Crinoids: Ancient Organisms, Modern Chemistry[‡]

Yunjiang Feng, Shahan Khokhar and Rohan A. Davis^{*}

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Griffith Institute for Drug Discovery, Griffith University, Brisbane, QLD 4111, Australia. E-mail: r.davis@griffith.edu.au

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Abstract

The ancestors of present-day crinoids are thought to be some of the earliest echinoderms, with fossil records dating back to the early Paleozoic Era (Ordovician Period, 505 – 440 million years ago). Their bright colours have been noted for over 100 years, and are attributed to a series of polyketide-derived pigments. Some crinoid metabolites display a range of biological activities, including cytotoxicity and fish anti-feedant activity. This review is divided into two parts. Part 1 is encyclopaedic in scope, collating information on the >50 known metabolites isolated from crinoids, including their taxonomic source, collection location, chemical structure and biological activities. During the compilation of this data, two distinct themes emerged. Firstly, there is little variation in the class of metabolites produced by crinoids, irrespective of their species or geographic origin. Secondly, the complete and unambiguous assignment of crinoid metabolite structures has been, in many cases, a difficult task. This has been due to a lack of spectroscopic technology available in the past, the presence of proton-poor chemical structures, or both. Thus, Part 2 provides a critical discussion of crinoid chemistry, including the biosynthetic origin of crinoid pigments, as well as the pitfalls and solutions experienced by ourselves and other chemists when elucidating the chemical structures of crinoid metabolites.

1. **Introduction**
2. **Part 1: Crinoid Metabolites**
 - 2.1 **Crinoid Chemistry: Early Reports**
 - 2.2 **Anthraquinones**
 - 2.3 **Naphthopyrones**
 - 2.4 **Bisanthrone and Phenanthroperylene Quinones**
 - 2.5 **Miscellaneous**
 - 2.6 **Geographic Collection Sites of Crinoids that have Yielded Chemistry**
3. **Part 2: A Critical Discussion of Crinoid Chemistry**
 - 3.1 **Biosynthetic Origins**
 - 3.2 **Structure Elucidation - Problems and Solutions**
4. **Conclusions**
5. **Acknowledgements**
6. **Notes and References**

1. **Introduction**

Crinoids (Class: Crinoidea) are echinoderms that vaguely resemble flowering plants.¹ The fossil record indicates that crinoids are the most primitive of the present-day echinoderms, having flourished during both the Palaeozoic (544 – 248 million years ago) and Mesozoic (248 – 65 million years ago) Eras.^{1,2} Their surviving descendants have hardly changed in their appearance.¹ Approximately 700 known species exist today,³ typically existing in two forms: ‘stalked’ crinoids, or sea-lilies (representing approximately 100 known species, **Fig. 1**), and ‘unstalked’ crinoids, also known as comatulids or featherstars (approximately 600 species, **Fig. 2**).^{1, 3} Sea lilies are sessile and usually live at depths of >100 m,¹ where they attach themselves to rocks or to the ocean floor by means of a prominent stalk, which may grow to a length of up to 1 m.^{1, 4} Unstalked featherstars, however, are commonly encountered from the intertidal zone to the deep ocean, and also inhabit coral reefs.¹ In general, these shallow-water featherstars are found primarily in tropical waters.⁴ Featherstars are free-moving, and can swim or crawl short distances to escape predators.³ They can anchor themselves to the sea-floor by means of ‘claw-like’ cirri.⁴ Shallow water featherstars are primarily nocturnal,³ and they typically

inhabit small crevices in rocks, or spaces between coral during the day, emerging later at night to feed.³

Crinoid species usually have five arm bases, which bifurcate to form a total of ten crinoid arms.³ Further arm-branching may occur, with the result that some crinoids may have up to 200 arms.³ These arms can range from a few centimetres to almost 35 centimetres in some species.¹ Crinoids are capable of regenerating lost-limbs, similar to sea-stars, which helps them to survive predation by larger organisms. They have separate sexes, with ova and sperm shed into the water. After fertilization, the larvae attach themselves to a solid substrate such as rocks or coral, and begin the process of metamorphosis, which results in the generation of a small crinoid.¹

Crinoids feed on small organisms that are filtered through the water.³ This filtration occurs through their tube-like feet, with the food particles transported to the gut where digestion occurs. They feed primarily on microorganisms, such as algae, diatoms and larvae, as well as marine detritus.⁴ Unlike other echinoderms, crinoids live with their mouths orientated upwards.³ Being primarily sessile, crinoids are at risk of predation from large fish and other organisms.³ In addition to physical deterrents, such as hard, spike-like pinnules that protect the organism, crinoids are capable of producing toxic chemicals.⁴ These natural products have been shown to display fish anti-feedant activity, and are responsible for the rich colours of crinoids and their extracts.⁴ The toxicity can be attributed to a series of polyketide-derived, brightly coloured, heavily oxidised quinones, which usually contain an aliphatic carbon chain bonded to a quinone nucleus.⁵

Fig. 1 The stalked crinoid, *Porphyrocrinus cf. verrucosus*.

Fig. 2 The unstalked crinoid, *Cenometra bella*.

2. Part 1: Crinoid Metabolites

2.1 Crinoid Chemistry: Early Reports

The vivid colours of crinoids have been known for many years.⁶ However, records suggest that chemical investigations began with H. N. Moseley, a British naturalist who embarked on the Challenger science expedition (1872–1876), which circumnavigated the world.^{7, 8} Two pigments, named “purple pentacrinin” and “red pentacrinin” were isolated from stalked crinoids collected during this expedition as the Challenger passed through the East Indies, whereas a third pigment, “antedonin” was obtained from an unstalked comatulid.^{7, 8} The stalked crinoid pigments were obtained by extracting specimens of *Hypalocrinus naresianus*, *Endoxocrinus alternicirris* and *Metacrinus* sp. under mildly acidic conditions,^{7, 8} whereas “antedonin” was obtained from a purple unstalked crinoid collected from Cape York, Australia and identified by the authors as either *Comatula rotalaria* or *Validia rotalaria*.⁹ Spectral maxima were recorded in the visible light region for the compounds using a “spectroscope”.¹⁰

The acid-base properties of the pigments were also noted. One early study (1882) was by the naturalist, C. F. Krukenbery, who examined the ethanol extract from the Mediterranean crinoid *Antedon adriatica*.¹¹ Acidification of the solution resulted in a colour change from red to orange.¹¹ Further studies of this coloured extract were conducted by MacMunn in 1890, who surmised that the

“comatulin” pigment differed from “antedonin”.¹² MacMunn was also responsible for the examination of crinoids obtained from the Challenger expedition, including *Comatella stelligera*, which yielded a yellow coloured solution.^{8, 12}

2.2 Anthraquinones

Significant contributions to crinoid pigment chemistry were made by the Australian chemists Rideout and Sutherland in the 1960s, 70s and 80s,^{5, 8, 13-17} which culminated in a significant review on Crinoidea pigments in 1985.¹⁷ In all cases, the structural elucidation of the pigments was limited by the technology available at the time: UV/Vis and IR spectroscopy, mass spectrometry, comparison of TLC retention times to related known compounds and simple ¹H NMR experiments. The absolute configurations of many metabolites still require assignment or re-evaluation, as they were originally based on comparison of optical rotations to similar metabolites.¹³ This particular chiro-optical comparison method is fraught with issues that are discussed in the work of Monde, Kobayashi and Garson.¹⁸⁻²⁰ Thus, for the structures presented in this literature review, absolute configurations are defined (and depicted) in the structure when they have been supported by CD or NMR methodology.

Sutherland’s work began with the isolation of acyl derivatives of 2,4,5,7-tetrahydroxyanthraquinone, (including the first report of rhodocomatulin, **(1)** and 1,2,4,5,7-pentahydroxyanthraquinones from the crinoids *Comatula pectinata* and *C. cratera*.²¹ Some evidence for the hydroxylation pattern for these metabolites was provided through semi-synthetic studies conducted in the following decade.²² Further investigations by Sutherland suggested that the position of the butyryl side chain, originally assigned to position 2, was now clearly present at position 1 in rhodocomatulin **(1)**.²² *C. pectinata* and *C. cratera* from the South Queensland coast yielded two further derivatives: rhodocomatulin 5,7-dimethyl ether **(2)** and rhodocomatulin 7-methyl ether **(3)**. The structures of these substances were inferred again through chemical degradation techniques, and a total synthesis that was performed in 1987.^{8, 23}

Interestingly, later studies revealed that these crinoid pigments may be present as a mixture of water-soluble sulfate ester derivatives (e.g. **4**), as well as the sulfate-free hydroxy-anthraquinones.¹⁶ Further studies by Khokhar et al. in 2016 provided 2D and ¹³C NMR evidence to support the structures of **2** and **3**, which until then, were unreported.

Ptilometra australis (the ‘passion flower’) collected from South East Queensland and New South Wales was found to contain the new ptilometric pigments rhodoptilometrins (**5**), isorhodoptilometrins (**6**), and ptilometric acid (**7**).¹³ Ptilometric acid has also been obtained from the crinoid *Tropiometra afra hartlaub* collected from South Queensland waters.¹³ In these cases (**5–7**), a three-carbon alkyl chain is present at position 3 (compared to a four-carbon alkyl chain at position 1 in compound **1**). A re-isolation of **5** in 2009 from a crinoid, *Colobometra perspinosa*, collected from the Great Barrier Reef, led to a full NMR assignment following interpretation of both 1D and 2D NMR data, thus confirming its chemical structure.²⁴

A new pigment, rhodolamprometrin (**8**), was isolated from the crinoid *Lamprometra klunzingeri*, collected from the Coral Reef at Elat in the Gulf of Aqaba, and was characterised by UV, IR, MS and ¹H NMR.²⁵ Two new quinone sulfates (**9–10**) were isolated from the Japanese crinoids *Tropiometra afra macrodiscus* and *Oxycomanthus japonicus*, including an anthraquinone sulfated ptilometric acid derivative (**9**) and the naphthoquinone (**10**).²⁶ Compound **9** showed antifeedant activity on fish (concentration added to fish-food = 0.2–1.0% of total food weight).²⁶ Interestingly, the desulfated

compound, ptilometric acid (**7**), displayed no fish antifeedant activity, suggesting that the sulfate group is important for biological activity.

From *O. japonicus*, the known metabolites rhodolamprometrin (**8**), 2-acetylemodin (**11**), flaviolin (**12**), 1,3,6,8-tetrahydroxyanthraquinone (**13**) were also obtained.

A scarlet-coloured crinoid (stalked sea-lily) *Proisocrinus ruberrimus* collected by submersible (depth = 1800 m) in the Okinawa Trench resulted in the isolation of six new, water-soluble brominated and sulfated anthraquinones, proisocrinins A–F (**14–19**).²⁷ The low yields and proton-poor nature of these quinones meant that full ¹³C NMR assignments were not always possible (e.g. proisocrinins **16**, **18** and **19**). The proisocrinins were optically active, owing to the presence of a chiral centre at position 2'. While the proisocrinins were not assessed for biological activity, the authors suggested that these quinones may be used by the crinoid as a chemical defence mechanism.²⁷

The crinoid, *Comanthus bennetti*, from the Caroline Islands in the South-West Pacific Ocean, yielded five pigments, including rhodoptilometrin (**5**).²⁸ The minor constituents were determined to be: 1,6,8-trihydroxy-3-propyl-9,10-anthraquinone (later named crinemodin, **20**) 1,6,8-trihydroxy-3-(propionyl)-9,10-anthraquinone (**21**), 1,6,8-trihydroxy-6-(2'-hydroxypentyl)-9,10-anthraquinone (**22**), and 1,4,5,7-tetrahydroxy-2-(1'-hydroxypropyl)-9,10-anthraquinone (**23**).²⁸ Compound **20** had been previously isolated as a synthetic degradation product of ptilometric acid, however this was the first report of this anthraquinone as a natural product.¹³ The other new compounds had their structures determined through IR, mass spectrometric analysis, ¹H NMR spectroscopy and chemical degradation studies (no ¹³C NMR was reported). Compound **22** was considered unique because of the presence of a five-carbon side chain.²⁸

Two metabolites, including **5** and **20**, were isolated from an Australian crinoid (*Colobometra perspinosa*), which was collected from the Great Barrier Reef.²⁴ 2-[(Phenylacetyl)amino]ethanesulfonic acid (**24**) and 4-hydroxybutanoic acid (**25**) were also isolated during these studies. This was the first report of compound **24** as a natural product.²⁴ Moderate, non-selective

activity against MCF-7 (breast), SF-268 (CNS) and H460 (non-small cell lung) cancer cell lines was observed for compound **5** (GI_{50} = 41, 21 and 25 μ M, respectively) and compound **20** (GI_{50} = 72, 20 and 25 μ M, respectively).

Our own investigations into the chemistry of a crinoid, *Comatula rotalaria* (collected from the Torres Strait, situated between Queensland and Papua New Guinea) resulted in the isolation and characterization of the known compounds **2** and **3**.²⁹ Additionally, two related but new compounds, 12-desethylrhodocomatulin 7-methyl ether (**26**) and 12-desethylrhodocomatulin 5,7-dimethyl ether (**27**) were isolated and characterised.²⁹ In this study, the major component, **2**, showed no significant effects towards H460 and SF-268 cell lines at 10 μ M, and only minor growth inhibition (9%) towards MCF-7 cells at 10 μ M.²⁹

2.3 Naphthopyrones

Crinoid naphthopyrone pigments exist in linear or angular forms, which are differentiated by the orientation of the pyrone ring relative to the naphthalene group.³⁰

A yellow, phenolic, linear naphthopyrone sulfated-ester (**28**) was isolated from a specimen of *Comantheria perplexia* collected off the South Queensland coast. This ester, upon acid hydrolysis, yielded an artefact which was called comantherin (**29**).⁵ Acid hydrolysis of a mixture of other natural products from *C. perplexia* yielded additional artefacts, neocomantherin (**30**), and anhydrofonsecin (**31**).⁵

Angular naphthopyrones were reported by Sutherland in 1971.³⁰ A green specimen of the crinoid *Comanthus parvicirrus timorensis* collected from Moreton Bay, Queensland, Australia, contained the polar pigments, comaparvin 8-*O*-sulfate (**32**), 6-methoxycomaparvin 8-*O*-sulfate (**33**) and 6-methoxycomaparvin 5-methyl ether sulfate (**34**). Acid hydrolysis of these three compounds yielded the corresponding phenols, comaparvin (**35**), 6-methoxycomaparvin (**36**) and 6-methoxycomaparvin-5-methyl ether (**37**). The sulfate group was inferred to exist through acid hydrolysis of the extract and precipitation of BaSO₃ via addition of BaCl₂.³⁰ The sulfate esters (**32–34**) were observed to have fish-repellent activity, providing evidence that the quinones may play a chemical defence role in protecting the crinoid.¹⁵ Analysis and characterization of the hydrolysis products (i.e., comaparvin and its derivatives) was limited to ¹H NMR (60 MHz) spectroscopy.³⁰ The authors found evidence that transferal of the crinoid from the ocean to an organic solvent resulted in hydrolysis of the sulfate. In contrast, immersion of the crinoid in water instead of organic solvent retained the sulfate esters. In 1976 Rideout et al reported the generation of several permethylated derivatives of the natural products **35–37** by total synthesis; comparison of the ¹H NMR data of these synthetic analogues with the natural products supported the chemical structures that were originally assigned to these angular naphthopyrones.^{14, 31} The ¹³C NMR data for **36** and **37** was finally reported in the literature in 2008 by Folmer et al following isolation of these metabolites from a Fijian specimen of *C. parvicirrus*.^{14, 31} The same paper also reported the single-crystal X-ray diffraction structure for 6-methoxycomaparvin (**36**) and showed that both **36** and **37** completely inhibited TNF- α -induced NF- κ B activation and NF- κ B–DNA binding at MIC values of 303 and 284 μ M, respectively. Furthermore, the results showed that kinase IKK β was the major target of **36** and **37** along the NF- κ B activation pathway.^{14,}

In one study, comantherin (**29**), neocomantherin (**30**), comaparvin (**35**) and the new naphthopyrone 5,8-dihydroxy-6-methoxy-2-propyl-4*H*-naphtho[2,3-*b*]pyran-4-one (**38**, only ¹H NMR data reported) were isolated from the Western Australian collected *Comantheria briareus*, in addition to **8** (isolated from *Comatula solaris*).³²

Several benzochromenone pigments were obtained from the Papua New Guinean crinoid *Comantheria rotula*,³³ including the new compounds, benzo[*g*]chromenone dimer 9,9'-oxybisneocomantherin (**39**) and 8-*O*-methyl-neocomantherin (**40**).³³ Compound **39** inhibited tumour cell growth in the American National Cancer Institute (NCI) 60-cell line panel (GI₅₀ values 1.6–18.2 μM). Five other previously isolated benzochromenone pigments were also reported, including **29–31**, **35**, as well the known fungal metabolite TMC-256A1 / anhydrofonsecin (**31**).^{5, 32, 34} All compounds demonstrated hypoxia-inducer factor 1 (HIF-1) reporter inhibition, but did not decrease the production of HIF-1 target-secreted vascular endothelial growth-factor (VEGF).³³

Five new compounds [10-methoxyrubrofusarin (**41**), 5,6-dihydroxy-8,10-dimethoxy-2-propyl-4*H*-naphtho[2,3-*b*]pyran-4-one (**42**), 5,8-dihydroxy-6,10-dimethoxy-2-methyl-4*H*-naphtho[1,2-*b*]pyran-4-one (**43**), 8-hydroxy-5,6,10-trimethoxy-2-methyl-

4*H*-naphtho[1,2-*b*]pyran-4-one (**44**), and 5-*O*-methylcomaparvin (**45**)], and six known compounds [**31**, **35–38**, and TMC 256C1 (**46**)] were obtained from three crinoids, including an unidentified crinoid, *Comanthus parvicirrus* and *Capillaster multiradiatus*.³⁵ The collection sites of these specimens were not disclosed by the authors. These compounds were isolated during a high-throughput screening campaign that detected inhibition of the ABCG2-mediated transporter, a breast-cancer resistance protein implicated in chemotherapeutic drug resistance.³⁵ The angular naphthopyrones **35**, **37**, **43**, **45** and **46** all showed weak to moderate activity, with IC₅₀ values ranging from 5.9–20 μM.

2.4 Bisanthrone and Phenanthroperylene Quinones

Evidence suggests that bisanthrone crinoid pigments have been preserved in ancient crinoid fossils.⁶ For example, organic pigments with UV and mass spectrometric properties consistent with bisanthrones were isolated in the 1960s from a crinoid fossil, *Apiocrinus* sp.,^{17, 36} that was collected from Upper Jurassic Beds in North-Western Switzerland. These bisanthrone pigments were assigned the trivial names, fringelites A–F.³⁶ The fringelite structures have been occasionally revised, owing to continually improving spectroscopic techniques.^{17, 36, 37} Subsequent chemical studies in 2006 suggested that the fringelites were in fact derivatives of hypericin (**47**), a known plant metabolite obtained from the medicinal herb St John's Wort (*Hypericum perforatum*).^{37, 38}

More recently (in 2015), chemical investigations by Wolkenstein on crinoid fossils collected from Tanzania, France, the United Kingdom, Switzerland, Poland, Israel, Portugal, and Germany have unambiguously proven via HPLC-DAD-MS analysis (with authentic standards) that the spatial distribution of hypericinoid pigments, such as hypericin (**47**) and fringelite F (**48**), is almost

worldwide.² Owing to continuing structural ambiguities surrounding some of the fringelites and the constant revisions, only the chemical structure of fringelite F (**48**) is shown below.

Three bisanthrones (**49–51**) were successfully isolated from *Lamprometra palmata gyges*, which was collected from Moreton Bay in Queensland, Australia.¹⁷ These structures were determined as crinemodin bisanthrone (**49**), crinemodin-rhodoptilometrin bisanthrone (**50**), and rhodoptilometrin bisanthrone (**51**). Two additional components (**52** and **53**) were also reported. The structure of **52** was confirmed by total synthesis and comparison of TLC retention times to that of a natural sample, however NMR data were not presented and a mass spectrum could not be obtained.¹⁷ In this same paper, an investigation of *Zygometa microdiscus* collected from Moreton Bay (Queensland, Australia) yielded **5** and the chiral and meso isomers of crinemodin bisanthrone (**49**). This crinoid also yielded compound **54**, similar in structure to **52**, although limited structural data was made available.¹⁷ The authors also reported an investigation of a Caribbean *Comactinia meridionalis meridionalis* specimen, the extract of which was shown to contain compound **8**.¹⁷

The marine crinoid, *Himerometra magnipinna*, collected from the South China Sea, was found to contain the crinemodin-rhodoptilometrins bisanthrone (**50**).³⁹ This bisanthrone displayed minimal inhibitory activity towards a *Streptomyces* strain. Although this chemical had been previously described from the same species in 1985, ¹H and ¹³C NMR were reported for the first time.^{17, 39} Unusually, two bromotyrosine alkaloids were also obtained from this extract: the known sponge metabolite aerothionin (**55**)⁴⁰ and the new compound (+)-12-hydroxyl-homoaerothionin (**56**).³⁹ Aerothionin displayed low to moderate activity towards *Streptomyces* 85e.³⁹

From the stalked New Caledonian crinoid *Gymnocrinus richeri*, five new brominated phenanthroperylenequinones [gymnochromes A–D (**57–60**) and

isogymnochrome D (**61**)] were obtained.⁴¹ The absolute configurations of the stereogenic carbon centres of these five molecules, as well as their axial chiralities, were inferred from CD and NMR data that was compared to known perylenequinones. The configurations of the stereogenic centres in the side chains of **60** and **61** were determined through Horeau's method of kinetic resolution.⁴¹ Interestingly, compound **60** was found to be a diastereomer of **61**; the absolute configuration at C-16 and C-19 were both assigned as *R*, however the helicity of the pigments were opposite, imbuing an element of axial chirality. The authors noted that the green colour of the extracts might suggest that the pigments are present as phenoxide salts, possibly in the form of a metal complex with zinc.⁴¹ Gymnochrome D (**60**) and isogymnochrome D (**61**) have shown activity against the dengue fever virus with 50% reduction of foci (RF50) at concentrations of <1 μ M.⁴² Gymnochrome A (**57**) appears to alter the transmembrane electrical activity of frog heart muscle tissue.⁴³ There is still some debate over the location of the bromine atom in **58**, which could hopefully be resolved through modern spectroscopic techniques. Additional studies of the *n*-hexane/CH₂Cl₂ extract of *G. richeri* yielded a number of steroids, including cholesta-1,4-diene-3-one (**62**), which was reported from a marine organism for the first time, and several sulphated sterols (structures not shown) that were identified following solvolysis and GC-MS analysis.⁴⁴

The new compounds gymnochromes E (**63**) and F (**64**) were obtained from the deep-water crinoid, *Holopus rangii*, collected by deep-submersible from the South-West coast of Curacao.⁴⁵ Compound **63** was shown to be moderately cytotoxic towards NCI/ADR-Res and histone deacetylase-1, whereas **64** poorly inhibited NCI/ADR-Res. However, **64** was a moderate inhibitor of myeloid cell leukaemia sequence 1 (MCL-1). The configurations of the chiral centres present in the alkyl side chains were tentatively assigned by CD and NMR arguments.^{41, 45} The known emodic acid (**65**), as well as a new bromo-emodic acid derivative (**66**) were also isolated from this sample.⁴⁵

2.5 Miscellaneous

While the majority of secondary metabolites isolated from crinoids to date are highly coloured compounds such as the bisanthrones and anthraquinones, other natural products belonging to different structure classes have also been identified from crinoid extracts. In the previous sections two bromotyrosine alkaloids, that included the known sponge metabolite aerothionin (**55**)⁴⁰ and the new compound (+)-12-hydroxyl-homoaerothionin (**56**)³⁹ were mentioned, along with 4-[(phenylacetyl)amino]ethanesulfonic acid (**24**) and 4-hydroxybutanoic acid (**25**) (see section 2.2)²⁴ and the steroid, cholesta-1,4-diene-3-one (**62**) (see Section 2.4). Additional structure classes identified from crinoidea include the cerebroside CJP1 (**67**), which was isolated from the feather star *Comanthus japonica* that was collected at Koinoura, Japan. CJP1 (**67**) was shown to be a mixture of two L-myo-inositol-1-*O*-phosphoceramides, which both contained a C16:1 sphingosine unit, but differed in the fatty acid moiety with the major compound containing a C22:0 unit, while the minor lipid constituted a C24:0 moiety.⁴⁶

Further studies on the same sample identified glycosyl inositolphosphoceramide-type gangliosides CJP2 (68), CJP3 (69), and CJP4 (70), which were also isolated as homologue mixtures^{47, 48} and five ceramides [JC-1–JC-5 (72–76)] and four glucocerebrosides [JCer-1 – JCer-4, (77–80)].⁴⁹ JCer-1 was found to be identical to a metabolite isolated from the terrestrial plant, *Arisaema amurense*, which has been shown to display antihepatotoxic properties.⁵⁰ Whilst JC-2 and JCer-3 had been previously identified as components of natural products that were obtained from the soft coral *Lobophytum* species⁵¹ and the sea cucumber *Holothuria coronopertusa*,⁵² the 2004 crinoid paper by Inagaki et al⁴⁹ was the first to report the isolation of these sphingolipids. CJP1–CJP4 (67–70) were subsequently evaluated by the Higuchi group for their effects on neuritogenesis in a rat pheomycytoma cell line (PC12), and all four metabolites were shown to display neuritogenic activity in the presence of nerve growth factor at 10 μ M, with CJP4 (70) the most active in this assay.⁴⁸ Subsequent chemical studies on another Japanese feather star, *Comanthina schlegelii* (collected

from Okinawan waters near Hedo Cape) revealed the presence of CJP2 (**68**), plus a new ganglioside, which was named CSP2 (**71**).⁵³

The natural products discussed in this section highlight the possibility of other compound classes being identified from crinoidea. Additional chemical investigations on crinoids will undoubtedly identify further non-pigmented metabolites in the future.

2.6 Geographic Collection Sites of Crinoids that have Yielded Chemistry

Worldwide, crinoids currently consist of ~700 and ~190 accepted species and genera, respectively, however, few chemical investigations have been performed to date.⁵⁴ A search on the MarinLit database,⁵⁵ indicates that only 25 species belonging to 16 genera (*Capillaster*, *Colobometra*, *Comactinia*, *Comantheria*, *Comanthina*, *Comanthus*, *Comatula*, *Gymnocrinus*, *Holopus*, *Lamprometra*, *Liliocrinus*, *Oxycomanthus*, *Proisocrinus*, *Ptilometra*, *Tropiometra*, *Zygometa*) from the class Crinoidea have been worked on by marine natural product chemists. Furthermore, an analysis of the collection site details for crinoids that have been the source of chemistry (see **Fig. 3**) clearly shows that the Pacific Ocean, with an emphasis on the Western region of this Ocean, has been where the vast majority of collecting has been undertaken over the past 50 years. Countries such as Australia, Japan, and China have been the major sources of crinoid collections and subsequent chemical investigations. These data indicate that crinoids have been only superficially studied,

and collections in other parts of the world are clearly warranted in order to determine the chemical diversity of this particular marine organism.

Fig. 3 Collection sites (coloured tags) for the crinoids that have had chemistry reported in the literature. Adapted from MarinLit with permission from The Royal Society of Chemistry (<http://pubs.rsc.org/marinlit>)

3. Part 2: A Critical Discussion of Crinoid Chemistry

3.1 Biosynthetic Origins

Our own investigations into the secondary metabolism of Australian marine invertebrates resulted in the isolation of rhodocomatulin 5,7-dimethyl ether (**2**) and rhodocomatulin 7-methyl ether (**3**), in addition to four new analogues: 6-methoxyrhodocomatulin-7-methyl ether (**81**), 3-bromo-6-methoxy-12-desethylrhodocomatulin 7-methyl ether (**82**), 3-bromo-6-methoxyrhodocomatulin 7-methyl ether (**83**), and 3-bromo-rhodocomatulin 7-methyl ether (**84**), from an extract of the sponge *Clathria hirsuta*.²⁹

81 R₁ = *n*-propyl, R₂ = H, R₃ = H, R₄ = OMe

82 R₁ = Me, R₂ = Br, R₃ = H, R₄ = OMe

83 R₁ = *n*-propyl, R₂ = Br, R₃ = H, R₄ = OMe

84 R₁ = *n*-propyl, R₂ = Br, R₃ = H, R₄ = H

We were intrigued to find these crinoid-type metabolites (**2–3**, **81–84**) in a sponge extract, which raised some questions about their ultimate biosynthetic

origin, which included: (1) were these metabolites produced by the sponge itself; and (2) were they produced by a microbial symbiont (bacteria or fungus) associated with the sponge. Regardless of their biosynthetic origin, the quinone-type pigments described in this review are believed to be formed through known, well-established polyketide biosynthetic pathways.^{56, 57} The formation of emodin (**Fig. 4**) involves generation of a polyketide chain through a polyketide synthase enzyme, followed by successful aldol condensations and enolization to create aromaticity within the resultant six-membered rings, hydrolysis and decarboxylation, and finally, oxidation at the appropriate positions to form a *para*-anthraquinone.⁵⁶ An alternative folding (**Fig. 4**) of a similar-length polyketide chain, however, would result in the formation of an ether linkage, leading to the formation of a naphthopyrone such as anhydrofonsecin.⁵⁸ Radical coupling of a less-oxidised anthrone, followed by oxidation and further radical couplings could result in the formation of bisanthrones such as hypericin.⁵⁶

Fig. 4. Proposed biosynthesis of the major classes of crinoid quinones and pyrones.

3.2 Structure Elucidation - Problems and Solutions

While compiling this review, and in conducting our own research, we encountered three issues relating to the determination of crinoid metabolite structures. Firstly, the reports describing many of the structures isolated and characterised prior to the 1990's lacked detailed NMR spectroscopic data to support their claims (see **Table 1** in ESI).^{5, 8, 13, 14, 16, 17, 30} The NMR data for many compounds were typically limited to low-field ¹H NMR experiments, while a number of metabolites had neither ¹H nor ¹³C NMR data reported. This is to be expected, as routine high-field 1D and 2D NMR experiments were not made until the end of the 20th Century. Unfortunately, this literature deficiency made the characterization of some crinoid metabolites in our studies more difficult [e.g., our isolation of rhodocomatulin 5,7-dimethyl ether (**2**) and subsequent NMR data acquisition could not be easily compared to literature values]. To address this literature shortfall, it would be worth reinvestigating many earlier described metabolites, and also to subject them to modern NMR spectroscopic analysis. The availability of more sensitive instruments, cryo-probes, smaller NMR tubes and better pulse sequences would, as was the case with our studies, address this issue.

However, a second and related issue is the difficulty in establishing atomic connectivity by NMR experiments in some crinoid metabolites. Many of the anthraquinones described in this review are proton deficient and possess numerous non-protonated sp^2 hybridised carbon atoms.^{27, 29, 45} As shown in **Table 1** (see ESI), the majority of the quinone and pyrone metabolites have low proton to non-proton ratios (in the range of 0.30–0.83), therefore the number of proton signals in the NMR spectra is minimal. This can greatly limit the ability to elucidate structures by 2D NMR experiments, such as HMBC or ROESY/NOESY. In theory, ^{13}C - ^{13}C coupling experiments such as INADEQUATE or 1-1 ADEQUATE may provide solutions to issues regarding atomic connectivity, however larger amounts of material (> 50 mg) are often required. This precludes many crinoid metabolites [see rhodoptilometrins (**5**) in [23] for a rare exception]. Newer, longer-ranged coupling experiments (LR-HSQMBC) could possibly give answers to structural problems.⁵⁹

While X-ray crystallography has played a key role in the structure determination of numerous marine natural products over the decades, X-ray crystallographic studies on crinoid-derived metabolites is rare. To the best of our knowledge, only one crinoid metabolite, 6-methoxycomaparvin (**36**), has had X-ray crystallographic data reported.³¹ Our own experience with attempting to grow suitable crystals for X-ray studies on several rhodocomatulin-type metabolites has to date proved unsuccessful.

Due, in part to this lack of crystal growing success, we recently decided to apply DFT-NMR to the elucidation of several crinoid metabolites.²⁹ In our studies, we found that the experimental NMR data that we acquired for compound **27** was consistent with two possible structures, *ortho*-**27** and *para*-**27**.²⁹ Although biosynthetic arguments suggested that *para*-**27** was the correct structure, we felt that it would be a perfect opportunity to use this technique to differentiate between the two forms. Thus, theoretical ^1H and ^{13}C DFT-NMR shifts were computed for both *ortho*-**27** and *para*-**27**, and were compared to the experimental data for **27** using the DP4 statistical analysis.^{29, 60-62} With this technique, we were able to confirm with almost 100% certainty that **27** existed in the *para* form.

The third issue in crinoid metabolite structure elucidation is in the establishment of absolute configurations. As can be seen in Table 1 (see ESI), only 10 chiral compounds have had their absolute configuration/s determined. The absolute configuration of compounds **5–7** were deduced by the extension of empirical rules established by El Khadem and El-Shafei⁶³ and Mills,⁶⁴ while the configurations of **57–61**, **63** and **64** were assigned by the comparison of CD and NMR data. Examples of crinoid metabolites still requiring assignment of their absolute configurations include the proisocrinins (**14–19**), anthraquinones (**22–23**) and bisanthrones (**49–54**). Once again we believe that DFT calculations may yield positive results. The use of CD-DFT and VCD-DFT calculations is becoming more routine with each passing year, and there have been numerous reports describing successful applications in assigning absolute configurations in secondary metabolites by DFT methods.⁶⁵⁻⁶⁷

4. Conclusions

The chemistry of crinoids has been studied for over 140 years, however numerous questions remain unanswered. For example, many researchers are questioning the true biosynthetic origin of crinoid metabolites, with many hypothesising that some of these metabolites may indeed be produced by a microbial symbiont. Due to the ubiquity of various pigmented metabolites (e.g. quinones and naphthopyrones) across the crinoid species, which have likely diverged from a common ancestor, this endosymbiont relationship could be ancient. Further chemical and biological research into the microbial populations of present-day as well as fossilised crinoids is certainly warranted.

The difficulty associated with the structure elucidation of proton-poor crinoid-sourced natural products, will be alleviated, to a degree, by the use of modern NMR spectroscopic analysis. More sensitive instruments (e.g. higher field and/or cryo-probes), and improved and new pulse-sequences will certainly help address this issue. The rapid uptake of DFT-NMR, DFT-CD and DFT-

VCD methodologies by natural products chemists around the world will further support structure determination studies on Crinoidea secondary metabolites. Gaps in spectroscopic and spectrometric data for many of the metabolites isolated and only partially characterised prior to the 1990's will no doubt be filled by chemists with access to high-end instrumentation.

Furthermore, analysis of the taxonomy and collection sites of the crinoid specimens that have yielded chemistry to date has revealed that only 25 species have been investigated; most of which have been collected from the Western region of the Pacific Ocean. The vast majority of living crinoids remain not only an untapped chemistry resource, but also a unique biology repository. More studies on crinoids collected from previously un-investigated and/or under-represented geographic regions around the world (e.g. Africa, The Americas) is key to gaining a better understanding of the chemistry and biology of these unique invertebrates. Importantly this provides a huge opportunity for marine natural product chemists and their pharmacology collaborators to discover not only new chemistry, but also small molecules from the marine environment that may impact drug discovery and chemical biology research. With this in mind we have recently initiated a new research project in collaboration with the Queensland Museum (Marine Invertebrates Group) that will investigate the chemistry of >30 crinoid samples collected from the Great Barrier Reef and Torres Strait for new chemistry. Museum-sourced samples that have been stored in 70% EtOH / 30% H₂O will be dried then sequentially triturated with *n*-hexane, CHCl₃ and MeOH. NMR fingerprinting of the three crude extracts in combination with LC-MS and a MarinLit database⁵⁵ search will enable prioritization of samples for new chemistry. Triaged crinoids will then proceed to large-scale isolation studies and subsequent compound characterization and identification. Crinoidea samples that have different taxonomy to those previously studied and reported in the literature will be included in the >30 selected samples. Un-investigated genera such as *Oximetra*, *Comissa*, and *Liparometra* have already been chosen for a small pilot study at Griffith University, the results of which will help fine-tune this crinoid chemistry program, before larger sample sets (>30 specimens) are accessed. We hope that this approach will increase the chances of not only discovering new chemistry from this unique marine invertebrate, but will also shed light on

the chemical diversity of this taxonomic class. All pure compounds isolated during this project will be added to an Open Access Natural Product-based Screening Library* in order to facilitate drug discovery⁶⁸ and chemical biology research⁶⁹ with both national and international collaborators; this unique resource is housed at Compounds Australia (Griffith University).⁷⁰

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* This Open Access Compound Library currently consists of 512 pure natural products or semi-synthetic derivatives. Access to this resource can be organised through R.A.D. or Compounds Australia.

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