Synthesis and Biological Evaluation of 2- and 3-Aminobenzo[*b*]thiophene Derivatives as Antimitotic Agents and Inhibitors of Tubulin Polymerization

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Two new series of inhibitors of tubulin polymerization based on the 2-amino-3-(3,4,5-trimethoxybenzoyl)-benzo[*b*]thiophene molecular skeleton and its 3-amino positional isomer were synthesized and evaluated for antiproliferative activity, inhibition of tubulin polymerization, and cell cycle effects. Although many more 3-amino derivatives have been synthesized so far, the most promising compound in this series was 2-amino-6-methyl-3-(3,4,5-trimethoxybenzoyl)benzo[*b*]thiophene, which inhibits cancer cell growth at subnanomolar concentrations and interacts strongly with tubulin by binding to the colchicine site.

Introduction

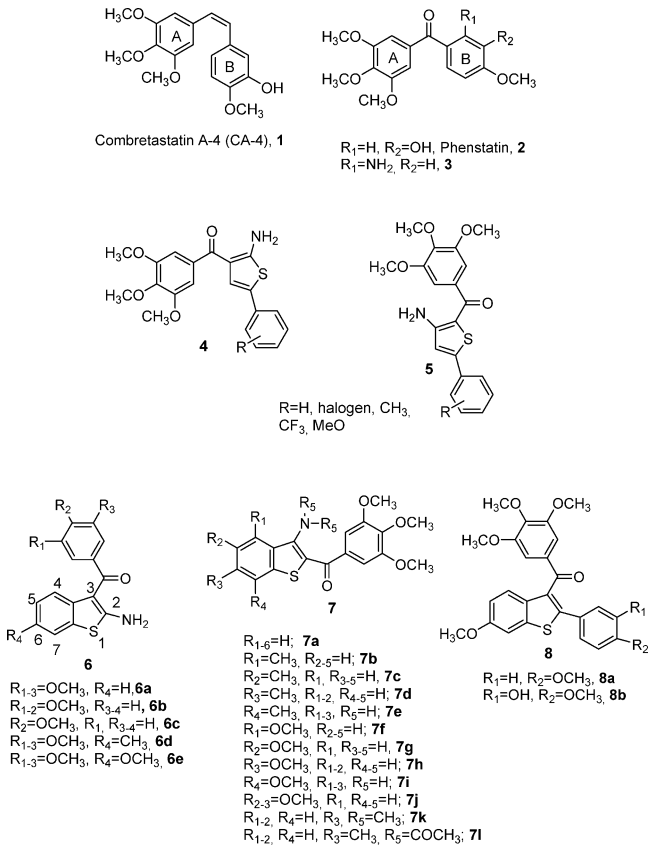
There has long been considerable interest in the discovery and development of novel small molecules able to affect tubulin polymerization.[1] More recently, it has been established that some tubulin-binding agents selectively target tumor vasculature and thus can also be considered vascular disrupting agents.[2] These compounds induce morphological changes in the endothelial cells of the tumor's blood vessels, resulting in their occlusion and therefore interruption of blood flow.[3]

One of the most important tubulin-binding agents is combretastatin A-4 (CA-4, **1**; Chart 1). CA-4, isolated from the bark of the South African tree *Combretum caffrum*,[4] is one of the well-known natural tubulin-binding molecules affecting microtubule dynamics. CA-4 strongly inhibits the polymerization of tubulin by binding to the colchicine site.[5] Because of its simple structure, a large number of CA-4 analogues have been developed and evaluated in SAR studies.[6] Among synthetic small-molecule tubulin inhibitors, replacement of the double bond of **1** with a carbonyl group furnished a benzophenone-type CA-4 analogue named phenstatin (**2**). This compound demonstrated interesting efficacy in a variety of tumor models while retaining the characteristics of **1**.[7] The 2-aminobenzophenone derivative **3** also strongly inhibited cancer cell growth and tubulin polymerization and caused mitotic arrest, as did **2**.[8]

The classical bioisosteric equivalence between benzene and thiophene prompted us recently to synthesize a series of thiophene derivatives in which a 2-aminothiophene system replaced the 2-aminobenzene moiety in the 2-aminophenstatin analogue **3**. Many of the 2-amino-3-(3,4,5-trimethoxybenzoyl)-5-phenylthiophene molecules with general structure **4** are potent inhibitors of tubulin polymerization.[9] These derivatives are as effective as their 2-(3,4,5-trimethoxybenzoyl)-3-aminothiophene isomeric analogues with general structure **5**.[10]

Our preliminary study revealed that a limited series of 2- and 3-aminobenzo[*b*]thiophenes (**6a**−**c** and **7a**,**d**) showed interesting activities in inhibiting microtubule polymerization and cell proliferation, with 3-amino-6-methyl-2-(3,4,5-trimethoxybenzoyl)benzo[*b*]thiophene (**7d**) being the most promising deriva-

Chart 1. Inhibitors of Tubulin Polymerization



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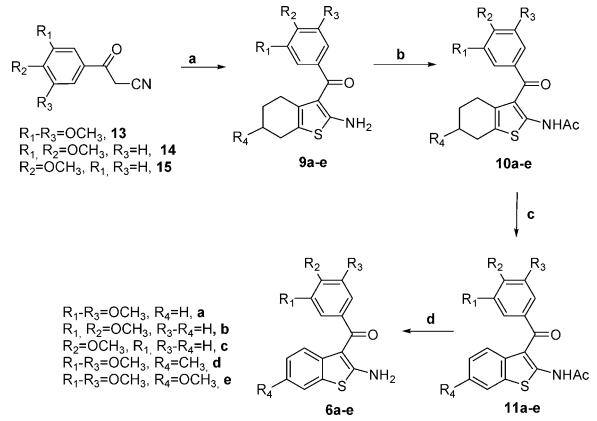
Scheme 1[a]



[a] Reagents: (a) cyclohexanone, 4-methylcyclohexanone or 4-methoxycyclohexanone, S_8, morpholine, EtOH, 70 °C for 1 h, then 18 h at rt; (b) Ac_2O, pyridine, rx; (c) 10% Pd/C moistened with 50% water, heating, 18 h; (d) 1 N NaOH, EtOH, rx, 2 h.

tive in this collection.[11] These compounds with general structures **6** and **7** are structurally related to **3**, in which the 2-aminophenyl moiety of this latter derivative was replaced by various 2- or 3-aminobenzo[*b*]thiophenes. The benzo[*b*]thiophenes **6** and **7** also represent benzofused analogues of 5-phenylthiophene derivatives **4** and **5**, respectively. Here, we report further work to improve the potency of this series of tubulin polymerization inhibitors.

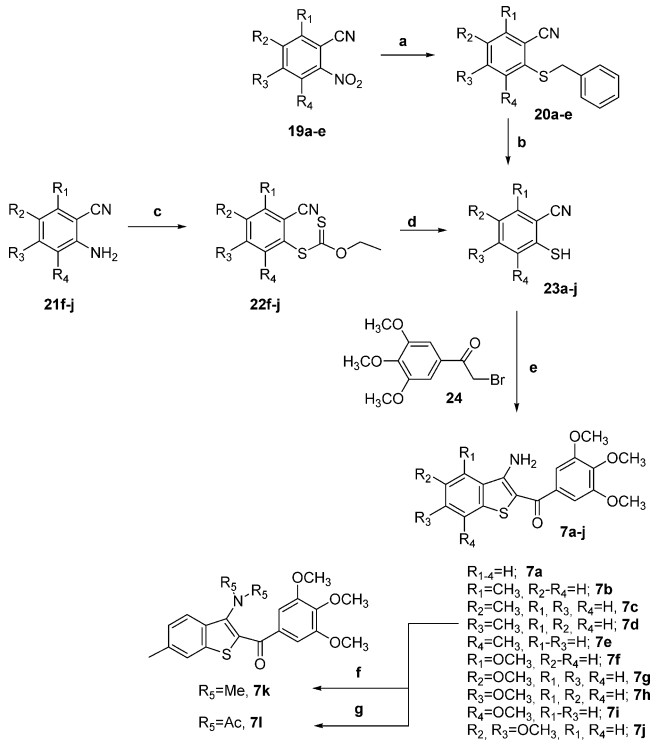
Chemistry

By the synthesis of **7b**−**j**, we focused on effects of electron-donating methyl and methoxy substituents at positions 4−7 of the 3-aminobenzo[*b*]thiophene skeleton. By the synthesis of **7k**−**l**, we evaluated whether the 3-amino substituent was important in restricting the conformation of the adjacent trimethoxybenzoyl moiety through an intramolecular hydrogen bond with the carbonyl oxygen.

The benzo[*b*]thiophene molecular skeleton is the core structure of a series of inhibitors of microtubule polymerization, which showed activity comparable with that of CA-4.[12] The most representative compounds in this series are derivatives with general structure **8**, which incorporates the 3-(3,4,5-trimethoxybenzoyl)-6-methoxybenzo[*b*]thiophene ring system. Previous studies have shown that the 6-methoxy substituent significantly contributes to maximize activity and mimics the 4-methoxy group in the B-ring of **1** and aminobenzophenones **2** and **3**.[8,12] Building upon these observations, as represented by the synthesis of **6e**, we were intrigued with studying the biological effects of replacing the aryl substituent with an amino group at the 2-position of the benzo[*b*]thiophene ring of **8a** and **8b**. Since the C-6 methoxy group proved to be favorable for bioactivity, by the synthesis of **6d** we evaluated the effect of replacing it with a weaker electron-releasing methyl group, which had been well-tolerated in a series of CA-4 analogues published previously.[13] Finally, through the synthesis of **6b** and **6c** we investigated the effect on antiproliferative activity of the 3,4,5-trimethoxybenzoyl group in the 2-aminobenzo[*b*]thiophene derivative **6a**.

Derivatives **6a**−**e** were synthesized in four steps, as shown in Scheme 1. The Gewald reaction[14] applied to *β*-ketonitriles **13**, **14**, and **15** and cyclohexanone, 4-methylcyclohexanone, or 4-methoxycyclohexanone furnished the 2-amino-3-aroyl-4,5,6,7-tetrahydrobenzo[*b*]thiophenes **9a**−**c**, **9d**, and **9e**, respectively. Acetylation of the amino group, using a mixture of acetic anhydride and pyridine, yielded **10a**−**e**, which afforded the

Scheme 2[a]



[a] Reagents: (a) $C_6H_5CH_2SH$, KOH, DMF; (b) $AlCl_3$, C_6H_6; (c) $NaNO_2$, HCl, 0 °C, then KS_2COEt, 65 °C; (d) NaOH, 65 °C; (e) **24**, K_2CO_3, $(CH_3)_2CO$, rx, 18 h; (f) MeI, NaH, DMF; (g) Ac_2O, Py, rx.

benzo[*b*]thiophene derivatives **11a**−**e** following aromatization by heating with 10% Pd/C. These latter compounds were transformed by ethanolysis into the final products **6a**−**e**.

The 3-amino-2-(3,4,5-trimethoxybenzoyl)benzo[*b*]thiophenes **7a**−**j** were prepared in excellent yields. Condensation was followed by intramolecular cyclization when 2-cyanothiophenol (**23a**) and methyl/methoxy substituted 2-mercaptobenzonitriles **23b**−**j** were reacted with 1-(3,4,5-trimethoxyphenyl)-2-bromoethanone (**24**), using potassium carbonate in refluxing acetone (Scheme 2).

2-Mercaptobenzonitrile (**23a**)[15] and methyl substituted 2-mercaptobenzonitriles **23b**−**e** were obtained by S-debenzylation of thioethers **20a**−**e** with aluminum chloride in benzene. These latter derivatives were obtained in good yields by condensation of the corresponding 2-nitrobenzonitriles **19a**−**e** with the potassium salt of phenyl methanethiol in cold aqueous DMF.[16] Methoxy substituted 2-mercaptobenzonitriles **23f**−**j** were synthesized by the Leuckart reaction[17] starting from the precursor amines **21f**−**j**. The method involved diazotation of **21f**−**i** with $NaNO_2$ in a MeOH/H_2O mixture of HCl/AcOH, followed by nucleophilic displacement of the diazonium moiety with potassium *O*-ethylxanthate to furnish the resulting arylxanthate **22f**−**j.** The subsequent hydrolysis with NaOH followed by acidification furnished the desired 2-mercaptobenzonitriles **23f**−**j**, which were used without further purification. Compound **7d** was bis-N-methylated with methyl iodide or acetylated with a mixture of acetic anhydride and pyridine to give **7k** or **7l**, respectively.

Results and Discussion

Table 1 summarizes the antiproliferative effects of benzo[*b*]-thiophene derivatives **6** and **7** against a panel of tumor cell lines with **1** as reference compound. Several derivatives demonstrated substantial inhibitory effects on the growth of murine leukemia

Table 1. In Vitro Inhibitory Effects of Compounds **6a**−**e**, **7a**−**l**, and CA-4 (**1**) against the Proliferation of Murine Leukemia (L1210), Murine Mammary Carcinoma (FM3A), and Human T-Lymphocyte (Molt/4 and CEM) Cells

	IC_{50} (nM)[a]			
compd	L1210	FM3A	Molt4/C8	CEM
6a[11]	90 ± 3	100 ± 0	73 ± 9	74 ± 15
6b[11]	3700 ± 60	4700 ± 250	1700 ± 200	1700 ± 500
6c[11]	>10000	>10000	9200 ± 200	>10000
6d	0.76 ± 0.06	0.09 ± 0.069	0.69 ± 0.02	0.52 ± 0.06
6e	4.4 ± 0.5	4.8 ± 0.7	4.1 ± 0.0	4.5 ± 0.5
7a[11]	350 ± 320	1800 ± 300	290 ± 10	310 ± 20
7b	>10000	>10000	>10000	>10000
7c	9500 ± 1300	>10000	4200 ± 1700	4600 ± 3400
7d[11]	58 ± 50	71 ± 5	34 ± 3	24 ± 2
7e	160 ± 10	360 ± 60	83 ± 6	98 ± 18
7f	>10000	>10000	>10000	>10000
7g	>10000	>10000	>10000	>10000
7h	39 ± 16	46 ± 12	10 ± 7	7.7 ± 2.9
7i	33 ± 29	27 ± 13	8.5 ± 1.4	8.9 ± 2.0
7j	150 ± 30	380 ± 120	87 ± 19	460 ± 440
7k	87 ± 1	84 ± 10	85 ± 1	90 ± 10
7l	150 ± 60	190 ± 100	75 ± 36	100 ± 10
CA-4 (**1**)	2.8 ± 1.1	42 ± 6	1.6 ± 1.4	1.9 ± 1.6

[a] IC_{50}: compound concentration required to inhibit tumor cell proliferation by 50%. Data are expressed as the mean ± SE from the dose response curves of at least three independent experiments.

(L1210), murine mammary carcinoma (FM3A), and human T-lymphoblastoid (Molt/4 and CEM) cells. In general, the antiproliferative activities of the compounds were greater against the Molt/4 and CEM cells compared with the two murine lines. The most active benzo[*b*]thiophene compound identified in this study was **6d**, which was more cytostatic than **1** in all four cell lines. Derivative **6d** inhibited the growth of L1210, FM3A, Molt/4, and CEM cancer cell lines with IC_{50} values of 0.76, 0.09, 0.69, and 0.52 nM, respectively.

Derivatives **6a**−**c** demonstrated that the 3′,4′,5′-trimethoxybenzoyl substitution in **6a** was crucial for potent cell growth inhibition. Its substitution with 3,4-dimethoxybenzoyl and 4-methoxybenzoyl moieties (**6b** and **6c**, respectively) led to the loss of growth inhibition activity. In terms of antiproliferative activity, **6a** and **7a**, which were both unsubstituted in the benzene portion of the benzo[*b*]thiophene ring, were substantially less active than derivatives with a methyl or methoxy group at C-6 or C-7.

In the 3-aminobenzo[*b*]thiophene derivatives, the results indicated that inhibition of cell growth was strongly dependent on the position of the methoxy or methyl moiety. A fairly dramatic difference was observed between C-4/5 and C-6/7 substituted compounds (**7b**,**c** and **7f**,**g** versus **7d**,**e** and **7h**,**i**). Invariably, the greatest activity occurred when the methyl and methoxy groups were located at the C-6 or C-7 position, the least when located at the C-4 or C-5 position. A comparison of substituent effects revealed that the replacement of the methoxy by a methyl group at the C-6 or C-7 position resulted in a slight reduction in antiproliferative activity. We observed the opposite effect in the isomeric 2-aminobenzo[*b*]thiophene series, where the substitution of C-6 methoxy with a C-6 methyl (**6e** and **6d**, respectively) caused a generally modest increase in activity (6- to 9-fold except in the FM3A cells) and yielded the most active compound of the whole series. Elimination of the C-6 substituent (**6a**) significantly decreased antiproliferative activity in all four cell lines. Specifically, by comparison of **6e** to **8a** and **8b**, the 2-amino group of **6e** is a good surrogate for the substituted phenyl rings present in the latter compounds.

The more extensive synthetic work thus far with the 3-amino derivatives permits us to analyze more extensively the effects of substitution in the benzo[*b*]thiophene ring on antiproliferative activity. Thus, **7b**, with a methyl group at C-4, had no antiproliferative activity and only marginal improvement occurred with the methyl group at C-5 (**7c**). Simply moving the methyl group to C-6 (**7d**) resulted in a highly active compound, while moving the methyl to C-7 (**7e**) resulted in a 2- to 4-fold loss of activity. With methoxy substituents, the differences between C-4/5 versus C-6/7 were even more dramatic. Both **7f** and **7g** were inactive, while **7h** and **7i** were the most active compounds in the 3-amino series. However, a moderate loss of activity was observed with methoxy substituents at C-6 and C-7 (**7j**). Activity generally intermediate between the activities of **7d** and **7e** were observed with **7k** and **7l**, the *N*,*N*-dimethylamino and *N*,*N*-diacetyl derivatives, respectively, of **7d**. Thus, the 3-amino hydrogens of **7d** cannot be considered essential for its antiproliferative activity.

Finally although only few compounds have been synthesized in the 2-amino series, thus far all 2-amino-3-(3,4,5-trimethoxybenzoyl)benzo[*b*]thiophenes are significantly more potent than the isomeric 3-amino compounds (compare **6a**,**d**,**e** with **7a**,**d**,**h**, respectively).

To investigate whether the antiproliferative activities of these compounds were related to an interaction with the microtubule system, **6a**,**d**,**e** and **7a**,**d**,**e**,**h**−**j** were evaluated for their inhibitory effects on tubulin polymerization and on the binding of [^{3}H]-colchicine to tubulin (Table 2).[18,19] With the exception of **7i**, there was a positive correlation between the inhibition of tubulin polymerization and antiproliferative activity. The most potent compound in this series was **6d**, with an IC_{50} of 0.76 μM. This is in agreement with **6d** being the compound with the greatest antiproliferative activity. Compounds **7d** and **7h** were as active as CA-4 as inhibitors of tubulin assembly, although both compounds were less active in their effects on cell growth..

Table 2. Inhibition of Tubulin Polymerization and Colchicine Binding by Compounds **6**−**7a**, **6**−**7d**, **6**−**7e**, **7h**−**j**, and CA-4

compd	tubulin assembly[a] IC_{50} ± SD (μM)	colchicine binding[b] % ± SD
6a	1.9 ± 0.1	25 ± 2
6d	0.76 ± 0.1	91 ± 2
6e	1.8 ± 0.2	66 ± 2
7a	2.4 ± 0.1	15 ± 1
7d	1.3 ± 0.2	60 ± 4
7e	2.6 ± 0.1	19 ± 3
7h	1.3 ± 0.1	60 ± 0.1
7i	2.1 ± 0.3	39 ± 1
7j	1.8 ± 0.1	16 ± 2
CA-4 (**1**)	1.2 ± 0.1	86 ± 3

[a] Inhibition of tubulin polymerization. Tubulin was at 10 μM. [b] Inhibition of [^{3}H]colchicine binding. Tubulin, colchicine, and tested compound were at 1, 5, and 1 μM, respectively.

In the colchicine binding studies, **6d**,**e** and **7d**,**h** potently inhibited the binding of [^{3}H]colchicine to tubulin, since 60−91% inhibition occurred with these agents at 1 μM with colchicine at 5 μM. Only **6d** was as potent as CA-4, which in these experiments inhibited colchicine binding by 86%.

Because molecules exhibiting effects on tubulin assembly should cause the alteration of cell cycle parameters with preferential G2-M blockade, flow cytometry analysis was performed to determine the effect of the most active compounds on K562 (human chronic myelogenous leukemia) cells. Cells were cultured for 24 h in the presence of each compound at the IC_{50} determined for 24 h of growth (**6a**, 150 nM; **6d**, 2.5 nM; **6e**, 20 nM; **7a**, 800 nM; **7d**, 80 nM; **7e**, 250 nM; **7h**, 25 nM; **7i**, 25 nM; **7j**, 950 nM), with CA-4 as reference compound.

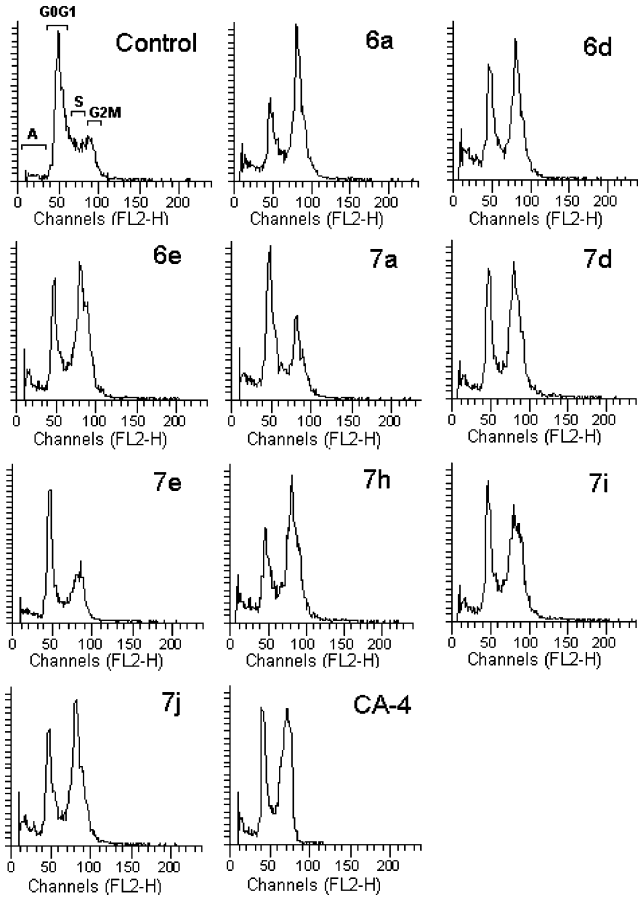


Figure 1. Effects of **6a**,**d**,**e**, **7a**,**d**,**e**,**h**−**j**, and CA-4 on DNA content/cell following treatment of K562 cells for 24 h. The cells were cultured without compound (control), with 2.5 nM or with compound used at the concentration leading to 50% cell growth inhibition after 24 h of treatment. Cell cycle distribution was analyzed by the standard propidium iodide procedure as described in the Experimental Section of Supporting Information. Sub-G0-G1 (A), G0-G1, S, and G2-M cells are indicated in the control panel.

Compounds **6a**,**d**,**e** and **7d**,**h**−**j** caused a marked increase in the percentage of cells blocked in the G2-M phase of the cell cycle, with a simultaneous decrease of cells in S and G0-G1. Compounds **7a** and **7e** were less effective as agents able to induce cell cycle changes (Figure 1). These data confirm that this class of molecules acts selectively on the G2-M phase of the cell cycle, as expected for inhibitors of tubulin assembly (Table 2).

Conclusion

The synthesis and biological evaluation of two classes of synthetic antitubulin compounds based on 2- and 3-(3′,4′,5′-trimethoxybenzoyl)benzo[*b*]thiophene skeletons are described. For the former series, for which many more compounds were available for study, the location of the methyl or methoxy substituent at the C-6 or C-7 position of the benzo[*b*]thiophene moiety was required for potency as an inhibitor of cell growth. This activity dramatically decreased when the methyl or methoxy groups were located at the C-4 or C-5 position. Compound **6d** constitutes an interesting antitubulin agent with the potential to be clinically developed for cancer treatment.. This compound was a potent inhibitor of the binding of [^{3}H]-colchicine to tubulin, and it was the most active antiproliferative agent and the most effective inhibitor of polymerization among the newly synthesized compounds. We also showed by flow cytometry that the most active compounds had cellular effects typical of agents that bind to tubulin, causing accumulation of cells in the G2-M phase of the cell cycle. Molecular modeling studies were also performed, and the proposed binding modes for **6d** and **7d** are consistent with the experimental data (see Supporting Information).

Experimental Section

General Procedure A for Synthesis of 9a−e. A mixture of aroylacetonitriles **13**−**15** (5 mmol), cyclohexanone, 4-methylcyclohexanone or 4-methoxycyclohexanone (5 mmol), morpholine (0.44 mL, 5 mmol), and sulfur (164 mg, 5 mmol) was heated at 70 °C for 1 h, then stirred at room temperature for 20 h. The mixture was evaporated and the residue diluted with dichloromethane (25 mL). After the mixture was washed with water (10 mL), the organic layer was dried and filtered and the solvent removed by evaporation. The crude product was purified by flash column chromatography and recrystallized from petroleum ether to give **9a**−**e**.

General Procedure B for Synthesis of 10a−e. Pyridine (10 drops) was added to a stirred solution of **9a**−**e** (4 mmol) in Ac_2O (10 mL). The solution was refluxed for 2 h, poured into water, and extracted with EtOAc (15 mL). The extract was washed successively with saturated aqueous $NaHCO_3$ (5 mL), water (5 mL), and brine (5 mL), dried, and concentrated in vacuo. The crude product was purified by flash column chromatography and recrystallized from petroleum ether to give **10a**−**e**.

General Procedure C for the Synthesis of 11a−e. A mixture of **10a**−**e** (2 mmol), 10% Pd/C (50% wet with water, 1.4 g), and $CHCl_3$ (20 mL) was stirred at room temperature for 10 min, followed by removal of the solvent by evaporation. The resulting powder was heated at 130 °C for 20 h, cooled to room temperature, and extracted with dichloromethane. The insoluble solids were removed by filtration, and the filtrate was concentrated in vacuo. The crude product was purified by flash column chromatography to furnish **11a**−**e**.

General Procedure D for the Synthesis of 6a−e. A mixture of **11a**−**e** (1 mmol), 1 N aqueous NaOH (1 mL, 1 mmol), and EtOH (10 mL) was refluxed for 5 h, and the solvent was removed by evaporation. The residue was triturated with water (5 mL) and extracted with dichloromethane (3 × 5 mL). The extract was washed with water to neutral reaction of the aqueous phase and dried. The residue after evaporation was purified by flash chromatography on silica gel and crystallized from petroleum ether to give **6a**−**e**.

Supporting Information Available: Detailed biological protocols, physical and spectroscopic data for **6a**−**e**, **7a**−**l**, **9a**−**e**, **10a**−**e**, **11a**−**e**, **20a**−**e**, **22f**−**j**, **23a**−**e**, **23f**−**j**; elemental analysis results of **6a**−**e** and **7a**−**l**; and molecular modeling studies of **6d** and **7d**. This material is available free of charge via the Internet at http://pubs.acs.org.

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