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MUNI

HABILITATION THESIS

SYNTHETICALLY MODIFIED COMPLEX NATURAL PRODUCTS

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Dedicated to my group, my teachers, and my family.

ABSTRACT

Natural products are endowed with diverse bioactivities. They have become an important source of life-saving medicines, particularly for infectious diseases and cancer. Natural products also grew indispensable in basic research, serving as tools to perturb fundamental biological processes. Frequently, structural modifications of the natural skeleton are necessary for optimizing the properties of the natural products (→ natural product analogs). Modern synthetic chemistry has been particularly enabling in this context, but the approach remains challenging for complex structures.

This thesis traces chronologically three different research projects performed in the Švenda group at Masaryk University since 2013, each tackling the synthetic complexity of a specific natural product class in the context of structural analog preparation and accompanying biological studies. The first part is devoted to the pseurotin family of fungal natural products characterized by a unique spirocyclic skeleton and diverse biological activities. The second part features a complex natural diterpene forskolin, an iconic molecule to practitioners of natural product synthesis and a common research tool in biology. The third part focuses on a family of potent natural antibiotics, bactobolins, which compromise the function of ribosomes and, thereby, normal protein synthesis. The thesis highlights different ideas in synthetic planning aimed at formulating concise and flexible de novo assembly of complex natural products and their analogs, as well as the challenges and successes in implementing these ideas in experimental practice.

ACKNOWLEDGMENTS

This thesis celebrates the dedicated experimental efforts of the talented students I had the pleasure of working with and whose original contributions are to be clearly acknowledged. The names of many of them appear within the original publications cited. I also wish to acknowledge the wonderful collaborations with other scientists over the years, both in chemistry and biology.

To me, the establishment of the Laboratories of Organic Synthesis and Medicinal Chemistry at Masaryk University, run jointly with my colleague Kamil Paruch, has been a success. I'm immensely thankful for the financial support that made it possible to conduct the research described herein. With genuine gratitude, I wish to thank Alfred and Isabel Bader and the Bader Philanthropies for their vision and support of organic chemistry in the Czech Republic.

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CONTEXT STATEMENT

Natural products, the specialized metabolites produced mainly by bacteria, fungi, and plants, have been fundamentally important to our society. Research on natural products yielded important advances in our knowledge and technical capabilities.[1] Multiple natural products became the basis of essential medicines in the management of life-threatening diseases (particularly cancer and infectious diseases).[1–3] Natural products were key in probing and answering fundamental questions in biology and helped establish the field known as chemical biology.[4,5] Their often remarkable, potent, and selective bioactivities have been explained through evolutionary arguments: ages-long biosynthesis and screening of natural products as part of the chemical warfare and communication between microorganisms.[6] Natural products are enriched in their ability to interact with biomolecules.[1]

The path from a natural product to a drug is, however, seldom straightforward. Molecules isolated from nature often require structural modifications, which may yield analogs superior to the natural product (recently dubbed 'supernatural'[7]) and help fulfill the stringent requirements during preclinical development. Likewise, formulating high-quality chemical biology probes[8] based on a natural product frequently calls for structural optimization. Our ability to manipulate natural product structures is, therefore, quintessential. Historically, semisynthesis (chemical modifications performed on a natural product) and de novo chemical synthesis (synthetic assembly of a natural product or analog from simple chemicals) have been the approaches explored the most. Engineering of deconvoluted biosynthetic pathways represents a more recent approach to modified natural products.[9] Depending on the complexity of the natural product, the type of modification required, and the approach pursued, this can be a straightforward or highly non-trivial undertaking. These challenges, when paired with the lack of financial incentives, made several big pharma companies move away from natural product-based drug discovery, most concerningly in the context of infectious diseases.[10]

The complexity of organic molecules was treated theoretically on numerous occasions. For this thesis, I adopt the view that divides complexity into molecular (intrinsic property) and synthetic (extrinsic property).[11,12] The molecular complexity does not change with time and is defined by chosen metrics such as atom count, connectivity, stereochemistry, heteroatom content, or symmetry (e.g., the Böttcher scale[13]). Synthetic complexity, on the other hand, *can* change with time as methods and strategies to prepare (synthesize) molecules evolve through insightful research studies by laboratories around the world. Based on a hard-earned experience (though not a rule), more molecularly complex structures tend to be more difficult to synthesize. Decades of groundbreaking research in natural product synthesis made de novo preparation of even the most intimidatingly complex natural products possible,[14,15] given sufficient resources and time. The challenge, however, is to render the synthetic approach to complex organic molecules efficient, flexible, and scalable.[16,17]

I highlight a few inspiring examples here to demonstrate the various roles organic synthesis has taken on over the years. The infamous and highly complex alkaloid strychnine, originally made in about 30 steps (longest linear sequence, LLS),[18] can now be prepared in as few as 6 steps (LLS),[19] a notable change in synthetic complexity with time. Fully synthetic radioactive and affinity probes of the natural product trapoxin

helped characterize important enzymes histone deacetylases.[20] Synthetic probes off the complex natural product FK506 advanced our understanding of the action of immunosuppressive agents.[21] Eribulin, a potent cancer drug, is a fully synthetic analog of the highly complex natural product halichondrin A.[22] Eravacyclin represents the first fully synthetic tetracycline-inspired antibiotic to enter clinical development and gain approval.[23] Holistic analysis of the chemical space occupied by bioactive natural products has inspired new strategies for molecular library assembly (e.g., diversity-oriented synthesis[24] and biology-oriented synthesis[25,26]). Rapid developments in machine learning and robotics made computer-aided (retro)synthesis,[27,28] again[29], the topic of interest.[30–32] It is through examples like these that one appreciates the tremendous impact natural products and synthetic chemists had and are set to have in science, technology, and medicine.

THESIS STRUCTURE

The three sections below trace chronologically three different research projects performed in my group at Masaryk University since 2013. Each project tackles the synthetic complexity of a specific natural product class in the context of analog preparation and accompanying biological studies. **Part 1** is devoted to the pseurotin family of fungal natural products characterized by a unique spirocyclic skeleton and diverse biological activities. **Part 2** features a complex natural diterpene forskolin, an iconic molecule to practitioners of natural product synthesis and a common research tool in biology. **Part 3** focuses on a family of potent natural antibiotics bactobolins produced by bacteria, which compromise the function of ribosomes and, thereby, normal protein synthesis. The individual parts are structured similarly and include a brief outline of the research problem, including the prior art, original contributions by my group, and critical conclusions. My aim here has not been to reproduce the research findings comprehensively and thus mirror the content of the original articles but rather to overview key ideas behind each project, particularly the logic of our synthetic planning and what the particular synthetic design enabled.

PART 1. PSEUDOROTINS

1A. INTRODUCTION TO THE RESEARCH PROBLEM

Pseurotins are complex secondary metabolites produced by various fungi. This natural product family is characterized by a signature γ-lactam–3(2*H*)furanone spirocyclic core (Scheme 1A).[33,34] Structural differences between members of the family arise through varied stereochemistry, levels of oxidation, and specific substitution attached to the spirocyclic core. Isotopic labeling determined that propionate, malonate, L-methionine, and L -phenylalanine units are used in the biosynthesis of pseurotin A.[35] The pseurotin biosynthetic machinery is a hybrid polyketide synthase (PKS) / non-ribosomal peptide synthetase (NRPS) and includes tailoring enzymes.[36–39] While pseurotins are not isolated as racemates, stereoisomeric pseurotins frequently occur, and Scheme 1A gives four illustrative examples. It is intriguing to ponder how stereochemical diversity within the family might arise. One plausible explanation invokes non-stereospecific steps in the biosynthetic pathway, for example, during the spirocyclization event. Stereochemical scrambling at the assembled pseurotin core might also occur and contribute to the stereoisomerism of pseurotins - a hypothetical retro-aldol–aldol reaction (C5–C9 bond) allows one to move freely between the four natural pseurotins shown in Scheme 1A. Studies have shown this to be a feasible process under mild conditions, at least with model compounds.[40] Stereochemical considerations are important beyond simple curiosity, as the biological activity of pseurotins is likely tied to their 3D structure.

Pseurotins were reported to display diverse bioactivities in vitro and in vivo, including cytotoxicity toward selected cancer cell lines, inhibition of angiogenesis and immunoglobulin E production, antiseizure activity, and more (reviewed in ref. 41) However, most of these effects were reported as initial observations, often not followed by mechanistic studies.[42] Robust structure–activity relationships for pseurotins are also not available. This is partly a consequence of the limited amounts of natural pseurotins obtained through isolation and challenges in selective modifications of their densely functionalized structures. It was possible to exploit the knowledge of biosynthesis and prepare modified pseurotins by feeding experiments (e.g., fluorinated analogs).[43] One notable study used semisynthesis to prepare about twenty new analogs of pseurotin A in the context of pseurotin-modulated immunoglobulin E production.[44]

The first fully synthetic route to a natural pseurotin was reported in 2002.[45] Since then, several non-enantioselective and enantioselective syntheses of individual pseurotins were disclosed. The state-of-the-art in the chemical synthesis of pseurotins was recently reviewed.[41] The main contributions came from the groups of Hayashi, Tanado, Tanabe and Misaki, Rovis, Kuramochi, and Han. The step counts (longest linear sequence, LLS) and overall yields of the published syntheses vary dramatically, ranging between 6 and 38 steps, depending on the pursued strategy and structure of the pseurotin.[41] Despite the successful development of several distinct synthetic routes to pseurotins, the preparation of fully synthetic analogs has been surprisingly little explored.[44]

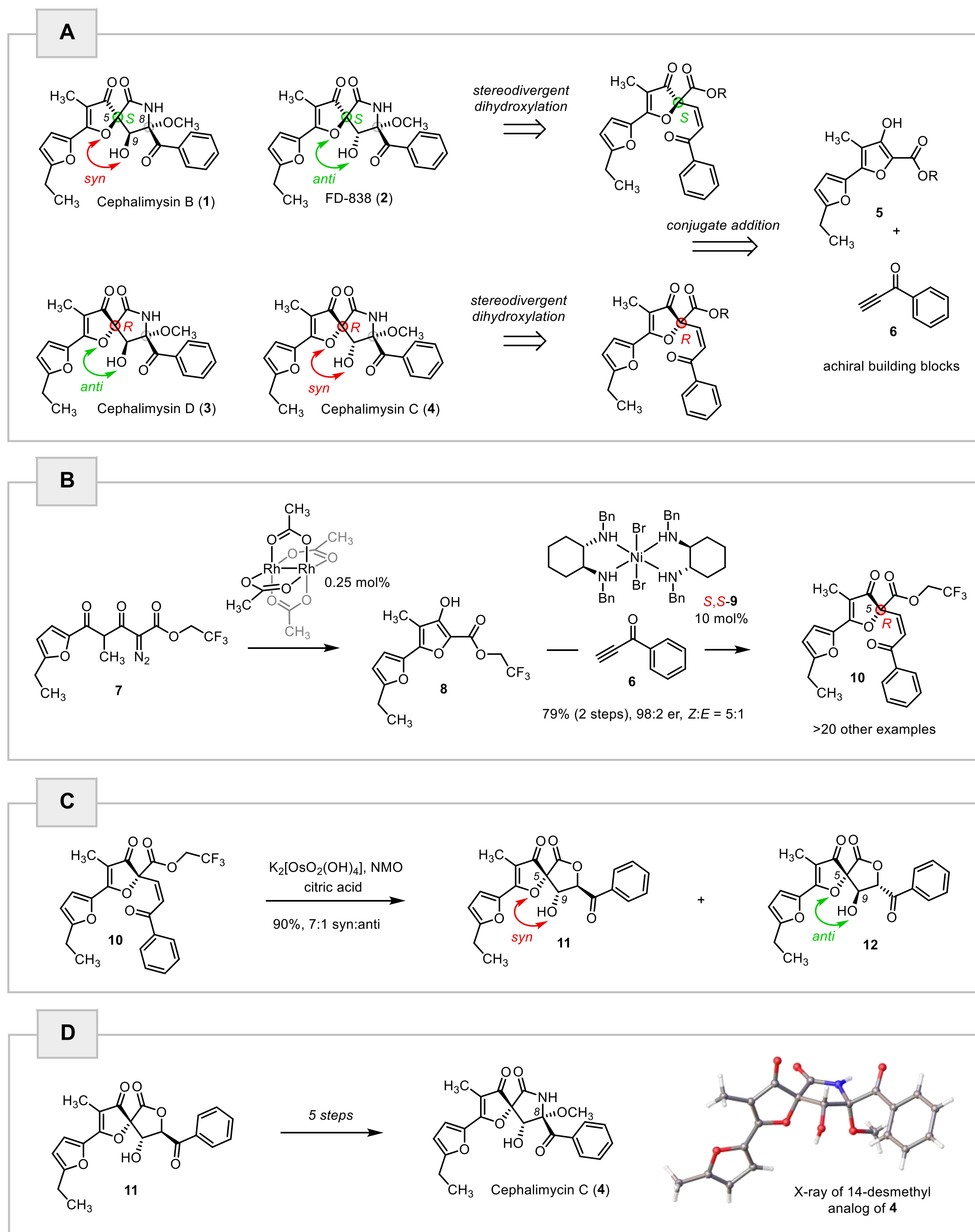
1B. ORIGINAL CONTRIBUTIONS FROM MY GROUP

We initiated the work on pseurotins in 2013, when the state-of-the-art was defined primarily through the work of Hayashi.[45,46] The logic of our approach to pseurotins emerged from the ambition to address the stereoisomerism commonly seen among pseurotins. The tetrad of pseurotins shown in Scheme 1A (**1**–**4**) became our testing ground. We envisioned that the absolute configuration of the spiro-carbon (C5) could arise from catalyst-controlled enantioselective conjugate addition of achiral 3(2*H*)-furanone (**5**) onto an alkynone (**6**) shown in Scheme 1A. The syn and anti relationships between the oxygen atom of the spiro-center (C5) and the neighboring secondary hydroxyl (C9) would be set through a stereodivergent dihydroxylation (see Scheme 1A for numbering). We expected that the stereocenter at C8 (shown in grey) would be possible to equilibrate (set) later in the synthesis.[47] The experimental execution of our synthetic plan is described next.

Cephalimysins B and C. To generate the substituted 2-alkoxycarbonyl-3(2*H*)-furanone **8**, we employed highly efficient rhodium-catalyzed cyclization[48,49] of the corresponding diazo carbonyl precursor **7** (Scheme 1B). The resulting furanone **8** was prone to air oxidation (hydroxylation) and was used directly in the subsequent conjugate addition to alkynone **6** in a single-flask process. The addition occurred smoothly in the presence of a catalytic amount of alkylamines but also silica, which we serendipitously found while monitoring the reaction progress and enantioselectivity. Later, we discovered that nickel(II)–diamine complex **9**, developed by Evans and Seidel for asymmetric Michael additions to nitrostyrenes[50], is an effective catalyst for the transformation and rendered the conjugated addition highly enantioselective (product **10**, 98:2 er, 5:1 *Z:E*, Scheme 1B). The method has a scope, which we demonstrated for over twenty other furanone–alkynone combinations.[51] The generally observed *Z* selectivity in the 1,4-adducts is likely the product of kinetic control during the protonation of an assumed allenolate intermediate (not shown).

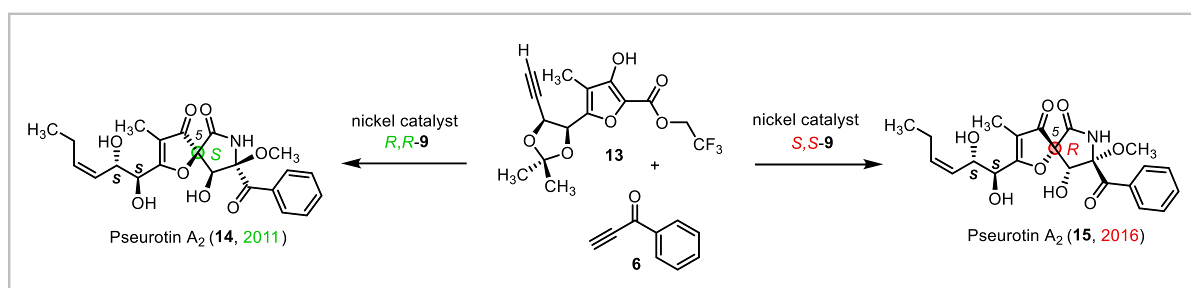
Our strategic choice of using the reactive trifluoroethyl ester transpires in Scheme 1C. The electron-deficient enone double bond of the 1,4-adduct **10** was dihydroxylated using a citric acid-modified Upjohn protocol,[52] with preference for the syn C5/C9 relationship (**11**:**12** 7:1; relative stereochemistry of both products confirmed by X-ray crystallography). Under the reaction conditions, the initially formed 1,2-diol underwent lactonization facilitated by the active trifluoroethyl ester. The potential problem of diol differentiation was thereby solved. We later made attempts to overturn the syn C5/C9 selectivity of dihydroxylation and preferentially obtain the anti relationship (diastereomer **12** and *ent*-**12** are potential precursors of cephalimycin D (**3**) and FD-838 (**2**)). Unfortunately, the Sharpless asymmetric dihydroxylation using various cinchona ligands or Upjohn oxidation with chiral citric acid analogs was unsuccessful. Changing the configuration of enone **10** (iodine-catalyzed $Z \rightarrow E$ isomerization) before dihydroxylation did not affect the preference for the C5/C9 syn relationship. Despite that, cephalimycin C (**4**) was readily synthesized in five steps from γ-lactone **11** having the syn configuration (Scheme 1D). The C8 stereocenter was established last during the introduction of the methoxy group (acid- or gold(III)-mediated process). The mixture of C8 epimers obtained was readily separated to provide pure cephalimycin C (**4**). That route represents the first enantioselective synthesis of this natural pseurotin

(12 steps, LLS).[53] At that time, it was also one of the shortest routes to a pseurotin. Diastereomeric cephalimycin B (**1**) was prepared analogously.



Scheme 1. Strategy for the synthesis of the pseurotin class of natural products.[53,51]

Pseurotin A2. After reporting our work on cephalimycins,[53] we were approached by researchers who identified antiseizure activity of naturally occurring pseurotin A2 using zebrafish and mouse models.[54] The authors were keen on exploring their findings further but were limited by the quantities of the natural product available through isolation. In planning to extend our chemistry to preparing this pseurotin, we found puzzling that two diastereomeric structures were proposed for pseurotin A2 (structures **14** and **15** in Scheme 2).[55,56] This was a problem well-fitted to our strategy. Specifically, catalysis by nickel(II)–diamine complex **9** could be used to set the spiro-carbon (C5) in either absolute configuration. As shown above, the stereochemical information at C5 is effectively relayed to the rest of the molecule. Importantly for us, both proposed pseurotin A2 structures feature a syn C5/C9 relationship. We found early on that the side-chain chirality of the required furanone partner **13** had little effect on the stereochemical outcome of the conjugate addition to **6** (Scheme 2). Accordingly, diastereomeric adducts (not shown) were selectively prepared using *S,S*-**9** and *R,R*-**9** as catalysts and elaborated to the 2011 and 2016 structures of pseurotin A2 (**14** and **15**, Scheme 2). The precise sequences had to be carefully choreographed due to the chemoselectivity problems associated with introducing the *Z*-configured side-chain alkene. This called for a late-stage Negishi-type sp–sp3 cross-coupling of a complex bromo alkyne,[56] then semi-hydrogenation. By comparing analytical data of the synthetic materials to those previously published in the literature for isolated pseurotin A2 (primarily NMR and CD spectroscopy),[55,56] we concluded that the 2016 stereoisomer **15** is the natural product.[57] However, it should not be too surprising if the stereoisomer **14** is isolated from natural sources in the future.



Scheme 2. Synthesis of both stereoisomeric structures proposed for pseurotin A2.[57]

Synthetic pseurotin analogs. In the next phase of the project, we demonstrated that our synthetic technology developed for natural pseurotins is useful for the preparation of structural analogs. This was achieved by changing the furanone and the alkynone components (see Scheme 1A). It was satisfying to see that the nickel(II)–diamine-catalyzed enantioselective conjugate addition methodology performed well for over twenty other furanone–alkynone combinations examined.[51] Extension to other classes of π-electrophiles, such as α-bromo enones and α-substituted nitroalkenes, was possible but required different catalyst systems (cupreine- and cupreidine-derived catalysts).[51] We prepared over forty new fully synthetic pseurotin analogs via our strategy and subjected those to various biological screenings.

One biological screen focused on the known immunomodulatory effects of pseurotins.[44] Collaborating with the laboratory of Lukáš Kubala at the Institute of Biophysics of the Czech Academy of Sciences, we found that some synthetic pseurotin analogs inhibited immunoglobulin E (IgE) production with a potency comparable to natural pseurotins A and D.[58] Kubala lab experiments revealed that the active inhibitors affect B-cell activation and phosphorylation of specific STAT proteins (Signal Transducer and Activator of Transcription). The latter step is obligatory in activating STATs, known transcription factors crucial for regulating the proliferation and differentiation of B-cells into immunoglobulin-producing plasma cells. The Kubala lab has attempted to further elucidate the mechanism, but the direct molecular target of pseurotins remains unknown, and it has been difficult to discern clear structure–activity relationships from this set of pseurotin analogs.[58]

1C. Critical summary

Our synthetic work on pseurotins led to the formulation of concise and efficient enantioselective routes to several members of this natural product family. We employed this method to complete the first enantioselective synthesis of cephalimycins B and C[53] and later of two stereoisomeric structures proposed for pseurotin A_2.[57] The pursued strategy proved effective in the synthesis of new pseurotin analogs. Developed methodology for enantioselective conjugate additions of 3(2*H*)-furanones to alkynones featuring nickel(II)-diamine catalysis should find use beyond this project.[51] One current limitation of our synthetic approach to pseurotins concerns the problem of stereochemically flexible dihydroxylation: the syn versus the anti C5/C9 relationship. Solving this problem would allow us to cover the full stereochemical matrix of pseurotins through a single approach. Despite this current limitation, our work yields pseurotins containing the syn C5/C9 relationship, while most other syntheses give pseurotins with the anti C5/C9 relationship.[41]

In the context of biological studies of pseurotins, we were able to show that synthetic pseurotin analogs can replicate some of the immunomodulatory effects of the more complex natural pseurotins. Furthermore, we gained insights into the cell-signaling pathways affected in B-cells after exposure to pseurotins (link to STAT signaling).[58] Nevertheless, it has been difficult to discern clear structure–activity relationships from these studies and identify a direct cellular target(s). Fundamental questions about the biological mechanisms of pseurotins, while partly advanced through our work, remain. From the chemical point of view, the signature spirocyclic scaffold of pseurotins is now readily accessible by synthesis. Being an underexplored chemotype, it should appeal to medicinal chemists.[59]

PART 2. FORSKOLIN

2A. INTRODUCTION TO THE RESEARCH PROBLEM

Forskolin (**19**, Scheme 4A) is a complex diterpene produced in the special root cork cells of the plant *Coleus forskohlii* as a small part of a rich mixture of various diterpenes (diterpenoids).[60] The biosynthetic pathway to forskolin involves the cyclase and oxidase stages. The complete carbon framework is built during the cyclase stage by the action of two diterpene synthases. Geranylgeranyl diphosphate is the substrate for key polyene cyclization and yields the tricyclic biosynthetic intermediate manoyl oxide.[61] The oxidase phase is complex and entails multiple C−H oxidations (positions C1, C6, C7, C9, and C11). A minimal set of P450 oxygenases has been identified.[60] Recently, the biosynthetic pathway was transferred to microbial organisms to give higher titers of manoyl oxide (10 mg/L)[62] and forskolin (40 mg/L).[60]

Forskolin (**19**) is most known for its ability to bind and allosterically stimulate membrane-associated adenylyl cyclases (isoforms denoted as AC1−AC9), enzymes responsible for the biochemical conversion of ATP to cAMP;[63] forskolin (**19**) is a cAMP booster. cAMP is a crucial second messenger behind various physiological processes, notably those acting through the therapeutically successful G-protein-coupled receptors (GPCRs).[64] While mechanistic details of the stimulatory effect are still a subject of research, forskolin (**19**) increases the affinity of the AC subunits making up the active site and induces conformational changes leading to enhanced catalysis (in synergy with G-protein subunit $G\alpha_s$).[65] This somewhat unique property among small molecule ligands made forskolin a frequently used tool in basic research[66] and a candidate in drug discovery. However, there are drawbacks to working with forskolin (**19**). For one, it is poorly water-soluble. Further, forskolin (**19**) affects multiple isoforms of adenylyl cyclase due to a high degree of conservation of the catalytic core[65] and was shown to interact with other cellular targets beyond adenylyl cyclases.[67] Isoform-selective modulators have long been sought to help probe and understand potentially different roles they might play physiologically (most are derived from genetic knock-out experiments).[68]

As signaled in the introduction to this thesis, structural modifications of natural products can fine-tune their properties. Exploiting the biosynthetic pathway represents one plausible route to forskolin analogs. In practice, it has been semisynthesis that yielded most new forskolin analogs.[69–72] The effort generated a lot of data, and, with it, insight into structure–activity relationships. The focus has been on modifying the C1, C6, and C7 hydroxyls of forskolin, understandably due to the ease of chemical manipulation (see Scheme 4A for numbering). The C6 and C7 positions were found to accommodate various substituents including bulky groups. Colforsin daropate is a water-soluble C6-modified semisynthetic analog approved for clinical use in Japan (acute heart failure).[73] In contrast, the free C1 hydroxy group was found to be essential for AC stimulation and, consequently, not a candidate site for manipulation (1-deoxyforskolin lacks the stimulatory property and served as the negative control in many studies). The absence of the tertiary C9-hydroxyl is tolerated as the resulting analog, 9-deoxyforskolin, remains a stimulator of adenylyl cyclases. Notwithstanding the importance of semisynthesis, the positions of forskolin that can be selectively altered within its complex structure are inherently limited. The available X-ray crystal

structures of the catalytically active subunits of AC with bound forskolin were used in rationalizing the activity of semisynthetic analogs.[65] It should be noted that these crystal structures, while consistent with most of the SAR data points, are proxies/models of the full adenylyl cyclase and should be treated with caution. The first structures of the full enzyme (specifically AC5, AC8, and AC9 isoforms) emerged only recently with the help of cryo-EM technology.[74–76] Also, until recently, standardized assays were unavailable to comprehensively query the isoform selectivity of small-molecule modulators (e.g., the full panel of AC isoforms expressed in a single cell line).[77]

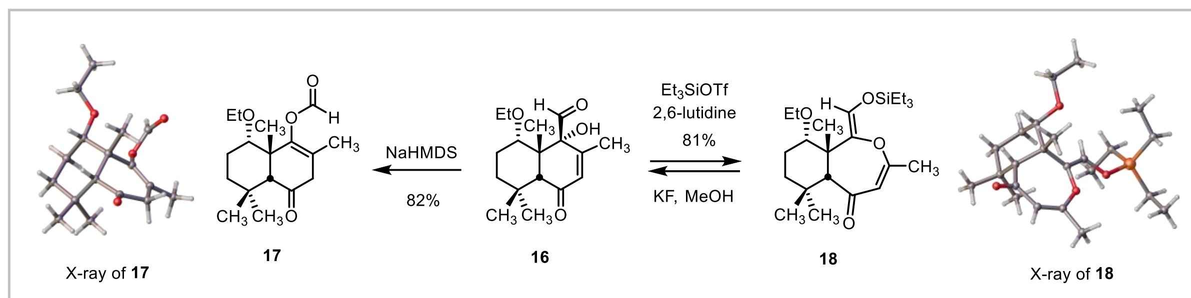
Fully synthetic analogs of forskolin (**19**) were considered to cover new modifications inconceivable by semisynthesis.[78] The challenge of synthesizing forskolin de novo (synthetic complexity) is the result of its densely substituted sp3-rich tricyclic scaffold, the presence of multiple oxygen-containing functional groups, and the complex stereochemistry (a total of eight stereocenters, four quaternary). Forskolin (**19**) can be prepared in the laboratory, as shown through the landmark works of Ziegler,[79] Corey,[80] Ikegami,[81] and more recently, Lett.[82,83] These routes are non-trivial for analog preparation because over 30 steps (LLS) are required to reach the target. We understood that designing an efficient, short, and flexible synthetic route to forskolin (**19**) would be a daring problem. Our route reported in 2017[84] and the 2019 route from Pronin lab[85] streamlined the preparation of forskolin (**19**) to 24 and 14 steps (LLS), respectively. An overview of our 2017 route to forskolin (**19**) and its application in analog synthesis is described below.

2B. Original contributions from my group

In 2014, when our laboratory initiated the development of a synthetic route to forskolin (**19**), the state-of-the-art required over 30 steps to obtain single-milligram quantities of this complex target in racemic form. We aimed at developing a route that would be shorter yet flexible enough to be useful in analog synthesis. Our synthetic planning is shown in Scheme 4A. The plan benefited from the pioneering study by scientists at Roche, who described Diels−Alder reactions of 2,6-dimethylbenzoquinone (**23**) with various 1,3-dienes as part of their synthesis of diterpene erigennol.[86,87] The authors demonstrated that a cyclopropyl ring within the diene component (see **22** in Scheme 4A) represents a masked form of the signature gem-dimethyl group of diterpenes. Curiously, they arrived at intermediates mapping well onto the A−B bicyclic ring system of forskolin (**19**) and were fully aware of the potential of their work: *"…man als potentielle zwischen-verbindungen fur eine forskolin synthese betrachten. Entsprechende verschuche sind im gange."* While the authors did not accomplish the synthesis of forskolin (**19**), their experimental findings proved invaluable to us.

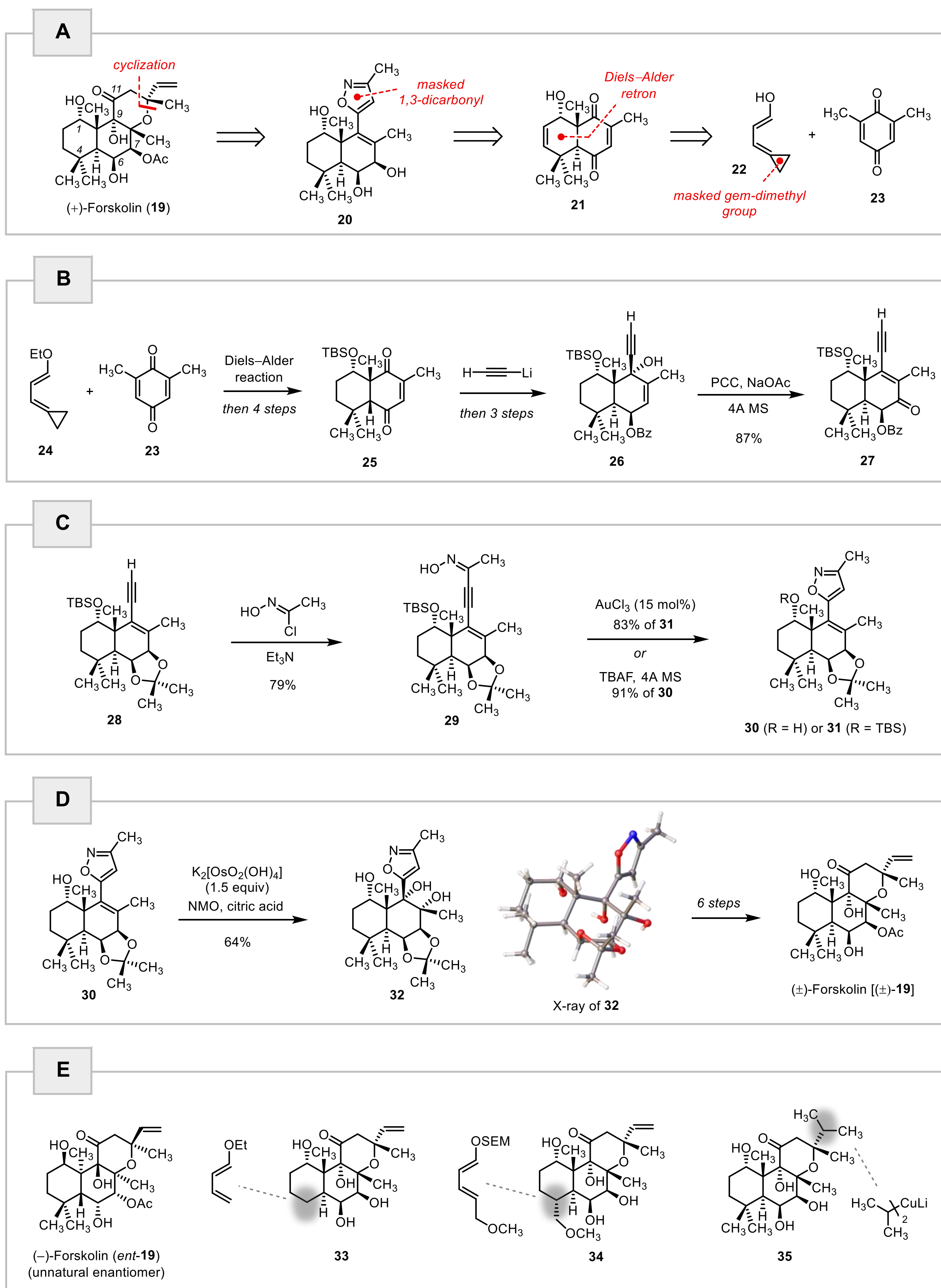
Our first approach to forskolin analogs. Our main question regarding the synthetic elaboration of the above-mentioned 2,6-dimethylquinone Diels−Alder adducts (e.g., **21** in Scheme 4A) was how to orchestrate and control chemoselectivity and stereoselectivity of the subsequent steps en route to forskolin (**19**). The early attempts were plagued by problematic reactivity caused by the proximity of several functional groups. Scheme 3 provides two illustrative examples. Exposure of bicyclic α-hydroxy aldehyde **16** (prepared from the corresponding Diels−Alder adduct in a few steps) to basic conditions unexpectedly delivered formate **17** in high yield. Separately,

attempted silylation of the hydroxy group on the same substrate (**16**) led to the ring-expanded product **18**. Both reaction outcomes were surprising to us. We speculated that the apparent rearrangement products **17** and **18** formed through mechanisms involving C–C bond cleavage of tentative epoxide intermediates.[88]



Scheme 3. Unexpected rearrangements of functionalized α-hydroxy aldehyde **16** during our early studies toward forskolin.[88]

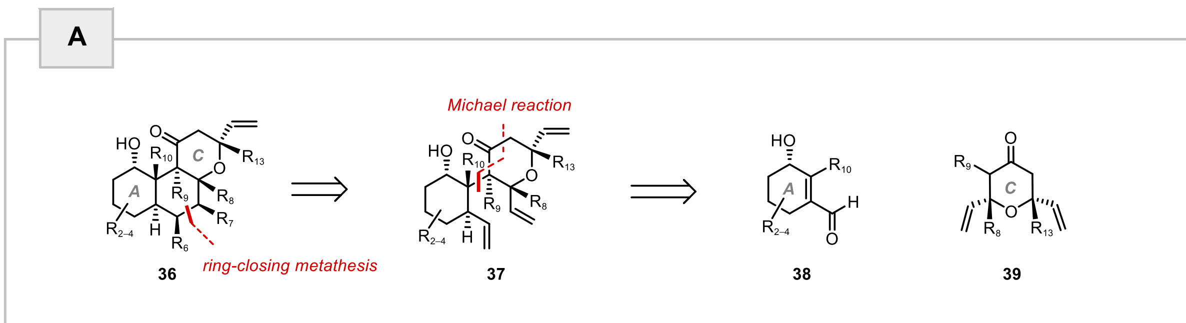
Solving these and other sometimes mind-boggling challenges was ultimately possible, and the 24-step synthetic route to forskolin (**19**) emerged (Scheme 4).[84] The specific sequence is discussed here only briefly. Diels–Alder adduct **25** prepared from quinone **23** and diene **24** was subjected to regioselective and diastereoselective acetylide addition, cis → trans ring isomerization, and strategic 1,3-oxidative transposition of **26** to yield enone **27** (Scheme 4B). Diastereoselective 1,2-reduction and protection provided advanced intermediate **28**. We viewed the pendant terminal alkyne in **28** as a substrate for (3+2) dipolar cycloaddition with acetonitrile oxide. The expected cycloadduct – isoxazole **31** – was targeted as a chemically robust masked form of a 1,3-diketone. It was interesting to discover that the reaction between alkyne **28** and acetonitrile oxide yielded alkynyl oxime **29** instead of the expected isoxazole **31** (Scheme 4C). This is an unusual reaction outcome and one we observed with several other nitrile oxides. We suspect that steric crowding around the alkyne **28** and the electron-rich character of the enyne π-system capable of stabilizing cationic intermediates might have contributed to the interrupted cycloaddition pathway. Fortunately, the alkynyl oxime **29** was readily isomerized (5-endo-dig cyclization) to give the desired isoxazole (**30**/**31**, Scheme 4C). The subsequent dihydroxylation of the hindered tetrasubstituted alkene **30** was challenging, requiring high loadings of osmium tetroxide and citric acid as a crucial additive.[52] We completed the synthesis and obtained over 100 mg of racemic forskolin (**19**).[84] Semipreparative HPLC was used to separate the enantiomers and demonstrate, for the first time, that the unnatural (–) enantiomer of **19** does not stimulate adenylyl cyclases. The natural (+) enantiomer of synthetic forskolin (**19**) matched the potency of the natural product. We published the work in 2017 as the most concise route to forskolin (24 steps, LLS).[84] Two years later, Pronin lab reported an even shorter route to this target featuring the bicyclic A–B intermediate used in our synthesis.[85] Pronin's innovation was primarily in a more concise construction of the A–B ring system (radical/polar crossover annulation as an alternative to the Diels–Alder reaction).

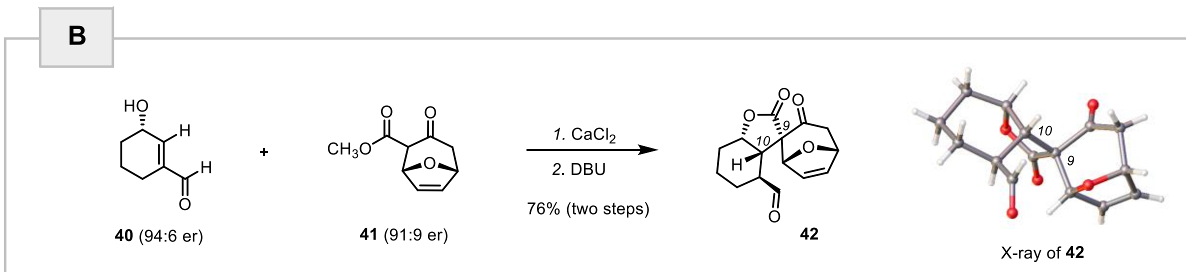


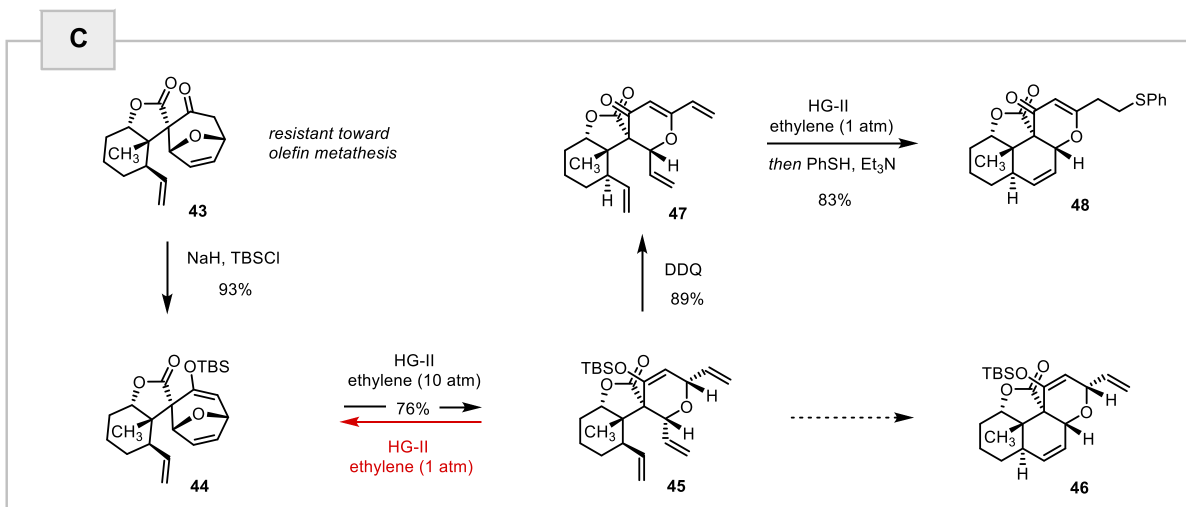
Scheme 4. Our first approach to the fully synthetic forskolin analogs.[84]

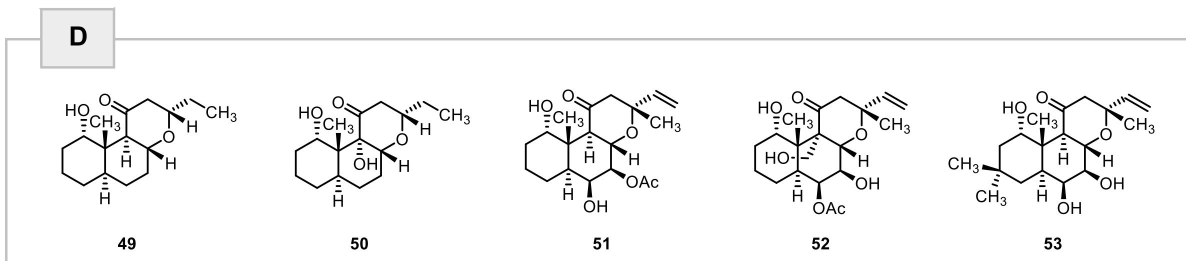
The ultimate ambition to prepare fully synthetic analogs of forskolin put our synthetic route to the test. We knew from the literature that modifications of the A-ring were little explored except for the C1 hydroxyl, which is, however, essential in the stimulation of adenylyl cyclase. Using 1-ethoxy-1,3-butadiene in the Diels–Alder step, we prepared forskolin analog **33** missing the gem-dimethyl group at the A-ring (Scheme 4E). The analog was an active stimulator of a subset of isoforms of adenylyl cyclase. Fueled by these findings, my colleague Kamil Paruch and his students utilized the established synthetic route to prepare several other A-ring-modified forskolin derivatives having activity toward adenylyl cyclase (e.g., **34** in Scheme 4E, unpublished results). This effort began to expand the available structure–activity relationships in new and exciting ways (ongoing). Admittedly, a slow rate of analog production has limited us, as each A-ring-modified forskolin analog requires structural change early in the synthesis. This line of thought led us to explore, in parallel, an alternative synthetic strategy for preparing forskolin analogs.

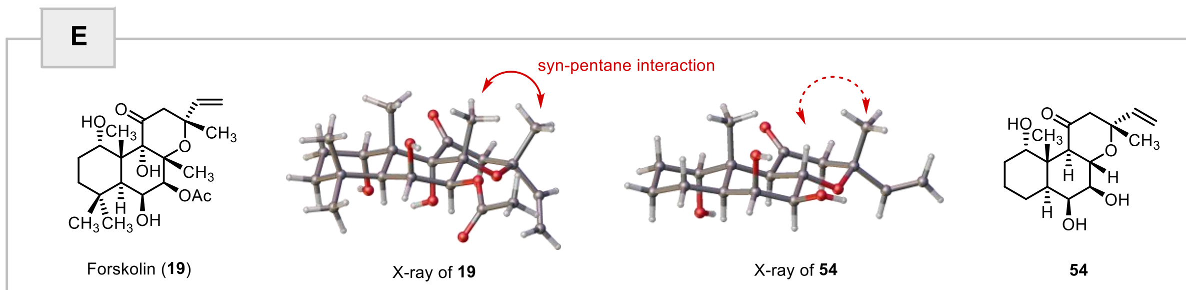
Our second approach to forskolin analogs. The essence of the alternative plan is presented graphically in Scheme 5A. We envisioned a convergent approach based on couplings of pre-functionalized A-ring and C-ring fragments (**38** and **39**, respectively) focusing on the A-ring diversity. We implemented the Michael reaction and ring-closing olefin metathesis as reactions enabling the fragment coupling. In experimental practice, it was critical to perform the Michael addition intramolecularly and use the more compact bicyclic β-keto ester form of the C-ring fragment (compare **39** and **41** in Scheme 5A, B). After implementing these modifications, the highly congested C9–C10 bond (see Scheme 5B for numbering) was established with near-perfect stereocontrol and good efficiency. This is illustrated for the specific fragment combination **40** + **41** in Scheme 5B. After transesterification and base-promoted Michael addition, the adduct **42** was obtained in 76% yield as a single diastereomer. The method was extended to other A-ring–C-ring fragment combinations and readily scaled up (gram scale).
From the Michael adducts, we initially envisioned that olefin metathesis could deliver the tricyclic structure of forskolin directly via a ring-opening/ring-closing metathetic cascade (Scheme 5C). The idea came short, however, because the bicyclic alkene **43** (prepared by Wittig reaction of the corresponding Michael adduct) was resistant to olefin metathesis using Hoveyda–Grubbs second-generation catalyst (HG-II)[89] and other ruthenium or molybdenum complexes (Scheme 5C). We solved this, at the time a major problem, by converting **43** into its silyl enol ether **44** (possibly increasing the ring strain) and using higher pressure of ethylene (10 atm, autoclave). The ring-opened product **45** was obtained in 76% yield. Subsequent C-ring oxidation to give **47** was used to prevent re-closure to the bicyclic alkene **44** (observed, see Scheme 5C) and thus direct the ring-closing metathesis toward tricycle **48** (product after capture by thiophenol). This carefully choreographed sequence worked well with this and other substrates. The limits of the method were met with substrates containing mono- and dimethylated C-ring at the bridgehead position(s), which was unfortunate considering the structure of forskolin (**19**). The methylation pattern of the C-ring, therefore, must be adjusted later in the synthesis.











Scheme 5. Our second approach to fully synthetic forskolin analogs.[90]

We elaborated tricyclic products such as **48** to the fully synthetic forskolin analogs in a few steps. We achieved the post-metathetic functionalizations with good chemo- and stereocontrol. Specific sequences varied and involved some interesting chemistry, as detailed in our article.[90] Selected fully synthetic forskolin analogs prepared this way are shown in Scheme 5D. The second approach is faster than our original route and seems promising in terms of flexibility (structural analogs).

In collaboration with the lab of Lukáš Kubala at the Institute of Biophysics of the Czech Academy of Sciences, we were eager to profile all prepared forskolin analogs from the second-generation approach. The Kubala lab established a FRET-based cAMP detection assay in human embryonic kidney cell cultures (HEK293) individually overexpressing eight AC isoforms (AC1–AC8; AC9 is only conditionally forskolin sensitive). Quite disappointingly, these new analogs displayed negligible stimulatory activity at micromolar concentrations despite the close structural similarity to the active analogs we made earlier (e.g., **33**). In trying to rationalize this outcome, we posited that the missing C8 methyl group in our synthetic analogs (see **49**–**53** in Scheme 5D) removes the 1,3-diaxial (syn-pentane) interactions normally present in forskolin (**19**). This has consequences particularly for the conformation of the C-ring, as can be appreciated from the side-by-side comparison of the X-ray crystal structures of **19** and **54** shown in Scheme 5E. Recognizing that the C-ring of forskolin is buried deeply within the AC binding site, changes to its conformation can be expected to affect binding. In the absence of a direct binding assay, however, we cannot rule out alternative explanations. Investigating chemistry to introduce the C8 methyl group would seem worthwhile.

2C. Critical summary

The forskolin project has been a significant effort in our group. We formulated and executed two independent synthetic strategies to prepare fully synthetic forskolin analogs. Many chemistry developments emerged over the course of this project. For example, interrupted alkyne–nitrile oxide cycloadditions yielding alkynyl oximes, construction of adjacent all-carbon quaternary stereocenters by intramolecular Michael addition, strain-driven ring-opening olefin metathesis of oxabicyclo[3.2.1]octenes, and more. Since bicyclic alkenes of the type studied here (e.g., **43**) are readily accessible by (4+3) cycloadditions[91] and feature prominently in natural product synthesis,[92] our findings on their reactivity in ring-opening olefin metathesis may be helpful to others. Importantly, the developed synthetic routes yielded a collection of new analogs of forskolin that were inconceivable through semisynthesis, thus painting new structure–activity relationships in the context of adenylyl cyclase stimulation. While it has been challenging to identify analogs with exquisite selectivity for a single isoform of adenylyl cyclase, we found that A-ring modifications lead to altered isoform selectivity relative to forskolin. Modifications in this region should be explored more in the future. We also learned about the apparent importance of substitution and conformational details at the C-ring. Overall, the forskolin project illustrates how the dedicated effort of synthetic chemists can reduce the synthetic complexity of highly complex targets, going from >40 steps down to 24 steps (our work) and, most recently, 14 steps (Pronin's work). There is room for

improvement, and further progress should make the preparation of fully synthetic forskolin analogs even more practical.

The forskolin project required the establishment of a full panel of individually overexpressed isoforms of adenylyl cyclase in a human cell line (HEK293). Previously, this non-trivial technology was not available in the field, and direct comparisons of published data were complicated, if possible at all. Our panel unified some of this data for the known compounds and served as a screening platform for fully synthetic analogs. Future search for new isoform-selective adenylyl cyclase modulators to explore the potentially isoform-dependent biology should benefit from this and other technologies.[93]

PART 3. BACTOBOLINS

3A. INTRODUCTION TO THE RESEARCH PROBLEM

Bactobolins are structurally unique secondary metabolites that have attracted considerable attention due to their potent antibiotic and antiproliferative activity.[94] The basic bactobolin scaffold is bicyclic, comprised of a δ-lactone and a cyclohexanone ring. The 3D structure of the most potent member of the family, bactobolin A (**56**), is shown in Scheme 6A. The δ-lactone ring of most bactobolins carries a signature dichloromethyl group and an axially positioned primary amine acylated by one or more amino acids (L-alanine). The cyclohexanone part contains a trans 1,2-diol and an enol of the β-keto ester unit. Bactobolin A (**56**) contains five contiguous stereocenters within the bicyclic scaffold, which presents a challenge to synthesis. Other members of the bactobolin family differ from bactobolin A in one or more ways, specifically (1) substitution at C3 (compare bactobolin A (**56**) and actinobolin (**55**)), (2) hydroxylation at C6, and (3) structure of the peptidyl substituent linked to the axial amine at C4. Recently discovered cryptic metabolites acybolins[95] feature complex peptidyl–fatty acid side chains attached to the amine at C4 (see below).

Biosynthetically, bactobolins are products of hybrid non-ribosomal peptide synthetase–polyketide synthase (NRPS–PKS) machinery.[96,97] 3-Hydroxy-4,4-dichlorovaline serves as an unusual extender unit and is derived from enzymatic chlorination and hydroxylation of L-valine (mechanistic details not clear). The bicyclic ring system is formed late in the biosynthesis through intramolecular aldol condensation and δ-lactonization to release the full product from the biosynthetic enzyme.

Bactobolins are potent antibiotics and antiproliferative agents.[94] They show sub-micromolar activity (MIC values) against Gram-positive and Gram-negative bacteria, including some from the so-called ESKAPE pathogens.[98] Antiproliferative effects of bactobolin A (**56**) against multiple cancer cell lines were demonstrated. Bactobolin A (**56**) inhibits protein synthesis (translation) by targeting bacterial and eukaryotic ribosomes.[99] In 2012, the Clardy lab analyzed ribosome mutations of the bactobolin-resistant bacterial strains to point the finger at the candidate binding site of bactobolin A (**56**) at the bacterial ribosome.[100] In 2015, Ramakrishnan and co-workers revealed molecular details of this binding site through X-ray crystallography and supported the mode of action at the molecular level.[101] Ribosome is a well-established target for antibiotics.[102] The distinct binding site makes bactobolins interesting in the context of new antibiotics and the issue of bacterial resistance. Poor selectivity of bacterial versus eukaryotic ribosome inhibition displayed by bactobolins is, however, a hurdle in considering the development of these natural products into clinically useful antibiotics.

Researchers have explored the structure–activity relationships of bactobolins in search of analogs with improved properties. Bactobolins can be biosynthesized on scale, and a pilot fermentation plant produced a multi-kilogram quantity of sulfate salt of (+)-actinobolin (**55**).[103] Semisynthetic modifications logically became the method of choice in preparing bactobolin analogs. Semisynthesis revealed some of the important structure–activity relationships. For example, the importance of L-alanine became apparent through the study by Munakata,[104] which evaluated various other amino

acids but arrived at analogs with inferior antibacterial activity. It is also clear now that substitution at C3, the position to which the signature dichloromethyl group is attached in bactobolin A (**56**), contributes to the potency of bactobolins. Nishimura and colleagues showed this in a series of papers.[105–107] Interesting was their finding that stepwise reductive dehalogenation of bactobolin A (**56**) gradually decreased the potency of the corresponding analogs. How the chlorine atoms help the potency has not been explained.

In addition to obtaining bactobolins by fermentation, several creative de novo syntheses of (+)-actinobolin (**55**, ≥17 steps (LLS))[108–114] were reported. Synthesis of (–)-bactobolin A (**56**, about 16 steps (LLS)) was accomplished only once by the Weinreb group.[115,116] De novo preparation of bactobolins can complement the semisynthetic approaches in the context of analog preparation but remains little explored.

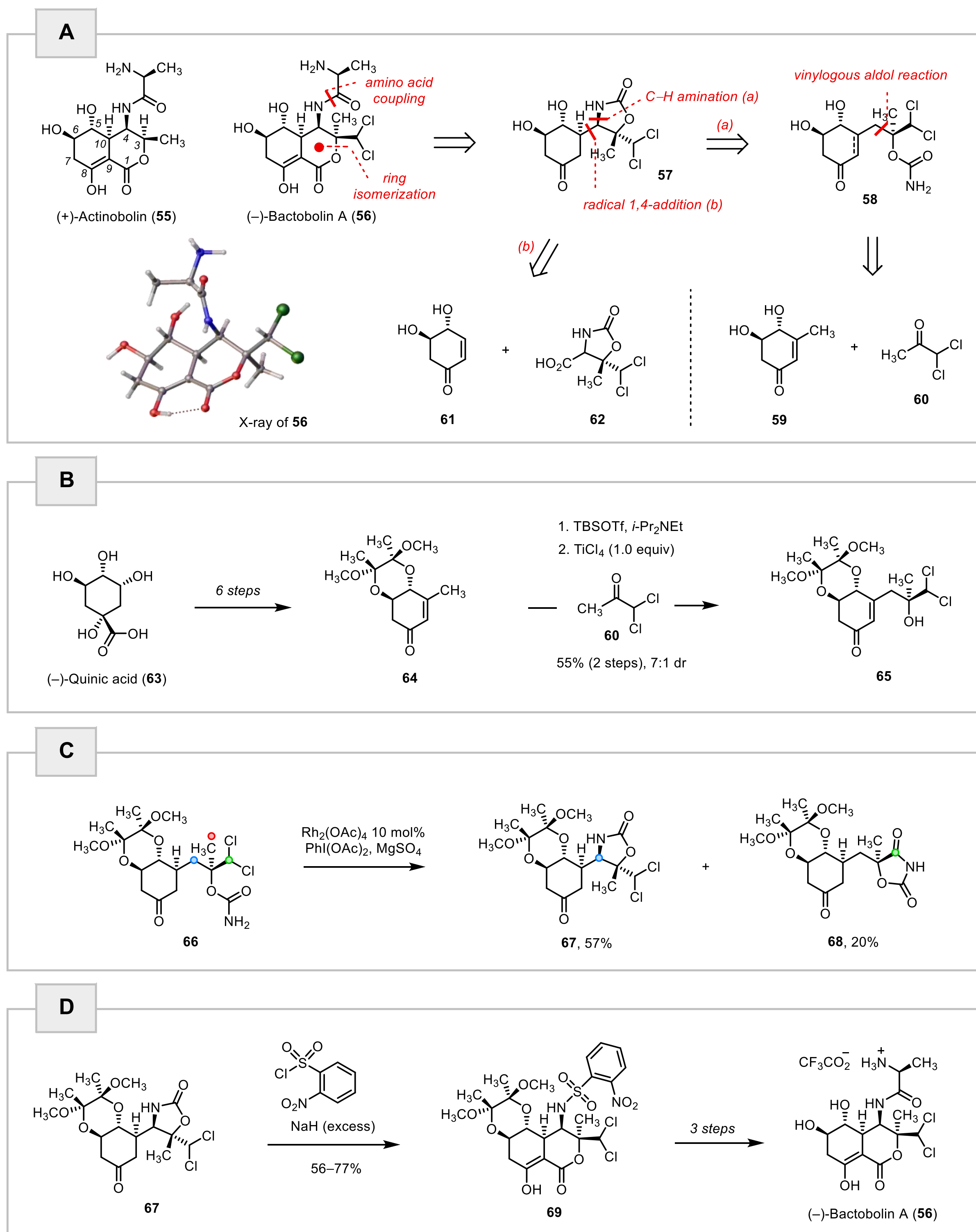
3B. Original contributions from my group

We established two synthetic routes to bactobolins. Substructure matching identified (–)-quinic acid (**63**) as the chiral pool starting material suitable for both routes, as it maps well onto the cyclohexane ring of bactobolins. To efficiently append the δ-lactone ring onto the cyclohexane ring of quinic acid, we targeted the oxazolidinone → δ-lactone rearrangement (**57** → **56**, Scheme 6A). This powerful yet underused transformation[115,117] offers a dramatic change in molecular topology. The cyclohexanone–oxazolidinone intermediate **57**, a substrate for the rearrangement, would be assembled by two strategies: one combining vinylogous aldol reaction and C–H amination, and the other involving radical 1,4-addition.

Our first-generation route to bactobolins. We published the first route in 2020,[118] and the synthetic sequence is overviewed in Scheme 6. Notable steps include a diastereoselective vinylogous Mukayiama aldol reaction[119] between enone **64** and 1,1-dichloroacetone (**60**) to set the challenging all-carbon quaternary stereocenter at C3, and rhodium-catalyzed diastereoselective DuBois C–H amination[120,121] to construct the oxazolidinone ring **67** (Scheme 6B, C). Site-selectivity of the amination step is interesting to analyze, as there are three types of C–H bonds within reach of the carbamate nitrogen (color-coded in Scheme 6C). While nitrene insertion into primary versus secondary C–H bonds can be predicted to favor the latter,[120] reactivity of the dichloromethyl C–H bond was uncharted territory. In practice, the desired site-selectivity was achieved (product **67**), with insertion into the dichloromethyl C–H bond occurring as a minor pathway (→ side product **68**, Scheme 6C). We later systematically studied the stereocontrol elements behind the observed high diastereoselectivity of the amination step (C4 carries two diastereotopic hydrogens).[118]

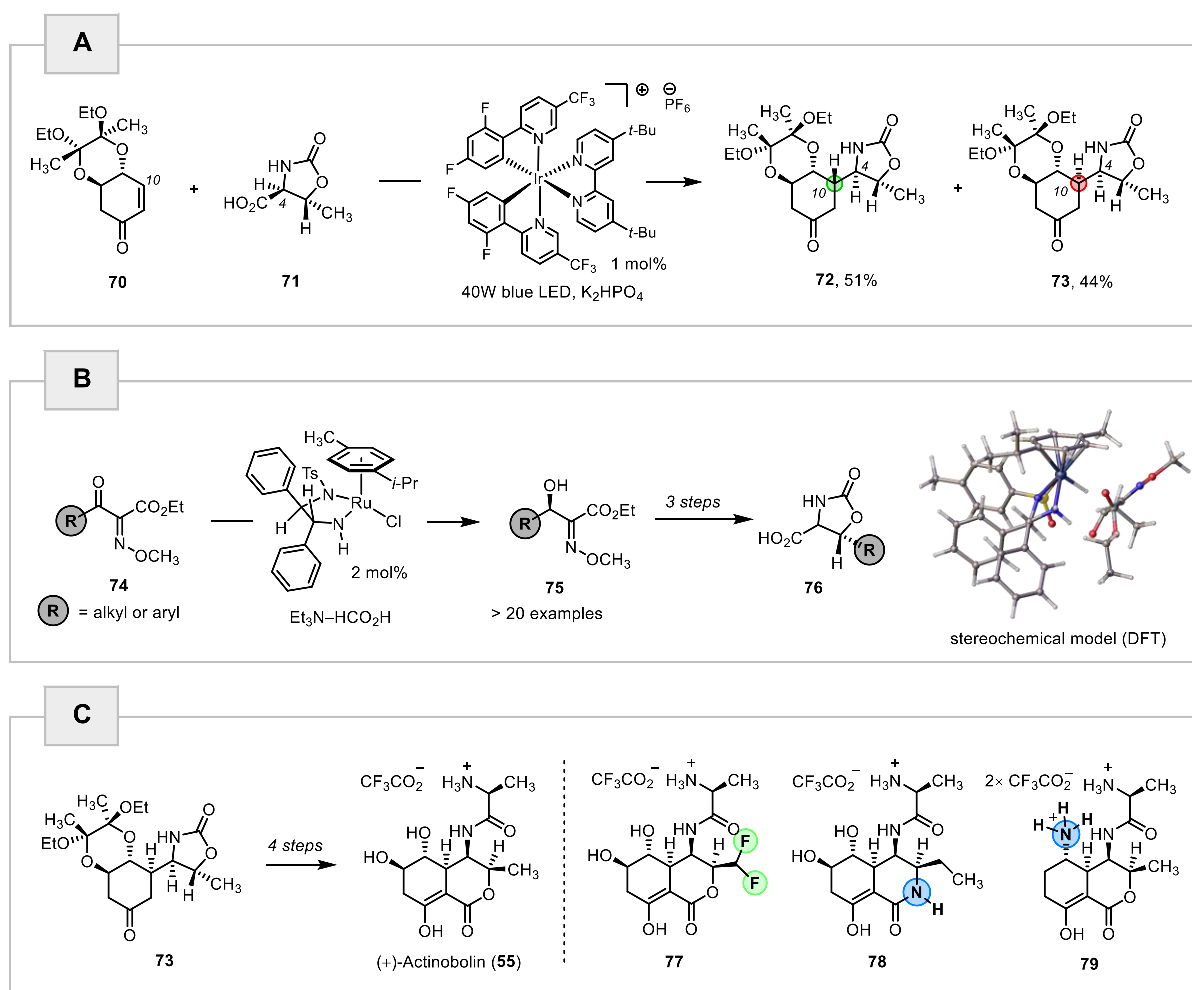
With the cyclohexanone–oxazolidinone intermediate **67**, we explored its rearrangement to lactone **69** (Scheme 6D). After some struggle, we developed a one-pot process, wherein the oxazolidinone is first *N*-sulfonylated (activated) and subsequently attacked by an enolate intermediate (DFT-calculated activation barrier for the intramolecular enolate attack is about 12 kcal/mol). Using the nosyl group (2-nitrobenzenesulfonyl) is worth pointing out, as previously, researchers struggled with the cleavage of other sulfonyl-based protecting groups from the analogous late-stage

intermediates.[117] The rearrangement established the bactobolin scaffold (**69**). The final steps involved cleavage of the nosyl group, coupling of protected L-alanine, and global deprotection. Overall, we prepared hundred-milligram quantities of (–)-bactobolin A (**56**) in 16 steps (LLS) and about 10% overall yield. It remains the most efficient synthetic route to this target.



Scheme 6. Our first-generation synthetic route to bactobolins.[118]

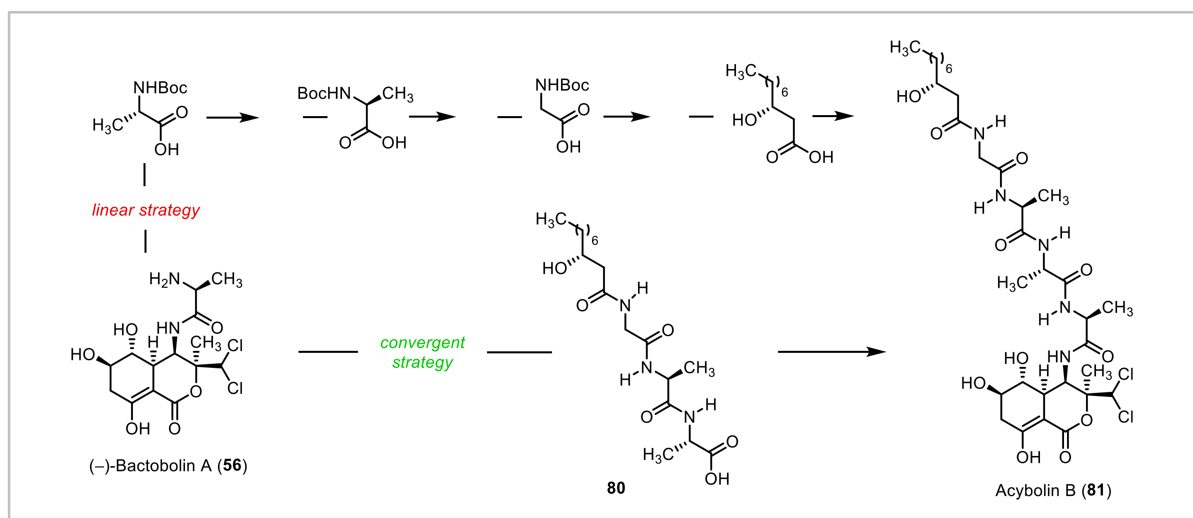
Our second-generation route to bactobolins. Our second route to bactobolins was published in 2022.[122] The approach was born out of the recognition that the key cyclohexanone–oxazolidinone intermediate **57**, originally prepared by the aldol and C–H amination chemistries, can be accessed more concisely by a radical-based fragment coupling (C10–C4 bond, Scheme 6A). This disconnection posed questions regarding chemo- and stereoselectivity but presented a unique opportunity to streamline the synthesis and render it modular.[123,124] In practice, L-threonine-derived oxazolidinone carboxylic acid **71** became the precursor of an α-amino radical, which we released through MacMillan's photoredox method.[125] Philicity[126] of the α-amino radical was well matched by the quinic acid-derived enone **70**, and the radical 1,4-addition (Giese reaction[127,128]) proceeded in close to quantitative yield (Scheme 7A). Two of four theoretically possible diastereomers were isolated (products **72** and **73**). Excellent facial selectivity occurred at the amino radical (C4) and, contrastingly, poor selectivity at the enone (C10). The diastereomers were easy to separate, and product **73** was used to complete the synthesis of (+)-actinobolin (**55**, 9 steps (LLS), 18% overall yield, Scheme 7C).[122]



Scheme 7. Our second-generation synthetic route to bactobolins and analogs thereof.[122]

Bactobolin analogs. The second route is significantly more concise and modular in design. The enone (**70**) and the oxazolidinone (**71**) fragments can be readily varied and yield the final analogs in about 5 steps. Following this logic, we prepared several novel bactobolin analogs, including **77**, **78**, and **79**, shown in Scheme 7C. I believe these examples illustrate well the unique capabilities of the developed synthetic approach compared to the prior art.
During the analog synthesis, we discovered a highly enantioselective Noyori–Ikaryia transfer hydrogenation[129] of α-methoxyimino-β-keto esters (**74**) as a method to prepare synthetic precursors (**75**) of enantiomerically enriched oxazolidinone fragments (Scheme 7B). The method was gauged on more than 20 examples and appears to have a broad substrate scope and functional group compatibility (manuscript in revision). The presence and configuration of the methoxyimino group were shown to be critical for the stereochemical outcome of the reduction. DFT calculated geometry of one of the low-energy transition states is shown in Scheme 7B (calculations done by Pavel Dub at Schrödinger, Inc., USA).
We determined the potency and selectivity of ribosomal inhibition for some of the synthetic bactobolin analogs in collaboration with Erik Boettger and Sven Hobbie at the University of Zurich (translation assay using bacterial ribosomes from *Mycobacterium smegmatis* and *Escherichia coli,* and eukaryotic ribosomes from rabbit reticulocytes). Selected data were already reported[122] and confirmed that potency and even ribosomal selectivity can be significantly perturbed by changing substitution at C3 of the bactobolin scaffold. Our work on this project is ongoing.

Acybolins. The latest synthetic development on the bactobolin project concerns the complex family of cryptic metabolites called acybolins discovered in 2016 by Seyedsayamdost lab.[95] These are biosynthesized only under specific conditions and were discovered upon exposure of *Burkholderia thailandensis* to the antibiotic trimethoprim. Inspection of the structure of acybolin B (**81**) in Scheme 8 immediately reveals the bactobolin A (**56**) within. Because of the complex side chain, acybolin B (**81**) and other acybolins do not fit into the bactobolin binding site and do not potently inhibit protein synthesis. The question of why bacteria biosynthesize acybolins is open and worthwhile investigating.
We developed two synthetic approaches to acybolins to provide more material for research on these natural products (only single milligrams were isolated from *B. thailandensis*). Our first route is linear and based on the step-by-step introduction of individual amino acids and the β-hydroxy fatty acid cap onto bactobolin A (**56**). The approach works but suffers from the typical drawbacks of a linear synthetic design. The second approach, more convergent,[11,130] appends the entire side chain **80** of acybolin B (**81**) onto bactobolin A (**56**). Through considerable experimentation, we found coupling conditions that are efficient and devoid of epimerization. Conveniently, no protecting groups are required in this one-step transformation (**56** + **80** $\rightarrow$ **81**, Scheme 8). Several other acybolins were synthesized analogously (manuscript in preparation).



Scheme 8. Linear and convergent routes to acybolins (unpublished results).

3C. CRITICAL SUMMARY

The two generations of syntheses of bactobolins developed by my group constitute the most concise de novo assemblies of these complex natural products. Our route to bactobolin A (**56**)[118] featured several interesting transformations, most notably the stereoselective aldol reaction of base-sensitive dichloroacetone, the C–H amination on a polyfunctionalized substrate, and the single-flask oxazolidinone → lactone rearrangement procedure. The route is an order of magnitude higher yielding (10% overall yield) than the prior art.[115,116] We later leveraged this aspect during the first synthesis of the complex cryptic metabolite acybolin B (**81**) and other members of the acybolin family. Ample quantities of synthetic material obtained will be useful during biological studies and, hopefully, in answering why acybolins are biosynthesized.

The second-generation route to bactobolins is particularly concise, and only 9 steps (LLS) were required to synthesize the naturally occurring (+)-actinobolin (**55**).[122] For reference, this is roughly half the number of steps required by the previous shortest approach.[110] Improving diastereoselectivity of the key radical 1,4-addition represents an obvious path to an improved overall yield of the sequence (currently 18%). I believe the technology represents a general solution to the synthesis of bactobolin analogs. The convergency and modularity of the approach delivered novel and fully synthetic analogs featuring deep-seated and unknown structural modifications. Whether this technology will solve the daring problem of ribosomal selectivity of bactobolins remains to be seen. The experiments systematically targeting different positions of the bactobolin scaffold are ongoing.

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