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Synthesis and activity of novel analogs of hemiasterlin as inhibitors of tubulin polymerization: modification of the A segment

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Abstract—Analogs of hemiasterlin (**1**) and HTI-286 (**2**), which contain various aromatic rings in the A segment, were synthesized as potential inhibitors of tubulin polymerization. The structure–activity relationships related to stereo- and regio-chemical effects of substituents on the aromatic ring in the A segment were studied. Analogs, which carry a *meta*-substituted phenyl ring in the A segment show comparable activity for inhibition of tubulin polymerization to **2**, as well as in the cell proliferation assay using KB cells containing P-glycoprotein, compared to those of **1** and **2**.
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Hemiasterlin[1] (**1**, Fig. 1) isolated from marine sponges,[2] is one member of a family of tripeptides consisting of three sterically congested amino acids. Hemiasterlin and its relatives have been found to be potent antimitotic agents that effectively inhibit tubulin polymerization by binding to the Vinca alkaloid site.[3] The total synthesis of **1** was achieved with a focus on the enantioselective synthesis of *N*-methyl-α,α-dimethyltryptophan (the A segment).[4] The phenyl analog (**2**, HTI-286), in which the N-methylindole in the A segment was replaced with a phenyl ring, was also synthesized, and its antimitotic properties were compared with those of **1**.[5] Although the biological profile of **2** as an inhibitor of tubulin polymerization is comparable to that of **1**, **2** showed better activity than **1**[6] against resistant cell lines. In addition, the synthesis of the A segment of **2** required fewer and relatively easier steps than that of **1**. Initial SAR (structure–activity relationship) involving stereochemical modifications of **2** was studied.[7] The general trends observed from the SAR study are: (1) changing the stereochemistry of the amino acids of the A–B–C segment from α*,β*,γ* = *S*,*S*,*S* resulted in reduction of the activity. (2) Both the *gem*-dimethyl and N-methyl groups in the A segment are necessary. (3) A sterically hindered alkyl substituent in the B segment is requisite for activity, as the *tert*-butyl group showed the best activity. (4) Removal of the N-methyl group in the C segment of **2** decreases the activity. (5) The stereochemistry of the olefin in the D segment prefers the E form.

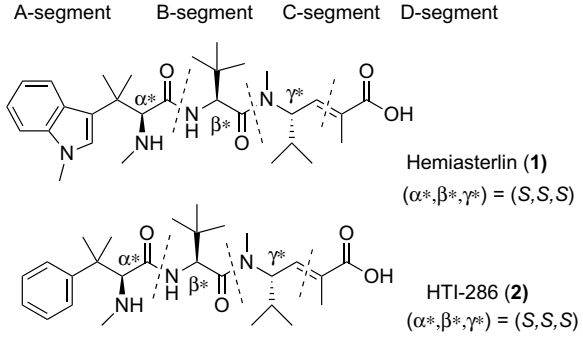

Figure 1.

Based on these observations, we next studied the SAR involving stereo- and regio-chemical effects of substituents on the aromatic ring in the A segment of **2**. Toward this goal, we prepared various A segments, in which the phenyl ring is substituted by methyl, halogen, and aryl

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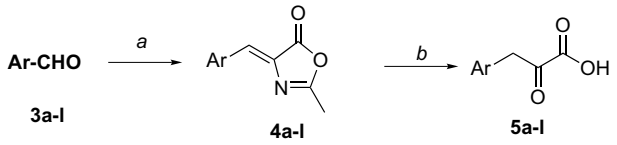


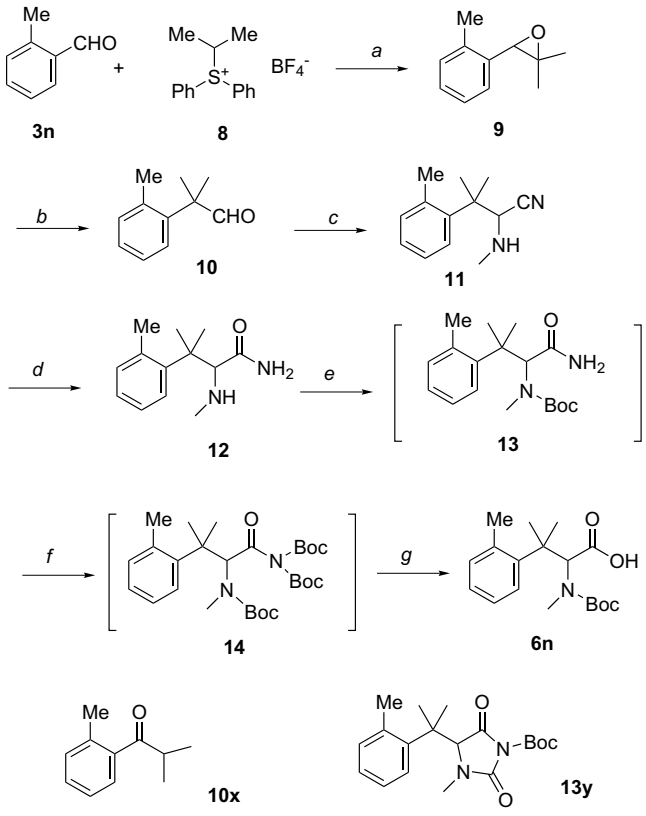
Compds	Ar
6a	3-Tolyl
6b	4-Tolyl
6c	3,4-Dimethylphenyl
6d	3,5-Dimethylphenyl
6e	3-Chlorophenyl
6f	4-Chlorophenyl
6g	3-Bromophenyl
6h	3-Fluorophenyl
6i	3,5-Difluorophenyl
6j	3-Trifluoromethylphenyl
6k	3,5-Di-trifluoromethylphenyl
6l	2-Thienyl
6m	3-Thienyl

Scheme 1. Reagents and conditions: (a) i. N-acetylglycine, NaOAc, Ac_2O, reflux, 5 h; (b) i. 1 N NaOH, 80 °C, ii. 5 N HCl, reflux, 5 h; (c) i. MeI, 5 N NaOH, THF, rt, ii. CH_3NH_2, THF, 55 °C, iii. BH_3-pyridine, 60 °C, 4 h.

groups at various positions (*ortho*, *meta*, *para*). The A segments thus prepared were coupled with the B–C–D segment, and the final analogs were evaluated for inhibition of tubulin polymerization. In this paper, we report our finding that introduction of a substituent at the *meta*-position of the phenyl ring, in general, increases potency relative to **2**.

The synthesis of a racemic A segment **6** follows the reported procedures[7] (Scheme 1). Aldehyde **3** was converted to the corresponding azlactone **4**,[8] which was then converted to the α-keto acid **5** by hydrolysis.[9] Dimethylation, followed by reductive amination, gave the desired racemic amino acid **6**.[10] This process allowed us to prepare *meta*- and *para*-tolyl-derivatives **6a** and **6b**.

In the synthesis of an *ortho*-tolyl A segment, *ortho*-tolualdehyde (**3n**) was converted to the corresponding keto acid using the procedure described earlier. An attempt to introduce dimethyl group to the keto acid, however, resulted in only mono-methylation at the benzylic position, presumably because the second methylation was inhibited by steric hindrance from the *ortho*-methyl in the phenyl ring as well as by the methyl at the benzylic position. An alternative route to the A segment **6n** was, therefore, developed (Scheme 2).[10] Aldehyde **3n** was converted to an epoxide **9** by treatment with the anion of isopropyldiphenylsulfonium tetrafluoroborate (**8**).[11] Ring opening/rearrangement of **9** in the presence of Lewis acid, using the conditions reported by Yamamoto and co-workers,[12] gave aldehyde **10** as the major product, along with a small amount of ketone **10x**, which was removed by chromatography. Reaction of **10** with potassium cyanide and methylamine gave aminonitrile **11**. Hydration of the cyano group in **11** to amide **12** with lithium hydroxide and hydrogen peroxide turned out to be very slow. Only a 25% yield of **12** was obtained, along with recovered **11**, after 5 days.



Scheme 2. Reagents and conditions: (a) LDA, DME, CH_2Cl_2, −78 °C then −40 °C, 15 h, 65%; (b) i. $(C_6F_5)_3B$, benzene, 60 °C, 89%; (c) $MeNH_2$, KCN, MeOM, H_2O, 1–2 days, 89%; (d) LiOH, H_2O_2, 2–5 days, 25%; (e) Boc_2O, MeCN, 4–5 days; (f) DMAP, *i*-Pr_2EtN; (g) NaOH, 88%.

Considerable effort was put into achieving the conversion of carboxamide **12** into the Boc-protected A segment **6n** by a one pot process, via the intermediates **13** and **14**. We found that it was crucial to protect the basic nitrogen in **12** to form intermediate **13**, before the amide nitrogen could be diacylated with the Boc groups. Acylation of the N-methyl group could be done in the absence of DMAP, but further acylation of the amide was extremely slow, and it took 4–5 days to form **13** completely. When the whole triacylation process was attempted in the *presence* of DMAP, the hydantoin **13y** was isolated, suggesting that under those conditions, the amide in **12**, rather than the N-methyl group, was first to be Boc-protected, and that the intermediate then spontaneously cyclized to **13y**. It was eventually found that if DMAP and Hünig's base were added *after* the completion of the Boc-protection of the basic amine to **13**, the desired tri-Boc product **14** was obtained. It could be converted, without isolation, to the corresponding racemic amino acid **6n** by treatment with aqueous base.

Coupling between the *meta*-tolyl containing A segment **6a** and the optically pure B–C–D segment **15**[13] under standard conditions (see Section 1) produced the protected tripeptide as a mixture of two diastereomers. They were separated by flash chromatography, to give the (*S*,*S*,*S*) diastereomer **16a** and the (*R*,*S*,*S*) isomer **17a**.[14] Hydrolysis of **16a** with aqueous lithium hydroxide solution, followed by acidification to pH 6, gave the tripeptide **18a**,[15] as a white solid. In a similar manner, **17a** was converted to **19a**.[15] The racemic A segment **6a** was thus transformed into two optically pure tripeptides, **18a** and **19a**. In parallel sequences, the *para*-tolyl A segment **6b** was converted to the optically pure **18b** and **19b** via intermediates **16b** and **17b**, respectively. Coupling between **6n** and **15**, followed by chromatographic separation, gave the tripeptides **16n** and **17n**. Hydrolysis of the esters, followed by removal of the Boc group, gave the optically pure tripeptides **18n** and **19n**, respectively.[15]

The antimitotic activities of **18a**, **19a**, **18b**, **19b**, **18n**, and **19n**, were measured by the inhibition of tubulin polymerization, as well as by proliferation assays with KB cell lines (KB-3-1, KB85, KBV1), using **1** and **2** as reference compounds (Table 1). Comparison of activities between **18a**, **19a**, **18b**, **19b**, **18n**, and **19n** should show the effect of a methyl group at the *ortho*-, the *meta*-, or the *para*-position, the effect of the configuration of the tripeptides (*SSS* vs *RSS*), as well as the effect of protecting groups (the protected vs the unprotected tripeptides). Excellent inhibition of tubulin polymerization was observed with all analogs mentioned above, with the exception of **17a**. For the proliferation assays with KB cell lines, the *SSS* diastereomers **18a**, **18b**, **18n**, as expected,[7] were found to be 10-fold more active (IC_{50} nM: KB-3-1 0.23–1.5) than the *RSS* isomers **19a**, **19b**, **19n** (IC_{50} nM: KB-3-1 6.9–17). The unprotected peptides **18a**, **19a** are more active than their esters **16a**, **17a**, although **16a** showed unusually high activity on the KB cell assay (IC_{50} nM: KB-3-1 22), compared with **17a**.[16] Among the *SSS* analogs, **18a**, **18b**, and **18n**, the *meta*-tolyl analog **18a** shows better KB activities (IC_{50} nM: KB-3-1, 0.23; KB85, 0.65; KBV1, 19) than the *para*- and the *ortho*-analogs, **18b** and **18n**. In addition, the potency of **18a** in KB cell lines is four-fold better than that of **2**.

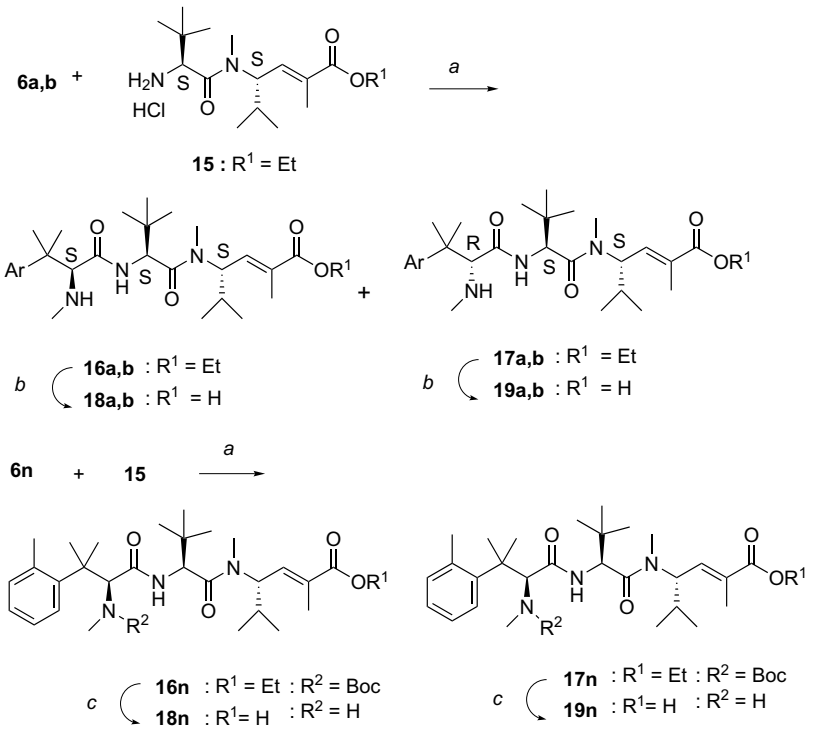
Table 1. Cellular proliferation assay profiles and inhibition of tubulin polymerization: comparison of analogs with *ortho*-, *meta*-, and *para*-tolyl substituted A segment

Compd	IC_{50} (nM)			Inhibition of tubulin polymerization %[d] (%)[e]
	KB-3-1[a]	KB85[b]	KBV1[c]	
1[f]	0.3	1.0	76	NA
2[f]	1.0	2.4	81	91
16a	21.9	525.3	2410	58 (94)
17a	263	1698	3000	26 (94)
18a	0.23	0.65	19	90 (89)
19a	7.9	19.9	446	88 (89)
18b	1.5	2.6	55	95 (94)
19b	17	27.5	1072	95 (94)
18n	0.9	1.8	56	91 (96)
19n	6.9	13.8	501	85 (96)

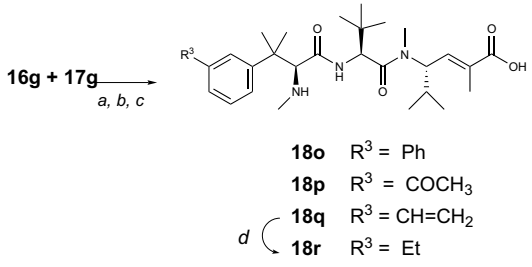
[a] IC_{50} (amount of drug needed to kill 50% of cells after 3 days continuous exposure) in human epidermoid cells, which contain very low levels of P-glycoprotein.
[b] IC_{50} in KB85 cells, which have moderate levels of P-glycoprotein.
[c] IC_{50} in KBV1 cells, which have very high levels of P-glycoprotein.
[d] Inhibition of MAP-rich tubulin polymerization at concentration 0.3 μM.
[e] Values of (%) are results for **2** in the same assay.
[f] Compounds **1** and **2** were used as references.

Based on these biological data, various A segments **6c**–**k**, which carry a substituent at the *meta* position, were prepared (Scheme 1). Similarly, the A segments carrying two regioisomeric thienyl groups **6l** and **6m** were also prepared. They were converted to the optically pure peptides **18c**–**m** and **19c**–**m** by the procedures described above (Scheme 3). The *meta* bromophenyl derivative (**16g** + **17g**) was further converted to optically pure analogs, such as the biphenyl (**18o**), the acetylphenyl (**18p**), the vinylphenyl (**18q**), and the ethylphenyl (**18r**) by the Pd(0) mediated coupling reactions and deprotection sequences (Scheme 4).[15]

The analogs thus prepared were evaluated for their antimitotic activities (Table 2). The analogs carrying the *meta*-substituted A segment showed consistently good activity in KB cell lines. The electronic effect of the phenyl ring of the A segment appears to have little influence on their activity, as seen in IC_{50} nM: KB 1.9 for **18p** and IC_{50} nM: KB-3-1 0.86 for **18q**. Among the analogs carrying a halogen substituted phenyl A segment (Cl, Br, F), **18e** (*meta*-Cl) and **18f** (*para*-Cl) showed better activities (IC_{50} nM: KB-3-1 0.58–1.7) than those of **18g** (Br) and **18h** (F) (IC_{50} nM: KB-3-1 1.8 and 0.8–1.2, respectively). The analogs carrying a sterically bulky substituent on the A segment, such as **18o**, **18p**, showed some reduction of activity (IC_{50} nM: KB 1.9), while substitution by a vinyl or ethyl group maintained activity (IC_{50} nM: KB-3-1 0.7–0.9). In order to increase the potential metabolic stability of these compounds, the analog **18j** carrying the A segment substituted with a trifluoromethylphenyl group was prepared. The potency of **18j** was, however, slightly less (IC_{50} nM: KB-3-1 0.65) than that of **18a**. Activities of the analogs carrying the A segment



Scheme 3. Reagents and conditions: (a) i. HOBT, EDC, NMM, DMF, rt, ii. chromatographic separation; (b) i. LiOH, MeOH, H_2O, rt, 15 h, ii. pH = 6, citric acid; (c) i. LiOH, MeOH, H_2O, rt, 15 h, ii. 4 N HCl, *p*-dioxane, 30 min, rt.



Scheme 4. Reagents and conditions: (a) for **18o**: phenylboronic acid, $Pd(Ph_3P)_4$, Na_2CO_3, DME, H_2O, reflux, 18 h; for **18p**: i. *n*-$Bu_3Sn(CH_2{=}CHOEt)$, $Pd(Ph_3P)_4$, toluene 120 °C, 4 h, ii. 2 N HCl, THF, rt, 15 h; for **18q**: *n*-$Bu_3Ti(CH{=}CH_2)$, $Pd(Ph_3P)_4$, CH_3CN, reflux, 20 h; (b) chromatographic separation; (c) i. LiOH, MeOH, 50 °C, 4–15 h, ii. citric acid, pH 6.0; (d) 10% Pd/C, EtOH.

substituted with dimethylphenyl groups (**18c**, **18d**) were as good as that of **2** (IC_{50} nM: KB-3-1 0.6–1.7), but **18k**, carrying the di-trifluoromethylphenyl A segment, somehow lost its potency (IC_{50} nM: KB-3-1 17). Against KB85 and KBV1 cells, which contain moderate and very high levels of P-glycoprotein, respectively, **18a**, **18c**, and **18d** showed better activity than **2** (IC_{50} nM: KBV1 19 for **18a**, 23–55 for **18c**, 38–50 for **18d** vs 81 for **2**).

Selected compounds (**16c**, **18a**, **18c**, **18e**, **18j**, **18l**), which met criteria of good cellular potency (KB-3-1) and activity in resistant cell lines (KB85, KBV1), were further evaluated in in vivo assays using Lox melanoma human xenograft models in athymic mice. Compound **2** was used as a reference (Table 3). Both the minimum effective dose (MED) and the maximum tolerated dose (MTD) were determined. Several compounds were further evaluated in xenograft models, using cell lines expressing P-glycoprotein transporters (HCT-15,

Table 2. Cellular proliferation assay profile and inhibition of tubulin polymerization of analogs

Compd	IC_{50} (nM)			Inhibition of tubulin polymerization %[d] (%)[e]
	KB-3-1[a]	KB85[b]	KBV1[c]	
2	1.0	2.4	81	91
16c	9.1	24	191	49 (92)
18c	0.4	1.13	39	88 (88)
18d	0.55	1.23	44	85 (88)
18e	1.14	3.2	91	91 (88)
18f	0.56	1.7	53	95 (96)
18g	1.8	4.1	148	90 (84)
18h	1.0	1.8	77	103(93)
18i	6.2	16.2	457	90 (93)
18j	0.65	1.7	42.7	98 (96)
18k	17	19	457	60 (93)
18l	2.0	3.9	162	92 (88)
18m	2.1	6.6	724	88 (96)
18o	1.9	5.4	149	86 (84)
18p	1.9	5.8	370	93 (87)
18q	0.86	2.0	130	88 (90)
18r	0.79	1.8	62	88 (90)

[a] IC_{50} (amount of drug needed to kill 50% of cells after 3 days continuous exposure) in human epidermoid cells, which contain very low levels of P-glycoprotein.
[b] IC_{50} in KB85 cells, which have moderate levels of P-glycoprotein.
[c] IC_{50} in KBV1 cells, which have very high levels of P-glycoprotein.
[d] Inhibition of MAP-rich tubulin polymerization at 0.3 μM concentration.
[e] Values of (%) are results for **2** in the same assay.

Table 3. Effect on human tumor xenografts in athymic mice

Compd	MED[a]	MTD[a]	HCT-15	DLD1	MX1W
2	0.2	1.6	Y[b]	Y	Y
16c	0.5	>2	NA	NA	NA
18a	<0.1	1.5	Y	Y	Y
18c	0.2	>1	NA	NA	NA
18e	0.3	3	N[c]	NA	Y
18g	1	4	NA	Y	NA
18j	1	>5	NA	NA	NA
18l	1	>5	NA	NA	NA

NA: not available.
[a] MED: the minimum effective dose; MTD: the maximum tolerated dose.
[b] Y = active.
[c] N = inactive.

DLD1, MX1W). The 3,4-dimethylphenyl derivative **18c** and the *meta*-chlorophenyl analog **18e** showed similar in vivo activity where MEDs were 0.2 and 0.3 mpk (milligram per kilogram), and MTDs were 1 and 3, respectively. Among the compounds tested, the *meta*-tolyl analog **18a** demonstrated the best in vivo activity. The MED of **18a** was <0.1 mpk, which was the most potent among the compounds tested, including **2** (MED = 0.2 mpk). In addition, the MTD value of **18a** was 1.5 mpk, which is ideal for a potential drug. In addition, **18a** showed almost zero tumor growth in the xenograft model, and was active in vivo using cell lines expressing P-glycoprotein (HCT-15, DLD1, MX1W).

In summary, we have synthesized various analogs related to **1** and **2**, in which the aromatic ring in the A segment is substituted with a variety of functional groups in various positions. The *meta*-tolyl substitution consistently showed increased activity both in a proliferation assay with KB cell lines, as well as comparable activity in inhibition of tubulin polymerization to that of **2**. Among the analogs thus synthesized, **18a** showed the best biological profile; in particular, its activity against resistant KB-3-1 cell lines seems ideal for a potential drug for the treatment of cancer.

Determination of inhibition of tubulin polymerization.[17] Bovine MAP-rich tubulin, PEM buffer [80 mM Na-PIPES (pH 6.9), 1 mM $MgCl_2$, and 1 mM EGTA], and GTP were purchased from Cytoskeleton (Denver, CO). MAP-rich tubulin (final concentration 1.5 mg/mL) was dissolved in cold PEM buffer containing 1 mM GTP (GPEM) and centrifuged at 12,000*g* for 10 min at 4 °C. The tubulin solution (100 μL/well) was added rapidly to wells of a low-volume, 96-well plate already containing duplicate aliquots (10 μL) of test compounds in GPEM. Final compound concentrations were 0.3 μM. Control wells contained the same final concentration of DMSO (0.3%). After initiation of the reaction, absorbance at 340 nm was measured every minute for 60 min at 24 °C using a SpectraMax Plus plate reader (Molecular Devices, Sunnyvale, CA).

Cellular proliferation assays. KB-3-1 cells[18] were plated in 96-well plates in 100-μL media at densities predetermined to produce 60–90% confluence at the time of analysis. Compounds, which were serially diluted into media as 2 × stocks, were added to cells in duplicate. After 3 days of incubation, cell survival was assessed by the SRB assay as described.[19]

In vivo model. Human tumor cell lines Lox melanoma ($1.5–7 \times 10^6$) are injected (sc) into athymic mice. When tumors attain a mass of 80–100 mg, animals are randomized into groups of 5 or 10 animals. Animals were treated (iv) with the indicated doses of compound in a saline formulation, that represent 80–95% of the maximal tolerated doses for the indicated schedule. Tumor growth is assessed by weekly measurement of the length (L) and width (W) of subcutaneous tumors [mg = $(L \times W^2)/2$]. Relative tumor growth (mean tumor mass on day measured divided by mean tumor mass on day 0) and $\%T/C$ (mean relative tumor growth of treated divided by that of control multiplied by 100) are calculated. Data are analyzed by Student's t-test.

1. Experimental

1.1. General procedure for coupling between 6 and 15

To a cooled (0 °C) solution of **6a** (1.1 mmol), hydroxybenzotriazole (1.1 mmol), and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (1.2 mmol) in anhydrous DMF (3–5 mL) was added N-methylmorpholine (1.4 mmol) via syringe, under an inert atmosphere. After stirring for 15 min at 0 °C, the cooling bath was removed, and the resulting mixture was stirred for 2–24 h (the reaction time depends on the solubility of **6** in DMF and the time for activation of the acid). The solution was cooled at 0 °C (ice bath), and was added a solution of **15** (1.0 mmol) in anhydrous DMF (3 mL). The cooling bath was removed, and the resulting mixture was stirred for 15–36 h at room temperature, under an inert atmosphere. The mixture was diluted with water, and the aqueous layer was extracted with ethyl acetate. The combined extracts were washed with saturated sodium chloride solution, dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was chromatographed (silica gel, flash column), to give **16** (*SSS*) and **17** (*RSS*). In general, the first eluate was a diastereomer of the *SSS* configuration, and the second an isomer of the *RSS* configuration.[14]

1.2. General procedure for preparation of 18 (or 19)

To a cooled (0 °C) solution of **16** (or **17**) in MeOH (24 mL/mmol) were added H_2O (8 mL/mmol) and aqueous LiOH solution (8 mL/mmol). The cooling bath was removed, and the resulting mixture was stirred at room temperature for 15 h. The solvent was removed in vacuo (bath temperature was kept below 30 °C), and the residue was dissolved in a small amount of H_2O. The aqueous solution was cooled to 0 °C, and acidified to pH 5.5–6.0 with aqueous citric acid solution. The precipitate was collected by filtration, and the solid was washed with cold water, and dried under high vacuum. Alternatively, the crude acidic residue was chromatographed using preparative HPLC.

1.3. Pd(0) mediated coupling reaction: preparation of 18n

To a solution of a mixture of **16g** and **17g** (0.43 g, 0.74 mmol) in ethylene glycol dimethyl ether (15 mL) and water (7.5 mL) were added tetrakis-(triphenylphosphine)palladium(0) (0.086 g, 0.074 mmol), phenylboronic acid (0.18 g, 1.5 mmol) and sodium carbonate (0.23 g, 2.2 mmol), at room temperature under nitrogen atmosphere. The resulting mixture was heated at reflux for 15 h, and then cooled. The mixture was diluted with ether, the organic layer was separated, and the aqueous layer was extracted with ether. The combined extracts were washed with saturated aqueous $NaHCO_3$ and saturated aqueous NaCl solution, dried over Na_2SO_4, filtered, and concentrated in vacuo. The desired ester **16n** (*SSS* configuration) was isolated by chromatography (flash column, silica gel, EtOAc/ether; 0.18 g, 42%). Compound **16n** (0.18 g, 0.31 mmol) was hydrolyzed with lithium hydroxide using the procedure described above. The product **18n** was purified by reverse-phase HPLC (elution from 5% acetonitrile/95% water containing trifluoroacetic acid to 100% acetonitrile, 60 min) as its trifluoroacetic acid salt (0.19 g, 79%).

Acknowledgements

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14. The absolute stereochemistry of the two diastereomeric isomers was determined by NMR studies and physical properties of the product as described in the literature: see Ref. 7.
15. All compounds described in this paper gave satisfactory analytical data (mass, IR, ^{1}H NMR, LC–MS, HPLC analysis).
16. Usually, the protected tripeptides gave activities comparable to those obtained for **17a**, which showed IC_{50} nM: KB-3-1 263–3000.
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