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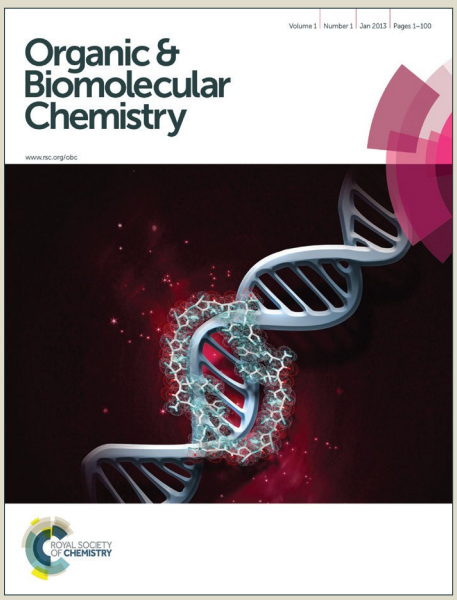

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ARTICLE

Complementary isonitrile-based multicomponent reactions for the synthesis of diversified cytotoxic hemiasterlin analogues

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A small family of structural analogues of the antimitotic tripeptides hemiasterlins have been designed and synthesized, as potential inhibitors of tubulin polymerization. The effectiveness of a multicomponent approach was fully demonstrated by applying complementary versions of the isocyanide-based Ugi reaction. Compounds strictly related to the lead natural products, as well as more extensively modified analogues, have been synthesized in a concise and convergent manner. In some cases, biological evaluation provided evidence for strong cytotoxic activity (six human tumor cell lines) and for potent inhibition of tubulin polymerization.

Introduction

Multicomponent reactions (MCRs) are convergent chemical processes that involve the one pot condensation of more than two reactants to form a product that incorporates most of each reagent, containing ideally all atoms. In addition to generating structural complexity with greater atom economy, they usually also offer the advantage of simplicity and synthetic efficiency over conventional chemical reactions.[1] In particular, isonitrile-based MCRs (IMCRs) are widely applied in diversity-oriented synthetic strategies, due to the considerable ability of isocyanides to undergo α-addition with electrophiles and nucleophiles and due to the various possibilities to exploit the different secondary reactions of the obtained α-adducts. Among IMCRs, the Ugi reaction has undergone developments over the years, and various modifications of the classic protocol have been used successfully. As a consequence, more than linear, peptide-like adducts can be obtained by introduction of unusual building blocks, by transformation of the MCR products using post-condensation reactions or by performing intramolecular IMCRs with bifunctional inputs.[2] Nevertheless, with regard to the target-oriented synthesis of natural products or their derivatives, the rational design of practical and versatile approaches employing MCRs, and in particular the Ugi reaction and its modifications, remained, until recently, a largely unexplored area of chemical research.[3] As a result of our interest in the MCR-based approach to conformationally constrained peptidomimetics,[4] in this work we show the use of complementary Ugi-type reactions for the synthesis of a small family of cytotoxic hemiasterlin analogues.

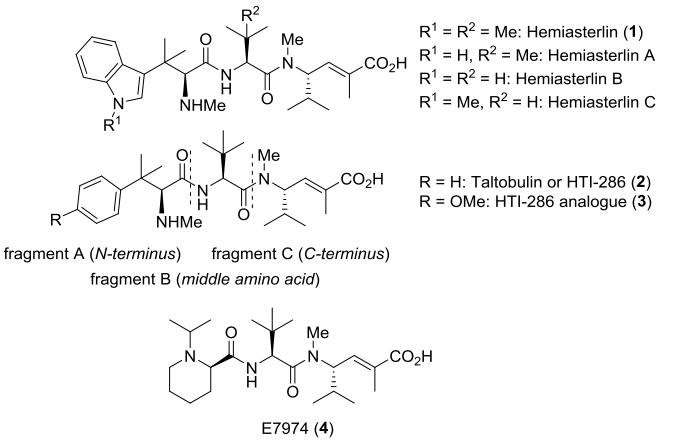

Figure 1. Tubulin polymerization inhibitors: natural hemiasterlins and synthetic analogues.

Hemiasterlins are a family of natural tripeptides, discovered and isolated from the South African marine sponge *Hemiastrella minor* some years ago.[5] The most active members of the family show cytotoxicity in the nanomolar range and are highly potent inhibitors of microtubule polymerization, binding in the vinca domain of tubulin.[6] Relative to other known antimitotic agents, hemiasterlins possess an attractive combination of structural simplicity and potent antimitotic

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activity, which makes them ideal targets for synthetic modification.[7]

Recently, synthetic analogues of hemiasterlin **1** (Figure 1), namely taltobulin (HTI-286) **2** and the closely related **3**,[8,9] wherein aryl groups replace the indol-3-yl substituent, and the piperidine-based E7974 **4**[10] advanced into clinical trials, due to their more potent in vivo cytotoxicity and antimitotic activity. Moreover, unlike taxanes and vincas, such synthetic derivatives are poor substrates for P-glycoprotein drug transporters and maintain toxicity towards cell lines with high expression of multidrug resistant (MDR) drug efflux pumps. Further, since **4** binds predominantly to the α-subunit of tubulin, with minor binding to the β-subunit, it offers significant promise of activity in taxane-resistant tumor types, regardless of whether the mechanism driving resistance is based on P-glycoprotein or tubulin mutations.[11]

Hemiasterlins and derivatives contain three highly modified amino acids (A, B and C segments, see Figure 1) and their successful synthesis has always relied on amide bond synthesis in a sterically challenged environment.[12] This approach has prevented more extensive structural modifications, for instance at the central (L)-valine or (L)-*tert*-leucine amino acid residue.

Since the Ugi reaction and its modifications are less sensitive to steric hindrance than peptide coupling, we envisioned that a multicomponent strategy could be suitable for generation of a wide range of hemiasterlin derivatives, also including non-peptidic analogues. By means of a Ugi four-component reaction (U-4CR), we achieved the synthesis of **5** (Figure 2), a compound closely related to taltobulin, in which we employed (L)-valine in place of (L)-*tert*-leucine, as it represents a variation that could allow substantial bioequivalence. By the same approach, we achieved also the unprecedented indole-based analogue **6**. Applying a Ugi-like three-component reaction (U-like-3CR), oxazole-based compounds **7-9** could be easily obtained. To the best of our knowledge, these compounds represent the first example of hemiasterlin analogues with major modifications of the central B core. Lastly, a Ugi-Joullié three-component reaction (U-J-3CR) allowed us to prove the applicability of the multicomponent approach for the synthesis of piperidine-based compounds, such as **10-12**, closely related to E7974.

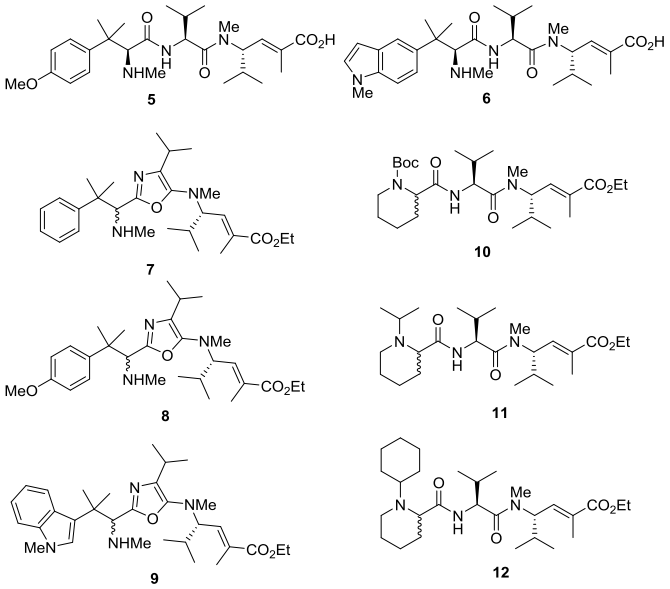
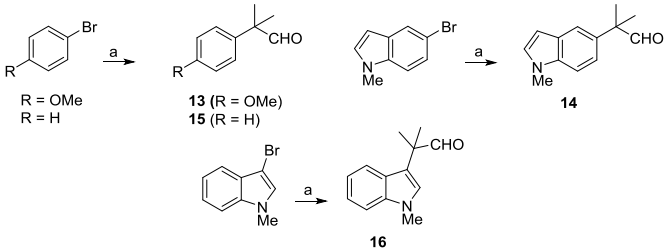


Figure 2. Structures of hemiasterlin analogues **5-12**.

Results and Discussion

The aldehyde components **13-16**, which were necessary in the U-4CR and U-like-3CR strategy, were prepared as described in Scheme 1. The syntheses relied on an allylpalladium-catalyzed α-arylation of isobutyraldehyde with the appropriate aryl or heteroaryl bromide, in the presence of catalytic Q-phos,[13] cleanly affording the desired aldehydes in yields up to 75%. Alternative palladium-catalyzed protocols, based on palladium diacetate as catalyst,[14] or involving vinylic acetates as coupling components,[15] proved to be less effective.



Scheme 1. Synthesis of aldehyde components **13-16**. Reagents and conditions: a) isobutyraldehyde, $[Pd(\eta^3\text{-allyl})Cl]_2$, Q-phos, Cs_2CO_3, THF, reflux (**13**: 75%; **14**: 57%; **15**: 50%; **16**: 46%).

Many synthetic procedures are reported for the preparation of isocyanides from α-amino acid esters hydrochlorides. In order to achieve the enantiomerically pure α-isocyanoacetate component **17** (Scheme 2), we selected a two-step sequence, involving formylation of the precursor by reaction with trimethyl orthoformate in neat conditions, followed by dehydration of the obtained α-*N*-formylamino acid methylester, using triphosgene as a mild dehydrating agent and *N*-methylmorpholine as base.[16] Trifluoroacetic acid and methylamine were chosen as suitable carboxylic acid and amine for the U-4CR process.

To preserve the optical purity of the isocyanoacetate, the Ugi reactions employing aldehydes **13** or **14** as carbonyl

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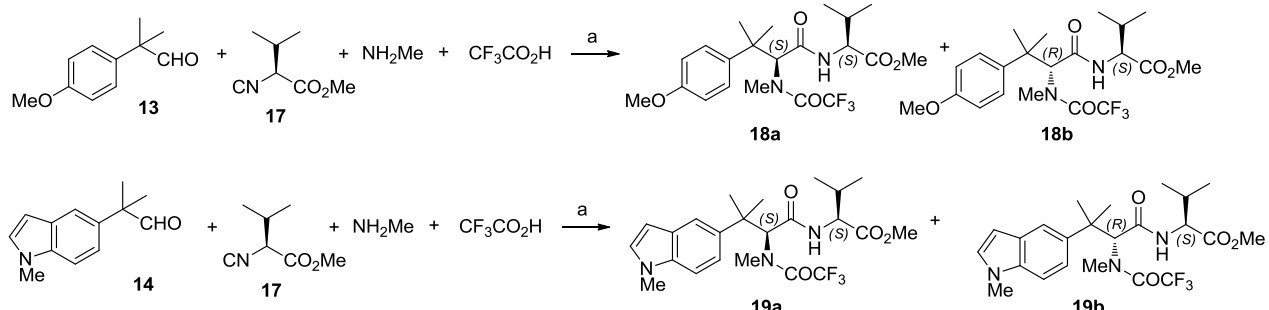
components were conducted after a precondensation time of 2 h between the aldehyde and methylamine, in the presence of $MgSO_4$ used as dehydrating promoter.[17] Ugi compounds **18** and **19** were obtained in good overall yields (63% for **18**, 75% for **19**), both as 1:1 diastereoisomeric mixtures, which could be easily separated by flash chromatography (FC).

Relying on a valuable literature suggestion,[18] the stereochemistry of both compounds **18** and **19** was postulated by NMR, and in particular performing the NOESY experiment on the separated **a** and **b** diastereoisomers. Besides, with the aim to unambiguously confirm the stereochemistry of these intermediates, we performed an X-ray diffraction analysis on compound **18b**, for which good diffracting single crystals were isolated from a methanol solution. The crystallographic structure of **18b** disclosed an (*R*,*S*)-configuration (Figure 3), leading us to select diastereoisomers **18a** and **19a** for continuing the synthesis, as the stereochemistry reported for potent taltobulin derivatives is (*S*,*S*,*S*).



Scheme 2. First multicomponent approach: the 4C-Ugi reaction. Reagents and conditions: a) MeOH, $MgSO_4$, rt (**18a**: 32%; **18b**: 31%; **19a**:37%; **19b**:38%).

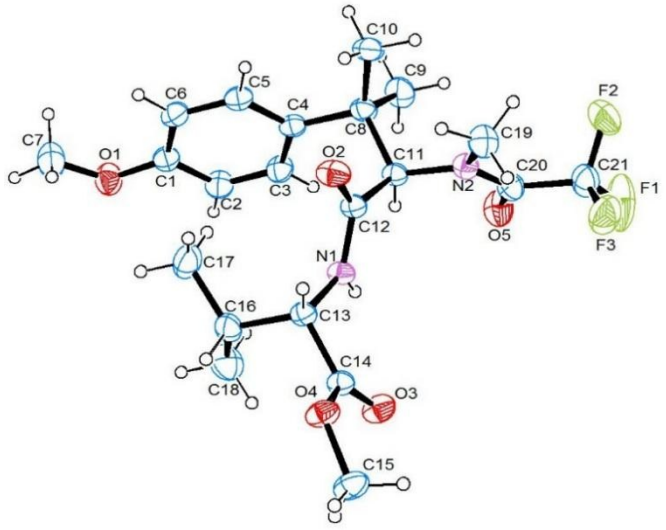
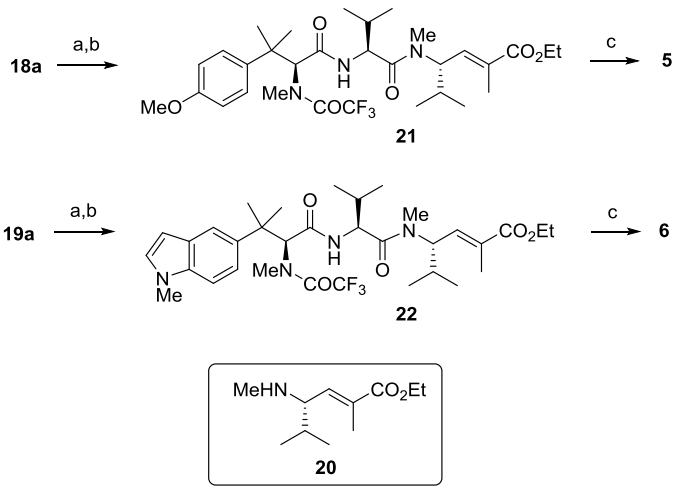


Figure 3. ORTEP[19] view of compound **18b**, *anti (R, S),* and the relative atom-numbering scheme (thermal ellipsoids at 40% probability).

To complete the synthesis, methyl esters **18a** and **19a** were carefully converted into the corresponding acids under mild basic conditions, with preservation of the trifluoroacetamide functional group, and then condensed with the known amino ester fragment **20**,[20] in acceptable yields by use of HTBU and DIPEA. From intermediates **21** and **22**, final compounds **5** and **6** were eventually recovered as amino acids by basic hydrolysis of both the ethyl ester and the trifluoroacetamide group.

With the aim of evaluating more extensively modified analogues, even compounds lacking amide bonds, we looked at a U-like-3CR and pursued the synthesis of oxazole-based compounds **7-9**, as depicted in Scheme 4. In this case, the key intermediate is the α-isocyanoacetamide **23**. In comparison to α-isocyanoacetates, α-isocyanoacetamides are much more configurationally stable. They show a higher Lewis basicity of the amide oxygen compared with that of the corresponding esters, and this should kinetically favor the cyclization step with the irreversible formation of the oxazole ring.[21] Isocyanopeptide **23** was efficiently prepared starting from amine **24**,[22] through intermediate formation of formamide **25** and subsequent dehydration using diphosgene at - 30 °C,[23] as depicted in Scheme 5. By stirring compound **23** with aldehydes **13**, **15** or **16** in the presence of methylamine and $MgSO_4$, we easily obtained final compounds **7**-**9**, in satisfactory yields as inseparable 1:1 to 1.5:1 mixture of diastereoisomers. Since for such extensively modified scaffolds the preliminary indication of activity can be considered the main goal, we performed the biological evaluation on the diastereoisomeric mixture (see below).



Scheme 3. Synthesis of analogues **5** and **6**. Reagents and conditions: a) LiOH, 50% aq MeOH, rt; then b) compound **20**, HBTU, DIPEA, CH_2Cl_2, rt (**21**: overall 58%; **22**: overall 52%). c) LiOH, 50% aq MeOH, 60 °C (**5**: 76%; **6**: 65%).

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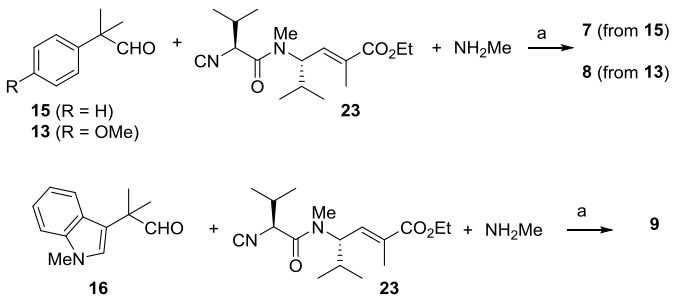
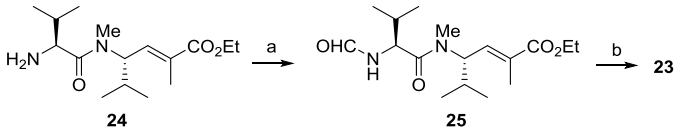
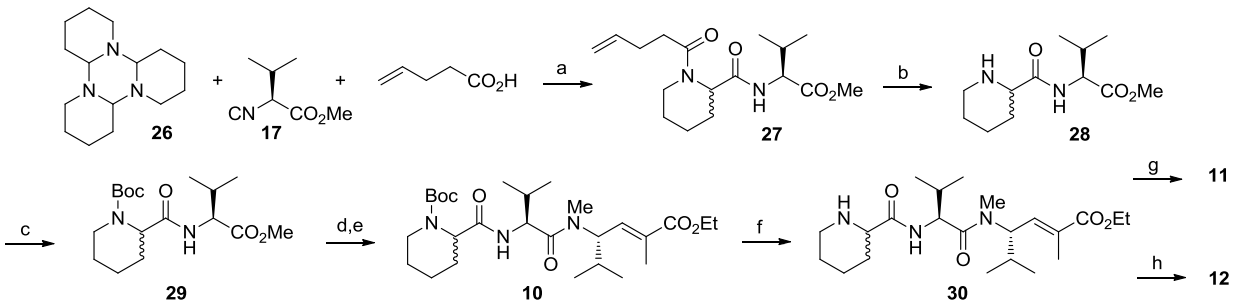


Scheme 4. Second multicomponent approach: the 3C-Ugi-like reaction. Synthesis of analogues **7-9.** Reagents and conditions: a) MeOH, $MgSO_4$, rt (**7**: 51%; **8**: 68%; **9**: 64%).



Scheme 5. Synthesis of the isocyanopeptide **23**. Reagents and conditions: a) Acetic formic anhydride, CH_2Cl_2, 0 °C to rt (**25**: quant. yield). b) *N*-methylmorpholine, diphosgene, THF, -30 °C to 0 °C (**23**: 80%).

In order to exploit the multicomponent strategy for the synthesis of piperidine-based E7974 analogues, we relied on the U-J-3CR, a modification of the Ugi protocol involving the use of cyclic imines and resulting in the synthesis of α-substituted nitrogen heterocycles. Being aware of the reported risk of isocyanoacetate epimerization related to the manner in which the cyclic imine was prepared, we followed the protocol of inducing a reversible trimerization of Δ1-piperideine, yielding crystalline and easily isolable tripiperideine **26**, as the starting component. Carrying out the multicomponent reaction of tripiperideine, isocyanoacetate **17** and 5-pentenoic acid as the acid component, we obtained the expected peptide **27** in good yield, as a 1:1 unseparable diastereoisomeric mixture. Unfortunately, this mixture could not be resolved at any stage of the synthesis of final compounds **11** and **12**. In our approach, the 5-pentenoic acid was chosen because the pentenoyl moiety can be selectively removed by iodolactonization[24] after the multicomponent reaction and the resulting secondary amine could be functionalized in various ways. Once the NH piperidine derivative **28** was synthesized, we looked at the reductive amination as a route to install selected lipophilic moieties on the piperidine ring. Therefore, after temporary Boc protection of the piperidine secondary nitrogen to give **29** and subsequent methylester hydrolysis and amide coupling with fragment **20**, we easily synthesized compound **10**. From **10**, Boc deprotection gave the key intermediate **30**. Reductive amination with acetone or cyclohexenone, by use of sodium triacetoxyborohydride and acetic acid, afforded, respectively, final compounds **11** and **12**.



Scheme 6. Third multicomponent approach: the 3C-Ugi-Joullié reaction. Synthesis of analogues **10-12.** Reagents and conditions: a) MeOH, rt (**27**: 46%). b) Iodine, aq $Na_2S_2O_3$, THF/H_2O, rt (**28**: 85%). c) $(Boc)_2O$, CH_2Cl_2, rt (**29**: 92%). d) LiOH, 50% aq MeOH, rt; then e) compound **20**, HBTU, DIPEA, CH_2Cl_2, rt (**10**: overall 47%). f) 50% TFA in CH_2Cl_2, rt (**30**: quant. yield). g) Acetone, $Na(OAc)_3BH$, AcOH, CH_2Cl_2, rt (**11**: quant. yield). h) Cyclohexenone, $Na(OAc)_3BH$, AcOH, CH_2Cl_2, rt (**12**: quant. yield).

Table 1. In vitro cell growth inhibitory effects.

Compd	GI_{50} [a](nM)					
	HT-29	HeLa	MCF-7	Jurkat	HL-60	RS4;11
HTI-286 (**2**)	0.4±0.05	0.3±0.06	2.0±0.6	0.2±0.08	0.4±0.1	0.3±0.1
5	3000±356	700±259	3750±943	176.7±28.5	34.3±5.6	430±224
6	8.0±2.4	11.2±0.5	7.3±1.7	0.8±0.1	1.1±0.1	2.3±0.3
7	12580±738	21300±2979	16800±4217	2333±120	3067±120	2967±418
8	23500±512	10580±5203	22300±1250	2441±203	923±79.3	2000±600
9	4700±711	8533±654	8300±1525	2433±296	3800±833	6833±917
10	36433±2882	13333±4826	13956±6233	4400±458	10166±1524	405±45
11	4.2±1.1	0.9±0.3	25.3±5.1	0.9±0.2	0.8±0.4	0.9±0.4
12	18780±7486	22760±1311	17160±1513	223.3±18.6	320±35.1	125.3±33

[a]GI_{50}= compound concentration required to inhibit tumor cell growth by 50%. Data are presented as the mean ± SE (Standard Error) from the dose-response curves of at least three independent experiments.

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Table 2. Inhibition of tubulin assembly and the binding of [^{3}H]vinblastine [^{3}H]dolastatin 10 and [^{3}H]halicondrin B.

	Inhibition of tubulin assembly	Inhibition of binding[b] of					
	IC_{50} (μM) ± SD[a]	[^{3}H]vinblastine		[^{3}H]dolastatin 10		[^{3}H]halichondrin B	
		% inhibition ± SD[a]					
		5 μM	20 μM	5 μM	20 μM	5 μM	20 μM
		inhibitor		inhibitor		inhibitor	
HTI-286 (**2**)	0.94±0.01	41±10	62±20	2±1	22±3	21±4	62±10
6	10±0.6	3±1	22±7	2±1	27±4	1±1	11±4
11	15±2	4±2	23±8	0	21	0	0

[a]SD = standard deviation. [b]Ligand binding studies were performed in 0.1 M 4-morpholinethanesulfonate (pH 6.9 in 1 M stock solution adjusted with NaOH)-0.5 mM $MgCl_2$ containing 10 μM tubulin (1.0 mg/ml), 10 μM radiolabeled ligand, and inhibitors as indicated. Reaction volume was 0.3 mL, incubation time 15 min at RT (around 20 °C). Ligands mixed prior to tubulin addition. Duplicate aliquots of each reaction mixture applied to syringe columns of Sephadex G-50 (superfine) swollen in 0.1 M Mes-0.5 mM $MgCl_2$. At least two experiments performed for each condition.

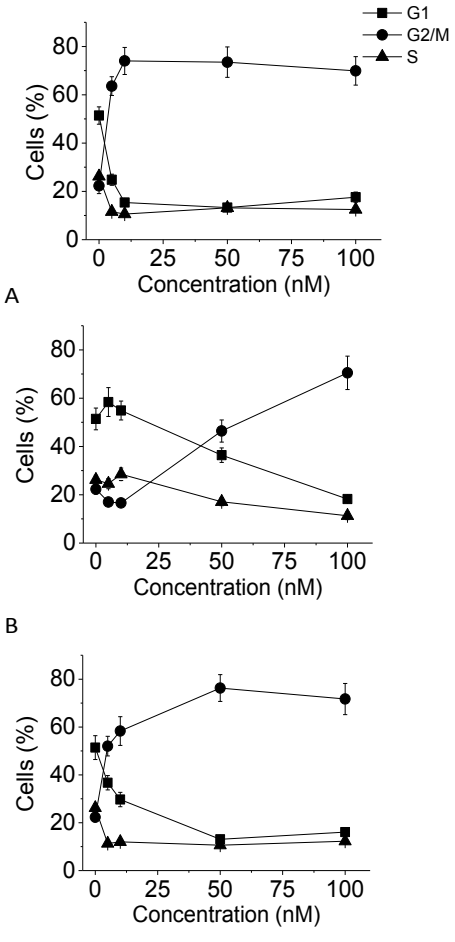

Figure 4. Percentage of cells in each phase of the cell cycle in HeLa cells treated with HTI-286 (**2**) (Panel A), **6** (Panel B) and **11** (Panel C) at the indicated concentrations for 24 h. Cells were fixed and labeled with propidium iodide and analyzed by flow cytometry as described in the experimental section.

Compounds **5**-**12** were evaluated in vitro for their cytotoxic activity against a panel of six human tumor cell lines, and the results are summarized in Table 1. Two of analogues synthesized during this work, namely compounds **6** and **11**, possessed cytotoxicity against all lines, though being 10-fold less active compared to the model compound HTI-286. The other compounds showed modest (compound **5**) activity or were practically devoid of any significant activity, having GI_{50} values in the micromolar range. The two highly active compounds **6** and **11** were also examined for their effects on tubulin polymerization and as inhibitors of the binding of [^{3}H]vinblastine, [^{3}H]dolastatin 10, and [^{3}H]halichondrin B to tubulin (Table 2). In these studies, they were found to be active as tubulin inhibitors, although less active than HTI-286 (compound **2**). Their reduced activity in the tubulin assays is in agreement with their reduced cytotoxicity as compared with **2** (compare data in Table 1 and 2). We think it most likely that their interactions with tubulin are similar to those of hemiasterlin (**1**) and HTI-286 (**2**). Compound **6** retains a high structural similarity to the natural product hemiasterlin **1**, highlighting the possibility that further modifications of the aromatic moiety in the first (A) amino acid segment will yield interesting and active agents. With regard to compound **11**, closely related structurally to E7974 (**4**), its potent activity suggests a marginal role of the piperidine ring stereogenic centre configuration, opening the way to more reliable and straightforward synthetic approaches. Lastly, the poor activity found with the oxazole-based derivatives **7**-**9** discourages further extensive modifications on the central (B) amino acid segment. In particular, the consistent structural modification brought by the presence of the oxazole ring caused a remarkable conformational bending, presumably forcing the

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molecule into a less favorable conformation with respect to bioactive compounds.

To demonstrate the presumptive antimitotic activity of **6** and **11**, based on their antitubulin activities, we analyzed their effects on cell cycle progression in HeLa cells. As shown in Figure 4, the two compounds caused a significant G2/M arrest in a concentration-dependent manner. In particular, compound **11** was very active, inducing cell cycle arrest at 5 nM, similar to the activity of HTI-286 (**2**). Compound **6** was less active, inducing a G2/M block only at 50 nM. The increase in the proportion of cells in the G2/M phase was accompanied by a sharp decrease in the proportion cells in the other phases of the cell cycle.

Conclusions

In summary, the preparation of new hemiasterlin derivatives was achieved, in which either the A or the B fragments was alternatively replaced. The procedures exploited multicomponent approaches, applied in three complementary isonitrile-based versions, and were highly valuable for the rapid and convergent synthesis of a small family of analogues. Our multicomponent approach was not previously used in preparing hemiasterlin analogues and allowed us to prepare compounds with unconventional modifications, such as compounds **7**-**9**. Biological evaluation confirmed that we had prepared two cytotoxic molecules, for which tubulin assembly inhibition and ligand binding studies were also performed, with activity for the two analogues obtained in these assays. The two analogues also caused a G2/M arrest in HeLa cells. We plan to continue our target-oriented synthesis programs, using addition strategies relying on MCRs. Our goal is to replace the multistep generation of sterically hindered amide functions with more reliable multicomponent assembly reactions.

Experimental section

General information

All commercial materials (Aldrich, Fluka) were used without further purification. All solvents were of reagent grade or HPLC grade. All reactions were carried out under a nitrogen atmosphere. All reactions were monitored by thin layer chromatography (TLC) on precoated silica gel 60 F254; spots were visualized with UV light or by treatment with a 1% aqueous $KMnO_4$ solution. Products were purified by flash chromatography (FC) on silica gel 60 (230–400 mesh). ^{1}H NMR spectra and ^{13}C NMR spectra were recorded on 300 and 400 MHz spectrometers. Chemical shifts are reported in parts per million relative to the residual solvent. ^{13}C NMR spectra have been recorded using the APT pulse sequence. Multiplicities in ^{1}H NMR are reported as follows: s = singlet, d = doublet, t = triplet, m = multiplet, br s = broad singlet. High-resolution MS spectra were recorded with an FT-ICR (Fourier Transform Ion Cyclotron Resonance) instrument, equipped with an ESI source.

General procedure for preparation of aldehydes 13-16. A solution of $[Pd(\eta^3\text{-allyl})Cl]_2$ (0.03 mmol) and Q-phos (0.06 mmol) in dry THF (10 mL) was prepared and stirred for 5 min at room temperature. Cs_2CO_3 (12 mmol), the required Br-benzene or Br-indole (6 mmol) and isobutyraldehyde (7 mmol) were then added. The reaction mixture was stirred for 18 h at 80 °C and then was diluted with EtOAc (20 mL) and filtered through a pad of Celite®. The filtrate was concentrated *in vacuo*, and the crude product was purified by (FC).

2-(4-Methoxyphenyl)-2-methylpropanal 13.[13] FC (7:3, *n*-hexane/DCM); 75% yield; yellow oil; R_f 0.27 (7:3, *n*-hexane/dichloromethane); ^{1}H NMR (300 MHz, $CDCl_3$) and ^{13}C NMR (75 MHz, $CDCl_3$) in accordance with the literature. HRMS (ESI) calcd for $C_{11}H_{15}O_2^+$ $[MH]^+$ 179.1067, found 179.1075.

2-Methyl-2-(1-methyl-1*H*-indol-5-yl)propanal 14. FC (7:3, *n*-hexane/DCM); 57% yield; oil; R_f 0.2 (1.5:1, *n*-hexane/dichloromethane); ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.50 (s, 1H), 7.56 (s, 1H), 7.26 (d, *J* = 8.5 Hz, 1H), 7.18-7.03 (m, 2H), 6.47 (d, *J* = 2.9 Hz, 1H), 3.75 (s, 3H), 1.53 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 202.7, 135.8, 131.8, 129.5, 128.8, 120.6, 118.8, 109.6, 101.1, 51.0, 33.6, 23.2 (2C); HRMS (ESI) calcd for $C_{13}H_{15}NNaO^+$ $[MNa]^+$ 224.1046, found 224.1054.

2-Methyl-2-phenylpropanal 15.[13] FC (7:3, *n*-hexane/DCM); 50% yield; yellow oil; R_f 0.2 (4:1, *n*-hexane/dichloromethane); ^{1}H NMR (300 MHz, $CDCl_3$) and ^{13}C NMR (75 MHz, $CDCl_3$) in accordance with the literature. HRMS (ESI) calcd for $C_{10}H_{12}NaO^+$ $[MNa]^+$ 171.0780, found 171.0792.

2-Methyl-2-(1-methyl-1*H*-indol-3-yl)propanal 16. FC (7:3, n-hexane/DCM); 46%; oil; R_f 0.2 (1.5:1, *n*-hexane/dichloromethane); ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.48 (s, br, 1H), 7.55 (d, br, *J* = 7.7 Hz, 1H), 7.32 (d, *J* = 7.8 Hz, 1H), 7.24 (t, br, *J* = 7.8 Hz, 1H), 7.10 (t, *J* = 7.7 Hz, 1H), 6.96 (s, br, 1H), 3.79 (s, br, 3H), 1.56 (m, br, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 202.3, 137.7, 130.9, 126.2, 121.9, 120.3, 119.4, 115.1, 109.6, 46.5, 32.8, 22.0 (2C); HRMS (ESI) calcd for $C_{13}H_{16}NO^+$ $[MH]^+$ 202.1226, found 202.1234.

(*S*)-Methyl 2-isocyano-3-methylbutanoate 17.[16] Prepared according to the literature.[16] Spectroscopic and optical rotatory power data as in the literature.[25]

(*S*)-methyl 2-((*S*)-3-(4-methoxyphenyl)-3-methyl-2-(2,2,2-trifluoro-*N*-methylacetamido)butanamido)-3-methylbutanoate 18a and **(*S*)-methyl 2-((*R*)-3-(4-methoxyphenyl)-3-methyl-2-(2,2,2-trifluoro-*N*-methylacetamido)butanamido)-3-methylbutanoate, 18b**. Aldehyde **13** (250 mg, 1.40 mmol) and methylamine (1 M in MeOH, 1.54 mL, 1.54 mmol) were dissolved in dry MeOH (2.8 mL), anhydrous $MgSO_4$ (1.26 g) was added, and the mixture was stirred for 2 h at 25 °C. Trifluoroacetic acid (128 mL, 1.68 mmol) and α-isocyanoacetate **17** (238 mg, 1.68 mmol) were added with a time gap of 20 minutes between the two additions. With all the reactants added, the mixture was stirred for 48 h. The reaction mixture was then concentrated in vacuo to give a residue that was purified by FC (4:1, *n*-hexane/ethyl acetate) to give **18a** (200 mg, 32%) and **18b** (194 mg, 31%). **18a**: white amorphous solid; R_f (9:1 *n*-hexane/ethyl acetate) 0.17; $[a]_D^{21}$ = + 46.4 (c = 0.1, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.42 (d, *J* = 8.7 Hz, 2H), 6.89 (d, *J* = 8.7 Hz, 2H),

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5.68 (d, br, *J* = 7.8 Hz, 1H), 5.47 (s, 1H), 4.30 (dd, *J* = 8.7, 4.9 Hz, 1H), 3.78 (s, 3 H), 3.69 (s, 3H), 3.26 (s, br, 3H), 1.97 (m, 1H), 1.61 (s, 3H), 1.41 (s, 3H), 0.74 (d, *J* = 6.8 Hz, 3H), 0.64 (d, *J* = 6.8 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.6, 168.0, 158.9 (q, *J* = 34.9 Hz), 158.5, 137.8, 127.5 (2C), 116.5 (q, *J* = 287.7 Hz), 113.8 (2C), 65.0, 57.1, 55.2, 42.0, 41.7, 33.7, 30.5, 27.5, 25.5, 18.8, 17.4; HRMS (ESI) calcd for $C_{21}H_{29}F_3N_2O_5^+$ $[MNa]^+$ 469.1921, found 469.1919. **18b**: white amorphous solid; R_f (9:1 *n*-hexane/ethyl acetate) 0.18; $[a]_D^{21}$ = + 26.3 (c = 0.1, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.39 (d, *J* = 8.7 Hz, 2H), 6.86 (d, *J* = 8.7 Hz, 2H), 5.82 (d, *J* = 8.1 Hz, 1H), 5.45 (s, 1H), 4.30 (dd, *J* = 8.4, 4.7 Hz, 1H), 3.77 (s, 3H), 3.65 (s, 3H), 3.22 (s, 3H) 1.96 (m, 1H), 1.61 (s, 3H), 1.41 (s, 3H), 0.70 (d, *J* = 6.8 Hz, 3H), 0.69 (d, *J* = 6.8 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.5, 167.7, 159.4 (q, *J* = 34.9 Hz), 158.4, 137.5, 127.7 (2C), 116.4 (q, *J* = 287.7 Hz), 113.8 (2C), 64.8, 57.2, 55.3, 52.2, 41.8, 33.5, 30.7, 27.2, 25.5, 18.7, 17.7; HRMS (ESI) calcd for $C_{21}H_{29}F_3N_2O_5^+$ $[MNa]^+$ 469.1921, found 469.1931.

(*S*)-methyl 3-methyl-2-((*S*)-3-methyl-3-(1-methyl-1*H*-indol-5-yl)-2-(2,2,2-trifluoro-*N*-methylacetamido)butanamido)butanoate 19a and **(*S*)-methyl 3-methyl-2-((*R*)-3-methyl-3-(1-methyl-1*H*-indol-5-yl)-2-(2,2,2-trifluoro-*N*-methylacetamido)butanamido)butanoate 19b**. Aldehyde **14** (250 mg, 1.24 mmol) and methylamine (1 M in MeOH, 1.37 mL, 1.37 mmol) were dissolved in dry MeOH (2.5 mL), anhydrous $MgSO_4$ (1.15 g) was added, and the mixture was stirred for 2 h at 25 °C. Trifluoroacetic acid (115 mL, 1.49 mmol) and α-isocyanoacetate **17** (210 mg, 1.49 mmol) were added with a time gap of 20 min between the two additions. With all the reactants added, the mixture was stirred for 48 h. The reaction mixture was then concentrated in vacuo to give a residue that was purified by FC (4:1, *n*-hexane/ethyl acetate) to give **19a** (215 mg, 37%) and **19b** (221 mg, 38%). **19a**: white amorphous solid; R_f (5.7:1 *n*-hexane/ethyl acetate) 0.17; $[a]_D^{21}$ = + 53.0 (c = 0.1, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.77 (s, br, 1H), 7.43 (dd, *J* = 8.8 and 2.0 Hz, 1H), 7.33 (d, br, *J* = 8.8 Hz, 1H), 7.04 (d, *J* = 2.9 Hz, 1H), 6.46 (d, br, *J* = 2.9 Hz, 1H), 5.75 (s, 1H), 5.65 (d, br, *J* = 7.8 Hz, 1 H), 4.22 (dd, *J* = 7.8 and 4.9 Hz, 1H), 3.77 (s, 3H), 3.60 (s, 3H), 3.31 (s, br, 3H), 1.83 (m, 1H), 1.72 (s, 3H), 1.45 (s, 3H), 0.59 (d, *J* = 6.8 Hz, 3H), 0.37 (d, *J* = 7.0 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.6, 168.4, 158.4 (q, *J* = 34.9 Hz), 136.9, 135.5, 129.5, 128.7, 120.0, 119.6, 116.5 (q, *J* = 287.7 Hz), 109.8, 101.0, 65.6, 57.2, 51.8, 42.2, 34.1, 32.8, 30.2, 28.3, 25.2, 18.7, 17.0; HRMS (ESI) calcd for $C_{23}H_{30}F_3N_3NaO_4^+$ $[MNa]^+$ 492.2081, found 492.2071. **19b**: white amorphous solid; R_f (5.7:1 *n*-hexane/ethyl acetate) 0.18; $[a]_D^{21}$ = + 30.2 (c = 0.1, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.69 (s, br, 1H), 7.40 (dd, *J* = 8.7 and 2.0 Hz, 1H), 7.31 (d, br, *J* = 8.7 Hz, 1H), 7.03 (d, *J* = 2.9 Hz, 1H), 6.43 (d, *J* = 2.9 Hz, 1H), 5.67 (d, br, *J* = 7.8 Hz, 1H), 5.58 (s, 1H), 4.25 (dd, *J* = 7.8 and 4.9 Hz, 1H), 3.77 (s, 3H), 3.56 (s, 3H), 3.31 (s, 3H), 1.78 (m, 1H), 1.71 (s, 3H), 1.48 (s, 3H), 0.53 (d, *J* = 6.8 Hz, 3H), 0.51 (d, *J* = 6.8 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.3, 167.9, 158.4 (q, *J* = 34.9 Hz), 136.5, 135.6, 129.4, 128.6, 120.1, 118.4, 116.5 (q, *J* = 287.7 Hz), 109.4, 101.2, 65.2, 57.2, 51.9, 42.3, 33.6, 32.8, 30.6, 27.9, 25.5, 18.3, 17.5; HRMS (ESI) calcd for $C_{23}H_{30}F_3N_3NaO_4^+$ $[MNa]^+$ 492.2081, found 492.2066.

(*S,E*)-Ethyl 2,5-dimethyl-4-(methylamino)hex-2-enoate 20.[20] Prepared according to the literature. Spectroscopic and optical rotatory power data as in the literature.

(*S,E*)-Ethyl 4-((S)-2-((S)-3-(4-methoxyphenyl)-3-methyl-2-(2,2,2-trifluoro-*N*-methylacetamido)butanamido)-*N*,3-dimethylbutanamido)-2,5-dimethylhex-2-enoate, 21. LiOH (24 mg, 1.0 mmol) was added to a suspension of methyl ester **18a** (88 mg, 0.2 mmol) in 50% aqueous methanol (v/v, 8 mL). The resulting mixture was stirred for 18 h at 25 °C and then was diluted with water (10 mL) and extracted with diethyl ether (2 x 7 mL). The aqueous layer was acidified to pH 2 – 3 with a 5% aqueous solution of H_3PO_4 and extracted with EtOAc (3 x 5 mL). The combined organic layers were dried over Na_2SO_4 and concentrated in vacuo to afford the crude acid intermediate, which was used in the condensation step without purification. HBTU (60 mg, 0.15 mmol) was added to a solution of the crude acid (60 mg, 0.14 mmol) in dry dichloromethane (3 mL). After 10 min, amine **20** (30 mg, 0.15 mmol) and DIPEA (30 mL, 0.17 mmol) in dry dichloromethane (3 mL) were added. The resulting reaction mixture was stirred for 24 h at 25 °C and then washed with a saturated aqueous solution of $NaHCO_3$ (two times), water and finally with a 5% aqueous solution of H_3PO_4. The resulting organic layer was dried over Na_2SO_4 and concentrated in vacuo. The crude residue was purified by FC (4:1, *n*-hexane/ethyl acetate) to give **21** (48 mg, 58%). Pale yellow oil; R_f (4:1, *n*-hexane/ethyl acetate) 0.25; $[a]_D^{23}$ = - 57.4 (c = 0.12, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39 (d, *J* = 8.8 Hz, 2H), 6.86 (d, *J* = 8.8 Hz, 2H), 6.61 (dq, br, *J* = 9.2 and 1.5 Hz, 1H), 6.09 (d, br, *J* = 8.6 Hz, 1H), 5.44 (s, 1H), 5.01 (dd, *J* = 10.5 and 9.2 Hz, 1H), 4.52 (dd, *J* = 8.6 and 6.8 Hz, 1H), 4.18 (q, *J* = 7.0 Hz, 2H), 3.77 (s, 3H), 3.15 (q, br, *J* = 1.7 Hz, 3H), 2.88 (s, 3H), 1.93-1.75 (m, br, 2H), 1.85 (d, *J* = 1.5 Hz, 3H), 1.54 (s, 3H), 1.40 (s, 3H), 1.28 (t, *J* = 7.0 Hz, 3 H), 0.88 (d, *J* = 6.6 Hz, 3H), 0.81 (d, *J* = 6.6 Hz, 3H), 0.78 (d, *J* = 6.8 Hz, 3H), 0.65 (d, *J* = 6.8 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.3, 167.9, 167.7, 158.4 (q, *J* = 34.9 Hz), 158.0, 138.2, 137.6, 132.9, 127.5 (2C), 116.6 (q, *J* = 287.7 Hz), 114.0 (2C), 65.0, 60.9, 56.4, 55.3, 54.0, 41.6, 33.5, 30.8, 30.3, 30.0, 27.3, 26.4, 19.4 (2C), 18.8, 17.3, 14.2, 13.7; HRMS (ESI) calcd for $C_{31}H_{46}F_3N_3NaO_6^+$ $[MNa]^+$ 636.3231, found 636.32423.

(*S,E*)-4-((*S*)-2-((*S*)-3-(4-methoxyphenyl)-3-methyl-2-(methylamino)butanamido)-*N*,3-dimethyl-butanamido-2,5-dimethylhex-2-enoic acid, 5. LiOH (16 mg, 0.64 mmol) was added to a suspension of ester **21** (50 mg, 0.08 mmol in 50% aqueous methanol (v/v, 3 mL). The resulting mixture was stirred for 18 h at 60 °C, then diluted with water (10 mL) and extracted with diethyl ether (2 x 10 mL). The aqueous layer was acidified to pH 2 – 3 with a 5% aqueous solution of H_3PO_4 and extracted with EtOAc (3 x 10 mL). The combined organic layers were dried over Na_2SO_4 and concentrated in vacuo to afford pure **5** (30 mg, 76%). Foam; $[a]_D^{23}$ = - 47.1 (c = 0.58, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.29 (d, *J* = 8.7 Hz, 2H), 7.28 (m, br, 1H), 6.89 (d, *J* = 8.7 Hz, 2H), 6.94-6.66 (m, br, 2H), 6.79 (dq, br, *J* = 9.9 and 1.5 Hz, 1H), 5.15 (dd, *J* = 9.9 and 5.3 Hz, 1H), 4.48 (d, *J* = 10.9 Hz, 1H), 3.98 (s, 1H), 3.82 (s, 3H), 3.24 (dhept, *J* = 10.9 and 6.7 Hz, 1H), 2.93 (s, 3H), 2.33 (s, 3H), 1.96 (s, br, 3H), 1.89 (m, 1H), 1.60 (s, 3H), 1.35 (s, 3H), 0.92-0.87 (m,

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9H), 0.86 (d, *J* = 6.6 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.3, 171.3, 169.6, 158.4, 140.5, 137.8, 131.8, 127.4 (2C), 113.9 (2C), 70.6, 58.4, 56.7, 55.3, 41.3, 31.8, 30.3, 29.8, 27.7, 27.0, 20.9, 19.7, 19.6, 19.5, 19.4, 13.5; HRMS (ESI) calcd for $C_{27}H_{44}N_3O_5^+$ $[MH]^+$ 490.3275, found 490.3270.

(*S*,*E*)-Ethyl 4-((*S*)-*N*,3-dimethyl-2-((*S*)-3-methyl-3-(1-methyl-1*H*-indol-5-yl)-2-(2,2,2-trifluoro-*N*-methyl acetamido)butanamido)butanamido)-2,5-dimethylhex-2-enoate, 22. LiOH (20 mg, 0.83 mmol) was added to a suspension of methyl ester **19a** (78 mg, 0.17 mmol) in 50% aqueous methanol (v/v, 7 mL). The resulting mixture was stirred for 18 h at 25 °C, then was diluted with water (10 mL) and extracted with diethyl ether (2 x 5 mL). The aqueous layer was acidified to pH 2 – 3 with a 5% aqueous solution of H_3PO_4 and extracted with EtOAc (3 x 5 mL). The combined organic layers were dried over Na_2SO_4 and concentrated in vacuo to afford the crude acid intermediate, which was used in the condensation step without purification. HBTU (76 mg, 0.20 mmol) was added to a solution of the crude acid (77 mg, 0.17 mmol) in dry dichloromethane (3.5 mL). After 10 min, amine **20** (40 mg, 0.20 mmol) and DIPEA (38 mL, 0.22 mmol) in dry dichloromethane (3.5 mL) were added. The resulting reaction mixture was stirred for 24 h at 25 °C, then was washed with a saturated aqueous solution of $NaHCO_3$ (two times), water and finally with a 5% aqueous solution of H_3PO_4. The resulting organic layer was dried over Na_2SO_4 and concentrated in vacuo. The crude residue was purified by FC (3:1, *n*-hexane/ethyl acetate) to give **22** (58 mg, 52%). Pale yellow foam; R_f (3:1, *n*-hexane/ethyl acetate) 0.28; $[\alpha]_D^{21}$= - 78.1 (c = 0.1, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.80 (s, br, 1H), 7.41 (d, br, *J* = 8.7 Hz, 1H), 7.34 (d, *J* = 8.7 Hz, 1H), 7.06 (d, *J* = 2.9 Hz, 1H), 6.64 (d, br, *J* = 8.9 Hz, 1H), 6.49 (d, *J* = 2.9 Hz, 1H), 6.14 (d, *J* = 8.2 Hz, 1H), 5.71 (s, 1 H), 5.04 (t, *J* = 9.9 Hz, 1H), 4.46 (t, *J* = 7.6 Hz, 1H), 4.21 (q, *J* = 6.7 Hz, 2H), 3.80 (s, 3H), 3.21 (s, 3H), 2.89 (s, 3H), 1.88 (s, 3H), 1.93-1.78 (m, 1H), 1.78-1.64 (m, 1H), 1.69 (s, 3H), 1.50 (s, 3H), 1.82 (t, *J* = 6.7 Hz, 3H), 0.91 (d, *J* = 6.5 Hz, 3H), 0.80 (d, *J* = 6.5 Hz, 3H), 0.72 (d, *J* = 6.7 Hz, 3H), 0.47 (d, *J* = 6.7 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.1, 168.9, 168.4, 158.9 (q, *J* = 35.3 Hz), 139.1, 137.4, 136.2, 133.5, 130.1, 129.3, 120.80, 119.1, 117.3 (q, *J* = 288.2 Hz), 110.3, 102.0, 66.3, 61.5, 57.0, 54.9, 42.7, 34.5, 33.5, 31.3, 30.9, 30.6, 30.6, 28.7, 27.0, 20.0, 19.9, 19.4, 17.9, 14.9; HRMS (ESI) calcd for $C_{33}H_{47}F_3N_4NaO_5^+$ $[MNa]^+$ 659.3391, found 659.3384.

(*S*,*E*)-4-((*S*)-*N*,3-dimethyl-2-((*S*)-3-methyl-3-(1-methyl-1*H*-indol-5-yl)-2-(methylamino)butanamido)butanamido) -2,5-dimethylhex-2-enoic acid, 6. LiOH (10 mg, 0.4 mmol) was added to a suspension of ester **22** (35 mg, 0.05 mmol in 50% aqueous methanol (v/v, 2 mL). The resulting mixture was stirred for 18 h at 60 °C, then diluted with water (10 mL) and extracted with diethyl ether (2 x 5 mL). The aqueous layer was acidified to pH 2 – 3 with a 5% aqueous solution of H_3PO_4 and extracted with EtOAc (3 x 8 mL). The combined organic layers were dried over Na_2SO_4 and concentrated in vacuo to afford almost pure **6** (17 mg, 65%). Pale yellow foam; $[a]_D^{20}$ = - 56.5 (c = 0.1, $CHCl_3$); ^{1}H NMR (400 MHz, CD_3OD) δ 7.67 (s, 1H), 7.38 (d, *J* = 8.7 Hz, 1H), 7.30 (d, br, *J* = 8.7 Hz, 1H), 7.17 (d, *J* = 3.2 Hz, 1H), 6.75 (d, br, *J* = 9.3 Hz, 1H), 6.44 (d, *J* = 3.2 Hz, 1H), 4.98 (dd, *J* = 10.2 and 9.3 Hz, 1H), 4.48 (d, *J* = 10.5 Hz, 1H), 4.34 (s, 1H), 3.81 (s, 3H), 3.05 (m, 1H), 3.03 (s, 3H), 2.25 (s, 3H), 2.01 (m, 1H), 1.90 (s, 3H), 1.66 (s, 3H), 1.49 (s, 3H), 0.91 (d, br, *J* = 6.7 Hz, 6H), 0.85 (d, br, *J* = 6.5 Hz, 3H), 0.80 (d, br, *J* = 6.7 Hz, 3H); ^{13}C NMR (100 MHz, CD_3OD) δ 172.4, 168.9, 168.0, 138.6, 136.8, 136.3, 134.3, 129.8, 128.4, 120.3, 118.5, 109.4, 101.9, 70.7, 58.7, 57.8, 42.0, 32.2, 31.1, 30.2, 29.7, 28.0, 27.7, 27.5, 19.1 (2C), 18.9, 18.8, 13. 2; HRMS (ESI) calcd for $C_{29}H_{45}N_4O_4^+$ $[MH]^+$ 513.3435, found 513.3422.

(4*S*,*E*)-Ethyl 4-((4-isopropyl-2-(2-methyl-1-(methylamino)-2-phenylpropyl)oxazol-5-yl)(methyl)amino)-2,5-dimethylhex-2-enoate, 7. A mixture of aldehyde **15** (50 mg, 0.34 mmol), methylamine (2 M solution in MeOH, 0.25 mL, 0.50 mmol) and $MgSO_4$ (20 mg) in MeOH (0.6 mL) was stirred for 2.5 h. Then isocyanide **23** (95 mg, 0.31 mmol) was added. After 48 h the reaction mixture was filtered through a pad of Celite® and concentrated in vacuo. The residue was purified by FC (1.5:1, *n*-hexane/ethyl acetate) to give **7** (73 mg, 51%) as a 1.5:1 unseparable mixture of two diastereoisomers. White foam; R_f 0.38 (1:1.5, *n*-hexane/ethyl acetate); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.56-7.18 (m, 5H), 6.67 (d, br, *J* = 9.8 Hz, 1H), 4.21 (q, *J* = 7.1 Hz, 2H), 3.76 (s, 0.6H), 3.73 (s, 0.4 H), 3.43 (m, 1H), 2.86 (m, 1H), 2.57 (s, 3H), 2.21 (s, 3H), 1.81 (s, 3H), 1.76 (m, 1H), 1.39 (s, 6H), 1.30 (t, *J* = 7.1 Hz, 3H), 1.22 (m, 6H), 0.91 (m, 3H), 0.84 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.3, 159.6, 149.9, 146.0, 140.1, 139.9, 135.0, 131.9, 129.3 (2C), 127.0 (2C), 126.5, 69.7, 67.3, 61.3, 42.6, 40.2, 35.8, 30.9, 26.5, 24.8, 24.3, 21.8 (2C), 19.9 (2C), 14.9; HRMS (ESI) calcd for $C_{28}H_{43}N_3NaO_3^+$ $[MNa]^+$ 492.3197, found 492.3209.

(4*S*,*E*)-Ethyl 4-((4-isopropyl-2-(2-(4-methoxyphenyl)-2-methyl-1-(methylamino)propyl)oxazol-5-yl)(methyl)amino)-2,5-dimethylhex-2-enoate, 8. A mixture of aldehyde **13** (34 mg, 0.19 mmol), methylamine (2 M solution in MeOH, 0.15 mL, 0.30 mmol) and $MgSO_4$ (15 mg) in MeOH (0.6 mL) was stirred for 2.5 h. Then isocyanide **23** (60 mg, 0.19 mmol) was added. After 48 h the reaction mixture was filtered through a pad of Celite® and concentrated in vacuo. The residue was purified by FC (7:3, *n*-hexane/ethyl acetate) to give **8** (66 mg, 68%) as a 1.5:1 unseparable mixture of two diastereoisomers. White foam; R_f 0.4 (1:1.5, *n*-hexane/ethyl acetate); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.24 (d, *J* = 8.8 Hz, 2H), 6.83 (d, *J* = 8.8 Hz, 2H), 6.67 (m, 1H), 4.21 (q, *J* = 7.1 Hz, 2H), 3.79 (s, 3H), 3.72 (s, 0.6H), 3.69 (s, 0.4 H), 3.43 (m, 1H), 2.88 (m, 1H), 2.61 (s, 3H), 2.21 (s, 3H), 1.81 (s, br, 3H), 1.76 (m, 1H), 1.43 (s, 6H), 1.30 (t, *J* = 7.1 Hz, 3H), 1.19 (m, 6H), 0.97 (m, 3H), 0.85 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.6, 159.2, 157.9, 149.6, 139.4, 138.2, 134.3, 130.1, 127.5 (2C), 113.4 (2C), 69.2, 66.7, 60.6, 55.2, 41.4, 39.6, 35.1, 30.3, 26.5, 24.9, 24.3 (2C), 21.8 (2C), 19.9 (2C), 13.1; HRMS (ESI) calcd for $C_{29}H_{45}N_3NaO_4^+$ $[MNa]^+$ 522.3302, found 522.3317.

(4*S*,*E*)-Ethyl 4-((4-isopropyl-2-(2-methyl-2-(1-methyl-1*H*-indol-3-yl)-1-(methylamino)propyl)oxazol-5-yl)(methyl)amino)-2,5-dimethylhex-2-enoate, 9. A mixture of aldehyde **16** (40 mg, 0.20 mmol), methylamine (2 M solution in MeOH, 0.15 mL, 0.30 mmol) and $MgSO_4$ (15 mg) in MeOH (0.6 mL) was stirred for 2.5 h. Then isocyanide **23** (65 mg, 0.21 mmol) was added. After 48 h the reaction mixture was filtered

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through a pad of Celite® and concentrated in vacuo. The residue was purified by FC (1.5:1, *n*-hexane/ethyl acetate) to give **9** (66 mg, 64%) as a 1:1 unseparable mixture of two diastereoisomers. Thick oil; R_f 0.38 (1:1.5, *n*-hexane/ethyl acetate); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.85 (d, *J* = 8.2 Hz, 0.5H), 7.83 (d, *J* = 8.2 Hz, 0.5H), 7.30 (d, *J* = 8.1 Hz, 1H), 7.22 (t, br, *J* = 8.1 Hz, 1H), 7.09 (t, br, *J* = 7.9 Hz, 1H), 6.88 (s, 1H), 6.72 (d, br, *J* = 9.8 Hz, 0.5H), 6.69 (d, br, *J* = 9.8 Hz, 0.5H), 4.21 (q, *J* = 7.1 Hz, 2H), 4.13 (s, 0.5H), 4.11 (s, 0.5H), 3.75 (s, 3H), 3.47 (m, 1H), 2.86 (m, 1H), 2.59 (s, 1.5H), 2.57 (s, 1.5H), 2.14 (s, 3H), 1.92-1.81 (m, 4H), 1.50 (s, 3H), 1.41 (s, 3H), 1.30-1.21 (m, 10H), 0.95 (m, 3H), 0.84 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.4, 160.2, 150.0, 140.2, 140.0, 135.1, 134.9, 127.6 (2C), 126.7, 122.7, 121.9, 119.3, 110.8, 68.3, 67.3, 61.3, 40.1, 40.0, 36.0, 33.3, 31.0, 27.7, 27.5, 25.7, 24.5, 22.5, 21.8, 20.1, 20.5, 18.3; HRMS (ESI) calcd for $C_{31}H_{46}N_4NaO_3^+$ $[MNa]^+$ 545.3462, found 545.3455.

(*S*,*E*)-Ethyl 4-((*S*)-2-amino-*N*,3-dimethylbutanamido)-2,5-dimethylhex-2-enoate, 24.[22] Prepared according to the literature. Spectroscopic and optical rotatory power data were in accord with the literature.

(*S*,*E*)-Ethyl 4-((*S*)-2-formamido-*N*,3-dimethylbutanamido)-2,5-dimethylhex-2-enoate, 25. Acetic formic anhydride (prepared by stirring 1 equiv of acetic anhydride and 1.1 equiv of formic acid for 2 h at 55 °C, 0.85 mL, 13.5 mmol) was added dropwise at 0 °C to a stirred solution of amine **24** (0.84 g, 2.8 mmol) in dichloromethane (10 mL), and the mixture was stirred for 18 h at room temperature. After elimination of all volatiles under reduced pressure, compound **25** was obtained (0.91 g, quantitative yield). Oil; R_f 0.4 (ethyl acetate); $[\alpha]^{21}_D$ = - 103.5 (c 1.3, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.21 (s, 1H), 6.62 (d, *J* = 9.2 Hz, 1H), 6.50 (d, br, *J* = 8.8 Hz, 1H), 5.09 (dd, *J* = 10.0 Hz and 9.4 Hz, 1H), 4.94 (dd, *J* = 7.0 Hz and 8.8 Hz, 1H), 4.19 (q, *J* = 7.1 Hz, 2H), 2.97 (s, 3H), 2.08-1.77 (m, 5H), 1.30 (t, *J* = 7.1 Hz, 3H), 0.85 (m, 12H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.1, 167.7, 161.3, 137.8, 133.1, 60.9, 56.7, 52.6, 32.0, 30.6, 29.9, 19.3 (2C), 18.7, 17.5, 14.2, 13.6; HRMS (ESI) calcd for $C_{17}H_{31}N_2O_4^+$ $[MH]^+$ 327.2278, found 327.2290.

(*S*,*E*)-Ethyl 4-((*S*)-2-isocyano-*N*,3-dimethylbutanamido)-2,5-dimethylhex-2-enoate, 23. Formamide **25** (0.90 g, 2.76 mmol) was dissolved in dry THF (40 mL), and *N*-methylmorpholine (1.13 mL, 10.2 mmol) was added. The resulting solution was cooled to - 30 °C, and diphosgene (0.2 mL, 1.66 mmol) in THF (1.5 mL) was added dropwise over a period of 15 min, while the temperature was maintained at - 30 °C. After addition of the diphosgene was completed, the solution was allowed to warm to 0 °C. An ice-cold saturated aqueous sodium bicarbonate solution (10 mL) was added, and the reaction mixture was stirred vigorously for 10 min. The product was extracted with EtOAc (25 mL), and the EtOAc phase was washed sequentially with a saturated aqueous sodium bicarbonate solution and brine. The organic layer was dried over Na_2SO_4 and evaporated in vacuo. The product was purified by FC (4:1, *n*-hexane/ethyl acetate) to give **23** (0.67 g, 80%). Yellow oil; R_f 0.26 (4:1, *n*-hexane/ethyl acetate); $[\alpha]^{19}_D$ = - 91.8 (c 1.1, CH_3OH); ^{1}H NMR (300 MHz, CD_3OD) δ 6.70 (d, *J* = 9.2 Hz, 1H), 4.97 (dd, *J* = 10.0 and 9.2 Hz, 1H), 4.70 (d, *J* = 5.9 Hz, 1H), 4.20 (q, *J* = 7.1 Hz, 2H), 2.94 (s, 3H), 2.30 (m, 1H), 1.90 (m, 1H), 1.86 (s, 3H), 1.29 (t, *J* = 7.1 Hz, 3H), 1.09 (m, 6H), 0.85 (m, 6H); ^{13}C NMR (75 MHz, CD_3OD) δ 169.2, 168.1, 159.4, 139.2, 134.5, 62.4, 62.0, 59.3, 32.5, 31.8, 31.4, 19.4-19.3 (3C), 18.5, 14.5, 13.8; HRMS (ESI) calcd for $C_{17}H_{28}N_2N_a$ O_3^+ $[MNa]^+$ 331.1992, found 331.2008.

α-Tripiperidein, **26**.[17] Prepared according to the literature. Spectroscopic data were in accord with the literature.

(2*S*)-Methyl 3-methyl-2-(1-(pent-4-enoyl)piperidine-2-carboxamido)butanoate, **27**. A solution of pent-4-enoic acid (579 μL, 5.67 mmol) and α-tripiperidein **26** (466 mg, 1.87 mmol) in dry MeOH (12 mL) was stirred for 10 min. Isocyanoacetate **17** (880 mg, 6.24 mmol) was added, and the mixture was stirred at 25 °C for 72 h. The solvent was removed in vacuo, and the crude mixture was taken up in EtOAc (15 mL) and washed with a saturated aqueous solution of $NaHCO_3$ (3 x 10 mL). The organic layers were dried over Na_2SO_4, and the solvent was concentrated in vacuo. The crude product was purified by FC (7:3 to 1.5:1 gradient, *n*-hexane/ethyl acetate) to give **27** (843 mg, 46%) as an unseparable 1:1 mixture of diastereoisomers. Yellow oil; R_f 0.29 (7:3, *n*-hexane/ethyl acetate); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.69-6.59 (m, 1H), 5.96 – 5.83 (m, 1H), 5.32 (d, br, *J* = 5.4 Hz, 0.5H), 5.26 (d, br, *J* = 5.4 Hz, 0.5H), 5.10 (d, m, *J* = 17.1 Hz, 1H), 5.03 (d, br, *J* = 10.0 Hz, 1H), 4.50 (dd, *J* = 5.4 and 3.9 Hz, 0.5H), 4.48 (dd, *J* = 5.0 and 3.2 Hz, 0.5H), 3.85 – 3.75 (m, 1H), 3.74 (1.5 H, s), 3.73 (1.5 H, s), 3.17 (dt, *J* = 13.2 and 3.2 Hz, 0.5H), 3.14 (dt, *J* = 13.2 and 3.2 Hz, 0.5H), 2.58 – 2.50 (m, 2H), 2.50-2.41 (m, 2H), 2.33 – 2.14 (m, 2H), 1.78 – 1.65 (m, 3H), 1.60–1.42 (m, 2H), 0.96 (d, *J* = 6.8 Hz, 1.5H), 0.93 (d, *J* = 6.8 Hz, 1.5H), 0.88 (d, *J* = 6.9 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.1 and 172.8 (1C), 172.5 and 172. 0 (1C), 171.3, 137.2, 115.4, 57.3, 52.1 and 52.0 (1C), 51.9 and 51.8 (1C), 43.8 and 43.7 (1C), 32.8 and 32.7 (1C), 31.0 and 30.7 (1C), 29.2, 25.5, 25.3 and 25.0 (1C), 20.4 and 20.3 (1C), 19.1, 17.7 and 17.6 (1C); HRMS (ESI) calcd for $C_{17}H_{28}N_2NaO_4^+$ $[MNa]^+$ 347.1941, found 347.1958.

(2*S*)-Methyl 3-methyl-2-(piperidine-2-carboxamido)butanoate, **28**. Iodine (117 mg, 0.46 mmol) was added to a solution of compound **27** (100 mg, 0.31 mmol) in THF/H_2O (6 mL, 31 v/v). After stirring for 30 min, aqueous $Na_2S_2O_3$ (20 mL, 1 M) was added, and the suspension thus obtained was stirred for 30 min. The mixture was then poured into an aqueous solution of $Na_2S_2O_3$/brine (20 mL 1:1 v/v) and extracted with EtOAc (3 x 20 mL). The combined organic layers were dried over Na_2SO_4 and concentrated in vacuo to give a yellow residue that was taken up in diethyl ether (10 mL) and washed with a 1 M aqueous solution of HCl (3 mL x 2). The aqueous phase was basified to pH 9 with a saturated aqueous solution of $NaHCO_3$ and extracted with dichloromethane (3 x 10 mL). The combined organic phases were dried over Na_2SO_4 and concentrated in vacuo to give compound **28** (64 mg, 85%) as an unseparable 1:1 mixture of diastereoisomers. Yellow oil; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.40–7.30 (m, 1H), 4.51 (t, br, *J* = 5.8 Hz, 0.5H), 4.49 (t, br, *J* = 5.4 Hz, 0.5H), 3.70 (s, 3H), 3.40–3.28 (m, 1H), 3.14–3.00 (m , 1H), 2.79–2.65 (m, 1H), 2.65-2.48 (m, 1H), 2.17 (oct, *J* = 5.9 Hz, 1H), 2.13–1.88 (m, 1H), 1.85-1.69 (m, 1H), 1.63–1.37 (m, 4H), 0.95 – 0.87 (m, 6H); ^{13}C NMR (75

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MHz, $CDCl_3$) δ 173.7 and 173.5 (1C), 172.5 and 172.4 (1C), 60.1 and 60.0 (1C), 59.9 and 59.8 (1C), 56.8 and 56.7 (1C), 45.5, 33.8, 31.9 and 31.20 (1C), 25.5, 23.5 and 22.6 (1C), 19.2 and 19.1 (1C), 18.7 and 18.0 (1C); HRMS (ESI) calcd for $C_{12}H_{22}N_2NaO_3^+$ $[MNa]^+$ 265.1523, found 265.1510.

***tert*-Butyl 2-(((*S*)-1-methoxy-3-methyl-1-oxobutan-2-yl)carbamoyl)piperidine-1-carboxylate**, **29**. Compound **28** (30 mg, 0.12 mmol) and Boc_2O (33 mg, 0.15 mmol) were dissolved in dichloromethane (0.5 mL) and stirred overnight. The mixture was washed with a saturated aqueous solution of $NaHCO_3$ (2 x 10 mL), a 5% aqueous solution of H_3PO_4 (2 x 10 mL) and brine (10 mL). The organic phase was dried over Na_2SO_4 and concentrated in vacuo to afford the crude product, which was purified by FC (9:1, *n*-hexane/ethyl acetate) to give compound **29** (39 mg, 92%) as an unseparable 1:1 mixture of diastereoisomers. Yellow oil; R_f 0.28 (9:1, *n*-hexane/ethyl acetate); ^{1}H NMR (300 MHz, $CDCl_3$, rotameric mixture of diastereoisomers) δ 6.60 (m, br, 0.5H), 6.47 (m, br, 0.5H), 4.78(m, br, 1H), 4.64 (d, br, *J* = 7.8 and 3.9 Hz, 1H), 4.28–3.89 (m, 1H), 3.72 (s, 3H), 2.85 (t, br, *J* = 12.7 Hz, 0.7H), 2.77 (m, br, 0.3H), 2.28 (m, br, 1H), 2.17 (m, 1H), 1.71– 1.32 (m, 5H), 1.48 (s, 3H), 1.47 (s, 6H), 0.93 (d, *J* = 6.8 Hz, 1.7H), 0.92 (d, *J* = 6.8 Hz, 1.3H), 0.87 (d, *J* = 6.8 Hz, 1.5H), 0.86 (d, *J* = 6.8 Hz, 1.5H); ^{13}C NMR (75 MHz, $CDCl_3$, rotameric mixture of diastereoisomers) δ 172.4 and 172.1 (1C), 171.3, 161.1, 80.6, 56.9, 52.1, 42.4 and 42.1 (1C), 31.30, 30.9, 28.4 (3C), 25.3, 24.9, 20.5, 19.0, 17.7 and 17.5 (1C); HRMS (ESI) calcd for $C_{17}H_{30}N_2NaO_5^+$ $[MNa]^+$ 365.2047, found 365.2038.

***tert*-Butyl 2-(((*S*)-1-(((*S,E*)-6-ethoxy-2,5-dimethyl-6-oxohex-4-en-3-yl)(methyl)amino)-3-methyl-1-oxobutan-2-yl)carbamoyl)piperidine-1-carboxylate, 10**. LiOH (9 mg, 0.37 mmol) was added to a suspension of methyl ester **29** (25 mg, 0.07 mmol) in 50% aqueous methanol (v/v, 2.5 mL). The resulting mixture was stirred for 18 h at 25 °C, then diluted with water (4 mL) and extracted with diethyl ether (2 x 4 mL). The aqueous layer was acidified to pH 2-3 with a 5% aqueous solution of H_3PO_4 and extracted with EtOAc (3 x 5 mL). The combined organic layers were dried over Na_2SO_4 and concentrated in vacuo to afford the crude acid intermediate (19 mg, 76%), which was used in the condensation step without purification. HBTU (15 mg, 40 mmol) was added to a solution of the crude acid (12 mg, 37 mmol) in dry dichloromethane (2 mL). After 10 min, amine **29** (8 mg, 40 mmol) and DIPEA (8 mL, 44 mmol) in dry dichloromethane (2 mL) were added. The resulting reaction mixture was stirred for 24 h at 25 °C and then was washed successively with a saturated aqueous solution of $NaHCO_3$ (two times), water and a 5% aqueous solution of H_3PO_4. The resulting organic layer was dried over Na_2SO_4 and concentrated in vacuo. The crude residue was purified by FC (7:3, *n*-hexane/ethyl acetate) to give **10** (12 mg, 62%) as an unseparable 1:1 mixture of diastereoisomers. White amorphous solid; R_f (7:3, *n*-hexane/ethyl acetate) 0.29; ^{1}H NMR (400 MHz, $CDCl_3$, rotameric mixture of diastereoisomers) δ 6.72–6.59 (m, 1H), 5.12–4.98 (m, 1H), 4.94–4.64 (m, 3H), 4.23 (q, *J* = 7.1 Hz, 1.2H), 4.22 (q, *J* = 7.1 Hz, 0.8H), 4.10-3.94 (m, 1H), 3.00 (s, 0.9H), 2.99 (s, 0.3H), 2.98 (s, 0.6H), 2.97 (s, 1.2H), 2.89-2.77 (m, 1H), 2.37-2.20 (m, 1H), 2.08-1.86 (2H), 1.91 (d, *J* = 1.4 Hz, 0.9H), 1.90 (d, *J* = 1.4 Hz, 1.3H), 1.89 (d, *J* = 1.4 Hz, 0.8H), 1.72-1.40 (m, 5H), 1.60 (s, br, 9H), 1.83 (t, *J* = 7.1 Hz, 1.8H), 1.82 (t, *J* = 7.1 Hz, 1.2H), 0.97-0.83 (m, 12H); ^{13}C NMR (100MHz, $CDCl_3$, rotameric mixture of diastereoisomers) δ 172.0 and 171.5 (1C), 171.6 and 171.1 (1C), 167.7, 157.6 and 156.7 (1C), 138.3, 132.9, 80.5, 60.8, 56.9, 56.3, 53.9, 42.6 and 41.2 (1C), 31.2 and 31.1 (1C), 30.4, 30.0, 28.4 (3C), 25.8, 24.9, 20.6, 20.1- 17.3 (4C), 14.3, 13.7 and 13.5 (1C); HRMS (ESI) calcd for $C_{27}H_{47}N_3NaO_6^+$ $[MNa]^+$ 532.3357, found 532.3366.

(4*S,E*)-Ethyl 4-((2*S*)-*N*,3-dimethyl-2-(piperidine-2-carboxamido)butanamido)-2,5-dimethylhex-2-enoate, 30. TFA (0.5 mL) was added to a solution of compound **10** (128 mg, 0.25 mmol) in dichloromethane (0.5 mL). The mixture was stirred for 1 h at 25 °C, and then the solvent was removed in vacuo to give a residue which was taken up with dichloromethane (5 mL) and washed three times with a 10% aqueous solution of Na_2CO_3. The organic layer was dried over Na_2SO_4 and concentrated in vacuo to give pure amine **30** as an unseparable 1:1 mixture of diastereoisomers (102 mg, quantitative yield). Colorless oil; ^{1}H NMR (400 MHz, $CDCl_3$, rotameric mixture of diastereoisomers) δ 7.70 (d, br, *J* = 8.2 Hz, 0.25H), 7.48 (d, br, *J* = 8.4 Hz, 0.5H), 7.36 (d, *J* = 8.9 Hz, 0.25H), 6.69-6.71 (m, 1H), 5.15- 4.92 (m, 1H), 4.86-4.63 (m, 1H), 4.23 (q, *J* = 7.1 Hz, 2 H), 3.69 (m, br, 0. 2H), 3.58 (m, br, 0.8H), 3.36-3.23 (m, 1H), 3.03 (s, 1H), 2.99 (s, 2H), 2.89 (m, 1H), 2.36-2.18 (m, 1H), 2.16-1.97 (m, 2H), 1.95-1.86 (m, 1H), 1.90 (s, br, 3H), 1.81 (m, br, 1H), 1.76-1.60 (m, 3H), 1.52 (m, 1H), 1.33 (t, *J* = 7.1 Hz, 3H), 1.05-0.82 (m, 12H); ^{13}C NMR (100 MHz, $CDCl_3$, rotameric mixture of diastereoisomers) δ 172.5 and 172.4 (1C), 172.2, 168.4, 138.9 and 138.7 (1C), 133.4, 61.6, 59.6 and 58.8 (1C), 57.8 and 57.5 (1C), 55.1 and 54.9 (1C), 45.3, 31.6 and 31.5 (1C), 31.1, 30.6, 29.4, 25.0 and 24.6 (1C), 23.4 and 23.1 (1C), 20.7-17.7 (4C), 14.9, 14.4; HRMS (ESI) calcd for $C_{22}H_{40}N_3O_4^+$ $[MH]^+$ 410.3013, found 410.3010.

(4*S,E*)-Ethyl 4-((2*S*)-2-(1-isopropylpiperidine-2-carboxamido)-*N*,3-dimethylbutanamido)-2,5-dimethylhex-2-enoate, 11. To a solution of sodium triacetoxyborohydride (55 mg, 0.26 mmol) in MeOH (0.5 mL) kept at 0 °C, acetic acid (16 ml, 0.26 mmol), acetone (19 ml, 0.26 mmol) and a solution of compound **30** (53 mg, 0.13 mmol) in MeOH (0.5 mL) were added. The mixture was stirred at ambient temperature for 18 h. The reaction was quenched with 0.5 N aqueous sodium potassium tartrate (4 mL), then diluted with dichloromethane (4 mL) and washed with aqueous saturated sodium bicarbonate (3 mL). The organic layer was dried over Na_2SO_4 and concentrated in vacuo to give pure **11** as an unseparable 1:1 mixture of diastereoisomers (59 mg, quantitative yield). White amorphous solid; ^{1}H NMR (400 MHz, $CDCl_3$, rotameric mixture of diastereoisomers) δ 7.50-7.22 (m, br, 1H), 6.66 (d, br, *J* = 9.4 Hz, 0.7H), 6.62 (d, br, *J* = 9.4 Hz, 0.3H), 5.13-4.99 (m, 1H), 4.80-4.62 (m, 1H), 4.22 (q, *J* = 7.0 Hz, 1.4H), 4.21 (q, *J* = 7.0 Hz, 0.6H), 3.22-2.92 (m, 1.5H), 2.99 (s, 1H), 2.98 (s, 1H), 2.97 (s, 0.7H), 2.96 (s, 0.3H), 2.85 (m, br, 0.5H), 2.74 (m, br, 1H), 2.38-2.13 (m, br, 1H), 2.13-1.81 (m, br, 2H), 1.91 (d, br, *J* = 1.2 Hz, 1.5H), 1.90 (d, br, *J* = 1.2 Hz, 0.9H), 1.87 (d, br, *J* = 1.2 Hz, 0.6H), 1.76-1.37 (m, br, 4H), 1.82 (t, *J* = 7.0 Hz, 2.1H), 1.81 (t, *J* = 7.0

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Hz, 0.9H), 1.24 (m, br, 2H), 1.04-0.79 (m, 18H); ^{13}C NMR (100 MHz, $CDCl_3$, rotameric mixture of diastereoisomers) δ 175.9 and 175.8 (1C), 172.5 and 172.3 (1C), 168.4, 139.1, 133.5 and 133.4 (1C), 65.6 and 64.8 (1C), 61.5, 57.4 and 56.9 (1C), 54.3 and 53.6 (1C), 51.9, 43.2, 31.6, 31.2 and 31.0 (1C), 30.6, 26.0 and 25.5 (1C), 24.2, 23.9, 20.6-18.4 (6C), 14.9, 14.4; HRMS (ESI) calcd for $C_{25}H_{45}N_3NaO_4^+$ $[MNa]^+$ 474.3302, found 474.3318.

(4*S*,*E*)-Ethyl 4-((2*S*)-2-(1-cyclohexylpiperidine-2-carboxamido)-*N*,3-dimethylbutanamido)-2,5-dimethylhex-2-enoate, 12. To a solution of sodium triacetoxyborohydride (30 mg, 0.14 mmol) in MeOH (0.5 mL) kept at 0 °C, acetic acid (9 ml, 0.14 mmol), cyclohexanone (14 mg, 0.14 mmol) and a solution of compound **30** (30 mg, 0.07 mmol) in MeOH (0.5 mL) were added. The mixture was stirred at ambient temperature for 18 h. The reaction was quenched with 0.5 N aqueous sodium potassium tartrate (2 mL), then diluted with dichloromethane (2 mL) and washed with aqueous saturated sodium bicarbonate (2 mL). The organic layer was dried over Na_2SO_4 and concentrated in vacuo to give pure **12** as an unseparable 1:1 mixture of diastereoisomers (34 mg, quantitative yield). White amorphous solid; ^{1}H NMR (400 MHz, $CDCl_3$, rotameric mixture of diastereoisomers) δ 7.52 (m, br, 0.65H), 7.41 (m, br, 0.35H), 6.68-6.59 (m, 1H), 5.15-4.92 (m, br, 1H), 4.87-4.58 (m, 0.7H), 4.58-4.43 (m, 0.3H), 4.20 (q, *J* = 7.0 Hz, 1.3H), 4.19 (q, *J* = 7.0 Hz, 0.7H), 3.66-3.51 (m, 2H), 2.96 (s, 2H), 2.88 (s, 1H), 2.34 (t, *J* = 6.8 Hz, 2H), 2.07-1.49 (m, 15H), 1.37-1.10 (m, 15H), 0.98-0.76 (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$, mixture of diastereoisomers) δ 171.7 and 171.2 (1C), 167.8 and 167.2 (2C), 138.5, 132.8, 70.3, 60.8, 56.9 and 56.3 (1C), 53.6, 47.5, 42.0, 35.6 (2C), 31.4 and 30.3 (1C), 29.9, 29.8, 29.7, 27.0 (2C), 25.5, 25.0, 24.2, 19.5 and 19.4 (4C), 14.2, 13.8 and 13.7 (1C); HRMS (ESI) calcd for $C_{28}H_{49}N_3NaO_4^+$ $[MNa]^+$ 514.3615, found 514.3608.

Biological studies

Antiproliferative assays. Human T-cell leukemia (Jurkat), human B-cell leukemia (RS4;11) and human promyelocitic leukemia (HL-60) cells were grown in RPMI-1640 medium (Gibco, Milano, Italy). Human cervix carcinoma (HeLa), human colon adenocarcinoma (HT-29), and human breast cancer (MCF-7) cells were grown in DMEM medium (Gibco, Milano, Italy). Both media were supplemented with 115 units/mL of penicillin G (Gibco, Milano, Italy), 115 μg/mL of streptomycin (Invitrogen, Milano, Italy) and 10% fetal bovine serum (Invitrogen, Milano, Italy). All cell lines were purchased from ATCC. Stock solutions (10 mM) of the different compounds were obtained by dissolving them in DMSO. Individual wells of a 96-well tissue culture microtiter plate were inoculated with 100 μL of complete medium containing $8x10^3$ cells. The plates were incubated at 37 °C in a humidified 5% CO_2 incubator for 18 h prior to the experiments. After medium removal, 100 μL of fresh medium containing the test compound at different concentrations was added to each well and incubated at 37 °C for 72 h. Cell viability was assayed by the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide test and absorbance was measured at 560 nm using a Victor3 TM 1420 Multilabel Counter (PerkinElmer, Waltham, MA, USA). The GI_{50} was defined as the compound concentration required to inhibit cell proliferation by 50%.

Effects on tubulin polymerization and on ligand binding to tubulin. The preparation of electrophoretically homogeneous bovine brain tubulin was as described previously.[26] To evaluate the effect of the compounds on tubulin assembly *in vitro*, varying concentrations of compounds were preincubated with 10 μM bovine brain tubulin in glutamate buffer at 30 °C and then cooled to 0 °C. After addition of 0.4 mM GTP, the mixtures were transferred to 0 °C cuvettes in a recording spectrophotometer and warmed to 30 °C. Tubulin assembly was followed turbidimetrically at 350 nm. The IC_{50} was defined as the compound concentration that inhibited the extent of assembly by 50% after a 20 min incubation. The assay was described previously in detail.[27] The ability of the test compounds to inhibit [^{3}H]vinblastine (from Perkin-Elmer, Boston MA), [^{3}H]dolastatin 10 (supplied by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, National Cancer Institute, Gaithersburg MD) and [^{3}H]halichondrin B (custom synthesized[28]) binding to tubulin was measured as describedpreviously centrifugal gel filtration chromatography.[28] Briefly, experiments were performed in 0.1 M 4-morpholinethanesulfonate (pH 6.9 in 1 M stock solution adjusted with NaOH)-0.5 mM $MgCl_2$ containing 10 μM tubulin (1.0 mg/ml), 10 μM radiolabeled ligand, and inhibitors at different concentrations. Reaction volume was 0.3 mL, incubation time 15 min at rt (around 20 °C). Ligands were mixed prior to tubulin addition. Duplicate aliquots of each reaction mixture were applied to syringe columns of Sephadex G-50 (superfine) swollen in 0.1 M Mes-0.5 mM $MgCl_2$ (pH=6.9).

Flow cytometric analysis of cell cycle distribution. $5x10^5$ HeLa cells in exponential growth were treated with different concentrations of the test compounds for 24 h. After the incubation period, the cells were collected, centrifuged and fixed with ice-cold ethanol (70%). The cells were then treated with lysis buffer containing RNAse A and 0.1% Triton X-100, and then stained with propidium iodide. Samples were analyzed on a Cytomic FC500 flow cytometer (Beckman Coulter). DNA histograms were analyzed using MultiCycle® for Windows (Phoenix Flow Systems).

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