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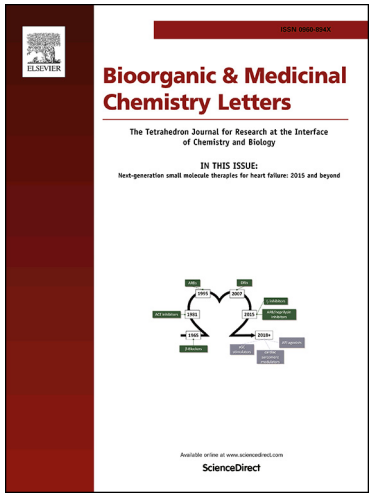

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Synthesis of C-ring-modified blebbistatin derivatives and evaluation of their myosin II ATPase inhibitory potency

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ABSTRACT

(*S*)-Blebbistatin is a micromolar myosin II ATPase inhibitor that is extensively used in research. In search of analogs with improved potency, we have synthesized for the first time C-ring modified analogs. We introduced hydroxymethyl or allyloxymethyl functionalities in search of additional favorable interactions and a more optimal filling of the binding pocket. Unfortunately, the resulting compounds did not significantly inhibit the ATPase activity of rabbit skeletal-muscle myosin II. This and earlier reports suggest that rational design of potent myosin II inhibitors based on the architecture of the blebbistatin binding pocket is an ineffective strategy.

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(*S*)-Blebbistatin (*S*)-**1** is a micromolar ATPase inhibitor selective for myosin II and with extensive applications in research despite a number of physicochemical shortcomings (Figure 1).[1,2] We and others have previously established that modification of ring D,[3-7] and to a lesser extent ring A,[8] leads to analogs with superior research tool properties, such as (*S*)- 4'- nitroblebbistatin (*S*)-**2**, (*S*)-4'-aminoblebbistatin (*S*)-**3**, (*S*)-3'-hydroxyblebbistatin (*S*)-**4** and (*S*)-3'-aminoblebbistatin (*S*)-**5**. Myosin II has multiple physiological roles, such as in migration, neuronal functioning, biochemical signaling and gene transcription.[9] The protein is considered a potential therapeutic target in a diverse range of diseases and disorders, e.g. cancer metastasis,[9] methamphetamine use relapse,[10] viral infections,[11] glaucoma,[12] liver fibrosis[13] and thrombosis.[14] Potent and drugable inhibitors of particular isoforms of this protein could thus be valuable pharmacological tools.[9] However, obtaining significant potency enhancement *via* modification of the aforementioned rings D and A of (*S*)-blebbistatin (*S*)-**1** seems unattainable.[3,15,16] The goal of the present study was to examine the influence of small chemical modifications of ring C on ATPase inhibitory potency. No reports on the exploration of the structure-activity relationship (SAR) landscape of this part of the molecule have been published to date.

The co-crystal structure of (*S*)-blebbistatin (*S*)-**1** bound to *Dictyostelium discoideum* myosin II (PDB: 1YV3) was used to scout for possible favorable interactions with the residues lining the binding pocket.[17] Looking at Figure 2A, we hypothesized that *cis*-oriented hydrophilic moieties (e.g. hydroxymethyl) on C^2 of the blebbistatin scaffold might engage in hydrogen bonding with the carboxylate of the neighboring Arg238 residue. This additional bonding possibility would be absent in the *trans*-oriented diastereoisomers. Further, both *cis*- and *trans*-oriented larger functionalities (e.g. allyloxymethyl) might optimize filling of the binding pocket (Figure 2B). Given these observations and the ready availability of racemic pyroglutamic acid **9** (Scheme 1), the synthesis of both diastereoisomers of analogs (±)-**6** and (±)-**7** was envisioned.

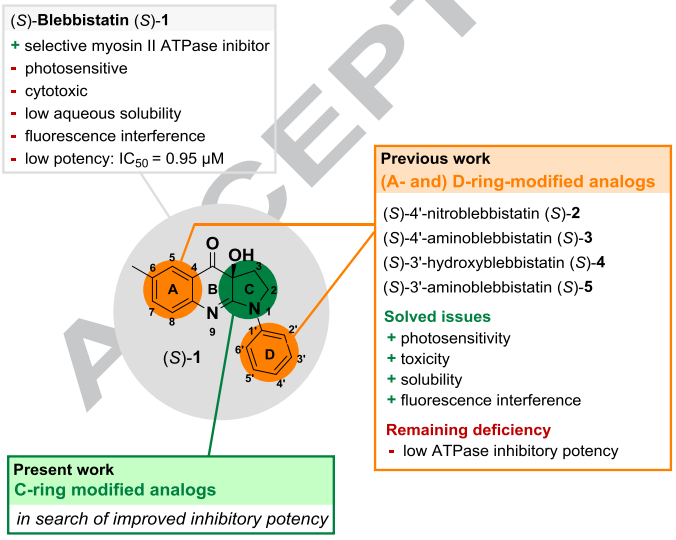


Figure 1. Structure and numbering system of (*S*)-blebbistatin (*S*)-**1**, most important analogs with improved biochemical tool properties and envisaged work in this study.

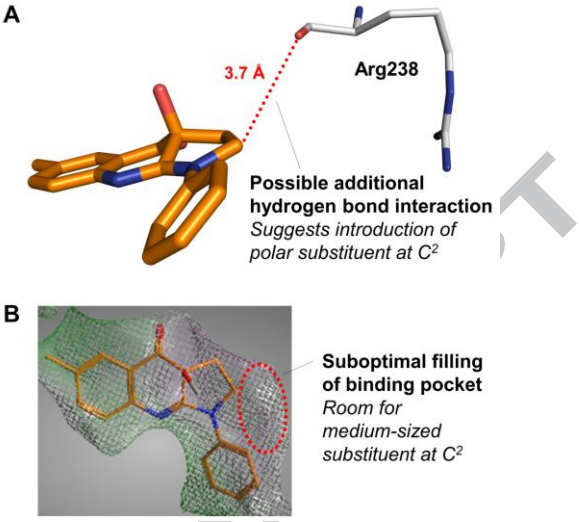
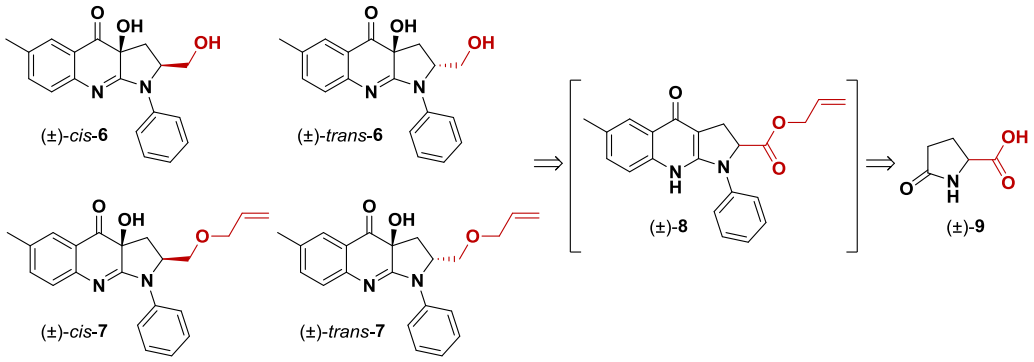
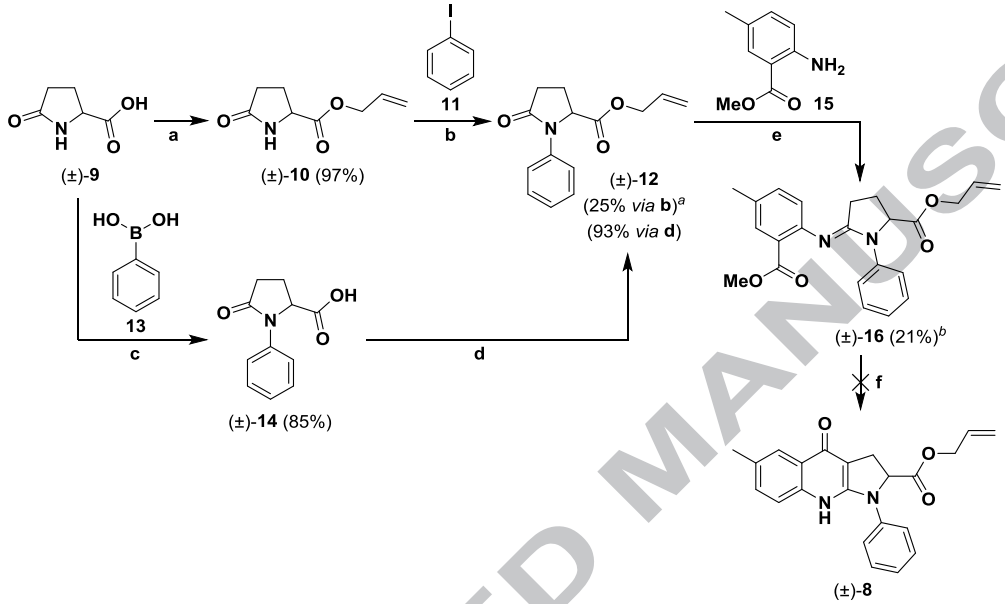


Figure 2. Rationale of the present work. **A.** The co-crystal structure of (*S*)-blebbistatin (*S*)-**1** bound to *Dictyostelium discoideum* myosin II (PDB: 1YV3)[17] suggests the possibility of hydrogen bonding between *cis*-oriented hydrophilic moieties (e.g. a hydroxymethyl group) at C^2 and the carboxylate oxygen of Arg238. **B.** Interaction surfaces (onset of Van der Waals clash) for the binding mode of (*S*)-blebbistatin (*S*)-**1**: pink indicates polar areas on the receptor surface, green indicates greasy areas.[18] Atom colors in A and B: orange: carbon, red: oxygen, blue: nitrogen, grey: carbon.

Due to the exploratory nature of this investigation, the syntheses in this letter start from racemic substrates and do not focus on obtaining enantiomerically pure compounds. The precursor (±)-pyroglutamic acid (±)-**9** was chosen as an ideal starting point for the synthesis of the proposed blebbistatin analogs (±)-**6**–**7** (Scheme 1). This strategy required the preparation of quinolinone (±)-**8** as a key intermediate. The pathway (Scheme 2) commenced with the synthesis of amide (±)-**12** which could be accessed *via* two separate routes. In a first method, allyl protection of the carboxyl group of (±)-pyroglutamic acid (±)-**9** (step **a**, 97%)[19] preceded Goldberg-type *N*-arylation of compound (±)-**10** with iodobenzene (**11**).[20] The rather low conversion of the latter step (step **b**, 25%) prompted us to use an alternative method for the preparation of amide (±)-**12**. In this approach, Chan-Lam-type *N*-arylation of the unprotected (±)-pyroglutamic acid (±)-**9** was performed with phenylboronic acid (**13**) (step **c**, 85%).[21,22] Subsequent allyl protection of the carboxyl group of compound (±)-**14** afforded amide (±)-**12** (step **d**, 93%) in a much higher yield than *via* the first method. Amide (±)-**12** was reacted with $POCl_3$ and amine **15** to furnish amidine (±)-**16** (step **e**, 21%) which was subsequently treated with LiHMDS to induce intramolecular cyclization. However, this resulted in a complex mixture, containing only trace amounts of quinolinone (±)-**17** (step **f**).



Scheme 1. Overview of the targeted C-ring-modified blebbistatin derivatives (±)-**6**–**7**, accessible from (±)-pyroglutamic acid (±)-**9** *via* key intermediate quinolinone (±)-**8**.

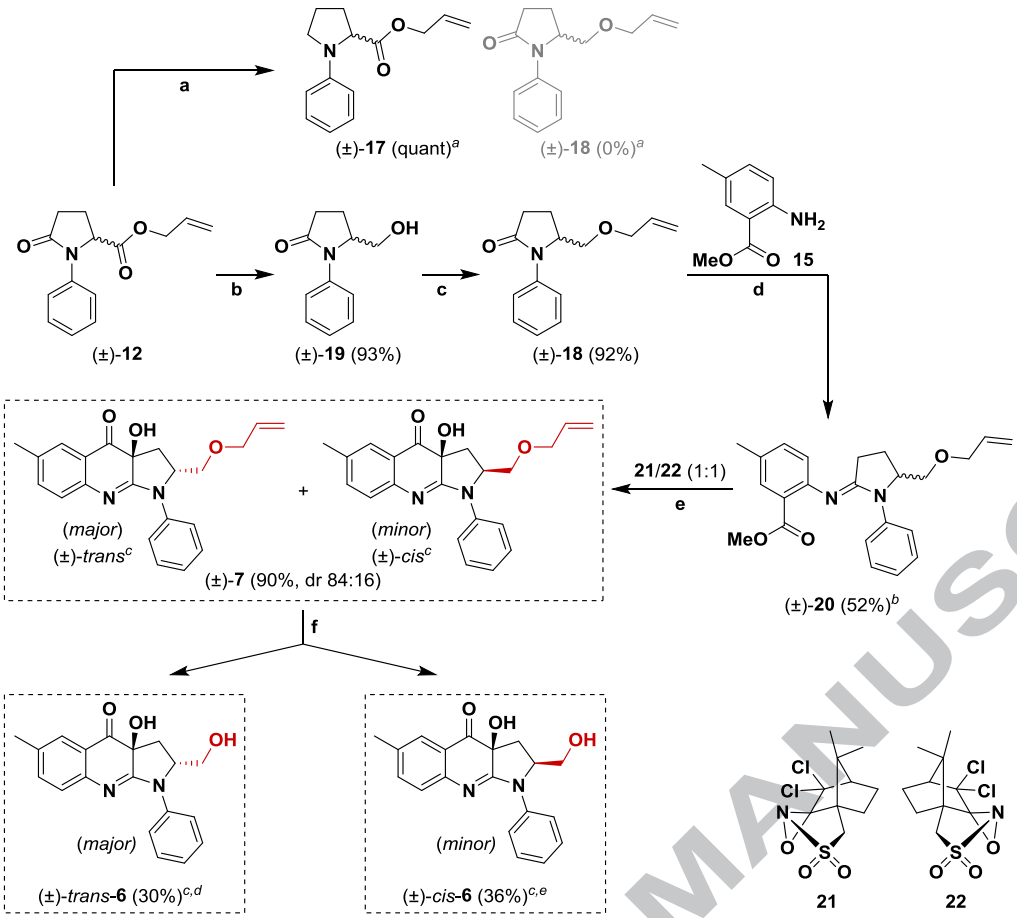


Scheme 2. Attempted synthesis of quinolinone (±)-**8**. Reagents and conditions: **a.** 1.2 equiv $SOCl_2$, allyl alcohol, rt, 17 h. **b.** 3× 0.1 equiv CuI, 3× 0.1 equiv tris(3,5-dimethyl-1*H*-pyrazol-1-yl)methane, 1.2 equiv iodobenzene (**11**), 2 equiv K_3PO_4, dry 1,4-dioxane, reflux, 5+16+48 h. **c.** 2 equiv DBU, 0.15 equiv CuTMEDA, 1.5 equiv phenylboronic acid (**13**), dry CH_3CN, rt, 48 h. **d.** 2× 1.2 equiv $SOCl_2$, allyl alcohol, rt, 9+15 h. **e.** 1) 2 equiv $POCl_3$, dry CH_2Cl_2, rt, 24 h; 2) 1.05 equiv amine **15**, dry CH_2Cl_2, 35 °C, 48 h. **f.** 2.1 equiv LiHMDS, dry THF, 0 °C, 2 h. [a] Conversion (not isolated). [b] Prior to aqueous work-up, the reaction mixture consisted of 55 mol% of amide (±)-**12** and 45 mol% of amidine (±)-**16**.

These side reactions presumably occurred due to the presence of an acidic hydrogen in the *α*-position of the allyl ester (±)-**16**. We thus opted to reduce the ester functionality in (±)-**12** (Scheme 3). Sakai *et al*. had reported on the direct and selective reduction of esters to the corresponding ethers in the presence of secondary amides with Et_3SiH and catalytic amounts of $InBr_3$.[23] We applied these conditions in an attempt to selectively convert the allyl ester in amide (±)-**12** to an allyl ether in amide (±)-**18** (Scheme 3, step **a**). A clean conversion to a sole product was obtained. Unfortunately, reduction of the tertiary amide, rather than the ester, occurred affording allyl ester (±)-**17**. No trace of amide (±)-**18** was observed. Therefore, a detour was made by first completely reducing the allyl ester in amide (±)-**12** to primary alcohol (±)-**19** (step **b**, 93%), after which the latter was allyl protected to yield amide (±)-**18** (step **c**, 92%).

Preparation of amidine (±)-**20** was achieved by the action of $POCl_3$ and amine **15** (step **d**, 52%). Next, sequential treatment with LiHMDS and an equimolar mixture of oxaziridines **21** and **22** resulted in a clean one-pot ring closure-α-hydroxylation sequence[3] towards (±)-2-(allyloxymethyl)blebbistatin (±)-**7**. (step **e**, diastereoisomeric ratio 84:16, 90%). This mixture of diastereoisomers was allyl deprotected with $Pd(PPh_3)_4$ and K_2CO_3, but only a 50% conversion toward (±)-2-(hydroxymethyl)blebbistatin (±)-**6** was obtained for each diastereoisomer (step **f**, 30–36%). Likely, a higher conversion could have been achieved by adding multiple portions of $Pd(PPh_3)_4$. By chance, we observed that a yellow precipitate persisted when trying to dissolve the crude reaction mixture in THF. This precipitate was isolated and identified as the minor diastereoisomer of compound (±)-**6** in pure form. The remaining major diastereoisomer was subsequently purified *via* automated flash chromatography.

The relative stereochemistry of both the major and minor diastereoisomer of (±)-2-(hydroxymethyl)blebbistatin (±)-**6** (and thus (±)-2-(allyloxymethyl)blebbistatin (±)-**7**) was determined with 1D-NOESY experiments (Figure 3), as crystallization efforts failed. These experiments revealed an interaction between H^2 and the hydroxyl hydrogen on C^{3a} in the major diastereoisomer. This NOE was absent in the minor diastereoisomer. These results suggest a *trans*- and *cis*-configuration for the major and minor diastereoisomer, respectively.



Scheme 3. Synthesis of (±)-2-(allyloxymethyl)blebbistatin (±)-**7**, (±)-*trans*-2-(hydroxymethyl)blebbistatin (±)-*trans*-**6** and (±)-*cis*-2-(hydroxymethyl)blebbistatin (±)-*cis*-**6**. Reagents and conditions: **a.** 0.1 equiv $InBr_3$, 10 equiv Et_3SiH, dry $CHCl_3$, 60 °C, 1 h. **b.** 3× 1.2 equiv $NaBH_4$, EtOH, rt, 4+4+2 h. **c.** 1) 1.5 equiv NaH, dry THF/DMF (2:1), 0 °C, 30 min; 2) 2 equiv allyl bromide, dry THF/DMF (2:1), rt, 1 h. **d.** 1) 2 equiv $POCl_3$, dry CH_2Cl_2, rt, 24 h; 2) 1.05 equiv amine **15**, dry CH_2Cl_2, 35 °C, 24 h. **e.** 1) 2.1 equiv LiHMDS, dry THF, 0 °C, 1.5 h; 2) 1.2 equiv oxaziridine **21**, 1.2 equiv oxaziridine **22**, dry THF, −15 °C, 15 h. **f.** 0.05 equiv $Pd(PPh_3)_4$, 6 equiv K_2CO_3, dry MeOH, reflux, 10 h. [a] Conversion (not isolated). [b] Prior to aqueous work-up, the reaction mixture consisted of 23 mol% of amide (±)-**18** and 77 mol% of amidine (±)-**20**. [c] The relative configuration was determined *via* 1D-NOESY experiments with the major and minor diastereoisomer of compound (±)-**6** (Figure 3). [d] 50% conversion of (±)-*trans*-**7** to (±)-*trans*-**6**. [e] 50% conversion of (±)-*cis*-**7** to (±)-*cis*-**6**.

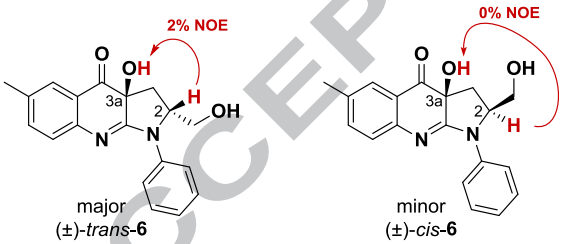


Figure 3. 1D-NOESY experiments of the major and minor diastereoisomer of (±)-2-(hydroxymethyl)blebbistatin (±)-**6** suggest a *trans*- and *cis*-configuration, respectively.

The myosin II ATPase inhibitory potency of the thus obtained blebbistatin derivatives (±)-*trans*-2-(hydroxymethyl)blebbistatin (±)-*trans*-**6**, (±)-*cis*-2-(hydroxymethyl)-blebbistatin (±)-*cis*-**6** and (±)-2-(allyloxymethyl)blebbistatin (±)-**7** (the latter containing an 84:16 mixture of the *trans*- and *cis*-diastereoisomer) was subsequently evaluated. To this extend, our in-house-developed steady-state ATPase assay against rabbit skeletal-muscle myosin II was applied.[3,4,15] Rabbit skeletal muscle myosin II is a suitable model system for the human protein, as crucial residues and hydrophobic areas in the binding site have been conserved across species, and experimental responses to (*S*)-blebbistatin and its analogs in the ATPase assay are highly similar for rabbit and human isoforms.[24] (*S*)-Blebbistatin (*S*)-**1**, obtained as earlier described,[3] was used as a benchmark (Figure 4). Contrary to what was expected based on the observation made in Figure 2A, (±)-*cis*-2-(hydroxymethyl)blebbistatin (±)-*cis*-**6** displayed no inhibition at a concentration as high as 100 µM, while (±)-*trans*-2-(hydroxymethyl)blebbistatin (±)-*trans*-**6** possessed an IC_{50} value of ~75 µM (vs 0.95 µM for (*S*)-blebbistatin (*S*)-**1**). Further, an 84:16 mixture of (±)-*trans*- and (±)-*cis*-2-(allyloxymethyl)-blebbistatin (±)-*trans*- and -*cis*-**7** showed no inhibition of the ATPase activity at 100 µM. These data reveal that small modification of ring C can have a large negative impact on the myosin II ATPase inhibitory potency. The results also indicate, as observed previously for A- and D-ring modification,[3,4,15] that rational modification of the blebbistatin scaffold based on analysis of residues lining the binding pocket (cfr. Figure 2A) is not straightforward. We recently investigated the inability of this approach to correctly predict ligand discrimination using an array of computational techniques.[24] It was concluded that structure-based methods perform poor because these techniques do not account

for steric and temporal (due to the kinetics of the chemo-mechanical cycle) restrictions in the path(s) leading toward the binding site, as had earlier been suggested.[3,15] In the same study, a ligand-based method using dissimilarity distances between compounds calculated by extended chemical fingerprints proved able to select for actives.[24]

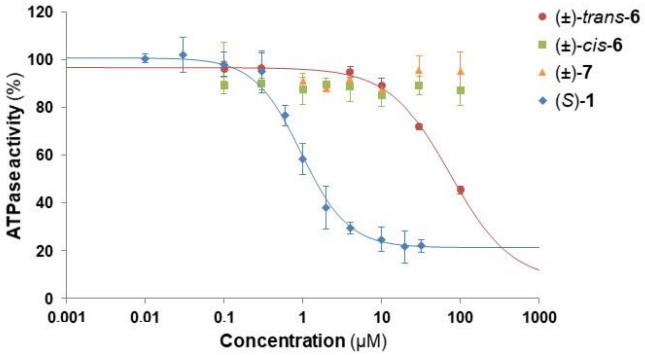


Figure 4. Overview of the myosin II ATPase inhibitory potency of (±)-*trans*-2-(hydroxymethyl)blebbistatin (±)-*trans*-**6**, (±)-*cis*-2-(hydroxymethyl)blebbistatin (±)-*cis*-**6** and (±)-2-(allyloxymethyl)blebbistatin (±)-**7** (the latter containing an 84:16 mixture of the (±)-*trans*- and (±)-*cis*-diastereoisomer), evaluated in a steady-state ATPase assay against rabbit skeletal-muscle myosin II. The data points represent the mean ± s.d. of at least three samples (N = 1). Concentrations exceeding 100 μM were not evaluated in this screen. As an approximation, the relative ATPase activity obtained for (*S*)-blebbistatin (*S*)-**1** at a concentration of 32.5 μM was used to set the lower asymptote of the 4-parameter logistic curve fitted to (±)-*trans*-2-(hydroxymethyl)blebbistatin (±)-*trans*-**6**.

In conclusion, the synthesis of a small set of blebbistatin derivatives with C-ring modifications at C^2 was attempted in order to (i) enable an additional hydrogen bonding interaction and (ii) optimize filling of the binding pocket. (±)-*Trans*-2-(hydroxymethyl)blebbistatin (±)-*trans*-**6**, (±)-*cis*-2-(hydroxy-methyl)blebbistatin (±)-*cis*-**6** and (±)-2-(allyloxymethyl)-blebbistatin (±)-**7** (an 84:16 mixture of the *trans*- and *cis*-diastereoisomer) were synthesized. The myosin II ATPase inhibitory potency of these analogs with small C-ring modifications was lower than that of parent compound (*S*)-blebbistatin (*S*)-**1**. Potency improvement from C-ring modification thus seems not straightforward, yet additional C-ring-modified analogs must be evaluated in order to test this hypothesis. Such compounds should preferentially be designed using ligand-based methods.

Acknowledgments

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Supplementary Material

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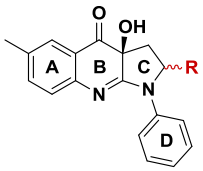

C-ring-modified blebbistatin derivatives

R = CH_2OH, $CH_2OAllyl$

Rabbit skeletal muscle myosin II ATPase IC_{50} *>75 µM*

Highlights

- First report on C-ring-modified blebbistatin analogs
- Incorporation of hydroxymethyl or allyloxymethyl functionalities
- Additional favorable interactions and optimal filling of binding pocket targeted
- No significant inhibition of rabbit skeletal muscle II ATPase activity
- Structure-based design strategies should be complemented by ligand-based insights