Extraction and identification of antioxidant polyhydroxynaphthoquinone pigments from the sea urchin, Psammechinus miliaris

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Extraction and identification of antioxidant polyhydroxynapthoquinone ~~pigments~~pigments from the sea urchin, *Psammechinus miliaris*

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INTRODUCTION

The presence of polyhydroxylated naphthoquinone (PHNQ) pigments in sea urchins has been known and studied for some years (e.g. Yoshida et al., 1959). These pigments have been named echinochromes and spinochromes depending on their original sources (Anderson et al., 1969)

A wide variety of sea urchin species are present throughout the world's oceans and many are caught to be processed and the gonads removed and consumed as the "Sushi" food stuff "Uni", which is highly regarded in Japanese cuisine. Although PHNQs are present in "Uni", they are enriched in the shells or tests and it has been suggested that they could find use as antioxidants in a similar fashion to other polyphenolic components from edible plants (Kuwahra et al, 2009). Indeed, PHNQs from sea urchin tests have been shown to be effective antioxidants in lipid peroxidation and food systems (Hatate et al, 2002; Kuwahara et al., 2009). As the shells and spines are effectively wastes after the gonads have been removed, these tests could be used as a source of food-grade antioxidants to replace currently-used artificial antioxidants in food products such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA). The PHNQs may also prove to have biological activities akin to those suggested for plant phenolics (see Hancock et al., 2008) and indeed some evidence for biomedical applications has been presented (Agafonova et al, 2011).

In Scotland, *Psammechinus miliaris* is found on bouldery sheltered shores of the west coast, occasionally in the intertidal and shallow subtidal, and can be locally very abundant (Kelly 2000). This species is commonly found around the United Kingdom but

also present in the Atlantic Ocean from Scandinavia to Morocco .A relatively small urchin, typically 40mm in test diameter it is a good candidate for aquaculture in terms of its ease of culture, omnivorous habit, and its production of gonads of acceptable colour and taste. However, its small size and lack of market acceptance has so far prevented its commercial development (Kelly and Cook 2001). The use of these urchins as a source of natural antioxidants could help the development of commercial cultivation of this species. This project examines the yield and composition of PHNQs available in tests from *Psammechinus miliaris* obtained from a Scottish sea loch.

Materials and Methods

Source of *Psammechinus miliaris*

Individual sea urchins were collected by Dr Adam Hughes from the sea loch, Loch Creran on the west coast of Scotland. At a site known at South Shian (56º31'N, 5º24'W) the animals were collected from the intertidal zone at low tide.

The test were separated from the gonads and washed in water. After air-drying, the samples were transported to Dundee. After freezing and freeze-drying, the individual samples were milled in a XXXX ball mill to a fine powder.

Extraction procedure

Three samples in a powdered form were weighed out into Pyrex tubes in three technical replicates at 1 g within an error range of ± 10 mg. To each of these samples 7 ml of 6M formic acid in 0.5 ml aliquots was added until no further reaction occurred. A vigorous reaction was observed between the acid and calcium carbonate in the shell releasing CO_2. A visual change from grey powder to red/brown solution and grey precipitate was seen. Samples were then vortexed before 5 ml of ethyl acetate was added, creating a visible immiscible layer. These were then centrifuged at 1500 rpm for 10 minutes to drive the partition of phenolic components into the solvent. The upper phase was then removed to larger labeled tubes. The extraction process was repeated with diethyl ether but little further pigment was obtained. To remove water, anhydrous sodium sulphate powder was added to the tubes before transferring the extract into clean glass tubes and drying in a centrifugal evaporator for 2 hours. The dried pigments were resuspended in 1 ml of 5 % aqueous acetonitrile.

Solid phase extraction

The solid phase extraction (GIGA C_{18}E SPE, Phenomenex, ETC) units were prepared by wetting the units with 80% acetonitrile in ultra pure water (UPW) which was then equilibrated with two volumes of 0.1% formic acid in UPW. The units were placed on a vacuum manifold to draw the sample through the SPE unit. The extract was added and the unbound fraction was collected under the units in labeled tubes. The units were washed with one volume of 0.1% formic acid then two volumes of UPW (total 14.5 ml) to ensure that any unbound material was removed. The collection tubes were changed and 5 ml of 80% acetonitrile/UPW solution added to units to elute bound material from SPE units.

Total Phenol Assay

This assay followed the procedure outlined previously (Deighton et al., 1999). The samples (in total volume of 250 µL) were prepared in triplicate and added to Folin Ciocalteu reagent (250 µL) in cuvettes and allowed to stand for 3 minutes. Saturated sodium carbonate (500 µL) to drive the reaction and left for one hour. A reference was made up of Folin Ciocalteu reagent (250 µL) and distilled water (250 µL). The absorbance at 750 nm was recorded and the phenol content estimated from a standard curve of gallic acid.

FRAP assay of antioxidant capacity

The antioxidant capacity was assessed using the FRAP assay (Deighton et al., 1999). The assay follows the formation of a colored TPTZ complex from the ferric ion.

The FRAP analysis was carried out in triplicate. A blank was made to calibrate the spectrophotometer. At time zero, 900 µL of FRAP reagent was added to 100 µL of sample in a cuvette and after exactly 4 minutes, the absorbance is read at 593nm. The values were calibrated against standards and the Ferric reducing ability was calculated.

Liquid Chromatography – Mass Spectrometric (LC-MS) analysis

Samples (20 µL) were analysed on a LCQ-DECA system, comprising a Surveyor autosampler, pump and photo diode array detector (PDAD) and a ThermoFinnigan mass spectrometer iontrap. The PDAD scanned three discrete channels at 280, 365 and 520 nm. Samples were eluted on a gradient of 5 – 40% acetonitrile over 30 min on a C18 column (Synergi HydroC18 with polar end capping, 2 mm X 150 mm, Phenomenex Ltd.) at 200 µL/min. The LCQ-DECA LC–MS was fitted with an electrospray ionisation interface and the samples were analyzed in positive and negative mode. There were 2 scan events; full scan analysis, followed by data dependent MS/MS of the most intense ions. The data dependent MS/MS used collision energies (source voltage) of 45% in wideband activation mode. The MS detector was tuned against standards cyanidin-*3-O*-glucoside (positive mode) and ellagic acid (negative mode).

LTQ Orbitrap LCMS

In addition to the above LCMS analysis, a second system, the LTQ Orbitrap XL LC–MS was fitted with an Acella 600 Pump, Acella PDA and Acella autosampler, was used for accurate mass spectrometry to assist structural characterisation and identification. The Orbitrap MS analysed selected samples in negative ion mode. There were two scan

events; FTMS full scan (80-2000) analysis followed by data-dependent MS/MS of the most intense ions using normalized collision energy of 35%. The Orbitrap MS provides the facility to measure accurate mass data to four decimal places. The capillary temp was set at 300 °C, with sheath gas at 40 p.s.i. and auxiliary gas at 5 p.s.i. Samples (10 µL) were applied to a C18 column (Synergi Hydro C18 with polar endcapping, 2 mm X 150 mm, Phenomenex Ltd.) and eluted using a linear gradient of 5% acetonitrile (0.1% formic acid) to 40% acetonitrile (0.1% formic acid) over 30 min at a rate of 200 µL/min. Exact mass data was used to assign potential structural formulae using the resident Xcalibur™ QualBrowser software (Thermo, UK).

Results

The extraction method followed the procedure of Kuwahara et al. (2009) which involved the use of concentrated acid solutions to dissolve the tests and extract the pigments with subsequent fractionation of pigments into ethyl acetate. The procedure produced brown-orange extracts that yielded UV spectra with maxima (475, 320 and 270 nm) characteristic of extracts containing polyhydroxylated naphthoquinone pigments (PHNQs) from other sea urchins (e.g. Yoshida, 1959; Moore et al., 1966; Anderson et al., 1969). The extracts from the three sea urchin samples gave similar total phenol values of around 1300-1500 µg GAE/g DW material (TABLE 1) and only sample B approached being significantly different from the others (T-test value = 0.105). However, there was substantial variation in the amount of phenolic material that fractionated into the ethyl fraction (from 52 – 65 % of total TPC).

It should be noted that some of the material that contributes to the TPC value of the original extracts will not be phenolic in nature as metal ions, sugars and other components are known to interfere with the redox reaction that underlies the Folin assay (George et al., 2005). Nevertheless, it seems that fractionation into ethyl acetate may be a substantial and initial source of variation.

The total phenol content of the original extracts was generally correlated with antioxidant activity as measured by the FRAP method (Fig. 1) and there was also a relationship between TPC, FRAP and the absorbance of the extracts at 475 nm. This suggests that TPC and FRAP, which effectively measure different aspects of antioxidant potential/reducing power (Huang et al., 2005) could be directly related to the pigment content. This also raises the possibility that the simple measurement of absorbance could substitute for their more complex measurements.

Initial studies using solid phase extraction using C18 units provided very clean material suitable for further LC-MS analysis but revealed that only ~ 30 % of the original TPC was recovered in the bound sample. This loss was not due to overloading of the units as re-application of the unbound material to re-equilibrated units did not increase recovery as seen with other phenol-rich berry samples (McDougall et al., 2009). There was little overall difference between the LC-MS analysis of the bound material and the original samples but the SPE-bound samples gave more reproducible and easily interpreted data.

Twelve peaks were found in the sea urchin extracts (Fig. 2). Six of these had considerable absorption at 450-500 nm (Fig. 2c) and were named P1-P6 for spinochrome-like pigments. The MS properties also were suggestive of seven spinochrome structures (Fig. 2 and Table 2).

Peak P2 had PDA spectra and MS properties indicative of spinochrome E (Kuhuwara et al., 2010; Zhou et al., 2011). Peak P3 was putatively identified as aminopentahydroxy-naphthoquinone (APHQ), P4 as spinochrome B and peak P5 as spinochrome C. Peak P6 was composed of two overlapping components (A & B) putatively identified as echinochrome A and spinochrome A. These components eluted from the C18 column in the same order as described by Zhou et al. (2011). All of these putative identifications were supported by the structural formulas predicted from the exact mass data (Table 2).

Peak P1 was a relatively minor peak but it gave a PDA spectrum with maxima at 475, 330 and 260 nm suggestive of a spinochrome pigment. It yielded an *m/z* spectrum with an M-H signal at 332.8 which yielded a MS^2 fragment at 253.0. This suggests that this may be a derivative of spinochrome E (peak P2) plus 80 amu. A neutral loss of 80 amu is characteristic of the loss of sulphate or phosphate groups but such sulphated or phosphorylated derivatives of spinochromes have not been described. However, the exact mass data gave a predicted molecular formula that matched the structure of sulphated spinochrome E

Peak 2 had absorbance maxima at 420, 300 and 262 nm and yielded an M-H signal at 300.8 with a major MS^2 fragment at 221.0. Again this neutral loss of 80 amu suggests a phosphorylated/sulphated derivative of spinochrome B, which was supported by the exact

mass data. Notably both these putative sulphated/phosphorylated derivatives are less well retained and elute earlier from the C18 matrix than their parent components as would befit more hydrophilic derivatives.

Peaks 1 and 3 both had PDA maxima at around 350, 280 and 250 nm. Peak 1 gave an M-H signal at 254.9 and a major fragment ion at 192.9, a neutral loss of 62. However, the nature of this derivative is not known. Peak 3 gave an M-H signal at 296.9 with MS^2 fragments at 234.9 (neutral loss of 62 amu) and 206.9 (neutral loss of 90 amu). The exact mass determinations for these peaks yielded putative molecular formulas ($C_{10}H_7O_8$ and $C_{12}H_9O_8$ respectively). Intriguingly, these are equivalent to spinochrome E + 2H and spinochrome C + H_2O respectively but further work is required to identify these relatively minor components.

Discussion

The yield of phenolic material obtained from the shells of this sea urchin was similar to previous reports at around 1-2 mg/g dry weight (Amarowisc et al., 2012). This is not large compared to the level of phenols in fruits and vegetables (REF) but is considerable as the shells could be construed as a waste material after removal of the gonads. The antioxidant capacity (FRAP values) was lower than those recorded for e.g. berries (Deighton et al., 2000) but these extracts are comparable to extracts from other sea urchins and could act as effective antioxidants *in vitro* (Kuwahara et al., 2009) The absorbance of the extracts at 475 nm was not closely related to TPC and this reflects that

not all phenolic components were pigmented (Fig. 2) and that other non-phenolic components may cross react in the Folins assay (George et al REF).

Despite their relatively low antioxidant capacity, the PHNQs could have particular bioactivities related to their structure. For example, a preparation rich in echinochrome A has found use in preventing reperfusion damage occurring during treatment of myocardial infarction (Lebedev et al., 2005)

The LCMS results confirm that this common species of sea urchin contained PHNQ pigments similar to other sea urchins. In fact, the main spinochrome and echinochrome derivatives were similar to those identified in the purple sea urchins, *Stronglycentrotus nudus* (Zhou et al., 2011) and *Anthocidaris crassipina* (Kuwahara et al., 2010). However the relative amounts were different which is not expected given that *Psammechinus* were red-brown and these species are purple. Indeed, differences in PHNQ composition have been noted in different coloured species (Amarowisz et al., 2012; Kol'stova & Krasovshaya, 2009).

The presence of small amounts of sulphated spinochromes was indicated by LCMS analysis. Such components have not been reported previously but sulphated hydroxyanthraquinones, such as comantherin sulphate, have been identified in crinoid species (Baker, 1976). Sea urchins certainly have the metabolic capacity to carry out sulphation reactions (e.g. Creange & Szego 1967) and sulphated glycosaminoglycans have key roles in sea urchin embryo development (Solursh & Katow 1982). However, the

role of such sulphated pigments is not known and further work is required to confirm the nature and extent of the modification.

The role of PHNQ pigments in sea urchin shells is not known. PHNQs accumulate only in the living dermis layer which overlays the inner layers of the shell, which are mainly composed of calcium carbonate. Therefore, the PHNQs are in contact with the external environment and may have a protective function. PHNQs have been shown to have anti-algal and anti-bacterial effects (Haug et al., 2002; Mischenko et al., 2005) and their accumulation in the shells may restrict infection.

They have been suggested to act as protectants against UV-induced damage due to their antioxidant capacity and their ability to absorb UV light. Indeed, non-pigmented albino variants of the sea urchin species, *Tripneustes ventricosus*, sought shade by covering themselves with turtle grass more frequently than the coloured wild-types and this response was enhanced in stronger sunlight (Kehas et al., 2005). Indeed, many sea urchins accumulate mycosporine-like amino-acids in their eggs presumably as UV-protectants (Adams & Shick, 1996) and PHNQ pigments could play a similar role in the adult. ~~Of course, it is also possible that coloured shells act as camouflage and reduce predation.~~

REFS TO FOLLOW!

M. Solursh, H. Katow

Initial characterization of sulfated macromolecules in the blastocoels of mesenchyme blastulae of *Strongylocentrotus purpuratus* and *Lytechinus pictus*

Dev. Biol., 94 (1982), pp. 326–336

Biochem J. 1967 March; 102(3): 898–904.
PMCID: PMC1270341

Sulphation as a metabolic pathway for oestradiol in the sea urchin *Strongylocentrotus franciscanus*

John E. Creange* and Clara M. Szego

Mycosporine-like Amino Acids Provide Protection Against Ultraviolet Radiation in Eggs of the Green Sea Urchin *Strongylocentrotus droebachiensis*

Nikki L. Adams, J. Malcolm Shick*

Photochemistry and Photobiology

Volume 64, Issue 1, pages 149–158, July 1996

ANY INPUT GRATEFULLY RECEIVED!

Table 1 Total phenol content of sea urchin tests

Sample	Original extract	Ethyl acetate fraction	% Recovery
SSa	1508 ± 114	969 ± 81	64.3
SSb	1261 ± 10	690 ± 58	57.1
SSc	1405 ± 42	731 ± 68	52.0

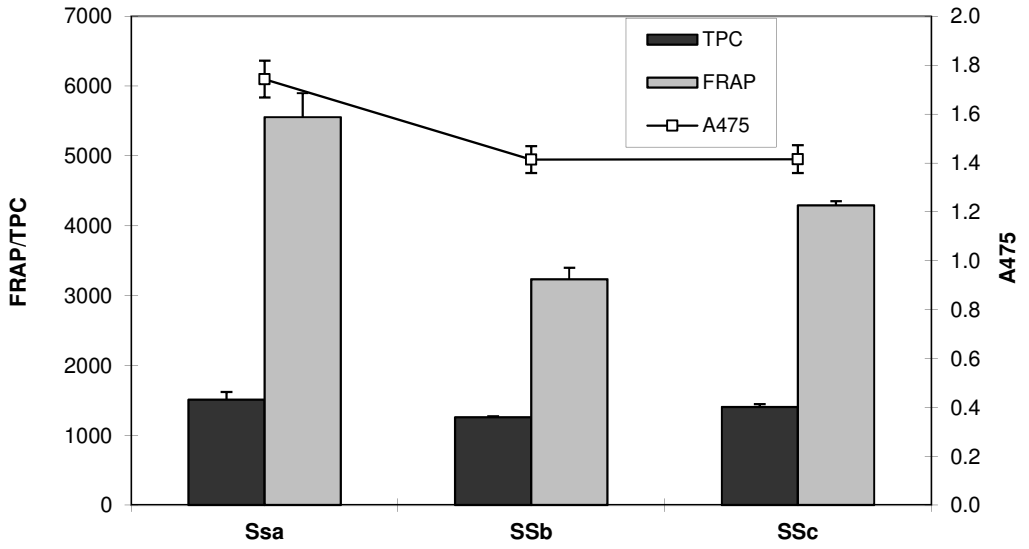
Table 2 LC-MS properties and putative identification of sea urchin pigments

Peak	T^R	PDA max.	*m/z* [M-H]$^-$	MS^2	Exact mass *m/z* [M-H]$^-$	MS^2	Predicted Formula	Δ amu	Putative Identification
P1	14.65	475, 330, 260	332.8, 253.1	253.0	**332.9553**, 252.9986	253.0931	$C_{10}H_5O_{11}S$	*0.001*	Spinochrome E sulphate derivative
P2	15.35	475, 350, 262	253.1	253.1, 206.8	**252.9986**	252.9734, 235.0416, 225.0691 **207.0698**	$C_{10}H_5O_8$	*0.001*	Spinochrome E (SpE)
P3	17.75	471, 369, 273	252.1	252.0	**252.0145**	234.1489, 224.0403, **206.1508**	$C_{10}H_6O_7N$	*0.001*	Aminopentahydroxy Napthoquinone (APHNQ)
P4	19.42	473, 289, 319, 267	221.1	221.1, 192.9, 176.9	**221.0089**	203.1320, 193.1710, 177.1205	$C_{10}H_5O_6$	*0.001*	Spinochrome B (SpB)
P5	25.84	467, 350, 285	279.1	279.1	279.0143	251.0401	$C_{12}H_7O_8$	*0.001*	Spinochrome C (SpC)
P6 A	28.87	507, 312, 250	265.1	265.1, 247.1, 210.0, 207.9, 193.1	**265.035**	265.1032*	$C_{12}H_9O_7$	*0.005*	Echinochrome A (EcA)
P6 B	28.87		263.1	263.0, 235.0	**263.0194**	263.1070	$C_{12}H_7O_7$	*0.001*	Spinochrome A (SpA)

Peak	T^R	PDA max.	*m/z* [M-H]$^-$	MS^2	Exact mass *m/z* [M-H]$^-$	MS^2	Predicted Formula	Δ amu	Putative ID
1	5.61	348, 295, 246	254.9	218.9, 192.9	**255.0144**	**237.0172**, 219.1156, 193.0897	$C_{10}H_7O_8$	*0.001*	ND
2	16.64	420, 300, 262	300.8, 221.1	221.0	**300.9657**, 221.0090	**221.0368**	$C_{10}H_5O_9S$	*0.001*	Spinochrome B sulphate derivative
3	18.29	365, 279, 250, 220	296.9, 235.0	234.9, 207.9	**297.0249**, 252.0147	**279.0732**, 235.1769, 206.8661	$C_{12}H_9O_9$	*0.001*	ND

All identifications are supported by Zhou et al. (2011). * - No MS^2 ions were detected. ND = not determined. RBD values were all around 7.5-8.5

Fig. 1 FRAP, total phenol content and A475 of extracts



FRAP is measured in µM Fe^{2+}/g D.W., TPC as µg GAE/g D.W. and A475 is the absorbance at 475 nm of the extracts.

Fig. 2 Identification of pigments in sea urchin extract (SS) by LCMS

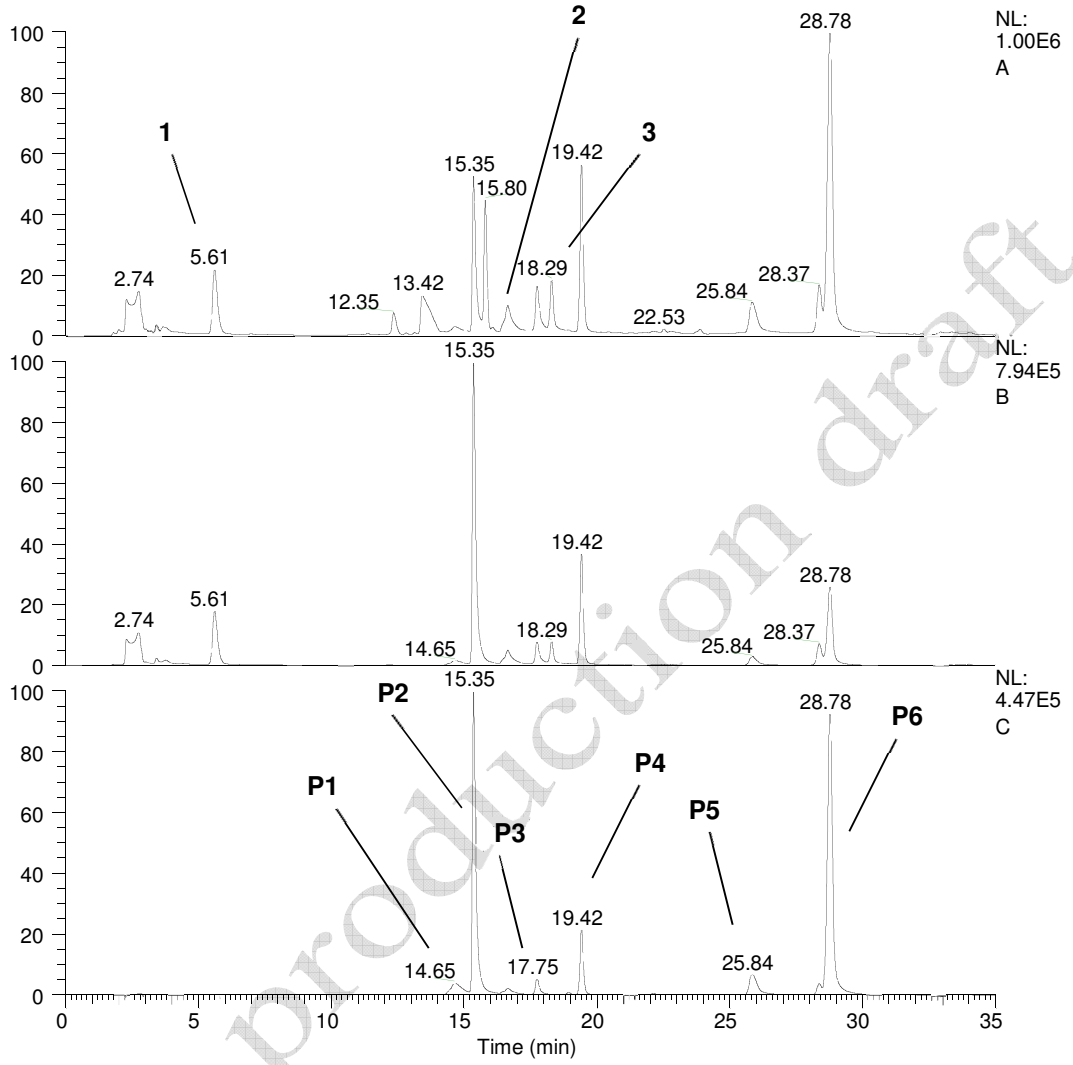
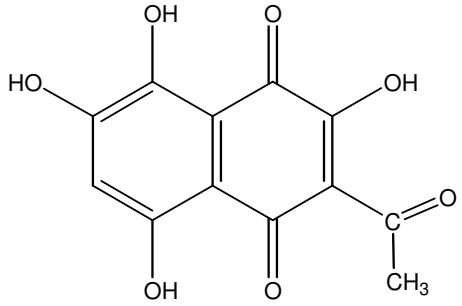
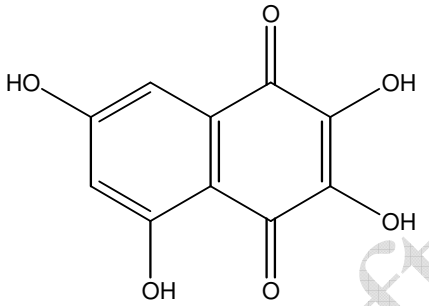


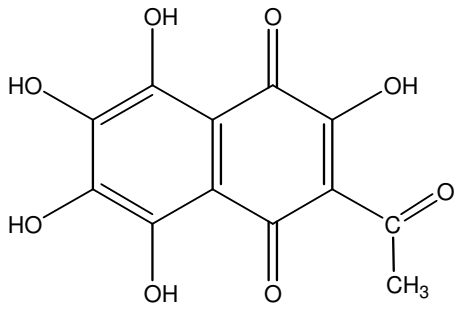
Diagram 1 Structure of spinochromes



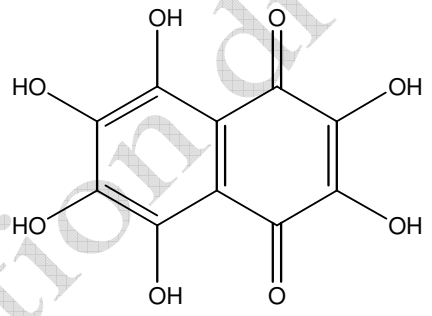
SpA



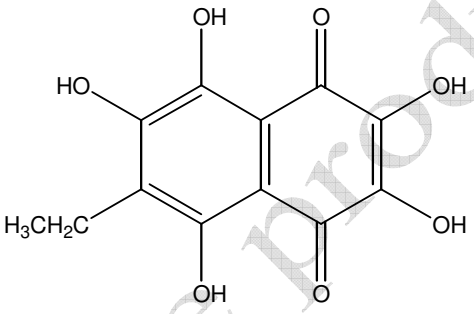
SpB



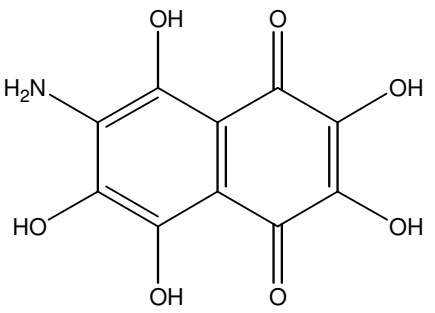
SpC



SpE



EcA



APHNQ

Literature Cited

Kelly, M. S. (2000). "The reproductive cycle of the sea urchin *Psammechinus miliaris* (Echinodermata : Echinoidea) in a Scottish sea loch." Journal of the Marine Biological Association of the United Kingdom **80**(5): 909-919.

Kelly, M. S. and E. J. Cook (2001). The ecology of Psammechinus miliaris. Edible Sea Urchins: Biology and Ecology, Elsevier Science**:** 217-224.