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New Cytotoxic 1,2,4-Thiadiazole Alkaloids from the Ascidian *Polycarpa aurata*

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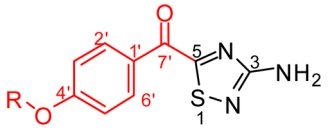
ABSTRACT



1 R = H
2 R = CH_3

Two new alkaloids, polycarpathiamines A and B (1 and 2), were isolated from the ascidian *Polycarpa aurata*. Their structures were unambiguously determined by 1D, 2D NMR, and HRESIMS measurements and further confirmed by comparison with a closely related analogue, 3-dimethylamino-5-benzoyl-1,2,4-thiadiazole (4), that was prepared by chemical synthesis. Compounds 1 and 2 both feature an uncommon 1,2,4-thiadiazole ring whose biosynthetic origin is proposed. Compound 1 showed significant cytotoxic activity against L5178Y murine lymphoma cells (IC_{50} 0.41 μM).

Marine invertebrates such as ascidians are prominent sources of a wide variety of natural products, many of them containing nitrogen atoms.[1]There is a high probability of discovering novel structures with unprecedented skeletons from these organisms as exemplified by ecteinascidin 743 (trabectedin), eudistomin C, dendrodoine, and lissoclinotoxin A.[2] Ascidians of the genus *Polycarpa* are known for producing various sulfur-containing alkaloids such as polycarpaurines A–C,[3] polycarpamines A–E,[4] the dimeric disulfide alkaloid polycarpine,[5] and derivatives[6] thereof. These compounds exhibited diverse biological activities, such as antifungal activity,[4] cytotoxic[3,6a,7] and antitumor activities,[7] inhibition of inosine monophosphate dehydrogenase,[5] inhibition of inflammatory cytokine production in lipopolysaccharide-stimulated RAW 264.7 cells,[8] and induction of apoptosis in JB6 cells through p53- and caspase 3-dependent pathways.[9] Thus, members

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(1) Blunt, J. W.; Copp, B. R.; Keyzers, R. A.; Munro, M. H. G.; Prinsep, M. R. *Nat. Prod. Rep.* **2013**, *30*, 237.
(2) (a) Rinehart, K. L.; Holt, T. G.; Fregeau, N. L.; Keifer, P. A.; Wilson, G. R.; Perun, T. J.; Sakai, R.; Thompson, A. G.; Stroh, J. G.; Shield, L. S.; Seigler, D. S.; Li, L. H.; Martin, D. G.; Grimmelikhuijzen, C. J. P.; Gäde, G. *J. Nat. Prod.* **1990**, *53*, 771. (b) Rinehart, K. L.; Kobayashi, J.; Harbour, G. C.; Hughes, R. G.; Mizsak, S. A.; Scahill, T. A. *J. Am. Chem. Soc.* **1984**, *106*, 1524. (c) Heitz, S.; Durgeat, M.; Guyot, M.; Brassy, C.; Bachet, B. *Tetrahedron Lett.* **1980**, *21*, 1457. (d) Litaudon, M.; Guyot, M. *Tetrahedron Lett.* **1991**, *32*, 911.
(3) Wang, W.; Oda, T.; Fujita, A.; Mangindaan, R. E. P.; Nakazawa, T.; Ukai, K.; Kobayashi, H.; Namikoshi, M. *Tetrahedron* **2007**, *63*, 409.
(4) Lindquist, N.; Fenical, W. *Tetrahedron Lett.* **1990**, *31*, 2389.
(5) Abbas, S. A.; Hossain, M. B.; van der Helm, D.; Schmitz, F. J.; Laney, M.; Cabuslay, R.; Schatzman, R. C. *J. Org. Chem.* **1996**, *61*, 2709.
(6) (a) Kang, H.; Fenical, W. *Tetrahedron Lett.* **1996**, *37*, 2369. (b) Wessels, M.; König, G. M.; Wright, A. D. *J. Nat. Prod.* **2001**, *64*, 1556.
(7) Popov, A. M.; Novikov, V. L.; Radchenko, O. S.; Elyakov, G. B. *Dokl. Biochem. Biophys.* **2002**, *385*, 213.
(8) Oda, T.; Lee, J.-S.; Sato, Y.; Kabe, Y.; Sakamoto, S.; Handa, H.; Mangindaan, R. E. P.; Namikoshi, M. *Mar. Drugs* **2009**, *7*, 589.
(9) Fedorov, S. N.; Bode, A. M.; Stonik, V. A.; Gorshkova, I. A.; Schmid, P. C.; Radchenko, O. S.; Berdyshev, E. V.; Dong, Z. *Pharm. Res.* **2004**, *21*, 2307.

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of this genus are interesting sources of potentially new bioactive metabolites.

In our search for bioactive metabolites from marine organisms,[10] we observed that the ethanolic extract of the ascidian *Polycarpa aurata*, collected in Indonesia, completely inhibited the growth of the murine lymphoma cell line L5178Y at a concentration of 10 μg/mL. Chromatographic separation afforded two new alkaloids (**1**–**2**) (Figure 1), together with four known compounds, which were identified as polycarpaurine C (**3**),[3] *N*,*N*-didesmethylgrossularine-1,[5] 4-methoxybenzoic acid,[6b] and *N*-methyl-2-(4-methoxyphenyl)-2-oxoacetamide[6b] based on their spectral data and on comparison with the literature. Herein, we describe the structure elucidation of the new compounds **1** and **2** and cytotoxicity of the isolated compounds.

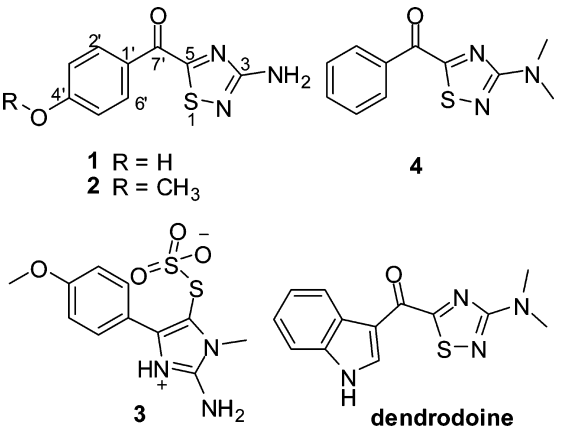


Figure 1. Structures of compounds **1**–**4** and dendrodoine.

Compound **1** was obtained as a dark yellow amorphous solid with a faint sulfur odor. The HRESIMS of **1** showed pseudomolecular ion peaks at m/z 222.0331 $[M + H]^+$, 244.0148 $[M + Na]^+$, and 465.0406 $[2M + Na]^+$ indicating the molecular formula $C_9H_7N_3O_2S$, bearing 8 double bond equivalents (8 DB). The ^{1}H NMR spectrum (DMSO-d_6) exhibited signals at δ_H 8.37 (2H, d, J = 8.8 Hz, H-2′/6′), and 6.94 (2H, d, J = 8.8 Hz, H-3′/5′), suggesting a typical AA′BB′ spin system of a *para* substituted benzene ring, and further signals at δ_H 10.79 (1H, br.s) for a hydroxy group and at δ_H 7.08 (2H, s) for an NH_2 group. The ^{13}C NMR data for C-1′–C-7′ (DMSO-d_6, Table 1) together with the HMBC correlations from H-2′/6′ to C-4′ (δ_C 163.8), C-3′/5′ (δ_C 115.6), C-6′/2′ (δ_C 133.6), C-1′ (δ_C 125.1), and C-7′ (δ_C 180.6), and from H-3′/5′ to C-1′, C-4′, and C-5′/3′, revealed the presence of a 4-hydroxybenzoyl moiety, which is consistent with the observation of a base peak at m/z 121 ($C_7H_5O_2^+$) in EI-MS. Apart from this unit, two carbon atoms (δ_C 185.7, 171.7), one NH_2 group (δ_H 7.08), as well as one sulfur, and two nitrogen atoms remained to be assigned according to the molecular formula. Since the benzoyl group accounts for 5 DB, the remaining 3DB have to originate from the $-C_2N_2S-NH_2$ moiety. This moiety (C_2N_2S) has to be cyclic, as otherwise a linear substructure with an alkyne group or cumulative double bonds must be formulated which is not supported by the carbon-13 chemical shifts. Theoretically, there are four possible heterocyclic ring structures for the $-C_2N_2S-$ moiety of **1** (h-1–h-4, Figure 2). A thorough literature search for the possible ring structures was carried out, and the chemical shifts for the carbons in these heterocyclic rings were either extracted based on reported values, or assigned by NMR measurements for those compounds that were commercially available (see Table S1 in the Supporting Information). The 1,2,4-thiadiazole substructure (h-3) was found to be the only possible heterocyclic ring in which one carbon resonates at around 170 ppm, and the second carbon at 180–190 ppm, which is very similar to the chemical shifts obtained for **1**. However, it was difficult to assign the positions of the NH_2 and benzoyl groups in this heterocyclic ring structure, since the sulfur bearing carbon (C_5) generally resonates at 180–190 ppm regardless of the substitution (Table S1). Thus, two possible structures (i.e., **1a** and **1b**) remained (Figure 2). We strongly favored **1a**, a guanidine containing molecule, whose biogenetic origin could be easily explained since this substructure is also present in polycarpaurine C (**3**), and *N*,*N*-didesmethylgrossularine-1 that were likewise isolated from *P. aurata*. Moreover, an indole alkaloid, dendrodoine, featuring a similar 3-dimethylamino-1,2,4-thiadiazole moiety had previously been reported to be from the ascidian *Dendrodoa grossularia*.[2c] A comparison of the ^{13}C NMR data of **1** with those of dendrodoine[2c,11] revealed close similarities in the 1,2,4-thiadiazole unit if one assumes that the original assignments for C-5 and the carbonyl group of dendrodoine are wrong and should be interchanged.[12] A ^{1}H–^{15}N HMBC measurement of **1** was performed to distinguish between the two possible structures **1a** and **1b** (Figure 2). The observed two three-bond correlations from the NH_2 group to two nitrogen atoms (δ_N 171.3, 202.2) clearly indicate **1a** to be the correct structure for compound **1**.

In order to further confirm the existence of this unusual heterocyclic ring structure in **1**, we synthesized an analogous structure, 3-dimethylamino-5-benzoyl-1,2,4-thiadiazole (**4**), which closely resembles **1** and dendrodoine, following a method reported in the literature.[11] The analogue was synthesized according to a two-step reaction: (1) the formation of 5-dimethylamino-1,3,4-oxathiazol-2-one, which was used to produce *N*,*N*-dimethylaminonitrile sulfide in the following step via thermolysis; (2) a 1,3-dipolar

(10) (a) Pham, C.-D.; Hartmann, R.; Müller, W. E. G.; de Voogd, N.; Lai, D.; Proksch, P. *J. Nat. Prod.* **2013**, *76*, 103. (b) Niemann, H.; Lin, W.; Müller, W. E. G.; Kubbutat, M.; Lai, D.; Proksch, P. *J. Nat. Prod.* **2013**, *76*, 121. (c) Lai, D.; Liu, D.; Deng, Z.; van Ofwegen, L.; Proksch, P.; Lin, W. *J. Nat. Prod.* **2012**, *75*, 1595.

(11) Hogan, I. T.; Sainsbury, M. *Tetrahedron* **1984**, *40*, 681.

(12) The original assignments (see ref 11) for C-5 at δ_C 175.7 and the carbonyl conjugated with an indole group at δ_C 187.8 were not consistent with the reported 1,2,4-thiadiazole derivatives (usually C-5 at 180–190 ppm; see Table S1), and the indole-3-carbonyl containing derivatives (e.g., Rhopaladin D, $\delta_{C=O}$ 177.4 (Sato, H.; Tsuda, M.; Watanabe, K.; Kobayashi, J. *Tetrahedron* **1998**, *54*, 8687), and such a carbonyl usually resonates at lower than 180 ppm).

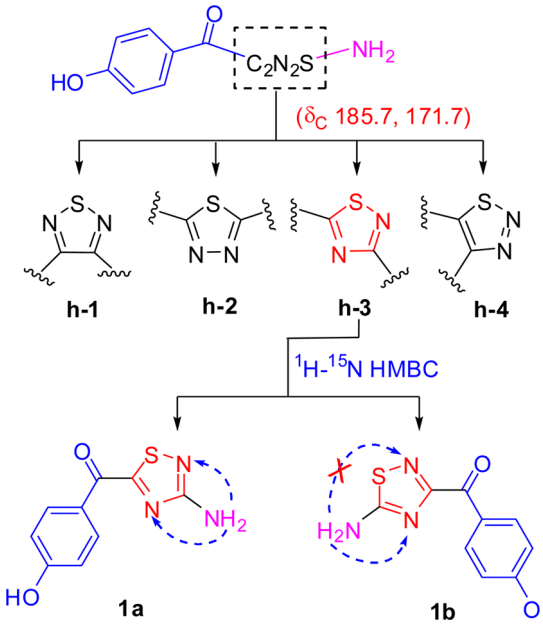


Figure 2. Assignment of the heterocyclic unit in **1** by analysis of the ^{13}C NMR chemical shifts and $^1H-^{15}N$ HMBC correlations.

cycloaddition between the nitrile sulfide generated *in situ* and benzoylcyanide (Scheme 1). This synthetic analogue (**4**) has two *N*-methyl groups, which are helpful in assigning the chemical shifts of the thiadiazole unit. The HMBC correlations from both *N*-methyl groups to C-3 (δ_C 172.5) and from H-2′/6′ to C-7′ (δ_C 182.9) allow the unambiguous assignments for C-3 and C-7′; thus the signal at δ_C 184.9 can only be assigned to C-5, which supports our assumption that the previously reported assignment for C-5 of dendrodoine was wrong. The strong similarity between the carbon chemical shifts of **1** and the synthetic analogue (**4**) (Table 1) in the thiadiazole unit independently supports the proposed structure for **1**. Therefore, compound **1** was determined as the new 3-amino-5-(4-hydroxybenzoyl)-1,2,4-thiadiazole and named polycarpathiamine A.

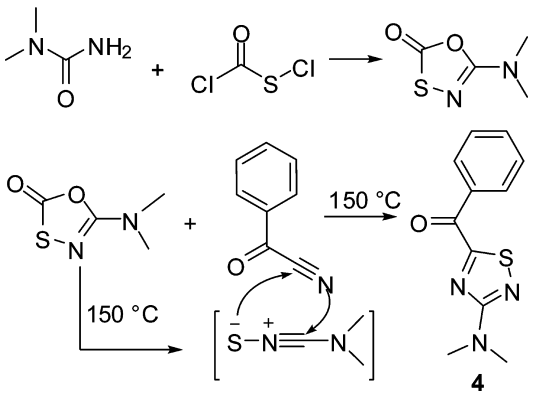
Table 1. ^{13}C NMR Data of **1**-**2**, and **4**

position	**1**[a]	**1**[b]	**2**[c]	**4**[d]
3	171.7 C	172.7 C	171.8 C	172.5 C
5	185.7 C	187.6 C	185.4 C	184.9 C
1′	125.1 C	127.2 C	126.6 C	134.0 C
2′ /6′	133.6 CH	134.7 CH	133.3 CH	130.6 CH
3′ /5′	115.6 CH	116.5 CH	114.3 CH	128.9 CH
4′	163.8 C	164.5 C	164.6 C	134.7 CH
7′	180.6 C	181.7 C	180.9 C	182.9 C
4′-OMe			55.8 CH_3	
3-NMe_2				38.6 CH_3

[a] Recorded at 100 MHz in DMSO-d_6. [b] Recorded at 150 MHz in acetone-d_6. [c] Recorded at 75 MHz in DMSO-d_6. [d] Recorded at 150 MHz in DMSO-d_6.

Compound **2** was obtained as a dark yellow amorphous solid with a slight sulfur odor similar to compound **1**. HRESIMS of **2** showed signals at *m*/*z* 236.0488 $[M + H]^+$ and 258.0307 $[M + Na]^+$, indicating the molecular formula $C_{10}H_9N_3O_2S$, which differs from that of **1** by a methyl substituent. The NMR data of **2** closely resemble those of **1**, except for a methoxy group (δ_H 3.90, δ_C 55.8) in **2** instead of a hydroxyl substituent in **1**. The methoxy group is located at C-4′, since it shows an HMBC correlation to C-4′. Thus, compound **2** was identified as 3-amino-5-(4-methoxybenzoyl)-1,2,4-thiadiazole and named polycarpathiamine B.

Scheme 1. Synthesis of 3-Dimethylamino-5-benzoyl-1,2,4-thiadiazole (**4**)

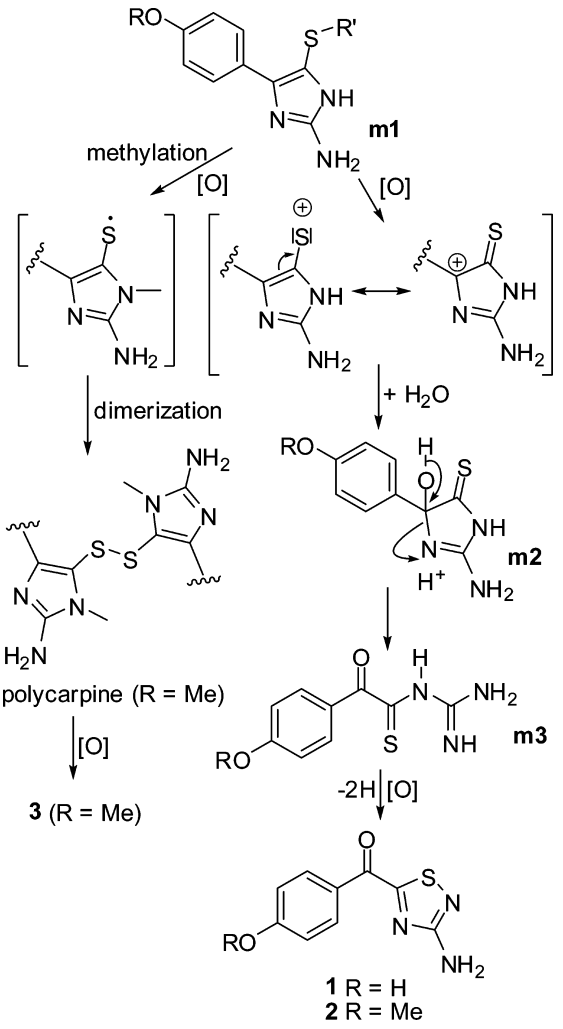


1,2,4-Thiadiazole alkaloids are rarely encountered in natural products. So far, only three natural products with this structural unit have been reported, including dendrodoine from the ascidian *Dendrodoa grossularia*,[2c] and a pair of enantiomers of an indole alkaloid from the root of *Isatis indigotica* in a recent paper.[13] The present study reports two new members of this class. It is worth mentioning that compounds **1** and **2** are the second and third example of 3-amino substituted 1,2,4-thiadiazole alkaloids from nature. Thus, their biosynthetic pathway should be different from that of the 1,2,4-thiadiazole alkaloid without a 3-amino substitution.[13] A plausible biosynthetic pathway for **1** and **2** is proposed (Scheme 2).

In light of the frequent occurrence of the dimeric disulfide alkaloid, polycarpine, in several ascidians from the genus *Polycarpa*,[3,5,6a] though not in the present study, we hypothesize a monomeric precursor (**m1**) being present in *P. aurata*, which would undergo methylation and oxidation to form a S-radical intermediate. A dimerization of this intermediate could afford polycarpine, in which the cleavage of the S−S or C−S bond accompanied by oxidation would give polycarpaurine B[3] or C (**3**), respectively. Further oxidation of **m1** and subsequent nucleophilic attack of water could produce an intermediate (**m2**), as similar derivatives with a 2-carbonyl group were already reported from ascidians.[5,6a] A follow-up ring-opening

(13) Chen, M.; Lin, S.; Li, L.; Zhu, C.; Wang, X.; Wang, Y.; Jiang, B.; Wang, S.; Li, Y.; Jiang, J.; Shi, J. *Org. Lett.* **2012**, *14*, 5668.

Scheme 2. Plausible Biosynthetic Pathway for **1** and **2**



reaction of **m2** would give the guanidine amide of 2-aryl-2-oxothioacetic acid (**m3**). A similar compound *N*-methyl-(4-methoxyphenyl)-2-oxothioacetamide was isolated from *P. aurata*.[5] Oxidative ring closure of **m3** would give rise to compounds **1** and **2**. Even though dendrodoine was isolated from an unrelated ascidian,[2c] it seems plausible that a similar biosynthetic pathway was also adopted here by using a precursor that features an indole group instead of the benzene group in **m1**, as suggested by the occurrence of several indole analogues[14] that resemble those hypothetical intermediates shown in Scheme 2.

All isolated compounds were examined for their effects on the growth of L5178Y mouse lymphoma cells *in vitro* using the MTT assay.[10a,15] Compound **1** and *N*,*N*-didesmethylgrossularine-1 inhibited the growth of L5178Y cells with IC_{50} values (μM) of 0.41 and 1.86, respectively, while the other compounds were not active ($IC_{50} > 10\ \mu$M). Compound **1** showed potent inhibitory activity in a submicromolar concentration, which is approximately 10-fold more active than the positive control kahalalide F (IC_{50} 4.3 μM). Apparently, the hydroxyl functional group at C-4′ of compound **1** should be unsubstituted in order to retain bioactivity since compound **2** was inactive. The unique structure and notable cytotoxicity of **1** make it an interesting starting point for cytotoxic drug development. Further SAR and biological investigations are ongoing.

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Supporting Information Available. Experimental procedures, ascidian material, extraction and isolation, characterization data of **1**–**2**, synthetic procedures and spectral data of **4**, cytotoxic assay, and copies of 1D and 2D NMR and HRMS spectra of **1**, **2**, and **4**. This material is available free of charge via the Internet at http://pubs.acs.org.

(14) Mancini, I.; Guella, G.; Pietra, F.; Debitus, C.; Duhet, D. *Helv. Chim. Acta* **1994**, *77*, 1886.

(15) Ashour, M.; Edrada, R.; Ebel, R.; Wray, V.; Watjen, W.; Padmakumar, K.; Müller, W. E. G.; Lin, W. H.; Proksch, P. *J. Nat. Prod.* **2006**, *69*, 1547.

The authors declare no competing financial interest.