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Biosynthesis and Structural Revision of Neomarinone

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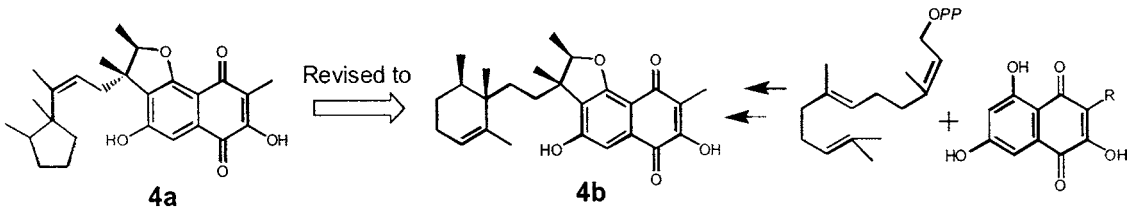
ABSTRACT



The biosynthesis of the meroterpenoid neomarinone from a marine actinomycete was probed through feeding experiments with ^{13}C-labeled precursors. NMR characterization of [U-$^{13}C_6$]glucose-enriched neomarinone led to the structural revision of structure 4a to 4b, which was confirmed by extensive 2D NMR spectrometry with unlabeled compound.

Meroterpenoids are natural hybrid compounds of mixed polyketide-terpenoid origin.[1] Bacterial natural products that result from this mixed biosynthesis are scarce and include a small group of prenylated naphthoquinones from actinomycetes that exhibit broad antibiotic and anticancer activities.[2] Members of this group include the monoterpenoid naphthoquinones naphterpene (**1**)[3] and furaquinocin C (**2**),[4] the sesquiterpenoid naphthoquinones marinone (**3a**)[5] and neomarinone (**4a**),[6] and disubstituted naphthoquinones belonging to the napyradiomycin family (Figure 1).[7] Labeling experiments support the mixed polyketide-terpenoid origin of these natural products. The naphthoquinone core in naphterpene and furaquinocin C is derived from a symmetrical acetate-derived pentaketide,[3,4b] such as 1,3,6,8-

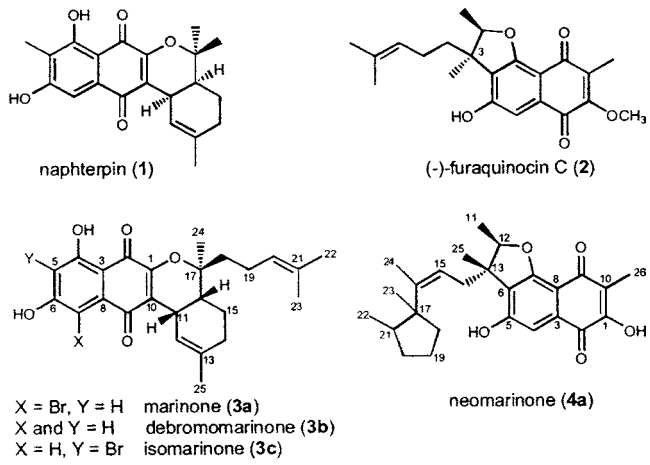


Figure 1. Structures of prenylated naphthoquinones produced by actinomycetes.

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tetrahydroxynaphthalene (THN), while the monoterpene unit of naphterpin is mainly derived from the mevalonate pathway that operates during secondary growth in the producing strain *Streptomyces aeriouvifer*.[8]

The marine sediment-derived actinomycete CNH-099 produces the marinone family of meroterpenoids, which comprises two different carbon skeletons.[6] Whereas marinone (**3a**) and its analogues (**3b**−**c**) contain a nonrearranged sesquiterpenoid residue formally attached via C1 of farnesyl pyrophosphate (FPP) to the naphthoquinone core,[5] neomarinone (**4a**) harbors a highly rearranged sesquiterpenoid moiety consisting of an unprecedented trisubstituted cyclopentane ring that is attached through C3 of FPP.[6,9] On the basis of the novel structure of the sesquiterpenoid unit in neomarinone, we set out to elucidate its biosynthesis through labeling studies with ^{13}C-labeled intermediates. The feeding experiments not only provided biosynthetic data but unexpectedly resulted in the modification of the published structure of neomarinone. Herein we report the biosynthesis of the marinones and a revised structure for neomarinone based on labeling studies and 2D NMR spectroscopy.

The biosynthesis of debromomarinone (**3b**), the major marinone produced by strain CNH-099, was predictable on the basis of previous work by Seto and co-workers on the structurally related naphterpene (**1**).[3] Nonetheless it served as a valuable internal standard for the elucidation of neomarinone biosynthesis.[10] As anticipated, feeding experiments with singly and doubly ^{13}C-labeled acetate confirmed that the naphthoquinone moiety of **3b** is derived from a symmetrical pentaketide intermediate like that in **1**. Although we consistently measured between 1% and 3% incorporation of labeled acetate into the pentaketide residue, the sesquiterpenoid unit was clearly not enriched (Table 1). This observation implied that the terpene is not made by the mevalonate pathway but rather by the recently established nonmevalonate pathway.[11] This is in contrast to naphterpene biosynthesis in which the monoterpene unit is derived from the mevalonate pathway.[8] Feeding experiments with [1-^{13}C]-glucose, [U-$^{13}C_6$]glucose, and [3-^{13}C]alanine, which is converted in vivo to [3-^{13}C]pyruvate, equally labeled the polyketide (via decarboxylation to ^{13}C-labeled acetate) and sesquiterpene (solely via the nonmevalonate pathway) units as illustrated in Figure 2.

Table 1. ^{13}C NMR Data of Debromomarinone (**3b**) and Incorporation Percentages of Labeled Precursors

		incorporation (%)			J_{CC} (Hz)	
carbon	δ_c ppm[a]	[1-^{13}C] acetate[b,c]	[3-^{13}C] alanine[b,d]	[1-^{13}C] glucose[b,d]	[1,2-^{13}C] acetate	[U-$^{13}C_6$] glucose
1	153.0		0.9	0.7	60, 69	60, 69
2	182.5	4.3			60[e]	60[e]
3	109.0		1.2	1.1	63[e]	64[e]
4	165.1	3.8			63, 70	63, 69
5	106.9		0.7	1.1	70[e]	70[e]
6	164.4	2.3			64, 70	63, 70
7	107.8		1.2	0.7	63, 68	63, 65
8	134.5	3.3			54, 66	54, 66
9	184.0		1.0	0.7	53, 58	53, 58
10	123.8	3.5			59, 69	58, 69
11	30.7			1.2		42
12	119.9					42
13	135.9					43
14	29.6					s[f]
15	20.0			1.3		34
16	37.2					34
17	82.9					42
18	36.5					s[f]
19	22.0			1.2		44
20	123.0					44
21	132.3					42
22	25.4					s[f]
23	17.4		1.4	0.8		42
24	22.4		1.2	0.9		42
25	23.3		1.3	0.9		43

[a] Referenced to $CDCl_3$. [b] Percent incorporation = (A − B)/B; A = intensity of enriched carbon and B = intensity of natural abundance carbon. [c] Incorporation relative to the natural abundance carbon at 184.0 ppm (C9). [d] Incorporation relative to natural abundance carbon at 182.5 ppm (C2). [e] This signal represents overlapping doublets. [f] s = enriched singlet.

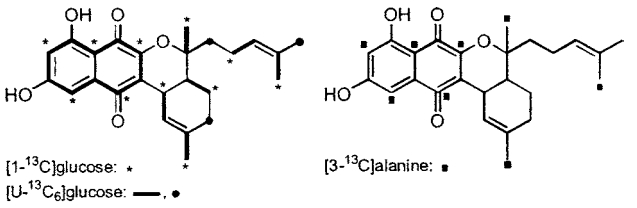

Figure 2. Incorporation of ^{13}C-labeled precursors in **3b**.

Feeding experiments with ^{13}C-labeled acetate similarly confirmed that the naphthoquinone unit of neomarinone is also derived from a symmetrical pentaketide. Administration of the general precursor [U-$^{13}C_6$]glucose resulted in the labeling of the sesquiterpene unit in neomarinone, which however was not completely consistent with the originally published structure of neomarinone (**4a**). Inspection of the

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(9) In addition to exhibiting moderate cytotoxicity,[6] neomarinone showed moderate antibiotic activities (MIC): *Staphylococcus aureus* ATCC29213 (MSSA), 4 μg/mL; *S. aureus* ATCC43300 (MRSA), >32 μg/mL; *Streptococcus pneumonia* ATCC 49619, >32 μg/mL; *Enterococcus faecalis* ATCC29212, 8 μg/mL; *Haemophilus influenzae* ATCC49766, >32 μg/mL; *Pseudomonas aeruginosa* ATCC27853, >32 μg/mL.

(10) Strain CNH-099 was grown in a seawater-based medium by a modification of the procedure of Fenical and co-workers.[6] Production cultures (15 × 100 mL media in 500-mL flasks) were maintained for 7 days with shaking at 37 °C. Labeled precursors (Cambridge Isotope Laboratories) were added to the cultures after 24, 48, and 72 h of growth in concentrations of 100 mg/L culture (450 mg total). The cultures were extracted with EtOAc (~4 L), and the dried extracts were subjected to silica gel flash column chromatography (Merck, grade 9385, 230−400 mesh). Debromomarinone and neomarinone typically eluted with solvent mixtures of 2:1 and 1:1 hexane/acetone. Fractions containing these compounds were combined, dried, and subjected to C_{18} (YMC, 20 mm × 250 mm, 10 μm) reversed-phase HPLC employing gradient elution from 30% MeCN in 0.15% TFA to 100% MeCN over 30 min at a flow rate of 9.5 mL/min with monitoring by UV at 254 nm. Neomarinone (~7 mg/L) and debromomarinone (~10 mg/L) eluted at 26 and 28 min, respectively.

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resulting ^{13}C NMR spectrum indicated five enhanced singlets and five pairs of enhanced and coupled doublets for the sesquiterpene unit (Table 2). Although four pairs of the coupled doublets between carbons C11−12 (40.1 Hz), C13−25 (35.5 Hz), C17−18 (34.9 Hz), and C21−22 (36.1 Hz) were consistent with structure **4a**, a 40-Hz doublet between the olefin C15 (δ 123.9) and the methylene C19 (δ 25.1) implied that these carbon atoms are directly bonded to each other rather than being four bonds apart as depicted in **4a** (Figure 3). Furthermore, the carbons flanking C15 in structure **4a** appeared only as enhanced singlets in the [U-$^{13}C_6$]glucose-enriched sample. The one-bond correlation between C15 and C19 in **4a** was confirmed by an INADEQUATE experiment, which clearly showed a strong correlation between these carbon atoms, as well as the other coupled carbons in the sesquiterpene and polyketide residues. As a consequence, the [U-$^{13}C_6$]glucose labeling pattern of neomarinone suggested that the original structure **4a** be revised as **4b** (Figure 3).[12]

Table 2. ^{13}C NMR Data of Neomarinone (**4a** and **4b**) and Incorporation Percentages of Labeled Precursors

		incorporation (%)			J_{CC} (Hz)	
carbon **4b**/**4a**	δ_c ppm[a]	[1-^{13}C] acetate[b,c]	[3-$^{1\,3}$C] alanine[b,c]	[1-^{13}C] glucose[b,c]	[1,2-$^{1\,3}$C] acetate	[U-$^{13}C_6$] glucose
1/1	153.6	1.0			54, 75	54, 75
2/2	180.4		0.6	0.7	55[e]	55[e]
3/3	131.6	1.9			55, 65	55, 65
4/4	107.9		1.5	0.4	63[e]	64[e]
5/5	157.7	3.6			62, 74	62, 74
6/6	127.3		3.8	0.2	61, 74	61, 74
7/7	159.9	3.1			61, 72	61, 72
8/8	107.9		1.5	0.4	58, 71	58, 71
9/9	182.9	3.8			56, 58	56, 57
10/10	120.3		0.3	0.7	55, 74	55, 74
11/11	15.1			0.8		40
12/12	86.3					40
13/13	46.0					36
14/14	31.0					s[f]
15/18	30.5			0.9		35
16/17	39.7					35[d]
17/21	32.8					36
18/20	26.6					s[f]
19/19	25.1			1.0		40
20/15	123.9					40
21/16	138.9					s[f]
22/24	18.8					s[f]
23/23	20.9		7.7	0.8		s[f]
24/22	15.7		7.8	0.7		36
25/25	19.7		8.0	0.8		36
26/26	8.6					

[a] Referenced to DMSO-d_6. [b] Percent incorporation = (A − B)/B; A = integrated signal of enriched carbon and B = integrated signal of natural abundance carbon. [c] Percent incorporation relative to the nonincorporated carbon at 8.6 ppm (C26). [d] Data collected in acetone-d_6 due to signal overlap with DMSO-d_6. [e] This signal represents overlapping doublets as verified by INADEQUATE NMR. [f] s = enriched singlet.

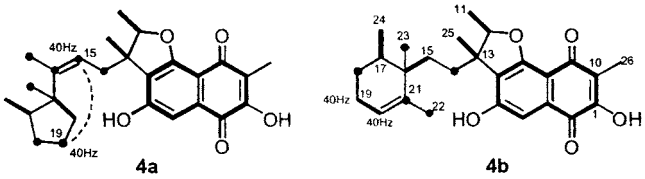

Figure 3. Labeling patterns of [U-$^{13}C_6$]glucose in **4a** and **4b**. Carbons 15 and 19 in **4a** appear as enhanced and coupled doublets (J_{CC} = 40 Hz), implying that these carbon atoms are directly bonded to each other as shown in **4b**.

To provide support for the revised structure **4b**, unlabeled neomarinone was independently reassigned by NMR, as the original description[6] of neomarinone was lacking comprehensive 2D NMR data for the side chain region in question. Proton and carbon NMR data, along with COSY and gradient-enhanced HMQC and HMBC spectroscopy, confirmed that the naphthoquinone moiety of the molecule and the adjacent furan ring were correctly assigned.[13] COSY correlations between H14 (δ 1.86) and methylene protons at δ 1.16 and 1.32, and not with the olefin at δ 5.35 as expected for structure **4a,** were consistent with the revised structure **4b**. The olefinic proton rather coupled to the C19 methylene proton H19a as part of a multispin system linking C20 through C24 in **4b**. Strong two- and three-bond HMBC correlations from the three methyl groups on the cyclohexene ring allowed for the majority of the sesquiterpene to be unambiguously assigned. Key HMBCs from methylene H14 to C12, C13, and C15 in the furan group and to C15 in the side chain and from the olefin H20 to C16, C18, and C22 in the cyclohexene ring confirmed the structure as **4b** and were not compatible with the previously reported **4a**.

The *cis* relative stereochemistry of the methyl groups C23 and C24 was deduced by comparison of their ^1H NMR chemical shifts in $CDCl_3$ with those of other natural products containing a similarly substituted cyclohexene ring. Chemical shifts of the methyl protons H23 (δ 0.81) and H24 (δ 0.83) favored the *cis* relative stereochemistry as reported for the sponge metabolite ageline A[14] (both signals at δ 0.85) rather than a *trans* stereochemical relationship as reported for another sponge metabolite (+)-subersin[15] (δ 0.94 and 1.03). Similarly, the *cis* stereochemistry of methyl groups attached to the furan ring was determined by comparison of ^1H NMR chemical shifts recorded in $CDCl_3$ with those reported for (−)-furaquinocin C[4] and the synthetic product (+)-3-epi-

(12) As a result of the structure revision, the numbering scheme for the original structure **4a** has been changed to reflect the revised structure **4b**.

(13) ^1H NMR (DMSO-d_6) δ (multiplicity, assignment, coupling constants in Hz, *COSYs*, HMBCs): 0.73 (s, H23, C15, C16, C17, C21), 0.77 (d, H24, 6.5 Hz, *H17*, C16, C17, C18), 1.13 (s, H25, C6, C12, C13, C14), 1.14 (m, H14a, *H15b*), 1.16 (m, H15a, *H14a*, *H14b*, C16, C21), 1.30 (d, H11, 6.0 Hz, *H12*, C12, C13), 1.32 (m, H15b, *H14a*, *H14b*, C16, C23), 1.36 (m, H18, *H17*, *H19a*, *H19b*, C17, C19), 1.51 (s, H22, C16, C20, C21), 1.69 (m, H17, *H18*, *H24*, C16, C18, C23, C24), 1.82 (s, H26, C1, C9, C10), 1.84 (m, H19a, *H18*, *H20*), 1.86 (m, H14b, *H15a*, *H15b*, C12, C13, C15, C25), 1.90 (m, H19b, *H18*, C20), 4.65 (q, H12, 6.0 Hz, *H11*, C6, C7, C13, C14, C25) 5.35 (brs, H20, *H19a*, C16, C18, C22), 7.04 (s, H4, C2, C3, C5, C6, C8, C13).

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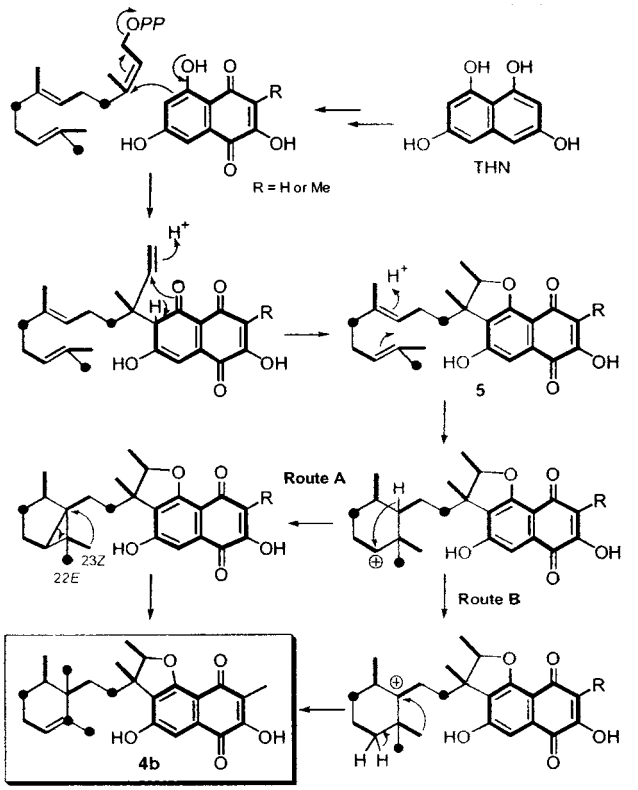
furaquinocin C.[16] Chemical shifts of H11 (δ 1.47) and H25 (δ 1.27) compared favorably with those of the *cis* oriented methyls in (+)-3-epifuraquinocin C (δ 1.47 and 1.30) rather than the *trans* oriented methyls in (−)-furaquinocin C (δ 1.51 and 1.47), supporting the relative stereochemistry originally reported for neomarinone.[6] The configuration of the cyclohexene ring relative to the furan residue could not be unequivocally determined by nuclear Overhauser NMR experiments due to the numerous overlapping signals in DMSO and $CDCl_3$.

With the revised structure of neomarinone (**4b**) in place, we characterized [3-^{13}C]alanine- and [1-^{13}C]glucose-enriched neomarinone by ^{13}C NMR to shed additional light on the biosynthesis of the sesquiterpenoid side chain. In addition to labeling the naphthoquinone core via [2-^{13}C]acetate, [3-^{13}C]alanine labeled three side chain carbons (C23, C24, and C25), whereas [1-^{13}C]glucose further labeled C11, C15, and C19 (Table 2). Taken together with the [U-$^{13}C_6$]glucose biosynthetic results, these data are consistent with a non-mevalonate pathway to the sesquiterpenoid unit in neomarinone involving two intact and one rearranged isoprene units. Attachment of C3 of FPP to the polyketide involving an initial C-prenylation, as seen for the related furaquinocin antibiotics from *Streptomyces* sp. KO-3988,[4b] would yield the furan intermediate **5** (Scheme 1). Conversely, O-prenylation of the C5- or C7-hydroxy groups followed by Claisen rearrangement also provides the precursor of **5** (not shown). Proton-assisted cyclization of the linear diene in **5** yields the putative cyclohexyl carbocation, which after deprotonation and methyl migration provides neomarinone (**4b**). The C23 methyl group in **4b** originates from the methyl group in the *Z* and not the *E* configuration at C21 in **5**, as C21, C22, and C23 all appear as enhanced singlets in the ^{13}C NMR spectrum of [U-$^{13}C_6$]glucose-derived **4b** (Table 2).

The biosynthesis of the naphthoquinone core common among the marinones must proceed through a symmetrical pentaketide intermediate such as THN to satisfy the observed labeling pattern with [1,2-$^{13}C_2$]acetate. Recently, it was demonstrated that THN is the product of the type III polyketide synthase THN synthase, which appears to be common among actinomycetes.[17] Flaviolin, a known auto-oxidation product of THN,[18] either directly or methylated at C10 via *S*-adenosyl methionine may serve as an intermediate

Scheme 1. Proposed Biosynthesis of Neomarinone **(4b)**



in neomarinone biosynthesis (Scheme 1). We recently cloned and sequenced a THN synthase homologous gene from strain CNH-099 in our work toward cloning the (neo)marinone biosynthetic gene cluster(s) for future metabolic engineering of bacterial meroterpenoids for drug discovery.

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Supporting Information Available: NMR spectra of unlabeled and ^{13}C-labeled neomarinone. This material is available free of charge via the Internet at http://pubs.acs.org.

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