Bi[3,5-dimethyl-5-(hydroxymethyl)-2-oxomorpholin-3-yl] (DHM-3 Dimer). A Water-Soluble, One-Electron Reducing Agent[1]

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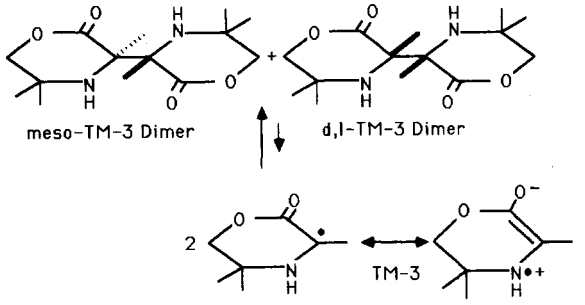
Received December 10, 1986

The synthesis and reactivity of the water-soluble, one-electron reducing agent bi[3,5-dimethyl-5-(hydroxymethyl)-2-oxomorpholin-3-yl] (DHM-3 dimer, **8**) are described. DHM-3 dimer was formed as a mixture of diastereoisomers from the photoreduction of 5,6-dihydro-3,5-dimethyl-5-(hydroxymethyl)-2*H*-1,4-oxazin-2-one (**3A**) in 2-propanol, and a major diastereoisomer **8A** was isolated in pure form. Oxazinone **3A**, which exists in equilibrium with a ring–chain tautomer, 3,5-dimethyl-8-aza-3,7-dioxabicyclo[3.2.1]octan-2-one (**3B**), was obtained from condensation of 2-amino-2-methyl-1,3-propanediol (**1**) with ethyl pyruvate (**2**). DHM-3 diastereoisomeric dimers equilibrate through facile bond homolysis via captodative radical 3,5-dimethyl-5-(hydroxymethyl)-2-oxomorpholin-3-yl (DHM-3). In the presence of an easily reducible substrate, DHM-3 reacts as a one-electron-reducing agent. Reductions of ferriin (an Fe^{3+}–1,10-phenanthroline complex), methylviologen (paraquat), 1,1-diphenyl-2-picrylhydrazyl (DPPH), potassium ferricyanide, molecular oxygen, and daunomycin by DHM-3 are described. The rates of these reductions, with the exception of the rates of reduction of molecular oxygen and daunomycin, are the rates of bond homolysis of DHM-3 dimer. The rate constant is solvent dependent and is highest in the most polar solvent, water, a result consistent with captodative resonance stabilization of the DHM-3 radical. Reduction of oxygen gives hydrogen peroxide less rapidly than bond homolysis, and reduction of daunomycin (**11**) gives 7-deoxydaunomycinone (**13**) via a consecutive first-order rate law. In the absence of a reducible substrate, DHM-3 dimer undergoes slow disproportionation to a mixture of **3A**, **3B**, and the two stereoisomers of 3,5-dimethyl-5-(hydroxymethyl)-2-oxomorpholine (**4A**, **4B**). The products of oxidation or disproportionation of DHM-3 hydrolyze in water: **3A** and **3B** give 2-amino-2-methyl-1,3-propanediol (**1**), pyruvic acid, and the two diastereoisomers of 2-carboxy-2,4-dimethyl-4-(hydroxymethyl)oxazolidine (**9A**, **9B**); **4A** and **4B** give *d,l-N*-[bis(hydroxymethyl)ethyl]alanine (**10**). Because of its water solubility, redox activity, and low animal toxicity, DHM-3 dimer is proving to be a useful antidote for some quinone antitumor drugs such as daunomycin.

We have observed that the radical dimer bi(3,5,5-trimethyl-2-oxomorpholin-3-yl) (TM-3 dimer) is an effective,



nontoxic antidote for the clinically important anthracycline antitumor drugs daunomycin and Adriamycin.[2] Antidotal activity presumably resides in the ability of TM-3 dimer to reduce the anthracyclines to their pharmacologically inactive 7-deoxyaglycons.[3] The actual reducing agent is the radical 3,5,5-trimethyl-2-oxomorpholin-3-yl (TM-3), produced from spontaneous bond homolysis of the dimer.[4,5] Bond homolysis of TM-3 dimer is facilitated by the captodative resonance interaction,[6] push–pull stabilization,[7] or merostabilization,[8] as shown below. Medicinal application of TM-3 dimer has been hampered by its low water solubility. As a result of earlier studies of substituent effects on the bond homolysis of TM-3 dimer,[9] we proposed bi[3,5-dimethyl-5-(hydroxymethyl)-2-oxomorpholin-3-yl] (DHM-3 dimer, **8**) as a water-soluble derivative with a sufficiently weak 3–3′ bond to be a superior antidote for the anthracyclines. Animal studies have now demonstrated that high Adriamycin dose–DHM-3 dimer rescue therapy increases the response of L-1210 leukemia to Adriamycin by a factor of 5.[10] DHM-3 dimer also has potential as an effective, nontoxic antidote for extravasation injury to skin incident to therapy for six anthracycline antitumor drugs and mitomycin C.[11] We describe here the synthesis, bond homolysis, and hydrolysis of DHM-3 dimer (**8**) and reduction of daunomycin with DHM-3 dimer.

Results and Discussion

Synthesis of DHM-3 Dimer. Bi[3,5-dimethyl-5-(hydroxymethyl)-2-oxomorpholin-3-yl] (DHM-3 dimer, **8**) was prepared as a mixture of at least five out of a possible six diastereoisomers in 50–66% yield by photoreduction of 5,6-dihydro-3,5-dimethyl-5-(hydroxymethyl)-2*H*-1,4-oxazin-2-one (**3A**) in 2-propanol. One diastereoisomer (**8A**) predominated and was isolated by crystallization and subsequently purified by recrystallization. The dimer structure was established from spectroscopic data, reported

(1) Support from the National Institutes of Health (Grant CA-24665), DHHS, and the National Science Foundation (Grant CHE-8419718) is acknowledged. We also thank Dr. Sergio Penco for a sample of daunomycin hydrochloride.

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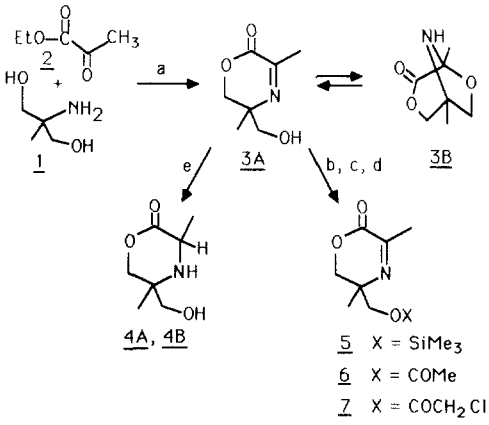
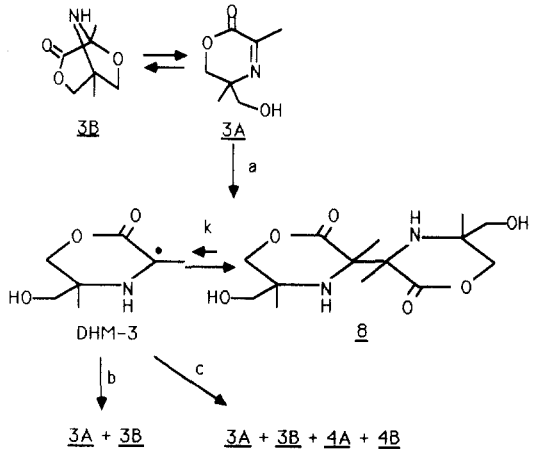
Scheme I[a]



[a] Reagents: (a) heat; (b) chlorotrimethylsilane and bis(trimethylsilyl)trifluoroacetamide; (c) acetic anhydride and pyridine; (d) chloroacetic anhydride and pyridine; (e) hydrogen and palladium on carbon.

Scheme II[a]



[a] Reagents: (a) 2-propanol and $h\nu$; (b) oxidizing agents such as oxygen, DPPH, ferriin, paraquat, or potassium ferricyanide; (c) heat.

in the Experimental Section, and the chemical reactivity is described below.

Oxazinone **3A** was formed in 58% yield from condensation of 2-amino-2-methyl-1,3-propanediol (**1**) with ethyl pyruvate (**2**) in refluxing butanol. Oxazinone **3A** exists in equilibrium with a ring–chain tautomer, 1,5-dimethyl-8-aza-3,7-dioxabicyclo[3.2.1]octan-2-one (**3B**), resulting from intramolecular nucleophilic addition of the hydroxy substituent of **3A** to the imine functional group. Ring–chain tautomerization leading to a bicyclic system such as **3B** has not been observed previously.[12] Furthermore, the only other report of the heterobicyclic ring system of **3B** concerns the product of the addition of maleic anhydride to nitrosocyclohexene followed by rearrangement.[13] Although **3A** and **3B** could not be separated, equilibration was observed by UV and ^{1}H NMR spectroscopy. The equilibrium constant was determined by integration of the methyl singlets and varied from 1 to 5, depending upon solvent, with the open-chain tautomer predominating in polar solvents. The equilibrium constant was used to calculate an approximate molar extinction coefficient of 110 M^{-1} cm^{-1} for the n–π^* band of **3A** in the region of 320 nm. The structure of **3A** and equilibrium of **3A** with **3B** were further established by quantitative conversions of the mixture of **3A** and **3B** to derivatives of **3A**. Reaction with chlorotrimethylsilane and bis(trimethylsilyl)trifluoroacetamide gave 5,6-dihydro-3,5-dimethyl-5-[[(trimethylsilyl)oxy]methyl]-2*H*-1,4-oxazin-2-one (**5**); reaction with acetic anhydride in pyridine gave 5-(acetoxymethyl)-5,6-dihydro-3,5-dimethyl-2*H*-1,4-oxazin-2-one (**6**); and reaction with chloroacetic anhydride and pyridine gave 5-[(chloroacetoxy)methyl]-5,6-dihydro-3,5-dimethyl-2*H*-1,4-oxazin-2-one (**7**). Furthermore, catalytic hydrogenation of **3A** plus **3B** with palladium on carbon as the catalyst gave a 1:1 mixture of the two diastereoisomers of 3,5-dimethyl-5-(hydroxymethyl)-2-oxomorpholine (**4A**, **4B**). These reactions are summarized in Scheme I. Just as the derivatization and hydrogenation of the mixture of **3A** and **3B** yielded products resulting exclusively from **3A**, the photoreduction of the mixture gave a good yield of DHM-3 dimer from excitation of the n–π^* band of **3A**.

Equilibrium of DHM-3 dimer with persistent radical 3,5-dimethyl-5-(hydroxymethyl)-2-oxomorpholin-3-yl (DHM-3) was established by EPR spectroscopy. A solution of **8** in methanol showed a characteristic 24-line signal both at ambient temperature and at 60 °C, with g = 2.004 and hyperfine coupling to the 3-methyl protons, the nitrogen, and the NH proton. That the complex product mixture was composed entirely of the stereoisomers of **8** was supported by the following observation. Dissolution of **8A** gave the thermodynamic mixture of diastereoisomers through bond homolysis and radical combination, as indicated by comparison of the ^{1}H NMR spectrum at equilibrium with the spectrum obtained from equilibration of any mixture of diastereoisomers.

Reactivity of DHM-3 Dimer. DHM-3 dimer behaves similarly to TM-3 dimer with respect to air oxidation and disproportionation.[4] Reaction of **8A** or the mixture of diastereoisomers of **8** in acetonitrile with molecular oxygen at 25 °C over a 5-day period gave a quantitative yield of the equilibrium mixture of **3A** and **3B**. The byproduct of the oxidation was hydrogen peroxide, which reached a maximum of 70% in approximately 30 h. Hydrogen peroxide was detected spectroscopically as its titanium tetrachloride complex. A quantitative yield of hydrogen peroxide was not achieved, possibly because of slow reaction of hydrogen peroxide with DHM-3, as observed earlier with TM-3 in the analogous air oxidation,[14] and because of slow disproportionation of hydrogen peroxide during the long reaction times. Similar, although faster, air oxidation was observed in water, methanol, and 2-propanol. The air oxidation was accompanied by a characteristic three-line EPR signal with g = 2.006 and a_N = 14.5 g. Based upon studies of the air oxidation of TM-3,[14] this signal reflects the intermediates 3,5-dimethyl-3-hydroperoxy-5-(hydroxymethyl)-2-oxomorpholin-4-yl radical. Heating of DHM-3 dimer (**8**) in degassed acetonitrile at 70 °C gave only products characteristic of disproportionation of DHM-3, namely a 1/1 mixture of oxazinone **3A** and its ring–chain tautomer **3B** and the morpholines **4A** and **4B**. Disproportionation was complete in about 50 h. The synthesis and reactions of DHM-3 dimer are summarized in Scheme II.

The first-order rate constant for bond homolysis of the major diastereoisomer of DHM-3 dimer (**8A**) was measured in several solvents with various oxidizing agents as traps for the DHM-3 radical. The most successful trapping agent in acetonitrile and water was ferriin, a complex of Fe^{3+} and 1,10-phenanthroline, which could even be used

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Table I. Rate Constants for Bond Homolysis of DHM-3 Dimer 8A at 25 °C as a Function of Medium and Radical Trapping Agent

8a,[a] %	solvent (pH)	trapping agent	k, s^{-1} (σ)	$t_{1/2}$
90	acetonitrile	DPPH	1.4×10^{-5}	13.8 h
90	acetonitrile	ferriin	1.6×10^{-5} (1.4×10^{-7})	12.0 h
100	acetonitrile	ferriin	1.43×10^{-5} (1.1×10^{-7})	13.5 h
90	methanol	DPPH	1.2×10^{-3}	580 s
90	methanol (8.1)[b]	paraquat	1.1×10^{-3}	630 s
90	water	ferriin	8.5×10^{-3} (1.5×10^{-3})	82 s
90	water (7.4)[c]	ferriin	7.9×10^{-3} (0.7×10^{-3})	88 s
100	water (7.4)[c]	ferriin	6.5×10^{-3} (1.6×10^{-4})	107 s
90	water (7.4)[c]	$K_3Fe(CN)_6$	7.2×10^{-3} (0.4×10^{-3})	96 s

[a] The balance of the material was a diastereoisomer of **8A**. [b] Buffered with 1/1 Tris–Tris-HCl. [c] Containing 0.13 M sodium chloride and buffered with 2.1×10^{-3} M Tris and 1.1×10^{-3} M Tris-HCl.

to trap DHM-3 efficiently in competition with molecular oxygen. Ferriin was reduced quantitatively to ferroin, a complex of Fe^{2+} and 1,10-phenanthroline, and DHM-3 was oxidized to **3A** plus **3B**. The rate constant was shown to be independent of the ferriin concentration. The other trapping reactions employed were reduction of 2,2-diphenyl-1-picrylhydrazyl (DPPH) to 2,2-diphenyl-1-picrylhydrazine, reduction of paraquat (methylviologen) to paraquat radical cation, and reduction of ferricyanide to ferrocyanide. Paraquat and DPPH were the reagents of choice in methanol. The kinetic measurements utilized the spectral differences between the trapping agent and its reduction product. The rate constants for bond homolysis are summarized in Table I and range approximately from 1.5×10^{-5} s^{-1} in acetonitrile to 7×10^{-3} s^{-1} in water. The increase in rate by a factor of nearly 500 in protic solvent is characteristic of oxomorpholinyl radicals and has been rationalized in terms of solvation of the radicals, which are polar as result of the captodative resonance interaction.[15] Recent calculations confirm that the resonance effect is magnified in a polar solvent.[16] In methanol, the rate constant for bond homolysis of DHM-3 dimer is somewhat lower than the rate constant for bond homolysis of TM-3 dimer, the latter being equal to 3.36×10^{-3} s^{-1} at 25 °C in methanol.[3] This difference in rate is consistent with an earlier observation of the reduction in the rate of bond homolysis of TM-3 dimer stemming from inductive effects of electron-withdrawing substituents at position 5.[9]

The rate of bond homolysis of a thermodynamic mixture of the diastereoisomers of DHM-3 dimer in pH 7.4 water was also observed with ferriin as the radical trapping agent. The reaction was no longer simple first order and showed a significant decrease in rate as a function of time, with the first, second, third, and fifth half-lives as follows: 61, 70, 110, and 462 s, respectively. Clearly, some of the minor diastereoisomers cleave a little more easily and some with more difficulty than the major diastereoisomer.

The activation parameters for bond homolysis of **8A** in pH 7.4 water were determined from the temperature dependence of the rate constant and are reported in Table II. Extrapolation to physiological temperature gives a half-life of 15 s for the major diastereoisomer of 8.

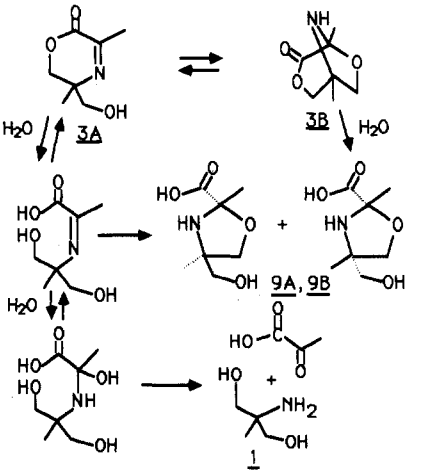
The hydrolysis of **3A** plus **3B** was also investigated because of possible relevance to future studies of the in vivo metabolism of DHM-3 dimer. As presented below, oxazinone **3A** is the product of oxidation of 8 by daunomycin and, as reported above, the product of oxidation by molecular oxygen. Monitoring by ^1H NMR spectroscopy showed that the mixture of **3A** and **3B**, when reacted with deuterium oxide, was completely hydrolyzed after 70 h to a mixture of the diastereoisomers of 2-carboxy-2,4-dimethyl-4-(hydroxymethyl)oxazolidine (**9A**, **9B**), 2-amino-2-methyl-1,3-propanediol (**1**), and pyruvic acid in the ratio 0.7/0.3/1/1. Attempted isolation of **9A** and **9B** by distillation or preparative GLC afforded only regenerated **3A** plus **3B**. A mechanism for the hydrolysis of **3A** in equilibrium with **3B** is proposed in Scheme III. Pyruvic acid and amino diol **1** most likely occur via sequential hydrolyses of the lactone and imine functional groups of **3A**. Oxazolidines **9A** and **9B** can result from lactone hydrolysis of **3A** followed by cyclization, and **9B** might also result from hydrolysis of the lactone functionality of **3B**. Because the oxazolidone stereochemistry is fixed in **3B**, hydrolysis of the lactone functional group of **3B** would not yield **9A**. Further hydrolysis of the oxazolidine rings is not proposed, since complete hydrolysis to pyruvic acid and amino diol **1** does not occur even after 51 days. Furthermore, pyruvic acid and amino diol **1** do not react in deuterium oxide to give any of **3** or **9**.

Table II. Activation Parameters for Bond Homolysis of 8A in pH 7.4 Water[a]

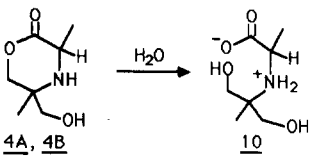
$A = 5 \times 10^{17}$ s^{-1}	$\Delta H^{\ddagger} = 26.6$ kcal/mol
$E_a = 27.2$ kcal/mol	$\Delta S^{\ddagger} = 20.5$ cal/K·mol
	$\Delta G^{\ddagger} = 20.6$ kcal/mol

[a] The rate constants used in the calculation of activation parameters are as follows: $(0.224 \pm 0.0015) \times 10^{-3}$ (5.1 °C), $(2.80 \pm 0.08) \times 10^{-3}$ (20.2 °C), $(5.95 \pm 0.16) \times 10^{-3}$ (25.0 °C), and $(12.8 \pm 0.4) \times 10^{-3}$ s^{-1} (30.0 °C). The correlation coefficient for the Arrhenius plot is 1.00.

Scheme III



Morpholines **4A** and **4B**, the reduction products from disproportionation of **8** or catalytic hydrogenation of **3A**, are hydrolyzed more easily than **3A** and **3B**. Even in the solid state and in the presence of moist air, **4** hydrolyzed at the lactone functional group to give *d,l-N*-[bis(hydroxymethyl)ethyl]alanine (**10**), characterized from spectral data.



Reduction of daunomycin (**11**) with the pure diastereoisomer of DHM-3 dimer (**8A**) was studied kinetically in methanol as described earlier for the reduction with *d,l-*

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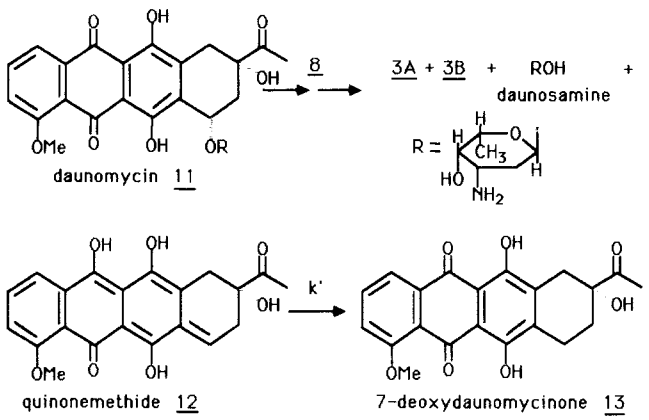
Scheme IV



TM-3 dimer.[3] The reaction is consecutive first order, with the two slow steps being bond homolysis of **8A** and tautomerization of the quinone methide intermediate (**12**) resulting from glycosidic cleavage of the hydroquinone of daunomycin. Steps leading to the quinone methide intermediate are all relatively fast reactions. The ultimate products were the 7-deoxyaglycon, 7-deoxydaunomycinone (**13**), the amino sugar daunosamine, and **3A** plus **3B**. Formation and destruction of the quinone methide **12** at 25 °C was monitored at 618–620 nm, a region of the visible spectrum where only **12** absorbed. Nonlinear least-squares analysis of the absorbance vs. time data as described previously gave a rate constant, k, of 1.1×10^{-3} s^{-1} for bond homolysis, in agreement with the independent measurement reported in Table I, and a pseudo-first-order rate constant, k', of 1.05×10^{-2} s^{-1} for protonation of the quinone methide, in agreement with the earlier measurement involving TM-3 dimer as the reducing agent.[3] Reduction of **11** with a 10-fold excess of a mixture of the diastereoisomers of DHM-3 dimer in pH 8.1 buffered water at 10 °C showed rapid formation of **12** with subsequent pseudo-first-order decay of the absorption at 618–620 nm. Linear least-squares treatment of ln $(A - A_\infty)$ vs. time gave a first-order rate constant of 2.8×10^{-2} s^{-1} for tautomerization of the quinone methide to 7-deoxydaunomycinone. Hence, the half-life for the reduction at 10 °C is approximately 25 s. Although these reductions were performed under anaerobic conditions, DHM-3 dimer can also reduce daunomycin in aqueous medium without degassing, in part because oxygen is not a particularly efficient oxidizing agent for DHM-3 radical. The reduction of daunomycin by DHM-3 is summarized in Scheme IV.

In summary, we report here the synthesis of a well-behaved, organic, water-soluble, one-electron reducing agent, which is an effective antidote for many quinone antitumor drugs. The material is nontoxic in animal models, due in part, perhaps, to its ability to participate readily in redox reactions and the product of its oxidation to undergo facile hydrolytic degradation to innocuous materials such as pyruvic acid and 2-amino-2-methyl-1,3-propanediol. We can think of no equivalent reducing agent in organic chemistry. In inorganic chemistry, DHM-3 dimer mimics in many respects the one-electron redox chemistry of dithionite.[17]

Experimental Section

General Remarks. IR spectra were recorded on a Perkin-Elmer Model 337 spectrometer. A Hewlett-Packard 8450A rapid-scan spectrometer was used for obtaining UV–visible spectral data; extinction coefficients reported below have units of M^{-1} cm^{-1}. ^1H NMR spectra were obtained with a Varian EM-390 90-MHz, a Chem Magnetics 200 200-MHz, or a Bruker 250-MHz spectrometer. Chemical shifts are reported (ppm) downfield from internal tetramethylsilane or, if in water, 3-(trimethylsilyl)-propanesulfonic acid sodium salt, and coupling constants are given in hertz. EPR measurements were made with a Varian 109E spectrometer equipped with field/frequency lock. Mass spectra were obtained with a VG Instruments 7070 EQ-HF high-resolution mass spectrometer equipped with FAB inlet system. GLC analyses and preparative chromatographies were performed with a Varian Aerograph Model 1700 gas chromatograph with a helium flow rate of 1 mL/s, using the following columns: (A) 5% SE30 on Chromosorb W HP 60/80 mesh, 4.3 m × 6.4 mm (o.d.); (B) 8% FS1265 on Chromosorb W HP 60/80 mesh, 3.6 m × 6.4 mm (o.d.). Capillary GLC analyses were performed with a Hewlett-Packard 5790A gas chromatograph equipped with an SE54 5% phenylsilicone–95% methylsilicone 30-m capillary column with a hydrogen flow rate of 2 mL/min. Analytical HPLC was performed with a Tracor 950 HPLC pump equipped with a Tracor 970A variable-wavelength UV–visible detector. The column, 25 cm × 4.6 mm (i.d.), was packed with C18 RSIL 10-μm reversed-phase packing. HPLC solvents were Fisher HPLC grade. Microanalyses were performed by Atlantic Microlab, Atlanta, GA, or Galbraith Laboratories, Knoxville, TN. TLC was done with EM Reagents' precoated silica gel 60 F-250 sheets. Spots were detected by UV or by spraying with ninhydrin or phosphomolybdic acid. Flash chromatographies were performed according to Still, Kahn, and Mitra[18] and dry-column chromatographies according to Harwood,[19] both using Merck 0.040–0.063-mm silica gel. Freeze–pump–thaw degassing was performed at liquid nitrogen temperature at 5×10^{-6} Torr through three cycles, followed by sealing under vacuum. Cells, degassed via the freeze–thaw method, were constructed as described previously.[20] The pH 7.4 buffered water used in the experiments, which was isotonic with blood serum, was 0.13 M in sodium chloride, 2.1×10^{-3} M in tris(hydroxymethyl)aminomethane (Tris), and 1.1×10^{-2} M in tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl). Reagent-grade chemicals were supplied by Aldrich Chemical, Milwaukee, WI, or Sigma Chemical, St. Louis, MO. All solvents were reagent spectranalyzed grade, or spectrograde, from J. T. Baker Chemical, Phillipsburg, NJ, or Fisher Scientific, Fair Lawn, NJ.

Condensation of 2-Amino-2-methyl-1,3-propanediol with Ethyl Pyruvate: 5,6-Dihydro-3,5-dimethyl-5-(hydroxymethyl)-2*H*-1,4-oxazin-2-one (3A) and Its Ring Tautomer 3B. 2-Amino-2-methyl-1,3-propanediol (**1**; 10.5 g, 0.10 mol) and ethyl pyruvate (**2**; 11.6 g, 0.10 mol) were dissolved in 200 mL of 1-butanol. The solution was refluxed for 45 min under a nitrogen atmosphere in a flask equipped with a Dean–Stark trap to remove water and ethanol. The distillate (50 mL) was collected in the trap. After cooling to ambient temperature, the solvent was rotary evaporated and the residue vacuum distilled. A mixture of oxazinone **3A** and its cyclic tautomer **3B** (8.2 g total) was collected as a very viscous, colorless oil, bp 120–135 °C (0.4 Torr). A second fraction of 1.9 g was collected at 135–155 °C and subsequently purified by dry-column chromatography using ethyl acetate as eluent to give an additional 0.90 g of product (total yield 58%). TLC gave only one spot [R_f 0.5 (ethyl acetate), 0.8 (acetonitrile), 0.3 (5% methanol in dichloromethane)]. GLC gave only one peak (A, 160 °C, R_t = 4 min; B, 160 °C, R_t = 6 min). Capillary GLC gave only one peak at 150 °C, R_t = 3.5 min. HPLC chromatography using 75/25 acetonitrile–water, at a flow rate of 2 mL/min, gave only one peak with R_t = 3 min. An attempt to resolve the mixture of **3A** and **3B** by flash chromatography, using 5% methanol in dichloromethane, failed. The mixture of **3A** and **3B** showed the following spectral absorptions: IR (neat) 3350, 2930, 1730, 1640, 1460, 1485, 1235, 1120, 1020 cm^{-1}; UV, see following experiments; ^1H NMR (acetone-d_6) (**3A**) 1.16 (s, 3 H), 2.12 (s, 3 H), 3.48 (d, J = 11, 1 H), 3.56 (d, J = 11, 1 H), 4.27 (d, J = 12, 1 H), 4.45 (d, J = 12, 1 H), (**3B**) 1.31 (s, 3 H), 1.49 (s, 3 H), 3.40 (dd, J = 8, 2.5, 1 H), 4.01 (d, J = 8, 1 H), 4.16 (dd, J = 10, 2.5,

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1 H), 4.22 (dd, J = 10, 2, 1 H; 2-Hz coupling disappeared upon addition of D_2O), **3A/3B** = 1.2; ^1H NMR ($CDCl_3$) (**3A**) 1.24 (s, 3 H), 2.29 (s, 3 H), 3.59 (d, J = 11, 1 H), 3.68 (d, J = 11, 1 H), 4.26 (d, J = 12, 1 H), 4.49 (d, J = 12, 1 H), (**3B**) 1.35 (s, 3 H), 1.65 (s, 3 H), 3.46 (dd, J = 8, 2, 1 H), 4.08 (d, J = 8, 1 H), 4.17 (d, J = 10, 1 H), 4.27 (dd, J = 10, 2, 1 H), **3A/3B** = 1; ^1H NMR (CD_3CN) (**3A**) 1.14 (s, 3 H), 2.16 (s, 3 H), 3.45 (d, J = 12, 1 H), 3.50 (d, J = 12, 1 H), 4.24 (d, J = 12, 1 H), 4.41 (d, J = 12, 1 H), (**3B**) 1.27 (s, 3 H), 1.51 (s, 3 H), 3.39 (dd, J = 7, 2.5, 1 H), 4.01 (d, J = 7, 1 H), 4.17 (br, 2 H), **3A/3B** = 1; ^1H NMR (benzene-d_6) (**3A**) 0.90 (s, 3 H), 2.12 (s, 3 H), (**3B**) 0.52 (s, 3 H), 1.58 (s, 3 H), **3A/3B** = 1; ^1H NMR (pyridine-d_5) (**3A**) 1.32 (s, 3 H), 2.30 (s, 3 H), (**3B**) 1.21 (s, 3 H), 1.81 (s, 3 H), **3A/3B** = 3; ^1H NMR (Me_2SO-d_6) (**3A**) 1.07 (s, 3 H), 2.09 (s, 3 H), (**3B**) 1.20 (s, 3 H), 1.43 (s, 3 H), **3A/3B** = 5; ^1H NMR (2-propanol-d_8) (**3A**) 1.20 (s, 3 H), 2.20 (s, 3 H), (**3B**) 1.33 (s, 3 H), 1.57 (s, 3 H), **3A/3B** = 4; ^1H NMR (methanol) (**3A**) 1.19 (s, 3 H), 2.21 (s, 3 H), (**3B**) 1.30 (s, 3 H), 1.56 (s, 3 H), **3A/3B** = 4; ^1H NMR (D_2O) (**3A**) 1.20 (s, 3 H), 2.24 (s, 3 H), (**3B**) 1.34 (s, 3 H), 1.61 (s, 3 H), **3A/3B** = 4.5; ^1H NMR (trifluoroacetic acid) (**3A**) 1.70 (s, 3 H), 2.88 (s, 3 H), **3A/3B** > 25; EI mass spectrum (70 eV), m/z (relative intensity) 157 M^+ (8), 98 (22), 82 (25), 72 (82), 57 (base). Anal. Calcd for $C_7H_{11}NO_3$: C, 53.49; H, 7.05; N, 8.91. Found: C, 53.47; H, 7.03; N, 9.20.

Studies on the 3A ⇔ 3B Equilibration and the Molecular Extinction Coefficient of 3A at Its UV Maximum. (a) In Acetonitrile-d_3. A 0.100 M solution of **3A + 3B** was prepared and kept at 25 °C. The ^1H NMR spectrum of the solution was monitored vs. time. Comparison of the relative peak intensities of the methyl singlets (1.14 and 2.16 ppm for **3A**, 1.27 and 1.51 ppm for **3B**) gave a **3A/3B** ratio of 65/35 at time zero, which decreased slowly to 1/1 in about 20 h. In the meantime, aliquots of the solution were diluted with acetonitrile, and UV spectra were taken. The apparent ϵ at the maximum (323 nm) changed from an initial 73 to a final value of 59. After equilibration, both NMR and UV remained unchanged for at least several days. Calculated ϵ_{323} for **3A** was 113. UV monitoring of an 8.7×10^{-3} M solution of the same batch showed a slower equilibration, with an initial ϵ of 73.3 to a final value of 55 in 35 h. UV monitoring of a 5.1×10^{-3} M solution of a different batch at 25 °C showed a faster equilibration, with an initial ϵ of 73 to a final value of 54 in 6 h. A 0.70 M solution of another batch showed, by ^1H NMR spectroscopy, a **3A/3B** ratio equal to 1 soon after dilution.

(b) In Me_2SO. A 6.0×10^{-2} M solution of a mixture of **3A + 3B** gave an initial ϵ of 80 at the maximum (323 nm), which remained stable for at least 10 min. After 3 days, ϵ was 95 and did not change for at least 2 days more. An NMR spectrum of the same batch in Me_2SO-d_6 gave a **3A/3B** ratio of ca. 5, by comparison of the intensities of the methyl singlets (1.07 and 2.09 ppm for **3A**, 1.20 and 1.49 ppm for **3B**). Calculated ϵ for **3A** was ca. 110.

(c) In Methanol. A 9.0×10^{-2} M solution of **3A + 3B** at 20 °C gave an initial ϵ of 77 at the maximum (318 nm). The absorbance reached its maximum value (ϵ 83) after 30 min and then decreased slowly, probably due to solvolysis. The extinction coefficient was 75 after 18 h and 57 after 4 days more. An ^1H NMR spectrum taken 15 min after dissolution gave a **3A/3B** ratio of ca. 4, by comparison of the intensities of the methyl singlets (1.19 and 2.21 ppm for **3A**, 1.30 and 1.56 ppm for **3B**). Calculated ϵ for **3A** was ca. 110.

(d) In Water. A 5.0×10^{-3} M solution of **3A + 3B** gave an ϵ of 86 at its maximum (311 nm) 1 min after dissolution. The value of ϵ increased to a maximum of 90 after ca. 3 min. Subsequently, the absorbance decreased slowly due to hydrolysis (see below). The maximum at 311 nm disappeared in ca. 3 days; a residual absorption (ϵ 10) remained. A UV spectrum of **3A + 3B** in pH 7.4 water at 25 °C showed similar behavior. The ϵ value measured soon after dissolution was 80; it rose to 91 in 1 min and then decreased slowly to 10 in ca. 20 h. The ^1H NMR spectrum of a 4.5×10^{-2} M solution of **3A + 3B** in D_2O was monitored vs. time. The relative amounts of **3A** and **3B** were determined by comparison of the relative intensities of the methyl singlets (1.20 and 2.24 ppm for **3A**; 1.34 and 1.61 ppm for **3B**). Recording of spectra began 8 min after dissolution and was continued until **3A** and **3B** had disappeared completely, which occurred in ca. 3 days; hydrolysis had occurred, as reported below. The **3A/3B** ratio was 4.5 throughout the experiment. Calculated ϵ for **3A** was ca. 110.

(e) In 2-Propanol. A freshly prepared 1.4×10^{-5} M solution of **3A + 3B** gave an ϵ value of 75 at its maximum (323 nm), which increased to 95 in 10 h. No further changes were observed in 7 days.

(f) In Ethanol. A freshly made 1.2×10^{-2} M solution of **3A + 3B** gave an absorption maximum at 320 nm with ϵ 71.

Hydrolysis of 5,6-Dihydro-3,5-dimethyl-5-(hydroxymethyl)-2*H*-1,4-oxazin-2-one. The decomposition of **3A + 3B** in aqueous solution reported in the preceding paragraph was investigated by 200-MHz ^1H NMR spectroscopy as follows: (a) A mixture of **3A** and **3B** (5.0 mg) was dissolved in 0.70 mL of D_2O to give a 4.5×10^{-2} M solution. Spectra were recorded after 0.13, 0.5, 3.5, 6.5, 27.5, 70, and 95 h and after 51 days. During this time, the sample was kept at ambient temperature. The two pairs of methyl singlets of **3A** and **3B** (**3A** 1.20, 2.24 ppm; **3B** 1.34, 1.61 ppm), whose ratio was 4.5 throughout the experiment, slowly decreased in intensity and virtually disappeared in ca. 70 h. Five singlets at 1.16, 1.26, 1.55, 1.58, and 2.36 ppm, in a 1.0/1.8/1.0/0.6/1.2 ratio, appeared instead. In the meantime, complete disappearance of any absorption in the CH_2OCO region at 4.1–4.6 ppm occurred, and an increased absorption in the CH_2OR region at 3.4–4.1 ppm appeared. No meaningful changes were observed in the spectrum for at least 48 days, except for a slow decrease in the intensity of the 2.36 ppm peak, which eventually disappeared. The pH of the final solution was ca. 6.5. (b) In a separate experiment, the spectrum of an equivalent amount of pyruvic acid and 2-amino-2-methyl-1,3-propanediol (**1**), 5.7×10^{-2} M in both species in D_2O, was monitored. The species gave separate signals as follows: (1) 1.18 (s, 3 H), 3.55 (d, J = 11, 2 H), 3.60 (d, J = 11, 2 H), (pyruvic acid) 2.36 ppm (s, 3 H). The 2.36 ppm singlet slowly faded to 10% of its initial intensity in 24 h. No other changes in the spectrum were observed for at least 50 days. (c) A control experiment showed that the rate of D/H exchange of the CH_3CO protons of pyruvic acid in D_2O is a function of pH, occurring to an extent of 25% in 24 h at pH 7. In the pH 4–7 range the rate of exchange was faster at higher pH and slower at lower pH with virtually no exchange at pH 4. (d) The chemical shifts of the peaks of **1** depend on the pH. In fact, pure **1** at 5.7×10^{-2} M concentration in D_2O gave the methyl singlet at 0.90 ppm. Minor shifts were also observed in the 3.6 ppm region of the spectrum. (e) On the 51st day after the beginning of the investigation, the solution from experiment a was mixed with a portion of the solution from experiment b and the ^1H NMR spectrum recorded. This spectrum was essentially identical with the sum of the spectra of each of the two solutions, with some slight changes in chemical shifts, presumably due to a slight change in pH. Comparison with the previous spectra led to the following analysis of the spectrum arising from the products of hydrolysis of **3A + 3B**: (1) 1.26 (s, 3 H), 3.59 (d, J = 12, 2 H), 3.64 (d, J = 12, 2 H), (pyruvic acid) 2.36 (s, 3 H), (**9A**, major isomer) 1.16 (s, 3 H), 1.55 (s, 3 H), (**9B**, minor isomer) 1.26 (s, 3 H), 1.58 ppm (s, 3 H). The CH_2OH portion of **9A** and **9B** gave a complex pattern in the 3.4–4.0 ppm region, partially overlapped with the signals arising from **1**. The approximate ratio for **1**/pyruvic acid/**9A**/**9B** was 1/1/0.7/0.3. An attempt to isolate **9A** and **9B** from the reaction mixture obtained from a larger scale experiment (1 g of **3A + 3B** in 5 mL of water) was unsuccessful. Rotary evaporation of the water at 40 °C (0.5 Torr) gave a viscous residue with a complex NMR spectrum virtually identical with that reported above. However, either GLC (A, 160 °C, major peak with R_t = 4 min) or vacuum distillation (0.1 Torr) of the residue gave a mixture of **3A + 3B**, as shown by the ^1H NMR spectra.

Derivatives of 3A. (a) Trimethylsilyl Ether 5. To 85 mg (5.4×10^{-1} mmol) of oxazinone **3A + 3B** in 1 mL of acetonitrile-d_3 were added 38 μL (33 mg, 3.0×10^{-1} mmol) of chlorotrimethylsilane and 158 μL (153 mg, 5.9×10^{-1} mmol) of bis(trimethylsilyl)trifluoroacetamide, with exclusion of moisture. The NMR spectrum, taken after 10 min, showed only two methyl singlets, downfield from the $SiMe_3$ signal, at 1.2 and 2.2 ppm. GLC (B, 135 °C) gave only one peak with R_t = 6 min. A sample of pure **5** obtained by preparative GLC showed the following spectral properties: ^1H NMR (CD_3CN) 0.07 (s, 9 H), 1.13 (s, 3 H), 2.15 (s, 3 H), 3.52 (d, J = 9, 1 H), 3.62 (d, J = 9, 1 H), 4.25 (d, J = 11, 1 H), 4.37 ppm (d, J = 11, 1 H); IR (neat) 2960, 1745, 1645,

1255, 1105, 875, 847, 755 cm^{-1}; UV (acetonitrile) λ_{max} 322 nm (ϵ 126); mass spectrum (positive-ion FAB, glycerol matrix) $(M + 1)^+$ 230. Anal. Calcd for $C_{10}H_{19}NO_3Si$: C, 52.37; H, 8.35; N, 6.11. Found: C, 52.28; H, 8.40; N, 6.12.

(b) Acetate of 3A (6). To a solution of 330 mg (2.1 mmol) of oxazinone **3A + 3B** in 3 mL of acetonitrile-d_3 were added 0.25 mL of dry pyridine and 400 μL (430 mg, 4.2 mmol) of acetic anhydride. After several hours, the NMR spectrum showed that the peaks due to the starting material had been quantitatively replaced by the peaks expected for the acetate. GLC (B, 160 °C) gave only one peak with R_t = 4.5 min. Pure **6** obtained by preparative GLC or vacuum distillation [bp 150 °C (0.3 Torr)] gave the following spectral absorptions: ^1H NMR (CD_3CN) 1.18 (s, 3 H), 1.95 (s, 3 H), 2.15 (s, 3 H), 4.05 (d, J = 11, 1 H), 4.18 (d, J = 11, 1 H), 4.28 (d, J = 12, 1 H), 4.43 ppm (d, J = 12, 1 H); IR (neat) 2950, 1735, 1640, 1375, 1235, 1120, 1050, 905 cm^{-1}; UV (acetonitrile)λ_{max} 322 nm (ϵ 107); EI mass spectrum (70 eV) m/z (relative intensity) 199, M^+ (3), 157 (2), 127 (20), 114 (24), 99 (28), 98 (24), 82 (34), 72 (base). Anal. Calcd for $C_9H_{13}NO_4$: C, 54.26; H, 6.58; N, 7.03. Found: C, 54.16; H, 6.61; N, 7.00.

(c) Chloroacetate of 3A (7). Compound **7** was obtained in quantitative yield by using the same procedure as described for the preparation of **6**, with the following exceptions: chloroacetic anhydride replaced acetic anhydride, and complete reaction occurred after 2 h at ambient temperature. A pure sample obtained by preparative GLC (B, 180 °C, R_t = 6 min) had the following spectral properties: ^1H NMR ($DCCl_3$) 1.31 (s, 3 H), 2.28 (s, 3 H), 4.08 (s, 2 H), 4.17 (d, J = 12, 1 H), 4.43 (d, J = 12, 1 H), 4.29 (d, J = 12, 1 H), 4.36 ppm (d, J = 12, 1 H). Anal. Calcd for $C_9H_{12}ClNO_4$: C, 46.26; H, 5.18; N, 5.99; Cl, 14.69. Found: C, 45.92; H, 5.18; N, 5.77; Cl, 14.3.

Catalytic Hydrogenation of 5,6-Dihydro-3,5-dimethyl-5-(hydroxymethyl)-2*H*-1,4-oxazin-2-one: 3,5-Dimethyl-5-(hydroxymethyl)-2-oxomorpholine (4A + 4B). A solution of **3A + 3B** (0.95 g, 6.05 mmol) in 25 mL of dry ethyl acetate was hydrogenated in the presence of 5% palladium on carbon (0.24 g) under 1 atm at ambient temperature. After 1 mol of hydrogen had been absorbed (24 h), the solvent was rotary evaporated from the filtered solution, and the white residue was analyzed by GLC (A, 160 °C). Less than 5% (peak area) of starting material (R_t = 4 min) was detected. One broad peak (R_t = 5.5 min) was present, accounting for ca. 90% of the total area exclusive of the solvent peak. NMR showed only traces of impurities along with peaks due to a 1/1 mixture of **4A** and **4B** (see below). A 1/1 mixture of pure **4A** and **4B** was obtained by preparative GLC (A, 180 °C, R_t = 4 min) or vacuum distillation [bp 190 °C (0.3 Torr)]. The purified mixture of stereosiomers had the following physical and spectroscopic properties: mp 98–102 °C; IR (KBr) 3225, 2946, 1735, 1440, 1370, 1280, 1165, 1125, 1095, 1055, 850 cm^{-1}; ^1H NMR (CD_3CN) 1.05 (s, 3 H) 1.09 (s, 3 H), 1.22 (d, J = 6, 3 H + 3 H), 3.23 (d, J = 10, 1 H), 3.29 (d, J = 10, 1 H), 3.34 (s, 2 H), 3.61 (q, J = 7, 1 H), 3.72 (q, J = 6, 1 H), 4.04 (d, J = 12, 1 H), 4.06 (d, J = 12, 1 H), 4.25 (d, J = 12, 1 H), 4.26 ppm (d, J = 12, 1 H); upon addition of 1 drop of D_2O the two superimposed doublets at 1.22 ppm shifted slightly and split into one doublet at 1.23 ppm (J = 6 Hz) and one doublet at 1.24 ppm (J = 8 Hz). A freshly made solution of **4A + 4B** in D_2O gave the following: ^1H NMR 1.12 (s, 3 H), 1.16 (s, 3 H), 1.31 (d, J = 6, 3 H + 3 H), 3.46 (s, 2 H), 3.58 (s, 2 H), 4.26 (d, J = 12, 1 H), 4.28 (d, J = 12, 1 H), 4.41 ppm (d, J = 12, 1 H + 1 H). (Within a few hours of its preparation, the solution of **4A + 4B** gave an ^1H NMR spectrum typical of **10**, described below.) Anal. Calcd for $C_7H_{13}NO_3$: C, 52.82; H, 8.23; N, 8.80. Found: C, 52.25; H, 8.39; N, 8.74. As the elemental analysis of **4** suggests, **4A** and **4B** are very sensitive to humidity. In fact, upon exposure to air they changed into crystalline *d,l*-*N*-[bis(hydroxymethyl)ethyl]alanine (**10**), which was not soluble in acetonitrile or methanol but very soluble in water. It had the following physical and spectroscopic properties: mp 194–6 °C dec; ^1H NMR spectrum (D_2O) 1.23 (s, 3 H), 1.54 (d, J = 8, 3 H), 3.67 (s, 4 H), 3.83 ppm (q, J = 7, 1 H); IR (KBr) 3150, 2830, 1605, 1560, 1445, 1380, 1120, 1065 cm^{-1}. Anal. Calcd for $C_7H_{15}NO_4$: C, 47.45; H, 8.53; N, 7.90. Found: C, 46.74; H, 8.58; N, 7.71. (The low percent carbon is probably an indication that some hydration of the zwitterionic amino acid occurred.)

Photoreduction of 5,6-Dihydro-3,5-dimethyl-5-(hydroxymethyl)-2*H*-1,4-oxazin-2-one (3A): Bi[3,5-dimethyl-5-(hydroxymethyl)-2-oxomorpholin-3-yl] (DHM-3 Dimer, 8). A procedure similar to that described for the synthesis of TM-3 dimer was employed.[4] Oxazinone **3A + 3B** (6.0 g, 19 mmol) was dissolved in 500 mL of 2-propanol. The solution was irradiated with a 450-W mercury immersion lamp through a Pyrex filter. Throughout the reaction, the photochemical apparatus was immersed in a bath kept at 0 °C, and nitrogen was bubbled through the solution. The disappearance of the oxazinone chromophore was monitored by UV spectroscopy. After 24 h, when the UV maximum at 323 nm had disappeared, the irradiation was interrupted and the solvent quickly rotary evaporated without delay at ambient temperature, first at 15–20 Torr and then at 0.5 Torr. The residue was dissolved in 70 mL of chloroform, and the solution was stored in a freezer at −18 °C. The white precipitate that separated slowly (15 min to several days) was collected by suction filtration, washed thoroughly with cold chloroform, and dried under vacuum to give 3–4 g (50–66% yield) of a white powder (**8**; mixture of diastereoisomers) whose melting point varied from 80–90 to 120–130 °C, depending on the preparation and the time of collection of the precipitate. TLC (9/1 dichloromethane–methanol) showed three to five spots with R_f 0.2–0.4. Traces of oxazinone, if present, appeared with R_f 0.6. Relative size of the spots varied from batch to batch. Attempts to resolve the crude mixture of stereoisomers by flash chromoatography using methanol–dichloromethane mixtures at ambient temperature or methanol–diethyl ether mixtures at −15 °C gave only partial resolution. However, preferential crystallization occurred, at times, in the freezer and yielded mixtures in which the same stereoisomer was the major component. In these cases, further rapid recrystallization from acetonitrile (ca. 6 mL for 0.1 g) gave a virtually pure stereoisomer (**8A**) with the following properties: mp 145–147 °C; TLC R_f 0.25; UV in acetonitrile, featureless above 210 nm; IR (KBr) 3340, 2940, 1710, 1455, 1285, 1220, 1040, 770, 715 cm^{-1}; ^1H NMR (oxygen-free acetonitrile-d_3) 1.01 (s, 6 H), 1.41 (s, 6 H), 2.3 (br s, 2 H), 3.39 (d, J = 11, 2 H), 3.52 (d, J = 11, 2 H), 4.08 (d, J = 12, 2 H), 4.13 ppm (d, J = 12, 2 H); ^1H NMR (oxygen-free Me_2SO-d_6) 0.94 (s, 6 H), 1.31 (s, 6 H), 2.34 (br s, 2 H), 3.23 (dd, J = 12, 5, 2 H), 3.39 (dd, J = 12, 5, 2 H), 4.03 (br s, 2 H), 4.92 ppm (t, J = 5, 2 H); the 5-Hz couplings disappeared upon addition of D_2O; in oxygen-free D_2O, soon after dissolution at 5 °C, 1.07 (s, 6 H), 1.45 (s, 6 H), multiline patterns at 3.40–3.75 and 4.10–4.40 ppm superimposed with many peaks due to rapid formation of other stereoisomers; positive-ion FAB mass spectrum, glycerol matrix, 317 $(M + 1)^+$. Anal. Calcd for $C_{14}H_{24}N_2O_6$: C, 53.15; H, 7.65; N, 8.85. Found: C, 53.00; H, 7.70; N, 8.84. The mixtures of diastereoisomers of **8** from different batches had IR spectra identical with the spectrum of **8A**. NMR spectra of the mixtures taken in oxygen-free acetonitrile-d_3 each showed 11 singlets between 0.99 and 1.62 ppm, with different intensities depending on the batch. After a few days at ambient temperature, all batches gave the same spectrum. The most intense peaks in this area were at 1.01, 1.18, 1.41, and 1.50 ppm, medium intensity peaks at 0.99, 1.03, 1.19, and 1.53 ppm; and least intense peaks at 1.56, 1.59 and 1.62 ppm. Complex overlapping patterns were also visible in the 3.24–3.57 and 3.96–4.55 ppm regions. Similar behavior was observed in oxygen-free Me_2SO-d_6, with the spectrum showing 14 methyl singlets. The most intense occurred at 0.94, 1.11, 1.31, and 1.38 ppm, medium intensity peaks appeared at 0.91, 0.93, 0.96, 1.10, 1.29, 1.37, and 1.41 ppm, and the least intense peaks occurred at 1.43, 1.46, and 1.48 ppm. The NMR spectra, taken in oxygen-free D_2O (sample degassed at 0 °C (15 Torr) and sealed under nitrogen) and after a fast equilibration (1 h at ambient temperature), each gave 12 methyl singlets the most intense occurred at 1.05, 1.07, 1.21, 1.45, 1.55, and 1.57 ppm; those of medium intensity at 1.23 and 1.45 ppm; and those of least intensity at 1.26, 1.54, 1.61 and 1.63 ppm. After that time, peaks arising from the products of disproportionation (see below) and/or their hydrolysis products (**3A 1, 2, 9A, 9B, 10**) appeared in the NMR spectrum, slowly replacing the peaks of **8**. A freeze–pump–thaw degassed solution of **8** in methanol at ambient temperature gave a very weak 24-line EPR signal typical of morpholinyl radicals of similar structure.[9,21] The signal became stronger at 60 °C and had a g value of 2.004 and the following hyperfine splitting

(21) Gaudiano, G.; Sweeney, K.; Haltiwanger, R. C.; Koch, T. H. *J. Am. Chem. Soc.* **1984**, *106*, 7628.

constants: a_{CH_3} = 10.15, a_N = 6.4, a_{NH} = 6.4 G. Similar, but weaker, spectra were obtained in chloroform, acetonitrile, and 2-propanol, respectively, at 50–60 °C and even in the presence of air. However, in these solvents and in the presence of oxygen at room temperature, a fairly intense three-line spectrum was obtained, with g = 2.006 and a_N = 14.5 G; a very similar spectrum was also obtained for TM-3 radical.[14] Anal. Calcd for $C_{14}H_{24}N_2O_6$ (8): C, 53.15, H, 7.65; N, 8.85. Found: C, 53.03; H, 7.67; N, 8.84.

Kinetics of Bond Homolysis of 8A. UV–visible experiments were performed with 1-cm quartz cells. When freeze–pump–thaw degassing procedure was necessary, a two-compartment cell was used as described earlier.[20] Rate constants were obtained by linear least-squares fitting of ln ($A - A_\infty$) vs. time. The results are reported in Table I.

(A) In Methanol. Diastereoisomer **8A** (1.03 mg, 3.26×10^{-3} mmol) was dissolved in 50 mL of dichloromethane with stirring; 1.0 mL (6.5×10^{-5} mmol) of the solution was introduced via syringe into the cuvette compartment of the cell, and the solvent was evaporated with a stream of nitrogen. 2,2-Diphenyl-1-picrylhydrazyl hydrate (DPPH; 1.63 mg, 4.14×10^{-3} mmol) was dissolved in 50 mL of methanol; 2.5 mL (2.07×10^{-4} mmol) was added to the degassing compartment of the cell. After freeze–thaw degassing, the apparatus was sealed under vacuum and transferred to the thermostated cell holder of the spectrometer, maintained at 25.0 ± 0.1 °C, and held for 10 min at 90° to its normal position. Diastereoisomer **8A** and the DPPH solutions were then rapidly mixed by shaking the cell holder for 10 s. The average absorbance of the solution at 514–516 nm (maximum of DPPH) was automatically recorded as a function of time over a period of 1 h (6 half-lives).

(B) In Buffered Methanol. DHM-3 dimer **8A** (2.18 mg, 6.9×10^{-3} mmol) was dissolved in 50 mL of acetonitrile; 0.80 mL (1.1×10^{-4} mmol) of the solution was introduced via syringe into the cuvette compartment of the cell, and the solvent was evaporated with a stream of nitrogen. Methylviologen hydrate (3.36 mg, 1.3×10^{-2} mmol) was dissolved in 25 mL of a methanolic solution of 1.5 mg (1.25×10^{-2} mmol) of Tris and 1.98 mg (1.25×10^{-2} mmol) of Tris-HCl; 2.1 mL (1.1×10^{-3} mmol of methylviologen) of this solution was introduced into the degassing compartment of the cell. After freeze–thaw degassing, the cell was sealed under vacuum, temperature-equilibrated at 25.0 ± 0.1 °C, and treated as described under A. The average absorbance at the 604–608-nm maximum of reduced methylviologen was automatically recorded over a period of 1 h (6 half-lives).

(C) In Acetonitrile. (a) Using DPPH as Trapping Agent. The same procedure was followed as described under A, using 1.54×10^{-2} mg (4.9×10^{-5} mmol) of **8A** and 5.58×10^{-2} mg (1.4×10^{-4} mmol) of DPPH in 3.0 mL of acetonitrile. Average absorbance at 518–520 nm was automatically recorded over a period of 40 h (3 half-lives).

(b) Using Ferriin as Trapping Agent. A 2.5×10^{-2} M solution of ferrin was prepared by dissolving 48.5 mg (0.12 mmol) of ferric nitrate nonahydrate and 72 mg (0.36 mmol) of 1,10-phenanthroline in 10 mL of water. A portion of this solution (100 μL) was added to 24.9 mL of acetonitrile to make a 1.0×10^{-4} M ferriin solution. No absorption maximum due to any ferroin at 508 nm was observed. Only a residual absorbance of 0.016 was found as tail absorption from the ferriin. After 2 days in the dark, the solution showed no change in A_{508}. After 2 days more, A_{508} was 0.020. If the solution was kept in the presence of light, A_{508} became 0.073 in 2 days. A 2.0 mL (2.0×10^{-4} mmol) quantity of the fresh 10^{-4} M solution of ferriin was introduced into a UV cell provided with screw cap and silicone rubber septum. The cell was kept for 10 min at 25.2 ± 0.1 °C in a thermostated cell holder, which replaced the standard cuvette holder of the spectrometer. In the meanwhile, a 3.2×10^{-3} M solution of **8A** was prepared by dissolving 0.30 mg (9.5×10^{-4} mmol) of **8A** in 0.30 mL of acetonitrile; 20 μL (6.3×10^{-5} mmol) of this solution was introduced via syringe through the septum in the cuvette. The average absorbance at 506–510 nm was automatically recorded as a function of time over a period of 56 h (4 half-lives).

(D) In Water. A portion (80 μL) of the 2.5×10^{-2} M stock solution of ferriin prepared for experiment C, part b, was diluted to 25 mL with redistilled water. The resulting 8.0×10^{-5} M solution, with A_{508} = 0.03, showed A_{508} = 0.04 after 1 day. The kinetic experiment was run as described under C, part b, except that 2.0 mL (1.6×10^{-4} mmol) of the 8.0×10^{-5} M solution of ferriin and 1.84×10^{-2} mg (5.8×10^{-5} mmol) of **8A**, dissolved in 20 μL of acetonitrile, were used.

(E) In pH 7.4 Buffered Water. (a) Using Ferriin as Trapping Agent. The experiment was run as described under D, except that water, pH 7.4, was used to prepare the 8.0×10^{-5} M ferriin solution.

(b) Using Potassium Ferricyanide as Trapping Agent. Stereoisomer **8A** (0.11 mg, 3.5×10^{-4} mmol) dissolved in 0.50 mL of acetonitrile was introduced into the cuvette compartment of the cell and the solvent evaporated as described under B. Potassium ferricyanide (4.75 mg, 1.44×10^{-2} mmol) was dissolved in 25 mL of pH 7.4 buffer. The solution showed A = 0.587 at the 418-nm maximum of the ferricyanide ion. The kinetic experiment was run with 2.0 mL (1.16×10^{-3} mmol) of this solution after freeze–thaw degassing as described under A. The average absorbance at 416–420 nm was automatically recorded as a function of time at 25.0 ± 0.1 °C over a period of 0.5 h (19 half-lives). An experiment run in the presence of oxygen gave different results, with a poor fit to the first-order rate law because of an unsteady A_∞. Apparently, back-oxidation of ferrocyanide to ferricyanide had occurred. A separate control experiment showed that oxidation of ferrocyanide by hydrogen peroxide, a product of air oxidation of **8A**, is a much slower reaction than oxidation of **8A** by ferricyanide.

Activation Parameters for Bond Homolysis of 8A in pH 7.4 Buffered Water. Rate constants were measured as described under E, part a, of the preceding experiment at 5.1, 20.2, 25.0, and 30.0 ± 0.1 °C, using the same stock solutions of **8A** and ferriin for the experiments at all four temperatures. The rate constants are reported in the first footnote to Table II. The measurement at 20 °C was also performed with double the ferriin concentration, and a k of $(2.9 \pm 0.1) \times 10^{-3}$ s^{-1} was obtained. Calculations were based on reactions that proceeded to approximately 80% completion. The Arrhenius activation energy (E_a) and the frequency factor (A) were obtained from the least-squares slope and intercept of the Arrhenius plots (see Table II). Correlation coefficient was 1.00. The enthalpy of activation was calculated as $E_a - RT$, where R is the gas constant and T the average temperature of the measurement (280 K). The entropy of activation was calculated as $R \ln (ANh/eRT)$, where N is Avogadro's number, h is Planck's constant, and e = 2.718.

Kinetics of Bond Homolysis of 8 (Mixture of Stereoisomers). The experiment was run as follows, using a mixture of stereoisomers of **8** previously equilibrated in acetonitrile. DHM-3 dimer **8** (0.50 g, 1.6×10^{-2} mol) was mixed with 30 mL of HPLC-grade acetonitrile in a tube provided with attachment for a vacuum line. After freeze–thaw degassing (three cycles), the tube was sealed under vacuum. The tube was warmed in a steam bath to dissolve **8** (5 min) and then kept at 30 °C for 44 h. At this time, the tube was opened and the solvent rotary evaporated at ambient temperature at 0.5 Torr. The UV spectrum of a solution of the white solid residue showed that only 3% of the starting material had changed into oxazinone. The NMR spectrum showed the same features seen in the spectrum obtained by equilibration of any batch of **8** or **8A** in acetonitrile-d_3 solution, as reported above. The amount of **8** used for the kinetic experiment was 0.020 mg (6.3×10^{-5} mmol) dissolved in 20 μL of acetonitrile. The experiment was run as described under E, part a, of the experiment concerning the kinetics of bond homolysis of **8A**. The ferriin solution was prepared by dissolving 14.9 mg (7.5×10^{-2} mmol) of 1,10-phenanthroline and 10.1 mg (2.5×10^{-2} mmol) of ferric nitrate nonahydrate in 25 mL of pH 7.4 buffered water; 2.5 mL of the solution was then diluted to 25 mL with the same buffer to obtain a 1.0×10^{-4} M solution of ferriin. A portion of this solution (2.0 mL) was used for the experiment, which was run under the same conditions as described under C, part b, of the related experiment focusing on **8A**. The absorbance data were automatically recorded over a period of 1 h. Half-lives were calculated by comparison with the absorbance measured after 2 h, which was taken as A_∞. The increase in absorbance between 1 and 2 h was only 0.6% of total absorbance. The first, second, third, and fifth half-lives were as follows: 61, 70, 110, and 462 s, respectively.

Disproportionation of Bi[3,5-dimethyl-5-(hydroxymethyl)-2-oxomorpholin-3-yl] (8). A solution of 15 mg ($4.7 \times$

10^{-2} mmol) of **8** in 0.70 mL of acetonitrile-d_3 in an NMR tube provided with attachment for a vacuum line was freeze–thaw degassed (two cycles) and sealed under vacuum. The tube was kept in a thermostated bath at 70 °C, and NMR spectra were recorded at different intervals of time. Slowly, the peaks corresponding to the original mixture of diastereoisomers of **8** decreased in intensity, while new peaks arose corresponding to the oxazinone **3A**, its tautomer **3B**, and a 1/1 mixture of morpholines **4A** and **4B**. The disproportionation was complete in about 50 h. All, and only, the peaks corresponding to the products of disproportionation were present in the final spectrum.

Oxidation of Bi[3,5-dimethyl-5-(hydroxymethyl)-2-oxomorpholin-3-yl] (8) by Molecular Oxygen. (A) UV Experiments. (a) In Acetonitrile. Pure stereoisomer **8A** (6.1 mg, 1.93 × 10^{-2} mmol) was dissolved in 10 mL of acetonitrile saturated with air (oxygen molar concentration was ca. 1.5 × 10^{-3});[22] 3.0 mL of the solution was introduced into a 1-cm quartz cell kept in a thermostated cell holder at 25.1 ± 0.1 °C. The cell was stoppered with a screw cap provided with a silicone rubber liner. The absorbance at the 323-nm maximum of **3A** was monitored for a period of 40 h (3 half-lives). The cell was opened periodically to let air in and shaken to provide enough oxygen for full oxidation. The absorbance of the solution slowly increased to maximum value (0.21) after 5 days, an indication of quantitative formation of **3A** + **3B** with an apparent ϵ of 55. In the meantime, the bulk solution, which also was kept at 25.0 °C in a thermostated bath, was analyzed periodically for hydrogen peroxide by a slight modification of the method described by Wolfe:[23] 5–2.0-μL aliquots of the solution were mixed with 1.0 mL of 0.08 M titanium tetrachloride in 6 N hydrochloric acid and brought to a total volume of 3.0 mL with pure acetonitrile. As a yellow color developed, due to the formation of a $TiCl_4$–H_2O_2 complex, the absorbance at its 420-nm maximum was measured. From comparison with the absorbance of standard solutions of the hydrogen peroxide–titanium tetrachloride complex (ϵ 800), the amount of hydrogen peroxide formed in moles per mole of **8** was found to be 13% after 2.8 h, 23% after 5.9 h, 58% after 30.5 h, 68% after 48 h, and 25% after 144 h. In a similar experiment done with a mixture of diastereoisomers of **8**, 70% of the theoretical amount of hydrogen peroxide was obtained in ca. 27 h at ambient temperature.

(b) In Water. DHM-3 dimer **8** (6.6 mg, 2.1 × 10^{-2} mmol) was dissolved in 10 mL of water. The solution was kept at ambient temperature in the presence of air. The UV spectrum of the solution showed the rise of an absorption with a maximum of 0.43 at 310 nm, corresponding to **3A**. The maximum, reached after 86 min, subsequently decreased in intensity and disappeared in 64 h. Analyses of hydrogen peroxide were performed as follows: 1.0-mL aliquots of the solution were mixed with 2 mL of the titanium tetrachloride solution described above, and water was added to make a volume of 5.0 mL. Reading of the absorbance at 412 nm gave the concentration of hydrogen peroxide by comparison with standard solutions of hydrogen peroxide–titanium tetrachloride complex (ϵ 670). The amount of hydrogen peroxide formed was found to be 44% after 23 min, 60% after 44 min, 55% after 86 min, and 1.5% after 64 h. The absorbance at 412 nm did not change appreciably for at least 30 min, indicating that the acidic solution of titanium tetrachloride quenched the oxidation of **8**. In fact, no yellow color developed when **8** was dissolved directly in the titanium chloride solution. In a similar experiment run with **8A** in pH 7.4 buffered water at 25 °C in the presence of excess of oxygen provided by frequent shaking, 80% of the theoretical amount of hydrogen peroxide was found. The absorbance at 310 nm due to **3A** reached its maximum after 10 min.

(c) In Methanol or 2-Propanol. Solutions of **8** in methanol or 2-propanol in the presence of air showed a fairly rapid rise of an absorption band with maxima at 318 and 323 nm, respectively.

(B) NMR Experiments. (a) In Acetonitrile-d_3. DHM-3 dimer **8** (5.5 mg, 1.7 × 10^{-2} mmol) was dissolved in 1.0 mL of acetonitrile-d_3 in an NMR tube. A small amount of cyclohexane was added as internal reference. The tube was capped and kept in a bath thermostated at 25 °C. Occasionally, the cap was temporarily removed to admit air and the solution shaken to provide constant presence of oxygen. The oxidation was monitored via the changes in the NMR spectra. In 2–3 days, a virtually quantitative oxidation to oxazinone **3A** + **3B** was observed. A 100-μL sample removed from the solution after ca. 70% of the oxazinone had been formed was analyzed for hydrogen peroxide content. The analysis showed 73% hydrogen peroxide formation (1.04 mol/mol of oxidized **8**).

(b) In D_2O. An ^1H NMR spectrum of **8** in D_2O recorded soon after dissolution showed the typical complex pattern arising from the several diastereoisomers of **8**. Upon shaking in the presence of air, a fast change occurred; within 1 h the peaks of **8** disappeared completely and were replaced by the peaks of **3A** + **3B** (see above). A subsequent change in the spectrum occurred, whereby the peaks of oxazinone **3A** + **3B** were gradually replaced by the peaks arising from the products of hydrolysis of **3A** + **3B**, as reported above. A small peak at 1.91 ppm, due to acetic acid, also appeared. Formation of acetic acid was explained by the following separate experiment. A 0.1 M solution of pyruvic acd was prepared by dissolving 26 mg (0.3 mmol) of pyruvic acid in 3.0 mL of deuterium oxide. A small amount of *tert*-butyl alcohol was added as internal reference. The pH (1.8) was adjusted to 4 by addition of a few drops of a 1 M solution of sodium hydroxide in deuterium oxide. The NMR spectrum showed a singlet at 2.36 ppm for the MeCO protons. To 1 mL (0.1 mmol) of the solution was added 25 μL (0.025 mmol) of a 10 M solution of hydrogen peroxide. Gas evolution was observed. A spectrum recorded after a few minutes showed a remarkable decrease of the 2.36-ppm peak and the appearance of a strong peak at 1.96 ppm, whose intensity increased upon addition of a small amount of acetic acid.

(c) In Me_2SO-d_6. An ^1H NMR spectrum of **8** recorded soon after dissolution showed the typical complex pattern arising from the several diastereoisomers of **8**. When oxygen was bubbled through the solution, a change occurred in the spectrum whereby the peaks corresponding to **8** disappeared and were replaced by the peaks corresponding to a 5/1 mixture of **3A** and **3B**. The oxidation was complete in ca. 15 h.

Reaction of Bi[3,5-dimethyl-5-(hydroxymethyl)-2-oxomorpholin-3-yl] (8) with Daunomycin (11). (A) Using 8A in Methanol. Pure stereoisomer **8A** (5.8 mg, 1.8 × 10^{-2} mmol) was dissolved with stirring in 100 mL of dichloromethane in a nitrogen atmosphere. A 1-mL aliquot (1.8 × 10^{-4} mmol) of the solution was transferred via syringe into the cuvette of a two-compartment cell. The solvent was then evaporated with a stream of nitrogen. Daunomycin (**11**) hydrochloride (8.83 mg, 1.56 × 10^{-2} mmol) was dissolved in 100 mL of a methanolic solution of 18.9 mg (0.156 mmol) of Tris and 24.7 mg (0.156 mmol) of Tris-HCl. A 2.5-mL aliquot (3.9 × 10^{-4} mmol of **11**) of this solution was introduced via syringe into the degassing chamber of the cell. After freeze–thaw degassing (four cycles), the cell was sealed under vacuum and placed in the theromstated cell holder at 25.1 ± 0.1 °C for about 15 min prior to mixing the solution with **8A**. After mixing, the average absorbance at 618–620 nm[3] was automatically recorded vs. time over a period of 4000 s. From the absorbance change between time 24 and 300 s, a nonlinear least-squares treatment of the data gave[3] a first-order rate constant for bond homolysis of **8A** equal to 1.1 × 10^{-3} s^{-1} (σ 3 × 10^{-5}); a pseudo-first-order rate constant for protonation of the tautomer **12** of 7-deoxydaunomycinone (**13**) equal to 1.05 × 10^{-2} s^{-1} (σ 5 × 10^{-4}); and an ϵ value for **12** equal to 11 980 (σ 657). When the cell was opened, TLC, using dichloromethane–methanol (9/1 v/v) as eluent, showed only two spots with R_f 0 and 0.7, the former corresponding to unreacted **11** and the lattter to 7-deoxydaunomycinone (**13**), as confirmed by comparison with an authentic sample of **13**.

(B) Using a Mixture of Stereoisomers (8) in Buffered Water. A solution of **8A** (1.38 mg, 4.4 × 10^{-3} mmol) in 0.50 mL of oxygen-free methanol was introduced into the cuvette of a two-compartment cell. The solvent was then quickly evaporated under vacuum, so that the total time **8A** was left in the presence of methanol at ambient temperature was ca. 5 min (ca. 0.5 half-lives). A 2.1 × 10^{-4} M solution of daunomycin (**11**) was prepared by dissolving 11.9 mg (2.1 × 10^{-2} mmol) of its hydrochloride salt in 100 mL of a pH 8.1 aqueous buffer, 10^{-3} M in both

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Tris and Tris-HCl. A 2.0-mL aliquot (4.2×10^{-4} mmol of 11) of the solution was ussed for the experiment, which was run in the same way as reported above, the only difference being the tempeature which was kept at 10.0 ± 0.1 °C. From the absorbance change at 618–620 nm, a linear least-squares treatment of the data, recorded between times 164 and 224 s, gave a pseudo-first-order rate constant for protonation of the tautomer 12 of 7-deoxydaunomycinone equal to 2.8×10^{-2} s^{-1} (σ 3.1×10^{-3}). The initial 164 s allowed for the production of enought DHM-3 to reduce all the daunomycin.

The Luffariellins, Novel Antiinflammatory Sesterterpenes of Chemotaxonomic Importance from the Marine Sponge *Luffariella variabilis*

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Received January 22, 1987

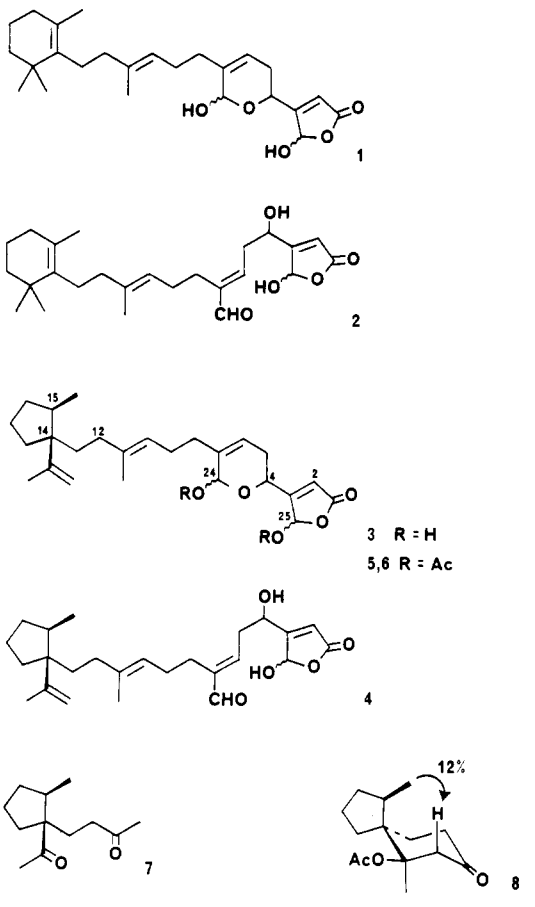
In some specimens of *Luffariella variabilis*, manoalide (1) and secomanoalide (2) are replaced, either totally or partially, by luffariellin A (3) and luffariellin B (4), respectively. The structures of luffariellin A (3) and luffariellin B (4) were elucidated by interpretation of spectral data and chemical transformations. Both luffariellin A and luffariellin B are potent antiinflammatory agents.

In 1980 and 1981, de Silva and Scheuer[1,2] reported the isolation of manoalide (1) and secomanoalide (2) from the Palauan sponge *Luffariella variabilis*. Manoalide (1) was subsequently found to have antiinflammatory properties and to irreversibly inhibit the enzyme phospholipase A_2.[3] In order to provide sufficient manoalide (1) for continued pharmacological evaluation, we made an extensive collection of *L. variabilis* from three locations in Palau. We were surprised to find that a small number of samples contained two new metabolites, luffariellin A (3) and luffariellin B (4) in place of manoalide (1) and secomanoalide (2) (Chart I). Specimens containing the luffariellins could only be distinguished by examining the ^{1}H NMR spectra of crude extracts since all specimens were physically indistinguishable and gave identical TLC patterns.

Luffariellin A (3) was obtained as an oil and was isomeric with manoalide (1). The ultraviolet absorption at 230 nm (ϵ 4800) and infrared bands at 3310, 1780, and 1762 cm^{-1} were assigned to a γ-hydroxybutenolide moiety. The 360-MHz ^{1}H NMR spectra of both manoalide (1) and luffariellin A (3) are highly solvent dependent and are therefore difficult to interpret. In CCl_4 or purified $CDCl_3$ solution, two diastereoisomers were observed and the H-2, H-4, H-6, H-24, and H-25 signals were all doubled, while in slightly acidic $CDCl_3$ solution, a single set of broad signals was obtained. The same is true for the ^{13}C NMR spectra (Table I). Nonetheless, when recorded under identical conditions, the ^{1}H and ^{13}C NMR spectra of 1 and 3 are identical for those signals assigned to the C-1 to C-11 region.

The ^{1}H NMR spectrum of luffariellin A (3) contains two methyl signals at δ 0.70 (d, 3 H, J = 7 Hz) and 1.68 (s, 3 H) and olefinic signals at δ 4.64 (s, 1 H) and 4.82 (s, 1 H) but lacks the signals at δ 0.99 (s, 6 H) and 1.65 (s, 3 H) found in the ^{1}H NMR spectrum of manoalide (I). The ^{13}C NMR spectrum confirmed the presence of a 1,1-disubstituted olefinic bond [δ 148.0 (s), 111.16 (t)] and revealed

Chart I



the presence of a fully substituted carbon atom [δ 55.1 (s)] and a methine carbon atom [δ 41.8 (d)]. These data could be accommodated by the replacement of the 2,6,6-trimethylcyclohexenyl ring system in manoalide (1) by a 1-isopropenyl-2-methylcyclopentane ring system in luffariellin A (3).

The relative stereochemistry at C-14 and C-15 was determined by NOEDS measurements on derivatives of luffariellin A (3). Treatment of luffariellin A (3) with acetic

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