New Natural Product Isolation and Comparison of the Secondary Metabolite Content of Three Distinct Samples of the Sea Hare *Aplysia dactylomela* from Tenerife[1]

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Three distinct samples of the sea hare *Aplysia dactylomela* from two different locations around Tenerife (Spain) were investigated for their secondary metabolite content. The investigation resulted in the isolation of five new natural products (**1**, **2**, **5**−**7**), a number of compounds known from red algae of the genera *Laurencia* (**4**, **8**−**14**) and *Plocamium* (**17**−**19**), and three known sea-hare metabolites (**3**, **15**, **16**). This is the first report of monoterpenes (**17**−**19**) from *A. dactylomela*. All structures were determined mainly by spectroscopic methods (1D and 2D NMR, MS, IR). Compounds **11**, **14**, **15**, and **17** demonstrated significant cytotoxicity toward three cancer cell lines (HM02, HEP G2, and MCF 7; IC_{50} for **11**, 7.0 to <1.0 μg/mL; **14**, <1.0 μg/mL; **15**, 17 to <1.0 μg/mL; **17**, 1.0 to 1.5 μg/mL). Compounds **3**, **8**, **9**, **11**, **15**, **18**, and **19** exhibited activity in a brine shrimp bioassay in the range of 100% lethality within 24 h to 40% after 48 h. Metabolites **11**, **14**, **15**, and **19** showed moderate antimicrobial activities (2−25 mm total/growth inhibition of several organisms), with **19** also being strongly algicidal (MIC 7−11 μg/filter disk).

Sea hares are known to store and enrich secondary metabolites from their diet. They are therefore a rich source of a variety of natural products, often found in higher concentrations than in the source food plants.[2] As a direct consequence, many researchers have investigated sea hares for their bioactive metabolite content.[3] For this purpose large quantities of animals were often collected to yield only minute quantities of natural products.[4] It is thus possible that the isolated compounds were originally located only in a few of the collected animals. Although these problems are apparently known, there is little information on qualitative and quantitative variation of metabolites in sea hares. The main theme of many reports concerning sea hares addresses the question of the defensive role of the sequestered metabolites.[2,5,6] As part of a more extensive project, de Nys et al. described the different bioaccumulation of five metabolites by *Aplysia parvula*.[2] The present investigation focused on the qualitative and semiquantitative determination of natural products in *Aplysia dactylomela* Rang 1828, (Gastropoda, Aplysiidae). A total of five animals from the same (3 animals and 1 animal) and different (1 animal) collection sites were compared with regard to their natural product content.

Results and Discussion

Three distinct samples of *A. dactylomela* were exhaustively extracted with CH_2Cl_2 and MeOH. Resultant extracts were separated by repeated column chromatography and HPLC. Purification was guided by TLC and ^{1}H NMR analysis and led to the isolation and identification of 19 secondary metabolites (**1**−**19**, Chart 1).

Structure Solution and Isolates. The ^{13}C NMR and mass spectral data of dactylopyranoid (**1**) indicated it to have the molecular formula $C_{20}H_{33}BrO_2$, which implied four elements of unsaturation within the molecule. ^{13}C NMR data allowed the presence of one carbon−carbon double bond (146.6 d, 125.5 t, ppm) to be deduced as the only multiple bond in **1**; therefore, the molecule is tricyclic. Further analysis of ^{13}C NMR spectral data revealed the presence of four quaternary carbons (85.7, 84.7, 76.4, 73.5 ppm) attached to oxygen, three methine (58.3, 56.5, 48.7 ppm), six methylene (41.0, 39.1, 35.2, 32.2, 27.7, 25.5 ppm), and five methyl groups (27.3, 24.6, 20.7, 19.2, 18.1 ppm) in **1**. These data, coupled with the IR and MS spectral information, indicated there to be two ether functions in the molecule. The ^{1}H NMR resonances for the methyl groups of **1** were present as one doublet (0.92 ppm) and four singlets (1.41, 1.36, 1.32, 1.28 ppm), which established their connections to one methine and up to four quaternary carbons, respectively. An ^{1}H NMR signal at 4.09 (dd, 1H, J = 4.1, 10.2 Hz) and the ^{13}C NMR resonance at 56.5 (d) ppm, indicated this proton to be part of a CHBr moiety.

After all proton resonances were assigned to those of their directly bonded carbon atoms via a HMQC measurement, it was possible to deduce several molecular fragments (Figure 1). Thus, from the ^{1}H−^{1}H COSY spectrum of **1** four spin systems could be deduced. Coupling deduced between H_2-1 and H-2 afforded fragment 1. Furthermore, couplings were observed between H_2-4 and H_2-5 and between H_2-5 and H-6, leading to fragment 2. Further, couplings were observed between H_3-16 and H-15 and between H-15 and H-10, which in turn, coupled with H_2-9, while H_2-9 was further coupled to H_2-8 (fragment 3). Coupling between H_2-12 and H_2-13 afforded fragment 4 (Figure 1). These fragments could then be united to the planar structure of dactylopyranoid (**1**) by interpretation of the HMBC spectrum of **1**. Thus, starting with fragment 3, cross-peaks in the HMBC spectrum were seen between the resonance for H_3-16 and those for C-10, C-14, and C-15. The resonances for C-14 and C-15 gave further cross-peaks to the signal for H_3-17, H_3-17 showed further HMBC couplings to the resonances for C-13, which connected fragment 3 and fragment 4. Further, HMBC cross-peaks between the resonances for C-12 and H_3-18, between the signals for H_3-18 and C-11 and C-10 led to the first ring. Furthermore, HMBC couplings between the resonances for H_3-19 and C-8, C-6, and C-7 were observed, allowing

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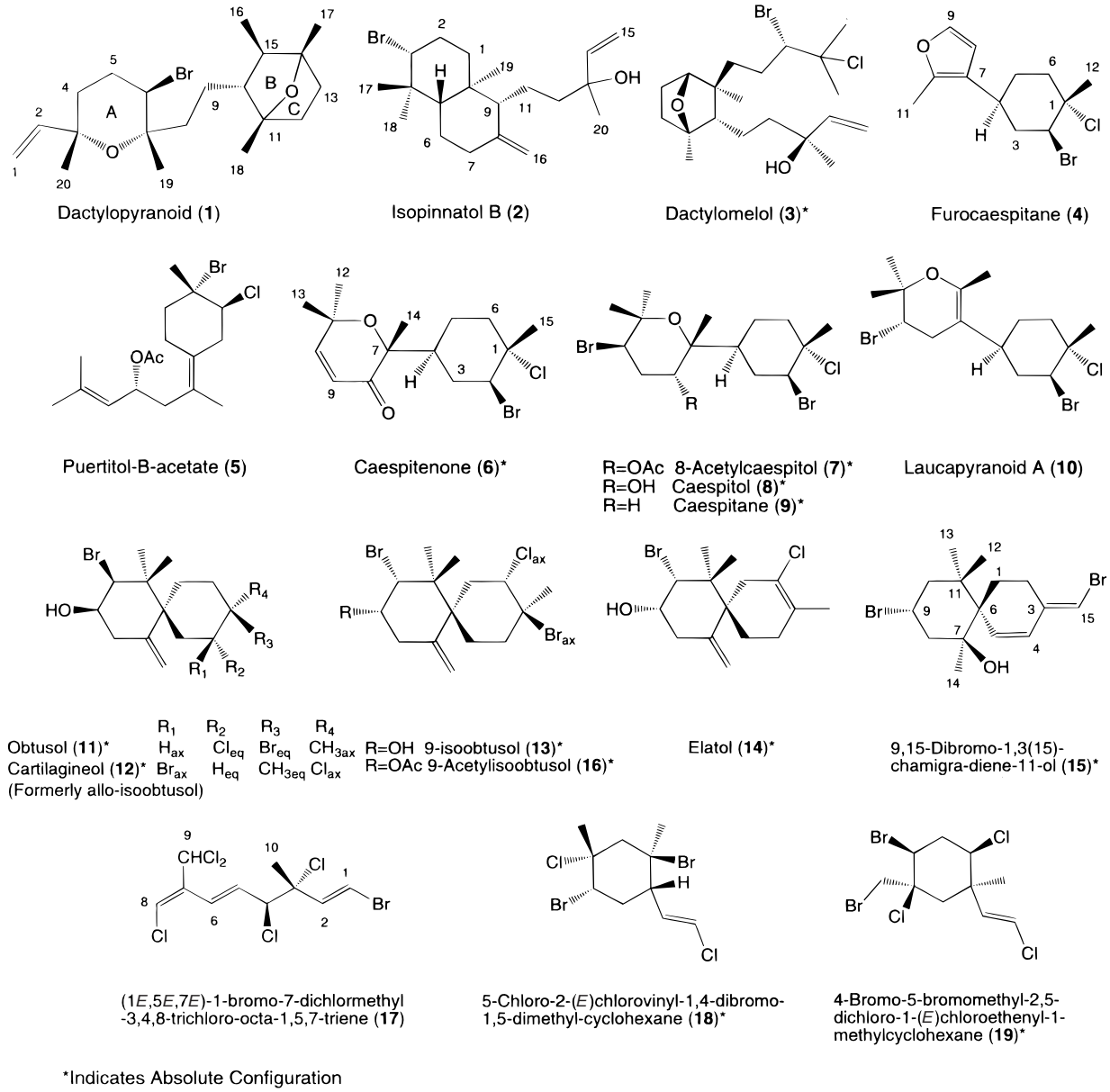
Chart 1



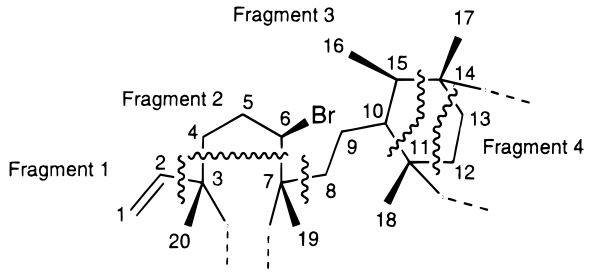


Figure 1. Structure solution of **1** based on ^{1}H−^{1}H-COSY and HMBC spectral data.

fragment 2 to be joined with the part of **1** already deduced. The connection of fragment 1 to C-3 was possible from the HMBC cross-peaks observed between the resonances for H_3-20 and those of C-2, C-3, and C-4.

The four remaining undetermined bonds within the molecule thus had to be associated with the two ether bridges, which, in principle, could be formed in three different ways, that is, between (a) C-3→C-7 and C-11→C-14; (b) C-3→C-11 and C-7→C-14; or (c) C-3→C-14 and C-7→C-11 (Figure 1). NOEs observed between CH_3-20 and CH_3-19 suggested close proximity of these methyl groups, best realized if C-3 and C-7 are linked via oxygen. ^{1}H NMR coupling constants for H-6 (4.09 [dd, 1H, J = 4.1, 10.2] ppm) are typical for an axially oriented proton in a six-membered ring, further supporting the C-3−O−C-7 connectivity. With this connectivity established, the other ether linkage had to form between C-11 and C-14. Comparison of the ^{13}C NMR chemical shifts of rings B and C (58.3, C-10; 85.7, C-11; 48.7, C-15; 20.7, C-18 ppm) with those for dactylomelol (**3**) (56.0, 87.5, 45.1, 21.7 ppm) also supported this deduction.

The relative stereostructure of dactylopyranoid (**1**) was deduced from the results of difference NOE measurements. Thus, irradiation at the resonance frequency of H_3-20 caused enhancement of the signals for H_3-19, H_β-4, H_β-5, H_2-1, and H-2; in turn, irradiation at the resonance frequency of H_3-19 caused enhancement of the signals for H_3-20, H_β-5, and H_2-8. These results indicated CH_3-19 and CH_3-20 to be axial and on the same side of the molecule. The coupling constants of H-6 [4.09 (dd, 1H, J = 4.1, 10.2 Hz) ppm] indicated it to be axial and α-oriented and the bromine to be equatorial and β-oriented. Further NOEs,

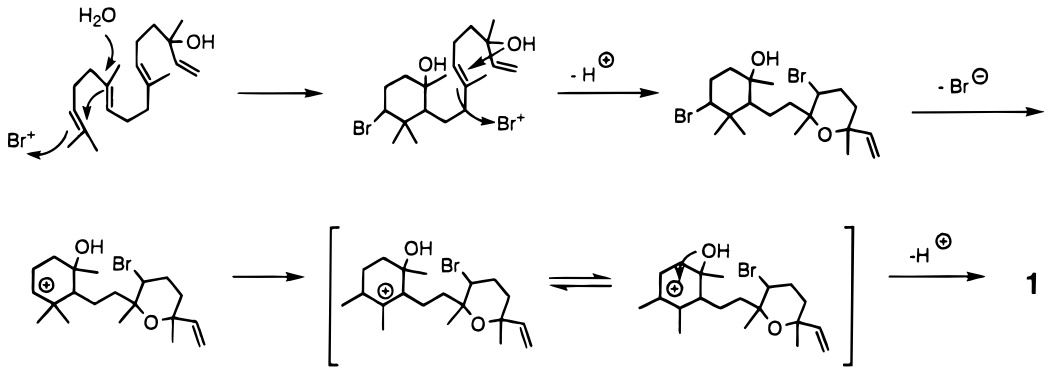


Figure 2. Possible biogenesis of compound **1**.

were observed between H_3-17 and H_3-16 and H_2-13. H_3-16 also demonstrated NOEs to H-10 and H_3-17, indicating CH_3-16, CH_3-17, and H-10 to be on the same side of the molecule. The oxygen bridge between C-11 and C-14 forced the six-membered carbocyclic ring into boat conformation, and thus, the methyl groups CH_3-17 and CH_3-18 are equatorial and β-oriented. The fact that H_3-17 showed a NOE to H_3-16 suggested that H_3-16 was also equatorial. The stereochemistry of the rings A and B of dactylopyranoid could not be correlated in a relative sense due to free rotation around the C-8−C-9 bond, and so it is possible that the relative stereochemistry for either ring A or rings B and C is opposite to that shown.

Compound **1** is a new structure probably biogenetically related to laurencianol, isolated from the red alga *Laurencia obtusa*.[7] Dactylopyranoid (**1**) may, as proposed for laurencianol,[8] derive from a "regular" head-to-tail arrangement of four isoprene units, followed by migration of a methyl group after bromine solvolysis and finally formation of a second ether bridge (Figure 2).

The second new compound, isopinnatol B (**2**), has the molecular formula $C_{20}H_{33}BrO$ based on ^{13}C NMR and mass spectral data, and thus has four elements of unsaturation. Its ^{13}C and ^1H NMR spectra showed the presence of two exo (147.4 s, 107.3 t, ppm) carbon−carbon double bonds (145.2 d, 111.6 t, ppm) as the only multiple bonds within **2**; hence the molecule is bicyclic. Further analysis of ^{13}C NMR spectral data revealed the presence of 3 × C (73.5, 40.0, 39.8 ppm), 3 × CH (69.7, 56.9, 56.0 ppm), 6 × CH_2 (41.2, 40.3, 38.2, 31.6, 25.7, 17.8 ppm), and 4 × CH_3 groups (30.8, 27.8, 18.2, 14.4 ppm). This indicated, taking the molecular formula into account, that there had to be a hydroxyl group (^{13}C, 73.5 s, ppm) within the molecule, as was substantiated by an absorption band in the IR spectrum at 3465 cm^{-1}. The ^1H NMR resonances for the 4 × CH_3 groups of isopinnatol B (**2**) were all present as singlets, indicating them to be connected to quaternary carbons. The 4.03 (dd, 1H, J = 5.1, 12.2 Hz) ppm resonance in the ^1H NMR spectrum was associated with a −CHBr function (^{13}C, 69.7 d, ppm). All protons were correlated with their directly bonded carbon atom via an HMQC spectrum, and from these data and the $^1H-^1H$ COSY spectrum several molecular fragments could be deduced. Thus, from the $^1H-^1H$ COSY spectrum of **2**, four spin systems were obvious. Coupling between H_2-15 and H-14 afforded fragment 1. Further, couplings were observed between H_2-12 and H_2-11, which in turn coupled with H-9 (fragment 2). A third fragment could be deduced from the coupling observed between H_2-7 and H_2-6, which further coupled to H-5 (fragment 3). Couplings between H_2-1 and H_2-2 and between H_2-2 and H-3 gave fragment 4. HMBC correlations then allowed the planar structure of **2** to be deduced. Thus, starting with fragment 1, cross-peaks were observed between the resonances for C-14 and H_3-20, which in turn had cross-peaks with the resonances for C-13 and C-12, connecting fragment 1 to fragment 2. The signal for H_2-16 demonstrated HMBCs with those of C-9 and C-7, fragment 3 could thus be added to the partial structure of **2**. The resonances for C-5 showed HMBC cross-peaks with those of H_3-17 and H_3-18. The signals for the protons of both methyl groups also had further cross-peaks with the resonances for C-4 and C-3, connecting fragment 4 with the extended partial molecule. Further HMBC cross-peaks were seen between the resonances for C-1 and H_3-19, which in turn gave cross-peaks to the resonances for C-5, C-9, and C-10. At the end of this analysis only C-13 remained with a "free" bond, indicating the OH group to reside at this position. The ^{13}C NMR chemical shift of C-13 (73.5 ppm) and comparison of the NMR chemical shifts for the side chain with the corresponding data of dactylomelol (**3**) (72.5 ppm) supported this deduction.

The relative stereostructure of the decalin system of **2** was deduced from $^1H-^1H$ coupling constants and difference NOE experiments. NOEs were detected between H_3-17, H-3, and H-5, indicating these protons to be located on the same side of the molecule (β). H_3-19 showed NOEs to H_2-11 and H_3-18, showing these groups all to be on the same side of **2** and α, and that H_3-18 and H_3-19 are axially oriented. The proton coupling constants of H-3 (J = 5.1, 12.2 Hz) evidenced the axial position for this proton and indicated the decalin to be *trans*-fused. The ^{13}C NMR chemical shift of C-19 (14.4 ppm), being typical for a bridgehead methyl group in a *trans*-fused decalin system, further supported the previous deduction.

A literature search based on the planar structure of **2** led to a report in which the isolation of pinnatol B, a molecule with the same planar structure but with a different optical rotation, was described ($[\alpha]_D$ −7.6°, as compared to $[\alpha]_D$ +20.2° for **2**).[9] Unfortunately, the spectroscopic data reported for pinnatol B[9] do not contain any ^{13}C NMR data, and so a detailed comparison between the two molecules was not possible. The minimal data (0.96 [6H, s], 1.08 and 1.27 [each 3H, s], 4.52 and 4.71 [each 1H, t, J = 2.0 Hz] ppm) published, however, do not compare well with those for **2**.

Compound **2** is a new diterpene based on a labdane skeleton, probably produced by a red alga from the genus *Laurencia* on which the sea hares were grazing. Biosynthetically, **2** could derive from geranylgeranyl pyrophosphate.

Based on ^{13}C NMR and mass spectral data, the new natural product caespitenone (**6**) has the molecular formula $C_{15}H_{22}BrClO_2$. Its ^{13}C NMR spectrum revealed the presence of one carbonyl, a keto group (198.5 s ppm), and one

Table 1. ^{13}C NMR Data of Compounds **1**, **2**, **6** (100 MHz), **9** (75.5 MHz), **13** (100 MHz), **15** (100 MHz), **16** (100 MHz), and **17**, (100 MHz) (all δ in ppm Relative to $CDCl_3 = 77.0$)[a]

carbon	1	2	6	9	13	15	16	17
1	128.5 (t)[b]	40.3 (t)	71.6 (s)	71.7 (s)	33.9 (t)	26.5 (t)	33.9 (t)	110.2 (d)
2	146.6 (d)	31.6 (t)	63.6 (d)	63.9 (d)	65.2 (d)	21.9 (t)	65.1 (d)	138.5 (d)
3	73.5 (s)	69.7 (d)	34.6 (t)	36.0 (t)	71.2 (s)	138.7 (s)	71.1 (s)	71.4 (s)
4	35.2 (t)	40.0 (s)	46.3 (d)	51.1 (d)	33.4 (t)	132.6 (d)	33.2 (t)	68.7 (d)
5	27.7 (t)	56.0 (d)	24.0 (t)	23.3 (t)	25.4 (t)	128.3 (d)	25.5 (t)	131.3 (d)
6	56.5 (d)	25.7 (t)	42.2 (t)	42.7 (t)	43.8 (s)	46.2 (s)	43.7 (s)	124.5 (d)
7	76.4 (s)	38.2 (t)	80.7 (s)	74.3 (s)	147.2 (s)	78.3 (s)	147.2 (s)	135.9 (s)
8	41.0 (t)	147.4 (s)	198.5 (s)	35.2 (t)	39.1 (t)	49.2 (t)[c]	35.2 (t)	69.5 (d)
9	25.5 (t)	56.9 (d)	122.6 (d)	28.2 (t)	69.7 (d)	46.8 (d)	71.5 (d)	124.4 (d)
10	58.3 (d)	39.8 (s)	155.1 (d)	58.2 (d)	76.2 (d)	49.3 (t)[c]	66.4 (d)	25.3 (q)
11	85.7 (s)	17.8 (t)	71.5 (s)	75.1 (s)	43.5 (s)	41.2 (s)	43.8 (s)	
12	32.2 (t)	41.2 (t)	30.5 (q)	31.1 (q)	24.7 (q)	29.4 (q)[d]	24.6 (q)	
13	39.1 (t)	73.5 (s)	28.8 (q)	23.6 (q)	25.2 (q)	25.0 (q)[d]	25.1 (q)	
14	84.7 (s)	145.2 (d)	24.9 (q)	22.7 (q)	113.7 (t)	30.2 (q)	114.6 (t)	
15	48.7 (d)	111.6 (t)	23.8 (q)	24.0 (q)	33.0 (q)	106.1 (d)	33.1 (q)	
16	19.2 (q)	107.3 (t)					170.2 (s)	
17	18.1 (q)	30.8 (q)					21.1 (q)	
18	20.7 (q)	18.2 (q)						
19	24.6 (q)	14.4 (q)						
20	27.3 (q)	27.8 (q)						

[a] All assignments are based on extensive 1D and 2D NMR measurements (HMBC, HMQC, COSY). [b] Multiplicities determined by DEPT (s = C, d = CH, t = CH_2, q = CH_3). [c,d] Assignments may be interchanged.

carbon−carbon double bond (155.1 d, 122.6 d ppm) and accounted for two of the four elements of unsaturation indicated by the molecular formula; the molecule is bicyclic. Further analysis of the ^{13}C NMR spectral data revealed the presence of 3 × C (80.7, 71.6, 71.5 ppm), 2 × CH (63.6, 46.3 ppm), 3 × CH_2 (42.2, 34.7, 24.0 ppm), and 4 × CH_3 groups (30.5, 28.8, 24.9, 23.8 ppm). The 4.36 (dd, 1H, J = 4.6, 12.2 Hz) ppm resonance in the ^1H NMR spectrum was associated with a −CHBr function (^{13}C, 63.6 d, ppm). In the $^1H-^1H$ COSY spectrum of caespitenone (**6**) two $^1H-^1H$ spin systems could be discerned. Coupling between H-9 and H-10 afforded fragment 1, and couplings from H-2 through H_2-3 to H_2-6 delineated fragment 2. HMBC cross-peaks between the resonances for H_3-12 and H_3-13 and those of C-10 and C-11 positioned the *gem*-dimethyl function. The resonances for H-10 had an HMBC cross-peak with those of C-8, and so positioned the carbonyl group. The resonance for H_3-14 had HMBC cross-peaks with those of C-4, C-7, and C-8, indicating C-7 to bond with C-4, C-8, and C-14. A cross-peak, albeit of weaker intensity, was also observed from the signal for H_3-14 to that of C-9. HMBC cross-peaks between the resonance for H_3-15 and those of C-1, C-2, and C-6, positioned C-1 and established the first ring. The chlorine atom was located at C-1 as evidenced by the ^{13}C chemical shift of C-1 (71.6 ppm), which is very similar to the ^{13}C NMR chemical shift of the corresponding atom in caespitol (**8**). The remaining oxygen atom had thus to be positioned between C-11 and C-7, closing the second ring.

Comparison of the ^{13}C NMR data of the C-1 to C-6 part of the molecule and of CH_3-15 of **6** with those of comparable atoms in **7** and **8** showed them to be in good agreement, differences being less than ±1.6 ppm (see Table 1). The ^1H NMR data of semisynthetic **6** given in the literature[10] (1.32 [s, 3H, H-14], 1.36 and 1.41 [2s, 3H each, H-12 and H-13], 1.66 [s, 3H, H-15], 4.34 [dd, 1H, J = 4.5, 12.3 Hz, H-2], 5.89 [d, 1H, J = 10.6 Hz, H-10], 6.63 [d, 1H, J = 10.6 Hz, H-9] ppm), especially coupling constants, were comparable to those of natural **6**, except for the assignments of H-9 and H-10. These data also indicated **6** to have the identical relative stereochemistry as **7** and **8**. This is the first report of caespitenone (**6**) as a natural product, it being formerly produced semisynthetically from its corresponding alcohol caespitol (**8**) by oxidation with Jones's reagent.[10]

Compounds **5** and **7** are also new as natural products. The corresponding alcohols having been described as metabolites of the red alga *L. obtusa* and *L. caespitosa*, respectively. Both compounds were produced semisynthetically by acetylation of the corresponding naturally occurring alcohols.[11,12] Spectroscopic data for the semisynthetic compounds are given in the literature and are in agreement with those found for naturally occurring **5** and **7**.

Together with the new natural products (**1**, **2**, **5**−**7**), the known compounds **3**, **4**, and **8**−**19** were also isolated from the three specimens of *A. dactylomela*. These compounds are known as secondary metabolites from *Laurencia* spp., (**4**, **8**−**14**),[10,12−17] from *A. dactylomela* (from Tenerife, **15** and **3**; and from the Caribbean Sea, **16**),[18,19,20] and from specimens of the red alga *Plocamium* sp., (**17**−**19**).[21,22] This is the first report of monoterpenes (**17**−**19**) from *A. dactylomela*.

In this study, complete 1D and 2D NMR investigations of all isolates were made and revealed that the ^{13}C NMR assignments of caespitane (**9**) and isoobtusol (**13**) required revision. For compounds **15** and **17**, no ^{13}C NMR data are published. All new and newly assigned ^{13}C NMR data are presented in Table 1. Furthermore, ^1H NMR data of compounds **4**, **7**−**9**, **11**, **13**, and **16** are either incomplete, unassigned, or in need of revision, so they were recorded and assigned and are presented in Table 2.

As compounds **5**, **7**, and **16** have never been reported from any algal species, it is not unlikely that the acetylation of ingested algal-derived alcohols has been undertaken by the sea hares. This proposal is supported by the studies of the chemical relationship between *A. dactylomela* and *L. claviformis* (Easter Island, Chile),[23] and those into *Stylocheilus longicauda* and its major food source, the cyanobacterium *Microcoleus lyngbyaceus*.[5] These studies showed the animals to contain acetylated compounds that were not detectable in the nutritional source; the corresponding alcohols could, however, be found in both organisms, sea hare and diet.

Secondary Metabolite Distribution/Variation. The secondary metabolite content of three distinct samples of *A. dactylomela* (CT195A [1 animal], CT195A1 [3 animals], CT195A2 [1 animal]), collected at two different locations, was clearly dependent on the site of collection (see Table 3). Samples collected at the same location (CT195A1,

Table 2. ^{1}H NMR Data for Compounds **1**, **2**, **4**, **6**−**9**, **11**, **13**, and **16** (400 MHz, $CDCl_3$, δ Relative to Residual $CHCl_3$ in $CDCl_3$ = 7.26 ppm, *J* in Hz)[a]

proton	**1**	**2**	**4**	**6**	**7**
1	5.11 (dd, 1H, *J* 1.5, 17.3) 4.94 (dd, 1H, *J* 1.5, 10.7)	1.22 (m, 1H), 1.80 (m, 1H)			
2	5.84 (dd, 1H, *J* 10.7, 17.3)	2.17 (m, 2H)	4.44 (dd, 1H, *J* 4.1, 12.5)	4.36 (dd, 1H, *J* 4.6, 12.2)	4.23 (dd, 1H, *J* 4.1, 12.2)
3		4.03 (dd, 1H, *J* 5.1, 12.2)	2.02 (m, 1H), 2.33 (m, 1H)	2.02 (m, 1H), 2.30 (m, 1H)	1.61 (m 1H), 1.87 (m, 1H)
4	1.76 (m, 2H)		2.62 (dddd, 1H, *J* 4.1, 4.1, 12.5, 12.5,)	2.02 (m, 1H)	1.86 (m, 1H)
5	2.23 (m, 2H)	1.22 (m, 1H)	1.59 (m, 1H), 1.76 (m, 1H)	1.40 (m, 1H), 1.58 (m, 1H)	1.26 (m, 1H), 1.89 (m 1H)
6	4.09 (dd, 1H, *J* 4.1, 10.2)	1.46 (m, 1H), 1.80 (m, 1H)	2.18 (m, 1H), 2.46 (m, 1H)	2.02 (m, 1H), 2.30 (m, 1H)	2.05 (ddd, 1H, *J* 3.6, 3.6, 13.7) 2.42 (ddd, 1H, *J* 3.1, 3.1, 13.7)
7		2.39 (ddd, 1H, *J* 2.5, 4.1, 12.7) 1.97 (ddd, 1H, *J* 4.6, 12.7, 12.7)			
8	1.65 (m, 2H)		6.18 (d, 1H, *J* 1.5)		4.65 (dd, 1H, *J* 2.5, 3.6)
9	1.41 (m, 2H)	1.53 (d, 1H, *J* 10.6)	7.22 (d, 1H, *J* 1.5)	5.91 (d, 1H, *J* 10.2)	2.31 (ddd, 1H, *J* 3.6, 4.1, 14.8) 2.47 (ddd, 1H, *J* 2.5, 13.2, 14.8)
10	1.14 (m, 1H)			6.84 (d, 1H, *J* 10.2)	4.12 (dd, 1H, *J* 4.1, 13.2)
11		1.36 (m, 2H)	2.22 (s, 3H)		
12	1.90 (m, 1H) 1.41 (m, 1H)	1.72 (dd, 1H, *J* 4.6, 13.2) 1.26 (m, 1H)	1.78 (s, 3H)	1.43 (s, 3H)[d]	1.33 (s, 3H)
13	1.57 (m, 2H)			1.37 (s, 3H)[d]	1.40 (s, 3H)
14		5.90 (dd, 1H, *J* 10.7, 17.3)		1.34 (s, 3H)	1.19 (s, 3H)
15	1.34 (m, 1H)	5.20 (dd, 1H, *J* 1.0, 17.3) 5.05 (dd, 1H, *J* 1.0, 10.7)		1.68 (s, 3H)	1.67 (s, 3H)
16	0.92 (d, 3H, *J* 6.6)	4.84 (s, 1H) 4.55 (s, 1H)			
17	1.28 (s, 3H)	1.07 (s, 3H)			2.15 (s, 3H)
18	1.36 (s, 3H)	0.94 (s, 3H)			
19	1.41 (s, 3H)	0.72 (s, 3H)			
20	1.32 (s, 3H)	1.27 (s, 3H)			
others		1.56 (brs, 1H, OH)			

proton	**8**	**9**	**11**[b]	**13**	**16**
1			not assigned[c]	2.80 (dd, 1H, *J* 4.0, 15.6) 3.08 (brd, 1H, *J* 15.6)	2.82 (dd, 1H, *J* 3.6, 15.8) 3.16 (dd, 1H, *J* 5.1, 15.8)
2	4.37 (dd, 1H, *J* 4.6, 12.7)	4.33 (dd, 1H, *J* 4.3, 12.4)	not assigned[c]	4.45 (m, 1H)	4.45 (dd, 1H, *J* 3.6, 5.1)
3	1.63 (ddd, 1H, *J* 12.6, 12.6, 12.6), 2.28 (m, 1H)	1.74 (m, 1H), 2.30 (m, 1H)			
4	1.89 (m, 1H)	1.42 (m, 1H)	4.70 (dd, 1H, *J* 4.4, 11.2)	1.87 (m, 1H), 2.25 (m, 1H)	1.85 (m, 1H), 2.22 (m, 1H)
5	1.24 (m, 1H), 1.89 (m, 1H)	1.25 (m, 1H), 1.81 (m, 1H)	not assigned[c]	1.82 (m, 1H), 2.03 (m, 1H)	1.80 (m,1H), 2.05 (m, 1H)
6	2.05 (ddd, 1H, *J* 4.1, 4.1, 13.5), 2.41 (ddd, 1H, *J* 3.3, 3.3, 13.5)	2.40 (ddd, 1H, *J* 3.2, 3.2, 13.5), 2.01 (ddd, 1H, *J* 13.5, 13.5, 3.2)			
8	3.57 (m, 1H)	1.61 (ddd, 1H, *J* 4.3, 13.5, 13.5), 1.45 (ddd, 1H, *J* 3.8, 3.8, 13.5)	2.49 (dd, 1H, *J* 2.4, 14.2) 2.62 (dd, 1H, *J* 3.1, 14.2)	2.42 (dd, 1H, *J* 5.4, 12.6) 2.69 (dd, 1H, *J* 4.1, 12.6)	2.38 (dd, 1H, *J* 4.1, 12.2) 2.95 (dd, 1H, *J* 12.2, 12.2)
9	2.23 (m, 1H), 2.52 (ddd, 1H, *J* 2.0, 13.2, 14.2)	2.11 (m, 1H), 2.26 (m, 1H)	4.11 (m, 1H)	3.66 (m, 1H)	4.82 (ddd, 1H, *J* 4.1, 4.1, 12.2)
10	4.29 (dd, 1H, *J* 4.6, 13.2)	3.83 (dd, 1H, *J* 4.3, 12.4)	4.47 (d, 1H, *J* 3.0)	4.42 (dd, 1H, *J* 1.9, 3.8)	4.43 (dd, 1H, *J* 1.5, 4.1)

Table 2 (Continued)

proton	**8**	**9**	**11**[b]	**13**	**16**
12	1.31 (s, 3H)	1.28 (s, 3H)	1.08 (s, 3H)	1.34 (s, 3H)	1.31 (s, 3H)
13		1.40 (s, 3H)	1.08 (s, 3H)	1.06 (s, 3H)	1.11 (s, 3H)
14		1.19 (s, 3H)	5.05 (s, 1H), 5.39 (s, 1H),	4.93 (s, 1H), 5.17 (s, 1H)	4.98 (s, 1H), 5.20 (s, 1H)
15		1.68 (s, 3H)	1.83 (s, 3H)	1.92 (s, 3H)	1.92 (s, 3H)
17					2.09 (s, 3H)
others	1.59 (d, 1H, *J* 6.6, OH)		2.20 (d, 1H, *J* 1.9, OH)		

[a] All assignments are based on extensive 1D and 2D NMR measurements (HMBC, HMQC, COSY). [b] 300 MHz. [c] Data could not be unambiguously assigned as no $^{1}H-^{13}C$ shift-correlated 2D NMR spectra were recorded. [d] Assignments may be interchanged.

Table 3. Secondary Metabolites Isolated (**1**−**19**) from Three Distinct Samples of *Aplysia dactylomela*

	location		
	San Juan de la Rambla	Punta del Hidalgo	
compound	CT195A[a]	CT195A1[b]	CT195A2[a]
1		0.005	
2	0.017[c]		
3	0.032		
4		0.005	0.020
5	0.030		
6		0.006	
7	0.019	0.018	0.183
8	0.031	0.049	0.153
9	0.006	0.006	0.006
10	0.005	0.005	0.004
11	0.082		
12	0.005		
13	0.002		
14	0.189		
15		0.005	
16	0.041		
17	0.018		0.010
18	0.078		0.003
19			0.011
total	0.56	0.1	0.39

[a] Single animal (CT195A: 28 g, CT195A2: 34 g). [b] Three animals (CT195A1: 84 g). [c] All numbers are expressed as percentage (%) of dry weight.

CT195A2) also differed significantly from each other in terms of their secondary metabolite content (see Table 3). Differences between the samples were first indicated by GC−MS measurements of the CH_2Cl_2 extracts of each sample. Of the three samples, sample CT195A showed the greatest secondary metabolite variety and also the highest concentration of natural products, with sample CT195A1 having the lowest secondary metabolite variety as well as the lowest total concentration of natural products. The total concentration of secondary metabolites (CT195A, 0.56%; CT195A1, 0.1%; and CT195A2, 0.39%) also differs significantly between the samples.

Sample CT195A was collected from the beach wrack at San Juan de la Rambla (north Tenerife) and contained elatol (**14**), obtusol (**11**), and the monoterpene **18** as the major constituents of the CH_2Cl_2 extract. In the other two samples, CT195A1 and CT195A2, collected off Punta del Hidalgo (northeastern region of the island), 8-acetylcaespitol (**7**) and caespitol (**8**) were present as the major secondary metabolites. These two compounds were also found in the first sample (CT195A), but only as minor components. Comparison of the CH_2Cl_2 extracts shows that several metabolites are common to all three samples (**7**−**10**), while others are present in only one of the samples (**1**−**3**, **5**, **6**, **11**−**16**, **19**).

These results may be explained by different bioaccumulation of algal metabolites by individual sea hares. Such a case was reported by de Nys et al., for the red alga *Delisea pulchra* and *A. parvula* sea hares grazing on it.[2] The difference in the secondary-metabolite content of the samples collected from the same location may result from such individual differences. Another explanation for these results may be the varied dietary sources of the sea hares around the island. As the two collection sites were approximately 35 km apart, it was no surprise to find that the secondary metabolite content of the CH_2Cl_2 extract of sample CT195A was markedly different from the other two samples. The fact that the sea hares are able to swim with their parapodia and perhaps feed at different locations before coming back to the beach area could be seen as a further reason for the different chemical content of samples CT195A1 and CT195A2.

Table 4. Cytotoxicity (IC_{50} values in μg/mL) of Compounds **1**−**11**, and **14**−**19** toward Cultured Cancer Cell Lines[a]

compound	HM02[b]	HEP G2[c]	MCF 7[d]
11	7.0[e]	<1.0	1.5
14	<1.0	<1.0	<1.0
15	17	<1.0	<1.0
17	1.1	1.0	1.5

[a] Compounds **1**−**10**, **16**, **18**, **19** were tested and found to be inactive. [b] HM02 = gastric carcinoma. [c] HEP G2 = liver carcinoma. [d] MCF 7 = breast carcinoma. [e] IC_{50} = 50% growth inhibition concentration.

In any event, the finding of some metabolites in only one of the samples indicates that relatively large numbers of animals would need to be harvested to ensure that reasonable amounts (5−10 mg) of less abundant metabolites are obtained. This approach to collection is, however, in an ecological sense, somewhat irresponsible and not a recommended collection strategy. A point that also has to be remembered is that, it is possible, purely by chance, that a single individual in a population of hundreds may be the only one to contain a given metabolite, hence, harvesting many individuals may not lead to the isolation of more of a particular substance.

Biological Activities. The isolated compounds were tested in several bioassays for their biological activity: antimicrobial activity against *Escherichia coli, Bacillus megaterium* (bacteria), *Eurotium repens, Fusarium oxysporum, Mycotypha microspora,* and *Microbotryum violacea* (fungi), and algicidal activity against *Chlorella fusca*. Inhibition of reverse transcriptase of the human immunodeficiency virus type 1 (HIV-1-RT), tyrosine kinase $p56^{lck}$ (TK), nematicidal activity (*Caenorrhabditis elegans*), antiplasmodial (*Plasmodium falciparum*) and antitrypanosomal (*Trypanosoma brucei rhodesiense, Trypanosoma cruzi*) activities, cytotoxic properties (selected cancer cell lines), and lethalities (brine shrimp assay) were also assessed.

Compounds **11**, **14**, **15**, and **17** demonstrated cytotoxicity toward the cell lines HM02 (gastric carcinoma), HEP G2 (liver carcinoma), and MCF 7 (breast carcinoma) at con-

Table 5. Antibacterial, Antifungal, Antialgal Activities and Brime Shrimp Lethalities of Compounds **1**−**19**[a]

compound	bacteria[b]	fungi[c]	alga[d]	*A. salina*[e]
2	na[f]	na	na	animals became hyperactive
5	na	Mv WH1[g]; Er WH2	na	na
8	na	na	WH3	70%
9	na	na	WH1	40%
11	na	Mm TH6[h];	WH3	80%
14	Bm TH2	Mv TH3; Fo WH7; Er WH25; Mm WH25	na	100% (24 h)
15	Bm TH1	Mv TH3; Er WH2; Mm WH1	na	100% (24 h)
16	na	Er WH1	na	na
17	Bm WH2	na	TH1	na
18	Bm WH1	Er WH1; Mv WH1	na	90%
19	na	Mv TH1; Mm TH2	TH16 (MIC 7−11 μg)[i]	100%

[a] Compounds **12** and **13** were not tested; compounds **1**, **3**, **4**, **6**, **7**, **10** were tested and found inactive in all these systems. [b] Tested against *Bacillus megaterium* (Bm) and *Escherichia coli* (Ec), diethyl phthalate as positive control: inhibition zone 10 mm (Bm) and 1 mm (Ec), respectively. Test concentration: 50 μg/disk. [c] Tested against *Eurotium repens* (Er), *Fusarium oxysporum* (Fo), *Microbotryum violacea* (Mv), and *Mycotypha microspora* (Mm), miconazol as positive control: inhibition zone 20 mm (Er), 3 mm (Fo), 32 mm (Mv), and 5 mm (Mm), respectively. Test concentration: 50 μg/disk. [d] Tested against *Chlorella fusca*, miconazol (M), and diethyl phthalate (D) as positive controls: inhibition zones 4 mm (M) and 13 mm (D). Test concentration: 50 μg/disk. [e] Lethality rate of *Artemia salina* after 48 h, if no other time is shown. Test concentration: 0.5 mg/mL. [f] na = not active. [g] WH # = Growth Inhibition in mm, few colonies of the test organism were growing within the inhibition zone. [h] TH # = Total inhibition in mm, no colonies of the test organism were growing within the inhibition zone. Inhibition zones were measured from the edge of the filter disks. [i] MIC = minimum inhibition concentration.

centrations of 17 μg/mL and lower (Table 4). Metabolite **14** showed cytotoxicity also toward L-6 muscle myoblast cells with a minimum inhibition concentration (MIC) of 3.3 μg/mL, and antitrypanosomal activity toward *T. cruzi* (IC_{50} 0.92 μg/mL, MIC 3.3 μg/mL), suggesting it to be generally cytotoxic. Compound **19** was found to be weakly cytotoxic toward Lu1 (human lung cancer; IC_{50} 12.9 μg/mL), KB (human oral epidermoid carcinoma; IC_{50} 13.3 μg/mL), and ZR-75−1 (hormone dependent human breast cancer; IC_{50} 7.8 μg/mL) cells.[24] All other compounds were found to be inactive in these tests (IC_{50} >25 μg/mL). The monoterpene **19** showed good activity against the microalga *Chlorella fusca* (Table 5). Investigation of the antitubercular activity of **19** demonstrated it to be moderately active, with a MIC of 32 μg/mL toward *Mycobacterium tuberculosis* and 34 μg/mL toward *Mycobacterium avium*.[24]

Many of the isolates showed weak antimicrobial and algicidal properties (Table 5). Six compounds (**8**, **11**, **14**, **15**, **18**, **19**) showed activity in the brine shrimp lethality assay, and five compounds (**8**, **11**, **14**, **15**, **19**) exhibited nematicidal effects toward *Caenorrhabditis elegans*. An inhibition of the reverse transcriptase of HIV type 1 and tyrosine kinase $p56^{lck}$, and an antiplasmodial activity could not be detected for any of the isolates.

For a number of the isolated compounds biological activities have already been reported in the literature. Caespitol (**8**), obtusol (**11**), and isoobtusol (**13**) showed weak to very weak cytotoxic activity against HeLa cells (cervical cancer) with IC_{50} values of 100 μg/mL, 50 μg/mL, and 10 μg/mL, respectively.[25] Cartilagineol (**12**) and elatol (**14**) were also found to be cytotoxic toward A-549 (nonsmall lung cancer), HT-29 (human colon carcinoma), and MEL-28 (melanoma) cells (IC_{50} 1.0 μg/mL, 0.25 μg/mL, 1.0 μg/mL, respectively, for **12**, and IC_{50} 0.1 μg/mL for **14**), and against P-388 cells (murine lymphoid neoplasm), with IC_{50} values of 5.0 μg/mL and 1 μg/mL, respectively.[26] Compounds **14**, **16**, and **19** are reported to have weak antimicrobial activity.[17,25,27,28] All of these findings are in agreement with our results. 9-Acetylisoobtusol (**15**) and the monoterpene **18** are reported as being cytotoxic toward HeLa cells with IC_{50} values of 4.5 μg/mL and 1 μg/mL, respectively.[25] These two compounds showed no effects in the currently employed test systems.

It is worthwhile mentioning that small changes in all chemical structures can have a pronounced influence on bioactivity. Compounds **7** and **8**, for example, only differ by the acetyl group, and **8** is algicidal and lethal to brine shrimp and *C. elegans*, while **7** is completely inactive in the same assays (Table 5). This finding may also relate to solubility. This may be the case for 9-acetylisoobtusol (**16**) and obtusol (**11**) as well. The cytotoxicity data for **11** and **16** reported in the literature, however, gave a contrary result concerning their activity toward HeLa cells.[25]

Experimental Section

General Experimental Procedures. The general experimental procedures were carried out as previously described.[29]

Collection and Isolation. One sample (single animal, number CT195A) of *A. dactylomela* Rang, 1828, was collected in August 1995, from the beach wrack of San Juan de la Rambla on Tenerife (Canary Islands, Spain). Two samples (three animals, number CT195A1, and a single animal, number CT195A2) of *A. dactylomela* were collected at the same time from the ocean at Punta del Hidalgo, Tenerife. Collected material was stored at −20 °C until used.

Animal tissue was freeze-dried and extracted with CH_2Cl_2 (2 L) and then with MeOH (2 L). Solvents were removed in vacuo and the extracts filtered through a pad (5 mm thick) of Si gel. The MeOH-extract was extracted with CH_2Cl_2, and the two lipophilic fractions were combined. The lipophilic extract was applied to a vacuum liquid column (VLC; Si gel) and eluted, using a solvent gradient from hexane to EtOAc and thereafter with MeOH. Individual metabolites were isolated from the various VLC fractions by repeated HPLC [Si gel, 1−10% $(CH_3)_2CO$ or EtOAc in hexane].

Biological Tests. The antimicrobial,[30] TK inhibition,[31] RT inhibition,[32] antimalarial,[33] antitrypanosomal,[34] cytotoxic,[35] antitubercular,[36,37] brine shrimp,[38] and nematicidal[38] assays were carried out as previously described.

Dactylopyranoid (1): $[\alpha]_D$ −14.0° (*c* 0.42, $CHCl_3$); IR ν_{max} 2960, 2870, 1580, 1450, 1380, 1120, 1070, 1030; ^{13}C NMR, see Table 1; ^1H NMR, see Table 2; EIMS *m/z* (rel int %) 386 (16), 384 (16) [M^+], 371 (4), 369 (4), 357 (4), 355 (4), 305 (72) [M^+ − Br], 287 (26) [M^+ − Br − H_2O], 211 (24), 179 (32), 161 (36), 139 (50), 109 (62), 95 (54), 81 (90); HREIMS *m/z* 384.1656 (calcd for $C_{20}H_{33}{}^{79}BrO_2$, 384.1656).

Isopinnatol B (2): $[\alpha]_D$ +20.2° (*c* 0.48, $CHCl_3$); IR ν_{max} 3465, 3415, 2970, 2850, 1450, 1385, 1370, 1155, 920, 890; ^{13}C NMR, see Table 1; ^1H NMR, see Table 2; EIMS *m/z* (rel int %) 352 (10), 350 (10) [M^+ − H_2O], 337 (22), 335 (22) [M^+ − H_2O − CH_3], 324 (20), 322 (20), 271 (36) [M^+ − H_2O − Br], 255 (12), 215 (14), 203 (22), 187 (16), 175 (16), 161 (14), 149 (18), 135

(100), 119 (24), 107 (36), 93 (38); HREIMS *m/z* 350.1600 (calcd for $C_{20}H_{31}{}^{79}Br$, 350.1609).

Dactylomelol (3): $[\alpha]_D$ −34.9° (*c* 0.3, $CHCl_3$), lit.[20] −31.3° (*c* 0.7, $CHCl_3$); the remaining physical and spectral properties of **3** in agreement with those published.[20]

Furocaespitane (4): $[\alpha]_D$ ±0.0° (*c* 0.19, $CHCl_3$); ^1H NMR, see Table 2; the remaining physical and spectral properties of **4** in agreement with those published.[13,15]

Puertitol-B-acetate (5): $[\alpha]_D$ +35.6° (*c* 0.23, $CHCl_3$); IR ν_{max} 2930, 1730, 1445, 1370, 1015, 960; HREIMS *m/z* 318.0570 (calcd for $C_{15}H_{22}{}^{79}Br^{35}Cl$, 318.0571); the remaining physical and spectral properties of **5** in agreement with those published.[11]

Caespitenone (6): $[\alpha]_D$ +36.3° (*c* 0.47, $CHCl_3$); IR ν_{max} 2930, 2860, 1740, 1680, 1460, 1375, 1270, 1185, 1090, 820, 725; ^{13}C NMR, see Table 1; ^1H NMR, see Table 2; CIMS *m/z* (rel int %) 370 (26), 368 (100), 366 (74) $[M + NH_4]^+$, 353 (22), 351 (66), 349 (52) $[M + H]^+$, 332 (42), 330 (42), 233 (78), 96 (94).

8-Acetylcaespitol (7): $[\alpha]_D$ ±0.0° (*c* 0.34, $CHCl_3$); ^1H NMR, see Table 2; the remaining physical and spectral properties of **7** in agreement with those published.[12,13]

Caespitol (8): $[\alpha]_D$ +4.3° (*c* 0.44, $CHCl_3$); ^1H NMR, see Table 2; the remaining physical and spectral properties of **8** in agreement with those published;[12−14] absolute configuration described elsewhere.[10]

Caespitane (9): $[\alpha]_D$ +34.1° (*c* 0.22, $CHCl_3$), lit.[10] +39.8° (*c* 0.89, $CHCl_3$); ^{13}C NMR, see Table 1; ^1H NMR, see Table 2; the remaining physical and spectral properties of **9** in agreement with those published.[10]

Laucapyranoid A (10): $[\alpha]_D$ +25.4° (*c* 0.13, $CHCl_3$), lit.[10] +0.5° (*c* 0.87, $CHCl_3$); the remaining physical and spectral properties of **10** in agreement with those published.[10]

Obtusol (11): $[\alpha]_D$ +14.3° (*c* 0.38, $CHCl_3$), lit.[16] +10° (no further information published); ^1H NMR, see Table 2; the remaining physical and spectral properties of **11** in agreement with those published.[13,16,39]

Cartilagineol (12): the physical and spectral properties of **12** in agreement with those published.[40]

Isoobtusol (13): ^{13}C NMR, see Table 1; ^1H NMR, see Table 2; the remaining physical and spectral properties of **13** in agreement with those published.[16,39]

Elatol (14): $[\alpha]_D$ +94.2° (*c* 0.24, $CHCl_3$), lit. +75.4° (*c* 1.01, $CHCl_3$),[27] +83.5° (*c* 0.365, MeOH),[39] +90° (no further information published);[39] the remaining physical and spectral properties of **14** in agreement with those published.[17,41−43]

9,15-Dibromo-1,3(15)-chamigradiene-7-ol (15): $[\alpha]_D$ −44.5° (*c* 0.38, $CHCl_3$), lit.[18] −64° (c 0.29, $CHCl_3$); ^{13}C NMR see, Table 1; the remaining physical and spectral properties of **15** in agreement with those published.[18]

9-Acetylisoobtusol (16): $[\alpha]_D$ +57.9° (*c* 0.38, $CHCl_3$), lit. +57.0° (*c* 0.405, $CHCl_3$),[19] +51.0° (*c* 0.514, $CHCl_3$),[19] ^{13}C NMR, see Table 1; ^1H NMR, see Table 2; the remaining physical and spectral properties of **16** in agreement with those published.[19]

(1*E*,5*E*,7*E*)-1-Bromo-7-dichloromethyl-3,4,8-trichlorooctatriene (17): $[\alpha]_D$ −22.1° (*c* 0.14, $CHCl_3$), lit.[21] −22.9° (*c* 0.9, $CHCl_3$); ^{13}C NMR, see Table 1; the remaining physical and spectral properties of **17** in agreement with those published.[21]

5-Chloro-2-(*E*)chlorovinyl-1,4-dibromo-1,5-dimethylcyclohexane (18): $[\alpha]_D$ −46.1° (*c* 0.345, $CHCl_3$), lit.[22] −35.7° (*c* 1.13, $CHCl_3$); the remaining physical and spectral properties of **18** in agreement with those published.[22]

4-Bromo-5-bromomethyl-2,5-dichloro-1-(*E*)chloroethenyl-1-methylcyclohexane (19): $[\alpha]_D$ −56° (*c* 0.1, $CHCl_3$), lit. −43.8° (*c* 1.01, $CHCl_3$),[22] −67.8° (*c* 1.33, $CHCl_3$);[28] the remaining physical and spectral properties of **19** in agreement with those published.[22,28]

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