Synthesis and Biological Activity of Chimeric Structures Derived from the Cytotoxic Natural Compounds Dolastatin 10 and Dolastatin 15†

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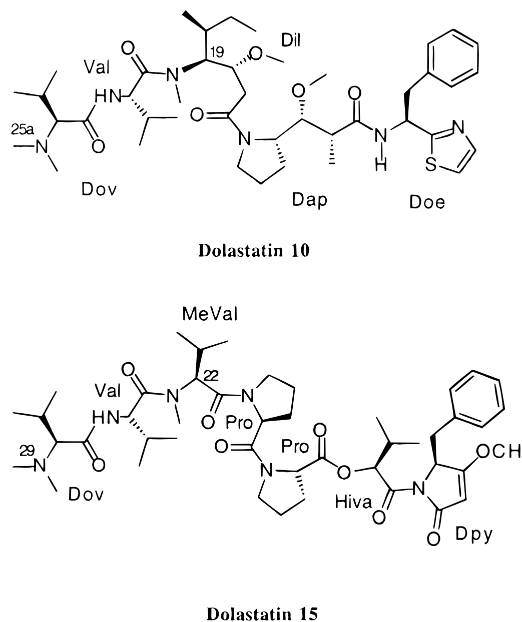
The natural cytotoxic compounds dolastatins 10 and 15 exhibit great similarities in structure and in their biological activity profiles. Two compounds (**1** and **2**) formed by interchanging the dolaisoleuine residue of dolastatin 10 and the MeVal-Pro dipeptide of dolastatin 15 were synthesized in order to evaluate the possible equivalence of these units. These compounds can be considered as chimeras of dolastatins 10 and 15 formed by the N-terminal part of the former and the C-terminal part of the latter and vice versa. Both analogues exhibited a marked decrease in their cytotoxic activity but showed similar differential cytotoxicity with regard to the cell lines assayed compared with the parent compounds. HT-29 cell line was the least sensitive one. However, this activity was in the nanomolar level and close to that of vincristine. The differences in their effect on tubulin polymerization were less pronounced. We confirmed the already known crucial role of the Dil residue in this assay. The nonequivalence of the Dil unit and the MeVal-Pro dipeptide probably reflects modification in the relative positions of the *N*-dimethylamino and the phenyl moieties.

Introduction

Dolastatins are a family of antineoplastic pseudopeptides isolated from the sea hare *Dolabella auricularia*. Most of them were originally identified by the group of Pettit,[1−7] and more recently the number of active metabolites present in this mollusk was extended by the groups of Yamada[8−14] and Pettit.[15] Among them, dolastatins 10 and 15 show promising antitumoral capacity. They are both highly potent inhibitors of the proliferation of murine and human leukemia cell lines[16−19] and also inhibitors of hematopoietic progenitor cells.[20] These compounds typically behave as antimitotic agents.[21,22] Dolastatin 10 inhibits in vitro microtubule assembly by binding to the tubulin β subunit near the vinca domain in a similar fashion as the noncytotoxic pseudopeptide phomopsin A.[23] Like vinblastin, the binding of dolastatin 10 to tubulin induces aggregation in vitro, but at higher concentration than that which inhibits cell proliferation.[24] A similar study has not been reported for dolastatin 15. However, both compounds[22] as well as several analogues[25,26] induce disruptive effects on the cytoplasmic microtubule network.



Dolastatin 10

Dolastatin 15

The similarity between the cellular effects of dolastatin 10 and dolastatin 15 is probably a reflection of their structural analogies.[22] A formal comparison of both structures shows that, first, the N-termini are identical from N-25a to C-19 for dolastatin 10 and from N-29 to C-22 for dolastatin 15; second, the C-termini are constituted of phenylalanine-derived residues where the carboxylic acid group is replaced by a heterocycle; and third, a pyrrolidine occupies the center of both molecules. These considerations have led us to the assumption that the Dil−Dap or Pro−Pro bond might act as a hinge in the middle of the molecules. To evaluate this hypothesis, we envisaged the synthesis of compounds **1** and **2** formed by replacing the MeVal-Pro

† The different residues of dolastatins 10 and 15 were named according to refs 2 and 3. Abbreviations follow IUPAC−IUB Joint Commission on Biochemical Nomenclature for amino acids and peptides (*Eur. J. Biochem.* **1984**, *138*, 9−37). Additional abbreviations used herein are as follows: BroP, tris(dimethylamino)bromophosphonium hexafluorophosphate; COMMOD, 2,2′-carbonylbis(3,5-dioxo-4-methyl-1,2,4-oxadiazolidine); DIEA, diisopropylethylamine; DMAP, 4-(dimethylamino)pyridine; IPCC, isopropenyl chlorocarbonate; PyBOP, (1*H*-benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; PyBroP, tripyrrolidinobromophosphonium hexafluorophosphate; PyCloP, tripyrrolidinochlorophosphonium hexafluorophosphate; TEA, triethylamine; TFA, trifluoroacetic acid.

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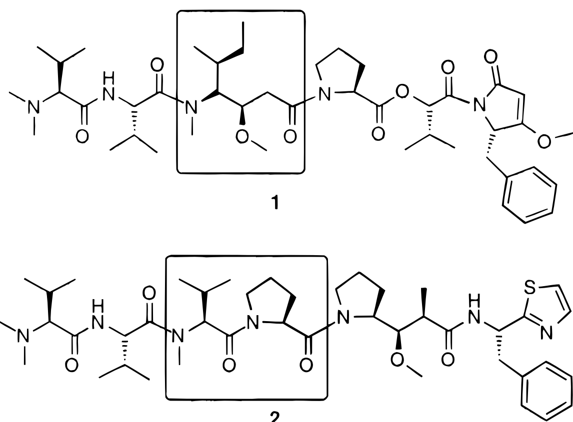
dipeptide in dolastatin 15 by the central Dil residue of dolastatin 10 and vice versa. These molecules could be considered as chimeric structures associating the dolastatin 10 N-terminus and the dolastatin 15 C-terminus in the case of **1** and vice versa in the case of **2**. We report here our results concerning the synthesis of these compounds and the evaluation of their biological activity.



Chemistry

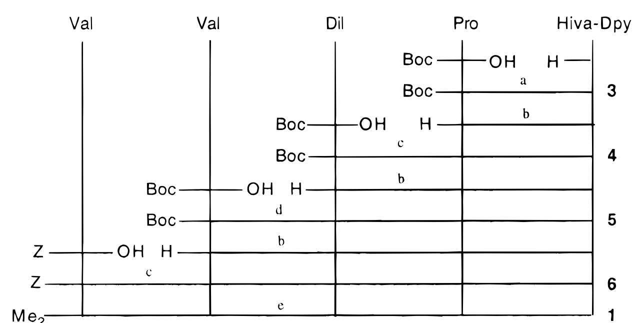
Compound **1** was obtained by following a stepwise peptide synthesis starting from the previously described compound Hiva-Dpy (Scheme 1).[27] The unusual amino acids were all synthesized according to previously described procedures.[27,28]

To prepare compound **2** we decided to proceed by fragment coupling between protected peptides Val-Val-MeVal-Pro and Dap-Doe. Thus, the tetrapeptide **10** was elaborated as depicted in Scheme 2.

Hydrogenolysis of **9** in the presence of formaldehyde followed by treatment with TFA yielded the free tetrapeptide **11** (Scheme 3). Unfortunately, we were unable to couple this compound to the Dap-Doe fragment under various conditions. We tried to circumvent this difficulty by coupling Dap-Doe before elaborating the tertiary amine function. Thus, the ester function of **9** was hydrolyzed by TFA treatment to furnish **12** (Scheme 3). Coupling of this compound with Dap-Doe yielded **14**. Hydrogenolysis of the Z group by using Pd catalysts was unsatisfactory, whereas deprotection under acidic conditions (HBr/AcOH) led surprisingly to extensive breaking of the MeVal-Pro amide bond to give the tetrapeptide **14**. These latter results can be explained in the light of Anteunis's studies concerning the sensitivity of imino acids to acid-catalyzed peptolysis.[29,30]

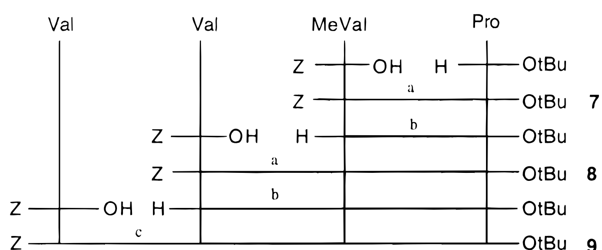
Finally, the compound **2** was synthesized according to a stepwise Fmoc strategy as depicted in Scheme 4.

Scheme 1[a]



[a] Reagents: (a) IPCC, TEA, DMAP, 5 °C; (b) TFA; (c) PyBOP, DIEA; (d) PyCloP, DIEA; (e) HCHO, H_2, Pd/C.

Scheme 2[a]



[a] Reagents: (a) PyBroP, DIEA; (b) H_2, Pd/C; (c) BOP, DIEA.

Evaluation of Biological Activity

Cytotoxic activities of analogues **1** and **2** were tested on either human (HT-29, MCF7) or murine (L1210) cell lines and compared with those of the parent compounds and vincristine, another natural antimitotic agent taken as reference. Drug effects on tubulin polymerization were also evaluated. The results are given in the Table 1.

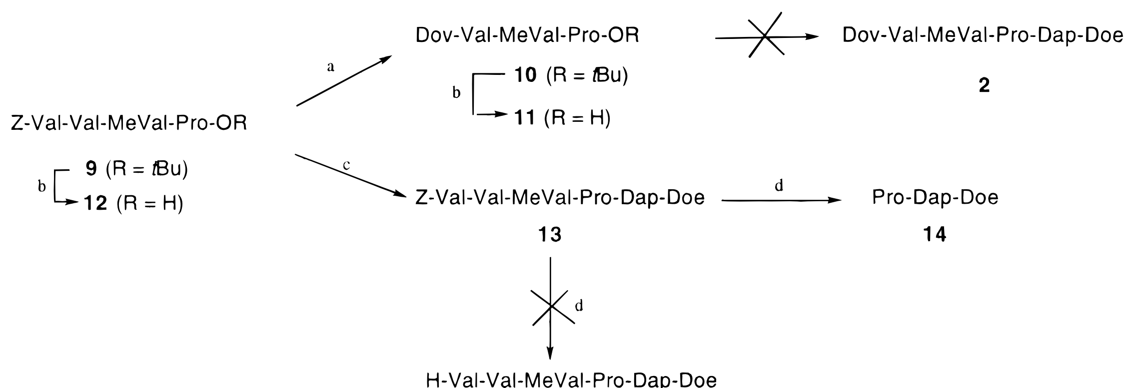
Both analogues, just like dolastatins 10 and 15 and vincristine, showed similar differential cytotoxicity with regard to the cell lines assayed, HT-29 being the least sensitive one, particularly in the case of **1**. Both analogues exhibited a loss of activity by comparison with the parent compounds. This loss was uniformly within the range of about 2 orders of magnitude except for **1** against the HT-29 cell line where the loss of activity was even greater. Nevertheless, all the IC_{50} values were at the nanomolar level and in the same range as that of vincristine.

On the other hand, the inhibition of tubulin polymerization by these analogues could not be correlated with their effects on cell growth. Interestingly, the IC_{50} value of dolastatin 10 was similar to that of compound **1**, which possesses the N-terminal part of dolastatin 10 (2.2 vs 3.6), and the IC_{50} value of dolastatin 15 was similar to that of compound **2**, which possesses the N-terminal part of dolastatin 15 (17 vs 13).

Discussion

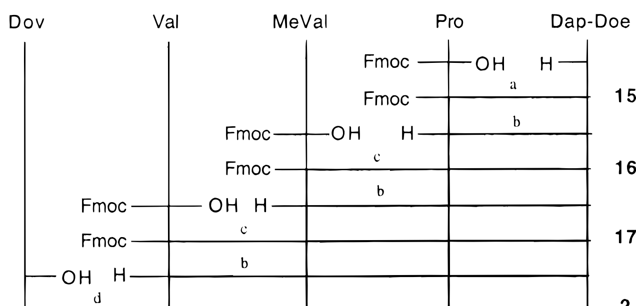
The development of synthetic routes to dolastatin 10[28,31−33] and dolastatin 15[27,34] has permitted preliminary studies of structure−activity relationships.[35−39] However, most of the studies reported so far have dealt with modifications of the side chains. In this context, it has been shown that there is only a limited correlation between inhibition of tubulin polymerization and in vitro cell growth.[35,36] Moreover, various modifications of the Dil or Dap units lead to opposite effects whether assayed in vitro[36] or in vivo.[37] These discrepancies could suggest the involvement of complex interaction mechanisms and also differential transport into cells or intracellular metabolism. Nevertheless, these studies have led to the elucidation of some key features of the structure−activity relationship: in the case of dolastatin 10, Dil unit with an erythro configuration seems to be crucial for both antitumoral and antitubulin activities; a phenyl moiety at the C-terminus and at least one

Scheme 3[a]



[a] Reagents: (a) HCHO, H_2, Pd/C; (b) TFA; (c) Dap-Doe, PyBroP, DIEA; (d) HBr/AcOH.

Scheme 4[a]



[a] Reagents: (a) PyBroP, DIEA; (b) DEA; (c) BroP, DIEA; (d) COMMOD.

Table 1. Inhibition of Cell Growth (IC_{50}, nM) and in Vitro Tubulin Polymerization (IC_{50}, μM)

compound	cell line HT-29	MCF7	L1210	tubulin polymerization
dolastatin 10	0.06	0.03	0.03	2.2
dolastatin 15	0.29	0.04	0.13	17
vincristine	2.06	0.84	0.73	1.8
1	396 (1365)[a]	0.19 (5)[a]	1.58 (12)[a]	3.6
2	11.4 (190)[b]	2.48 (83)[b]	2.72 (90)[b]	13

[a] Ratio IC_{50}(**1**)/IC_{50}(dolastatin 15). [b] Ratio IC_{50}(**2**)/IC_{50}(dolastatin 10).

N-methyl group at the N-terminus are necessary to maintain the activity in both compounds. In the case of dolastatin 15, almost no data have been published concerning modifications of the backbone although several modifications have been revendicated in a patent.[40] Surprisingly, whereas residue deletion is detrimental to cytotoxicity in the case of dolastatin 10,[36] the shortened LU103793 analogue retains the ability of dolastatin 15 to inhibit not only cell proliferation in vitro and in vivo but also tubulin polymerization.[26] Thus, backbone modifications introduced into compounds **1** and **2** could provide another insight into how the Dil unit or the MeVal-Pro dipeptide acts upon the biological activity of both dolastatins 10 and 15.

Coupling the N-terminal part of dolastatin 10 to the C-terminal part of dolastatin 15 (compound **1**) and vice versa (compound **2**), i.e., a Dil versus MeVal-Pro permutation, resulted in a marked decrease in cytotoxic activity (Table 1) depending on the cell line. However, all IC_{50} values (except for the activity of **1** on the HT-29 cell line) remained at the nanomolar level and in the same range as that of vincristine. Comparison of the IC_{50} ratios of **1** to dolastatin 15 and **2** to dolastatin 10 shows that the replacement of MeVal-Pro by Dil in dolastatin 15 and Dil by MeVal-Pro in dolastatin 10 led to more or less identical decreases. From this point of view, these two units seem to induce an almost equivalent perturbation relative to the parent compounds.

The difference between the two compounds was less pronounced by considering the effects on in vitro tubulin polymerization. As already observed by other authors, this activity did not correlate with the cytotoxic potency as both compounds exhibited an inhibitory effect midway between that of dolastatins 10 and 15. Substitution of the MeVal-Pro dipeptide by the Dil residue into dolastatin 15 (compound **1**) led to a lower inhibition constant (3.6 instead of 17 μM), while the introduction of the MeVal-Pro dipeptide into dolastatin 10 (compound **2**) decreased this inhibition (13 instead of 2.2 μM). Our results are in agreement with the findings of Bai et al. who showed that the Dil residue is critical in the tubulin assays and that the N-terminal tripeptide of dolastatin 10 is responsible for nearly the entire antitubulin activity of the molecule.[36]

In the absence of a well-defined biological mechanism, the understanding of the structure–activity relationships of dolastatins is not easy. The ability of dolastatin 10 to bind to tubulin is now well-documented.[24] However, the molecular mechanisms involved are unknown. The assumption that dolastatin 15 binds to tubulin in the same site is based on its structural analogy to dolastatin 10 and on its inhibitory effect on GTP hydrolysis.[22] Within this context, one could assume that conformational modifications are at the origin of the differences between both analogues and their parent compounds. Several approaches to the conformational analysis of dolastatin 10 have been tried based on X-ray diffraction analysis[41] or theoretical molecular design.[42,43] NMR studies[41,44] showed that, in solution, dolastatin 10 exists in two different conformations corresponding to a cis–trans isomerization of the Dil-Dap amide bond, conferring an important bend shape around the Dil-Dap junction. Adversely, in solution all amide bonds are trans in dolastatin 15 thus inducing an extended form of the molecule.[45] The difference between **1** and **2** and their parent compounds could result from perturbation of such an equilibrium. Similarly, if one considers the distal *N*-dimethylamino and phenyl moieties, which were shown to be crucial for the activity of both natural

compounds, they are 19 bonds apart in dolastatin 10 and 21 bonds apart in dolastatin 15. Permutation of the MeVal-Pro and Dil units led to both analogues **1** and **2** with a 20-bond backbone. Thus, our biological results could be interpreted in terms of a structural model where both pharmacophores (the phenyl and the dimethylamino groups) are maintained at an optimum distance. Such a model was already discussed by Bai et al.[21] and could also explain the activity of the analogue LU103793.[26]

In conclusion, although dolastatin 10 and dolastatin 15 exhibit some analogies, our study shows that the Dil unit and the MeVal-Pro dipeptide are not equivalent on the basis of their effects on cell growth nor on the basis of their effects on tubulin polymerization. It is conceivable that they play a determinant role in both phenomena through the induction of an optimal conformation of the drugs. Alternatively, the 4- versus 5-bond backbone could position differently the N- and C-termini. These hypotheses are currently being addressed by structural studies on compounds **1** and **2** and backbone modifications of dolastatins 10 and 15 leading to blocked structures.

Experimental Part

HPLC analyses were performed using an Ultrabase C8 (5 μm, 4.6 × 150 mm; Shandon) column and mixtures of 0.1% TFA in water (solvent A) and 0.1% TFA in CH_3CN (solvent B) at a flow rate of 1.5 mL/min. Preparative HPLC was performed using a Hypersil C18 (10 μm, 25 × 500 mm) column and mixtures of water (solvent A) and CH_3CN (solvent B) at a flow rate of 9 mL/min. Column chromatography was performed on 70−200-μm silica gel. ^{1}H NMR spectra were recorded at 360 MHz.

Coupling General Procedure. To a solution of P-Aaa-OH (1 equiv) and H-Bbb-P′ (1 equiv) in ethanol-free CH_2Cl_2 (2 mL/mmol) were added successively DIEA (3.5 equiv) and the coupling reagent (1.5 equiv). After stirring for 1 h (unless specified otherwise) at room temperature, the reaction mixture was concentrated to dryness under reduced pressure. The residue was dissolved in AcOEt (10 mL/mmol), washed twice with 5% $KHSO_4$ (equal to one-tenth the volume of the organic phase), water (ibid.), 5% $NaHCO_3$ (ibid.), and saturated brine (ibid.), dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. Further purification was performed by column chromatography on silica gel and crystallization. Samples for elemental analysis were dried at 78 °C on KOH for 12 h.

Dov-Val-Dil-Pro-Hiva-Dpy (1). Compound **6** (0.30 g, 0.33 mmol) was solubilized in a mixture of MeOH (4 mL) and water (3 mL) and hydrogenolyzed over 10% Pd on charcoal (60 mg) in the presence of 30% aqueous formaldehyde (2.1 mL) for 48 h at room temperature and atmospheric pressure. The reaction mixture was filtered and concentrated under reduced pressure to furnish an oily residue (0.25 g) which was chromatographed (CH_2Cl_2/MeOH, 90:10). **1** was obtained as a white solid (0.122 g, 46%): mp 98−100 °C; $[\alpha]^{20}_D$ 25° (*c* 1, MeOH); R_f 0.17 (AcOEt/hexane/AcOH, 70:30:1); FABMS *m*/*e* (relative intensity) 812 (M + H, 37), 780 (M − MeOH + H, 7), 685 (Val-Dil-Pro-Hiva-Dpy + H, 2), 586 (Dil-Pro-Hiva-Dpy + H, 4), 401 (Pro-Hiva-Dpy + H, 6), 186 (Dil + H, 20), 154 (Dil − MeOH + H, 20), 128 (Dov, 15), 100 (Val + H, base), 72 ($H_2N{=}CH^+$iPr, 95), 70 (pyrrolinium, base). Anal. ($C_{44}H_{69}N_5O_9$) C, H, N.

Dov-Val-MeVal-Pro-Dap-Doe (2). The Fmoc group of **17** (81 mg, 89 μmol) was removed as described for **16** to yield the free peptide (62 mg). Dov (22 mg, 140 μmol) was activated using COMODD according to a previously described procedure[28] and coupled to the preceding free peptide to yield **2** (39 mg, 70%) as a white powder after preparative HPLC (gradient from 20% to 80% B in 90 min) and lyophilization: mp 190 °C dec; $[\alpha]^{20}_D$ −60° (*c* 0.1, MeOH); t_R 7.9 min (gradient from 20% to 80% in 15 min); FABMS *m*/*e* (relative intensity) 810 (M + H, 10), 471 (H-Pro-Dap-Doe + H, 5), 437 (Dov-Val-MeVal-Pro$^+$, 3), 340 (Dov-Val-MeVal$^+$, 33), 138 (H-Dap$^+$ − MeOH, 53), 100 ($Me_2N^+{=}CH$iPr, 100). Anal. ($C_{43}H_{67}N_7O_6S$) C, H, N.

Boc-Pro-Hiva-Dpy (3). Following the procedure described by Zeggaf et al.,[46] to a cooled (−5 °C) solution of Boc-Pro (0.90 g, 4.12 mmol), Hiva-Dpy (1.25 g, 4.12 mmol) prepared according to Patino et al.,[27] TEA (0.58 mL, 4.12 mmol), and DMAP (0.15 g) in ethanol-free CH_2Cl_2 (8 mL) was dropped a solution of IPCC (0.45 mL, 4.12 mmol) in ethanol-free CH_2Cl_2 (2 mL). After stirring for 30 min at −5 °C and for 4 h at room temperature, the reaction mixture was worked up as usual to yield **3** which was crystallized in AcOEt/hexane (2.00 g, 97%): mp 168−169 °C (lit.[34] 158−159 °C); $[\alpha]^{20}_D$ 135° (*c* 1, MeOH); R_f 0.60 (AcOEt/hexane/AcOH, 40:60:1); ^{1}H NMR (DMSO-d_6) δ 0.88 (d, J = 6.8 Hz, 3 H), 0.99 (d, J = 6.8 Hz, 3 H), 1.38 (s, 9 H), 1.78−1.98 (m, 2 H), 2.05−2.20 (m, 2 H), 2.21−2.31 (m, 1 H), 2.98 (dd, J_1 = 13.7 Hz, J_2 = 3.0 Hz, 1 H), 3.32 (dd, J_1 = 13.7 Hz, J_2 = 8.6 Hz, 1 H), 3.39−3.46 (m, 2 H), 3.80 (s, 3 H), 4.34 (dd, J_1 = 3.0 Hz, J_2 = 8.6 Hz, 1 H), 4.91−4.93 (m, 1 H), 5.10 (s, 1 H), 5.78 (d, J = 2.8 Hz, 1 H), 7.01−7.04 (m, 2 H), 7.17−7.24 (m, 3 H). Anal. ($C_{27}H_{36}N_2O_7$) C, H, N.

Boc-Dil-Pro-Hiva-Dpy (4). Compound **3** (2.00 g, 4.00 mmol) was treated with TFA for 30 min. The solvent was evaporated under reduced pressure to yield the amino-free compound as an oil (2.03 g). This compound was coupled to Boc-Dil (1.43 g, 4.95 mmol) by using PyBOP. After purification by column chromatography (AcOEt/hexane, 30:70), **4** was crystallized in AcOEt/hexane (2.42 g, 88%): mp 155−156 °C; $[\alpha]^{20}_D$ 94° (*c* 1, MeOH); R_f 0.47 (AcOEt/hexane, 40:60); FABMS *m*/*e* (relative intensity) 686 (M + H, 9), 586 (Dil-Pro-Hiva-Dpy + H, 7), 401 (Pro-Hiva-Dpy + H, 7), 286 (Boc-Dil, 6), 91 (tropylium, 22), 57 (tBu$^+$, 40). Anal. ($C_{37}H_{55}N_3O_9$) C, H, N.

Boc-Val-Dil-Pro-Hiva-Dpy (5). Compound **4** (0.60 g, 0.90 mmol) was treated with 3 N HCl in dioxane solution (3 mL) for 1 h at room temperature. The mixture was lyophilized to yield the HCl salt of the amino-free compound as a white powder (0.55 g). This compound was coupled to Boc-Val (0.33 g, 1.35 mmol) by using PyCloP. After the mixture stirred for 12 h, 1 equiv of Boc-Val, 1 equiv of PyCloP, and 2 equiv of DIEA were added. The reaction was complete within 24 h. After purification by column chromatography (AcOEt/hexane, 70:30), **5** was crystallized in AcOEt/hexane (0.51 g, 88%): mp 125−130 °C; $[\alpha]^{20}_D$ 50° (*c* 1, MeOH); R_f 0.42 (AcOEt/hexane/AcOH, 70:30:1); FABMS *m*/*e* (relative intensity) 785 (M + H, 10), 685 (Val-Dil-Pro-Hiva-Dpy + H, 20), 586 (Dil-Pro-Hiva-Dpy + H, 3), 401 (Pro-Hiva-Dpy + H, 5), 186 (Dil + H, 24), 154 (Dil − MeOH + H, 25), 100 (Val + H, 55), 72 ($H_2N{=}CH^+$-iPr, 60), 70 (pyrrolinium, 70), 57 (tBu$^+$, base). Anal. ($C_{42}H_{63}N_4O_{10}$) C, H, N.

Z-Val-Val-Dil-Pro-Hiva-Dpy (6). Compound **5** (0.50 g, 0.64 mmol) was treated with TFA for 30 min. The solvent was evaporated under reduced pressure to yield the amino-free compound as an oil (0.50 g). This compound was coupled to Z-Val by using PyBOP. After purification by column chromatography (AcOEt/hexane, 70:30), **6** was crystallized in AcOEt/hexane (0.42 g, 72%): mp 115−118 °C; $[\alpha]^{20}_D$ 29° (*c* 1, MeOH); R_f 0.39 (AcOEt/hexane/AcOH, 70:30:1); FABMS *m*/*e* (relative intensity) 918 (M + H, 5), 886 (M − MeOH + H, 3), 685 (Val-Dil-Pro-Hiva-Dpy + H, 1), 586 (Dil-Pro-Hiva-Dpy + H, 2), 100 (Val + H, 25), 91 (tropylium, base), 72 ($H_2N{=}CH^+$iPr, 60), 70 (pyrrolinium, 35). Anal. ($C_{50}H_{71}N_5O_{11}\cdot 2H_2O$) C, H, N.

Z-MeVal-Pro-OtBu (7). Z-MeVal (1.00 g, 3.80 mmol) was coupled to Pro-OtBu (0.78 g, 4.5 mmol) by using PyBroP to yield **7** (1.21 g, 76%) as a white solid: mp (Et_2O/hexane); $[\alpha]^{20}_D$ −129° (*c* 1, MeOH); R_f 0.49 (AcOEt/hexane, 50:50); FABMS *m*/*e* (relative intensity) 419 (M + H, 35), 285 (MeVal-Pro-OtBu + H, 11), 91 (tropylium, base); ^{1}H NMR (DMSO-d_6) (two conformers in a ratio of 2:1) major conformer δ 0.79 (d, J = 6.6 Hz, 3 H), 1.38 (s, 9 H), 1.71−1.92 (m, 3 H), 2.12−2.20 (m, 2 H), 2.81 (s, 3 H), 3.54−3.66 (m, 2 H), 4.11−4.17 (m, 1 H),

4.50 (d, $J = 10.9$ Hz, 1 H), 5.05 and 5.18 (AB, $J = 12.1$ Hz, 2 H), 7.30−7.40 (m, 5 H); minor conformer (distinguishable signals) δ 0.92 (d, $J = 6.6$ Hz, 3 H), 1.36 (s, 9H), 2.78 (s, 3 H), 4.33 (d, $J = 10.8$ Hz, 1 H), 5.08 and 5.14 (AB, $J = 12.9$ Hz, 2 H). Anal. ($C_{23}H_{34}N_2O_5$) C, H, N.

Z-Val-MeVal-Pro-OtBu (8). Compound **7** (1.10 g, 2.63 mmol) was solubilized in MeOH (30 mL) and hydrogenolyzed over 10% Pd on charcoal (0.22 g) in the presence of 12 N HCl (90 μL) during 16 h at room temperature and atmospheric pressure. The reaction mixture was filtered and concentrated under reduced pressure to furnish the ammonium chlorhydrate as a white solid (0.88 g, 99%) which was coupled to Z-Val by using PyBroP. After purification by chromatography (AcOEt/hexane, 35:65), **8** was obtained as a pale-yellow oil (1.28 g, 93%): $[\alpha]^{20}_D$ −162° (*c* 1, MeOH); R_f 0.53 (AcOEt/pentane/AcOH, 30:70:1); FABMS, *m*/*e* (relative intensity) 518 (M + H, 12), 384 (M − Z + 2 H, 5), 347 (Z-Val-MeVal$^+$, 100), 285 (H-MeVal-Pro-OtBu + H, 10), 257 (HO_2C-Val-MeVal$^+$, 10), 213 (H-Val-MeVal$^+$, 10), 172 (H-Pro-OtBu + H, 5), 91 (tropylium, 100), 86 ($MeHN^+$=CHiPr, 95), 70 (pyrrolinium, 25), 57 (tBu$^+$, 25); ^{1}H NMR (DMSO-d_6) δ 0.70 (d, $J = 6.7$ Hz, 3 H), 0.79 (d, $J = 6.7$ Hz, 3 H), 0.88 (d, $J = 6.7$ Hz, 6 H), 1.38 (s, 9 H), 1.70−1.80 (m, 2 H), 1.89−1.99 (m, 2 H), 2.08−2.20 (m, 2 H), 3.29 (s, 3 H), 3.47−3.54 (m, 1 H), 3.64−3.70 (m, 1 H), 4.10 (dd, $J_1 = 8.7$ Hz, $J_2 = 3.3$ Hz, 1 H), 4.22 (t, $J = 8.6$ Hz, 1 H), 4.94 (d, $J = 10.9$ Hz, 1 H), 4.98 and 5.07 (AB, $J = 12.7$ Hz, 2 H), 7.29−7.36 (m, 5 H), 7.52 (d, $J = 8.6$ Hz, 1 H). Anal. ($C_{28}H_{43}N_3O_6$) C, H, N.

Z-Val-Val-MeVal-Pro-OtBu (9). Compound **8** (1.20 g, 2.32 mmol) was solubilized in MeOH (30 mL) and hydrogenolyzed over 10% Pd on charcoal (0.24 g) in the presence of 12 N HCl (80 μL) during 16 h at room temperature and atmospheric pressure. The reaction mixture was filtered and concentrated under reduced pressure to furnish the ammonium chlorhydrate as a white solid (0.96 g, 99%) which was coupled to Z-Val by using BOP to lead to **9** as a white foam (1.10 g, 84%): $[\alpha]^{20}_D$ −155° (*c* 1, MeOH); R_f 0.51 (AcOEt/pentane/AcOH, 50:50:1); FABMS *m*/*e* (relative intensity) 617 (M + H, 10), 446 (Z-Val-Val-MeVal$^+$, 65), 356 (HO_2C-Val-Val-MeVal$^+$, 15), 285 (H-MeVal-Pro-OtBu + H, 20), 172 (H-Pro-OtBu + H, 10), 91 (tropylium, 100), 86 ($MeHN^+$=CHiPr, 75), 72 (H_2N^+=CHiPr, 55), 70 (pyrrolinium, 25), 57 (tBu$^+$, 50); ^{1}H NMR (DMSO-d_6) δ 0.67 (d, $J = 6.7$ Hz, 3 H), 0.80 (d, $J = 6.7$ Hz, 6 H), 0.82 (d, $J = 6.7$ Hz, 3 H), 0.87 (d, $J = 6.7$ Hz, 6 H), 1.38 (s, 9 H), 1.71−1.80 (m, 2 H), 1.87−2.00 (m, 3 H), 2.08−2.20 (m, 2 H), 2.99 (s, 3 H), 3.47−3.54 (m, 1 H), 3.64−3.70 (m, 1 H), 3.94 (dd, $J_1 = 6.9$ Hz, $J_2 = 8.9$ Hz, 1 H), 4.10 (dd, $J_1 = 5.2$ Hz, $J_2 = 8.6$ Hz, 1 H), 4.48 (t, $J = 8.6$ Hz, 1 H), 4.92 (d, $J = 10.9$ Hz, 1 H), 5.01 (s, 2 H), 7.21 (d, $J = 8.6$ Hz, 1 H), 7.22−7.35 (m, 5 H), 8.00 (d, $J = 8.6$ Hz, 1 H). Anal. ($C_{33}H_{52}N_4O_7 \cdot H_2O$) C, H, N.

Dov-Val-MeVal-Pro-OtBu (10). Compound **9** (1.00 g, 1.60 mmol) was solubilized in MeOH (30 mL) and hydrogenolyzed over 10% Pd on charcoal (0.24 g) in the presence of 30% aqueous formaldehyde (10.3 mL) during 48 h at room temperature and atmospheric pressure. The reaction mixture was filtered and concentrated under reduced pressure to furnish **10** as a white solid (0.98 g, 98%): mp 84−86 °C (AcOEt/pentane); $[\alpha]^{20}_D$ −149° (*c* 1, MeOH); R_f 0.32 (AcOEt/hexane, 70:30); FABMS *m*/*e* (relative intensity) 511 (M + H, 50), 340 (Dov-Val-MeVal$^+$, 25), 100 (Me_2N^+=CHiPr, 100); ^{1}H NMR (DMSO-d_6) δ 0.69 (d, $J = 6.7$ Hz, 3 H), 0.70 (d, $J = 6.7$ Hz, 3 H), 0.81 (d, $J = 6.7$ Hz, 3 H), 0.86 (d, $J = 6.7$ Hz, 3 H), 0.88 (d, $J = 6.7$ Hz, 3 H), 0.92 (d, $J = 6.7$ Hz, 3 H), 1.38 (s, 9 H), 1.70−1.80 (m, 2 H), 1.86−2.08 (m, 3 H), 2.11−2.20 (m, 2 H), 2.20 (s, 6 H), 3.03 (s, 3 H), 3.47−3.54 (m, 1 H), 3.65−3.71 (m, 1 H), 4.09 (dd, $J_1 = 5.5$ Hz, $J_2 = 8.5$ Hz, 1 H), 4.51 (t, $J = 8.6$ Hz, 1 H), 4.65 (d, $J = 6.2$ Hz, 1 H), 4.95 (d, $J = 10.9$ Hz, 1 H), 8.01 (d, $J = 8.6$ Hz, 1 H). Anal. ($C_{27}H_{50}N_4O_5$) C, H, N.

Dov-Val-MeVal-Pro-OH (11). Ester **10** (51 mg, 0.10 mmol) was treated with TFA during 30 min, and the solvent was evaporated under reduced pressure. The residue was solubilized in 2 N HCl and lyophilized to yield the hydrochloride salt of **11** as a white powder (50 mg, 100%): FABMS *m*/*e* (relative intensity) 455 (M + H, 60), 340 (Dov-Val-MeVal$^+$, 45), 100 (Me_2N^+=CHiPr, 100).

Z-Val-Val-MeVal-Pro-OH (12). Compound **9** (55 mg, 0.090 mmol) was treated with 3 N HCl in dioxane (1.5 mL). After 5 h, the mixture was lyophilized to yield **12** as a white powder.

Z-Val-Val-MeVal-Pro-Dap-Doe (13). Compound **12** (27 mg, 0.080 mmol) was coupled to Dap-Doe prepared according to Roux et al.[28] by using PyBroP to yield **13** as a white gum (49 mg, 82%) after preparative HPLC (gradient from 40% to 90% B in 60 min) and lyophilization: R_f 0.25 (CH_2Cl_2/MeOH, 70:30); t_R 8.1 min (gradient from 40% to 90% in 15 min); FABMS *m*/*e* (relative intensity) 916 (M + H, 10), 543 (Z-Val-Val-MeVal-Pro$^+$, 10), 447 (H-Val-MeVal-Pro-Dap$^+$ − MeOH, 90), 446 (Z-Val-Val-MeVal$^+$, 100), 356 (HO_2C-Val-Val-MeVal$^+$, 15), 333 (Z-Val-Val$^+$, 10), 234 (Z-Val$^+$, 40), 188 (Doe − NH, 45), 138 (H-Dap$^+$ − MeOH, 20), 114 (H-MeVal$^+$, 15), 91 (tropylium, 100), 86 ($MeHN^+$=CHiPr, 100), 72, (H_2N^+=CHiPr, 100), 70 (pyrrolinium, 70). Anal. ($C_{49}H_{69}N_7O_8S$) N. C: calcd, 64.24; found, 63.85. H: calcd, 7.59; found, 7.10.

H-Pro-Dap-Doe (14). To a cooled solution of **13** (30 mg, 0.032 mmol) in CH_2Cl_2 (0.5 mL) was added 30% HBr/AcOH (0.5 mL). After the mixture stirred for 1 h, the solvent was evaporated under reduced pressure and the residue purified by preparative HPLC (gradient from 10% to 80% B in 60 min) to yield **14** as a brown gum after lyophilization (10 mg, 64%): t_R 5.1 min (gradient from 20% to 80% in 15 min); FABMS *m*/*e* (relative intensity) 471 (M + H, 83), 373 (H-Dap-Doe + H, 19). Anal. ($C_{25}H_{34}N_4O_3S$) C, H, N.

Fmoc-Pro-Dap-Doe (15). Fmoc-Pro-OH (0.210 g, 0.60 mmol) was coupled to HCl-Dap-Doe prepared according to Roux et al.[28] by using PyBroP to furnish **15** as a white gum (0.185 g, 90%): R_f 0.36 (CH_2Cl_2/MeOH, 95:5); t_R 13.5 min (gradient from 20% to 80% in 20 min). Anal. ($C_{40}H_{44}N_4O_5S$) C, H, N.

Fmoc-MeVal-Pro-Dap-Doe (16). A solution of compound **15** (0.185 g, 0.27 mmol) in DMF (1 mL) was treated with diethylamine (0.3 mL) at room temperature for 30 min. After evaporation of the solvent, the resulting amino-free peptide was obtained as a pale-yellow oil (0.124 g) and coupled to Fmoc-MeVal-OH (0.180 g, 0.52 mmol) prepared according to Freidinger by using BroP reagent to furnish **16** as a white gum (0.188 g, 92%): R_f 0.43 (AcOEt); t_R 14.5 min (gradient from 20% to 80% in 20 min); FABMS *m*/*e* (relative intensity) 806 (M + H, 20), 433 (Fmoc-MeVal-Pro$^+$, 10), 188 (Doe − NH, 9), 138 (H-Dap$^+$ − MeOH, 10), 179 (fluorenylmethylenium, 100). Anal. ($C_{46}H_{55}N_5O_6S$) C, H, N.

Fmoc-Val-MeVal-Pro-Dap-Doe (17). By following the procedure described for the preparation of **16**, **16** (0.188 g, 0.24 mmol) led to **17** (0.160 g, 80%): R_f 0.30 (AcOEt); t_R 14.4 min (gradient from 20% to 80% in 20 min); FABMS *m*/*e* (relative intensity) 905 (M + H, 8), 471 (H−Pro-Dap-Doe + H, 8), 435 (Fmoc-Val-MeVal$^+$, 50), 138 (H-Dap$^+$ − MeOH, 60), 179 (fluorenylmethylenium, 100). Anal. ($C_{51}H_{64}N_6O_7S$) C, H, N.

Purification of Microtubule Protein and Assembly Assay. Microtubule proteins were purified from bovine brain by cycles of assembly at 37 °C and disassembly at 0 °C essentially according to the method of Shelanski.[47] Fresh bovine brains were cooled on ice, minced, and homogenized in buffer A (100 mM MES, 0.5 mM $MgCl_2$, 1 mM EGTA, pH 6.6). After centrifugation at 90000*g* for 45 min at 4 °C, the supernatant was mixed with an equal volume of buffer B (buffer A, 8 M glycerol, 1 mM GTP) and warmed at 37 °C for 30 min. The polymerized tubulin was collected as a pellet by centrifugation at 98000*g* for 1 h at 30 °C. The pellet was resuspended in buffer A, homogenized, and chilled at 4 °C for 30 min. Centrifugation of the suspension at 90000*g* for 30 min at 4 °C led to a supernatant which was mixed with buffer B and then warmed at 37 °C for 30 min. After centrifugation for 60 min at 30 °C, the supernatant was suspended in buffer A and 0.5 M GTP and stored at −80 °C until required. Immediately before use, the protein solution was mixed with buffer A and 1 mM GTP to give a concentration of 2 mg of protein/mL, and the solution was kept on ice. Assembly was evaluated by measuring turbidity at 350 nm and 37 °C.[48]

Assembly was initiated by a temperature jump from 4 to 37 °C. Each drug was added as a solution in DMSO. The IC_{50} is defined as the drug concentration inducing a 50% inhibition of the rate of assembly.

Inhibition of Cell Growth. L1210 cells were provided by the National Cancer Institute and HT-29 and MCF7 cells by the American Type Culture Collection (Frederick, MD). Cells were cultivated in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 10 mM HEPES buffer (pH 7.4). Cytotoxicity was measured by the microculture tetrazolium assay as described.[49] Cells were exposed to graded concentrations of the compounds (nine serial dilutions in triplicate) for about four doubling times, i.e., 48 h (L1210), 96 h (HT-29), or 168 h (MCF7). Results are expressed as IC_{50}, the concentration which reduced by 50% the optical density of treated cells with respect to untreated controls.

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