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Total Synthesis and Biological Evaluation of Rakicidin A and Discovery of a Simplified Bioactive Analogue

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Abstract: *We report a concise asymmetric synthesis of rakicidin A, a macrocyclic depsipeptide that selectively inhibits the growth of hypoxic cancer cells and stem-like leukemia cells. Key transformations include a diastereoselective organocatalytic cross-aldol reaction to build the polyketide portion of the molecule, a highly hindered ester fragment coupling reaction, an efficient Helquist-type Horner–Wadsworth–Emmons (HWE) macrocyclization, and a new DSC-mediated elimination reaction to construct the sensitive APD portion of rakicidin A. We further report the preparation of a simplified structural analogue (WY1) with dramatically enhanced hypoxia-selective activity.*

Tumor hypoxia is tightly associated with chemo- and radioresistance, increased metastatic potential, and poor prognosis in a number of cancer sub-types.[1] Evidence connects hypoxia to the capacity of cancer cell populations to form new tumors—defined by the content of stem-like cancer cells—through activation of a latent transcriptional program called the epithelial–mesenchymal transition (EMT).[2] These insights have elevated master hypoxic regulators such as mediators of the ER stress response,[3,4a] the TSC1/2-mTOR-RHEB axis,[4] and the hypoxia-inducible factors HIF-1/HIF-2[5] to high-priority therapeutic targets. Mechanistic studies of novel small molecules that specifically target hypoxic/stem-like cancer cells may reveal new pathway addictions that are critical in these cells. The natural product rakicidin A (**1**, Figure 1 A) is a macrocyclic depsipeptide from *Micromonospora sp.*[6] with selective toxicity towards hypoxic cancer cells[7] and gleevec-resistant hypoxia-adapted chronic myelogenous leukemia (CML) cells.[8] The latter is a model system for CML stem cells. The mechanism of action of rakicidin A (**1**) is unknown, and preliminary data indicate that the compound does not perturb the transcriptional activity of HIF-1,[7] nor does it appear to undergo bioreductive activation.[7,9] Structurally, **1** is characterized by a complex architecture comprising a polyketide unit and three amino acids: Sarcosine, β-hydroxyasparagine (β-HOAsn), and the rare 4-amido-2,4-pentadienoate (APD) group. The combination of interesting structure, potent biological activity, and unresolved mechanistic questions prompted us to investigate rakicidin A.

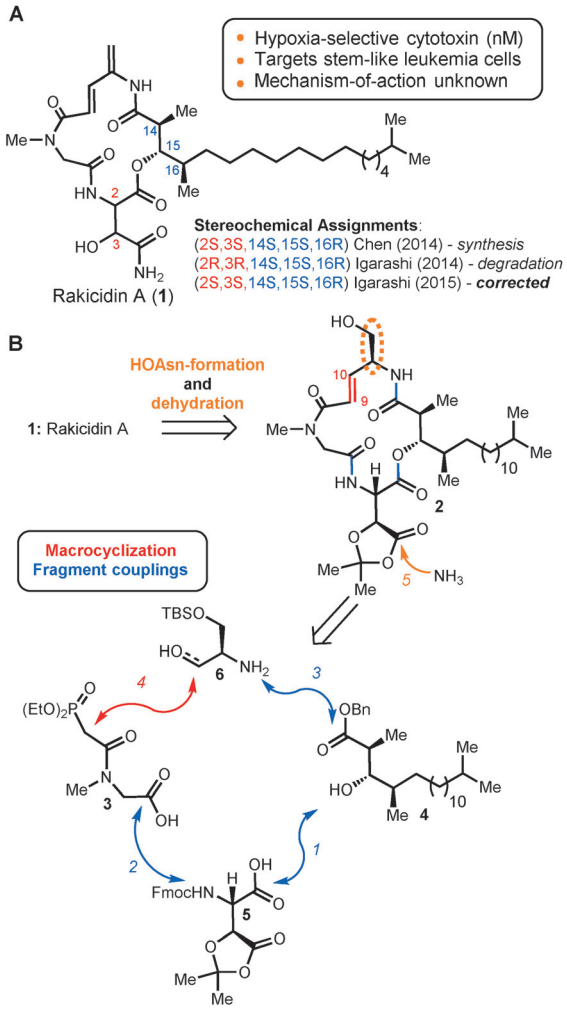

Figure 1. A) Chemical structure and key features of rakicidin A. B) Retrosynthetic analysis of rakicidin A. Colored numbers represent the sequence of fragment couplings and macrocyclization.

Since the stereochemistry of **1** was undetermined at the onset of our studies, we conducted a combination of NOE and conformational analyses of our previously developed model system of the macrocyclic ring,[10] as well as surveys of the literature (see the Supporting Information) in order to prioritize stereoisomers for synthesis.

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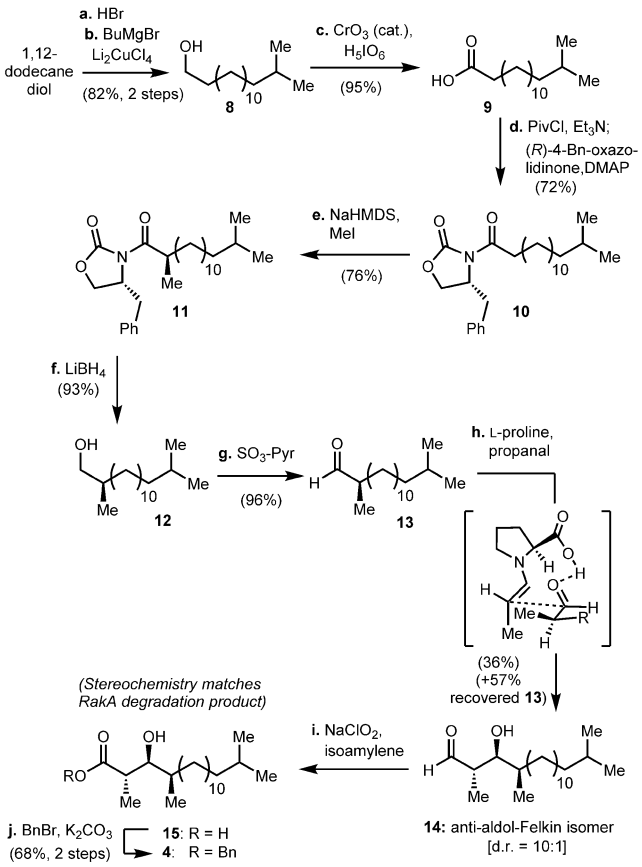
This analyses converged upon (2*R*,3*R*,14*R*,15*R*,16*S*) as the most likely configuration of natural rakicidin A,[11,12] and we initially targeted this particular isomer. Recently, two reports appeared, both claiming assignment of the configuration of rakicidin A.[13,14] Through synthesis of a compound with spectroscopic data matching those of the natural product, Chen and co-workers arrived at a (2*S*,3*S*,14*S*,15*S*,16*R*) configuration,[13] whereas Igarashi and co-workers paradoxically concluded the stereochemistry to be (2*R*,3*R*,14*S*,15*S*,16*R*) by direct degradation of the natural product and comparison with authentic standards (Figure 1 A).[14] This latter assignment, however, was corrected earlier this year to corroborate a (2*S*,3*S*,14*S*,15*S*,16*R*) configuration for rakicidin A, and it was thus clear that our initial synthesis in fact had targeted *ent*-rakicidin A. Herein, we report our unique route to the natural isomer of rakicidin A, along with biological evaluation and the discovery of a simplified bioactive rakicidin A analogue.

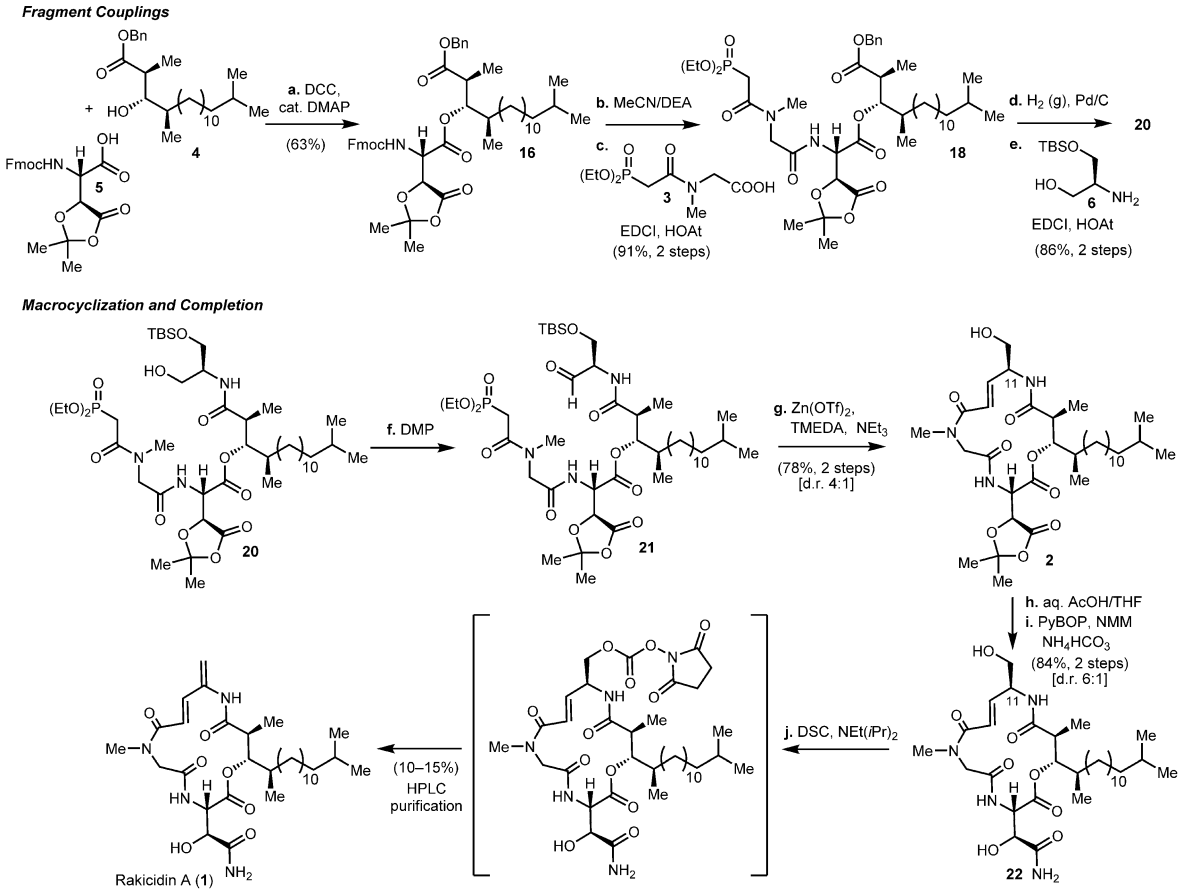
Although we have previously demonstrated in a model system that the APD group can be constructed directly by a Horner–Wadsworth–Emmons (HWE) reaction between a β-amidophosphonate and a 2-amidoacrolein-derivative,[10] it was deemed too challenging to handle this sensitive functionality during several synthetic steps. Consequently, dehydration would be delayed to the final step of the synthesis (Figure 1 B).[15] Considering macrocyclization sites to access **2**, we finally opted for pursuing an intramolecular HWE reaction for building the *trans*-C9–C10 double bond because of the relatively unhindered setup and the potential templating effects of the required metalloenolate intermediate. The cyclization precursor would be constructed by three fragment couplings, the first of which being an intermolecular ester coupling reaction[16] between a β-hydroxyaspartate-building block **5** and the secondary carbinol of β-hydroxy ester **4**. The (14*S*,15*S*,16*R*) isomer of **4** constitutes an *anti*-aldol-Felkin motif that should be accessible through asymmetric aldol methodology. The *threo*-(2*S*,3*S*) enantiomer of β-hydroxyaspartic acid can be accessed through a known enantioresolution of the commercial racemate.[17]

We first devised an efficient route from 1,12-dodecanediol to chiral aldehyde **13** (Scheme 1). The first stereogenic center at the C4 position of fragment **4** (C16 in **1**) was installed through an asymmetric methylation employing Evans (*R*)-oxazolidinone.[18] This was followed by reductive cleavage of the auxiliary and Parikh–Doering oxidation[19] to give enantiopure aldehyde **13**. Working initially on *ent*-**4**, we found that an Oppolzer aldol reaction[20] using superstoichiometric $TiCl_4$ could be employed to selectively prepare the desired *anti-syn* stereotriad. In our hands, however, this method lacked reproducibility, notably on a large scale, resulting occasionally in drastically reduced yields and selectivity. Poor solubility of **13** at low temperature was a major issue. We therefore considered alternatives,[21] and to our delight, we found that the proline-catalyzed cross-aldol reaction[22] between propanal and **13**, despite the double stereocontrol, proceeded with high diastereoselectivity. The typical yields ranged from 30–40 % after chromatography, however, owing to its operational simplicity, relative step-economy, and cost-effectiveness, this solution is superior to the Oppolzer aldol reaction sequence, at least for this particular target structure. Pinnick oxidation and benzyl ester formation completed an efficient route to β-hydroxy ester **4**.



Scheme 1. Synthesis of β-hydroxyester **4**. Reagents and conditions: a) 48 % aq. HBr (1.7 equiv), toluene, reflux, 65 h; b) *i*BuMgBr (3.0 equiv), Li_2CuCl_4 (0.013 equiv), THF, −78 °C to RT, 100 min; c) H_5IO_6 (3.0 equiv), CrO_3 (0.012 equiv), aq. CH_3CN, 0 °C to RT, 8 h; d) PivCl (1.1 equiv), NEt_3 (3.0 equiv), THF, 0 °C to RT, 50 min; then DMAP (0.3 equiv), (*R*)-4-Benzyl-2-oxazolidinone (1.2 equiv), THF, 50 °C, 5 h; e) NaHMDS (1.2 equiv), THF, −78 °C, 1 h; then MeI (5.0 equiv), −78 °C, 3 h; f) $LiBH_4$ (2.0 equiv), MeOH (1.5 equiv), THF, 0 °C to RT, 7 h; g) SO_3-Pyr (4.0 equiv), NEt_3 (5.0 equiv), CH_2Cl_2/DMSO, 0 °C 1 h; h) propanal (2.0 equiv, syringe pump), L-proline (0.2 equiv), DMF, 0 °C, 64 h; i) $NaClO_2$ (4.0 equiv), isoamylene (10 equiv), *t*-BuOH/H_2O, 0 °C, 17 h; j) BnBr (2.5 equiv), K_2CO_3 (3.0 equiv), DMF/CH_2Cl_2, RT, 48 h. *i*BuMgBr = isobutyl magnesium bromide, PivCl = pivaloyl chloride, DMAP = 4-dimethylaminopyridine, NaHMDS = sodium hexamethyldisilazide, Pyr = pyridine, THF = tetrahydrofuran, DMF = dimethylformamide, DMSO = dimethylsulfoxide.

Proceeding with the key ester coupling, we encountered serious problems and came up short trying to achieve the coupling between various protected forms of (2*S*,3*S*)-β-HOAsn or (2*S*,3*S*)-β-HOAsp with hindered alcohol **4**. We had anticipated challenges in this reaction,[16] but not even traces of product could be isolated. Searching for ways to reduce steric crowding as much as possible, we discovered that tying together the β-OH and β-COOH groups as a lactone ketal[23] uniquely enabled reactivity in this coupling (Scheme 2).



Scheme 2. Amide fragment couplings and completion of synthesis. Reagents and conditions: a) DCC (2.4 equiv), DMAP (0.3 equiv), DMF/CH_2Cl_2 (9:1), 0 °C, 5 h, b) MeCN/DEA (1:1), RT, 30 min; c) **3** (2.5 equiv), EDCI (2.6 equiv), HOAt (2.6 equiv), NEt(*iPr*)$_2$ (3.0 equiv), RT, 16 h; d) 10% Pd/C, H_2, EtOH, RT, 2 h; e) **6** (2.0 equiv), EDCI (1.5 equiv), HOAt (1.5 equiv), NEt(*iPr*)$_2$ (2.0 equiv), rt, 16 h; f) DMP (2.0 equiv), CH_2Cl_2, RT, 1 h; g) $Zn(OTf)_2$ (4.0 equiv), TMEDA (2.0 equiv), NEt_3 (5.0 equiv), THF, 45 °C, 16 h; h) AcOH/H_2O/THF (2:2:1), 45 °C, 24 h; i) PyBOP (1.2 equiv), NMM (4.0 equiv), NH_4HCO_3 (3.0 equiv), THF, 0 °C to RT, 24 h; j) DSC (1.5 equiv), NEt(*iPr*)$_2$ (2.0 equiv), MeCN/THF, 45 °C, 24 h. DCC = Dicyclohexylcarbodiimide, DMAP = 4-dimethylaminopyridine, EDCI = 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide, HOAt = 1-Hydroxy-7-azabenzotriazole, DMP = Dess–Martin periodinane, TMEDA = *N*,*N*,*N*′,*N*′-Tetramethylethane-1,2-diamine, PyBOP = (Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate, NMM = *N*-methylmorpholine, DSC = *N*,*N*′-Disuccinimidyl carbonate, DEA = Diethylamine.

After extensive optimization, we converged on a DCC-coupling method that reproducibly generated the required ester **16** in yields of around 60%, typically with recovery of alcohol **4**. The lactone ketal protecting group may find use in other syntheses of natural products containing β-HOAsn or β-HOAsp functionalities. With fragment **16** in hand, we could proceed with the construction of the two amide bonds (Scheme 2) and in both instances, efficient EDCI-mediated couplings were achieved to give the phosphonate **20** in an excellent yield (79%, 4 steps). The macrocyclization sequence was initiated with DMP oxidation of the primary alcohol, followed by exposure of aldehyde phosphonate **21** to conditions that would enable the HWE cyclization. We finally achieved an efficient (*E*)-selective macrocyclization under the soft-enolization conditions originally described by Helquist in the presence of TMEDA/$Zn(OTf)_2$ and triethylamine.[24] These conditions also reproducibly resulted in removal of the TBS ether, delivering advanced macrocycle **2** as a 4:1 mixture of diastereomers[25] in 78% yield over the three transformations. At this stage, the lactone ketal could be unmasked to install the primary amide functionality. Exposure of macrocycle **2** to 2 M NH_3 in 2-propanol allowed selective opening of the ketal to generate the β-HOAsn group but, unfortunately, it was generated as a 1:1 mixture of epimers most likely at C3. We attribute this stereochemical scrambling to the strongly basic conditions. Scouting for an alternative sequence for efficient introduction of the primary amide, we found that deprotection to the α-hydroxy acid could be carried out cleanly by using aqueous AcOH in THF, and when this material was directly subjected to a PyBOP-mediated coupling with ammonium bicarbonate, β-hydroxy amide **22** was formed in high yield.

The final dehydration of the primary alcohol also turned out to be difficult. Mesylation with MsCl curiously resulted in a non-mesylate derivative that still contained an sp^3 center at C11. We tentatively assign this material as the corresponding chloride, although subjection of the compound to HRMS analysis paradoxically only returned the mass matching that of rakicidin A. Elimination from this intermediate under basic conditions was not successful. Finally, we discovered

that dehydration to the sensitive APD functionality could be achieved in a one-pot reaction employing *N*,*N*′-disuccinimidyl carbonate (DSC)[26] at slightly elevated temperature, likely through the intermediacy of a mixed NHS carbonate (Scheme 2).

Purification and isolation of the natural product proved to be very challenging and resulted in low yields of the analytically pure material even though the dehydration appeared to proceed smoothly (by TLC analysis), with full conversion of the starting material after 24 hours. In accord with the observations of Chen and co-workers,[13] rakicidin A appears not to be stable in the organic solvents used for standard purifications (see the Supporting Information), and we observed extensive degradation after flash column chromatography and subsequent concentration (or lyophilization) from these solvents.[27] Final purification by RP–HPLC (eluting with MeCN/H_2O) and subsequent lyophilization delivered pure rakicidin A as a white powder, which could be readily manipulated. The synthetic material matched all spectroscopic data reported for the natural product (see the Supporting Information). Attempted DSC dehydration of epimer (3*R*)-**22**, generated as a side product in our initial NH_3-mediated lactone ketal opening, resulted in complete decomposition.

We investigated the performance of rakicidin A (**1**) in comparative cell viability assays with PANC-1 pancreatic carcinoma cells under normoxic (20% O_2) and hypoxic (0–0.5% O_2) growth conditions (Figure 2A). Synthetic rakicidin A causes potent and selective growth inhibition under hypoxic conditions ($GI_{50} = 36 \pm 1.4$ nM, $SI = 9.8 \pm 1.0$), in accord with the data initially reported for the natural product.[7] Interestingly, the hypoxia selectivity of rakicidin A exceeds that of tirapazamine ($GI_{50} = 17 \pm 1.3$ μM, $SI = 6.4 \pm 1.3$), an established bioreductively triggered cytotoxin used as a positive control. The combination of a macrocycle-embedded weak electrophile (APD)[15b] and a pendent long lipophilic chain means that rakicidin A shows significant structural similarity to the syrbactins (e.g. cepafungin I, Figure S2 in the Supporting Information), a family of potent inhibitors of the eukaryotic 20S proteasome.[28] However, rakicidin A (**1**) does not act through this mechanism, as demonstrated by the fact that none of the proteolytic activities of the proteasome were inhibited by **1** (10 μM) in direct biochemical assays (Figure 2B and Figure S2). In further support of this conclusion, we found that bortezomib, an FDA-approved proteasome inhibitor, did not display hypoxia-selective growth inhibition (Figure 2B). Indeed, specific small-molecule inhibitors of several additional pathways known to be important in the hypoxic state failed to phenocopy the activity of rakicidin A in PANC-1 cells (Figure S3). We found that the APD unit is critical since its removal in compound **22** results in a complete loss of activity (Figure 2D). We next generated a structure (WY1) encompassing only the APD unit and the lipopeptide chain. Despite the increased conformational freedom of this compound, the synthesis and notably the macrocyclization proceed with high efficiency (Figure 2C and

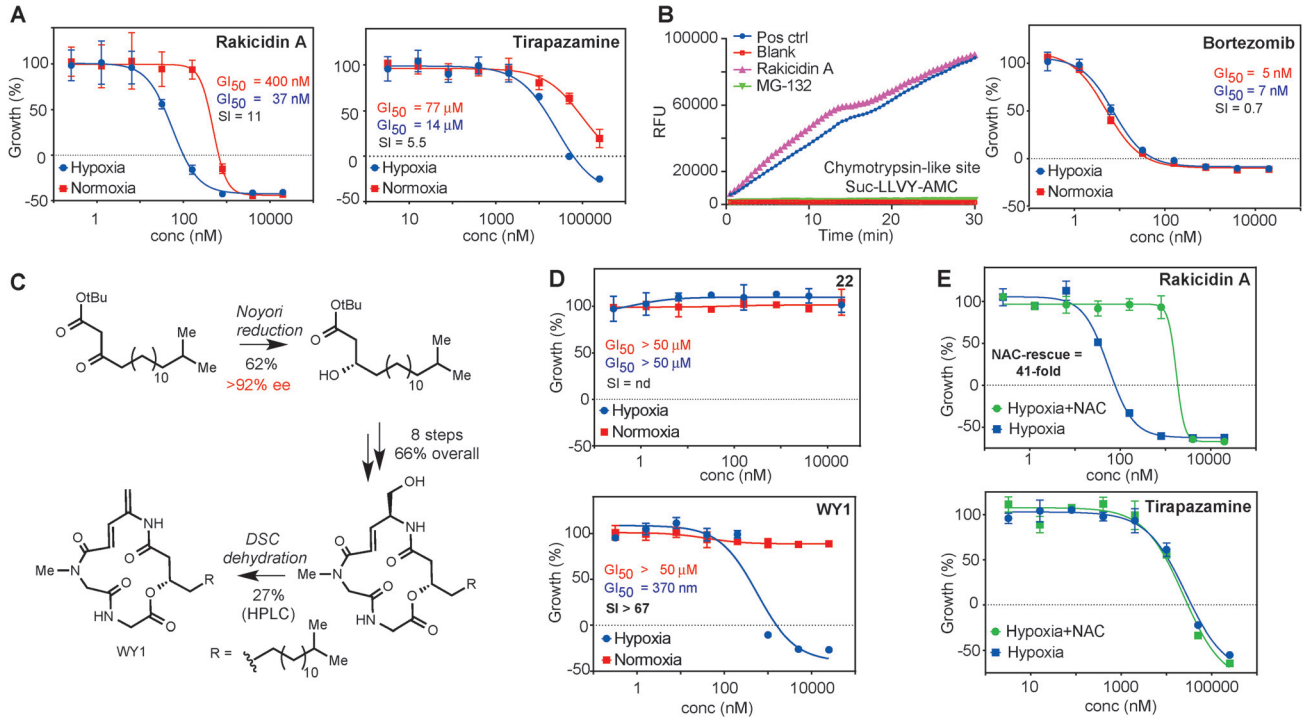


Figure 2. Biological evaluation of synthetic rakicidin A and analogues. A) Representative growth inhibition curves in PANC-1 cells evaluated under normoxic (20% O_2, red) and hypoxic (0.1–0.5% O_2, blue) culture conditions for 48 h. Bioreductively activated cytotoxin tirapazamine is the positive control. B) Hypoxia-selective activity of bortezomib and in vitro 20S proteasome inhibition assay with an AMC-conjugated substrate specific for the chymotrypsin-like activity. C) Synthetic route to WY1, a rakicidin A analogue comprising only the APD and lipid functionalities (for details see Supporting Information and Scheme S1). D) Growth inhibition by compound **22** (Scheme 2) and WY1 in PANC-1 cells. E) Effect of *N*-acetylcysteine (NAC) on growth inhibition by rakicidin A and tirapazamine under hypoxic conditions. SI = Selectivity index.

Scheme S1 in the Supporting Information). Cell-based evaluation of WY1 revealed that this minimal APD-cyclolipodepsipeptide (APD-CLD) is sufficient to afford hypoxia-selective growth inhibition. Although the potency is approximately 10-fold reduced during hypoxia ($GI_{50} = 352 \pm 21$ nM, Figure 2D) compared to rakicidin A, WY1 is completely inactive under normoxic conditions up to 25 μM ($SI > 70 \pm 3.8$). This remarkable increase in selectivity clearly indicates that rakicidin A has at least two separable mechanisms of toxicity. Finally, we evaluated the effect of augmenting the hypoxia-induced reductive stress through co-treatment with the glutathione-precursor *N*-acetylcysteine (NAC, Figure 2E). We found that the activity of tirapazamine in hypoxic PANC-1 cells was not further enhanced by NAC but that the activity of rakicidin A was dramatically reduced. The mechanistic basis for this finding remains to be determined.

In conclusion, we have developed a modular asymmetric synthesis of rakicidin A and have discovered a simplified analogue with enhanced hypoxia-selective activity. Our synthetic route includes novel solutions to the preparation of the key functionalities. Rakicidin A appears to act through a non-canonical pathway upon which hypoxic cancer cells show an increased dependence.

Acknowledgements

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[27] We have isolated material by FC (pure by TLC) that would equate a yield of 47%, which is similar to that reported by Chen and co-workers (Ref. [13]) for their mesylation-elimination sequence on the C11 epimer of alcohol **22**. In our hands, attempted dissolution of this TLC-pure material in [D_6]DMSO afforded a > 90% pure NMR spectrum of rakicidin A but nearly 3/4 of the material (by mass) did not dissolve and thus very likely represents a degraded compound.
[28] a) M. Groll, B. Schellenberg, A. S. Bachmann, C. R. Archer, R. Huber, T. K. Powell, S. Lindow, M. Kaiser, R. Dudler, *Nature* **2008**, *452*, 755 – 758; b) D. Krahn, C. Ottmann, M. Kaiser, *Nat. Prod. Rep.* **2011**, *28*, 1854 – 1867.

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