Synechoxanthin, an Aromatic C_{40} Xanthophyll that Is a Major Carotenoid in the Cyanobacterium *Synechococcus* sp. PCC 7002

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A major aromatic, dicarboxylate carotenoid (>15% of total) was isolated from the euryhaline cyanobacterium *Synechococcus* sp. PCC 7002. This compound, which was given the common name synechoxanthin (**1**), has been assigned the structure (all-*E*) χ,χ-caroten-18,18′-dioic acid by a combination of spectroscopic (UV−vis, FT-IR, ^{1}H and ^{13}C NMR, LC-MS) and chemical methods. This discovery conclusively establishes that some cyanobacteria are capable of synthesizing aromatic carotenoids.

Aryl carotenoids were first discovered in the marine sponge *Reniera japonica*,[1] a brightly colored, orange-red sponge that grows in abundance in the Sea of Japan. Three bicyclic aromatic compounds, renieratene (χ,ϕ-carotene), isorenieratene (ϕ,ϕ-carotene), and renierapurpurin (χ,χ-carotene), were all initially isolated from this organism. The discovery of monocyclic ϕ,ψ-chlorobactene in a variety of green sulfur bacteria followed,[2] and monocyclic χ,ψ-okenone was subsequently identified as a common pigment in purple sulfur bacteria.[3] The bicyclic carotenoid ϕ,ϕ-isorenieratene was later proven to be identical to leprotene from mycobacteria;[4] it was first isolated from *Streptomyces mediolani* and is also produced by various other *Streptomyces* species.[5,6] Isorenieratene is also produced as a major carotenoid in brown-colored, green sulfur bacteria that synthesize bacteriochlorophyll *e*.[7,8] Because of their association with photosynthetic green and purple sulfur bacteria, aromatic carotenoids have become important biomarkers.[9,10] The diagenetic products of aromatic C_{40} carotenoids have been used as biomarkers to predict the occurrence of anaerobic environments in the ancient oceans.[11] Aromatic carotenoids have further been studied as models to understand the preservation pathways of sedimentary carbon.[12] The organisms previously known to synthesize aromatic carotenoids therefore include green sulfur bacteria, purple sulfur bacteria, and actinomycetes.[6,13] In contrast, cyanobacteria have long been assumed to be devoid of aromatic carotenoids.[14,15]

Here we describe the isolation and structural characterization of a previously unreported, aromatic carotenoid diacid from the cyanobacterium *Synechococcus* sp. PCC 7002. In recognition of its origin, we have given this compound the common name synechoxanthin. Synechoxanthin occurs as one of the major pigments (>15% of total carotenoids) in the wild-type strain of this organism; it is found in several other cyanobacteria, including the freshwater and soil organisms *Synechocystis* sp. PCC 6803 and *Nostoc* sp. PCC 7120.[16] Additionally, renierapurpurin was isolated in smaller quantities from *Synechococcus* sp. PCC 7002 cells grown into stationary phase.

Whole *Synechococcus* sp. PCC 7002 cells were extracted with acetone/methanol (7:2, v/v) with sonication. Examination of the crude extract by reversed-phase HPLC revealed a highly polar carotenoid compound that eluted much earlier than myxoxanthophyll from this organism (Figure 1). This compound, synechoxanthin (compound **1** in Figure 2), was isolated by extraction from whole cells in 70% (v/v) MeOH/H_2O, followed by extraction into CH_2Cl_2. The free acid was then purified by preparative HPLC. The free acid was treated with trimethylsilyldiazomethane in MeOH to form the dimethyl ester (**2**), which was repurified by preparative HPLC. Synechoxanthin was not reduced by $NaBH_4$, had no chemically modifiable hydroxyl groups, and was unaffected by saponification. Visible spectra, including λ_{max} and spectral fine structure (%III/II),[17] of the reduction ($LiAlH_4$) product (**3**) were consistent with that of renierapurpurin (**4**). The visible spectrum of **1** was similar to that of **3** and **4** but had a 4-nm red shift consistent with carbonyl functions at the 18,18′ positions (Figure 3).[13] LC-MS analysis of **1** gave a molecular mass of 588 Da, whereas compound **2** had a mass of 616 Da. FT-IR analysis of **2** showed that synechoxanthin dimethyl ester had bands at 1710, 1290, 1130, and 970 cm^{-1}. Similar bands are detected in methylbenzoate (1730, 1280, 1110, and 970 cm^{-1}) and are assigned to the C═O stretch, C−O stretches, and O═C−O bending vibrations of an aryl ester.[18]

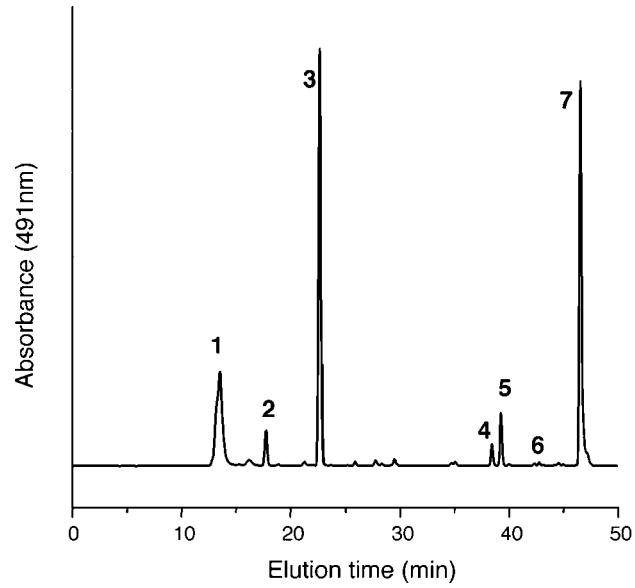

Figure 1. Chromatogram showing HPLC separation of pigments extracted from *Synechococcus* sp. PCC 7002 grown to early stationary phase: 1. synechoxanthin (compound **1**, Figure 2); 2. myxoxanthophyll; 3. zeaxanthin; 4. cryptoxanthin; 5. echinenone/chlorophyll *a*; 6. renierapurpurin (compound **4**, Figure 2); 7. β-carotene. Structures of all other compounds may be found in ref 16.

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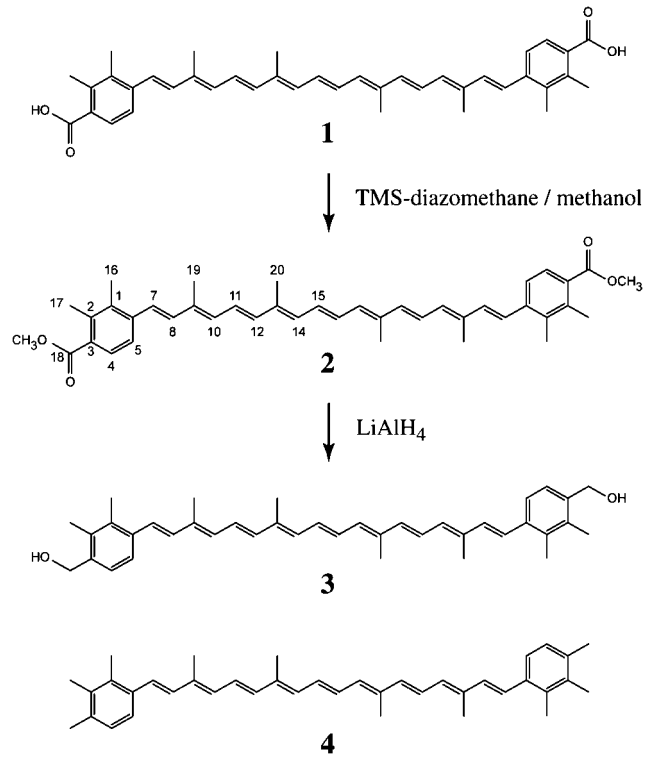

Figure 2. Structures of synechoxanthin [(all-*E*) χ,χ-caroten-18,18′-dioic] (**1**), synechoxanthin dimethyl ester [(all-*E*) dimethyl χ,χ-caroten-18,18′-dioate] (**2**), χ,χ-caroten-18,18′-diol (**3**), and renierapurpurin [(all-*E*) χ,χ-carotene] (**4**).

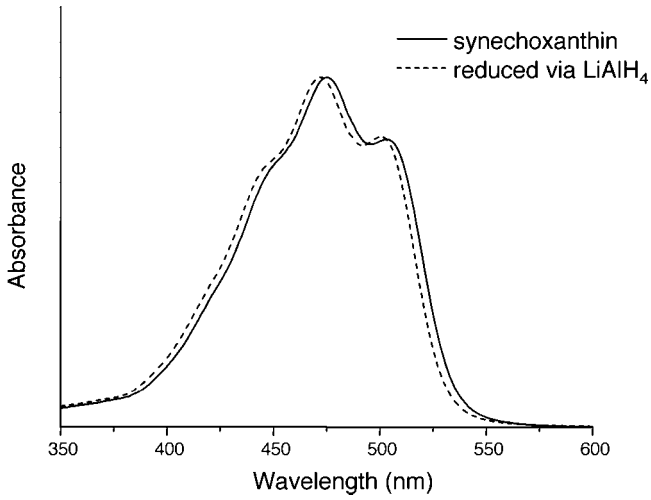

Figure 3. In-line UV−vis absorption spectra of synechoxanthin (solid line) and its $LiAlH_4$ reduction product, χ,χ-caroten-18,18′-diol (dashed line).

To elucidate the structure, ^1H NMR data were collected on the free acid (**1**), and ^1H and ^{13}C NMR data were collected on the dimethyl ester (**2**).

The one-dimensional ^1H NMR spectrum of **1** collected in DMSO-d_6 contained a broad peak at ~12.75 ppm (Supporting Information Figure S1), a shift that is characteristic of carboxylic acid protons. Saturation of the water line by rf irradiation at 3.55 ppm resulted in the disappearance of the peak at 12.75 ppm, consistent with its assignment to labile protons. The ^1H NMR spectrum of **2** in CD_2Cl_2 (Supporting Information Figure S2) displayed a total of five resolved methyl signals and nine signals downfield of 6 ppm and was therefore indicative of 2-fold symmetry. The covalent structure of **2** was established by homodecoupling experiments, rotating-frame

Table 1. ^1H and ^{13}C NMR Spectroscopic Data for Dimethyl χ,χ-Carotene-18,18′-dioate (**2**)

position	δ (ppm)	multiplicity and *J* (Hz)
H-4	7.57	d, $J(4,5) = 8.2$
H-5	7.40	d
H-7	6.89	d, $J(7,8) = 15.7$
H-8	6.83	d
H-10	6.39	m, $J(10,11) = 11.5$, $J(10,19) \approx 0.9$
H-11	6.73	dd, $J(11,12) = 14.8$
H-12	6.47	m, $J(12,20) < 0.9$
H-14	6.33	m, $J(14,15) \sim 11.9$
H-15	6.69	m, $J(15,15') \sim 14.3$
H-16	2.33	s
H-17	2.47	s
H-18	na	
H-19	2.08	d
H-20	2.01	d
H-18e	3.86	s
C-1	135.7	
C-2	138.2	
C-3	130.1	
C-4	127.6	
C-5	123.0	
C-6	140.7	
C-7	125.7	
C-8	137.2	
C-9	136.4	
C-10	134.3	
C-11	125.4	
C-12	139.2	
C-13	137.2	
C-14	133.7	
C-15	130.9	
C-16	16.0	
C-17	17.5	
C-18	169.3	
C-19	13.2	
C-20	13.1	
C-18e[a]	52.2	

[a] 18e refers to the ester methyl at C-18.

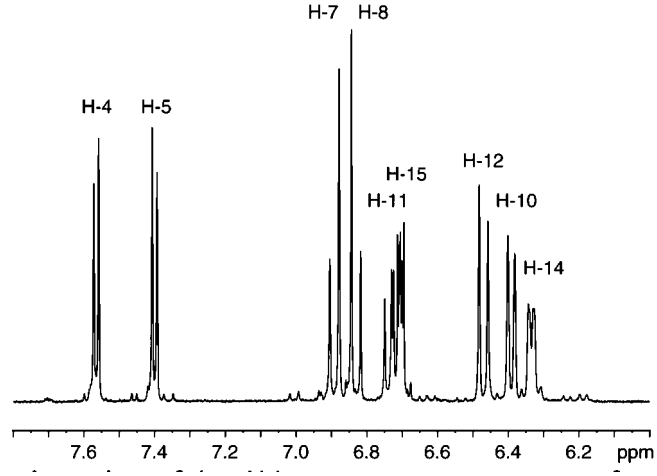

Figure 4. Vinylic-aromatic region of the 600 MHz proton spectrum of synechoxanthin dimethyl ester in CD_2Cl_2, 25 °C. The numbering corresponds to **2** in Figure 2; chemical shifts and *J* values are listed in Table 1. Additional NMR data are presented in the Supporting Information.

nuclear Overhauser effect (ROE) difference spectra, and $^1H-^{13}C$ correlation experiments (Supporting Information Figures S3−S18). Chemical shifts and coupling constants for **2** are listed in Table 1.

Figure 4 shows the aromatic and vinylic region of the ^1H spectrum. The downfield-shifted protons at 7.57 and 7.40 ppm are *J*-coupled to each other (~8 Hz) and to no other protons. The HMBC data (Supporting Information Figure S7) contained a correlation between 7.57 and 169 ppm, a shift consistent with a

carbonyl carbon. Long-range correlations to four additional quaternary carbons were observed from 7.57 and 7.40 ppm (Supporting Information Figure S8); these described an aromatic ring. A long-range correlation between the proton at 7.40 ppm and the carbon bearing the proton at 6.89 ppm placed the 7.40 ppm proton *ortho* to the polyene chain. Additional correlations placed methyl groups *ortho* and *meta* to the chain. Thus, the data indicated a χ substitution pattern of the benzene ring. The chemical shifts calculated for C-4 and C-5 with CH_3 at C-1, CH_3 at C-2, $COOCH_3$ at C-3, and polyene at C-6 of the benzene ring agreed with the observed values (Supporting Information Table S1 and Figure S20). The location of the methyl groups on the polyene chain (at C-9 and C-13) was determined with the HMBC data. Stronger coupling was detected through the double bond than the *s-trans* bond (Supporting Information Figure 9). These results unambiguously established the covalent structure of **2** as dimethyl χ,χ-carotene-18,18′-dioate (Figure 2). The all-*E* configuration was apparent through the *trans* $^3J_{H-H}$ coupling values and ROE effects (Supporting Information Figures S11−18). The *trans* geometry at the 15−15′ bond was supported by the 14.3 Hz vicinal coupling constant required in the simulation of the AA′BB′ spin system (Supporting Information Figure S19). $^3J_{H-H}$ coupling across the single 14−15 bond was 11.9 Hz, in agreement with trends observed in other carotenoids.[19] The UV−vis absorption spectrum and HPLC retention time were consistent with the all-*E* configuration. Furthermore, we have established that all-*trans* β-carotene is the biochemical precursor for the synthesis of synechoxanthin.[16,20]

Renierapurpurin (**4**) was identified as a minor compound that accumulated in cells grown to stationary phase. Its elution time by HPLC was similar to that of isorenieratene, although its visible absorption spectrum was nearly identical to that of the reduction product of synechoxanthin (**3**) and to the reported absorption spectrum of renierapurpurin.[21] The molecular mass of **4** was determined to be 528 Da. The ^{1}H NMR spectrum confirmed the structure of **4** as renierapurpurin (χ,χ-carotene). Renierapurpurin is presumed to be a precursor to synechoxanthin.

The demonstration that *Synechococcus* sp. PCC 7002 synthesizes the aromatic carotenoid synechoxanthin establishes that this cyanobacterium is the first organism proven to be capable of *de novo* production of a χ,χ-carotenoid. Although the presence of aromatic carotenoids has been reported in some marine sponges, the source of these compounds has usually been attributed to bacterial symbionts.[22] Recent work has identified an astounding array of sponge-associated cyanobacteria, including members of the genera *Synechoccoccus*, *Prochlorococcus*, and *Oscillatoria* among others.[23] Therefore, it is possible that cyanobacterial symbionts are the source of the χ,χ-carotene found in marine sponges.

Perhydro-renierapurpurin (renierapurpurane), a diagenetic product of χ,χ-carotenoids, has been used alongside other preserved carotanes as geochemical biomarkers for photosynthesis.[11,24] Because these compounds have generally been believed to be synthesized by photosynthetic sulfur bacteria, it has been suggested that these compounds could be used to identify ancient anaerobic environments.[11] Although monocyclic χ,ψ-carotenes have been isolated from purple sulfur bacteria,[13] no photosynthetic sulfur bacterium has yet been described that produces χ,χ-carotenes. The production of synechoxanthin by *Synechococcus* sp. PCC 7002 establishes that cyanobacteria can synthesize aromatic carotenoids, and this brings into question the origin of diagenetic products of renierapurpurin in the fossil record.[11,25]

Experimental Section

General Experimental Procedures. UV/vis spectra were recorded on a Genesis 10 spectrophotometer or were recorded in-line during HPLC by diode array detector (Agilent model G1315B). ^{1}H and inverse-detected ^{13}C data were collected on a Bruker DRX-600 spectrometer operating at a ^{1}H frequency of 600.18 MHz and equipped with a triple axis gradient TXI shielded probe. For mass analyses an Applied Biosystems Sciex 3200 Q-Trap LC/MS/MS system was used in either positive atmospheric pressure chemical ionization (APCI) mode or electrospray ionization (ESI) mode.

For NMR analyses, the sample of synechoxanthin was ~1.0 mM in DMSO-d_6, and the sample of synechoxanthin dimethyl ester was ~1.6 mM in CD_2Cl_2. 1D spectra for integration and *J* measurement were collected with a resolution of 0.18 Hz (32 k data points, spectral width of 6 kHz). The relaxation delay was 5 s. 1D-ROE (CAMELSPIN) difference spectra[26] were collected by inverting the signal of interest with a 32 ms sinc pulse. Locking was achieved with a succession of 180° pulses (10 kHz) sustained for 150 to 600 ms. A total of 32 transients were acquired for each frequency, and the relaxation delay was 4 s. Spectra were collected in pairs (on/off resonance). ^{1}H−^{13}C HMQC data[27] were collected in the phase-sensitive mode (TPPI[28]). Acquisition parameters were 4 k points and a spectral width of 5.3 kHz in the ^{1}H dimension and 278 real points and a spectral width of 23 kHz in the indirect dimension. The delay τ was 3.57 ms (short-range) or 50 ms (long-range). GARP decoupling[29] (80 μs) was applied to ^{13}C nuclei during acquisition. Relaxation time was 2 s. ^{1}H−^{13}C HMBC data[30] were collected with gradient selection, magnitude mode, with a τ delay of 3.57 ms. ^{1}H−^{13}C H2BC data[31] were collected in the phase-sensitive mode (echo−antiecho) with a third-order low-pass *J* filter. Acquisition parameters were 4 k points and a spectral width of 5.3 kHz in the ^{1}H dimension and 180 complex points and a spectral width of 23 kHz in the indirect dimension with GARP decoupling (80 μs) during acquisition. Relaxation time was 2 s. Data were processed with XWIN-NMR (Bruker, Biospin) and analyzed with Sparky.[32] Chemical shifts were referenced indirectly to $CHDCl_2$ at 5.32 ppm (^{1}H) and 53.8 ppm (^{13}C). Spinworks[33] was used to simulate the proton spectrum shown in Figure 4, in particular the 14−15−15′−14′ system.

Bacterial Material. *Synechococcus* sp. PCC 7002 strains were grown axenically at 38 °C in 30 mL tubes, or 12 L carboys with constant illumination by standard fluorescent lamps (250 μmol photons m^{-2} s^{-1}) and constant bubbling with 1% (v/v) CO_2 balanced with air. A *crtR* mutant,[20] deficient in the synthesis of 3,3′-hydroxy carotenoids, was used to produce samples for NMR experiments, because this mutant strain synthesizes fewer xanthophylls but produces more synechoxanthin than the wild type. Unless otherwise specified, cells were harvested in midexponential phase by centrifugation (20 min, 5500*g*, 4 °C) and frozen until required.

Isolation of Carotenoids. General precautions for work with carotenoids were taken.[34] For isolation of synechoxanthin, cells were extracted with 70% (v/v) MeOH/H_2O via sonication, then pelleted by centrifugation. The supernatant contained large amounts of synechoxanthin, but was largely devoid of other pigments. Synechoxanthin was then extracted into CH_2Cl_2, and fractions were pooled and dried under N_2 in glass. The pigment was resuspended and purified by preparative HPLC or treated with trimethysilyldiazomethane in MeOH to generate the diester (**2**) and then purified by preparative HPLC.

HPLC Separation. Pigments were separated by HPLC (Agilent model 1100) equipped with a diode array detector (model G1315B) and controlled with Agilent ChemStation software (Agilent Technologies, Palo Alto, CA) on a 25 cm by 4.6 mm, 5 μm Discovery C18 analytical column or a 25 cm by 10 mm semipreparative column (Supelco, Bellefonte, PA). A gradient elution method was used. Solvent A was H_2O/MeOH/CH_3CN (62.5:21:16.5) containing 10 mM NH_4OAc. Solvent B was 50% MeOH, 30% EtOAc, and 20% CH_3CN. The gradient was [time, %B][0, 20][10, 70][40, 100][50, 100]. Flow rates were 1 mL min^{-1} for the analytical column and 3.5 mL min^{-1} for the semipreparative column.

Synechoxanthin ((all-*E*) χ,χ-caroten-18,18′-dioic) (1): UV−vis (MeOH) λ_{max} 442 (sh), 470, 497 nm (%III/II = 4);[17] (pyridine) 463 (sh), 494, 522 nm (%III/II = 2); (DMSO) 463 (sh), 494, 522 nm (%III/II = 2); (HPLC system, 13 min) 447 (sh), 475, 503 nm (%III/II = 9); CIMS *m/z* 589 [M$^+$ + 1].

Synechoxanthin dimethyl ester ((all-*E*) dimethyl χ,χ-caroten-18,18′-dioate) (2): methyl ester of **1** produced by treatment of **1** with (trimethylsilyl)diazomethane in MeOH; UV−vis (HPLC system, 34 min) λ_{max} 451 (sh), 477, 503 nm (%III/II = 0); ^{1}H NMR (CD_2Cl_2, 600 MHz) see Table 1; HMQC correlations, H-4/C-4; H-5/C-5; H-7/C-7; H-8/C-8; H-10/C-10; H-11/C-11; H-12/C-12; H-14/C-14; H-15/C-15; H_3-16/C-16; H_3-17/C-17; H_3-18e/C-18e; H_3-19/C-19; H_3-20/C-20; H2BC correlations, H-4/C-5; H-5/C-4; H-7/C-8; H-8/C-7; H-10/C-11; H-11/C-10; H-11/C-12; H-12/C-11; H-14/C-15; H-15/C-14; HMBC

correlations, H_3-16 to C-1, C-2, C-6; H_3-17 to C-1, C-2, C-3; H-4 to C-2, C-6, C-18; H-5 to C-1, C-3, C-7; H-7 to C-5, C-9; H-8 to C-6, C-19, C-10; H_3-19 to C-8, C-9, C-10; H-10 to C-8, C-12, C-19; H-11 to C-13; H-12 to C-14, C-20; H_3-20 to C-12, C-13, C-14; H-14 to C-12, C-15′; H-15 to C-13, C-14′; 1D-ROE connectivities, H-4/H-5; H-5/H-8; H-7/H_3-16; H-7/H_3-19; H-8/H-10; H-10/H-12; H-11/H_3-19; H-11/H_3-20; H-12/H-14; H-15/H_3-20; H-14/H-15′; H_3-16/H_3-17; CIMS *m/z* 617 [$M^+ + 1$] (79), 585 (100), 571 (4), 553 (28), 439 (5).

(all-*E*) 18,18′-Dihydroxy-χ,χ-carotene (3): produced either from **1** or **2** by reduction with lithium aluminum hydride; UV−vis (HPLC system, 20 min) λ_{max} 446 (sh), 472, 500 nm (%III/II = 13); CIMS *m/z* 561 [$M^+ + 1$].

Renierapurpurin [(all-*E*) χ,χ-carotene] (4): minor product from *Synechococcus* sp. PCC 7002; UV−vis (benzene) λ_{max} 457 (sh), 484, 516 nm (%III/II = 20), (HPLC system, 42 min) λ_{max} 446 (sh), 472, 500 nm (%III/II = 12); ^{1}H NMR (CD_2Cl_2, 500 MHz) δ 7.25 (1H, d, J = 7.75 Hz, H-5), 6.96 (1H, d, J = 7.75 Hz, H-4), 6.89 (1H, d, J = 15.92, H-7), 2.30 (1H, s, Me, H-16), 2.28 (1H, s, Me, H-18), 2.20 (1H, s, Me, H-17), 2.07 (1H, s, Me, H-19); CIMS *m/z* 529 [$M^+ + 1$] (100), 409 (19), 396 (12), 277 (36).

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Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

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