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4,9-DIACETOXYUDOTEAL: A LINEAR DITERPENE ALDEHYDE FROM THE GREEN ALGA *HALIMEDA OPUNTIA*

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Key Word Index—*Halimeda opuntia*; marine green algae; phytochemistry; diterpenes; 4,9-diacetoxyudoteal; $^1H/^{13}C$ NMR

Abstract—A new linear diterpene aldehyde, 4,9-diacetoxyudoteal, was isolated from the marine green alga *Halimeda opuntia*, and its structure determined by spectral methods.

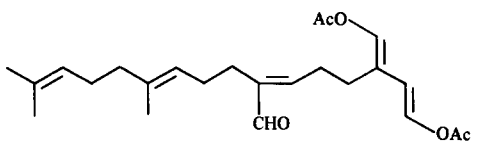
Marine green algae have proved to be a source of a variety of terpenoids and nitrogenous compounds [1]. Among these are a small group of linear sesqui- and diterpenes having in common a 1,4-diacetoxybuta-1,3-diene moiety [2–6]. Three of these, rhipocephalin [4], rhipocephenal [4] and udoteal (**1**) [6], were found to be toxic [4] to pomacentrid fishes and to induce feeding avoidance behavior in one such herbivorous species [4, 6]. In our continuing studies [7] directed towards the isolation of biologically-active compounds from marine organisms, we have isolated a more highly oxygenated form of udoteal (**1**), namely 4,9-diacetoxyudoteal (**2**), from the green alga *Halimeda opuntia* from Puerto Rico.

Chromatography of the chloroform extract of air-dried algae on Sephadex LH-20 followed by reverse-phase high-performance liquid chromatography afforded **2** (0.02% of dry wt) as an optically active, $[\alpha]_D - 16.6°$, unstable, colorless oil, which quickly turned light yellow if left exposed to air, and which was unstable to chromatography on silica gel. Although a molecular ion was not observed in the mass spectrum of **2**, a formula of $C_{28}H_{38}O_9$ was established from the exact mass of the highest ion observed, 458.2304 ($[M - HOAc]^+$; $C_{26}H_{34}O_7$; calculated 458.2305), when considered together with the presence of 28 signals in the ^{13}C NMR spectrum and four acetate resonances in the ^1H NMR spectrum. Carbonyl absorptions were observed at 1760, 1745 and 1685 cm^{-1}, compatible with enol acetate, alkyl acetate and α,β-unsaturated aldehyde or ketone groups.

The ^{13}C NMR spectrum, with carbon multiplicities determined by the attached proton test (APT) [8], confirmed the presence of an aldehyde group (δ 195.9, *d*), four acetate carbonyls (δ 168.6, 169.5, 171.4, 172.3, each *s*) and ten olefinic carbons (four –C= and six –CH=) which accounted for all ten degrees of unsaturation implied by the molecular formula. Hence an acyclic structure was inferred for **2**.

Comparison of the ^1H NMR spectrum of **2** in C_6D_6 vs $CDCl_3$ revealed that the proton resonances were more completely resolved in C_6D_6, and hence these values are recorded around structure **2** for discussion (see Experimental for data in $CDCl_3$). An *E,Z*-1,4-diacetoxybutadiene moiety (C-1, C-2, C-3, C-17) was deduced from the coupled doublets at δ 7.95 and 5.76 plus the singlet at 7.32, values nearly identical to those observed in $CDCl_3$ for this same moiety in **1** [6]. This assignment was substantiated by the observation of a nuclear Overhauser effect (NOE) between the H-2 and H-17 signals. Additionally, this NOE established the *Z*-configuration for the 3,17-double bond and infers a predominant *s-trans* orientation for C-2, C-3.

The C-4 to C-6 unit was established by the couplings confirmed for H-4 to H-6 (see **2**). The aldehyde group was positioned at C-7 to form the α,β-unsaturated carbonyl system indicated by the IR data (see above) and to account



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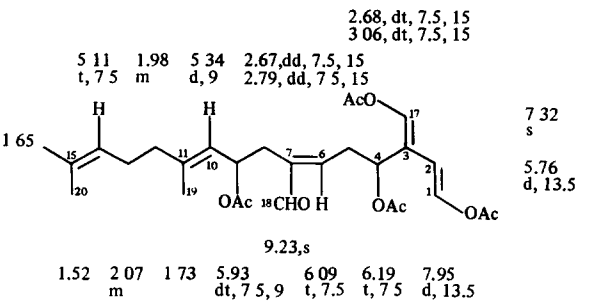

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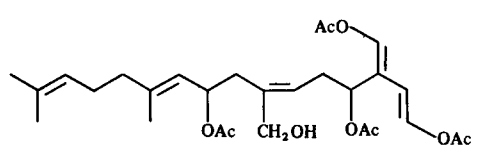

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for the low-field position of H-6 (δ 6.09 in C_6D_6; 6.39 in $CDCl_3$). A NOE was observed between the aldehyde proton and H-6, thus clearly establishing a 6*E* configuration.

The segment C-8 to C-11 plus C-19 was also deduced from proton interactions (confirmed by decoupling) shown with structure **2**. Irradiation of the δ 5.34 signal (H-10) sharpened markedly the δ 1.73 vinyl methyl signal, thus identifying which methyl resonance was associated with this trisubstituted double bond. Irradiation of this δ 1.73 methyl resonance produced a NOE for H-9, thus confirming a 10*E*-arrangement. Irradiation of H-10 (δ 5.34) simplified slightly, but unmistakably, a distorted 2H-triplet at δ 1.98 (H-12), thereby providing evidence for connectivity between the quaternary C-11 atom and the C-12 methylene group.

The terminal isopentenyl group follows from biogenetic considerations, from the confirmation of coupling between H-13 and H-14, and from nuclear Overhauser enhancement between H-16 (δ 1.65) and H-14. The C-12, C-13 connection was verified by decoupling of the corresponding proton resonances (skewed quartet and triplet, respectively).

Combination of the above partial structures gleaned from the ^{1}H NMR data yields structure **2** with the stereochemistry shown. Although no coupling or NOE enhancements were observed to indicate joining C-7 specifically to C-8 and C-4 to C-3, this bonding is required to account for the occurrence of the H-4 resonance (δ 6.19) as a triplet. Additionally, of course, the assigned arrangement provides a regular isoprenoid skeleton.

It is interesting to note that in the ^{1}H NMR spectrum of **2** in $CDCl_3$ the diastereotopic protons at C-8 appear as a sharp two-proton doublet in contrast to the distinct pair of double-doublets observed in C_6D_6; the diastereotopic protons at C-5 exhibit different chemical shifts in both solvents. H-6 is shifted upfield in C_6D_6 relative to $CDCl_3$ (δ 6.09 vs 6.39, respectively), while H-9 moved downfield in C_6D_6 relative to $CDCl_3$ (δ 5.93 vs 5.48, respectively).

Reduction of **2** with sodium borohydride in tetrahydrofuran at room temperature yielded the alcohol **3**, which showed IR absorptions at 1730 and 1740 cm^{-1} and contained an AB quartet in the ^{1}H NMR spectrum at δ 4.01 for the C-18 allylic alcohol methylene group. Also consistent with the reduction was the occurrence of the H-6 proton resonance at δ 5.54, in contrast to δ 6.09 in the aldehyde **2**. The H-8 protons in **3** exhibited diastereotopic behavior in both $CDCl_3$ and C_6D_6, whereas in **2** the double-doublet characteristic of the diastereotopic protons was observed only in C_6D_6.

EXPERIMENTAL

^{1}H NMR spectra were recorded on Nicolet 300 MHz and Varian XL-300 MHz spectrometers; ^{13}C spectra were recorded at 75 MHz on a Varian XL-300 spectrometer, all proton chemical shifts are reported in ppm (δ) downfield from internal TMS. HPLC separations were carried out using an Applied Science 5 μm C_{18}-column with a Waters Associates Model R401 differential refractometer.

Isolation of opuntiol (**2**). *Halimeda opuntia* was collected by hand at −1 to −3 m at La Parguera, Puerto Rico in June 1982. The sun-dried specimens (325 g) were ground to a coarse powder and extracted with $CHCl_3$ at room temp. for 7 days. Chromatography of this extract (2.86 g) on Sephadex LH-20 (450 g, 2″ × 3¼′) using $CHCl_3$–MeOH (1:1) yielded several dark-green fractions, followed by a light-yellow fraction (0.24 g) which contained **2**. Purification of a portion of this fraction (70 mg) by HPLC using a reverse-phase column (C_{18}) and MeOH–H_2O (80:20) as eluent afforded **2** (24 mg) as an unstable colorless oil: $[\alpha]_D$ −16.6° (*c* 2.5; $CHCl_3$); IR ($CHCl_3$) cm^{-1}: 3020, 2920, 2840, 2720, 1760, 1745, 1685, 1620, 1430, 1360, 1230, 1190, 1080, 1020, 930, UV $\lambda_{max}^{95\%\,EtOH}$ nm (ε): 254 (27000), 236 sh (23000); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.53 (*s*, 3H), 1.6 (*s*, 6H), 1.9 (*s*, 3H), 1.98 (*m*, 4H), 2.02 (*s*, 3H), 2.1 (*s*, 3H), 2.13 (*s*, 3H), 2.52 (*d*, 2H, *J* = 7.5 Hz), 2.68 (*dt*, 1H, *J* = 15, 7.5 Hz), 3.0 (*dt*, 1H, *J* = 15, 7.5 Hz), 4.98 (*t*, 1H, *J* = 7.5 Hz), 5.03 (*d*, 1H, *J* = 9 Hz), 5.48 (*dt*, 1H, *J* = 9, 7.5 Hz), 5.79 (*d*, 1H, *J* = 13.5 Hz), 5.94 (*t*, 1H, *J* = 7.5 Hz), 6.39 (*t*, 1H, *J* = 7.5 Hz), 7.25 (*s*, 1H), 7.58 (*d*, 1H, *J* = 13.5 Hz), 9.28 (*s*, 1H), ^{13}C NMR (CD_3OD): δ 195.9 (*d*, *J* = 171 Hz), 172.3 (*s*), 171.4 (*s*), 169.5 (*s*), 168.6 (*s*), 152.3 (*d*, *J* = 157 Hz), 142.7 (*s*), 141.8 (*s*), 139.1 (*s*), 136.4 (*d*, *J* = 190 Hz), 132.7 (*s*), 125 (*d*, *J* = 151 Hz), 124.1 (*d*, *J* = 158 Hz), 120 (*s*), 110.5 (*d*, *J* = 155 Hz), 71.8 (*d*, *J* = 146 Hz), 70 (*d*, *J* = 151 Hz), 40 (*t*, *J* = 125 Hz), 34.1 (*t*, *J* = 130 Hz), 30.5 (*t*, *J* = 130 Hz), 27.7 (*t*, *J* = 127 Hz), 26.4, 21.4, 20.9, 20.5 (2C), 18.2, 17.7 (all *q*'s); MS (70 eV) *m/z* (rel. int.): 458 $[M-HOAc]^+$ (1.7), 416 $[M-HOAc-CH_2CO]^+$ (3), 398 $[M-2HOAc]^+$ (6.5), 356 $[M-2HOAc-CH_2CO]^+$ (36.5), 338 $[M-3HOAc]^+$ (13.5), 296 $[M-3HOAc-CH_2CO]^+$ (19.7), 287 (18.4), 227 (33.9), 135 (100); high resolution mass measurement: $[M-HOAc]^+$ 458.2304; $C_{26}H_{34}O_7$ requires 458.2305.

Reduction of **2**. A saturated soln (0.4 ml) of $NaBH_4$ in THF was added over 1 hr to a stirred soln of 4,9-diacetoxyudoteal (7 mg) in THF at room temp, and the soln was stirred an additional hr. A few drops of H_2O were added. THF was removed under vacuum, and the residue was extracted with $CHCl_3$. Evaporation of the solvent left a residue which was purified by reverse phase HPLC to yield **3** (2.9 mg) as a colorless oil; $[\alpha]_D$ −25.1° (*c* 0.14; $CHCl_3$); IR cm^{-1}: 3550 (*br*), 1730, 1740; ^{1}H NMR ($CDCl_3$, 300 MHz). δ 1.6 (3H, *s*, H-16), 1.68 (3H, *s*, H-20), 1.69 (3H, *s*, H-19), 1.99, 2.07, 2.16, 2.19 (3H each, acetates), 2.04 (4H, *m*, H-12, 13), 2.26, 2.56 (1H each, *dd*, *J* = 7.5, 15 Hz, H-8, 8′), 2.42, 2.71 (1H each, *dt* each, *J* = 7.5, 7.5, 15 Hz, H-5, 5′), 5.06 (1H, *t*, *J* = 7.5 Hz, H-14), 5.16 (1H, *br d*, *J* = 9 Hz, H-10), 5.46 (1H, *t*, *J* = 7.5 Hz, H-6), 5.64 (1H, *dt*, *J* = 6, 6.9 Hz, H-9), 5.83 (1H, *d*, *J* = 13.5 Hz, H-2), 5.86 (1H, *t*, *J* = 7.5 Hz, H-4), 7.04 (2H, *br s*, H-18), 7.2 (1H, *s*, H-17), 7.6 (1H, *d*, *J* = 13.5 Hz, H-1); C_6D_6: δ 1.52 (3H, *s*, H-20), 1.65 (3H, *s*, H-16), 1.72 (3H, *s*, H-19), 1.57, 1.62, 1.70, 1.71 (3H each, *s*, acetates), 1.97, (2H, *m*, H-12), 2.08 (2H, *m*, H-13), 2.62 (1H, *dt*, *J* = 7.5, 7.5, 15 Hz, H-5), 2.90 (1H, *dt*, *J* = 7.5, 7.5, 15 Hz, H-5′), 2.67, 2.83 (1H each, *dd* each, *J* = 7.5, 15 Hz, H-8, 8′), 4.02, 3.99 (2H, *d* each, *J* = 15 Hz, H-18, 18′), 5.12 (1H, *t*, *J* = 7.5 Hz, H-14), 5.36 (1H, *d*, *J* = 9 Hz, H-10), 5.83 (1H, *d*, *J* = 13.5 Hz, H-2), 5.99 (1H, *dt*, *J* = 6, 6, 9 Hz, H-9), 6.22 (1H, *t*, *J* = 7.5 Hz, H-4), 7.31 (1H, *s*, H-17), 7.94 (1H, *d*, *J* = 13.5 Hz, H-1); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 16.71, 17.54, 20.50 (2C), 20.82, 21.15, 25.52 (all *q*), 36.13, 31.12, 33.71, 39.33, 66.86 (all *t*), 69.11 (*d*), 70.46 (*d*), 109.3 (*d*), 118.89 (*s*), 122.81 (*d*), 122.88 (*d*), 123.58 (*d*), 131.74 (*s*), 134.10 (*d*), 136.96 (*d*), 137.99 (*s*), 140.94 (*s*), 167.35 (*s*), 167.98 (*s*), 170.23 (*s*), 170.46 (*s*); MS (70 eV) *m/z* 400 $[M-120]^+$.

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THE EFFECT OF CHLOROPHYLL-BLEACHING HERBICIDES ON GROWTH, CAROTENOID AND DIOSGENIN LEVELS IN CELL SUSPENSION CULTURES OF *DIOSCOREA DELTOIDEA*

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Key Word Index—*Dioscorea deltoidea*; Dioscoreaceae; suspension cultures; diosgenin production; phenylpyridazinone herbicides; norflurazon; amitrole; difunon.

Abstract—Bleaching herbicides of the phenylpyridazinone group (norflurazon, metflurazon, SAN 9774 and SAN 9785) and difunon and amitrole were added to cell suspension cultures of *Dioscorea deltoidea* at 1 μM. Difunon inhibited growth and carotenoid biosynthesis and metflurazon inhibited growth. Norflurazon was the only herbicide tested that influenced diosgenin production. Norflurazon increased the rate of diosgenin biosynthesis so that 180 mg/l were obtained after 14 days of incubation as compared to 30 days for the control to reach the same level.

INTRODUCTION

Diosgenin is used in the commercial production of female steroid hormones and steroidal pharmaceuticals. The highest level of the steroidal sapogenin diosgenin, are found in cell suspension cultures of *Dioscorea deltoidea* A-51 when growth has ceased [1–3]. The early biosynthetic precursors of diosgenin are common to many other metabolites (phytol, carotenoids, chlorophyll, terpenes, etc.) [4] and we surmised that inhibiting the formation of one or more of these metabolites might increase the level of diosgenin.

Some herbicides of the phenylpyridazinone group (metflurazon and norflurazon) have been shown to inhibit carotenoid biosynthesis in higher plants [5–7], while others inhibit photosynthetic electron transport [7]. The primary action of norflurazon, metflurazon [6] and difunon [8] is to inhibit the enzyme phytoene synthetase [6]. Amitrole [9] and difunon also have bleaching effects. Amitrole has been shown to inhibit cyclisation of lycopene to α- and β-carotene [9]. The herbicides used are shown in Fig. 1.

RESULTS AND DISCUSSION

Effects of various herbicides on growth and carotenoid biosynthesis in D. deltoidea *suspension cultures*

Different herbicides were added to cell suspensions of *D. deltoidea* on the second day of growth (Fig. 2). Difunon and norflurazon inhibited growth four days after addition and upon further incubation there was a decrease in biomass (Fig. 2a). Amitrole did not inhibit growth. Norflurazon caused a total inhibition of carotenoid biosynthesis three days after addition and difunon five days after addition (Fig. 2b). Amitrole also inhibited carotenoid biosynthesis five days after addition and after that the carotene concentration decreased. These results show that the herbicides were able to influence both growth and carotenoid biosynthesis in cell suspension cultures of *D. deltoidea*.

Other phenylpyridazinones were added to cell suspensions of *D. deltoidea* on the second day of growth and their influence on growth was measured on the fourth and tenth day (Table 1). SAN 9758 did not influence growth.