Design, Syntheses, and Evaluations of Bicyclomycin-Based Rho Inactivators

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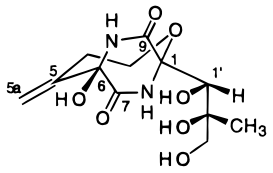
The commercial antibiotic bicyclomycin (**1**) has been shown to target the essential transcriptional termination factor rho in *Escherichia coli*. Little is known, however, about the bicyclomycin binding site in rho. A recent structure−activity relationship study permitted us to design modified bicyclomycins that may irreversibly inactivate rho. The four compounds selected were C(5a)-(4-azidoanilino)dihydrobicyclomycin (**3**), C(5a)-(3-formylanilino)dihydrobicyclomycin (**4**), C(5)−norbicyclomycin C(5)-*O*-(4-azidobenzoate) (**5**), and C(5)-norbicyclomycin C(5)-*O*-(3-formylbenzoate) (**6**). In each of these compounds the inactivating unit was placed at the C(5)−C(5a) site in bicyclomycin. In compounds **3** and **5** an aryl azide moiety was used as a photoaffinity label whereas in **4** and **6** an aryl aldehyde group was employed as a reductive amination probe. The synthesis and spectral properties of **3**−**6** are described. Chemical studies demonstrated that **3** and **4** were stable in D_2O and CD_3OD (room temperature, 7 d), while **5** and **6** underwent significant change within 1 d. Biochemical investigations showed that **3** and **4** retained appreciable inhibitory activities in rho-dependent ATPase and transcription termination assays. In the ATPase assay, I_{50} values for **1**, **3**, and **4** were 60, 135, and 70 μM, respectively. Correspondingly, the I_{50} values for **5** and **6** were >400 and 225 μM, respectively. In the transcription termination assay, compounds **1**, **3**, and **4** all prevented (≥97%) the production of rho-dependent transcripts at 40 μM, whereas little (≤15%) inhibition of transcription termination was observed for **5** and **6** at this concentration. Antimicrobial evaluation of **3**−**6** showed that none of the four compounds exhibited antibiotic activity at 32 mg/mL or less against W3350 *E. coli*. The combined chemical and biochemical studies led to our further evaluation of **3** and **4**. Photochemical irradiation (254 nm) of **3** in the presence of rho led to a 29−32% loss of rho ATPase activity. Attempts to confirm the irreversible adduction of **3** to rho by electrospray mass spectrometry were unsuccessful. No higher molecular weight adducts were detected. Incubation of rho with **4** at room temperature (4 h) followed by the addition of $NaBH_4$ led to significant losses (>62%) of rho ATPase activity. Analyses of the **4**−rho modified adduct showed appreciable levels of adduction (∼40%). Mass spectrometric analyses indicated a molecular weight for the adduct of approximately 47 410, consistent with a modification of a rho lysine residue by **4**. Compound **4** was selected for additional studies.

Bicyclomycin (**1**) is a structurally unique antibiotic[1−4] whose primary target in *Escherichia coli* is the transcription termination factor rho.[5] We have demonstrated that **1** reversibly binds to rho and as a result inhibits both ATPase and transcription termination activities.[6,7] Upon prolonged incubation with excess drug, multiple covalent modification of the protein occurs.[8]



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The exact binding site of bicyclomycin in rho is unknown, although several studies have provided structural information concerning rho. Rho protein is composed of six identical 47-kDa proteins of 419 amino acids[9] each in a proposed planar, hexagonal D_3 symmetry.[10−12]

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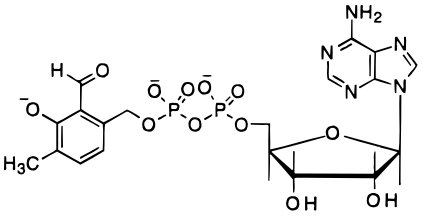
Investigations show that rho possesses primary RNA, secondary RNA, and ATP binding domains.[13−18] The *N*-terminal 151 amino acids have been assigned to the primary RNA binding site[19] whereas the ATP binding domain is believed to extend from residues 160 to 340 and contain sequence similarity to the *E. coli* β subunit of F1 ATPase and adenylate kinase.[15] The secondary RNA binding site has not been identified. We showed that bicyclomycin binding affects the secondary RNA binding site,[7] and mutations conferring bicyclomycin resistance (M218K, S266A, G337S) were located at or near the ATP domain.[5] These studies suggest that part of the bicyclomycin, ATP, and secondary RNA binding domains are close to each other. We report herein the design and evaluation of a modified bicyclomycin derivative that efficiently modifies a lysine residue(s) in rho and inactivates the protein.

Results and Discussion

A. Choice of Probes. In a recent structure−activity relationship (SAR) study, we demonstrated that modification of either the C(1) triol group[20] or the piperazinedione group within the [4.2.2] bicyclic ring system[21] led to a significant loss in inhibitory activity of the bicyclomycin analogue in rho-dependent ATPase[22] and in transcription termination assays.[23] These findings showed that both structural sites played important roles in the antibiotic's binding to rho. Correspondingly, selective modification of the C(5)−C(5a) site in **1** led to bicyclomycin analogues exhibiting excellent inhibitory activities in these assays.[24] Accordingly, we installed a rho irreversible inactivating unit at the C(5)−C(5a) locus in bicyclomycin.

Numerous examples have been reported of functional groups which affect enzyme inactivation.[25] We focused in this study on photoaffinity and reductive amination probes since they have been used extensively and successfully to target receptor sites within proteins. The phenylazido group was selected as the photoaffinity unit.[26] Irradiation of this moiety generates nitrenes, which can undergo insertion reactions in amino acid residues surrounding the receptor binding site. Moreover, the high extinction coefficient (ε) and photochemical quantum yield (φ) at 250−280 nm of aryl azides allow photolysis to occur rapidly with minimal ultraviolet damage to the protein.

Reductive amination probes require a lysine group to be present at or near the receptor binding site close to the reactive aldehyde group to permit imine formation and subsequent conversion to the amine by reduction. We chose the aryl aldehyde moiety as the reductive amination probe because this group provides an attractive balance between stability and reactivity. In a relevant study, Platt and co-workers utilized the adenine nucleotide analogue **2** to identify the ATP binding domain in rho.[14]



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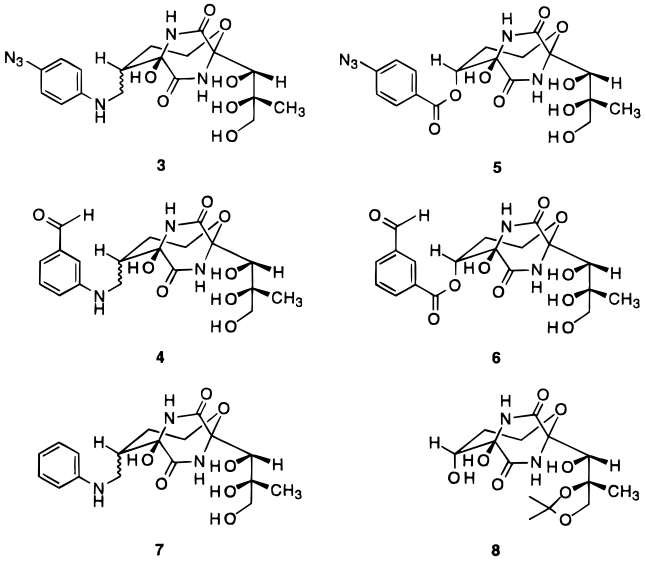
Compounds **3**−**6** were selected for synthesis and evaluation. Our SAR studies demonstrated that the C(5a) aryl dihydrobicyclomycin derivatives bound tightly to rho.[24] In particular, we observed C(5a)-(anilino)dihydrobicyclomycin (**7**) possessed an I_{50} value of 120 μM in the ATPase assay, comparing favorably to bicyclomycin (I_{50} = 60 μM). This finding, coupled with the commercial availability of aniline derivatives, prompted us to choose **3** and **4** as potential photoaffinity and reductive amination probes, respectively. Our high-yield synthesis of norbicyclomycin-5-ol C(2′),C(3′)-acetonide (**8**)[27] led us also to synthesize the photoaffinity probe **5** and reductive amination agent **6**.



3 **5** **4** **6** **7** **8**

B. Synthesis and Structural Characterization of Bicyclomycin Probes. Compounds **3** and **4** were prepared using the method employed for **7**[24] (Scheme 1). Dissolution of bicyclomycin C(2′),C(3′)-acetonide[28,29] (**9**)

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Scheme 1. Preparation of Compounds 3 and 4

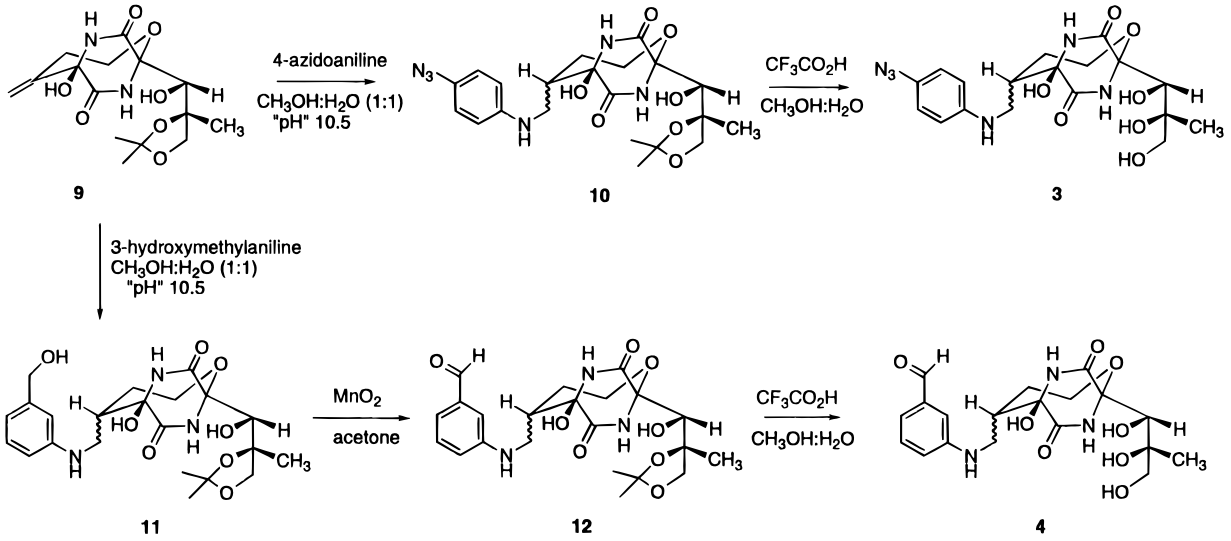
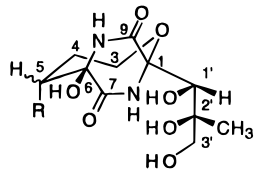


Table 1. Select ^{1}H NMR and ^{13}C NMR Spectral Properties for Rho Photoaffinity and Reductive Amination Reagents



	^{1}H NMR[a]					^{13}C NMR[b]				
compd	C(4)*H*H′	C(4)H*H*′	C(5)H	C(5a)*H*H′	C(5a)H*H*′	C(3)	C(4)	C(5)	C(5a)	C(6)
3	1.80−2.10 (m)		2.28−2.40 (m)	2.94 (dd, 9.3, 13.8)	3.50−3.65 (m)	62.6	29.9	50.6	44.2	83.9
4	1.85−2.10 (m)		2.30−2.45 (m)	3.02 (dd, 9.6, 13.8)	3.62−3.85 (m)	62.6	29.9	50.7	43.6	83.8
5[c]	1.85−2.00 (m)		5.06 (t, 3.9)			57.3	31.1	78.7		80.8
6[d]	2.10−2.35 (m)		5.47 (t, 3.9)			59.4	31.9	80.3		82.4

[a] The number in each entry is the chemical shift value (δ) observed in ppm relative to Me_4Si, followed by the multiplicity of the signal and the coupling constant(s) in hertz. All spectra were recorded at 300 MHz, and the solvent used was CD_3OD unless otherwise indicated. [b] The number in each entry is the chemical shift value (δ) observed in ppm relative to Me_4Si. All spectra were recorded at 75 MHz, and the solvent used was CD_3OD unless otherwise indicated. [c] The solvent used was DMSO-d_6. [d] The solvent used was CD_3OD:D_2O (1:1).

and 4-azidoaniline·HCl in 50% aqueous methanol adjusted to "pH" 10.5 yielded the Michael addition adduct **10** as a mixture of diastereomers (>10:1, NMR analysis). Deprotection of its acetonide group in **10** at room temperature (2 h) with trifluoroacetic acid furnished photoaffinity probe **3**. A similar procedure was employed for the preparation of a precursor to **4**. Dissolution of **9** and 3-(hydroxymethyl)aniline in 50% aqueous methanol ("pH" 10.5) warmed to 50 °C gave the addition adduct **11**. Oxidation of the benzyl alcohol group in **11** with MnO_2[30] produced **12**, which upon deprotection of its acetonide group yielded reductive amination probe **4**. The major diastereomer in **3** (**10**) and **4** (**11**, **12**) has been assigned as C(5)-*R* on the basis of the finding that the ^{1}H and ^{13}C NMR spectral data for **3** and **4** were similar to that reported for **7**.[24] The stereochemical identity of **7** was determined from X-ray crystallographic analysis of the corresponding acetonide derivative.[24]

Both **5** and **6** were prepared by coupling norbicyclomycin-5-ol C(2′),C(3′)-acetonide[27] (**8**) with the requisite carboxylic acid (Scheme 2). Ozonolysis of a methanolic solution of **9** followed by catalytic reduction (10% Pd/C) gave **8** as a single diastereomer. X-ray crystallographic analysis of **8** revealed the stereochemical orientation of C(5) as (*R*).[27] Treatment of a tetrahydrofuran solution of **8** with either 4-azidobenzoic acid or 3-formylbenzoic acid, using dicyclohexylcarbodiimide, (dimethylamino)-pyridine, and triethylamine, gave **13** and **14**, respectively. Deprotection of **13** and **14** furnished **5** and **6**, respectively.

Satisfactory spectroscopic data (IR, ^{1}H NMR, ^{13}C NMR, low- and high-resolution mass) were obtained for compounds **3**−**6**. Table 1 contains key ^{1}H NMR and ^{13}C NMR chemical shift values for these bicyclomycin derivatives. Similar NMR patterns were observed for **3** and **4**, as well as for **5** and **6**.

C. Stability Studies of Bicyclomycin Probes. An important requirement for the use of probes **3**−**6** in bicyclomycin−rho studies is that these reagents do not undergo fragmentation during rho binding, protein modification, and protein digestion. If this requirement is met amino acid residue(s) surrounding the bicyclomycin binding domain may be identified. Accordingly, we determined the stability of these compounds in CD_3OD and D_2O by ^{1}H NMR spectroscopy and TLC analyses (data not shown). Compounds **3** and **4** did not undergo change in either solvent at room temperature for 1 week. Dissolution of **5** and **6** in either CD_3OD or D_2O led to the gradual hydrolysis of these bicyclomycin derivatives to **15**, as evidenced by the upfield shift of the C(5) methine signal to δ 3.82−3.90.[27] The approximate $t_{1/2}$ value for **5**

(29) Zhang, Z.; Kohn, H. *J. Chem. Soc., Chem. Commun.* **1994**, 1343−1344.
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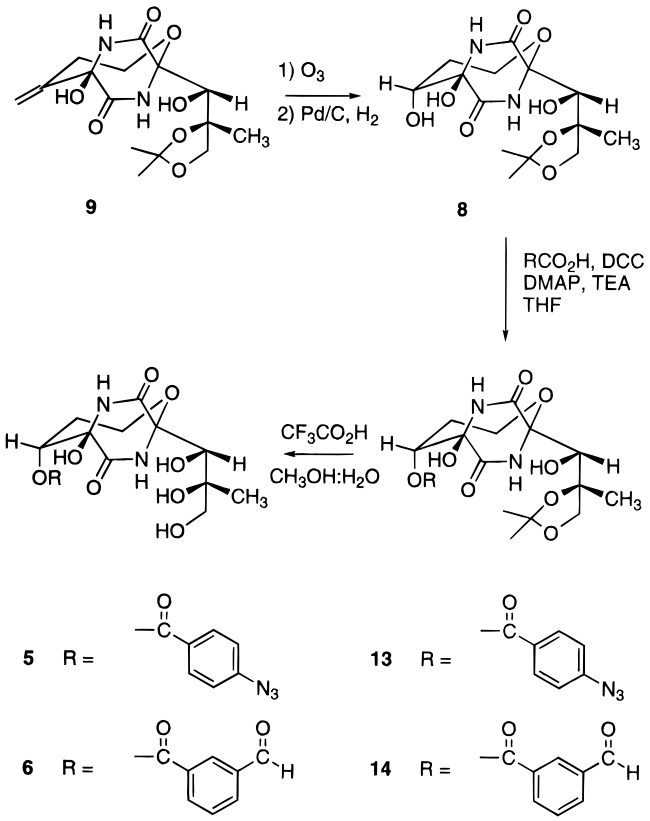
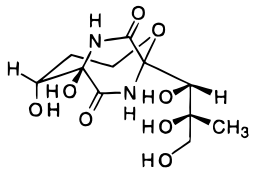
Scheme 2. Preparation of Compounds 5 and 6



and **6** in CD_3OD (22 °C) was 12 h and in D_2O (22 °C) was 24 h (^1H NMR analyses).



These studies indicated that compounds **3** and **4** were stable in protic solvents in the absence of added reactants and that probes **5** and **6** underwent cleavage of the bicyclomycin unit in CD_3OD and D_2O. This finding indicated that the use of **5** and **6** to identify the bicyclomycin binding site could be hampered by hydrolysis of the C(5) aryl ester bond.

D. Biochemical and Biological Properties of Bicyclomycin Probes. The inhibitory properties of compounds **1** and **3**−**6** in the poly C-dependent rho ATPase assay[22] are provided in Figure 1, and these data are summarized along with the transcription termination assay[23] results in Table 2. The I_{50} values for **3** and **4** were 135 and 70 μM, respectively, and they compared favorably to bicyclomycin (60 μM).[6] Compound **6** only moderately inhibited rho hydrolysis of ATP (I_{50} = 225 μM), and **5** was a weak inhibitor (I_{50} > 400 μM). Two patterns emerged from this limited data set. First, the aniline-based Michael addition adducts **3** and **4** inhibited rho-dependent ATPase activity to a greater extent than their bicyclomycin ester counterparts, **5** and **6**. The I_{50} values for **3** and **4** were 135 and 70 μM, respectively, and the I_{50} values for **5** and **6** were >400 and 225 μM, respectively. Second, the aryl aldehyde bicyclomycin derivatives **4** and **6** were more potent inhibitors of poly C-dependent ATPase processes than the corresponding aryl azide adducts **3** and **5**. The I_{50} value for **4** was 70 μM, while that for **3** was 135 μM, and the I_{50} value for **6** (I_{50} = 225 μM) was lower than that observed for **5** (I_{50} > 400 μM). Evaluation of **3**−**6** in the rho-dependent transcription termination assay led to similar results (Table 2). Bicyclomycin, **3**, and **4** all prevented the production of rho-dependent transcripts at 40 μM (≥97%), while little inhibition of transcription termination was observed for **5** and **6** at this concentration (≤15%). Accordingly, the I_{50} values for **3** and **4** were >10, <40, and ~10 μM, respectively, while the I_{50} values for **5** and **6** exceeded 100 μM. The corresponding I_{50} value for bicyclomycin was 5 μM.

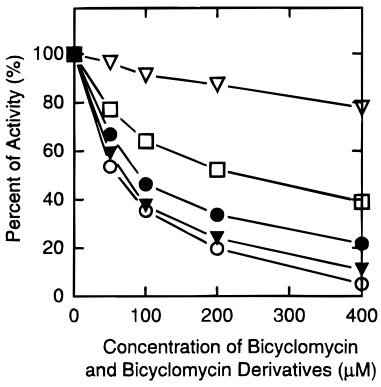


Figure 1. A Plot of the relative percent ATPase activity of rho in the presence of **1**: (○), **3** (●), **4** (▼), **5** (▽), **6** (□).

These results demonstrated that bicyclomycin C(5a) aryl amine derivatives **3** and **4** inhibit rho more efficiently than bicyclomycin C(5) benzoate derivatives **5** and **6** and were consistent with the previous SAR findings.[24] We have attributed the increased inhibitory properties of the aryl-substituted aldehyde derivatives **4** and **6** over the aryl azide adducts **3** and **5** to the adverse binding interactions accompanying the placement of the polar azide residue on the aromatic ring.[24]

The antimicrobial properties of **3**−**6** are listed in Table 2. None of the four compounds exhibited antibiotic activity (MIC > 32 mg/mL) against W3350 *E. coli* in the filter disc assay.[31] These results paralleled the result previously observed for **7**.[24]

The enhanced binding of **3** and **4** with rho compared to that of **5** and **6**, along with the ease with which **5** and **6** underwent hydrolysis, led us to select **3** and **4** for further study.

E. Studies on the Labeling of Rho by Photoaffinity Bicyclomycin Probe 3. The effectiveness of bicyclomycin irreversible inactivating probe **3** to covalently modify the drug-binding pocket in rho was assessed by the poly C-dependent ATPase assay.[22] Solutions (pH 7.9) containing **3** (1 mM) and rho with ATP were irradiated (254 nm, 15 min) and then extensively dialyzed to remove any unbound **3**. A similar experiment was done with 4-azidoaniline (**16**) in place of **3**. Comparison of the results obtained from **3** with **16** permitted us to assess the effect of random rho modification on poly C-dependent ATPase activity and provided a preliminary measurement of the site specificity of **3**. Efforts to increase the extent of protein modification by increasing the concentration of **3** (5 mM) and by the incremental

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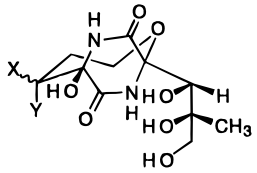
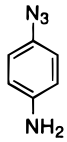
Table 2. Biochemical and Biological Activities of Photoaffinity and Reductive Amination Reagents



			inhibition of ATPase activity[a]		TT activity[d]		
compd	X	Y	I_{50} (μM) (BCM)[b]	400 μM (%) (BCM)[c]	I_{50} (μM)[e]	40 μM (%)[f]	MIC[g] (mg/mL)
1		CH_2	60	5	~5	100	0.25
3	H	*p*-$N_3C_6H_4N(H)CH_2$	135 (60)	78 (95)	>10, <40	97	>32 (0.25)
4	H	*m*-(C(O)H)$C_6H_4N(H)CH_2$	70 (60)	89 (95)	~10	100	>32 (0.25)
5	H	*p*-$N_3C_6H_4C(O)O$	>400 (60)	22 (95)	>100	0	>32 (0.25)
6	H	*m*-(C(O)H)$C_6H_4C(O)O$	225 (60)	61 (95)	>100	15	>32 (0.25)

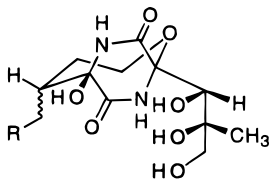
[a] Activity measured using the ATPase assay (ref 22) and described in an earlier paper (ref 6). [b] The I_{50} value is the average 50% inhibition concentration determined from duplicate tests. The corresponding value obtained from bicyclomycin in a concurrently run experiment is provided in parentheses. [c] The percent inhibition of ATPase activity at 400 μM. The corresponding value obtained from bicyclomycin in a concurrently run experiment is provided in parentheses. [d] Activity in the transcription termination assay was determined using the method of T. Platt and co-workers (ref 23) and described in an earlier paper (ref 7). [e] The I_{50} value is the average concentration for termination of rho-dependent transcripts determined from duplicate tests. [f] The estimated amount of transcription termination at 40 μM. [g] MIC value is the average minimum inhibitory concentration of the test compound determined from duplicate tests against W3350 *E. coli* using a filter disc assay (ref 31). The number in parentheses is the corresponding value obtained from bicyclomycin in a concurrently run experiment.

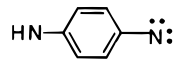
addition of **3** (2 × 1 mM) over a 15 min time period did not produce any appreciable changes in the level of protein inactivation (data not shown). We included DTT (1 mM) and mercaptoethanol (1 mM) in the reactions to minimize random protein modification.[26b] Use of higher concentrations of mercaptoethanol (20 mM) led to little probe−rho bonding (data not shown).

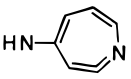


16

The results of the experiments are presented in Figure 2. UV irradiation of rho solutions in the absence of an aryl azide did not produce any detectable loss of rho ATPase activity (Figure 2, lanes 1 and 2). A comparable result was observed when aryl azide **16** (1 mM) was added to the reaction solution (Figure 2, lanes 2 and 4). Correspondingly, irradiation of a rho solution containing bicyclomycin photoaffinity probe **3** (1 mM, 5 mM) led to a 29−32% loss of ATPase activity (Figure 2, lanes 6 and 8). Significantly, lower levels of rho ATPase inactivation (10%) were observed when rho was treated with **3** in the dark (Figure 2, lanes 5 and 7). The observed losses in ATPase activity were consistent with levels typically reported in aryl azide−protein photolabeling experiments.[26b] Attempts to verify that the loss of rho ATPase activity was associated with covalent modification were unsuccessful. Photolysis of **3** was expected to initially give the singlet aryl nitrene **17** and then the ring-expanded ketene imine azepine **18**.[26b] Insertion reactions stemming from **17** or nucleophilic addition reactions from **18** would lead to an increase in the molecular weight of rho by 408. We observed no evidence by electrospray mass spectrometry that the rho−**3** photolysis experiments led to protein adduction compared with controls (data not shown). We concluded that either the photoproduct was unstable to the conditions of storage and analysis[26b] or that photolysis of the mixture of rho and **3** led to partial protein degradation without formation of an adduct with the intact protein.



17 R = 

18 R = 

Attempts to induce **3** modification of rho by including poly C in the reaction proved unsuccessful (data not shown). Poly C is known to bind tightly to rho[10,15,32] and induce a protein conformational change.[16,33] We observed that irradiation of rho solutions containing poly C (24 μM) without **3** led to noticeable losses of ATPase activity (50−60%). This result was consistent with an earlier study showing that poly C is cross-linked to rho by UV light.[16] Inclusion of **3** with poly C in the photolysis experiments did not lead to a significant further loss of rho ATPase activity.

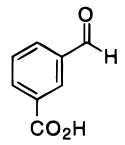
F. Studies on the Labeling of Rho by Reductive Amination with Bicyclomycin Probe 4. The effectiveness of reductive amination probe **4** to selectively modify rho was determined by comparing the ATPase activity after dialysis of $NaBH_4$ treated solutions[34] containing rho and **4** with those of rho and 3-carboxybenzaldehyde (**19**).

Figure 3 shows the effect of reductive amination of rho with either **4** or **19** in the absence of poly C and the

(32) (a) Galluppi, G. R.; Richardson, J. P. *J. Mol. Biol.* **1980**, *138*, 513−539. (b) McSwiggen, J. A.; Bear, D. G.; von Hippel, P. H. *J. Mol. Biol.* **1988**, *199*, 609−622.

(33) Bear, D. G.; Andrews, C. L.; Singer, J. D.; Morgan, W. D.; Grant, R. A.; von Hippel, P. H.; Platt, T. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 1911−1915.

(34) Means, G. E.; Feeney, R. E. *Anal. Biochem.* **1995**, *224*, 1−16.



19

presence of ATP. We adopted the following protocol. The rho solution containing the aromatic aldehyde was first incubated in the dark at room temperature for 4 h followed by the addition of excess $NaBH_4$. The solution was allowed to stand at room temperature for 20 min and was dialyzed overnight (5 °C) to remove any unbound **4**. The extent of protein modification was determined by the ATPase activity assay,[22] and these results were compared with control experiments conducted without $NaBH_4$. We observed no appreciable losses in poly C−dependent ATPase activity for rho solutions treated with $NaBH_4$ compared with untreated $NaBH_4$ solutions (Figure 3, lanes 2 and 3). No detectable protein inactivation was found when **19** (1 mM) was included in the reaction with $NaBH_4$ (Figure 3, lane 6). Use of **4** in place of **19** led to significant decreases (72%) in rho ATPase activity (Figure 3, lanes 3 and 9). Moreover, significantly decreased levels of inactivation were observed for **4** when $NaBH_4$ was excluded from the reaction (Figure 3, lane 8). These results indicated that **4**-mediated rho inactivation proceeds by $NaBH_4$ reduction of an intermediate imine. Further evidence in support of imine formation was the reduced levels of ATPase activity in the absence of $NaBH_4$ (Figure 3, lane 8).

An $NaBH_4$-treated sample obtained from rho and **4** (1 mM) in the absence of ATP, found to have 26% rho ATPase activity, was examined by electrospray mass spectrometry. We observed two proteins in an approximate 1:0.7 ratio that corresponded to approximately 47 008 and 47 410 Da (Figure 4). We have assigned these to rho and the **4**−rho reduced imine product since reductive amination of rho by **4** leads to an increase in the molecular weight of rho by 407.

The detection of the 47 410 Da signal does not permit us to conclude that bonding proceeded at a specific site in rho. Information concerning the specificity and site selectivity of reductive amination probe **4** is being obtained by mass spectral analysis of the tryptic digest of **4**-modified rho samples.[35] Complementary information can be obtained by radiodetection of the HPLC tryptic digest of radiolabeled **4**-rho. Our syntheses of C(5a)-(anilino)dihydrobicyclomycin enzyme inactivators (*e.g.*, **3** and **4**) provide an attractive opportunity to introduce a radiolabel into the compound, and we have tested this possibility. These compounds are formed by Michael addition of a substituted aniline to the ring-opened form of bicyclomycin C(2′),C(3′)-acetonide (**9**) followed by trapping of a proton from the solvent (CH_3OH:H_2O (1:1)) at C(5) and deprotection of the acetonide group. Accordingly, use of CH_3OT and T_2O in place of CH_3OH and H_2O, respectively, would allow generation of the corresponding C(5) radiolabeled probes. The feasibility of this approach has been examined by treating **9** with 4-azidoaniline in CD_3OD:D_2O (1:1) to give **10**-*d*. In the ^1H NMR spectrum of **10**-*d* the C(5a) protons appeared as two doublets (δ 2.92, J = 13.8 Hz; δ 3.61, J = 13.8 Hz) rather than the pair of doublets of doublets found for **10**. Dissolution of **10**-*d* in a buffered aqueous solution (pH 8.0) at room temperature for 2 d followed by recovery of the starting material did not produce detectable C-D → C-H exchange at C(5) (data not shown).

(35) Riba, I.; Gaskell, S. J.; Cho, H.; Park, H.; Widger, W. R.; Kohn, H. Unpublished results.

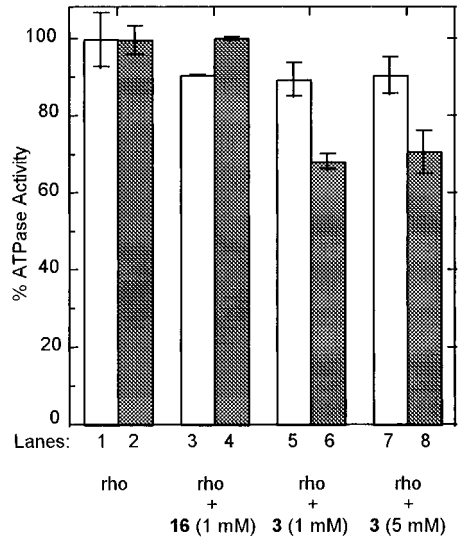


Figure 2. Histogram depicting the percentages of rho ATPase activity after photochemical activation in the presence and absence of **3** and **16**. The solutions were successively irradiated with UV light (254 nm) for 0.25 h (25 °C) and then dialyzed. The reactions were (1) no antibiotic and no UV; (2) no antibiotic and UV; (3) **16** and no UV; (4) **16** and UV; (5) **3** (1 mM) and no UV; (6) **3** (1 mM) and UV; (7) **3** (5 mM) and no UV; (8) **3** (5 mM) and UV. Figure code: open, no UV; shaded, UV.

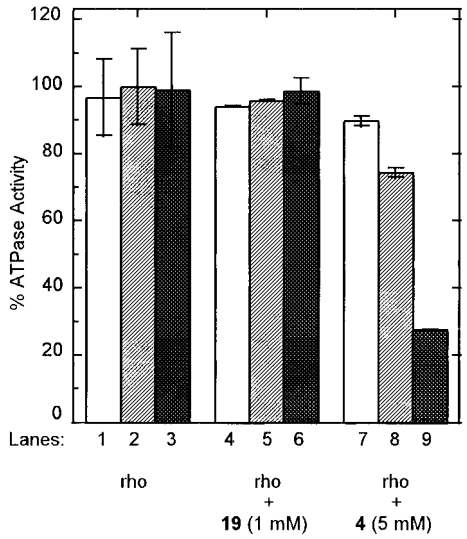


Figure 3. Histogram depicting the percentages of rho ATPase activity after reductive amination with either **4** or **19** in the presence of ATP (1 mM) using $NaBH_4$. The solutions were incubated in select cases for 4 h at room temperature, treated with $NaBH_4$ in select cases, and dialyzed. The reactions were: (1) no antibiotic, no incubation, and no $NaBH_4$; (2) no antibiotic, with incubation, and no $NaBH_4$; (3) no antibiotic, with incubation and $NaBH_4$; (4) **19** (1 mM), no incubation, and no $NaBH_4$; (5) **19** (1 mM), with incubation, and no $NaBH_4$; (6) **19** (1 mM), with incubation and $NaBH_4$; (7) **4** (5 mM), no incubation, and no $NaBH_4$; (8) **4** (5 mM), with incubation, and no $NaBH_4$; (9) **4** (5 mM), with incubation and $NaBH_4$. Figure code: open, no incubation, no $NaBH_4$; shaded, with incubation and no $NaBH_4$; solid, with incubation and $NaBH_4$.

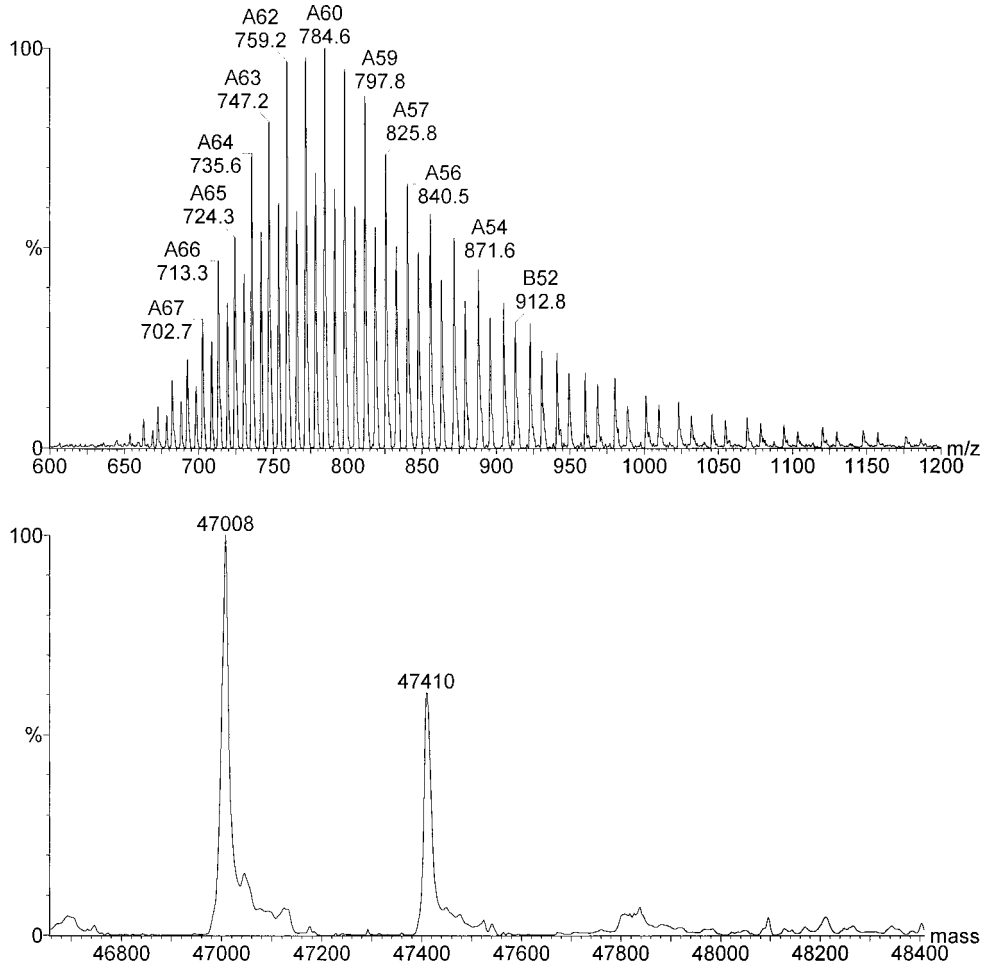
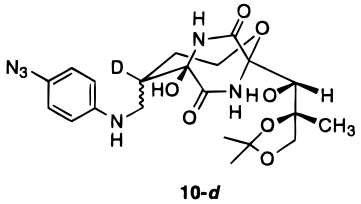


Figure 4. Electrospray mass spectrometric analysis of an $NaBH_4$-treated sample containing rho and **4** (1 mM). Upper panel: mass/charge ratio spectrum as recorded, showing multiple charge states of each component. Lower panel: spectrum obtained by maximum entropy processing (Ferrige, A. G.; Seddon, M. J.; Jarvis, S. *Rapid Commun. Mass Spectrom.* **1991**, *5*, 374−377) of the acquired data to achieve deconvolution and transformation to a mass scale.



10-*d*

Conclusions

These studies document that probes **3** and **4** retained excellent inhibitory activity in rho-dependent assays, providing preliminary evidence that these agents bind to the bicyclomycin binding pocket in rho. Photochemical activation of **3** in the presence of rho and ATP led to reduced rho−ATPase activity after dialysis compared with controls. Efforts to corroborate **3** adduction by mass spectrometry were unsuccessful. Correspondingly, treatment of a rho solution containing **4** with $NaBH_4$ led to an appreciable loss of rho-dependent ATPase activity after dialysis. This finding coupled with the detection of a higher-molecular-weight adduct by mass spectrometry provided preliminary information that **4** binds to the bicyclomycin binding pocket close to a lysine residue. Future studies will be directed toward determining the site and specificity of rho bonding and documenting that **4** bonds at the bicyclomycin binding domain in the protein. Identification of the lysine residue would provide structural information concerning both the sites of antibiotic and secondary RNA binding.[17,18]

Experimental Section[36]

Preparation of C(5a)-(4-Azidoanilino)dihydrobicyclomycin C(2′),C(3′)-Acetonide (10). To a 50% methanolic aqueous solution (2 mL) of **9**[28,29] (26 mg, 0.076 mmol) was added 4-azidoaniline·HCl (19 mg, 0.11 mmol). The "pH" was adjusted to 10.5 with an aqueous 0.1 N NaOH solution. The solution was stirred at room temperature (24 h), and the "pH" was adjusted to 7.0 with an aqueous 5% HCl solution, and then the solvent was removed *in vacuo*. The residue was purified by column chromatography (SiO_2, 10% MeOH−$CHCl_3$) to give **10** as a mixture of diastereomers (>10:1, NMR analysis): 13 mg (33%); R_f 0.60 (10% MeOH−$CHCl_3$); mp 120 °C dec; FT-IR (KBr) 3385 (br), 3313 (br), 2111, 1688, 1511, 1044 cm^{-1}; ^1H NMR (CD_3OD) δ 1.36 (s, 3 H), 1.46 (s, 6 H), 1.80−2.10 (m, 2 H), 2.28−2.40 (m, 1 H), 2.93 (dd, J = 9.0, 14.1 Hz, 1 H), 3.61 (dd, J = 4.5, 14.1 Hz, 1 H), 3.71 (d, J = 8.4 Hz, 1 H), 3.81 (dd, J = 8.4, 13.8 Hz, 1 H), 4.00−4.10 (m, 2 H), 4.56 (d, J = 8.4 Hz, 1 H), 6.68 (d, J = 9.0 Hz, 2 H), 6.81 (d, J = 9.0 Hz, 2 H); ^{13}C NMR (CD_3OD) 24.9, 26.8, 28.3, 30.4, 44.6, 50.7, 64.1, 73.2, 73.4, 84.0, 86.4, 88.8, 111.7, 115.3, 120.8, 129.7, 147.5, 167.9, 171.9 ppm; MS (+CI) 477 $[M + 1]^+$; M_r (+CI) 477.208 13 $[M + 1]^+$ (calcd for $C_{21}H_{29}N_6O_7$ 477.209 77).

Preparation of C(5)-Deuterated C(5a)-(4-Azidoanilino)dihydrobicyclomycin C(2′),C(3′)-Acetonide (10-*d*). Using the preceding procedure and utilizing a CD_3OD-D_2O

(36) For the general experimental procedures employed, see ref 20.

(1:1) solution (1 mL), **9**[28,29] (5 mg, 0.015 mmol) and 4-azidoaniline hydrochloride (5 mg, 0.029 mmol) gave **10**-*d* as a mixture of diastereomers (>10:1, NMR analysis). The "pD" was adjusted to 10.9 with a 0.1 N NaOD D_2O solution. The "pD" of the solution was determined from the observed pH meter reading by using the relationship pD = pH meter reading + 0.4.[37] The isolated yield for **10**-*d* was 1 mg (13%): R_f 0.60 (10% MeOH−$CHCl_3$); ^{1}H NMR (CD_3OD) δ 1.37 (s, 3 H), 1.46 (s, 6 H), 1.86 (dd, J = 8.4, 16.5 Hz, 1 H), 2.04 (dd, J = 8.1, 16.5 Hz, 1 H), 2.92 (d, J = 13.8 Hz, 1 H), 3.61 (d, J = 13.8 Hz, 1 H), 3.72 (d, J = 8.4 Hz, 1 H), 3.81 (dd, J = 8.1, 13.5 Hz, 1 H), 4.02−4.10 (m, 2 H), 4.46 (d, J = 8.4 Hz, 1 H), 5.34 (d, J = 8.7 Hz, 2 H), 6.82 (d, J = 8.7 Hz, 2 H); MS (+CI) 478 [M + 1]$^+$; M_r (+CI) 477.208 79 [M]$^+$ (calcd for $C_{21}H_{27}DN_6O_7$ 477.208 22).

Preparation of C(5a)-(3-(Hydroxymethyl)anilino)dihydrobicyclomycin C(2′),C(3′)-Acetonide (11). Using the procedure described for **10** and utilizing **9**[28,29] (100 mg, 0.29 mmol) and 3-(hydroxymethyl)aniline (72 mg, 0.44 mmol) gave **11** as a single diastereomer (NMR analysis) after treatment at 50 °C (5 h): yield, 105 mg (78%); mp 150−180 °C; FT-IR (KBr) 3390 (br), 3302 (br), 1687, 1608, 1044 cm^{-1}; ^{1}H NMR (CD_3OD) δ 1.37 (s, 3 H), 1.46 (s, 6 H), 1.80−2.10 (m, 2 H), 2.32−2.47 (m, 1 H), 2.95 (dd, J = 9.0, 14.1 Hz, 1 H), 3.60−3.73 (m, 2 H), 3.81 (dd, J = 8.1, 13.5 Hz, 1 H), 4.00−4.10 (m, 2 H), 4.45−4.48 (m, 3 H), 6.55−6.70 (m, 3 H), 7.07 (t, J = 7.0 Hz, 1 H); ^{13}C NMR (CD_3OD) 24.8, 26.8, 28.2, 30.4, 44.6, 50.7, 64.2, 65.6, 73.2, 73.5, 84.1, 86.3, 88.8, 111.7, 112.9, 113.4, 117.0, 130.1, 143.5, 149.8, 167.8, 171.9 ppm; MS (+CI) 466 [M + 1]$^+$; M_r (+CI) 466.217 97 [M + 1]$^+$ (calcd for $C_{22}H_{32}N_3O_8$ 466.218 94).

Preparation of C(5)-Norbicyclomycin C(2′),C(3′)-Acetonide C(5)-*O*-(4-Azidobenzoate) (13). To an anhydrous THF solution (1 mL) of **8**[27] (5 mg, 0.015 mmol) were added 4-azidobenzoic acid (3.5 mg, 0.019 mmol), dicyclohexylcarbodiimide (7 mg, 0.034 mmol), (dimethylamino)pyridine (2 mg, 0.016 mmol), and triethylamine (10 μL, 0.071 mmol), and the solution was stirred at room temperature (5 h). The solvent was removed *in vacuo*, and the residue was purified by preparative TLC (10% MeOH−$CHCl_3$) to give **13**: yield, 3 mg (41%); mp 125−130 °C; R_f 0.60 (10% MeOH−$CHCl_3$); FT-IR (KBr) 3452 (br), 3320 (br), 2128, 1716, 1603, 1268 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.25 (s, 3 H), 1.35 (s, 3 H), 1.40 (s, 3 H), 1.95−2.10 (m, 2 H), 3.60−3.85 (m, 2 H), 3.90−4.07 (m, 2 H), 4.31 (d, J = 8.1 Hz, 1 H), 5.14 (t, J = 4.2 Hz, 1 H), 5.86 (d, J = 8.1 Hz, 1 H), 7.03 (s, 1 H), 7.26 (d, J = 8.4 Hz, 2 H), 7.97 (d, J = 8.4 Hz, 2 H), 8.12 (s, 1 H), 9.00 (s, 1 H); ^{13}C NMR (DMSO-d_6) 24.6, 26.1, 27.8, 30.9, 58.6, 70.6, 71.2, 78.4, 80.8, 85.0, 87.3, 109.3, 119.2, 126.1, 131.3, 144.6, 163.7, 167.0, 167.4 ppm; MS (+CI) 492 [M + 1]$^+$; M_r (+CI) 492.172 00 [M + 1]$^+$ (calcd for $C_{21}H_{26}N_5O_9$ 492.173 05).

Preparation of C(5)-Norbicyclomycin C(2′),C(3′)-Acetonide C(5)-*O*-(3-Formylbenzoate) (14). Using the preceding procedure and utilizing **8**[27] (50 mg, 0.15 mmol), 3-carboxybenzaldehyde (35 mg, 0.23 mmol), dicyclohexylcarbodiimide (50 mg, 0.24 mmol), (dimethylamino)pyridine (10 mg, 0.08 mmol), and triethylamine (100 μL, 0.71 mmol) gave **14** as a single diastereomer (NMR analysis) after treatment at room temperature (3 h): yield, 30 mg (43%); mp 152−157 °C; R_f 0.50 (10% MeOH−$CHCl_3$); FT-IR (KBr) 3447 (br), 1699, 1603, 1384, 1271, 1190, 1073 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.26 (s, 3 H), 1.37 (s, 3 H), 1.40 (s, 3 H), 2.00−2.10 (m, 2 H), 3.65−3.80 (m, 2 H), 3.90−4.05 (m, 2 H), 4.32 (d, J = 8.1 Hz, 1 H), 5.21 (t, J = 4.5 Hz, 1 H), 5.86 (d, J = 8.1 Hz, 1 H), 7.07 (s, 1 H), 7.78 (t, J = 7.8 Hz, 1 H), 8.10−8.70 (m, 3 H), 8.43 (s, 1 H), 9.03 (s, 1 H), 10.07 (s, 1 H); ^{13}C NMR (DMSO-d_6) 24.7, 26.1, 27.9, 31.0, 58.9, 70.6, 71.2, 78.8, 80.9, 85.1, 87.5, 109.4, 129.7, 130.1, 130.6, 134.1, 134.9, 136.4, 163.7, 166.9, 167.5, 195.5 ppm; MS (+CI) 479 [M + 1]$^+$; M_r (+CI) 479.165 40 [M + 1]$^+$ (calcd for $C_{22}H_{27}N_2O_{10}$ 479.166 57).

Preparation of C(5a)-(4-Azidoanilino)dihydrobicyclomycin (3). To a 50% aqueous methanolic solution (2 mL) of **10** (10 mg, 0.02 mmol) was added trifluoroacetic acid (1 drop), and the solution was stirred at room temperature (2 h). The solvent was removed *in vacuo*, and the residue was purified by preparative TLC (10% MeOH−$CHCl_3$) to give **3** as a mixture of diastereomers (>10:1, NMR analysis): yield, 7 mg (76%); R_f 0.20 (10% MeOH−$CHCl_3$); mp 190 °C dec; FT-IR (KBr) 3384 (br), 3275 (br), 2112, 1686, 1512, 1405 cm^{-1}; ^{1}H NMR (CD_3OD) δ 1.33 (s, 3 H), 1.80−2.10 (m, 2 H), 2.28−2.40 (m, 1 H), 2.94 (dd, J = 9.3, 13.8 Hz, 1 H), 3.51−3.85 (m, 4 H), 4.00−4.10 (m, 2 H), 6.68 (d, J = 9.0 Hz, 2 H), 6.81 (d, J = 9.0 Hz, 2 H); ^{13}C NMR (CD_3OD) 24.2, 29.9, 44.2, 50.6, 62.6, 68.5, 72.2, 78.1, 83.9, 89.5, 115.3, 120.8, 129.6, 147.5, 168.5, 172.4 ppm; MS (+CI) 437 [M + 1]$^+$; M_r (+CI) 437.178 40 [M + 1]$^+$ (calcd for $C_{18}H_{25}N_6O_7$ 437.178 47).

Preparation of C(5a)-(3-Formylanilino)dihydrobicyclomycin (4). To an acetone solution (1 mL) of **11** (15 mg, 0.032 mmol) was added MnO_2 (10 mg), and the reaction mixture was stirred at 40 °C (2 h). The solvent was removed *in vacuo*, the crude product **12** was dissolved in a 50% aqueous methanolic solution (1 mL), and then 1 drop of trifluoroacetic acid was added. The solution was stirred at 40 °C (1 h), and then the solvent was removed *in vacuo*. The residue was purified by preparative TLC (10% MeOH−$CHCl_3$) to give pale yellow **4** as a mixture of diastereomers (>10:1, NMR analysis): yield, 5 mg (36%); mp 134-136 °C; R_f 0.16 (10% MeOH−$CHCl_3$); FT-IR (KBr) 3433 (br), 1685, 1436, 1207, 1141 cm^{-1}; ^{1}H NMR (CD_3OD) δ 1.34 (s, 3 H), 1.85−2.10 (m, 2 H), 2.30−2.45 (m, 1 H), 3.02 (dd, J = 9.6, 13.8 Hz, 1 H), 3.54 (d, J = 11.4 Hz, 1 H), 3.62−3.85 (m, 3 H), 4.00−4.15 (m, 2 H), 6.90−7.38 (m, 4 H), 9.84 (s, 1 H); ^{13}C NMR (CD_3OD) 24.2, 29.9, 43.6, 50.7, 62.6, 68.5, 72.3, 78.7, 83.8, 89.5, 113.2, 119.7, 120.3, 130.7, 139.0, 150.6, 168.5, 172.4, 195.0 ppm; MS (−CI) 423 [M]$^-$; M_r (−CI) 423.166 46 [M]$^-$ (calcd for $C_{19}H_{25}N_3O_8$ 423.164 17).

Preparation of C(5)-Norbicyclomycin C(5)-*O*-4-(Azidobenzoate) (5). Using the procedure for **3** and utilizing **13** (15 mg, 0.03 mmol) gave **5** as a single diastereomer (NMR analysis): yield, 7 mg (50%); mp 152−160 °C; R_f 0.50 (20% MeOH−$CHCl_3$); FT-IR (KBr) 3436 (br), 2129, 1700, 1603, 1273 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.08 (s, 3 H), 1.85−2.00 (m, 2 H), 3.20−3.40 (m, 2 H), 3.50−3.65 (m, 1 H), 3.75 (m, 2 H), 4.39 (t, J = 5.7 Hz, 1 H), 5.06 (t, J = 3.9 Hz, 1 H), 5.16 (s, 1 H), 5.23 (d, J = 7.5 Hz, 1 H), 6.73 (s, 1 H), 7.16 (d, J = 6.0 Hz, 2 H), 7.89 (d, J = 6.0 Hz, 2 H), 8.68 (s, 1 H), 9.02 (s, 1 H); ^{13}C NMR (DMSO-d_6) 23.8, 31.1, 57.3, 66.5, 70.3, 77.0, 78.7, 80.8, 87.6, 119.1, 126.1, 131.4, 144.5, 163.7, 167.4, 167.9 ppm; MS (+CI) 452 [M + 1]$^+$; M_r (+CI) 452.143 30 [M + 1]$^+$ (calcd for $C_{18}H_{22}N_5O_9$ 452.141 75).

Preparation of C(5)-Norbicyclomycin C(5)-*O*-(3-Formylbenzoate) (6). Using the procedure for **3** and utilizing **14** (25 mg, 0.05 mmol) gave **6** as a single diastereomer (NMR analysis): yield, 13 mg (43%); mp 138−145 °C; R_f 0.20 (10% MeOH−$CHCl_3$); FT-IR (KBr) 3423 (br), 3273 (br), 1698, 1407, 1274, 1193 cm^{-1}; ^{1}H NMR (CD_3OD:D_2O (1:2)) δ 1.36 (s, 3 H), 2.10−2.35 (m, 2 H), 3.58 (d, J = 11.4 Hz, 1 H), 3.70 (d, J = 11.4 Hz, 1 H), 3.80−3.95 (m, 1 H), 4.08 (s, 1 H), 4.15−4.30 (m, 1 H), 5.47 (t, J = 3.9 Hz, 1 H), 7.70−8.52 (m, 4 H), 10.03 (s, 1 H); ^{13}C NMR (CD_3OD:D_2O (1:2)) 23.9, 31.9, 59.4, 67.7, 71.6, 78.2, 80.3, 82.4, 88.8, 130.8, 131.1, 132.0, 135.6, 136.7, 137.3, 166.2, 170.2, 170.7, 195.6 ppm; MS (−CI) 437 [M − 1]$^-$; M_r (−CI) 437.120 53 [M − 1]$^-$ (calcd for $C_{19}H_{21}N_2O_{10}$ 437.119 62).

Studies on the Photoaffinity Labeling of Rho by Bicyclomycin Probe 3. A solution (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 (10 μg, 1 μM), ATP (1 mM), **3** (1 mM), DTT (1 mM), and mercaptoethanol (1 mM) was irradiated (254 nm) using a Mineralight UVG-54 lamp (UVP Inc.) at 25 °C (0.25 h). The reaction was then dialyzed (5 °C, 20 h) against 100 mM NaCl, 10 mM Tris·HCl (pH 7.6), 5% glycerol, 0.1 mM EDTA, and 0.1 mM DTT and then assayed. The percentage inactivation of rho was determined by measuring the initial velocity of ATPase activity using the previously described protocol[20,22] with rho (1 μg), poly C (24 μM), ATP (250 μM), and 0.5 μCi [γ-^{32}P]ATP. A second reaction was conducted using **16** in place of **3**. The results are presented in Figure 2.

Studies on the Reductive Amination of Rho by Bicyclomycin Probe 4. A solution (0.2 mL) containing the buffer (40 mM Tris·HCl, pH 7.9, 50 mM KCl, 12 mM $MgCl_2$, 0.1 mM

(37) Bates, R. G. *Determination of pH: Theory and Practice*, 2nd ed.; Wiley: New York, 1973; pp 375−376.

EDTA, 0.1 mM DTT), rho (10 μg), ATP (1 mM), and **4** (5 mM) was incubated at 25 °C (4 h). An aqueous solution (20 μL) of $NaBH_4$ (600 mM) was added, and the reaction was allowed to stand at 25 °C (20 min). The reaction was dialyzed (5 °C, 20 h) against 100 mM NaCl, 10 mM Tris·HCl (pH 7.6), 5% glycerol, 0.1 mM EDTA, and 0.1 mM DTT and assayed. The percentage inactivation of rho was determined by measuring the initial velocity of ATPase activity using the previously described protocol[20,22] with rho (1 μg), poly C (24 μM), ATP (250 μM), and 0.5 μCi [γ-^{32}P]ATP. A second reaction was conducted using **19** in place of **4**. The results presented in Figure 4 were done using **4** (1 mM) in the absence of ATP. Electrospray mass spectra were recorded using a Quattro tandem quadrupole mass spectrometer upgraded to Quattro II specifications (Micromass UK Ltd., Altrincham, UK). The electrospray capillary potential was 3.0−3.9 kV, and the cone was held at 20−30 V. The resolution was set to give a peak width at half height of 0.4 *m*/*z* unit for the monoisotopic peak of a singly charged ion. Analytes were introduced at a 5 μL/min flow rate using a syringe driver (Harvard Apparatus, South Natick, MA). Data were collected and processed using Micromass MassLynx software running on a Celebris XL 590 personal computer (Digital Equipment Corporation, Maynard, MA).

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra of all new compounds prepared in this study (17 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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