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Supplemental information

Enniatin A inhibits the chaperone Hsp90 and unleashes the immune system against triple-negative breast cancer

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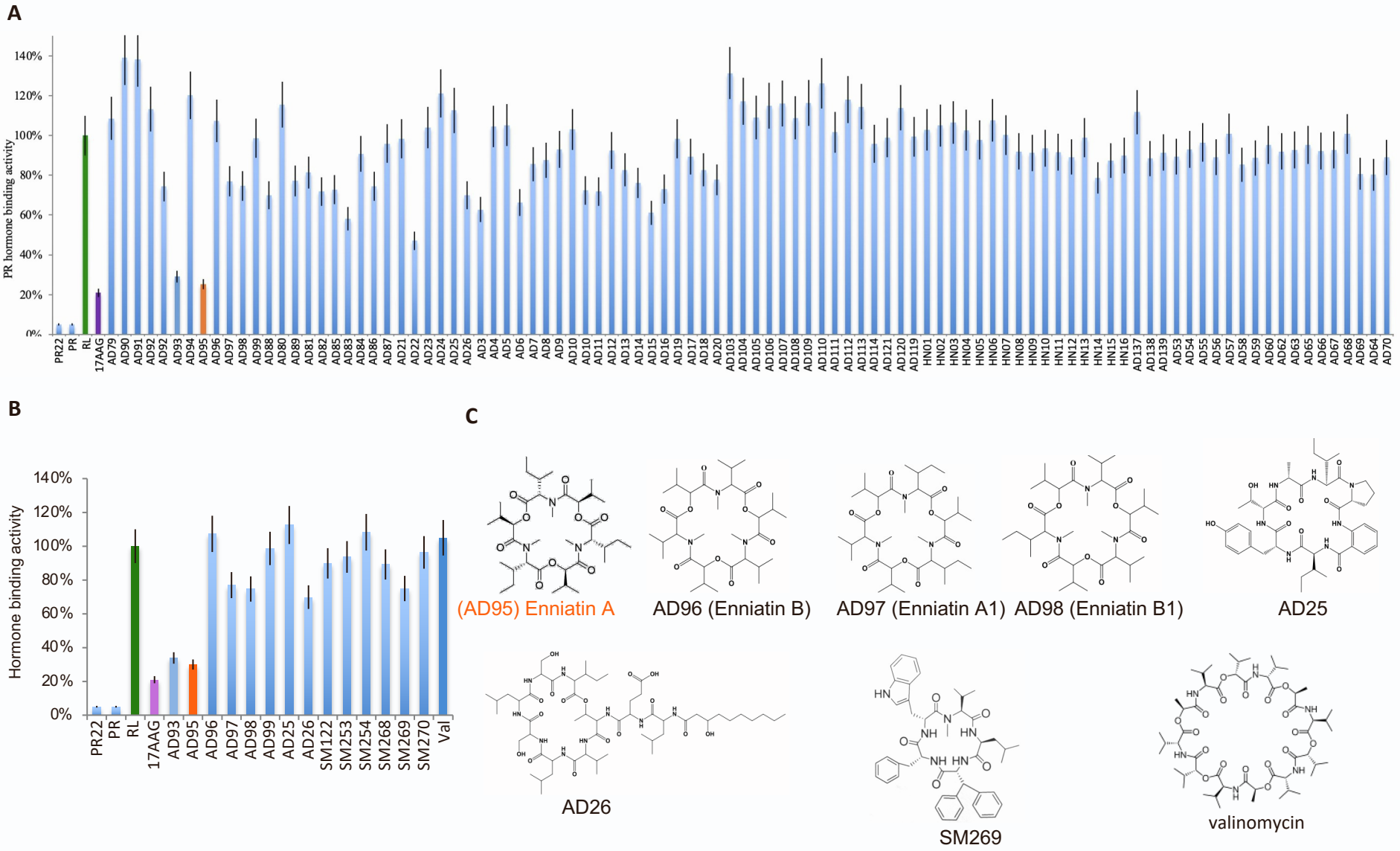
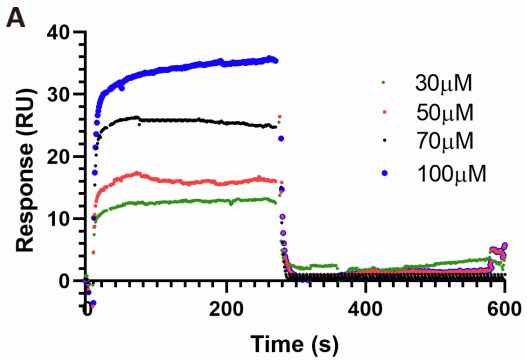
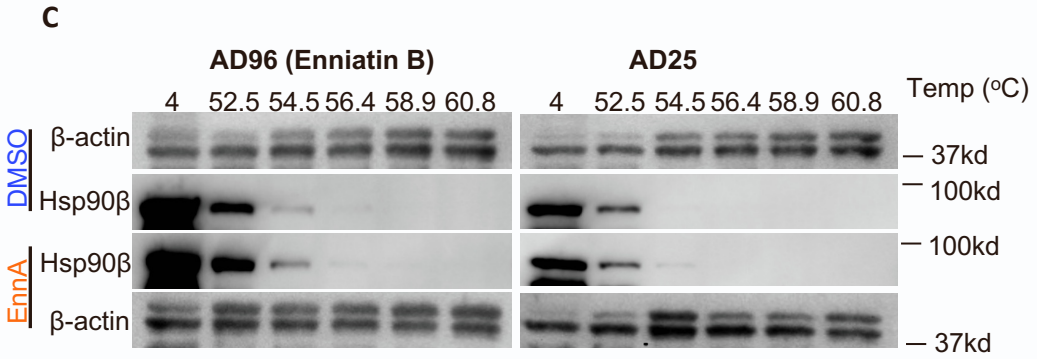


Supplementary Fig. 1. EnnA inactivates the Hsp90 machine in vitro, related to Fig. 1. A.) Example of data obtained from screening 125 natural products. Data are represented in the percent of samples containing rabbit reticulocyte lysate (RL) and DMSO without 10 μM drug added. Of interest are samples with no drugs (green), and those treated with the positive control 17-AAG (purple) and EnnA (orange). B.) Comparison of cyclic peptide effects on PR reconstitution assay. C.) Structures of a few cyclic peptides tested in B. SM compounds were graciously provided by Dr. Shelli R. McAlpin (University of New South Wales, Sydney , Australia).





B

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Y.Hsp82      TKPLWTRNPSDITQEEYNAFYKSISNDWEDPLYVKHFSVEGQLEFRAILFIPKRAPFDLF  332
H.Hsp90b     TKPIWTRNPDDITQEEYGEFYKSLTNDWEDHLAVKHFSVEGQLEFRALLFIPRRAPFDLF  344
H.Hsp90a     TKPIWTRNPDDITNEEYGEFYKSLTNDWEDHLAVKHFSVEGQLEFRALLFVPRRAPFDLF  352

H.Trap1      VSR--ELGSSVALYSRKVLIQTKATDILPKWLRFIRGVVDSEDIPLNLSRELLQESALIR  382
H.Grp94      DEYGSKKSDYIKLYVRRVFITDDFHDMMPKYLNFVKGVVDSDDLPLNVSRETLQQHKLLK  458
Y.Hsp82      ESK--KKKNNIKLYVRRVFITDEAEDLIPEWLSFVKGVVDSEDLPLNLSREMLQQNKIMK  390
H.Hsp90b     ENK--KKKNNIKLYVRRVFIMDSCDELIPEYLNFIRGVVDSEDLPLNISREMLQQSKILK  402
H.Hsp90a     ENR--KKKNNIKLYVRRVFIMDNCEELIPEYLNFIRGVVDSEDLPLNISREMLQQSKILK  410

H.Trap1      KLRDVLQQRLIKFFIDQSKKDAEKYAKFFEDYGLFMREGIVTATEQEVKEDIAKLLRYES  442
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Y.Hsp82      VIRKNIVKKLIEAFNE-IAEDSEQFEKFYSAFSKNIKLGVHEDTQ--NRAALAKLLRYNS  447
H.Hsp90b     VIRKNIVKKCLELFSE-LAEDKENYKKFYEAFSKNLKLGIHEDST--NRRRLSELLRYHT  459
H.Hsp90a     VIRKNLVKKCLELFTE-LAEDKENYKKFYEQFSKNIKLGIHEDSQ--NRKKLSELLRYYT  467

H.Trap1      SALPSGQLTSLSEYASRMRAGTRNIYYLCAPNRHLAEHSPYYEAMKKKDTEVLFCFEQFD  502
H.Grp94      SHHP-TDITSLDQYVERMKEKQDKIYFMAGSSRKEAESSPFVERLLKKGYEVIYLTEPVD  573
Y.Hsp82      TKSV-DELTSLTDYVTRMPEHQKNIYYITGESLKAVEKSPFLDALKAKNFEVLFLTDPID  506
H.Hsp90b     SQSG-DEMTSLSEYVSRMKETQKSIYYITGESKEQVANSAFVERVRKRGFEVVYMTEPID  518
H.Hsp90a     SASG-DEMVSLKDYCTRMKENQKHIYYITGETKDQVANSAFVERLRKHGLEVIYMIEPID  526

H.Trap1      ELTLLHLREFDKKKLISVETDIVVDHYKEEKFEDRSPAAECLSEKETEELMAWMRN-VLG  561
H.Grp94      EYCIQALPEFDGKRFQNVAKEGVKFDESEKT-----KESREAVEKEFEPLLNWMKDKALK  628
Y.Hsp82      EYAFTQLKEFEGKTLVDITKD-FELEETDEE-----KAEREKEIKEYEPLTKALKE-ILG  559
H.Hsp90b     EYCVQQLKEFDGKSLVSVTKEGLELPEDEEE-----KKKMEESKAKFENLCKLMKE-ILD  572
H.Hsp90a     EYCVQQLKEFEGKTLVSVTKEGLELPEDEEE-----KKKQEEKKTKFENLCKIMKD-ILE  580

H.Trap1      SRVTNVKVTLRLDTHPAMVTVLEMGAARHFLRMQQLAKTQ------EERAQLLQPTLEIN  615
H.Grp94      DKIEKAVVSQRLTESPCALVASQYGWSGNMERIMKAQAYQTGKDISTNYYASQKKTFEIN  688
Y.Hsp82      DQVEKVVVSYKLLDAPAAIRTGQFGWSANMERIMKAQALR---DSSMSSYMSSKKTFEIS  616
H.Hsp90b     KKVEKVTISNRLVSSPCCIVTSTYGWTANMERIMKAQALR---DNSTMGYMMAKKHLEIN  629
H.Hsp90a     KKVEKVVVSNRLVTSPCCIVTSTYGWTANMERIMKAQALR---DNSTMGYMAAKKHLEIN  637
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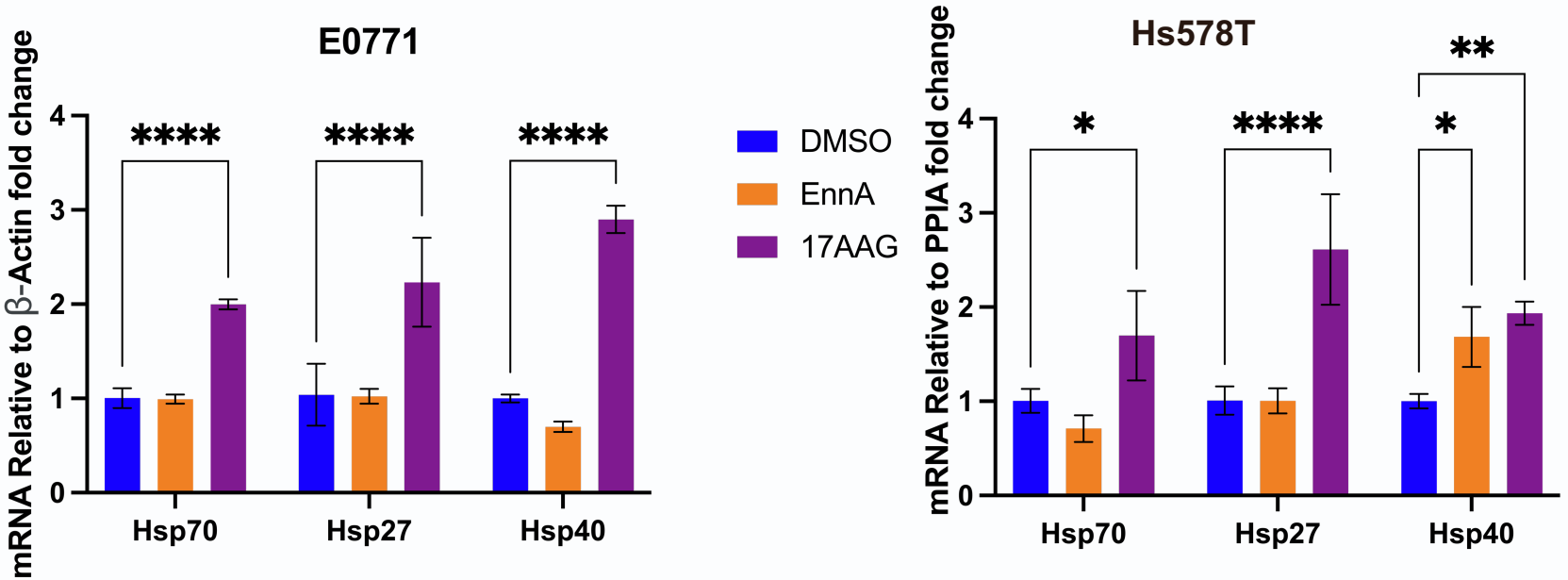
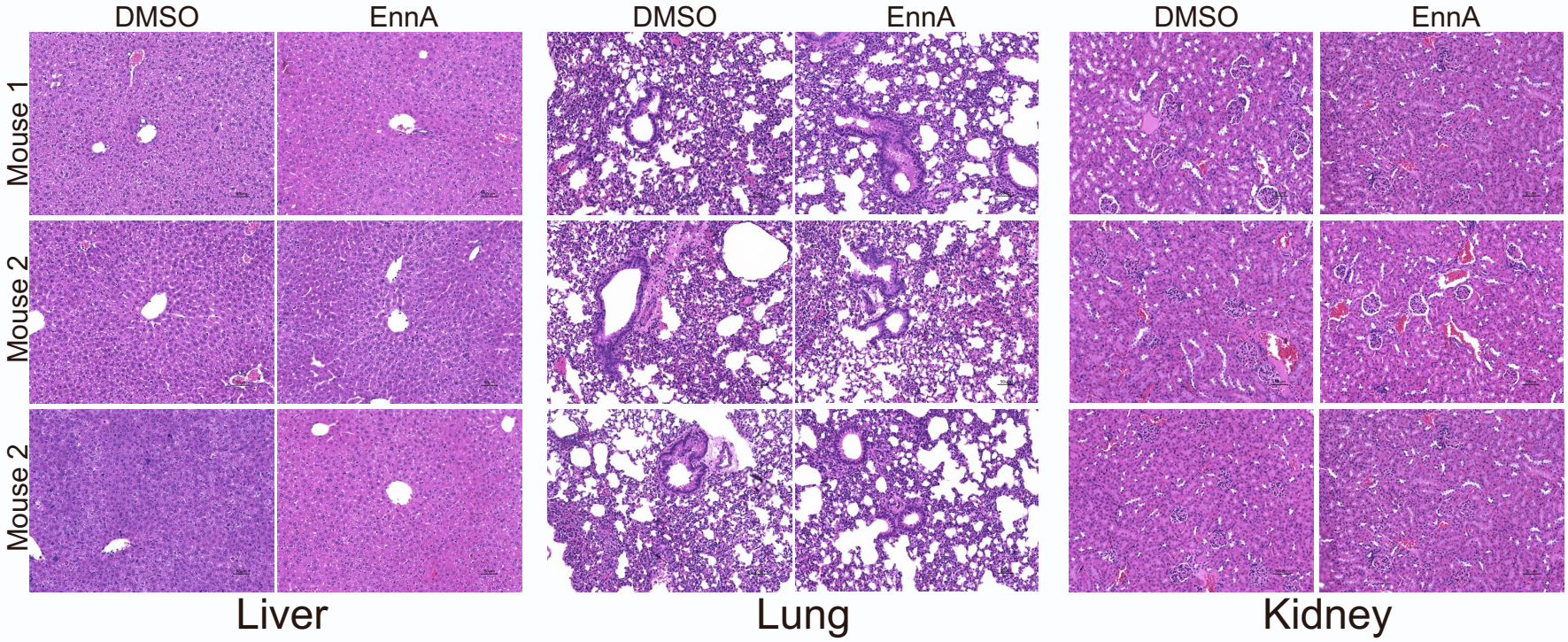
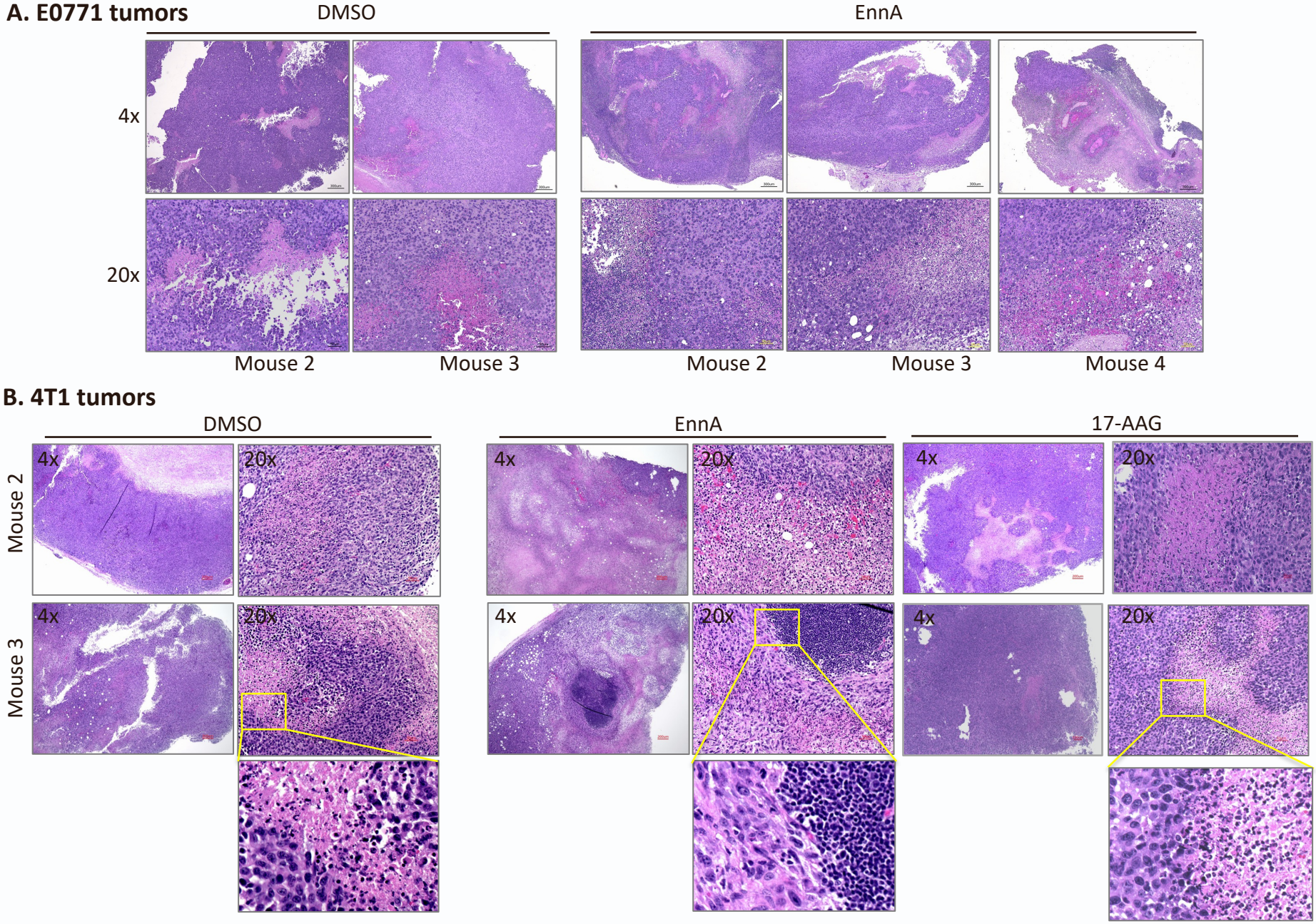
Supplementary Fig. 2. EnnA binding to Hsp90, related to Fig. 1. A.) SPR analysis of EnnA/HSP70 interaction. HSP70 was immobilized on the microchip surface. Overlay of sensorgrams recorded at increasing EnnA concentrations. B.) Primary sequence alignment of human TRAP1, Grp94, Hsp90α Hsp90β, and yeast Hsp82. C.) Cellular Thermal Shift Assay (CETSA) was performed with E0771 cells in the presence of 20 μM AD96 and 20 μM AD25 for 90 mn. Cell lysates were separated by SDS-PAGE and analyzed by Western blotting.



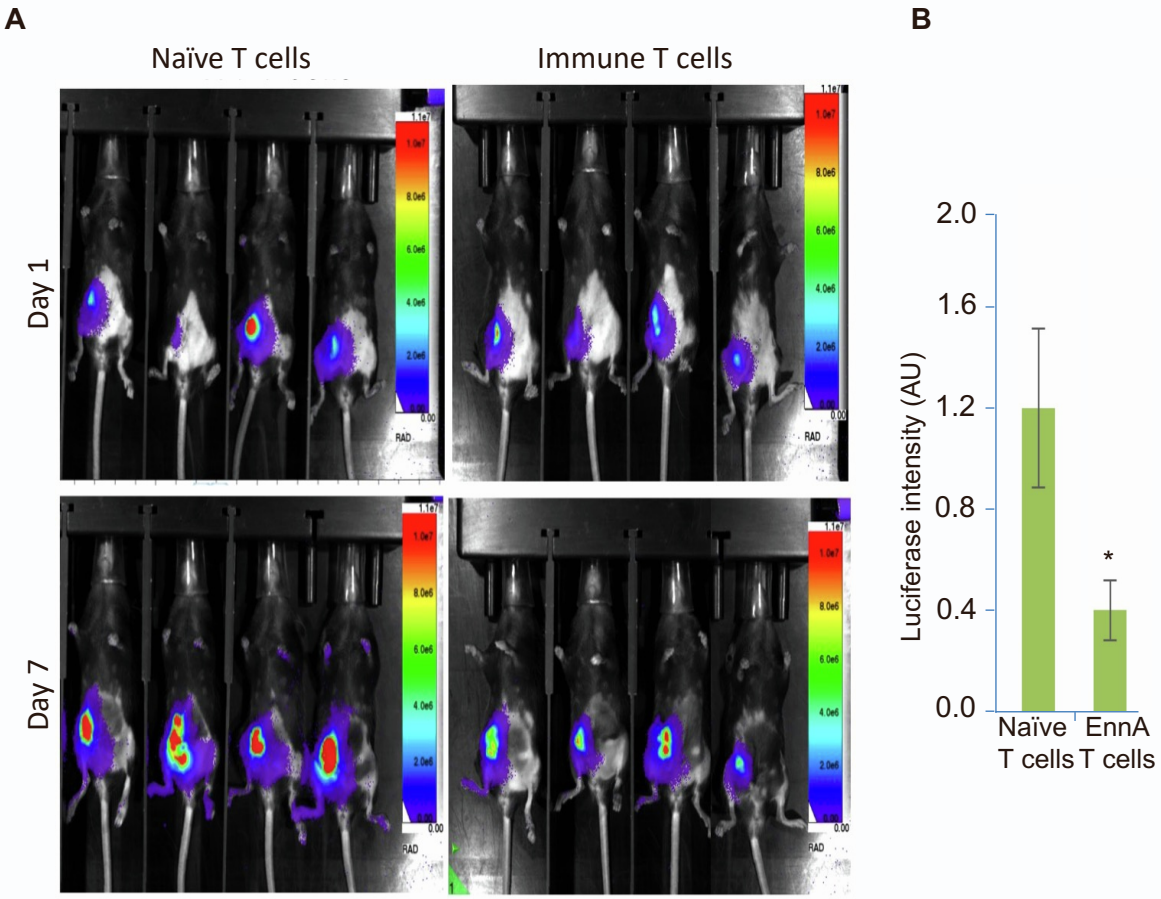
Supplementary Fig. 3. EnnA does not induce of heat shock response (HTS), related to Fig. 2. RTqPCR of Hsp70, Hsp27, and Hsp40 genes were performed using E0771 and Hs578T cell lines treated with DMSO, 5 μM EnnA, 0.5 μM 17-AAG for 24h. Data are represented as a mean ± S.E.M. *P<0.01; ****P<0.0001 by two-way ANOVA.



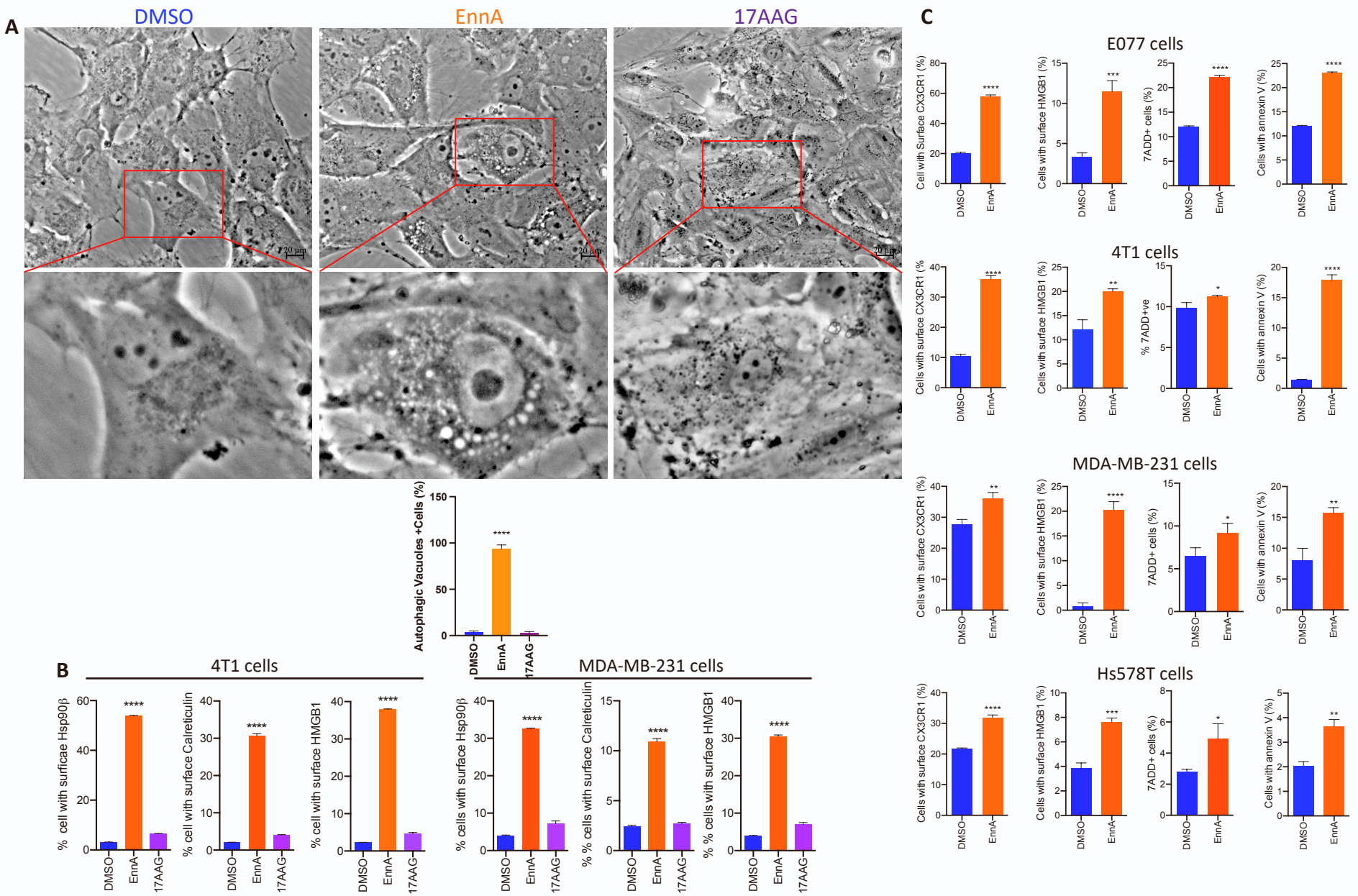
Supplementary Fig. 4. EnnA does not induce obvious *in vivo* toxicity in mice, related to Fig. 3. Evaluation of microscopic tissue toxicity caused by EnnA treatment. H&E staining of livers, Lungs, and kidneys from three mice per group sacrificed on day 12 (Fig. 3A) after intraperitoneal injection of the vehicle or EnnA (10 mg/kg) every 48h. The scale bar represents 50 μm.



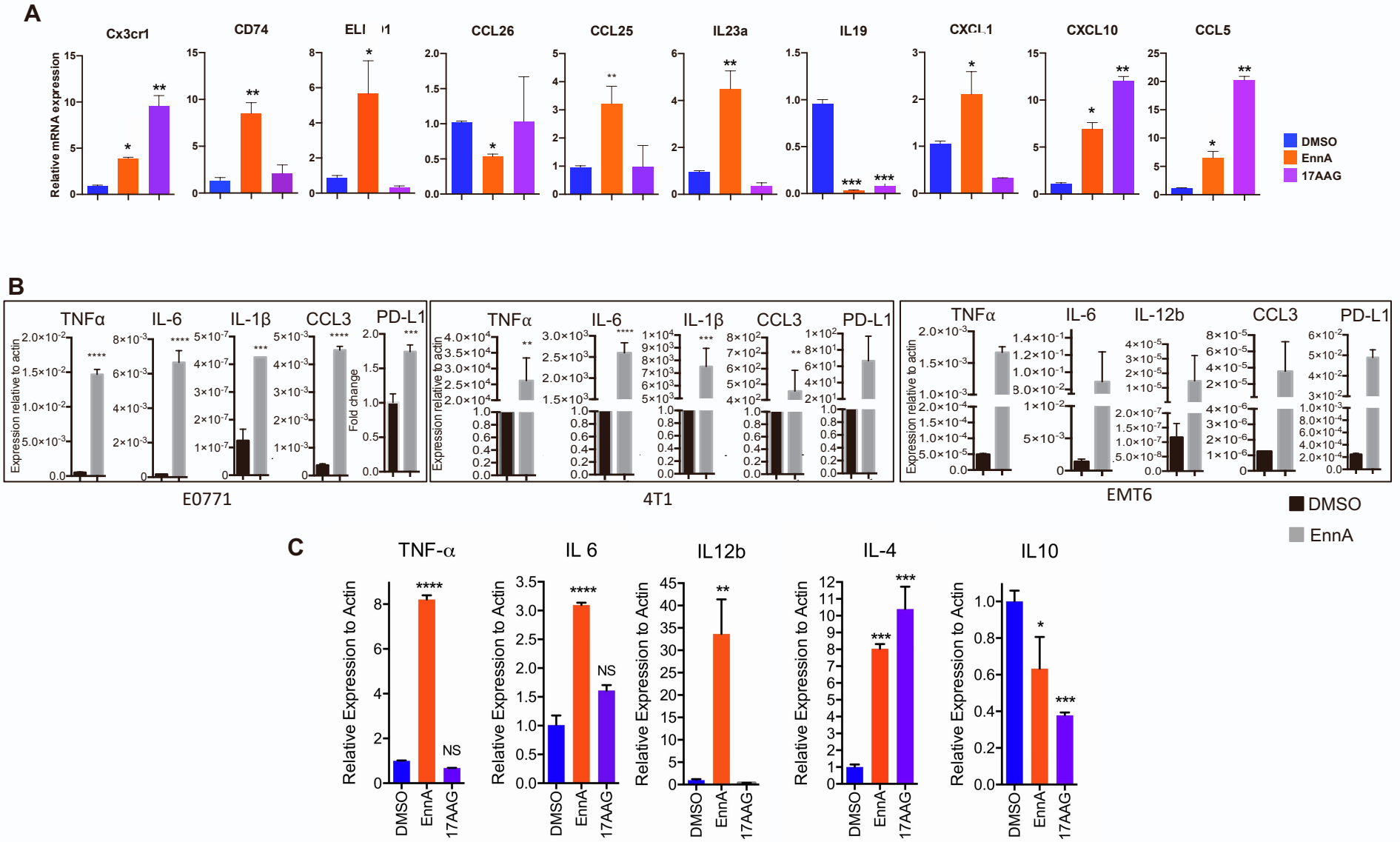
Supplementary Fig. 5. EnnA's anti-tumor activity, related to Fig. 3. H&E staining of E0771 (A) and 4T1 (B) tumors from animals treated with DMSO, EnnA (10 gm/kg) or 17-AAG (25 mg/kg). Two representative samples from each group are shown.



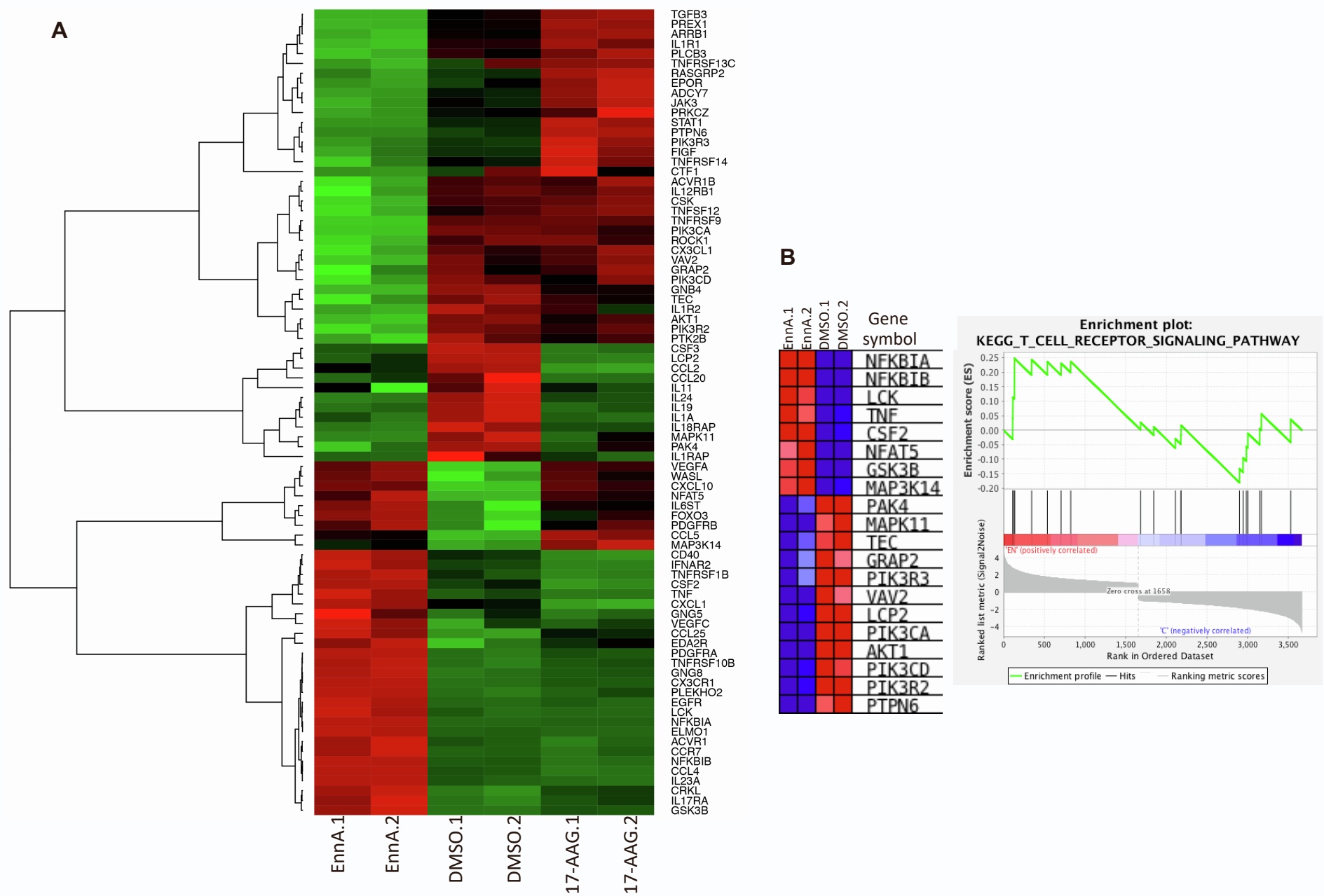
Supplementary Fig. 6, EnnA-induced anti-tumor immunity is transferable, related to Fig. 4. A.) Adoptive T cell transfer significantly reduced tumor growth. $2x10^6$ T cells isolated from spleens of E0771 tumor-bearing mice treated with EnnA or tumor-free mice were transferred into naive mice implanted with $5x10^4$ E0771 cells. B.) Quantification of tumor size in A at day 7 using luciferase intensity as readout.



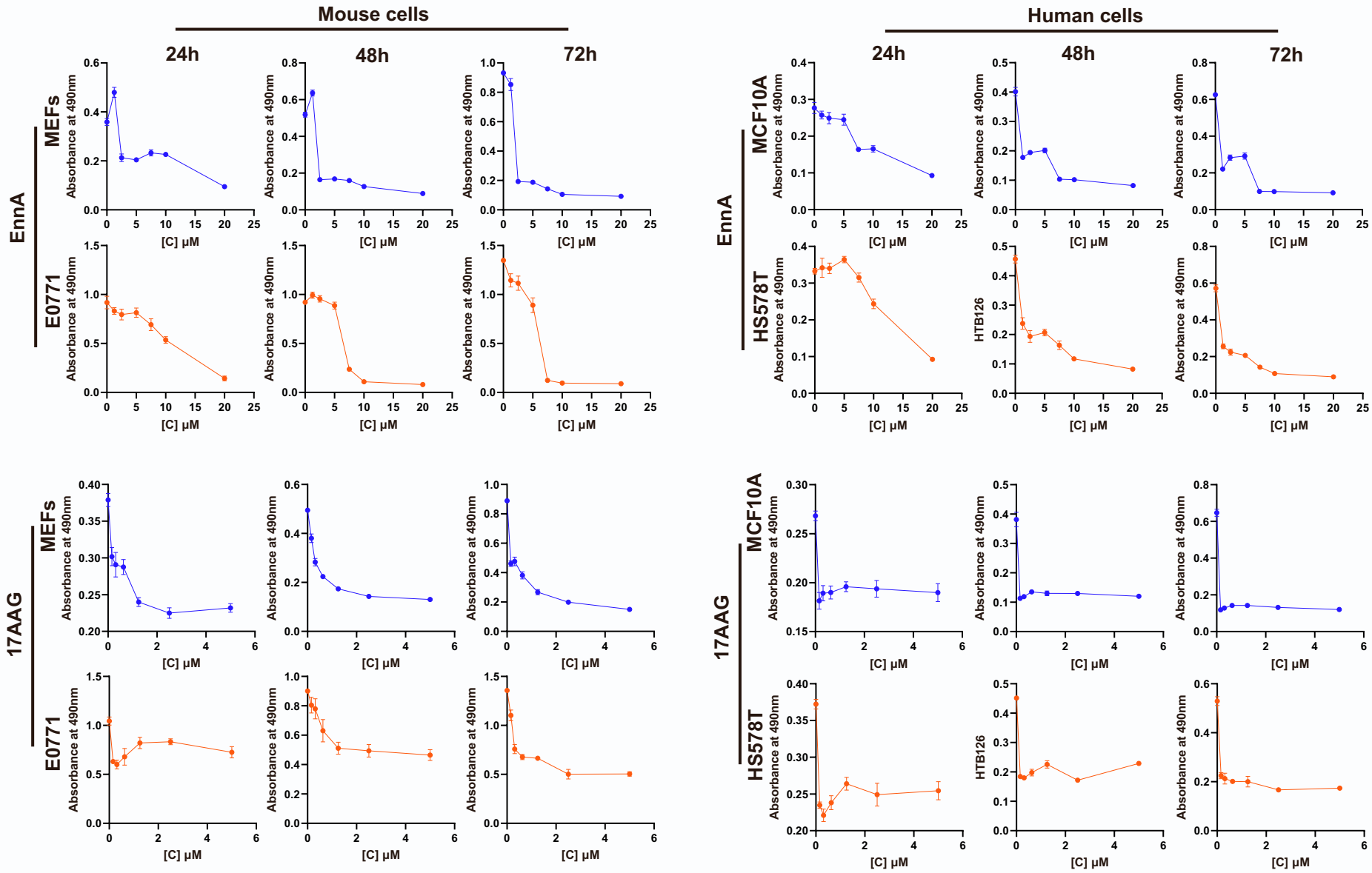
Supplementary Fig. 7. EnnA induces immunogenic cell death feature in vitro, related to Fig. 5. A.) Light microscopy of Hs578T cells treated with DMSO, 2.5 μM EnnA, or 17AAG for 24h. B.) Flow cytometry analysis of indicated cells treated with 7.5 μM EnnA or 0.5 μM 17-AAG for 24h. Cell surface staining protocol with no permeabilization step was used to detect Hsp90β, calreticulin, and HMGB1. C.) Indicated cell lines were treated with 7.5 μM EnnA for 24h. Cell surface staining protocol was implemented to detect, CX3CR1, HMGB1. Annexin V. 7AAD staining was used to assess cell death.



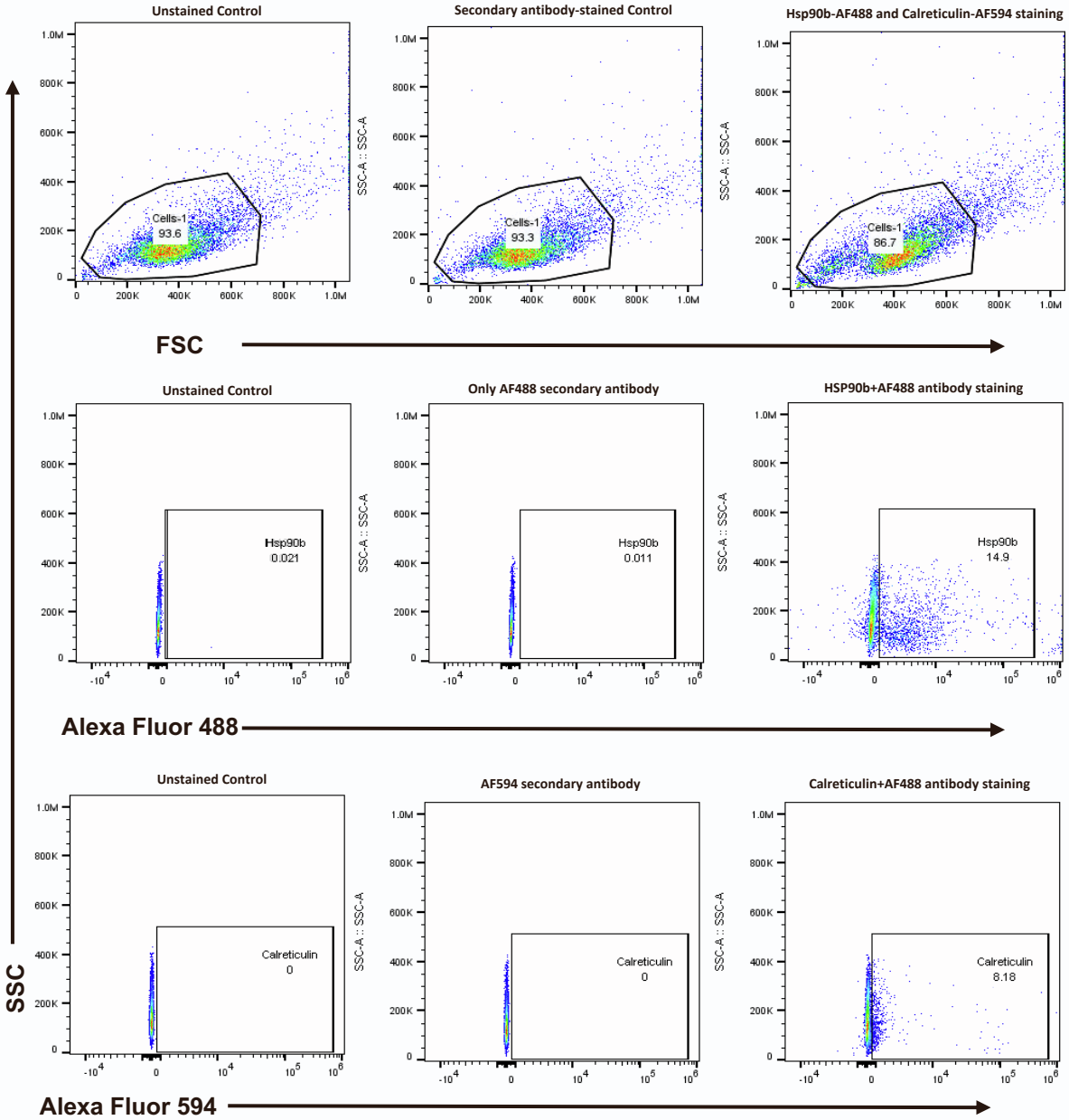
Supplementary Fig. 8. EnnA modulates E0771 cell transcriptome to activate the immune system, related to Fig. 6. A.) RTqPCR validating top and relevant genes identified by RNA-seq. B.) RTqPCR of cytokines from E0771, 4T1, and EMT6 murine breast cancer cell lines treated with DMSO and EnnA 48h. E0771, 4T1, and EMT6 cells were treated with 5, 1.25, and 2,5 μM EnnA, respectively. C.) Comparison of the effects of EnnA and 17-AAG on cytokine expression in E0771 cells.



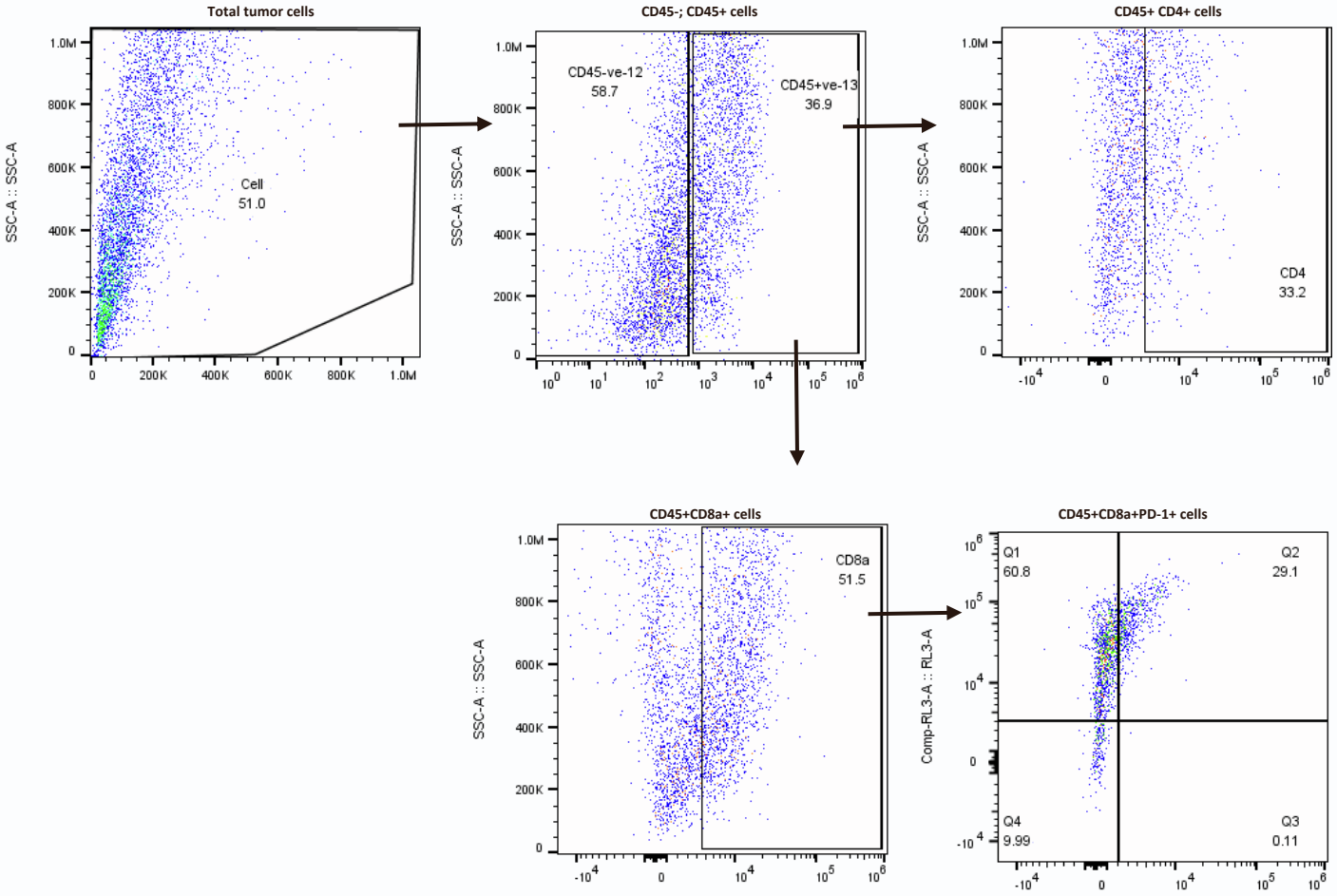
Supplementary Fig. 9. EnnA modulates E0771 cell transcriptome to activate the immune system, related to Fig. 6. A.) Heatmap based on RNA-seq data comparing the impact of EnnA and 17-AAG on immune pathways. 1&2 are biological duplicates of cells treated with DMSO, EnnA, or 17-AAG. Red and green indicate up- and down-regulated genes respectively. B.) Plots of Heatmap and Enrichment Score by GSEA on immune response involved Kegg pathways as in Figure 6F.



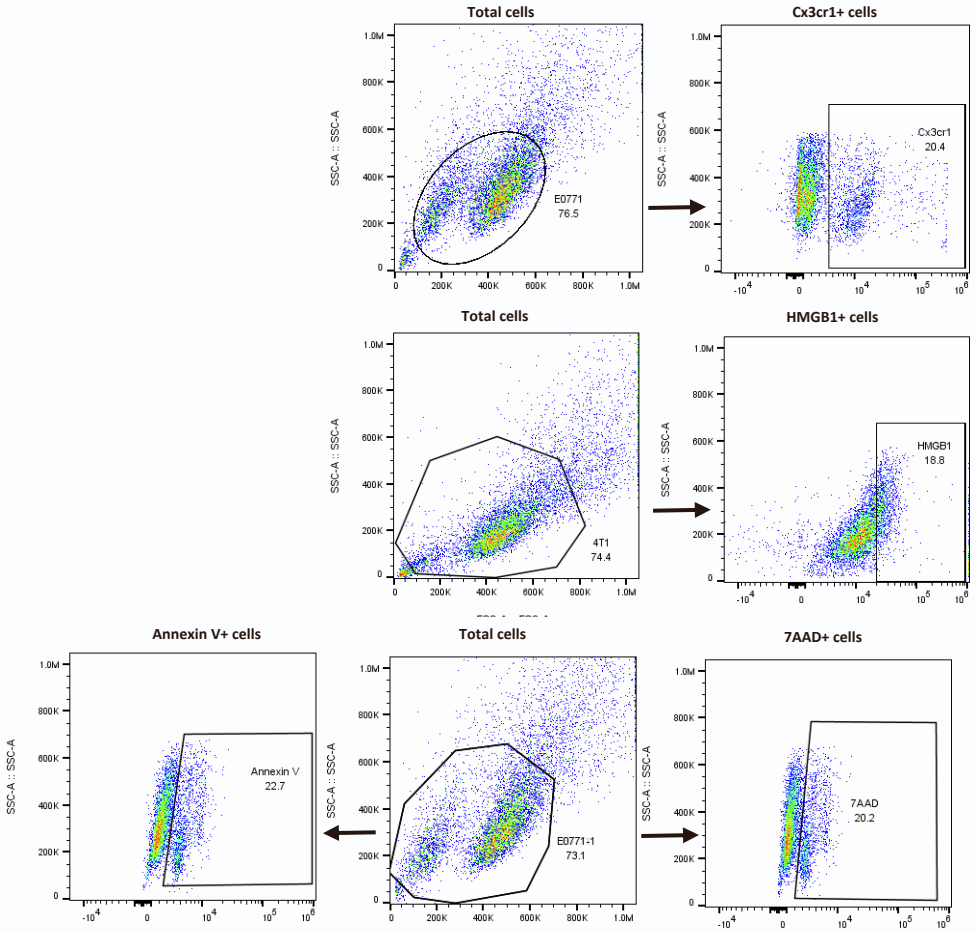
Supplementary Fig. 10. EnnA cytotoxicity *in vitro*. Normal mouse embryonic fibroblasts (MEFs) and human MCF10A, as well as cancer mouse E0771 and human Hs578T cell lines were treated with various EnnA concentrations (20, 10, 7.5, 5, 2.5, 1.25 & 0 µM) or 17AAG (5, 2.5, 1.25, 0.62, 0.31, 0.15 & 0 µM) for 24, 48 and 72 hours. DMSO was used as a control. Cell viability was determined by measuring the absorbance at 490 nm of the MTT reagent following the manufacturer's instructions.



Supplementary Fig. 11. Gating strategy, related to STAR Methods. Forward and side scatter detector voltage settings were adjusted based on the position of cells and then the cluster of E0771 cells was gated to exclude cell debris. Cells were gated for Alexa Fluor 488-Hsp90β and Alexa Fluor 595-Calreticulin. Unstained and only secondary antibody-stained cells (Mouse-AF488 & Rabbit-AF594) were used as a control to exclude any non-specific signal and correct gating purpose.



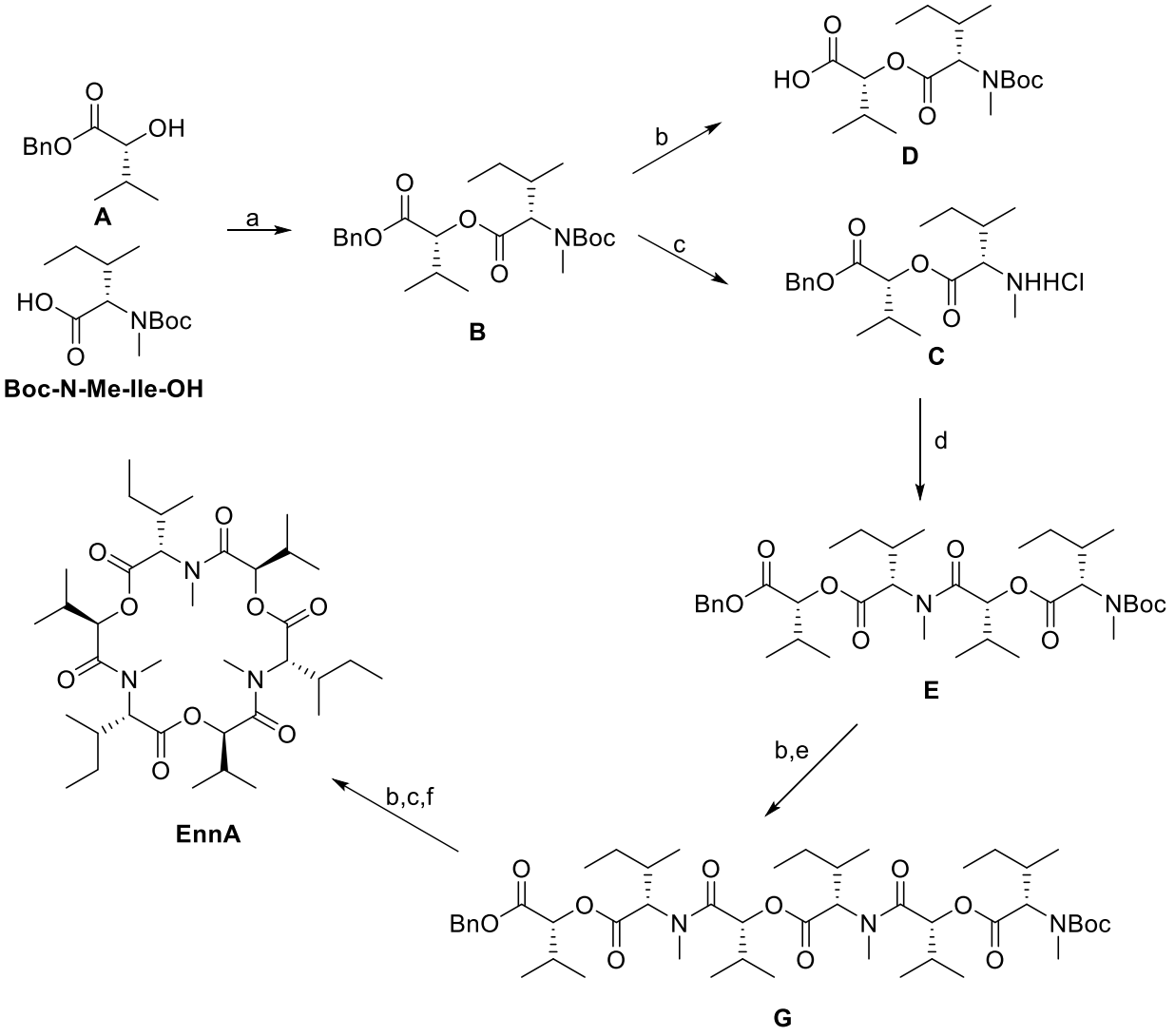
Supplementary Fig. 12. Gating strategy, related to STAR Methods. Forward and side scatter detector voltage settings were adjusted based on the position of E0771 tumor single-cell suspension and then the cluster of cells was gated to exclude cell debris. Cells were gated for BV605 CD45+ cells. CD45+ cells were further gated to separate Alexa Fluor 700 CD8a+ and APC-Cy7 PD-1+ cells. CD45+ cells were gated for separating PE-Cy7 CD4+ cells. CD4+ cells were further gated to identify CD25+ and Foxp3+ cells. Unstained and single antibody-stained cells were used as a control to exclude any non-specific signal and compensation for correct gating purposes.



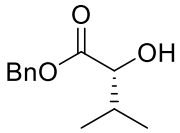
Supplementary Fig. 13. Gating strategy, related to STAR Methods. Forward and side scatter detector voltage settings were adjusted based on the position of cells and then clusters of the appropriate cells were gated to exclude cell debris. Cells were gated by Alexa Fluor 488-Cx3cr1+ and Alexa Fluor 488-HMGB1+cells. Unstained and only secondary antibody-stained cells (Mouse-AF488) were used as a control to exclude any non-specific signal and correct gating purpose. Cells were also gated to separate Annexin V+ and 7AAD+ cells to detect apoptosis.

Data S1/Methods S1: Synthesis of Enniatin A, related to STAR Methods.

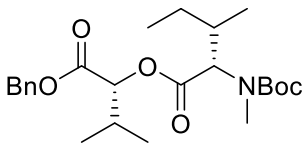
Chemistry General. ^{1}H NMR were recorded at 400 (Bruker AVIIIHD 400 MHz NMR with a broadband X-channel detect gradient probe) or 500 MHz (Avance AVIII 500MHz spectrometer with a dual carbon/proton cryoprobe) and ^{13}C were recorded at 125 MHz (Bruker AVIII spectrometer equipped with a cryogenically-cooled carbon observe probe); chemical shifts are reported in δ (ppm) relative to the internal standard ($CHCl_3$, 7.26 ppm or 77.2 ppm). HRMS spectra were recorded with a LCT Premier (Waters Cor., Milford, MA). The purity of Enniatin A was determined by HPLC (Agilent 1100 series quaternary pump; 80% MeCN/20% Water; Agilent C-18 column, 4.6x150mm, 5µM) with UV detection at 214 nm. All biologically tested compounds were determined to be >95% pure. TLC analysis was performed on glass backed silica gel plates and visualized by UV light or molybdenum stain. All reactions were performed using dry solvents under an inert atmosphere, unless otherwise stated.



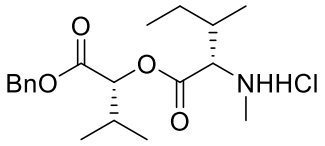
Scheme 1. Synthesis of Enniatin A. Reagents: (**a**) EDCI, DMAP, DCM; (**b**) H_2, 10% Pd/C, EtOAc; (**c**) 4N HCl in Dioxane; (**d**) Ghosez's Regaent, Hunig's base, DCM; (**e**) Ghosez's Reagent, Hunig's Base, **C**, DCM. (**f**) Ghosez's reagent, Hunig's base, DCM



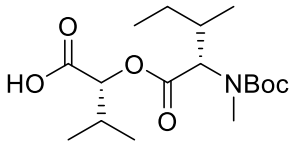
benzyl (R)-2-hydroxy-3-methylbutanoate (**A**). Cs_2CO_3 (1.72 g, 5.3 mmol, 0.5 eq.) was added portion wise to a stirred solution of (*R*)-2-hydroxy-3-methylbutanoic acid (1.25 g, 10.6 mmol, 1 eq.) in wet MeOH (19.6 mL) and H_2O (3.9 mL) at room temperature. The solution was allowed to stir for 30 minutes at which point the solvent was removed via rotary evaporation followed by co-evaporation of toluene (3 x 15 mL) to remove water. The residue was then dissolved in dry DMF (15.7 mL, 0.675 M) and cooled to 0˚C. Benzyl bromide (1.32 mL, 11.1 mmol, 1.05 eq.) was added dropwise and the reaction was stirred for 12 h at room temperature. EtOAc (30 mL) and H_2O (20 mL) were added. The organic layer was washed with H_2O (5 x 20 mL). The organic layer was dried over Na_2SO_4 and the solvent removed. The residue was purified via flash chromatography (EtOAc:Hexanes, 1:9) to afford the title compound. Yield 2.06 g (93%), clear oil. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.40 – 7.33 (m, 5H), 5.27 – 5.18 (m, 2H), 4.09 (dd, *J* = 6.2, 3.5 Hz, 1H), 2.69 (d, *J* = 6.2 Hz, 1H), 2.10 (pd, *J* = 6.9, 3.5 Hz, 1H), 1.01 (d, *J* = 6.9 Hz, 3H), 0.83 (d, *J* = 6.8 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 174.9, 135.2, 129.0, 128.8, 128.7, 128.6, 128.5, 75.0, 67.3, 32.2, 18.8, 15.9. HRMS (ESI) *m/z* $[M+Na]^+$ for $C_{12}H_{16}O_3Na$ 231.0997, found 231.1004.



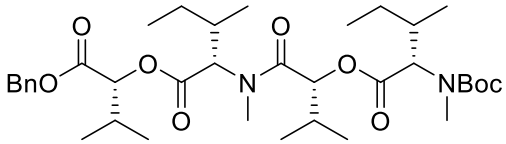
(R)-1-(benzyloxy)-3-methyl-1-oxobutan-2-yl N-(tert-butoxycarbonyl)-N-methyl-L-isoleucinate (**B**). EDCI (2.27 g, 11.9 mmol, 1.2 eq.) was added to a stirred solution of **A** (2.06 g, 9.9 mmol, 1 eq.), N-(tert-butoxycarbonyl)-N-methyl-L-isoleucine (2.67 g, 10.9 mmol, 1.1 eq.), and DMAP (1.57 g, 12.8 mmol, 1.3 eq.) in dry DCM (30 mL) at 0˚C and stirred for 12 h. 1N HCl (40 mL) was added to the reaction. The organic layer was separated and washed with water (40 mL) and saturated $NaHCO_3$ (40 mL). The organic layer was dried over Na_2SO_4 and solvent removed via rotary evaporation. The residue was purified via flash chromatography (EtOAc:Hexanes, 9:1) to yield the title compound. Yield 3.5 g (81%), clear oil. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.39 – 7.27 (m, 5H), 5.16 (q, *J* = 12.2 Hz, 2H), 4.87 (d, *J* = 4.0 Hz, 1H), 4.54 (dd, *J* = 109.9, 10.5 Hz, 1H), 2.79 (d, *J* = 19.8 Hz, 3H), 2.25 (s, 1H), 1.98 (s, 1H), 1.45 (s, 10H), 1.17 – 1.02 (m, 1H), 1.00 – 0.84 (m, 12H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 171.3, 171.0, 169.4, 169.2, 156.3, 155.8, 135.5, 135.4, 128.7, 128.6, 128.5, 128.5, 80.3, 80.0, 67.0, 67.0, 63.1, 62.0, 33.5, 33.4, 30.7, 30.2, 30.1, 28.5, 25.2, 19.0, 18.9, 17.2, 16.0, 15.9, 10.9, 10.4. HRMS (ESI) *m/z* [M+K] for $C_{24}H_{37}NO_3K$ 474.2258, found 474.2262.



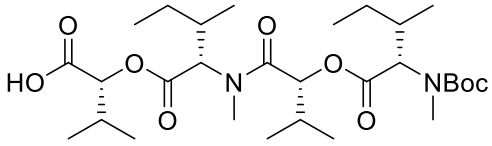
(R)-1-(benzyloxy)-3-methyl-1-oxobutan-2-yl methyl-L-isoleucinate hydrochloride (**C**). 4N HCl in Dioxane (5.8 mL, 23 mmol, 10 eq.) was added to **B** (1 g, 2.3 mmol, 1 eq.) at 0˚C. The reaction was stirred for 12 h at room temperature. The solvent was removed and the residue was co-evaporated with toluene (3 x 10 mL) to yield the title compound. Yield 705 mg (82%), amorphous white solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.40 – 7.31 (m, 5H), 5.25 – 5.08 (m, 2H), 5.01 (d, *J* = 3.8 Hz, 1H), 3.76 (d, *J* = 3.9 Hz, 1H), 2.67 (s, 3H), 2.54 – 2.44 (m, 1H), 2.39 – 2.29 (m, 1H), 1.64 – 1.53 (m, 2H), 1.46 – 1.37 (m, 1H), 1.16 (dd, *J* = 6.9, 2.2 Hz, 3H), 1.06 – 0.98 (m, 5H), 0.95 (d, *J* = 6.9 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 168.4, 167.2, 134.8, 128.8, 128.7, 128.6, 78.9, 67.6, 65.9, 36.3, 33.1, 29.8, 26.7, 19.1, 16.8, 14.8, 11.6. HRMS (ESI) *m/z* $[M-Cl]^+$ for $C_{19}H_{30}NO_4$ 336.2175, found 336.2184.



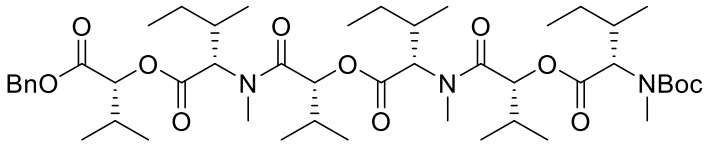
(R)-2-((N-(tert-butoxycarbonyl)-N-methyl-L-isoleucyl)oxy)-3-methylbutanoic acid (**D**). 10% Pd/C (100 mg) was added to a solution of **B** (1 g, 2.3 mmol) in EtOAc (15 mL) and stirred under a hydrogen atmosphere for 3h. The reaction mixture was filtered through a pad of celite and washed with DCM (3 x 10 mL). The filtrate was concentrated to yield the title compound. Yield 770 mg (97%) as a clear oil. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 5.07 – 4.86 (m, 1H), 4.51 – 4.26 (m, 1H), 2.84 (s, 3H), 2.31 (s, 1H), 2.10 – 1.96 (m, 1H), 1.54 – 1.41 (m, 9H), 1.18 – 1.05 (m, 1H), 1.07 – 0.86 (m, 13H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 171.0, 170.8, 62.9, 33.5, 33.1, 30.3, 29.9, 28.3, 25.2, 25.0, 18.9, 17.0, 16.9, 16.1, 15.8, 10.8, 10.2. HRMS (ESI) *m/z* $[M+Na]^+$ for $C_{17}H_{32}NO_6Na$ 368.2044, found 368.2053.



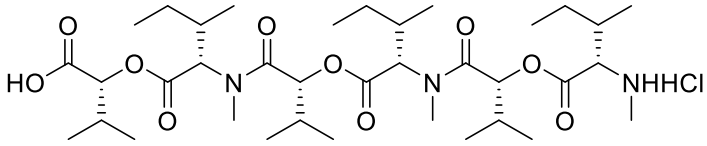
(R)-1-(benzyloxy)-3-methyl-1-oxobutan-2-yl N-((R)-2-((N-(tert-butoxycarbonyl)-N-methyl-L-isoleucyl)oxy)-3-methylbutanoyl)-N-methyl-L-isoleucinate (**E**). 1-chloro-*N*,*N*,2-trimethylprop-1-en-1-amine (Ghosez's Reagent, 0.16 mL, 1.2 mmol, 1.1 eq.) was added to a stirred solution of **D** (375 mg, 1.1 mmol, 1 eq.) in DCM (4.2 mL) at 0˚C. The reaction was stirred for 15 minutes at this temperature before the addition of C (413 mg, 1.1 mmol, 1 eq.) and Hunig's base (0.7 mL, 4.0 mmol, 3.6 eq.) in DCM (4.2 mL). The reaction was allowed to warm to room temperature over 12 hours. The reaction was quenched via the addition of 1N HCl (15 mL) and the organic layer was washed with water (20 mL) and saturated $NaHCO_3$ (20 mL). The organic layer was dried over Na_2SO_4 and the solvent removed. The residue was purified via flash chromatography (EtOAc:Hexanes, 1:4) to provide the title compound. Yield 587 mg (81%) as a clear oil. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.40 – 7.29 (m, 5H), 5.24 – 5.01 (m, 4H), 4.88 (dd, *J* = 19.1, 4.1 Hz, 1H), 4.51 (dd, *J* = 98.4, 10.2 Hz, 1H), 2.98 (d, *J* = 4.7 Hz, 2H), 2.94 – 2.81 (m, 4H), 2.34 – 2.19 (m, 1H), 2.16 – 1.91 (m, 2H), 1.63 – 1.53 (m, 1H), 1.45 (s, 9H), 1.35 (s, 1H), 1.15 – 0.79 (m, 27H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.9, 170.7, 170.5, 170.1, 169.8, 169.6, 169.4, 168.8, 156.5, 155.8, 135.5, 135.2, 128.8, 128.7, 128.7, 128.6, 128.6, 128.5, 80.3, 79.9, 77.6, 76.2, 75.7, 75.1, 67.3, 67.0, 65.1, 63.3, 62.1, 60.6, 33.9, 33.8, 33.5, 33.4, 31.9, 31.9, 30.9, 30.4, 30.2, 30.0, 30.0, 29.7, 28.5, 25.5, 25.4, 25.4, 25.1, 25.1, 19.6, 19.5, 19.0, 18.8, 17.4, 17.3, 17.3, 17.1, 16.1, 16.0, 15.9, 15.8, 10.9, 10.9, 10.5. HRMS (ESI) *m/z* $[M+Na]^+$ for $C_{36}H_{58}N_2O_9Na$ 685.4040, found 685.4037.



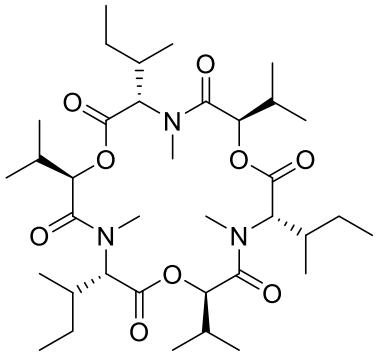
(6S,9R,12S,15R)-6,12-di((S)-sec-butyl)-9,15-diisopropyl-2,2,5,11-tetramethyl-4,7,10,13-tetraoxo-3,8,14-trioxa-5,11-diazahexadecan-16-oic acid (**F**). 10% Pd/C (50 mg) was added to a solution of **E** (587 mg, 0.9 mmol) in EtOAc (10 mL) and stirred under a hydrogen atmosphere for 12 h. The reaction was then filtered through a pad of celite and washed with DCM (3 x 15 mL). The filtrated was concentrated to provide the title compound. Yield 495 mg (quant.). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 5.24 – 5.12 (m, 1H), 5.02 – 4.86 (m, 2H), 4.55 (dd, *J* = 200.1, 9.7 Hz, 2H), 3.18 – 2.73 (m, 7H), 2.37 – 2.24 (m, 2H), 2.05 (s, 2H), 1.47 (s, 9H), 1.15 – 1.03 (m, 2H), 1.03 – 0.84 (m, 24H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 171.3, 170.5, 170.2, 170.1, 169.9, 81.1, 75.3, 63.1, 62.3, 60.6, 60.3, 34.9, 32.9, 32.2, 31.8, 30.9, 29.9, 29.9, 28.4, 26.0, 25.3, 24.7, 24.5, 18.9, 18.5, 18.0, 17.3, 17.1, 15.9, 15.8, 15.7, 15.5, 11.4, 10.4, 10.2. HRMS (ESI) *m/z* $[M+Na]^+$ for $C_{29}H_{52}N_2O_9Na$ 595.3571, found 595.3571.



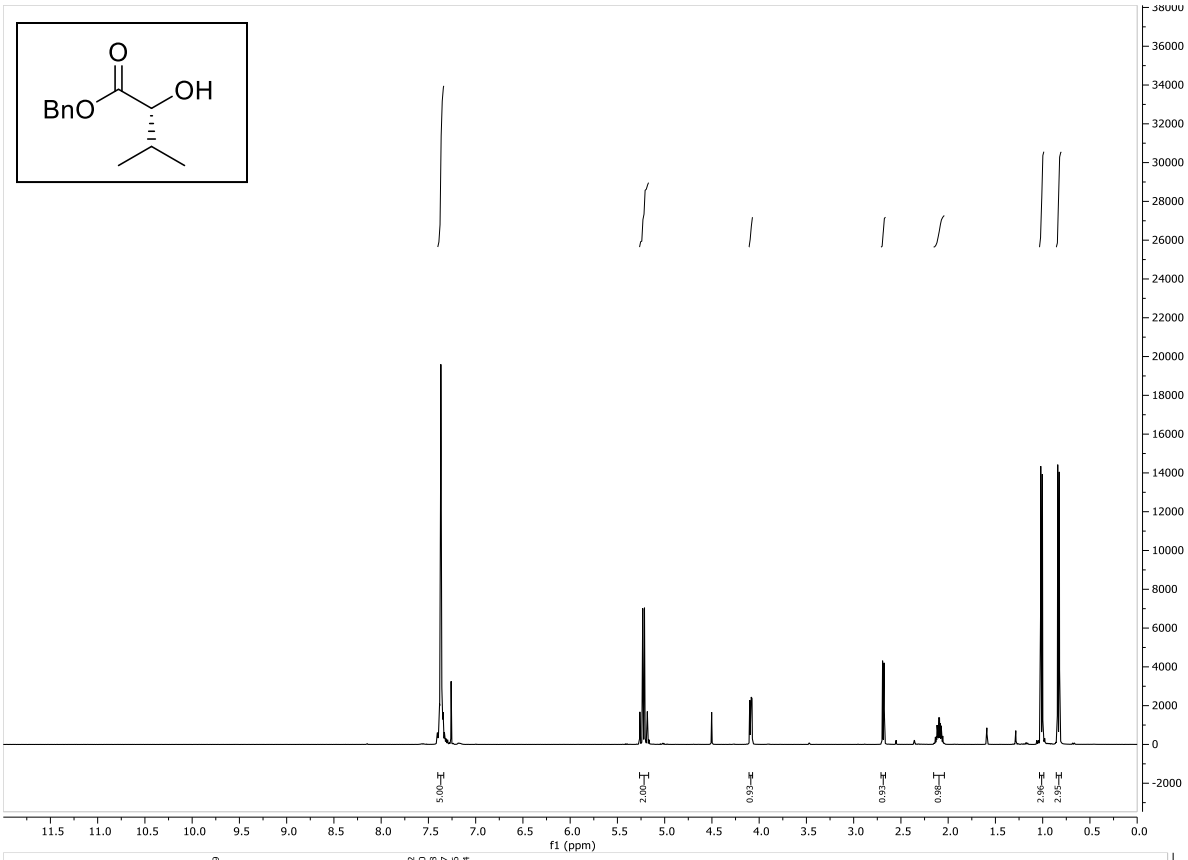
(R)-1-(benzyloxy)-3-methyl-1-oxobutan-2-yl N-((6S,9R,12S,15R)-6,12-di((S)-sec-butyl)-9,15-diisopropyl-2,2,5,11-tetramethyl-4,7,10,13-tetraoxo-3,8,14-trioxa-5,11-diazahexadecan-16-oyl)-N-methyl-L-isoleucinate (**G**). 1-chloro-*N,N*,2-trimethylprop-1-en-1-amine (Ghosez's Reagent, 0.14 mL, 1 mmol, 1.2 eq.) was added to a stirred solution of **F** (495 mg, 0.9 mmol, 1 eq.) in DCM (4.1 mL) at 0˚C. The reaction was stirred for 15 minutes at this temperature before the addition of **C** (320 mg, 0.9 mmol, 1 eq.) and Hunig's base (0.54 mL, 3.1 mmol, 3.6 eq.) in DCM (4.1 mL). The reaction was allowed to warm to room temperature over 12 hours. The reaction was quenched via the addition of 1N HCl (15 mL) and the organic layer was washed with water (20 mL) and saturated $NaHCO_3$ (20 mL). The organic layer was dried over Na_2SO_4 and the solvent removed. The residue was purified via flash chromatography (EtOAc:Hexanes, 1:3) to provide the title compound. Yield 557 mg (69%) as a clear oil. ^{1}H NMR (400 MHz, Chloroform-d) δ 7.39 – 7.30 (m, 5H), 5.21 (dd, J = 12.2, 2.5 Hz, 1H), 5.16 – 5.08 (m, 2H), 5.07 – 4.99 (m, 2H), 4.93 – 4.84 (m, 1H), 4.51 (dd, J = 97.9, 10.1 Hz, 1H), 4.13 (d, J = 13.5 Hz, 1H), 3.14 (s, 2H), 3.01 – 2.91 (m, 4H), 2.90 – 2.77 (m, 3H), 2.41 – 2.21 (m, 2H), 2.20 – 1.88 (m, 6H), 1.59 (s, 2H), 1.45 (d, J = 1.7 Hz, 9H), 1.42 – 1.35 (m, 2H), 1.03 – 0.83 (m, 36H). ^{13}C NMR (126 MHz, CDCl3) δ 170.7, 170.6, 170.3, 170.0, 169.9, 169.3, 156.3, 135.4, 128.6, 128.6, 128.5, 128.5, 128.4, 80.1, 79.7, 66.9, 65.1, 63.2, 62.0, 60.7, 60.5, 35.1, 33.8, 33.4, 33.2, 32.4, 31.7, 30.9, 30.3, 30.1, 29.6, 29.4, 28.4, 25.3, 25.0, 19.5, 19.3, 18.9, 18.7, 17.1, 16.9, 16.7, 15.9, 15.7, 11.6, 10.9, 10.7, 10.5. HRMS (ESI) *m/z* $[M+Na]^+$ for $C_{48}H_{79}N_3O_{12}Na$ 912.5561, found 912.5533.



(3S,6R,9S,12R,15S,18R)-3,9,15-tri((S)-sec-butyl)-6,12,18-triisopropyl-8,14-dimethyl-4,7,10,13,16-pentaoxo-5,11,17-trioxa-2,8,14-triazanonadecan-19-oic acid hydrochloride (**H**). 10% Pd/C (50 mg) was added to a solution of **G** (557 mg, 0.63 mmol) in EtOAc (10 mL). The reaction was stirred under a hydrogen atmosphere for 12 h. The reaction was then filtered through a pad of celite and washed with DCM (3 x 20 mL). The filtrate was concentrated to provide the title compound which was used in the subsequent step without further purification. 4N HCl in dioxane (1.7 mL, 7 mmol, 10 eq.) was added to residue (546 mg, 0.7 mmol, 1 eq.) at 0˚C. The reaction was stirred for 12 h at room temperature. The solvent was evaporated to provide the title compound. Yield 535 mg (quant.) as a clear oil. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 5.12 (d, *J* = 11.4 Hz, 1H), 4.95 (d, *J* = 7.5 Hz, 1H), 4.87 (d, *J* = 6.9 Hz, 1H), 4.61 (d, *J* = 6.0 Hz, 1H), 3.18 (s, 3H), 3.11 (s, 3H), 2.80 (s, 3H), 2.54 (p, *J* = 6.7 Hz, 1H), 2.41 (d, *J* = 9.8 Hz, 1H), 2.27 – 2.01 (m, 2H), 1.58 (ddd, *J* = 10.3, 7.4, 2.9 Hz, 1H), 1.53 – 1.44 (m, 2H), 1.40 – 1.28 (m, 1H), 1.13 – 0.83 (m, 42H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 171.8, 170.3, 170.2, 170.1, 168.3, 168.1, 78.4, 77.7, 65.6, 59.1, 35.5, 34.9, 33.5, 32.9, 30.4, 30.3, 29.5, 29.4, 26.5, 24.5, 24.4, 18.8, 18.5, 18.3, 18.0, 18.0, 17.9, 16.9, 15.1, 13.7, 11.4, 9.9. HRMS (ESI) *m/z* $[M-HCl+Na]^+$ for $C_{36}H_{65}N_3O_{10}Na$ 722.4568, found 722.4544.



(3S,6R,9S,12R,15S,18R)-3,9,15-tri((S)-sec-butyl)-6,12,18-triisopropyl-4,10,16-trimethyl-1,7,13-trioxa-4,10,16-triazacyclooctadecane-2,5,8,11,14,17-hexaone (**Enniatin A**). 1-chloro-*N*,*N*,2-trimethylprop-1-en-1-amine (Ghosez's Reagent, 0.12 mL, 0.9 mmol, 1.2 eq.) was added to a stirred solution of **H** (535 mg, 0.9 mmol, 1 eq.) in DCM (145 mL) at 0˚C. The reaction was stirred for 15 minutes at this temperature before the addition of Hunig's base (0.38 mL, 2.1 mmol, 3 eq.). The reaction was stirred at room temperature for 18 h. Solvent was removed via rotary evaporation until the volume totaled 10 mL. 1N HCl (20 mL) was added and the organic layer was washed with water (20 mL) and saturated $NaHCO_3$ (20 mL). The organic layer was dried over Na_2SO_4 and the solvent removed. The residue was purified via preparative HPLC chromatography to provide **Enniatin A** as an amorphous white solid (255 mg, 42%). Purity: 98.05%, T_R = 9.77 min. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 5.12 (d, *J* = 8.6 Hz, 3H), 4.63 (d, *J* = 9.5 Hz, 3H), 3.10 (s, 9H), 2.31 – 2.20 (m, 3H), 2.13 – 2.02 (m, 3H), 1.52 – 1.39 (m, 3H), 1.13 – 1.04 (m, 3H), 1.02 – 0.92 (m, 29H), 0.87 (t, *J* = 7.4 Hz, 10H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.5, 169.3, 75.8, 62.0, 34.1, 33.3, 30.0, 25.5, 18.8, 18.6, 16.4, 11.1. HRMS (ESI) *m/z* $[M+H]^+$ for $C_{36}H_{63}N_3O_9$ 682.4643, found 682.4647.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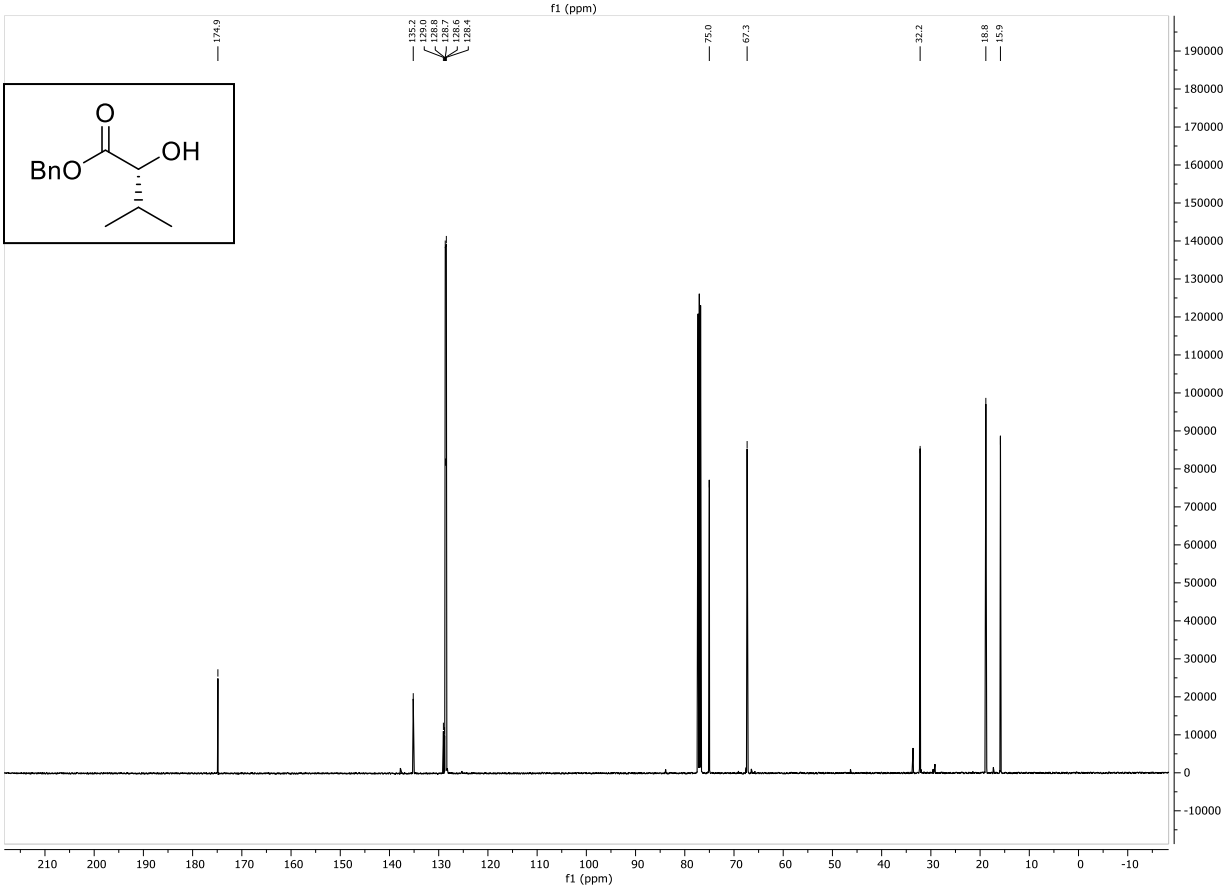
0.93
0.98
2.96
2.95
f1 (ppm)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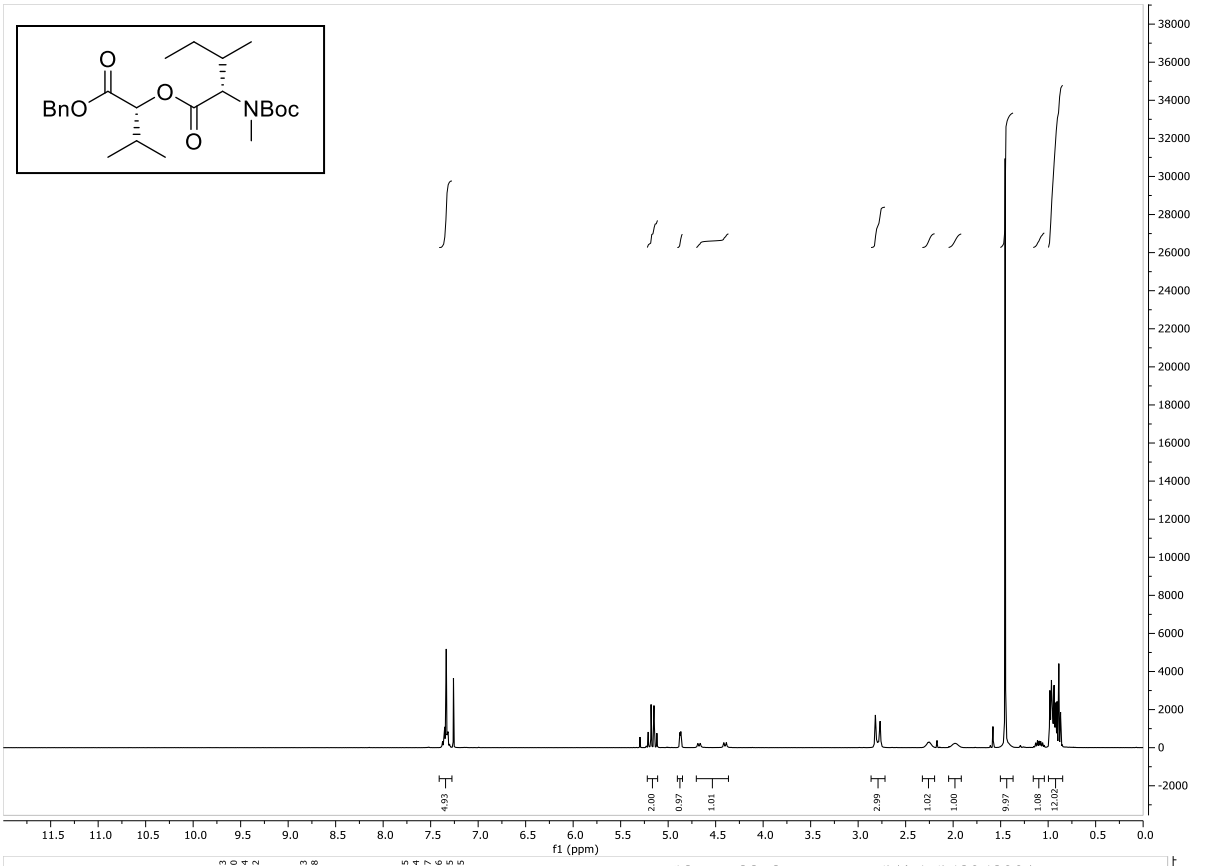
128.8
128.7
128.6
128.4
75.0
67.3
32.2
18.8
15.9
O
BnO
OH
f1 (ppm)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O
O
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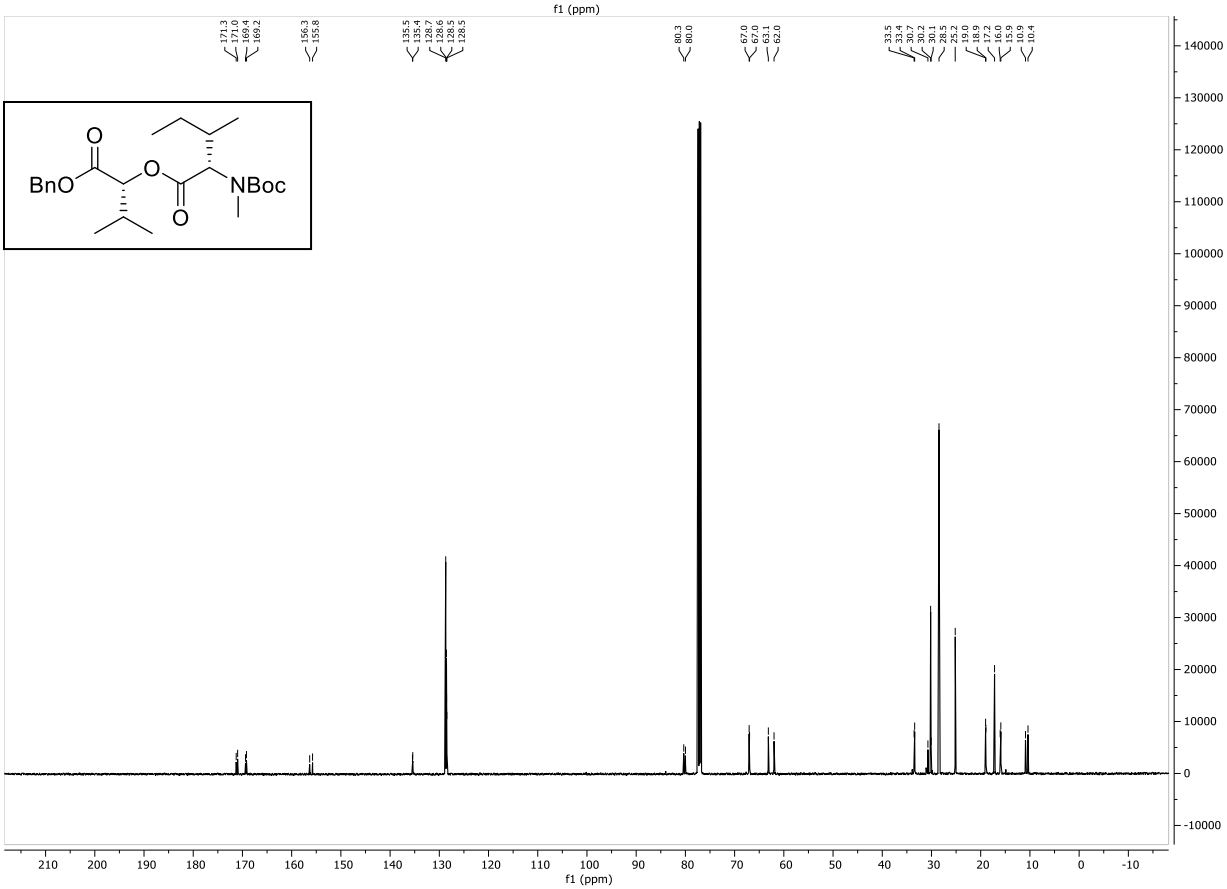
2.99
1.02
1.00
9.97
1.08
12.03
f1 (ppm)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O
O
O
NBoc
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171.0
169.4
169.2
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155.8
135.5
135.4
128.7
128.6
128.5
128.5
80.3
80.0
67.0
67.0
63.1
62.0
33.5
33.4
30.7
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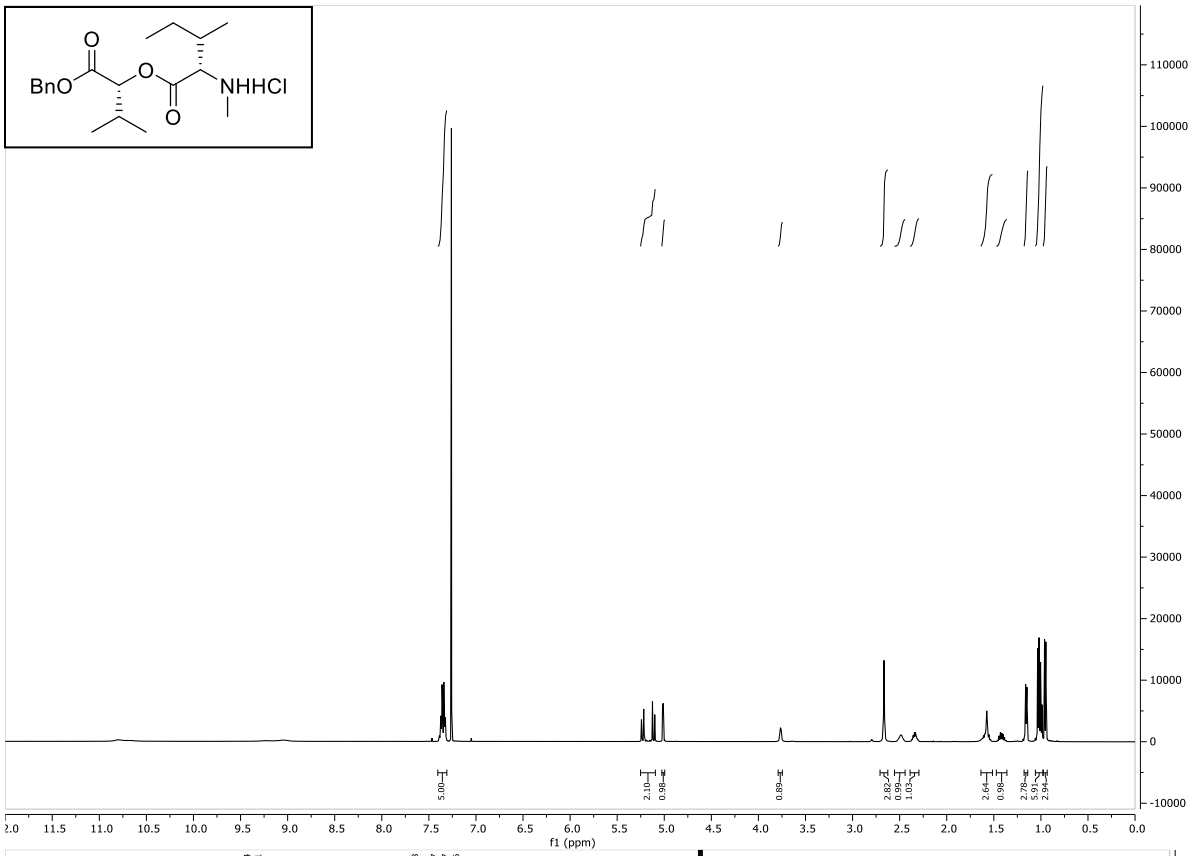
25.2
19.0
18.9
17.2
16.0
15.9
10.9
10.4
f1 (ppm)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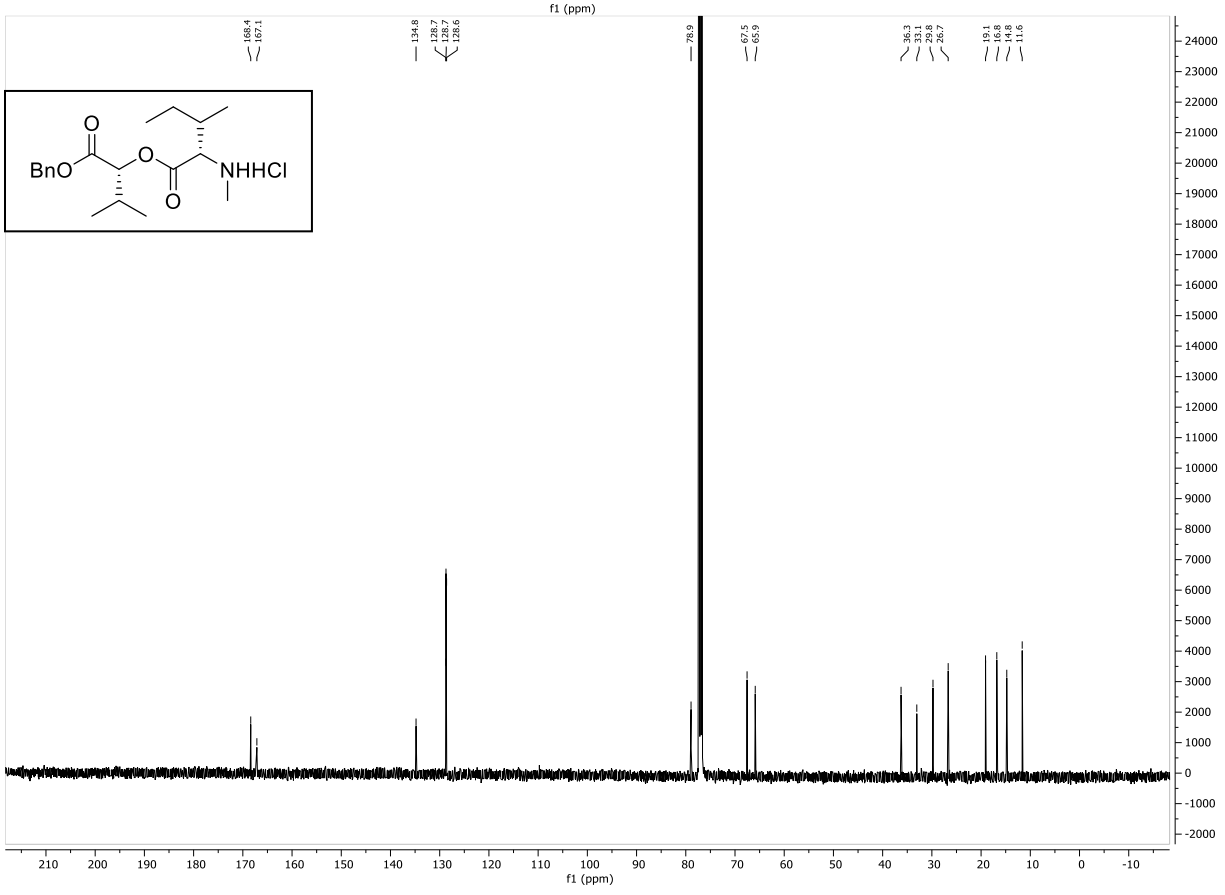
O
O
NHHCl
f1 (ppm)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167.1
134.8
128.7
128.7
128.6
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67.5
65.9
36.3
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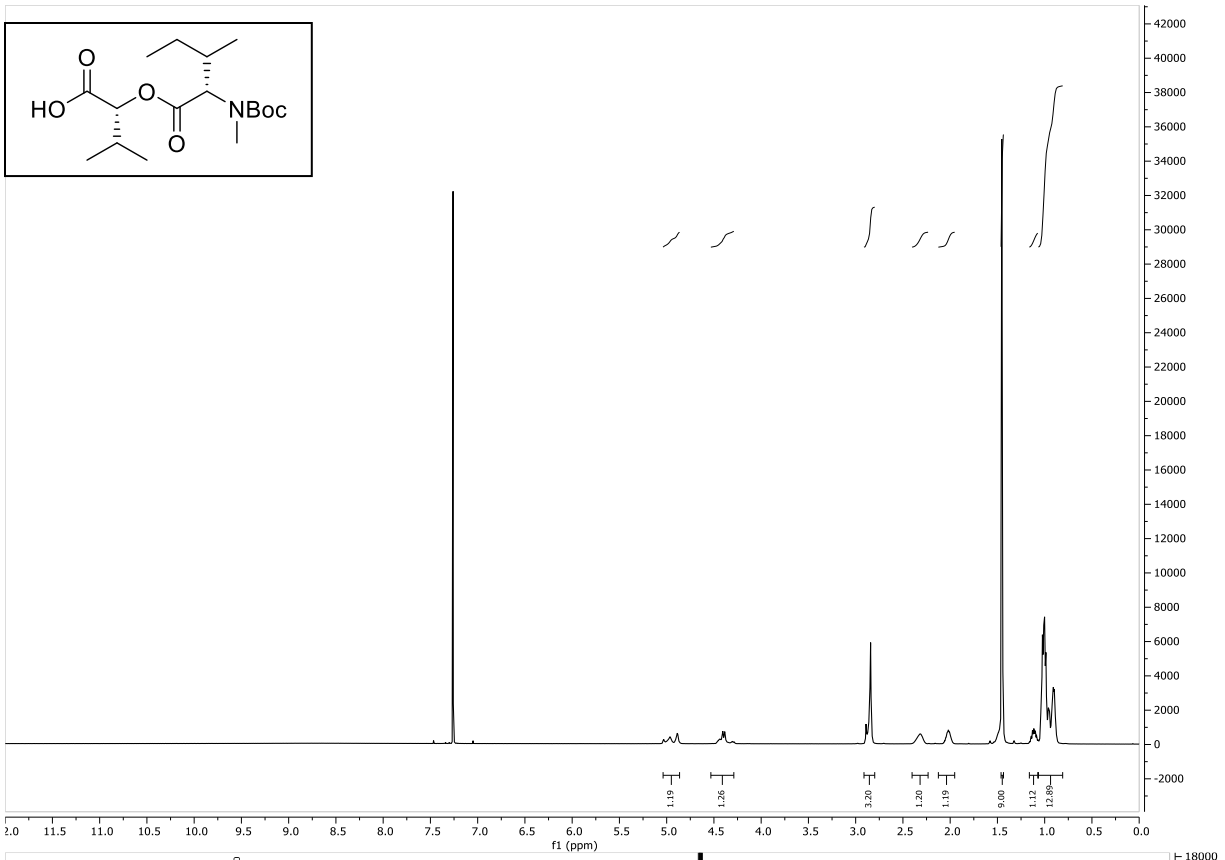
29.8
26.7
19.1
16.8
14.8
11.6
BnO
O
O
O
NHHCl
f1 (ppm)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1.20
1.19
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1.12
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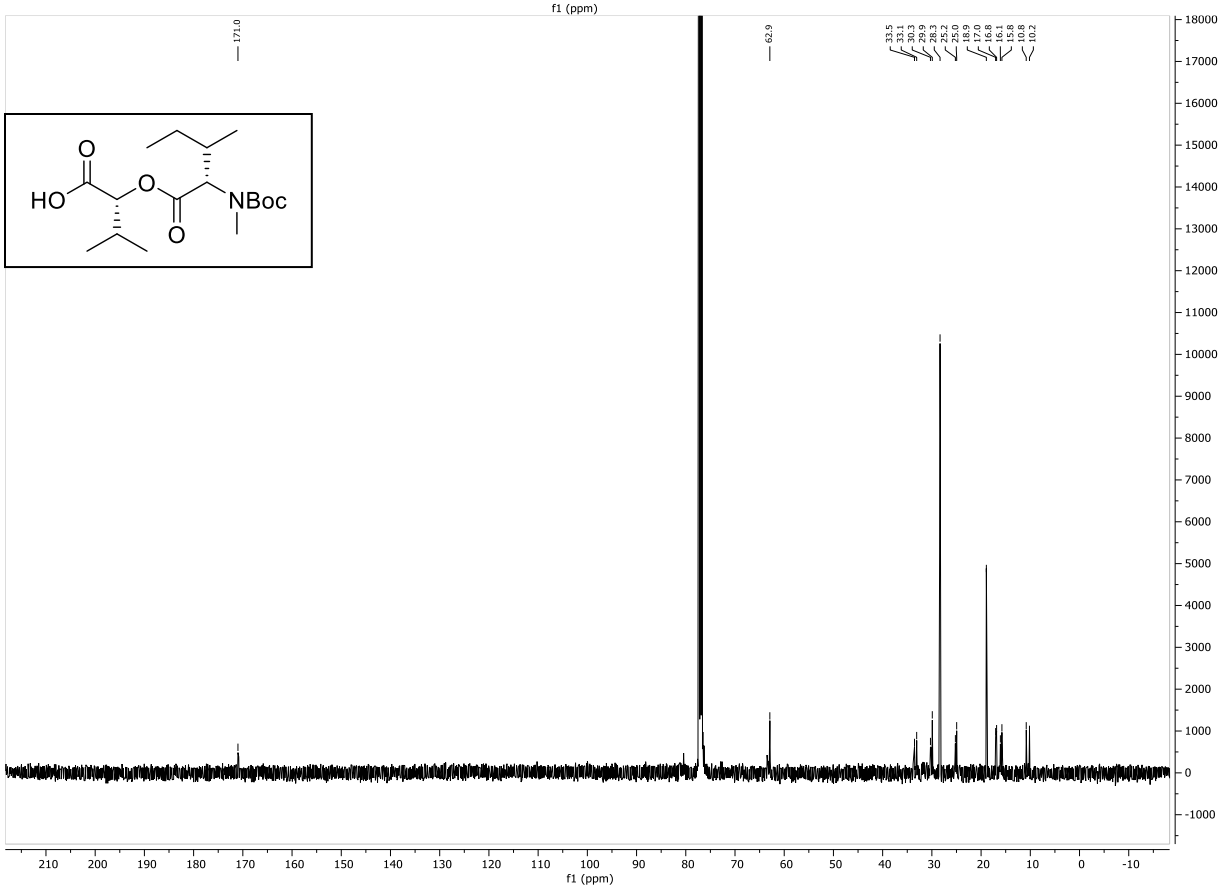
f1 (ppm)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62.9
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33.1
30.3
29.9
28.3
25.2
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18.9
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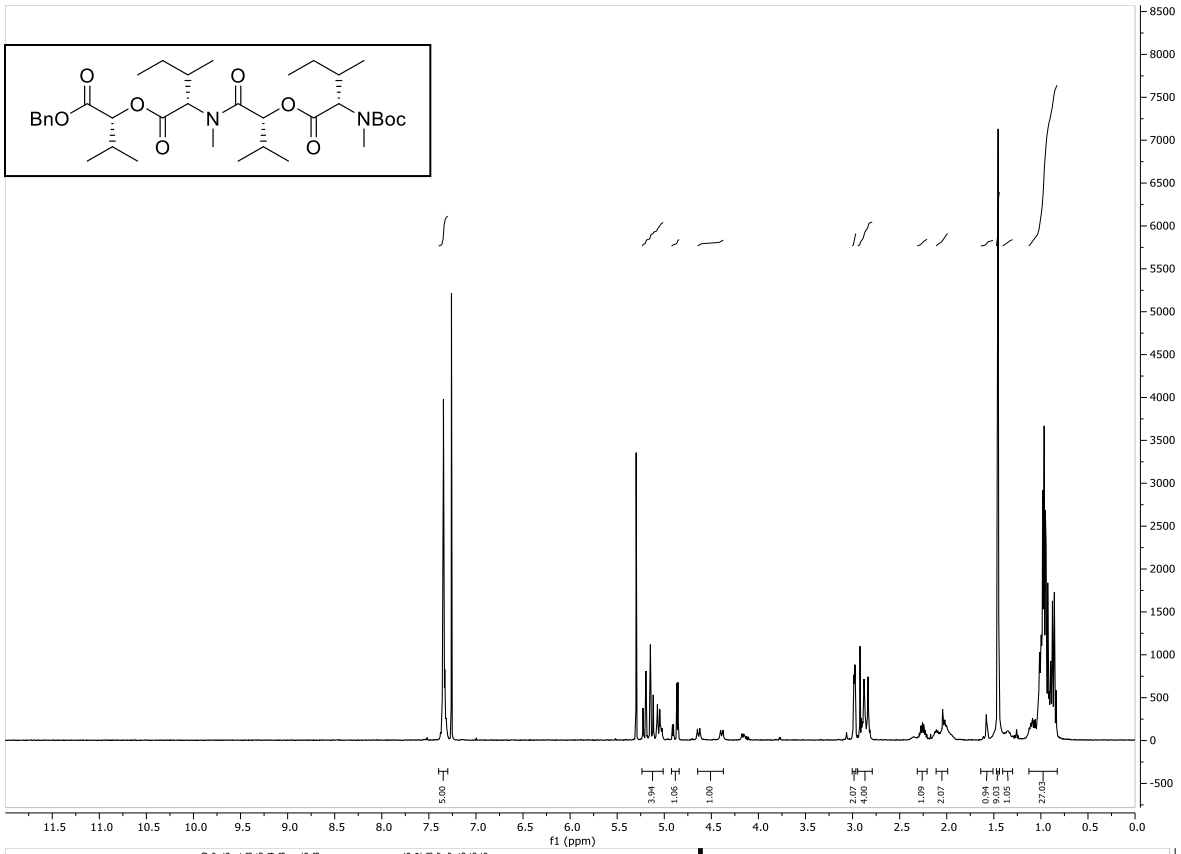
16.8
16.1
15.8
10.8
10.2
HO
O
O
O
NBoc
f1 (ppm)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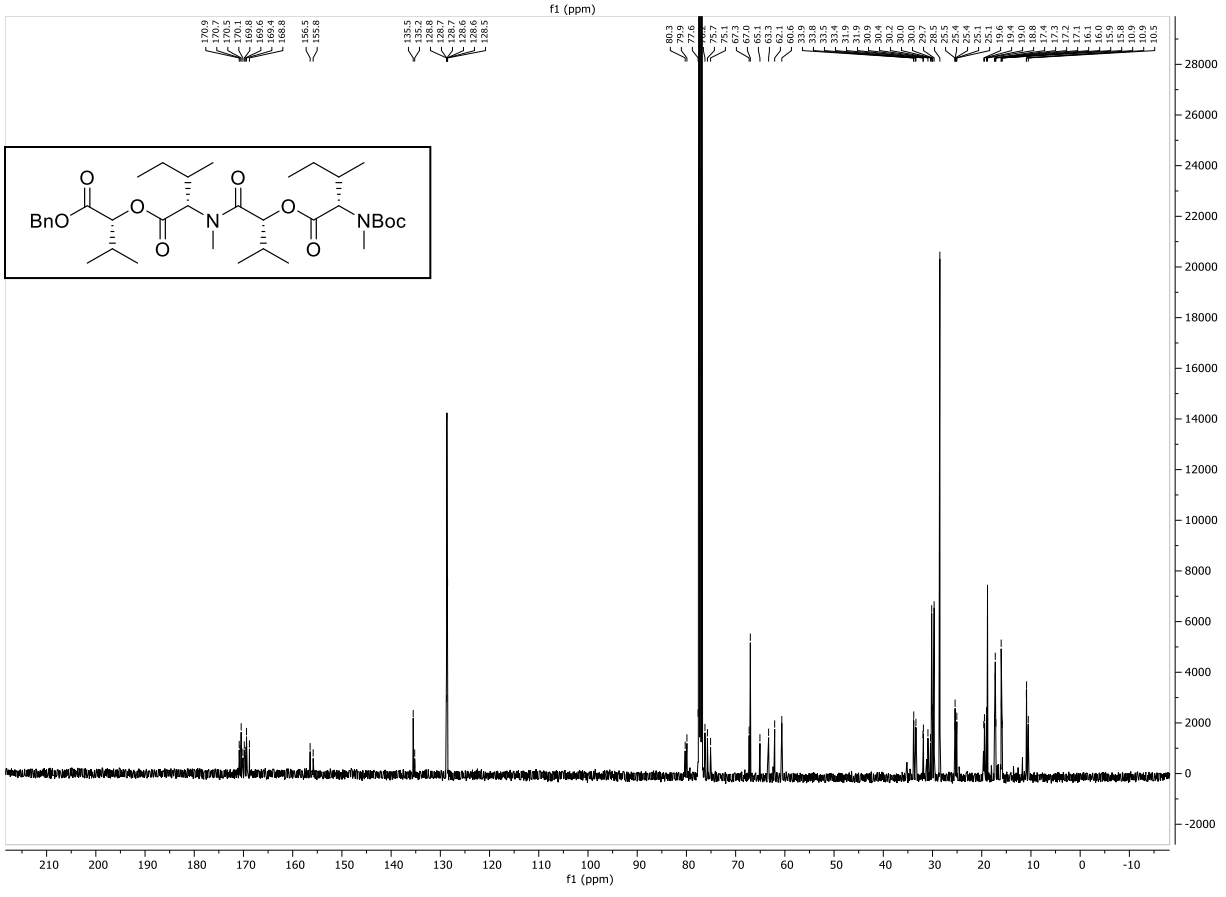
O
N
O
O
O
NBoc
f1 (ppm)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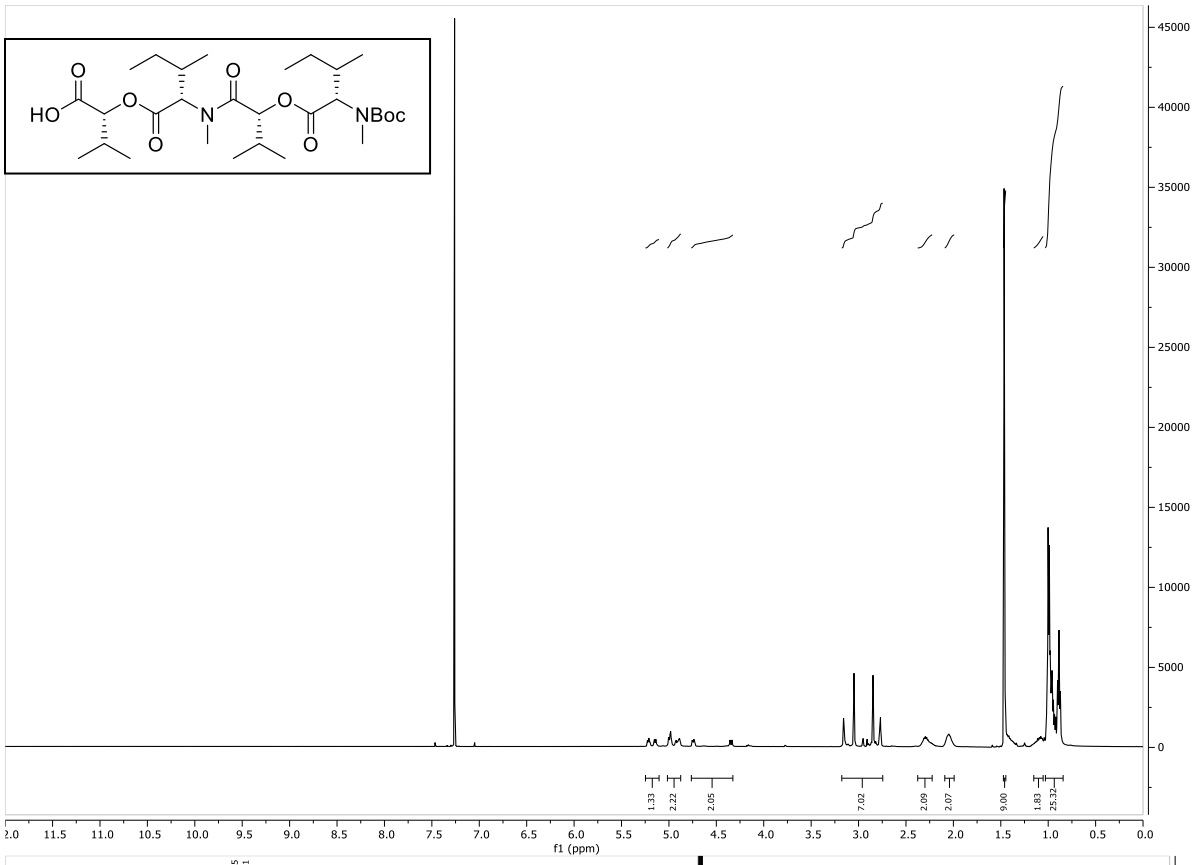
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O
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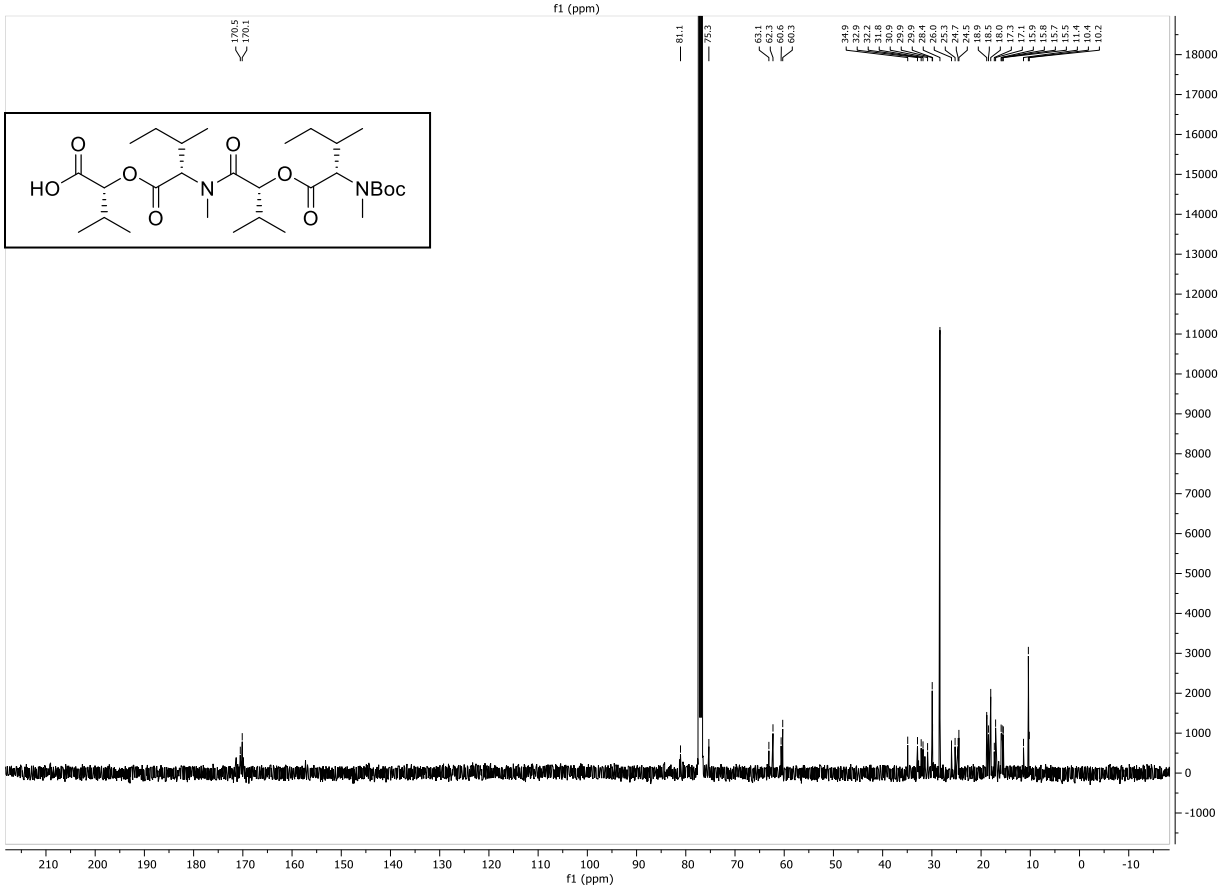
O
O
O
NBoc
f1 (ppm)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O
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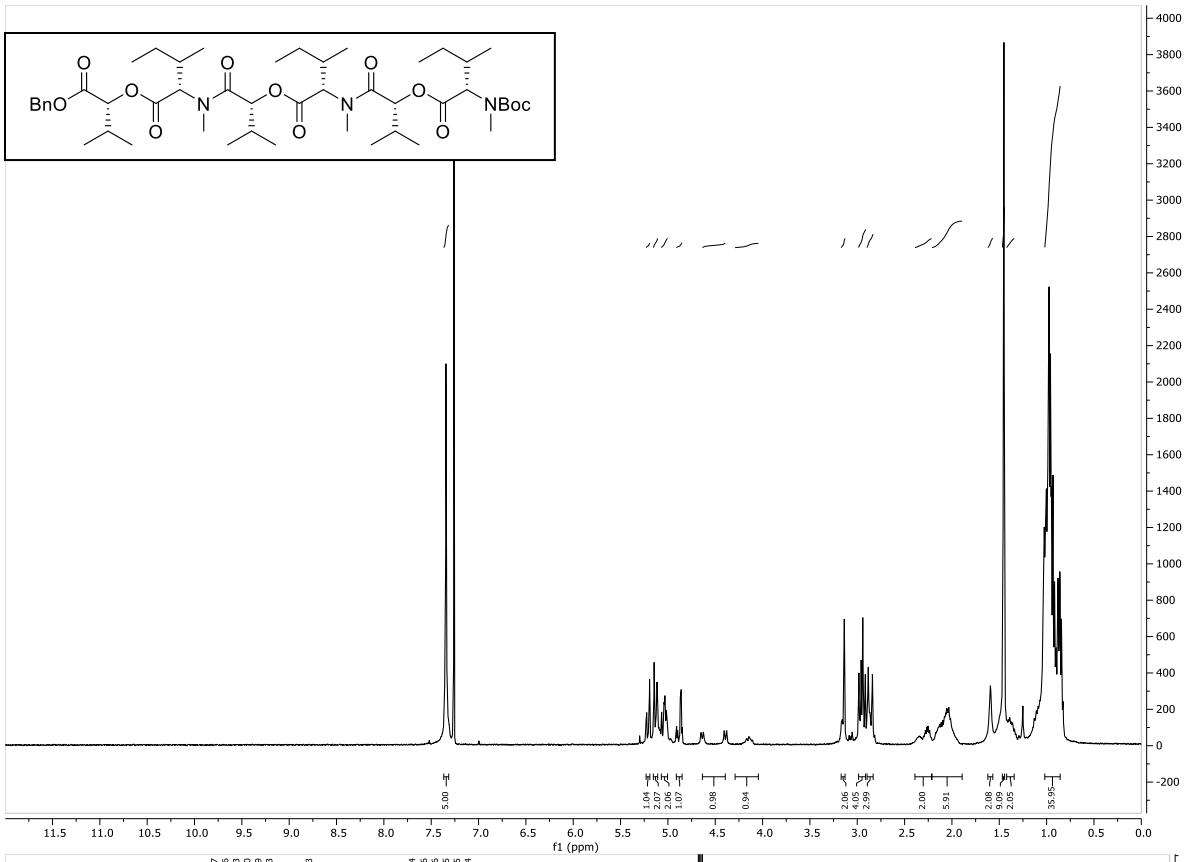
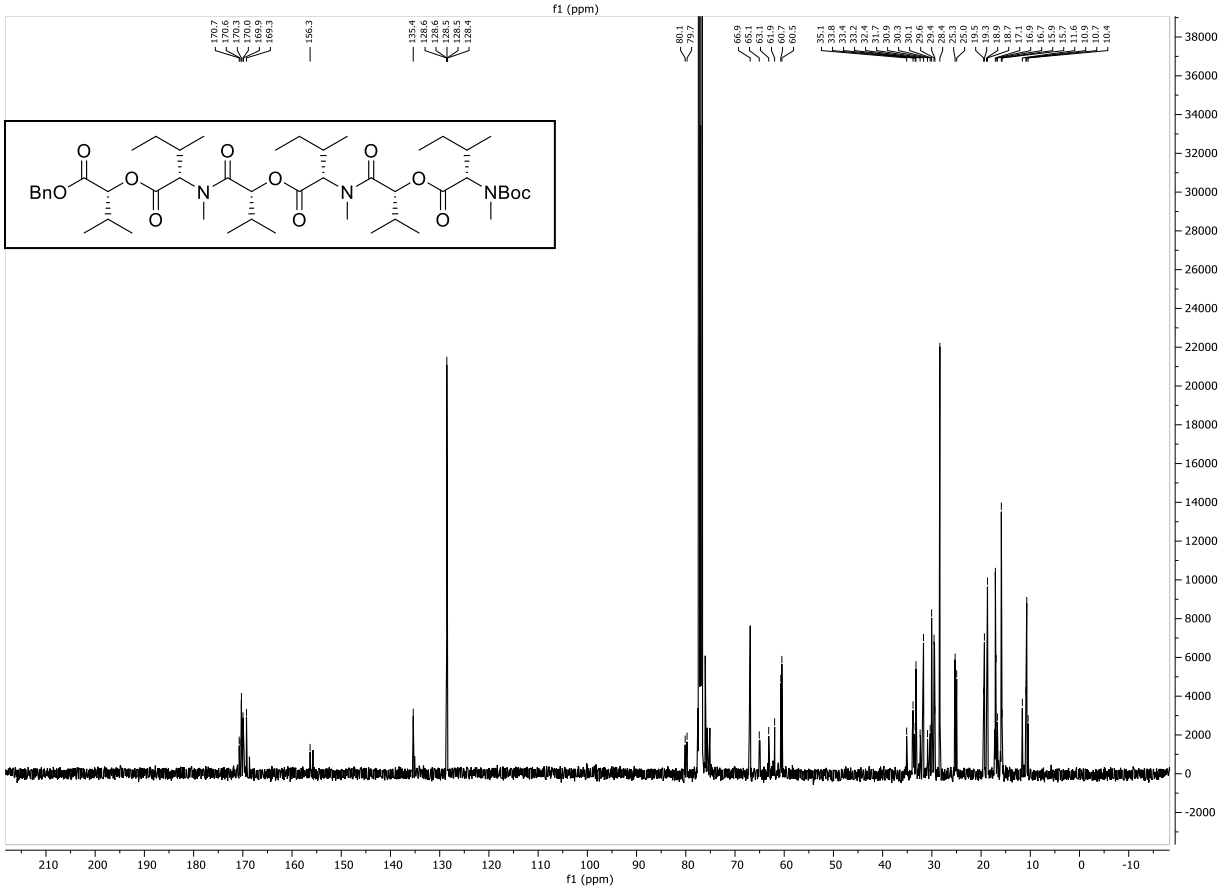
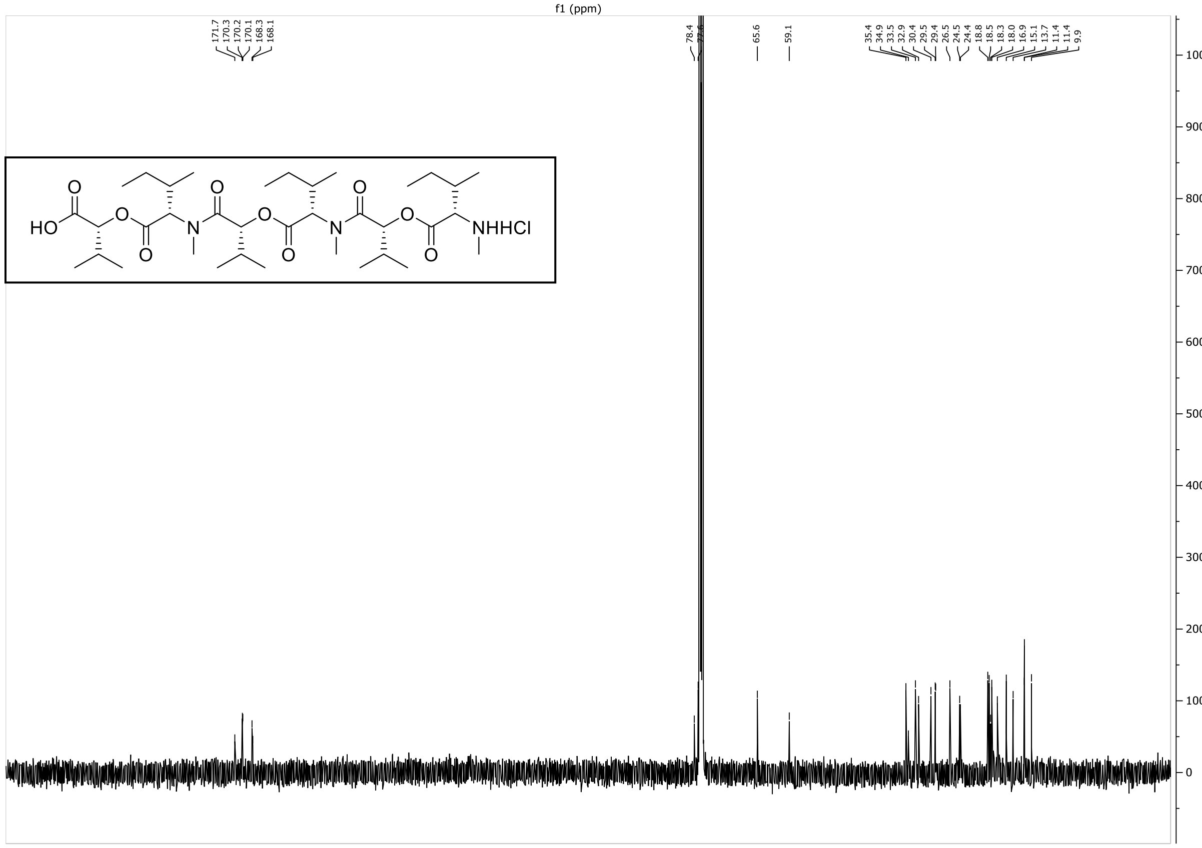
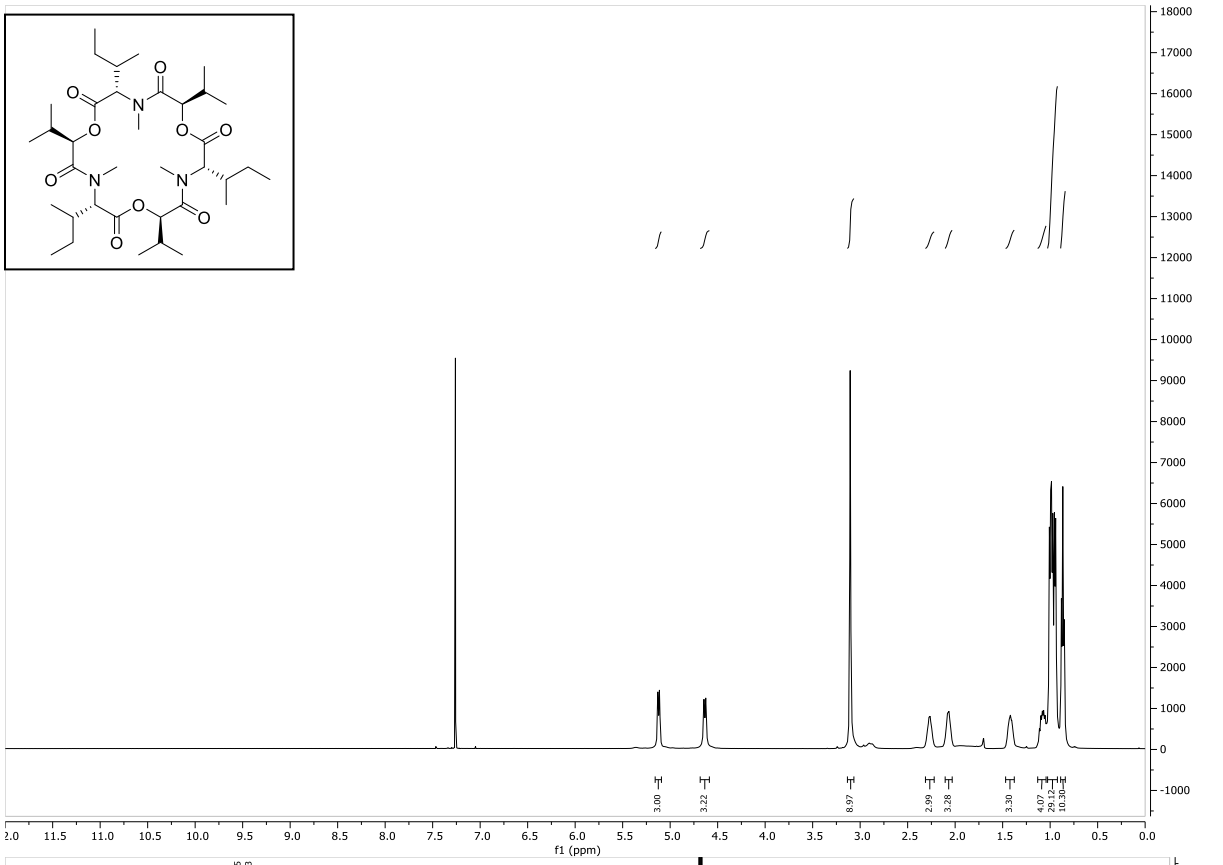
O
O
O
NBoc
f1 (ppm)


BnO
NBoc
f1 (ppm)


BnO
NBoc
f1 (ppm)

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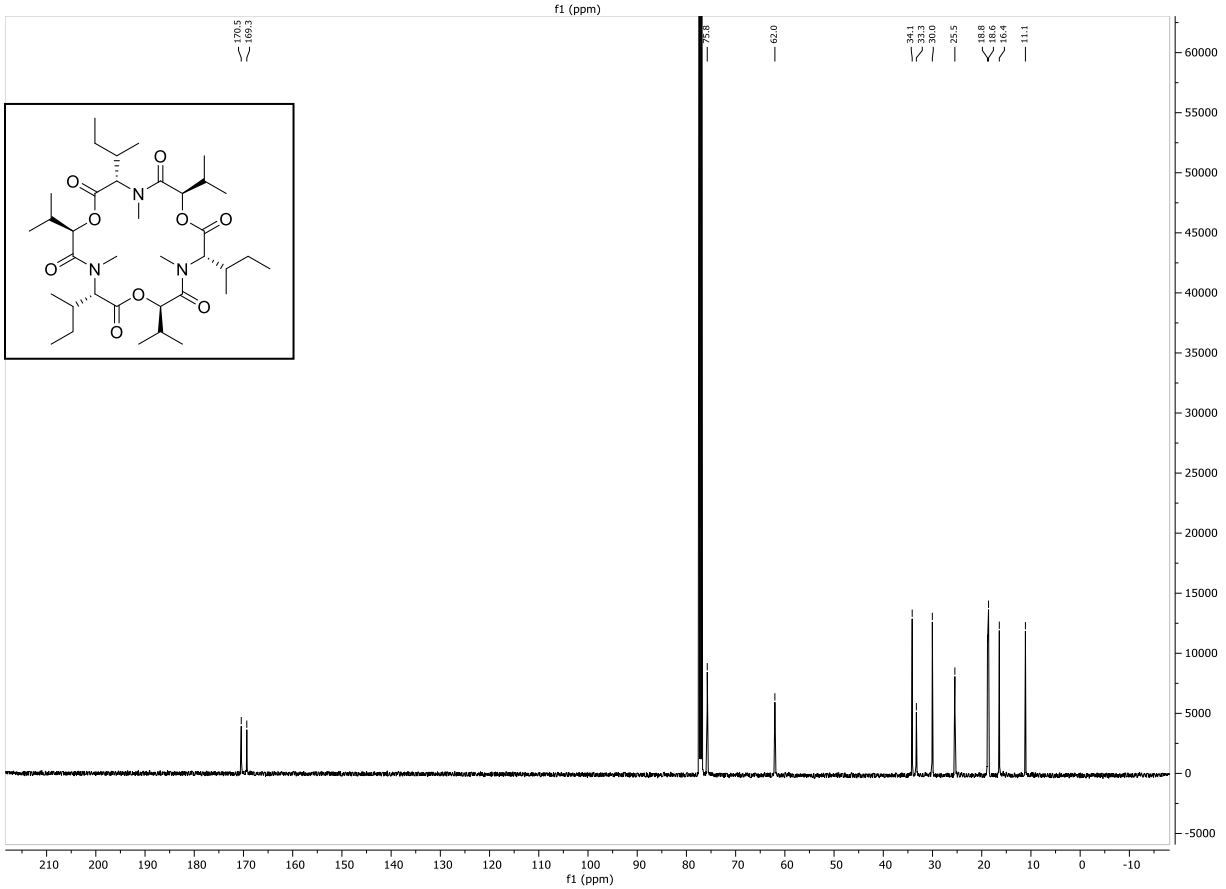
f1 (ppm)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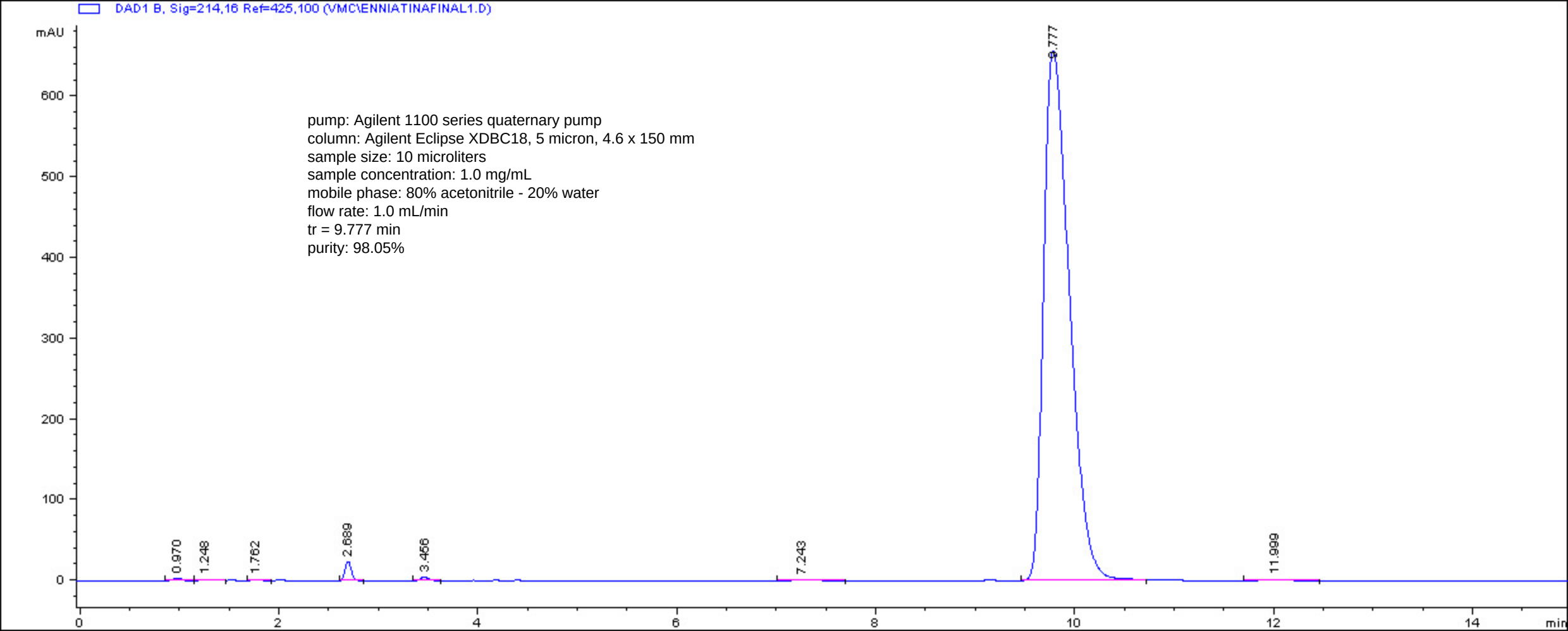
f1 (ppm)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300
200
100
0
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sample size: 10 microliters
sample concentration: 1.0 mg/mL
mobile phase: 80% acetonitrile - 20% water
flow rate: 1.0 mL/min
tr = 9.777 min
purity: 98.05%
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1.248
1.762
2.689
3.456
7.243
9.777
11.999
0
2
4
6
8
10
12
14
min