5a-Formylbicyclomycin: Studies on the Bicyclomycin−Rho Interaction†

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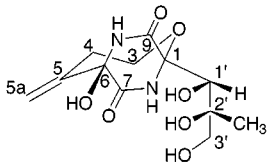
ABSTRACT: Bicyclomycin (**1**) is a commercial antibiotic whose primary site of action is the rho transcription termination factor. A new bicyclomycin irreversible inactivator, 5a-formylbicyclomycin (**3**), was prepared to provide information concerning the bicyclomycin-rho inactivation process and the drug's binding pocket within rho. The apparent I_{50} value for **3** was 35 μM, showing that **3** was a more effective inhibitor of rho poly C-dependent ATPase activity than **1** ($I_{50} = 60$ μM). Mechanistic studies demonstrated that **3** inhibited poly C-dependent ATP hydrolysis, in part, by a reversible, noncompetitive pathway with respect to ATP ($K_i = 62$ μM). Incubation of **3** with rho led to efficient imine formation. Adding excess **1** to solutions containing **3** and rho prevented imine formation, demonstrating that **1** and **3** bind to the same active site in the protein. The **3**-rho imine was stabilized by either ATP or ADP or by both, and was converted to the nonreversible **3**-rho amine adduct upon treatment with $NaBH_4$. Mass spectrometric analysis of the amine provided a stoichiometry of approximately five bound **3** per rho hexamer indicating the number of bicyclomycin binding sites for the rho hexamer is between five and six. Monomer exchange experiments using modified **3**-rho amine and wild type rho demonstrated that no more than two modified subunits per rho hexamer are sufficient to halt poly C-dependent rho ATPase activity.

The increasing number of antibiotic-resistant microbes has led researchers to search for structurally unique antibacterial agents that have novel modes of action (*1*). Bicyclomycin (**1**) is one such agent. This drug possesses a broad spectrum



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of antimicrobial activity against Gram-negative bacteria including *Escherichia coli*, *Klebsiella*, *Shigella*, *Salmonella*, *Citrobacter*, *Enterobacter cloacae*, and *Neisseria gonorrheae* (*2*−*5*) and the Gram-positive bacterium *Micrococcus luteus* (*6*). The use of bicyclomycin to treat nonspecific diarrhea in humans and bacterial diarrhea in animals has led to its commercial introduction under the trade name Bicozamycin.[1]

Recently, we demonstrated that the primary site of action of bicyclomycin in *E. coli* is the essential cellular protein rho transcription termination factor (*7*). Transcription termination is a regulatory process that controls gene expression (*8*, *9*). In Gram-negative bacteria, transcription termination proceeds by one of two major mechanisms. In one process, nascent RNA is released at intrinsic termination sites along the genome. In the second mechanism, rho factor terminates transcription (*10*). Rho is a hexameric protein containing identical 47-kDa subunits arranged in either D_3 (*11*−*14*), C_3 (*15*), or C_{3-6} (*15*) symmetry. Rho first binds to specific cytidine-rich areas on the newly synthesized RNA and then tracks toward the 3′ end of the RNA where the stalled RNA polymerase rests on the transcription bubble (*16*). The movement of rho toward the RNA polymerase is fueled by RNA-dependent ATPase activity (*10*). In a poorly understood mechanism, which may involve rho helicase activity (*17*), rho factor disrupts the polymerase complex and terminates transcription (*18*−*20*). We have shown that **1** inhibits rho-dependent in vitro transcription termination processes by a reversible pathway (*21*, *22*). Bicyclomycin affects rho translocation toward the RNA elongation complex by interfering with the RNA tracking mechanism and by preventing RNA-dependent ATP hydrolysis (*22*).

The precise rho-bicyclomycin binding site has not been identified. In the past 2 years, preliminary structural informa-

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[1] The commercial name is licensed to Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan.

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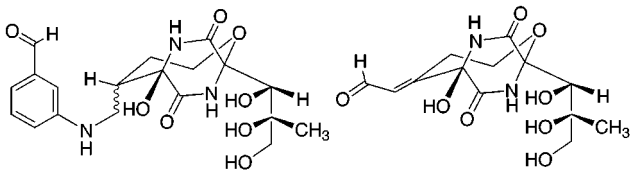
tion has been secured through the use of the bicyclomycin irreversible inactivator, 5a-(3-formylanilino)dihydrobicyclomycin (*23*, *24*) (**2**), and through kinetic studies of rho mutants insensitive to bicyclomycin (*25*). In 1998, we reported that incubation of rho with **2** followed by $NaBH_4$ treatment led to appreciable losses of rho ATPase activity (*24*). Mass spectrometric analysis indicated the presence of two proteins in an approximate 1.2:1 ratio whose masses corresponded to wild-type rho (47 010 Da) and lysine-modified rho (47 417 Da), respectively. Trypsin digestion of the rho sample followed by HPLC separation and tandem mass spectrometry analysis identified the site of modification as Lys-181 (*24*). Kinetic and biochemical analysis of mutant rho proteins conferring bicyclomycin resistance showed that two, M219K and G337S, exhibited enhanced binding to ribo$(C)_{10}$ in the poly(dC)-ribo$(C)_{10}$-stimulated ATPase assay and altered RNA specificities in the ATPase assay, while another, S266A, did not (*25*). We interpreted our results in the context of a proposed model of rho based on the bovine F_1-ATP synthase structure (*26*). The mutations map to the putative, secondary RNA binding site and to the likely ATP hydrolysis site leading us to suggest that **1** binds to a deep cleft in rho that includes the catalytic site for ATP hydrolysis (*25*).



2 **3**

Identifying the pathway for bicyclomycin inhibition of rho and the bicyclomycin binding pocket remains an essential objective in our efforts to understand this drug's action. Neither is the number of bicyclomycin binding sites in hexameric rho known nor is there information concerning the minimum level of bicyclomycin occupancy necessary to halt the movement of rho toward the RNA polymerase. In this paper, we report on studies of the synthesis and evaluation of the reductive amination probe, 5a-formylbicyclomycin (**3**). We demonstrate that **3** is a highly potent inactivator of rho, and we provide information on the mechanism of the inactivation process. Evidence is presented that **3** is capable of inactivating at least five of the six subunits, thereby defining the stoichiometry of the **3**-rho complex. We further show that adduction of one-to-two subunits is sufficient to prevent poly C-dependent rho ATPase activity. In the accompanying paper (*27*), the **3**-rho adduction site is identified, and the information is used to further define the site of bicyclomycin binding to rho and to provide mechanistic insights into rho-dependent transcription termination processes.

MATERIALS AND METHODS

General Procedures. Low-resolution and high-resolution (CI) MS[2] studies were run at the University of Texas at Austin by Dr. M. Moini. The low-resolution MS studies were run on a Finnigan MAT TSQ-70 instrument and the high-resolution MS studies conducted on a Micromass ZAB-E mass spectrometer tuned to a resolution of 10 000 (10% valley definition). Electrospray mass spectrometry (ESI-MS) analyses were performed using a Quattro tandem quadrupole mass spectrometer, upgraded to Quattro II specifications (Micromass UK Ltd., Manchester, U.K.). The capillary potential was held between 3 and 4 kV, and the cone was set at 20−30 V. (All potentials are reported relative to the skimmer lens.) Rho protein was isolated from *E. coli* AR 120 containing the overexpressing plasmid p39-ASE, which has been corrected for the K155E substitution seen in the original p39-AS plasmid (*28*). Rho purity was determined by SDS−PAGE, and concentrations were determined according to the Lowry protein determination. The concentrations reported for the rho protein samples correspond to monomer concentrations. Poly C concentrations were estimated by determining the average size of the RNA using denaturing PAGE electrophoresis. The average length was estimated as 300 nt; a solution of 40 nM was saturating (data not shown) and was used in the kinetic assays. [γ-^{32}P]ATP (NEN no. NEG002H) was purchased from Dupont New England Nuclear (Doraville, GA); nucleotides were obtained from Sigma. Polyethyleneimine (PEI) thin-layer chromatography plates used for ATPase assays were purchased from J. T. Baker, Inc. (Phillipsburg, NJ). Preparative thin-layer chromatographies (PTLC) were run on precoated silica gel slides (20 × 20 cm; Sigma Z12272−6).

*5a-Formylbicyclomycin C(2′),C(3′)-Acetonide (**6**).* To an anhydrous tetrahydrofuran (THF) solution (15 mL) of 5a-(hydroxy)methylbicyclomycin C(2′),C(3′)-acetonide (**4**) (24 mg, 0.063 mmol) was added Dess−Martin periodinane (*29*) (33 mg, 0.077 mmol). The reaction mixture was stirred at 25 °C (1 h), filtered (glass wool), and concentrated in vacuo. The residue was redissolved in anhydrous THF and purified by PTLC (20% MeOH−$CHCl_3$) to afford **6** as a colorless film (19 mg, 79%): mp 205−210 °C (dec); R_f 0.58 (20% MeOH−$CHCl_3$); IR (KBr) 3439 (br), 3303 (br), 2992, 2926, 1701, 1630, 1395, 1242, 1200, 1135, 1074, 1044, 872 cm^{-1}; ^{1}H NMR (DMF-d_7) δ 1.39 (s, 6 H, C$(CH_3)_2$), 1.43 (s, 3 H, C(2′)CH_3), 2.90−2.98 (m, 1 H, C(4)*H*H′), 3.52−3.59 (m, 1 H, C(4)H*H*′), 3.76 (d, J = 8.1 Hz, 1 H, C(3′)*H*H′), 3.90 (dd, J = 6.2, 8.9 Hz, 1 H, C(3)*H*H′), 4.09 (dd, J = 6.2, 8.9 Hz, 1 H, C(3)H*H*′), 4.22 (d, J = 8.1 Hz, 1 H, C(1′)H), 4.42 (d, J = 8.1 Hz, 1 H, C(3′)H*H*′), 6.00 (d, J = 8.1 Hz, 1 H, C(1′)OH), 6.48 (d, J = 7.2 Hz, 1 H, C(5a)H), 7.62 (s, 1 H, C(6)OH), 7.85 (s, 1 H, N(10)H or N(8)H), 9.01 (s, 1 H, N(8)H or N(10)H), 10.12 (d, J = 7.2 Hz, 1 H, C(5b)H), the structural assignments were in agreement with the ^{1}H-^{1}H COSY experiment; ^{13}C NMR (DMF-d_7) 25.2 (C(2′)*C*H_3), 27.0 (C(*C*H_3)$_2$), 28.5 (C(*C*H_3)$_2$), 29.2 (C(4)), 64.9 (C(3)), 72.9 (C(3′), C(1′)), 83.3 (C(6)), 86.4 (C(2′)), 88.7 (C(1)), 110.9 (*C*$(CH_3)_2$), 129.0 (C(5a)), 162.1 (C(5)), 167.7 and 169.5 (C(7), C(9)), 193.2 (C(5b)) ppm; MS (+CI) 371 [M + 1]$^+$; M_r (+CI) 371.1454 [M + 1]$^+$ (calcd for $C_{16}H_{23}N_2O_8$ 371.1454).

*5a-Formylbicyclomycin (**3**) (Method A).* To an anhydrous THF solution (15 mL) of 5a-(hydroxy)methylbicyclomycin (**5**) (45 mg, 0.14 mmol) was added Magtrieve (*30*) (345 mg,

[2] Abbreviations: ATP-γ-S, adenosine 5′-*O*-(3-thiotriphosphate); COSY, correlated spectroscopy; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ESI-MS, electrospray mass spectrometry; IR, infrared; *m*/*z*, mass/charge; MS, mass spectrometry; NMR, nuclear magnetic resonance; PEI, polyethyleneimine; PTLC, preparative thin-layer chromatography; THF, tetrahydrofuran; tris, tris(hydroxymethyl)-aminomethane.

4.11 mmol). The reaction mixture was stirred at 80 °C (6 h), filtered (glass wool), and concentrated in vacuo. The residue was redissolved in anhydrous THF and purified by PTLC (20% MeOH−$CHCl_3$) to afford **3** as a colorless film (12 mg, 27%); R_f 0.30 (20% MeOH−$CHCl_3$); ^{1}H NMR (THF-d_8) δ 1.30 (s, 3 H, C(2′)CH_3), 2.80 (dd, J = 9.3, 16.8 Hz, 1 H, C(4)*H*H′), 3.42−3.62 (m, 4 H, C(4)H*H*′, C(3′)H_2, C(3)*H*H′), 3.86 (dd, J = 8.7, 13.8 Hz, 1 H, C(3)H*H*′), 3.95 (t, J = 6.6 Hz, 1 H, C(3′)OH), 4.03 (d, J = 7.1 Hz, 1 H, C(1′)H), 4.73 (d, J = 7.1 Hz, 1 H, C(1′)OH), 4.76 (s, 1 H, C(2′)OH), 6.45 (d, J = 7.2 Hz, 1 H, C(5a)H), 6.51 (s, 1 H, C(6)OH), 8.20 (s, 1 H, N(10)H or N(8)H), 9.05 (s, 1 H, N(8)H or N(10)H), 9.97 (d, J = 7.2 Hz, 1 H, C(5b)H), the structural assignments were in agreement with the ^{1}H-^{1}H COSY experiment; ^{13}C NMR (DMF-d_7) 24.4 (C(2′)*C*H_3), 26.5 (C(4)), 63.6 (C(3)), 68.0 (C(3′)), 71.8 (C(1′)), 78.0 (C(2′)), 83.1 (C(6)), 89.1 (C(1)), 128.6 (C(5a)), 167.3 and 169.5 (C(7), C(9)), 193.0 (C(5b)) ppm, the C(5) signal was not detected and is believed to be beneath the solvent peak; MS (+CI) 331 [M + 1]$^+$; M_r (+CI) 331.1144 [M + 1]$^+$ (calcd for $C_{13}H_{19}N_2O_8$ 331.1141).

*5a-Formylbicyclomycin (**3**) (Method B).* Trifluoroacetic acid (TFA) (2 drops) was added to a solution of **6** (16 mg, 0.043 mmol) in H_2O (2 mL). The resulting solution was stirred at 25 °C (18 h), and then the pH was adjusted from 1.8 to 3.3 using aqueous sodium hydroxide (0.5 M). The reaction solution was then concentrated in vacuo. The residue was redissolved in 1-propanol (2 mL), and then the solution was maintained at 25 °C (18 h). The solution was concentrated in vacuo, and the residue was redissolved in THF and purified by PTLC (20% MeOH−$CHCl_3$) to afford **3** (4.5 mg, 28%): ^{1}H NMR (THF-d_8) δ 1.30 (s, 3 H, C(2′)CH_3), 2.80 (dd, J = 9.0, 16.2 Hz, 1 H, C(4)*H*H′), 3.42−3.54 (m, 4 H, C(4)H*H*′, C(3′)H_2, C(3)*H*H′), 3.85 (dd, J = 8.7, 13.2 Hz, 1 H, C(3)H*H*′), 3.96 (t, J = 6.6 Hz, 1 H, C(3′)OH), 4.03 (d, J = 6.9 Hz, 1 H, C(1′)H), 4.74 (d, J = 6.9 Hz, 1 H, C(1′)-OH), 4.77 (s, 1 H, C(2′)OH), 6.45 (d, J = 7.2 Hz, 1 H, C(5a)H), 6.52 (s, 1 H, C(6)OH), 8.20 (s, 1 H, N(10)H or N(8)H), 9.05 (s, 1 H, N(8)H or N(10)H), 9.96 (d, J = 7.2 Hz, 1 H, C(5b)H), the structural assignments were in agreement with the ^{1}H-^{1}H COSY experiment; ^{13}C NMR (THF-d_8) 29.2 (C(4)), 63.7 (C(3)), 68.3 (C(3′)), 71.9 (C(1′)), 77.5 (C(2′)), 82.9 (C(6)), 89.3 (C(1)), 128.9 (C(5a)), 160.4 (C(5)), 166.9 and 169.5 (C(7), C(9)), 191.2 (C(5b)) ppm, the C(2′)*C*H_3 signal was not detected and is believed to be beneath the solvent peak.

*5a-Dipropylacetal Bicyclomycin (**7**).* HCl was added to a solution of **6** (41 mg, 0.11 mmol) in H_2O (5 mL) until a pH of 1.7 was reached. The solution was stirred at 25 °C (4 h) and then the solution pH was adjusted to 3.3, using aqueous NaOH (1 M). The reaction was concentrated in vacuo, the residue dissolved in 1-propanol (5 mL), and then the solution was maintained at 25 °C (20 h). The pH of the solution was raised to 7.5 with aqueous NaOH (1 M) before concentration in vacuo. The residue was triturated with anhydrous THF (2×) and the solvent filtered (glasswool) to afford **7** (30 mg, 63%), as a white hygroscopic solid: R_f 0.43 (20% MeOH−$CHCl_3$); IR (KBr) 3413 (br), 3262 (br), 2965, 2937, 2879, 1694, 1401, 1130, 1067, 776 cm^{-1}; ^{1}H NMR (THF-d_8) δ 0.86 and 0.87 (t, J = 7.2 Hz, 3 H, C(5b)(OCH$_2$CH$_2$C*H*$_3$)$_2$), 1.26 (s, 3 H, C(2′)CH_3), 1.49 (m, 4 H, C(5b)(OCH$_2$C*H*$_2$-CH$_3$)$_2$), 2.43 (dd, J = 8.7, 16.2 Hz, 1 H, C(4)*H*H′), 2.81 (dd, J = 6.9, 16.2 Hz, 1 H C(4)H*H*′), 3.28−3.85 (m, 9 H, C(5b)(OC*H*$_2$CH$_2$CH$_3$)$_2$), C(3′)H_2, C(3)H_2, C(3′)OH), 3.96 (d, J = 7.0 Hz, 1 H, C (1′)H), 4.66 (d, J = 7.0 Hz, 1 H, C(1′)-OH), 4.73 (s, 1 H, C(2′)OH), 5.10 (d, J = 5.8 Hz, 1 H, C(5b)H), 6.05 (d, J = 5.8 Hz, 1 H, C(5a)H), 6.10 (s, 1 H, C(6)OH), 8.05 and 8.85 (s, 1 H, N(8)H, N(10)H); ^{13}C NMR (THF-d_8) 11.2 and 11.3 (C(5b)(OCH$_2$CH$_2$*C*H$_3$)$_2$), 24.0 (C(5b)-(OCH$_2$*C*H$_2$CH$_3$)$_2$), 26.4 (C(2′)*C*H_3), 29.9 (C(4)), 64.2 (C(3)), 66.3 (C(5b)(O*C*H$_2$CH$_2$CH$_3$)$_2$), 68.3 (C(3′)), 71.9 (C(1′)), 77.5 (C(2′)), 82.6 (C(6)), 89.2 (C(1)), 99.0 (C(5b)), 129.1 (C(5a)), 144.5 (C(5)), 167.2 and 170.8 (C(7), C(9)) ppm; MS (+CI) 433 [M + 1]$^+$; M_r (+CI) 433.2109 [M + 1]$^+$ (calcd for $C_{19}H_{32}N_2O_9$ 433.2186).

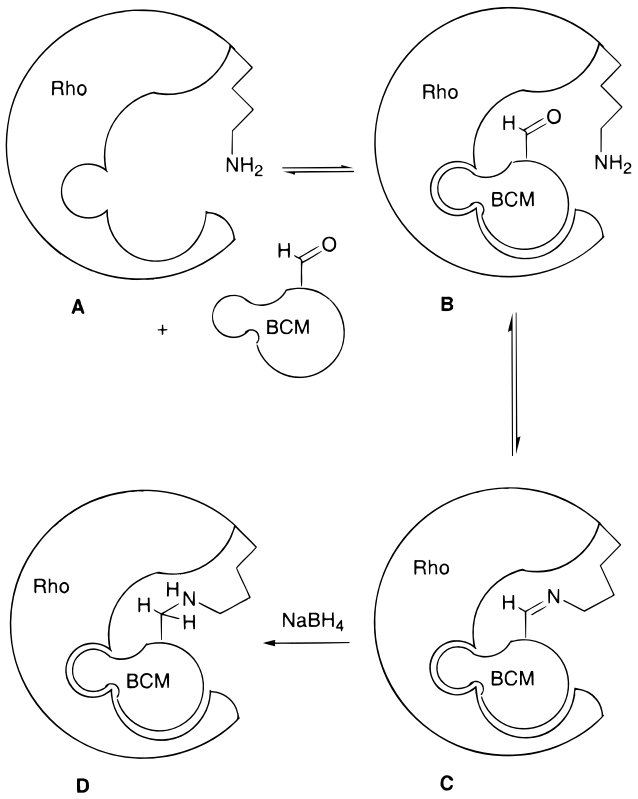


FIGURE 1: Proposed mode of action of reductive amination probe **3**.

General Procedure for Poly C-dependent Rho ATPase Activity (GP 1). The ATPase activity assays were carried out in 100 μL reactions containing 40 mM Tris·HCl, pH 7.9, 50 mM KCl, 12 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, 0.07 mg bovine serum albumin, 0.25 mM ATP, 0.5 μCi of [γ-^{32}P]ATP, 40 nM poly C and 100 nM rho. Reactions were preincubated at 32 °C for 90 s prior to the addition of ATP. Aliquots (2 μL) were removed at various times (15, 30, 45, 60, 75 s) during the reaction and spotted onto PEI TLC sheets. [γ-^{32}P]ATP and $^{32}P_i$ were separated by chromatography on the PEI sheets using 0.75 M KH_2PO_4, pH 3.5, as the mobile phase. The developed TLC plates were used to expose PhosphorImager plates (10−20 min) and scanned using a Fuji BAS Bio Imaging Analyzer; the radioactive spots were analyzed using the Macintosh BAS analysis program. The initial rates of reactions were determined by plotting the amount of ATP hydrolyzed versus time.

*General Procedure for Reductive Amination of Rho by 5a-Formylbicyclomycin (**3**) (GP 2).* A solution (0.2 mL) containing the buffer (40 mM Tris·HCl, pH 7.9, 50 mM KCl,

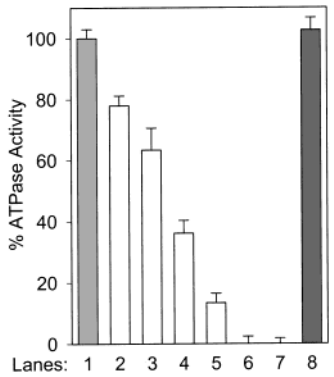


FIGURE 2: Histogram depicting the percentages of rho ATPase activity after reductive amination by various concentrations of 5a-formylbicyclomycin (**3**). The reaction was conducted using a solution (0.2 mL) containing reaction buffer, rho (1 μM), poly C (40 nM), and ATP (1 mM). The solution was maintained at 25 °C (4 h), treated with a 20-μL solution of $NaBH_4$ (600 mM), and then dialyzed. The reactions contained the following additional substrates: (1) no **3**; (2) **3** (2.5 μM); (3) **3** (5 μM); (4) **3** (10 μM); (5) **3** (15 μM); (6) **3** (25 μM); (7) **3** (50 μM); (8) (*E*)-2-pentenal (100 μM).

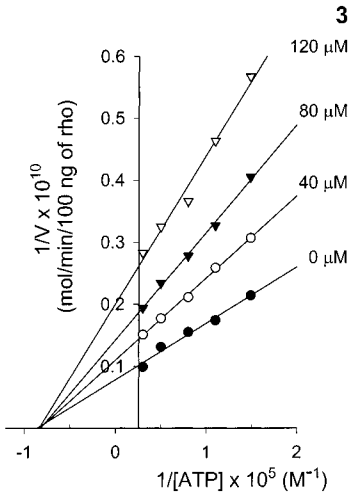


FIGURE 3: Double reciprocal plot of the poly C-dependent ATPase activity of rho with varying concentrations of 5a-formylbicyclomycin (**3**).

12 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol (DTT)), rho (1 μM), ATP (1 mM), poly C (40 nM), and **3** (40 μM) was incubated at 25 °C (4 h). An aqueous 600 mM $NaBH_4$ solution (20 μL) was then added and the reaction was allowed to stand at 25 °C (20 min). The reaction mixture was dialyzed (4 °C, 20 h) against 100 mM NaCl, 10 mM Tris·HCl (pH 7.6), 5% glycerol, 0.1 mM ethylenediaminetetraacetic acid (EDTA), and 0.1 mM DTT. The percentage inactivation of rho was determined by measuring the initial velocity of ATPase activity using the general procedure GP 1 and adding poly C (40 nM), ATP (250 μM), and 0.5 μCi [γ-^{32}P]ATP.

Reductive Amination of Rho using Various Concentrations of 5a-Formylbicyclomycin (3). General procedure GP 2 for the reductive amination of rho was employed using **3** (2.5, 5, 10, 15, 25, 50 μM) and (*E*)-2-pentenal (100 μM). The results are presented in Figure 2.

Kinetics of 5a-Formylbicyclomycin (3) Rho Inhibition. ATPase activity assays were carried out using general procedure GP 1 with 100 ng of rho except **3** (0, 40, 80, 120 μM) was added to the reaction solution containing various ATP concentrations (6.7, 9.1, 12.5, 20, 33.3 μM). The initial rates for each ATP concentration plus or minus inhibitor were plotted as a double reciprocal plot. The results are presented in Figures 3 and S2.

Mass Spectrometry: Detection of 5a-Formylbicyclomycin (*3*)*-Modified Rho*. The reductive amination of rho (3 μM) by **3** (400 μM) was conducted using general procedure GP 2 in the presence of poly C (120 nM) and ATP (3 mM). The samples were sent to UMIST for ESI-MS analysis in dialysis buffer at room temperature. The samples were desalted using a C8 protein trap cartridge (Michrom BioResources, Inc., CA), with elution in acetonitrile/water (80/20 by volume with 0.1% formic acid). A portion of the eluate was used directly for MS analysis. Samples were introduced via a syringe driver (Harvard Apparatus, South Natick, MA) at a constant flow rate of 5 μL/min. Mass spectra were recorded at a resolution set to give a peak width at half-height of 0.7 *m*/*z* units for a monoisotopic peak of a singly charged ion. The *m*/*z* range 200−1600 was repetitively scanned at 14 s per scan. Spectra were accumulated for 2 min under the control of Masslynx software (Micromass) and were processed using the maximum entropy approach (*31*). The results are presented in Figure 4.

Subunit Exchange Experiment. The reductive amination of rho (5 μM) by **3** (750 μM) was conducted using general procedure GP 2 in the presence of poly C (200 nM) and ATP (5 mM). After dialysis (4 °C, 20 h), 100-μL aliquots of modified rho were centrifuged through Bio Spin 6 columns for further purification. Solutions (0.2 mL) containing the buffer (40 mM Tris·HCl, pH 7.9, 50 mM KCl, 12 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT), ATP (1 mM), varying concentrations of fresh rho and modified rho, and poly C (40 nM/1 μM of protein) were incubated at 25 °C (1 h). Samples were assayed for their poly C-dependent rho ATPase activity using general procedure GP 1. The results are presented in Figure 5.

Number of Modified Subunits Needed for Rho Inactivation. The reductive amination of rho (5 μM) by **3** (750 μM) was conducted using general procedure GP 2 in the presence of poly C (200 nM) and ATP (5 mM). After dialysis (4 °C, 20 h), 100-μL aliquots of modified rho were centrifuged through Bio Spin 6 columns for further purification. Solutions (0.2 mL) containing the buffer (40 mM Tris·HCl, pH 7.9, 600 mM KCl, 12 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT) and varying concentrations of fresh rho and modified rho were incubated at 37 °C (6 h). Samples were assayed for their poly C-dependent rho ATPase activity using general procedure GP 1. The results are presented in Figure 6.

Reductive Amination of Rho by 5a-Formylbicyclomycin (3) in the Presence of Bicyclomycin (1). General procedure GP 2 for the reductive amination of rho was employed using **3** (30 μM) and **1** (100, 500, 1000, 2000, 5000 μM) in the absence of ATP with an incubation time of 90 min. The results are presented in Figure S1 (Supporting Information).

Time Dependency for Rho Inactivation by 5a-Formylbicyclomycin (3). The modification of rho (1 μM) by **3** (15 μM) was conducted using general procedure GP 2 (without $NaBH_4$ reduction) in the presence of poly C (40 nM) and ATP (1 mM) with incubation times of 5 min, 30 min, 1 h, 2 h, 4 h, 8 h, and 24 h. After passage through a Bio-Spin 6 column the samples were assayed for their poly C-dependent

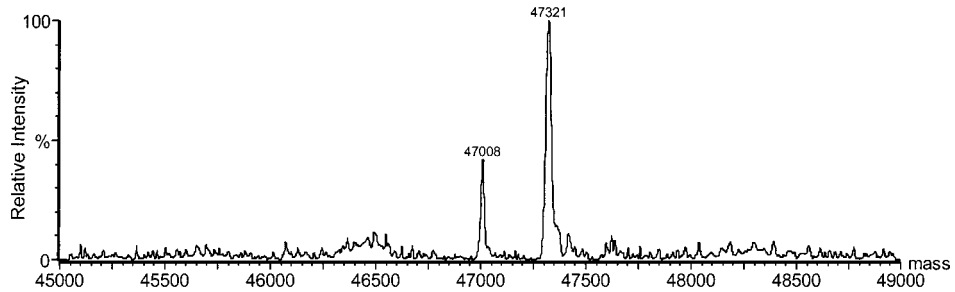


FIGURE 4: Mass spectrometric analysis of the product of reductive amination of rho with **3**. The spectrum was obtained by maximum entropy processing of mass/charge ratio data.

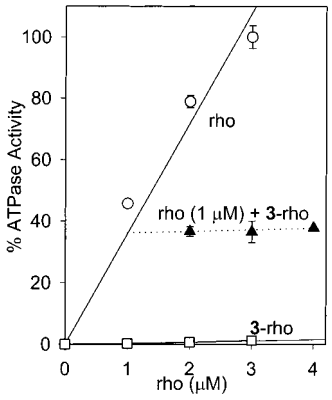


FIGURE 5: Monomer exchange experiment. The reductive amination of rho (5 μM) by **3** (750 μM) was used to produce extensively modified rho (79%). The activities of rho (○) and modified rho (□) were assayed at concentrations of 1, 2, and 3 μM. Mixtures (▲) containing rho (1 μM) and varying concentrations of modified rho (1, 2, and 3 μM) were incubated in the presence of poly C (40 nM/1 μM of protein) and ATP (1 mM) at 25 °C (1 h). The dotted line represents the activity predicted by the addition of the activity of modified rho to that of rho (1 μM).

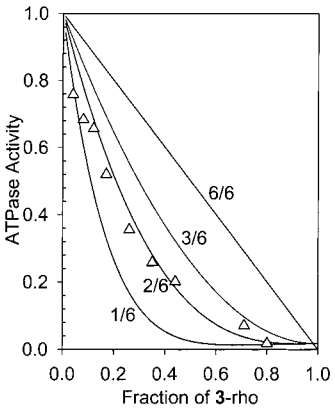


FIGURE 6: Number of modified subunits needed to inactivate rho. The reaction was conducted using a solution (0.2 mL) containing reaction buffer (0.6 M KCl), rho and **3**-rho amine (total concentration 1 μM). The solution was maintained at 37 °C (6 h) and then assayed (△). The four theoretical curves correspond to the ATPase activity (y) expected when 1 in 6, 2 in 6, 3 in 6, or every subunit needs to be modified to stop rho-mediated ATP hydrolysis. They were generated using the equation $y = (1 - x)^n + 0.02x$ for values of x from 0 to 1 and $n = 1, 2, 3, 6$.

rho ATPase activity using general procedure GP 1. The results are presented in Figure S3.

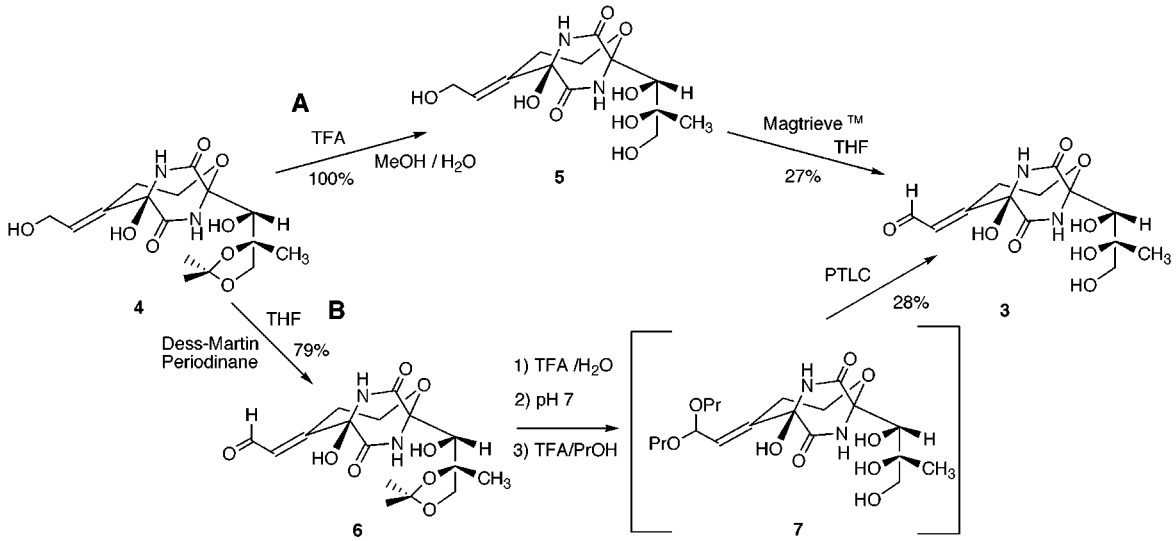
***3*-Rho Imine Adduct: Stability Versus Time.** Solutions (0.2 mL) containing the buffer (40 mM Tris·HCl, pH 7.9, 50 mM KCl, 12 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT), rho (1 μM), ATP (1 mM), poly C (40 nM), and **3** (100 μM) were incubated at 25 °C (5 h). Aliquots (100 μL) were centrifuged at 3000 rpm (4 °C, 5 min) using a Bio-Spin 6 column that had been prewashed (4×) with ATPase buffer (0.5 mL). The centrifuged filtrates were then dialyzed at room temperature for various times (0, 2, 4, 8, 12, 16, 20, 24 h). The recovered samples were weighed and normalized for their weight differences, and then their poly C-dependent rho ATPase activity was determined using general procedure GP 1. The results are presented in Figure S4.

*Effect of Adenine Nucleotides on the Stability of the **3**-Rho Imine Adduct.* Solutions (0.2 mL) containing the buffer (40 mM Tris·HCl, pH 7.9, 50 mM KCl, 12 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT), rho (3 μM), poly C (120 nM), and **3** (400 μM) were incubated at 25 °C (2 h). Aliquots (100 μL) were centrifuged at 3000 rpm (4 °C, 5 min) using a Bio-Spin 6 column, diluted to 1 mL and left standing at 25 °C (24 h) in the presence of the following adenine nucleotides: none, AMP (1 mM), ADP (1 mM), adenosine 5′-*O*-(3-thiotriphosphate) (ATP-γ-S) (1 mM), and ATP (10 μM, 100 μM, 1 mM). The samples were assayed for their poly C-dependent rho ATPase activity using general procedure GP 1. The results are presented in Figure S5.

Stability of the **3**-Rho Amine Adduct. Solutions (0.2 mL) containing the buffer (40 mM Tris·HCl, pH 7.9, 50 mM KCl, 12 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT), rho (1 μM), ATP (1 mM), poly C (40 nM), and **3** (100 μM) were incubated at 25 °C (90 min). Water or an aqueous 600 mM $NaBH_4$ solution was then added (20 μL) to designated samples. Aliquots (100 μL) were centrifuged at 3000 rpm (4 °C, 5 min) using a Bio-Spin 6 column that had been prewashed (4×) with ATPase buffer (0.5 mL). The centrifuged filtrates were then assayed directly (GP 1) or dialyzed at room temperature for 24 h. The recovered samples were weighed, normalized for their weight differences and then their poly C-dependent rho ATPase activity was determined using general procedure GP 1. The results are presented in Figure S6.

*Determination of the Extent of **3**-Rho Imine Formation upon Addition of **3** to Rho.* Solutions (0.2 mL) containing the buffer (40 mM Tris·HCl, pH 7.9, 50 mM KCl, 12 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT), rho (21.3 or 100 nM), ATP (100 or 250 μM), poly C (40 nM), and **3** (50 μM) were incubated at 32 °C (75 s). Aliquots (100 μL) were centrifuged at 3000 rpm (4 °C, 5 min) using a Bio-Spin 6 column that had been prewashed (4×) with ATPase buffer (0.5 mL). The recovered samples were weighed, normalized for their weight differences and then their poly C-dependent rho ATPase activity was determined using general procedure GP 1.

Scheme 1: Preparation of 5a-Formylbicyclomycin (**3**)



RESULTS

Choice of Probe 3 Design. Compound **3** was designed to act as rho-irreversible inactivator by complexing to the drug binding site (Figure 1, e.g., **B**), by imine (Schiff-base) formation with a nearby lysine residue (e.g., **C**), and then by irreversible adduction upon treatment with $NaBH_4$ (e.g., **D**). Bicyclomycin derivative **3** differs from **2** (*23*) in several important aspects. In probe **3** the aldehyde unit was directly attached and was in conjugation with the C(5)-C(5a) exomethylene group whereas in **2**, the aldehyde unit was part of a 3-aminobenzaldehyde moiety appended to the bicyclomycin ring skeleton through the saturated C(5)-C(5a) linkage. Accordingly, in **3**, the distance between the aldehyde unit and the [4.2.2] ring core was reduced, compared with **2**. Furthermore, the number of conformational states accessible to the aldehyde group in **3** was diminished compared with **2**.

Synthesis and Structural Characterization of Probe 3. We envisaged that selective oxidation of the primary alcohol groups in 5a-(hydroxy)methylbicyclomycin C(2′), C(3′)-acetonide (*32*) (**4**) would permit the synthesis of bicyclomycin irreversible inactivator **3**. Compound **4** can be prepared from bicyclomycin in four steps with a 35% overall yield.

Two routes were developed for the synthesis of **3**, and they differed in the sequence of the oxidation and deprotection steps (Scheme 1). In the first procedure (method A), the acetonide group in **4** was removed to give **5** (*32*) and then the allylic alcohol group selectively oxidized with Magtrieve (*30*) to provide **3**. Use of MnO_2 (*33*) in place of Magtrieve gave **3**, but in lower yields (data not shown). In the second route (method B) the allylic alcohol group in **4** was first oxidized with 1,1,1-triacetoxy-1,1-dihydro-1,2-benziodoxol-3(1H)-one (*29*) (Dess−Martin periodinane) to give **6**. Other oxidants (Magtrieve, MnO_2 (*33*), TPAP plus O_2 (*34*), PCC) provided the aldehyde **6**, but at lower levels (data not shown). Careful deprotection of the C(2′), C(3′)-acetonide group in **6** followed by treatment with propanol and TFA gave the dipropyl acetal **7** in situ. Compound **7** was converted to **3** during PTLC workup. We found that the intermediate generation of **7** simplified the purification of bicyclomycin probe **3**.

The ^1H NMR, ^{13}C NMR, and mass spectral data for **3** were consistent with the proposed structural assignment. Key signals in the ^1H NMR spectrum were the downfield, coupled signals for the aldehyde (δ 9.96) and vinylic (δ 6.45) protons of the α,β-unsaturated aldehyde unit (*35*). The corresponding signals in the ^{13}C NMR spectrum were observed at 191.2 and 128.9 ppm (*36*).

Biochemical and Biological Studies. (1) Initial Evaluation. Previous studies (*31*, *37*, *38*) demonstrated that the poly C-dependent rho ATPase assay (*39*) serves as a reliable test to measure the inhibitory effects of bicyclomycin analogues. Furthermore, we have found (*32*, *37*, *38*) that every compound showing poor or no activity in the poly C-dependent ATPase assay also exhibited little or no antibiotic activity against W3350 *E. coli* in the filter disk assay (*40*). Accordingly, **3** was first evaluated in the poly C-dependent ATPase assay. We found that **3** was an excellent inhibitor. The *apparent* I_{50} value for **3** was 35 μM, which is lower than that of bicyclomycin (*7*) (I_{50} = 60 μM). The activity of **3** was further evaluated in the antimicrobial filter disk assay. We observed antibiotic activity against W3350 *E. coli* only at 32 mg/mL. The corresponding minimum inhibitory concentration (MIC) for bicyclomycin was 0.32 mg/mL.

The I_{50} value observed for **3** in the poly C-dependent ATPase assay suggested that this bicyclomycin analogue efficiently bound to hexameric rho. We then asked whether it could serve as an irreversible inactivator after Schiff-base formation with a nearby lysine residue and after reduction (e.g., Figure 1D). Incubation of rho (1 μM) and poly C (40 nM) with varying concentrations of **3** (25 °C, 4 h) followed by $NaBH_4$ reduction led to the permanent loss of ATPase activity upon overnight dialysis (Figure 2). Poly C was included in the reaction to ensure formation of the rho hexamer. Greater than 63% loss of ATPase activity occurred at concentrations ≥10 μM and reached 100% inhibition of ATPase activity with 25 μM of **3**. Use of high concentrations (100 μM) of (*E*)-2-pentenal in place of **3** gave no detectable loss of poly C-dependent ATPase activity (Figure 2, lane 8). These collective findings provided initial evidence that **3** served as a potent in vitro inhibitor of rho function.

(2) 5a-Formylbicyclomycin (3) Binds to the Bicyclomycin Binding Pocket in Rho. If **3** is to serve as a bicyclomycin-

specific irreversible inactivator it is necessary to verify that it binds at the bicyclomycin binding site. Accordingly, we repeated the reductive amination experiments in the presence of bicyclomycin. Incubation of rho and **3** with increasing amounts of bicyclomycin (0−5 mM) followed by treatment with $NaBH_4$ and dialysis led to progressively higher levels of rho activity (Figure S1). Using 5 mM of **1** we observed near full recovery of poly C-dependent ATPase activity. These findings demonstrated that **1** competed with **3** for the drug binding site in rho. The high levels (5 mM) of **1** required to block permanent $NaBH_4$-mediated inactivation by **3** have been attributed to the intermediate formation of a *covalent* **3**-rho imine complex (see section 3b).

*(3) Studies on the 5a-Formylbicyclomycin (**3**)-rho Imine Formation Process.* The projected first steps of the **3**-inactivation process are reversible binding with the protein (e.g., Figure 1**B**) followed by imine formation (e.g., Figure 1**C**). Our earlier studies with probe **2** showed that imine production is rapid but that imine hydrolysis to regenerate rho occurs within minutes ($t_{1/2} \approx 15$ min) at 25 °C (*41*). We also found that including ATP in these reactions led to *lower* amounts of imine product (*41*).

Four aspects of the 5a-formylbicyclomycin (**3**)-rho imine formation process were examined: (a) binding of **3** to rho, (b) ease with which the Schiff base was generated, (c) stability of the 5a-formylbicyclomycin (**3**)-rho imine adduct, and (d) effect of ATP and adenine nucleotides on imine production and maintenance.

*(a) 5a-Formylbicyclomycin (**3**)-rho Binding Process*. Information about the binding process was determined from the kinetics of **3** inhibition of rho poly C-dependent ATPase activity. Figure 3 is a plot of 1/(rate of ATPase activity) versus 1/[ATP] at various concentrations of **3**. Our findings are consistent with the notion that **3** inhibits poly C-dependent ATP hydrolysis, in part, by a reversible, noncompetitive pathway with respect to ATP (see next section). This result is comparable to previous reports for **1** (*21*) and **2** (*24*). Both compounds inhibited ATP hydrolysis by a reversible, noncompetitive pathway with respect to ATP. The K_i values for **1**, **2**, and **3** obtained from the replot of the slopes versus drug concentrations were 20 (*21*), 27 (*24*), and 62 μM (Figure S2), respectively, while the K_m for ATP were 11.0 ± 0.5 (*21*), 28.0 ± 0.9 (*24*), and 11.4 ± 0.2 μM, respectively.

*(b) Relative Ease of 5a-Formylbicyclomycin (**3**)-rho Imine Formation*. The high K_i value for **3** was surprising in light of the observed low I_{50} value. We suspected that the I_{50} value might reflect both the binding of **3** to rho (e.g., Figure 1**B**) and the imine formation (Figure 1**C**). Accordingly, we sought an *approximate* assessment of the extent of imine formation at the conclusion of the I_{50} and kinetic studies (32 °C, 75 s) when moderately high concentrations of **3** were used. Treatment of a rho solution (100 nM) with **3** (50 μM) under conditions that approximated the I_{50} measurement conditions (poly C (40 nM), ATP (250 μM)) followed by removal of excess **3** by spin-column chromatography (4 °C) gave 77% of inactivated rho in the poly C-dependent rho ATPase assay compared with the control (data not shown). Similarly, when 21.3 nM rho and 50 μM **3** were used under conditions that approached the kinetic studies (poly C (40 nM), ATP (100 μM)) a 70% loss of poly C-stimulated ATPase hydrolysis was observed (data not shown). These findings demonstrated that the effectiveness of **3** in inhibiting rho function is derived, in large part, by its ability to form a covalent imine adduct.

The facility with which rho adduction occurred with **3** led us to determine the relative time course for imine production at 25 °C with a low concentration of 5a-formylbicyclomycin (15 μM). We observed the steady inactivation of rho under these conditions (incubation followed by spin-column chromatography) with only 8% residual poly C-ATPase activity remaining after 24 h (Figure S3).

*(c) Stability of the 5a-Formylbicyclomycin (**3**)-rho Imine*. The relative stability of the 5a-formylbicyclomycin-rho imine was determined by measuring the recovery in the ATPase activity of a preformed solution of the imine as a function of dialysis time. Accordingly, a rho solution containing the putative Schiff base was prepared, excess **3** removed by spin-column chromatography and the solution was divided into eight dialysis tubes. Each aliquot (100 μL) was dialyzed at 25 °C against 1 L of buffer for various times (Figure S4). We observed complete recovery of rho activity only after 24 h. These results show that the Schiff base for **3** is more stable than that obtained from **2** (*41*). Recovery of rho activity from the **2**-rho complex occurred within 50 min when 10-μL aliquots were mixed with 100 μL of buffer solutions (25 °C).

Our finding that **3**-rho imine formation occurred rapidly (Figure S3) and that the Schiff base was stable (Figure S4) led us to ask what was the minimum concentration of 5a-formylbicyclomycin needed to observe appreciable losses in rho activity. We found a solution containing rho (1 μM), poly C (40 nM), and ATP (1 mM) and only 5 μM **3** maintained at 25 °C (30 h) led to 83% loss of poly C-dependent rho ATPase activity compared with the control. This level of inactivation far exceeded those observed for comparable levels of bicyclomycin. We estimate that 150 μM **1** would be required to reach the same reduction of poly C-dependent ATPase activity observed with **3** and that a rho solution containing only 5 μM **1** would retain >90% of ATPase activity.

(d) Effect of ATP and Adenine Nucleotides on Imine Production and Maintenance. We first determined if ATP affected the extent of **3**-rho adduction. Incubation (room temperature, 1 h) of a poly C (40 nM) solution containing rho (1 μM) and **3** (40 μM) with or without ATP (1 mM) followed by the addition of $NaBH_4$ led to comparable losses of poly C-dependent ATPase activities (74.4 ± 0.3%) (data not shown). This result indicated that the presence of ATP or its hydrolyzed product, ADP, did not catalyze **3**-rho imine formation. We then asked if the **3**-rho imine adduct was stabilized by adenine nucleotides. Accordingly, a poly C solution of the **3**-rho imine was prepared and incubated with either AMP (1 mM), ADP (1 mM), the slowly hydrolyzable ATP analogue, ATP-γ-S (*42*, *43*) (1 mM), or ATP (10 μM-1 mM) (Figure S5). The **3**-rho imine solutions were maintained at 25 °C for 24 h and the poly C-dependent ATPase activity measured. We found that the solutions containing either no adenine nucleotides or AMP regained their ATPase activity (Figure S5, lanes 3, 4), while in those containing high concentrations of ADP, ATP-γ-S or ATP (Figure S5, lanes 5, 6, 8, 9) we saw no loss of rho inhibition. Finally, the solution containing 10 μM ATP (Figure S5, lane 7) had a moderate loss in rho inhibition. These results demonstrated

that the **3**-rho imine adduct was stabilized by ATP-γ-S and by either ATP or ADP or by both.

*(4) Studies of the 5a-Formylbicyclomycin (**3**)-Rho Inactivation Process.* An important objective was to determine the maximum number of bicyclomycin binding sites within hexameric rho and to learn the minimum number of bicyclomycin-disabled protein subunits needed to inactivate rho function. The efficiency of **3**-rho imine formation and the ease with which the imine underwent $NaBH_4$ reduction to the amine provided us with a unique opportunity to examine these questions. These objectives required us first to determine if the **3**-rho amine (e.g., Figure 1**D**) generated upon $NaBH_4$ reduction was stable enough to permit these investigations.

*(a) Stability of the Amine Generated Upon $NaBH_4$ Reduction of the 5a-Formylbicyclomycin (**3**)-Rho Imine.* The relative activities in the poly C-dependent rho ATPase assay of **3**-rho imine and **3**-rho amine samples were compared after spin column chromatography and dialysis (Figure S6). We found complete recovery of ATPase activity in the **3**-rho imine preparation after spin-column chromatography followed by dialysis (25 °C, 24 h) (Figure S6, lane 5). Correspondingly, $NaBH_4$ reduction of **3**-rho imine followed by spin-column chromatography provided a modified rho sample that underwent little recovery of activity ($\leq$10%) upon dialysis (Figure S6, lane 6). These findings demonstrated that the covalent amine generated upon $NaBH_4$ reduction of **3**-rho imine was sufficiently stable for mass spectral studies.

*(b) Maximum Stoichiometry for 5a-Formylbicyclomycin (**3**)-hexameric Rho Complexes*. We determined the upper limit of **3** adduction to rho under reductive amination conditions using high concentrations of 5a-formylbicyclomycin. Treatment of rho (3 μM) with poly C (120 nM), ATP (1 mM) and **3** (400 μM) at room temperature (4 h) followed by $NaBH_4$ reduction and dialysis provided a rho sample that showed about 99% loss of poly C-dependent ATPase activity, compared with the control. ESI-MS gave two principal signals that corresponded with wild-type rho (47 008 Da) and the **3**-rho amine adduct (47 321 Da), which was 313 mass units (theoretical value 314) higher than wild-type rho (Figure 4). The approximate ratio of **3**-modified rho to wild-type rho was 4:1. Repetition of this experiment (10×) led to similar results. Noticeably absent in the mass spectrum were peaks corresponding with higher order **3**-modified rho adducts. Comparable findings were obtained with lower concentrations of **3** (45, 90 μM). The absence of peaks corresponding with multiple **3** adducts suggested that the reaction was site specific.

The finding that 79% of the rho subunits underwent reductive amination with levels of **3** exceeding 45 μM to give **3**-rho adducts suggested that approximately five of the six subunits are modified. Key to establishing this stoichiometry is demonstrating that rho does not dissociate to give either smaller multimeric or monomeric species under the reaction conditions. Careful studies have provided the conditions (i.e., buffer, temperature, ligands) that promote rho subunit dissociation and those that help stabilize rho as the hexamer (*44*). Conditions were employed in our **3**-rho reductive amination experiments known to stabilize rho as the hexamer. Specifically, we included ATP and poly C in the reaction solution, maintained the salt concentration at 50 mM KCl, and employed rho concentrations between 1 and 3 μM. Nonetheless, it is important to document that little or no dissociation of hexameric rho occurred during our experiments. Fortunately, Richardson and Ruteshouser have described a convenient protocol to monitor rho dissociation and monomer exchange among different rho proteins (*45*). Accordingly, we incubated wild-type rho with varying amounts of modified **3**-rho amine using conditions (room temperature, 1 h) similar to those employed for **3**-rho imine formation (room temperature, 4 h). The poly C-dependent ATPase activities of the rho solutions were measured and plotted (Figure 5). We found that the ATPase activities of the binary mixtures were additive. If subunit exchange of **3**-modified rho with wild-type rho had proceeded then depressed values for the cumulative ATPase activity would have been observed. These findings indicated that hexameric wild-type rho solutions are stable under the employed reaction conditions and dissociation, and recombination with **3**-modified rho subunits was minimal. Repetition of this experiment at higher temperatures (37 °C) and for longer mixing times (4 h) led to small deviations from the theoretical cumulative ATPase activity values (data not shown). These results are consistent with the notion that the hexamer is stable under the conditions used to generate the **3**-rho imine and the **3**-rho amine modified hexamers.

*(5) Determination of the Minimum Number of 5a-Formylbicyclomycin (**3**)-Disabled Rho Subunits Necessary to Inhibit Rho Functional Activity*. We have learned that five of the six monomers within rho can each bind a single molecule of **3**. This finding provides information concerning the maximum stoichiometry of **3**-rho modification but not the *minimum* number of modified subunits needed to inhibit rho function. In 1986, Richardson and Ruteshouser also described a protocol to estimate this value for wild-type and mutant rho mixtures using the poly C-dependent ATPase assay (*45*). Varying ratios of wild-type and mutant rho proteins were mixed under conditions that permitted subunit exchange and then the samples were dialyzed with subsequent reestablishment of the hexameric structure. The ATPase activity was measured and compared with theoretical curves of the ATPase activity for samples of randomized rho hexamers where one-to-six mutant monomers inactivated the hydrolysis of ATP.

We have adopted the Richardson and Ruteshouser procedure (*45*) in our study. Varying ratios of extensively modified **3**-rho (79%) and wild-type rho were mixed and then incubated at 37 °C (6 h) in aqueous 0.6 M KCl solutions in the absence of ATP. These conditions are sufficient to dissociate poly C-bound hexameric rho (*46*).[3] The reaction solutions were dialyzed (4 °C, 24 h) and then the activity of the solutions measured in the poly C-dependent ATPase assay. Figure 6 shows the activity curves plotted as a function of the **3**-modified rho along with the predicted curves for complexes containing one in six, two in six, three in six, and all six inactivated subunits. The data came closest to the curves in which a rho hexamer containing 16.7−33.3%

[3] A fixed 1:1 ratio of **3**-rho amine and wild-type rho were incubated at 37 °C for various times (0−22 h) in 0.6 M KCl solution, dialyzed, and then the poly C-dependent ATPase activity was measured. The ATPase activities steadily decreased for the first 3 h and then leveled off for the remaining 19 h.

3-modified rho subunits (one or two subunits per hexamer) is incapable of efficiently hydrolyzing ATP to ADP and P_i. Although preferential subunit association of modified subunits could influence the degree of inactivation, this finding demonstrated that inactivation of all six rho subunits was not necessary to eliminate rho ATPase activity. Since ATP hydrolysis is necessary for the movement of rho toward the RNA polymerase, **3**-mediated cessation of ATPase activity must lead to the loss of rho transcription termination.

DISCUSSION

Mechanistic studies on the mode of action of bicyclomycin have revealed that the rho transcription termination factor is the primary drug target in *E. coli (7*, *22*, *24*, *25*). Rho functions by binding tightly to the nascent RNA followed by translocation toward the RNA polymerase, leading to the disruption of the elongation complex and release of RNA (*16*−*20*). The tracking to the polymerase is projected to proceed at the secondary RNA binding site in rho and requires hydrolysis of ATP.

Three primary (tight) and three secondary (weak) ATP binding sites in hexameric rho have been identified (*47*, *48*). Recent studies have reported that the secondary site catalyzes ATP hydrolysis (*48*) and that disabling one of the ATP binding sites is sufficient to inhibit rho hexameric ATPase activity (*49*, *50*).

Our studies have shown that bicyclomycin inhibits rho-mediated ATP hydrolysis by a reversible pathway that is noncompetitive with respect to ATP (*21*) and that drug binding to rho disrupts RNA binding at the secondary RNA site (*22*). We have not determined (1) the stoichiometry of the bicyclomycin-hexameric rho complex, (2) the minimum number of bicyclomycin-inactivated rho subunits required for cessation of protein function, or (3) the bicyclomycin binding domain within rho. Irreversible inactivator **3** provided information on these issues.

Compound **3** was evaluated in the rho-mediated poly C-dependent ATPase assay (*39*) and compared with bicyclomycin. The *apparent* inhibitory activity of **3** (I_{50} = 35 μM) exceeded that of **1** (I_{50} = 60 μM) (*7*). Kinetic studies demonstrated that **3** inhibited rho by preventing ATP hydrolysis, in part, by a reversible, noncompetitive pathway with respect to ATP similar to **1** (Figure 3). The K_i value for **3** determined from the replot of either the slopes or the intercepts was 62 μM (Figure S2), which was three times higher than **1** (K_i = 20 μM) (*21*). The surprising effectiveness of **3** in the I_{50} measurement has been attributed to the facile formation of the corresponding imine (Figure 1**C**) upon **3** binding to rho and the stability of the imine. We further learned that solutions containing ATP, ATP-γ-S and ADP could stabilize the **3**-rho imine adduct (Figure S5). Correspondingly, we found that **3**-rho imine solutions slowly regained poly C-dependent ATPase activity in the absence of any adenine nucleotide or in the presence of AMP alone. Since rho inactivation by **3** is estimated to be 95% by the poly C-mediated ATPase assay (Figure S5, lane 2), ATP hydrolysis to ADP and P_i slowly proceeds throughout the 24-h reaction period. This time is sufficient to convert all the ATP to ADP. Accordingly, we are uncertain if ATP alone is sufficient to stabilize the **3**-rho imine adduct. These findings are consistent with the notions that ATP, ADP, or both are required for hexamer stabilization (*44*) and **3**-rho imine maintenance or that ATP (ADP) binding induces a conformational change (*14*) favorable for imine stabilization.

The remarkable stability of the imine generated from **3** and rho provided us with the opportunity to probe the potential number of bicyclomycin binding sites within hexameric rho and to assess the minimum number of bicyclomycin-inactivated subunits required to shut down rho-mediated ATPase activity. Incubation of rho with excess **3** in the presence of poly C and ATP followed by $NaBH_4$ provided a stable product. Mass spectrometry showed the presence of a major product at 47 321 Da along with a minor amount of unmodified wild-type rho in an approximate 4:1 ratio, respectively (Figure 4). The experiments were run under conditions that allowed the hexamer to remain intact with minimal amounts of subunit exchange (Figure 5). Significantly, the absence of multiple **3**-rho monomer adducts provided evidence that the initial imine formation process was selective. The approximate 79% conversion of rho to singly modified **3**-rho subunits establishes the maximum stoichiometry of the **3**-rho complex, prior to reduction, to be near five per hexamer. Assuming that the binding properties of **3** and **1** are similar and knowing that **3** competes with **1** for binding to rho (Figure S1), we suspect that the stoichiometry for bicyclomycin is between five and six per rho hexamer. This finding is reminiscent of data reported for F_1-ATP synthase, which has 43% similarity with rho (*51*). X-ray crystallographic analysis showed only five adenine nucleotides bound to the $\alpha_3\beta_3$ hexamer. The remaining ATP binding pocket located at the interface of an α and β subunit was distorted. We have proposed that bicyclomycin binds near the rho catalytic ATP hydrolysis site (*25*), which suggests that small topological changes in one of the ATP binding pockets or other regions in rho might inhibit either **3**-rho binding or **3**-rho imine formation.

Our finding that the maximum stoichiometry for the **3**-hexameric rho complex was near five differed from that reported for high concentrations of **2** (1 mM) (*23*). Here the maximum stoichiometry was approximately 2.7 equivalents of **2** per rho hexamer. We suspect that the lower stoichiometry observed for **2** is due, in part, to the differences in the stabilities of the generated imines. We found that the imine produced from **3** was significantly more stable than that produced from **2**. Accordingly, we suspect that the stoichiometry observed in the present study provides a better estimation of the bicyclomycin rho complex. In agreement with this notion of rho imine stabilities, we observed that higher concentrations of **1** were required to displace **3** than **2** (*24*) (Figure S1).

We obtained information concerning the minimum number of inactivated subunits within hexameric rho necessary to halt rho-mediated poly C-dependent ATP hydrolysis. Samples of wild-type rho were incubated with varying molar ratios of the **3**-rho amine adduct under conditions favorable to subunit exchange[3] and then evaluated (poly C ATPase activity). Comparison of the observed activities with those theoretically predicted (Figure 6) indicated that statistically one or two **3**-inactivated rho subunits per rho hexamer are sufficient to prevent ATP hydrolysis. This result is consistent with the reports of Richardson (*45*), Stitt (*49*), and Platt (*50*), indicating that disabling only a fractional number of the subunits within rho is sufficient to inactivate the protein.

This study establishes (1) that reductive probe **3** is an efficient rho inhibitor that provides high levels of singly modified rho subunits, (2) that approximately five of the six subunits are modified, (3) that ATP, ADP, or both stabilize the 3-rho imine, and (4) that inactivation of no more than two of the six subunits is sufficient to shut down rho-dependent ATPase activity. This information, when integrated with the site of **3** adduction, permits new insights into the mechanism of rho inhibition and rho transcription termination processes.

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SUPPORTING INFORMATION AVAILABLE

Figures S1−S6 providing data on the effect of bicyclomycin on **3** binding, the kinetics of **3**-rho binding, the time course for **3**-rho imine formation, the stability of the **3**-rho imine and **3**-rho amine adducts, and the effect of adenine nucleotides on the stability of the **3**-rho imine. ^{1}H and ^{13}C NMR spectra are provided for all new compounds prepared in this study. This material is available free of charge via the Internet at http://pubs.acs.org.

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