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Isolation and structure identification of new alkaloids from the sponge *Rhabdastrella rowi*

Isolasi and Identifikasi struktur senyawa alkaloid baru dari spons *Rhabdastrella rowi*

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Abstrak

Telah dilakukan penelitian tentang komponen kimia spons laut *Rhabdastrella rowi* yang dikoleksi dari Bali, Indonesia. Penelitian ini bertujuan untuk mengisolasi dan mengidentifikasi struktur metabolit sekunder spons tersebut dan menguji aktivitas sitotoksisnya terhadap kultur sel limfoma tikus L5178Y.

Isolasi dilakukan dengan menggunakan berbagai tehnik kromatografi. Metode spektroskopi resonansi magnetic inti serta spektrometri massa digunakan untuk mengidentifikasi struktur kimia isolat. Pengujian sitotoksisitas pada kultur sel limfoma tikus L5178Y dilakukan dengan menggunakan *MTT assay*.

Penelitian ini mengungkap keberadaan dua senyawa alkaloid baru quinolin-4-ol (**1**) and quninazolin-4-amine (**2**) sebagai komponen minor *Rhabdastrella rowi.* Kedua senyawa tersebut tidak menunjukkan aktivitas sitotoksisitas terhadap kultur sel limfoma tikus L5178Y.
Kata kunci: *Rhabdastrella rowi*, spons, alkaloid

Abstract

Chemical investigation on marine sponge *Rhabdastrella rowi* collected from Bali, Indonesia has been performed. This study was aimed to isolate and to identify structures of the sponge secondary metabolites as well as to test their cytotoxicity activity on mouse lymphoma cell line L5178Y.

Isolation procedure was performed by using different chromatography techniques. NMR spectroscopy and mass spectrometry methods were used to identify the compounds chemical structures. Cytotoxicity of the isolates was tested on mouse lymphoma cell line L5178Y by using the microculture tetrazolium (MTT) assay.

This study yielded two new alkaloids, quinolin-4-ol (**1**) and quninazolin-4-amine (**2**) which were found as minor constituents of *Rhabdastrella rowi*. Both compounds were observed as inactive against mouse lymphoma cell line L5178Y.
Key words: *Rhabdastrella rowi*, sponge, alkaloids

Introduction

As the central part of Indo West Pacific region, Indonesia is known to possess high marine biodiversity (Van Soest, 1989). Increased feeding pressure from fishes and possibly from predatory invertebrates as well as higher microbial infection rate in tropical areas might be an explanation for the high abundance of structurally diverse secondary metabolites that are found in sponges from this region compared to other ecosystem of the world (Proksch *et al*., 2002). Considering the unfulfilled need for chemotherapeutic agent to overcome new and more complicated diseases worldwide, chemical studies on Indonesian sponges are as they represent a potential source of new drugs from the sea or compounds that may serve as lead structures for future drug development.

Marine sponges of genus *Rhabdastrella* are commonly distributed in Asian tropical oceans, such as shallow coral reefs in southern China, New Caledonia and the Philippines (Lu *et al*., 2004; Rao *et al*., 1997; Tasdemir *et al*., 2002; Bourguet-Kondracki *et al*., 2000). Only the species *R. globostelata* has been examined chemically of which a series of potential bioactive isomalabaricane-type nortriterpenoids and triterpenoids were isolated (Lu *et al*., 2004; Rao *et al*., 1997; Tasdemir *et al*., 2002; Bourguet-Kondracki *et al*., 2000; Fouad *et al*., 2006). Chemical constituents of *Rhabdastrella rowi* have not yet been reported. Therefore we chose this sponge for a detailed chemical investigation.

Methodology

Sponge Material

Sponge sample was collected on October 2003, from offshore Menjangan Island, Bali, Indonesia at a depth of 12 m by means of SCUBA. It was directly preserved in ethanol after harvesting. A voucher specimen for taxonomic identification was deposited in the Zoological Museum in Amsterdam, under reg. no. ZMAPOR1829.

Isolation procedure

The chemicals used in the detection and isolation methods were anisaldehyde (4-methoxybenzaldehyde), hydrochloric acid and concentrated sulphuric acid (all provided by Merck, Darmstadt, Germany); ammonium hydroxide, glacial acetic acid, (Fluka, Seelze, Germany); dimethylsulfoxide, trifluoroacetic acid 99%, extra pure (Acros® organic, Geel, Belgium).

Solvents used for separation techniques were dichloromethane; ethyl acetate; *n*-hexane; methanol. These solvents were purchased from the Institute of Chemistry HHU Düsseldorf. They were distilled before using and special grade were used for spectroscopic measurements. Others solvent used were *n*-butanol and acetonitrile (Fluka, Seelze, Germany), ethanol (Merck, Darmstadt, Germany).

Column chromatography was performed on silica gel (0.040-0.063 mm; Merck, Darmstadt, Germany) or on Sephadex LH20 (Merck, Darmstadt, Germany). For HPLC analysis, samples were injected into an HPLC system equipped with a photodiode array detector (Dionex, München, Germany). Routine detection was at λ 235, 254, and 340 nm. The separation column (125 x 4 mm i.d.) was prefilled with Eurospher 100-C18, 5μm (Knauer, Berlin, Germany). Separation was achieved by applying a linear gradient from 90% H_2O (pH 2.0) to 100% MeOH over 40 min. TLC analysis was carried out using aluminium sheet precoated silica gel 60 F254 or on glass precoated RP-18 F254 plates (Merck, Darmstadt, Germany).

Sponge tissue was separated from the supernatant (ethanol) and dried at room temperature. Dried sponge tissue (184 g) was ground and extracted exhaustively with methanol. After removing the solvent under reduced pressure, the methanol extract was combined with the ethanol extract to yield a total weight of 44 g. DAD-HPLC and LC/MS as well as TLC was used to guide the isolation procedure.

The extract was first partitioned between *n*-hexane and 90% MeOH-water to yield a hexane fraction (0.7 g). Residue was partitioned between ethyl acetate and H_2O to obtain ethyl acetate fraction (1.4 g), and finally water phase was partitioned against *n*-BuOH to obtain n-butanol (2.3 g) and water fractions (18 g). Investigation of the fractions by analytical HPLC indicated that only ethyl acetate fraction which was of interest for further analysis.

The ethyl acetate fraction was chromatographed over a silica gel G60 (vacuum liquid chromatography) using eluents with increasing polarity from 100% *n*-hexane to 100% ethyl acetate followed by 100% dichloromethane to 100% MeOH which gave 17 fractions. Fraction 6 was chromatographed over a Sephadex LH20 column using MeOH as an eluent which resulted to 24 sub-fractions. Sub-fraction 10 was subjected to semi preparative HPLC RP18 by gradient elution of methanol-water as mobile phase to obtain compound **1** (1 mg) and **2** (1 mg). The program used

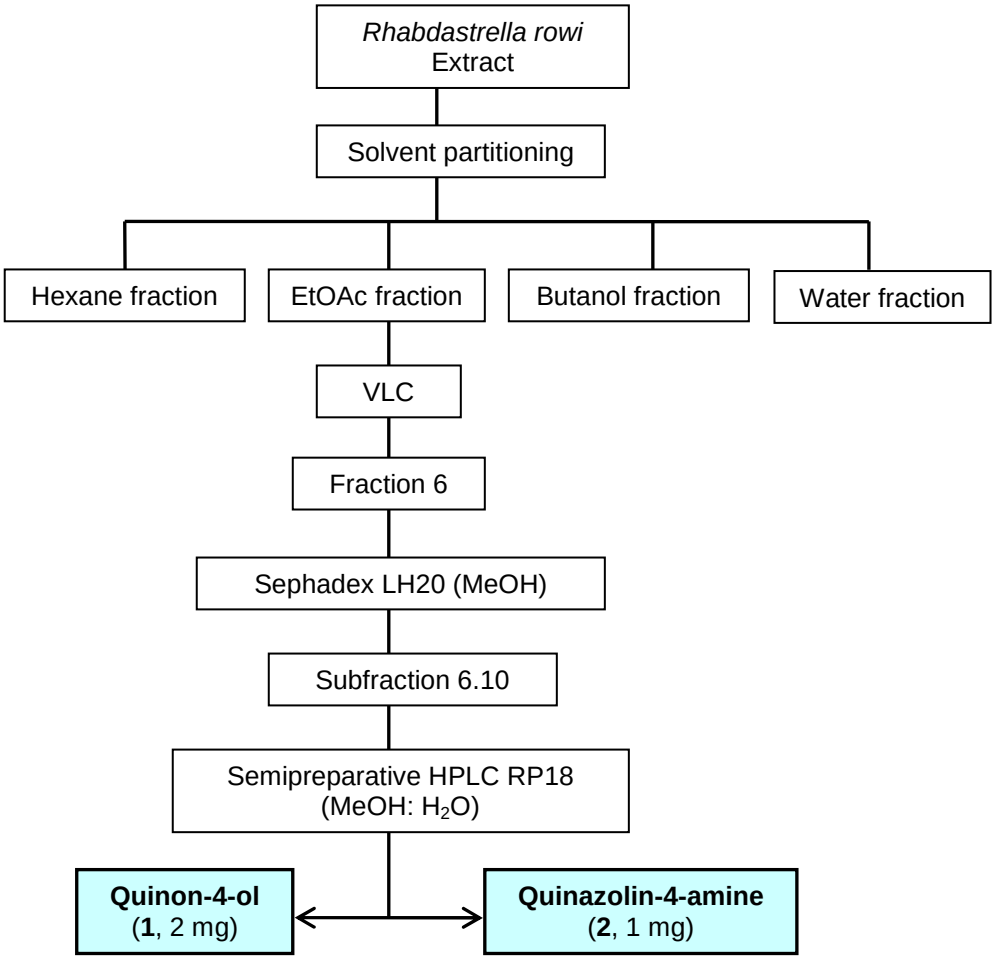

Figure 1. Isolation scheme of *Rhabdastella rowi* metabolites

for the solvent system was as follows, 0' – 8': MeOH 10%; 8' – 15': 10% - 50% MeOH; 15' – 20': 50 - 100% MeOH; 20' – 23': 100% MeOH; 23' – 25': 10% MeOH.

Structure identification

^{1}H and ^{13}C NMR spectra were recorded at 300°K on Bruker DPX 300, ARX 400, 500 or AVANCE DMX 600 NMR spectrometers using deuterated methanol or DMSO-d_6 (Eurisotop, France) as solvents. All 1D and 2D spectra were obtained using the standard Bruker software. ESI mass spectra were obtained on a ThermoFinnigan LCQ DECA mass spectrometer coupled to an Agilent 1100 HPLC system equipped with a photodiode array detector.

Cytotoxicity assay

Isolates were tested for cytotoxicity against L5178Y mouse lymphoma cells by using the microculture tetrazolium (MTT) assay and compared to that of untreated controls as described by Carmichael and collaborators (1987).

Results And Discussion

Rhabdastrella rowi (Dendy) is a member of sponge genus Rhabdastrella (phylum Porifera; class Demospongiae; order Astrophorida; family Ancorinidae; genus *Rhabdastrella* (Uriz, 2002)). Preliminary investigation on the crude extract of *Rhabdastrella rowi* yielded fatty acids as major constituents and no isomalabaricane type compounds were detected which have frequently been described for other sponges of

Figure 2. *Rhabdastrella rowi*

this genus (Lu *et al.*, 2004; Tasdemir *et al.*, 2002; Bourguet-Kondracki *et al.*, 2000; Fouad *et al.*, 2006). This was evident from its analytical HPLC chromatogram which gave no hint to the presence of isomalabaricane type compounds. Bioassays done on the crude extract revealed no biological activity. Presence of trace amounts of alkaloids was indicated by analytical HPLC. Further isolation was then guided by chemical profile data.

Figure 3. shows the retention times of isolated compounds **1** and **2** at roughly 12.9 min and 15.5 min, respectively, as well as their UV absorption spectra measured by on line detection. It is evident that **1** is slightly more polar than **2** as it was eluted earlier. Moreover, a slight bathocromic shift of peak I (240.5 nm) was detected in the UV spectrum of **2** as well as an additional peak at 262.5 nm compared to the spectrum of **1**. On the other hand, the UV maximum of **1** at 315.7 nm is slightly more intense in comparison to that of **2**.

LC/MS experiment done on both compounds showed the molecular ion peak of **1** in the positive mode as 146 mU and in negative mode at m/z 144. On the other hand, compound **2** only showed a rather extensive fragmentation and gave m/z 146 as molecular ion $[M+H]^+$. This finding suggests the same molecular weight for both alkaloids at m/z 145 (Fig.4a & b).

^{1}H-NMR and ^{1}H-^{1}H COSY experiments were performed in order to elucidate the structures of **1** and **2** (Fig.7-10). ^{1}H-NMR spectrum of **1** in deuterated methanol showed six proton resonances in the aromatic region. ^{1}H–^{1}H COSY experiment revealed a presence of two spin systems suggesting two aromatic rings. One spin system correlated the protons at δ_H 7.98 (1H, d, J = 7.2 Hz, H-2) to the proton at δ_H 6.33 (1H, d, J = 7.2 Hz, H-3). The second spin system showed a typical ABCD system and suggested a presence of an *ortho* disubstituted benzene ring. Deshielded effect was exhibited by H-9 (δ_H 8.25, 1H, d, J = 8.2 Hz) and H-2 which implied the presence of a quinoline unit, while shielded effect experienced by H-3 could be explained by a hydroxyl substitution in *ortho* position. Thus compound **1** was determined as **quinolin-4-ol** (Fig.5).

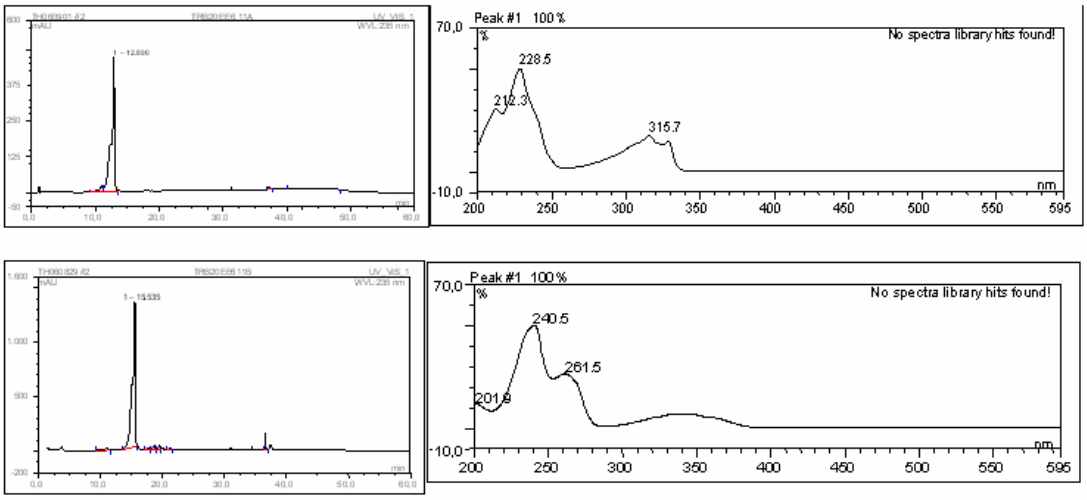

Figure 3. Chemical profiles of **1** (upper) and **2** (lower) in DAD HPLC

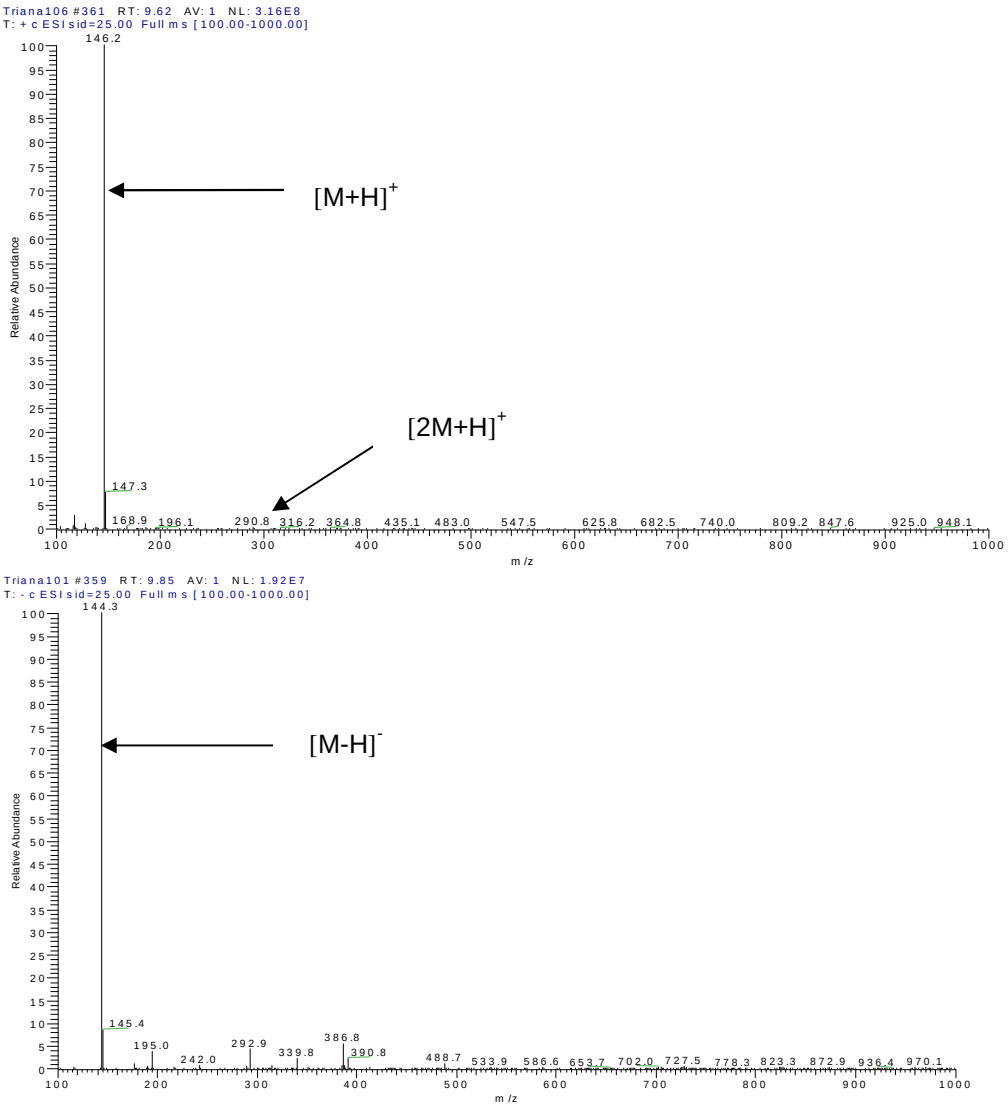

Figure 4a. ESIMS profiles of compounds 1 .

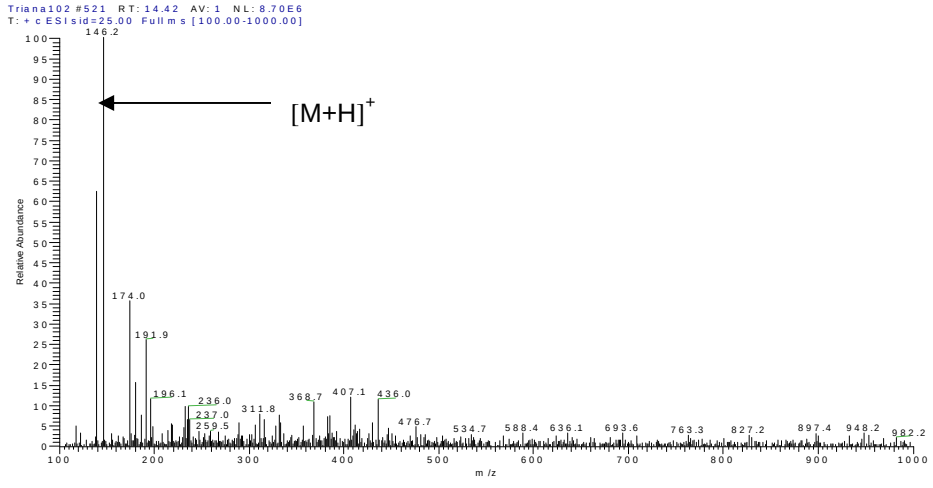

Figure 4b. ESIMS profiles of compounds 2 .

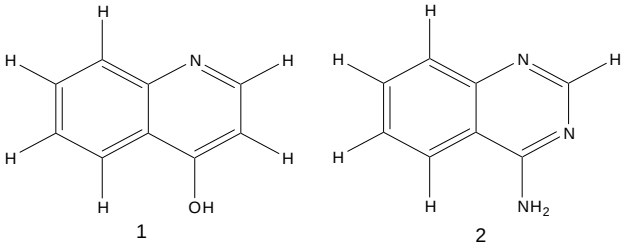

Figure 5. Quinolin-4-ol (**1**, left) and quninazolin-4-amine (**2**, right)

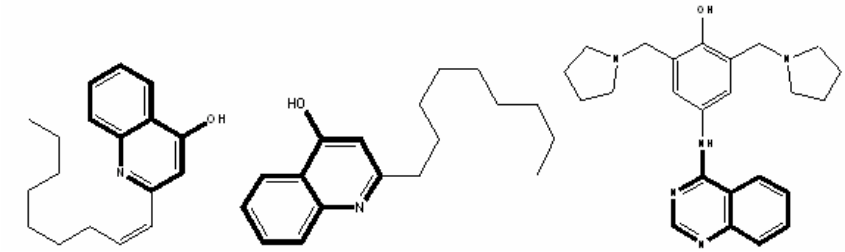

Figure 6. 4-hydroxy-2-(1-nonenyl)quinoline (left), 4-hydroxy-2-nonylquinolin (middle) (Debitus *et al*., 1998) and changrolin (right) (Li *et al*., 1979)

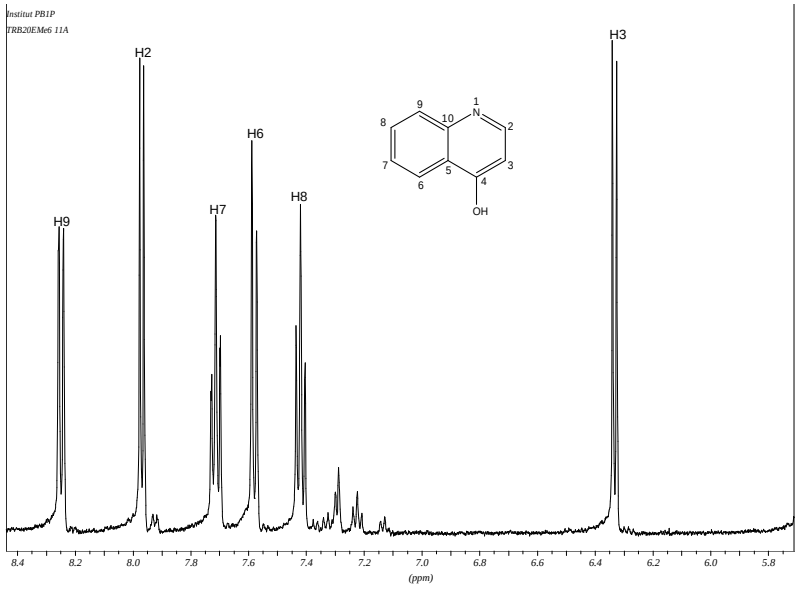

Figure 7. ^{1}H-NMR spectrum of **1** in MeOD (500 MHz)

A similarity in UV absorption pattern of both compounds suggested a related core structure for both compounds. ^{1}H-NMR spectrum of **2** in DMSO-d_6 showed a presence of 6 peaks integrated for 7 protons. ^{1}H–^{1}H COSY experiment revealed only one spin system. It coupled the protons of an ABCD ring system i.e., δ_H 8.28 (1H, d, J = 7.8 Hz, H-9) to the proton at δ_H 7.81 (2H, dd, J = 7.0 Hz, 7.8 Hz, H-8) to the proton at δ_H 7.31 (1H, dd, J = 7.2 Hz, 7.0 Hz, H-7) and at δ_H 7.76 (1H, d, J = 7.2 Hz, H-6). This finding also suggested an *ortho*

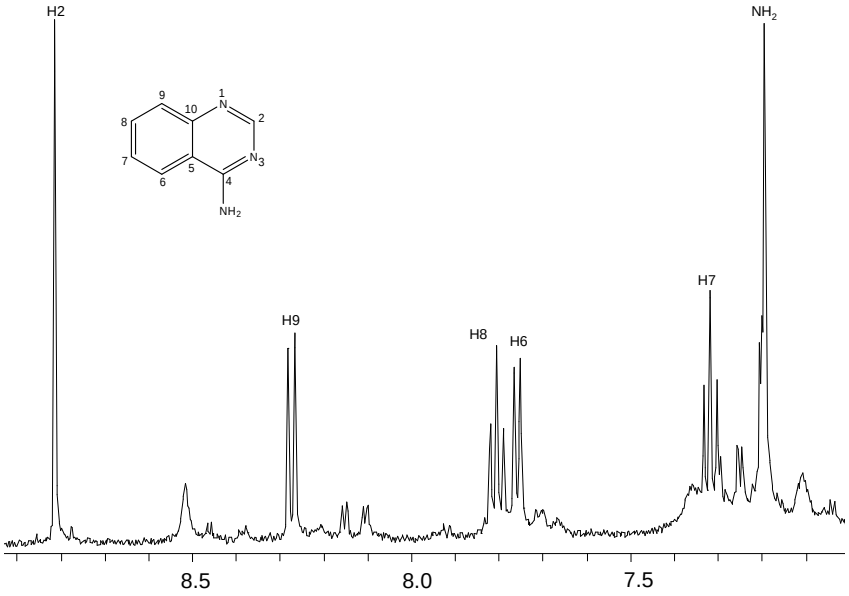

Figure 8. ^{1}H-NMR spectrum of **2** in DMSO-d_6 (500 MHz)

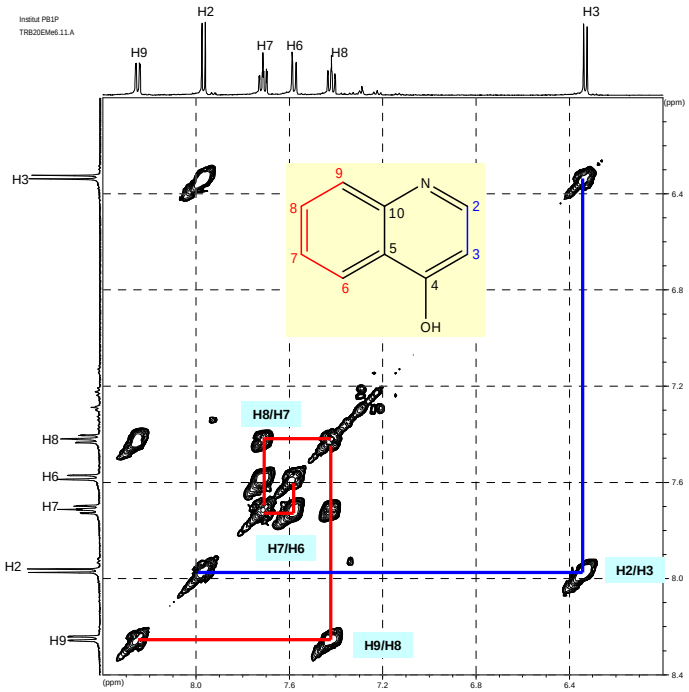

Figure 9. ^{1}H-^{1}H COSY spectrum of **1** in MeOD (500 MHz)

disubstituted benzene ring as found in **1**. Deshielded effect experienced by H-9 suggested presence of an electron withdrawing substituent at an *ortho* position. Detection of an aromatic NH_2 signal at δ_H 7.18 and a deshielded singlet at δ_H 8.82 suggested that the second ring might be a pyrimidine-4-amine. It explained the chemical shift difference of the

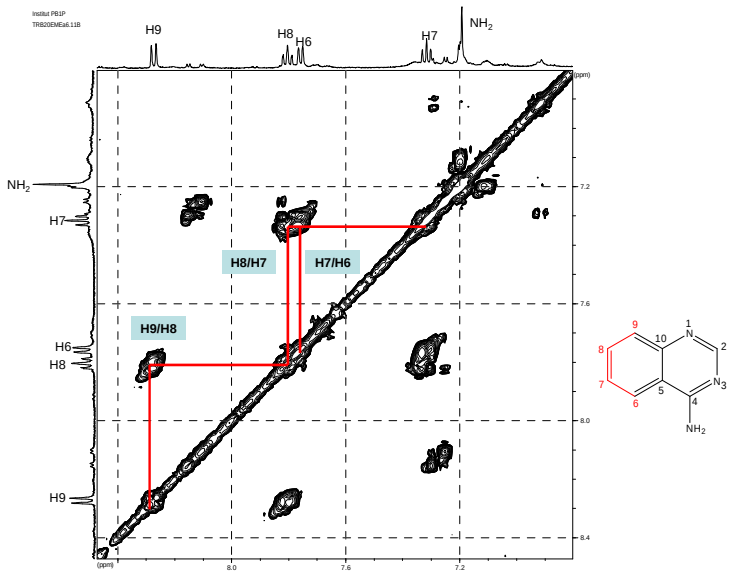

Figure 10. ^{1}H-^{1}H COSY spectrum of **2** in DMSO-d_6 (500 MHz)

Table I. ^{1}H-NMR data of compound **1** [a] and **2** [b]

No	Compound 1		Compound 2	
	δ	Integration, multiplicity, *J* in Hz	δ	Integration, multiplicity, *J* in Hz
2	7.98	1H, d, 7.2	8.81	1H, s
3	6.33	1H, d, 7.2	-	-
6	7.59	1H, d, 8.2	7.75	1H, d, 7.2
7	7.73	1H, dd, 8.2, 7.3	7.31	1H, dd, 7.2, 6.9
8	7.42	1H, dd, 7.6, 7.9	7.81	1H, dd, 8.2, 7.0
9	8.25	1H, d, 8.2	8.35	1H, d, 7.9
NH_2	-	-	7.19	2H, b s

[a] Data were recorded in MeOD, at 500 MHz; [b] Data were recorded in DMSO-d_6, at 500 MHz

ortho disubstituted benzene ring protons of **2** in comparison to **1** (Table I). Like for **1**, ^{13}C-NMR spectra could not be measured for **2** due to the limited amounts isolated. Based on the NMR and ESIMS data, compound **2** was determined as quinazolin-4-amine (Fig.5).

To the best of our knowledge, neither quinolin-4-ol (**1**) nor quinazolin-4-amine (**2**) has been reported from natural sources. Further analyses such as ^{13}C-NMR and high resolution mass spectrometry methods are recommended to ensure the structures of both compounds. Structurally closely related compounds such as 4-hydroxy-2-(1-nonenyl)quinoline or 4-hydroxy-2-nonylquinolin (Fig.6) are known as metabolites produced by a bacterium isolated from a marine sponge *Suberea creba* (Debitus *et al.*, 1998) whereas another compound structurally related to **2** that had been named changrolin was reported as constituent of a Chinese plant, *Dichroa febrifuga.* Changrolin has potent anti-arrhythmic activity (Li *et al.*, 1979)

Result of the cytotoxicity assay indicated that both compounds were inactive when tested against mouse lymphoma cell line L5178Y.

Conclusion

Two minor alkaloids, quinolin-4-ol (**1**) and quinazolin-4-amine (**2**) were isolated from extract of an Indonesian marine sponge *Rhabdastrella rowi*. Both alkaloids have not been reported before from natural sources. The fact that structurally closely related natural products are known from a sponge-associated bacterium and the very low yield of the alkaloids obtained

from *R. rowi* suggest that the compounds might likewise be metabolites of microorganisms that are harboured by the sponge.

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