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Structure and absolute configuration of 3-alkylpiperidine alkaloids from an Indonesian sponge of the genus *Halichondria*

I. Wayan Mudianta [a,b], Peter L. Katavic [a], Lynette K. Lambert [c], Patricia Y. Hayes [a], Martin G. Banwell [d], Murray H.G. Munro [e], Paul V. Bernhardt [a], Mary J. Garson [a,*]

[a] *School of Chemistry and Molecular Biosciences, The University of Queensland, Brisbane, QLD 4072, Australia*
[b] *Department of Analytical Chemistry, Faculty of Mathematics and Natural Sciences, Ganesha University of Education, Bali, Indonesia*
[c] *Centre for Magnetic Resonance, The University of Queensland, Brisbane, QLD 4072, Australia*
[d] *Research School of Chemistry, Institute of Advanced Studies, The Australian National University, Canberra, ACT 0200, Australia*
[e] *Department of Chemistry, University of Canterbury, Private Bag 4800, Christchurch, New Zealand*

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ABSTRACT

Chemical analysis of an Indonesian sponge sample has provided three new 3-alkylpiperidine alkaloids, tetradehydrohaliclonacyclamine A, its mono-*N*-oxide derivative, and a 2-*epi* isomer. The absolute structure of tetradehydrohaliclonacyclamine A has been established by X-ray crystallography from anomalous dispersion effects using Cu radiation, which determined that the absolute configuration is 2*S*, 3*S*, 7*S*, 9*S* while an HPLC study revealed that the alkaloid is enantiomerically pure.

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1. Introduction

Over thirty different 3-alkylpiperidine-based carbon frameworks have been discovered in metabolites isolated from sponges of the order Haplosclerida. Representatives include the haliclamine/cyclostellettamine, ingenamine, madangamine, ircinal, manzamine, halicyclamine, sarain, petrosin, xestospongin/araguspongine and aragupetrosine skeletons.[1,2] Previously we reported the isolation of four bioactive alkylpiperidine alkaloids, the haliclonacyclamines A to D (**1**–**4**) (Fig. 1), from a *Haliclona* species collected at Heron Island (Australia);[3,4] our X-ray crystallographic studies have recently established that (−)-haliclonacyclamine A (**1**) and (+)-haliclonacyclamine B (**2**) each have the absolute configuration 2*R*, 3*R*, 7*R*, 9*R*.[5] Two additional haliclonacyclamine metabolites, E (**5**) and F (**6**), and a series of C-22 hydroxylated metabolites known as the arenosclerins have been isolated from the Brazilian sponges *Arenosclera braziliensis*[6] and *Pachychalina alcaloidifera*.[7] All of these metabolites share two connecting chains ('spacer groups') of ten and twelve carbons, respectively, in length, but show variation in double bond locations. The identical carbon skeleton is present in halicyclamine A (**7**), isolated by two separate research groups from an Indonesian *Haliclona* sp.[8] and a Japanese *Amphimedon* sp.,[9] and in halichondramine (**8**), isolated from the Red Sea sponge *Halichondria* sp.[10] The related halicyclamine B (**9**), whose relative stereochemistry has been secured by an X-ray analysis, possesses two spacer groups that each contain only eight carbon atoms.[11] Differences in the relative configuration at the methine centres of these various alkaloids may be of biosynthetic significance.

We now report the isolation and characterization of a new alkaloid tetradehydrohaliclonacyclamine A (**10**) from the Indonesian sponge *Halichondria* sp., together with its *N*-oxide derivative **11** and the 2-*epi* isomer **12**. X-ray crystallographic determination of the absolute configuration of **10** was undertaken using anomalous dispersion effects, while an HPLC study has confirmed that **10** is present in a single enantiomeric form in the sponge.

2. Results and discussion

A large encrusting, dark green sponge (genus *Halichondria*) was collected in Tulamben Bay, Bali, Indonesia at a depth of 20 m, and

* Corresponding author: Tel.: +61 7 33653605; fax: +61 7 33654273.
E-mail address: m.garson@uq.edu.au (M.J. Garson).

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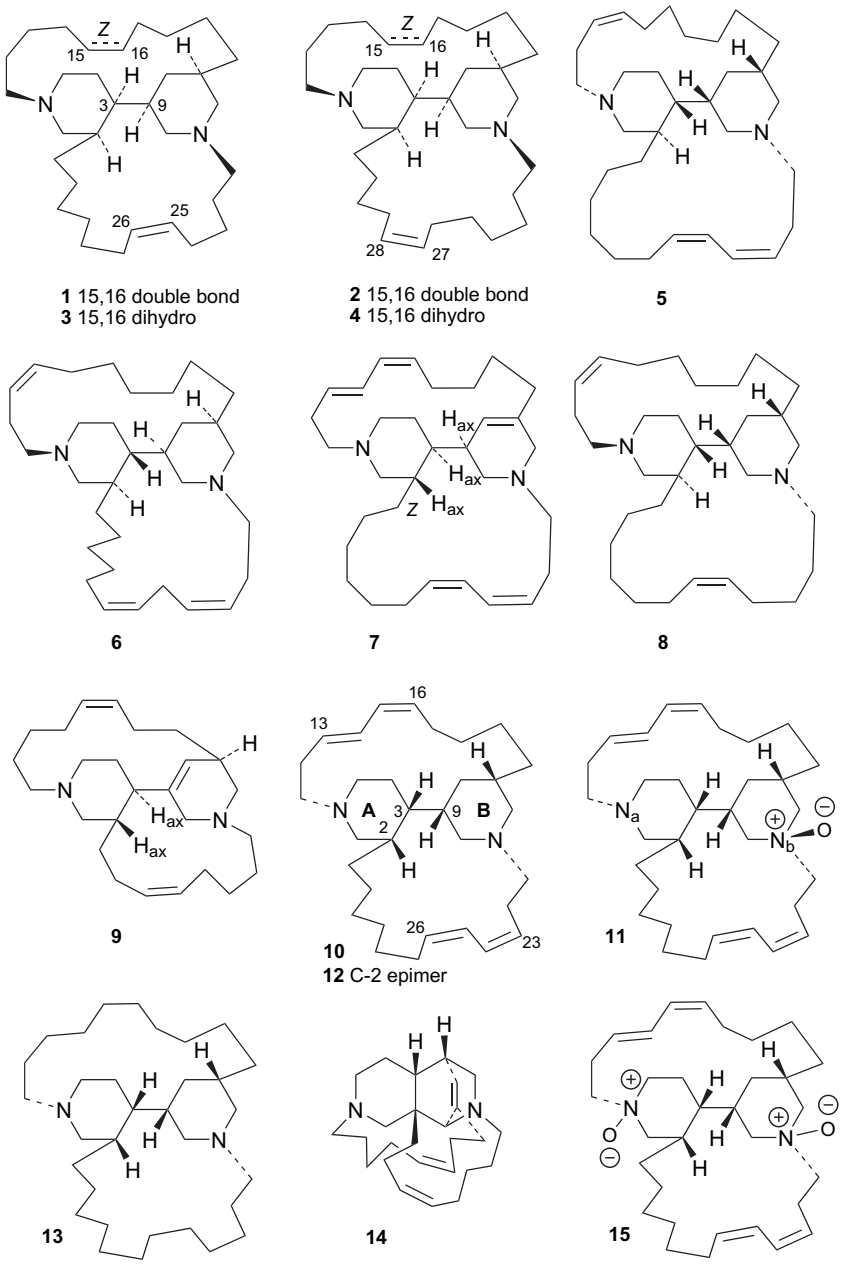

Figure 1. Structures of some 3-alkylpiperidine alkaloids.

kept frozen prior to extraction with DCM/MeOH (1:1). The crude extract was subjected to normal phase flash chromatography followed by normal phase HPLC using hexanes/EtOAc/Et_3N (60:35:5). The new compound **10** was isolated as a white solid, and had a molecular formula of $C_{32}H_{52}N_2$ inferred from HRESIMS.

The ^1H NMR spectrum showed severely overlapping signals particularly in the δ_H 1.0–1.7 region, however eight alkene protons were observed in the downfield region and were consistent with four double bonds. Inspection of ^{13}C and DEPT NMR spectra of **10** showed the presence of 32 carbon signals including eight double bond carbons between δ_C 122.6 and 135.5, four methine carbons at δ_C 36.6, 38.8, 42.7 and 46.6, and six distinctive methylenes adjacent to nitrogen atoms between δ_C 47.8 and 61.1. There were no methyl or quaternary carbon signals apparent from the preliminary spectra, and all these data implied that the structure of **10** was a 3-alkylpiperidine alkaloid.

An *E,Z* diene, apparent from signals at δ_H 5.65 (dt, J=7.5, 14.1 Hz, H-13), 6.32 (dd, J=14.1, 11.5 Hz, H-14), 5.99 (dd, J=11.5, 10.6 Hz, H-15) and 5.23 (dt, J=8.6, 10.6 Hz, H-16), was positioned in the C_{10} spacer group from DQFCOSY, 1D-TOCSY and HMBC data. DQFCOSY correlations from H-13 at δ_H 5.65 identified the H-12 protons, and then H-11 at δ_H 3.00 (2H, m), while an HMBC correlation from H-13 to C-11 at δ_C 56.5 was observed. In 1D-TOCSY experiments (mixing time 100 ms), the signals of protons H-11, H-12a[†], H-12b, H-14, H-15, H-16 and H-17 were apparent when H-13 was irradiated, while irradiation of H-16 detected the signals for protons H-17, H-18, H-19 and H-20. Individual proton and carbon chemical shifts for C-17 to C-20 were deduced from the HMBC and HSQC data.

A second diene system of *Z,Z* configuration (δ_H 5.45 (dt, J=4.2, 10.6 Hz, H-23), 6.11 (dd, J=10.6, 11.7 Hz, H-24), 6.32 (dd, J=11.7, 10.9 Hz, H-25) and 5.35 (dt, J=5.4, 10.9 Hz, H-26)), was apparent in the C_{12} spacer group. DQFCOSY correlations starting from the signal

[†] a and b denote downfield and upfield resonances, respectively, of a geminal pair.

at δ_H 5.45 for H-23 led to H-22 at δ_H 2.86/1.81, and to H-21 at δ_H 2.93, while a HMBC correlation linked H-23 to C-21. In 1D-TOCSY experiments, the signals of proton H-21, H-22a and H-22b were apparent when H-23 was irradiated together with the signals of H-24, H-25, H-26, H-27a, and H-27b, while irradiation of H-26 resulted in the detection of the overlapping signals corresponding to H-29 and H-30 together with H-27 and H-28.

The starting point for assembly of piperidine ring A was the well-resolved signal for H-1a at δ_H 2.62 (t, J=11.6 Hz), which showed DQFCOSY connectivity to H-2 at δ_H 1.63, while C-2 at δ_C 42.7 displayed HMBC correlations to H-1a, and to the methylene signals at δ_H 1.15 and 1.56 assigned to H-32 and H-4b, respectively. Correlations between H-4a and H-5a at δ_H 2.92, and between H-2 and H-32 were evident in the DQFCOSY spectrum while HMBC correlations linked C-5 at δ_C 47.8 to H-1a, H-1b, H-3 and H-11, C-3 at δ_C 38.8 to H-1a, H-1b, and H-5b, C-32 to H-1a, H-1b and H-3, and importantly C-11 to H-5. 1D-TOCSY irradiation of H-1a (mixing time 20 ms) revealed signals corresponding to H-1b, H-2, H-3 and H-32, while signals for H-4a, H-4b, H-5a, H-5b, H-30 and H-31 also appeared when the mixing time was extended to 100 ms. Identical 1D-TOCSY experiments were performed on haliclonacyclamine A (**1**) and provided a comparable set of proton signals (Fig. 2).

In ring B, a distinctive upfield signal at δ_H 0.77 (q, J=12.3 Hz) was assigned to H-8b by comparison with the data for haliclonacyclamine A (**1**) and B (**2**),[3,4] and provided H-7 at δ_H 1.41 and H-9 at δ_H 1.59 from DQFCOSY data. Other correlations linked H-7 to H-6b at δ_H 2.01 along with H-9 to H-10b at δ_H 2.49. HMBC correlations were observed for C-10 at δ_C 61.1 to H-8b, to H-6b, and to H-21 of the spacer group, and for C-6 at δ_C 57.7 to H-8b, to H-21, and to H-20b at δ_H 1.04. Both H-6b and its geminal partner H-6a at δ_H 2.53 showed HMBC correlations with C-8, C-20 and C-21. 1D-TOCSY irradiation (mixing time 20 ms) of H-8b detected H-7, H-8a and H-9 while protons H-6a, H-6b, H-10a, H-10b, H-20a and H-20b were visible when the mixing time was extended to 100 ms. 1D-TOCSY irradiation of the H-8b signal of haliclonacyclamine A (**1**) gave comparable results (Fig. 2).

The two piperidine rings were linked on the basis of HMBC correlations; C-8 and C-10 were found to show HMBC coupling to H-3, while C-9 correlated to H-4. A connection between H-3 and H-9 was not evident in the DQFCOSY spectrum and suggested that the dihedral angle between them was close to 90°.[3,4] Consequently, there were no H2BC cross peaks observed between H-3 and C-9 or between H-9 and C-3. DQFCOSY correlations between H-7 and H-20, together with HMBC correlations for H-20 to C-6, C-7 and C-8, and for H-7 to C-20 positioned the C10 alkyl chain, while the C_{12} spacer group was linked to C-2 of the bis-piperidine core from the HMBC and DQFCOSY correlations mentioned earlier. Full ^{1}H and ^{13}C assignments are summarized in Table 1.

The relative configuration of ring B was identical to that of haliclonacyclamines A–D,[3,4] and arenosclerins A and C,[6] with the protons H-7, H-8b and H-9 all axially disposed. The well-defined quartet for H-8b at δ_H 0.77 presented a 12.3 Hz coupling to each of H-7, H-8b and H-9, while H-8b showed NOESY correlations to H-10a and H-6b, and H-7 was correlated to H-9. In ring A, the triplet signal at δ_H 2.62 for H-1a had an 11.6 Hz coupling to its geminal partner H-1b and also to H-2, therefore H-1a and H-2 were *trans*-diaxial. In the NOESY spectrum, H-1a correlated to H-5b, however there were no correlations observed between H-3 and either H-1a or H-5b. The 1D-TOCSY data revealed that H-3 was a multiplet showing small couplings and suggested that this proton was equatorial rather than axial. The relative configuration of ring A is thus the same as haliclonacyclamine A-D[3,4] but differs with that deduced for haliclonacyclamine E or arenosclerins A and C.[6] When joined, the two rings adopt a perpendicular orientation with a vicinal dihedral angle between H-3 and H-9 close to 90° as suggested by the DQFCOSY data.[3,4] NOESY couplings were observed between H-1a and both H-8b and H-10a. The overall relative configuration of the bis-piperidine system in **10** is shown in Figure 3; both rings possess chair conformations, and the nitrogen lone pairs are in the more stable axial position.[12]

The molecular structure of **10** was confirmed by single crystal X-ray analysis of crystals grown from a solution in hexanes/EtOAc (1:3) (Fig. 4). The structure determined at 130 K was monoclinic ($P2_1$) and comprised two independent molecules in the asymmetric unit (with atom labels 'a' and 'b') that differ in the conformation of the methylene carbons in the region C18a/b to C20a/b. The greatest apparent difference was in the positions of the methylene carbons C19a and C19b, as illustrated in Figure 4B. The *E,Z* and *Z,Z* diene units are apparent at C-13/C-16 and C-23/C-26 in the C_{10} and C_{12} spacer groups, respectively. Each alkylpiperidine ring adopts a chair conformation with H-2, H-7 and H-9 all axial, while H-3 was equatorial.

In addition to confirming the relative configuration of **10** the absolute configuration of **10** was determined by the Bijvoet analysis of Hooft et al. using Cu-Kα radiation and a high redundancy of data,[13] (see Experimental section), which has been shown to be effective in the absolute structure determination of light atom compounds, such as the haliclonacyclamines.[5] In similar light atom compounds the conventional Flack parameter[14] used for absolute structure determination is often inconclusive due to its poor precision. Indeed, the Flack parameter for the structure of **10** was −0.1(±0.3), and could not be used in this case to discriminate between enantiomers. By contrast the Bijvoet pair analysis of Hooft et al.[13] yielded a probability $P2$=1.000 that the correct enantiomorph was chosen for a two-state model (one enantiomer or the other). There are four stereogenic C-atoms highlighted in Figure 4 as C2a/b, C3a/b, C7a/b and C9a/b. These data revealed the absolute configuration of tetradehydrohaliclonacyclamine A (**10**) was 2*S*, 3*S*, 7*S* and 9*S*, and thus was the opposite configuration to the haliclonacyclamines A and B for which 2*R*, 3*R*, 7*R* and 9*R* configuration had previously been determined[5] by the same crystallographic analysis employed here.

Catalytic hydrogenation of **10** with Pd/C/H_2 gave a sample of the fully saturated derivative **13** of $[\alpha]_D^{22}$ −20.9 (*c* 0.205, $CHCl_3$); this optical rotation value is similar in magnitude but *opposite in sign* to the values for samples of *ent*-**13** prepared previously from either haliclonacyclamine A ($[\alpha]_D^{22}$ +24.9 (*c* 0.45, CH_2Cl_2))[3] or from haliclonacyclamine C ($[\alpha]_D^{22}$ +12.7 (*c* 0.28, CH_2Cl_2)).[4] We propose the name perhaliclonacyclamine to describe **13** rather than tetrahydrohaliclonacyclamine A[3] or dihydrohaliclonacyclamine C[4] since this is independent of the origin of the sample. The spectroscopic correlation further verified the Hooft crystallographic analysis method[13] that was employed in studies of **3**, **4**[5] and **10**. We note in passing that the 293 K crystal structure of **10** is orthorhombic, but this has no bearing on the present results. The full details of this structure will be published separately.[15]

An optical rotation measurement of **10** prior to recrystallization gave +19.9 (*c* 0.68, $CHCl_3$) while the $[\alpha]_D$ values of the crystals and the residual mother liquors were +19.4 (*c* 0.515, $CHCl_3$) and +19.1 (*c* 0.835, $CHCl_3$), respectively. When an HPLC analysis was run using a DAICEL chiral OD-H column (100% IPA at 0.30 mL/min), the trace (Fig. 5) clearly showed the (+) isomer of **10**, while the peak shape was not consistent with the presence of any (−)-**10**. It was concluded that tetradehydrohaliclonacyclamine A (**10**) appears to be a single enantiomer. Our recent X-ray crystallographic analyses of both haliclonacyclamines A (**1**) and B (**2**) also suggested that each of these metabolites is a single enantiomer.[5] These results contrast with the observations of Kobayashi et al. that the pentacyclic metabolite keramaphidin B (**14**) is a mixture of enantiomers. They found crystals of **14** to be racemic, while the mother liquors were enriched in the (−) isomer.[16]

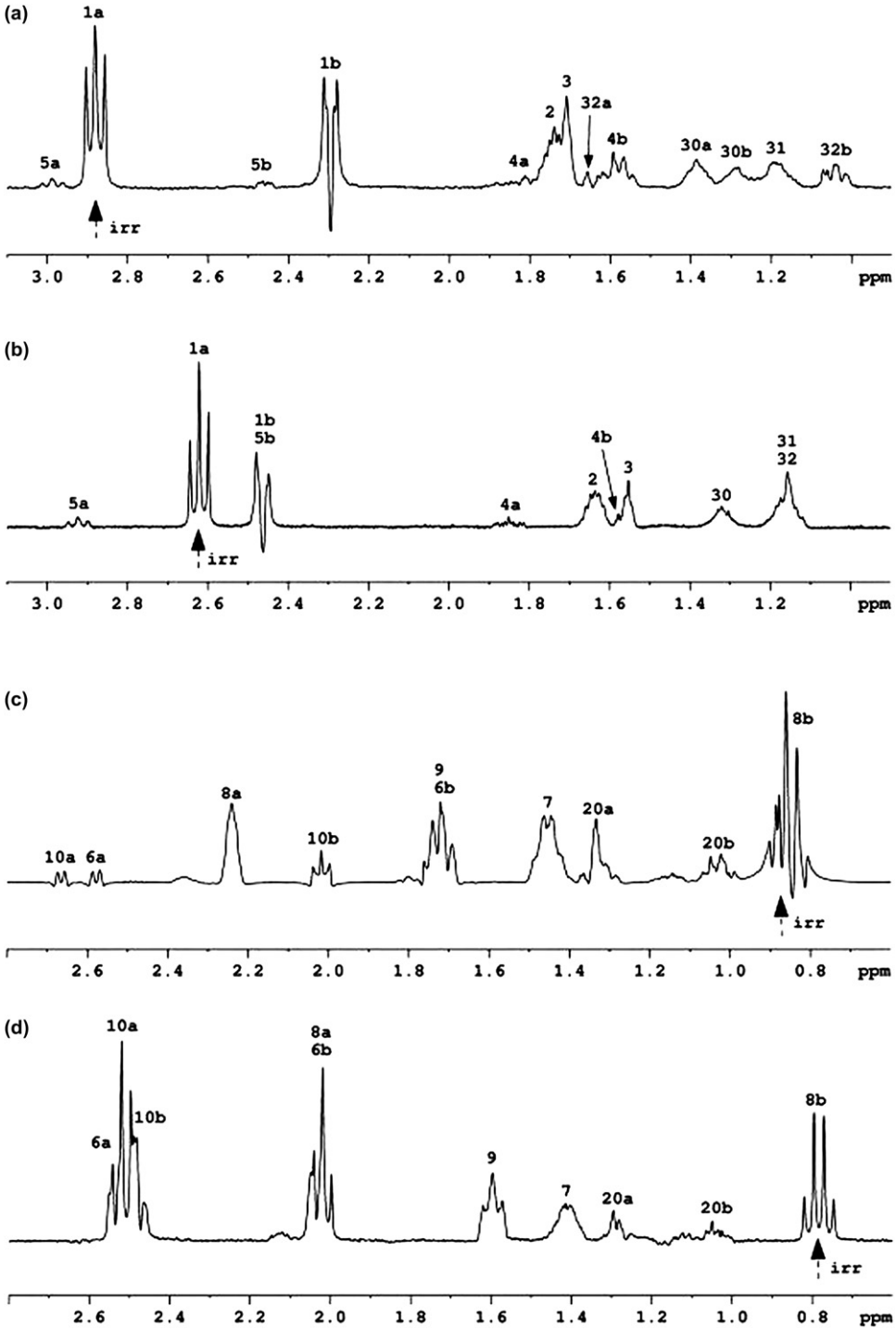

Figure 2. 1D-TOCSY spectra for haliclonacyclamine A (**1**) and tetradehydrohaliclonacyclamine A (**10**) showing the effect of irradiating: (a) H-1ax of haliclonacyclamine A; (b) H-1ax of tetradehydrohaliclonacyclamine A; (c) H-8ax of haliclonacyclamine A; (d) H-8ax of tetradehydrohaliclonacyclamine A. All spectra were acquired with a mixing time of 100 ms.

A second alkaloid **11** isolated in small quantity had a molecular formula of $C_{32}H_{52}N_2O$ inferred from HRESIMS. The ^1H and ^{13}C NMR spectra were fully assigned by 2D NMR analysis, and by comparison with the data for **10** and for the haliclonacyclamines. In the ^1H NMR spectrum of **11**, irradiation of a signal at δ_H 3.52 (2*H*) (mixing time 80 ms) led to enhancement of signals for the H-23/H-26 diene. This identified the signal as belonging to H-21, however the δ_H value was downfield compared to δ_H 2.93 for H-21 of **10**. Likewise in the ^{13}C NMR spectrum, three carbons at δ_C 73.8 (C-21), 69.0 (C-6) and 72.0 (C-10) were noticeably more downfield than in **10**. These data all suggested a mono-*N*-oxide associated with ring B of the alkaloid. 1D-TOCSY irradiation of the upfield quartet at δ_H 0.88, assigned to H-8ax by comparison with the data for **10**, revealed signals at δ_H 2.50 and 2.77, assigned to H-7 and H-9, respectively. The appearance of H-8ax implied that H-7 and H-9 were both axial. In the A ring, H-2 was axial since H-1ax was a triplet (J=11.6 Hz), while H-3/C-3 had chemical shift values (δ_H 1.53, δ_C 38.8) closely similar to those of **10** (δ_H 1.55, δ_C 38.8), which suggested that H-3 was equatorial. Treatment of **10** with 1 equiv of MCPBA yielded a complex mixture of the two possible mono-*N*-oxides, the bis-*N*-oxide

Table 1
NMR assignments for tetradehydrohaliclonacyclamine A (**10**), its N^b-oxide (**11**), and 2-*epi* isomer (**12**)

C	Tetradehydrohaliclonacyclamine A (**10**)				N^b-oxide (**11**)		2-*epi* isomer (**12**)	
	^{1}H (mult., *J*)[a,b]	^{13}C, ppm[c]	HMBC[d,e]	DQFCOSY	^{1}H (mult., *J*)[a,b]	^{13}C, ppm[f]	^{1}H (mult., *J*)[a,b]	^{13}C, ppm[g]
1	2.62 (t, 11.6) 2.46 (br d, 11.6)	52.9	3, 11, 32	2	2.64 (t, 11.6) 2.48 (m)	52.9	2.85 (m) 2.22 (m)	55.0
2	1.63 (m)	42.7	1a, 1b, 3, 4a	1, 32	1.72 (m)	43.0	1.46 (m)	33.4
3	1.55 (m)	38.8	1a, 1b, 2, 4a, 4b, 5a, 5b	4a	1.53 (m)	38.8	1.48 (m)	37.6
4	1.85 (m) 1.56 (m)	37.0	5a, 5b	3, 5a	1.87 (m) 1.77 (br d, 11.9)	37.6	1.80 (m) 1.22 (m)	29.1
5	2.92 (m) 2.46 (m)	47.8	1a, 1b, 3, 4a, 4b, 11	4a	2.96 (m) 2.51 (m)	47.8	2.88 (m) 2.41 (dt, 5.0, 10.7)	50.3
6	2.53 (m) 2.01 (t, 11.0)	57.7	8a, 8b, 10a, 10b, 20b, 21	7	2.71 (t, 11.7) 2.88 (m)	69.0	2.65 (m) 2.07 (t, 10.7)	58.6
7	1.41 (m)	38.3	6a, 6b, 8a, 8b, 9, 20a, 20b	6a, 6b, 8a, 8b, 20	2.50 (m)	32.7	1.25 (m)	38.2
8	2.03 (dd, 12.3, 10.8) 0.77 (q, 12.3)	36.6	3, 6a, 6b, 10a, 10b, 20a, 20b	7, 9	2.14 (m) 0.88 (q, 12.3)	35.5	1.95 (m) 0.65 (q, 12.4)	34.3
9	1.59 (t, 12.3)	46.6	4a, 4b, 8a, 8b, 10a, 10b	8a, 8b, 10a, 10b	2.77 (br t, 12.3)	38.0	1.48 (m)	44.2
10	2.52 (m) 2.49 (m)	61.1	3, 6a, 6b, 8a, 8b, 21	9	2.82 (m) 3.09 (t, 12.1)	72.0	2.63 (m) 1.91 (t, 10.7)	59.9
11	3.00 (m)	56.5	5a, 5b, 13	12	3.05 (m) 3.03 (m)	57.0	2.85 (m) 2.75 (m)	55.9
12	2.42 (m) 2.29 (m)	29.5	11, 13	11, 13	2.48 (m) 2.29 (m)	29.5	2.29 (m) 2.24 (m)	29.7
13	5.65 (dt, 7.5, 14.1)	135.5	11, 12, 15	12, 14	5.71 (dt, 7.1, 14.6)	136.2	5.69 (dt, 7.2, 14.8)	135.0
14	6.32 (dd, 14.1. 11.5)	125.5	12, 15, 16	13, 15	6.34 (dd, 14.6, 11.4)	125.3	6.35 (dd, 11.0, 14.8)	126.2
15	5.99 (dd, 11.5, 10.6)	129.9	13, 14, 17, 18	14, 16	6.03 (dd, 11.4, 10.4)	129.7	5.99 (dd, 11.0, 10.7)	129.4
16	5.23 (dt, 8.6, 10.6)	129.4	14, 17, 18	15, 17	5.26 (dt, 8.3, 10.4)	129.5	5.22 (dt, 8.8, 10.7)	129.8
17	2.12 (m)	26.4	15, 18, 19	16, 18	2.16 (m) 2.19 (m)	25.5	2.18 (m)	27.0
18	1.44 (m) 1.30 (m)	28.0	16, 19a, 19b	—	1.45 (m) 1.25 (m)	27.5	1.33 (m) 1.25 (m)	27.9
19	1.29 (m) 1.12 (m)	24.6	17, 20a, 20b	—	1.28 (m) 1.22 (m)	23.7	1.45–1.20 (m) [f]	-[j]
20	1.30 (m) 1.04 (m)	33.1	6b, 7, 8a, 8b, 18a, 18b	—	1.34 (m) 1.06 (m)	32.0	1.33 (m) 1.04 (m)	33.1
21	2.93 (m)	57.0	6a, 6b	—	3.52 (br)	73.8	2.98 (dt, 3.0, 12.0) 2.65 (m)	57.5
22	2.86 (m) 1.81 (br d, 14.2)	23.4	23, 24	23	2.68 (m) 2.77 (br t, 12.2)	23.5	2.15 (m)	25.9
23	5.45 (dt, 4.2, 10.6)	132.9	21, 22, 25	22, 24	5.50 (m)	128.3	5.47 (m)	132.5
24	6.11 (dd, 11.7, 10.6)	122.6	22, 25, 26	23, 25	6.23 (t, 11.3)	123.7	6.17 (t, 11.0)	123.3
25	6.32 (dd, 11.7, 10.9)	124.5	23, 24, 27	24, 26	6.34 (t, 11.3)	125.3	6.29 (t, 11.0)	123.9
26	5.35 (dt, 5.4, 10.9)	132.2	24, 27, 28	25, 27	5.50 (m)	134.3	5.47 (m)	132.4
27	2.33 (m) 1.95 (m)	25.6	25, 26, 28, 29, 30	26, 28	2.40 (m) 2.04 (m)	24.8	2.58 (m) 2.22 (m)	24.7
28	1.45 (m)	28.1	27, 29, 30	27a	1.50 (m) 1.40 (m)	27.5	1.45–1.20 (m)[h]	-[j]
29	1.21–1.14 (m)[h]	28.6[i]	28, 31	—	1.22 (m)[h] 1.40 (m)	29.5[i]	1.45–1.20 (m)[h]	-[j]
30	1.35–1.27 (m)[h]	28.7[i]	28, 31, 32	—	1.22 (m) 1.35 (m)[h]	28.5[i]	1.45–1.20 (m)[h]	-[j]
31	1.17 (m)	25.7	29, 30	—	1.19 (m) 1.16 (m)	25.2	1.45–1.20 (m)[h]	-[j]
32	1.15 (m)	32.3	1a, 1b, 2, 3	2	1.15 (m) 1.04 (m)	32.3	1.33 (m) 1.22 (m)	33.0

[a] 500 MHz, $CDCl_3$ referenced to ^{1}H at δ 7.24 ppm.
[b] Coupling constant in hertz (Hz).
[c] 100 MHz, $CHCl_3$ referenced to ^{13}C at δ 77.0 ppm.
[d] HMBC connectivity from C to H.
[e] Correlations observed for one bond J_{C-H} of 145 Hz and long range J_{C-H} of 8 Hz.
[f] 187.5 MHz, $CHCl_3$ referenced to ^{13}C at δ 77.0 ppm.
[g] 125 MHz, $CHCl_3$ referenced to ^{13}C at δ 77.0 ppm.
[h] Signals may be interchangeable.
[i] Signals may be interchangeable.
[j] The three signals at 28.8, 28.7, and 27.9 ppm and the two signals at 26.4 and 25.0 ppm could not be individually assigned.

together with unreacted amine. However the bis-*N*-oxide **15** was cleanly prepared from reaction of **10** with 2 equiv MCPBA. ^{1}H and ^{13}C NMR data for H-6, H-10 and H-21 of **15** were fully consistent with those shown by alkaloid **11**. Recently 3,4-dihydromanzamine J *N*-oxide has been isolated from the Okinawan marine sponge *Amphimedon* sp.[17]

A second minor metabolite **12** was isolated from the sponge extract by flash chromatography followed by several rounds of NP HPLC. The molecular formula of $C_{32}H_{52}N_2$ suggested that the metabolite was a stereoisomer of **10**. Full ^{1}H and ^{13}C assignments were completed as for **10** and for **11**. In particular, an edited HSQC experiment located four methine carbons at δ_C 33.4, 37.6, 38.2 and 44.2, and identified their attached protons. The quartet appearance of the upfield signal for H-8ax at δ_H 0.65 indicated that the stereochemistry of the neighbouring methines (H-7 and H-9) remained the same as in **10**. Therefore, the stereochemistry at either C-2 or at C-3, or even at both positions, differed in **12**. 1D-TOCSY irradiation of H-8ax, and of a signal at δ_H 2.41 assigned to

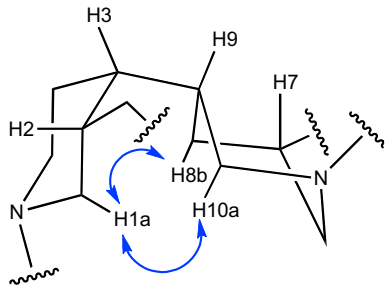

Figure 3. Relative configuration of the bis-piperidine ring system of **10** showing inter ring NOEs.

H-5ax, secured identification of the remaining protons of the individual piperidine rings. These TOCSY experiments gave data consistent with the equatorial placement of the H-3 proton since there was no enhancement of ring A protons on irradiation of H-8ax, or of ring B protons on irradiation of H-5ax, nor any evidence of a large diaxial coupling for either of H-2 or H-3. The H-1ax signal presented as a multiplet rather than the distinctive triplet observed in **10** and **11**, which suggested that H-2 was equatorial rather than axial. Finally, when compared with values for **10**, differences were observed in the ^{13}C assignments of ring A of **12**. The chemical shift of C-2 (δ_C 33.4) was shielded by 9.3 ppm compared to C-2 of **10**, while C-3 was 1.2 ppm upfield, C-4 was 7.9 ppm upfield, and C-5 was 2.5 ppm downfield of the values shown by these carbons in **10**. These chemical shift differences are similar to the chemical shift differences reported for haliclonacyclamine E (**5**) by Berlinck[6] compared to the data for haliclonacyclamines A-D.[3,4] On this basis, **12** was suggested to be the 2-*epi* isomer of **10**, however there was insufficient compound available for recrystallization and X-ray study.

The biosynthesis of tetradehydrohaliclonacyclamine A (**10**) is proposed as shown in Figure 6. A nicotinic acid chain starter unit leads to the formation of a series of C_{11} and C_{13} chains, each with a carboxyl terminus. Condensation of these alkyl chains, which must involve decarboxylation, gives the macrocyclic intermediate **16**, now with C_{10} and C_{12} spacer groups. Next an intramolecular Diels–Alder, as first suggested by Baldwin and Whitehead,[18] produces intermediate **17**, closely related to keramaphidin B (**14**). The tetradehydrohaliclonacyclamine A skeleton is then generated by ring opening, involving cleavage (arrow b) of the bond joining

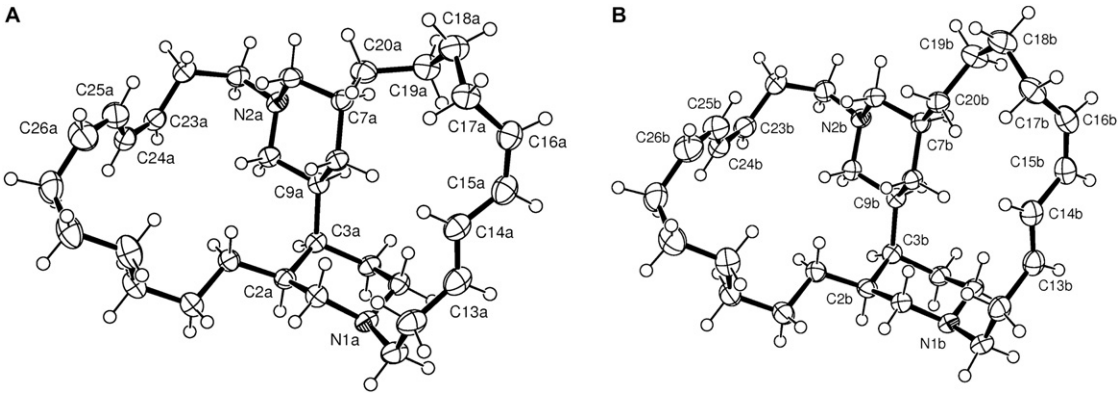

Figure 4. ORTEP views of the two crystallographically independent forms (A, B) of **10** defined in the low temperature monoclinic form. The distinctly different conformations are indicated by the atoms labelled C18a/b–C20a/b.

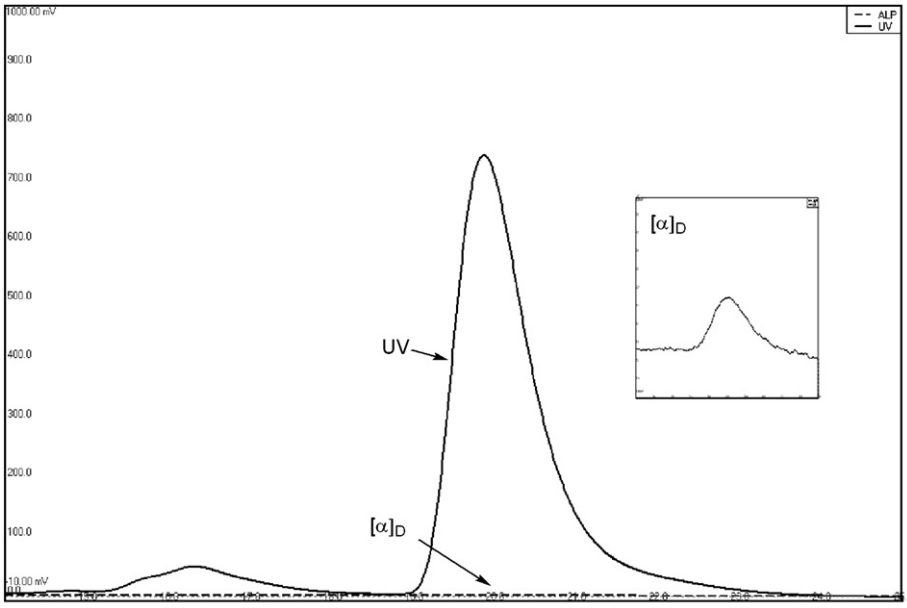

Figure 5. Enantioselective HPLC trace for **10** showing a single enantiomer of the metabolite DAICEL OD-H column 250×4.6 mm; 100% ^{i}PrOH. Trace shows (solid line) UV detection at 254 nm and (dotted line or insert) $[\alpha]_D$ trace.

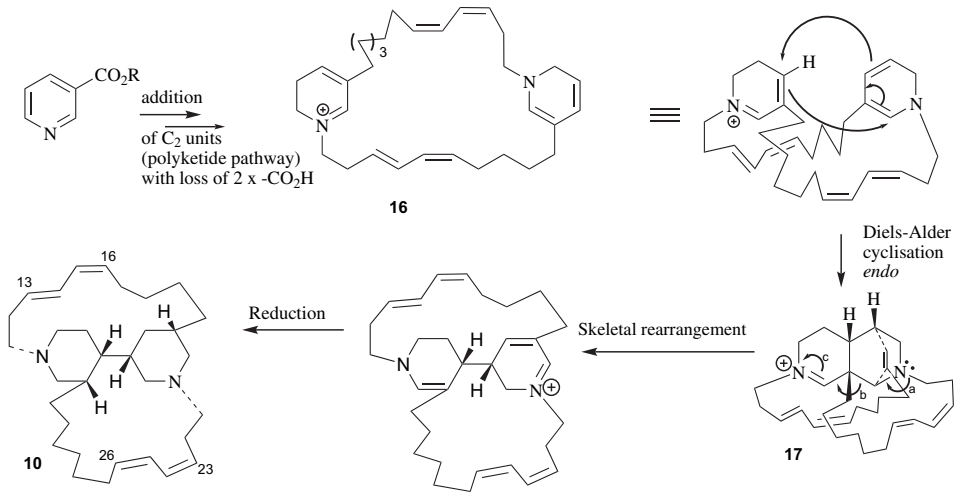

Figure 6. Proposed biosynthesis of tetradehydrohaliclonacyclamine A (**10**).

the two carbons that become C-3 and C-6 in **10**, and reduction of the C-1/C-2, C-6 imine and C-7/C-8 double bonds. Detection of a single enantiomeric form for the haliclonacyclamines,[5] and now also for tetradehydrohaliclonacyclamine A is consistent with the involvement of an enantioselective Diels–Alderase enzyme[19–23] in the biosynthesis rather than a stepwise (ionic) mechanism. Interestingly, the absolute configuration of tetradehydrohaliclonacyclamine A is opposite to that of the haliclonacyclamines. Both enantiomeric series have previously been reported in the 3-alkylpiperidine series of alkaloids.[19]

Tetradehydrohaliclonacyclamine A (**10**) and its bis-*N*-oxide **15** were evaluated for antitumour activity against the P388 cell line. Compound **10** exhibited an IC_{50} of 1.8 μg/mL whereas **15** was not active at the concentrations tested.

3. Conclusions

This study reported three new 3-alkylpiperidine alkaloids, tetradehydrohaliclonacyclamine A (**10**), the mono-*N*-oxide **11**, and a C-2 epimer **12**, from the Indonesian sponge *Halichondria* sp. The relative configuration was deduced by coupling constant analysis combined with 1D-TOCSY data, and confirmed by an X-ray crystallographic analysis of **10**. This crystallographic study also determined the absolute configuration for **10** using anomalous dispersion effects, and was found to be the opposite of that determined for haliclonacyclamine metabolites isolated from the Australian sponge *Haliclona* sp. 628. Catalytic hydrogenation of **10** gave a sample of perhydrohaliclonacyclamine A (**13**) whose optical rotation was equal in magnitude, but opposite in sign, to those of samples of **13** prepared by hydrogenation of haliclonacyclamine A (**1**) or of haliclonacyclamine C (**3**). An HPLC study using a chiral HPLC column showed that tetradehydrohaliclonacyclamine A was a single enantiomer. A biosynthetic scheme consistent with all these stereochemical observations is proposed.

4. Experimental

4.1. General experimental procedures

Optical rotations were obtained using a Perkin–Elmer 241-MC polarimeter. 1D and 2D NMR spectra were acquired using Bruker Avance 400, Bruker Avance 500 or Bruker Avance 750 instruments. NMR spectra were obtained in deuterochloroform at 298 K, and were internally referenced to $CHCl_3$ at δ_H 7.24 or $CDCl_3$ at δ_C 77.0. Positive ion electrospray mass spectra (LRESMS) were determined using a Bruker Esquire HCT instrument or (HRESMS) using a MicroTof Q instrument each with a standard ESI source. Samples were introduced into the source using MeOH as solvent. Normal phase HPLC was carried out using a Waters 515 pump with a Phenomenex (Luna-NH_2 5 μ; 250×10 mm) column and a Gilson 132 series RI detector with EtOAc/hexanes/Et_3N as solvent. Et_3N was distilled prior to use. Chiral HPLC analysis was performed using an Agilent 1200 series instrument equipped with a multiwavelength UV and optical rotation detector, and with a DAICEL chiral OD-H column (25.0×0.46 cm) using ^{i}PrOH (100%) as solvent.

4.2. Biological material

A sponge sample provisionally identified as *Halichondria* sp. was collected from Tulamben Bay, Bali, using SCUBA at a depth of 20 m on 15 January 2008. The sample was taken back to the laboratory and stored at −20 °C until extraction.

4.3. Extraction and isolation of alkaloids 10, 11 and 12

The specimen of *Halichondria* sp. (frozen weight 500 g) was extracted exhaustively with DCM/MeOH (1:1), and the combined extracts (13.7 g) dried under vacuum, then sequentially partitioned into hexanes, DCM, and EtOAc fractions. A portion (2.7 g) of the DCM extract was subjected to SiO_2 flash chromatography with gradient elution (hexanes→EtOAc; plus 1% Et_3N) to give fifteen fractions. Fraction 10 (145 mg) eluting in hexanes/EtOAc (1:1) was subjected to NP HPLC (hexanes/EtOAc/Et_3N 60:35:5) to give compound **10** (56 mg). Alkaloid-containing fractions eluting in 100% EtOAc or EtOAc/MeOH (1:1) were combined and subjected to a DCM/1 M aq HCl solution (10 mL each) partition. The aqueous layer was then basified to pH 10 with K_2CO_3 and extracted three times with DCM (10 mL). The organic layer was separated, dried over Mg_2SO_4, filtered and evaporated to yield additional alkaloid (43.5 mg). A portion of this sample (22 mg) was subjected to SiO_2 flash chromatography with isocratic elution (DCM/MeOH/Et_3N 80:15:5) to yield additional tetradehydrohaliclonacyclamine A (**10**) (13 mg) and tetradehydrohaliclonacyclamine A mono-*N*-oxide (**11**) (0.7 mg). The 2-*epi* isomer **12** was isolated from another portion of the DCM extract (480 mg) using SiO_2 flash chromatography eluting with hexanes/EtOAc/Et_3N (75:20:5). The fractions containing **12** eluted before the fractions containing **10** and were combined (6.3 mg) and subjected to repetitive NP HPLC (hexanes/EtOAc/Et_3N 60:35:5), which yielded compound **12** (1.1 mg). Crystals of **10** were grown slowly from hexanes/EtOAc (1:3) at −4 °C using the vapour diffusion method. Before inducing crystallization, it was essential to

remove any residual traces of halogenated solvents remaining from the earlier NMR studies.

*4.3.1. Tetradehydrohaliclonacyclamine A (**10**).* White needles, mp 116–121 °C; $[\alpha]_D^{22}$ +19.4 (*c* 0.515, $CHCl_3$); ^1H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz), see Table 1; HRESMS *m*/*z* 465.4203 $[M+H]^+$ (calcd for $C_{32}H_{52}N_2$, 465.4209).

*4.3.2. Tetradehydrohaliclonacyclamine A mono-N-oxide (**11**).* White solid; $[\alpha]_D^{22}$ +9.2 (*c* 0.035, $CHCl_3$); ^1H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 187.5 MHz), see Table 1; HRESMS *m*/*z* 481.4153 $[M+H]^+$ (calcd for $C_{32}H_{53}N_2O$, 481.4158).

*4.3.3. 2-epi-Tetradehydrohaliclonacyclamine (**12**).* White solid; $[\alpha]_D^{20}$ +6.6 (*c* 0.083, $CHCl_3$); ^1H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz), see Table 1; HRESMS *m*/*z* 465.4191 $[M+H]^+$ (calcd for $C_{32}H_{53}N_2$, 465.4203).

4.4. Preparation of tetradehydrohaliclonacyclamine bis-*N*-oxide (15)

3-Chloroperoxybenzoic acid (77% max, Sigma–Aldrich, 6.6 mg, 0.0289 mmol) was added to a solution of **10** (6.1 mg, 0.0132 mmol) in DCM (1.5 mL) at 0 °C and stirred for 1 h at this temperature. The reaction mixture was extracted with aq K_2CO_3 (10%, 2×1 mL) and the combined organic layers were dried over Mg_2SO_4 filtered and evaporated to yield tetradehydrohaliclonacyclamine bis-*N*-oxide (**15**) (2.3 mg).

*4.4.1. Tetradehydrohaliclonacyclamine A bis-N-oxide (**15**).* White solid; $[\alpha]_D^{22}$ +7.6 (*c* 0.115, $CHCl_3$); ^1H NMR ($CDCl_3$, 500 MHz) 0.86 (1H, q, 12.1, H-8ax), 1.0–1.6 (16H, m), 1.68 (1H, br d, 14.8), 1.75 (1H, br d, 11.3, H-8eq), 2.04 (1H, br, H-27), 2.13 (1H, br, H-17), 2.25 (1H, br, H-17), 2.38 (1H, q, 12.0, H-27), 2.56-2.67 (4H, m), 2.74–2.94 (10H, m), 3.11 (1H, t, 12.0, H-10ax), 3.19 (1H, t, 12.0, H-1ax), 3.47 (1H, t, 11.2, H-5ax), 3.53 (2H, t, 5.6 H-21), 3.59 (2H, t, 5.6, H-11), 5.35 (1H, q, 9.6, H-16), 5.51 (2H, m, H-23/H-26), 5.64 (1H, dt, 6.4, 13.1, H-13), 6.05 (1H, t, 11.0, H-15), 6.26 (1H, t, 11.0, H-24), 6.37 (2H, m, H-14/H-25); ^{13}C NMR ($CDCl_3$, 100 MHz) 134.7 (C-26), 131.5 (C-16), 131.3 (C-13), 128.9 (C-15), 128.0 (C-23), 127.4 (C-25), 125.7 (C-14), 123.6 (C-24), 73.5 (C-21), 73.2 (C-11), 70.7 (C-10), 68.5 (C-6), 65.2 (C-1), 60.1 (C-5), 38.1 (C-9), 35.4 (1C, either C-2 or C-3), 34.3 (2C, C-8 and either C-2 or C-3), 31.5 (C-7), 31.3, 31.0, 29.5, 29.2, 27.9, 27.7 (2C), 27.4, 25.68 (C-17), 25.60 (C-22), 25.45, 23.4 (C-22); HRESMS *m*/*z* 497.4083 $[M+H]^+$ (calcd for $C_{32}H_{53}N_2O_2$, 497.4102).

4.5. Preparation of perhaliclonacyclamine (13)

A solution of **10** (4.8 mg, 0.0103 mmol) and Pd/C (10%, 3 mg) in MeOH (1.5 mL) was stirred under an atmosphere of dihydrogen at room temperature, while the reaction course was monitored by +LRESIMS. After 24 h, the reaction mixture was filtered under vacuum through a small pad of Celite that was then rinsed with MeOH followed by DCM. The filtrate was evaporated to yield a product (4.1 mg), which was purified by NP HPLC (Luna-NH_2 5μ, hexanes/EtOAc/Et_3N (65:30:5)) to yield perhydrohaliclonacyclamine (2 mg).

*4.5.1. Perhydrohaliclonacyclamine (**13**)*[3,4]. White waxy solid; $[\alpha]_D^{22}$ −20.9 (*c* 0.205, $CHCl_3$); ^1H NMR ($CDCl_3$, 500 MHz) 0.90 (1H, q, 12.2, H-8ax), 0.93 (1H, m, H-20), 1.15–1.58 (39H, m), 1.70 (1H, bd, 13.5, H-4eq), 1.75–1.95 (6H, m), 2.14 (1H, t, 11.4, H-10ax), 2.38–2.47 (3H, m), 2.50–2.85 (7H, m), 2.95 (1H, dt, 2.9, 14.2, H-5ax); ^{13}C NMR ($CDCl_3$, 100 MHz) 60.7 (C-6), 60.3 (C-10), 58.4 (C-22), 57.1 (C-11), 53.2 (C-1), 46.9 (C-5), 45.5 (C-9), 41.5 (C-2), 38.3 (C-8), 37.8 (C-7), 36.5 (C-4), 35.7 (C-3), 34.1 (C-20), 33.6 (C-23), 29.3, 27.91, 27.85, 27.77, 27.69, 27.60, 27.24, 27.07, 26.8 (2C), 26.5, 26.3, 26.2, 25.72, 25.67, 25.60, 22.0, 21.5 (C-12); +LRESIMS (*m*/*z*, rel intensity) 473.49 ($M+H^+$, 66%), 237.24 ($(M+2H)^{2+}$, 33%).

4.6. X-ray crystallography

X-ray data were collected on an Oxford Diffraction Gemini S Ultra CCD diffractometer employing Cu-Kα radiation (λ=1.54180 Å) and operating in the ω scan mode within the range $2<\theta<125°$. The crystal temperature was maintained at 130 K with an Oxford Cryosystems 600 Series Cryostream Cooler. Data reduction and corrections for decay and an analytical absorption were performed with the CrysAlisPro package (vers. 171.33.34d, Oxford Diffraction). The structure was solved by direct methods with SHELXS and refined by full matrix refinement on F^2 with SHELXL[24] implemented within the WinGX graphical user interface.[25] Thermal ellipsoid plots were produced with the program ORTEP3.[26] Absolute structure determination was performed using the Bijvoet analysis reported by Hooft et al.[13] implemented within the PLATON program.[27] A total of 4304 Bijvoet pairs were analysed using Student's t-statistics and a parameter $\nu=10$. The Hooft *y* parameter was −0.26 with an estimated standard deviation of 0.18. The probability that the correct enantiomorph was chosen (*P*2) was 1.000.

4.7. Crystal data

Tetradehydrohaliclonacyclamine A: $C_{32}H_{54}N_2$, M=464.76, monoclinic, space group $P2_1$ (No. 4), a=9.8074(1) Å, b=15.9505(2) Å, c=18.3186(2) Å, β=92.449(1)°, V=2863.01(6) Å^3, Z=4, D_c=1.078 g cm^{-3}, T=130 K, μ=0.457 mm^{-1}, $F(000)$=1032, colourless prism (0.67×0.32×0.19 mm); total reflections 44,198, unique reflections 9051 (R_{int}=0.0451). Final refinement: data/restraints/parameters 9051/1/613, goodness-of-fit on F^2=1.010, R_1=0.0349 (for 7712 obs. reflections $I>2\sigma(I)$), wR_2=0.0857 (all data), Flack parameter −0.1(3). Crystallographic data in CIF format have been deposited with the Cambridge Crystallographic Data Centre (CCDC 742927).

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