Trapping of the Quinone Methide from Reductive Cleavage of Menogaril with the Nitrogen Nucleophile Imidazole[1]

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Anaerobic reduction of menogaril, 7-*con*-*O*-methylnogarol (**1**), a semisynthetic antitumor drug in clinical trials, with 10 mol % of *meso*-bi(3,5,5-trimethyl-2-oxomorpholin-3-yl) (*meso*-TM-3 dimer, **4**) in methanol at ambient temperature in the presence of a large excess of imidazole yielded 58% of a mixture of *con*- and *dis*-7-imidazoyl-7-deoxynogarol (**8**) and 13% 7-deoxynogarol (**7**). Similar reduction of **1** with 30 mol % of a mixture of the diastereoisomers of bi(3,5-dimethyl-5-(hydroxymethyl)-2-oxomorpholin-3-yl) (DHM-3 dimers, **9**) in water yielded 54% **8** and 33% **7**. The reaction is proposed to occur by the Fisher chain mechanism with reductive activation to the quinone methide state **2** followed by nucleophilic addition of imidazole and subsequent oxidation of adduct hydroquinone **14** by starting menogaril. These experiments represent the first trapping of an anthracycline quinone methide state with a nitrogen nucleophile.

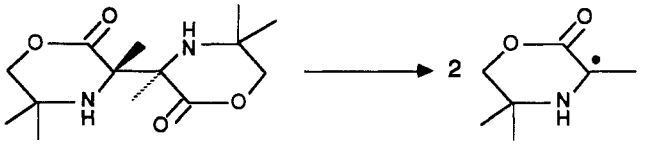
Introduction

The anthracycline antitumor drugs, as illustrated most notably by daunomycin, adriamycin, aclacinomycin A, and menogaril, are proposed to be bioreductively activated to quinone methide states.[3,4] Quinone methides result from elimination after reduction to the hydroquinone state as illustrated for menogaril (**1**) in Scheme I[5-8] and are possibly reactive with nucleophilic sites in biological macromolecules at the 7 position.[6] Covalent binding to macromolecules, possibly followed by catalytic production of reactive oxygen species,[9] might explain part of the observed cytotoxicity. The anthracycline electrophore has been shown to be an efficient catalyst for the reduction of oxygen with production of reactive oxygen species.[9,10] Quinone methide states have been observed as transients from in vitro reduction of all of the above-mentioned anthracyclines.[5,7,8,11] In protic solvents, quinone methides readily tautomerize to the corresponding 7-doexyaglycons, a reaction heavily competitive with nucleophilic trapping. Nucleophilic trapping has indeed been shown to occur in the formation of the quinone methide dimers which are obtained from reduction of aclacinomycin A,[7] 11-deoxydaunomycin,[8] and menogaril.[11] Trapping by nucleophiles other than by the quinone methide itself has been successful only with mercaptan nucleophiles such as cysteine derivatives in a short-chain-type reaction process first demonstrated by Fisher and co-workers.[6,12] The chain mechanism is needed because the nucleophilic addition generates a hydroquinone state which can expel the added nucleophile unless it is subsequently oxidized to the quinone state. In the Fisher chain process[6] the starting quinone serves as the oxidizing agent and the oxidation is the chain-carrying step.

We have recently demonstrated that reduction of menogaril with substoichiometric quantities of reducing agent yields a long-lived quinone methide (**2**) which is observable when produced in methanol solvent but not when produced in water solvent.[11] Observability is a function of the rate of formation from the hydroquinone, which is slower in water than in methanol. The major pathway for disappearance of the quinone methide at pH 8 is slow dimerization at the 7 position, which occurs in methanol at ambient temperature with a rate constant of 11 M^{-1} s^{-1}. In contrast, the major pathway for disappearance of the quinone methide from reduction of daunomycin or adriamycin under similar conditions is relatively rapid, pseudounimolecular tautomerization to the 7-deoxyaglycon, occurring with rate constants of 0.013 and 0.011 s^{-1}, respectively.[5,8] As a consequence of the long lifetime of **2**, nucleophilic tapping with *N*-acetylcysteine, a reaction competing with dimerization, gave yields as high as 85%.[11] This level of success prompted us to examine nucleophilic trapping in methanol and water with the nitrogen nucleophile imidazole which would model trapping by the imidazole group of the amino acid residue, histidine, in proteins. The nitrogen nucleophile imidazole was selected because it is a protein nitrogen nucleophilic site not protonated at physiological pH.

Results and Discussion

Menogaril was reduced in the presence of imidazole (**3**) in a Tris-buffered, anaerobic methanol solution at ambient temperature with *meso*-bi(3,5,5-trimethyl-2-oxomorpholin-3-yl) (**4**, TM-3 dimer). The reducing agent



meso-TM-3 Dimer, 4 TM-3, 5

operates by bond homolysis of **4**, occurring with a first-order rate constant of 2.4×10^{-3} s^{-1} at ambient temperature in methanol solvent,[13] to yield the one-electron reducing

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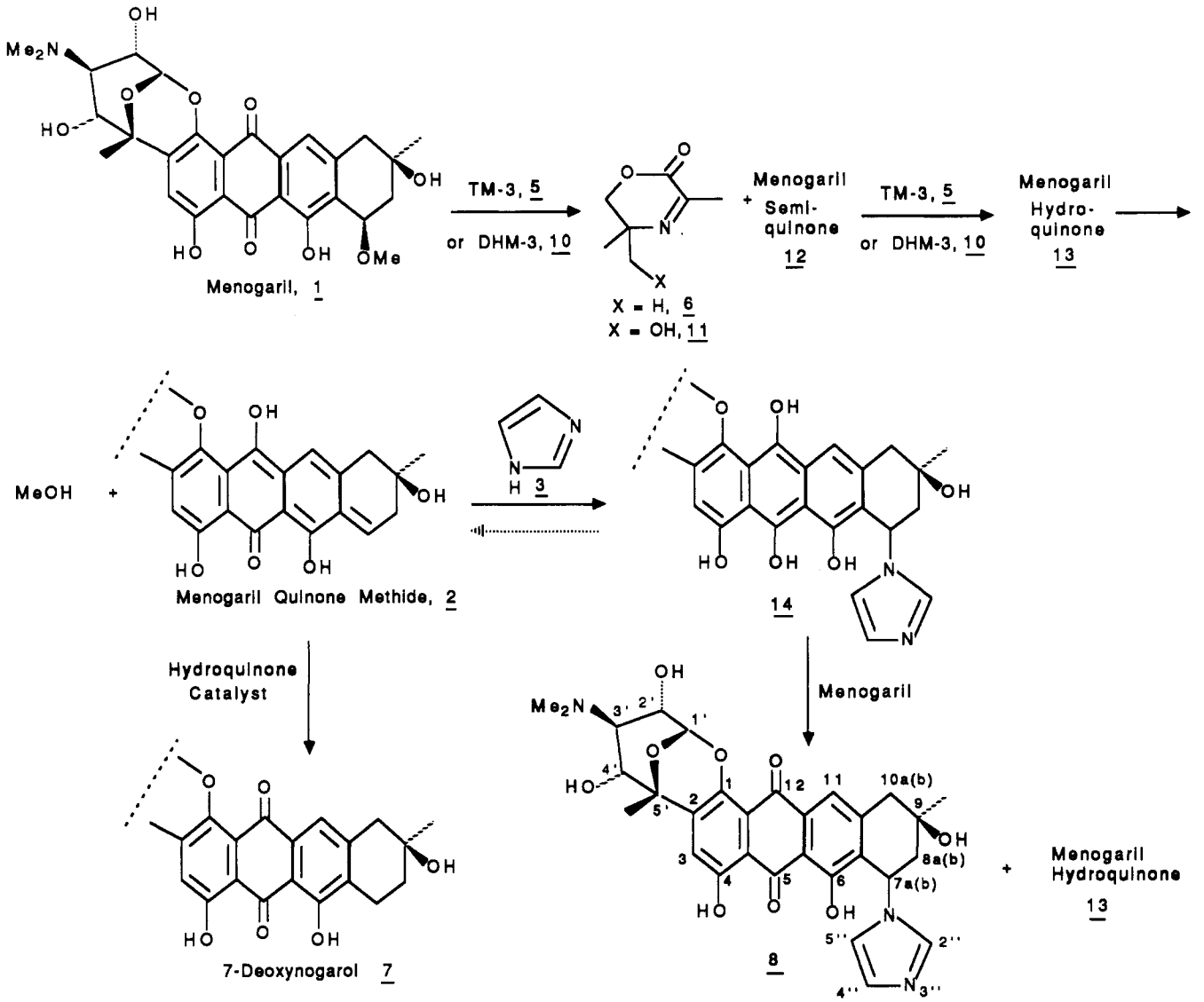
Scheme I



agent 3,5,5-trimethyl-2-oxomorpholin-3-yl (**5**, TM-3).[14] The byproduct of reduction is 5,6-dihydro-3,5,5-trimethyl-1,4-oxazin-2-one (**6**). The reducing agent stoichiometry that optimized the yield of quinone methide trapping with *N*-acetylcysteine was 10 mol %. A large excess of imidazole, 200 times the concentration of menogaril, and a long reaction time, 6 days, were used to accommodate the Fisher chain mechanism and the anticipated low rate constants for the propagation steps. HPLC analysis of the reaction mixture showed 23% unreacted menogaril (**1**), 13% 7-deoxynogarol (**7**), traces of two unidentified side products, and 58% of the diastereoisomeric products of imidazole trapping of the quinone methide, *con*- and *dis*-7-imidazoyl-7-deoxynogarol (**8**).

The diastereomers **8** were isolated from the other anthracycline materials and most of the imidazole by a series of solvent extractions. Subsequent recrystallization from chloroform yielded a sample of one diastereomer of **8**. The imidazole adducts were characterized from spectroscopic data on the mixture and the pure isomer. The most significant data were the high-field, one-dimensional, and COSY ^{1}H NMR spectra, assigned in the Experimental Section, and the exact mass FAB molecular ion. Substitution with an imidazole group at the 7 position of menogaril was evident from imidazole resonances with expected chemical shifts and couplings, a downfield one-proton resonance for the proton at the 7 position with coupling to the protons at the 8 position. The remainder of the ^{1}H NMR spectrum was similar to that observed for menogaril. The two diastereomers each showed distinct resonances for protons at the 7, 8, 10, and dimethylamino positions. Molecular models suggest that the dis isomer with the oxabicyclo ring in the favored chair conformation would have the dimethylamino group in the shielding region created by the imidazole ring current.[15] Consequently, the resonance for the dimethylamino group would appear upfield from the dimethylamino resonance for the con isomer. The absolute stereochemistry of the remainder of the molecule is based upon the known structure of members of the nogalamycin and nogamycin families.[16,17]

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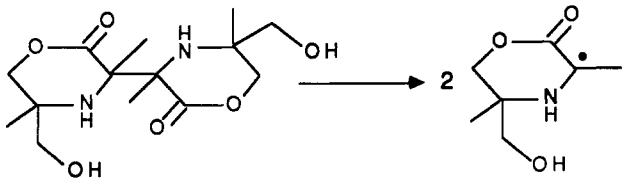
7-Deoxynogarol was identified by comparison with an authentic sample.

Trapping of the quinone methide by imidazole in pH 8.2 buffered water containing 10% dimethylformamide to facilitate dissolution of menogaril was also achieved. In this experiment the reducing agent was a mixture of the diastereoisomers of the water-soluble equivalent to TM-3 dimer, bi(3,5-dimethyl-5-(hydroxymethyl)-2-oxomorpholin-3-yl) (DHM-3 dimer).[18] Again the reducing



DHM-3 Dimers, 9 DHM-3, 10

agent is the radical, DHM-3, from bond homolysis of the dimer. The rate constant for bond homolysis in water of the major diastereoisomer present is 6.5×10^{-3} s^{-1}, approximately twice the rate constant for bond homolysis of *meso*-TM-3 dimer in methanol solvent.[18] The byproduct of reduction is 5,6-dihydro-3,5-dimethyl-5-(hydroxymethyl)-1,4-oxazin-2-one (11).[18] The reduction was performed in a manner similar to the reduction in methanol except that 30 mol % reducing agent was employed. The yield of imidazole adduct was 54% and the balance of the product mixture was predominantly 7-deoxynogarol (7).

A mechanism for formation of the imidazole adducts and 7-deoxynogarol is shown in Scheme I and is based upon earlier kinetic studies of reductive activation of menogaril with TM-3 dimer in methanol solvent.[11] Semiquinone (12) and hydroquinone (13) states are produced by sequential one-electron reductions by TM-3 or DHM-3 radicals; alternatively, the hydroquinone state can result from disproportionation of the semiquinone state.[4,19] Elimination of methanol gives the quinone methide 2, which has been previously characterized by its UV–vis absorptions at 375 and 602 nm.[11] Nucleophilic addition of imidazole at the 7 position of 2, which is most likely reversible,[7] gives the adduct in its hydroquinone state (14). Oxidation of 14 by starting menogaril yields adduct in its quinone state (8) and menogaril in its hydroquinone state, the chain-carrying step. In competition with nucleophilic trapping followed by oxidation is tautomerization of quinone methide 2 to 7-deoxynogarol (7), catalyzed by any hydroquinone present. The catalytic tautomerization was established earlier from kinetic measurements of the decay of quinone methide 2 in the absence of good nucleophiles.[11]

The formation of imidazole adduct 8 represents the first trapping of a quinone methide from reductive activation of an anthracycline by a nitrogen nucleophile. The trapping was successful primarily because the quinone methide 2 is very long lived in both methanol and water solvents. The reactions that compete with adduct formation are dimerization and tautomerization to 7-deoxynogarol (7). The tautomerization reaction is very slow except when hydroquinones which catalyze the reaction are present. Hydroquinones are formed from the initial reduction of menogaril (1), from nucleophilic addition to the quinone methide 2, and from oxidation of the adduct hydroquinone 14 by menogaril. The dimerization reaction is second order, and the rate constant is modest. Consequently, the rate of dimerization is slow unless the concentration of quinone methide is high. Under the reaction conditions employed here, the concentrations of both the quinone methide and the various hydroquinones were maintained low.

This observation of reaction of the quinone methide 2 with imidazole and the earlier observation of reaction with the sulfur nucleophilic site in *N*-acetylcysteine and *N*-acetylcysteine methyl ester indicate that bioreductive activation of menogaril might lead to significant levels of covalent attachment of the drug to SH and imidazole groups in proteins. In vivo, the probability for a quinone serving as the oxidizing agent in the chain-carrying step may not be high. However, as Fisher and co-workers have suggested,[6] the oxidizing agent needed for converting the adduct hydroquinone 14 to the adduct quinone 8 might also be molecular oxygen. Efficient reactivity of 2 with nucleophilic sites in proteins is consistent with the low levels of menogaril and its metabolites recovered in animal[20] and human[21] metabolic studies. Only menogaril and *N*-demethylmenogaril were detected in human plasma, bile, and urine. Biliary and urinary excretion accounted for only 2–4% and 5%, respectively, of the daily dose.[21] Recovered drug and fluorescent metabolites in organs and extracellular fluid accounted for only 10% of the menogaril administered to rabbits.[20]

In conclusion, trapping of the quinone methide from reductive activation of menogaril with the nitrogen nucleophile imidazole has been achieved. This represents the first isolation and characterization of an anthracycline–quinone methide–nitrogen nucleophile adduct. The trapping experiment was successful because of the long lifetime of the menogaril quinone methide state when formed with substoichiometric quantities of reducing agent. A long quinone methide lifetime is characteristic of quinone methides derived from 11-deoxyanthracyclines, most notably, 11-deoxydaunomycin, aclacinomycin A, and menogaril. Consequently, such quinone methides are likely to bind to nitrogen and sulfur nucleophilic sites in proteins and, possibly, nucleophilic sites in nucleic acids upon bioreductive activation. An important question for the future is what is the role of covalent binding to proteins in tumor response and in the important side effects, most notably for menogaril, myelosuppression and gastrointestinal toxicity.[22] Fisher and Aristoff have recently suggested that poor DNA-binding drugs such as menogaril might exert their antitumor effects at least in part through topoisomerase inhibition.[23] Possibly, such inhibition involves covalent bond formation to nucleophilic sites in the topoisomerases.

Experimental Section

General Remarks. UV–vis spectra were recorded with a Hewlett-Packard 8450 diode array spectrometer. ^1H NMR spectra were obtained with a Chemagnetics 200 MHz, a Varian Gemini 300 MHz, or Varian 500 MHz instrument. Chemical shifts are reported in ppm on the δ scale with tetramethylsilane or chloroform as an internal reference, and coupling constants are given in hertz. Mass spectral data were obtained with a VG Instruments 7070 EQ-HF high-resolution mass spectrometer equipped with a FAB inlet system. HPLC was performed with a Hewlett-Packard Model 1090 chromatograph equipped with a diode array

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UV–visible detector and data-processing work station. A 2.1 × 100 mm, Hypersil ODS, 5-μm column was used for analyses. The initial composition of the HPLC eluting solution was 60% v/v aqueous pH 4 buffer (3% ammonium formate adjusted to pH 4.0 with formic acid) and 40% methanol. The solvent delivery system was programmed to change gradually to 45% buffer in 3 min and then to 15% buffer in 8 min, at a flow rate of 0.5 mL/min. The chromatography was stopped after 15 min. The percentages of anthracyclines reported from HPLC analyses are the area percentages of the peaks resulting from compounds absorbing at 480 nm. Freeze–pump–thaw–sonnicate degassing was performed at liquid nitrogen temperature at 2×10^{-6} Torr. No metal needles were used in transferring the solutions of reactants or products. All the experiments with menogaril were run protected from direct light. *meso*-Bi(3,5,5-trimethyl-2-oxomorpholin-3-yl) (meso-TM-3 dimer, **4**) was prepared by photoreductive dimerization of 5,6-dihydro-3,5,5-trimethyl-1,4-oxazin-2-one (**6**) and separated from the *dl* isomer by low-temperature flash chromatography.[24] Bi-(3,5-dimethyl-5-(hydroxymethyl)-2-oxomorpholin-3-yl) (DHM-3 dimer, **9**) as a mixture of diastereoisomers was prepared by photoreductive dimerization of 5,6-dihydro-3,5-dimethyl-5-(hydroxymethyl)-1,4-oxazin-2-one (**11**).[18] Menogaril (**1**) and 7-deoxynogarol (**7**) were gifts of the Upjohn Co., Kalamazoo, MI. Imidazole (**3**) was obtained from Matheson Coleman & Bell, Norwood, OH. Tris(hydroxymethyl)aminomethane (Tris) and tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) were purchased from Sigma Chemical Co., St. Louis, MO. Reagent grade solvents were purchased from Fisher Scientific Co., Fair Lawn, NJ, and redistilled before use. HPLC-grade solvents were also purchased from Fisher Scientific.

Reaction of the Quinone Methide 2 from Reduction of Menogaril (1) with Imidazole (3) in Methanol. A Pyrex apparatus consisting of a 250-mL round-bottom flask provided with a small tube, 0.9 cm o.d. × 3.5 cm long fused at 90° in the upper part of the neck was fabricated. The top of the flask was also coaxially fused to a 0.9-cm o.d. tube for connecting to the vacuum line with an Ultra Torr union. *meso*-TM-3 dimer (**4**, 1.9 mg, 6.7×10^{-3} mmol) dissolved in 1 mL of dichloromethane was transferred into the small compartment of the flask, and the solvent evaporated with a stream of nitrogen. Menogaril (**1**, 36 mg, 6.6×10^{-2} mmol) was suspended in 150 mL of a methanolic buffer, 8.8×10^{-2} M in Tris and 1.1×10^{-1} M in Tris-HCl, containing 900 mg (13.2 mmol) of imidazole (**3**). The suspension was transferred into the large part of the flask, which contained a tiny magnet. The mixture was deoxygenated with a stream of nitrogen, freeze–thaw–degassed through three cycles, and then sealed with a torch. The sealed reaction flask was warmed to 50 °C for 1 min to let most of 1 dissolve. The solution was then mixed with the TM-3 dimer, quickly cooled to ambient temperature, and magnetically stirred for several hours until complete dissolution of 1 had occurred. The solution was then kept in the dark at ambient temperature for 6 days. The flask was opened, and the clear solution rotary evaporated at 20–30 °C. Then 30 mL of water was added to the residue. The aqueous solution analyzed by HPLC showed 58% of 7-imidazoyl-7-deoxynogarol (**8**) (two partially overlapped peaks with retention times of 1.0 and 1.3 min, in a 1:1 ratio, corresponding to the two stereoisomers con-*8* and dis-*8*), 23% of unreacted 1, retention time 5.2 min, 13% of 7-deoxynogarol (**7**), retention time 5.5 min, and two minor side products, with retention times of 6.7 and 9.4 min, both showing the typical anthracycline chromophore. The aqueous solution was washed five times with 40 mL each of benzene to remove oxazinone **6** and all the anthracyclines but **8**, which was then extracted from the aqueous solution with five extractions with 40 mL each of dichloromethane. The combined dichloromethane extracts were washed three times with 10 mL each of water to remove most of the extracted imidazole. The combined aqueous washings were reextracted with 40 mL of dichloromethane. Finally this extract was washed with 5 mL of water and combined with the original five dichloromethane extracts. HPLC, eluting as described above, showed only the overlapped peaks corresponding to *con*-**8**, and *dis*-**8**, 98% pure. The combined extracts were filtered by gravity and the solvent rotary evaporated to give 24 mg of a residue that by NMR and UV analysis appeared to contain ca. 40% of imidazole and 60% of a 1:1 mixture of the two stereoisomers. The mixture was easily dissolved in 2 mL of deuteriochloroform. The solution was kept overnight in the freezer, which caused a separation of a solid material (17 mg). The solid was quickly recrystallized from 1.5 mL of deuteriochloroform; 3.5 mg of most likely pure *dis*-**8** (retention time 1.1 min) was obtained as orange crystals. The material had the following properties: UV (methanol) λ_{max}, nm (ϵ, L/mol·cm) 234 (4.8×10^4), 259 (2.3×10^4), 291, sh (9.1×10^3), 476 (1.4×10^4); ^{1}H NMR (500 MHz, $CDCl_3$) δ 12.10 and 12.05 (br s, phenolic OH), 7.58 (slightly broadened s, very small $J_{2'',4''}$ and $J_{2'',5''}$ from COSY, H2″), 7.32 (s, H11), 7.13 (s, H3), 7.01 (slightly broadened s, very small $J_{4'',2''}$ and $J_{4'',5''}$ from COSY, H4″), 6.80 (slightly broadened s, very small $J_{5'',2''}$ and $J_{5'',4''}$ from COSY, H5″), ca. 5.8 (overlapped with H7, $J_{1',2'}$ not resolved but observed from COSY, H1′), 5.77 (m, overlapped with H1′, $J_{7,8a}$ and $J_{7,8b}$ not resolved but observed from COSY, H7), 4.14 (dd, $J_{2',1'} = 3$, $J_{2',3'} = 10$, H2′), 3.53 (d, $J_{4',3'} = 10$, H4′), 3.15 [d, $J_{10a,10b} = 17$, H10a(b)], 2.96 [d, $J_{10b,10a} = 17$, H10b(a), long-range coupling also seen with H8a, $J < 1$], 2.58 [m, $J_{8a,8b}$, $J_{8a,10b}$ and $J_{8a,7}$ not resolved but observed from COSY, H8a(b)], 2.46 [s, $N(CH_3)_2$], 2.34 [m, overlapped with N(CH3)2, $J_{3',2'}$ and $J_{3',4'}$ not resolved but observed from COSY, H3′], 2.06 [A part of ABX pattern, $J = 10, 13$, H8b(a)], 1.72 (s, C5′–CH_3), 1.48 (s, C9-CH_3); the second isomer (most likely *con*-**8**, retention time 1.3 min) showed distinct peaks at δ 5.57 (slightly broadened d, $J =$ 6, H7), 2.94 [d, $J = 18$, H10a(b)], 2.51 [s, $N(CH_3)_2$], 2.22 [dd, $J = 6, 14$, H8a(b)]; some of the chemical shifts varied from sample to sample, most likely due to differences in trace condentrations of DCl; mass spectrum, positive ion FAB (matrix, racemic mixture of dithiothreitol), m/z 578 ($M + H^+$); negative ion FAB, m/z 577 (M^-); exact mass of ($M + H^+$) for $C_{30}H_{32}N_3O_9$ 578.2166; calcd 578.2139 amu.

In a blank experiment, a Tris-buffered methanol solution 10^{-4} M in menogaril and 10^{-2} M in imidazole was left at ambient temperature for 28 days. At the end of this time period, HPLC analysis showed menogaril as the only anthracycline species present in solution.

Reaction of the Quinone Methide 2 from Reduction of Menogaril (1) with Imidazole (3) in Water. A Pyrex apparatus consisting of 1-mL and 5-mL compartments similar to the one described above was used. Bi(3,5-dimethyl-5-(hydroxymethyl)-2-oxomorpholin-3-yl) (DHM-3 dimer, **9**, 2.70 mg, 8.53×10^{-3} mmol) was transferred to a 50-mL volumetric flask, and the flask was brought to volume with acetonitrile. The smaller compartment of the cell was loaded with 0.70 mL (0.12×10^{-3} mmol) of the DHM-3 dimer solution, and the acetonitrile was then removed with a stream of nitrogen. Menogaril (1, 2.30 mg, 4.25×10^{-3} mmol) was dissolved in dimethylformamide (2.12 mL) by stirring for 10 min; 1.00 mL of this solution was transferred to a 10-mL volumetric flask. Imidazole (**3**, 27.2 mg, 400×10^{-3} mmol) was added, and the flask was brought to volume with 100 mM aqueous Tris/Tris-HCl buffer, pH 8.2. The menogaril–imidazole solution (2.0 mL) was loaded into the other compartment of the two-compartment apparatus. The aqueous solution was freeze–pump–thaw–sonnicate degassed through four cycles, and the apparatus was sealed with a torch. The contents of the two compartments were mixed at ambient temperature, and the mixture was allowed to react for 3 days in the dark at 25 ± 0.1 °C. The cell was then opened, and the contents were analyzed by reverse-phase HPLC using the column and eluting conditions as described above. The chromatogram showed two partially resolved peaks at 1.0 and 1.3 min in a 1:1 ratio. These were identified as *con*- and *dis*-7-imidazoyl-7-deoxynogarol (**8**) by co-injection with the sample isolated from the reaction in methanol solvent and purity evaluation of the peak by the diode array detector. The HPLC yield of the imidazole adduct was 54%. The remainder of the mixture consisted of 33% 7-deoxynogarol (**7**), 2.9% menogaril (**1**), and minor unidentified side products eluting at 2.6 (2.1%), 2.8 (1.3%), 6.8 (1.5%), 7.4 (1.4%), and 9.7 min (3.9%).

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