## Potent antitumor agents: Sansalvamide A derivatives

Katerina Otrubova<sup>1</sup>, Thomas J. Styers<sup>1</sup>, Po-Shen Pan<sup>1</sup>, Rodrigo Rodriguez<sup>1</sup>,

Kathleen L. McGuire<sup>2</sup>\*, Shelli R. McAlpine<sup>1</sup>\*

<sup>1</sup>5500 Campanile Drive, Department of Chemistry and Biochemistry, San Diego, CA 92182-

1030, <sup>2</sup>5500 Campanile Drive, Department of Biology, San Diego, CA 92182-4630

## **Synthesis**

**General Remarks.** All coupling reactions were performed under argon atmosphere with the exclusion of moisture. All reagents were used as received. Anhydrous methylene chloride Dri Solv (EM) and Anhydrous Acetonitrile Dri Solv (EM) were bought from VWR, and were packed under nitrogen with a septum cap. Diisopropylethylamine (DIPEA) was purchased from Aldrich, packaged under nitrogen in a sure seal bottle. The coupling agent HATU and PyAOP were purchased from Perspective:Applied Biosystems at 850 Lincoln Center Dr. Foster City, CA 94404, Telephone: 1-800-327-3002. The coupling agents TBTU, and PyBROP were purchased from NovaBiochem. DEPBT [3(diethoxyphosphoryloxy)-1,2,3-bezotriazine-4(3H)] was purchased from Aldrich (order number 49596-4). The <sup>1</sup>H NMR spectra were recorded on a Varian at 500 MHz. LCMS were obtained at San Diego State University using HP1100 Finnnigan LCQ. Flash column chromatography was performed on 230-400 mesh 32-74  $\mu$ m 60 Å silica gel from Bodman Industries.

**General peptide synthesis.** All peptide coupling reactions were carried out under argon with dried solvent, using methylene chloride for dipeptide and tripeptide couplings and acetonitrile for all other peptide couplings. The amine (1.1 equivalents) and acid (1 equivalent) were weighed into a dry flask along with 3 equivalents of DIPEA and 1.1 equivalents of TBTU.\* Anhydrous methylene chloride was added for a 0.1M solution. The solution was stirred at room temperature and reactions were monitored by TLC. Reactions were run for 4 - 24 hours before working up by washing with saturated ammonium chloride. (Note: if acetonitrile was used for the reaction, methylene chloride was added to reaction upon workup and then the resulting solution was washed with ammonium chloride). After back extraction of aqueous layers with methylene chloride, organic layers were combined, dried over sodium sulfate, filtered and concentrated. Flash chromatography using 0-100% ethyl actetate-hexane gave our desired peptide.

\* Some coupling reactions would not go to completion using only TBTU and therefore HATU, and/or DEPBT were used. In a few cases 1.1 equivalents of all three coupling reagents were used.

**General Amine deprotection.** Amines were deprotected using 20% TFA in methylene chloride (0.1M) with two equivalents of anisole. The reactions were monitored by TLC, where the TLC sample was first worked up in a mini-workup using DI water and methylene chloride to remove TFA. Reactions were allowed to run for 1-2 hours and then concentrated *in vacuo*.

**General Acid deprotection.** Acids were deprotected using 4 equivalents of lithium hydroxide (or enough was added until  $pH=\sim11$ ) in methanol (0.1 M). The peptide was placed in a flask, along with lithium hydroxide and methanol and stirred overnight. Within 12 hours the acid was usually deprotected. Work-up of reactions

involved the acidification of reaction solution using HCl to pH = 1. The aqueous solution was extracted three times with methylene chloride, and the combined organic layer was dried, filtered and concentrated *in vacuo*.

Macrocyclization procedure (in situ). All pentapeptides were acid and amine deprotected using HCl (8 drops per 0.3mmols of linear pentapeptide) in THF (0.05M). Addition of anisole (2 equivs) was added to the reaction and the reaction was stirred at room temperature. The reaction typically took 4 days, although it was checked after 24 hours via LCMS and TLC. The LCMS would usually show the reaction was ~50% complete after the first day. Addition of four drops of HCl per 0.3mmoles of pentapeptide, stirring at RT overnight and checking the reaction gave an LCMS that showed ~75% completion. An additional 2 drops of HCl were added on the third day. On the fourth day verification of the presence of the free amine and free acid and disappearance of the starting linear protected pentapeptide permitted workup. The reaction was concentrated in vacuo and the crude, dry, double deprotected peptide (free acid and free amine) was dissolved in a minimum solution of THF: acetonitrile: methylene chloride (2:2:1 ratio). Three coupling agents were used: DEPBT, HATU, and TBTU (~0.5 to 0.75 equivalents each). These coupling agents were dissolved in a calculated volume of dry 40% THF, 40% acetonitrile, and 20% methylene chloride that would give a 0.007 M solution when included in the volume used for the deprotected peptide. The coupling agents were then added to the deprotected peptide solution. DIPEA (6 equivs or more in order to neutralize the pH) were then added to the reaction. It is important to recognize that the coupling agents are typically not very soluble in acetonitrile, which is why a combination of solvents is used.

After 1 hour, TLC and LCMS (where the LCMS sample was worked up prior to injection) were taken and a clear distinct product spot was visible. The comparison for Rf value in the product spot on TLC was the protected linear pentapeptide. The reactions were always complete after 2 hours, and monitoring the starting material deprotected pentapeptide via LCMS was the easiest method of determining completion. Upon completion, the reaction was worked up by washing with ammonium chloride. After back extraction of aqueous layers with large quantities of methylene chloride, the organic layers were combined, dried over sodium sulfate, filtered and concentrated. All macrocycles were purified by initially running a crude plug of compound with EthylAcetate/Hexane solvent system on silica gel, then running a column on the isolated product. Finally, if necessary reverse phase HPLC was used for additional purification using a gradient of acetonitrile and DI water with 0.1% TFA.

## **BioAssays**

## **Thymidine Uptake Assays**

Proliferation of the HT29 colon cancer cell line was tested in the presence and absence of the compounds using <sup>3</sup>H-thymidine uptake assays. Cells treated with the compounds were compared to DMSO-treated controls for their ability to proliferate as indicated by the incorporation of <sup>3</sup>H-thymidine into their DNA. Cells were cultured in 96 well plates at a concentration of 50,000 cells/well. The media was McCoy's 5a with L-glutamine, 10% fetal bovine serum and antibiotics. After incubation for approximately 6 hours, the compounds were added. The compounds were dissolved in DMSO at a final concentration of 2 mM and tested at the concentrations indicated in the manuscript. The DMSO concentration was held constant in all wells at 2.5%. After the cells had been incubated with the compounds for 24 hours, 1µCi <sup>3</sup>H-thymidine per well was added and the cells were cultured for an additional 16 hours (for the cells to have a total of 40 hours with the drug), at which time the cells were harvested using a PHD cell harvester from Cambridge Technology Incorporated. The samples were then counted in a scintillation counter for 2 minutes each using ScintiVerse universal scintillation fluid from Fisher. Decreases in <sup>3</sup>H-thymidine incorporation, as compared to controls, are an indication that the cells are no longer progressing through the cell cycle or synthesizing DNA, as is shown in the studies presented.





Compound 1 Cyclized 1a-2a-3a-4a-5a (MW=585)



Compound 2 Cyclized 1a-2a-3c-4a-5a (MW=599)



Compound 3 Cyclized 1b-2b-3d-4b-5b (MW=599)



Compound 4 Cyclized 1b-2a-3a-4a-5a (MW=585)



Compound 5 Cyclized 1a-2b-3a-4a-5a (MW=585)



Compound 6 Cyclized 1a-2a-3b-4a-5a (MW=585)



Compound 7 Cyclized 1a-2a-3a-4b-5a (MW=585)



Compound 8 Cyclized 1b-2b-3b-4b-5a (MW=585)



Compound 9 Cyclized 1a-2a-3d-4a-5a (MW=600)



Compound 10 Cyclized 1a-2d-3a-4a-5a (MW=600)



Compound 11 Cyclized 1a-2c-3a-4a-5a (MW=600)



Compound 12 Cyclized 1b-2b-3b-4b-5b (MW=600)



Compound 1 NMR in CD<sub>3</sub>OD Cyclized 1a-2a-3a-4a-5a



Compound 5 NMR in CD<sub>3</sub>OD Cyclized 1a-2b-3a-4a-5a



Compound 6 NMR in CD<sub>3</sub>OD Cyclized 1a-2a-3b-4a-5a