Evidence for de Novo Biosynthesis of the Polyketide Fragment of Diaulusterol A by the Northeastern Pacific Dorid Nudibranch *Diaulula sandiegensis*

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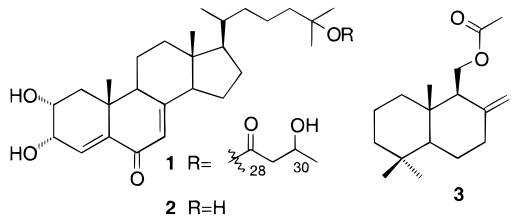
Stable isotope incorporation experiments using sodium [1,2-$^{13}C_2$]acetate have demonstrated that the 3-hydroxybutyrate substituent of diaulusterol A (**1**) is biosynthesized de novo by the dorid nudibranch *Diaulula sandiegensis*. There was no evidence for acetate incorporation into the steroid portion of **1**, nor were radiolabeled mevalonate or cholesterol incorporated.

Skin extracts of many dorid nudibranchs contain structurally novel secondary metabolites.[1] It has often been proposed, and in some instances experimentally verified, that these secondary metabolites play a role in protecting the shell-less mollusks from predation.[2] Most nudibranch skin extract secondary metabolites are sequestered unchanged from dietary sponges, coelenterates, or bryozoans.[2,3] Several nudibranch species in the sub-order Doridacea have been shown to be capable of de novo biosynthesis of the terpenoid and polyketide secondary metabolites found in their skin extracts.[4,5] The ability to make defensive allomones de novo should increase the range of habitat available to a particular dorid species by eliminating dietary dependence on "secondary metabolite rich" prey organisms.

The dorid nudibranch *Diaulula sandiegensis* is commonly encountered in rocky subtidal habitats along the western coast of North America from Alaska to Cabo San Lucas.[6] Skin extracts of specimens collected at many different sites in British Columbia (BC), extending from the Alaskan border to the southern end of Vancouver Island, have consistently yielded two steroidal metabolites, diaulusterol A (**1**) and B (**2**).[7] Specimens of *D. sandiegensis*



collected in southern California have yielded a series of chlorinated acetylenes that are not present in BC specimens.[8] Recently, trace amounts of **2** were found in Californian specimens of *D. sandiegensis*.[9]

The observation that specimens of *D. sandiegensis* collected at numerous geographically diverse sites in BC always contained diaulusterols A (**1**) and B (**2**), along with a failure to find a dietary source for the compounds, suggested that sterols **1** and **2** were being made de novo by *D. sandiegensis*.[5,10] Diaulusterol A (**1**) has a number of structural features that are of biosynthetic interest. These include the 2α,3α-dihydroxy substituents, the cross-conjugated ketone at C-6, and the 3-hydroxybutyrate substituent at C-25 of the steroidal skeleton. Prompted by the structural novelty of diaulausterol A (**1**) and an interest in the ecological implications of de novo biosynthesis by dorid nudibranchs, precursor incorporation studies were undertaken with *D. sandigensis*.

Stable isotope incorporation experiments with *D. sandiegensis* followed the general protocol used by our group in recent investigations of terpenoid and polyketide biosynthesis in dorid nudibranchs.[5] Independent feeding experiments using [1,2-$^{13}C_2$]acetate were conducted in the months of April, August, and December in order to control for seasonal effects. For each experiment, specimens of *D. sandiegensis* were collected by scuba on rocky reefs in Barkley Sound, BC, and transported back to Vancouver where they were maintained in Barkley Sound seawater at 12 °C. Throughout a period of 17 days following collection, seven feedings of precursor were administered to each nudibranch via syringe injection through its dorsum into the hepatopancreas. At the end of this period, the nudibranchs were removed from the aquarium seawater and immersed whole in MeOH to generate a skin extract. The concentrated MeOH skin extract was partitioned between H_2O and EtOAc. Fractionation of the EtOAc soluble materials via sequential application of silica gel flash chromatography and reversed-phase HPLC gave pure diaulusterol A (**1**). Due to the limited number of animals used in each feeding experiment, it was not possible to isolate significant amounts of diaulusterol B (**2**), a minor metabolite usually found in only 15% of the yield of **1**.[7]

Examination of the ^{13}C NMR spectra of diaulusterol A (**1**) isolated from each of the April, August, and December acetate feeding experiments revealed the same pattern of incorporation in all instances. The complete absence of flanking doublets around the ^{13}C resonances assigned to the steroid fragment of diaulausterol A (**1**) indicated that either no intact acetate incorporation had occurred in this portion of the molecule or that the levels of incorporation were below the detection limits of the experiment. However, intense doublets and more complex multiplets were observed flanking all four of the ^{13}C resonances assigned to the 3-hydroxybutyrate substituent (Figure 1), indicating that this portion of diaulusterol (**1**) had been synthesized de novo by *D. sandiegensis*. Analysis of the coupling constants measured for the doublets flanking the side chain resonances showed that intact acetate units had been incorporated at C-28/C-29 (J = 57.0 Hz) and C-30/C-31 (J = 39.4 Hz) as expected for a simple diketide fragment. The

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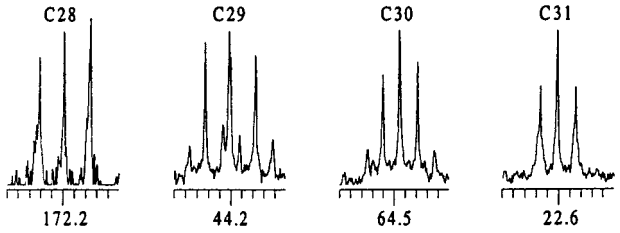

Figure 1. ^{13}C NMR resonances (125 MHz, C_6D_6) for selected carbons of diaulusterol A (**1**) labeled with sodium [1,2-$^{13}C_2$]acetate. All expansions span a 1.00 ppm range and are normalized to a common central peak height and then truncated for ease of viewing.

specific incorporation for this molecular fragment was found to be 3.0%.

The complex multiplets flanking the C-29 and C-30 resonances (Figure 1) can be attributed to the presence of individual molecules of **1** having incorporated intact acetate units at both the C-28/C-29 and C-30/C-31 positions. This is consistent with an earlier quantitative analysis of [1,2-$^{13}C_2$]acetate incorporation into luteone by the nudibranch *Cadlina luteomarginata*, where it was found that only a small percentage of the isolated molecules were synthesized during the labeling experiment but these new molecules have high specific incorporations of precursors.[5e] Under these circumstances, there is a relatively high probablity of two intact acetate units being adjacent to each other in individual molecules of the biosynthetic product leading to more complex coupling patterns.

The stable isotope feeding experiments described above have clearly shown that the polyketide side chain of diaulusterol A (**1**) is biosynthesized de novo by *D. sandiegensis*. However, these feeding experiments failed to provide any evidence for the origin of the steroidal skeleton of **1**. There are several possible explantions for the lack of incorporation of labeled acetate into the steroidal skeleton. The simplest explantion is that the feeding experiments were simply not carried out in a fashion conducive to incorporation of acetate into the steroid fragment. Similar difficulties were observed in labeling studies of albicanyl acetete (**3**), the major metabolite the dorid *Cadlina luteomarginata*. In a series of feeding experiments carried out at various times of the year, high specific incorporations of labeled acetete were consistently observed in the acetyl residue of **3**, but only during egg laying season were detectable levels of incorporation observed in the terpenoid fragment.[5e]

Internal regulation in the biosynthetic pathway leading to diaulusterol A (**1**) may have prevented incorporation of acetate into the steroid portion. Steroid biosynthesis is known to be controlled and limited at the step of conversion of 3-hydroxy-3-methylglutaryl (HMG) CoA to mevalonate. Regulation of the enzyme HMG-CoA reductase occurs in vertebrate metabolism and is believed to operate in invertebrates as well.[11] Therefore, the uptake of the early biosynthetic precursor acetate may have been too inefficient for ^{13}C NMR detection. To test for this possibility, specimens of *D. sandiegensis* were injected with 1.85 MBq of [2-^{14}C]mevalonate. Diaulusterol A (**1**) isolated from this experiment failed to exhibit radioactivity above background levels.

An alternate explanation for the lack of acetate and mevalonate labeling in the steroid fragment of **1** would be that diaulusterol B (**2**) is sequestered from a dietary source and simply acylated by *D. sandiegensis*. However, no dietary source of **2** has been found. A slightly more complex explanation would involve conversion of common sterols such as cholesterol present in the diet of *D. sandiegensis* into the functionalized diaulusterol steroid fragment followed by acylation. These last two possibilities assume a mixed dietary and de novo biosynthetic origin for diaulusterol A (**1**), which from an evolutionary perspective is somewhere between the unaltered sequestration and total de novo biosynthetic strategies employed by other dorid nudibranchs to generate their skin extract secondary metabolites. Thus, [4-^{14}C]cholesterol (1.85 MBq) was fed to specimens of *D. sandiegensis* and **1** isolated. Scintillation counting of purified **1** demonstrated that cholesterol was not incorporated within detectable levels.

The positive incorporation of labeled acetate into the 3-hydroxybutyrate fragment of diaulusterol A (**1**) indicates that *Diaulula sandiegensis* performs at least some aspects of de novo biosynthesis in the production of **1**. *D. sandiegensis* joins a growing list of dorid nudibranchs that are capable of de novo biosynthesis of secondary metabolites. Interestingly, all of these species are found in temperate ocean waters where there is a smaller diversity of prey species and where the secondary metabolism of the available prey species is not as rich as in typical tropical prey species.

Experimental Section

General Experimental Procedures. ^1H and ^{13}C NMR spectra were recorded in C_6D_6 on a Bruker AMX-500 spectrometer at 500 and 125 MHz, respectively. Chemical shifts are reported in ppm downfield from TMS. Scintillation counting was conducted on a Beckman LS6000IC instrument.

Stable Isotope Feeding Experiments. Specimens of *D. sandiegensis* (April: 27 animals; August: 22 animals; December: 18 animals) were collected by scuba from several reefs at −1 to −15 m in Barkley Sound, BC, and transported to Vancouver in chilled seawater. The nudibranchs were kept without food in 12 °C Barkley Sound seawater for a period of 17 days. During this time seven 100−200 μL doses of 550 mM sodium [1,2-$^{13}C_2$]acetate in distilled water was administered to the hepatopancreas of each nudibranch by syringe injection through the left dorsum.

Isolation of Diaulusterol A (1). After 17 days the animals were extracted whole with MeOH (2×) and MeOH/CH_2Cl_2 1:1 (4×). The extracts were combined and reduced in vacuo, and the resulting residue was partitioned between H_2O (200 mL) and EtOAc (3 × 200 mL). The EtOAc layers were combined, dried over anhydrous magnesium sulfate, and concentrated in vacuo. Flash silica gel column chromatography (eluent: acetone/CH_2Cl_2 1:1) followed by reversed-phase HPLC (eluent: MeOH/H_2O 4:1) of the EtOAc soluble materials gave pure diaulusterol A (**1**) (2−10 mg per stable isotope feeding experiment).

Calculation of Specific Incorporation. The peak areas of central singlets and flanking multiplets were determined by integration using Bruker WIN NMR. Percent specific incorporation was calculated for C-28 using the following formula:

$$\% \text{ si} = 1.1\% \times \frac{\text{(peak area of flanking doublets)}}{\text{(peak area of central singlet)}}$$

The C-29 and C-31 resonances were overlapped by the C-13 and C-15 resonances, respectively, and the C-30 resonance was complicated by the presence of a triplet (as well as the expected doublet and singlet), which overlapped with the central singlet. Therefore, the specific incorporation of acetate into the 3-hydroxybutyrate moiety was based upon the calculations for C-28.

Radioactive Isotope Feeding Experiments. [2-^{14}C]-Mevalonate as Precursor. Three specimens of *D. sandiegensis* were collected in September 1997 in Barkley Sound and transported to Vancouver as above. On the third, fifth, seventh, and ninth days following collection, each specimen was injected with 100 μL of [2-^{14}C]mevalonate solution, which

consisted of 1.85 MBq of [2-^{14}C]mevalonate *N,N,N′,N′*-dibenzylethylenediamine (DBED) salt, dissolved in 1.2 mL of distilled H_2O. On the eleventh day, the specimens were immersed in MeOH. Extracts of *D. sandiegensis* from this experiment were spiked with 0.8 mg of pure unlabeled diausterol A (**1**), and isolation was conducted as described above. After one round of purification by HPLC, a 1.0% aliquot of purified **1** (1.2 mg total) displayed scintillation counts comparable to blank samples (36 DPM vs 20−100 DPM background).

[4-^{14}C]Cholesterol as Precursor. Three specimens of *D. sandiegensis* were collected and transported as above. On the third, fifth, and seventh days following collection, each specimen was injected with 50 μL of [4-^{14}C]cholesterol solution, which consisted of 1.85 MBq of [4-^{14}C]cholesterol dissolved in 50 μL of DMSO and diluted with 400 μL of H_2O. By the ninth day following collection, one nudibranch had died and was discarded. The two remaining specimens were immersed in MeOH, the extract was spiked with 0.8 mg of pure unlabeled **1**, and isolation of **1** was performed as described above. After one round of purification by HPLC, a 1.0% aliquot of purified **1** (0.5 mg total) produced scintillation counts within the range of blank samples (95 DPM vs 20−100 DPM background).

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References and Notes

(1) Faulkner, D. J. *Nat. Prod. Rep.* **1997**, *14*, 259−302 and previous reviews cited therein.
(2) Faulkner, D. J. Chemical Defenses of Marine Molluscs. In *Ecological Roles of Marine Natural Products*; Paul, V. J., Ed.; Comstock Publishing Associates: Ithaca, NY, 1992; pp 119−163.
(3) Schulte, G. R.; Scheuer, P. J. *Tetrahedron* **1982**, *38*, 1857−1863.
(4) (a) Cimino, G.; de Rosa, S.; de Stephano, S.; Sodano, G.; Villani, G. *Science* **1983**, *219*, 1237−1238. (b) Cimino, G.; Sodano, G. Biosynthesis of Secondary Metabolites in Marine Molluscs. In *Topics in Current Chemistry*; Scheuer, P. J., Ed.; Springer-Verlag: Berlin, 1993; Vol. 167, pp 77−115.
(5) (a) Graziani, E. I.; Andersen, R. J. *J. Am. Chem. Soc.* **1996**, *118*, 4701−4702. (b) Graziani, E. I.; Andersen, R. J.; Krug, P. J.; Faulkner, D. J. *Tetrahedron* **1996**, *52*, 6869−6878. (c) Graziani, E. I.; Andersen, R. J. *J. Chem. Soc., Chem. Commun.* **1996**, 2377−2378. (d) Kubanek, J.; Andersen, R. J. *Tetrahedron Lett.* **1997**, *38*, 6327−6330. (e) Kubanek, J.; Graziani, E. I.; Andersen, R. J. *J. Org. Chem.* **1997**, *62*, 7239−7246.
(6) Behrens, D. W. *Pacific Coast Nudibranchs*, 2nd ed.; Sea Challengers: Los Osos, California, 1991.
(7) Williams, D. E.; Ayer, S. W.; Andersen, R. J. *Can. J. Chem.* **1986,** *64*, 1527−1529.
(8) Walker, R. P.; Faulkner, D. J. *J. Org. Chem.* **1981**, *46*, 1475−1478.
(9) Kubanek, J.; Andersen, R. J.; Faulkner, D. J. Unpublished results.
(10) Faulkner, D. J.; Molinski, T. F.; Andersen, R. J.; Dumdei, E. J.; de Silva, E. D. *Comp. Biochem. Physiol.* **1990**, *97C*, 233−240.
(11) Voogt, P. A. Lipids: Their Distribution and Metabolism. In *The Mollusca*; Hochachka, P. W., Ed.; Academic Press: New York, 1983; Vol. 1, pp 329−370.

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