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Stereochemical challenges in characterizing nitrogenous spiro-axane sesquiterpenes from the Indo-Pacific sponges *Amorphinopsis* and *Axinyssa*

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Abstract—An investigation was conducted to identify the structures and bioactive properties of five compounds isolated from the Halichondrida sponges *Amorphinopsis foetida* and *Axinyssa aplysinoides*. All compounds possessed the spiro-axane sesquiterpene core and all were substituted at C-2 with nitrogen containing functionality. The stereochemistry of one known compound has been revised to (2*R*,5*R*,10*S*)-2-formamido-6-axene (**3**). It exhibited mild selective solid tumor and mild antibacterial activity and was found from *Axinyssa*. A second known substance whose stereochemistry has also been revised, (2*R*,5*R*,10*S*)-2-isothiocyanato-6-axene (**4**) plus its undescribed diastereomer (**5**) were isolated from *Amorphinopsis*. Both sponges were the source of two new *N*-phenethyl-2-formamido-6-axene diastereomeric compounds **6** and **7**. No solid tumor or antibacterial activity was found for **4**–**7**.

1. Introduction

The strategy of using an in vitro cell-based assay to identify solid tumor selectivity[1] continues to be a robust tool in our quest to investigate unusual constituents of Indo-Pacific sponges.[2] An initial stimulus for this work was the observation of large inhibition zones (at 10 μg/mL per disk) against murine colon-38 cells versus much reduced zones against murine leukemia (L1210) cells for extracts obtained from two related Indo-Pacific sponges. The taxa responsible for this data belonged to different genera but were in the same family consisting of the Papua New Guinea *Amorphinopsis foetida*[3] (order Halichondrida, family Halichondriidae) and the Vanuatu *Axinyssa aplysinoides*.[4] This parallel bioactivity pattern suggested that similar metabolites might be present in both sponges because nitrogen containing terpenes, which often possess many structural variants, are commonly isolated from sponges of the order Halichondrida. The most unique terpenoids of this set are compounds containing isocyanide, isothiocyanate, thiocyanate, and formamide moieties.[5]

Our pursuit of the major constituents from *A. foetida* and *Axinyssa aplysinoides* became of priority when mass spectrometry data indicated that nitrogenous spiro-axane sesquiterpene metabolites were to be isolated. The current literature shows that there are 15 sponges and/or nudibranch-derived spiro-axane compounds of general structure **A** (all shown in Table S1, Supplementary data) divided between 14-substituted compounds with *N*-functionality and one compound with an OH group.[5] Use of diagnostic ^{13}C NMR shifts simplifies the dereplication of a spiro-axane skeleton and facilitates the defining of the sites for heteroatom attachment at C-1, C-2, or C-6. Alternatively, establishing the configuration at each of the possible chiral centers (C-1, C-2, C-5, C-7, and C-10) can be more difficult. However, there are four sponge-derived compounds that provide important stereostructural templates for the members of this series and these are (+)-axisonitrile-3 (**1a**),[6] (+)-axenol (**1b**)[7] [*syn* (+)-gleenol], (−)-10-*epi*-axisonitrile-3 (**2a**),[8] and (+)-exiguamide (**2b**),[8b,9] whose absolute configurations at C-5, C-6, C-7, and C-10 were established after extensive experimentation including total synthesis. Relevant to the stereochemical challenges we encounter was that each of the preceding compounds possessed the 10*S* configuration, whereas the configuration varied from 5*R* in **1a** and **1b** to 5*S* in **2a** and **2b**. The biosynthetic assembly of the

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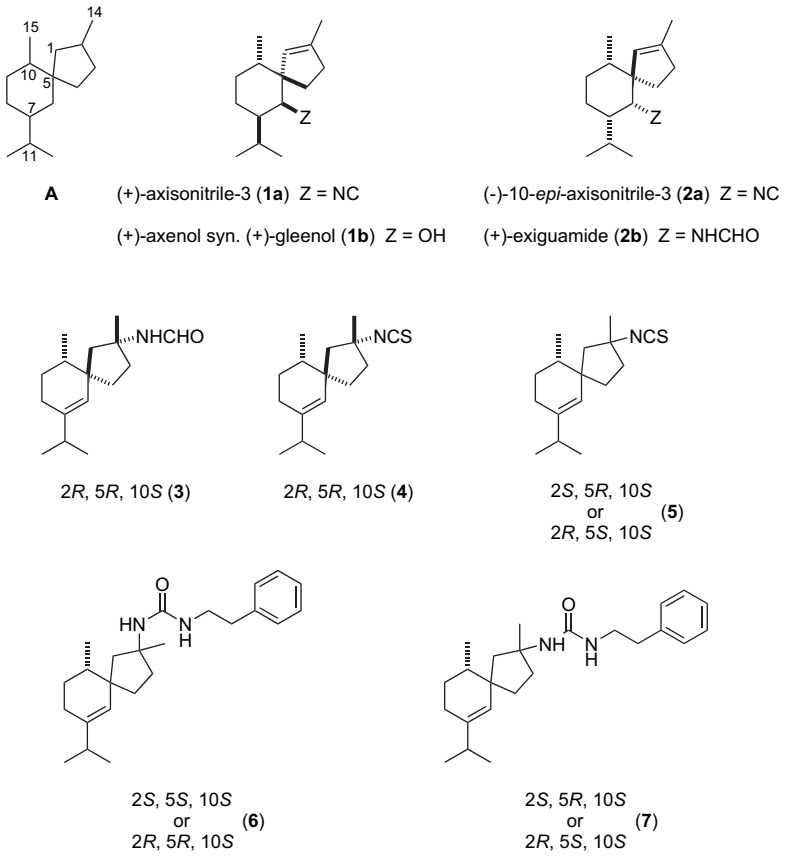
sponge-derived spirobicyclic ring system **A** produces an invariant absolute configuration at C-10, which is consistent with the 10*S* or 10*S** stereochemistry deduced for nine additional metabolites[10] (see Table S1, Supplementary data) and contrasts with the 10*R** stereochemistry previously assigned for **3** and **4**.[11] In contrast, a 10*R* stereochemistry has been found for spiro-axenes isolated from plants including pine,[12] juniper,[13] and cryptmeria trees[14] and a marine algae.[15] Thus, the challenges we faced were to determine the location of the nitrogen substituent and then set the configurations for each of the compounds isolated. In view of the above precedents, all the natural products isolated herein from sponges were assigned with 10*S* chirality.

Described below are properties of five compounds obtained including revision of the configurations for the known substances 2-formamido-6-axene (**3**) and 2-isothiocyanato-6-axene (**4**),[11] a new 2-isothiocyanato-6-axene diastereomer **5**, and a set of new diastereomeric compounds, *N*-phenethyl-2-formamido-6-axenes **6** and **7**. Also discussed below is that our data were consistent with two stereoisomeric possibilities for **5**–**7** with each possessing the 10*S* configuration.



A

(+)-axisonitrile-3 (**1a**) Z = NC

(-)-10-*epi*-axisonitrile-3 (**2a**) Z = NC

(+)-axenol syn. (+)-gleenol (**1b**) Z = OH

(+)-exiguamide (**2b**) Z = NHCHO

2*R*, 5*R*, 10*S* (**3**)

2*R*, 5*R*, 10*S* (**4**)

2*S*, 5*R*, 10*S* or 2*R*, 5*S*, 10*S* (**5**)

2*S*, 5*S*, 10*S* or 2*R*, 5*R*, 10*S* (**6**)

2*S*, 5*R*, 10*S* or 2*R*, 5*S*, 10*S* (**7**)

2. Results and discussion

Investigated first were two semi-pure extract fractions of *Axinyssa aplysinoides* (coll. no. 03411, Vanuatu), which included the hexane soluble sample (coded as XFH) and the dichloromethane soluble material (coded as XFD). Reversed-phase HPLC on both (see Fig. S1, Supplementary data) afforded known **3**,[11] previously isolated from *Amorphinopsis* (syn. *Trachyopsis*) *aplysinoides* ($[\alpha]_D^{25}$ obsd/lit.=+17.4/+14.8) whose ^{1}H NMR ($CDCl_3$) spectra contained doubled peaks ascribed to a mixture of amide rotamers in ratio of 6:4. Further fractionation on the XFD guided by LCMS screening revealed a subfraction containing two isomeric compounds exhibiting a *m*/*z* of 369.3 $[M+H]^+$. Additional HPLC purification afforded small quantities of **6** and **7** (~0.5 mg each) presumed to have a spiro-axane core but, by NMR, without multiple rotameric forms. Their structures were elucidated after re-isolation of more material as described below.

The sponge *A. foetida* (coll. no. 00381, Papua New Guinea) was examined next and the focus was on the butanol semi-pure extract fraction (coded as WB, Fig. S2, Supplementary data). Zone of inhibition data from the disk diffusion solid tumor whole cell assay indicated that this fraction was active against colon-38 cells. HPLC purification afforded 15 fractions (coded as H1–H15), which were then re-assayed. Three HPLC fractions (coded as H4–H6) exhibited the largest inhibition zones. Further LC screening of all the fractions by evaporative light scattering (ELSD), UV, and ESIMS detection was used to identify possible spiro-axane containing samples. Attention was given to two fractions (coded as H7 and H12) with *m*/*z* peaks of 369.3 and 264.2 amu, respectively. Repeated HPLC of the latter fraction yielded the previously reported compound 2-isothiocyanato-6-axene (**4**),[11] previously isolated from *Amorphinopsis* (syn. *Trachyopsis*) *aplysinoides* ($[\alpha]_D^{25}$ obsd/lit.=−22.4/−13.0) and its undescribed diastereomer 2-isothiocyanato-6-axene (**5**)

($[\alpha]_D^{25}$=+44.1). Similarly, HPLC of the former fraction yielded additional samples of **6** (5 mg) and **7** (6 mg).

The structures of **3** (HRESIMS *m/z* 250.2093 $[M+H]^+$ requiring molecular formula $C_{16}H_{27}NO$) and **4** (HRESIMS *m/z* 264.1706 $[M+H]^+$ requiring molecular formula $C_{16}H_{25}NS$) were confirmed as being known by comparing their properties to those in the literature.[11] On re-examining the argument for the previous assignment of the 2*R**,5*R**,10*R** configuration proposed for **3** and **4**,[11] based primarily on NOE data, it was clear that other plausible arrangements had not been ruled out. Based on the biosynthetic analysis presented above, the four possibilities we envisioned consisted of 2*S*,5*S*,10*S*; 2*R*,5*R*,10*S*; 2*R*,5*S*,10*S*; or 2*S*,5*R*,10*S*. An NOE correlation we observed for **4** from H-6 to H_3-14, shown in Figure 1, was used to rule out the latter two. Consistent with the literature,[11] we observed other key NOE enhancements (Fig. 1) from H-6 (δ 5.14) to H-1_b (δ 1.68), H-3_b (δ 1.81), H-4_b (δ 1.43),[16] and H_3-14 (δ 1.51) indicating that these atoms were on the same side of the molecule, but this did not differentiate between the remaining two possibilities. The interpretation of these results was further complicated by the presence of two conformers for the cyclohexene ring of **4** in which H_3-15 was pseudo-axial or pseudo-equatorial. Modeling experiments for all four conformers (Fig. 1) provided two sets of predicted *J* values: (a) H_3-15(eq) $^3J_{9-10}$=7.2 and 5.7 Hz versus (b) H_3-15(ax) $^3J_{9-10}$=2.5 and 2.1 Hz. Comparison of these data to that observed experimentally, $^3J_{9-10}$=6.8 and 2.9 Hz, indicated that significant populations were present for both conformations. Thus, either possibility of 2*S*,5*S*,10*S* or 2*R*,5*R*,10*S* was consistent with the NOE and *J* data.

The results of semi-synthesis provided additional information to resolve this uncertainty. This process began with the LAH reduction of the isothiocyanate **4**, yielding **8**, which was then hydrogenated to afford **9**. The ^{1}H NMR data collected supported the cis arrangement of the equatorial isopropyl at C-7 (based on the diagnostic $\delta_{C\text{-}11}$ 33.0)[17] and the axial methyl at C-10 in **9**. A complex ^{1}H NMR multiplet was observed for H-10 (δ 1.69) with a *J* value sum of 27.1 Hz (see, Fig. S3, Supplementary data). Simulation of the ddq patterns expected for H-10 as a function of variation in its geometry gave a *J* value sum of 33.1 Hz for axial (^3J=9.4, 2.4, 7.1 Hz) versus a *J* value sum of 26.5 Hz for equatorial (^3J=3.7, 1.5, 7.1 Hz). The next step was to obtain and interpret NOE data for **9**. The key result consisted of a strong enhancement from H-4_a to H_3-15 (axial), which required C-4 to be equatorial. Finally, these data indicated the configuration of **9** as 2*R*,5*R*,7*R*,10*S*, which translated into reversal of assignments at C-10 versus that previously reported for **4**.[11] These data also placed in question the previous assignments for **3**. In summary, the stereochemistry of **3** and **4** is now revised to 2*R*,5*R*,10*S* as both compounds were previously determined to have the same relative stereochemistry after they were co-isolated from the same sponge.[11] The logical next step of conducting a side-by-side analysis of **3** and **4** isolated here versus those from the Faulkner repository was considered. However, a search of the UC San Diego compound bank indicated that these compounds were missing.

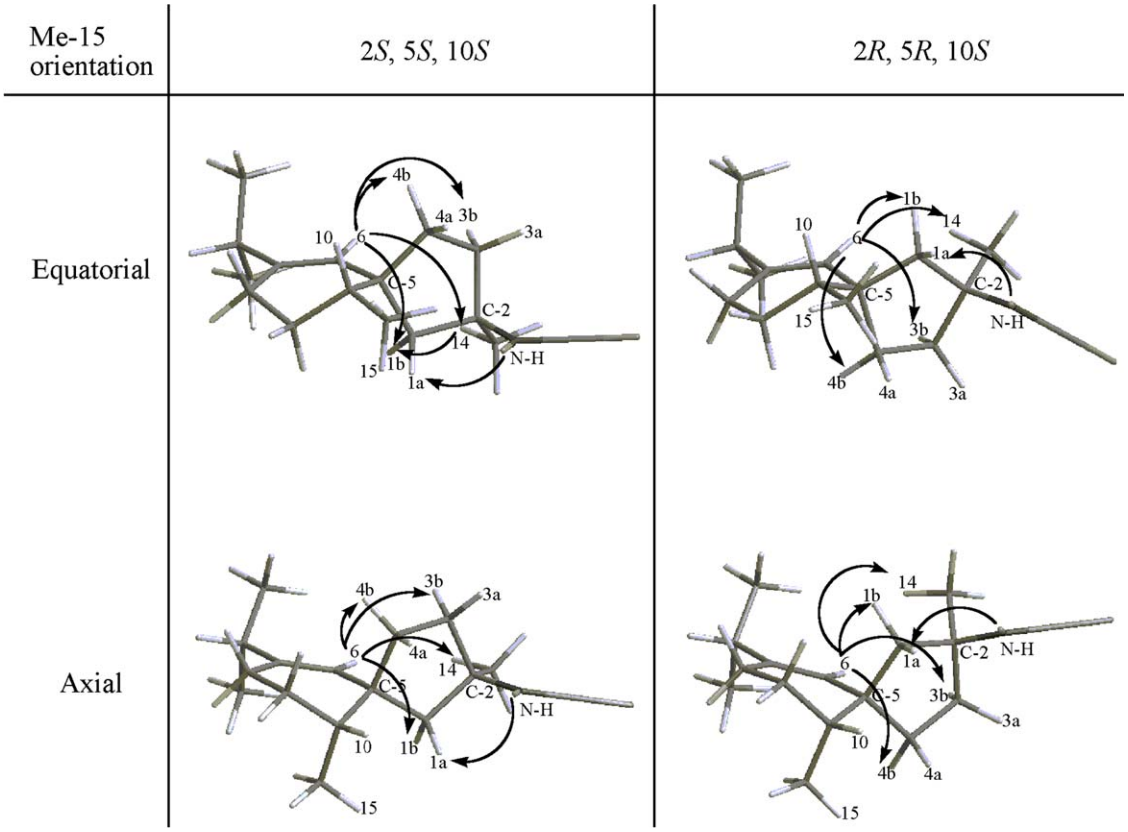
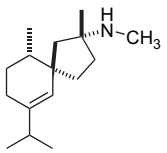
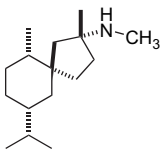


Figure 1. NOE correlations for candidate structures of **4**.



2*R*, 5*R*, 10*S* (**8**)



2*R*, 5*R*, 10*S* (**9**)

The NMR data and stereochemical conclusions discussed above provided an important reference to evaluate the new compounds that were isolated. Compounds **4** and **5** possessed identical molecular formulas and their ^{1}H and ^{13}C NMR data, shown in Table 1, are also extremely similar. Two-dimensional NMR data for **5** including gCOSY, gHMQC, and gHMBC (Table 1) indicated that it had the same atom connectivity as in **4**, however, their optical rotations were of different overall sign and magnitude, **4** $[\alpha]_D^{25}=-22.4$ versus **5** $[\alpha]_D^{25}=+44.1$, indicating that one or more stereocenters were different between this pair.

With the stereochemistry of **4** defined above, there were now three possibilities to be considered for **5**: 2*S*,5*S*,10*S*; 2*S*,5*R*,10*S*; or 2*R*,5*S*,10*S*. The important NOE correlation observed in **4** from H-6 to H_3-14 was not in the data set for **5**, which allowed elimination of the first possibility listed above. In parallel to the situation with **4**, all of the additional NOE data collected for **5** were consistent with the two remaining possibilities. These data included NOE enhancements from H-6 to H-1_a (δ 1.98) and H-4_b (δ 1.63), indicating that these atoms were on the same molecular face. Also important to note is that in **4** the NOE from H-6 was to H-1_b (δ 1.68), indicating that the isothiocyanate and methyl groups at C-2 are on opposite sides of the five-membered ring than that determined for **4**. Subsequently, the changes in the relative shifts of the diastereotopic protons at C-1 and the differences in their NOE correlations to H-6 in **4** versus **5** gave indications to which side of the molecule these atoms were on, however, they were not of real diagnostic value in determining *R* or *S* stereochemistry for the C-2/C-5 positions. Identical synthetic modifications performed on **4** were carried out on **5**, however, overlapping signals and low yields precluded obtaining the NOE data.

The structures of **6** and **7** were deduced as outlined below. A molecular formula of $C_{24}H_{36}N_2O$ (HRESIMS *m/z* 369.2881 $[M+H]^+$), requiring seven degrees of unsaturation, was established for **6**. Dereplication using this formula as a search seed gave 200 compounds as hits, but only one compound *N*-phenethyl-*N*′-2-trachyopsanylurea (**10**)[18] was a natural product and this nitrogenous sesquiterpene was also isolated from an Indo-Pacific *Axinyssa aplysinoides*. Comparison of the ^{1}H and ^{13}C NMR data between **6** (shown in Table 2) and **10** indicated that the phenethyl urea side chain was also present in the former. The remaining NMR signals were also consistent with the presence of a spiro-axane sesquiterpene residue with the nitrogenous group attached at C-2, as seen in **3**–**5**. The elements of the gross structure shown were further confirmed from gCOSY and gHMBC correlations. The formula of $C_{24}H_{36}N_2O$ (HRESIMS *m/z* 369.2879 $[M+H]^+$) was established for **7**, which is identical to that of **6**. The NMR properties of **7** including 2D correlations and ^{13}C shifts were identical to those observed for **6** (Table 2). Minor but significant differences were observed for the ^{1}H NMR shifts of **7** versus **6** for H-3, H-4, H-8, and H-9. Thus, it was clear that **6** and **7** were diastereomers probably differing in the configuration at C-2 and/or C-5. The results of 1D NOE experiments were used to rule out two of the four stereoisomer possibilities. For example, an NOE enhancement at H_3-14 was found when irradiating the vinylic H-6 of **6**, indicating that H_3-14 and H-6 were on the same side of the molecule. This was consistent with a 2*S*,5*S*,10*S* or

Table 1. NMR data[a] for compounds **4** and **5** in $CDCl_3$

Position	**4**			**5**			
	δ_C	δ_H (*J* in Hz)	NOE	δ_C	δ_H (*J* in Hz)	gHMBC	NOE
1	55.5	2.09 dd (1.5, 14.7) 1.68 d (14.7)	 1_a, 6, 14	54.2	1.98 dd (14.1, 2.0) 1.74 d (14.1)	2, 5, 14	1_b, 6 1_a, 3_b, 10, 14, 15
2	68.0			68.5			
3	41.2	2.01 dddd (1.6, 4.2, 6.4, 12.7) 1.81 m		42.2	2.05 m 1.60 m	1, 2, 4, 5	3_b, 4_a, 6
4	35.1	1.46 m 1.43 m	6, 15 6, 15	34.0	1.77 m 1.63 m	1, 2, 3, 10	15
5	46.4			46.5			
6	127.3	5.14 s	1_b, 3_b, 4_b, 11, 12, 13, 14	128.6	5.45 s	1, 5, 8, 10, 11	1_a, 4_b, 11, 12, 13
7	140.5			140.1			
8	22.5	1.92 m 1.82 m		24.4	1.90 ddd (1.5, 5.9, 7.2)	9, 10	9_a, 9_b, 10, 11, 12, 13, 15
9	27.8	1.93 m 1.63 m		28.6	1.58 m 1.36 dddd (7.2, 7.2, 9.3, 13.3)	5, 7, 8, 15	 4_a, 15
10	36.9	1.70 ddq (2.9, 6.8, 6.8)		37.4	1.45 ddq (2.4, 9.3, 6.8)	4, 5, 9	1_b, 8, 15
11	35.0	2.11 sept (6.8)		34.9	2.15 sept (6.8)	6, 7, 8, 12, 13	4_a, 6, 8, 12, 13
12	21.7	0.96 d (6.8)	6, 8_a, 8_b, 11	21.7	0.99 d (6.8)	7, 11	6, 8, 11
13	21.5	0.96 d (6.8)	6, 8_a, 8_b, 11	21.6	0.99 d (6.8)	7, 11	6, 8, 11
14	28.5	1.51 s		27.8	1.49 s	1, 2, 3	1_b
15	15.2	0.90 d (6.8)	1_a, 4_a, 8_a, 10	15.8	0.85 d (6.8)	5, 9, 10	1_b, 4_a, 9_a, 9_b, 10
16	129.2			129.3			

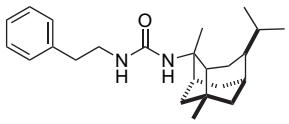
[a] Measured at 500 MHz (^{1}H) and 125 MHz (^{13}C). H_a=downfield proton, H_b=upfield proton.

Table 2. NMR data[a] for compounds **6** and **7** in MeOH-d_4

Position	6						7	
	δ_C	δ_H (J in Hz)	gCOSY	gHMBC	TOCSY	NOE	δ_C	δ_H (J in Hz)
1	53.9	2.04 d (14.2)				1_b	52.4	2.05 dd (1.0, 14.2)
		1.58 d (14.2)		2, 5, 14		1_a		1.60 d (14.2)
2	59.7						60.2	
3	39.7	1.84 m		1, 2, 4	4		40.4	1.92 m
		1.71 m						1.56 ddd (7.3, 8.7, 12.7)
4	34.0	1.76 m		2, 3, 5, 10	3		33.3	1.70 ddd (5.3, 7.4, 12.9)
		1.3 m						1.42 m
5	45.8						45.8	
6	128.5	5.26 s		1, 5, 8, 10, 11		14, 12, 13, 11	129.7	5.40 s
7	139.2						138.4	
8	22.4	1.92 m		9, 10			23.3	1.89 m
		1.85 m						
9	27.5	1.66 m		5, 7, 8, 15			28.1	1.62 m
		1.42 m						
10	36.8	1.6 m	15	4, 5, 9	15		37.3	1.46 m
11	34.7	2.12 sept (6.5)	12, 13	6, 7, 8, 12, 13	12, 13	6	34.7	2.11 sept (6.6)
12	20.5	0.98 d (7.0)	11	7, 11	11	6	20.5	0.95 d (7.0)
13	20.4	0.98 d (7.0)	11	7, 11	11	6	20.4	0.95 d (7.0)
14	26.7	1.38 s		1, 2, 3		6	25.9	1.36 s
15	14.3	0.87 d (7.0)	10	5, 9, 10	10		14.5	0.87 d (7.0)
16	158.9						159.0	
17	40.7	3.30 m	18	16, 18, 19			40.8	3.30 m
18	36.1	2.73 t (7.2)	17	17, 19			36.2	2.75 dt (2.5, 7.2)
19	139.4						139.5	
20	128.0	7.26 m		19, 21, 22	21, 22		128.0	7.26 m
21	128.4	7.19 m		19, 20, 22	20, 22		128.4	7.20 m
22	125.7	7.17 m		20, 21	20, 21		125.7	7.17 m

[a] Measured at 500 MHz (^{1}H) and 125 MHz (^{13}C).

2*R*,5*R*,10*S* configuration. Conversely, irradiation of H-6 on **7** showed no enhancement to H_3-14, which, by analogy to the arguments for **5**, would be consistent with a configuration of 2*S*,5*R*,10*S* or 2*R*,5*S*,10*S*.



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3. Conclusion

The combination of bioactivity and LCMS screening was the key factor that allowed us to interrelate the parallel chemistry from two distantly related Indo-Pacific sponges. This came through the isolation of sesquiterpenes **3**, **6**, and **7** from *Axinyssa aplysinoides* versus **4–7** obtained from *A. foetida*. The observation of diastereomeric pairs (**4/5** and **6/7**) adds another element of distinctiveness to this study. We believe that the three new spiro-axane structures reported here along with the 15 others previously described in the literature represent challenging scaffolds for total synthesis because to date there are only a few such examples in the literature, the preparation of (−)-axisonitrile-3 (**1**),[19] (+)-axenol,[20] and (−)-axenol[21] [*syn* (−)-gleenol[22,23]]. The scant availability of the natural products reported above warrants further synthetic studies to: (a) further affirm the absolute stereochemistry of **3–5**, and (b) fully determine the absolute stereochemistry of **5–7**.

Others have noted the difficulties we encountered in defining the compound(s) responsible for the cytotoxicity of the initial extract, which eventually yielded nitrogenous sesquiterpenes. This was the circumstance in the attempt to isolate bioactive constituents from *Axinyssa aplysinoides* active in an assay against DNA-repair deficient yeast mutants,[18] and was also problematic in the study of antimitotic compounds from another collection of this same species.[24] Our search for the potent cytotoxins from these sponges is continuing, and it is clear that **3** was only mildly solid tumor selective while it also exhibited mild antibacterial activity against *Staphylococcus epidermidis* (ATTC no. 12228) and *Enterococcus durans* (ATTC no. 11576) with MIC's of 0.1 and 0.1 mg/mL, respectively. Finally, no antibacterial activity was found for **4–7**.

4. Experimental

4.1. General experimental procedures

Optical rotations were obtained on a JASCO DIP-370 digital polarimeter. UV measurements were recorded on a diode array spectrometer. The NMR spectra were recorded on a 500 spectrometer, operating at 499.9 and 125.7 MHz for ^{1}H and ^{13}C, respectively. High-resolution mass measurements were obtained on a bench-top ESI-TOF mass spectrometer. HPLC was performed at ambient temperature with Alltech Alltima columns of 5 μm ODS (250 mm×10 mm). Some extraction work was performed using an accelerated solvent extractor (ASE) system, which uses high pressure (~1700 psi) and elevated temperature (~100 °C) to assist in compound extraction.

4.2. Animal material

The sponge *A. foetida* (coll. no. 00381)[3] was collected in December 2000 using SCUBA at a depth of 30 ft in the

Madang region of Papua New Guinea. Voucher samples have been deposited at the Zoological Museum of Amsterdam (ZMA POR. 17553). An abovewater photograph of the sponge is available from the Crews laboratory. The sponge *Axinyssa aplysinoides* (coll. no. 03411)[4] was collected in November 2003 by hand using SCUBA at depths of 30–60 ft in the Mele Bay region of Vanuatu. Voucher samples have been deposited at the Zoological Museum of Amsterdam (ZMA POR. 17767). Abovewater and underwater photographs of the sponge are available from the Crews laboratory.

4.3. Extraction and isolation

Both sponges were preserved by soaking in 1:1 ethanol/seawater for 24 h, decanting, and vacuum sealing while transporting back to the laboratory at ambient temperature. The Vanuatu sponge (coll. no. 03411, 1.5 kg wet wt) was extracted using the ASE to give three fractions. The procedure for extraction with the ASE is as follows. The preserved sponge was allowed to air dry (~48 h). After this, the sponge was dissected into small pieces and partitioned using the ASE by first extracting with hexanes (3×), then with CH_2Cl_2 (3×), and finally with MeOH (3×). The CH_2Cl_2 fraction (XFD) (780 mg) was subjected to preparative reversed-phase HPLC (49:50:1 acetonitrile/water/isopropanol to 99:1 acetonitrile/isopropanol) over 50 min to yield 11 fractions. Fraction 6 yielded compound **3** (78 mg). Fraction 8 (32 mg) was further purified using reversed-phase semi-preparative HPLC (isocratic 75:35 acetonitrile/water both with 0.1% formic acid) to yield 20 fractions. H7 contained compound **3** (24 mg), H10 contained **6** (0.6 mg), and H11 contained **7** (1.0 mg).

The Papua New Guinean sponge (coll. no. 00381, 1.5 kg wet wt) was extracted using a Kupchan style solvent partition method. The field preserved sponge was first extracted with MeOH (3×). The resulting oil was then partitioned between water and CH_2Cl_2. The aqueous layer was extracted with butanol and the butanol layer was evaporated in vacuo to yield a brown gum. The gum was subjected to reversed-phase HPLC using a gradient solvent system of 10:90 methanol/water to 100% methanol over 60 min to afford 15 fractions (H1–H15). Fraction H7 (24.7 mg) was run on HPLC using a gradient solvent system of 40:60 methanol/water to 60:40 methanol/water over 50 min resulting in five fractions. The fourth fraction (H7H4) contained a mixture of both **6** and **7**. Fraction H7H4 was subjected to a shallow gradient HPLC run using a solvent system of 45:55 methanol/water to 50:50 methanol/water over 50 min resulting in **6** (5.2 mg) and **7** (6.3 mg) in pure form. Fraction H12 from the crude WB was run on HPLC to yield **4** (7.0 mg) and **5** (5.5 mg).

4.4. Antibacterial assay

Three different bacterial strains were employed including *Escherichia coli*, *S. epidermidis* (ATTC no. 12228), and *E. durans* (ATTC no. 11576). Minimum inhibitory concentrations (MIC) against these three bacteria were measured using a micro broth dilution test in 96-well microtiter plates with 0.2 mL per well. The maximum concentration of **3** used was 400 μg/mL, and this was serially diluted down to 6.25 μg/mL. The microtiter plates were inoculated with 0.1 mL of overnight cultures that were diluted and adjusted to give concentrations of 10^5–10^6 CFU/mL (per well) and a final volume of 0.2 mL. The 96-well microtiter plates were then incubated at 37 °C overnight for 24 h. A growth control was included to demonstrate the viability of the inoculum in each assay plate. Penicillin G and vancomycin were included as positive controls and DMSO was used as a negative control. The MIC values were determined by visual inspection as the minimum concentration of compound gives 100% inhibition of bacterial growth.

4.4.1. (2*R*,5*R*,10*S*)-2-Formamido-6-axene (3). Colorless solid, $[\alpha]_D^{25}$ +17.4 (*c* 1.5, $CHCl_3$); NMR data were in accordance with literature values; HRESIMS *m/z* 250.2089 $[M+H]^+$ (calcd for $C_{16}H_{27}NO$+H: 250.2093).[11]

4.4.2. (2*R*,5*R*,10*S*)-2-Isothiocyanato-6-axene (4). Colorless oil, $[\alpha]_D^{25}$ −22.4 (*c* 1.0, $CHCl_3$); for ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 264.1706 $[M+H]^+$ (calcd for $C_{16}H_{25}NS$+H: 264.1702).

4.4.3. 2-Isothiocyanato-6-axene (5). Colorless oil, $[\alpha]_D^{25}$ +44.1 (*c* 1.0, $CHCl_3$); for ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 264.1706 $[M+H]^+$ (calcd for $C_{16}H_{25}NS$+H: 264.1702).

4.4.4. *N*-Phenethyl-2-formamido-6-axene (6). Colorless solid, $[\alpha]_D^{25}$ −18.6 (*c* 0.1, MeOH); for ^1H and ^{13}C NMR data, see Table 2; HRESIMS *m/z* 369.2881 $[M+H]^+$ (calcd for $C_{24}H_{36}N_2O$+H: 369.2900).

4.4.5. *N*-Phenethyl-2-formamido-6-axene (7). Colorless solid, $[\alpha]_D^{25}$ +38.6 (*c* 0.1, MeOH); for ^1H and ^{13}C NMR data, see Table 2; HRESIMS *m/z* 369.2879 $[M+H]^+$ (calcd for $C_{24}H_{36}N_2O$+H: 369.2900).

4.4.6. (2*R*,5*R*,10*S*)-2-*N*-Methyl-6-axene (8). To a suspension of 10 mg LAH in 20 mL dry ether was added dropwise **4** (3.0 mg) in 250 μL dry ether. This was then allowed to reflux overnight. Workup consisted of cooling to 0 °C then adding 0.25 mL 10% NaOH followed by 0.5 mL H_2O. Vacuum filtration followed by HPLC resulted in **8** (1.0 mg). Colorless oil; ^1H NMR ($CDCl_3$) δ 9.20 (N-H), 5.31 (H-6), 2.62 (H-16), 2.15 (H-11), 1.78 (H-1_b), 1.43 (H-14), 0.98 (H-12, H-13), 0.92 (H-15); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 141.8 (C-7), 126.9 (C-6), 65.7, 50.9, 46.3, 37.3, 36.5, 35.0, 33.7, 27.9, 27.6, 23.7, 23.4, 21.6 (C-12 or C-13), 21.5 (C-12 or C-13), 15.2 (C-15); HRESIMS *m/z* 236.2369 $[M+H]^+$ (calcd for $C_{16}H_{29}N$+H: 236.2373).

4.4.7. (2*R*,5*R*,10*S*)-2-*N*-Methyl axane (9). A solution of **8** (1.0 mg) in MeOH (0.5 mL) along with 2.0 mg of 10 wt % Pd/C was placed under 3 atm of H_2 and shaken overnight to yield **9** (0.5 mg). Colorless oil; ^1H NMR ($CDCl_3$) δ 9.19 (N-H), 2.57 (H-16), 2.08 (H-3_a), 1.98 (H-1_a), 1.92 (H-4_a), 1.79 (H-1_b), 1.78 (H-3_b), 1.69 (H-10), 1.40 (H-4_b, H-8_a, H-11), 1.27 (H-6_a, H-9_a), 1.13 (H-6_b, H-8_b, H-9_b), 0.89 (H-15), 0.86 (H-12, H-13); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 66.3 (C-2), 48.2 (C-1), 46.6 (C-5), 40.4 (C-7), 38.0 (C-10), 37.4 (C-4), 35.3 (C-3), 34.6 (C-6), 33.0 (C-11), 30.0 (C-9), 27.4 (C-16), 25.0 (C-14), 23.0 (C-8), 20.0 (C-12 or C-13), 19.8 (C-12 or C-13), 15.0 (C-15); HRESIMS *m/z* 238.2533 $[M+H]^+$ (calcd for $C_{16}H_{31}N$+H: 238.2529).

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Supplementary data

A complete list of sponge/nudibranch-derived spiro-axane compounds, compound isolation diagrams, comparison of simulated and experimental H-10 multiplets for **9**, and proton NMR spectra of **4–7**. Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2006.08.070.

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