Insights into the myosin II inhibitory potency of A-ring modified (*S*)-blebbistatin analogs

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Abstract Myosin II is an interesting target for therapeutic intervention, as it is involved in a large number of motility-based diseases. (*S*)-Blebbistatin is a known micromolar inhibitor of this protein. A new series of (*S*)-blebbistatin derivatives with a modified A-ring were synthesized and their myosin II inhibitory properties were evaluated *in vitro*. In this way, we gained insight into the influence of structural modifications in this part of the scaffold on myosin II inhibitory potency. Our results indicate there are few possibilities for potency enhancement *via* ring A modification of the blebbistatin scaffold.

Keywords blebbistatin, A-ring modification, myosin II

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(*S*)-Blebbistatin (*S*)-**1** (Figure 1) is a well-known and widely used micromolar inhibitor of myosin II.[1] Given the multiple roles of this protein in a diverse range of motility-based diseases,[2–7] it is a promising target for therapeutic intervention. We have previously reported on the feasibility of improving (*S*)-blebbistatin's myosin II inhibitory potency and physicochemical properties.[8] We have also generated and analyzed SAR data on D-ring modified analogs.[9] These studies have shown that D-ring modification enables fine-tuning of (*S*)-blebbistatin's physicochemical properties, but potency enhancement cannot be pursued in this manner.[8–12]
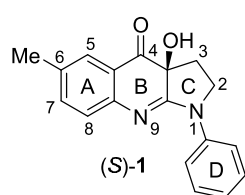
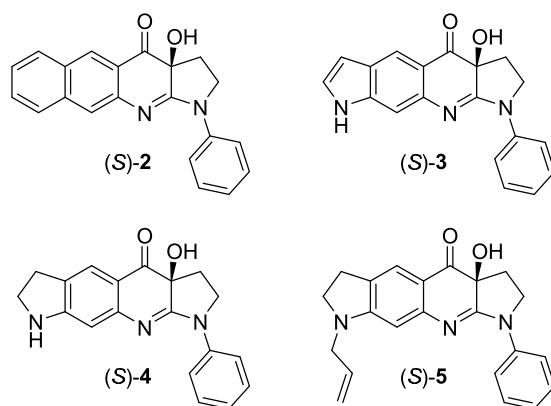**Figure 1.** State-of-the-art on SAR-information of the (*S*)-blebbistatin scaffold and present work.[8–14]

In the present report, our search for improved activity focused on the impact of structural changes in ring A. Analysis of the co-crystal structure of (*S*)-blebbistatin (*S*)-**1** bound to the metastable state of *Dictyostelium discoideum* myosin II (PDB: 1YV3)[15] shows that the residues in closest proximity to rings AB are Tyr261, Thr474, Tyr634, Gln637 and Leu641 (Figure 2). Extending the (aromatic) ring system in this part of the molecule therefore has potential to improve binding affinity through additional hydrophobic interactions and π-π stacking with Tyr261. Lucas-Lopez et al. previously reported that small-sized substituents are

of little influence at the C^5, C^6 and C^7 positions, but are undesired at the C^8 position.[13,14] In this study, we incorporated larger cyclic substituents fused at positions C^6 and C^7. Analog (*S*)-**2** was envisioned to accommodate π-π stacking with Tyr261. To overcome potential solubility issues associated with the latter compound, a more polar analog (*S*)-**3** was also prepared. Indoline (*S*)-**4** and *N*-allyl protected synthetic intermediate (*S*)-**5** were included as aliphatic counterparts.
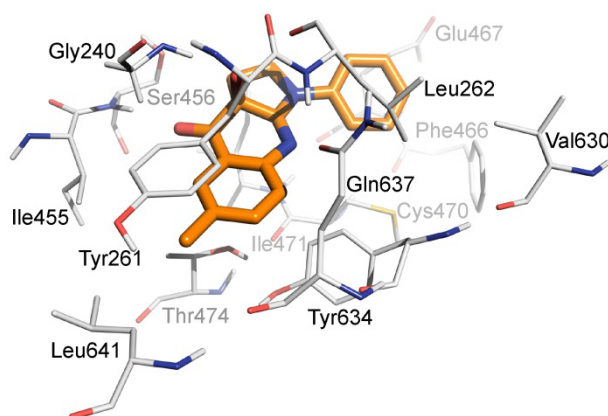

Figure 2. Co-crystal structure of (*S*)-blebbistatin (*S*)-**1** bound to the metastable state of *Dictyostelium discoideum* myosin II (PDB: 1YV3)[15] indicates possible additional π-π stacking interactions with Tyr261 by extending the aromatic system in ring A of the scaffold.

(*S*)-Benzo[*h*]blebbistatin (*S*)-**2** was prepared *via* a route that was optimized previously by us.[8,9] The synthesis started from commercially available 3-amino-2-naphthoic acid (**6**) (Scheme 1), which was converted to methyl ester **7** upon treatment with sulfuric acid in MeOH (step (a), 96%). Reaction of pyrrolidinone **8** with $POCl_3$ and amine **7** resulted in a 75% conversion to amidine **9** and isolated yield of 65% (step (b)). Intramolecular ring closure of the latter compound was induced after deprotonation by LiHMDS (step (c)). Asymmetric α-hydroxylation of intermediate **10** using Davis' oxaziridine methodology yielded analog (*S*)-**2** (step (d), 76%, ee 72%). A single recrystallization from CH_3CN afforded enantiopure (*S*)-benzo[*h*]blebbistatin (*S*)-**2** (ee >99%).

The synthesis of (*S*)-(*N*-allyl-2,3-dihydro-1*H*)-pyrrolo[3,2-*h*]blebbistatin (*S*)-**5**, (*S*)-(2,3-dihydro-1*H*)-pyrrolo[3,2-*h*]blebbistatin (*S*)-**4** and (*S*)-(1*H*)-pyrrolo[3,2-*h*]blebbistatin (*S*)-**3** required the preparation of precursor **16**, which was synthesized in an analogous way as described by Showalter et al. (Scheme 2).[16] In short, nitration of methyl 3-methyl-4-nitrobenzoate (**12**) resulted in a 85:15 mixture of methyl 5-methyl-2,4-dinitrobenzoate (**13**) and its isomer methyl 3-methyl-2,4-dinitrobenzoate. Isolation of compound **13** out of this
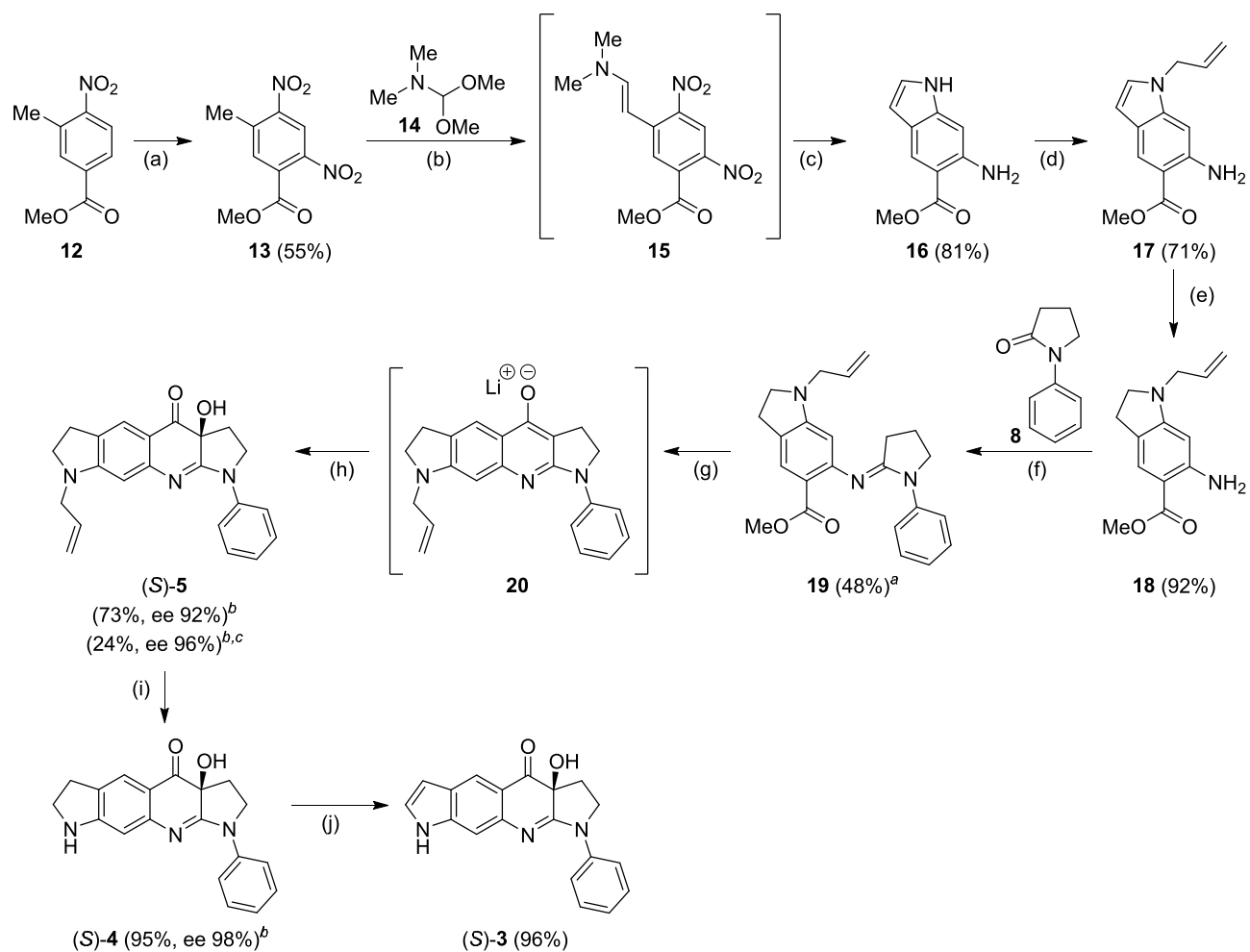
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mixture of isomers proved difficult. Efforts to purify it *via* normal phase and reversed phase automated flash chromatography both failed and initial recrystallization attempts in 2-propanol were not successful either. Recrystallization parameters (solvent volume, timing) appeared to be crucial and eventually we were able to isolate methyl 5-methyl-2,4-dinitrobenzoate (**13**) in 55% yield (step (a)). Condensation with reagent **14** gave enamine **15** (step (b)), which was reductively cyclized to precursor **16** (step (c), 81%) without intermediate isolation. Subsequent protection with allyl bromide yielded a crude mixture of unreacted starting material and mono- and diallylated regioisomers, out of which indole **17** was purified *via* reversed phase automated flash chromatography (step (d), 71%). Reduction with NaCNBH$_3$ in glacial acetic acid yielded indoline **18** (step (e), 92%). The synthesis of amidine **19** proved difficult due to the rather low nucleophilic propensity of aniline **18** (step (f), 48%). One-pot intramolecular cyclization (step (g)) and enantioselective hydroxylation (step (h), 73%, ee 92%) afforded highly optically enriched (*S*)-(*N*-allyl-2,3-dihydro-1*H*)-pyrrolo[3,2-*h*]blebbistatin (*S*)-**5** upon recrystallization from CH$_3$CN (ee 96%). (*S*)-(2,3-Dihydro-1*H*)-pyrrolo[3,2-*h*]blebbistatin (*S*)-**4** was obtained after successful allyl deprotection (step (i), 95%, ee 98%). Finally, oxidation with MnO$_2$ yielded (*S*)-(1*H*)-pyrrolo[3,2-*h*]blebbistatin (*S*)-**3** (step (j), 96%).
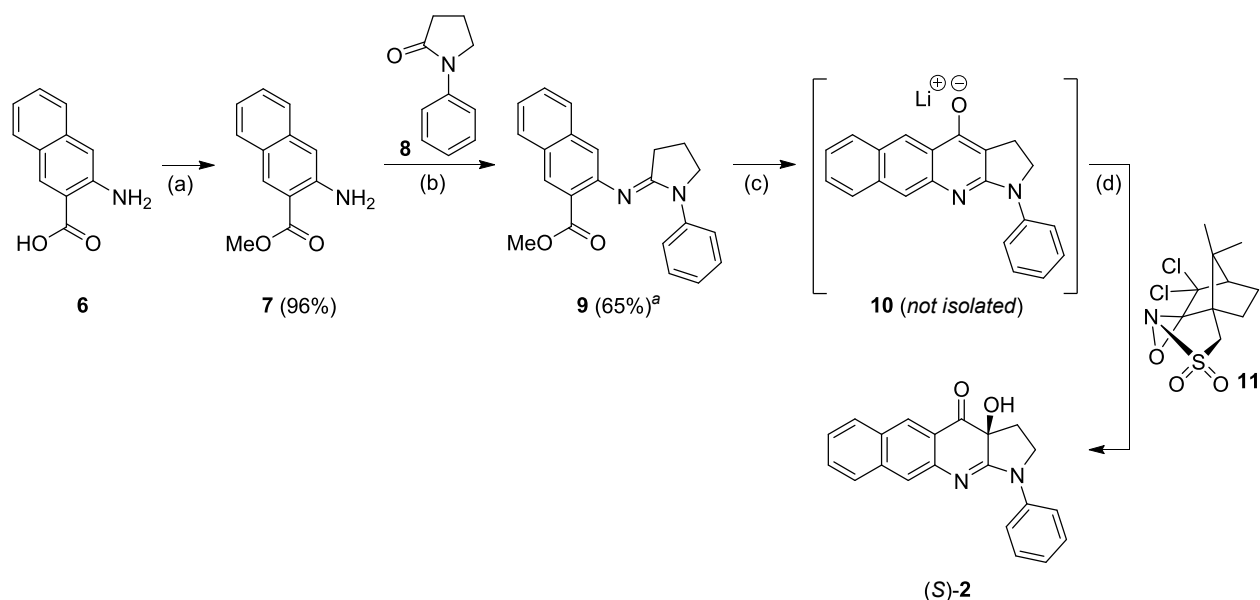
Scheme 1. Synthesis of (*S*)-benzo[*h*]blebbistatin (*S*)-**2**. Reagents and conditions: (a) H$_2$SO$_4$, MeOH, reflux, 48 h; (b) (1) **8**, POCl$_3$, CH$_2$Cl$_2$, rt, 24 h, (2) **7**, CH$_2$Cl$_2$, 35 °C, 48 h; (c) LiHMDS, THF, 0 °C, 1.5 h; (d) **11**, THF, −15 °C, 16 h. ^{a} The reaction mixture initially

Scheme 2. Synthesis of (*S*)-(*N*-allyl-2,3-dihydro-1*H*)-pyrrolo[3,2-*h*]blebbistatin (*S*)-**5**, (*S*)-(2,3-dihydro-1*H*)-pyrrolo[3,2-*h*]blebbistatin (*S*)-**4** and (*S*)-(1*H*)-pyrrolo[3,2-*h*]blebbistatin (*S*)-**3**. Reagents and conditions: (a) HNO$_3$, H$_2$SO$_4$, −20 °C, 20 h; (b) **14**, 1,4-dioxane, reflux, 20 h; (c) Pd/C, H$_2$ (1 bar), 1,4-dioxane/MeOH (5:2), rt, 16 h; (d) (1) NaH, DMF, 0 °C, 30 min, (2) allyl bromide, DMF, 0 °C, 30 min; (e) NaCNBH$_3$, glacial acetic acid, rt, 4h; (f) (1) **8**, POCl$_3$, CH$_2$Cl$_2$, rt, 24 h, (2) **18**, CH$_2$Cl$_2$, 35 °C, 3 days; (g) LiHMDS, THF, 0 °C, 1.5 h; (h) **11**, THF, −15 °C, 16 h; (i) *N,N'*-dimethylbarbituric acid, Pd(PPh$_3$)$_4$, CH$_2$Cl$_2$, reflux, 6 h; (j) MnO$_2$, DMF, rt, 25 min. [a] The reaction mixture initially consisted of 44 mol% of **8** and 56 mol% of **19**. [b] Determination of ee *via* chiral HPLC analysis by comparison of (*R*)-enantiomer enriched fractions. [c] After recrystallization from CH$_3$CN.

(*S*)-Blebbistatin derivatives (*S*)-**2**–**5** were evaluated for their myosin II inhibitory properties in a steady-state ATPase assay using rabbit skeletal muscle myosin II and (*S*)-blebbistatin (*S*)-**1** was used as a benchmark. Dose-response curves are presented in Figure 3 and half-maximum

inhibitory concentrations (IC_{50}) are summarized in Table 1. Extending the aromatic system of ring A of the blebbistatin scaffold ((S)-**2** and (S)-**3**) did not result in improved binding affinity. The aliphatic ring systems in analogs (S)-**4** and (S)-**5** too had a negative impact on myosin II inhibitory activity. Moreover, Table 1 shows there is no correlation between compound solubility and potency. Our data indicate there is little tolerance toward linear extension of the blebbistatin scaffold at the side of ring A. In addition, Lucas-Lopez et al. observed that moving the small methyl group to C^8 resulted in a reduced potency and *in silico* analysis shows no room for fused substituents at C^5 and C^{6}.[14] Thus, other modifications of ring A are also unlikely to result in improved potency. This SAR-investigation, focused on ring A, complements earlier data on ring D.[8–12] Taken together, little room for potency enhancement remains except for ring C.
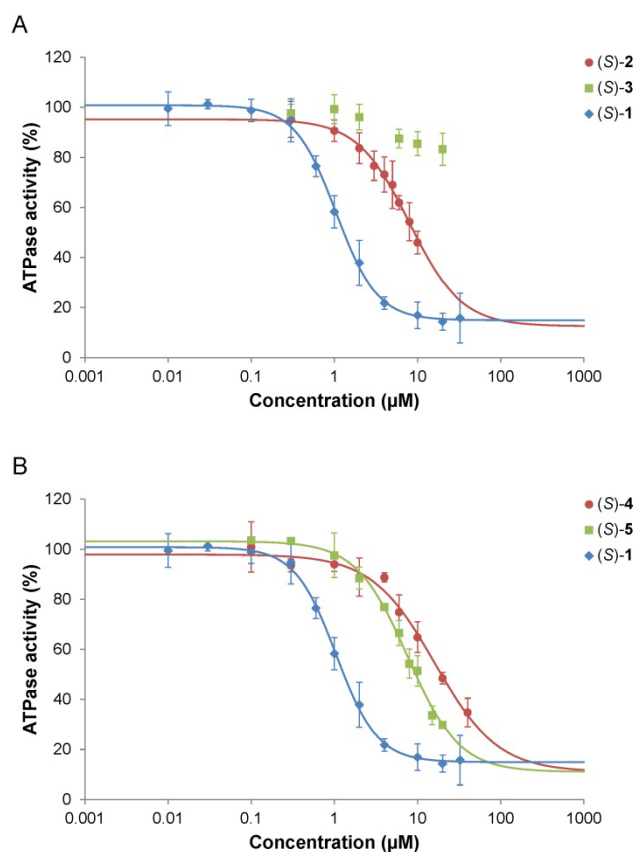
Figure 3. Overview of the myosin II inhibitory properties of compounds (S)-**2–5**, evaluated in a steady-state ATPase assay with rabbit skeletal muscle myosin II. Examples of dose-response curves and 4-parameter logistic curve fitting obtained for (A) (S)-benzo[h]blebbistatin (S)-**2** and (S)-(1H)-pyrrolo[3,2-h]blebbistatin (S)-**3** and (B) (S)-(2,3-dihydro-1H)-pyrrolo[3,2-h]blebbistatin (S)-**4** and (S)-(N-allyl-2,3-dihydro-1H)-pyrrolo[3,2-h]blebbistatin (S)-**5**. Data points represent mean ± s.d. of at least three samples (N = 1). (S)-blebbistatin (S)-**1** is shown

as a benchmark. Concentrations exceeding 10 µM, 40 µM and 20 µM caused compounds (*S*)-**2**, (*S*)-**4** and (*S*)-**5**, respectively, to precipitate in the assay buffer. 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 fitted curve for these compounds.

Table 1. Evaluation of the myosin II inhibitory properties of compounds (*S*)-**1**–**5**: half-maximum inhibitory concentrations (IC_{50}) for the steady-state ATPase activity of rabbit skeletal muscle myosin II and solubility in the latter assay.

Compound	$IC_{50}{}^{a}$ (µM)	Solubility in ATPase assay (µM)[b]
(*S*)-**1**	1.02 ± 0.05	<100
(*S*)-**2**	7.97 ± 0.02	<15
(*S*)-**3**	$>20^{c}$	<40
(*S*)-**4**	14.5 ± 2.2	<50
(*S*)-**5**	8.46 ± 1.22	<40

[a] Data represent mean ± s.d. of two independent experiments. [b] Concentrations resulting in compound precipitation. [c] Highest compound concentration used was 20 µM, as concentrations exceeding 20 µM resulted in compound precipitation in the assay buffer.

In conclusion, a new series of (*S*)-blebbistatin derivatives with a modified A-ring was developed in this study. The myosin II inhibitory properties of (*S*)-benzo[*h*]blebbistatin (*S*)-**2**, (*S*)-(1*H*)-pyrrolo[3,2-*h*]blebbistatin (*S*)-**3**, (*S*)-(2,3-dihydro-1*H*)-pyrrolo[3,2-*h*]blebbistatin (*S*)-**4** and (*S*)-(*N*-allyl-2,3-dihydro-1*H*)-pyrrolo[3,2-*h*]blebbistatin (*S*)-**5** were evaluated in a steady-state ATPase assay with rabbit skeletal muscle myosin II and proved less potent than the parent compound. Therefore, potency enhancement *via* modification of ring A of the blebbistatin scaffold seems unattainable. Future attempts to improve (*S*)-blebbistatin's potency will focus on ring C.

Acknowledgement

A. Supplementary material

Materials and methods, ^{1}H and ^{13}C NMR spectra, chiral HPLC chromatograms.

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