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14-Membered Macrocyclic Ring-Derived Toolbox: The Identification of Small Molecule Inhibitors of Angiogenesis and Early Embryo Development in Zebrafish Assay

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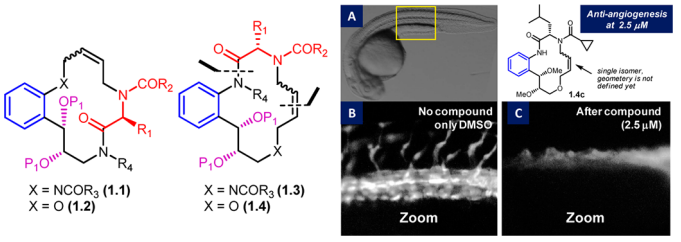
ABSTRACT



A highly practical and modular synthesis to obtain a diverse 14-membered ring-based macrocyclic toolbox is achieved. These compounds were further tested in zebrafish assays related to early embryonic development, angiogenesis, and neurogenesis, respectively. 1.4c was identified as an antiangiogenesis agent.

The need to assemble a small molecule toolbox with compounds that are more natural-product-like to search for modulators of protein−protein interactions[1−3] and dissectors of pathways[4,5] has grown in recent years. In particular, interest in the macrocyclic natural products is rising because they provide diverse functionality and stereochemical complexity in a conformationally preorganized ring structure.[6] These features might be useful for high affinity and selectivity for protein targets, while preserving sufficient bioavailability to approach intracellular targets. Despite these valuable characteristics and the proven success of several marketed macrocycle drugs derived from natural products, this structural class has been poorly explored within drug discovery.[1] Certainly, this testifies to the need to develop practical and modular methods that allow us to build a toolbox with a diverse set

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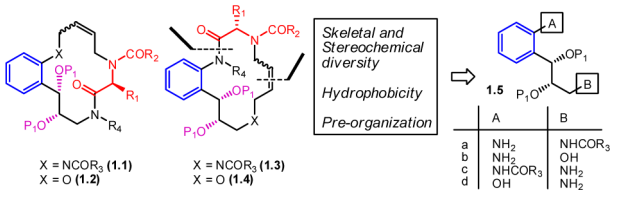
Scheme 1. A Modular Approach To Access a Diverse Set of 14-Membered Ring Macrocyclic Compounds



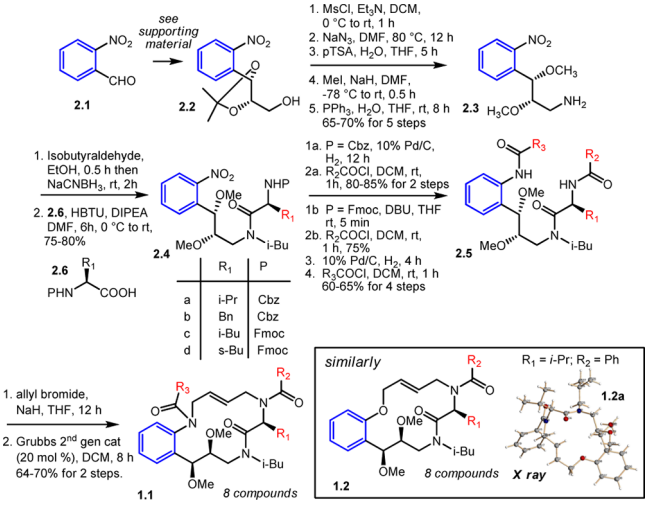
of macrocyclic compounds to explore their biological functions.[7−15]

With this objective, we were interested in developing a modular synthesis method to access different types of 14- membered ring macrocyclic compounds as shown in Scheme 1. In our approach, we were interested in developing a method that is simple and practical in nature, and in our design strategy, we can introduce skeletal diversity and modulate various functional groups, i.e. through D- or L-amino acids and Sharpless chemistry.[16] The proposed natural product-inspired compounds are more closely related to natural products in terms of 3D shapes and the dense display of chiral functional groups. One of the major advantages is that they are easy to explore in regards to stereochemical and skeletal diversity and the chemical space around the scaffold and are easy to synthesize on a gram scale in a reasonable time scale.[7,8,17]

In our modular design strategy, we had the option to bring the amino acid functionality either through the aromatic amine or from the aliphatic side chain (see macrocyclic targets **1.1**−**1.4**). Further, variation in the side chain, i.e. R_2−R_4, on the macrocyclic skeleton can also be achieved through selective alkylation and amidation, respectively. For our initial studies, we focused on a 14-membered ring skeleton because there are several examples of the presence of this ring in a wide variety of bioactive natural products, such as radicicol,[18] pochonin,[19] hypothemycin,[20] aigialomycin, and other related compounds (structures not shown). Four macrocyclic targets (**1.1**−**1.4**) were to be obtained from a common starting material (**1.5**) which is easy to access, and both enantiomers could be obtained in several gram quantities in a short duration. This can further allow us to explore the stereochemical diversity on a macrocyclic ring.

As shown in Scheme 2, 2-nitrobenzaldehyde was converted to an α,β-unsaturated carboxylester by a Horner−Wadsworth−Emmons reaction and then subjected to a Sharpless asymmetric dihydroxylation reaction, giving an enantiopure dihydroxyl derivative. Following the acetonide protection of the diol, the carboxylester was then reduced with lithium borohydride to give primary alcohol **2.2**. Primary amine **2.3** was then obtained from **2.2** in four steps: (i) the conversion of alcohol to azide by mesylation with methane sulfonyl chloride and then by treating with sodium azide, (ii) the deprotection of actonide, (iii) 1,2-diol methylation with methyl iodide, and, finally, (iv) the reduction of azide by a Staudinger reaction to give primary amine **2.3**. This was converted to a secondary amine by imino-reduction with isobutyraldehyde, which was then coupled with different *N*-protected amino acids (**2.6**) under HBTU conditions to obtain compounds **2.4**. With compounds having *N*Cbz as the protecting group, the nitro reduction and *N*Cbz removal were performed in a single

Scheme 2. Synthesis of 14-Membered Macrocycles **1.1** and **1.2**



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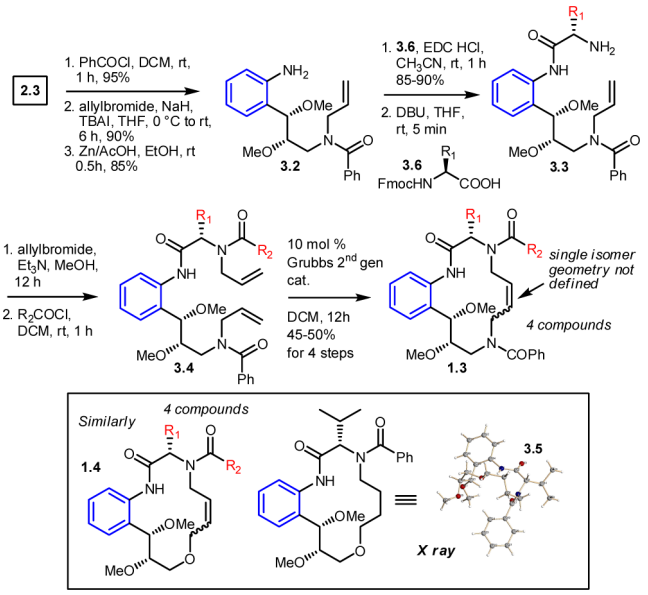
step with 10% Pd/C under hydrogen conditions to obtain diamines, which were then converted to bisamides on reaction with different acid chlorides. Compounds having *N*Fmoc were converted into **2.5** in four steps: (i) the removal of *N*Fmoc with DBU, (ii) amidation with different acid chlorides, (iii) reduction of nitro to amine with 10% Pd/C, and (iv) amidation with different acid chlorides. Macrocyclic compounds, **1.1**, were obtained from the corresponding bisamides in two steps, i.e. bisallylation with allylbromide and NaH followed by ring-closing metathesis[21−23] using the Grubbs second generation catalyst (G-II). In this series, eight macrocyclic compounds were synthesized, and in all cases, the *trans* olefin geometry was obtained as determined by NMR (see the Supporting Information (SI)).

In a similar manner, using 2-benzyloxybenzaldehyde as the starting material, we completed the synthesis of eight macrocyclic compounds **1.2**. As before, the *trans* olefin geometry was obtained following the "stitching technology". The detailed synthesis for **1.2** is provided in the SI. In one case (**1.2a**), we could further obtain the X-ray to confirm the macrocyclic skeleton.

In another series, macrocycles **1.3** (Scheme 3) were obtained from the key intermediate **2.3** in eight steps as follows. Primary amine **2.3** was converted to an amide with benzoyl chloride and then by allylation on amide −*N*H followed by the reduction of an aromatic nitro to give the aromatic amine **3.2**. This was coupled with *N*-protected amino acids (**3.6**) using EDC•HCl and then subjected to *N*Fmoc removal to obtain **3.3**. Monoallylation (allyl bromide, MeOH) of **3.3** was followed by an amidation to obtain the key bisallyl product **3.4**, needed for the stitching technology. Four macrocyclic compounds **1.3** were obtained using the ring-closing technology (Grubbs second generation catalyst, G-II). In this series, although we obtained a single isomer (determined by HPLC-MS), the olefin geometry remains to be determined. Similarly, four macrocycle compounds **1.4** were obtained (see the SI), and in one case, we could obtain the X-ray of the reduced double bond macrocyclic product **3.5** to further confirm our assignments.

A small molecule toolbox containing 85 compounds was then subjected to a search for chemical probes affecting epiboly during early embryonic development,[24−26] angiogenesis,[27,28] and neurogenesis[29] in zebrafish embryo-based assays.[30,31] These assays are well-documented in the literature,[28,32−34] and the detailed experimental procedure is provided in the SI. It was interesting to observe that among all the compounds tested from this collection, we identified a novel small molecule (**1.4c**, Figure 1) that specifically inhibited the trunk angiogenesis (see Figure 1B and C) at 2.5 μM without affecting either epiboly or neurogenesis. Embryos exposed to 2.5 μM of this small molecule (**1.4c**) displayed normal epiboly movement and neurogenesis, and an overall embryonic development was also not affected. The embryos showed a complete lack of migration of early endothelial cells (i.e., tip and stalk cells) to form an early intersegmental vessel of the trunk.

Scheme 3. Synthesis of 14-Membered Macrocycles **1.3** and **1.4**



In another parallel study, we identified four active compounds (**S$_{27}$d**, **2.5c** and **1.1c**, **1.2f**) that inhibited epiboly cell movements during early embryonic development (Figure 2). The delay in epiboly was clearly seen in embryos exposed to 5.0 μM of the small molecules; however these embryos did complete epiboly and developed normally without any visible effects on angiogenesis and neurogenesis. At higher concentrations, such as 10.0 μM, epiboly did not begin leading to the lethality of the embryo. The mechanism underlying the inhibition of angiogenesis and epiboly will be characterized using both zebrafish and other cell based assays.

To summarize, we report a practical and modular approach for accessing a diverse set of 14-membered ring

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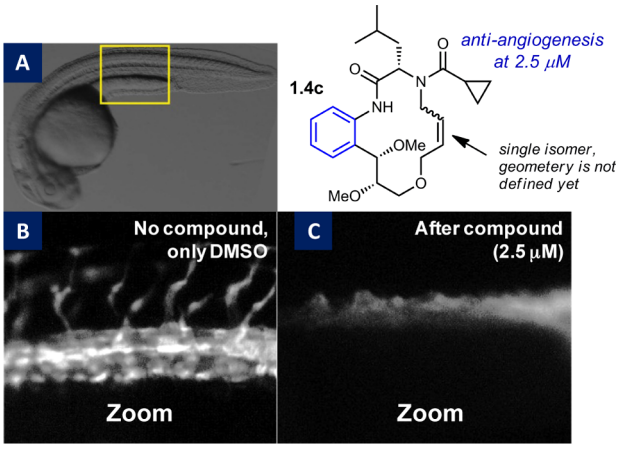

Figure 1. Zebrafish angiogenesis assay: (A) Wild-type zebra fish embryo at 30 hpf of development, region zoomed in panels B and C is shown by a yellow box; (B) zoom section of wild-type or vehicle treated embryo; (C) zoom section after treatment with compound **1.4c**.

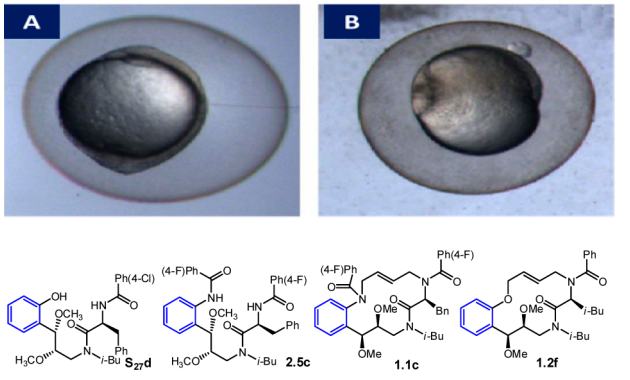

Figure 2. Zebrafish early embryonic development assay: (A) DMSO exposed embryos at 10 hpf of development; (B) small molecule exposed embryos causing a delay in epiboly.

macrocyclic compounds. The ease of the synthesis and the reported modular methodology are the two attractive features in assembling a macrocyclic chemical toolbox to explore its value. The presence of two contiguous stereogenic hydroxyl group derivatives and an amino acid moiety (note: in the present study only natural amino acids were utilized) in the macrocyclic ring architecture allow access to this unique set of compounds. With the goal of going beyond the conventional chemical space in the drug discovery arena where most compounds are rich in sp^2 character, the present method provides a good entry to access a diverse set of 14-membered macrocyclic compounds with variation in the display of different functional groups. Furthermore, to explore their biological effects, we utilized zebrafish embryonic screening assays. These rapid assay procedures allowed us to quickly identify the effects of small molecules on various biological processes. The effects produced by these novel molecules provided a platform for using chemical biology approaches to understand basic biological processes such as epiboly and angiogenesis. Extensive validation procedures need to be developed to characterize the effect produced and to elucidate the mechanism of action. Further, work is needed to understand the deep impact of these functional small molecules in the context of developing a new class of inhibitors of angiogenesis as well as the inhibitors of early embryonic development.

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Supporting Information Available. The detailed synthesis procedure and characterization of all new compounds are thoroughly provided. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.