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Structure and Absolute Configuration of Jurassic Polyketide-Derived Spiroborate Pigments Obtained from Microgram Quantities

Klaus Wolkenstein,*[,†,⊥] Han Sun,[‡,⊥] Heinz Falk,[§] and Christian Griesinger[‡]

[†]Department of Geobiology, Geoscience Centre, University of Göttingen, Goldschmidtstraße 3, 37077 Göttingen, Germany
[‡]Department of NMR Based Structural Biology, Max Planck Institute for Biophysical Chemistry, Am Fassberg 11, 37077 Göttingen, Germany
[§]Institute of Organic Chemistry, University of Linz, Altenbergerstraße 69, 4040 Linz, Austria

Supporting Information

ABSTRACT: Complete structural elucidation of natural products is often challenging due to structural complexity and limited availability. This is true for present-day secondary metabolites, but even more for exceptionally preserved secondary metabolites of ancient organisms that potentially provide insights into the evolutionary history of natural products. Here, we report the full structure and absolute configuration of the borolithochromes, enigmatic boron-containing pigments from a Jurassic putative red alga, from samples of less than 50 μg using microcryoprobe NMR, circular dichroism spectroscopy, and density functional theory calculations and reveal their polyketide origin. The pigments are identified as spiroborates with two pentacyclic *sec*-butyl-trihydroxy-methyl-benzo[*gh*]-tetraphen-one ligands and less-substituted derivatives. The configuration of the *sec*-butyl group is found to be (*S*). Because the exceptional benzo[*gh*]tetraphene scaffold is otherwise only observed in the recently discovered polyketide clostrubin from a present-day *Clostridium* bacterium, the Jurassic borolithochromes now can be unambiguously linked to the modern polyketide, providing evidence that the fossil pigments are almost originally preserved secondary metabolites and suggesting that the pigments in fact may have been produced by an ancient bacterium. The borolithochromes differ fundamentally from previously described boronated polyketides and represent the first boronated aromatic polyketides found so far. Our results demonstrate the potential of microcryoprobe NMR in the analysis of previously little-explored secondary metabolites from ancient organisms and reveal the evolutionary significance of clostrubin-type polyketides.

An intriguing class of boron-containing pigments, named borolithochromes, has recently been discovered in distinctly pink-colored, more than 150-million-years-old specimens of the Jurassic putative red alga *Solenopora jurassica* (Figure 1A).[1] The borolithochromes are exceptional in containing the element boron, which is rarely found in natural products,[2−4] in the preservation of polar moieties, which rarely survive in organic molecules of Jurassic age,[5−7] and by the fact that the pigments are directly associated with a Jurassic macroorganism. However, because of the tiny amounts of individual pigments in the fossil material, their molecular weights of >700 Da, and the inherently low mass sensitivity of conventional NMR spectroscopy, no structure elucidation could be achieved, leaving the chromophoric system and the geometry of the boron binding groups a mystery. The molecules have been preliminarily characterized as boric acid esters with two phenolic moieties as ligands by means of mass spectrometry.[1] By solvolysis reactions, it has been shown that the complex mixture of borolithochrome isomers and homologues is composed of combinations of a relatively small number of homologous ligands with different substitution patterns, and circular dichroism (CD) measurements revealed a perpendicular orientation of the ligands and a resulting C_2 symmetry or C_2 pseudosymmetry of the molecules.[1] Using the recent microcryoprobe NMR methodology, a technique allowing full characterization of natural products even on the nanomole scale,[8] here we describe the full structure and absolute configuration of the enigmatic boron-containing Jurassic pigments, reveal their polyketide origin, and link them to a present-day polyketide antibiotic.

To elucidate the chemical structure of the borolithochromes, it was necessary to isolate at least several micrograms of individual borolithochromes in sufficient purity. For this purpose, a large amount of distinctly pink-colored *Solenopora* material (0.8 kg) was treated according to a modified procedure reported previously (see Supporting Information).[1] The main borolithochromes **1**, **2a**/**2b**, **3a**/**3b**, **4**, **5a**/**5b**, **6**−**8**, **9a**/**9b**, among several series of homologous pigments and their isomers, could be isolated (6−57 μg) by high-performance liquid chromatography (HPLC) from an intensely crimson-colored pigment isolate (8.1 mg) and were subjected to NMR using 1.7 mm micro- and microcryoprobes (Figure 1B).

The least substituted structure among the pigments is borolithochrome G (**1**). High-resolution electrospray ionization−MS (HR-ESI−MS) data supported a molecular formula of $C_{48}H_{32}O_8B$, but only 16 proton signals where observed in the ^{1}H NMR spectrum, confirming the proposed symmetry of the molecule and indicating the presence of two identical ligands. Corresponding to each ligand, the ^{1}H NMR spectrum of **1** showed signals for a hydroxy group at δ_H 14.39 ppm, eight aromatic protons, and signals for an isopropyl group. Because

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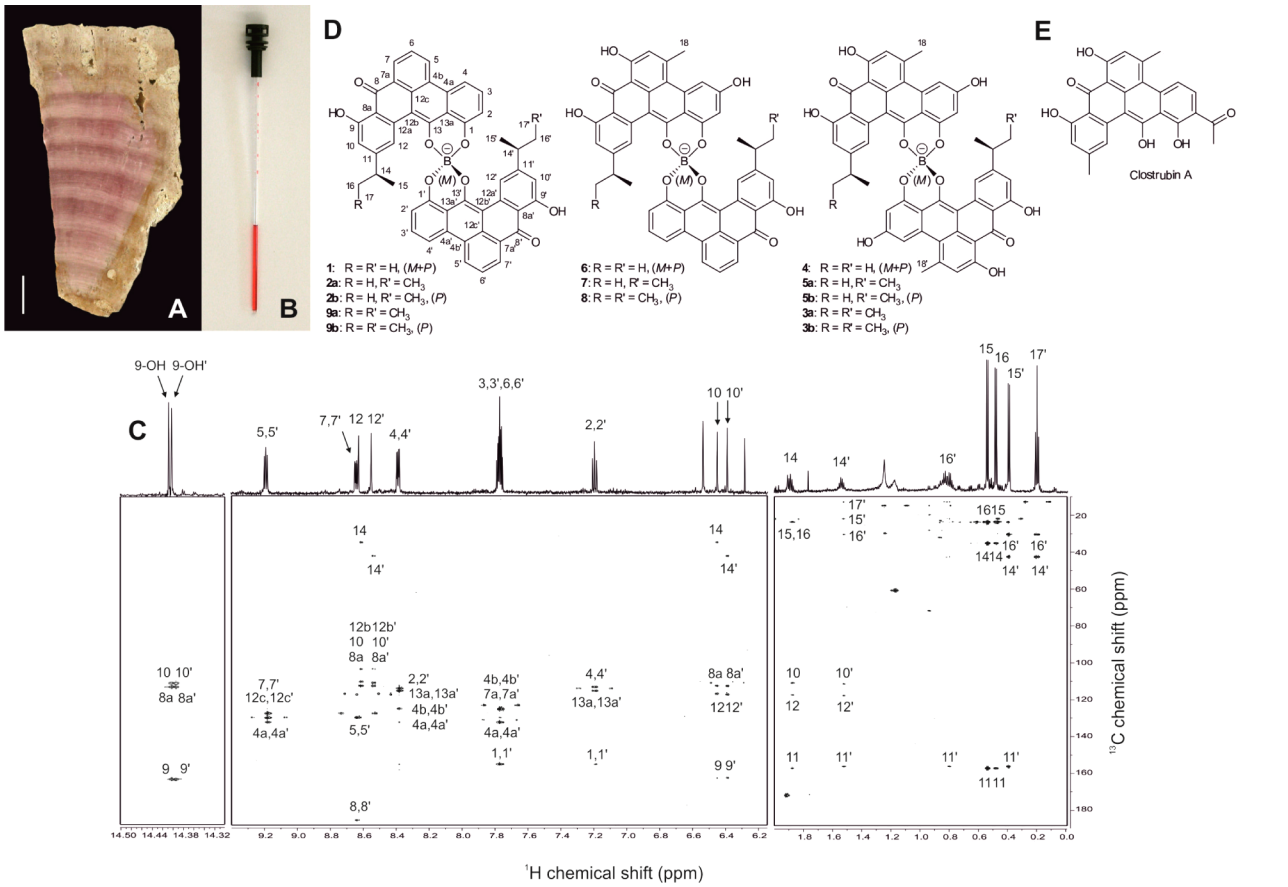

Figure 1. Structural elucidation of fossil boron-containing organic pigments (borolithochromes) isolated from the Jurassic putative red alga *Solenopora jurassica* and comparison with the present-day aromatic polyketide clostrubin from the anaerobic bacterium *Clostridium beijerinckii*. (A) Slab of *S. jurassica* with exceptional preservation of borolithochromes (SMNS P24204), Upper Jurassic, France. Scale bar, 1 cm. (B) 1.7 mm NMR tube with 38 μg of borolithochrome C1 (**3a**) dissolved in 40 μL of DMSO-d_6. (C) ^{1}H NMR (upper) and HMBC spectrum (lower) of borolithochrome H1 (**2a**) (800 MHz, DMSO-d_6) showing ^{1}H−^{13}C long-range correlations. (D) Chemical structures of the borolithochromes showing the exceptional benzo[*gh*]tetraphene scaffold. **1**, **4**, and **6** are racemates [racemic mixture of (*M*)- and (*P*)-enantiomer]; **2a**/**2b** and **5a**/**5b** are (*M*,14′*S*)/(*P*,14′*S*) configured diastereomers; **3a**/**3b** and **9a**/**9b** are (*M*,14*S*,14′*S*)/(*P*,14*S*,14′*S*) configured diastereomers. From the borolithochromes **7** and **8**, only the (*M*,14′*S*) and (*P*,14*S*,14′*S*) configured diastereomers, respectively, could be isolated in sufficient purity for NMR spectroscopic assignment. (*M*) and (*P*) denote the configuration of the chiral axis of the borate. (E) Chemical structure of clostrubin A.

only tiny amounts of **1** could be isolated, its structural assignment was deduced by comparison with the homologue **2a** with one ligand identical to those in **1**.

Borolithochrome H1 (**2a**) and its isomer borolithochrome H2 (**2b**), with molecular formulas of $C_{49}H_{34}O_8B$, showed two sets of proton signals in their ^{1}H NMR spectra (Figure 1C): one set of 16 signals with very similar chemical shifts to those of **1**, and a second set of 18 signals for a homologous ligand including the signals for a *sec*-butyl group. Extensive COSY, TOCSY, NOESY, HSQC, and HMBC analysis of **2a** (Figure 1C, Figure S1) (for details, see Supporting Information) suggested a pentacyclic carbon skeleton for the ligands, which was identified as a benzo[*gh*]tetraphene, and established for **1**, **2a**, and **2b** the structures shown in Figure 1, panel D. To verify the proposed structures, the experimental HMBC and COSY correlations of **2a** were evaluated using COCON simulations (see Supporting Information).[9] After exclusion of constitutional alternatives for the ligands, only two constitutions remained, one of them being **2a**. The constitution of **2a** was finally confirmed by comparison of the experimental ^{13}C chemical shifts with those calculated by density functional theory (DFT) (Figure S3A,B).

The most substituted pigments that could be isolated in sufficient quantities for structure elucidation are borolithochrome C1 (**3a**) and its isomer borolithochrome C2 (**3b**) with molecular formulas of $C_{52}H_{40}O_{12}B$. The 1D and 2D NMR data (see Supporting Information) indicated that **3a** as well as **3b** contain two identical ligands and differ from **2a** and **2b** by the presence of a *sec*-butyl group in both ligands and two additional hydroxy and one methyl group in each of the ligands (Figure 1D). The constitution of **3a** was finally confirmed by DFT calculations (Figure S3C).

To determine the configuration of the chiral axis and the *sec*-butyl groups in the pigments, experimental CD spectra and NMR chemical shifts were compared with those obtained by DFT calculations. In contrast to the racemic compound **1** showing no optical activity, the chromatographically resolvable isomers **2a** and **2b** exhibit a chiral center in addition to their axial chirality and showed optical activity, indicating that they are diastereomers. The CD spectra of **2a** and **2b** showed opposite sign and typical exciton couplets (Figure S4A,B). By comparing the measured CD spectra with those obtained by quantum chemical CD calculations, the configuration of **2a** and **2b** at the boron was determined as (*M*) and (*P*), respectively (Figure S4A,B). Moreover, the configuration of the chiral

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center C14′ must be the same in both compounds since otherwise a (*M*,14′*R*)-**2a** + (*P*,14′*S*)-**2b** enantiomeric or (*P*,14′*R*)-**2b** + (*M*,14′*S*)-**2a** enantiomeric situation would be present, which in both cases would exclude the observed separation by nonenantioselective HPLC and the optical activity of the compounds. CD measurements of **3a** and **3b** and comparison with their calculated spectra indicated that the configuration of these diastereomers at the boron is also (*M*) and (*P*), respectively (Figure S4C,D), confirming previous results deduced from coupled oscillator theory.[1] Furthermore, the chiral centers C14 and C14′ must have the same configuration within **3a** and **3b** since their NMR spectra indicate the presence of symmetric molecules, and also between the compounds, since the compounds could be separated by nonenantioselective HPLC and show CD spectra with opposite sign.

Because the CD spectra of the borolithochromes are predominantly defined by the configuration at the boron and thus only small differences in the calculated CD spectra of the (*M*,14′*R*)/(*M*,14′*S*) and (*M*,14*R*,14′*R*)/(*M*,14*S*,14′*S*) stereoisomers are observable (Figure S4), the absolute configuration of the chiral centers C14 and C14′ could not be determined by CD spectroscopy. However, because of the exceptional structure of the borolithochromes with two orthogonally coordinated aromatic ligands in close distance to each other, it is expected that ring currents induced by the aromatic rings of one ligand will influence the chemical shifts of the protons in the *sec*-butyl group of the opposite ligand in dependence of the configuration of the borate. Indeed, characteristic chemical shift differences are observed between the methyl protons H15′ and H17′ in **2a** and **2b** as well as in **3a** and **3b** (Table S3), always with a larger chemical shift difference in the (*P*)-configured (**2b**, **3b**) than in the corresponding (*M*)-configured diastereomers (**2a**, **3a**). By conformational analysis of the *sec*-butyl group and comparison of experimental and DFT-calculated chemical shifts, the configuration of the chiral center C14′ in **2a**/**2b** and the chiral centers C14 and C14′ in **3a**/**3b** were assigned as (*S*) (for details, see Supporting Information).

The structures of the further borolithochromes **4**, **5a**/**5b**, **6**−**8**, **9a**/**9b** could be assigned by comparison of their HR-ESI−MS, ^{1}H NMR, and CD data with those of the closely related **1**, **2a**/**2b**, and **3a**/**3b**. Because the same patterns in the ^{1}H chemical shift differences as observed for **2a**/**2b** and **3a**/**3b** can be observed in all *sec*-butyl bearing borolithochromes (Table S3), we conclude that the configuration of the chiral centers C14 and C14′ is always (*S*).

The observed specific chirality of the *sec*-butyl groups provides unequivocal evidence for a biological origin of the borolithochrome ligands. Moreover, the characteristic succession of oxygen-containing functional groups on alternate carbon atoms in the higher substituted borolithochrome ligands strongly suggests that the fossil pigments are aromatic polyketides. In contrast to the wide occurrence and diversity of structural types of present-day polyketides,[10] only very few examples are known from the fossil record: flavonoids from Miocene leaves,[11] the quincyte pigments (*peri*-xanthenoxanthene quinones) from Eocene lake deposits,[12] and hypericinoid pigments (phenanthroperylene quinones) from Jurassic and Triassic crinoids[6,7] (Figure S7).

To our knowledge, the borolithochromes are the first boronated aromatic polyketides discovered so far. Their structures differ fundamentally from previously described boronated polyketides, which are all macrodiolides from present-day bacteria (Figure S8).[2−4] In boron-containing natural products, typically the boron is bound via vicinal dianionic oxygen-containing binding groups,[2−4,13] whereas in the borolithochromes, the ligands are esterified via their *peri* hydroxy groups to form a bis-six-membered spiroborate, which is unprecedented among natural products. The boron in the borolithochromes may have been introduced biogenically as in the case of the present-day macrodiolide polyketides, but it also cannot be excluded that the boron may have been introduced during diagenesis (see discussion in ref 1).

Beyond the exceptional occurrence of boron, the most astonishing structural feature of the borolithochromes is their highly unusual benzo[*gh*]tetraphene carbon skeleton, a carbon skeleton, which until now has not been observed in any fossil compound. Moreover, during the time we elucidated the structure, it was also unknown from any present-day natural product. However, surprisingly, in a recent independent study of the anaerobic bacterium *Clostridium beijerinckii*, a polyketide antibiotic with striking structural similarities to the borolithochromes was found, named clostrubin A.[14] Clostrubin shows the same benzo[*gh*]tetraphene scaffold and even a similar substitution pattern as observed in the higher substituted borolithochrome ligands (Figure 1D,E) (in ref 14, the carbon skeleton of clostrubin erroneously has been termed benzo[*a*]-tetraphene). Recently, the structure of clostrubin has also been confirmed by total synthesis.[15] Further natural products with similarities in the carbon skeleton are other rare aromatic polyketides: benzanthrones isolated from *Aloe vera* and *Cassia garrettiana*,[16] the naphthanthracene derivative resistomycin from *Streptomyces resistomycificus*,[17] and the benzo[*a*]pyrene derivatives arenicochromine and benzopyrenomycin from *Arenicola marina* and *Streptomyces lavendula*, respectively.[18,19] The results of structural elucidation in comparison with the structures of present-day natural products unambiguously demonstrate that the borolithochromes are almost unchanged[20] polyketide secondary metabolites and constitute an unprecedented class of fossil pigments.

Although any isotope-labeling experiments are excluded for natural products of extinct organisms, the likely biosynthesis of the borolithochrome chromophore may be inferred in analogy to the proposed model for the biosynthesis of the closely related clostrubin A[14] (Figure S9). However, different from clostrubin, the presence of branched alkyl groups indicates that the borolithochromes were biosynthesized from a nonacetate starter unit. Deduced from the known chemical mechanisms of present-day polyketide chain assembly,[21] it would be likely that the (*S*)-configured *sec*-butyl groups of the borolithochromes are due to (*S*)-2-methylbutyryl-coenzyme A units derived from L-isoleucine.

The structure and occurrence of the borolithochromes raises intriguing questions on the organisms that produced them. The fossil species *S. jurassica* is generally assigned to a once widespread, but now extinct group of coralline red alga (solenoporaceans, Phylum Rhodophyta),[1,22] although the solenoporaceans are currently considered a heterogeneous group and Lower Paleozoic forms have been interpreted as chaetetid sponges.[22,23] However, the nonpyrrolic borolithochromes differ fundamentally from the pigments typically known from red algae: phycoerythrin with the linear tetrapyrrole chromophore phycoerythrobilin, chlorophylls, carotenoids, and the brominated phenolic floridorubins.[24] The remarkable present-day occurrence of clostrubin, with a highly characteristic chemical structure that is very similar to

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those of the borolithochrome ligands, suggests that the fossil pigments originally may have been produced by an ancient bacterium. The borolithochromes have been exclusively found in the Jurassic macroorganism *S. jurassica*, indicating a close association of hypothetical primary producing bacteria with this species. However, natural products produced by associated (symbiotic) bacteria are, in contrast to marine invertebrates, rather untypical for macroalgae,[25] with the presumable polyketide lobophorolide as the only suspected example.[26] An alternative explanation would be that the borolithochromes originated from bacteria that degraded dead organic material of *S. jurassica* and became fossilized *in situ*. However, it is very striking that borolithochromes are not found in other fossil organisms from the same localities where pink-colored *S. jurassica* specimens are found. Because the taxonomic assignment of fossil organisms is generally based on morphological features of preserved hard parts, the assignment of many extinct organisms including representatives of the solenoporaceans cannot be determined with absolute certainty and often is controversial. Although the observed morphological features of *S. jurassica* are in agreement with the current classification as calcareous red algae, this assignment may be scrutinized in further studies.

The borolithochromes are a rare exception of almost originally preserved fossil secondary metabolites and provide direct evidence that complicated polyketides with structural characteristics only merely known from present-day organisms were already biosynthesized since Jurassic time, providing rare insights into natural product diversity of ancient life and demonstrating the evolutionary significance of clostrubin-type polyketides. Moreover, the results show that fossil polyketides may be more common in well-preserved macrofossils than previously thought.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b08191.

Experimental details, spectroscopic data for all new compounds, configurational analysis, Cartesian coordinates of stationary points, additional information on previously known polyketides, proposed pathway for the biosynthesis of the borolithochrome chromophore (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: klaus.wolkenstein@geo.uni-goettingen.de.

Present Address

H.S., Leibniz-Institut für Molekulare Pharmakologie, Robert-Rössle-Str. 10, 13125 Berlin, Germany.

Author Contributions

⊥These authors contributed equally to this work.

Notes

The authors declare no competing financial interest.

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