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ISOLATION OF THE NICKEL-CHLORIN CHELATE TUNICHLORIN FROM THE SOUTH PACIFIC OCEAN SEA HARE *DOLABELLA AURICULARIA*

GEORGE R. PETTIT,* DARKO KANTOCI, DENNIS L. DOUBEK, BRUCE E. TUCKER,

Cancer Research Institute and Department of Chemistry, Arizona State University, Tempe, Arizona 85287-1604

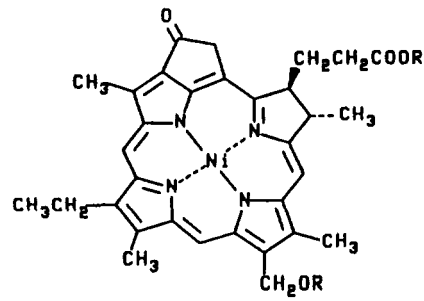
WILLIAM E. PETTIT, and ROBERT M. SCHROLL

Department of Chemistry, Eastern Washington University, Cheney, Washington 99004

ABSTRACT.—The Papua New Guinea shell-less mollusc *Dolabella auricularia* has been found to contain a series of green to blue-green chlorins. One of these compounds was found to be the nickel chelate tunichlorin [**1**] which was isolated previously only from the Caribbean tunicate *Trididemnum solidum*. Discovery of tunichlorin [**1**] in a sea hare suggests that its occurrence in algae-consuming marine animals may be more common than earlier realized, and it may have a role in electron transfer or other metabolic processes.

The isolation of new oxygen (1,2) and nitrogen (3–7) heterocyclic compounds from various molluscs has been increasing. Most of these compounds display a variety of interesting and potentially useful biological properties. Over the past twenty years we have been investigating antineoplastic constituents of the green sea hare *Dolabella auricularia* Engel (Aplysiidae) collected in the western Indian Ocean. We routinely encountered a series of yellow-green to blue-green pigments originally presumed to arise from the sea hare's consumption of algae chlorophyll. With discovery (8,9) of the marine tunicate blood constituent tunichrome B-1, a yellow vanadium-complexing peptide derived from 3,4,5-trihydroxyphenylalanine, with its green (V^{+3}) to blue (V^{+4}) oxidation levels (10,11), and characterization of the blue-green tunicate pigment-(Ni^{+2}) tunichlorin (12) from *Trididemnum solidum* (~10^{-5}% yield), the *Dolabella* chlorins seemed worthy of investigation.

On prolonged storage the green-colored components of *D. auricularia* from various collections appeared to become even more complex mixtures. Hence, a 1985 collection (Papua New Guinea) of *D. auricularia* (ca. 500 kg wet wt) preserved in MeOH until 1989 was employed for the present study. The MeOH and subsequent CH_2Cl_2/MeOH extracts were combined and concentrated. A CH_2Cl_2 fraction prepared from this extract was separated, employing a series of gel permeation and partition chromatographic separations on Sephadex LH-20 [MeOH and *n*-hexane–toluene–MeOH (3:1:1)] and Si gel columns eluted with $Me_2CO \rightarrow CHCl_3$-MeOH (4:1) to afford 49 mg (ca. 10^{-5}% yield) of tunichlorin [**1**]. The structure **1** was primarily established from spectral analyses using high field (300 MH_z) ^{1}H-nmr and ^{13}C-nmr and hrfab mass data. Further confirmation was obtained by methylation (12) of tunichlorin in MeOH containing *p*-toluenesulfonic acid to provide dimethyl tunichlorin [**2**]. The methylated product exhibited spectroscopic characteristics consistent with that previously reported (12).



1 R=H
2 R=Me

Both the eims and the hrfabms indicated the presence of nickel in the *D. gigas* chlorin (potentially biologically significant). To confirm this observation, the *D. gigas* tunichlorin specimen was subjected to qualitative and quantitative metal analyses using energy dispersive X-ray fluorescence (XRF) and graphite furnace atomic absorption spectroscopy, respectively. These techniques verified that no vanadium was present and established nickel as the chelating metal. The quantitative nickel analysis was also revealing for other reasons. The sample used in the quantitative nickel analysis appeared chromatographically pure and displayed the appropriate ms, uv, and ^{1}H- and ^{13}C-nmr features, but only 5% nickel was found instead of the calculated 9.8%. A total ion chromatogram of this sample showed it to contain 50.8% tunichlorin accompanied by five other components ranging from 2.2 to 17.6% of the total integrated areas. While not further pursued, these results were consistent with the large number of closely related chlorophyll derivatives observed during the *D. auricularia* tunichlorin isolation and the previous result that five such components were isolated from *T. solidum* (12). Purification of tunichlorin has been reported (12) to be very difficult, with rapid decomposition even at low temperature under N_2.

As noted by Bible *et al.* (12), conversion of chlorin (13) to nickel chelates is usually difficult, with other metals generally preferred. Indeed, tunichlorin is the only natural nickel chlorin known to date (12). Unless tunichlorin [**1**] is a trace exogenous artifact of *D. auricularia*, it may play an important role in electron transport or other metabolic requirements of such molluscs. The scope of nickel chlorin occurrence in invertebrates and a specific biochemical study of tunichlorin metabolic functions in *Dolabella* should provide useful and interesting results.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—All solvents were redistilled. Evaporation of solvents was performed under reduced pressure on a rotary evaporator at 40°. Analtech Si gel GF (0.25 mm) plates were used for tlc. Stationary phases used for cc were Sephadex LH-20 obtained from Pharmacia, Uppsala, Sweden and E. Merck (Darmstadt) Si gel (70–230 μm, or 40–63 μm).

The uv spectra were obtained in MeOH solution with a Hewlett-Packard 8450A UV/VIS spectrophotometer. Nmr spectra were recorded in $CDCl_3$ (also the internal standard) with a Varian Gemini 300 MHz instrument. The eims were recorded with a Finnigan-MAT 312 unit. The SP-SIMS fabms were recorded with a KRATOS MS 50 instrument in the NSF regional mass spectrometry facility at the University of Nebraska. The energy dispersive XRF data were collected with a Kevex instrument (model 7000 data system) equipped with an RH side window X-ray tube operating at 20 kV, current 2.0 mA. The characteristic X-rays were collected by a lithium-drifted silicon detector with a resolution of 175 eV based on the full width at half maximum measurement of Fe Kα at 6.4 KeV. A Varian model AA-375 atomic absorption spectrophotometer fitted with VWR Scientific hollow-cathode lamps and a Varian CRA-90 carbon rod atomizer was used for the quantitative nickel analyses.

ISOLATION OF TUNICHLORIN [**1**].—Approximately 500 kg (wet wt) of *D. auricularia* was recollected (original collection in 1983) in the Ysebel Channel between New Ireland and New Hanover, Papua New Guinea in 1985 and preserved in MeOH until 1989. A voucher specimen is in the ASU-CRI. After exhaustive extraction with CH_2Cl_2/MeOH, the combined CH_2Cl_2 fraction (1863 g) obtained (14,15) from the MeOH and chlorocarbon-alcohol extractions was subjected to separation in CH_2Cl_2-MeOH (3:2) on a column of Sephadex LH-20. The resulting green-colored fractions were combined to provide 74 g of dark green oil. The oil was dissolved in CH_2Cl_2 (500 ml) and washed with citric acid (20%, 2×300 ml) and dried (Na_2SO_4). Solvent removal gave 62 g of green oil. The oil was triturated with *n*-hexane (400 ml followed by 300 ml) to provide 14.6 g of dark green oily product which was further purified by chromatography on SiO_2 (0.063–0.200 mm, column 3.5×65 cm) and elution with Me_2CO (2000 ml). The top portion of the SiO_2 bed (20 cm, brown in color) was removed by this means, and the green color was eluted with $CHCl_3$-MeOH (4:1). Solvent was removed from the green fraction (1500 ml) to give 2.6 g of a dark green oil. New LH-20 Sephadex (250 g) was swollen in MeOH overnight and degassed. The Sephadex was washed (equilibrated) with *n*-hexane–toluene–MeOH (3:1:1) (2.0 liters). The deep green oil (2.6 g) was dissolved in MeOH (3 ml) and applied to the Sephadex column. Elution with *n*-hexane–toluene–MeOH

(3:1:1) gave in the final fractions tunichlorin (49 mg): hrfabms (3-NBA Matrix) $[M]^+$ 595.1809 (calcd for $C_{32}H_{33}N_4NiO_4$, 595.1855). The uv, eims, hrfabms, ^{1}H-nmr, and ^{13}C-nmr data corresponded to those reported (12) for tunichlorin [**1**].

A mixture composed of green fractions (35.9 mg) for the final isolation of tunichlorin was dissolved in 0.07 M MeOH *p*-toluenesulfonic acid (50 ml). The solution was stirred (in the dark under argon at 40°) for 30 min with monitoring by Si gel tlc [$CHCl_3$-MeOH (9:1)]. The methylation product was isolated as previously described (12) to provide 12.4 mg, and the reaction was repeated to afford a total of 71.9 mg. Purification of the crude dimethyltunichlorin was achieved on a LOBAR column (size A, 310–25, LiChroprep Si 60, 40–63 μm) and elution with *n*-hexane–Me_2CO (4:1) using a Gilson 305 pump (flow rate of 5 ml/min) to yield 37.4 mg. The purification was repeated on a size B LOBAR column to give 6.1 mg of dimethyltunichlorin [**2**]: uv (1.26 mg/100 ml MeOH, 2.022×10^{-5} M); λ (log ε) nm, 295 (4.16), 368 (4.44), 393 (4.57), 416 (4.62), 600 (3.93), 646 (4.65). The ^{1}H- and ^{13}C-nmr spectra and mass analyses (ei and hrfab) were consistent with those expected (12) for dimethyltunichlorin [**2**].

QUALITATIVE TUNICHLORIN NICKEL ANALYSIS.—A qualitative analysis for the presence of both nickel and vanadium was performed by energy dispersive XRF. An MeOH solution of tunichlorin was spread over a thin piece of Mylar and allowed to evaporate. The doped film was then radiated for 388 sec with the secondary radiation from a germanium target. Nickel Kα and Kβ peaks were both found well above the background. The presence of vanadium was not detected.

A quantitative analysis for both nickel and vanadium was performed by graphite furnace atomic absorption spectroscopy. The stock solutions of each metal were prepared with standards. All test solutions were prepared immediately prior to use by dilution with distilled deionized H_2O and absolute MeOH in equal amounts. Measurements were made on 5 μl samples, which were dried at 95° for 50 sec, ashed for 30 sec at 1000°, and atomized for 20 sec at 2600°. The temperature for atomization was chosen based on its effectiveness for analyzing both elements (16,17). Pyrolytic graphite was chosen for use in furnace parts as it has shown substantially improved results over non-pyrolytic graphite for both Ni and V (17,18).

The need for background correction was determined by an examination of absorption peaks for both metals with and without background correction. Standards of varying concentration along with samples of the unknown were analyzed. The peaks showed no difference between modes. The standards were run in quadruplicate, consecutively, in descending concentration. For each analysis the spectrophotometer was run in both peak area and peak height mode. Peak height mode provided better results and was used to plot calibration curves. The sensitivity of the integration method is similar to, or less than, that obtained by the peak-height method (19).

The tunichlorin sample was prepared for analysis by adding 0.1 mg to 120 μl of the same MeOH/H_2O solution. After recording the standards the tunichlorin absorbance was determined five times and the results were averaged. No absorption for vanadium was detected. However, there was considerable absorption when testing for nickel. The absorbance of the final solution was 1.561 (standard deviation of 0.0052) corresponding to a nickel concentration of 5.975 μg/μl. Therefore, we found 0.0050 mg of nickel per 0.1 mg of sample giving a total concentration of 5% Ni (wet wt) with 9.8% calculated for nickel tunichlorin.

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