Interaction of a Cyclostreptin Analogue with the Microtubule Taxoid Site: The Covalent Reaction Rapidly Follows Binding[⊥]

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The natural product cyclostreptin reacts covalently and stoichiometrically with microtubules, at either of two amino acid residues of β-tubulin, Thr-218 or Asn-226, but much less extensively and only at Thr-218 in unpolymerized tubulin. It was found that 8-acetylcyclostreptin (8AcCS) induces tubulin assembly in a manner almost identical with that of cyclostreptin. We therefore synthesized [^{14}C-acetyl]8AcCS and studied the kinetics of its interaction with glutaraldehyde-stabilized microtubules and with unassembled tubulin. With the microtubules, we found that 8AcCS bound rapidly, with a minimal (unmeasurable with the radiolabeled analogue) lag prior to the occurrence of the covalent reaction. Apparent reaction rate constants for the overall reaction ranged from 6.2×10^2 M^{-1} s^{-1} at 0 °C to 5.6×10^3 M^{-1} s^{-1} at 20 °C. The rate constants obtained at 0 and 10 °C indicate an activation energy for the reaction of about 27 kcal/mol, while those obtained at 10 and 20 °C indicate an activation energy of about 7.7 kcal/mol. With the unpolymerized tubulin, we did find a minimal covalent reaction occurred without apparent microtubule assembly, but a substantial reaction only occurred following assembly. In conclusion, the radiolabeled 8AcCS shows that an extensive covalent interaction of ligand with tubulin requires microtubule assembly and that the covalent reaction occurs rapidly after the initial binding interaction.

The natural world has served as a rich source of highly cytotoxic compounds that interfere with normal microtubule (MT) function in cells.[1] Compounds discovered thus far have varying mechanisms of action, binding at numerous sites on the tubulin molecule. While for the most part these interactions have been noncovalent with a wide range of binding affinities, with at least two natural products covalent binding modes have been described. The plant-derived compound ottelione A inhibits MT assembly by binding in the colchicine site of tubulin, and it most likely alkylates Cys-239 of β-tubulin.[2] In contrast, cyclostreptin (structure shown in Figure 1), which was originally isolated from the growth medium of a *Streptomyces* species as FR182877,[3] induces MT assembly and binds in the taxoid site.[4,5] Cyclostreptin, in comparison with other taxoid site compounds, only weakly promotes tubulin polymerization,[5] but it quantitatively and stoichiometrically reacts covalently with MTs at either of two amino acid residues of β-tubulin.[6] These are Asn-226, located at the taxoid site, and Thr-218, which is located on the outer MT surface at a pore near the taxoid site. This probably occurs by nucleophilic attack by the amino acids, when properly oriented, on the strained bridgehead olefin of cyclostreptin at C-17.

Among the intriguing properties of cyclostreptin was minimal ability to induce tubulin assembly at lower temperatures (<20 °C) and, relative to paclitaxel and docetaxel, apparently reduced affinity for the taxoid site at 4 °C versus 30 °C.[5] We desired radiolabeled cyclostreptin to study its binding reactions with tubulin in greater detail, but its synthesis was not feasible. An alternate approach was suggested by initial structure–activity studies of cyclostreptin. We found that 6-acetylcyclostreptin, 8-acetylcyclostreptin (8AcCS; structure in Figure 1), and 6,8-diacetylcyclostreptin all seemed to be comparable to cyclostreptin in their abilities to induce MT assembly. In the acetylation reaction that produced these compounds, we found that the major product was the 8AcCS, presumably because the 6-hydroxyl group is masked by the conformation of cyclostreptin. Further work, as described here, showed that 8AcCS was modestly more active than cyclostreptin as an inducer of tubulin assembly. We therefore prepared radiolabeled 8AcCS ([^{14}C]8AcCS; position of radiolabel shown in Figure 1) and studied its binding and covalent interaction with β-tubulin and with microtubules. We wished to determine whether there was any kinetic lag between the binding of 8AcCS to tubulin or microtubules and the covalent reaction of the analogue with tubulin. If such a lag existed, it might help us understand why cyclostreptin, despite its irreversible reaction with tubulin, is less potent than paclitaxel.

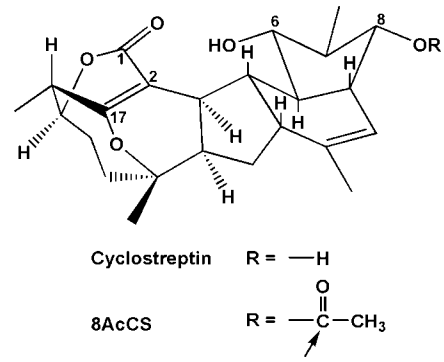

Figure 1. Molecular structures of cyclostreptin and 8AcCS. The arrow indicates the radiolabeled carbon atom in [^{14}C]8AcCS.

Results and Discussion

Our initial studies with 8AcCS were in an assembly system that included MT-associated proteins (MAPs). An example of the relative activities of cyclostreptin, 8AcCS, and paclitaxel in enhancing MT assembly with MAPs is shown in Figure 2A. Tubulin and drug concentrations were all 15 μM. As in previous studies,[5] cyclostreptin was less active than paclitaxel. There appeared to be partial cold reversibility of paclitaxel-enhanced assembly, as compared with little reversibility with cyclostreptin or 8AcCS. There was nevertheless partial assembly with paclitaxel at 0 °C. In the

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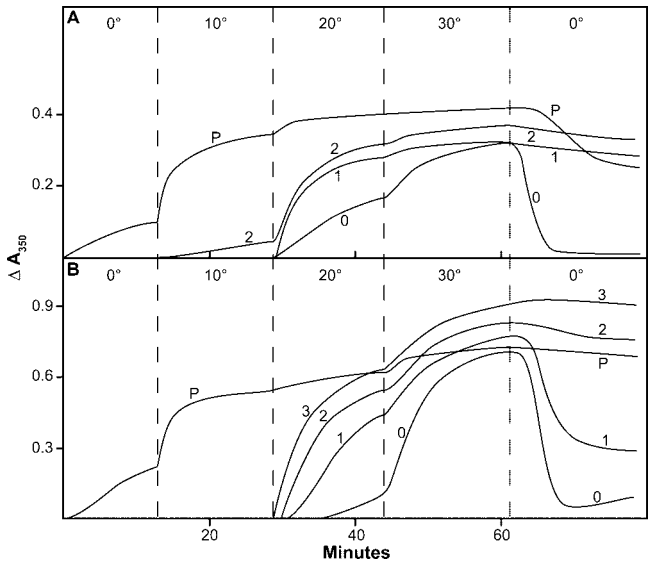

Figure 2. Comparison of the effects of 8AcCS with those of cyclostreptin and paclitaxel on tubulin assembly. Drugs were added to otherwise complete reaction mixtures following establishment of baselines at 350 nm in the spectrophotometer. The temperature was initially at 0 °C and set at the indicated temperatures at the times indicated by the vertical dashed lines. (A) Assembly dependent on MAPs. Each 0.25 mL reaction mixture contained 15 μM (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 $MgCl_2$, 2% (v/v) dimethyl sulfoxide, and either no further addition (curve 0), 15 μM paclitaxel (curve P), 15 μM cyclostreptin (curve 1), or 15 μM 8AcCS. (B) Assembly dependent on glycerol. Each 0.25 mL reaction contained 30 μM tubulin, 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, 3% (v/v) dimethylsulfoxide, and either no further addition (curve 0), 30 μM paclitaxel (curve P), or 10 (curve 1), 30 (curve 2), or 60 (curve 3) μM 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 [^{14}C]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/Mg^{2+},[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 μM vs 15 μM with MAPs), and Figure 2B compares the reactions that occurred without drug and with 10, 30, and 60 μM 8AcCS and with 30 μM 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 substoichiometric concentration of 10 μM 8AcCS, there was partial polymer disassembly at 0 °C.

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 ± SD			
total[b]	0.14 ± 0.03	0.38 ± 0.05	0.53 ± 0.05	0.93 ± 0.09
covalent[c]	0.13 ± 0.01	0.35 ± 0.03	0.46 ± 0.06	0.81 ± 0.05

[a] Reaction mixtures contained 0.25 mg/mL (2.0 μM taxoid binding sites) tubulin in glutaraldehyde-fixed MTs, 2.5 μM [^{14}C]8AcCS, 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, 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 $MgCl_2$, 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.

We initially evaluated the interaction of [^{14}C]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 [^{14}C]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 [^{14}C]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 [^{14}C]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:

$$[^{14}\text{C}]8\text{AcCS} + \text{MT site} \rightarrow [^{14}\text{C}]8\text{AcCS–MT site} \quad (1)$$

and solving

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

through analysis of the data with the FITSIM software package,[9] the following apparent second-order rate constants were obtained: 6.2 ± 0.2 (SE) × 10^2 M^{-1} s^{-1} at 0 °C, 3.5 ± 0.1 × 10^3 M^{-1} s^{-1} at 10 °C, and 5.6 ± 0.3 × 10^3 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

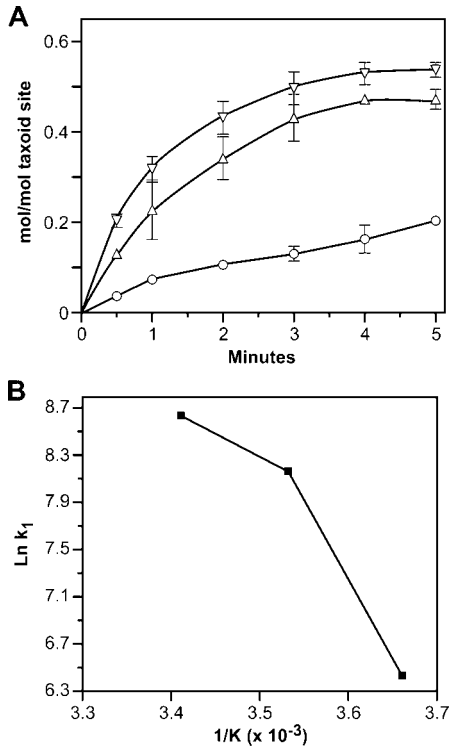

Figure 3. (A) Kinetics of interaction of [^{14}C]8AcCS with glutaraldehyde-fixed MTs. Each reaction mixture contained MTs at 0.25 mg/mL (2.0 μM taxoid binding sites) and [^{14}C]8AcCS at 2.5 μM. Reaction mixtures also contained 3.4 M glycerol, 6 mM $MgCl_2$, 1.0 mM GTP, 1.0 mM EGTA, 10 mM sodium phosphate buffer at pH 6.8, and 3% (v/v) dimethylsulfoxide. 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 [^{14}C]8AcCS would react with tubulin prior to assembly. Initial experiments were performed with 5 μM tubulin and 5 μM [^{14}C]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 °C, higher bound ligand was recovered from the guanidine hydrochloride columns than the native columns, but the difference is not statistically significant.

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
	mol 8AcCS/mol tubulin ± SD			
total[b]	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 [^{14}C]8AcCS, 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, 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 $MgCl_2$, 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.

It was of interest to determine how the covalent reaction of [^{14}C]8AcCS with tubulin correlated with tubulin assembly. In planning an experiment with 30 μM tubulin and 30 μM [^{14}C]8AcCS to be performed in multiple cuvettes in a spectrophotometer, however, we found that insufficient [^{14}C]8AcCS remained for such experiments. We therefore reduced the [^{14}C]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 [^{14}C]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 cyclostreptin[6] 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

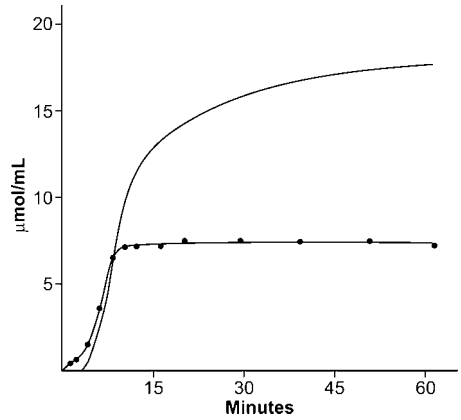

Figure 4. Covalent interaction of [^{14}C]8AcCS with tubulin correlated with turbidity development. Baselines were established in multiple cuvettes held at 0 °C containing the same reaction mixture, consisting of 30 μM tubulin, 10 μM [^{14}C]8AcCS, 3.4 M glycerol, 6 mM $MgCl_2$, 1.0 mM GTP, 1.0 mM EGTA, 10 mM sodium phosphate buffer at pH 6.8, and 3% (v/v) dimethylsulfoxide. The temperature was rapidly increased to 20 °C, assembly was followed turbidimetrically, and 150 μL 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 turbdity 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 50 000 rpm for 15 min at 20 °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 μmol/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 cyclostreptin[6] 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 MAPs[10] and synthetic cyclostreptin[11] 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 cyclostreptin[11] (ca. 3 mg, 7.5 μmol) was dissolved in CH_2Cl_2 (150 μL). 2,4,6-Collidine (20 μL, 150 μmol) was added, and the resulting mixture was cooled to 0 °C with an ice/water bath. Acetyl chloride was added in 1 μL 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 × 1 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 → 3:2 → 1:1 hexanes−EtOAc) to afford 8AcCS (ca. 1.1 mg): ^{1}H NMR (CD_3OD, 500 MHz) δ 5.33 (1H, s), 4.68 (1H, d, J = 4.7 Hz), 4.48 (1H, brs), 3.58 (1H, dd, J = 9.1, 5.3 Hz), 3.51 (1H, d, J = 9.0 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 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 Hz), 1.14 (3H, d, J = 6.9 Hz); HRMS m/z 443.2433 $[M + H]^+$ (calcd for $C_{26}H_{35}O_6$ 443.2434); m/z 465.2241 $[M + Na]^+$ (calcd for $C_{26}H_{34}O_6Na$, 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 Hz) in cyclostreptin to 4.68 ppm (d, J = 4.7 Hz) in 8AcCS (both taken in CD_3OD). The C-6 proton was relatively invariant [(3.46 ppm, dd, J = 11.0, 6.6 Hz) vs (3.58 ppm, dd, J = 9.1, 5.3 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 Hz) and 4.61 ppm (dd, J = 9.0, 4.6 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 μmol), 2,4,6-collidine (26 μL, 200 μmol) in CH_2Cl_2 (200 μL), and 5 mCi of [1-^{14}C]acetyl chloride (Moravek Biochemicals, 57 mCi/mmol, 7.8 μL, 7.1 mg, 88 μmol) mixed with 14.4 μL 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}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 °C/s and falls at about 6 °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 μL 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 μL 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 13 × 100 mm openings.

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