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SYNTHESIS OF SOME RIFAMYCIN DERIVATIVES AS INHIBITORS OF AN RNA-INSTRUCTED DNA POLYMERASE FUNCTION

Allan N. Tischler, Urs R. Joss* and Melvin Calvin

Laboratory of Chemical Biodynamics and Department of Chemistry,

University of California, Berkeley**

ABSTRACT

Several new derivatives of the antibiotic rifamycin SV have been prepared in the search for potent inhibitors of an RNA-instructed DNA polymerase function. It was observed that derivatives containing large, hydrophobic substituents bound to the 3-position of the rifamycin molecule are particularly potent inhibitors. Derivatives containing nitroxyl and dansyl functionalities were synthesized as potentially useful labeled rifamycins.

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* Fellow of the Elsa U. Pardee Foundation for Cancer Research, 1971-72. Present address: Friedrich Miescher Institut, Basel, Switzerland.

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INTRODUCTION

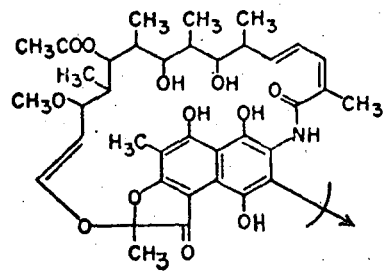
With the discovery of RNA-instructed DNA polymerase (RIDP)[1] came the attractive hypothesis proposing the DNA provirus of the tumor virus RNA as the genetic material ultimately responsible for cell transformation by tumor viruses. If this is so, then inhibition of the RIDP, which would be required for the synthesis of the DNA provirus, would be an effective means of preventing transformation of cells inoculated with tumor viruses. A similar comment holds for the more recently formulated protovirus modification.[2] Certain derivatives of rifamycin SV have been shown to be promising inhibitors of RIDP in *in vitro* studies.[3] Moreover, some of these derivatives have been shown to reduce the incidence of transformation in both BALB/3T3 cells[4] and normal rat kidney (NRK) cells[5] infected with murine sarcome virus (MSV).

In this paper, we present the synthesis of several new derivatives of rifamycin SV, some of which, especially (17) are more potent inhibitors of RIDP than the best derivatives previously synthesized and studied.[3,6] Based on the results of the derivatives presented here[7] and others[3], we propose that *in vitro* inhibition of RIDP is favored by large and hydrophobic substituents attached to the 3-position of rifamycin SV. Derivatives with 3-substituents meeting both criteria are especially active.

The drugs whose synthesis is described in this paper were either prepared by the condensation of rifaldehyde with the appropriate hydrazine or by acylation of N-desmethylrifampicin (6) in the 4-position of the

piperazine ring. Figure 1 presents the basic rifamycin structure and the structures of some common rifamycin derivatives. The structures of all of the products and intermediates were assigned on the basis of IR and NMR. UV and ESR were also taken when appropriate.

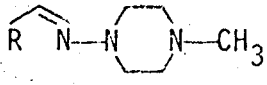
Figure 1



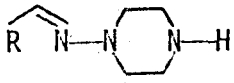
= R

R-H rifamycin SV

rifaldehyde



rifampicin



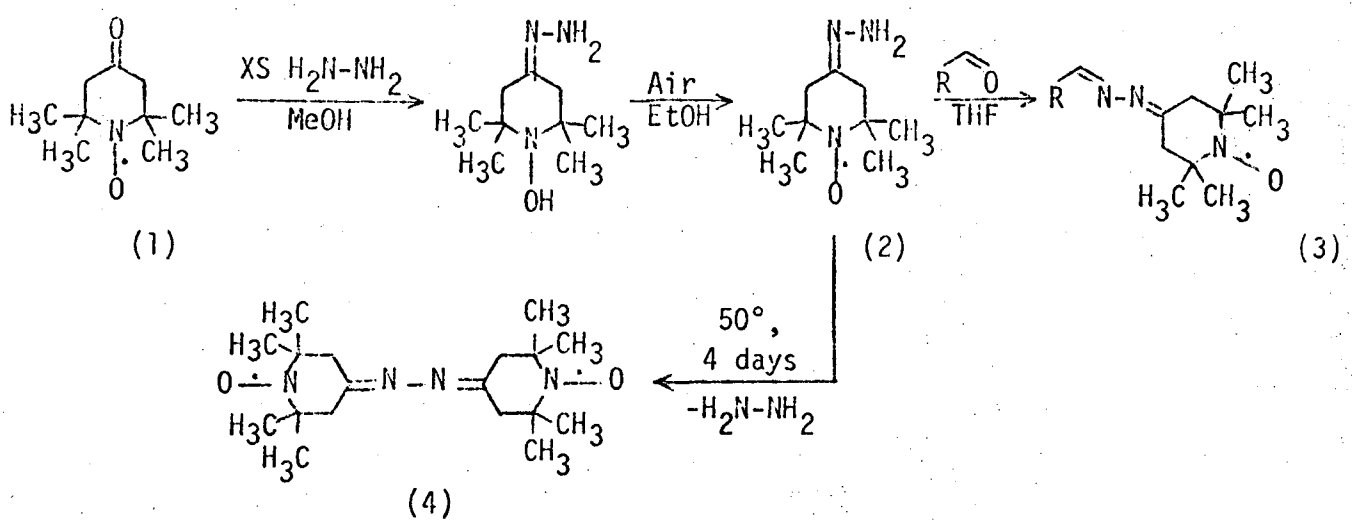
N-desmethylrifampicin

Structure of rifamycin and derivatives

DISCUSSION AND RESULTS

Because of its potential use in RIDP purification and as a tracer in biological systems, we undertook the synthesis of two spin-labeled derivatives (3 and 8) and a fluorescent derivative (9) of rifamycin SV. The synthesis of (3) is outlined in Scheme 1.

Scheme 1

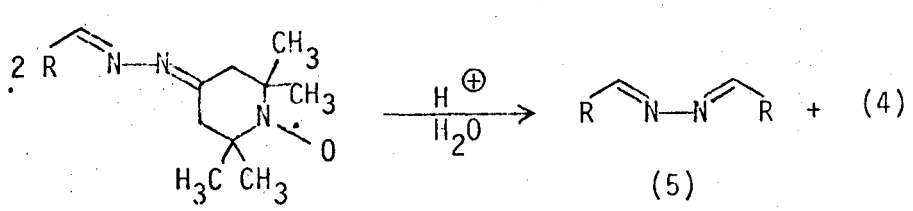


When N-oxyl-2,2,6,6-tetramethylpiperidine-4-one (1) is treated with a large excess of hydrazine in methanol, two reactions occur simultaneously; formation of the hydrazone of the keto group and reduction of the oxyl group to the hydroxylamine. The reaction proceeds with the evolution of nitrogen as a result of the latter reaction. Removal of the excess hydrazine followed by air oxidation in ethanol results in the oxidation of the hydroxylamine, yielding the oxyl hydrazone (2).

Condensation of this hydrazine with rifaldehyde affords the spin-labeled drug. Both (2) and (3) give the expected nitroxyl triplet in the ESR. The hydrazone (2) was never isolated in completely pure form. It slowly disproportionates to the azine dimer (4) This proved to be no hindrance as (4) does not react with rifaldehyde. When (2) is evacuated at 50°C for four days, the azine dimer, which gives an ESR consistent with a biradical,[8] is quantitatively formed. Spin-label compound (3) was found to be unsatisfactory as it shows little inhibition of RIDP and as it slowly decomposes with loss of its ESR signal. Presumably an oxidation-reduction takes place between the oxyl group and the hydroquinone of the rifamycin chromophore.

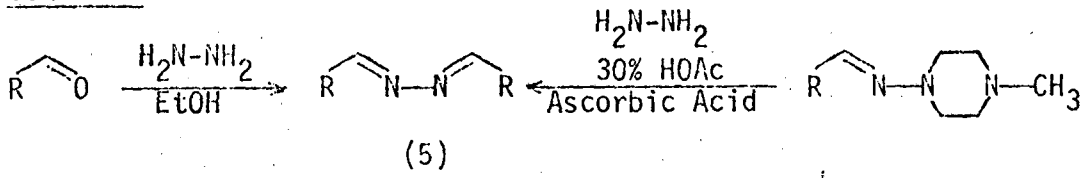
(3) was found to readily disproportionate in aqueous acid to give a bright red compound identified as the azine of rifaldehyde (rifamazine) on the basis of its IR and NMR, Rast molecular weight determination and UV. In the latter, one sees a bathochromic shift due to extension of conjugation across two rifamycin chromophores (Scheme 2). The rifamazine (5) was found to be a potent inhibitor of RIDP.

Scheme 2



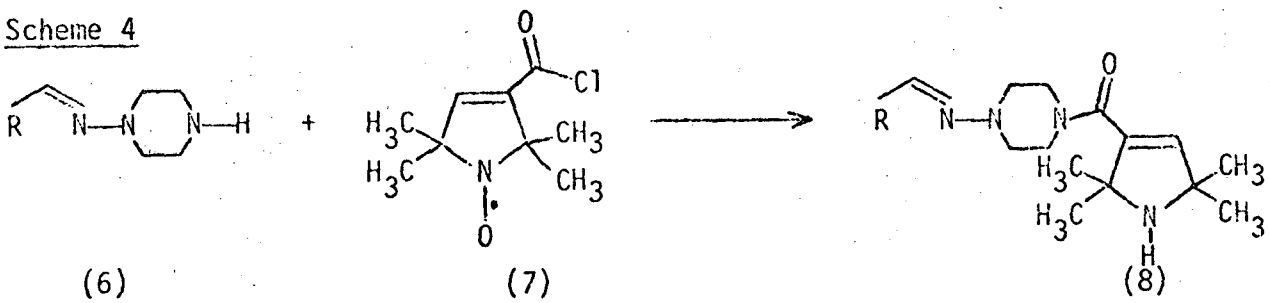
(5) can be more conveniently prepared either by the reaction of rifaldehyde with hydrazine, or by the reaction of rifampicin with hydrazine under hydrolyzing conditions (Scheme 3). Both routes afford the azine dimer in near quantitative yield.

Scheme 3



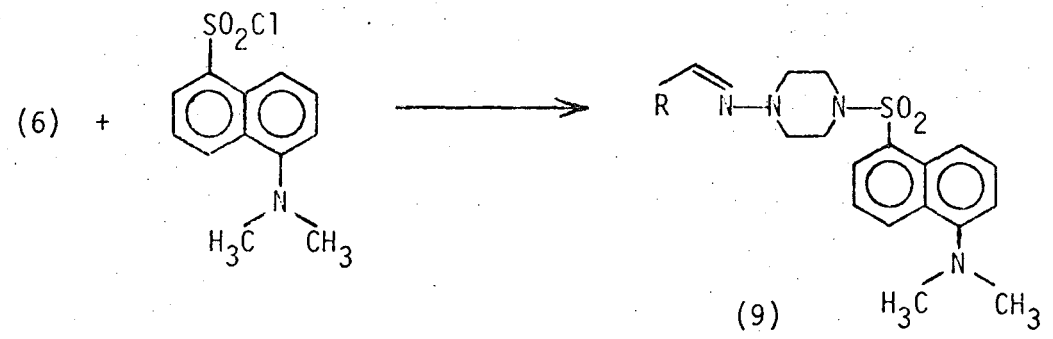
The second spin-labeled rifamycin derivative (8) was synthesized by acylation of (6) with the acid chloride (7) of 1-oxyl-2,2,5,5-tetramethyl-3-carboxypyrroline (Scheme 4). It is stable in pure form and like (3) it shows a three line ESR signal with somewhat broader lines than observed for nitroxide radicals with smaller molecular weight.

Scheme 4



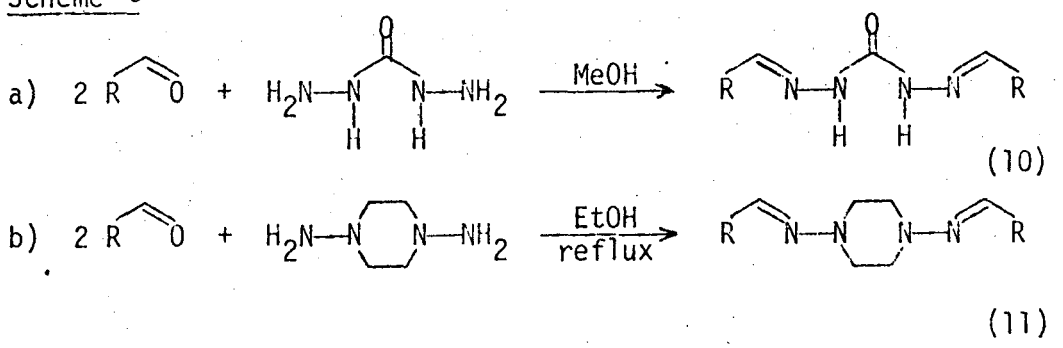
The dansyl derivative (9) was prepared by acylation of (6) with dansyl chloride (Scheme 5). (9) shows only approximately 1% of the expected fluorescence in organic solvents at around 520 nm, presumably due to quenching by the naphthalene system of the rifamycin chromophore. While (8) was found to be an ineffective inhibitor, (9) was found to be very effective.

Scheme 5



The high degree of RIDP inhibition shown by (5) encouraged us to look further at rifamycin dimers. Two additional dimers were thus prepared from readily available bifunctional compounds (Scheme 6). Urea derivative (10) (Rif-urea) was prepared by the condensation of rifaldehyde with carbohydrazide and the piperazine derivative (11) (Dirifampin) was prepared by the condensation of rifaldehyde with N,N'-diaminopiperazine. Both of these dimers were also found to be potent inhibitors of RIDP.

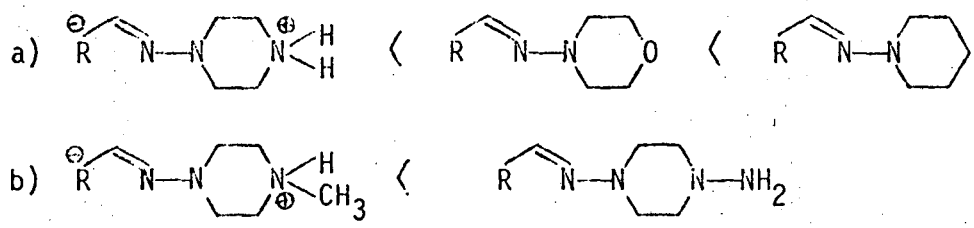
Scheme 6



The question arose as to whether the activity of these dimers is due to two chromophores in one molecule or one chromophore with one very bulky group bound to it. A test of the latter possibility, which seemed more reasonable to us, would involve the synthesis and evaluation of derivatives

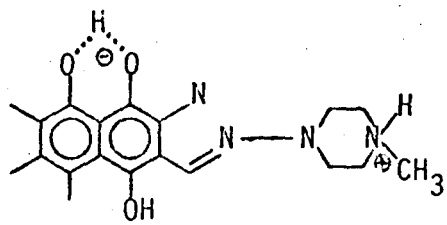
with large (preferably cyclic) groups attached to the 3-position. In addition, the work of Gallo[3] and Green[3] suggested to us that, substituent size being approximately equal, hydrophobic "tails" are more effective than hydrophilic. For example, Figure 2 gives sets of compounds listed in increasing order of activity. Desmethylrifampicin and rifampicin

Figure 2



Increasing Order of Activity of Rifamycin Derivatives

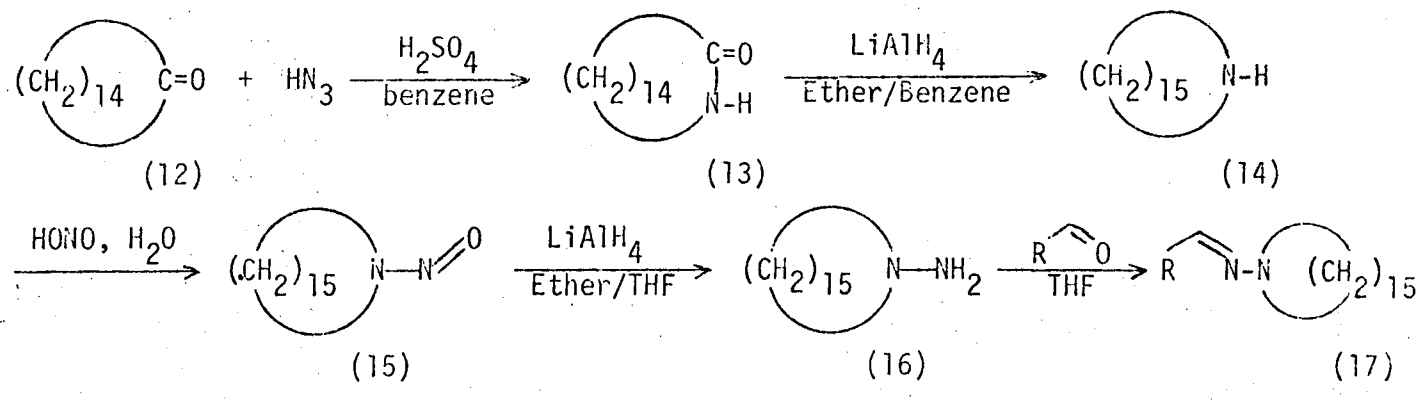
are known to be zwitterionic at pH = 7.8, the pH at which RIDP activity is measured, with the amine being protonated (pKa $\approx$ 10) and the hydrojuglone system existing as the anion (pKa $\approx$ 2.6).



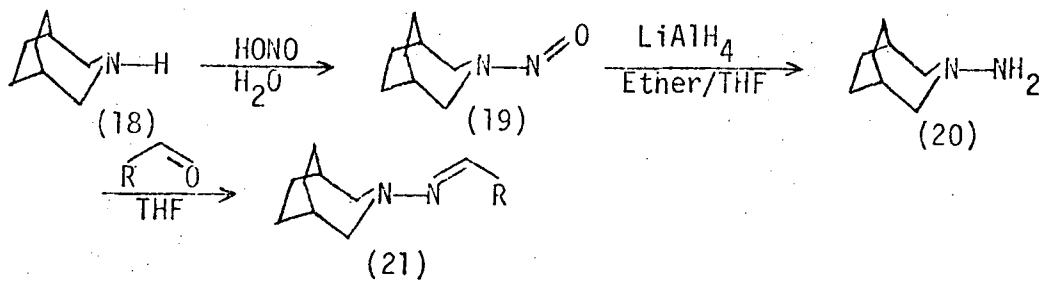
The analogous nitrogen in N-aminodesmethylrifampicin would not be expected to be largely protonated since it is a hydrazine (pKa $\approx$ 6-7).

From the above it was inferred that in addition to being large, hydrophobicity might also be desirable. As a result, two new drugs were prepared following this reasoning. Their preparations are outlined in Schemes 7 and 8.

Scheme 7



Scheme 8



Cyclopentadecanone (12) undergoes the Schmidt reaction to give the cyclic amide (13), which is readily reduced by lithium aluminum hydride to azacyclohexadecane (14). This secondary amine is converted to N-aminoazacyclohexadecane (16) by nitrosation to N-nitrosoazacyclohexadecane (15) followed by $LiAlH_4$ reduction. Analogously, 3-azabicyclo-[3.2.2]nonane (18) is converted to N-amino-3-azabicyclo[3.3.2]nonane (20) by nitrosation to N-nitroso-3-azabicyclo[3.2.2]nonane (19) followed by $LiAlH_4$ reduction. These two hydrazines (16 and 20) were condensed with rifaldehyde in THF to yield the corresponding hydrazones (17) rifaza-

cyclo-16) and (20) (rifazabicyclo-9), respectively. Both drugs were found to be very potent inhibitors of RIDP. Rifazacyclo-16 is the most potent drug tested by this laboratory to date.[7]

EXPERIMENTAL

IR spectra were taken on a Perkin-Elmer Model 137 and 257 grating infrared spectrometer. NMR spectra were recorded on a Varian Associates Model HR-220 instrument. Chemical shifts are reported in δ (ppm downfield). EPR spectra were taken on a Varian Associates Spectrometer Model E-3. UV spectra were recorded on a Cary Model 14 spectrophotometer. Thin-layer chromatography (TLC) was done on Eastman Chromagram 6060 Silica Gel sheets. Rifamycin derivatives used as precursors were kindly supplied by Gruppo Lepetit S.p.A., Milan, Italy.

N-oxyl-2,2,6,6-tetramethylpiperidine-4-one-hydrazone. N-oxyl,2,2-6,6-tetramethylpiperidine-4-one (1.00 g, 0.0058 moles) was dissolved in methanol (3 ml). The solution was cooled to 0° and hydrazine hydrate 2.90 g, 0.058 moles) was added to it dropwise. The flask was fitted with an air lock, and then allowed to stand at room temperature for 5 days. All volatile material was removed under vacuum. The resultant pale yellow oil, which gave no ESR signal and one spot by the thin-layer chromatography (silica gel in both dioxane and chloroform) was dissolved in 95% ethanol (15 ml) and stirred vigorously open to the air, for 24 hrs. Removal of the solvent afforded a bright yellow liquid (0.90 g, 84% of theory) which gave both ESR and IR consistent with the proposed structure. The hydrazone was used without further purification.

Di-N-oxyl-2,2,6,6-tetramethylpiperidine-4-one)azine. N-oxyl 2,2,6,6-tetramethylpiperidine-4-one hydrazone (0.50 g, 0.0027 moles) was heated at 50° under vacuum for 4 days, afforing a yellow crystalline product (0.45 g), m.p. = 173.77. A degassed THF solution gave a typical binitroxyl spectrum. IR was consistent with the proposed structure.

Anal. Calcd: C, 64.25%; H, 9.59%; N, 16.65%. Found:, C, 64.12%; H, 9.72%; N, 16.49%.

Spin-label (3). Hydrazone,(2)(0.17 g, 0.0010 moles) dissolved in 95% ethanol (3.5 ml) was added to a solution of rifaldehyde (0.500 g, 0.00069 moles) in 95% ethanol (10.5 ml) and the resultant mixture was stirred at room temperature for 15 hrs.

A column of alumina(activity 1) was prepared with a bed volume of 100 ml of 3:1 ethyl acetate-ethanol (by volume). The above reaction mixture was added to the column and then eluted with 3:1 ethyl acetate-ethanol until the wash gave no signal by ESR. The contents of the column were then placed in a flask and extracted with three portions of 100% ethanol (100 ml each). Removal of the solvents under vacuum afforded 0.43 g of the spin-labeled drug, (3) ESR, IR and UV are consistent with the assigned structure. TLC: Rf = 0.43 (dioxane).

Spin-label (8). Equal weights of (6) and acid chloride (7) were mixed as 10% solutions in chloroform. After completion of the reaction, which was checked by TLC, the reaction mixture was directly chromatographed on silica gel (BioSil-A, BioRad Laboratories, Richmond, Calif.) with benzene-chloroform. Yield: 90% [with respect to (6)]. TLC: Rf = 0.50 (ether:ethanol:ethyl acetate 1:1:1). UV in DMSO: 342 nm (ε- 26400), 485 nm (ε - 13200). EPR: g value: 2.0051, hyperfine splitting constant: 13.7 gauss.

Dansyl derivative (9). To a mixture of 200 mg of (6), 1 ml benzene and 5 ml 1 M bicarbonate buffer (pH 8.7), a solution of 70 mg of dansyl chloride in 1 ml of benzene was added. The mixture was heated to 40° and stirred at this temperature until completion of the acylation, which was checked by TLC. the organic phase was dried with sodium sulfate and chromatographed on SiO_2 (BioSil-A) with chloroform-ether. Yield: 80% [with respect to (6')]. TLC: Rf = 0.56 (ether:ethanol:ethyl acetate 1:1:1). UV in DMSO: 341 nm (ε - 25700), 483 nm (ε - 11900).

Rifamazine - Method A. Rifaldehyde (0.100 gm, 0.000138 moles) was dissolved in 95% ethanol (17 ml). To it was added 0.100 M hydrazine in 95% ethanol (2.80 ml, 0.00028 moles). the pH of the resultant solution was adjusted to 6.0 with 0.10 M HCl. Within 5 min a red precipitate was observed. The solution was then stirred for an additional hour, after which water (20 ml) was added. The solution was filtered, the red precipitate washed with 50% aqueous ethanol and dried under vacuum. Yield: 0.098 g (100%). TLC: Rf = 0.28 (ether:ethanol:ethyl acetate 1:1:1). UV in ethanol: 228 nm (ε - 54200), 358 nm (ε = 33500), 505 nm (ε =20000).

Rifamazine - Method B. Rifampicin (0.100 g, 0.000122 moles) 0.20 M aqueous hydrazine (3.0 ml, 0.00060 moles) and ascorbic acid (0.025 g) was dissolved in 30% aqueous acetic acid (25 ml). The solution was stirred in the dark for 5 days at room temperature. The resultant red precipitate was collected by filtration, washed with ethanol and dried under vacuum. Yield: 0.085 g (97.5%).

Rifurea. Rifaldehyde (0.100 g, 0.000138 moles) and carbohydrazide (0.00585 g, 0.000065 moles) were dissolved in methanol (10 ml). After stirring 4 hrs at room temperature, water (10 ml) was added dropwise to

affect crystallization. The orange precipitate was collected and washed twice (50% ethanol) by centrifugation. The product was dried under vacuum. Yield: 0.068 g (67%). TLC: Rf = 0.33 (ether:ethanol:ethyl acetate 1:1:1). UV in ethanol: 232 nm (ε - 51500), 337 nm (ε - 38800), 475 nm (ε - 20000).

Dirifampin. Rifaldehyde (0.100 g, 0.000138 moles) and N,N'-diaminopiperazine dihydrate (0.00988 g, 0.000065 moles) were dissolved in ethanol (12 ml). The reaction vessel was fitted with a condenser and then heated to reflux for 2.5 hrs. An orange precipitate was observed soon after reaching reflux. The solution was then cooled to 0° to complete precipitation. The precipitate was collected and washed (100% ethanol) by centrifugation and then dried under vacuum.
Yield: 0.085 g (84%). TLC: Rf = 0.75 (ethanol). UV in ethanol: 234 nm (ε - 32700), 348 nm (ε - 29900), 476 nm (ε - 17700).

2-Azacyclohexadecanone. Cyclopentadecanone (4.50 g, 0.0201 moles) and hydrazoic acid (16.9 ml of 1.25 M HN_3 in benzene) in benzene (30 ml) was added dropwise to an ice cold mixture of sulfuric acid (15.5 ml) and benzene (47 ml) with stirring. The temperature was maintained below 10°. After the addition, the ice bath was removed, and the reaction mixture was stirred for another 20 min. Ice water (100 ml) was then added. The benzene layer was separated and the aqueous layer was washed once with benzene (15 ml). The two benzene solutions were combined and washed once with 1.0 N KOH (50 ml) and dried over Na_2SO_4. The benzene was removed under vacuum, affording a white crystalline solid, which was recrystallized from 50% aqueous acetone. Yield: 4.30 g (89%), m.p. - 131-134°.

Azacyclohexadecane. 2-Azacyclohexadecanone (4.30 g, 0.0179 moles) dissolved in benzene (12 ml) was added dropwise to a stirred mixture of

$LiAlH_4$ (0.70 g, 0.018 moles) in ether (12 ml) at a rate to maintain a gentle reflux. Reflux was then maintained by heating for 15 hrs. The sequence water (0.70 ml), 15% NaOH (0.70 ml), water (2.1 ml) was added dropwise to the reaction mixture. Benzene (10 ml) was then added and the reaction mixture was filtered and washed with additional benzene. The filtrate was dried over Na_2SO_4 and evaporated under vacuum affording the product as a waxy solid, m.p. = 45-47.5°. Yield: 3.90 g (96%).

N-Nitrosoazacyclohexadecane. Concentrated HCl (1.35 ml, 0.0167 moles) was slowly added to a mixture of azacyclohexadecane (3.00 g, 0.0133 moles) and water (3.0 ml) at 0°. The reaction flask was then fitted with a thermometer and heated to 65°. A solution of $NaNO_2$ (1.02 g, 0.0167 moles) in water (3.0 ml)) was then added dropwise at a rate which maintained the temperature between 65-70°. This temperature was maintained by heating for an additional 5 minutes after the addition. The reaction mixture was then cooled to 25° and titrated with 15% NaOH to pH = 7.0. The organic layer was removed by extraction with three portions of benzene (15 ml each). The benzene solutions were combined, treated with Na_2SO_4 and decolorizing carbon, filtered and evaporated under vacuum. The N-nitroso compound resulted as a pale yellow, low melting solid, m.p. = 32-34°. Yield: 3.02 g (90%).

N-Aminoazacyclohexadecane. N-nitrosoazacyclohexadecane (2.95 g, 0.0116 moles) in ether (12 ml) was added dropwise to a stirred mixture of $LiAlH_4$ (0.50 g, 0.013 moles) in ether (6 ml) at a rate to maintain a gentle reflux. Reflux was then maintained by heating for 15 hrs, after which the sequence water (0.50 ml), 15% NaOH (0.50 ml), water (1.50 ml) was slowly added dropwise. Benzene (10 ml) was then added, and the reaction mixture was filtered and washed with additional benzene. The fil-

trate was dried over Na_2SO_4 and evaporated under vacuum affording the product as a waxy solid, m.p. = 31.5-34°. Yield: 2.58 g (93%).

Rifazacyclo-16. Rifaldehyde (0.190 g, 0.000262 moles) and N-aminoazacyclohexadecane (0.0630 g, 0.000262 moles) were dissolved in THF (12 ml) from which oxygen had been removed by bubbling in nitrogen. The solution was stirred at 25° for 48 hrs, after which the solvent was removed under vacuum. The resulting orange solid was recrystallized from petroleum ether (40 ml). Yield: 0.205 g (81%). TLC: Rf = 0.87 (tetrahydrofuran). UV in ethanol: 227 nm (ε - 24100), 279 nm (ε - 22300), 350 nm (ε - 20500), 479 nm (ε - 12900).

Acetone Derivative of N-Aminoazacyclohexadecane. N-aminocyclohexadecane (0.10 g, 0.00042 moles) was dissolved in acetone (10 ml) and stirred at 25° for 48 hrs. Removal, under vacuum, of the excess acetone yielded a light yellow oil which gave the expected NMR and IR for the acetone hydrazone derivative. Yield: 0.12 g (100%).

N-Nitroso-3-azabicyclo[3.2.2]nonane. 3-Azabicyclo[3.2.2]nonane (10.0 g, 0.080 moles) was slowly added to ice cold co centrated HCl (8.12 ml, 0.100 moles). The flask was then fitted with a thermometer and heated to 65°. A solution of $NaNO_2$ (6.0 g, 0.10 moles) in water (18 ml) was added dropwise at a rate which maintained the temperature between 65-70°. This temperature was maintained by heating for 10 min after the addition. The reaction mixture was cooled to 25°. The yellow precipitate that resulted was collected by filtration, dissolved in ether (100 ml) and treated with Na_2SO_4 and decolorizing carbon. The filtered solution was evaporated under vacuum affording the N-nitroso compound as a pale yellow solid. Yield: 6.0 g (49%).

N-Amino-3-azabicyclo[3.2.2]nonane. N-nitroso-3-azabicyclo[3.2.2]-nonane (3.00 g, 0.0195 moles) in ether (10 ml) was added dropwise to a stirred mixture of $LiAlH_4$ (0.75 g, 0.020 moles) in ether (10 ml) and THF (10 ml) at 0°. Ten minutes after the addition, the reaction was refluxed for 15 hrs, after which the sequence water (0.75 ml), 15% NaOH (0.75 ml) water (2.25 ml) was added dropwise. The reaction mixture was then filtered, and the precipitated hydroxides were washed twice with ether (10 ml each). The filtrate was dried over Na_2SO_4 and evaporated under vacuum. The bicyclic hydrazine (2.5 g, 90%) was obtained as a white solid. The compound was further purified by sublimation at 1 atm on a steam bath, giving white plates, m.p. - 58-70°, with decomposition.

Rifazabicyclo-9. Rifaldehyde (0.100 g, 0.00138 moles) and N-amino 3-azabicyclo[3.2.2]nonane (0.0193 g, 0.000138 moles) were dissolved in THF (12 ml). The solution was stirred for 24 hrs at 25°. The solvent was then removed under vacuum. The product, which resulted as an orange solid, was recrystallized from ethyl acetate. Yield: 0.071 g (61%).
TLC: Rf = 0.54 (tetrahydrofuran).

N-Amino-N-desmethylrifampicin. Rifaldehyde (1.00 g, 0.00138 moles) in THF (50 ml) was added dropwise to a stirred solution of N,N'-diamino-piperazine dihydrate (3.80 g, 0.028 moles), in water (50 ml). The addition was made over the period of one hr. The reaction was stirred for an additional hour after the addition. Half of the reaction volume was evaporated under vacuum. The remaining portion was extracted once with chloroform (100 ml). The chloroform solution was evaporated under vacuum affording the product as an orange powder. Yield: 0.98 g (83%).
TLC: Rf = 0.45 (ethanol). UV in ethanol: 237 nm (ε - 25500), 340 nm (ε - 21200), 479 nm (ε - 11500).

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TECHNICAL INFORMATION DIVISION
LAWRENCE BERKELEY LABORATORY
UNIVERSITY OF CALIFORNIA
BERKELEY, CALIFORNIA 94720