Full Papers

Puupehenone Congeners from an Indo-Pacific *Hyrtios* Sponge

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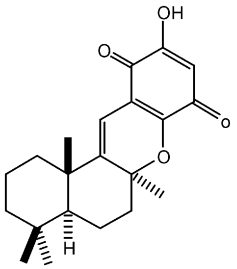
An investigation of the constituents from an Indonesian *Hyrtios* sponge has provided new insights about the chemistry and biology of the puupehenones, a unique class of merosesquiterpenes. The parent compound, puupehenone (**2**), has been repeatedly encountered in sponges from four distinct orders. In this study we characterized three compounds, (+)-(5*S*,8*S*,9*R*,10*S*)-20-methoxypuupehenone (**3**), (+)-(5*S*,8*S*,10*S*)-20-methoxy-9,15-ene-puupehenol (**4**), and (+)-(5*S*,8*S*,9*R*,10*S*)-15,20-dimethoxypuupehenol (**5**). Their structures were supported by complete sets of spectroscopic data along with comparisons to literature properties. While **5** was observed in the crude extracts, it was also heat labile and could be converted at 35 °C to a mixture of **3** and **4**. The possibility that **3**, **4**, and **5** are formed from **2** by a series of methanol additions is discussed. The bioactivity of these compounds in soft-agar cytotoxicity tests was also explored.

Introduction

When mixed terpene−shikimate biosynthetic pathways are at work, the resultant products can be quite distinctive. Sponge-derived metabolites of this type often possess intriguing structure and functionalization patterns. One representative set are the highly unsaturated tetracyclic merosesquiterpene families headed by (+)-cyclospongiaquinone (**1**), reported in 1978,[1] and (+)-puupehenone (**2**),



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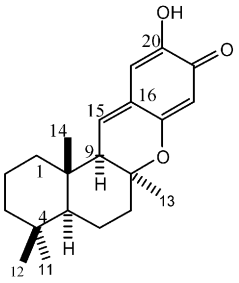

2

described one year later.[2] The compounds of these two families have interesting physical and biological properties that are largely dictated by the variation in their conjugation patterns and oxygen functionalization. For example, the highly electrophilic quinone-methide present in **2** facilitates the formation of C/D ring analogues[3] while also contributing an avenue for biological action.[4] These along with other factors motivated our further study of the puupehenone class.

The puupehenones have been the subject of sustained interest during the past two decades, as 11 additional analogues have been described and studied.[5] The current bioorganic chemical understanding of this class consists of the following information. The absolute stereochemistry of **2** has been defined as 5*S*, 8*S*, 9*R*, 10*S*.[6] This heterocyclic framework can be efficiently accessed by sequences designed for its total synthesis.[7] As expected, the quinone-methide site of **2** is the locus of numerous nucleophilic 1,6-conjugate addition reactions. Finally, the putative *in vitro* bioactivity properties of the puuphenones are unusually diverse and vary from antifungal, antimalarial, antimicrobial, antituberculosis, antiviral, cytotoxic, to immunomodular.[2,4,5b,c,8]

Prior to the initiation of this work, minute samples of (+)-**2** and a corresponding dimer were cataloged in the UC Santa Cruz repository of sponge-derived compounds. Additional puupehenones were encountered during the project described herein, whose initial goal was to isolate inhibitors of endoplasmic reticulum (ER) formation.[9] An Indonesian sponge extract of a *Hyrtios* sp. (collect. no. 95653) active in the ER assay was investigated and yielded two new puupehenone analogues, (+)-(5*S*,8*S*,9*R*,10*S*)-20-methoxypuupehenone (**3**) and (+)-(5*S*,8*S*,10*S*)-20-methoxy-9,15-ene-puupehenol (**4**), and a third compound, (+)-(5*S*,8*S*,9*R*,10*S*)-15,20-dimethoxypuupehenol (**5**), which appeared to be the methoxy analogue of a compound previously published but without accompanying experimental data.[5a] Unfortunately, the ER assay was discontinued prior to the completion of the compound characterizations. Toward the end of the research attention shifted to interrogating possible biogenetic relationships between puupehenone and its analogues. A nonenzyme-mediated equilibrium could interconvert general structures **A**−**D** as shown in Figure 1. The separate results of Scheuer[5b] and Zjawiony[3] clearly dem-

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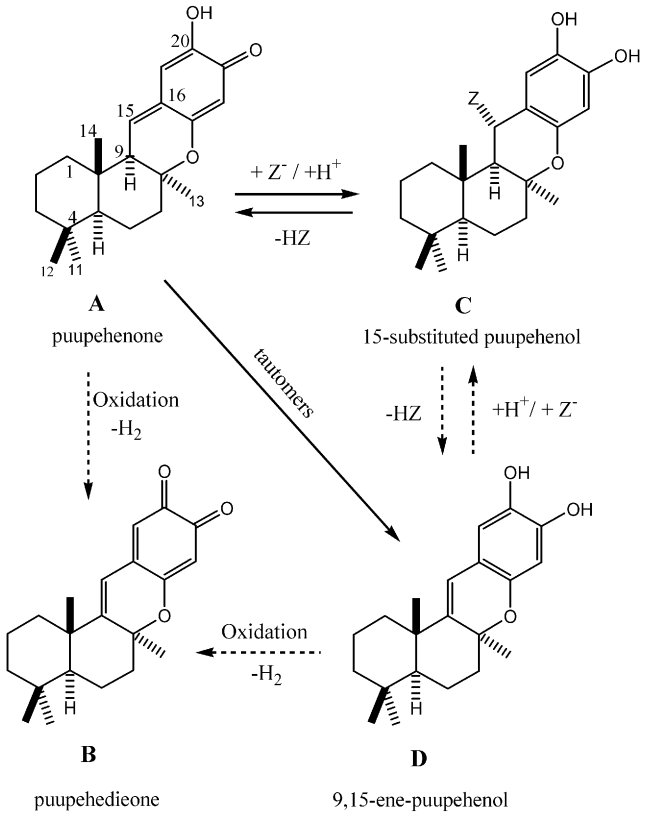

Figure 1. Possible biogenetic relationships among puupehenone analogues.

onstrated the ability of nucleophiles to initiate room-temperature transformation of **A** to **C**. Unclear was the extent that the labile quinone-methide present in **2** and in three other analogues (21-chloropuupehenone, 21-bromopuupehenone, or 15-cyanopuupehenone) actually represented a biosynthetic endpoint. Also unknown was the propensity to add methanol during extraction or an elimination reaction on **C** to generate **A** and/or **D**. Finally, structure **D** could be formed from **A** and represents a biosynthetic endpoint or an alternative source of **B**. Described below are the structures, biological properties, and reactions that interconvert these compounds.

Results and Discussion

Our initial approach to establish structures of the three compounds isolated began with dereplication efforts.[10] Puupehenone (**2**)[5b] was never observed in this work, but it served as a lead structure. The ^{1}H NMR spectrum of the CH_2Cl_2 partition fraction, which soon became the focus of the subsequent isolation steps, contained approximately 10 methyl singlets in the region δ 0.6−1.2 and several double-bond resonances as singlets from δ 5.8 to 6.8. These data were indicative of unsaturated, polycyclic meroterpenoids. Fractions obtained from silica chromatography were each examined by NMR to identify those rich in such constituents. Each purified compound was subsequently examined by HRMS in an effort to rapidly establish the molecular formula.

The initial candidate for complete structure elucidation was compound **5**, which eluted first during semipreparative chromatography. The HRFABMS confirmed the molecular formula as $C_{23}H_{34}O_4$ (*m*/*z* 374.2452 for $[M]^+$). The NMR APT-MF established as $C_{23}H_{33}$, of unsaturation number 7, also indicated the presence of an OH group. The three double bonds, which collectively contained only two un-

Table 1. Comparison of the ^{13}C NMR Data of Puupehenone (**2**) to its Analogues **3**−**5** in $CDCl_3$ at 125 MHz

no.	**2**[5b]	**3**	**4**	**5**
1	40.0[a]	40.1 (t)	39.3 (t)	40.0 (t)
2	18.1[a]	18.2 (t)	17.5 (t)	18.6 (t)
3	40.7[a]	40.8 (t)	42.2 (t)	41.9 (t)
4	33.3	33.4 (s)	33.9 (c)	33.4 (s)
5	53.8	53.9 (d)	44.1 (d)	55.3 (d)
6	18.4[a]	18.5 (t)	19.1 (t)	18.4 (t)
7	39.2[a]	39.3 (t)	31.1 (t)	41.0 (t)
8	78.8	78.5 (s)	76.7 (s)	75.1 (s)
9	54.8	54.9 (d)	149.9 (s)	53.6 (d)
10	41.6	41.7 (s)	38.7 (c)	37.1 (s)
11	33.7	33.8 (q)	32.9 (q)	33.9 (q)
12	21.9	22.0 (q)	21.3 (q)	22.1 (q)
13	28.0	28.2 (q)	25.1 (q)	27.3 (q)
14	15.0	15.2 (q)	25.6 (q)	14.5 (q)
15	140.4	138.9 (d)	114.1 (d)	73.9 (d)
16	129.3	129.0 (s)	116.5 (s)	114.5 (s)
17	162.8	161.1 (s)	146.4 (s)	148.6 (s)
18	105.1	105.4 (d)	103.6 (d)	103.6 (d)
19	182.0	182.3 (s)	145.6 (s)	146.5 (s)
20	147.5	151.8 (s)	141.1 (s)	141.2 (s)
21	106.1	108.7 (d)	109.0 (d)	111.8 (d)
22		55.4 (q)	56.7 (q)	56.4 (q)
23				56.2 (q)

[a] Literature assignments at C1, C2, C3, C6, and C7 were revised by analogy with those of **3**.

coupled protons, meant that four rings were present. A series of literature searches using these constraints plus the requirement for two methoxy groups and four aliphatic methyl singlets eventually drew our attention to a 1983 publication by Scheuer.[5a] This paper contained a brief recapitulation of the puupehenone (**2**) NMR properties and incomplete spectroscopic properties of a demethoxy analogue of **5**. Tables 1 and 2 summarize the entire data set for **5**. Noteworthy are the diagnostic ^{13}C NMR Me shifts (δ 33.9, C11; δ 22.1, C12; δ 27.3, C13; δ 14.5, C14), which are virtually identical to those of **2** and allow the fused ABC-rings to be established as *trans*−*cis*. Applying the results of Capon[6] to establish absolute stereochemistry of the puupehenones, we conclude this compound to be (+)-(5*S*,8*S*,9*R*,10*S*)-15,20-dimethoxypuupehenol (**5**).

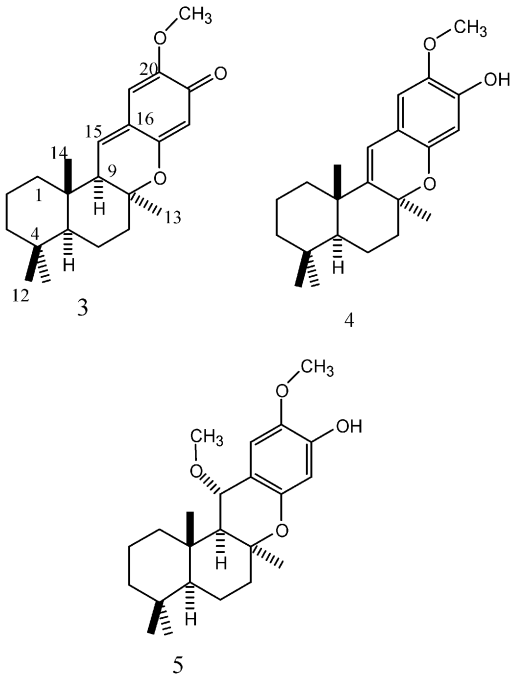

Table 2. Comparison of the ^{1}H NMR Data of 20-Puupehenone (**2**) to its Analogues **3**−**5** Obtained in $CDCl_3$ at 500 MHz

no.	**2**[5b]	**3**	**4**	**5**
1	1.15 (1H, m)	1.15 (1H, m)	1.27 (1H, d, 7.5)	1.08 (1H, dt, 3, 13)
	1.70 (1H, m)	1.65 (1H, brd, 13)	2.04 (1H, m)	1.96 (1H, brd, 13)
2	1.51 (2H, m)	1.40 (1H, m)	1.45 (2H, m)	1.40 (1H, m)
		1.53 (1H, m)		1.56 (2H, m)
3	1.40 (1H, m)	1.15 (1H, m)	1.15 (1H, dt, 13, 4)	1.15 (1H, m)
	1.45 (1H, m)	1.40 (2H, m)	1.43 (1H, m)	1.40 (1H, m)
5	0.96 (1H, m)	0.92 (1H, dd, 3, 9.5)	1.41 (1H, m)	0.95 (1H, d, 13)
6	1.54 (2H, m)	1.53 (2H, m)	1.73 (1H, m)	1.56 (2H, s)
			1.98 (1H, m)	
7	2.17 (1H, dd, 11.4, 2.7)	2.12 (1H, dd, 2.5, 12)	2.01 (1H, m)	2.09 (1H, dd, 3, 7)
	1.52 (1H, m)	1.51 (1H, m)	2.18 (1H, bq, 11)	1.55 (1H, m)
9	2.04 (1H, d, 6.9)	2.00 (1H, d, 7)		1.52 (1H, s)
11	0.84 (3H, s)	0.88 (3H, s)	0.87 (3H, s)	0.91 (3H, s)
12	0.82 (3H, s)	0.81 (3H, s)	0.96 (3H, s)	0.81 (3H, s)
13	1.23 (3H, s)	1.18 (3H, s)	1.35 (3H, s)	1.21 (3H, s)
14	0.91 (3H, s)	0.79 (3H, s)	1.22 (3H, s)	0.63 (3H, s)
15	6.65 (1H, d, 6.9)	6.59 (1H, d, 7)	6.08 (1H, s)	4.08 (1H, s)
18	5.86 (1H, s)	5.98 (1H, s)	6.46 (1H, s)	6.35 (1H, s)
21	6.20 (1H, s)		6.56 (1H, s)	6.73 (1H, s)
22		3.70 (3H, s)	3.80 (3H, s)	3.45 (3H,s)
23				3.83 (3H,s)

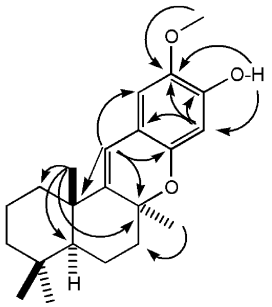

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Figure 2. HMBC correlations.

The second compound isolated, **3**, was rapidly characterized as the methoxy derivative of **2**. Its molecular formula of $C_{22}H_{30}O_3$, established by HRFABMS (*m*/*z* 343.2289 to $[M + H]^+$), differed from that of **2** by a C_1H_2. The APT-MF of $C_{20}H_{30}$ and similarity of the aromatic ring ^{13}C shifts to that of **2** (see Tables 1 and 2) suggested the OH at C20 was replaced by an OCH_3. The quinone-methide moiety was evident from the seven low-field ^{13}C NMR resonances including the C═O (δ 180.3, s) and the two relatively shielded CH shifts (δ 108.7, C18, d; δ 105.4, C21, d). Other diagnostic resonances included ^{13}C NMR CH_3 shifts (δ 33.8, C11; δ 22.0, C12; δ 28.2, C13; δ 15.2, C14), which are virtually identical to those of **2** and, as above, substantiated the fused ABC-rings. Also supporting this assignment was a ^{1}H NMR AB doublet (J = 7 Hz) between a vinylic δ 6.59 (H15) and an aliphatic proton δ 2.00 (H9). These features plus the assumption of a unified biosynthetic pathway to the compounds in hand supported the stereochemistry and name as (+)-(5*S*,8*S*,9*R*,10*S*)-20-methoxypuupehenone (**3**).

The final compound **4**, of molecular formula $C_{22}H_{30}O_3$ (*m*/*z* 342.2201 $[M]^+$ by HRFABMS), was isomeric to **3**. The absence of a C═O signal, but presence of eight vinyl carbons and three singlet protons, indicated the vinylic unsaturation was present as an aromatic ring and a conjugated trisubstituted double bond. Additionally, the ^{1}H NMR spectrum displayed a single OCH_3 with the four characteristic methyl ^{13}C NMR shifts of the fused ABC-rings. The various substructures were quickly joined with the aid of the HMBC data, with the most relevant correlations shown in Figure 2. Biogenetic arguments were again employed to complete the stereochemical assignment as (+)-(5*S*,8*S*,10*S*)-20-methoxy-9,15-ene-puupehenol (**4**).

Conclusions

There are very few examples in the literature in which *an identical* sponge-derived meroterpene compound is reported from disparate taxonomic classes.[10,11] Not widely recognized is that puupehenone (**2**) occurs from both shallow and deep water sponges divided among four taxonomic orders. Table 3 provides a detailed outline of this unexplained, yet significant pattern. Our results can be added to those of Table 3 because the isolation of 20-methoxypuupehenone (**3**), undoubtedly derived from **2**, indicates this biosynthetic pathway is functional in the *Hyrtios* sponge we studied. Finally, the taxonomic occurrence of **2** provides a contrast to previously discussed patterns of meroterpenoid distribution. For example, it has been noted that sesquiterpene quinones are useless for chemosystematics, since different, unrelated sesquiterpene quinone structures have been reported from sponges in six orders (Hadromerida, Halichondrida, Haplosclerida, Dictyoceritida, Dendroceratida, and Verongida).[12] Future applications of molecular approaches to sponge classification may provide some answers to this paradox.[13]

The puupehenone analogues isolated in this work, **3**, **4**, and **5**, are closely related and potentially interconvertible by the general reactions outlined in Figure 1. Aside from our work, none of the previous isolation papers report mixtures of general structures **A**−**D**.

By ^{1}H NMR we observed both **3** and **5** (approximately 1:1) in both the crude extract and in solvent partition fractions. In addition, **5** was heat labile and at 35 °C converted to a mixture of **3** (90%) and **4** (10%), and on standing **3** was completely transformed to **4**. This implies that analogues of general structure **C**[5b,14] could serve as an additional source of structures **A** (i.e., puupehenone) accompanied by **D**. Alternatively, there is the possibility that **3**, **4**, and **5** isolated in this study were formed from **2** by a series of methanol additions and the tautomerization outlined in Figure 3. We were unable to further explore this possibilty by conducting a fresh extraction with non-methanolic solvents because of a lack of fresh sponge material.

Unfortunately, the close structural relationship between **2**, previously indicated to be a potent cytotoxin, and the compounds **3**−**5** isolated here did not translate into an extension of bioactivity. None of the three compounds reported above were active in the Valeriote *in vitro* soft-

Table 3. Taxanomic Distribution of Puupehenone (**2**)-Containing Sponges[a]

Dendroceratida	Dictyoceratida	Verongida	Haplosclerida
Dysideidae (Gray)	Thorectidae (Bergquist)	Aplysinellidae (Bergquist)	Petrosiidae (Van Soest)
Dysidea (DW)[b]	*Hyrtios* (SW)	undescribed genus (SW)	*Stronglophora* (DW)
Australia[c]	Hawai'i[d]	Hawai'i[f]	Caribbean[g]
	New Caledonia[e]		

[a] Codes: SW and DW are shallow (<150 ft) and deep (>400 ft), respectively, with taxonomy from the "Spongeguide" available from Hooper, J.N.A.: www.qmuseum.qld.gov.au/organisation/sections/SessileMarineInvertebrates/index.asp. [b] *Dysidea* is sometimes included in the order Dictyoceratida; see: Jaspars, M.; Jackson, E.; Lobkovsky, E.; Clardy, J.; Diaz, M. C.; Crews, P. *J. Nat. Prod.* **1997**, *60*, 556−561. [c] Ref 6. [d] Ref 5c. [e] Ref 5d. [f] Ref 5b. [g] Ref 5e.

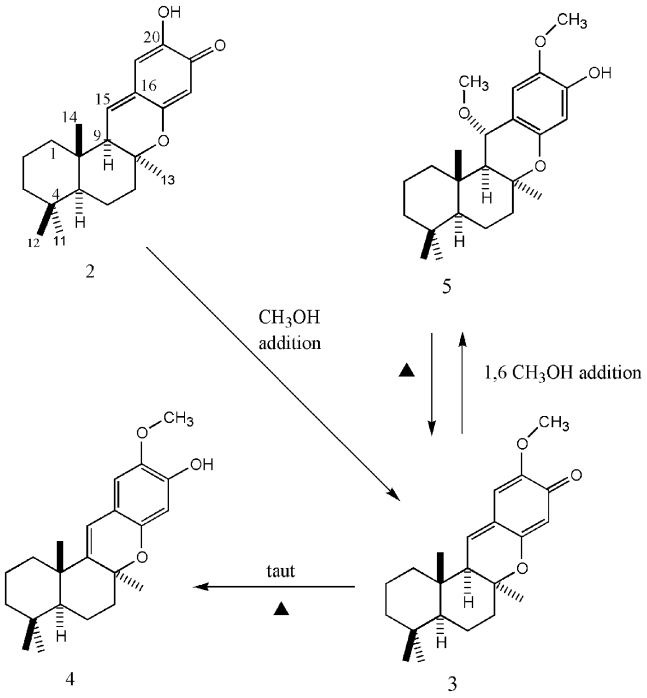

Figure 3. Possible chemical relationships of puupehenone (**2**) to the compounds isolated.

agar disk diffusion assay screen employing human L1210 leukemia or murine colon 38 or human colon H116 tumors.[15] These data alongside the anti-tuberculosis SAR data reported by El Sayed[4] collectively indicate that the quinomethide chromophore, present in puupehenonone (**2**), is required for bioactivity and that substitution of H15 is acceptable, but replacement of the C20−OH is not tolerated.

Experimental Section

General Experimental Procedures. Optical rotations were run on a DIP 370 (Jasco) digital polarimeter, UV was obtained from an HP 8455 diode array spectrophotometer, and IR was recorded on a Varian 1600 Series FTIR spectrometer. The NMR spectra ($CDCl_3$) were recorded at 500 MHz (^1H) and 125.7 MHz (^{13}C). Final NMR assignments were based on previously published data of puupehenone (see Tables 1 and 2) and 2D NMR data derived from HMQC, HMBC, and $^1H-^1H$ COSY. MS data were obtained on a VG 70-SE-4F and a VG Quattro. Chromatography was performed using Sephadex LH-20 (gel permation) and ODS (reversed-phase: HPLC). HPLC was performed with a 10 μm ODS column.

Biological Material, Collection, and Identification. The *Hyrtios* sp. (Thorectidae, Dictyoceratida), collect. no. 95653 (1 kg wet wt), was collected from Togian Island in Tomini Bay, north Sulawesi, Indonesia, at a depth of 30 feet (N 00°16.835′, E 121°38.436′). This massive sponge (4−6 cm thick) is densely covered with thin, long conules (100 μm × 1 mm, width × length), which give it a hispid appearance. The ectosome is light burgundy with a tan-yellowish endosome. The sponge is compressible in consistency. Its skeleton consists of a sturdy fibroreticle densely cored by sand and foreign spicules. Primaries (350−500 μm in diameter) reach the surface, forming the sharp conules. Secondaries (110−150 μm in diameter) form regular reticulation toward the surface. The fibers are so fully charged of material that their nature (laminated, stratified, or clear) is obscured. However, at the surface some lamination is evident in parts of the conules that are clear of debris. Many of the conules split at the end. The identification of this species as *Hyrtios*[11] is mainly based on the sturdy and regular nature of the debris-filled fiber skeleton, the apparent laminated fibers (evident at the surface), and the general massive construction of this sponge body. The characteristics are similar to those indicated by Bergquist, in which the conule tips are tan in color due to the embedded sand.[12] A voucher as well as an underwater photo is available from PC.

Extraction and Isolation of Puupehenones. The sponges were initially preserved according to our standard procedure as described previously.[13] The sponge was soaked three times in ethanol. Parallel concentration of these extracts afforded a brown, amorphous slurry. Resuspension of this material in water/CH_2Cl_2 afforded an organic layer, which was evaporated to yield an oil. Standard solvent partitioning afforded fractions: hexanes (0.4 g), CH_2Cl_2 (3.0 g), and CH_3OH (0.9 g). The CH_2Cl_2 fraction was the only one active in the ER assay, and its ^1H NMR contained both high- and low-field resonances. A portion of the CH_2Cl_2 fraction (1.5 g) was applied to a Sephadex LH-20 silica column (sample in CH_2Cl_2) and eluted with CH_2Cl_2/CH_3OH to give eight fractions (no./wt in mg): S1 (84.3), S2 (47.8), S3 (48.8), S4 (56.6), S5 (115.1), S6 (371.9), S7 (43.4), S8 (9.4). The fraction S6 was next subjected to flash chromatography (silica) eluting with hexanes/EtOAc (80:20) to afford six fractions (no./wt in mg): S6F1 (6.9), S6F2 (177.4), S6F3 (3.2), S6F4 (119.4), S6F5 (33.6), S6F6 (5.1). A second flash chromatography run (silica) again eluting with hexanes/EtOAc (80:20) afforded three fractions (from S6F#/wt in mg): S6F4 (**3**) (80 mg); S6F4-II-F1 (**4**) (177 mg); and S6F2 (**5**) (80 mg).

(+)-(5*S*,8*S*,9*R*,10*S*)-20-Methoxypuupehenone (3): yellow oil; $[\alpha]_D^{24}$ +37° (*c* 1.52, MeOH); UV λ_{max} (CH_2Cl_2) 232 and 322 (ϵ 8800 and 6900, respectively); IR ν_{max} (film) 3200−3600, 2948, 1622, 1500, 1456, 1374 cm^{-1}; ^{13}C and ^1H data shown in Tables 1 and 2 and in the Supporting Information; HRFABMS found *m*/*z* 343.2289 $[M + H]^+$ ($C_{22}H_{30}O_3$, Δ1.6 mmu of calcd).

(+)-(5*S*,8*S*,10*S*)-20-Methoxy-9,15-ene-puupehenol (4): a rose-white power; $[\alpha]_D^{24}$ +41° (*c* 4.68, MeOH); UV λ_{max} (CH_2Cl_2) 234 and 324 (ϵ 16700 and 9500, respectively); IR ν_{max} (film) 3560 3420, 2950, 2920, 2870, 2840, 1625, 1600 cm^{-1}; ^{13}C and ^1H data shown in Tables 1 and 2 and in the Supporting Information; HRFABMS found *m*/*z* 342.2201 $[M^{\bullet}]^+$ ($C_{22}H_{30}O_3$, Δ0.6 mmu of calcd).

(+)-(5*S*,8*S*,9*R*,10*S*)-15,20-Dimethoxypuupehenol (5): colorless oil; ^{13}C and ^1H data shown in Tables 1 and 2 and in the Supporting Information; ESIMS(+) *m*/*z* 373 $[M - 2H]^+$; HRFABMS found *m*/*z* 374.2452 $[M + H]^+$ ($C_{23}H_{34}O_4$, Δ0.5 mmu of calcd).

Reaction of 15,20-Dimethoxypuupehenol (5) to Form 3 and 4. A small sample (<1 mg) of **5** was placed in 1 mL of CH_2Cl_2 and heated at 35 °C for 8 h. Evaporation gave an oil, which when analyzed by ^1H NMR showed it was a mixture of **3** (90%) and **4** (10%). Samples of **5** on prolonged standing in $CDCl_3$ were observed to give similar mixtures of **3** and **4**;

eventually $CDCl_3$ samples of **3** were completely converted to **4**. For data that illustrate this process see spectra S11A and S11B in the Supporting Information.

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Supporting Information Available: NMR spectra of **3**, **4**, and **5** (^1H, ^{13}C NMR, 2D NMR, and FAB^+MS) are available. This material is available free of charge via the Internet at http://pubs.acs.org.

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