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New 9-Thiocyanatopupukeanane Sesquiterpenes from the Nudibranch *Phyllidia varicosa* and Its Sponge-Prey *Axinyssa aculeata*

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Two new 9-thiocyanatopupukeanane sesquiterpene isomers were isolated as major metabolites from the MeOH extract of the sponge *Axinyssa aculeata* and from its nudibranch predator *Phyllidia varicosa*. The presence of the sesquiterpenes was monitored by GC−MS, and the structures were confirmed by both 1D and 2D NMR spectroscopy. The isolated sesquiterpenes were found to be toxic toward brine shrimp at LC_{50} of 5 ppm. At a dose level of 20 μg, they were found to be weakly and moderately active against *B. subtilis* and *C. albicans*, respectively.

Cyano- and isothiocyanosesquiterpene metabolites of the phyllidiids *Phyllidia* and its sponge-prey *Axinyssa* have been reported to be potent antimalarial and antifouling agents.[1−4] It has also been hypothesized that phyllidiids sequester sesquiterpene metabolites from their specific sponge-prey to protect their naked, soft body from predation. This further suggested the key role of sesquiterpenes as chemical defense emissaries for both the nudibranchs and the sponge.[1,5,6,8,9] It was later shown that sesquiterpene isocyanides were the metabolites responsible for this toxic response.[10] Similar compounds have been isolated from specimens of the same species collected from Sri Lanka,[11] from the Philippines,[12] and from the Okinawan waters.[2]

Chemical studies on the sponge *Hymeniacidon* sp. revealed that it was the source of the substance sequestered by its nudibranch predator *Phyllidia varicosa*, and the metabolites were elucidated as 9- and 2-isocyanopupukeanane.[10] These findings established the selective accumulation of a chemical defense agent in the nudibranch diet.[1] It was also shown that *P. varicosa* feeding on the sponge *Halichondria* sp. sequestered the metabolites 4α-isocyanogorgon-11-ene and 4α-formamidogorgon-11-ene.[12] The association between sponges of the genus *Axinyssa* and the nudibranch *P. varicosa* is well known.[13] Sponges of the genus *Axinyssa* sp. have been reported to contain unusual sesquiterpenes.[14] Some of these were found to be structurally similar to the sesquiterpenes isolated from *P. varicosa*,[3] which has been recently confirmed to be a specialist predator on sponges producing isocyanide sesquiterpenes.[14a]

P. varicosa constitutes one of the most frequently encountered phyllidiid species in the coral reefs of Thousand Islands, Indonesia.[7] The nudibranch *P. varicosa* and its sponge-prey *Axinyssa aculeata* were collected by scuba at depths of 5−25 m from a coral reef near Pramuka Island, Thousand Islands National Park, Indonesia. Two new stereoisomeric-related thiocyanate pupukeanane sesquiterpenes were isolated from the MeOH sponge extract and from the different dissected parts (e.g., mantle, digestive gland, and foot) of the nudibranch by a series of normal-phase column chromatography using different ratios of hexanes and EtOAc as eluent. The sesquiterpenes were found to be the major metabolites in both the sponge and nudibranch. Their presence was monitored by GC−MS and the structures were confirmed by both 1D and 2D NMR spectroscopy. Together with the sesquiterpenes, ergosta-4,22,25-trien-3-one (**6**) was isolated from the nudibranch. Ergosta-4,22,25-trien-3-one has been described to be produced only synthetically, and this is the first account that it is isolated as a natural product.[16]

The thiocyanatopupukeanane sesquiterpenes (**1** and **2**) were isolated as an epimeric mixture. The presence of the two isomers was detected by GC coupled to a FID detector. Both compounds gave a molecular weight of 263, and their MS spectra also indicated identical fragmentation patterns. The base peak was observed at *m*/*z* 205, demonstrating the loss of a −SCN function. HREIMS data established the molecular formula $C_{16}H_{25}SN$. The co-occurrence of the two epimers for 9α- and 9β-thiocyano metabolites was clearly evident from their ^{1}H and ^{13}C NMR spectra showing separated resonances for H-9, C-9, C-10, and C-2 for each of the epimers. Proton and carbon assignments for each isomer were inferred by 2D ^{1}H,^{1}H-COSY, HMBC, and HMQC (Table 1). The ROESY data confirmed the presence of two epimers at C-9 (see below).

The basic structures of compounds **1** and **2**, as well as their ^{1}H and ^{13}C NMR data, were comparable to those of 9-isothiocyanopupukeanane (**3**) and 9-isocyanopupukeanane epimers (**4** and **5**), which were previously isolated from another sponge species of the genus *Axinyssa* and its predator of the genus *Phyllidiella*.[3,10,17] The presence of a thiocyanate function at C-9 as opposed to an isothiocyanate group was confirmed by inspection of the ^{13}C NMR spectrum and comparison with established data already reported.[3,10,17] The ^{13}C chemical shifts for the epimers **1** and **2** were comparable with those of **3** and epimers **4** and **5**. The major difference in chemical shift was observed at C-16 of the respective congeners. For compound **3** the chemical shift at ca. δ 125 referred to the presence of an isothiocyanate group,[13a] while the chemical shift at ca. δ 155 as in **4** and **5** indicated the occurrence of an isocyanide moiety.[13b,c,18] For epimers **1** and **2**, the carbon shift at δ 113.5 characterized the presence of a thiocyanate function as described for 2- and 4-thiocyanatopupukeanane.[3] Epimers **1** and **2** can be distinguished from each other by the difference in ^{13}C chemical shifts for C-2 (δ 46.9 for **1**, δ 55.0 for **2**) and C-10 (δ 35.5 for **1**, δ 27.3 for **2**). This pattern of

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Table 1. NMR Data for 9-Thiocyanatopupukeanane (**1**) and 9-*epi*-9-Thiocyanatopupukeanane (**2**) in $CDCl_3$

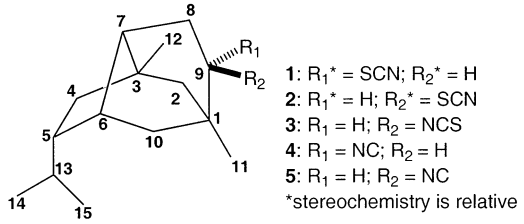
	1					**2**				
	δ_C	(mult.)	δ_H	(mult., *J*)[a]	HMBC (δ_H to δ_C)	δ_C	(mult.)	δ_H	(mult., *J*)[a]	HMBC (δ_H to δ_C)
1	33.1	(C)				33.0	(C)			
2	46.9	(CH_2)	1.58	(ddd, 14.1, 3.5[c], 1.8[c])	C-1, C-3, C-5, C-6, C-9	55.0	(CH_2)	1.37	(dd, 13.8, 1.8[c])	C-1, C-3, C-4,C-7, C-11
			1.00	(ddd, 14.0, 2.3[c], 1.3[c])				1.25	(ddd, 13.5, 3.5[c], 1.5[c])	
3	39.1	(C)				38.9	(C)			
4	48.3	(CH_2)	1.83	(ddd, 13.3, 10.3, 2.8[c])	C-2, C-3, C-5	48.3	(CH_2)	1.85	(ddd, 13.3, 10.3, 2.8[c])	C-2, C-3, C-5
			1.14	(dd, 13.5, 8.0)				1.16	(dd, 13.5, 8.0)	
5	49.3	(CH)	1.40	(dddd, 12.5, 10.4, 8.2, 4.8)		49.6	(CH)	1.44	(dddd, 12.5, 10.5, 7.8, 4.9)	
6	38.3	(CH)	2.11	(br dddd, 6.3, 5.5, 4.5, 3.5)	C-4	38.3	(CH)	2.09	(bm)	C-4
7	44.3	(CH)	1.33	(ddd, 6.2, 3.8, 2.5)		43.7	(CH)	1.35	(dd, 6.0, 3.0, 2.5)	C-5
8	28.9	(CH_2)	2.35	(ddd, 15.5, 10.5, 3.8)	C-1, C-6, C-7, C-9	28.9	(CH_2)	2.40	(ddd, 15.7, 10.8, 3.0)	C-1, C-6, C-7, C-9
			1.72	(ddd, 15.5, 6.8, 2.5)				1.89	(ddd, 15.7, 4.4, 3.0)	
9	56.5	(CH)	3.40	(ddd, 10.6, 6.6, 2.3[c])	C-1, C-8, C-12, C-16	56.2	(CH)	3.30	(ddd, 10.8, 4.2, 2.5[c])	C-1, C-2, C-8, C-11, C-16
10	35.5	(CH_2)	1.46	(ddd, 13.5, 5.5, 3.5[c])	C-11, C-1, C-3[b], C-7, C-4[b]	27.3	(CH_2)	1.65	(ddd, 14.8, 10.3, 3.5[c])	C-7
			1.38	(dd, 13.0, 3.5)				1.17	(dd, 14.5, 2.5[c])	C-11, C-13[b],C-7, C-2[b]
11	26.5	(CH_3)	0.93	(s)	C-1, C-2, C-10	26.8	(CH_3)	0.92	(s)	C-1, C-2, C-9, C-10
12	26.0	(CH_3)	1.01	(s)	C-2, C-3, C-4, C-7	26.2	(CH_3)	0.98	(s)	C-2, C-3, C-4, C-7
13	29.7	(CH)	1.52	(ddq, 12.5, 6.4, 6.4)		29.6	(CH)	1.56	(ddq, 12.5, 6.4, 6.4)	C-5, C-14, C-15
14	21.6	(CH_3)	0.84	(d, 6.4)	C-5, C-13, C-15	21.6	(CH_3)	0.85	(d, 6.4)	C-5, C-13, C-15
15	21.6	(CH_3)	0.84	(d, 6.4)	C-5, C-13, C-14	21.5	(CH_3)	0.83	(d, 6.4)	C-5, C-13, C-14
16	113.5	(C)				113.5	(C)			

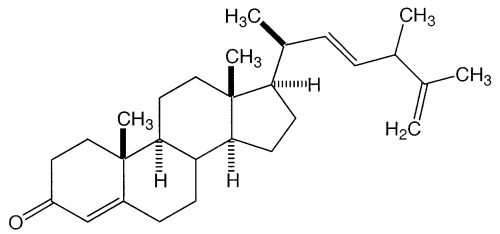
[a] The chemical shifts were assigned from a combination of 2D HMQC, HMBC, and ^{1}H,^{1}H-COSY data. Coupling constants were evaluated from a 2D *J*-resolved ^{1}H spectrum. [b] Cross-peaks observed with weak intensity which signified ^4J couplings. [c] Long-range ^4J 'w' couplings were observed in the 2D ^{1}H,^{1}H-COSY spectrum for **1**: $^4J_{2A-10A}$ (3.5 Hz), $^4J_{2B-9}$ (2.3 Hz), $^4J_{2A-11}$ (1.8 Hz), $^4J_{2B-12}$ (1.3 Hz) and for **2**: $^4J_{2A-10A}$ (3.5 Hz), $^4J_{10B-9}$ (2.5 Hz), $^4J_{2A-12}$ (1.8 Hz), $^4J_{2B-11}$ (1.5 Hz).

differences was also observed in the co-occurrence of **4** and **5** in the nudibranch *Phyllidia bourguini*.[17] The signals for C-2 and C-10 of **2** are compatible with those in **3** and **5**, indicating the same configuration of their functional group. The presence of the two epimers was observed from the 2D COSY spectrum as presented by a four-bond "w" coupling between H-9 (δ_H 3.40) and H-2 (δ_H 1.00) in **1**, while epimer **2** showed a similar coupling between H-9 (δ_H 3.30) and H-10 (δ_H 1.16). Coupling constants were evaluated from a 2D *J*-resolved ^{1}H spectrum: 2.3 Hz ($^4J_{9-2B}$) for epimer **1** while epimer **2** gave a coupling constant of 2.5 Hz ($^4J_{9-10B}$). Additional evidence of the presence of the two epimers was also provided by the significant differences in the coupling constants for CH_2-8 to H-9 in the respective epimers. For epimer **1**, H-9 gave coupling constants of 10.6 Hz ($^3J_{8A-9}$) and 6.6 Hz ($^3J_{8B-9}$), while **2** gave coupling constants of 10.8 Hz ($^3J_{8A-9}$) and 4.2 Hz ($^3J_{8B-9}$).

The relative stereochemistry in both epimers was confirmed from a 2D ROESY spectrum. The correlation of δ_H 3.40 (H-9) with δ_H 1.38 (CH_2-10B) attested to the α-configuration of the −SCN group in **1**, which indicated that H-9 was *cis* to H-10B. Correlation of δ_H 3.30 (H-9) with δ_H 1.25 (CH_2-2B) confirmed the *β*-configuration of the −SCN group for **2**, where H-9 was *cis* to H-2B. Similar responses were also observed in the NOESY spectra of epimers **4** and **5**.[17]

The co-occurrence of epimers **1** and **2** in the sponge and different body parts of the nudibranch was quantified by GC analysis. The sponge extract showed a higher concentration of the thiocyanate metabolite **2** compared to congener **1** in a ratio of 60:40. Higher concentration of epimer **2** was also found in the mantle of *P. varicosa*, where it was approximately twice that of its congener **1**. In the digestive





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gland, however, the epimeric mixture was found to be in a ratio of 1:1. Epimer **2** was found to accumulate in the mantle, which could be a defensive mechanism that protects the organisms from other predators. The decrease in concentration of epimer **2** in the digestive gland may also be a defensive mechanism of the nudibranch to cope with the self-toxicity of consuming the more toxic epimer.

Since separation of the compounds **1** and **2** was not successful, both compounds were bioassayed as epimeric mixtures. The ratios of epimers **1** and **2** present in the mixture were quantified by GC analysis from aliquots of the test samples used for the bioassays. At a dose of 200 ppm, different ratios of the epimeric mixture of compounds **1** and **2** were subjected to a brine shrimp assay. A mixture

ratio of 30:70 for epimer **1** versus epimer **2** showed a mortality rate of 90% (LD_{50} 5 ppm), while a 50:50 ratio resulted in a 35% mortality rate. Increased concentration of epimer **2** in the mixture gave a significant increase in the biological activity. These results indicated that epimer **2** was more toxic than **1**. At a dose level of 20 μg, the 30:70 **1** to **2** mixture was found to be weakly and moderately active against *B. subtilis* and *C. albicans*, respectively.

Isocyanide congeners have always been reported together with their isothiocyanate derivatives due to an interconversion process in the presence of a peroxidase or rhodanese enzyme which was postulated from a radio-labeled ^{14}C feeding experiment in sponges.[13] This metabolic conversion has been inferred by studies performed on *Ciocalypta* sp.[13] To date, there are fewer thiocyanate marine sesquiterpenes reported in the literature than isothiocyanate and isocyanide sesquiterpenes,[13,19] and this is the first report of a 9-thiocyanatopupukeanane described as a marine natural product.

Experimental Section

Animal Materials. Both the phyllidiid and the sponge were collected by hand with the use of scuba at depths of 5−25 m from the coral reefs of Pramuka Island, Thousand Islands National Park, Indonesia. The phyllidiid was dissected into several parts: mantle, digestive gland, foot, etc. The sponge and each body part of the phyllidiid were preserved in MeOH. The sponge and the phyllidiid were identified as *Axinyssa* cf. *aculeata* (family Halichondridae, order Halichondrida) and *Phyllidia varicosa* (family Phyllidiidae, order Nudibranchia). A voucher sponge specimen has been deposited in the Zoological Museum, University of Amsterdam, under the registration no. ZMA POR.10885, whereas a voucher phyllidiid specimen has been stored in the Taxonomy Laboratory of the Biology Department, University of Indonesia, under the registration no. Phyl.varicosa.141100.

Extraction and Isolation. The sponge and each part of the phyllidiid were preserved and repeatedly extracted with MeOH. The resulting total MeOH extract of the sponge was subjected to solvent−solvent partitioning between EtOAc and H_2O. The resulting EtOAc sponge extract and the MeOH extracts of the mantle and digestive gland of *P. varicosa* were then further chromatographed by vacuum liquid chromatography (VLC) through step gradient elution commencing with a nonpolar solvent (hexanes) followed by addition of increasing amounts of a more polar solvent (EtOAc) to each successive solvent fraction. Compounds **1** and **2** were isolated as an epimeric mixture from the nonpolar fraction, which was eluted at a solvent ratio of 95:5 (hexane/EtOAc). Separation of the epimeric compounds **1** and **2** was not successful in using normal-phase column chromatography even at different ratios of hexanes and EtOAc or by addition of a third solvent like MeOH. Utilizing flash column chromatography also did not work to separate the two compounds. Fusetani's group[17] used a chiral normal-phase HPLC column to separate mixtures of 9-isocyanopupukeanane epimers (**4** and **5**). However, this kind of separation setup is not available in our laboratory. Ergosta-4,22,25-trien-3-one (**6**) was isolated from the more polar fractions but only from the nudibranch.

The fractions and the isolated compounds were monitored on an Agilent 6850 GC system (150 °C at 0 min to 300 °C at 80 min). ^1H and ^{13}C NMR spectra (chemical shifts in ppm) were recorded in $CDCl_3$ on a Bruker Avance DMX 600 NMR spectrometer. Mass spectra (EI) were measured on a Finnigan MAT 8430 mass spectrometer and on a Series II gas chromatography 5890 mass selective detector 5972. ROESY spectra were recorded in $CDCl_3$ on a Bruker Avance DPX 500 NMR spectrometer.

9-Thiocyanatopupukeanane (1 and 2): ^1H NMR, ^{13}C NMR, and HMBC data, see Table 1; EIMS (42 eV) *m/z* $[M]^+$ 263.2 (33), 205.2 (100), 161.2 (20) 149.1 (70), 135.1 (35), 107.1 (55), 93.1 (50), 81.1 (30); HREIMS *m/z* 263.1752 (calcd for $C_{16}H_{25}SN$, 263.1708).

Brine Shrimp Toxicity Assays. Eggs of *Artemia salina* (Dohse, Aquaristik GmbH, Bonn, Germany) were hatched in a small tank filled with artificial seawater (Sera Sea-Salt, Aquaristik GmbH, Bonn, Germany). After 48 h, the phototropic nauplii were collected, 20 brine shrimps were transferred to each sample vial using a pipet, and artificial seawater was added to make 5 mL. After 24 h, mortality at each dose was determined and compared to controls.

Antimicrobial Assays. Samples were dissolved in dichloromethane and loaded onto sterile filter paper disks of 5 mm diameter (Oxoid Ltd) at a dose of 10 and 20 μg. The impregnated disks were then placed on the agar plates previously inoculated with *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, and *Candida albicans*. Solvent blanks were run against each test organism. The plates were incubated at 37 °C for 24 h, then antimicrobial activity was recorded as the clear zones of inhibition surrounding the disk, of which the diameter was measured in mm. A test sample is considered active when the zone of inhibition is greater than 7 mm.

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