Renillafoulins, Antifouling Diterpenes from the Sea Pansy *Renilla reniformis* (Octocorallia)

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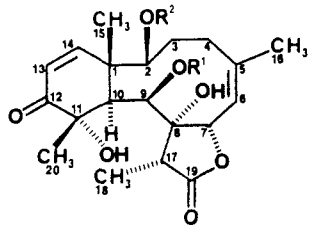
Extracts of the Atlantic sea pansy *Renilla reniformis*, which inhibit the settlement of barnacle larvae, have been found to contain three new diterpenes: renillafoulins A (1), B (2), and C (3). All three compounds inhibit barnacle settlement, with EC_{50} values ranging from 0.02 to 0.2 μg/mL. The structures were elucidated by using two-dimensional NMR data and spectroscopic comparisons to briarein-type diterpenes isolated from other Pennatulacea. Single-crystal X-ray diffraction data for 3 confirmed the structure.

Barnacles are well-known to the maritime industry as fouling organisms. Toxic materials used to control fouling by barnacles have included metallic copper and lead and coatings containing mercury, copper, or arsenic.[1] Organotin coatings, the latest biocides of choice, are currently under criticism. Environmental concerns over the use of these toxic metals have prompted investigations into alternative methods of inhibiting fouling by these organisms.[2] Studies of the response of barnacle larvae to allelochemicals, using a settlement assay, have demonstrated the presence of both settlement inducers and inhibitors in homogenates of octocorals.[3] We report here the structures of three diterpenes (1–3) isolated from *Renilla*



1 $R^1 = R^2 =$ Ac-

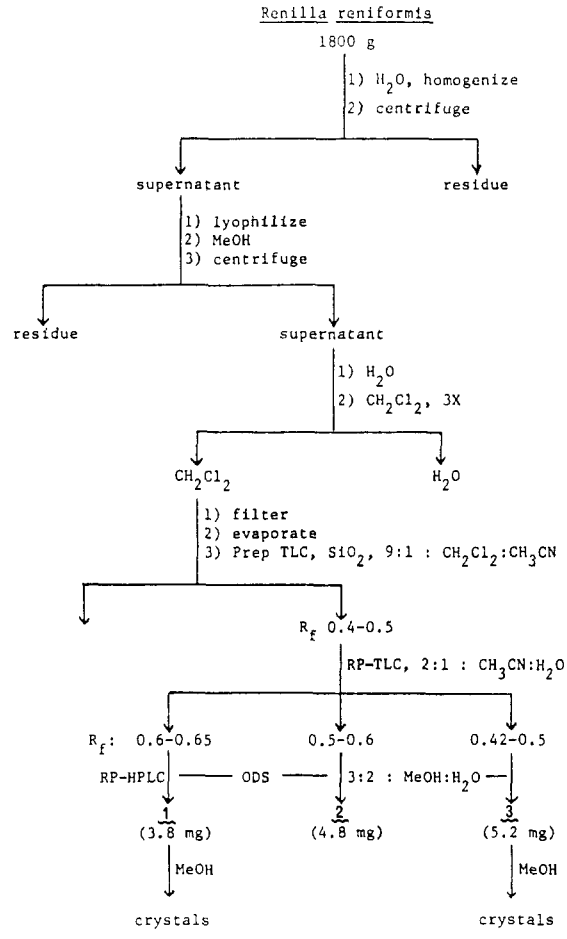
2 $R^1 =$ Ac, $R^2 = C_2H_5CO$-

3 $R^1 =$ Ac, $R^2 =$ n-C_3H_7CO-

reniformis (common sea pansy, a pennatulacean octocoral), which inhibit settlement of larvae of the barnacle *Balanus amphitrite amphitrite* Darwin 1854.[4] These compounds are related to briarein-type diterpenes found in other Pennatulacea.[5]

Inhibitory compounds (including 1–3) were isolated from *Renilla reniformis* by using a barnacle settlement assay to guide purification.[6] The methanol-soluble fraction of the aqueous extract was partitioned between methylene chloride and water (Scheme I). The methylene chloride soluble compounds were separated by preparative silica gel TLC (9:1 CH_2Cl_2/CH_3CN). A bioactive, UV quenching band was further separated on reversed-phase preparative TLC (2:1 CH_3CN/H_2O) to give three bands containing

Scheme I



renillafoulins A (1), B (2), and C (3). These three compounds inhibited barnacle larval settlement with EC_{50} values of 0.02–0.2 μg/mL.[6] Further purification was accomplished on reversed-phase HPLC (3:2 $MeOH/H_2O$). Compound 2 was a colorless oil, while 1 and 3 gave colorless crystals from methanol.

Renillafoulin A (1), the smallest of the three, had the molecular formula $C_{24}H_{32}O_9$ by high-resolution fast atom bombardment mass spectrometry (HRFABMS) and renillafoulins B and C (2 and 3) had molecular formulas $C_{25}H_{34}O_9$ and $C_{26}H_{36}O_9$, respectively. The homology indicated by the molecular formulas was confirmed by the ^1H and ^{13}C NMR spectra (Tables I and II), which were nearly identical for the three compounds except for acetate

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Table I. ^{1}H NMR Data for Compounds 1–8

	δ, multiplicity[a] (J, Hz)							
proton	1[b]	2[b]	3[b]	4[c]	5[c]	6[d]	7[e]	8[f]
H-2	4.62, d (6.9)	4.62, d (6.7)	4.63, d (6.8)	4.83, d (7)	4.83, d (7)	5.03, d (7)	5.72 (9)	6.21, d (15.3)
H-3a	1.85, td (14, 4)	1.84, td (14, 4)	1.83, td (14, 5)	2.93, dt (15, 5)	2.92, dt (15, 5)	1.66, m	5.63 (12, 9)	5.62, d (15.0)
H-3b	1.72, d br (15)	1.71, d br (15)	1.73, d br (15)	1.55, m	1.57, m	1.66, m		
H-4a	2.66, td (15, 5)	2.67, td (15, 5)	2.66, td (15, 5)	2.48, d br (15)	2.47, d br (14)	2.56, m	5.99 (12)	5.68, d (2.3)
H-4b	2.53, d br (14)	2.53, d br (15)	2.53, d br (15)	1.95, m	1.95, m	2.56, m		
H-6	5.50, d br (10.1)	5.50, d br (9.5)	5.50, d br (10.0)	5.36, d (10)	5.36, d br (10)	5.42, m	5.26 (3.7, 2, 1)	5.56, s br
H-7	5.18, d (10.3)	5.18, d (9.8)	5.18, d (9.9)	5.47, d (10)	5.47, d (10)	5.42, m	5.01 (3.7)	5.04, s br
H-9	5.82, d (3.6)	5.82, d (3.7)	5.81, d (3.7)	4.54, dd (7, 4)	4.52, dd (7, 4)	5.99, s	5.49 (7.3)	5.51, s br
H-10	2.80, d (3.5)	2.80, d (3.6)	2.80, d (3.6)	2.64, s br	2.62, s br	2.97, s br	2.82, m	2.71, s br
H-13	6.04, d (10.7)	6.03, d (10.7)	6.03, d (10.7)	5.18, dd (3, 2)	5.16, s br		5.90 (11)	6.07, d (10.2)
H-14	6.42, d (10.6)	6.42, d (10.7)	6.42, d (10.7)	5.30, s br	5.26, s br	4.76, s br	6.61 (11)	6.58, d (10.0)
15-CH_3	1.24, s	1.24, s	1.23, s	1.29, s	1.29, s	0.98, s	1.18, s	1.43, s
16-CH_3	1.89, s br	1.90, s br	1.90, s br	2.01, s	2.02, s	2.00, s br	5.96 (2, 1)	5.59, s
							6.12 (1, 1)	5.58, s
H-17	2.31, q (7.0)	2.31, q (7.0)	2.33, q (6.8)	3.16, q (7)	3.16, q (7)		2.41 (7, 7, 7)	2.84, q (7.8)
18-CH_3	1.17, d (7.0)	1.17, d (7.3)	1.17, d (7.0)	1.23, d (7)	1.20, d (7)	1.21, d (7)	1.21 (7)	1.27, d (7.8)
20-CH_3	1.45, s	1.45, s	1.45, s	1.89, s	1.87, s	2.00, s br	1.30 (7)	1.42, s
OH	4.77, s (exch)	4.80, s (exch)	4.78, s (exch)	1.65, s	1.63, m			3.85, s br (exch)
OH	5.00, s (exch)	5.05, s (exch)	5.01, s (exch)	2.93, d (7)	3.13, d (7)			
OAc	2.23, s	2.23, s	2.23, s	2.01		2.17, s	2.20	2.16, s
other	2.12, s (3 H)	2.39, q (2 H, 7.6)	2.36, t (1 H, 6.8)	1.96	2.28, dq (16, 7)	2.03, s (3 H)	1.98	2.10, s (3 H)
			2.33, t (1 H, 6.8)		1.07, t (7)	1.96, s (3 H)		1.97, s (3 H)
		1.17, t (3 H, 7.6)	1.68, m (2 H)		2.20, t (7)			
			0.97, t (3 H, 7.5)		2.18, t (7)			
					1.57, m			
					0.97, t (7)			
					0.90, t (7)			

[a] s = singlet, d = doublet, t = triplet, q = quartet, br = broad, exch = exchangeable proton. [b] 360 MHz; $CDCl_3$. [c] Reference 8; $CDCl_3$. [d] Reference 9; $CDCl_3$. [e] Reference 10. [f] Reference 11; $CDCl_3$.

Table II. ^{13}C NMR Data for Renillafoulins A (1), B (2), and C (3)

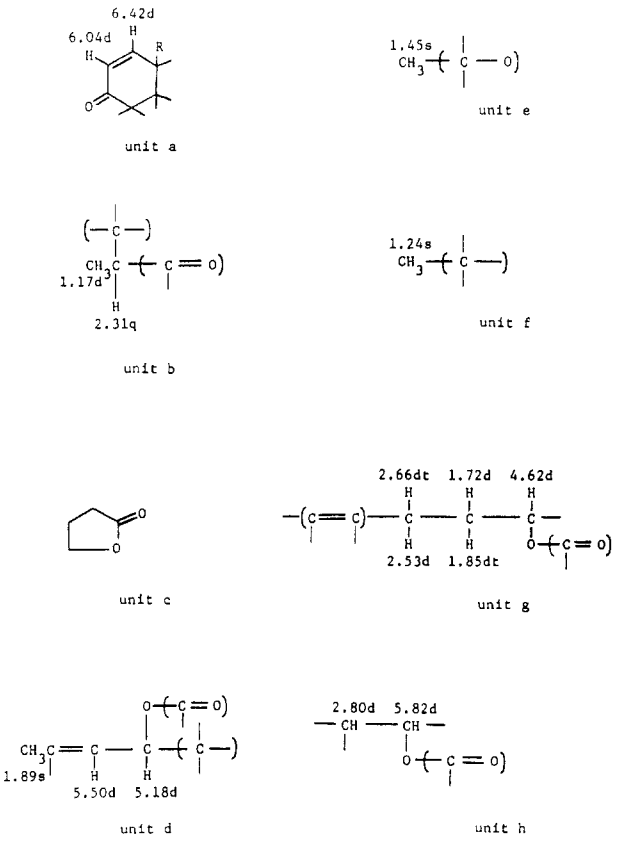
carbon	1[a]	2[a]	3[a]	4[b]	6[c]	8[d]
C-1	46.6 (s)[e]	46.6 (s)	46.6 (s)	47.1 (s)	44.5 (s)	41.8 (s)
2	79.0 (d)*[f]	78.7 (d)*	78.6 (d)*	70.9 (d)*	73.5 (d)*	144.5 (d)
3	31.8 (t)#	31.8 (t)#	31.8 (t)#	31.6 (t)#	28.5 (t)	131.5 (d)
4	28.3 (t)#	28.2 (t)#	28.2 (t)#	28.9 (t)#	26.7 (t)	73.5 (d)
5	143.1 (s)	143.3 (s)	143.2 (s)	140.0 (s)+	134.7 (s)	141.5 (d)
6	120.6 (d)+	120.4 (d)+	120.4 (d)+	120.6 (d)	120.5 (d)#	65.7 (d)
7	67.7 (d)	67.5 (d)	67.6 (d)	78.4 (d)*	70.4 (d)*	79.1 (d)
8	81.1 (s)	81.0 (s)	81.0 (s)	82.9 (s)	81.8 (s)	81.0 (s)
9	78.3 (d)*	78.3 (d)*	78.3 (d)*	68.2 (d)	79.1 (d)*	77.6 (d)
10	43.0 (s)	42.9 (s)	42.9 (s)	41.6 (d)	40.3 (s)	44.9 (d)
11	76.0 (s)	75.9 (s)	76.0 (s)	147.6 (s)+	147.0 (s)	80.6 (s)
12	199.6 (s)	199.6 (s)	199.7 (s)	117.5 (d)	117.7 (d)#	195.5 (s)
13	121.9 (d)+	121.8 (d)+	121.8 (d)+	74.9 (d)*	31.9 (t)	126.1 (d)
14	156.6 (d)	156.7 (d)	156.7 (d)	72.7 (d)*	74.8 (d)*	154.5 (d)
15	14.1 (q)	14.1 (q)	14.1 (q)	15.9 (q)	14.5 (q)	22.9 (q)
16	28.2 (q)	28.2 (q)	28.2 (q)	27.9 (q)	27.5 (q)	115.5 (t)
17	43.6 (d)	43.4 (d)	43.5 (d)	43.9 (d)	43.8 (d)	48.8 (d)
18	6.7 (q)	6.7 (q)	6.7 (q)	6.9 (q)	7.0 (q)	9.3 (q)
19	176.1 (s)	176.2 (s)	176.2 (s)	173.7 (s)	176.6 (s)	176.2 (s)
20	24.9 (q)	24.9 (q)	25.0 (q)	24.5 (q)	24.3 (q)	20.7 (q)
$OCOCH_3$	168.8 (s)	168.8 (s)	168.8 (s)	171.2 (s)	171.3 (s)	168.9 (s)
	21.6 (q)	21.6 (q)	21.6 (q)	21.4 (q)	21.4 (q)	21.0 (q)
other	169.9 (s)	173.4 (s)	172.6 (s)		170.5 (s)	169.5 (s)
	21.0 (q)	27.8 (t)	36.4 (t)	170.9 (s)	169.8 (s)	
		9.3 (q)	18.5 (t)	21.2 (q)	21.4 (q)	21.0 (q)
			13.7 (q)		21.2 (q)	21.0 (q)

[a] 90 MHz; $CDCl_3$. [b] Reference 8; $CDCl_3$. [c] Reference 9; $CDCl_3$. [d] Reference 11; $CDCl_3$. [e] Multiplicities by SFORD or DEPT: s = singlet, d = doublet, t = triplet, q = quartet. [f] Signals with identical superscripts within a columm (*, #, +) may be interchanged.

signals in **1**, propionate signals in **2**, and *n*-butyrate signals in **3** (see tables). We shall direct our attention first to the simplest compound, **1**.

The ^{13}C NMR spectrum of **1** showed the required 24 carbons, while DEPT spectra located 30 of the 32 protons. All 32 protons were observed in the ^{1}H NMR spectrum, which contained two exchangeable hydroxyl protons at δ 5.00 and 4.77. The ^{13}C NMR spectrum indicated oxygenated methine carbons at δ 79.00, 78.31, and 67.68 and oxygenated nonprotonated carbons at δ 81.08 and 76.00. Carbon resonances at δ 199.61, 176.12, 169.92, and 168.80 are appropriate for a conjugated ketone, a γ-lactone, and

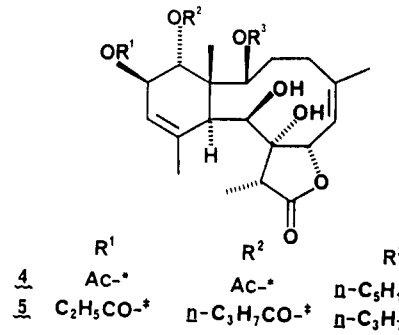
Chart I



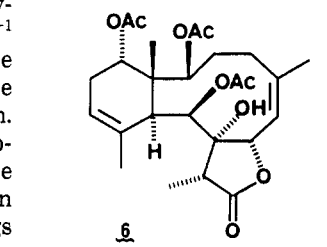
two ester carbonyls, respectively.[7] The IR spectrum contained a broad band at 3360 cm^{-1}, confirming the hydroxyl groups, and bands at 1773, 1740, and 1696 cm^{-1} corresponded to the γ-lactone, the two esters, and the conjugated ketone. These groups account for all nine oxygens and locate six of the nine elements of unsaturation. The enone alkene and one additional alkene unit are observed in carbons at δ 120.6, 121.9, 156.6 (all methine carbons) and 143.1 (nonprotonated carbon). In addition to these seven units of unsaturation, two additional rings must be present. ^1H NMR data, including COSY analysis, were used to assemble segments of the remaining structure.

Six methyl groups were indicated for **1**, with signals at δ 2.23 (s, 3 H) and 2.12 (s, 3 H) assigned to two acetate esters. The 20 carbons remaining after the diacetate assignment, including four unassigned methyl resonances, suggested a diterpene (bicyclic) structure. The unsaturated ketone was further characterized as being α,β-unsubstituted, γ-fully substituted (-COCH=CHC<) by the AB quartet at δ 6.04 and 6.42 (J = 10.7 Hz), with the coupling constant suggesting a cyclohexenone ring (unit a). The methyl protons at δ 1.17 (d, 3 H, J = 7.0 Hz) were coupled to a methine proton at δ 2.31 (q, 1 H, J = 7.0 Hz) (unit b); this was assigned to an α-methyl γ-lactone (on unit c) or α-methyl ketone (on unit a). The methyl group at δ 1.89 (br s, 3 H) was slightly coupled to a proton giving a doublet at δ 5.50 (br d, 1 H, J = 10.1 Hz), which was coupled to a proton giving a doublet at δ 5.18 (d, 1 H, J = 10.3 Hz). This indicated a methyl group on a trisubstituted olefin bearing an allylic ester (unit d). The remaining methyl groups at δ 1.45 (s, 3 H) and 1.24 (s, 3 H) were uncoupled (units e and f). The coupling of the methine proton at δ 4.62 (d, 1 H, J = 6.9 Hz) with two of the four mutually coupled protons at δ 2.66 (td, 1 H, J = 15, 5 Hz), 2.53 (br d, 1 H, J = 15 Hz), 1.85 (td, 1 H, J = 15, 5 Hz), and 1.72 (br d, 1 H, J = 15 Hz) indicated an isolated three-carbon chain with an oxygenated substituent (unit g). From the chemical shifts at δ 2.66 and 2.53, the left-hand end of unit g must be attached to an unsaturated group (C=C, C=O), but this cannot be in unit a (which is too highly substituted) or unit c (which would not allow a terpene skeleton). Thus, g must link to d to give g–d (>CCH(OCO—)CH=C(CH_3)CH_2CH_2CH(OCO—)—). To join this to a cyclohexenone (unit a) requires, then, at least an eight-membered ring. Remaining in the ^1H NMR spectrum of **1** are two mutually coupled protons at δ 5.82 (d, 1 H, J = 3.6 Hz) and 2.80 (d, 1 H, J = 3.6 Hz); these are assigned to H-9 and H-10, respectively (unit h).

These units described—the α-methyl γ-lactone, the cyclohexenone, and the methyl olefin attached to an oxygenated three-carbon chain—are found in one or more of the pennatulacean diterpenes cavernuline (**4**) and caver-



	R^1	R^2	R^3
4	Ac-*	Ac-*	n-C_5H_{11}CO-*
5	C_2H_5CO-‡	n-C_3H_7CO-‡	n-C_3H_7CO-‡



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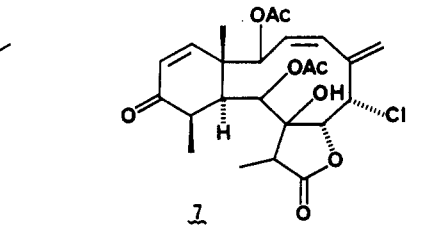

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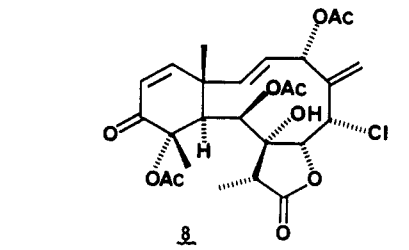

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nulinene (**5**),[8] stylatulide lactone (**6**),[9] ptilosarcenone (**7**),[10] and erythrolide B (**8**),[11] all of which share the same carbon skeleton. Each gives NMR spectral data consistent with structure **1** for renillafoulin A (Tables I and II).

The regiochemistry of the assignment of the acetoxy substituents to C-2 and C-9 rather than C-8 and C-11 is based on the chemical shifts of the methine protons, while

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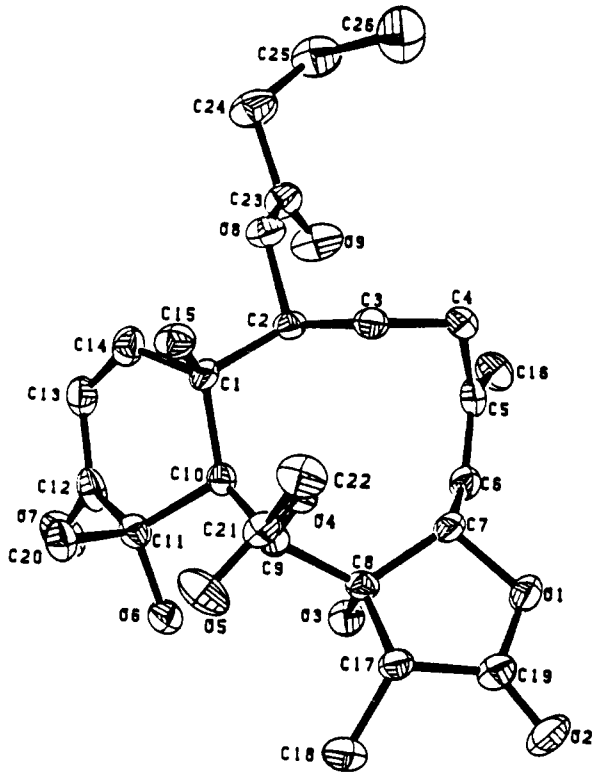

Figure 1. Computer-generated perspective drawing of **3**. Hydrogens are omitted for clarity.

the relative stereochemistries indicated for **1–3** are consistent with spectral comparisons to the metabolites **4–8**, which are mutually consistent.

As noted above, renillafoulins B (**2**) and C (**3**) differ from **1** by replacement of an acetate with a propionate and a butyrate, respectively. The locations of the homologous esters are not defined by the above analysis, but **2** and **3** are presumed to be single regioisomers, since their ^1H and ^{13}C NMR spectra contain only one of the two acetate resonances present in **1**. The α-methylene protons of the butyrate ester in **3** possess multiplicity thus far unexplained by variable-temperature NMR studies.

X-ray Studies. As noted above, the propionate and butyrate locations in **2** and **3** were not assigned by chemical evidence and stereochemical assignments were based mainly on analogy (and consistency). A single-crystal X-ray structure analysis of **1** proved to be difficult, since the crystal contained two molecules in the asymmetric unit; consequently, the manuscript was originally submitted with the structures shown (**1–3**) but without indication of stereochemistry at C-11 and without a decision between C-2 and C-9 for propionylation–butyrylation. More recently, the crystal structure of **3** has been solved, and the regiochemistries and relative stereochemistries are as shown (**1–3** and Figure 1). The absolute stereochemistry is assumed to be that shown, by analogy to the consistent absolute stereochemistries of erythrolide B (**8**),[11] briarein A,[12] and brianthein Y.[13]

Experimental Section

NMR spectra were recorded on Varian XL-200, Nicolet NT-360 and NT-470, and General Electric QE-300 FT spectrometers using deuteriochloroform as solvent and internal standard; ^1H and ^{13}C NMR data are reported in Tables I and II. Mass spectra were run on either a ZAB SE spectrometer operating in the fast atom bombardment mode, using xenon atoms at 3–10 keV and a matrix of dithiothreitol/dithioerythritol,[14] or an MAT 731 spectrometer operating in the field desorption mode. Infrared and UV spectra were obtained on IBM IR/32 and Perkin-Elmer Lambda 3 UV/vis spectrophotometers, respectively.

Isolation of Renillafoulins A (1), B (2), and C (3). Preliminary efforts to isolate the active compounds have been reported.[6] Whole sea pansies collected off Beaufort, NC, were homogenized in water (1:3 w/v). The solution was decanted, centrifuged, and lyophilized. The resulting dried material was suspended in methanol, centrifuged, and diluted 1:3 with water. This aqueous solution was extracted three times with methylene chloride and the organic layer was filtered and flash evaporated. The yellow residue was subjected to preparative TLC on silica gel using methylene chloride/acetonitrile (9:1). The band at R_f 0.40–0.50 (assay active, UV quenching) was subjected to reversed-phase TLC using acetonitrile/water (2:1) to give three bands at R_f 0.60–0.65, 0.50–0.60, and 0.42–0.50, yielding compounds **1**, **2**, and **3**, respectively (5×10^{-4} % wet wt each).[15] These compounds were further purified by reversed-phase HPLC (Econosphere ODS; 4.6 × 250 mm) using methanol/water (3:2) to give ca. 4–5 mg of each compound. Compounds **1** and **3** were ultimately crystallized from methanol.

Renillafoulin A (1): UV (MeOH) λ_{max} 203 nm (ϵ 15300), 220 (9500); IR (neat) 3359, 1773, 1740, 1696, 1211 cm^{-1}. Anal. Calcd for $C_{24}H_{33}O_9$: M_r 465.2125 (M + H). Found: M_r 465.2089 (M + H) (HRFABMS).

Renillafoulin B (2): UV (MeOH) λ_{max} 204 nm (ϵ 12600), 219 (9400); IR (neat) 3360, 1773, 1740, 1696, 1215 cm^{-1}. Anal. Calcd for $C_{25}H_{35}O_9$: M_r 479.2281 (M + H). Found: M_r 479.2261 (M + H) (HRFABMS).

Renillafoulin C (3): UV (MeOH) λ_{max} 202 nm (ϵ 13000), 220 (7300); IR (neat) 3374, 1773, 1740, 1734, 1696, 1215 cm^{-1}. Anal. Calcd for $C_{26}H_{37}O_9$: M_r 493.2438 (M + H). Found: M_r 493.2470 (M + H) (HRFABMS). Details of the X-ray crystal structure determination can be found in the supplementary material.

Renillafoulin A (**1**) was found to be inactive against *Escherichia coli*, *Bacillus subtilis*, *Saccharomyces cerevisiae*, and *Penicillium atrovenetum* in paper disk–agar assays using 50 μg of **1** per 6.5-mm disk. At this concentration, **1** was also inactive in a biochemical prophage induction assay[16] and showed no cytotoxicity or antiviral activity against monkey kidney cells (CV-1), herpes simplex virus, type 1, or vesicular stomatitis virus.[17]

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Collection of the *Renilla reniformis*, preparation of the crude extract, and settlement assays were performed by Dr. D. Rittschof and A. R. Schmidt, Duke University. Single-crystal X-ray structure determinations were performed by Dr. S. R. Wilson at the University of Illinois.

Registry No. 1, 104715-93-7; 2, 104715-94-8; 3, 104715-95-9.

Supplementary Material Available: Description of the X-ray diffraction determination of 3, including final atomic positional parameters (Tables 3 and 4), thermal parameters (Table 5), and selected bond distances and bond angles (Table 6), (6 pages); observed and calculated structure factors (Table 7) (12 pages). Ordering information is given on any current masthead page.

Further Kinetic Evidence for the Competitive Rotational and Inversional *Z*–*E* Isomerization of Substituted Azobenzenes

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The first-order rate constant for thermal *Z*–*E* isomerization of 4-(dimethylamino)-4′-nitroazobenzene was measured in various solvents at different temperatures and pressures. The temperature dependence of the activation volume was qualitatively different in different solvents. The Arrhenius plots were linear for ethanol and methanol but deviated upward at high temperatures for benzene and dioxane. These results unequivocally indicate that there are two competing reaction mechanisms in the *Z*–*E* isomerization of the azobenzene.

It is generally agreed that the thermal *Z*–*E* isomerization of azobenzenes proceeds through a transition state in which one of the nitrogen atoms is sp-hybridized. This mechanism is usually called "inversion". Conclusive experimental evidence for the inversion mechanism is the facile isomerization of an azobenzene unit incorporated into a ring system[1,2] in which the rotation of the phenyl ring around the nitrogen–nitrogen bond is extremely difficult for steric reasons. The decrease in polarity during activation[3,4] and the absence of a solvent effect on the activation parameters[5] are also in accord with the inversion mechanism. Theoretical calculations also provide support for inversion.[6,7]

In a previous report,[8] it was concluded that the thermal *Z*–*E* isomerization of *p*-aminoazobenzenes proceeds by two routes as shown in Scheme I. Inversion about a nitrogen atom effects isomerization via the activated complex **1**. In the route via **2**, the nitrogen–nitrogen π-bond is ruptured heterolytically, and rotation around the remaining σ-bond gives the *E* isomer (rotation mechanism). This conclusion was based mainly on the effects of solvent and pressure on the isomerization kinetics of several substituted azobenzenes. For example, the isomerization rate of 4-(dimethylamino)-4′-nitroazobenzene (NMe_2-NO_2-AB) increased rapidly with increasing polarity of the reaction medium. These results seem to reflect a large polarity increase during activation, and for this reason the dipolar transition state **2** was proposed.

Recently, Nishimura et al.[9] proposed that the isomerization of push–pull substituted azobenzenes in polar solvents proceeds by a single path through the intermediate resonance hybrid **3** (Chart I). Their proposal was based mainly on a correlation between the absorption maxima of the ortho-substituted *E* isomers of NMe_2-NO_2-AB and their free energies of activation for the *Z*–*E* isomerization. They took this correlation to indicate coplanarity of the two phenyl rings in the transition state and concluded that the nonplanar transition state **2** is not compatible with their observations. They also measured the reaction volumes for the isomerization in relatively nonpolar solvents[10] and found that the reaction volume was slightly

Scheme I

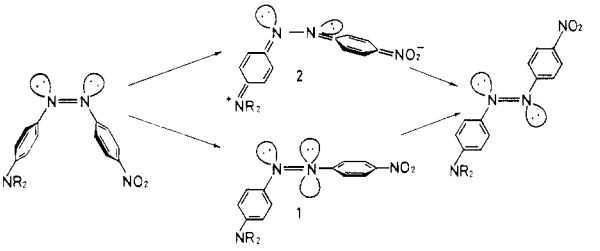
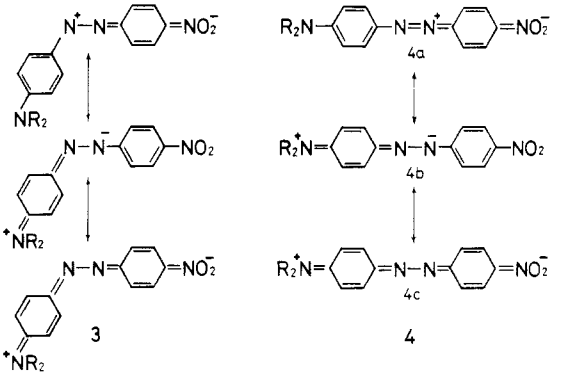


Chart I



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