H). 2.86 (t, *J*=7.8Hz, 2H), 3.14 (t, *J*=7.8Hz, 2H), 5.59 (t, *J*=2Hz, 1H); HPLC: t_R=1.61min.

Benzyl 3-(1-(3-(2-cyclobutylideneacetamido)benzoyl) piperidin-4-yl) benzylcarbamate (39): A solution of compound **38** (0.27g, 2.41mmol) in DMF (8mL) was charged with 3-(1-(3-aminobenzoyl) piperidin-4-yl) benzylcarbamate **37** (1.09g, 2.41mmol), PyBOP (2.5g, 4.82mmol) and DIPEA (1.1mL, 6.02mmol) and stirred at RT for 15h under N_2 atmosphere. The reaction mixture was quenched with water (5mL) and extracted with EtOAc (3 x 30mL) and the combined organic fractions were washed with water (10mL), brine (10mL), dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo* resulting in crude material. The crude compound was purified by chromatography on silica gel (100-200 mesh) [eluting with 0→80% EtOAc in hexanes] to obtain 0.84g (65% yield) of compound **39** as a white solid. ^{1}H NMR (400MHz, DMSO-d_6): δ=1.40-1.92 (br, 4H), 2.0-2.15 (m, 2H), 2.70-2.90 (m, 4H), 3.00-3.20 (m, 3H), 3.70 (br, 1H), 4.19 (d, *J*=6.0Hz, 2H), 4.62 (brs, 1H), 5.05 (s, 2H), 5.80 (s, 1H), 7.00-7.50 (m, 10 H), 7.61 (d, *J*=8.0Hz, 1H), 7.73 (s, 1H), 7.77-7.83 (m, 1H), 9.92 (s, 1H); MS (ES+): *m/z*=560.33 $[M+Na]^+$; LCMS calcd. for $C_{33}H_{35}N_3O_4$: 537.66, (M+Na) found 560; HPLC: t_R=2.93min.

Benzyl 3-(1-(3-(2-hydroxy-2-(1-hydroxycyclobutyl)acetamido)benzoyl)piperidin-4 yl) benzylcarbamate (40): A solution of **39** (0.012g, 0.022mmol) in acetone (2mL) and H_2O (0.3mL) was charged with OsO_4 (4% aqueous solution, 6 µL, 0.009mmol) and stirred for 10min at RT. Then NMO (50% aqueous solution, 6 µL, 0.026mmol) was added and allowed to stir at RT for another 15h. The reaction mixture was quenched with 10% aqueous sodium bisulphite solution and stirred for 1h at RT. The aqueous layer was extracted with EtOAc (3 x 20mL), dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by chromatography on silica gel (60-120 mesh) [eluting with 30% EtOAc in *n*-hexane] resulting in 0.011g, 91% yield of compound **40** as semi solid. ^{1}H NMR (400MHz, $CDCl_3$): δ=1.50-2.30 (m, 9H), 2.50-2.70 (br, 2H), 2.72-3.00 (br, 2H), 3.12 (br, 2H), 3.75-3.95 (m, 2H), 4.36 (d, *J*=5.6Hz, 2H), 4.84 (br, 1H), 5.14 (s, 2H), 7.00-7.50 (m, 12H), 7.72 (s, 1H), 8.86 (s, 1H); MS (ES+): *m/z*=594.56 $[M+Na]^+$; LCMS calcd. for $C_{33}H_{37}N_3O_6$: 574.67, (M+Na) found 594; HPLC: t_R=2.46min.

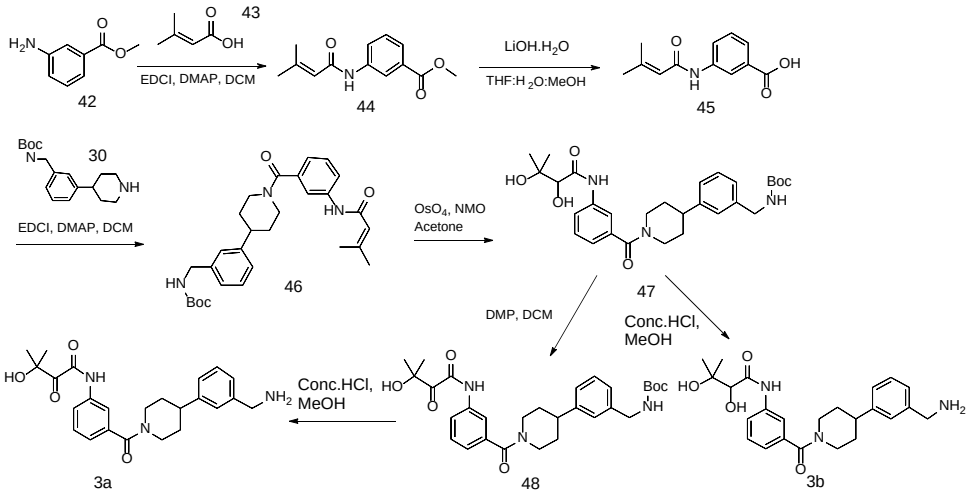
***N*-[3-[4-[3-(Aminomethyl)phenyl]piperidine-1-carbonyl]phenyl]-2-(1-hydroxycyclobutyl)-2-oxo-acetamide (41):** A solution of compound **40** (0.011g, 0.019mmol) in DCM (10mL) was charged with Dess Martin periodinane (0.24g, 0.57mmol) and stirred at RT for 2h under N_2 atmosphere. The reaction mixture was quenched with carbonate resin, filtered through cotton and the filtrate was concentrated *in vacuo*. The crude product was purified by preparative TLC [eluting with 100%

EtOAc] resulting in 0.01g (9% yield) of compound **41** as an oil. MS (ES+): *m/z*=570.20 $[M+H]^+$; LCMS calcd. for $C_{33}H_{35}N_3O_6$: 569.6, (M+1) found 569.3; HPLC: t_R=2.45min.

***N*-(3-(4-(3-(Aminomethyl)phenyl)piperidine-1-carbonyl) phenyl)-2-(1-hydroxycyclobutyl)-2-oxoacetamide (2a):** A solution of compound **41** (9mg, 0.016mmol) in $CHCl_3$ (5mL) was charged with TMSI (1 drop) at RT and stirred for 16h under N_2 atmosphere. An additional amount of TMSI was added (2 drops) and the reaction mixture was stirred at RT for an additional 6h. The reaction mixture was quenched with aq. 0.5 M ammonium formate solution (3mL), and organic layer was separated out. The aqueous layer was lyophilized and the compound further purified by prep. HPLC resulting in 4.7mg (47% yield) of compound **2a** as a TFA salt. ^{1}H NMR (400MHz, CD_3OD): δ=1.55 –2.00 (m, 4H), 2.02-2.20 (m, 2H), 2.35-2.65 (m, 3H), 2.80-3.20 (m, 2H), 3.10-3.30 (br, 3H, merged in the solvent peak), 3.87 (br, 1H), 4.10 (s, 2H), 7.18-7.50 (m, 6H), 7.62 (d, *J*=8.0Hz, 1H), 7.85 (s, 1H); MS (ES+): *m/z*=436.48 $[M+H]^+$; LCMS calcd. for $C_{25}H_{29}N_3O_4$: 435.52, (M+1) found 436; HPLC: t_R=1.58min.

***N*-(3-(4-(3-(Aminomethyl)phenyl)piperidine-1-carbonyl)phenyl)-2-hydroxy-2-(1-hydroxycyclobutyl) acetamide (2b):** A solution of compound **40** (0.011g, 0.019mmol) in MeOH (4mL) was charged with 10% Pd/C (0.016g) and stirred under hydrogen atmosphere at RT for 16h. The reaction mixture was filter through a pad of celite washed with MeOH and the filtrate was concentrated *in vacuo.* The crude was purified by preparative HPLC to give 4mg of **2b** as a colorless oil in 37% yield. ^{1}H NMR (400MHz, CD_3OD): δ=1.50-1.90 (m, 5H), 1.90-2.22 (m, 3H), 2.35-2.45 (m, 1H), 2.51-2.65 (m, 1H), 2.90-3.15 (m, 2H), 3.20-3.32 (m, 2H), 3.88 (br, 1H), 4.10 (s, 2H), 4.17 (s, 1H), 7.18 (d, *J*=7.6Hz, 1H), 7.26-7.50 (m, 5H), 7.59 (d, *J*= 8.4Hz, 1H), 7.90 (s, 1H); MS (ES+): *m/z*=460.30 $[M+Na]^+$; LCMS calcd. for $C_{25}H_{31}N_3O_4$: 437.53, observed (M+Na) 460.3.

Supplementary Scheme 3. Synthesis of 3a and 3b:



Methyl 3-(3-methylbut-2-enoylamino)benzoate (44): A solution of 3-methylbut-2-enoic acid (2.5g, 25mmol) in DCM (30mL) was charged with methyl 3-aminobenzoate (4.5g, 30mmol), EDCI (7.2g, 37.5mmol) and DMAP (1.5g, 12.5mmol) and stirred at RT for 15h under N_2 atmosphere. The reaction mixture was washed with water (20mL) and 2N HCl and the organic layer was separated, dried over anhydrous Na_2SO_4, filtered, and concentrated *in vacuo*. The crude product was washed with diethyl ether (3 x 10mL) resulting in 4.5g (77% yield) of compound **44** as an off-white solid. ^{1}H NMR (400MHz, $CDCl_3$): δ=1.89 (s, 3H), 2.23 (s, 3H), 3.90 (s, 3H), 5.73 (s, 1H), 7.31-7.50 (m, 2H), 7.75 (d, *J*=7.2Hz, 1H), 7.92 (br, 1H), 8.06 (s, 1H); MS (ES+): *m/z*=234.10 $[M+H]^+$; LCMS calcd. for $C_{13}H_{15}NO_3$: 233.27, m/z (M+1) found 234; HPLC: t_R=2.48min.

3-(3-Methylbut-2-enoylamino)benzoic acid (45): A solution of **44** (4.5g, 19.3mmol) in a mixture of THF (10mL), H_20 (10mL) MeOH (5mL) was charged with LiOH monohydrate (2.43g, 58mmol) and stirred at RT for 16h. The organic solvent was removed *in vacuo* and the aqueous was acidified with 10% citric acid solution to yield a white precipitate, which was collected by filtered, washed with hexanes (20mL) and dried under vacuum resulting in 4g (96% yield) of compound **45** as

a white solid. ^{1}H NMR (400MHz, DMSO-d_6): δ=1.86 (s, 3H), 2.15 (s, 3H), 5.86 (s, 1H), 7.40 (t, *J*=7.6Hz, 1H), 7.59 (d, *J*=7.6Hz, 1H), 7.82 (d, *J*=7.6Hz, 1H), 8.27 (s, 1H), 10.0 (s, 1H), 12.8 (br, 1H); MS (ES+): *m/z*=219.90 $[M+H]^+$; LCMS calcd. for $C_{12}H_{13}NO_3$: 219.24, (M+1) observed 219.9; HPLC: t_R=1.84min.

***tert*-Butyl *N*-[[3-[1-[3-(3-methylbut-2-enoylamino)benzoyl]-4-piperidyl]phenyl]methyl]carbamate (46):** A solution of **45** (0.03g, 0.136mmol) in DCM (2mL) was charged with *tert*-butyl 3-(piperidin-4-yl)benzyl carbamate (**30**) (0.047g, 0.16mmol), EDCI (0.039g, 0.2mmol) and DMAP (0.008g, 0.07mmol) and stirred at RT for 15h under N_2 atmosphere. The reaction mixture was washed with water (20mL), 2N HCl, and brine. The organic layer was separated dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo resulting in 0.027g (40% yield) of compound **46** which was used in the next step without further purification. ^{1}H NMR (400MHz, $CDCl_3$): δ=1.46 (s, 9H), 1.66-1.90 (br, 4H), 1.91 (s, 3H), 2.22 (s, 3H), 2.70-2.90 (m, 1H), 3.13 (br, 1H), 3.90 (br, 1H), 4.30 (d, *J*=4.8Hz, 2H), 4.87 (br, 2H), 5.74 (s, 1H), 7.00-7.40 (m, 7H), 7.50-7.70 (m, 3H); MS (ES+): m/z=514.40 $[M+Na]^+$; LCMS calcd. for $C_{29}H_{27}N_3O_4$: 491.63, (M+Na) found 514.4; HPLC: t_R=2.76min

***tert*-Butyl *N*-[[3-[1-[3-[(2,3-dihydroxy-3-methyl-butanoyl)amino]benzoyl]-4-piperidyl]phenyl]methyl]carbamate (47):** A solution of **46** (0.027g, 0.055mmol) in acetone (2mL) and H_2O (0.3mL) was charged with OsO_4 (4% aqueous solution, 0.013mL, 0.0022mmol) and stirred for 10min at RT followed by addition of NMO (50% aqueous solution, 0.015mL, 0.066mmol) and stirred at RT overnight. The reaction mixture was quenched with 10% aqueous sodium bisulphite and stirred for 1h at RT. The aqueous layer was extracted with EtOAc (3x15mL) and the combined organic layers were dried over anhydrous Na_2SO_4, filtered, and concentrated *in vacuo* resulting in crude compound. The crude product was purified by chromatography silica gel (100-200 mesh) [eluting with 60→80% EtOAc in *n*-hexane] resulting in 0.017g (60% yield) of compound **47** as an off-white solid. ^{1}H NMR (400MHz, CDCl3): δ=1.20 (s, 3H), 1.34 (s, 3H), 1.46 (s, 9H), 1.70-2.00 (br, 4H), 2.70-3.25 (br, 3H), 3.76 (s, 1H), 3.90 (br, 1H), 4.31 (s, 2H), 4.50-5.00 (br, 2H), 7.10-7.50 (m, 8H), 7.69 (bs, 1H), 8.90 (bs, 1H); MS (ES+): *m/z*=548.15 $[M+Na]^+$; LCMS calcd. for $C_{29}H_{39}N_3O_6$: 525.65, (M+Na) found 548.15; HPLC: t_R=2.4min.

***tert*-Butyl *N*-[[3-[1-[3-[(3-hydroxy-3-methyl-2-oxo-butanoyl)amino]benzoyl]-4-piperidyl]phenyl] methyl]carbamate (48):** A solution of **47** (0.01g, 0.019mmol) in DCM (5mL) was charged with Dess- Martin periodinane (0.24g, 0.57mmol) and stirred at RT for 2h under N_2 atmosphere. The reaction mixture was quenched with carbonate resin (1mmol eq), filtered through cotton and DCM filtrate was concentrated *in vacuo* resulting in crude compound which was further purified by preparative TLC [eluting with 100% EtOAc] resulting in 0.018g (18%) yield of compound **48** as an off-white solid. MS (ES+): *m/z*=546.10 $[M+Na]^+$; LCMS calcd. for $C_{29}H_{37}N_3O_6$: 523.6, (M+1) observed 523.3; HPLC: t_R=2.59min.

***N*-[3-[4-[3-(Aminomethyl)phenyl]piperidine-1-carbonyl]phenyl]-3-hydroxy-3-methyl-2-oxo-butanamide (3a):** A solution of **48** (0.01g, 0.019mmol) in MeOH (2.5mL) was charged with conc. HCl (0.12mL) and stirred at RT for 16h. The MeOH was evaporated *in vacuo* and the crude compound was purified by preparative HPLC resulting in 4.8mg (60% yield) of compound **3a** as TFA salt. ^{1}H NMR (400MHz, CD_3OD): δ=1.24 (s, 3H), 1.29 (s, 3H), 1.65-2.10 (br, 4H), 2.88-3.10 (m, 2H), 3.20-3.30 (br, 2H), 3.90 (br, 1H), 4.11 (s, 2H), 7.20-7.50 (m, 6H), 7.61 (d, *J* =8.0Hz, 1H), 7.90 (s, 1H); MS (ES+): m/z=446.10 $[M+MeOH]^+$; LCMS calcd. for $C_{24}H_{29}N_3O_4$: 423.5, (M+1) found 423.2; HPLC: t_R=1.44min.

***N*-(3-(4-(3-(Aminomethyl)phenyl)piperidine-1-carbonyl)phenyl)-2,3-dihydroxy-3-methylbutanamide (3b):**

A solution of **47** (0.03g, 0.057mmol) in MeOH (1.5mL) was charged with conc. HCl (0.36mL) and stirred at RT for 16h. The reaction mixture was concentrated *in vacuo* and purified by preparative HPLC (TFA salt) to give 12mg of **3b** in 48% yield. ^{1}H NMR (400MHz, CD_3OD): δ=1.28 (s, 6H), 1.70-2.01 (br, 4H), 2.85-3.00 (m, 2H), 3.20-3.40 (m, 1H), 3.66 (s, 1H), 3.88 (brs, 2H), 4.11 (s, 2H), 7.16-7.50 (m, 6H), 7.60 (d, *J*=8.0Hz, 1H), 7.90 (s, 1H); MS (ES+): m/z=426.05 $[M+H]^+$; LCMS calcd. for $C_{24}H_{31}N_3O_4$: 425.53, found 426 (M+1).

References

1. Levell, J., et al., *Structure based design of 4-(3-aminomethylphenyl)piperidinyl-1-amides: novel, potent, selective, and orally bioavailable inhibitors of beta II tryptase.* Bioorg Med Chem 2005. **13**(8): p. 2859-72.
2. Butterfield, J.H., et al., *Establishment of an immature mast cell line from a patient with mast cell leukemia.* Leuk. Res., 1988. **12**(4): p. 345-355.

Supplemental Figures

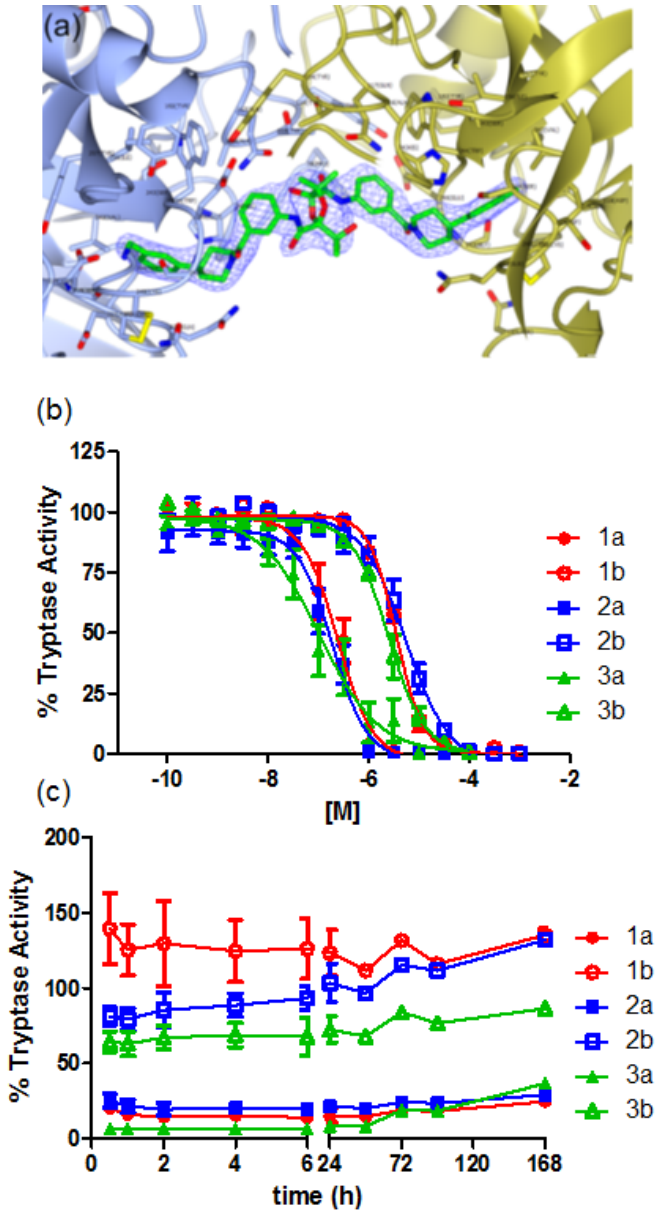


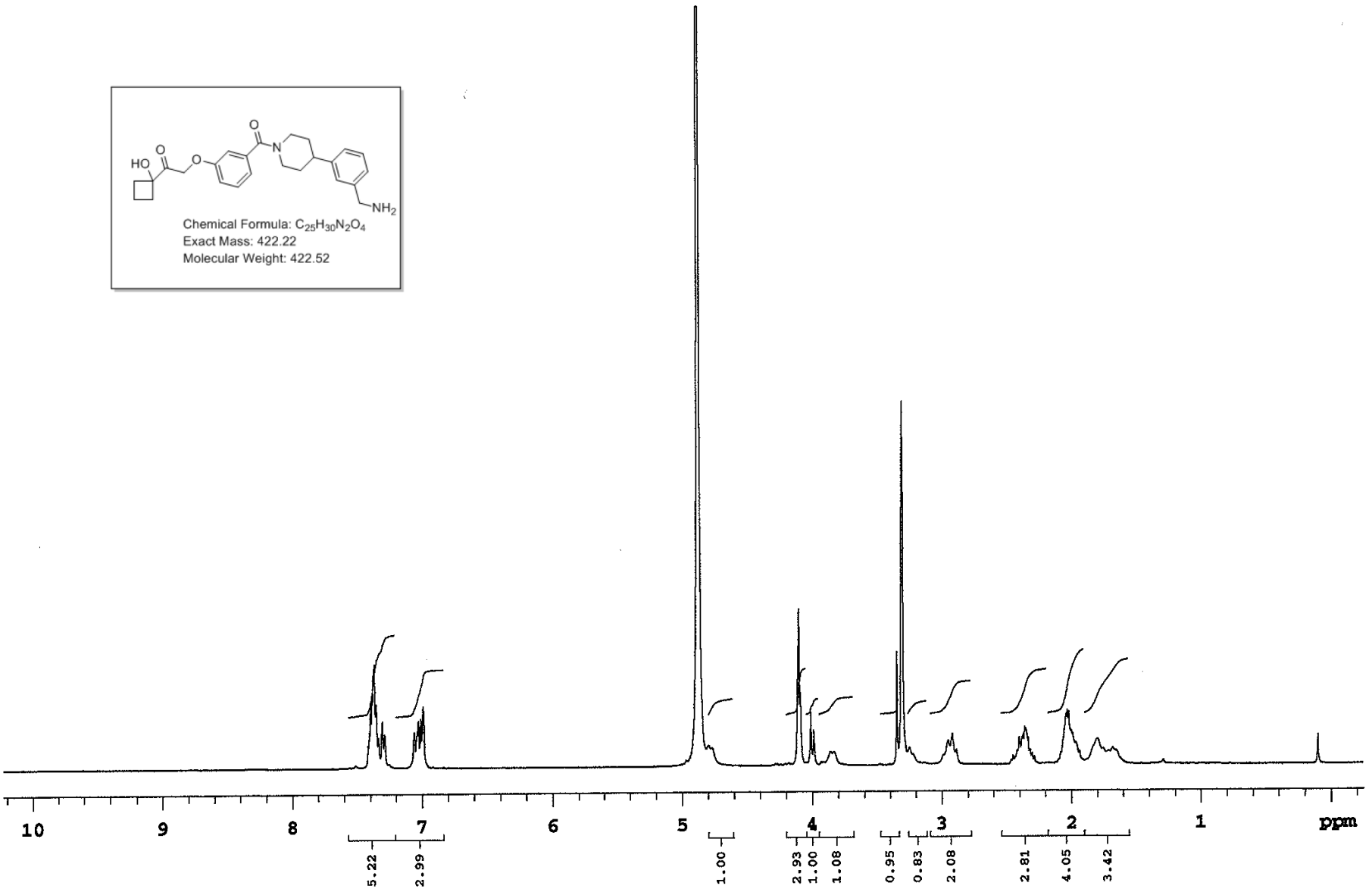
Figure S1. (a) $2F_o$-F_c electron density maps for Compound 3a bound to tryptase. The corresponding density map for Compound 2a is given in Fig. 2c. Compound densities are contoured at the 1σ level, illustrated in light blue mesh. Side-chains for tryptase within 7Å of the ligand are depicted explicitly. The separate tryptase monomer is colored tan and blue. Resolution information and other structural parameters for the crystal structures are given in Table S3. Images were generated with CCP4mg [S. McNicholas, E. Potterton, K. S. Wilson and M. E. M. Noble; Acta Cryst. D67: 386-394 (2011)]. **(b)** Dose response curves demonstrate significant leftward shift of the homodimerizing compounds relative to the non-dimerizable controls. IC_{50}s and fold-improvements are given in figure 2. **(c)** The high stability of tryptase's proteolytic activity at room temperature enabled reversibility studies of compounds to be conducted over an extended period of time. After the removal of excess unbound inhibitor from tryptase by a gel filtration spin-column we monitored the recovery of enzymic activity. Monomeric inhibitors were readily dissociated under these conditions to immediately restore full tryptase activity, while less than 25% of activity was recovered after 7 days with homodimeric compounds.

	Homodimeric Hydroxyketone Coferons	
	2a	**3a**
PDB ID	4MPU	4MPV
Cell dimensions *a*=*b*, *c* (Å)	78.157, 165.039	78.490, 165.481
Resolution (Å)[†]	50-1.65 (1.71-1.65)	30-2.30 (2.38-2.30)
R_{sym}[‡]	0.058 (0.66)	0.094 (0.64)
Average I/σI	25.7 (2.0)	20.3 (2.6)
Completeness (%)	99.7 (99.9)	99.9 (100)
Redundancy	3.0 (3.0)	4.8 (4.8)
Resolution (Å)	1.65	2.3
No. reflections[§]	67095	25193
R_{factor} , R_{free}	0.178 (0.216)	0.172 (0.230)
Protein atoms	3832	3832
Water molecules	515	154
Compound atoms	64	62
rmsd bond lengths (Å)	0.024	0.016
rmsd bond angles (°)	3.646	1.839

Table S1

X-ray data collection and refinement statistics. [†]The highest resolution shells are shown in parentheses. [‡]$R_{sym} = \Sigma |I_i - \langle I \rangle| / \Sigma I_i$ where I_i is the intensity of a measurement and <I> is the average intensity for that reflection. [§]Of these reflections, 5% are used for the R_{free} calculation.

^{1}H-NMR spectrum of compound 1a recorded on a Varian vnmrs400 spectrometer (400 MHz, CD_3OD)



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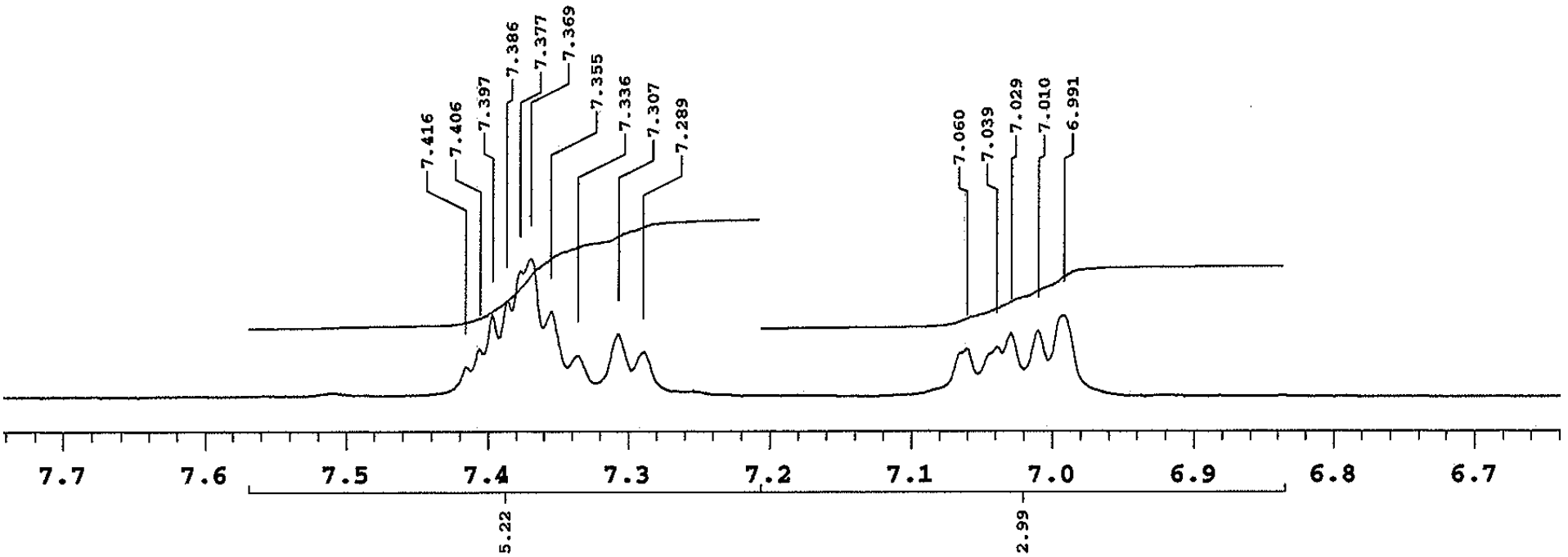

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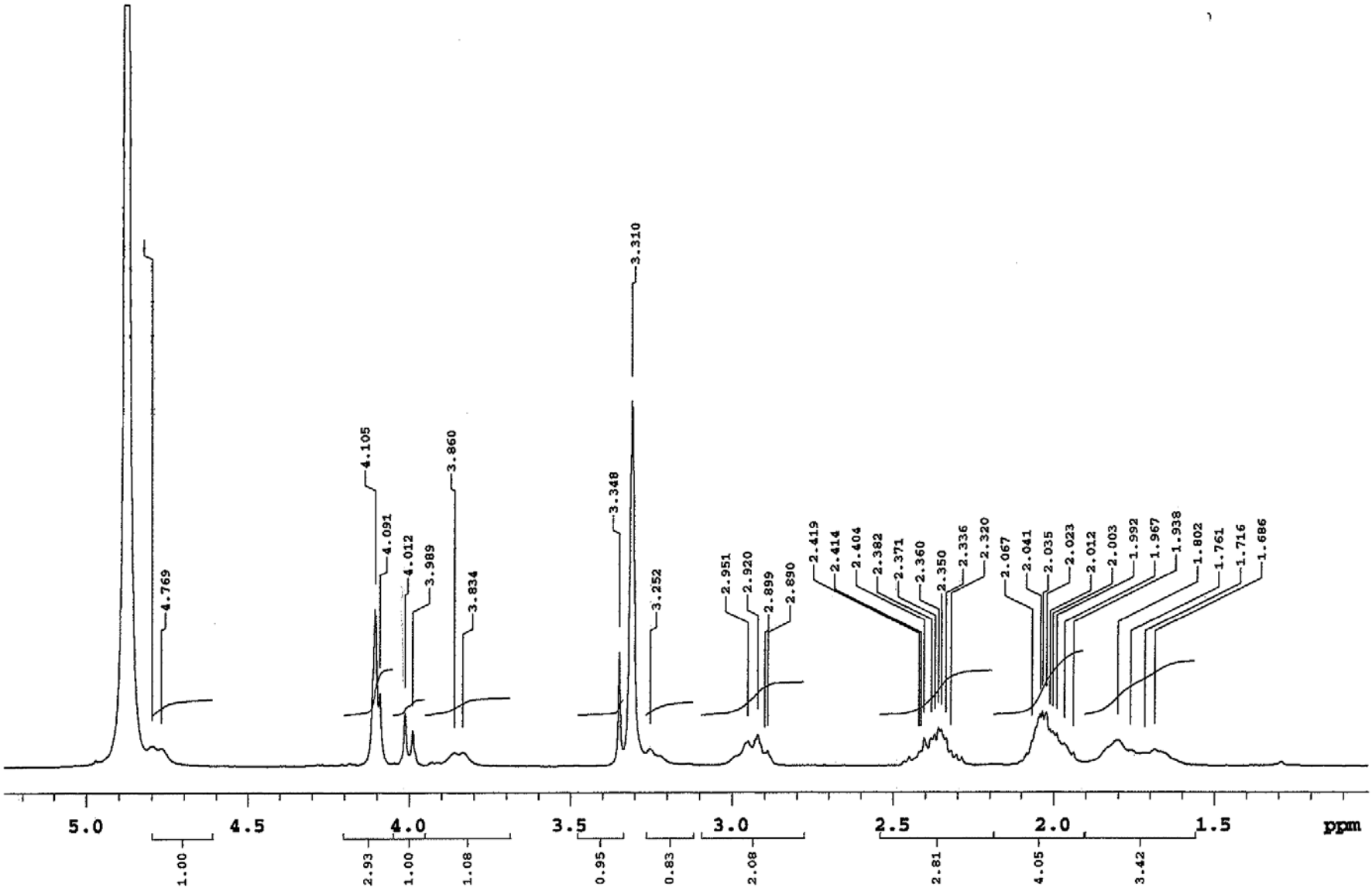

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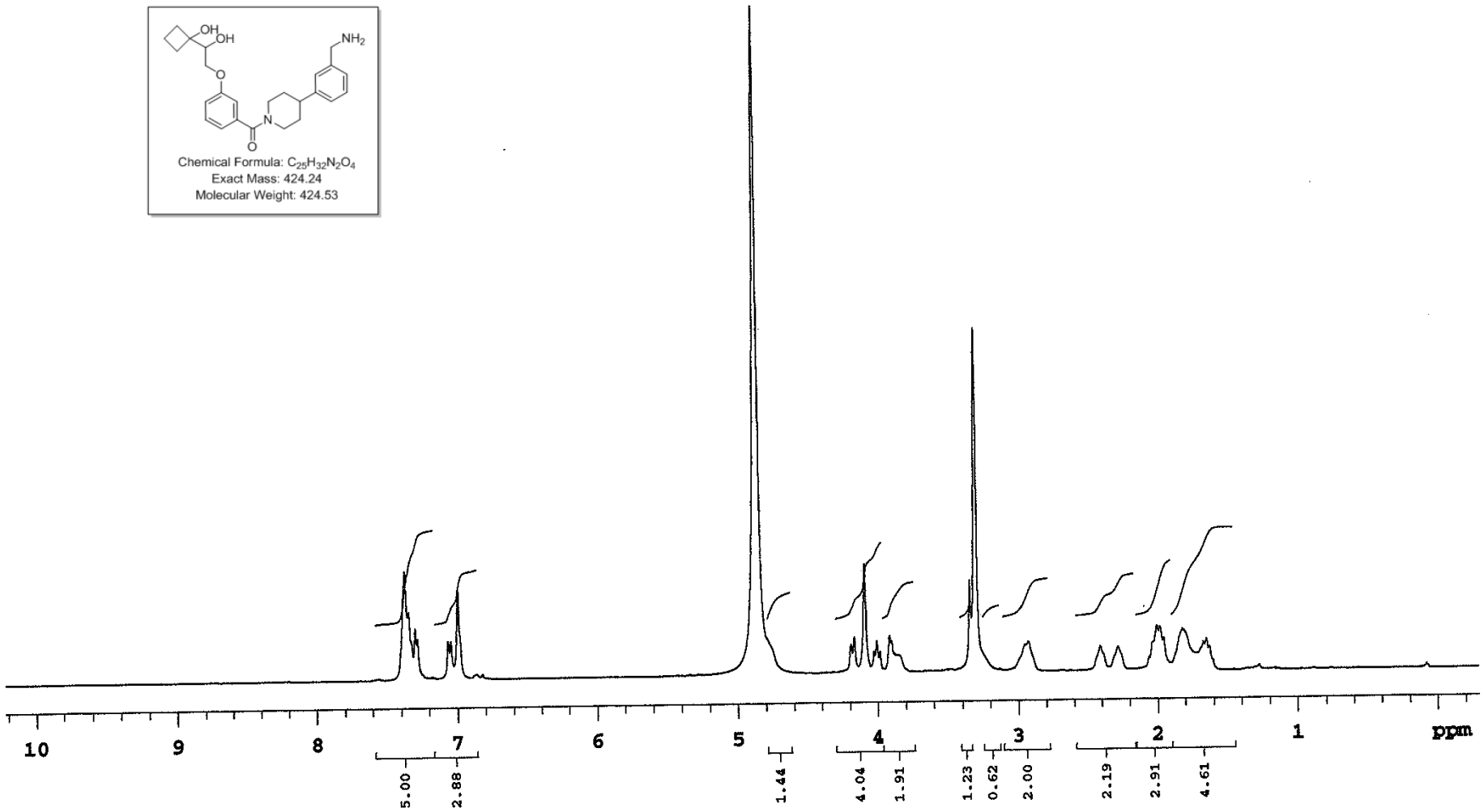

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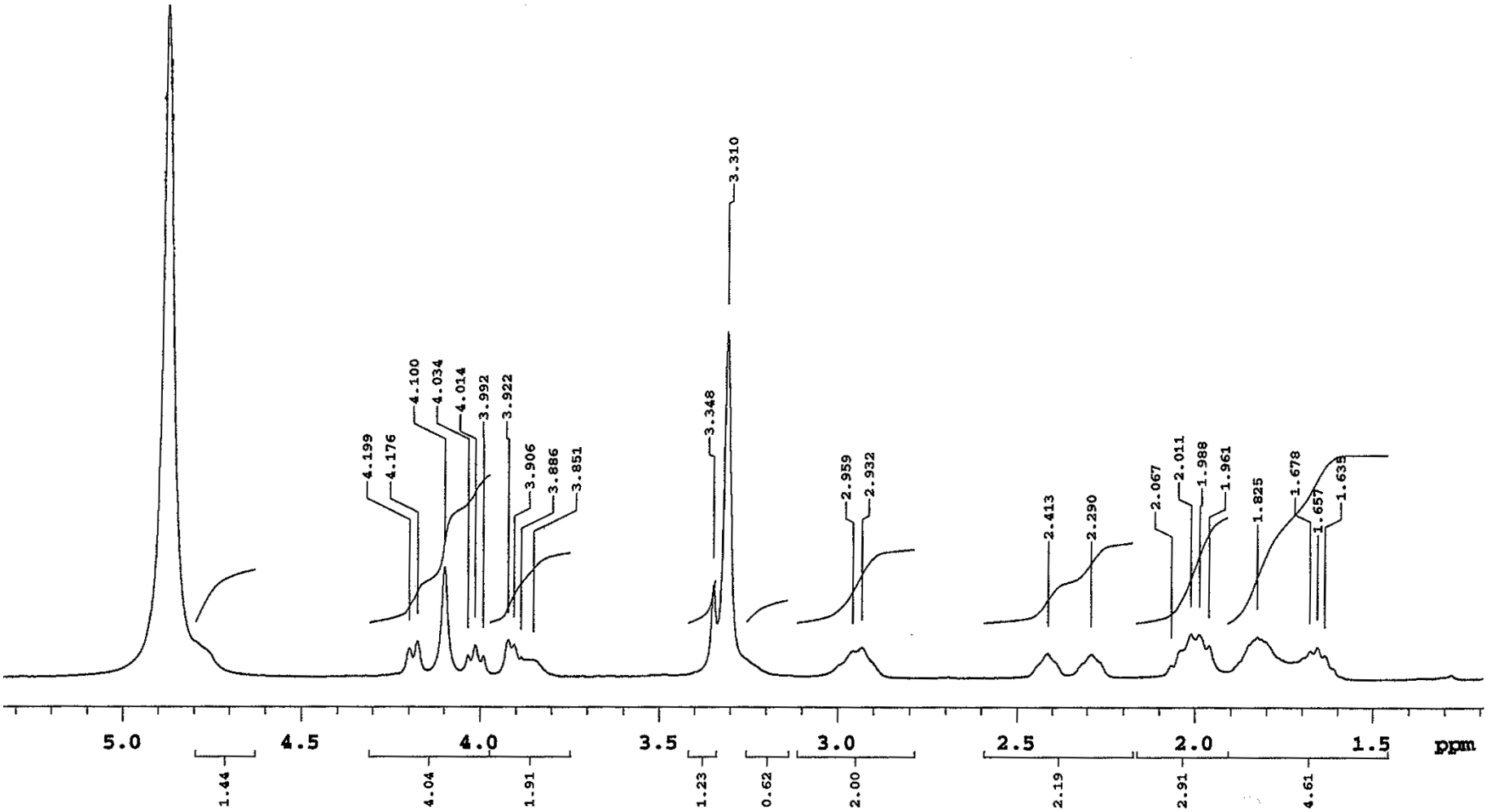

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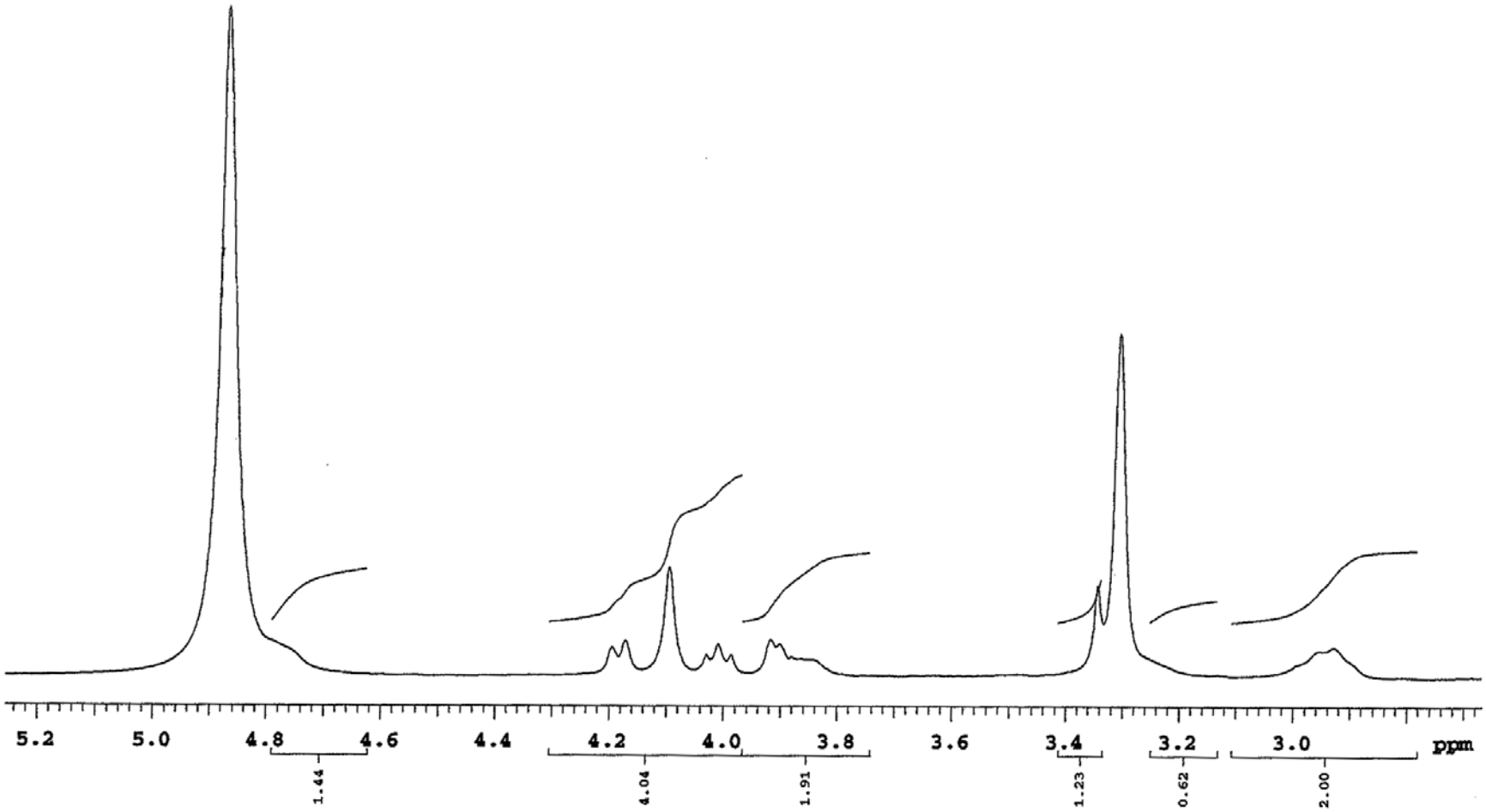

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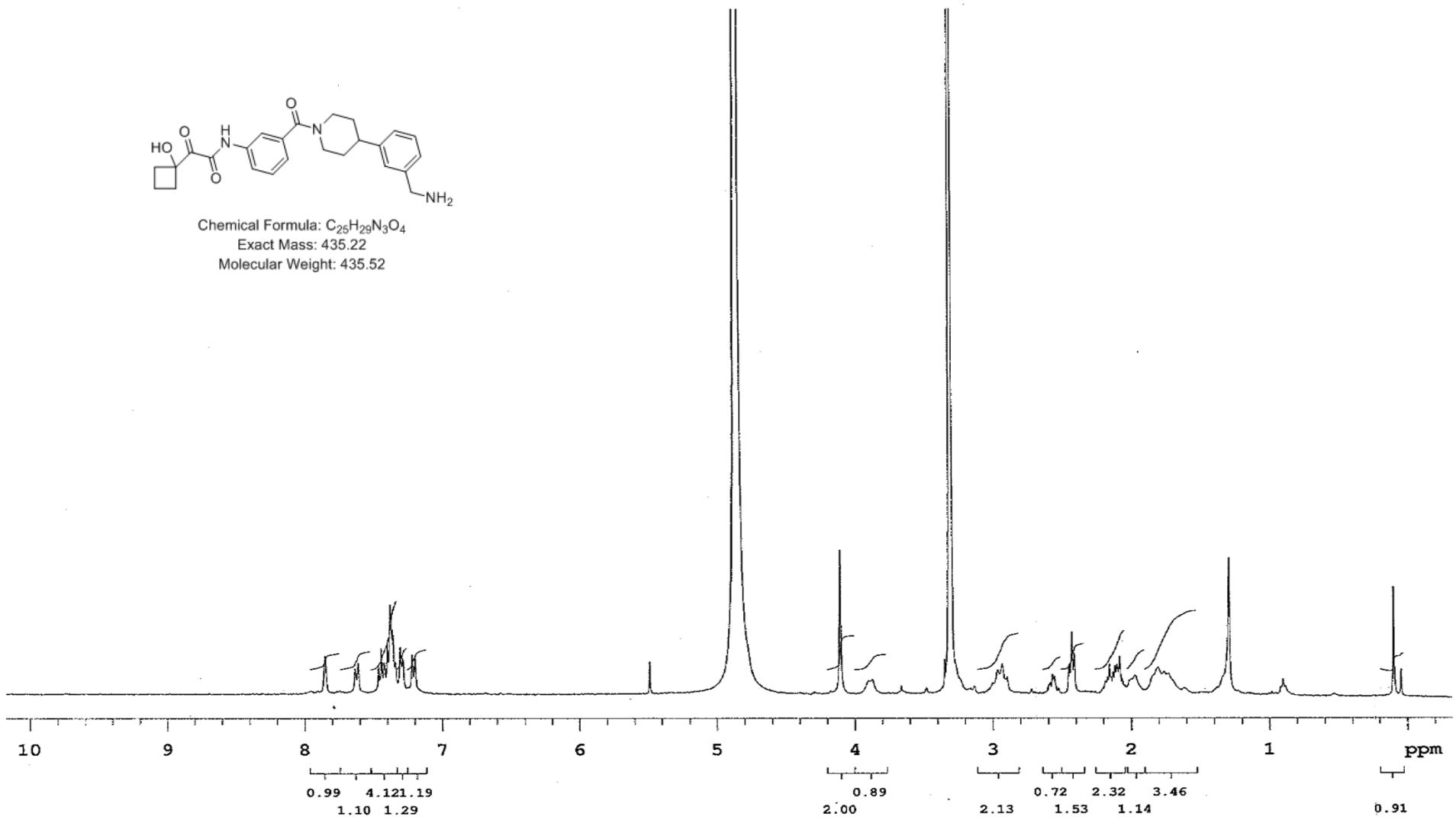

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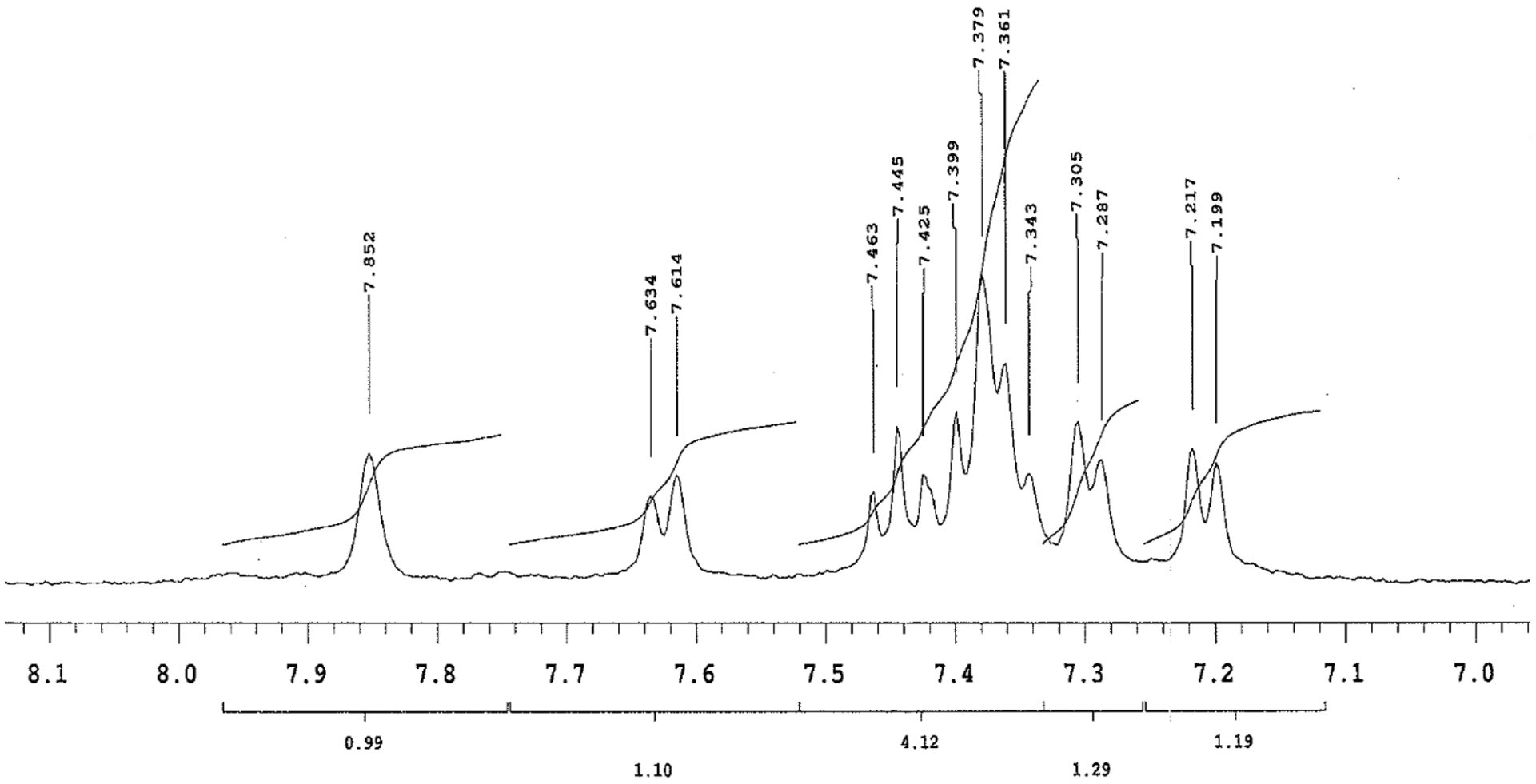

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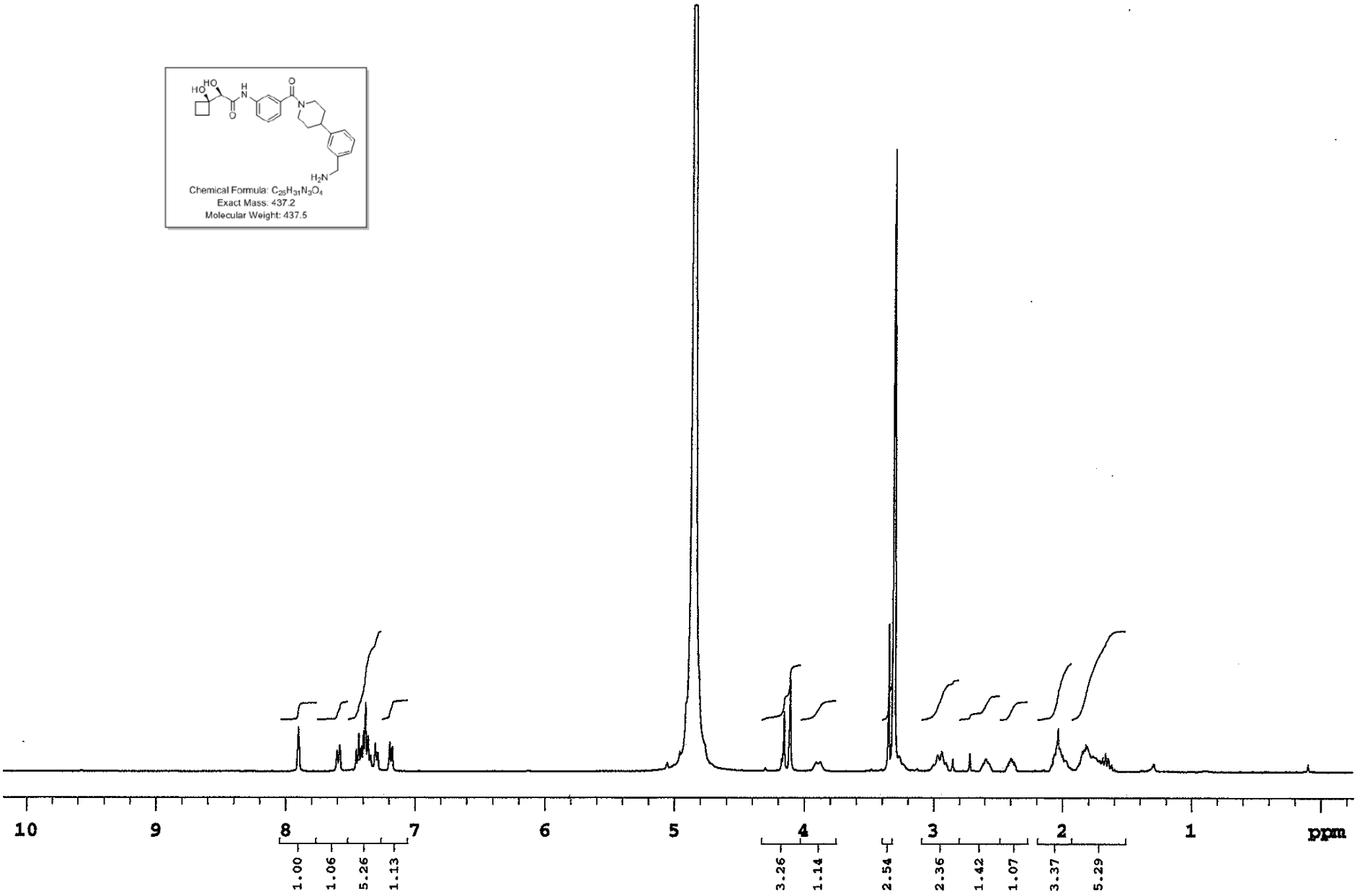

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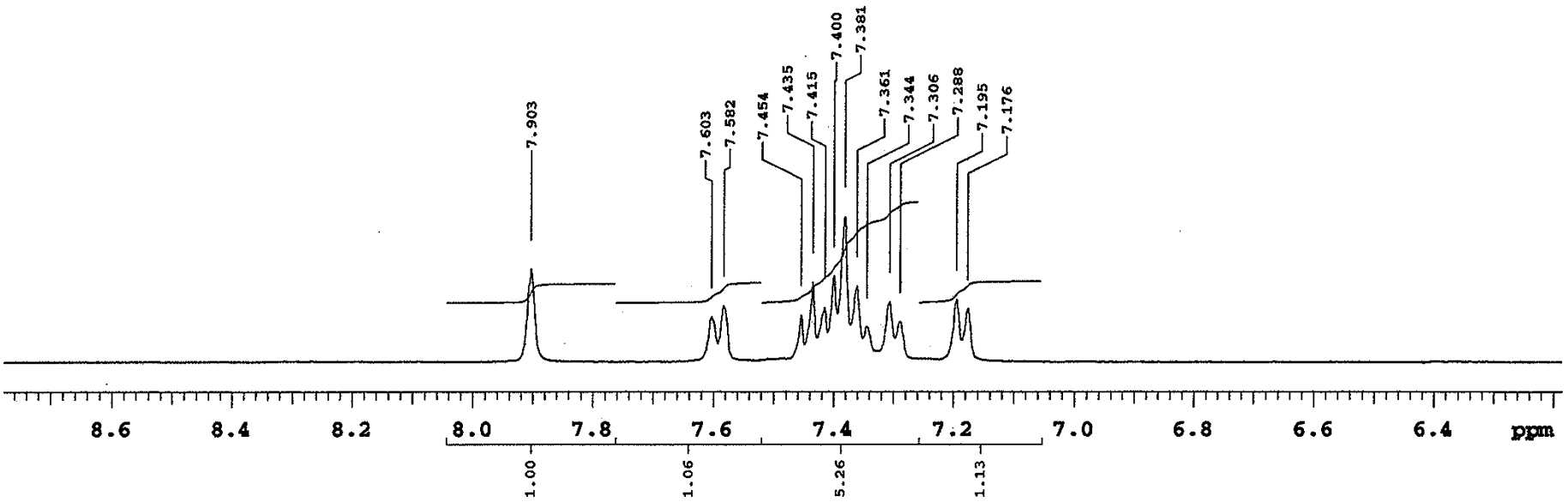

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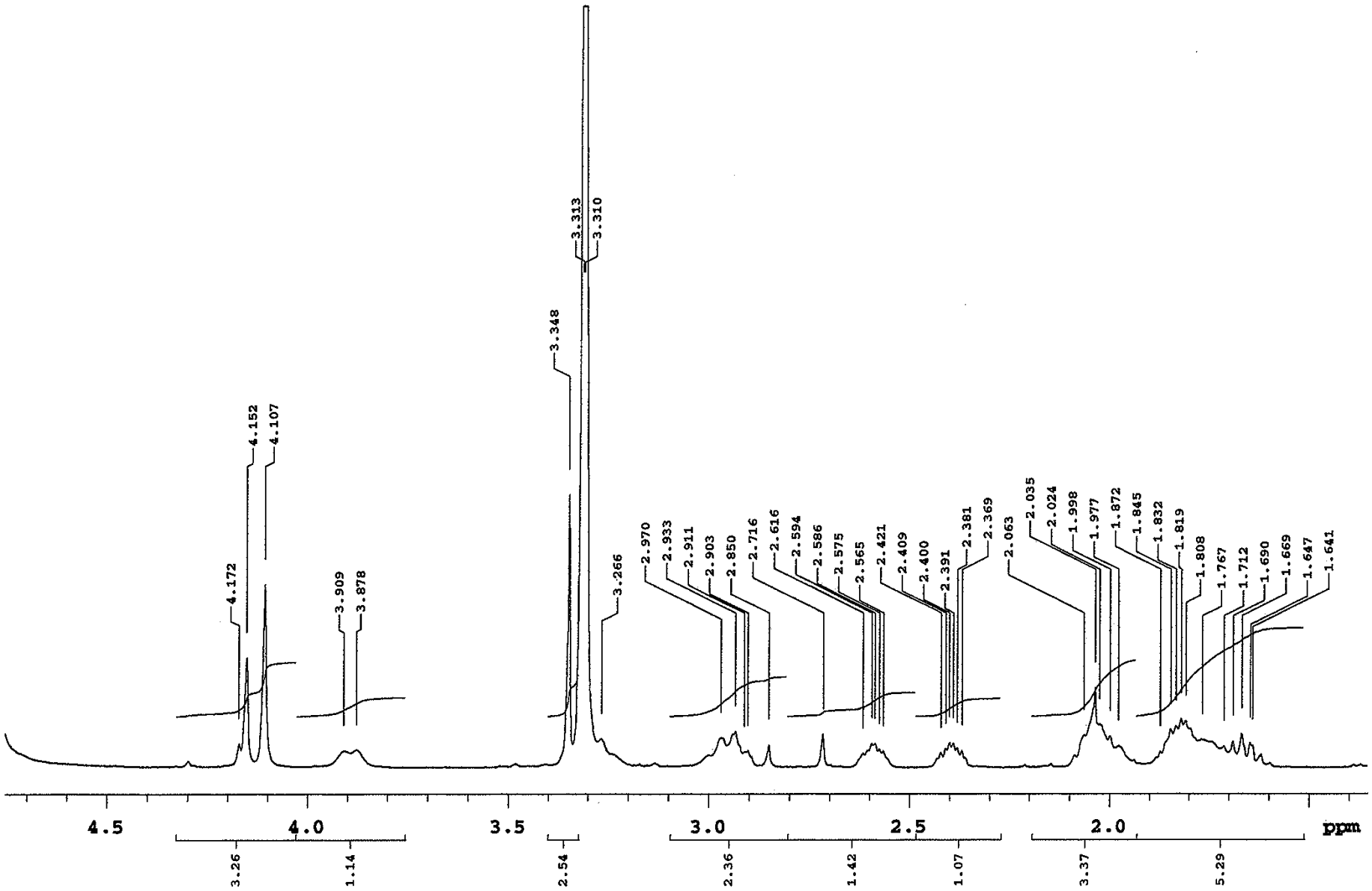

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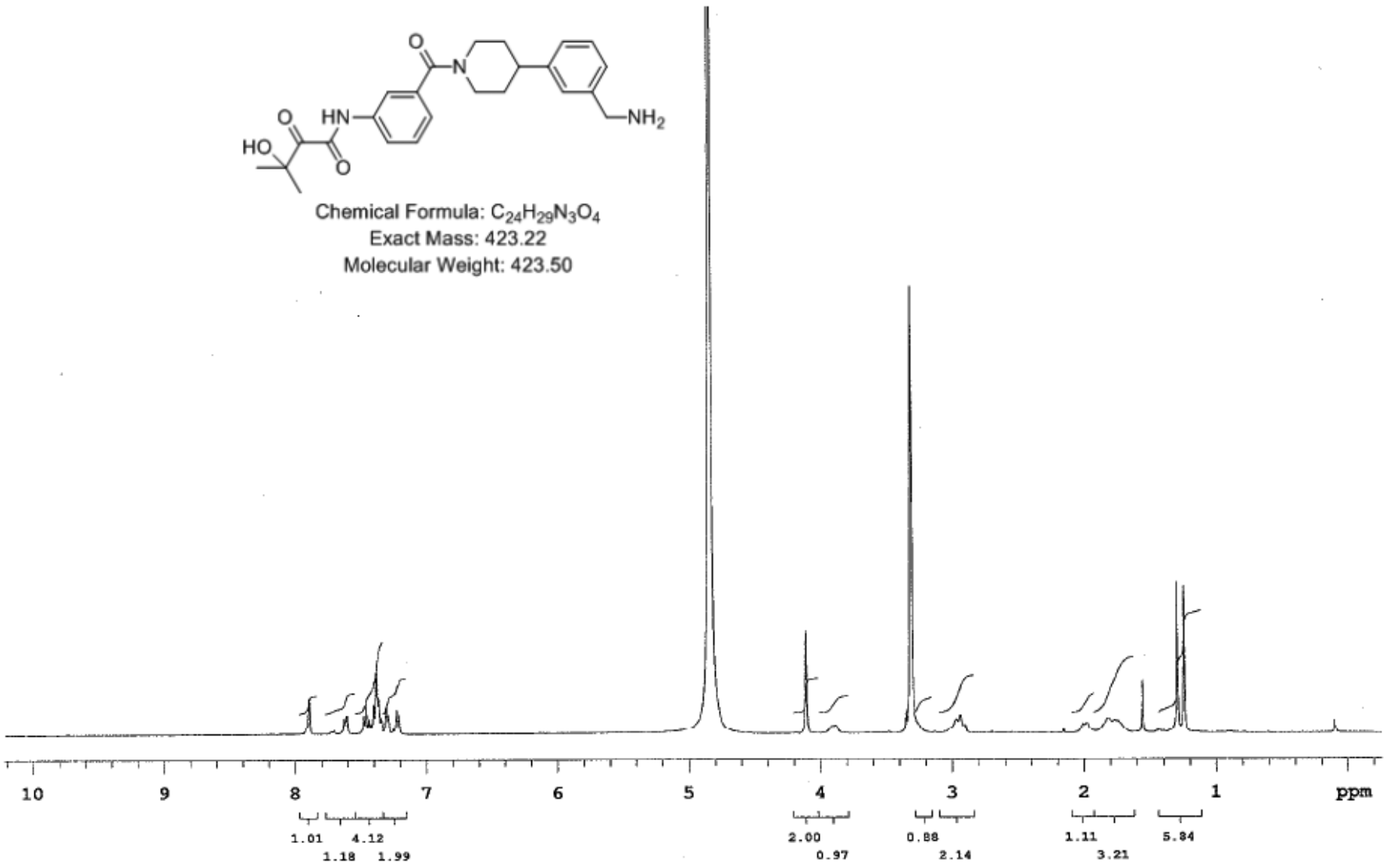

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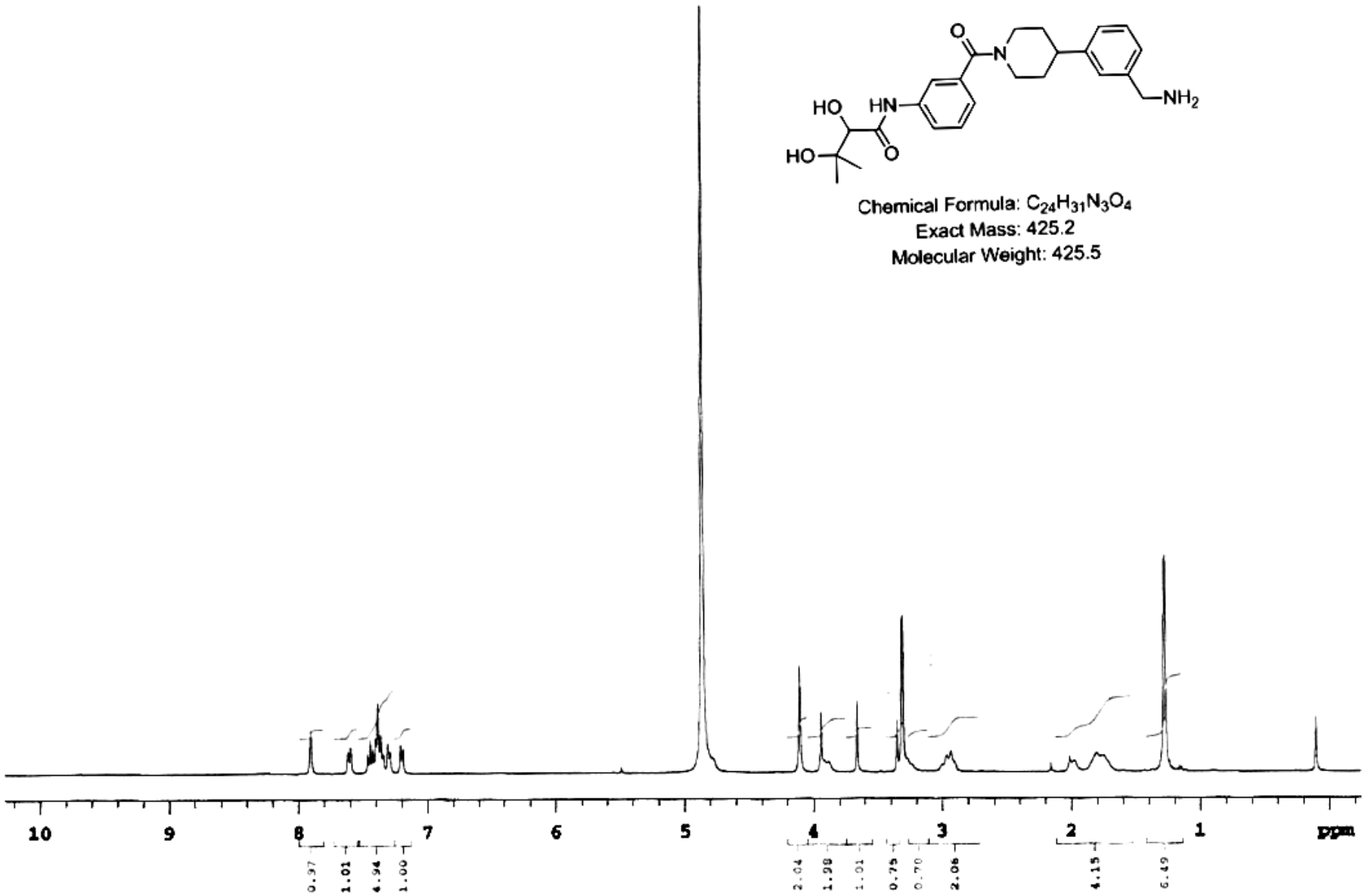

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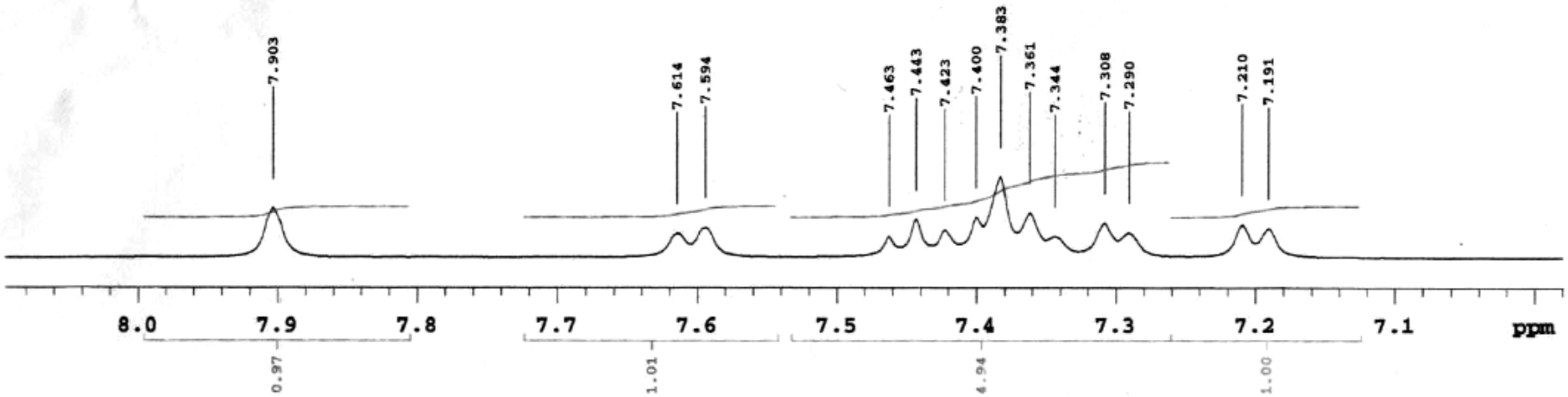

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