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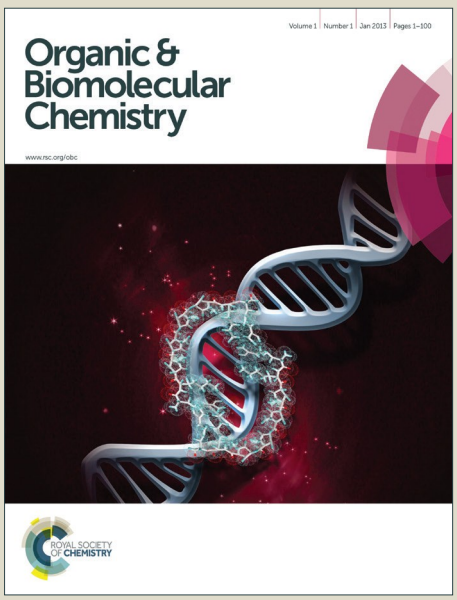

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ARTICLE

Synthesis and evaluation of hybrid molecules targeting the vinca domain of tubulin

Cite this: DOI: 10.1039/xoxxoooooox

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Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/xoxxoooooox

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Hybrids of vinca alkaloids and phomopsin A, linked by a glycine pattern, have been synthetized in one or two steps, by an insertion reaction. These compounds have been elaborated in order to interact with both "vinca site" and "peptide site" of the vinca domain in tubulin. Two out of three hybrids are potent inhibitors of microtubules assembly and they present good cytotoxicity against different cell lines. Molecular modelling studies show they could bind, within the vinca domain, in similar spatial regions as that of vinca and phomopsin thanks to the flexibility provided by the glycine linker used to elaborate these hybrids.

Introduction

Microtubules play a crucial role in eukaryotic cells. They are essential in the development and maintenance of cell shape, in the transport of different components, in cell signalling and in cell division, as they form the mitotic spindle.[1] Microtubules are dynamic polymers of heterodimers of α and β tubulin that both bind to GTP. During polymerisation, after the tubulin dimer is incorporated into the microtubule, the molecule of GTP bound to the β-tubulin subunit eventually hydrolyses into GDP. Various drugs can alter this dynamic behaviour either by stabilizing or destabilizing microtubules thereby causing cell death by apoptosis.[1,2] They are classified according to their binding site on tubulin (either that of taxanes, colchicine, laulimalide,[3,4,5,6] vinca alkaloids or the newly reported maytansine[7]).

Vinca alkaloids,[8,9] like natural vinblastine **1** and synthetic vinorelbine **2** or vinflunine **3** (Figure 1), are major anticancer agents. They prevent tubulin polymerization into microtubules by binding in the vinca site of the vinca domain that is located at the interface between two tubulin heterodimers close to the GTP/GDP nucleotide exchange site of the β subunit.[10,11]

Other peptide-type molecules are known to bind within the tubulin vinca domain. For example, phomopsin A **4**, a natural hexapeptide and unnatural *rac*-octahydrophomopsin A **5** (Figure 1) are very potent tubulin assembly inhibitors.[12] Their binding site called "peptide site" in the vinca domain partly overlaps with that of the vinca alkaloids.[13]

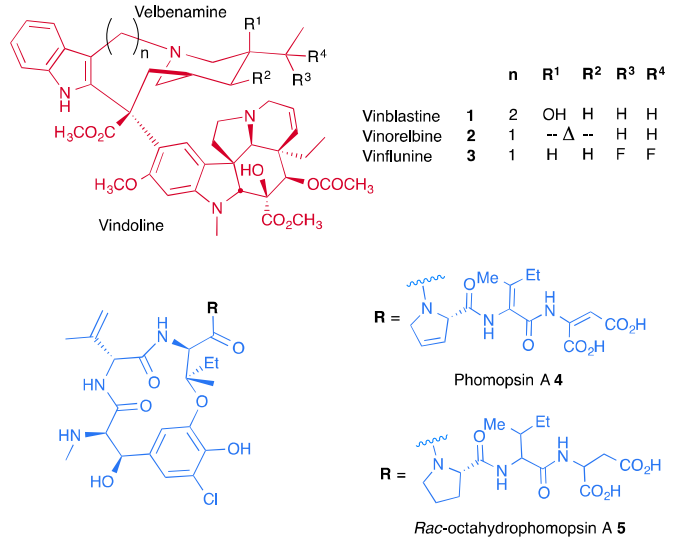


Fig. 1 Examples of vinca alkaloids, phomopsin A **4** and its unnatural analogue **5**

Indeed, the north velbenamine moiety of vinblastine **1** and the cyclic core of phomopsin A **4** occupy the same area, while the south vindoline moiety of vinblastine **1** and the lateral chain of phomopsin A **4** are oriented in opposite directions, phomopsin A **4** interacting with $Tyr_{\beta 1}224$, one of the amino acid that sandwiches the GDP/GTP nucleotide exchangeable site (Figure 2).

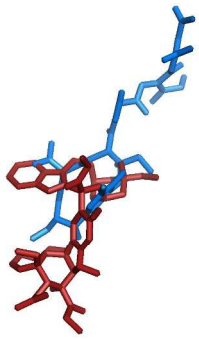


Fig. 2 Superimposition of vinblastine **1** (red) and phomposin **4** (blue) in their respective binding sites in tubulin

We have been involved for a few years, in the elaboration of hybrid structures of vinca and phomopsin that could occupy both binding sites in order to explore the vinca domain and eventually elaborate more potent and specific derivatives.[14,15,16] Recently, we reported the one-pot synthesis of vinca-phomopsin hybrids **6**)[17] in which the phomopsin lateral chain was grafted in C-8' on a 7'-*homo*-anhydrovinblastine core (Figure 3). Molecular modelling studies, supported by their very good activities on tubulin, were consistent with a very nice superimposition of the hybrid structures **6** with both vinblastine and phomopsin resulting in a good orientation in the vinca domain, near the GTP hydrolysis site.

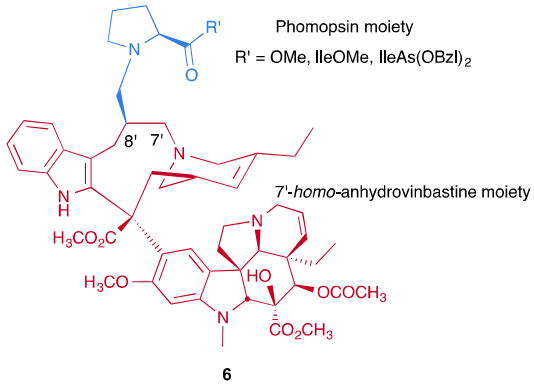
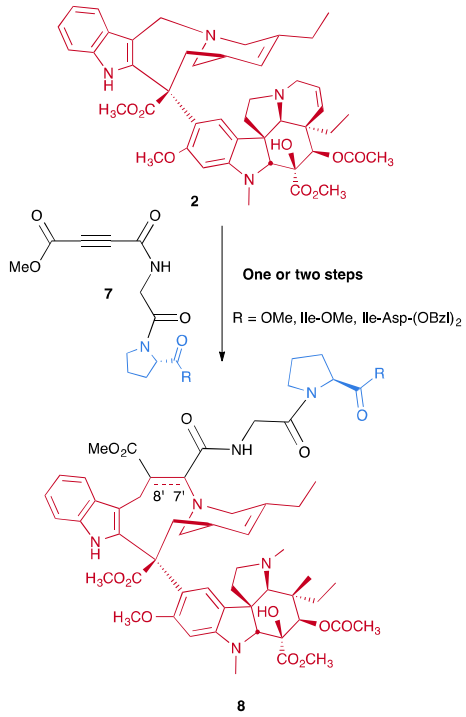


Fig. 3 Hybrids **6** of vinca and phomopsin clasped on C-8' of 7'-*homo*-anhydrovinblastine[13]

In parallel, we showed that functionalization of the C-7' position of 7'-*homo*-anhydrovinblastine derivatives was much more beneficial to the biological activity than that in C-8' and that steric hindrance at C-7' has no detrimental effect on biological activity.[18]

Thus, we wish to report the elaboration of a new family of hybrids[19] whose 7'-*homo*-anhydrovinblastine core carries the phomopsin lateral chain in C-7'. The synthetic pathway involves an insertion of activated alkynes into vinorelbine **2** gramine bridge that we recently disclosed.[18] This reaction takes advantage of the selective reactivity of the gramine bridge of vinorelbine **2** that, after a Michael addition on an acetylene and fragmentation, leads to a reactive alkylideneindoleninium ion that is trapped intramolecularly to give inserted compounds.[18]

In this work, various acetylenes **7** (Scheme 1) carrying the peptide lateral chain of phomopsin were prepared and stapled to vinorelbine **2**, leading in one or two steps to the desired new hybrids **8**.



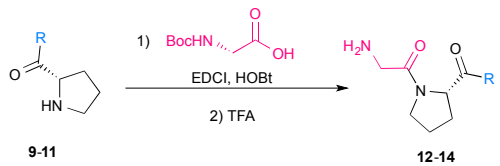
Scheme 1 Target hybrids **8** of vinca and phomopsin grafted on C-7' of 7'-*homo*-anhydrovinblastine

Results and discussion

Chemistry

We previously showed that insertion of unsymmetrical ester amide alkynes on the gramine bridge of vinorelbine **2**, was totally regioselective and directed by the ester moiety.[18] We used this remarkable result to design three asymmetrical activated alkynes **7** functionalized on the one hand by a methyl ester and on the other hand by an amide chain that includes the phomopsin lateral chain linked by a flexible glycine pattern. Indeed, the regioselectivity of the insertion should provide the desired hybrids with the phomopsin chain inserted on C-7'.

As *rac*-octahydrophomopsin A **5** is as potent as phomopsin A **4** on tubulin and the synthesis of the phomopsin unsaturated tripeptide side chain requires a multi-step procedure,[20] we used L-proline, L-isoleucine and L-aspartic acid instead of their unsaturated equivalents. Peptide chains of different length Gly-Pro-OMe **12**, Gly-Pro-Ile-OMe **13** and Gly-Pro-Ile-Asp-$(OBn)_2$ **14** were thus synthesized by a classical procedure (Scheme 2).

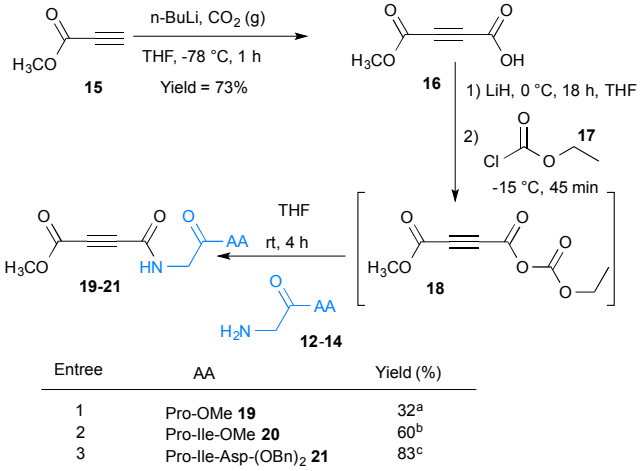


Entree	Cmpd	Product	R	Yield (%)
1	**9**	**12**	OMe	92
2	**10**	**13**	Ile-OMe	83
3	**11**	**14**	Ile-Asp-$(OBn)_2$	64

Scheme 2 Last step of peptides **12-14** synthesis

Once the peptide chains were synthetized, their coupling with 4-methoxy-4-oxobut-2-ynoic acid **16** was envisaged. Compound **16**[21] was obtained in one step from commercial methyl propiolate **15** by a modified procedure of Hall and coworkers[21a] replacing dry ice (which, in our hands resulted in poor yields of desired **16**) by gaseous CO_2.

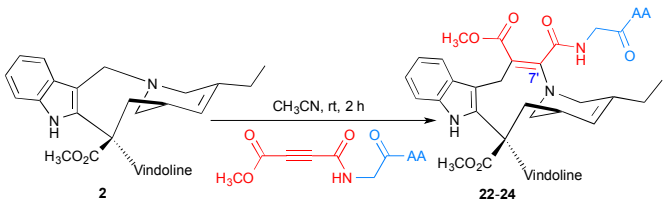
With classical peptide coupling conditions, only the products of Michael addition of the amines **12-14** on compound **16** could be isolated. The only effective approach was to draw on the work of Coppola *et al.* on *N*-substituted propynamides[22] and to prepare the transient anhydride **18** highly sensitive to nucleophilic attacks. Thus, 4-methoxy-4-oxobut-2-ynoic acid **16** was deprotonated and reacted with ethyl chloroformate **17** before the addition of amines **12-14** to give the desired acetylenes **19-21** (Scheme 3). It should be noted that the yield of the reaction was much higher when a large excess of anhydride was used (32% with 1 equivalent of **18** versus 81% with 10 equivalents).



Entree	AA	Yield (%)
1	Pro-OMe **19**	32[a]
2	Pro-Ile-OMe **20**	60[b]
3	Pro-Ile-Asp-$(OBn)_2$ **21**	83[c]

Scheme 3 Acetylene synthesis. [a] 1 eq of anhydride was used [b] 3 eq of anhydride were used [c] 10 eq of anhydride were used

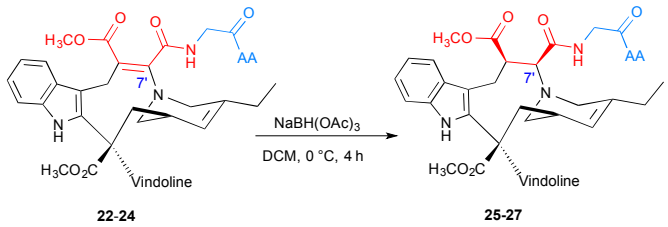
Asymmetric acetylenes **19-21** were reacted with vinorelbine **2** in acetonitrile, at room temperature for two hours (Scheme 4). As expected, the regioselectivity of the insertion was total and the expected β-enamino esters **22-24** could be isolated. The moderate yields of the reaction probably result in part from the complexity of the molecules and because loss of material was observed during the purification by flash chromatography.



Cmpd	AA	Yield (%)
22	Pro-OMe	31
23	Pro-Ile-OMe	21
24	Pro-Ile-Asp-$(OBn)_2$	26

Scheme 4 Synthesis of hybrids **22-24**

The hybrids **22-24** were then reduced to the β-amino esters **25-27** in the presence of $NaBH(OAc)_3$. The ^1H NMR spectra of the crude mixtures show the presence of only one compound. By analogy with our former results, the absolute configuration of the two new stereocenters was assumed to be 7'-*S*, 8'-*R*, as similar key chemical shifts and nOe correlations were observed for compounds **25-27** and β-amino esters previously synthesised.[18]



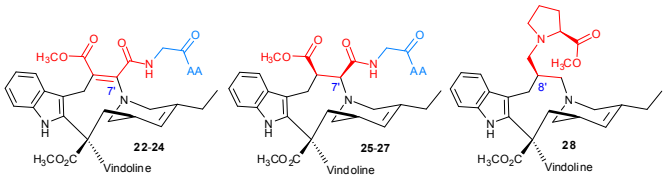
Cmpd	AA	Yield (%)
25	Pro-OMe	60
26	Pro-Ile-OMe	51
27	Pro-Ile-Asp-$(OBn)_2$	93

Scheme 5 Synthesis of hybrids **25-27**

Biological Evaluation

Cytotoxicity and biological activities on tubulin were evaluated for compounds **22-27** (Table 1). Compounds **22, 23, 25** and **27** (entries 3, 4, 6, 7) displayed significant inhibitory activity on microtubule assembly. Compound **25** was as active as vinblastine **1** and two times less potent than vinorelbine **2**. Nevertheless, the size of the added groups should not be too large in view of the total loss of activity on tubulin observed for compounds **24** and **27**, and already noted for hybrids **6** (when phomopsin is grafted on C-8').[17] Generally speaking, both cytotoxic and tubulin activities of reduced hybrids **25-27** are quite similar to those observed for hybrids in C-8' (compare, for instance, entry 6 and entry 9 in Table 1)Surprisingly anyway, the largest reduced hybrid **27** with no activity on tubulin, showed an interesting cytotoxicity (150, 100 and 140 nM on U-87, HCT-116 and K562 respectively) together with the shortest one **25** (150 and 250 nM), even if they were less cytotoxic than vinorelbine **2** and vinblastine **1**.

Table 1 Biological activity of hybrids **22-27**



Entry	Cpd	$IC_{50\text{-tubulin}}$ (μM)[a]	$IC_{50\text{-U87}}$ (nM)[b]	$IC_{50\text{-HCT116}}$ (nM)[b]	$IC_{50\text{-K562}}$ (nM)[b]
1	**VLB**	2.1	1.0	2	10
2	**VNL**	0.7	3.5	15	6
3	**22**	3.0	2500	2000	1500
4	**23**	4.1	850	500	600
5	**24**	>100.0	300	150	350
6	**25**	1.7	150	250	250
7	**26**	5.2	250	700	400
8	**27**	>100.0	150	100	140
9	**28**	1.0	250	700	120

VLB: vinblastine **1**; VLN: vinorelbine **2**; [a] IC50 is the concentration of a compound that inhibits 50% of the rate of microtubule assembly (concentration in tubulin = 3 mg/mL) [b] IC50 measures the drug concentration required for the inhibition of 50% cell proliferation after 72 h of incubation.

To check whether the mechanism of growth arrest is the same for the most cytotoxic compounds **25** and **27**, we assayed the changes in the cell cycle profile compared to vinorelbine **2** (10 nM) by treating asynchronously growing K562 cells with 500 nM of compound **25** or **27** (Figure 4). Thus, like vinorelbine **2** (59.1% of cells arrested), hybrids **25** (63.0% of cells arrested) and **27** (55.3% of cells arrested) inhibit the cell cycle progression at the G2-M phase, when compared to the 19% of cells arrested in G2-M in the control. This confirmation of a similar mechanism of action means that the large peptide lateral chain of hybrid **27** is probably partially hydrolysed by esterases in the cells giving a smaller hybrid that can interact with tubulin.

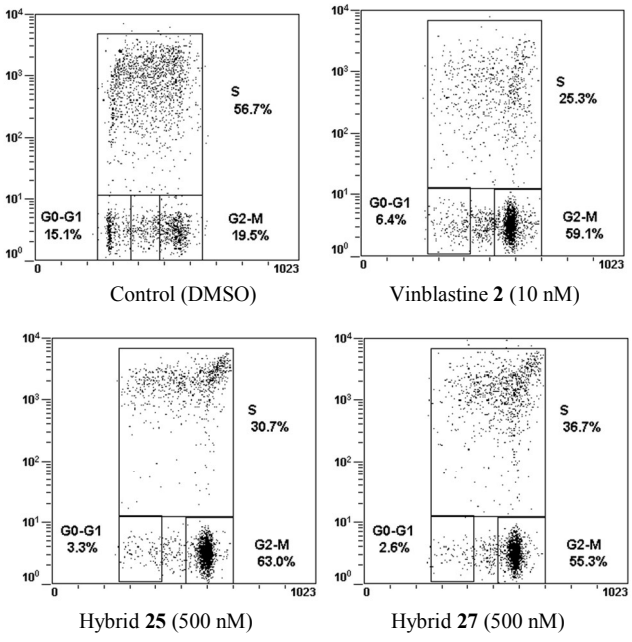


Fig. 4 Effect of compounds **25** and **27** and vinorelbine **2** on cell-cycle distribution in chronic myelogenous leukemia K562, measured by bromodeoxyuridine (BrdU) uptake and propidium iodide (PI) staining.

Molecular modelling

The structural knowledge provided by the crystal structures of $\alpha_1\beta_1$:$\alpha_2\beta_2$-tubulin dimers, stabilized by colchicine and an RB3 stathmin-like domain, in complex with either **1**[10] or **4**[13] solved at ~4.0 Å resolution has allowed us to propose molecular models for the binding of some 7'-*homo*-anhydrovinblastine derivatives to tubulin.[17,18] This modelling work has now been extended to compounds **22** and **25** possessing longer substituents appended to C-7'. The models presented here show the feasibility of their binding at the interface between two tubulin dimers assembled head-to-tail and rationalize available structure-activity relationships. Thus, the finding that esters longer than methyl carboxylate at C-8' bring about a dramatic decrease in potency in this series[18] is accounted for because the alkoxy moiety is buried in a relatively small lipophilic pocket lined by the hydrophobic side-chains of $Leu_{\alpha2}248$ and $Val_{\alpha2}250$ (Figure 5). On the other hand, the carbonyl oxygen of the amide attached to C-7' can establish a good hydrogen bond to the amide nitrogen of $Tyr_{\beta1}224$, located on the N-terminus of helix H7[23] whereas the remaining peptide chain is projected into the space region that is occupied by the acyclic half of **4** in its complex with tubulin.[13] This appears to be the reason why this position accepts the bulky substituents present in **22-23** and **25-26**, which can sample a variety of locations at the β_1:α_2-tubulin inter-dimer interface thanks to the flexibility provided by the glycine linker (Fig. 1 in Supp. Info). Nonetheless, the much bulkier substituents present in **24** and **27** are detrimental for tubulin binding. This finding is in consonance with results from Boger and coworkers[24] showing steric tolerance to bulky alkyl and aryl groups bonded to C-20' in a series of urea derivatives of **1**. In fact, our modelling results suggest that both types of substituents are likely to occupy similar spatial regions (Fig. 4 in Supp. Info).

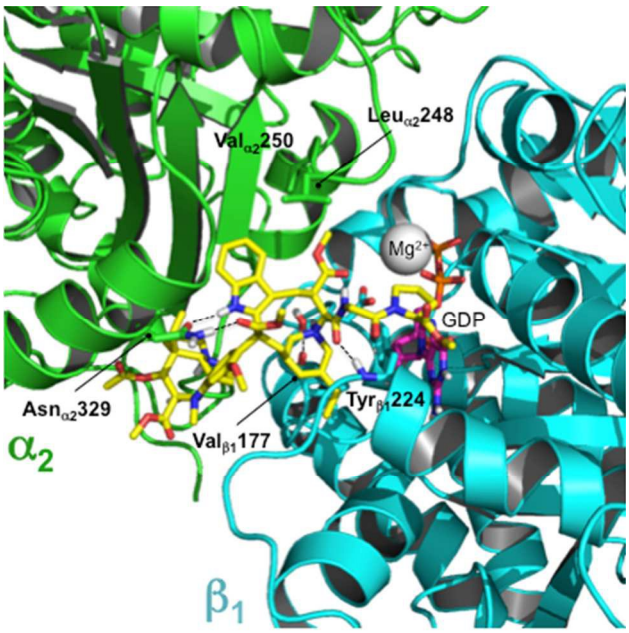


Fig. 5 Close-up view of the vinca domain at the interface between β_1-tubulin (cyan) and β_2-tubulin (green) subunits showing bound **25** (C atoms in yellow) as seen in a representative structure from the molecular dynamics simulation of the complex. Selected residues involved in the proposed stabilizing interactions are labelled. Broken lines represent hydrogen bonds.

The tubulin-**25** complex is stabilized at the inter-dimer interface by a number of interactions involving the vindoline core: in common with **1**, (a) the charged amino group in the velbenamine moiety (N-6') is hydrogen-bonded to a water molecule that is fixed in place by the backbone carbonyl of Val$_{\beta1}$177 and the carboxylate of Asp$_{\beta1}$179 (in loop T5), whose side chain was shown to change orientation relative to the apo form;[10] (b) the indole NH-17' (on ring B') and the ester carbonyl C-23' (on ring C') are engaged in a bidentate and highly directional hydrogen-bonding interaction with the side-chain carboxamide of Asn$_{\alpha2}$329; and (c) the ethyl group on ring D' stacks on the phenyl ring of Tyr$_{\beta1}$224, which sits on the guanine base of GDP present in the nucleotide-binding site.
Finally, although the unsaturation between positions 7′ and 8′ in **22** affects the puckering of ring D' relative to that found in **25** (Supporting Information), both analogues share essentially the same anchoring points at the binding site and project the peptide chain into the interfacial region in a similar way.

Conclusions

Six hybrids **22-27** of 7'-*homo*-anhydrovinblastine and octahydrophomopsin **5** were synthesized starting from vinorelbine **2**. In these hybrids, lateral peptide chains of various length, mimicking that of phomopsin A, were grafted on C7' of the vinca moiety using a glycine linker. Biological evaluation on tubulin revealed that their ability to inhibit its polymerization is tightly related a) to the length of the added peptide chain (the smaller the better) and b) to the nature of the upper motive in the vinca part (β-amino esters **25-26** are more potent than β-enamino esters **22-24**). These findings could be rationalized by molecular modelling studies which suggest that the peptide chains are located in the space region that is occupied by the acyclic half of **4** and that the flexible glycine linker enables a variety of locations of these chains at the β_1:α_2-tubulin inter-dimer interface.
Interestingly, the largest reduced hybrids **27**, inactive on tubulin, was found as cytotoxic as the most potent one **25**, probably due to a hydrolysis of the large peptide chain within the cells.

Experimental section

General Synthetic Procedure A
N-methylmorpholine (NMM; 1 eq.) was added to a solution of an amino acid with a free amine $NH_2CHRCOOR_1$ (1 eq.) in dichloromethane. After stirring for 5 min, an amino acid with a free carboxylic acid BocNHCHRCOOH (1 eq.), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI; 1.2 eq.) and hydroxybenzotriazole (HOBt; 1.2 eq.) were added. After 2 h of stirring at room temperature, the reaction mixture was quenched with a saturated solution of sodium hydrogen carbonate. The aqueous layer was extracted with dichloromethane. The combined organic layers were washed with brine. The extracts were then dried over magnesium sulfate and evaporated under reduced pressure. The residue was purified by flash chromatography with dichloromethane/methanol (99.5:0.5 to 95:5) to afford the desired peptide as colourless oil, which was dissolved in a 1/3 mixture of dichloromethane/TFA. After 30 min of stirring, toluene was added and the reaction mixture was evaporated to afford quantitatively the trifluoroacetate salt of the deprotected peptide that was used without further purification.
General Synthetic Procedure B
Activated alkyne (1.1 eq.) was added to a solution of vinorelbine (1 eq.) in acetonitrile. The reaction mixture was stirred for 2 h at room temperature. The solvent was evaporated under reduced pressure. The resulting residue was purified by column chromatography on alumina with ethyl acetate/acetone (1:0 to 4:1). The second fraction provided the corresponding vinca derivatives.
General Synthetic Procedure C
$NaBH(OAc)_3$ (3 eq.) was added to a solution of the corresponding enamino-ester (1 eq.) in dichloromethane (0.2 mL) at 0 °C. The reaction mixture was stirred for 4 hours at 0 °C. The resulting mixture was diluted with dichloromethane and washed with a saturated sodium carbonate solution. Solvent removal under reduced pressure provided pure amino esters.
4-methoxy-4-oxobut-2-ynoic acid **16**: Methyl propiolate was dissolved in 25 mL dry THF and cooled to -78°C. *n*-BuLi solution (2.5 M in hexanes, 4.0 mL, 10 mmol) was added dropwise via syringe and stirring was continued at -78°C for 1 hour followed by the addition of a CO_2 filled balloon. The reaction was allowed to warm to room temperature, and subsequently stirred for 1 hour. The solvent was removed *in vacuo*. Water (25 mL) and hexane (25 mL) were added. The layers were separated, and the aqueous layer was acidified by HCl (2 M, aq.) to pH~2. The aqueous layer was then back-

extracted with ethyl acetate. The organic layers were combined and washed with brine. The upper layer was dried over magnesium sulfate and filtered. Solvent evaporation under reduced pressure gave **16** (935 mg, 73% yield) as a brown oil.^{1}H NMR (300 MHz, $CDCl_3$) δ 9.21 (br s, 1H, H-6), 3.81 (s, 3H, H-1).^{13}C NMR (75 MHz, $CDCl_3$) δ 178.0 (C-5), 152.5 (C-2), 75.4 (C-3), 71.9 (C-4), 53.5 (C-1).

Dipeptide **12**: The general procedure A was followed using NMM (627 µL, 5.7 mmol, 1 eq.), L-Pro-OMe (944 mg, 5.7 mmol, 1 eq.), Boc-Gly-OH (1.0 g, 5.7 mmol, 1 eq.), EDCI (1.31 g, 6.84 mmol, 1.2 eq.) and HOBt (930 mg, 6.84 mmol, 1.2 eq.). After 2 h of stirring at room temperature in dichloromethane (54 mL), the reaction mixture was quenched with a saturated solution of sodium hydrogen carbonate. The residue was purified by flash chromatography on silica gel using dichloromethane/methanol (99.5:0.5 to 95:5) to afford the desired dipeptide as colourless oil.

According to the general procedure, this was later deprotected with a mixture of TFA/dichloromethane (14 mL/7 mL) to afford the trifluoroacetate salt of the deprotected peptide **12** (976 mg, 92% yield). $[\alpha]_D^{25}$ = -41 (*c* 0.1, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.9 (br s, 3H, H-1), 4.49 (dd, J=8.7 and 3.3 Hz, 1H, H-8), 3.92 (s, 2H, H-2), 3.68 (s, 3H, H-10), 3.59-3.51 (m, 1H, H-5α), 3.50-3.40 (m, 1H, H-5β), 2.30-2.20 (m, 1H, H-7α), 2.09-1.98 (m, 3H, H-6 and H-7β). ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.1 (C-9), 165.1 (C-3), 161.2 (C-11), 116.4 (C-12), 59.6 (C-8), 52.8 (C-10), 46.5 (C-5), 41.1 (C-2), 29.0 (C-7), 24.6 (C-6). HRMS-ESI calcd for $C_8H_{15}N_2O_3$ 187.1077, found 187.1172.

Tripeptide **13**: The general procedure A was followed using NMM (181 µL, 1.65 mmol, 1 eq.), dipeptide Pro-Ile-OMe (400 mg, 1.65 mmol, 1 eq.), Boc-Gly-OH (289 mg, 1.65 mmol, 1 eq.), EDCI (380 mg, 1.98 mmol, 1.2 eq.) and HOBt (267 mg, 1.98 mmol, 1.2 eq.). After 2 h of stirring at room temperature in dichloromethane (18 mL), the reaction mixture was quenched with a saturated solution of sodium hydrogen carbonate. The residue was purified by flash chromatography on silica gel using dichloromethane/methanol (99.5:0.5 to 95:5) to afford the desired tripeptide as colourless oil.

According to the general procedure, this was later deprotected with a mixture of TFA/dichloromethane (4.7 mL/1.6 mL) to afford the trifluoroacetate salt of the deprotected peptide **13** (410 mg, 83% yield). $[\alpha]_D^{25}$ = - 30 (*c* 0.1, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.05 (d, J = 9.0 Hz, 1H, H-9), 4.40-4.35 (m, 1H, H-4), 3.92-3.85 (m, 1H, H-10), 3.68 (s, 3H, H-16), 3.53 (s, 2H, H-2), 3.41-3.31 (m, 1H, H-7α), 3.25-3.14 (m, 1H, H-7β), 2.35-2.29 (m, 1H, H-5α), 2.08-1.85 (m, 2H, H-5β and H-11), 1.70-1.59 (m, 2H, H-6), 1.39-1.31 (m, 1H, H-12α), 1.12-1.05 (m, 1H, H-12β), 0.97-0.95 (m, 6H, H-13 and H-14). ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.1 (C-15), 172.3 (C-8), 166.3 (C-3), 161.2 (C-17), 116.4 (C-18), 60.3 (C-4), 56.1 (C-10), 51.8 (C-16), 47.1 (C-7), 43.5 (C-2), 37.6 (C-11), 30.8 (C-5), 25.8 (C-6), 25.0 (C-12), 15.5 (C-14), 11.4 (C-13). HRMS-ESI calcd for $C_{14}H_{26}N_3O_4$ 300.1918, found 300.2013.

Tetrapeptide **14**: The general procedure A was followed using NMM (13 µL, 0.113 mmol, 1 eq.), tripeptide Pro-Ile-Asp-$(OBn)_2$ (60 mg, 0.11 mmol, 1 eq.), Boc-Gly-OH (20 mg, 0.11 mmol, 1 eq.), EDCI (26 mg, 0.14 mmol, 1.2 eq.) and HOBt (18 mg, 0.14 mmol, 1.2 eq.). After 2 h of stirring at room temperature in dichloromethane (70 mL), the reaction mixture was quenched with a saturated solution of sodium hydrogen carbonate. The residue was purified by flash chromatography on silica gel using dichloromethane/methanol (99.5:0.5 to 95:5) to afford the desired tetrapeptide as colourless oil.

According to the general procedure, this was later deprotected with 37 mL of 1/3 mixture of TFA/dichloromethane to afford the trifluoroacetate salt of the deprotected peptide **14** (42 mg, 64% yield). $[\alpha]_D^{25}$ = - 27 (*c* 0.1, $CHCl_3$). ^{1}H (300 MHz, $CDCl_3$) δ 7.92 (br s, 3H, H-1), 7.87-7.77 (m, 2H, H-9 and H-16), 7.32-7.26 (m, 10H, H-22-H-26 and H-30-H-34), 5.06 (d, J=3.4 Hz, 2H, H-29), 5.00 (d, J=4.2 Hz, 2H, H-20), 4.86 (dt, J=7.4 and 5.1 Hz, 1H, H-17), 4.48 (dd, J=6.9 and 4.2 Hz, 1H, H-4), 4.16-4.07 (m, 1H, H-10), 3.31 (t, J=5.9 Hz, 1H, H-7α), 3.12 (t, J=5.9 Hz, 1H, H-7β), 3.00 (dd, J=17.0 and 5.5 Hz, 1H, H-18α), 2.85 (dd, J=17.0 and 5.5 Hz, 1H, H-18β), 2.83 (s, 2H, H-2), 2.15-2.05 (m, 1H, H-5α), 2.01-1.91 (m, 3H, H-5β and H-6), 1.88-1.75 (m, 1H, H-11), 1.44-1.35 (m, 1H, H-12α), 1.15-1.01 (m, 1H, H-12β), 0.78 (d, J = 7.1 Hz, 3H, H-14), 0.77 (t, J=7.3 Hz, 3H, H-13). ^{13}C NMR (75 MHz, $CDCl_3$): δ 172.1 (C-8), 170.9, 170.2 (C-19 and C-27), 166.4 (C-15), 161.0 (C-36), 160.8 (C-3), 135.2 (C-21 and C-29), 128.9 (C-23, C-25, C-31 and C-33), 128.5 (C-24 and C-32), 127.5 (C-22, C-26, C-30 and C-34), 117.6 (C-35), 68.0, 67.3 (C-20 and C-29), 61.3 (C-4), 59.2 (C-10), 49.1 (C-17), 46.9 (C-7), 43.4 (C-2), 36.2 (C-18), 35.9 (C-11), 29.7 (C-5), 25.3 (C-6), 24.7 (C-12), 14.8 (C-14), 10.6 (C-13). HRMS-ESI calcd for $C_{31}H_{41}N_4O_7$ 581.2959, found 581.2946.

Alkyne **19**: A solution of 4-methoxy-4-oxobut-2-ynoic acid (100 mg, 0.78 mmol, 1.03 eq.) in 1.0 mL of THF was cooled to 0 °C, under argon. Lithium hydride (6.5 mg, 0.82 mmol, 1.08 eq.) was added by portions. The reaction mixture was stirred for 18 h at room temperature. After 18 h, ethyl chloroformate (72 µL, 0.76 mmol, 1 eq.) was added at -15 °C and the reaction mixture was stirred for 45 min at -15 °C. Amine **12** (141 mg, 0.76 mmol, 1 eq.) was added at 0 °C and stirring continued for 4 h allowing the mixture to warm to room temperature. The solvent was removed under reduced pressure and the residue was dissolved in ethyl acetate. A saturated aqueous solution of sodium carbonate was added, and the two phases were separated. The organic phase was dried over magnesium sulfate, filtered, and concentrated under reduced pressure to give a brown oil that was purified by column chromatography on silica gel using ethyl acetate to afford compound **19** (72 mg, 32% yield) as colourless oil. $[\alpha]_D^{25}$ = - 80 (*c* 0.1, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.55-4.47 (m, 1H, H-12), 4.13-4.07 (m, 2H, H-7), 3.80 (s, 3H, H-14), 3.71 (s, 3H, H-1), 3.62-3.53 (m, 1H, H-10α), 3.50-3.41 (m, 1H, H-10β), 2.29-2.12 (m, 1H, H-9α), 2.10-1.96 (m, 3H, H-9β and H-11). ^{13}C NMR (75 MHz, $CDCl_3$) δ 175.4 (C-2), 172.2 (C-5), 167.5 (C-13), 165.6 (C-8), 92.1 (C-4), 77.4 (C-3), 59.3 (C-12), 53.5 (C-14), 52.6 (C-1), 46.1 (C-9), 42.5 (C-7), 29.3 (C-11), 24.8 (C-10). HRMS-ESI calcd for $C_{13}H_{17}N_2O_6$ 297.1081, found 297.1048.

Alkyne **20**: A solution of 4-methoxy-4-oxobut-2-ynoic acid (291 mg, 2.27 mmol, 3.09 eq.) in 1.0 mL of THF was cooled to

0 °C, under argon. Lithium hydride (19 mg, 2.37 mmol, 3.24 eq.) was added by portions. The reaction mixture was stirred for 18 h at room temperature. After 18 h, ethyl chloroformate (239 µL, 2.20 mmol, 3 eq.) was added at -15 °C and the reaction mixture was stirred for 45 min at -15 °C. Amine **13** (220 mg, 0.73 mmol, 1 eq.) was added at 0 °C and stirring continued for 4 h allowing the mixture to warm to room temperature. The solvent was removed under reduced pressure and the residue was dissolved in ethyl acetate. A saturated aqueous solution of sodium carbonate was added, and the two phases were separated. The organic phase was dried over magnesium sulfate, filtered, and concentrated under reduced pressure to give a brown oil that was purified by column chromatography on silica gel using ethyl acetate to afford compound **20** (180.2 mg, 60 % yield) as colourless oil. $[\alpha]_D^{25}$ = - 47 (*c* 0.1, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.15 (d, J=8.0 Hz, 1H, H-14), 4.53-4.47 (m, 1H, H-15), 4.17-4.09 (m, 3H, H-7α and H-12), 3.85 (s, 3H, H-1), 3.73 (s, 3H, H-21), 3.58 (td, J=9.3 and 3.7 Hz, 1H, H-9α), 3.44 (q, J=8.7 Hz, 1H, H-9β), 2.23-2.13 (m, 1H, H-11α), 2.36-2.27 (m, 1H, H-10α), 2.08-1.98 (m, 1H, H-10β), 2.04-1.93 (m, 1H, H-11β), 1.94-1.85 (m, 1H, H-16), 1.51-1.34 (m, 1H, H-17α), 1.25 (t, J=, 1H, H-6), 1.21-1.09 (m, 1H, H-17β), 1.12-1.05 (m, 1H, H-7β), 0.95-0.86 (m, 6H, H-18 and H-19). ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.2 (C-20), 170.7 (C-8), 170.5 (C-5), 166.5 (C-13), 150.5 (C-2), 76.0 (C-4), 74.2 (C-3), 61.3 (C-12), 60.3 (C-15), 53.4 (C-1), 52.2 (C-21), 46.5 (C-9), 42.3 (C-7), 37.7 (C-16), 27.6 (C-11), 25.1 (C-17), 24.8 (C-10), 15.5 (C-19), 11.6 (C-18). HRMS-ESI calcd for $C_{19}H_{28}N_3O_7$ 410.1922, found 410.2005.

Alkyne **21**: A solution of 4-methoxy-4-oxobut-2-ynoic acid (100 mg, 0.78 mmol, 10.63 eq.) in 1.0 mL of THF was cooled to 0 °C, under argon. Lithium hydride (6.5 mg, 0.82 mmol, 11 eq.) was added by portions. The reaction mixture was stirred for 18 h at room temperature. After 18 h, ethyl chloroformate (72 µL, 0.76 mmol, 10.3 eq.) was added at -15 °C and the reaction mixture was stirred for 45 min at -15 °C. Amine **14** (43 mg, 0.07 mmol, 1 eq.) was added at 0 °C and stirring continued for 4 h allowing the mixture to warm to room temperature. The solvent was removed under reduced pressure and the residue was dissolved in ethyl acetate. A saturated aqueous solution of sodium carbonate was added, and the two phases were separated. The organic phase was dried over magnesium sulfate, filtered, and concentrated under reduced pressure to give a brown oil that was purified by column chromatography on silica gel using ethyl acetate to afford compound **21** (42.5 mg, 83 % yield) as colourless oil. $[\alpha]_D^{25}$ = - 30 (c 0.1, $CHCl_3$).^{1}H NMR (300 MHz, $CDCl_3$) δ 7.31-7.20 (m, 10H, H-27-H-31 and H-35-H-39), 6.87 (d, J=8.2 Hz, 1H, H-14), 6.82 (d, J=8.2 Hz, 1H, H-21), 5.06 (s, 2H, H-33), 5.00 (d, J=4.0 Hz, 2H, H-25), 4.85 (dt, J=8.2 and 4.4 Hz, 1H, H-22), 4.43 (dd, J=7.9 and 2.4 Hz, 1H, H-12), 4.21 (dd, J=8.2 and 5.8 Hz, 1H, H-15), 4.04 (dd, J=13.8 and 4.0 Hz, 2H, H-7), 3.74 (s, 3H, H-1), 3.54-3.45 (m, 1H, H-9α), 3.40-3.30 (m, 1H, H-9β), 3.04 (dd, J=17.2 and 4.5 Hz, 1H, H-23α), 2.80 (dd, J=17.2 and 4.5 Hz, 1H, H-23β), 2.20-2.10 (m, 1H, H-11α), 2.10-2.00 (m, 1H, H-10α), 1.97-1.89 (m, 2H, H-10β and H-11β), 1.86-1.75 (m, 1H, H-16), 1.41-1.30 (m, 1H, H-17α), 1.19 (t, J=7.3 Hz, H-6), 1.10-0.96 (m, 1H, H-17β), 0.82-0.74 (m, 6H, H-18 and H-19). ^{13}C NMR (75 MHz, CDCl3) δ (ppm) 171.0 (C-32 and C-24), 170.9 (C-20), 170.8 (C-13), 170.3 (C-2), 166.7 (C-5), 135.1 (C-26 and C-34), 128.8 (C-28, C-30, C-36, C-38), 128.7 (C-29 and C-37), 128.6 (C-27, C-31, C-35 and C-39), 77.5 (C-4), 74.5 (C-3), 67.9, 67.2 (C-25 and C-33), 60.7 (C-12), 58.1 (C-14), 53.5 (C-15), 48.8 (C-22), 46.7 (C-9), 42.5 (C-7), 37.6 (C-16), 36.4 (C-23), 28.5 (C-11), 25.0 (C-10 and C-17), 15.6 (C-19), 11.7 (C-18). HRMS-ESI calcd for $C_{36}H_{43}N_4O_{10}$ 691.2974, found 691.2968.

Hybrid **22**: The general procedure B was followed using alkyne **19** (35 mg, 0.12 mmol, 2 eq.) and vinorelbine (47 mg, 0.06 mmol, 1 eq) in 0.5 mL of anhydrous acetonitrile. After 2 h of stirring at room temperature, the solvent was evaporated under reduced pressure. The resulting residue was purified by column chromatography on silica gel using ethyl acetate/acetone (1:0 to 4:1) to afford compound **22** (20 mg, 31% yield) as yellow powder. $[\alpha]_D^{25}$ = -30 (*c* 0.1, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$) 7.93 (s, 1H, H-17'), 7.53 (d, J=7.5 Hz, 1H, H-11'), 7.05 (t, J =7.5 Hz, 1H, H-14'), 7.00 (d, J=7.5 Hz, 1H, H-15'), 6.97 (t, J=7.5 Hz, 1H, H-13'), 6.66 (s, 1H, H-14), 6.58 (br s, 1H, H-28'), 6.11 (s, 1H, H-17), 5.83 (dd, J=10.4 and 4.3 Hz, 1H, H-7), 5.48 (s, 1H, H-4), 5.27 (d, J=10.0 Hz, 1H, H-6), 5.10 (br s, 1H, H-3'), 4.51 (d, J=14.6 Hz, 1H, H-9'β), 4.49-4.48 (m, 1H, H-35'), 4.16 (d, J=14.6 Hz, 1H, H-9'α), 4.10-4.08 (m, 2H, H-29'), 3.99 (d, J=13.0 Hz, H-20'β), 3.78 (s, 6H, H-22 and H-37'), 3.77 (s, 3H, H-25), 3.76 (s, 1H, H-2), 3.73-3.72 (m, 1H, H-35') 3.71 (s, 3H, H-26'), 3.60-3.59 (m, 1H, H-1'β), 3.57 (s, 3H, H-24'), 3.56-3.54 (m, 1H, H-32'α) 3.48-3.45 (m, 2H, H-5'β and H-32'β), 3.38 (dd, J=16.0 and 4.0 Hz, 1H, H-8β), 3.29 (td, J=10.0 and 4.1 Hz, 1H, 10α), 3.04 (d, J=16.0 Hz, 1H, H-5'α), 2.86 (dd, J=14.0 and 3.5 Hz, 1H, H-20'α), 2.79 (d, J=16.0 Hz, 1H, H-8α), 2.70 (s, 3H, H-23), 2.62 (s, 1H, H-19), 2.50 (q, J=10.0 Hz, 1H, H-10β), 2.20-2.18 (m, 2H, H-1'α and H-33'α), 2.08 (s, 3H, H-27), 2.03-2.00 (m, 3H, H-33'β and H-34'), 1.88 (q, J=7.5 Hz, 2H, H-21'), 1.81-1.75 (m, 1H, H-11α), 1.51-1.49 (m, 1H, H-2'), 1.36-1.29 (m, 1H, H-1β), 1.25-1.22 (m, 2H, H-20α and 20β), 0.94 (t, J=7.0 Hz, 3H, H-22'), 0.74 (t, J=7.5 Hz, 3H, H-21). ^{13}C NMR (150 MHz, CD_3CN) δ 175.6 (C-23'), 174.0 (C-36'), 173.4 (C-24), 172.6 (C-25'), 171.9 (C-26), 168.1 (C-30'), 167.0 (C-27'), 159.8 (C-16), 156.9 (C-7'), 154.8 (C-18), 139.8 (C-4'), 136.9 (C-16'), 132.8 (C-18'), 131.7 (C-6), 130.5 (C-11'), 126.1 (C-14), 125.9 (C-7), 125.1 (C-15), 124.3 (C-3'), 123.1 (C-14'), 122.4 (C-13), 121.1 (C-12'), 120.4 (C-13'), 116.1 (C-8'), 115.9 (C-10'), 112.5 (C-15'), 95.5 (C-17), 84.8 (C-2), 81.1 (C-3), 77.9 (C-4), 66.8 (C-19), 60.2 (C-35'), 57.2 (C-19'), 57.0 (C-22), 55.6 (C-20'), 54.7 (C-12), 53.2 (C-24'), 53.1 (C-37'), 53.0 (C-25), 52.9 (C-26'), 51.7 (C-8), 51.4 (C-10), 51.2 (C-5'), 47.4 (C-32'), 46.1 (C-11), 44.3 (C-5), 42.8 (C-29'), 39.3 (C-23), 36.0 (C-2'), 35.2 (C-1'), 32.3 (C-20), 30.2 (C-34'), 28.7 (C-21'), 26.8 (C-9'), 25.9 (C-33'), 21.7 (C-27), 13.1 (C-22'), 9.0 (C-21). HRMS-ESI calcd for $C_{58}H_{71}N_6O_{14}$ 1075.5023, found 1075.5083.

Hybrid **23**: The general procedure B was followed using alkyne **20** (17 mg, 0.04 mmol, 2 eq.) and vinorelbine (17 mg, 0.02

mmol, 1 eq) in 0.2 mL of anhydrous acetonitrile. After 2 h of stirring at room temperature, the solvent was evaporated under reduced pressure. The resulting residue was purified by column chromatography on silica gel using ethyl acetate/acetone (1:0 to 4:1) to afford compound **23** (5.3 mg, 21% yield) as yellow powder. $[\alpha]_D^{25}$ = -33 (*c* 0.1, $CHCl_3$) (500 MHz, $CDCl_3$) δ (ppm) 7.93 (s, 1H, H-17'), 7.53 (d, J=8.0 Hz, 1H, H-11'), 7.05 (t, J =7.5 Hz, 1H, H-14'), 7.00 (d, J=7.5 Hz, 1H, H-15'), 6.97 (t, J=7.5 Hz, 1H, H-13'), 6.68 (s, 1H, H-14), 6.59 (t, J=4.0 Hz, 1H, H-28'), 6.11 (s, 1H, H-17), 5.83 (dd, J=10.4 and 4.3 Hz, 1H, H-7), 5.47 (s, 1H, H-4), 5.27 (d, J=10.0 Hz, 1H, H-6), 5.18 (br s, 1H, H-3'), 4.58-4.46 (m, 3H, H-9'β, H-35' and H-38'), 4.25 (dd, J=18.5 and 4.0 Hz, H-29'β) 4.18 (d, J=14.3 Hz, 1H, H-9'α), 4.02 (d, J=12.8 Hz, H-20'β), 3.94 (dd, J=18.5 and 4.0 Hz, H-29'α), 3.78 (s, 3H, H-22), 3.78 (s, 3H, H-25), 3.76 (s, 1H, H-2), 3.75 (s, 3H, H-26'), 3.72 (s, 3H, H-44'), 3.68 (d, J=5.0 Hz, 1H, H-1'β), 3.62-3.58 (m, 1H, H-32'α), 3.57 (s, 3H, H-24'), 3.43 (d, J=13.8 Hz, 1H, H-5'β), 3.38-3.35 (m, 1H, H-32'β), 3.34 (dd, J=16.8 and 4.9 Hz, 1H, H-8β), 3.28 (td, J=10.0 and 4.0 Hz, 1H, H-10α), 3.07 (d, J=16.8 Hz, 1H, H-5'α), 2.85 (dd, J=14.0 and 3.5 Hz, 1H, H-20'α), 2.78 (d, J=16.8 Hz, 1H, H-8α), 2.70 (s, 3H, H-23), 2.61 (s, 1H, H-19), 2.47 (q, J=9.0 Hz, 1H, H-10β), 2.32-2.28 (m, 1H, H-11β) 2.22-2.13 (m, 2H, H-1'α and H-33'α), 2.08 (s, 3H, H-27), 1.94-1.84 (m, 3H, H-33'β and H-34'), 1.71-1.85 (m, 1H, 11α), 1.70-1.59 (m, 4H, H-21', H-39'), 1.53-1.51 (m, 1H, H-2'), 1.42-1.29 (m, 3H, H-20α, H-20β and H-40'β), 1.19-1.13 (m, 1H, H-40'α), 0.94 (t, J=8.0 Hz, 3H, H-22'), 0.91-0.84 (m, 6H, H-41' and H-42'), 0.74 (t, J=8.1 Hz, 3H, H-21). ^{13}C NMR (150 MHz, CD_3CN): δ 175.4 (C-23'), 173.3 (C-43'), 173.1 (C-24), 172.7 (C-36'), 172.4 (C-25'), 171.8 (C-26), 168.6 (C-30'), 166.7 (C-27'), 159.3 (C-16), 156.6 (C-7'), 154.4 (C-18), 139.5 (C-4'), 136.6 (C-16'), 132.4 (C-18'), 131.5 (C-6), 130.2 (C-11'), 125.9 (C-14), 125.6 (C-7), 124.6 (C-13), 123.9 (C-3'), 122.9 (C-14'), 121.9 (C-15), 120.7 (C-12'), 120.0 (C-13'), 115.8 (C-10'), 114.3 (C-8'), 112.3 (C-15'), 95.1 (C-17), 84.2 (C-2), 79.8 (C-3), 77.5 (C-4), 66.0 (C-19), 61.2 (C-35'), 57.9 (C-19'), 57.0 (C-38'), 55.2 (C-20'), 54.4 (C-12), 53.0 (C-44'), 52.9 (C-24'), 52.8 (C-25), 52.8 (C-26'), 51.3 (C-8), 50.9 (C-10), 50.9 (C-5'), 47.5 (C-32'), 45.9 (C-11), 43.9 (C-5), 42.7 (C-29'), 38.9 (C-23), 38.2 (C-39'), 35.7 (C-2'), 34.9 (C-1'), 30.6 (C-20), 29.5 (C-34'), 28.4 (C-21'), 26.2 (C-9'), 26.1 (C-40'), 25.7 (C-33'), 21.5 (C-27), 16.3 (C-42'), 12.8 (C-22'), 12.1 (C-41'), 8.9 (C-21). HRMS-ESI calcd for $C_{64}H_{82}N_7O_{15}$ 1188.5863, found 1188.5980.

Hybrid **24**: General procedure B was followed using alkyne **21** (42 mg, 0.06 mmol, 2 eq.) and vinorelbine (24 mg, 0.03 mmol, 1 eq) in 0.5 mL of anhydrous acetonitrile. After 2 h of stirring at room temperature, the solvent was evaporated under reduced pressure. The resulting residue was purified by column chromatography on silica gel using ethyl acetate/acetone (1:0 to 4:1) to afford compound **24** (12 mg, 26% yield) as yellow powder. $[\alpha]_D^{25}$ = -25 (*c* 0.1, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm) 7.89 (s, 1H, H-17'), 7.47 (d, J=8.0 Hz, 1H, H-11'), 7.29-7.15 (m, 10H, H-50'-H-54' and H-58'-H-62'), 7.01-6.93 (m, 3H, H-14', H-15', H-37'), 6.90 (t, J=8.0 Hz, 1H, H-13'), 6.87-6.82 (m, 1H, H-44'), 6.67 (br s, 1H, H-28'), 6.60 (s, 1H, H-14), 6.04 (s, 1H, H-17), 5.77 (dd, J=10.0 and 4.5 Hz, 1H, H-7), 5.40 (s, 1H, H-4), 5.21 (d, J=10.0 Hz, 1H, H-6), 5.10 (br s, 1H, H-3'), 5.03 (dd, J=16.4 and 13.1 Hz, 2H, H-48'), 4.97 (q, J=12.3 Hz, 2H, H-56'), 4.85-4.81 (m, 1H, H-45'), 4.42 (d, J=14.1 Hz, 1H, H-9'β), 4.40 (d, J=7.0 Hz, 1H, H-35'), 4.18 (m, 2H, H-29'β and H-38'), 4.10 (d, J=14.1 Hz, 1H, H-9'α), 3.95 (d, J=12.8 Hz, H-20'β), 3.88 (dd, J=18.3 and 3.6 Hz, H-29'α), 3.72 (s, 3H, H-22), 3.71 (s, 3H, H-25), 3.69 (s, 1H, H-2), 3.67 (s, 3H, H-26'), 3.53-3.50 (m, 2H, H-1'β and H-32'α), 3.50 (s, 3H, H-24'), 3.37 (d, J=13.8 Hz, 1H, H-5'β), 3.35-3.33 (m, 1H, H-32'β), 3.30 (dd, J=16.8 and 4.9 Hz, 1H, H-8β), 3.21 (td, J=9.0 and 4.4 Hz, 1H, 10α), 3.04 (dd, J=18.8 and 4.9 Hz, 1H, H-46'α), 2.99 (d, J=16.8 Hz, 1H, H-5'α), 2.84-2.76 (m, 2H, H-20'α and H-46'β), 2.71 (d, J=16.8 Hz, 1H, H-8α), 2.63 (s, 3H, H-23), 2.55 (s, 1H, H-19), 2.42 (q, J=9.0 Hz, 1H, H-10β), 2.14-2.06 (m, 3H, H-11α, H-1'α and H-33'α), 2.03 (s, 3H, H-27), 2.00-1.96 (m, 1H, H-33'β), 1.94-1.85 (m, 2H, H-34'), 1.80-1.73 (m, 5H, H-2', H-21', H-39' and H-11), 1.72-1.66 (m, 1H, H-20β), 1.37-1.22 (m, 2H, H-20α and H-40'β), 1.06-0.95 (m, 1H, H-40'α), 0.85 (t, J=8.0 Hz, 3H, H-22'), 0.79-0.70 (m, 6H, H-41' and H-42'), 0.67 (t, J=8.1 Hz, 3H, H-21). ^{13}C NMR (150 MHz, $CDCl_3$): δ 174.2 (C-23'), 172.0 (C-43'), 171.9 (C-24), 171.8 (C-36'), 171.1 (C-25'), 171.0 (C-47' and C-55'), 170.8 (C-26), 170.7 (C-36'), 167.3 (C-27'), 167.0 (C-30'), 158.0 (C-16), 154.4 (C-7'), 153.2 (C-18), 137.6 (C-4'), 135.1 (C-16'), 130.7 (C-18'), 129.4 (C-6), 129.1 (C-11'), 128.7, 128.6, 128.5 (C-49'-C-54' and H-57'-H-62'), 124.8 (C-7), 124.3 (C-14), 123.4 (C-13), 123.2 (C-3'), 122.4 (C-14'), 120.7 (C-15), 120.3 (C-12'), 119.4 (C-13'), 115.7 (10'), 115.0 (C-8'), 110.5 (C-15'), 94.3 (C-17), 83.4 (C-2), 79.7 (C-3), 76.4 (C-4), 67.8 (C-48'), 67.1 (C-56'), 66.0 (C-19), 60.4 (C-35'), 58.0 (C-38'), 56.1 (C-19'), 55.9 (C-22), 54.3 (C-20'), 53.3 (C-12), 52.6 (C-25), 52.5 (C-24'), 52.3 (C-26'), 50.7 (C-8), 50.5 (C-10), 50.0 (C-5'), 48.5 (C-45'), 46.6 (C-32'), 44.9 (C-11), 42.8 (C-5), 42.2 (C-29'), 38.3 (C-39'), 38.0 (C-23), 37.6 (C-2'), 36.3 (C-46'), 34.5 (C-1'), 30.9 (C-20), 29.8 (C-34'), 27.6 (C-21'), 25.0 (C-33'), 24.9 (C-9'), 24.8 (C-40'), 21.3 (C-27), 15.4 (C-42'), 12.2 (C-41'), 11.6 (C-22'), 11.5 (C-40'), 8.7 (C-21). HRMS-ESI calcd for $C_{81}H_{97}N_8O_{11}$ 1469.6915, found 1469.5588.

Hybrid **25**: General procedure C was followed using $NaBH(OAc)_3$ (6 mg, 0.028 mmol, 3 eq.) and enamino ester **22** (10 mg, 0.009 mmol, 1 eq.) in dichloromethane (0.1 mL) at 0 °C. The reaction mixture was stirred for 4 hours at 0 °C. The resulting mixture was diluted with dichloromethane and washed with a saturated sodium carbonate solution. Solvent removal under reduced pressure provided pure amino ester **25** (6 mg, 60% yield) as white powder. $[\alpha]_D^{25}$ = -21 (*c* 0.1, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.01 (s, 1H, H-17'), 7.46 (d, J=7.9 Hz, 1H, H-12'), 7.07-6.94 (m, 4H, H-13'-H-15' and H-28'), 6.51 (s, 1H, H-14), 6.07 (s, 1H, H-17), 5.76 (dd, J=10.1 and 5.1 Hz, 1H, H-7), 5.43 (s, 1H, H-4), 5.20 (d, J=10.1 Hz, 1H, H-6), 5.06 (d, J=4.7 Hz, 1H, H-3'), 4.45 (dd, J=7.9 and 2.6 Hz, 1H, H-35'), 4.31 (dd, J=16.6 and 6.1 Hz, 1H, H-29'α), 3.89 (d, J=16.5 Hz, 1H, H-9'β), 3.82 (dd, J, 18.2 and 5.0 Hz, H-29'β), 3.74 (s, 3H, H-22), 3.73 (s, 3H, H-25), 3.72 (s, 3H, H-26'), 3.70-3.68 (m, 1H, H-20'β), 3.68 (s, 1H, H-2), 3.66-3.63 (m, 1H,

H-8'), 3.65 (s, 3H, H-37'), 3.62-3.56 (m, 1H, H-32'), 3.56 (s, 3H, H-24'), 3.52-3.48 (m, 1H, H-20'α), 3.46-3.41 (m, 2H, H-7' and H-9'α), 3.37-3.33 (m, 3H, H-8α and H-5'), 3.36-3.29 (m, 1H, H-1'β), 3.30-3.25 (m, 1H, H-8β), 3.20-3.17 (m, 1H, H-32'), 2.82-2.78 (m, 1H, H-10β), 2.76 (d, J=15.0 Hz, 1H, H-5'α), 2.69 (s, 3H, H-23), 2.67-2.59 (m, 2H, H-34'), 2.61 (s, 1H, H-19), 2.40 (td, J=9.8 and 7.3 Hz, 1H, H-10α), 2.34-2.29 (m, 3H, H-1'α and H-11), 2.02-1.95 (m, 3H, H-33'), 1.91-1.80 (m, 2H, H-21'), 1.82-1.75 (m, 1H, H-20β), 1.37-1.30 (m, 1H, H-2'), 1.30 (s, 3H, H-27), 1.30-1.25 (m, 1H, H-20α), 0.87 (t, J=7.2 Hz, 3H, H-22'), 0.69 (t, J=7.8 Hz, 3H, H-21). ^{13}C NMR (125 MHz, $CDCl_3$) δ 175.1 (C-23'), 174.1 (C-36'), 172.2 (C-24), 171.1 (C-25'), 170.4 (C-26), 167.2 (C-30'), 163.7 (C-27'), 158.5 (C-16), 154.1 (C-18), 138.4 (C-4'), 134.5 (C-16'), 131.8 (C-18'), 130.3 (C-6), 129.7 (C-11'), 125.7 (C-15), 124.6 (C-7), 124.0 (C-14), 123.2 (C-13), 123.1 (C-14'), 122.7 (C-3'), 119.9 (C-12'), 119.5 (C-13'), 109.3 (C-10'), 94.7 (C-17), 83.7 (C-2), 79.8 (C-3), 76.6 (C-4), 66.3 (C-19), 66.2 (C-7'), 59.0 (C-35'), 57.1 (C-19'), 56.2 (C-22), 54.2 (C-12), 53.8 (C-24'), 53.5 (C-37'), 52.6 (C-25), 52.5 (C-26'), 52.5 (C-8), 50.9 (C-10), 50.6 (C-8'), 50.5 (C-5'), 49.4 (C-20'), 49.1 (C-32'), 46.2 (C-11), 44.9 (C-5), 41.8 (C-29'), 38.5 (C-23), 36.8 (C-2'), 34.8 (C-1'), 32.1 (C-20), 29.9 (C-34'), 28.6 (C-21'), 24.9 (C-33'), 22.9 (C-27), 19.6 (C-9'), 12.3 (C-22'), 8.66 (C-21). HRMS-ESI calcd for $C_{58}H_{73}N_6O_{14}$ 1077.5179, found 1077.5258.

Hybrid **26**: General procedure C was followed using $NaBH(OAc)_3$ (2.8 mg, 0.015 mmol, 3 eq.) and enamino ester **23** (5.4 mg, 0.005 mmol, 1 eq.) in dichloromethane (0.1 mL) at 0 °C. The reaction mixture was stirred for 4 hours at 0 °C. The resulting mixture was diluted with dichloromethane and washed with a saturated sodium carbonate solution. Solvent removal under reduced pressure provided amino ester **26** (3 mg, 51% yield) as white powder. $[\alpha]_D^{25}$ = -32 (*c* 0.1, $CHCl_3$). ^{1}H NMR (600 MHz, CD_3CN) δ 8.64 (s, 1H, H-17'), 7.81 (d, J=8.3 Hz, 1H, H-28'), 7.35 (d, J=8.3 Hz, 1H, H-12'), 7.16 (d, J=8.3 Hz, 1H, H-15'), 7.12 (d, J=8.5 Hz, 1H, H-37'), 7.01 (t, J=8.3 Hz, 1H, H-14'), 6.90 (t, J=8.3 Hz, 1H, H-13'), 6.79 (s, 1H, H-14), 6.27 (s, 1H, H-17), 5.78 (dd, J=10.5 and 4.9 Hz, 1H, H-7), 5.35-5.31(m, 1H, H-6), 5.17 (d, 1H, H-4), 5.07 (d, J=5.5 Hz, 1H, H-3'), 4.44-4.37 (m, 1H, H-35'), 4.29-4.25 (m, 1H, H-38'), 4.03-3.95 (m, 3H, H-1'β, H-8' and H-29'α), 3.83 (d, J=3.7 Hz, 1H, H-29'β), 3.78 (s, 3H, H-22), 3.68 (s, 3H, H-44'), 3.68 (s, 3H, H-25), 3.60 (s, 3H, H-26'), 3.56 (s, 1H, H-2), 3.54-3.51 (m, 1H, H-32'α), 3.52 (s, 3H, H-24'), 3.45-3.40 (m, 2H, H-32'β), 3.37 (dd, J=15.5 and 5.1 Hz, 1H, H-20'β), 3.37-3.34 (m, 1H, H-8β), 3.29-3.21 (m, 3H, H-7', H-9'β and H-1'α), 3.15 (d, 1H, H=14.8 Hz, H-5'β), 2.94 (s, 1H, H-19), 2.78 (d, J=14.8 Hz, 1H, H-5'α), 2.77 (d, J=15.4 Hz, 1H, H-8α), 2.30-2.27 (m, 1H, H-9'α), 2.00-1.95 (m, 2H, H-11), 1.94 (s, 3H, H-27), 1.94-1.89 (m, 4H, H-33' and H-34'), 1.89-1.83 (m, 3H, H-10 and H-20'α), 1.83-1.80 (m, 2H, H-21'), 1.80-1.74 (m, 1H, H-39'), 1.57-1.51 (m, 1H, H-20β), 1.40-1.34 (m, 1H, H-40'α), 1.34-1.29 (m, 1H, H-20α), 1.24 (s, 1H, H-2'), 1.06-1.02 (m, 1H, H-40'β), 0.88 (t, J=7.2 Hz, 3H, H-22'), 0.85-0.75 (m, 6H, H-41' and H-42'), 0.58 (t, J=7.4 Hz, 3H, H-21). ^{13}C NMR (150 MHz, CD_3CN) δ 176.0 (C-23'), 175.6 (C-24), 173.8 (C-43'), 173.1 (C-25'), 172.5 (C-36'), 171.1 (C-26), 168.4 (C-30'), 164.8 (C-27'), 159.1 (C-16), 154.0 (C-18), 140.7 (C-4'), 136.7 (C-16'), 131.8 (C-18'), 131.2 (C-6), 129.6 (C-11'), 125.5 (C-15), 126.0 (C-14), 124.5 (C-7), 123.3 (C-13), 123.6 (C-3'), 122.9 (C-14'), 120.0 (C-12'), 119.6 (C-13'), 109.2 (C-10'), 94.4 (C-17), 83.4 (C-2), 81.8 (C-3), 77.2 (C-4), 69.6 (C-7'), 65.9 (C-19), 60.5 (C-35'), 58.0 (C-8'), 57.8 (C-38'), 57.1 (C-19'), 56.8 (C-22), 54.5 (C-12), 52.7 (C-24'), 52.6 (C-44'), 52.6 (C-25), 52.5 (C-26'), 51.1 (C-8), 50.9 (C-10), 50.6 (C-5'), 49.4 (C-20'), 47.3 (C-32'), 44.4 (C-11), 43.8 (C-5), 42.3 (C-29'), 38.5 (C-23), 37.6 (C-40'), 37.5 (C-1'), 36.8 (C-2'), 31.1 (C-20), 29.6 (C-34'), 28.5 (C-21'), 25.6 (C-33'), 22.9 (C-27), 20.0 (C-9'), 16.1 (C-42'), 12.1 (C-41'), 11.9 (C-22'), 8.66 (C-21). HRMS-ESI calcd for $C_{64}H_{84}N_7O_{15}$ 1190.6020, found 1190.6141.

Hybrid **27**: General procedure C was followed using $NaBH(OAc)_3$ (2.8 mg, 0.015 mmol, 3 eq.) and enamino ester **24** (6.8 mg, 0.005 mmol, 1 eq.) in dichloromethane (0.1 mL) at 0 °C. The reaction mixture was stirred for 4 hours at 0 °C. The resulting mixture was diluted with dichloromethane and washed with a saturated sodium carbonate solution. Solvent removal under reduced pressure provided pure amino ester **27** (6.8 mg, 93% yield) as white powder. $[\alpha]_D^{25}$ = -36 (*c* 0.1, $CHCl_3$)^{1}H NMR (600 MHz, CD_3CN) δ 8.65 (s, 1H, H-17'), 7.38-7.30 (m, 11H, H-12', H-50'–H-54' and H-58'–H-62'), 7.26-7.20 (m, 1H, H-44'), 7.19 (d, J=7.1 Hz, 1H, H-15'), 7.02 (t, J=7.1 Hz, 1H, H-14'), 7.00-6.99 (m, 1H, H-28'), 6.92 (t, J=7.1 Hz, 1H, H-13'), 6.79 (s, 1H, H-14), 6.28 (s, 1H, H-17), 6.21-6.15 (m, 1H, H-37'), 5.79 (dd, J=10.4 and 4.9 Hz, 1H, H-7), 5.30 (d, J=10.4 Hz, 1H, H-6), 5.19 (s, 1H, H-4), 5.13-5.01 (m, 5H, H-3', H-48' and H-56'), 4.84-4.79 (m, 1H, H-45'), 4.41-4.34 (m, 3H, H-9' and H-35'), 4.24-4.16 (m, 2H, H-29'), 4.05-3.99 (m, 3H, H-1'β and H-20'), 3.89 (s, 3H, H-22), 3.70 (s, 3H, H-25), 3.69 (s, 3H, H-26'), 3.60-3.54 (m, 1H, H-32'α), 3.55 (s, 1H, H-2), 3.52 (s, 3H, H-24'), 3.50-3.42 (m, 1H, H-32'β), 3.30-3.25 (m, 2H, H-5'β and H-1'α), 3.20-3.15 (m, 1H, H-11α), 2.92-2.85 (m, 3H, H-46' and H-8β), 2.82 (s, 1H, H-19), 2.71-2.57 (m, 4H, H-5'α, H-39' and H-10), 2.48-2.45 (m, 1H, H-11β), 2.20-2.15 (m, 1H, H-8α), 2.07-2.05 (m, 1H, H-33'α), 1.98-1.91 (m, 4H, H-23, H-33'β), 1.88-1.79 (m, 2H, H-34'), 1.75-1.70 (m, 2H, H-21'), 1.41-1.33 (m, 1H, H-20α), 1.27 (s, 3H, H-27), 1.21-1.18 (m, 1H, H-2'), 1.11-1.05 (m, 1H, H-20β), 0.88 (q, J=6.7 Hz, 2H, H-40'), 0.85-0.77 (m, 4H, H-41' and H-42'), 0.76 (t, J=7.2Hz, 3H, H-22'), 0.63 (t, J=8.1 Hz, 3H, H-21). ^{13}C NMR (150 MHz, CD_3CN) δ 176.1 (C-23'), 175.1 (C-43'), 173.2 (C-24), 171.7 (C-36'), 171.1 (C-25'), 171.4 (C-47' and C-55'), 170.8 (C-26), 167.3 (C-27'), 167.0 (C-30'), 159.5 (C-16), 153.6 (C-18), 140.7 (C-4'), 134.3 (C-16'), 130.8 (C-18'), 130.4 (C-6), 129.0 (C-11'), 128.7, 128.6, 128.5 (C-49'-C-54' and H-57'-H-62'), 125.1 (C-14), 124.6 (C-15), 124.2 (C-7), 122.7 (C-13), 123.2 (C-3'), 122.7 (C-15'), 121.7 (C-14'), 118.7 (C-12'), 118.6 (C-13'), 111.2 (C-10'), 93.3 (C-17), 82.7 (C-2), 79.9 (C-3), 76.3 (C-4), 67.1 (C-48'), 66.4 (C-56'), 66.2 (C-7'), 64.5 (C-19), 60.4 (C-35'), 58.0 (C-8'), 57.5 (C-38'), 55.3 (C-19'), 55.1 (C-23), 54.0 (C-20'), 53.9 (C-12), 53.0 (C-25), 52.5 (C-24'), 52.3 (C-26'), 50.9 (C-8), 50.3 (C-10), 50.1 (C-5'), 48.7 (C-45'), 46.4 (C-11), 46.3 (C-32'), 43.4 (C-5), 42.1 (C-29'), 38.0 (C-39'), 37.9 (C-

23), 37.5 (C-1'), 36.0 (C-2'), 35.9 (C-46'), 31.2 (C-20), 28.3 (C-34'), 27.5 (C-21'), 24.3 (C-9'), 23.9 (C-40'), 21.5 (C-33'), 16.2 (C-42'), 12.9 (C-41'), 11.3 (C-22'), 11.0 (C-40'), 9.3 (C-21). HRMS-ESI calcd for $C_{81}H_{99}N_8O_{18}$ 1471.7072, found 1471.7134.

Inhibition of Tubulin Assembly

The drug, dissolved in DMSO at different concentrations, was added to a solution of free tubulin (obtained from sheep brain and prepared according to a published procedure[25]) at 0 °C. Then the solution was placed in a temperature controlled cell at 37 °C (microtubule assembly) and the increase of the optical density was monitored in a UV spectrophotometer at 350 nm (the maximum was reached in about 1 minute). The maximum rate of assembly was recorded and compared to a drug-free sample. The IC_{50} of the compound was calculated from the effect of several concentrations and compared to the IC_{50} of vinorelbine obtained within the same day with the same tubulin preparation.

Cell culture and proliferation assay

Cancer cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA) and were cultured according to the supplier's instructions. Human K562 leukemia cells and HCT116 colorectal carcinoma cells were grown in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and 1% glutamine. U87-MG human glioblastoma cells were grown in Dulbecco minimal essential medium (DMEM) containing 10% FCS and L-glutamine. Cell lines were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Cell viability was assessed using Promega CellTiter-BlueTM reagent (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, the cells were seeded in 96-well plates ($2.5x10^3$ cells/well) containing 50µL of growth medium. After 24 hours of culture, the cells were supplemented with 50 µL of medium containing different concentrations of the tested compound dissolved in DMSO (less than 0.1% in each preparation). After 72 hours of incubation, 20 µL of resazurin[26] was added for 1.5 h before recording fluorescence (λex = 560 nm, λem = 590 nm) using a microtiter plate fluorimeter. The IC_{50} corresponds to the concentration of compound that induced a 50% decrease in fluorescence of drug-treated cells compared with untreated cells. Experiments were performed in triplicate.

Cell Cycle Analysis

The cell cycle distribution of K562 was evaluated by flow cytometry as described previously.[27]

Computational methods

Compounds **22** and **25** were model-built using vinblastine (CSD entry MAYWIS[28]) as a template and their geometries were optimized using the AM1 Hamiltonian as implemented in Gaussian 09 (Gaussian, Inc., Wallingford, CT). Charges were then assigned to individual atoms by fitting the quantum mechanically calculated (RHF/6-31G*//RHF/3-21G*) molecular electrostatic potential to a point charge model. Consistent bonded and nonbonded AMBER parameters for ligand atoms were assigned by analogy or through interpolation from those already present in the AMBER database (ff10). The conformational space for **22** and **25** was sampled in neutral aqueous solution by immersing each molecule in a box containing ~2300 TIP3P water molecules and two Cl^- ions and running molecular dynamics (MD) simulations for 40 ns using the *pmemd* module from the AMBER12 suite of programs (http://ambermd.org/). Periodic boundary conditions were applied and electrostatic interactions were treated using the smooth particle mesh Ewald method with a grid spacing of 1 Å. The cutoff distance for the nonbonded interactions was 10 Å, the SHAKE algorithm was applied to all bonds, and an integration step of 2.0 fs was used throughout. Monitoring the root-mean-square deviations (RMSD) from the initial geometries over the course of the trajectories using the *ptraj* module showed no major changes in puckering for the ring systems but the expected variation in the flexible side chain (Figs. SI).

Two different β_1:α_2-tubulin dimers were used as the protein target, one extracted from the 4.0 Å-resolution PDB entry 1Z2B (obtained after soaking **1** into the tubulin complex reported in PDB entry 1SA0) and another from the more recent 2.3 Å-resolution crystal structure of a similar complex with epothilone A bound to β_2-tubulin and no ligand at the inter-dimer interface (PDB entry 4I50).[29] The reason for this, apart from the higher resolution of the latter complex, was that we detected some phi/psi anomalies at Leu227 of β_1-tubulin in 1Z2B, a residue that is critically near the bound ligand of interest and also because we wanted to assess the ability of the force field and our simulation conditions to drive the conformational rearrangement described for the T7 loop containing $Asp179_{\beta1}$ upon binding of **1** (*cf.* 1Z2B *vs* 1SA0). First we simulated the explicitly solvated β_1:α_2-tubulin-**1** complexes (~37300 TIP3P water molecules and 34 Na^+ ions) under the same conditions previously reported[17,18] to validate our protocol, which was shown to yield stable trajectories over 10 ns (Fig SI). Then **25** was docked within the vinca domain by superimposing the vindoline scaffold onto that of **1** in the corresponding relaxed and cooled complexes and the same MD protocol was followed. The molecular graphics program PyMOL version 1.3 (Schrodinger, LLC, 2010) was employed for visualization and molecular editing.

Acknowledgements

Jacques Fahy (Pierre Fabre Laboratories) is gratefully acknowledged for a generous gift of vinorelbine. O.G. gratefully acknowledges the ED425 "Innovation Therapeutique, du Fondamental à l'Appliqué" for a grant and L'Oreal Foundation, UNESCO and the French Academy of Science for her "Pour les Femmes et la Science" prize. We gratefully thanks Jean-François Gallard (ICSN) for the NMR spectra.

Notes and references

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Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

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