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RESEARCH ARTICLE

Enantioselective Synthesis and Biological Evaluation of Sanglifehrin A and B and Analogs

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Abstract: Sanglifehrin A and B are immunosuppressive macrocyclic natural products endowed with and differentiated by a unique spirocyclic lactam. Here, we report an enantioselective total synthesis and biological evaluation of sanglifehrin A and B and analogs. Access to the spirocyclic lactam was achieved via convergent assembly of a key pyranone intermediate followed by a stereo-controlled spirocyclization. The 22-membered macrocyclic core was synthesized by ring-closing metathesis in the presence of 2,6-bis(trifluoromethyl) benzeneboronic acid (BFBB). The spirocyclic lactam and macrocycle fragments were united by a Stille coupling to furnish sanglifehrin A and B. Additional sanglifehrin B analogs with variation at the C40 position were additionally prepared. Biological evaluation revealed that the **2-CF$_3$** analog of sanglifehrin B exhibited higher anti-proliferative activity than the natural products sanglifehrin A and B in Jurkat cells. Both natural products induced higher-order homodimerization of cyclophilin A (CypA), but only sanglifehrin A promoted CypA complexation with inosine-5'-monophosphate dehydrogenase 2 (IMPDH2). The synthesis reported here will enable further evaluation of the spirolactam and its contribution to sanglifehrin-dependent immunosuppressive activity.

Introduction

The natural products cyclosporin A (CsA), FK506, and rapamycin heralded transformative improvements in organ transplant success rates and remediation of autoimmune diseases in the clinic[1] via unique mechanisms of action involving formation of higher-order protein complexes.[2] For example, CsA mediates a ternary complex between cyclophilin A (CypA) and calcineurin, which inhibits signal transduction during T cell activation.[1c, 3] In screening for novel immunosuppressants that target CypA, the sanglifehrin class of macrolides were discovered from isolates of *Streptomyces sp.* A92-308110 in 1999.[4] Sanglifehrin A (**1**) and B (**2**) are composed of a 22-membered macrocycle linked to a structurally unique and highly substituted [5,5] spirolactam (**Figure 1A**). The macrocycle mediates the binding interaction with CypA,[5] and consists of an exocyclic (*E*,*E*)-1,3-diene polyketide backbone fused to a tripeptide, which is composed of valine, *m*-tyrosine, and an unusual β-substituted piperazic acid residue.

Sanglifehrin A (**1**) and B (**2**) possess mild anti-proliferative effects against T cells and B cells (mixed lymphocyte IC_{50}, **1** = 170 nM; **2** = 102 nM), indicating that these compounds may decrease the toxic side effects of CsA (mixed lymphocyte IC_{50} = 10.6 nM).[6] However, although sanglifehrin A (**1**) and B (**2**) are potent ligands for several members of the cyclophilin family,[4a] further mechanistic investigation revealed that sanglifehrin A (**1**) possesses a mechanism of action that is distinct from CsA. In contrast to CsA, sanglifehrin A (**1**) does not inhibit calcineurin activity upstream of IL-2 expression and instead blocks late-stage IL-2-dependent proliferation of T cells.[7] Mechanistic studies have further shown that sanglifehrin A (**1**) induces NF-κB-mediated p53 activation, stalls cell proliferation at the G1–S phase transition,[8] and induces mitochondrial dysfunction.[9] These effects may occur through a united target or through several targets, including binding interactions with CypA or cyclophilin D (CypD),[9a] which may be accompanied by the formation of higher-order complexes such as a sanglifehrin A (**1**)–CypA homodimer[5c] or a ternary complex between CypA and IMPDH2 that is stabilized by sanglifehrin A (**1**).[10] However, whether the immunosuppressive activity of sanglifehrin A (**1**) requires these higher-order complexes or cyclophilin binding itself is inconclusive. Competitive displacement of sanglifehrin A (**1**) from CypA has no effect on immunosuppressive activity[7] and oxidative cleavage of the C26–C27 olefin affords a macrocycle that maintains CypA binding, but is non-immunosuppressive.[8a] These sanglifehrin-derived CypA macrocyclic ligands are under evaluation as non-immunosuppressive anti-virals.[8c, 11] By contrast, beyond the initial reports,[6] additional mechanistic studies with sanglifehrin B (**2**) have not been reported to date.

Sanglifehrin A (**1**) and B (**2**) possess differential cyclophilin binding profiles and anti-proliferative effects,[4a] indicating that the structural differences in their respective spirolactams play a crucial role in tuning the activity of the sanglifehrins. While the structure-activity relationship (SAR) of the sanglifehrin macrocycle has been explored,[5a, 5b] SAR of the spirolactam has not been evaluated. Since the discovery of the sanglifehrins, total synthesis of sanglifehrin A (**1**) has been reported by the Nicolaou[12] and Paquette[13] groups, along with many other efforts.[14] Here, we report the development of a versatile approach to the spirolactam, which culminated in the total synthesis of sanglifehrin A (**1**) and B (**2**) and biological evaluation of the protein–protein interactions that are differentially mediated by these compounds. Furthermore, we utilized this synthetic route to develop analogs at the C40 position and report a more potent analog of sanglifehrin B, **2-CF$_3$**. This versatile route will enable

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further investigation into the mechanistic contribution of the spirolactam and drive the development of novel immunosuppressants based on the sanglifehrin family.

Results and Discussion

In designing a synthetic approach to sanglifehrin A (**1**) and B (**2**), we adopted a retrosynthetic disconnection of C25–C26 inspired by prior art[12-13] to afford two key fragments, the spirolactam **3** and the iodide **4** (**Figure 1A**). Prior approaches to access the spirolactam **3a** employed a linear route[12-13] that may limit access to spirolactam analogs like **3b** or those with alternative substitution patterns about positions C33–C40. We therefore envisioned that the spirolactam structures **3a** and **3b** could be elaborated from the common pyranone intermediate **5** through a 6-*exo-trig* Michael cyclization followed by stereo-controlled reduction at C35 (**Figure 1B**).

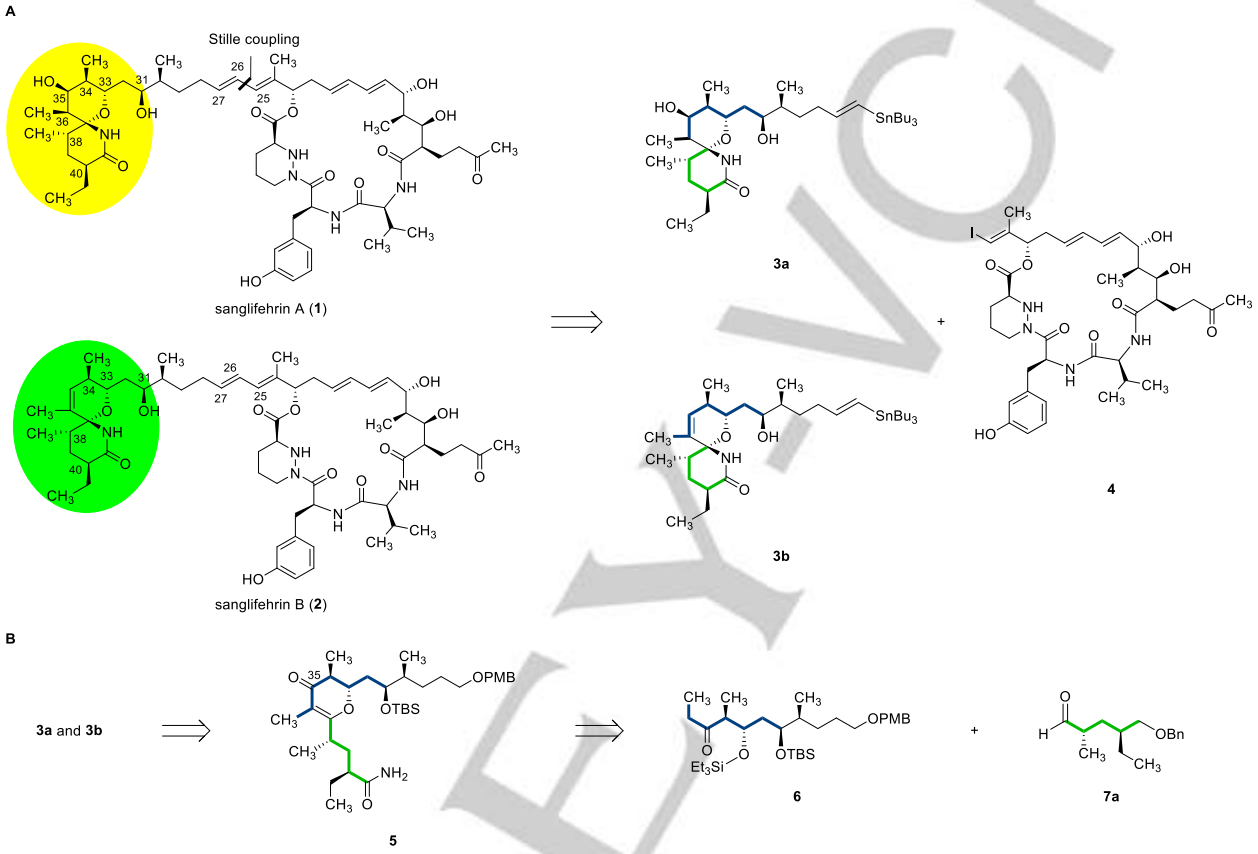
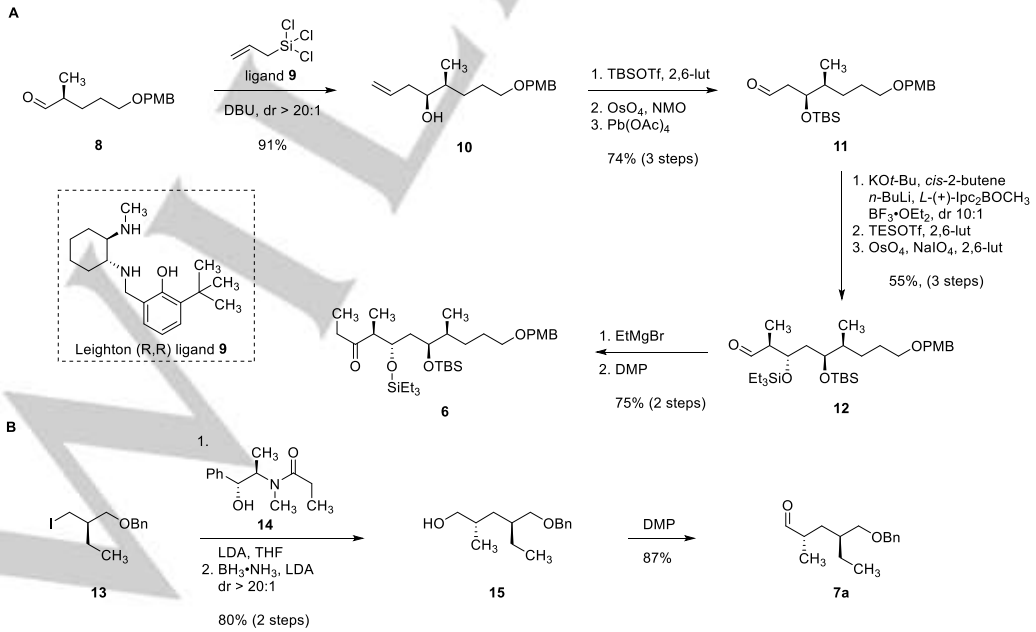


Figure 1. A. Structures and retrosynthetic analysis of sanglifehrin A (**1**) and B (**2**). **B.** Retrosynthetic approach to the spirolactam **3a** and **3b**.



Scheme 1. A. Synthesis of the ketone **6**. **B.** Synthesis of the aldehyde **7a**.

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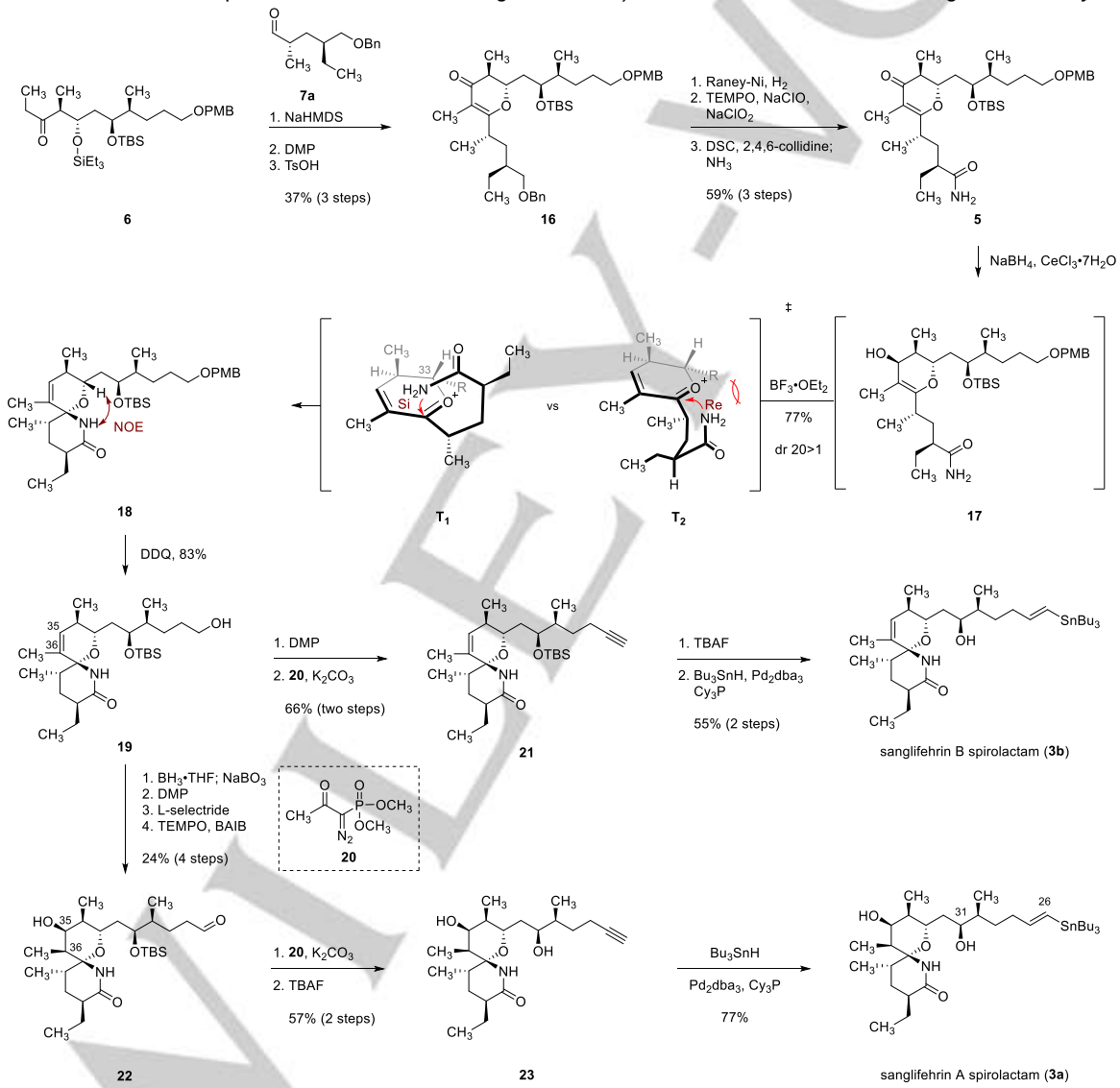
The pyranone **5** is easily accessed by assembly of the functionalized ketone **6** and the aldehyde **7a**. A convergent strategy that unites **6** and **7a** at a later stage decouples installation of each stereocenter, such that each stereocenter may be strategically elaborated independently, to maximize overall synthetic flexibility for derivatization of the substitution pattern about the spirolactam **3** in the future.

Synthesis of the ketone **6** was initiated by a one-pot Leighton allylation[15] of the readily accessible aldehyde **8**[16] with the (*R*,*R*)-Leighton ligand **9** to provide the alcohol **10** in 91% yield with excellent diastereoselectivity (dr > 20:1, **Scheme 1A**). Silylation of the alcohol **10** with *t*-butyldimethylsilane triflate (TBSOTf) and an oxidative cleavage sequence afforded the desired aldehyde **11** in high yield over three steps (74%). Subsequent Brown crotylation[17] established the requisite chiral centers in **12** in good yield with diastereoselectivity (74%, dr 10:1). After silyl protection of the resulting alcohol with triethylsilane triflate (TESOTf), an optimized $NaIO_4$-mediated oxidative cleavage[18] of the bulky alkene was performed to provide the aldehyde **12** in good yield (74%, two steps). Grignard addition of an ethyl group to the aldehyde **12** followed by oxidation using Dess-Martin periodinane provided the ketone **6** in a good yield (75%) over two steps.

An efficient sequence for synthesis of the corresponding aldehyde **7a** was developed using Myers asymmetric alkylation[19] (**Scheme 1B**). Accordingly, the readily accessible iodide **13**[20] was reacted with the lithiated pseudoephedrine propionate **14**, followed by removal of the chiral auxiliary by reaction with lithium amidohydroborate generated *in situ* to afford the primary alcohol **15** in good yield and excellent diastereoselectivity (80%, dr > 20:1). The alcohol **15** was oxidized to give the aldehyde **7a**.



Scheme 2. Synthesis of the spirolactam **3a** and **3b** of sanglifehrin A (**1**) and B (**2**), respectively.

With the two precursors in hand, we next turned to constructing the spirolactam moiety. Aldol cross-coupling of the ketone **6** with the aldehyde **7a** followed by oxidation afforded a 1,3-diketone intermediate, which was immediately subjected to acid-catalyzed cyclization[21] at 50 ˚C to furnish the 4-pyranone **16** in good yield (37%, **Scheme 2**). A regioselective debenzylation of **16** was performed under one atm pressure of hydrogen gas in the presence of Raney nickel to unmask the primary alcohol, which was then converted to the corresponding primary amide **5** through 2,2,6,6-tetramethylpiperidin-1-yl)oxidanyl (TEMPO) mediated oxidation,[22] and an one-pot amide transformation promoted by *N*,*N*'-disuccinimidyl carbonate (DSC). Attempts at direct

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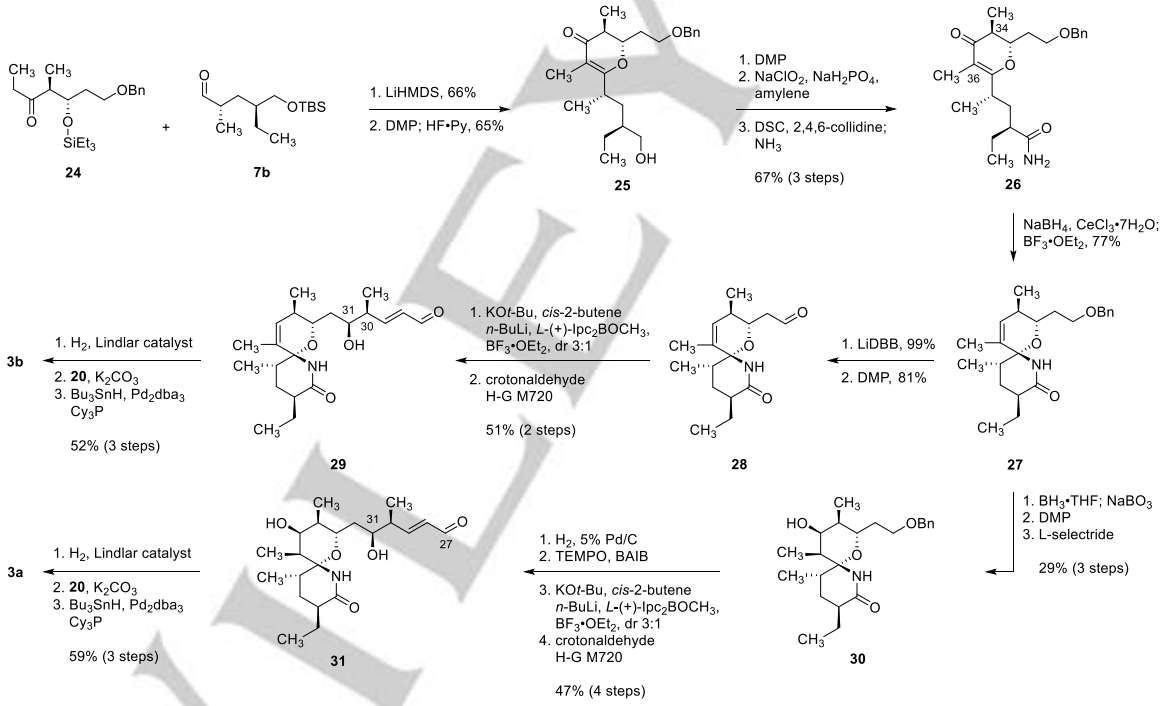
spirocyclization via Michael addition from the amide **5** proved unproductive. Therefore, the enone in **5** was subjected to 1,2-reduction with $NaBH_4$ in the presence of $CeCl_3•7H_2O$ to afford the vinyl alcohol **17**, which we observed spontaneously cyclize to the spirolactam **18** as a single diastereomer via Ferrier rearrangement[23] in low yield (35%), along with recovery of the alcohol **17**. The 2,6-*cis* stereochemistry was comfired by a Nuclear Overhauser Effect (NOE) experiment. The spirocyclization was subsequently optimized by subjection of the crude **17** to catalytic $BF_3•OEt_2$ at –78 ˚C, which greatly improved the yield without compromising stereocontrol (77%). The excellent diastereoselectivity may be as a result of both kinetic and thermodynamic control. The facial selectivity of oxocarbenium in the transition states is favored for the desired diastereomer.[24] In the transition state $\mathbf{T_1}$, the steric clash of the side chain and A 1,3-strain interactions between the two methyl groups are minimized on electrophilic trapping with the primary amide when approaching from the *Si* face, in contrast to $\mathbf{T_2}$, where steric interactions arise during approach of the primary amide from the *Re* face. The less stable twist boat carbocation as alternative intermediate in transition state also leads to the desired stereochemical outcome (**Figure S1**). In addition, the desired diastereomer as 2,6-*cis*-dihydropyran is thermodynamically favored (**Figure S2**, **Scheme S1**).

With the spirolactam core synthesized, our focus moved to functionalization of the spirolactam **18** with a stannane for Stille cross coupling via regioselective hydrostannation of a terminal alkyne. Therefore, oxidative removal of *p*-methoxybenzyl (PMB) was carried out using 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) to afford the primary alcohol **19** in good yield (83%). After oxidation, the resulting aldehyde was subjected to Seyferth-Gilbert homologation[25] using the Bestmann-Ohira reagent **20** to afford the terminal alkyne **21** (66%, two steps). Following deprotection of the TBS group, palladium-catalyzed hydrostannation was performed using tricyclohexylphosphine (Cy_3P)[26] as a ligand to provide the stannane **3b** towards sanglifehrin B (**2**) (55% yield, two steps).

Access to the corresponding sanglifehrin A spirolactam **3a** was achieved by functionalization of the olefin across positions C35–C36 of **19** to install the secondary alcohol in **22** (**Scheme 2**). Accordingly, a stereo-controlled hydroboration-oxidation[27] tactic installed the desired stereochemistry on C36 exclusively, albeit with inverted stereochemistry on the hydroxyl group on C35. Subsequently, a two-step sequence of oxidation and reduction using L-selectride resulted in the desired stereochemistry on C35 exclusively (60%, two steps).[14i] After TEMPO-mediated oxidation using (diacetoxyiodo)benzene (BAIB),[28] the aldehyde **22** was converted to the stannane **3a** in a similar manner to that used to access the stannane **3b**.



Scheme 3. Alternative synthetic route to the spirolactam **3a** and **3b** of sanglifehrin A (**1**) and B (**2**), respectively.

Alternatively, we envisioned that elaborating the C26–C31 spacer after construction of the spirolactam would allow access to sanglifehrin analogs with different connectivity (**Figure 1A**). Thus, the ketone **24** was elaborated via a similar sequence as described above to afford the spirolactam **27** (**Scheme 3**). The spirolactam **27** was subjected to radical-mediated debenzylation using lithium 4,4'-di-*tert*-butylbiphenylide (LiDBB, Freeman's reagent),[29] followed by Dess-Martin oxidation to smoothly deliver the aldehyde **28** in excellent yields (99%, 81%, respectively). The aldehyde **28** was elaborated to install the stereocenters on C30–C31 using Brown crotylation (65%, dr 3:1), and the resulting terminal alkene was subjected to cross metathesis with crotonaldehyde using Hoveyda-Grubbs Second Generation (H-G II) catalyst to afford the α,β-unsaturated aldehyde **29** in a good yield (78%). A chemoselective hydrogenation using Lindlar catalyst afforded the hemiketal,[30] which was readily converted to the stannane **3b**. The sanglifehrin A stannane **3a** was prepared in a similar manner from **27** after manipulation of the oxidation state of the olefin.

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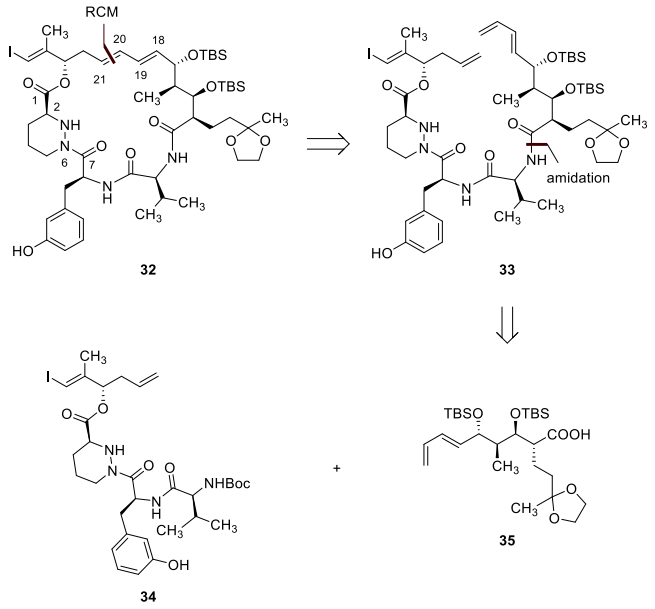
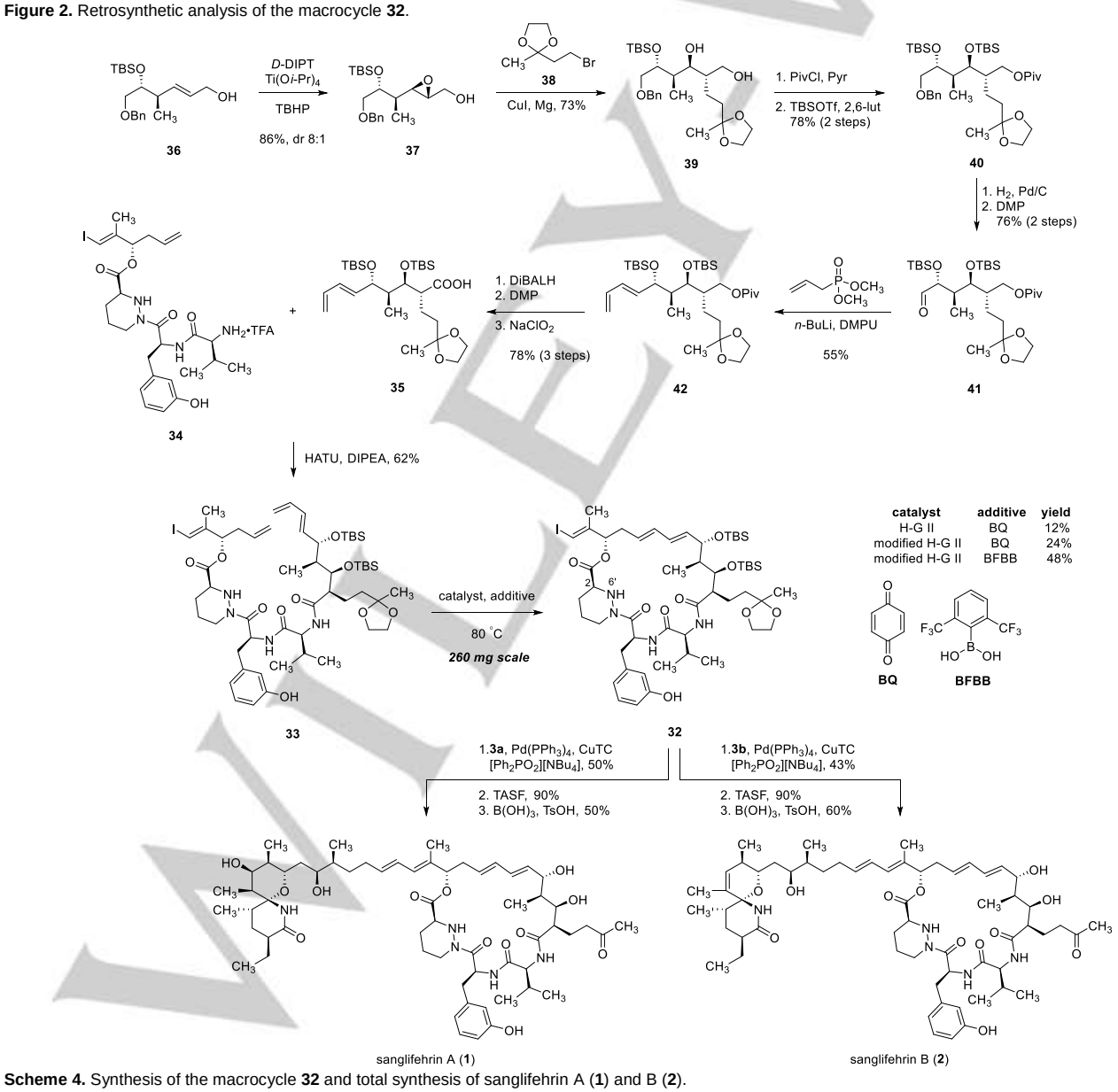


Figure 2. Retrosynthetic analysis of the macrocycle **32**.

We next focused on synthesis of the macrocycle **32**. Despite efforts to access **32** via an intramolecular Stille coupling[12] or macrolactonization,[13] we found that material throughput was challenging. Therefore, we were attracted to the recent reports of a ring-closing metathesis (RCM) strategy to provide sufficient access to the macrocyclic core[11, 14a, 14f] for the preparation of sanglifehrin A (**1**) and B (**2**). We further elected to use silyl protection for the diol and a ketal to mask the ketone in **32**, which would allow for stepwise deprotection and avoid elimination of the C17-hydroxyl group, which we observed in our early studies. Retrosynthetic disconnection across C20–C21 yields the tetraene **33**, which could be prepared by amide coupling between the tripeptide **34** and the diene **35** (**Figure 2**).

The sequence leading to the polyketide-derived fragment **35** commenced from the reported allyl alcohol **36**,[31] which was subjected to Sharpless epoxidation[32] to yield the epoxide **37** (86%, dr = 8:1, **Scheme 4**). Ring opening of the epoxide[33] with the bromide **38**[34] in the presence of anhydrous copper iodide successfully delivered the diol **39** in good yield (73%).[35]



Scheme 4. Synthesis of the macrocycle **32** and total synthesis of sanglifehrin A (**1**) and B (**2**).

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After sequential protection of the diol **39**, the intermediate **40** was debenzylated and oxidized to afford the aldehyde **41**. Installation of the requisite diene on the aldehyde **41** was carried out through a Horner-Wadsworth-Emmons reaction (HWE) with diethyl allylphosphonate in the presence of *N*,*N*′-dimethylpropyleneurea (DMPU).[36] The resulting diene **42** was functionalized to afford the acid **35**, by a sequence of pivalate deprotection and oxidation. The coupling partner tripeptide **34•TFA** was prepared through a sequence reported by Nicolaou[12] from the corresponding allyl alcohol. Amide coupling between the acid **35** and the tripeptide **34•TFA** using hexafluorophosphate azabenzotriazole tetramethyl uronium (HATU) yielded the tetraene **33** (62%).

The tetraene **33** was converted to the macrocycle **32** via a RCM reaction. In initial conditions, **33** was treated with the H-G II catalyst in the presence of benzoquinone (BQ) at 80 ˚C to achieve the macrocycle **32** in 12% yield (**Scheme 4**). Use of the modified H-G II catalyst improved the yield of **32** by two-fold (24%).[37] Notably, we observed oxidation of **33** or **32** across position C2–N6' to the imine, which may be due to an intramolecular reaction between C2–N6' moiety with the ruthenium carbene formed *in situ*. This position was previously reported to be oxidized on standing in air.[14f] To circumvent the undesired imine formation, we attempted to mask *N*6' using Lewis acids [e.g., chloride dicyclohexylborane,[38] or titanium (IV) isopropoxide],[39] but these additives led to decomposition. Serendipitously, we found that the yield of the RCM reaction to afford the macrocycle **32** was significantly improved in the presence of a catalytic amount of 2,6-bis(trifluoromethyl)benzeneboronic acid (BFBB, 48%) on 260 mg scales. Interestingly, addition of stoichiometric amounts of BFBB to the reaction did not further improve the yield,[40] possibly due to accelerating decomposition of the catalyst.[41]

Finally, the Stille-Migita cross coupling of the vinyl iodide **32** with the stannane **3a** under conditions reported by Fürster *et al.*[42] was performed to successfully give the coupling product in 50% yield. For the final deprotection of the ketal and silyl groups, the TBS groups were first removed by tris(dimethylamino)sulfonium difluorotrimethylsilicate (TASF)[43] in a good yield and the final deprotection of the ketal was conducted using catalytic *p*-toluenesulfonic acid (TsOH) in the presence of boric acid to afford sanglifehrin A (**1**) along with the corresponding interchangable hemiketal isomers in a 2:1 ratio (8.8 mg, 50% yield, **Scheme 4**, **Figure S3**). The ratio of the ketal to hemiketal was in agreement with previous reports.[30] In a similar manner, sanglifehrin B (**2**) was obtained in 23% yield over three steps (3.0 mg). The stepwise deprotection improved the overall yield as compared to use of sulfuric acid[12] or stoichiometric TsOH[13, 30] for deprotection of the intramolecular ketal. Synthetic sanglifehrin A (**1**) and sanglifehrin B (**2**) are in agreement with the NMR data in the literature.[4b, 30][44]

We proceeded to evaluate the biological activity of sanglifehrin A (**1**) and B (**2**) to probe whether the spirolactam promoted differential activity. Sanglifehrin A (**1**) and sanglifehrin B (**2**) both displayed moderate anti-proliferative effects in Jurkat cells, with sanglifehrin B (**2**) being more potent (**1**: IC_{50} = 4.2 μM; **2**: IC_{50} = 0.8 μM, **Figure 3B**). Enthused by this finding, we further investigated additional analogs based on sanglifehrin B using our synthetic route. We synthesized two C40 analogs of sanglifehrin B (**2**), which yielded a more potent analog **2-CF₃** (IC_{50} = 0.24 μM), in which the ethyl substituent was replaced by a trifluoroethyl group (**Figure 3A, 3B**). These data indicate that structural modification about the spirolactam, and specifically at the C40 position, may further enhance the activity of sanglifehrin analogs against Jurkat cells.

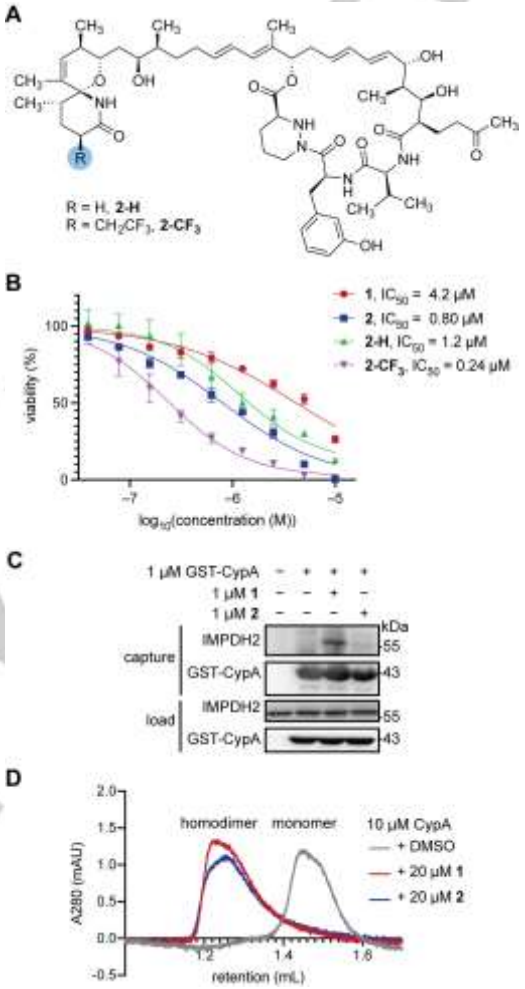

Figure 3: Biological evaluation of sanglifehrin A (**1**), B (**2**), and analogs. **A.** Structures of the sanglifehrin B C40 analogs **2-H** and **2-CF₃**. **B.** Antiproliferative effects in Jurkat cells as measured in a 72 h MTT assay. **C.** Co-enrichment of IMPDH2 with recombinant GST-CypA from a Jurkat lysate treated with the indicated compounds. **D.** Characterization of CypA dimerization state in the presence of the indicated compounds as determined by size-exclusion chromatography.

We next investigated whether the higher-order protein complexes that are mediated by sanglifehrin A (**1**) are recapitulated by sanglifehrin B (**2**) and therefore potential targets for further mechanistic investigation.[5c, 10] We first evaluated of the higher-order protein complexe between Cyp A and IMPDH2, which have previously been reported to form a ternary complex in the presence of sanglifehrin A. The data revealed that while sanglifehrin A (**1**) promotes an interaction between CypA and IMPDH2 *in vitro*, sanglifehrin B (**2**) induces a minimal CypA–IMPDH2 interaction (**Figure 3C**). By contrast, both sanglifehrin A (**1**) and sanglifehrin B (**2**) drive nearly complete homodimerization on interaction with CypA in vitro (**Figure 3D**). These data indicate that the spirolactam plays a key role in mediating the higher-order protein complexes formed by sanglifehrin A (**1**) and B (**2**), and suggests additional targets may be involved in the anti-

proliferative activity of sanglifehrin B (**2**). These data are the first mechanistic efforts comparing sanglifehrin A (**1**) to sanglifehrin B (**2**) and additional spirolactam analogs, revealing biologic effects mediated by the structural differences about the spirolactam.

Conclusion

In conclusion, we developed a convergent and enantioselective synthetic route toward sanglifehrin A (**1**) and the first total synthesis of sanglifehrin B (**2**), which enabled preparation of additional analogs and characterization of the effects of the spirolactam on the resulting protein complexes underlying their biological activity. Strategic assembly of the spirolactam via the common pyranone **5** enables derivatization of novel spirolactam analogs, such as those at position C40, to illuminate the mechanism of action of the sanglifehrin class of natural products. In addition, synthesis of the macrocycle **32** through RCM with the addition of BFBB to suppress the formation of the imine byproduct improved access to the natural product. Biological evaluation reveals that although sanglifehrin B (**2**) is more potent than sanglifehrin A (**1**) in Jurkat cells, sanglifehrin A (**1**) preferentially forms higher-order protein complexes between CypA and IMPDH2, while both natural products promote homodimerization with CypA. In addition, a more potent analog was discoveried by synthesizing two additional analogs of sanglifehrin B on C40, suggesting the dervatization on C40 would serve as starting point for further SAR investigation. Further insights into the mechanism of action of the sanglifehrin family will be enabled by evaluation of novel sanglifehrin analogs derivatized at other positions about the spirolactam (C33–C40), which are now accessible via the reported synthetic pathway.

Acknowledgements

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Conflict of Interest
The authors declare no conflict of interest.

Keywords: Sanglifehrin A • Sanglifehrin B • Cyclophilin• Total Synthesis• Natural product

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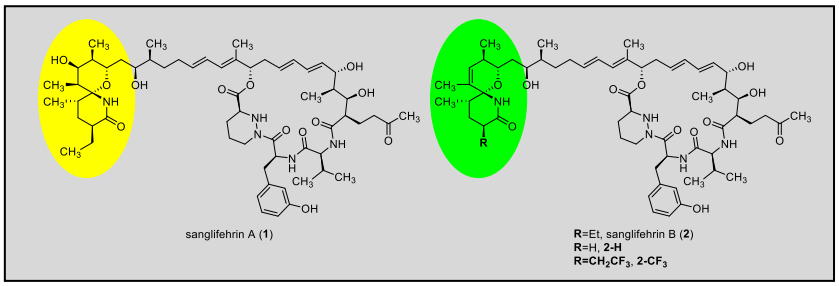

The immunosuppressive natural products, sanglifehrin A and B, bearing a 22-membered macrocycle appended with a unique highly-substituted spirolactam, pose formidable challenges to synthetic accessibility and derivatization. A versatile strategy toward the spirolactam via convergent assembly of fully-functionalized fragments culminated in the total synthesis of sanglifehrin A and sanglifehrin B and preparation of additional analogs at position C40 for evaluation of biological activity in Jurkat cells and stabilization of protein–protein interactions.