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Structure of the Pigment of the Red Sea Nudibranch *Hexabranchus sanguineus*

Yuewei Guo, Margherita Gavagnin, Ernesto Mollo, Enrico Trivellone, Guido Cimino*

Istituto per la Chimica di Molecole di Interesse Biologico-CNR,[1] Via Toiano, 6 - 80072, Arco Felice (Na), Italy

and Issa Fakhr

National Research Center, Tahrir Str. Dokki, Cairo, Egypt

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Abstract: The structure of hurghadin (**1**), a pigment from the Red Sea nudibranch *Hexabranchus sanguineus*, was elucidated by interpretation of spectral data and by chemical method.

Spanish dancer nudibranch *Hexabranchus sanguineus* is a large brillantly coloured shell-less sea slug (Gastropoda: Opisthobranchia). The metabolite pattern of *H. sanguineus* and its eggmasses was heavily characterized by a suite of unusual oxazole-containing macrolides[2-5] which play a defensive role[6] and was found also responsible for the cytotoxic and antimicrobial activities.[4,5] Similar metabolites were also isolated from the sponge *Halichondria* sp.,[4] diet of the nudibranch. Recently, in the course of our studies on the chemical ecology of nudibranch molluscs, we have examined the *H. sanguineus* and its eggmasses collected from the Red Sea. Careful chromatographic separation of the mantle Et_2O extract of the animal resulted in the isolation of a new pigment, named hurghadin (**1**), closely related to actinioerythrin (**2**), the first example of bisnorcarotenoid diester previously isolated from the sea anemone *A. equina*.[8]

H. sanguineus was collected by SCUBA at Hurghada, Egypt, in 1995 and kept frozen until processed. The animal (one specimen) was carefully dissected into two body parts: the mantle and the digestive gland/gonad. Each portion, together with mucoid exudate and eggmasses of *H. sanguineus*, was extracted with acetone. All these four Et_2O-soluble fractions of the Me_2CO extracts were similarly composed of a mixture of pigments, sterols, and macrolides on the basis of TLC analysis. However, the pigment (SiO_2; petroleum/Et_2O 1:1, R_f 0.72) was observed exclusively present in the skin part. In order to obtain this pigment, the Et_2O extract of the mantle (30 mg) was chromatographed on a Si gel column eluting first with petroleum ether and increasing amounts of Et_2O and then with Et_2O/MeOH (97:3). The pigment-containing fractions eluted with petroleum ether/Et_2O (7:3) were further purified by Si gel chromatography using petroleum ether and $CHCl_3$ as eluants affording pure hurghadin (**1**) (3.1 mg). Si gel column chromatography separation of the other three Et_2O extracts using first Et_2O then Et_2O/MeOH (98:2) as eluants to obtain, from each column, the same macrolide, kabiramide C,[3] as a major metabolite.

Hurghadin (**1**)[7] was obtained as a deep-red powder. Its IR spectrum showed the presence of conjugated carbonyl (1703 cm^{-1}) and ester carbonyl (1744 cm^{-1}) which were supported by ^{13}C-NMR resonances at δ 200.1 and δ 173.4, respectively. Furthermore, the presence of many low-field NMR resonances (δ_H 6.3-7.0; δ_C 120-170) along with VIS maxima of (**1**) [λ 498 nm (Me_2CO), ε 50000] implied that hurghadin is a esterified

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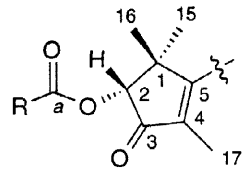
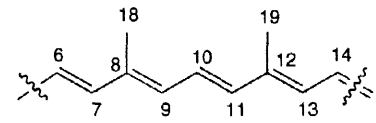
carotenoid. Extensive analysis of the NMR spectra (^{1}H-^{1}H COSY, TOCSY, HMQC, HMBC, NOESY, NOE difference, decoupling) of hurghadin (**1**) revealed the presence of the partial structures A and B.

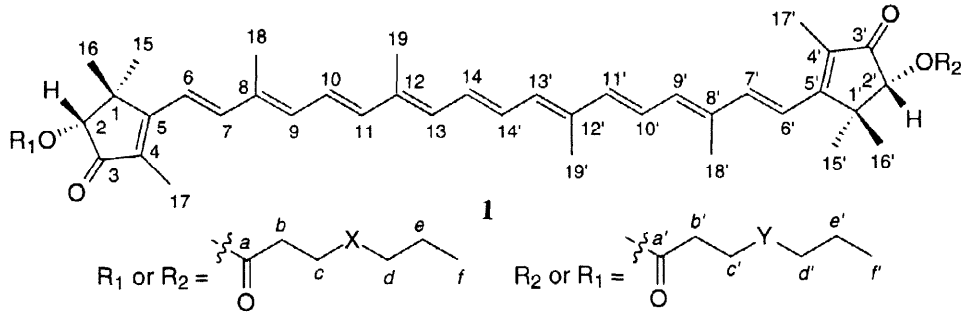


A



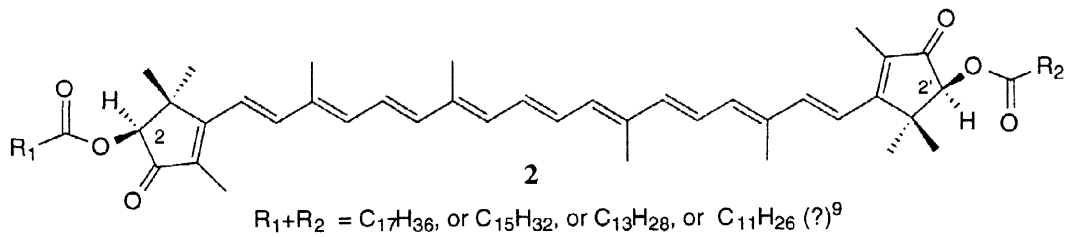
B

The 1,1,4-trimethyl-cyclopent-4,5-en-3-one moiety (partial structure A) of hurghadin was elucidated predominately by HMBC experiments. The significant long-range ^{1}H-^{13}C correlations between C-1 (δ 44.4) and H-2 (δ 5.17), H_3-15 (δ 1.16), H_3-16 (δ 1.43); between C-3 (δ 200.1) and H-2 and H_3-17 (δ 1.92); between C-5 (δ 169.6) and H_3-15, 16, 17, and also between the ester carbonyl (δ 174.3, C-*a*) and H-2 were observed leading to unambiguous assignments for partial structure A. The relative configuration of C-2, as depicted in A, was established by NOESY and NOE difference experiments. Irradiation of the methine proton at δ 5.17 (H-2) enhanced the signal at δ 1.43 (H_3-16) indicating that both H-2 and H_3-16 are *cis* oriented. For the partial structure B, the characteristic polyene signals of a *β*-carotene type carotenoid were observed by extensive analysis of 2D NMR spectra:[7] four allylic methyl carbons (C-18, 18' and C-19, 19'), four quaternary carbons (C-8, 8' and C-12, 12'), and fourteen olefinic methine carbons (C-6, 6', C-7, 7', C-9, 9', C-10, 10', C-11, 11', C-13, 13', C-14, 14'). These assignments were secured by a series of spin decoupling experiments. Finally, the partial structures A and B were connected through a linkage between C-5 and C-6 on the basis of the HMBC correlations observed between C-5 (δ 169.6) and H-6 (δ 6.38) and H-7 (δ 6.96) to afford the gross structure of hurghadin (**1**).



1

R_1+R_2 = $C_{27:2}$+$C_{20:1}$ or $C_{20:2}$+$C_{18:0}$ or $C_{20:1}$+$C_{18:1}$ or $C_{19:0}$+$C_{18:1}$ or $C_{18:1}$+$C_{18:0}$

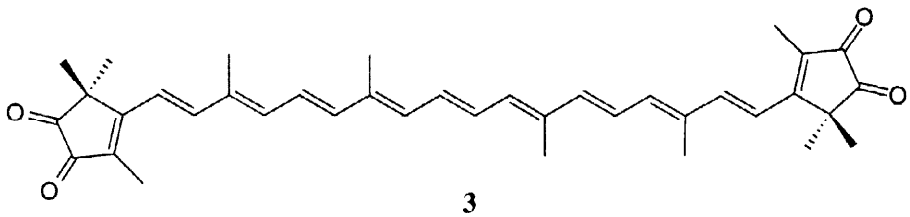


2

R_1+R_2 = $C_{17}H_{36}$, or $C_{15}H_{32}$, or $C_{13}H_{28}$, or $C_{11}H_{26}$ (?)[9]

In order to confirm the proposed structure and to disclose the differences between **1** and **2**, hurghadin was saponified by analogy with actinioerythrin.[8] Careful alkaline hydrolysis resulted in the isolation of the

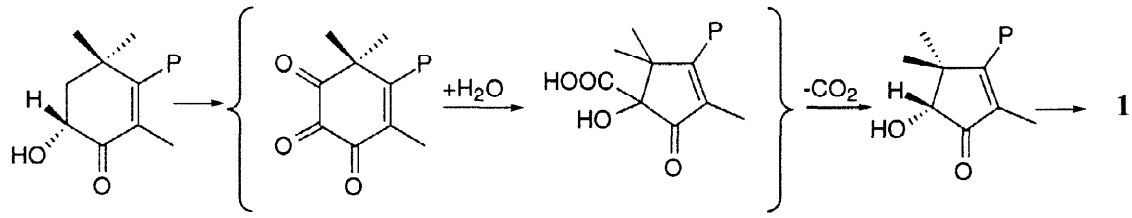
tetraketone derivative **3**[10] which resulted identical to violerythrin,[8] the saponification product of **2**, by detailed analysis of its 2D-NMR spectra as well as decoupling experiments.



3

Regarding the composition of acyl parts R_1 and R_2, the positive FABMS spectrum of **1** suggests that it is a mixture of several esters. Molecular weights of 1249, 1125, 1113 and 1099 are ascribed to the four main esters and indicated that R_1 and R_2 groups together contain 47, 38, 37, and 36 carbon atoms which include three, two and one double bonds, respectively, in one or both of these groups to explain the molecular weights. NMR spectrum of hurghadin also provides some evidence for these structural requirements: δ 5.35 (olefinic protons of isolated double bonds); δ 2.83 (br t, methylene between two double bonds). The NMR spectrum further supports the presence of methylene groups adjacent to the ester carbonyl function (multiplet at δ 2.46, ca. 4H, H_2-*b*, *b'*), methylenes in saturated environment (δ 1.26), and terminal methyls (δ 0.90, ca. 6H, H_3-*f*, *f'*) suggesting an unbranched long chain aliphatic nature for R_1 and R_2. The mixture of fatty acids obtained after saponification of hurghadin (**1**) were methylated with CH_2N_2 and then the methyl esters were subjected to GC-MS analysis.[11] Six fatty acid methyl esters ($C_{18:0}$, $C_{18:1}$, $C_{19:0}$, $C_{20:1}$, $C_{20:2}$, and $C_{27:2}$) were recognized by either the comparison with the standards or the interpretation of their EIMS spectra. This result was in good agreement with the FABMS and NMR data of hurghadin according to the formulated structure (**1**).

Finally, the absolute stereochemistry at C-2 and C-2' of hurghadin was determined as 2*R*, 2'*R*, inverted of those of actinioerythrin (**2**),[12] by analysis of the CD profile of **1**, opposite of that of actinioerythrin (**2**).



P = polyene chain

Scheme 1. The probable biogenetic pathway of hurghadin (**1**)

Analoguously with actinioerythrin,[8] hurghadin (**1**) could be also biogenetically derived, *in vivo*, from 3*R*, 3'*R*-astaxanthin, a common carotenoid present in many animal tissues,[13] involving a benzylic rearrangement followed by loss of C-2 as CO_2 and acylation (**Scheme 1**).

This is the first structural study of the pigment of *H. sanguineus*. Interestingly, **1** was found predominantly present in the mantle of the animal where it was observed the scarcity of the macrolides, e.g. kabiramide C,[3] typical defensive compounds of the nudibranch, respect to other parts of the animal. This fact suggests that **1**, apart from the responsibility for the red colour of the animal, probably also plays an alternative defensive role. Further studies should be conducted to answer it.

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7. **Hurghadin (1)**: A deep-red powder; VIS λ_{max} nm 498 (ε=50000); IR (liquid film) ν_{max} 2927, 1744, 1703, 1536, 1460, 1305, 1159, 968 cm^{-1}; FABMS *m/z* 1099, 1113, 1125, 1249 [all $(M+H)^+$]; CD (CH_2Cl_2) $[\Delta\varepsilon]_{267}$ - 0.651, $[\Delta\varepsilon]_{282}$ + 0.317, $[\Delta\varepsilon]_{306}$ - 1.046, $[\Delta\varepsilon]_{354}$ +1.752; ^{1}H-NMR ($CDCl_3$) 5.17 (2H, s, H-2, 2'), 6.38 (2H, d, *J*=16 Hz, H-6, 6'), 6.96 (2H, d, *J*=16 Hz, H7, 7'), 6.52 (2H, d, *J*=14 Hz, H-9, 9'), 6.66 (2H, m, H-10, 10'), 6.70 (2H, m, H-13, 13'), 6.35 (2H, m, H-14, 14'), 1.16 (6H, s, H_3-15, 15'), 1.43 (6H, s, H_3-16, 16'), 1.92 (6H, s, H_3-17, 17'), 2.03 (6H, s, H_3-18, 18'), 2.01 (6H, s, H_3-19, 19'), 2.46 (m, H_2-*b*, *b'*), 1.70-1.73 (m, H_2-*c*, *c'*), 1.27 (m, H_2-*d*, *d'*), 1.30 (m, H_2-*e*, *e'*), 0.90 (m, H_3-*f*, *f'*); and ^{13}C-NMR ($CDCl_3$) δ 44.4 (s, C1, C1'), 80.4 (d, C2, C2'), 200.1 (s, C3, C3'), 132.1 (s, C4, C4'), 169.6 (s, C5, C5'), 119.1 (d, C6, C6'), 144.3 (d, C7, C7'), 136.9 (s, C8, C8'), 140.9 (d, C9, C9'), 124.7 (d, C10, C10'), 119.1 (d, C6, C6'), 140.9 (d, C11, C11'), 136.9 (s, C12, C12'), 131.1 (d, C13, C13'), 134.5 (d, C14, C14'), 23.4 (q, C15, C15'), 25.9(q, C16, C16'), 9.6 (q, C17, C17'), 12.4 (q, C18, C18'), 12.2 (q, C19, C19'), 173.4 (s, C-*a*, C-*a'*), 34.2 (t, C-*b*, C-*b'*), 25.0 (t, C-*c*, C-*c'*), 32.7 (t, C-*d*, C-*d'*), 22.5 (t, C-*e*, C-*e'*), 14.0 (q, C-*f*, C-*f'*).
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9. The molecular composition of acyl parts of actinioerythrin (**2**) for the M.W. 780, reported in ref. 8, is incorrect probably due to an erroneous calculation.
10. **Violerythrin (3)**: ^{1}H-NMR ($CDCl_3$) δ 6.58 (2H, m, H-6, 6'), 7.16 (2H, d, *J*=16 Hz, H-7, 7'), 6.58 (2H, m, H-9, 9'), 6.72 (2H, H-10, 10'), 6.58 (2H, m, H-11, 11'), 6.74 (2H, m, H-13, 13'), 6.41 (2H, m, H-14, 14'), 1.41 (12H, s, H_3-15, 15' and 16, 16'), 2.10 (12H, s, H_3-17, 17' and H_3-18, 18'), 2.05 (6H, s, H_3-19, 19').
11. **Isolation and Identification of Fatty Acids of Hurghadin by GC-MS**: GC-MS analysis were carried out on a Hewlett Packard instrument consisting of an HP-GC 5890 series II apparatus equipped with a HP-5MS capillary column (30 m x 0.25 mm, 0.25 μm film thickness; cross-linked 5% HPME siloxane), and of an electron impact source operating at 70 eV and 250 °C. The column temperature was programmed to increase from 60 to 290 °C at a rate of 10 °C/min. The injector temperature was 250 °C and the detector temperature was 300 °C. The sample was injected into GC in $CHCl_3$. The mixture of fatty acids obtained after saponification of **1** was reacted with CH_2N_2 at r.t. for 20 min. and submitted to GC-MS analysis. Methyl esters of unsaturated unbranched $C_{14:1}$, $C_{16:1}$, $C_{18:1}$, $C_{20:1}$, and $C_{22:1}$ fatty acids were used as reference. The oleic acid ($C_{18:1}$, t_R=19.95 min) and a $C_{20:1}$ fatty acid (t_R=21.64 min) were identified unambiguously by comparison of both retention time and mass spectra with those of standards; the stearic acid ($C_{18:0}$, t_R=20.13 min) was recognized by comparison of EIMS spectrum with that of authentic sample, while other peaks were tentatively assigned as nonadecanoic acid ($C_{19:0}$, t_R=20.48 min), $C_{20:2}$ (t_R=21.64 min) and $C_{27:2}$ (t_R=26.64 min) fatty acids, respectively, by mainly interpretation of their EIMS spectra.
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