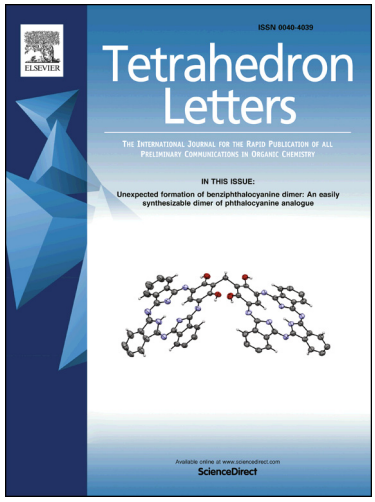

In pursuit of balgacyclamide A – Discovery of an oxazoline macrocycle with multiple myeloma cytotoxicity and penetration

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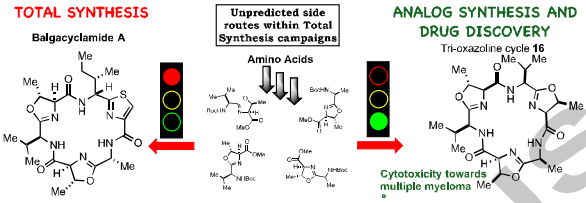

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In pursuit of balgacyclamide A – Discovery of an oxazoline macrocycle with multiple myeloma cytotoxicity and penetration

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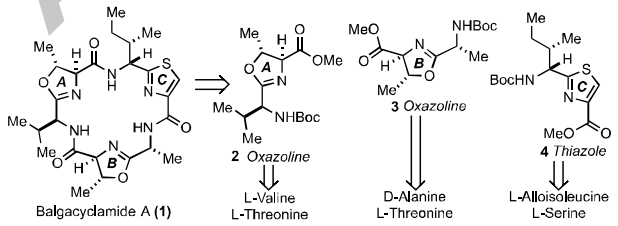
ABSTRACT

Four new oxazoline based macrocycles have been constructed, said cycles were found to possess varying cytotoxicity against six different cancer cell lines: IC_{50} values of 6.4 µM towards HeLa and 11.9 µM towards LnCaP being the most potent. Two of the four macrocycles were found to have marginal cytotoxicity against MM.1S and MM.1R, myeloma cancer cell lines, and further evaluation showed that they also possessed rapid cellular uptake and accumulation characteristics. Through the structure-activity relationship comparisons between the four compounds, it was found that the C6 position of the E-ring is amendable to substitution and could possibly serve as a conjugation site for the development of a selective delivery system to MM.1R.

1. Introduction

The balgacyclamides are an oxazoline and thiazole based family of natural products that were isolated from the aqueous methanolic extracts of *Microcystis aeruginosa* EAWAG 251 in 2013.[1] Their isolation was a part of a campaign in the efforts of identifying new compounds with antimalarial properties.[2-4] They belong to a larger class of cyclic peptides composed of oxazol(in)e or thiazol(in)e rings, as well as non-cyclized amino acids, which have been shown to possess a wide range of biological activities from antimalarial[5-7] to anticancer.[8, 9] It has been proposed that it is the presence of the heterocyclic ring components, either the thiazoline or thiazole rings that is responsible for the reported biological activity.[1-7] Balgacyclamide A (**1**, Scheme 1), composed of two oxazoline and one thiazole rings, was shown to have the greatest antiparasite activity towards *Plasmodium falciparum* K1 strain with an IC_{50} of 9.0 µM and displayed no cytotoxicity towards L6 rat myoblast cell line up to 150 µM. Given its antimalarial activity, reported low cytotoxicity towards myoblast cells, and the idea put forth by the isolation team that the heterocyclic component is critical for biological activity, our laboratory set forth in developing a synthetic route to this intriguing natural product and analogs.



Scheme 1. Proposed retrosynthetic route to balgacyclamide A.

2. Results and Discussion

Per the retrosynthetic route outlined in Scheme 1, it is envisioned that **1** can be assembled from the coupling and macrocyclization of oxazolines **2** and **3** and thiazole **4**. Future analogs can be assembled through the mismatching of the oxazoline units **2** and **3** and thiazole unit **4**, as well through the construction of other oxazol(in)e and thizaol(in)e units from other amino acids. Through this, initial efforts can be made towards substitutions about the C-ring as well as stereochemistry about the A/B-rings for biological investigations.

The construction of oxazoline units **2** and **3** can be accomplished from the coupling of the desired amino acids to access the corresponding dipeptides, with subsequent cyclization: L-valine and L-threonine to access **2**, and **3** from D-alanine and L-threonine. The assembly of the thiazole **4** will commence from the reported coupling of L-alloleucine to L-serine, cyclization to its corresponding oxazoline, ring opening and sulfur installation with hydrogen sulfide, and then cyclization followed by aromatization.[3, 10] We anticipate that through our efforts towards towards developing a total synthesis of **1** will also provide a methodological framework for constructing various analogs of **1**. Said analogs will allow for investigations to probe and elucidate what the effects of the various ring systems have within this class of natural products. Also, the stereo-chemical implications within

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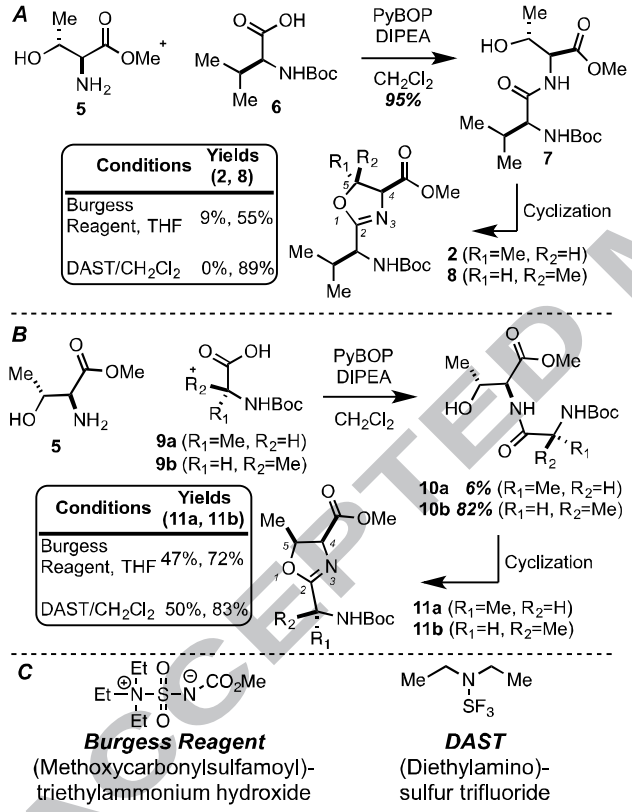
each ring system, and the importance/role of the heterocyclic rings can be investigated. It is envisioned that from the work performed, that new macrocycles analogous to **1** can be constructed, which could have unique cytotoxic and/or penetration characteristics not observed by the parent natural product or known family members.

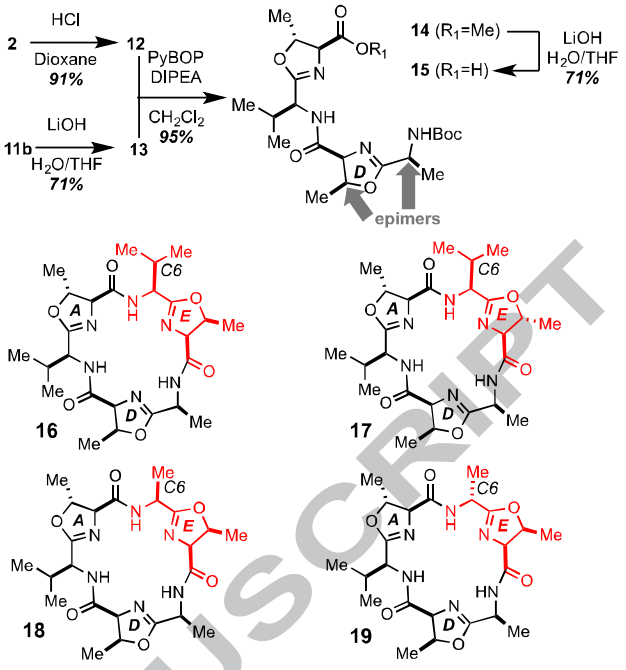
2.1 Synthesis

Construction of the A-ring commenced with the coupling of the methyl ester of L-threonine (**5**) to the *N-Boc* protected of L-valine (**6**) through a PyBOP mediated reaction to afford dipeptide **7** in 95% yield (Scheme 2A). With **7** in hand, the cyclization to afford oxazoline **2** was undertaken. While oxazoline **2** is required for accessing the balgacyclamide family, obtaining the C5 epimer within the oxazoline ring is also desirable in that it will allow for analog construction. Subjecting **7** to Burgess cyclization[11-15] conditions did afford the desired oxazoline **2**, albeit in a 9% yield. The major product of the Burgess cyclization was **8**, the epimer about C5, in 55% yield. All attempts to optimize this reaction failed to produce any increase in yield of the desired oxazoline **2**. Employing DAST,[10] a fluoride based cyclization reagent, also failed to provide **2**, but afford only epimer **8** in 89% yield.



Scheme 2. Construction of oxazoline ring **2/8** (A) and **11a/b** (B) formation from amino acids under Burgess and DAST conditions (C).

Assembly of the B-ring commenced in an analogous fashion as **2**, starting with the coupling of the methyl ester of L-threonine (**5**) to *N-Boc*-D-alanine (**9a**, Scheme 2B). Unexpectedly, the PyBOP coupling of these two amino acids failed to provide **10a** in any yields greater than 6%. This contrasted with the coupling of **5** to **9b**, L-alanine, which afforded **10b** in 82% yield. While the yields accessing the desired dipeptide **10a** cyclization was attempted nonetheless. To our surprise, the cyclization of **10a** failed to provide any of the desired cycle **3**, but rather only **11a**



Scheme 3. Assembly of di-oxazoline **15** (upper) from **2** and **11b** and the incorporation of oxazolines **8**, **2**, **11a**, and **11b** onto **15** (lower). Yields of each macrocycle from their corresponding oxazoline are illustrated below each macrocycle.

under both Burgess and DAST conditions in 47% and 72% yields, respectively. This result was highly unexpected, given that the cyclization of **7** under Burgess conditions gave accessed to the desired **2** (C5 R-stereochemistry). Similarly, the C5 S-stereochemistry selectivity (isomer **8**) was observed with the cyclization of **10b** to **11b**. Disappointingly, none of the desired oxazoline unit **3** was obtained under either condition attempted. Further optimization failed to provide the desired oxazoline **3** under these cyclization conditions. Variability in the oxazoline ring closure under both DAST and Burgess reagent conditions was observed. The Sn2 reaction to afford these rings is well documented,[10-15] but the failure of **2** to cyclize under DAST condition, whereas the other dipeptides successful cyclized was interesting. Cyclization of all dipeptides in this work proceeds through a strained conformational transition state. Possible implications on the variability of cyclization in these systems could arise from the isopropyl vs methyl substituents that could further impacting the said strained transition states via increased angular strain. Further investigations, and substrate scope, into these interesting cyclization results have not been conducted at this time.

Given the unexpected set back in accessing oxazoline **3**, the envisioned total synthesis of balgacyclamide A was abruptly halted. With numerous oxazoline units in-hand, efforts were then directed towards accessing analogs of balgacyclamide A, composed of three oxazoline units for biological studies. Starting with the desired oxazoline **2** (to give **12**), the coupling with **11b** was undertaken through saponification of **2**, Boc deprotection of **11b** (to give **13**), followed by the PyBOP mediated coupling to furnish the di-oxazoline unit **14** in 61% yield over 3 steps (Scheme 3). This unit mimics the A/B ring system of **1** in basic structure, but possess two epimeric centers within the D-ring compared to the desired B-ring.

Table 1. Effects on cell growth (IC_{50} μM) of different cancerous cell lines by compounds **16-19**.

Cell Type	IC_{50} (μM)			
	16	**17**	**18**	**19**
HeLa (cervical cancer)	75.9 ± 6.8	> 100	60.1 ± 9.5	6.4 ± 1.6
MIA PaCa-2 (pancreas cancer)	>100	>100	>100	>100
LnCaP (prostate cancer)	>100	34.6 ± 7.9	>100	11.9 ± 2.1
A549 (lung cancer)	60.9 ± 8.1	>100	61.6 ± 10.5	>100
MM.1S (blood cancer-Lambda myeloma)	45.8 ± 4.1	>100	52.7 ± 7.4	>100
MM.1R (blood cancer-multiple myeloma)	27.0 ± 2.0	>100	29.3 ± 9.3	>100

IC_{50}: inhibition concentration resulting in 50% reduction in cell growth, in μM; values are the mean ±SD from at least two triplicate cytotoxicity experiments assessed by Alamar Blue quantification.

Construction of the macrocycles commenced from the saponification of **14** to afford the free acid **15** in 71% yield. Each of the four-oxazoline units accessed in this work were then incorporated into scaffold **15**. The general procedure commenced with each oxazoline undergoing Boc deprotection, followed by coupling to **15** via PyBOP. Once coupled, the Boc group was removed, and this cycle was subjected to aminolysis conditions for macrocyclization, which gave access to cycles **16**-**19** with yields ranging from 5.3 to 10.9% from **15**.

2.2 Biological Evaluation - Cytotoxicity

To explore the biological activities of these cyclic oxazoline macrocycles, each one was screened against a panel of six cancerous cell lines (Table 1). Each compound was screened independently against the cancerous cell lines shown in Table 1. This was done in 384-well plates, at cell densities of 1,500 cells/well, at 37 °C for 72 hours, with the cells' viability being assessed by Alamar blue. Compound **19** was found to possess the greatest cytotoxicity towards HeLa (cervical cancer) and LnCaP (prostate cancer) with IC_{50} values of 6.4±1.6 and 11.9±2.1 μM, respectively. Compound **17** had little to no activity against the lines screened. The activities of **16** and **18** were the most intriguing, even though they both were shown to have marginal activity towards HeLa and A549. Unexpectedly, both **16** and **18** were shown to have activity towards MM.1S and MM.1R cell lines, immunoglobulin A Lambda myeloma and multiple myeloma, respectively. These cell lines represent cancers that are well-known as being difficult to treat, specifically multiple myeloma (MM.1R). While the modest cytotoxicities in these cell lines are not of considerable interest, the possible cellular uptake and accumulation within MM.1R elicited excitement.

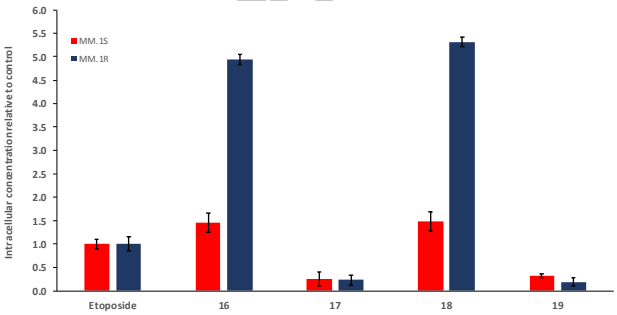


Figure 1. Relative intracellular concentrations of compounds **16-19** in MM.1S and MM.1R cells, as determined by LC/MS analysis. Data is graphed relative to etoposide. Cell lines were treated, independently, with 100 μM of each compound for 4h; cells were then lysed, and intracellular concentration was determined using integration area based upon standard curves. Error bars denote standard errors (*n*=3 independent experiments).

2.3 Biological Evaluation – Cellular Uptake Assays

The intracellular concentrations of compounds **16-19** were determined in both MM.1S and MM.1R by treating each cell line with 100 μM concentrations of each compound for four hours independently. The cell lysate was collected and subjected to LC/MS analysis, the relative amounts of each compound present in the lysate were detected by refractive index, and compound identification was confirmed by LC/MS pattern matching. The experimentally determined intracellular concentrations in both MM.1S and MM.1R for compounds **16-19** post 100 μM incubation in whole cells, relative to etoposide, are shown in Figure 1.

Of the compounds screened, the intracellular concentrations of **16** and **18** ranged from ~8 to 22-fold higher than those of compounds **17** and **19**. Both **16** and **18** also showed enhanced intracellular concentrations towards MM.1R compared to MM.1S, ~3.5-fold for **16** and ~2.7-fold for **18**. Structurally, both **16** and **18** are nearly identical, with the exception of an isopropyl group within **16** and methyl in **18** about the former α-carbons (position C6, Scheme 3). This may suggest that the C6 position within the E-ring can be altered to allow various substitutions, and may consequently serve as a potential site of conjugation onto cytotoxic species and/or other agents.

Compounds **16** and **18** were then evaluated towards MM.1R with regards to speed of cellular uptake and length of accumulation. Separate experiments were run for each time point and for each compound at 100 μM. Figure 2 illustrates the rapid

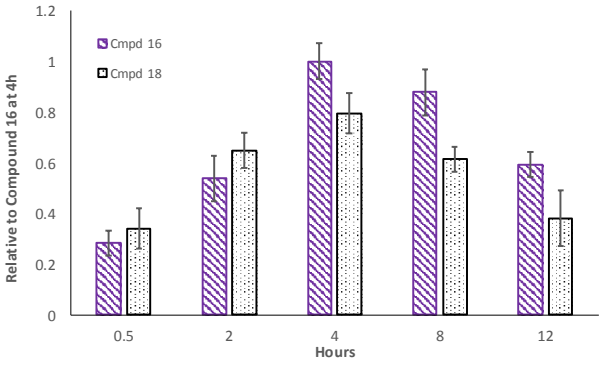


Figure 2. Relative intracellular concentrations of compounds **16** and **18** in MM.1R cells, as determined by LC/MS analysis over five independently run experiments. Data is graphed relative to the largest peak area obtained, compound **16** at four hours. Cell lines were treated independently with 100 μM of each compound for 4h; cells were then lysed, and intracellular concentration was determined using integration area based upon standard curves. Error bars denote standard errors (*n*=3 independent experiments).

uptake of both **16** and **18** into MM.1R, relative to the concentrations of **16** at the four hour evaluation point. Maximum accumulation of both compounds was observed at four hours, and over the next eight hours compounds **16** and **18** showed only a 49% and 62% reduction in intracellular concentration, respectively. Whether the reduction is by intracellular degradation or removal from the cell is currently being explored. Given the C6 variability between **16** and **18**, the rapid uptake by both in MM.1R, and the length of accumulation it could be envisioned that the C6 position could possibly be altered to allow for compound appendage as a drug delivery system towards this aggressive and difficult-to-treat form of cancer.

3. Conclusion

The synthetic efforts towards balgacyclamide A have given access to numerous oxazoline units, and while the synthesis of **1** has not yet been achieved, the oxazoline formed gave rise to four new tri-oxazoline macrocycles, **16-19**. Compounds **16** and **18** were shown to have marginal cytotoxicity against a panel of cancerous cell lines screened, but upon further evaluation, these two compounds were shown to have enhanced cellular uptake and accumulation within MM.1R, a multiple myeloma (MM) cancerous cell line. Given that MM is an incurable hematologic malignancy, the limited FDA approved drugs for its treatment, and the current limited therapeutic delivery to MM, these two compounds provide a new avenue for possible drug delivery via attachment about the C6 position (former α-carbon) of the E-ring. These results illustrate that the heterocyclic triazol(in)e systems are not directly required for biological activity, as suggested by the isolation team. Through an unexpected detour towards the total synthesis of balgacyclamide A, two new oxazoline-based cyclic peptides have been constructed and found to have selective penetration and accumulation characteristics into multiple myeloma.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2016.11.069.

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Highlights

- Assembly of new oxazoline based macrocycles
- Discovery of new cycles with diverse cytotoxicity
- Identification of new macrocycle with marginal cytotoxicity towards multiple myeloma
- Observed rapid accumulation within multiple myeloma