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# Synthesis of Sansalvamide A derivatives and their cytotoxicity in the MSS colon cancer cell line HT-29

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**Abstract**—We report the synthesis of thirty-six Sansalvamide A derivatives, and their biological activity against colon cancer HT-29 cell line, a microsatellite stable (MSS) colon cancer cell-line. The thirty-six compounds can be divided into three subsets, where the first subset of compounds contains L-amino acids, the second subset contains D-amino acids, and the third subset contains both D- and L-amino acids. Five compounds exhibited excellent inhibitory activity (>75% inhibition). The structure–activity relationship (SAR) of the compounds established that a single D-amino acid in position 2 or 3 gave up to a 10-fold improved cytotoxicity over Sansalvamide A peptide. This work highlights the importance of residues **2** and **3** and the role of D-amino acids in the extraordinary SAR for this compound class.


## 1. Introduction

Carcinogenesis in the colonrectum is thought to occur through two different pathways. The existing model suggests that 80–85% of colon cancers involve chromosomal instability, where point mutations are found in loci within RAS, p53, and other checkpoint proteins.[1] The remaining 15–20% of colon cancers involve a loss in the DNA mismatch repair system, which leads to point mutations in repetitive sequences. These repetitive sequences are known as microsatellites and occur in several important growth regulators. Mutations in these microsatellites lead to instability within these areas, which ultimately impacts the function of these growth regulator proteins. The two pathways are usually referred to as having microsatellite stability (MSS) or microsatellite instability (MSI), respectively.

Currently, only the MSS colon cancers are known to respond to chemotherapeutic drugs. The drug of choice for treatment, 5-fluorouracil (5-FU) [$IC_{50}$ = 5 μM], has severe side effects, making it desirable to develop a drug with improved efficacy. Because MSI colon cancers do not respond to 5-FU,[1,2] or to current chemotherapeutic drugs, finding new structures that target both cancer pathways would be very valuable.

It has been shown that Sansalvamide A (San A), which is a depsipeptide isolated from a marine fungus (*Fusarium* ssp.), exhibits anti-tumor activity.[3–5] Syntheses and evaluation of all-peptide analogs against HCT-116 (MSI) have revealed that several derivatives have greater potency against HCT-116 than the natural depsipeptide.[6–8] Previous work by our group[9] has shown that several derivatives are potent against HT-29 (MSS). Determining the structure–activity relationship (SAR) of this compound class will facilitate understanding of San A's mechanism of action in MSS cell lines. Further, very few analogs have been made (to date ~30 derivatives have been reported by us and others). Here, we describe the synthesis of 36 compounds, where 18 compounds are new structures not previously reported. The evaluation of these derivatives provides a clear structural motif

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important for cytotoxicity. Comparison of these to the current drug on the market emphasizes the relevance of San A as a new therapeutic class of compounds.

## 2. Results and discussion

### 2.1. Synthetic strategy

San A is composed of four hydrophobic amino acids and one hydrophobic hydroxy-acid. We report here the synthesis of thirty-six San A derivatives. All derivatives are peptide analogs, which involve the exchange of the hydroxy acid in position 4 to an amino acid (Fig. 1). We used a solution phase synthesis route because of the hydrophobic nature of the residues. Our convergent approach, involving two fragments (Fig. 1),[8] is amenable to inserting L- and D-amino acids systematically within San A. This route was also designed to facilitate large-scale synthesis in extensive biological studies.

Synthesis of thirty-six San A derivatives was completed using amino acids shown (Fig. 2) via the synthetic route outlined.

### 2.2. Synthesis

Using 2(1-*H*-benzotriazole-1-yl)-1,1,3-tetramethyluronium tetrafluoroborate (TBTU), and diisopropylethylamine (DIPEA), acid protected residue **1(a, b)** and N-Boc protected residue **2(a–i)** (Fig. 2) were coupled to give the dipeptides 1-2-Boc (80–94% yield). Deprotection of the amine on residue **2** using TFA gave the free amines **1** and **2** (~quantitative yields). Coupling of this dipeptide to monomer **3(a–h)** gave the desired tripeptides (*Fragment 1*) in good yields (80–95%).[11] The synthesis of *Fragment 2* was completed by coupling residue **4(a–d)** to residue **5(a–e)** to give the dipeptide **4**- and **5**-Boc (90–95% yield). The amine was deprotected on *Fragment 1* using TFA and the acid was deprotected in *Fragment 2* using lithium hydroxide. *Fragment 1* and *Fragment 2* were coupled using multiple coupling agents [8,10,12,13] yielding thirty-six examples of linear pentapeptides (66–90% yield).[11]

Cyclizing large macrocycles is usually very challenging, and typically the yields are low. The recent discovery of high-yielding conditions[14] provided most of the final macrocycles in good yields. Dissolving the linear pentapeptide in THF (0.05 M), addition of 2 equiv of anisole, and approximately eight drops concentrated HCl per

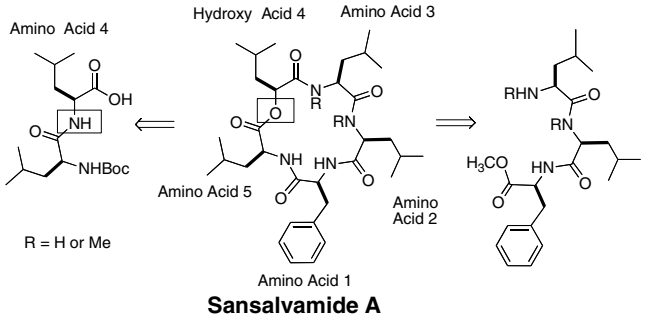


**Figure 1.** Retrosynthetic strategy.

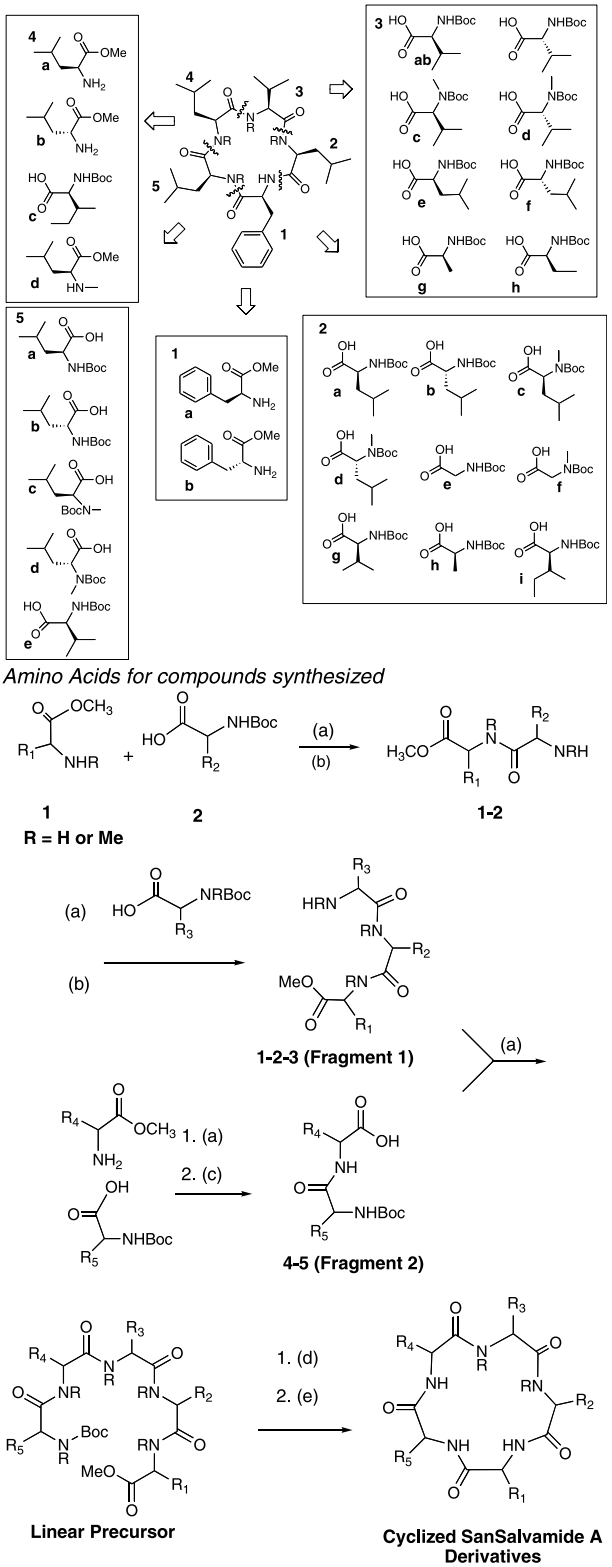


**Figure 2.** Synthesis of macrocycles. Reagents: (a) coupling agent (TBTU (1.2 equiv), and/or HATU (0.75 equiv)[10]), DIPEA (3 equiv), $CH_2Cl_2$ (0.1 M); (b) TFA (20%), anisole (2 equiv), $CH_2Cl_2$; (c) LiOH (4 equiv), MeOH; (d) HCl in THF (0.05 M), anisole (2 equiv); (e) HATU (0.7 equiv), DEPBT (0.7 equiv), TBTU (0.7 equiv), DIPEA (6 equiv), THF/$CH_3CN$/DCM (2:2:1) 0.007 M.

0.3 mmol of linear pentapeptide led to partially deprotected amine within 24 h. Four drops of HCl per 0.3 mmol of peptide were added. The reaction mixture was allowed to stir at room temperature and then checked after 24 h by LC–MS. Typically deprotection of the acid and amine was complete within four days.[15] Upon completion, the reaction is concentrated in vacuo and dried on the high-vac. The dried, crude, free-amine free-acid linear pentapeptide was dissolved in 2:2:1 ratio ofTrypanosoma THF/$CH_3CN$/$CH_2Cl_2$ (0.007 M). Addition of DIPEA (6 equiv), and three coupling agents (HATU, DEPBT, and TBTU 0.7 equiv ea) to reaction gave a clear solution. Reactions were usually complete in 4–6 h.[16] Workup with methylene chloride and ammonium chloride, concentration in vacuo, purification via flash chromotagraphy and subsequently LC–MS, provided the final products (yields ranged from 23% to 90% depending on the substrate).[17]

### 2.3. Structures of macrocyclic derivatives

We designed and synthesized three subsets of San A derivatives. The first subset of compounds contains all L-amino acids (compounds **1**–**18**, Fig. 3).

The second subset contain all D-amino acids (compounds **19**–**24**, Fig. 4).

The third subset contain all both L- and D-amino acids (compounds **25**–**36**, Fig. 5).

### 2.4. Thymidine uptake assays

The SAR was established using San A peptide as a control [compound (**1**)]. [$^3$H]thymidine uptake assays on HT-29 revealed five compounds, (**5**), (**26**), (**27**), (**32**), and (**33**), had greater than 75% inhibitory activity (Chart 1). Comparison of the three subsets showed that *subset 3*, which contains compounds with both D- and L-amino acids, has the most active macrocycles. Significantly, 63% (7/11) of the compounds in *subset 3* exhibit greater than 70% inhibition. By comparison, *subset 1* has 17% (3/18) and *subset 2* has 0% of compounds with greater than 70% inhibition. Clearly, *subset 3* contains the most intriguing pharmacophores.

Compounds (**5**), (**26**), (**27**), (**32**), and (**33**) have $IC_{50}$ values of 32 μM, 34 μM, 19 μM, 7.5 μM, and 33 μM, respectively (San A peptide $IC_{50}$ = 77 μM, and 5-FU = 5 μM) (Chart 2). Compound (32) not only exhibited 89% growth inhibition and a 10-fold increased potency compared to San A peptide [compound (**1**)], it is almost as potent as 5-FU.

### 2.5. Summary of structure–activity relationships

It has been proposed by Silverman and co-workers[7] that the *N*-methyl moieties may be important for antitumor activity of San A derivatives in the HCT 116 (MSI) cell line. With the exception of compound (**5**), our data suggest that in the MSS cancer cell line HT-29, the *N*-methyl moiety does not play a significant role in potency. Rather potency is enhanced when a single L-amino acid is exchanged to the enantiomeric D-amino acid. The four active compounds in *subset 3* are compounds (**26**) and (**33**), which contain D-amino acids in position 2, and compounds (**27**) and (**32**), which contain D-amino acids at position 3. Importantly, compounds (**4**) and (**32**) only differ by the exchange of an L-amino acid to a D-amino acid in position 3, yet (**32**) has a 40-fold increased potency over (**4**) [(**4**) $IC_{50}$ = 302 μM and (**32**) $|C_{50}$ = 7.5 μM].

Further, comparison of compound (**1**) to compounds (**2**), (**4**), and (**15**) shows that the inclusion of N-methyl groups on L-amino acids leads to a decrease in potency when in positions 1, 2, or 3. It also appears the SAR is related to a *single* D-amino acid in positions 2 or 3, but not multiple D-amino acids within the San A structure. For example, compounds (**20**) and (**22**), which contain all D-amino acids including the same *N*-methyl D-amino acids that enhance potency in compounds (**33**) and (**32**), are significantly less active than (**33**) or (**32**).

The only exception to our observed SAR is when a single N-methyl group is in position 5. This compound exhibited an unexpected and significant cytotoxicity. The synthesis of a new generation of compounds and testing on HT-29 will explore this trend. Interestingly, compounds that contain *N*-methyl moieties in position 5 combined with other positions [compounds (**3**), (**6**), and (**7**)] do not exhibit the same level of potency as compound (**5**). Perhaps having a single *N*-methyl in position 5 promotes a conformational change that enhances binding to the biological target.

### 2.6. Significance

In summary, five San A derivatives [(**5**), (**26**), (**27**), (**32**) and (**33**)] exhibit effective potency against an MSS colon cancer cell line (HT-29). Compound (**32**) has potency comparable to that of a current drug on the market (5-FU). Interestingly, *subset 3* contains four of the five most active compounds. This is the first thorough SAR exploration for an MSS colon cancer cell line, and it highlights the importance of compounds containing a single D- and four L-amino acids. The SAR seen in our data provides extraordinary promise in facilitating the design of new, potentially potent San A analogs. Evaluation of these derivatives provides a clear structural motif important for cytotoxicity. Further investigation will refine the roles of D- and *N*-methyl amino acids. Mechanistic and cytotoxicity assays of these compounds against other colon cancer cell lines are underway, and synthesis of next generation derivatives utilizing the information described here is also in progress. These results will be reported in due course.

### 2.7. Experimental procedures

**2.7.1. General remarks.** All coupling reactions were performed under argon. 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) and anhydrous THF were purchased from Aldrich,

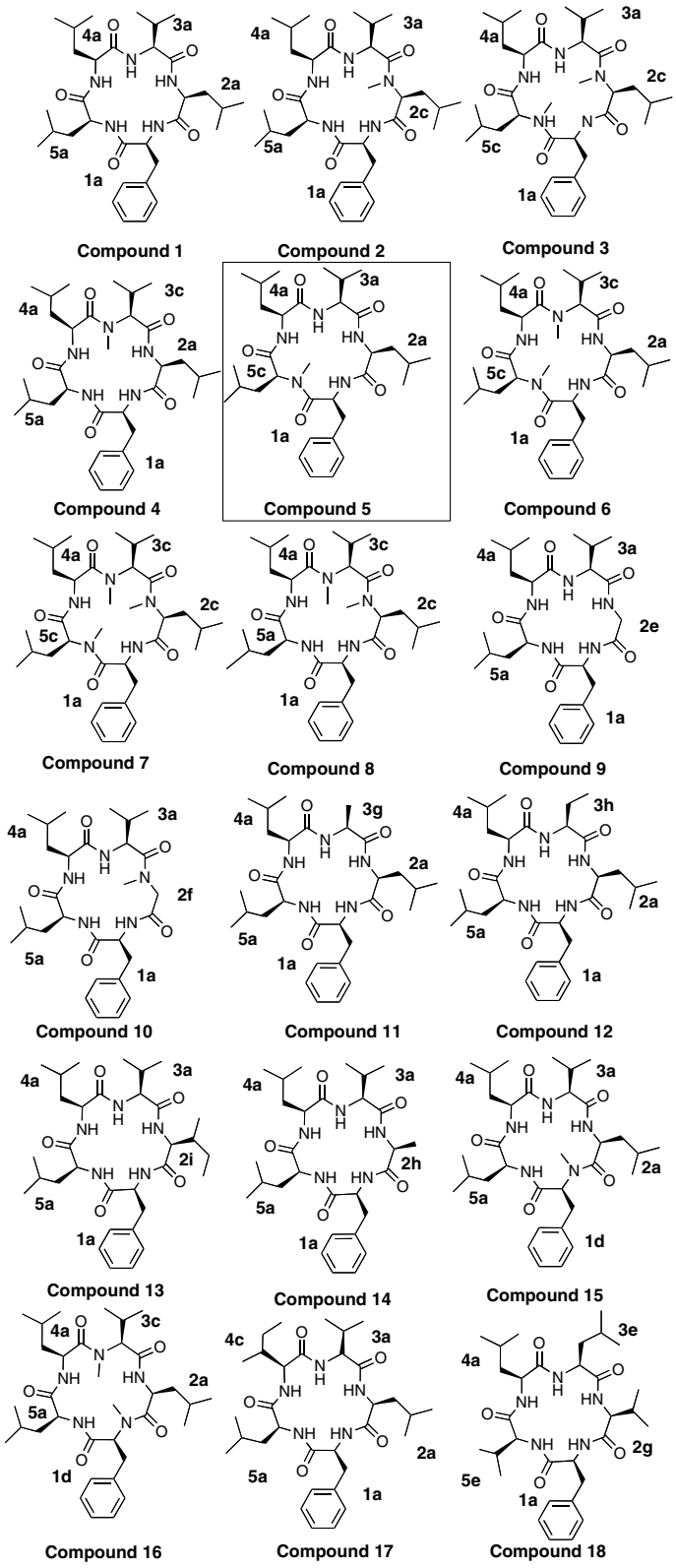


**Figure 3.** *Subset 1*: L-amino acid San A derivatives.

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 PyBOP were purchased from NovaBiochem. DEPBT [3(diethoxyphosphoryloxy)-1,2,3-benzotriazine-4(3*H*)] was purchased from Aldrich (order number 49596-4). The $^{1}$H NMR spectra were recorded on a Varian at 500 MHz. LC–MS were obtained at San Diego State University

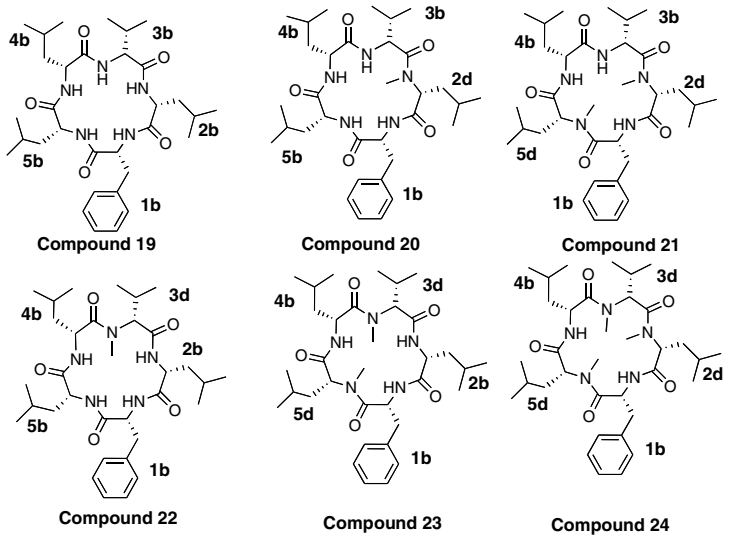


**Figure 4.** *Subset 2*: D-amino acid San A derivatives.

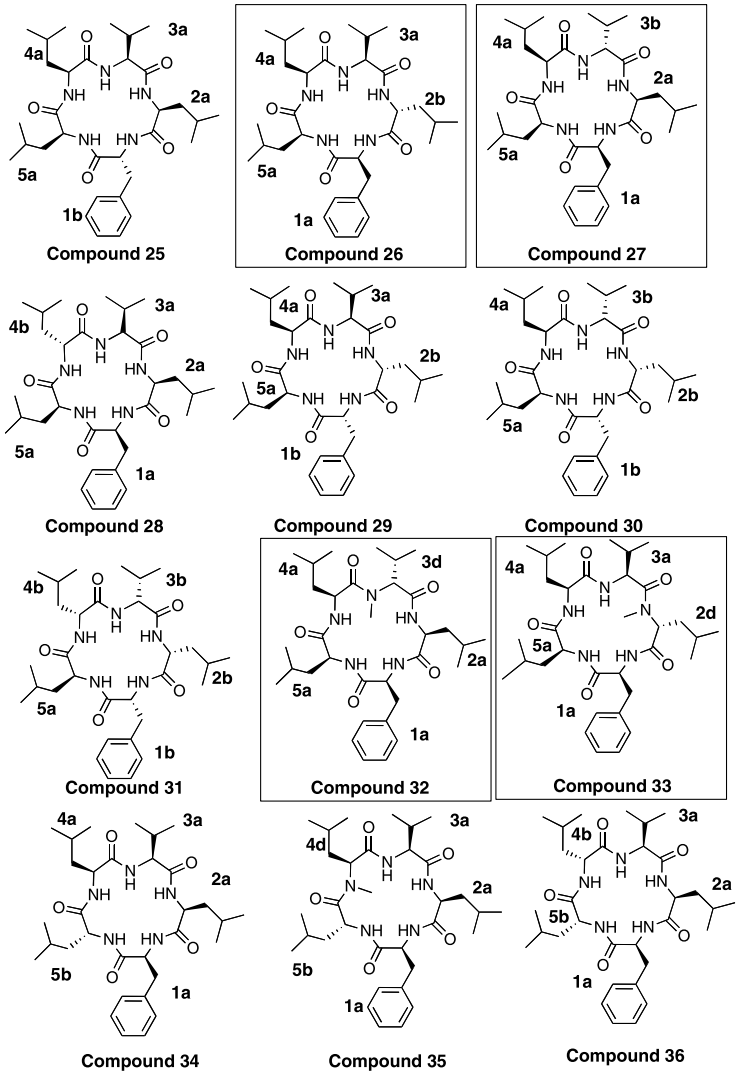


**Figure 5.** *Subset 3*: L- and D-amino acid San A derivatives.

using HP1100 Finnnigan LCQ. Flash column chromatography was performed on 230–400 mesh 32–74 μm 60 Å silica gel from Bodman Industries.

**2.7.2. General peptide synthesis.** All peptide coupling reactions were carried out under argon with dry solvent, using methylene chloride for dipeptide and tripeptide

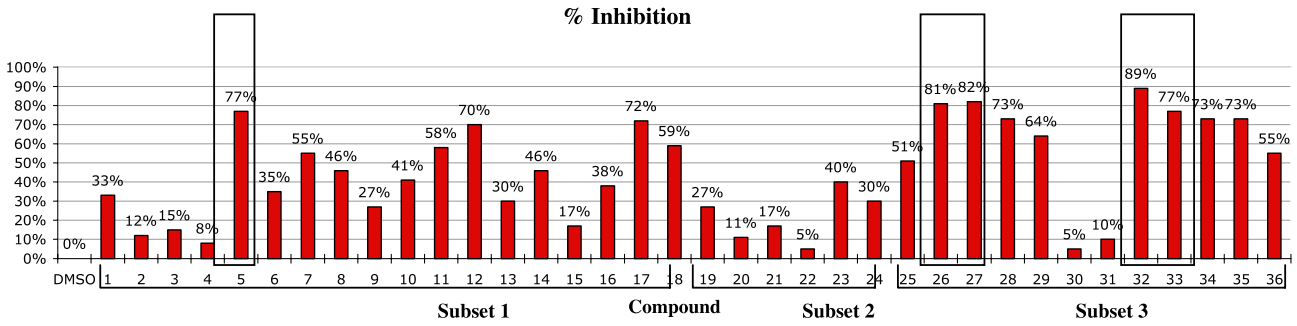


**Chart 1.** Activity of 36 compounds in colon cancer (50 μM) detected in $^{3}$H-thymidine uptake assays: Subset 1 = compounds **1-18**, Subset 2 = compounds 19-24, and Subset 3 = compounds 25-36. (Error bars are within approximately 2%).

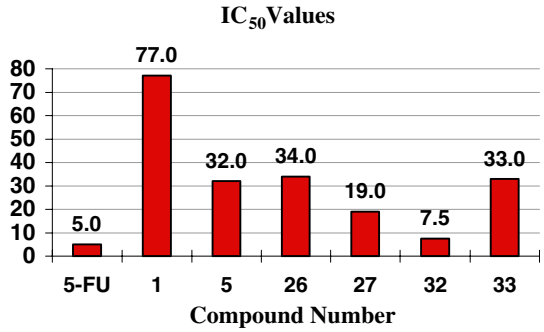


**Chart 2.** $IC_{50}$ of five most active compounds detected in [$^{3}$H]thymidine uptake assays.

couplings and acetonitrile for pentapeptide couplings. The amine (1.1 equiv) and acid (1 equiv) were weighed into a dry flask along with 4 equiv of DIPEA and 1.1 equiv of TBTU.[†] Anhydrous methylene chloride was added to generate a 0.1 M solution. The solution was stirred at room temperature and reactions were monitored by TLC. Reactions were run for 1 h before checking via TLC. If reaction were not complete additional 0.25 equiv was of HATU and TBTU were added. If reaction were complete, then workup was done by washing with saturated ammonium chloride. (Note: if acetonitrile was used for the reaction, methylene chloride was added to reaction upon workup and 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 a gradient of ethyl acetate–hexane gave our desired peptide.

**2.7.3. General amine deprotection.** Amines were deprotected using 20% TFA in methylene chloride (0.1 M) with 2 equiv 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 h and then concentrated in vacuo.

[†] Some coupling reactions would not go to completion using only TBTU and therefore ~0.25 equiv of HATU and/or DEPBT were used. In a few cases up to 1.1 equiv of all three coupling reagents were used.

**2.7.4. General acid deprotection.** Acids were deprotected using ~4 equiv of lithium hydroxide (or until pH ~11) in methanol (0.1 M). The peptide was placed in a flask, along with lithium hydroxide and methanol, and stirred overnight. Within 12 h the acid was usually deprotected. Workup 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.

**2.7.5. Macrocyclization procedure (in situ).** All pentapeptides were acid and amine deprotected using HCl (8 drops per 0.3 mmol of linear pentapeptide) in THF (0.05 M). Addition of anisole (2 equiv) was added to the reaction and the reaction mixture was stirred at room temperature. The reaction typically took 4 days, but TLC and LC–MS were used to monitor the reaction every 12 h. LC–MS data typically indicated the reaction was ~50% complete after the first day. Addition of four drops of HCl per 0.3 mmol of pentapeptide, stirring at RT overnight, and checking the reaction via LC–MS usually showed ~75% completion. 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 mixture 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 (DEPBT, HATU, and TBTU) were used at ~0.5 to 0.75 equiv 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 overall solution when included in the volume used for the deprotected peptide. The coupling agents was then added to the deprotected peptide solution. DIPEA (6 equiv or more in order to neutralize the pH) were then added to the reaction. The coupling agents are typically not very soluble in acetonitrile, which is why a combination of solvents is used.

After 1 h, TLC and LC–MS (where the LC–MS sample was worked up prior to injection) indicated that a product spot was developing. The comparison $R_f$ value in the product spot on TLC was the protected linear pentapeptide. The reactions were always complete after 2 h, and monitoring the starting material deprotected

pentapeptide via LC–MS 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, filtered, and concentrated. All macrocycles were purified by initially running a crude plug of compound using an ethylacetate/Hexane gradient 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.

### 2.8. Biological assay protocol

**2.8.1. Thymidine uptake assays.** Proliferation of the HT29 colon cancer cell line was tested in the presence and absence of the compounds using [$^3$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 [$^3$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 h, the compounds were added. The compounds were dissolved in DMSO for 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 h, 1 μCi [$^3$H]thymidine per well was added and the cells were cultured for an additional 16 h (for the cells to have a total of 40 h 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 min each using ScintiVerse universal scintillation fluid from Fisher. Decreases in [$^3$H]thymidine incorporation, as compared to controls, are an indication that the cells are no longer progressing through the cell cycle or synthesizing DNA.

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2006.04.031.

## References and notes

1. Boland, C. R.; Sinicrope, F. A.; Brenner, D. E.; Carethers, J. M. *Gastroenterology* **2000**, *118*, S115–S128.
2. Carethers, J. M.; Chauhan, D. P.; Fink, J. L.; Nebel, S.; Bresalier, R. S.; Howell, S. B.; Boland, C. R. *Gastroenterology* **1999**, *117*, 123–131.
3. Cueto, M.; Jensen, P. R.; Fenical, W. *Phytochemistry* **2000**, *55*, 223–226.
4. Hwang, Y.; Rowley, D.; Rhodes, D.; Gertsch, J.; Fenical, W.; Bushman, F. *Mol. Pharmacol.* **1999**, *55*, 1049–1053.
5. Belofsky, G. N.; Jensen, P. R.; Fenical, W. *Tetrahedron Lett.* **1999**, *40*, 2913–2916.
6. Lee, Y.; Silverman, R. B. *Org. Lett.* **2000**, *2*, 3743–3746.
7. Liu, S.; Gu, W. D. L.; Ding, X.-Z.; Ujiki, M.; Adrian, T. E.; Soff, G. A.; Silverman, R. B. *J. Med. Chem.* **2005**, *48*, 3630–3638.
8. Carroll, C. L.; Johnston, J. V. C.; Kekec, A.; Brown, J. D.; Parry, E.; Cajica, J.; Medina, I.; Cook, K. M.; Corral, R.; Pan, P.-S.; McAlpine, S. R. *Org. Lett.* **2005**, *7*, 3481–3484.
9. Otrubova, K.; Styers, T. J.; Pan, P.-S.; Rodriguez, R.; McGuire, K. L.; McAlpine, S. R. *Chem. Commun.* **2006**, 1033–1034.
10. Unpublished results from the Guy lab at UCSF and published results from our laboratory show that the use of several coupling reagents facilitates formation of the peptide bond in high-yields.
11. Dipeptide and tripeptide structures were confirmed using $^1$H NMR. All linear pentapeptides were confirmed using LC–MS and $^1$H NMR. (Note: $^1$H NMR were taken for cyclized peptides, but due to their complexity, they were not seen as the primary confirmation for cyclized compounds). See Supplementary material for spectra.
12. Bolla, M. L.; Azevedo, E. V.; Smith, J. M.; Taylor, R. E.; Ranjit, D. K.; Segall, A. M.; McAlpine, S. R. *Org. Lett.* **2003**, *5*, 109–112.
13. Liotta, L. A.; Medina, I.; Robinson, J. L.; Carroll, C. L.; Pan, P.-S.; Corral, R.; Johnston, J. V. C.; Cook, K. M.; Curtis, F. A.; Sharples, G. J.; McAlpine, S. R. *Tetrahedron Lett.* **2004**, *45*, 8447–8450.
14. Styers, T. J.; Rodriguez, R.; Pan, P.-S.; McAlpine, S. R. *Tetrahedron Lett.* **2006**, *47*, 515–517.
15. For details on the reaction conditions, see Supplementary material.
16. It was straightforward to follow the reactions via LC–MS as the starting material free-acid–free amine linear precursor would appear at 5.0–5.5 min and the cyclized product would appear between 6.1 and 7.0 min.
17. The thirty-six macrocyclic peptides have LC–MS spectra given in Supplementary material. In addition, data for intermediates involved in the synthesis of active compounds (**5**, **26**, **27**, **32**, and **33**) are also shown.