

Figure 2. Comparison of the effects of 8AcCS with those of cyclostreptin and paclitaxel on tubulin assembly. Drugs were added to otherwise complete reaction mixtures following establishment of baselines at 350 nm in the spectrophotometer. The temperature was initially at 0 °C and set at the indicated temperatures at the times indicated by the vertical dashed lines. (A) Assembly dependent on MAPs. Each 0.25 mL reaction mixture contained 15 μ M (1.5 mg/mL) purified tubulin, 1.0 mg/mL heat-treated MAPs, 0.1 M Mes (pH 6.9 with NaOH in 1.0 M stock solution), 0.4 mM GTP, 0.5 mM MgCl₂, 2% (v/v) dimethyl sulfoxide, and either no further addition (curve 0), 15 μ M paclitaxel (curve P), 15 μ M cyclostreptin (curve 1), or 15 μ M 8AcCS. (B) Assembly dependent on glycerol. Each 0.25 mL reaction contained 30 μ M tubulin, 3.4 M glycerol, 6 mM MgCl₂, 1.0 mM GTP, 1.0 mM EGTA, 10 mM sodium phosphate buffer at pH 6.8, 3% (v/v) dimethylsulfoxide, and either no further addition (curve 0), 30 μ M paclitaxel (curve P), or 10 (curve 1), 30 (curve 2), or 60 (curve 3) μ M 8AcCS.

drug-free control, partial assembly occurred at 20 °C, and the MTs formed were unstable at 0 °C. Cyclostreptin enhanced the reaction at 20 °C, and little further assembly occurred when the reaction temperature was raised to 30 °C. With 8AcCS, assembly differed little from that which occurred with cyclostreptin, except that a low level of assembly was observed at 10 °C.

This and similar studies with MAPs demonstrated that 8AcCS was equivalent to cyclostreptin in its interactions with tubulin, and we therefore synthesized [¹⁴C]8AcCS to evaluate binding and covalent interactions of this class of taxoid site agent with tubulin. Our earlier demonstration of the covalent binding of cyclostreptin to MTs employed polymer induced with glycerol/Mg²⁺,⁶ and in our initial experiments with the radiolabeled analogue, we demonstrated a rapid covalent reaction of the compound with glutaraldehyde cross-linked and stabilized MTs (see below). We wished also to evaluate potential binding interactions with unpolymerized tubulin. To minimize effects of changes in reaction conditions, we therefore examined effects of 8AcCS on tubulin assembly induced by glycerol. Assembly in the absence of drug required a higher tubulin concentration (we used 30 μ M vs 15 μ M with MAPs), and Figure 2B compares the reactions that occurred without drug and with 10, 30, and 60 μ M 8AcCS and with 30 μ M paclitaxel. Only with paclitaxel was there assembly at 0 °C, and the reaction was nearly complete at 10 °C. Under this reaction condition, the paclitaxel-enhanced polymer was stable at 0 °C. Without drug, there was a slight reaction at 20 °C, a much brisker reaction at 30 °C, with disappearance of polymer at 0 °C. The 8AcCS progressively enhanced the 20 °C reaction, with further assembly occurring at 30 °C with all concentrations of 8AcCS. Like paclitaxel, 8AcCS induced formation of polymer stable at 0 °C. Note that at the

Table 1. Comparison of the Binding and Covalent Reaction of 8AcCS with MTs^a

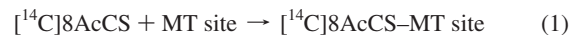
	reaction temperature			
	0 °C		20 °C	
	reaction time		reaction time	
	1 min	60 min	1 min	60 min
	mol 8AcCS/mol taxoid site \pm SD			
total ^b	0.14 \pm 0.03	0.38 \pm 0.05	0.53 \pm 0.05	0.93 \pm 0.09
covalent ^c	0.13 \pm 0.01	0.35 \pm 0.03	0.46 \pm 0.06	0.81 \pm 0.05

^a Reaction mixtures contained 0.25 mg/mL (2.0 μ M taxoid binding sites) tubulin in glutaraldehyde-fixed MTs, 2.5 μ M [¹⁴C]8AcCS, 3.4 M glycerol, 6 mM MgCl₂, 1.0 mM EGTA, 1.0 mM GTP, 10 mM sodium phosphate buffer at pH 6.8, and 3% (v/v) dimethylsulfoxide. Each experimental condition was evaluated three times. ^b Total binding was measured by applying aliquots of the reaction mixtures to microcolumns in which the Sephadex G-50 superfine was swollen in 3.4 M glycerol, 6 mM MgCl₂, 1.0 mM EGTA, 1.0 mM GTP, 10 mM sodium phosphate buffer at pH 6.8, and 3% (v/v) dimethylsulfoxide. ^c The covalent reaction was measured by mixing an aliquot of each reaction mixture with an equal volume of 8 M guanidine hydrochloride, and a portion of the mixture was applied to microcolumns in which the Sephadex G-50 superfine was swollen in 4 M guanidine hydrochloride.

substoichiometric concentration of 10 μ M 8AcCS, there was partial polymer disassembly at 0 °C.

We initially evaluated the interaction of [¹⁴C]8AcCS with glutaraldehyde cross-linked and stabilized MTs. These polymers are stable at low temperatures, can be stored for prolonged periods of time with minimal deterioration, and retain functional taxoid sites.^{7,8} We examined binding of [¹⁴C]8AcCS to these MTs by subjecting reaction mixtures to gel filtration chromatography under native and denaturing (4 M guanidine hydrochloride) conditions, with the former measuring total binding and the latter covalent binding to tubulin. In our first experiments, we compared binding for brief (1 min) and prolonged (1 h) reaction periods at 0 and 20 °C (Table 1). In these experiments we found that, although binding appeared to be more rapid and extensive at the higher temperature, similar amounts of [¹⁴C]8AcCS bound to the MTs whether native or denatured gel filtration was performed. In contrast, with radiolabeled paclitaxel negligible amounts of drug were recovered from the denatured columns as compared with the native columns (data not presented). We therefore concluded that [¹⁴C]8AcCS reacts with tubulin almost immediately after binding to the MT.

In subsequent experiments with MTs, we only followed the covalent interaction (examination of samples on the 4 M guanidine hydrochloride columns). This allowed us to examine in detail binding kinetics by stopping reactions with the denaturant. Reactions were examined at three reaction temperatures (0, 10, and 20 °C) (Figure 3A) and using the following model reaction:



and solving

$$d[^{14}\text{C}]8\text{AcCS-MT site}/dt = k_1[^{14}\text{C}]8\text{AcCS}[\text{MT site}] \quad (2)$$

through analysis of the data with the FITSIM software package,⁹ the following apparent second-order rate constants were obtained: 6.2 \pm 0.2 (SE) \times 10² M⁻¹ s⁻¹ at 0 °C, 3.5 \pm 0.1 \times 10³ M⁻¹ s⁻¹ at 10 °C, and 5.6 \pm 0.3 \times 10³ M⁻¹ s⁻¹ at 20 °C. Thus, at 10 and 20 °C, the rate constants were 5.8- and 9-fold greater than the rate constant at 0 °C, with the 20 °C rate constant 1.6-fold greater than the 10 °C rate constant.

When an Arrhenius plot was prepared from these data (Figure 3B), linearity was not observed. If only the 0 and 10 °C rate constants are used for the Arrhenius plot, an activation energy of 27 kcal/mol was obtained. A much lower value, 7.7 kcal/mol, was obtained from the values for the 10 and 20 °C rate constants. A nonlinear Arrhenius plot may indicate that, rather than a single

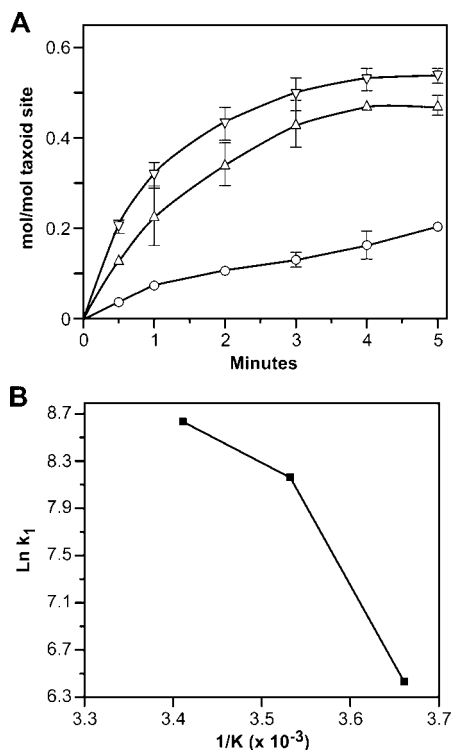


Figure 3. (A) Kinetics of interaction of [¹⁴C]8AcCS with glutaraldehyde-fixed MTs. Each reaction mixture contained MTs at 0.25 mg/mL (2.0 μM taxoid binding sites) and [¹⁴C]8AcCS at 2.5 μM. Reaction mixtures also contained 3.4 M glycerol, 6 mM MgCl₂, 1.0 mM GTP, 1.0 mM EGTA, 10 mM sodium phosphate buffer at pH 6.8, and 3% (v/v) dimethylsulfoxide. Reaction mixtures were incubated at 0 °C (○), 10 °C (Δ), or 20 °C (∇) for the indicated times, at which points 100 μL of the reaction mixture was rapidly mixed with 100 μL of 8 M guanidine hydrochloride. A 150 μL portion of each of these mixtures was applied to a microcolumn of Sephadex G-50 (superfine) equilibrated with 4 M guanidine hydrochloride. Radiolabel and protein in microcolumn filtrates were determined as described in the Experimental Section. (B) Arrhenius plot derived from the initial rates of the reactions shown in Figure 2A. See text for additional details.

reaction, two different reactions, either competitive or consecutive, with different activation energies were being examined.

If the two reactions were competitive, at the lower temperature the reaction with the lower activation energy would predominate, while at the higher temperature the reaction with the higher activation energy would predominate. However, this means that the observed activation energy should increase with temperature, contrary to what occurred here.

Therefore, the most plausible explanation for the apparent lower activation energy at the higher temperature is that there are two consecutive reactions with similar reaction rates, but different activation energies, specifically the binding reaction followed by the covalent reaction. Since binding must occur first, if its activation energy is higher, at low temperature it will be the observed reaction and the primary contributor to the observed activation energy. In contrast, at higher temperatures binding becomes fast, and the reaction with the lower activation energy becomes the rate-limiting step and thus is the one observed.

We also examined whether [¹⁴C]8AcCS would react with tubulin prior to assembly. Initial experiments were performed with 5 μM tubulin and 5 μM [¹⁴C]8AcCS in the glycerol system at 0 and 20 °C, but neither binding nor a covalent reaction occurred (data not shown). When the reactant concentrations were increased to 30 μM, however, slight binding was detected after a 1 h incubation on ice and an extensive reaction even at 1 min at 20 °C (Table 2). At 20

Table 2. Comparison of the Binding and Covalent Reaction of 8AcCS with Tubulin^a

	reaction temperature			
	0 °C		20 °C	
	reaction time		reaction time	
	1 min	60 min	1 min	60 min
total ^b	mol 8AcCS/mol tubulin ± SD			
	0.01 ± 0.002	0.06 ± 0.01	0.60 ± 0.20	0.81 ± 0.13
covalent ^c	not examined		0.70 ^d	0.88 ± 0.01

^a Reaction mixtures contained 3.0 mg/mL (30 μM) tubulin, 30 μM [¹⁴C]8AcCS, 3.4 M glycerol, 6 mM MgCl₂, 1.0 mM EGTA, 1.0 mM GTP, 10 mM sodium phosphate buffer at pH 6.8, and 3% (v/v) dimethylsulfoxide. Each experimental condition was evaluated twice. ^b Total binding was measured by applying aliquots of the reaction mixtures to microcolumns in which the Sephadex G-50 superfine was swollen in 3.4 M glycerol, 6 mM MgCl₂, 1.0 mM EGTA, 1.0 mM GTP, 10 mM sodium phosphate buffer at pH 6.8, and 3% (v/v) dimethylsulfoxide. ^c The covalent reaction was measured by mixing an aliquot of each reaction mixture with an equal volume of 8 M guanidine hydrochloride, and a portion of the mixture was applied to microcolumns in which the Sephadex G-50 superfine was swollen in 4 M guanidine hydrochloride. ^d The same value was obtained in both experiments.

°C, higher bound ligand was recovered from the guanidine hydrochloride columns than the native columns, but the difference is not statistically significant.

It was of interest to determine how the covalent reaction of [¹⁴C]8AcCS with tubulin correlated with tubulin assembly. In planning an experiment with 30 μM tubulin and 30 μM [¹⁴C]8AcCS to be performed in multiple cuvettes in a spectrophotometer, however, we found that insufficient [¹⁴C]8AcCS remained for such experiments. We therefore reduced the [¹⁴C]8AcCS concentration to 10 μM (see Figure 2B) and performed the experiment shown in Figure 4 two times, with no significant difference in the results obtained. At the conclusion of the experiment, residual reaction mixture was centrifuged to determine the proportion of tubulin that was polymerized (59%). This value was used to position the final turbidity point on the molar scale, and the covalently bound [¹⁴C]8AcCS at different time intervals was superimposed on the turbidity curve. At the two initial time points selected for sample withdrawal, there had been no turbidity development, but some covalent reaction had occurred. At the third time point, there still appeared to be a lag in turbidity development relative to the amount of covalent bond formation, while at the fourth time point the covalent reaction had reached a plateau, which superimposed on the turbidity curve at the fourth point and subsequently was substoichiometric to the amount of tubulin polymerized. The continuing turbidity development following apparent exhaustion of the 8AcCS indicates that unliganded tubulin was able to add to 8AcCS-containing seed polymers, as had been implied in the earlier experiment of Figure 2B by the partial instability of the corresponding polymer to 0 °C.

The covalent reaction of 8AcCs that occurred at 0 °C, in the absence of assembly, and at 20 °C prior to the onset of assembly is consistent with the prior observation with cyclostreptin⁶ that a low-level covalent reaction occurred only at Thr-218 with unpolymerized tubulin (both dimers and small oligomers), while the reaction with Asn-226 required MTs.

Summarizing the above findings, we have found that 8AcCs has properties similar to the natural product cyclostreptin in inducing formation of cold-stable MTs. Preparation of a radiolabeled form of 8AcCs has allowed us to demonstrate the close temporal association of the binding of 8AcCS to MTs and formation of a covalent bond between MTs and the bound 8AcCS. Considering the apparent minimal time interval between the binding of 8AcCS to microtubules and its covalent reaction with β-tubulin, it seems unlikely that this more complex mode of interaction can account

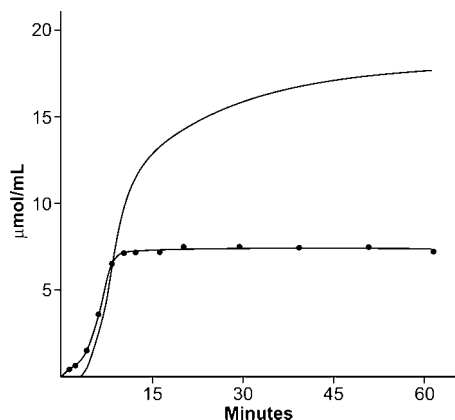


Figure 4. Covalent interaction of [^{14}C]8AcCS with tubulin correlated with turbidity development. Baselines were established in multiple cuvettes held at $0\text{ }^{\circ}\text{C}$ containing the same reaction mixture, consisting of $30\text{ }\mu\text{M}$ tubulin, $10\text{ }\mu\text{M}$ [^{14}C]8AcCS, 3.4 M glycerol, 6 mM MgCl_2 , 1.0 mM GTP, 1.0 mM EGTA, 10 mM sodium phosphate buffer at pH 6.8, and 3% (v/v) dimethylsulfoxide. The temperature was rapidly increased to $20\text{ }^{\circ}\text{C}$, assembly was followed turbidimetrically, and $150\text{ }\mu\text{L}$ aliquots were removed from one of the cuvettes at the indicated times (zero time was the point at which the temperature jump was initiated). The turbidity curves were superimposed on each other, and the aliquots were immediately mixed with an equal volume of 8 M guanidine hydrochloride. Half of each aliquot was analyzed by centrifugal gel filtration on each of two columns equilibrated with 4 M guanidine hydrochloride (see Experimental Section for further details). At the final time point, aliquots of the residual reaction mixture were removed for protein determination, and the remainder of the reaction mixture was centrifuged at $50\text{ }000\text{ rpm}$ for 15 min at $20\text{ }^{\circ}\text{C}$ in a Beckman 120.1 rotor in a Beckman Optima TLX miniultracentrifuge. Aliquots of the supernatant were removed for protein determination. In comparison with the initial protein concentration, it was determined that 59% of the tubulin was in the polymer pellet. This corresponded to $17.7\text{ }\mu\text{mol/mL}$ of tubulin, and the final point of the turbidity curve was placed at this level in the figure. The amounts of [^{14}C]8AcCS covalently bound to tubulin at each time point were plotted on the same molar scale.

for the reduced ability of cyclostreptin and 8AcCS to induce microtubule assembly relative to paclitaxel.

In fact, there appears to be a low-level covalent interaction of 8AcCS with tubulin prior to MT assembly. As in the study with cyclostreptin,⁶ this probably occurs at Thr-218 of β -tubulin, which is not at the taxoid site in the microtubule. In the microtubule, Thr-218 is located near a pore in the microtubule wall not far from the taxoid site. This finding implies that the enhanced nucleation that occurs with taxoid site drugs may derive from weak binding at or near Thr-218, and this binding permits more ready initiation of microtubule assembly by a tubulin–drug complex than by unliganded tubulin. Although we have demonstrated this interaction with cyclostreptin⁶ and 8AcCS because of the covalent bonds they form with β -tubulin, it seems likely that similar, more robust reactions occur between tubulin and taxoid site drugs, such as paclitaxel, that more potently induce assembly.

Finally, we should note that in cells and organisms the deficiency of cyclostreptin and 8AcCS in inducing microtubule assembly may not affect their abilities to disrupt microtubule dynamics. The compounds are added to systems with extensive, pre-existing microtubule networks, and as shown with cyclostreptin and cultured cells,⁶ they should bind avidly to such microtubules, as they do to the glutaraldehyde-fixed microtubules.

Experimental Section

General Experimental Procedures. Electrophoretically homogeneous bovine brain tubulin and MAPs¹⁰ and synthetic cyclostreptin¹¹ were prepared as described previously. Mildly cross-linked, stabilized microtubules were prepared as described previously,⁷ and the concentration of taxoid binding sites in the preparation was 80% (determined by maximum stoichiometry of binding of [^{14}C]8AcCS. Paclitaxel was generously provided by the Drug Synthesis and Chemistry Branch of the National Cancer Institute.

Synthesis of 8AcCS. Synthetic cyclostreptin¹¹ (ca. 3 mg , $7.5\text{ }\mu\text{mol}$) was dissolved in CH_2Cl_2 ($150\text{ }\mu\text{L}$). 2,4,6-Collidine ($20\text{ }\mu\text{L}$, $150\text{ }\mu\text{mol}$) was added, and the resulting mixture was cooled to $0\text{ }^{\circ}\text{C}$ with an ice/water bath. Acetyl chloride was added in $1\text{ }\mu\text{L}$ portions (ca. 2 equiv) until the cyclostreptin was consumed; the progress of the reaction was monitored by TLC. Aqueous 1 N HCl (1 mL) was added, and the resulting mixture was extracted with EtOAc ($3 \times 1\text{ mL}$). The combined organic extracts were washed with brine (2 mL), dried over MgSO_4 , and concentrated under a stream of nitrogen gas. The residue was purified by column chromatography (ca. 1.5 mL silica gel, 2:1 \rightarrow 3:2 \rightarrow 1:1 hexanes–EtOAc) to afford 8AcCS (ca. 1.1 mg): $^1\text{H NMR}$ (CD_3OD , 500 MHz) δ 5.33 (1H, s), 4.68 (1H, d, $J = 4.7\text{ Hz}$), 4.48 (1H, brs), 3.58 (1H, dd, $J = 9.1, 5.3\text{ Hz}$), 3.51 (1H, d, $J = 9.0\text{ Hz}$), 2.82 (1H, m), 2.69 (1H, m), 2.48 (1H, m), 2.35 (1H, m), 2.17 (1H, m), 2.10 (1H, dd, $J = 12.0, 6.7\text{ Hz}$), 2.03 (3H, s), 1.75–1.85 (4H, m), 1.73 (3H, s), 1.71 (1H, m), 1.53–1.67 (2H, m), 1.45 (3H, s), 1.22 (3H, d, $J = 7.5\text{ Hz}$), 1.14 (3H, d, $J = 6.9\text{ Hz}$); HRMS m/z 443.2433 [$\text{M} + \text{H}$]⁺ (calcd for $\text{C}_{26}\text{H}_{35}\text{O}_6$ 443.2434); m/z 465.2241 [$\text{M} + \text{Na}$]⁺ (calcd for $\text{C}_{26}\text{H}_{34}\text{O}_6\text{Na}$, 465.2253). The selective formation of the monoacetate of the C-8 hydroxyl group was confirmed on the basis of the change in chemical shift of the C-8 proton from 3.61 ppm (dd, $J = 5.8, 1.3\text{ Hz}$) in cyclostreptin to 4.68 ppm (d, $J = 4.7\text{ Hz}$) in 8AcCS (both taken in CD_3OD). The C-6 proton was relatively invariant [(3.46 ppm, dd, $J = 11.0, 6.6\text{ Hz}$) vs (3.58 ppm, dd, $J = 9.1, 5.3\text{ Hz}$), respectively]. Cyclostreptin (bis)acetate was made and characterized by Yoshimura and co-workers,¹² and the chemical shifts for the C-8 and C-6 protons were 4.70 ppm (d, $J = 4.4\text{ Hz}$) and 4.61 ppm (dd, $J = 9.0, 4.6\text{ Hz}$), respectively (in CD_2Cl_2) (personal communication, S. Yoshimura, Fujisawa Pharmaceutical Co., Ltd.). Selective monoacylation of the less hindered C-8 hydroxyl group under nearly identical conditions has been reported.¹³

Synthesis of [^{14}C]8AcCS. The same procedure used to prepare nonradiolabeled 8AcCS was followed to synthesize [^{14}C]8AcCS, using cyclostreptin (ca. 4 mg , $10\text{ }\mu\text{mol}$), 2,4,6-collidine ($26\text{ }\mu\text{L}$, $200\text{ }\mu\text{mol}$) in CH_2Cl_2 ($200\text{ }\mu\text{L}$), and 5 mCi of [^{14}C]acetyl chloride (Moravek Biochemicals, 57 mCi/mmol , $7.8\text{ }\mu\text{L}$, 7.1 mg , $88\text{ }\mu\text{mol}$) mixed with $14.4\text{ }\mu\text{L}$ of nonradiolabeled acetyl chloride (final specific activity, 20 mCi/mmol). The acetyl chloride was added portionwise in its entirety. Approximately 1.5 mg of purified product was obtained, and the $^1\text{H NMR}$ data were identical with those summarized above for nonradiolabeled 8AcCS.

Turbidimetry. Tubulin assembly was followed turbidimetrically at 350 nm in Gilford 250 spectrophotometers equipped with electronic temperature controllers. When a temperature change is initiated, the temperature rises at about $0.5\text{ }^{\circ}\text{C/s}$ and falls at about $6\text{ }^{\circ}\text{C/min}$.

Centrifugal Gel Filtration. The procedure was described in detail previously,¹⁴ except for centrifugation details (see below). The columns were prepared in tuberculin syringes plugged with custom-made polyethylene disks. The column matrix was Sephadex G-50 (superfine) swollen in the same buffer as used in the experiments (3.4 M glycerol, 6 mM MgCl_2 , 1.0 mM EGTA, 1.0 mM GTP, 10 mM sodium phosphate buffer at pH 6.8, 3% (v/v) dimethylsulfoxide) or in 4 M guanidine hydrochloride. For native binding studies, $150\text{ }\mu\text{L}$ samples were applied directly to the precentrifuged column bed, and the column was centrifuged again. For denatured binding studies, aliquots of reaction mixtures were added at the indicated times to an equal volume of 8 M guanidine hydrochloride. A $150\text{ }\mu\text{L}$ aliquot of the mixture was applied to the precentrifuged column bed, and the column was centrifuged again. Radiolabel in column filtrates was determined by scintillation counting, and protein was determined by the Bradford assay.¹⁵ The syringe columns were centrifuged, both before and after sample application, for 4 min at 2000 rpm in a Beckman Allegra 6KR centrifuge, using a GH-3.8A horizontal rotor. The adapters used were those with $30\text{ }13 \times 100\text{ mm}$ openings.

References and Notes

- (1) Hamel, E. In *Microtubule Targets in Cancer Therapy*; Fojo, A. T., Ed.; Humana Press: Totowa, NJ, 2008; in press.
- (2) Combeau, C.; Provost, J.; Lancelin, F.; Tournoux, Y.; Prod'homme, F.; Herman, F.; Lavelle, F.; Leboul, J.; Vuilhorgne, M. *Mol. Pharmacol.* **2000**, *57*, 553–563.
- (3) Sato, B.; Muramatsu, H.; Miyauchi, M.; Hori, Y.; Taskase, S.; Hino, M.; Hashimoto, S.; Terano, H. *J. Antibiot.* **2000**, *53*, 123–130.
- (4) Sato, B.; Nakajima, H.; Hori, Y.; Hino, M.; Hashimoto, S.; Terano, H. *J. Antibiot.* **2000**, *53*, 204–206.
- (5) Edler, M. C.; Buey, R. M.; Gussio, R.; Marcus, A. I.; Vanderwal, C. D.; Sorensen, E. J.; Díaz, J. F.; Giannakakou, P.; Hamel, E. *Biochemistry* **2005**, *44*, 11525–11538.
- (6) Buey, R. M.; Calvo, E.; Barasoain, I.; Pineda, O.; Edler, M. C.; Matesanz, R.; Cerezo, G.; Vanderwal, C. D.; Day, B. W.; Sorensen, E. J.; López, J. A.; Andreu, J. M.; Hamel, E.; Díaz, J. F. *Nat. Chem. Biol.* **2007**, *3*, 117–125.
- (7) Díaz, J. F.; Barasoain, I.; Andreu, J. M. *J. Biol. Chem.* **2003**, *278*, 8407–8419.
- (8) Buey, R. M.; Díaz, J. F.; Andreu, J. M.; O'Brate, A.; Giannakakou, P.; Nicolaou, K. C.; Sasmal, P. K.; Ritzén, A.; Namoto, K. *Chem. Biol.* **2004**, *11*, 225–236.
- (9) Barshop, B. A.; Wrenn, R. F.; Frieden, C. *Anal. Biochem.* **1983**, *130*, 134–145.
- (10) Hamel, E.; Lin, C. M. *Biochemistry* **1984**, *23*, 4173–4184.
- (11) Vanderwal, C. D.; Vosburg, D. A.; Weiler, S.; Sorensen, E. J. *J. Am. Chem. Soc.* **2003**, *125*, 5393–5407.
- (12) Yoshimura, S.; Sato, B.; Kinoshita, T.; Takase, S.; Terano, H. *J. Antibiot.* **2000**, *53*, 615–622; erratum *J. Antibiot.* **2002**, *55*, C-1.
- (13) Adam, G. C.; Vanderwal, C. D.; Sorensen, E. J.; Cravatt, B. F. *Agnew. Chem., Int. Ed.* **2003**, *42*, 5480–5484.
- (14) Hamel, E.; Lin, C. M. *J. Biol. Chem.* **1984**, *259*, 11060–11069.
- (15) Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248–254.

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