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Tubulin-guided dynamic combinatorial library of thiocolchicine–podophyllotoxin conjugates

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ABSTRACT

The use of tubulin as a target to influence the composition of the mixture from a dynamic combinatorial library, based on the disulfide bond exchange reaction, is described. ESI-FT-ICR-MS was used to determine the composition of the library. The heterodimeric compound amplified by this approach was used to design the homologous derivative with a two-carbon spacer in place of the disulfide function. The ability of the compounds to inhibit tubulin polymerization is reported and compared to thiocolchicine.

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1. Introduction

Tubulin and microtubules remain one of the most interesting targets in the design of new anticancer compounds.[1] In addition, increasing evidence supports a pathogenic role for tubulin in the neurodegeneration underlying several diseases, including Alzheimer's and Parkinson's disease,[2a,b] and the therapeutic potential of anti-tubulin drugs for treating neurodegeneration has been very recently proposed.[2c]

The discovery of new compounds that bind to tubulin is a challenging research aim with implications for the design and preparation of new high-quality collections of compounds useful for the study of the regulation of different ailments of modern society. The generation of divalent or multivalent compounds is a well-known strategy inspired by nature, that has produced a number of interesting results.[3] In our lab we accomplished the preparation of a number of multivalent conjugates, using as building blocks several natural products with well-documented pharmacological activity.[4]

Target-guided synthesis[5] emerged in the last decade as an important strategy that increases the versatility of combinatorial chemistry and in particular of Dynamic Combinatorial Libraries[6] (DCL), and has provided a demonstration of the evolutionary process that generates the tremendous molecular diversity of natural products.[7] In our earlier work, we have demonstrated the potential of the disulfide-exchange reaction to generate a dynamic library of thiocolchicine–podophyllotoxin adducts and the influence on the composition of this mixture induced by subtilisin or albumin.[8] Our next aim was to demonstrate the possibility of using tubulin to guide the evolutionary formation of divalent binders by favouring the structure that is thermodynamically most stabilized by non-covalent interactions with the biological target (Fig. 1).

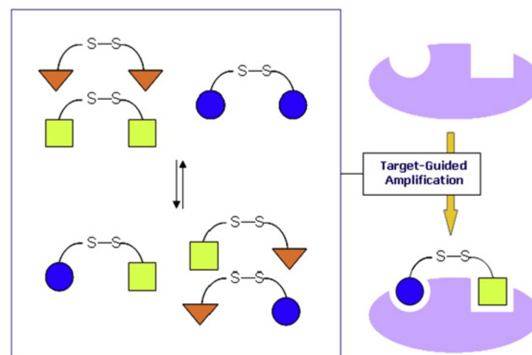

Fig. 1. General concept for the target-guided DCL based on the disulfide-exchange reaction.

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In this paper we report the use of thiocolchicine and podophyllotoxin (Fig. 2) for the generation of a dynamic combinatorial mixture of homo- and heterodimeric compounds based on the reversible formation of disulfide bonds.[9] The building blocks for the library were the homodimers obtained by condensation of thiocolchicine and podophyllotoxin with dicarboxylic acids incorporating a disulfide bond.

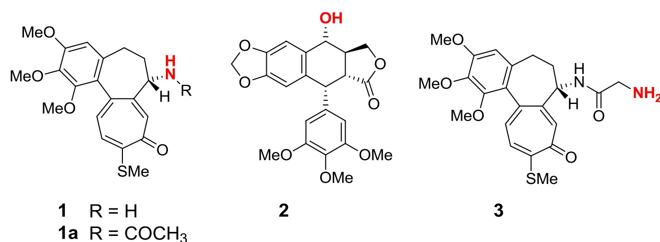


Fig. 2. Structure of deacetylthiocolchicine **1**, podophyllotoxin **2** and glycinoyl-deacetylthiocolchicine **3**.

Although thiocolchicine[10] and podophyllotoxin[11] recognize the same binding site, previous work in other areas has shown that homo- and heterodimers can have biological characteristics that are not merely the sum of the properties of the single entities.[3a] For this reason, we cannot predict if the interaction will be with single α- or β-tubulin, with the α-β heterodimer or with the microtubule assembly and this makes it of interest to consider conjugate compounds.

2. Results and discussion

We needed to generate a water soluble library, allowing us to work in aqueous conditions that would maintain the coherent structure of the biological target. Thus, our first task was to prepare a range of water soluble homodimeric starting compounds. We designed three different homodimers (Fig. 3) in which the spacer that connects the two entities presents primary amino and amide groups. They were obtained by condensation between podophyllotoxin **2**, deacetylthiocolchicine **1** and deacetylglycinyl-thiocolchicine **3** with *N*-Boc-cystine. The removal of the Boc group generated, respectively, compounds **4**,[4a] **5**[4a] and **6**, all of which had a satisfactory water solubility (1.2–1.5 mg/mL, as ditartrate salts). The three homodisulfides were mixed in a buffered solution at a concentration of 5×10^{-3} mM and maintained at room temperature for 96 h. The composition of the mixture was then evaluated by ESI-FT-ICR-MS (Supplementary data), which showed all six possible compounds due to the formation of the heterodimers **7**–**9** (Fig. 4). In parallel we prepared a second solution containing a cytoskeleton fraction to give a 2.1×10^{-6} M solution of tubulin. The analysis after 96 h revealed a marked alteration of the mixture composition (Table 1) and, in particular, we observed the amplification of the heterodimer **9**.

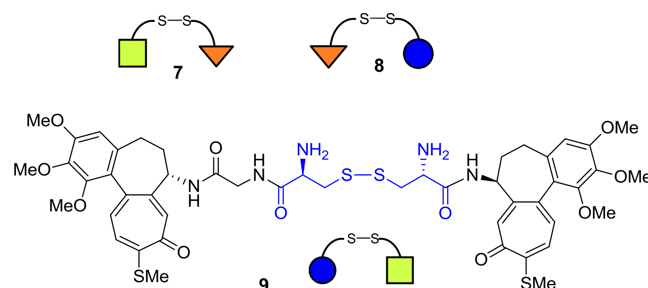


Fig. 4. Structures of compounds **7**–**9**.

Table 1
Comparison of the percentages of dimeric structures based on the relative intensity of the mass peaks in the presence or absence of tubulin

Compound	Mass peak	No biol. target	With biol. target
4	951.28422	58.3	37.5
5	1033.27563	3.8	—
6	1065.32572	27.6	44.6
7	992.27994	1.3	—
8	1049.29456	2.5	—
9	1008.30411	6.4	17.8

We then undertook the synthesis of compound **12** that resembles the structure of the selected compound **9** but with an ethylene group in the place of the liable disulfide bond to guarantee the binary structure in the cell medium. The condensation of the dicarboxylic acid **10**[4a] with deacetylthiocolchicine **1** gave adduct **11** that was subsequently reacted with glycinoylthiocolchicine to obtain, after Boc removal, the desired compound **12** (Scheme 1).

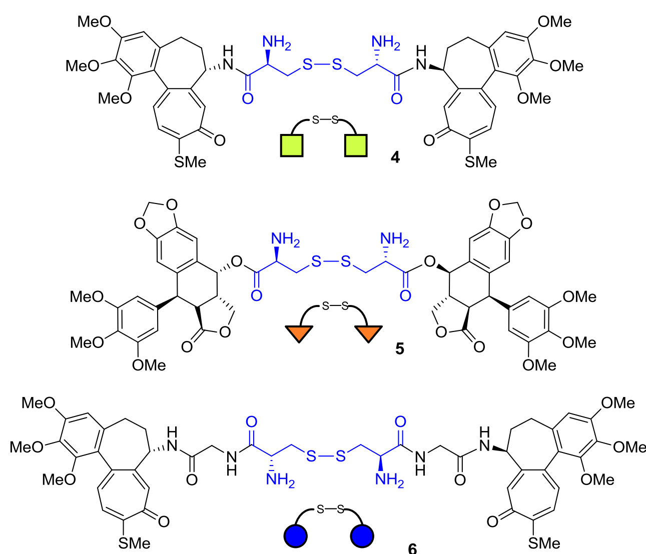
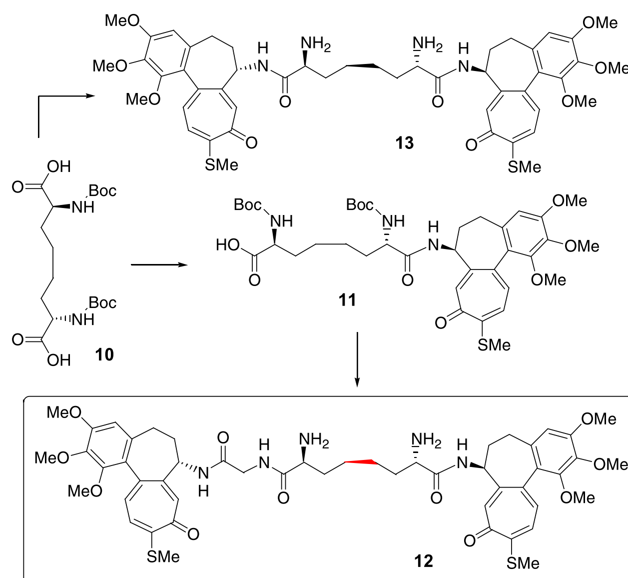


Fig. 3. Structures of compounds **4**–**6**.



Scheme 1. Synthesis of compound **12**.

To get an insight into the potential biological activity of **12** we investigated its ability to affect tubulin polymerization in vitro in comparison with the homodimers **4**–**6** and with the symmetric compound **13** that is missing the glycine framework. Bovine tubulin (purified from brain) was mixed with a standard solution of each sample in the absence of GTP. The solutions were incubated at 37 °C to allow slow-binding compounds to bind to the tubulin, and after 15 min GTP was added to initiate microtubule assembly. After 30 min the polymerized and the unpolymerized fractions were collected by centrifugation, separated by SDS-PAGE and quantified by densitometry as previously described.[4a] Compound **12** proved more active than two of the starting disulfide homodimers (**4** and **5**, Table 2), although the homodimer **6** had the highest activity. Comparison with analogue **13** underlines the importance of the glycine spacer. Furthermore, we demonstrated that inhibition of tubulin polymerization by compound **12** is dose-dependent (Fig. 5).

Table 2
Ratios of polymerized/unpolymerized tubulin obtained in the presence of compounds **4**, **5**, **6**, **12** and **13**

Compound (10 μM)	P/U tubulin (mean±SEM)
Control	10.2±0.36
4	5.9±0.14[b]
5	7.2±0.18[a]
6	3.9±0.12[c]
12	5.4±0.43[c]
13	9.2±0.21

[a] $p<0.05$.
[b] $p<0.002$.
[c] $p<0.001$ versus control, according to ANOVA, Fisher LSD post-hoc.

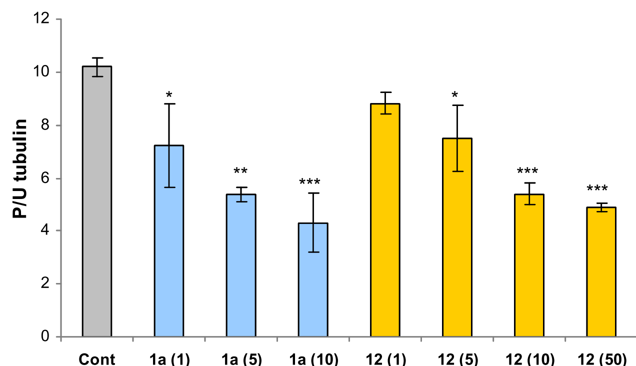


Fig. 5. Ratios of polymerized/unpolymerized tubulin obtained in the presence of different concentrations of thiocolchicine **1a** (1, 5 and 10 μM) and compound **12** (1, 5, 10 and 50 μM). Data are expressed as means±SEM of values of at least three independent experiments. *$p<0.05$, **$p<0.002$, ***$p<0.001$ versus control, according to ANOVA, Fisher LSD post-hoc.

3. Conclusion

Tubulin has been used as a biological target to guide the assembly of a binary compound in a DCL approach based on the disulfide-exchange reaction. The structure of the selected heterodimeric entity, derived from the precursor mixture of homodimers, inspired the design of the corresponding compound lacking the disulfide bond. Replacing the disulfide bond with an ethylene spacer resulted in a compound that inhibits the polymerization of tubulin in a dose-dependent manner. This result further demonstrates the power of target-guided synthesis used in conjunction with DCLs and underlines the pivotal role of weak interactions with biomacromolecules.

4. Experimental section

4.1. General

All reagents and solvents were reagent grade or were purified by standard methods before use. Column chromatography was carried out on flash silica gel (Merck 230–400 mesh). NMR spectra were recorded at 300/400 MHz (^{1}H) and at 75/100 MHz (^{13}C). FAB$^+$ mass spectra were recorded at an ionizing voltage of 6 Kev. ESI mass spectra were recorded on FT-ICR instrument.

*4.1.1. Thiocolchicine derivative (**3**).* To a solution of **1** (100 mg, 0.27 mmol) in CH_2Cl_2 (15 mL), *N*-Boc-glycine (95 mg, 0.59 mmol), DCC (334 mg, 1.62 mmol) and DMAP (66 mg, 0.54 mmol) were added. The reaction mixture was stirred at room temperature for 16 h, then filtered through Celite. The solvent was concentrated in vacuo and the residue was purified by column chromatography on silica gel (AcOEt/hexane 20:1) to afford the desired intermediate as a yellow amorphous solid (64 mg, 47%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.49 (1H, s), 7.35–7.38 (1H, m), 7.07–7.11 (1H, m), 6.54 (1H, s), 4.61–4.89 (3H, m), 3.41–3.93 (11H, m), 2.32–2.57 (5H, m), 1.94 (2H, m), 1.45 (9H, s). ^{13}C NMR (100 MHz, $CDCl_3$, detected signals): δ 182.3, 169.6, 158.2, 156.3, 154.5, 151.0, 141.8, 139.1, 135.5, 131.4, 128.1, 107.2, 80.6 (3C), 61.5, 61.3, 56.1, 52.1, 45.0, 36.4, 29.9, 28.3, 15.3. Anal. Calcd for $C_{27}H_{34}N_2O_7S$: C, 61.11; H, 6.46; N, 5.28. Found: C, 61.14; H, 6.41; N, 5.30. To a freshly prepared saturated solution of HCl in dioxane (21 mL) the previously obtained intermediate (130 mg, 0.26 mmol) was added and the reaction mixture was stirred at 0 °C for 1 h. The solvent was removed in vacuo and the residue was dissolved in CH_2Cl_2 and washed with saturated $NaHCO_3$. The combined organic layers were dried over sodium sulfate and concentrated in vacuo to afford **3** as a yellow amorphous solid (107 mg, 99%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.51 (1H, s), 7.91–7.92 (2H, m), 7.35–7.52 (2H, m), 3.93 (9H, s), 3.73 (2H, m), 3.47 (4H, m), 2.79–2.91 (4H, m), 1.94 (1H, m), 1.74 (1H, m). ^{13}C NMR (100 MHz, $CDCl_3$, detected signals): δ 182.3, 158.2, 156.3, 154.5, 151.0, 141.8, 139.1, 135.5, 131.4, 128.1, 107.2, 61.5, 61.3, 56.1, 52.1, 45.0, 36.4, 29.9, 15.3. Anal. Calcd for $C_{22}H_{26}N_2O_5S$: C, 61.38; H, 6.09; N, 6.51. Found: C, 61.42; H, 6.08; N, 6.48.

*4.1.2. Thiocolchicine derivative (**6**).* To a solution of $(Boc\text{-}cys\text{-}OH)_2$ (114 mg, 0.26 mmol) in dry THF/CH_3CN 8:1 (9 mL), compound **3** (236 mg, 0.055 mmol), DCC (163 mg, 0.79 mmol) and DMAP (32 mg, 0.26 mmol) were added. The reaction mixture was stirred at 50 °C for 5 h. The solvent was removed in vacuo and the residue was purified by column chromatography on silica gel (CH_2Cl_2/MeOH 14:1) to afford the desired intermediate as a yellow amorphous solid (307 mg, 92%). $[\alpha]_D^{20}$ −5.4 (*c* 0.84, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.55 (2H, d, *J* 6.85 Hz), 7.88 (2H, s), 7.66 (2H, s), 7.38 (2H, d, *J* 10.5 Hz), 7.16 (2H, d, *J* 10.5 Hz), 6.53 (2H, s), 5.89 (2H, d, *J* 4.90 Hz), 4.72–4.77 (4H, m), 4.09–4.16 (2H, m), 3.95 (6H, s), 3.91 (6H, s), 3.82–3.85 (2H, m), 3.65 (6H, s), 3.23–3.26 (2H, m), 3.01–3.02 (2H, m), 2.46 (6H, s), 2.45–2.47 (2H, m), 2.24–2.27 (4H, m), 1.84–1.88 (2H, m), 1.35 (18H, s); ^{13}C NMR (100 MHz, $CDCl_3$): δ 182.7, 171.6, 169.3, 159.4, 156.2, 154.6, 152.9, 151.9, 142.6, 139.8, 135.8, 135.0, 129.6, 127.8, 126.3, 108.4, 80.8, 55.1, 52.8, 44.5, 43.8, 37.1, 30.7, 29.0, 15.9. Anal. Calcd for $C_{60}H_{76}N_6O_{16}S_4$: C, 56.94; H, 6.05; N, 6.64. Found: C, 56.98; H, 6.01; N, 6.62. To a freshly prepared saturated solution of HCl in dioxane the previously obtained intermediate (43 mg, 0.03 mmol) was added and the reaction mixture was stirred for 2 h at 0 °C. The solvent was removed in vacuo and the residue was dissolved in CH_2Cl_2 and washed with saturated $NaHCO_3$. The combined organic layers were dried over sodium sulfate and concentrated in vacuo to afford **6** as a yellow amorphous solid (33.7 mg, 94%). $[\alpha]_D^{20}$ +0.2 (*c* 0.33, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.07 (2H, d, *J* 0.32 Hz), 8.58 (2H, br s), 7.68 (2H, s), 7.32 (2H,

d, *J* 10.3 Hz), 7.10 (2H, d, *J* 10.3 Hz), 6.52 (2H, s), 4.60–4.66 (6H, m), 4.39–4.43 (2H, m), 3.94 (6H, s), 3.90 (6H, s), 3.77–3.81 (2H, m), 3.68 (6H, s), 3.63–3.65 (2H, m), 3.16–3.19 (2H, m), 2.72–2.76 (2H, m), 2.45–2.53 (2H, m), 2.44 (6H, s), 2.22–2.29 (4H, m), 1.87–1.89 (2H, m); ^{13}C NMR (100 MHz, $CDCl_3$, detected signals): δ 182.3, 175.6, 169.6, 158.2, 153.5, 152.8, 151.0, 141.8, 139.1, 135.5, 134.5, 129.1, 127.1, 125.9, 107.2, 61.5, 54.9, 52.7, 45.0, 43.3, 35.5, 30.1, 15.3. Anal. Calcd for $C_{50}H_{60}N_6O_{12}S_4$: C, 56.37; H, 5.68; N, 7.89. Found: C, 56.40; H, 5.63; N, 7.86.

*4.1.3. Thiocolchicine derivative (**12**).* To a solution of **1** (18 mg, 0.051 mmol) in dry CH_2Cl_2 (4 mL) compound **10** (20 mg, 0.051 mmol), DCC (15 mg, 0.072 mmol) and DMAP (6 mg, 0.048 mmol) were added. The reaction mixture was stirred at room temperature for 20 h, then filtered through Celite and washed with saturated K_2CO_3. The combined aqueous layers were acidified and extracted with CH_2Cl_2. The combined organic layers were dried over sodium sulfate and concentrated in vacuo to afford compound **11** as a yellow amorphous solid (11 mg, 56%). ^1H NMR (300 MHz, $CDCl_3$): δ 7.74–7.76 (1H, m), 7.53–7.57 (1H, m), 7.00–7.10 (1H, m) 6.52 (1H, s), 4.18–4.22 (2H, m), 3.93 (1H, m), 3.89 (3H, s), 3.65 (6H, s), 3.42 (1H, m), 2.41 (3H, s), 2.26–2.30 (2H, m), 1.71–1.93 (2H, m), 1.43–1.68 (4H, m), 1.24 (18H, s), 0.81–0.93 (4H, m). Anal. Calcd for $C_{38}H_{53}N_3O_{11}S$: C, 60.06; H, 7.03; N, 5.53. Found: C, 60.09; H, 7.00; N, 5.51. To a solution of **11** (30 mg, 0.039 mmol) in dry CH_2Cl_2 (6 mL), compound **3** (18 mg, 0.042 mmol), DCC (13 mg, 0.06 mmol) and DMAP (5 mg, 0.042 mmol) were added. The reaction mixture was stirred at room temperature for 20 h then filtered through Celite. The solvent was removed in vacuo and the residue was purified by column chromatography on silica gel (AcOEt/hexane 8:2) to afford the desired intermediate as a yellow amorphous solid (28 mg, 59%). ^1H NMR (300 MHz, $CDCl_3$): δ 8.16–8.21 (2H, m), 7.65–7.78 (2H, m), 7.38–7.46 (2H, m), 6.98–7.12 (2H, m), 6.57 (2H, s), 4.68–4.75 (4H, m), 4.19–4.31 (4H, m), 3.85–4.04 (6H, m), 3.58–3.78 (12H, m), 2.88 (2H, s), 2.53 (6H, s), 1.9–2.35 (4H, m), 1.5–1.72 (4H, m), 1.37–1.49 (6H, m), 1.24 (18H, s). Anal. Calcd for $C_{60}H_{77}N_5O_{15}S_2$: C, 61.47; H, 6.62; N, 5.97. Found: C, 61.51; H, 6.59; N, 5.98. To a freshly prepared saturated solution of HCl in dioxane the previously obtained intermediate (7.5 mg, 0.0062 mmol) was added and the reaction mixture was stirred for 1 h at 0 °C. The solvent was removed in vacuo and the residue was dissolved in CH_2Cl_2 and washed with saturated $NaHCO_3$. The combined organic layers were dried over sodium sulfate and concentrated in vacuo to afford **12** as a yellow amorphous solid (4 mg, 57%). $[\alpha]_D^{24}$ −3.1 (*c* 0.17, $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$): δ 8.16–8.21 (2H, m), 7.65–7.78 (2H, m), 7.38–7.46 (2H, m), 6.98–7.12 (2H, m), 6.57 (2H, s), 4.68–4.75 (4H, m), 4.19–4.31 (4H, m), 3.85–4.04 (6H, m), 3.58–3.78 (12H, m), 2.88 (2H, s), 2.53 (6H, s), 1.9–2.35 (4H, m), 1.5–1.72 (4H, m), 1.37–1.49 (6H, m). ^{13}C NMR (100 MHz, $CDCl_3$) detected signals: δ 181.1, 171.1, 157.3, 155.3, 153.6, 52.7, 151.1, 141.6, 138.7, 133.6, 129.7, 125.9, 125.6, 108.3, 61.7, 60.8, 58.7, 56.1, 54.3, 44.0, 36.1, 34.5, 29.1, 22.8 15.5; HRMS (ESI): *m*/*z*: calcd for $C_{50}H_{61}N_5O_{11}S_2Na^+$: 1023.2192. Found: 1022,6579.

4.1.4. Disulfide-exchange reaction in the presence of tubulin. 5×10^{-3} mmol of **4**·tartrate, **5**·tartrate and **6**·tartrate were dissolved in 20 mL of a 1:1 mixture of PEM buffer [10% glycerol, 2 mM EGTA, 1 mM $MgCl_2$, 10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonicacid)] and water (HPLC purity grade). DMSO (1 mL) was added to the solution. The solution was divided in two equal amounts to give *Solution A* and *Solution B*. 5×10^{-3} mmol of **4**·tartrate, **5**·tartrate and **6**·tartrate were dissolved in water (10 mL) and DMSO (1 mL) and pH was adjusted to pH 8 by addition of Na_2CO_3 (satd sol) to obtain *Solution C*. A tubulin preparation that contained the microtubule-enriched fraction was prepared from PC12 cells following Triton-X 100 extraction as previously described.[12] The tubulin preparation (194 μL of Triton-X 100 insoluble fraction containing 2.25 mg of tubulin) was added to solution A (final concentration of tubulin was 2.1×10^{-6} M). Solutions B and C were used as references. The solutions were maintained at room temperature for 96 h then submitted to ESI-FT-ICR-MS.

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Supplementary data

Supplementary data associated with this article can be found in online version at doi:10.1016/j.tet.2011.07.038.

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