Organic & Biomolecular Chemistry

PAPER



Cite this: DOI: 10.1039/c4ob02114b

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

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Some hybrids of vinca alkaloids and phomopsin A, linked by a glycine pattern, have been synthesized in one or two steps, by an insertion reaction and shown to inhibit microtubule assembly. These compounds have been elaborated in order to interact with both the "vinca site" and the "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 that they could bind, within the vinca domain, in similar spatial regions as those of vinca and phomopsin thanks to the flexibility provided by the glycine linker used to elaborate these hybrids.

DOI: 10.1039/c4ob02114b www.rsc.org/obc

Received 3rd October 2014

Accepted 19th January 2015

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,^{3–6} vinca alkaloids or the newly reported maytansine⁷).

Vinca alkaloids,^{8,9} like natural vinblastine **1** and synthetic vinorelbine **2** or vinflunine **3** (Fig. 1), are major anticancer agents. They prevent tubulin polymerization into microtubules by binding to 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



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

(Fig. 1) are very potent tubulin assembly inhibitors.¹² Their binding site called the "peptide site" in the vinca domain partly overlaps with that of the vinca alkaloids.¹³

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 (Fig. 2).

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



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 $[\]dagger\, Electronic$ supplementary information (ESI) available. See DOI: 10.1039/ c4ob02114b

Organic & Biomolecular Chemistry



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



H₂CO₂(H₃CC DCOCH. CO_CH_ One or two steps R = OMe, Ile-OMe, Ile-Asp(Bn)-OBn MeO₂C H₂CO₂C OCOCH-H₂C(CO₂CH₂

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

Fig. 3 Hybrids 6 of vinca and phomopsin clasped on C-8' of 7'-homoanhydrovinblastine.17

eventually elaborate more potent and specific derivatives.¹⁴⁻¹⁶ 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 (Fig. 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.

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 the biological activity.¹⁸

Thus, we wish to report the elaboration of a new family of hybrids¹⁹ whose 7'-homo-anhydrovinblastine core carries the phomopsin lateral chain in C-7'. The synthetic pathway involves an insertion of activated alkynes into the 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.

Results and discussion

Chemistry

We previously showed that insertion of unsymmetrical ester amide alkynes in 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 lengths Gly-Pro-OMe 12, Gly-Pro-Ile-OMe 13 and Gly-Pro-Asp(Bn)-OBn 14 were thus synthesized by a classical procedure (Scheme 2).

Once the peptide chains were synthesized, their coupling with 4-methoxy-4-oxobut-2-ynoic acid 16 was envisaged.

Paper

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Scheme 2 Last step of peptides 12-14 synthesis.



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.

Compound 16^{21} was obtained in one step from commercially available methyl propiolate 15 by a modified procedure of Hall and coworkers^{21*a*} replacing dry ice (which, in our hands resulted in poor yields of desired 16) by gaseous CO₂.

Under 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 propynamides²² 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* 83% with 10 equivalents).

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.



Scheme 4 Synthesis of hybrids 22-24.



Scheme 5 Synthesis of hybrids 25-27.

The hybrids 22–24 were then reduced to the β -amino esters 25–27 in the presence of NaBH(OAc)₃ (Scheme 5). 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

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, and 7) displayed a significant inhibitory activity on the 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, entries 6 and 9 in Table 1). Surprisingly, 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.

To check whether the mechanism of growth arrest is the same for the most cytotoxic compounds 25 and 27, we assayed



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



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.

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 (Fig. 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.

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 a 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 series¹⁸ is accounted for because the alkoxy moiety is buried in a relatively small lipophilic pocket lined by the hydrophobic side-chains of Leu_{a2}248 and $Val_{\alpha 2}250$ (Fig. 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 $Tyr_{\beta 1}224$, located on the N-terminus of helix H7²³ 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



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.

locations at the β_1 : α_2 -tubulin inter-dimer interface thanks to the flexibility provided by the glycine linker (Fig. 1 in ESI†). Nonetheless, the much bulkier substituents present in 24 and 27 are detrimental for tubulin binding. This finding is in consonance with results by Boger and coworkers²⁴ 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 ESI†).

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_{β1}177 and the carboxylate of Asp_{β1}179 (in loop T5), whose side chain was shown to change its 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_{α2}329; and (c) the ethyl group on ring D' stacks on the phenyl ring of Tyr_{β1}224, which sits on the guanine base of GDP present in the nucleotide-binding site (Fig. 3 in ESI[†]).

Finally, although the unsaturation between positions 7' and 8' in 22 affects the puckering of ring D' relative to that found in 25 (Fig. 1 in ESI \dagger), 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 lengths, mimicking that of phomopsin A, were grafted on C-7' of the vinca moiety using a glycine linker. Biological evaluation on tubulin revealed that the 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 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 hybrid 27, inactive on tubulin, was found as cytotoxic as the most potent one 25, probably due to 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-(3dimethylaminopropyl)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 a 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 acetateacetone (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 a syringe and stirring was continued at -78 °C for 1 hour followed by the addition of a CO₂ 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 \sim 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. ¹H NMR (300 MHz, CDCl₃) δ 9.21 (br s, 1H, H-6), 3.81 (s, 3H, H-1). $^{13}\mathrm{C}$ NMR (75 MHz, CDCl₃) & 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 μ L, 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 a 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). $[\alpha]_{\rm D}^{25} = -41$ (*c* 0.1, CHCl₃). ¹H NMR (300 MHz, CDCl₃) 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-5 α), 3.50–3.40 (m, 1H, H-5 β), 2.30–2.20 (m, 1H, H-7 α), 2.09–1.98 (m, 3H, H-6 and H-7 β). ¹³C NMR (75 MHz, CDCl₃) 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 C₈H₁₅N₂O₃ 187.1077, found 187.1172.

Tripeptide 13. The general procedure A was followed using NMM (181 μ L, 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 a 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, CHCl₃). ¹H NMR (300 MHz, CDCl₃) 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-7 α), 3.25–3.14 (m, 1H, H-7 β), 2.35–2.29 (m, 1H, H-5 α), 2.08–1.85 (m, 2H, H-5 β and H-11), 1.70–1.59 (m, 2H, H-6), 1.39–1.31 (m, 1H, H-12 α), 1.12–1.05 (m, 1H, H-12 β), 0.97–0.95 (m, 6H, H-13 and H-14). ¹³C NMR (75 MHz, CDCl₃) 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 C₁₄H₂₆N₃O₄ 300.1918, found 300.2013.

Tetrapeptide 14. The general procedure A was followed using NMM (13 μ L, 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 a 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). $[\alpha]_{\rm D}^{25} = -27$ (*c* 0.1, CHCl₃). ¹H (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-7 α), 3.12 (t, I = 5.9 Hz, 1H, H-7 β), 3.00 (dd, I = 17.0 and 5.5 Hz, 1H, H-18 α), 2.85 (dd, J = 17.0 and 5.5 Hz, 1H, H-18 β), 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). ¹³C NMR (75 MHz, CDCl₃): 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 C₃₁H₄₁N₄O₇ 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 μ L, 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 was 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 a colourless oil. $[\alpha]_{D}^{25} = -80$ (c 0.1, CHCl₃). ¹H NMR (300 MHz, CDCl₃): 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). ¹³C NMR (75 MHz, CDCl₃) & 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 C₁₃H₁₇N₂O₆ 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 μ L, 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 was 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 a colourless oil. $\left[\alpha\right]_{D}^{25} = -47$ (c 0.1, CHCl₃). ¹H NMR (300 MHz, CDCl₃) 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-9 α), 3.44 (q, J = 8.7 Hz, 1H, H-9 β), 2.23–2.13 (m, 1H, H-11a), 2.36-2.27 (m, 1H, H-10a), 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-17 α), 1.25 (t, J = 7.3 Hz, 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). ¹³C NMR (75 MHz, CDCl₃) 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 C₁₉H₂₈N₃O₇ 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 $\mu L,$ 0.76 mmol, 10.3 eq.) was added at $-15~^{\rm o}C$ 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 was 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 a colourless oil. $\left[\alpha\right]_{D}^{25} = -30$ (c 0.1, CHCl₃). ¹H NMR (300 MHz, CDCl₃) & 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-23 α), 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-10ß and H-11ß), 1.86-1.75 (m, 1H, H-16), 1.41–1.30 (m, 1H, H-17 α), 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 C36H43N4O10 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 acetateacetone (1:0 to 4:1) to afford compound 22 (20 mg, 31% yield) as a yellow powder. $\left[\alpha\right]_{D}^{25} = -30$ (c 0.1, CHCl₃). ¹H NMR (500 MHz, CDCl₃) 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-8 β), 3.29 (td, J = 10.0 and 4.1 Hz, 1H, 10 α), 3.04 (d, J = 16.0Hz, 1H, H-5' α), 2.86 (dd, J = 14.0 and 3.5 Hz, 1H, H-20' α), 2.79 $(d, J = 16.0 \text{ Hz}, 1\text{H}, \text{H-}8\alpha), 2.70 (s, 3\text{H}, \text{H-}23), 2.62 (s, 1\text{H}, \text{H-}19),$ 2.50 (q, J = 10.0 Hz, 1H, H-10 β), 2.20–2.18 (m, 2H, H-1' α and H-33'a), 2.08 (s, 3H, H-27), 2.03-2.00 (m, 3H, H-33' 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-20 α and 20 β), 0.94 (t, J = 7.0 Hz, 3H, H-22'), 0.74 (t, J = 7.5 Hz, 3H, H-21). ¹³C NMR (150 MHz, CD₃CN) 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 C₅₈H₇₁N₆O₁₄ 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 a yellow powder. [α]_D²⁵ = -33 (*c* 0.1, CHCl₃) (500 MHz, CDCl₃) 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

Paper

Organic & Biomolecular Chemistry

H-38'), 4.25 (dd, J = 18.5 and 4.0 Hz, H-29' β) 4.18 (d, J = 14.3Hz, 1H, H-9'α), 4.02 (d, J = 12.8 Hz, H-20'β), 3.94 (dd, J = 18.5and 4.0 Hz, H-29'a), 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-10 α), 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, 11a), 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). ¹³C NMR (150 MHz, CD₃CN): 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 C₆₄H₈₂N₇O₁₅ 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 acetateacetone (1:0 to 4:1) to afford compound 24 (12 mg, 26% yield) as a yellow powder. $\left[\alpha\right]_{D}^{25} = -25$ (c 0.1, CHCl₃). ¹H NMR (500 MHz, CDCl₃) δ (ppm) 7.89 (s, 1H, H-17'), 7.47 (d, J = 8.0Hz, 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 $(d, J = 14.1 Hz, 1H, H-9'\beta), 4.40 (d, J = 7.0 Hz, 1H, H-35'), 4.18$ (m, 2H, H-29' β and H-38'), 4.10 (d, *J* = 14.1 Hz, 1H, H-9' α), 3.95 $(d, J = 12.8 Hz, H-20'\beta)$, 3.88 $(dd, J = 18.3 and 3.6 Hz, H-29'\alpha)$, 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-8 β), 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-8 α), 2.63 (s, 3H, H-23), 2.55 (s, 1H, H-19), 2.42 (q, J = 9.0 Hz, 1H, H-10 β), 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). ¹³C NMR (150 MHz, CDCl₃): 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 (C-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 C₈₁H₉₇N₈O₁₁ 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 a white powder. $\left[\alpha\right]_{D}^{25} = -21$ (c 0.1, CHCl₃). ¹H NMR (500 MHz, CDCl₃) 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'a), 3.46-3.41 (m, 2H, H-7' and H-9'a), 3.37-3.33 (m, 3H, H-8 α 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-10a), 2.34–2.29 (m, 3H, H-1'a 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-20 α), 0.87 (t, J = 7.2 Hz, 3H, H-22'), 0.69 (t, J = 7.8 Hz, 3H, H-21). ¹³C NMR (125 MHz, CDCl₃) 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 a white powder. $[\alpha]_{D}^{25} = -32$ (*c* 0.1, CHCl₃). ¹H NMR (600 MHz, CD₃CN) 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- $32'\alpha$), 3.52 (s, 3H, H-24'), 3.45–3.40 (m, 2H, H- $32'\beta$), 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, $J = 14.8 Hz, H-5'\beta$, 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-8 α), 2.30–2.27 (m, 1H, H-9'a), 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-20 α), 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). ¹³C NMR (150 MHz, CD₃CN) 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 C₆₄H₈₄N₇O₁₅ 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 a white powder. $\left[\alpha\right]_{D}^{25} = -36$ (c 0.1, CHCl₃). ¹H NMR (600 MHz, CD₃CN) 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-11 α), 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'a, 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, I = 7.2 Hz, 3H, H-22'), 0.63 (t, I = 8.1 Hz, 3H, H-21). ¹³C NMR (150 MHz, CD₃CN) 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 C81H99N8O18 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 procedure²⁵) at 0 °C. Then the solution was placed in a temperature controlled cell at 37 °C (microtubule assembly) and the increase in the optical density was monitored on 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 IC₅₀ 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's minimal essential medium (DMEM) containing 10% FCS and L-glutamine. Cell lines were maintained at 37 °C under a humidified atmosphere containing 5% CO₂. Cell viability was assessed using the Promega CellTiter-Blue™ reagent (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, the cells were seeded in 96-well plates $(2.5 \times 10^3 \text{ cells per well})$ containing 50 µL 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 resazurin²⁶ was added for 1.5 h before recording fluorescence ($\lambda_{ex} = 560 \text{ nm}$, $\lambda_{em} = 590 \text{ nm}$) using a microtiter plate fluorimeter. The IC₅₀ corresponds to the concentration of compound that induces a 50% decrease in fluorescence of drugtreated 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 MAYWIS²⁸) 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 of the rootmean-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 (Fig. 1 in ESI[†]).

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 Åresolution 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_{β 1} upon binding of **1** (*cf.* 1Z2B *vs.* 1SA0). First we simulated the explicitly solvated $\beta_1:\alpha_2$ -tubulin-1 complexes (~37 300 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. 2 in ESI[†]). 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. F.G. thankfully acknowledges financial support from CICYT (SAF2012-39760-C02-02) and Comunidad Autónoma de Madrid (S2010-BMD-2457). We gratefully thank Jean-François Gallard (ICSN) for the NMR spectra.

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