An Enzyme-Based Formaldehyde Assay and Its Utility in a Sponge Sterol Biosynthetic Pathway

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An enzyme-based assay has been developed and utilized to confirm the production of formaldehyde in the dealkylation of sterol side chains in a marine sponge. The enzyme used in the assay, formaldehyde dehydrogenase, is NAD^+ dependent, and the assay measures the production of NADH by determining the increase in fluorescence at 460 nm.

Sponges are responsible for the production of numerous novel sterols and are known to be capable of producing sterols by a variety of metabolic pathways.[1,2] Sponges can incorporate sterols from their diet, are capable of de novo cholesterol biosynthesis, and are capable of both side-chain alkylation and dealkylation. This metabolic plasticity in sterol biosynthesis appears to be unparalleled.[1]

Prior to the discovery of sterol side chain dealkylation in sponges, insects were thought to be the only group of organisms capable of this transformation.[3] A mechanism was proposed by Ikekawa[3] and, as shown in Scheme 1, is believed to involve the production of a 24(28) epoxide (**1a, b**) and loss of the C-24 alkyl substituent as an aldehyde. More recent work has indicated that a similar pathway operates in sponges.[4,5] In sponges, it has been shown that 24-alkyl sterols with 24(28) unsaturation (**2a**,**b**) were transformed to their epoxides (**1**), desmosterol (**3**) and cholesterol (**4**); epoxides **1a** and **1b** were transformed to **3** and **4**; and desmosterol was reduced to cholesterol.[4,5] On the basis of these observations, it seems reasonable to suggest that the alkyl group is lost in the form of an aldehyde (formaldehyde for **1a** and acetaldehyde for **1b**). However, there is no experimental documentation of the production of either of these aldehydes from insects or sponges. This paper describes the use of an enzyme-based assay for the determination of trace quantities of formaldehyde produced from the incubation of a cell-free extract of the sponge *Amphimedon compressa* (syn. *Haliclona rubens*, Niphatidea, Duchassaing & Michelotti, 1854) with 24-methylenecholesterol (**2a**).

Our goal was to develop an assay that could detect the presence of trace amounts of formaldehyde present in a sponge cell-free extract that had been incubated with a phytosterol. There are numerous methods available for the determination of formaldehyde such as the Nash reaction[6] in which acetylacetone yields 3,5-diacyl-1,4-dihydrolutidine upon reaction with ammonia and formaldehyde. The yield of this pyridine derivative is determined by measuring the intensity of absorbance at 412 nm. While this and other methods[7] have proven to be of value for formaldehyde detection, we felt that our sponge cell-free extract would be a sufficiently complex mixture that methods such as the Nash reaction would not be suitable.

Formaldehyde dehydrogenase is a specific nicotinamide adenine dinucleotide (NAD)-linked dehydrogenase, with a requirement for glutathione (GSH).[8] This enzyme facilitates the oxidation of formaldehyde to *S*-formylglutathione, which in microsomal systems, is subsequently hydrolyzed by *S*-formylglutathione hydrolase. NADH, the reduction product of the co-enzyme, is fluorescent, and thus, the amount of formaldehyde present in solution can be determined by conducting this reaction and then measuring the increase in fluorescence. This enzymatic process thus offers the potential for a selective and sensitive assay for formaldehyde as a tool in elucidating biosynthetic pathways.

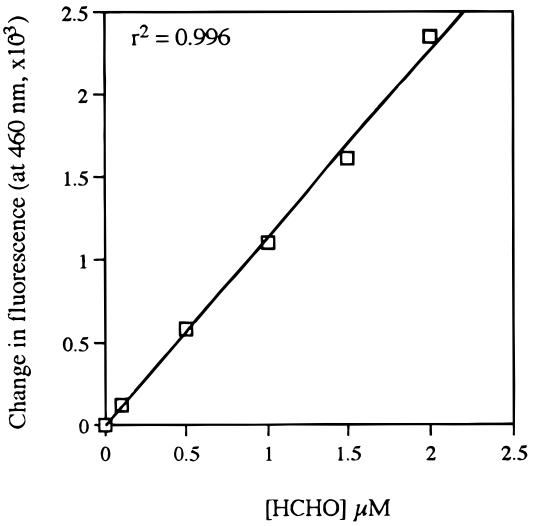

Figure 1. Fluorescence measurements.

Our formaldehyde assay was conducted by incubating a formaldehyde solution with reduced glutathione, NAD, and formaldehyde dehydrogenase in a phosphate buffer at pH 8.0. The increase in fluorescence was measured using a spectrofluorimeter with excitation at 350 nm and emission at 460 nm. To evaluate this assay, the increase in fluorescence was determined at different concentrations of formaldehyde. As is evident from Figure 1, there is a linear response of change in fluorescence to increased formaldehyde concentration over the range used.

To identify formaldehyde as a product of sterol side chain dealkylation in a sponge, 24-methylenecholesterol was incubated with a cell-free extract of the sponge and this mixture immediately subjected to the assay as described above. A local sponge, *A. compressa*, had proven to be capable of sterol side chain dealkylation[9] and was selected for this study. The sponge enzyme extract was prepared by cutting freshly collected sponge into ca. 1 cm^3 pieces and then homogenizing with a Tris/TES buffer (pH 7.8) and

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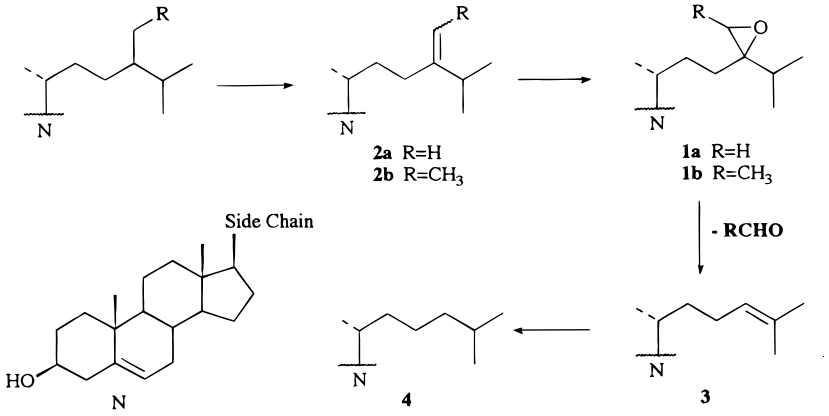
Scheme 1



liquid nitrogen in a Waring blender. Centrifugation (5000*g*) to remove insoluble debris resulted in a viable cell-free extract.

24-Methylenecholesterol (**2a**) was incubated with the sponge cell-free extract for 4 h and the resulting mixture subjected to the formaldehyde assay. The assay resulted in an increase in fluorescence at 460 nm of 0.5, corresponding to a 0.4 μM solution of formaldehyde (Figure 1).

A number of controls were performed to confirm that the observed increase in fluorescence was due to an enzymatic formation of formaldehyde during the incubation of 24-methylenecholesterol with the crude sponge enzyme extract. First, due to the complex nature of the sponge extract, background fluorescence levels were established. To achieve this, the untreated sponge cell-free extract was incubated with formaldehyde dehydrogenase, reduced glutathione, and NAD. This resulted in an increase in fluorescence of 0.07. Second, an incubation of 24-methylenecholesterol was conducted with the sponge cell-free extract that had been heated at 100 °C for 1 h, and subsequently this mixture was subjected to the formaldehyde assay. Again, the observed change in fluorescence was only 0.06. Further, to confirm that the observed increase in fluorescence was due to the action of formaldehyde dehydrogenase, the formaldehyde assay was performed with the sponge incubation mixture in the absence of FDH, resulting in a change in fluorescence of 0.04.

The significantly greater increase in fluorescence observed with the experimental conditions relative to that of the controls demonstrates for the first time the production of formaldehyde in sterol side-chain dealkylation. The described assay thus provides a sensitive and selective tool for formaldehyde detection and confirms the originally postulated mechanism shown in Scheme 1. The assay should prove to be of general utility in other complex systems.

Experimental Section

General Experimental Procedures. Formaldehyde dehydrogenase [EC 1.2.1.1], nicotinamide adenine dinucleotide (NAD), reduced glutathione (GSH), dithiothreitol (DTT), ethylenediamine tetraacetic acid (EDTA), bovine serum albumin (BSA), leupeptin, pepstatin A, and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma Chemical Co. Fluorescence measurements were recorded on a Perkin-Elmer LS50 spectrometer.

Cell-Free Extract Preparation of *A. compressa*. *A. compressa* was collected off the coast of Boca Raton, FL, at a depth of −15 m. A voucher specimen has been depositied at Florida Atlantic University. The sponge was maintained in aerated seawater during transit to the laboratory. A 100 g portion of the sponge was cut into ca. 1 cm^3 pieces and added to a blender containing liquid nitrogen with 200 mL of a Tris/TES buffer, at pH 7.8, containing 5 mM DTT, 5 mM EDTA, 10% BSA, 100 μg leupeptin, 100 μg pepstatin A, and 1 mM PMSF, and the mixture homogenized for 5 min. The homogenate was centrifuged at 5000*g* for 15 min, and the supernatant was stored at −80 °C.

Incubation of 24-Methylenecholesterol with the Cell-Free Extract of *A. compressa*. The cell-free extract of *A. compressa* (10 mL) was thawed and incubated with 24-methylenecholesterol (4 mg, 0.01 mmol) in a shaker water bath at 30 °C for 4 h. The mixture was maintained at 4 °C for 0.5 h and then subjected to the formaldehyde assay.

Assay with Formaldehyde Dehydrogenase. The formaldehyde assays were performed at 25 °C by preparing a solution of formaldehyde dehydrogenase (50 international units) in 0.5 mL of a solution of 33 mM Na_2HPO_4 (pH 7.5), 2 mM GSH, and 1 mM NAD. Fluorescence measurements were taken by adding 10 μL of FDH solution to 2.25 mL buffer (with GSH and NAD) and subsequently adding 0.25 mL of test solution (either a HCHO standard or sponge incubation mixture). Excitation was at 350 nm, and emission was measured at 460 nm.

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