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Design, synthesis, and biological testing of pyrazoline derivatives of combretastatin-A4

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Abstract—Fourteen *N*-acetylated and non-acetylated 3,4,5-tri- or 2,5-dimethoxypyrazoline analogs of combretastatin-A4 (**1**) were synthesized. A non-acetylated derivative (**5a**) with the same substituents as CA-4 (**1**) was the most active compound in the series, with IC_{50} values of 2.1 and 0.5 μM in B16 and L1210 cell lines, respectively. In contrast, a similar compound with an acetyl group at N1 of the pyrazoline ring (**6g**) showed poor activity in the cell lines studied. A cell-based assay indicated that compound **5a** caused extensive microtubule depolymerization with an EC_{50} value of 7.1 μM in A-10 cells while no activity was seen with the acetylated compound. Molecular modeling studies showed that these compounds possess a twisted conformation similar to CA-4 (**1**).

In recent years, there has been an intense effort to develop anti-cancer drugs that damage the tumor vasculature, obstruct blood flow, and ultimately kill the tumor. One class of compounds that have proven to be exceptional for such an approach is the combretastatins. Isolated from the African willow tree *Combretum caffrum*, combretastatin-A4 (**1**, CA-4, Fig. 1) binds to tubulin within the colchicine binding site, and it disrupts normal mitotic spindle functions.[1,2] Evidence suggests that its antivascular actions might be mediated through the vascular endothelial-cadherin signaling pathway.[3a] However, CA-4 (**1**) does come with drawbacks as a possible anti-tumor drug; the natural product has limited bioavailability and is poorly soluble in biological media.[3b] These limitations have led to the development of structural analogs of CA-4 such as the water-soluble phosphate prodrugs: CA-4P, AC-7739, and its amino acid derivative (AVE-8062), which have all shown encouraging potency and improved solubility.[1c] Research to develop new analogs with a more favorable therapeutic window is ongoing.[4a]

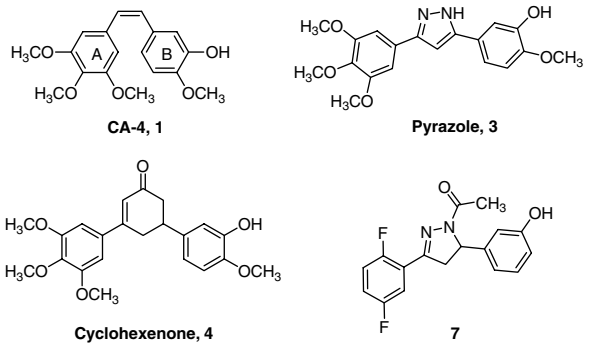

Figure 1. Structures of combretastatin A4 (CA-4, **1**), a pyrazole (**3**), a cyclohexenone (**4**), and a difluoro-analog (**7**).

The enone-containing chalcones (e.g., **2**, Scheme 1) have also been shown to inhibit tubulin polymerization.[2a,4] A number of substituted chalcones were synthesized within the authors' laboratory to study the effects of various functional groups on potency and solubility in biological media. Biological activity was observed with the chalcones, however further development was halted, as these

Keywords: Combretastatins; Tubulin; Anti-cancer; Pyrazolines; CC-1065; Duocarmycins; Pyrrolobenzodiazepines; Achiral; *seco*-Amino-CBI; Dimer.
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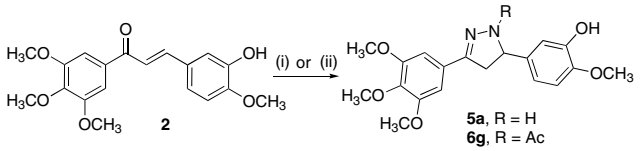


Scheme 1. (i) Hydrazine hydrate, ethanol, reflux, 16 h; (ii) hydrazine hydrate, acetic acid, reflux, 3 h.

compounds can undergo Michael reactions with biological nucleophiles, such as glutathione,[5] hence reducing their reliability as selective tubulin inhibitors.

Furanones, isoxazoles, imidazoles, triazoles, azetidinones, and pyrazole-containing (e.g., **3**, Fig. 1) analogs of CA-4 (**1**) have also been synthesized and the cytotoxicity results revealed varying degrees of potency amongst the different compounds.[6] Studies in the authors' laboratory revealed an unanticipated drop in potency of the pyrazole compounds. X-ray crystallography demonstrated that these analogs were planar, due to aromaticity of the pyrazole,[6f] compared to the twisted geometry of CA-4 (**1**).[6g] Thus the pyrazole loss of activity was directly related to an absence of twisted geometry.

As a result of these findings, the focus returned to alternative non-aromatic groups that would yield the necessary twisted structures required to maintain activity, yet continue to promote water solubility. A series of cyclohexenone derivatives (e.g., compound **4**, Fig. 1) were thus synthesized and their ability to inhibit the growth of L1210 and B16 cells (murine leukemia and melanoma cell lines, respectively) determined. Compound **4**, with the same substituents as CA-4 (**1**), proved to be the most potent (IC_{50} = 0.91 μM in L1210) and exposure to A-10 aortic cells produced a significant reduction in cellular microtubules (EC_{50} = 28 μM). Molecular modeling indicated that compound **4** adopts the required twisted geometry of CA-4 (**1**), suggesting that activity is related to the conformational shape of these compounds.[7] However, although the correct geometry for activity was introduced, these compounds are also Michael acceptors, hence likely to react with glutathione (ca. chalcones, mentioned previously). Therefore further structural designs of CA-4 analogs were investigated and are reported herein.

A series of pyrazoline analogs of CA-4 (e.g., **5**, **6**) were developed to address the previously discussed limitations: the non-aromatic, polar pyrazoline moiety should enable the molecules to adopt the correct geometry for activity and improve biological solubility, and Michael reactions should not present an issue. Fourteen pyrazolines with various substituents on the A-and B-rings were synthesized and assessed for biological activity. Specifically, three categories of compounds were synthesized: a series of non-acetylated 3,4,5-trimethoxypyrazolines (**5a**–**c**, Table 1), a range of *N*-acetylated 3,4,5-trimethoxypyrazolines (**6a**–**g**, Table 2), and a group of acetylated 2,5-dimethoxypyrazolines (**8a**–**c,** Table 3). The acetylated molecules (**6** and **8**) were also of interest as Hartman and colleagues recently showed that *N*-acetylated pyrazolines, such as compound **7** (Fig. 1), are inhibitors of kinesin spindle protein (KSP); potentially useful for the treatment of cancer.[8]

Table 1. Cytotoxicity data for non-acetylated 3,4,5-trimethoxypyrazolines (**4a**–**c**)

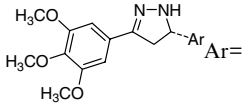
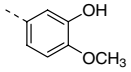
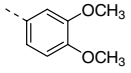
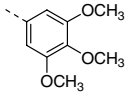
		IC_{50} (μM)	
		B16	L1210
 Ar=			
	**5a**	2.1	0.5
	**5b**	44	42
	**5c**	56	24
CA-4 (**1**)[13,14]		0.002	0.003

Table 2. Cytotoxicity data for acetylated 3,4,5-trimethoxypyrazolines (**5e**–**k**)

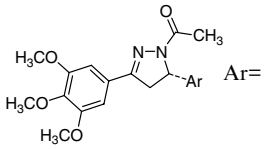
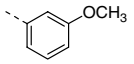
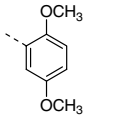
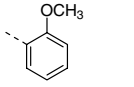
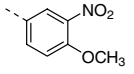
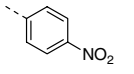
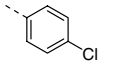
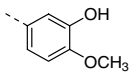
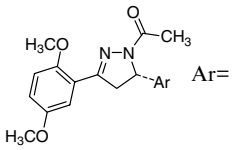
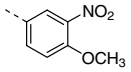
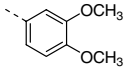
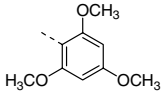
		IC_{50} (μM)	
		B16	L1210
 Ar=			
	**6a**	>100	44
	**6b**	>100	>100
	**6c**	>100	43
	**6d**	>100	52
	**6e**	51	42
	**6f**	35	30
	**6g**	>100	>100

Table 3. Cytotoxicity data for acetylated 2,5-trimethoxypyrazolines (**8a–c**)

		IC_{50} (μM)	
		B16	L1210
 Ar=			
	**8a**	58	40
	**8b**	62	28
	**8c**	69	33

The pyrazolines (**5a–c**) were synthesized by reaction of the required chalcone (e.g., **2**)[9] with hydrazine hydrate in refluxing ethanol as described in Scheme 1.[10] The acetylated compounds (**5a–g** and **8a–c**) were obtained using a published procedure,[11] by dissolving the chalcone (e.g., **2**) in acetic acid and adding hydrazine hydrate (Scheme 1).[12]

The conformation of compounds **5a** and **6g** (data not shown) was examined by molecular modeling studies using the suite of programs in MacSpartan, version '04. Upon optimization of the structure using molecular mechanics (MMFF) and molecular dynamics (equilibrium conformer search option and molecular mechanics), the structure was energy optimized using Hartree–Fock (3-21G), followed by density function theory (B3LYP and 6-31G*) calculations. The resulting conformation is depicted in Fig. 2a. For comparison, the conformation of CA-4 (**1**), determined using the same protocol and previously reported, is shown in Fig. 2b.[7] It is evident from these results that the pyrazoline core in compounds **5a** and **6g** provides the structure with some 'twist', which is in contrast with the planar shape of its counterpart pyrazole **3**.[6f]

Each of the synthesized pyrazolines was subjected to in-vitro cytotoxicity screening using a 72-h continuous exposure MTT assay.[6f,7] Concentrations of compounds that inhibited tumor cell growth by 50% relative to an untreated control, or IC_{50} (μM) values, for both B16 and L1210 cell lines (murine melanoma and leukemia, respectively) are shown in Tables 1–3.

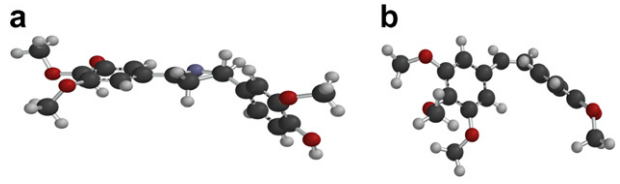


Figure 2. Molecular models of (a) compound **5a** and (b) CA-4 (**1**).

Cytotoxicity results for L1210 cells treated with each compound indicate that for both non-acetylated and acetylated pyrazolines, compounds with B-ring substituents analogous to CA-4 (**1**) (e.g., 3-hydroxy-4-methoxy) are the most potent (e.g., **5a** and **6g**) within the respective groups. However, the non-acetylated compound **5a** is significantly more active than its acetylated counterpart **6g** by a factor of ~50. Specifically, compound **5a** produced an $IC_{50} = 0.5$ μM against the growth of L1210 cells, albeit less than the published IC_{50} of 0.003 μM for CA-4 (**1**) in L1210 cells (Table 1).[13,14] In addition to its potency, compound **5a** has good solubility in biological media (ca. solubility >17.5 mM). Contrastingly, compound **6g**, with the same substituents as **5a**, except for an *N*-acetyl group present on the pyrazoline, revealed a large drop in activity ($IC_{50} > 24$ μM, Table 2). All other pyrazoline compounds gave IC_{50} values in the range of 24 to >100 μM against the growth of leukemia cells. The general trends reveal that having an acetyl group on the pyrazoline unit did not meet the desired goals. It decreased the cytotoxic potency of compounds shown in Table 2.

In the B16 cell line, the results followed a similar trend; the natural product CA-4 (**1**) was most active, with an IC_{50} of 0.002 μM.[7] The most potent pyrazoline compound was **5a** ($IC_{50} = 2.1$ μM, Table 1), confirming that the 3-hydroxy- and 4-methoxy groups in the B-ring are important for activity, perhaps, through binding to tubulin. The corresponding acetylated compound (3-hydroxy-4-methoxy) (**6g**) again produced a dramatic decrease in activity, with an IC_{50} of >100 μM. None of the other non-acetylated or acetylated compounds showed any significant biological activity.

Several *N*-acetylpyrazoline analogs possessing two methoxy groups at the *para* position of the A-ring were also synthesized and tested. Compound **8b** showed the greatest amount of activity in the L1210 cell line ($IC_{50} = 28$ μM, Table 3). In general, these compounds are not active and have comparable cytotoxicity to the trimethoxy equivalents (**6a–g**). This suggests that the methoxy groups on the A-ring play a minor role in endowing the compounds with biological activity.

Results from the cytotoxicity studies provide evidence that pyrazolines are good structural analogs of CA-4 (**1**), in terms of cytotoxicity and solubility in biological media. Moreover, substituents similar to that of CA-4 (**1**) on the pyrazoline compounds enhance cytotoxicity, but the presence of an acetyl group at N1 of the pyrazoline core is detrimental to activity. Evidently, a small change in the structure of compound **5a** (as in **6g**) had a major impact on activity, even though the effect on conformation was minimal. In order to gain insight into this striking difference in activity, both compounds were subjected to studies in which their ability to affect cellular microtubules was examined.

The effects of **5a** and **6g** on interphase cellular microtubules were evaluated in A-10 cells.[7,15] Compound **5a** was highly active, with an EC_{50} of 7.1 μM (effective concentration to cause a 50% loss of cellular microtubules). In

contrast, an EC_{50} value could not be determined with compound **6g**; and only a very modest loss (10%) of microtubules was observed at 40 μM. The difference in the microtubule disrupting properties exhibited by these two compounds could be rationalized by comparing the X-ray structure of a complex of colchicine with tubulin[16] and the molecular model of CA-4 (**1**) complexed with tubulin.[15] In both structures, the polar methoxy groups in positions 3,4-(A-ring) and 4′-(B-ring) point away from the active site, toward the solvent. As a result, the 3-carbon alkyl-bridge of colchicine and the *cis*-dihydro side of combretastatin (**1**) both face into the active site. According to these models, compound **5a** would fit into the active site more favorably than its acetylated congener because of steric hindrance imposed by the acetyl group. For comparison, CA-4 (**1**) is still the most active compound with an EC_{50} of 0.007 μM.[15]

In summary, pyrazoline compounds are suitable analogs of the combretastatins in terms of biological activity and aqueous solubility, and they have an advantage of being readily accessible. Furthermore, incorporating an acetyl group to N1 of the pyrazoline structure is detrimental to tubulin polymerization activity and diminishes cytotoxicity. Studies to further evaluate the anti-cancer properties of pyrazoline **5a** and related analogs are in progress and the results will be reported in due course.

Acknowledgments

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References and notes

1. (a) Pettit, G. R.; Bux, S.; Singh, B.; Niven, M. L.; Hamel, E.; Schimdt, J. M. *J. Nat. Prod.* **1987**, *50*, 119; (b) Chen, S.; Tan, U.; Chang, C.; Chen, T.; Lin, C.; Chang, Y.; Wang, C. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 101; (c) Pettit, G. R.; Singh, S. B.; Boyd, M. R.; Hamel, E.; Pettit, R. K.; Schimdt, J. M.; Hogan, F. *J. Med. Chem.* **1995**, *38*, 1666; (d) Gaukoger, K.; Hadfield, J. A.; Hepworth, L. A.; Lawrence, N. J.; McGown, A. T. *J. Org. Chem.* **2001**, *66*, 8135.
2. (a) Lawrence, N. J.; Rennison, D.; McGown, A. T.; Ducki, S.; Gul, L. A.; Hadfield, J. A.; Khan, N. *J. Comb. Chem.* **2001**, *3*, 421; (b) McGown, A. T.; Fox, B. W. *Anti-Cancer Drug Des.* **1989**, *3*, 249.
3. (a) Vincent, L.; Kermani, P.; Young, L. M.; Cheng, J.; Zhang, F.; Shido, K.; Lam, G.; Bompais-Vincent, H.; Zhu, Z.; Hicklin, D. J.; Bohlen, P.; Chaplin, D. J.; May, C.; Rafii, S. *J. Clin. Invest.* **2005**, *115*, 2992; (b) Galbraith, S. M.; Maxwel, R. J.; Lodge, M. A.; Tozer, G. M.; Wilson, J.; Taylor, N. J.; Stirling, J. J.; Sena, L.; Padhani, A. R.; Rustin, G. S. *J. Clin. Oncol.* **2003**, *15*, 2831.
4. (a) Hinnen, P.; Eskens, F. A. *Br. J. Cancer* **2007**, *96*, 1159; (b) Getahun, Z.; Jurd, L.; Ping, S. C.; Chii, M. L.; Hamel, E. *J. Med. Chem.* **1992**, *35*, 1058.
5. Dimmock, J. R.; Kandepu, N. M.; Hetherington, M.; Quail, J. W.; Pugazhenthi, U.; Sudom, A. M.; Chamankhah, M.; Rose, P.; Pass, E.; Allen, T. M.; Halleran, S.; Szydlowski, J.; Mutus, B.; Tannous, M.; Manavathu, E. K.; Myers, T. G.; De Clercq, E.; Balzarini, J. *J. Med. Chem.* **1998**, *41*, 1014.
6. (a) Kim, Y.; Nam, N.-H.; You, Y.-J.; Ahn, B.-Z. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 719; (b) Ohsumi, K.; Hatanaka, T.; Fujita, K.; Nakagawa, R.; Fukuda, Y.; Nihei, Y.; Suga, Y.; Morinaga, y.; Akiyama, Y.; Tsuji, T. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 3153; (c) Kaffy, J.; Monneret, C.; Mailliet, P.; Commerçon, A.; Pontinkis, R. *Tetrahedron Lett.* **2004**, *45*, 3359; (d) Wang, L.; Woods, K. W.; Li, Q.; Barr, K. J.; McCroskey, R. W.; Hannick, S. M.; Gherke, L.; Credo, R. B.; Hui, Y.-H.; Marsh, K.; Warner, R. J.; Lee, Y.; Zielinski-Mozng, N.; Frost, D.; Rosenberg, S.-H.; Sham, H. L. *J. Med. Chem.* **2002**, *45*, 1697; (e) Sun, L.; Vasilevich, N. I.; Fuselier, J. A.; Hocart, S. J.; Coy, D. H. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 2041; (f) LeBlanc, R.; Dickson, J.; Brown, T.; Stewart, M.; Pati, H.; VanDerveer, D.; Arman, H.; Harris, J.; Pennington, W.; Holt, H.; Lee, M. *Bioorg. Med. Chem.* **2005**, *13*, 6025; (g) Ducki, S.; Forrest, R.; Hadfield, J. H.; Kendall, A.; Lawrence, N. J.; McGown, A. T.; Rennison, D. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 1051.
7. Ruprich, J.; Prout, A.; Dickson, J.; Younglove, B.; Nolan, L.; Baxi, K.; LeBlanc, R.; Forrest, L.; Hills, P.; Holt, H.; Mackay, H.; Brown, T.; Mooberry, S.; Lee, M. *Lett. Drug Des. Disc.* **2007**, *4*, 144.
8. (a) Cox, C.; Breslin, M.; Mariano, B.; Coleman, P.; Buser, C.; Walsh, E.; Hamilton, K.; Huber, H.; Kohl, N.; Torrent, M.; Yan, Y.; Kuo, L.; Hartman, G. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 2041; (b) Fraley, M.; Garbaccio, R. M.; Arrington, K. L.; Hoffman, W. F.; Tasber, E. S.; Coleman, P. J.; Buser, C. A.; Walsh, E. S.; Hamilton, K.; Fernandes, C.; Schaber, M. D.; Lobell, R. B.; Tao, W.; South, V. J.; Yan, Y.; Kuo, L.; Prueksaritanont, T.; Shu, C.; Torrent, M.; Heimbrook, D. C.; Kohl, N. E.; Huber, H. E.; Hartman, G. D. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 1775; (c) Garbaccio, R. M.; Fraley, M. E.; Tasber, E. S.; Olson, C. M.; Hoffman, W. F.; Arrington, K. L.; Torrent, M.; Buser, C. A.; Walsh, E. S.; Hamilton, K.; Schaber, M. D.; Fernandes, C.; Lobell, R. B.; Tao, W.; South, V. J.; Yan, Y.; Kuo, L. C.; Prueksaritanont, T.; Slaughter, D. E.; Shu, C.; Heimbrook, D. C.; Kohl, N. E.; Huber, H. E.; Hartman, G. D. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 1780.
9. (a) Dickson, J.; Flores, L.; Stewart, M.; Holt, H. L., Jr.; LeBlanc, Regan; Lee, M. *J. Chem. Ed.* **2006**, *83*, 934; (b) Pati, H. N.; Holt, H. L., Jr.; LeBlanc, R.; Dickson, J.; Stewart, M.; Brown, T.; Lee, M. *Med. Chem. Res.* **2005**, *14*, 19.
10. The pyrazolines were synthesized as follows; compound **5a**: The required chalcone (e.g., **2**, 150 mg, 0.436 mmol) was suspended in ethanol (3 mL) and stirred at RT. Hydrazine hydrate (0.2 mL) was added and the mixture stirred at reflux for ~16 h. Upon cooling, the solid was collected by vacuum filtration, washed with water, and allowed to air dry overnight to yield **5a** as a white solid (162 mg, 78%), mp. 154 °C: ^1H ($CDCl_3$) 3.02 (dd, J = 16.5, 8 Hz, 1 H), 3.43 (dd, J = 16.5, 10.5 Hz, 1 H), 4.86 (dd, J = 10.5, 8 Hz, 1H), 5.90 (br s, 1H), 6.81 (d, J = 8 Hz, 1H) 6.85 (dd, J = 8, 2 Hz, 1H) 6.91 (s, 2H), 6.95 (d, J = 2 Hz, 1H); IR (neat) 3337, 2999, 2937, 2833, 1590, 1509, 1457, 1414, 1362, 1270, 1238, 1125, 1026, 1000, 756 cm^{-1}; LRMS direct probe, 358 (M^+, 100%), 343 (35%); HRMS calcd for $C_{19}H_{22}N_2O_5$ 358.1529 found 358.1527.
11. Lévai, A. *ARKIVOC* **2005**, *ix*, 344.
12. The acetylated pyrazolines were synthesized as follows using the published procedure[11]: Compound **6g**: The required chalcone (e.g., **2**, 344 mg, 1 mmol) was dissolved in acetic acid (15 mL) and hydrazine hydrate (0.16 mL, 5 mmol) was added and the solution stirred at reflux for ~3 h. The reaction mixture was poured over crushed ice (~100 mL) and the precipitate was collected by vacuum

filtration, washed with water, and allowed to air-dry for ~18 h to yield the pure material as a yellow solid (75 mg, 19%), mp. 188.1 °C: ^{1}H NMR ($CDCl_3$) 2.43 (s, 3H), 3.42 (dd, J = 4.4, 17.2 Hz, 1H), 3.72 (dd, J = 11.6, 17.2 Hz, 1H), 3.86 (s, 3H), 3.90 (s, 3H), 3.92 (s, 3H), 5.52 (dd, J = 4.0, 12 Hz, 1H), 6.78 (d, J = 3.2 Hz, 2H) 6.96 (s, 2H); IR (neat) 3350 (br), 3005, 2937, 2841, 1649, 1595, 1573, 1511, 1462, 1415, 1367, 1273, 1236, 1173, 1128, 1067, 1030, 1002, 761 cm^{-1}; LRMS direct probe, 400 (M^+, 100%), 358 (65%), 443 (25%); HRMS calcd for $C_{21}H_{24}N_2O_6$ 400.1634, found 400.1634.

13. Dorr, R. T.; Dvorakova, K.; Snead, K.; Alberts, D. S.; Salmon, S. E.; Pettit, G. R. *Invest. New Drugs* **1996**, *14*, 131.
14. Pettit, G. R.; Singh, S. B.; Hamel, E.; Lin, C. M.; Alberts, D. S.; Garcia-Kendall, D. *Experientia* **1989**, *45*, 209.
15. Kong, Y.; Grembecka, J.; Edler, M. C.; Hamel, E.; Mooberry, S. L.; Sabat, M.; Rieger, J.; Brown, M. L. *Chem. Biol.* **2005**, *12*, 1007.
16. Ravelli, R. B. G.; Gigant, B.; Curmi, P. A.; Jourdain, I.; Lachkar, S.; Sobel, A.; Knossow, M. *Nature* **2004**, *428*, 198.