# Synthesis and Biological Evaluation of a Focused Mixture Library of Analogues of the Antimitotic Marine Natural Product Curacin A

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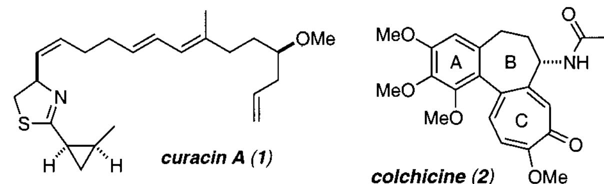
**Abstract:** The marine natural product curacin A served as the lead compound for the combinatorial synthesis of 6-compound mixture libraries. Fluorous trapping with a vinyl ether tag was used to streamline purification of the heterogeneous multicomponent reaction products and provide chemically clearly defined mixtures. The screening profile of one mixture library, **17mix**, was attractive enough to warrant the re-synthesis of the individual compounds, and an evaluation of their biological effects validated the composite data previously obtained on the product mixture. The most active of these compounds inhibited tubulin polymerization with an $IC_{50}$ of ca. 1 $\mu$M, showed an average growth inhibition activity $GI_{50}$ of ca. 250 nM, inhibited [$^3$H]colchicine binding to tubulin, and blocked mitotic progression at nanomolar concentrations. These compounds represent some of the most potent synthetic curacin A analogues identified to date but have simplified structures, greater water solubility, and increased chemical stability.

## Introduction

Natural products remain a significant source of promising lead structures for drug development.[1] In recent years, an expansion of the structural diversity pool by preparation of libraries of natural products or natural product-like molecules has become a major focus of combinatorial chemistry.[2] After completion of the total synthesis of the strongly antimitotic *Lyngbya majuscula* metabolite curacin A in 1996,[3] we remained intrigued by the impressive antiproliferative profile[4] of this marine natural product and the potential to use it as a scaffold for the development of novel tubulin polymerization inhibitors.[5,6]

Curacin A promotes arrest of the cell cycle at the $G_2$/M checkpoint and competitively inhibits the binding of [$^3$H]-colchicine to tubulin, and it can therefore be considered a colchicine site agent.[5,7] In addition to a large number of total syntheses of curacin A,[8] the attractive biological properties of this compound have led to numerous analogue studies. However, even minor changes in the structure of curacin A can lead to essentially inactive derivatives (Figure 1).[7,9] Critical issues for further pharmaceutical development of any sufficiently active analogue are increases in chemical stability and hydrophilicity and improved availability versus the natural product. Curacin

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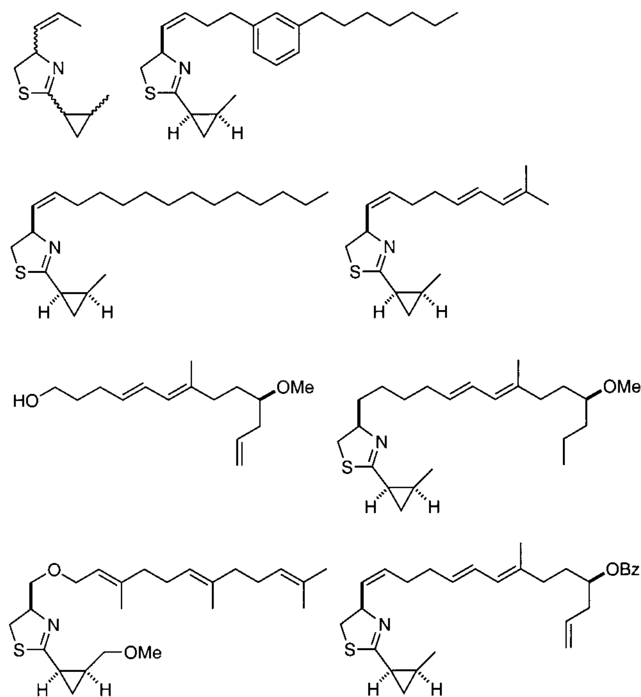



**Figure 1.** Examples of inactive curacin A analogues.

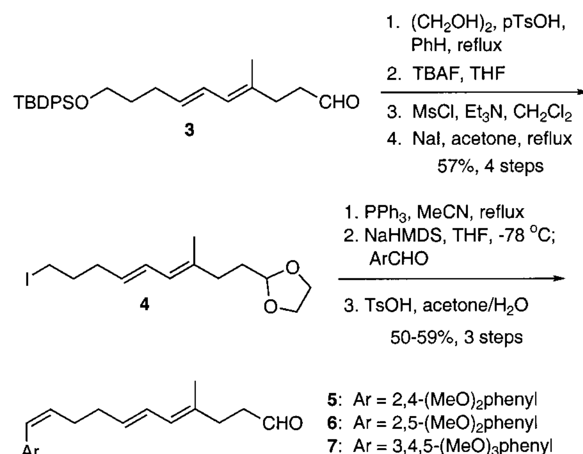
A is sensitive to oxidation, acids, and bases, and is readily irreversibly absorbed into plastic containers such as polyethylene 96-well plates.

## Results and Discussion

Since curacin A binds to the colchicine site on tubulin, and the alkenyl thiazoline moiety in **1** is largely responsible for the chemical instability of the natural product, we decided to substitute the heterocycle with electron-rich aromatics reminiscent of the A-ring in **2**. Furthermore, we planned to substitute the homoallylic methyl ether terminus of curacin A with a broad range of more hydrophilic benzylic alcohols but maintain the diene spacer unit, which has proven to be essential in the limited SAR studies of curacin A published to date.[4,7,9] Accordingly, three key building blocks **5**−**7** were prepared by standard solution synthesis from the readily available[3] aldehyde **3** (Scheme 1). After protection with ethylene glycol, desilylation, and mesylation, Finkelstein reaction with sodium iodide provided dioxolane **4** in 57% yield. The corresponding Wittig reagent was condensed with 2,4-dimethoxy-, 2,5-dimethoxy-, and 3,4,5-trimethoxybenzaldehyde, respectively, to give aldehydes **5**−**7** in 50−59% overall yields from **4** after acetal cleavage.[10]

The solution-phase combinatorial synthesis of mixtures for biological screening is not commonly used, but represents an efficient approach for rapid lead evaluation if the composition of the mixture is clearly defined and the throughput of the assay is limited.[11] In an effort to establish the validity of our analogue design, we prepared 7 mixtures of 6−9 compounds each by

**Scheme 1**




exposure of **5**−**7** as well as derivatives with other aryl substituents to a cocktail of 3−6 aryllithium reagents. The most biologically active mixtures were obtained from reaction of **5**−**7** with 2-lithiated furan, thiophene, benzofuran, benzothiophene, anisole, and 1,4-dimethoxybenzene (Scheme 2). The lithiation was performed directly on the mixture and was heteroatom-directed. Since a large excess (3 equiv each) of organolithium reagents in a 1:1:1:1:1:1 ratio was used to ensure fast and unselective aldehyde addition, the resulting alcohols **8mix**−**10mix** were heavily contaminated with excess Nu−H after aqueous workup. Fluorous quenching of the crude product with an excess of the recently developed vinyl ether **11** provided a convenient means to ensure rapid and thorough purification.[12,13] After simple liquid/liquid extraction with a mixture of the perfluorinated solvent FC-72 and MeCN/$H_2O$, the partially fluorinated acetals **12mix**−**14mix** were collected in the FC-72 layer; organic impurities remained in the organic phase and inorganic salts preferred the aqueous environment. Methanolysis of the fluorous extract and renewed postreaction fluorous/organic/aqueous liquid−liquid extraction finally provided pure 6-compound mixtures **15mix**−**17mix** in the organic phase,[14] while the fluorous phase contained the solvolysis products of protective group **11**.

In Table 1, the biochemical and cell growth activity of **15mix**−**17mix** is summarized. Each of the mixtures inhibited by >50% the 30 °C GTP-induced polymerization of glutamate-containing isolated bovine brain tubulin[15a] at concentrations <5 $\mu$M (summed concentration based on average molecular weight of the components), with both **16mix** and **17mix** showing high potency. Assays to determine if these compounds inhibited binding of [$^3$H]colchicine to tubulin at both 30 and 37 °C[15b] showed both **15mix** and **16mix** were significantly weaker at displacement than curacin A, whereas one or more of the

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(10) Aldehydes **5**−**7** were obtained as *all*-(*E*) isomers at the diene moiety in a ca. 5:1 ratio of (*Z*):(*E*) alkene stereoisomers after the Wittig condensation and characterized by $^1$H NMR and HRMS.

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(14) The three mixtures were characterized by LC-MS using a reverse-phase (C18) column and positive ionization electrospray mass spectral detection as well as by LC NMR. Each mixture contained the six expected products in close-to-equimolar ratio, with minor amounts (ca. 15−20%) of the (*E*)-alkene derivatives from the Wittig reaction. No organic impurities >5% were detected. Details of the LC NMR analysis will be reported elsewhere: Wipf, P.; Reeves, J. T.; Wilkinson, P. S. Bruker Report, in press.

**Table 1.** 50% Colchicine Binding Inhibitory Concentration (CBI), 50% Tubulin Polymerization Inhibition Concentration (TPI), and 50% Growth Inhibitory Concentrations ($GI_{50}$) for **15mix**−**17mix** and Discrete Compounds Comprising **17mix**[17]

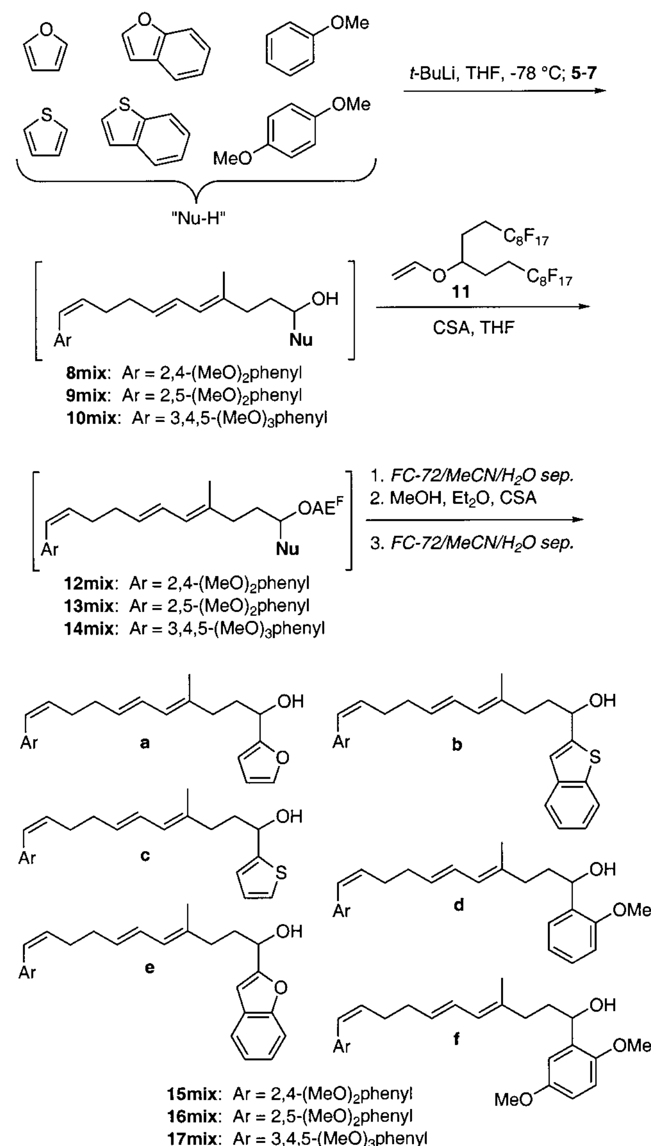
| | CBI[a] (μM) | | TPI[b] | $GI_{50}$ (μM)[c] | | |
|---|---|---|---|---|---|---|
| compd | 30 °C | 37 °C | $IC_{50}$ (μM) | MDA-MB231 (48 h) | PC-3 (48 h) | 2008 (48 h) |
| **15mix** | >250 | >250 | 4 | >50 | >50 | 36 ± 5 |
| **16mix** | >50 | 48 ± 5 | 2 | 5.2 ± 2 | >50 | 21 ± 4 |
| **17mix** | >50 | 26 ± 6 | 1.4 | 0.30 ± 1 | 0.80 ± 0.5 | 0.58 ± 0.2 |
| **17a** | 32 ± 4 | 9 ± 6 | 0.9 | 0.69 ± 0.1 | 8.0 ± 4 | 2.7 ± 1 |
| **17b** | 9 ± 7 | 19 ± 2 | 1.4 | 0.23 ± 0.06 | 0.62 ± 0.05 | 0.36 ± 0.001 |
| **17c** | 39 ± 10 | 9 ± 3 | 1.2 | 0.28 ± 0.04 | 0.38 ± 0.08 | 0.28 ± 0.02 |
| **17d** | 9 ± 5 | 9 ± 4 | 1.2 | 0.34 ± 0.03 | 0.62 ± 0.2 | 0.33 ± 0.02 |
| **17e** | 9 ± 4 | 28 ± 8 | 1.6 | 0.12 ± 0.07 | 0.31 ± 0.2 | 0.27 ± 0.05 |
| **17f** | >50 | >50 | >50 | 9.7 ± 1 | >50 | 22 ± 5 |
| curacin A | 0.9 ± 0.3 | 0.6 ± 0.1 | 0.78 ± 0.07 | 0.096 ± 0.06 | 0.050 ± 0.009 | 0.035 ± 0.007 |

[a] Values shown are means ($N = 9$ over four concentrations) ± standard deviations (SD) for incubation at 30 °C for 15 min and 37 °C for 10 min. [b] Average of two determinations except for curacin A, where $N = 7$ (±SD). [c] Means ($N = 4$ over 10 concentrations) ± SD.

compounds comprising **17mix** had significant concentration-dependent inhibitory activity at the colchicine site. Screening for growth inhibitory activities[15c] against a short series of human breast, prostate, and ovarian carcinoma cells confirmed the trends seen in the biochemical screens and, moreover, showed that **17mix** inhibited cell proliferation at submicromolar concentrations suggesting that one or more components might have potency comparable to that of curacin A.

In light of the intriguing activities in the tubulin polymerization and growth inhibition assays observed for **17mix**, we decided to synthesize the compounds **17a**−**f** individually and determine their specific potencies.[16,17] Analysis of the discrete compounds revealed that four of them, **17b**−**e**, had considerable potencies in all of the isolated protein and cell-based growth inhibition screens, and the 1-(2,5-dimethoxyphenyl)-containing **17f** was essentially inactive (Table 1). The furan-containing **17a**, although potent in the tubulin assays and against the breast carcinoma cell line, had reduced antiproliferative activity against the prostate and ovarian carcinoma cells. As comparison of

**Scheme 2**




(15) (a) Tubulin without microtubule-associated proteins was prepared from fresh bovine brains (Hamel, E.; Lin, C. M. *Biochemistry* **1984**, *23*, 4173). Reactions were carried out as described previously.[7] Tubulin (final concentration 10 μM.; 1 mg/mL) was preincubated with drugs dissolved in DMSO (4% v/v final concentration) and monosodium glutamate (0.8 M final concentration) for 15 min at 30 °C. The reaction mixture was cooled to 0 °C and GTP (0.4 mM final concentration) was added. Reaction mixtures were transferred to cuvettes at 0 °C in a Beckman-Coulter 7400 spectrophotometer reading absorbance at 350 nm. Baselines at 0 °C were established and temperature was quickly raised to 30 °C (in approximately 1 min with an electronically controlled Peltier temperature controller). The change in absorbance 20 min after samples reached 30 °C was used to calculate the extent of polymerization. The change in absorbance at this time point for vehicle plus no GTP was considered 100% assembly inhibition, while the change in absorbance for GTP plus vehicle was taken as 0% inhibition. Each series of determinations included positive and negative control determinations plus one determination made with 5 μM curacin A. (b) Using methods described previously (Kang, G.-J.; Getahun, Z.; Muzaffar, A.; Brossi, A.; Hamel, E. *J. Biol. Chem.* **1990**, *265*, 10255),[7] 5 μM [$^3$H]colchicine (2.3 TBq/mmol), drug (1, 5, 10, 50 or 250 μM), or vehicle (DMSO, 5% v/v) were incubated at 30 °C for 15 min or at 37 °C for 10 min with 1 μM tubulin in the presence of 1 M monosodium glutamate, 0.1 M glucose-1-phosphate, 1 mM $MgCl_2$, 1 mM GTP, and 0.5 mg/mL bovine serum albumin. The solutions were filtered through two stacks of DEAE-cellulose filters and the radioactivity in the filtrate was determined by scintillation spectrometry. Each series of determinations included positive controls of 1, 5, and/or 50 μM curacin A. (c) Cells were plated (500−1500 cells/well depending on the cell line) in 96-well plates and allowed to attach and grow for 48 h. They were then treated with vehicle (DMSO) or drug [50, 10, 2, 0.4, and 0.08 μM (for the mixtures, these would be the summed, apparent concentrations, i.e., approximately six times the concentration of each compound); 10, 2, 0.4, 0.08, and 0.016 μM for curacin A; then 1, 0.2, 0.04, 0.008, and 0.0016 μM for **17a**−**e** and curacin A] for the given times. One plate consisted entirely of cells used for a time zero cell number determination. The other plates contained eight wells of control cells, eight wells of medium, and each drug concentration tested in quadruplicate. Cell numbers were obtained spectrophotometrically (absorbance at 490 nm minus that at 630 nm) in a Dynamax plate reader after treatment with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS.; Owen's reagent) using *N*-methyldibenzopyurazine methyl sulfate (phenazine methosulfonate) as the electron acceptor.

(16) The synthesis of **17a**−**f** proceeded as shown in Schemes 1 and 2, but the final products were purified by column chromatography, and were characterized by $^1$H and $^{13}$C NMR, IR, and HRMS.

(17) As a negative control, single compounds of **15mix** were also resynthesized, and biological testing confirmed their lack of activity.

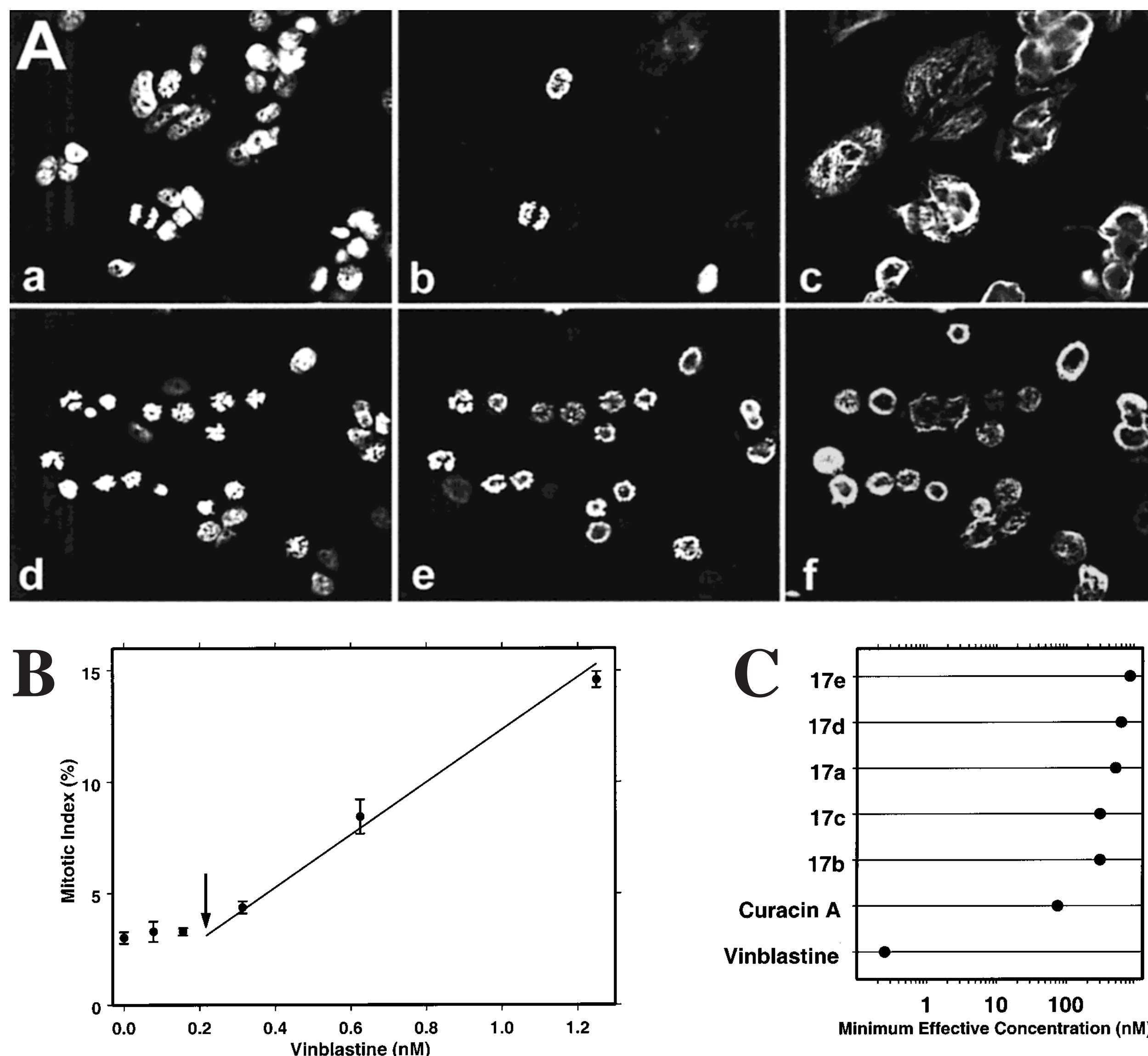



**Figure 2.** The effects of microtubule disrupting drugs on the microtubule cytoskeleton and the cell cycle of human carcinoma cells. (A) multiparameter fluorescence micrographs of untreated MCF-7 cells (a−c) and cells treated with compound **17b** (1 $\mu$M) for 16 h (d−f). Each set of three images, obtained from the same microscopic field, was used to map the size and shape of the cellular nuclei (a, d), a mitosis-specific phosphorylated core histone protein (b, e), and the microtubule cytoskeleton (c, f). Compound **17b** profoundly altered the morphology of the nuclei, the relative number of cells blocked in mitosis, and the fibrous structure of the microtubule cytoskeleton. (B) Determination of the minimum effective concentration of a drug to induce a measurable effect on the cell cycle of living tumor cells. MCF-7 carcinoma cells were treated with a range of vinblastine concentrations for 14 h followed by the measurement of the mitotic index. The minimum effective concentration of vinblastine to alter the cell cycle was determined graphically as the concentration (*x*-axis) where the linear portion of the dose response curve equaled the mitotic index of normally cycling cells (arrow). (C) Minimum effective concentration values of microtubule disrupting drugs.

**17mix** with **17a**−**f** clearly demonstrates, tubulin polymerization and cellular growth inhibition reflect the sum of the individual compounds that make up **17mix**. These results validate our mixture-based screening strategy.[17] In contrast, colchicine binding inhibition for **17mix** does not correlate as well with the activity of **17a**−**f**, possibly due to a combination of high-affinity (colchicine-site) and other low-affinity binding sites on tubulin for these compounds. Some less active curacin A analogues actually enhance the binding of [$^3$H]colchicine to tubulin, maybe via allosteric interactions. We are currently investigating the specific binding modes in detail, but these data confirm that while for some biological assays mixture analysis is highly relevant for a quick assessment of biological activities of a collection of compounds, more specific competitive binding studies can easily fail to adequately reflect the sum of the individual mixture components.

Further verification of the biological activity of compounds **17a**−**e** was obtained in living tumor cells by quantifying their effect on the mitotic index, essentially, counting the number of cells in the mitotic phase of the cell cycle. Antimitotic compounds increase the mitotic index, which was quantified immunohistochemically using an antibody to a mitosis-specific phosphorylated core histone protein (Mitotic Index HitKit (tm), Cellomics, Inc. (tm), Pittsburgh, PA).[18] MCF-7 human breast carcinoma cells were treated with increasing concentrations of compounds **17a**−**e** (7.8−1000 nM), curacin A (3.1−400 nM), and vinblastine (0.08−10 nM) for 14−16 h. The morphology

(18) Phosphorylation of a core histone occurs during mitosis and is linked to chromosome condensation. Thus, antimitotic agents cause an accumulation of cells containing the phosphorylated form of this protein. Wei, Y.; Mizzen, C. A.; Cook, R. G.; Gorovsky, M. A.; Allis, C. D. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 7480.

of the cellular nuclei and the microtubule cytoskeleton, as well as the phosphorylation of a core histone protein, were visualized using a multiparameter immunofluorescence approach (Figure 2; A). For example, compound **17b** induced a large increase in the fraction of cells exhibiting condensed chromatin and increased core histone phosphorylation (Figure 2; A: d and e). Furthermore, compound **17b** induced cell rounding and the generalized loss of structured microtubules in the same cells (Figure 2, A: f). The mitotic index in drug-treated cells was measured automatically using the ArrayScan® II System (Cellomics, Inc.). To assess the relative activity of compounds **17a−e** and two known microtubule disrupting drugs, vinblastine and curacin A, the minimum effective concentration of each drug was measured (Figure 2; B). Compounds **17a−d** were the most potent of the new agents with minimum effective concentrations in the submicromolar range and well within an order of magnitude of the activity of parent curacin A (Figure 2; C).

## Conclusions

Despite the relative structural complexity of the lead compound curacin A, we were able to devise an efficient synthetic strategy for the preparation of 6-compound mixture libraries that incorporate important structural elements of the marine natural product. Fluorous trapping with vinyl ether **11** was used to streamline purification of the heterogeneous multicomponent reaction products after the diversification step of the library synthesis and provide structurally defined mixtures. The screening profile of one mixture library, **17mix**, was attractive enough to warrant the synthesis of the individual components, and an evaluation of their biological effects validated the composite data previously obtained on the product mixture. A multiparameter high-content assay showed that compounds **17a−d** had significant activity in altering the cell cycle as well as the microtubule cytoskeleton in living human carcinoma cells. Five of the six compounds in **17mix** had significant activity in several other assays. The most active of these compounds, **17c** and **17e**, inhibited tubulin polymerization with an $IC_{50}$ of ca. 1 $\mu$M, showed an average growth inhibition activity $GI_{50}$ of ca. 250 nM, inhibited [$^{3}$H]colchicine binding to tubulin, and blocked mitotic progression at nanomolar concentrations. Compounds **17c** and **17e** represent therefore some of the most potent synthetic curacin A analogues identified to date, with an activity profile rivaling that of the natural product despite their simplified structure, greater water solubility, and increased chemical stability. Our analysis demonstrates that, depending on the type of assay used, the biological evaluation of mixtures can give highly relevant data that reflect the sum of the individual components.

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**Supporting Information Available:** Synthetic procedures and experimental data for **3−7**, **15mix**, **16mix**, **17mix**, and **17a−f** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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