Dotofide, a Guanidine-Interrupted Terpenoid from the Marine Slug *Doto pinnatifida* (Gastropoda, Nudibranchia)

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An unusual natural product (dotofide, **1**), in which the terpenoid skeleton is interrupted by a guanidine moiety was obtained from the marine slug *Doto pinnatifida*. The absolute configuration of compound **1** was deduced by ozonolysis and subsequent CD spectroscopy. *D. pinnatifida* subsists on the hydrozoan *Nemertesia antennina* (Cnidaria), which however does not contain dotofide, suggesting that the snail is able to perform the biosynthesis of the metabolite by itself. *D. pinnatifida* is grouped into the chemically hardly investigated taxon Cladobranchia (Opisthobranchia, Gastropoda). All members of this taxon completely lack a shell and are thought to employ toxic secondary metabolites or alternatively cnidocysts sequestered from their cnidarian prey organism as defence strategy.

Introduction

Marine snails without a protective shell are vulnerable soft-bodied animals and often employ toxic chemicals taken up from their food or synthesized by the animal itself as chemical defence. This sequestration of secondary metabolites from the prey, or the de novo synthesis is considered as a major driving force for speciation within Opisthobranchia.[1,2] Investigation of defence strategies within marine Opisthobranchia will thus aid to understand the evolution of this group of animals.

In marine gastropods, reduction or a complete loss of the shell is realized in the Opisthobranchia, potentially facilitating the occupation of new ecological niches and therefore supporting speciation within this group.[3] However, absence of a protective shell necessitates alternative defensive mechanisms. The most important defence strategies which ensure protection against predators involve cryptic appearance by mimicking colouration and shape of the food organism (see, for example, ref.[4]), formation of spicules in epidermal tissue,[5] uptake and use of nematocysts (stinging cells) from cnidarian food sources (found in Aeolidoidea),[6,7] and the uptake or synthesis of biochemicals encountered in many opisthobranch species (see, for example, ref.[8–11]).

Cladobranchia are an opisthobranch clade, which mostly feed on cnidarians, i.e. Anthozoa (hexa- and octocorals) and hydrozoans, whereas some members consume bryozoans. There is a tremendous wealth of information on the presence of secondary metabolites in opisthobranch groups closely related to the Cladobranchia, the so-called Anthobranchia.[1,10,11] In contrast, the natural product chemistry and ecology of the Cladobranchia has hardly been investigated (see review in ref.[7]).

Members of the cladobranch family Dotidae have been reported to exclusively feed on hydrozoans.[12] With nearly 70 described species, this extremely species-rich family with the only genus *Doto* Oken 1815 appears to be evolutionary very successful. *Doto* is characterized by the presence of one row of dorsal cerata, which very often are tuberculate (Figure 1). Underneath these tubercles, glandular cells are located.[13–15] Many of the species can only be distinguished by the shape and colour of the marks on the body and

Figure 1. *Doto pinnatifida* individual on its prey, the hydrozoan *Nemertesia antennina*.

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cerata. Defensive strategies within this genus do neither include the formation of spicules in epidermal tissue nor the uptake and use of nematocysts from cnidarian food sources.[16] Despite the small size of these animals (usually around 5 to 10 mm), it seems unlikely that Dotidae exclusively rely on cryptic appearance as a single defence strategy. It therefore appears plausible that species of this family protect themselves by predator-deterrent chemicals. However, the natural product chemistry has not been described for any member of this cladobranch family.

Results and Discussion

Chemical Investigation of *Doto pinnatifida*

D. pinnatifida (64 individual animals) collected in Spanish waters was extracted with methanol and the extract separated by vacuum liquid chromatography (VLC). The polar fractions contained compounds such as the known metabolites homarine, trigonelline, and purine derivatives such as adenine. The novel terpenoid **1** (Figure 2) was present in a non-polar VLC fraction eluting with 100% methanol from RP-18 material. After further purification on Sephadex LH-20 stationary phase, 2.1 mg of the pure compound were obtained as yellowish oil. On average each animal thus contains 0.033 mg of **1**.
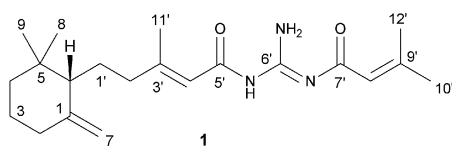
Figure 2. Structure of the unprecedented terpenoid **1** isolated from *Doto pinnatifida*.

The molecular formula of compound **1** was deduced by accurate mass measurement to be $C_{21}H_{33}N_3O_2$ and showed it to have seven elements of unsaturation. A sharp IR absorption at 3334 cm^{-1} evidenced the presence of an amino group and additional absorptions at 1620 cm^{-1} and 1689 cm^{-1} were indicative for carbonyl functions. The ^{13}C NMR spectroscopic data contained a total of 21 resonances attributable to five methyl, six methylene, and three methine groups, as well as seven quaternary carbons, as indicated by a dept 135 spectrum. These data also revealed the presence of six double bonds (3× C=C, one of which is an *exo*-methylene group, 1× C=N; 2× C=O); **1** was thus monocyclic. After assignment of all protons to their directly bonded carbon atoms via a HSQC measurement, it was possible to deduce minor fragments of the molecule from the results of a ^1H-^1H COSY measurement. Thus, analysis of the COSY spectrum of **1** gave evidence for connectivities from CH_2-2 to CH_2-4 and from CH-6 to CH_2-2′, respectively. A ^1H-^{13}C HMBC measurement permitted the planar structure of **1** to be further elaborated. Correlations from 8-H_3 and 9-H_3 to C-4, C-5 and C-6 showed that methyl groups CH_3-8 and CH_3-9 both are attached to C-5 and connected CH_2-4 to

CH-6 via C-5. HMBC correlations between 7-H_2 and C-1, C-2 and C-6 established *exo*-methylene group $\Delta^{1,7}$ and connected CH_2-2 to CH-6 via C-1, thus completing a cyclohexane ring, respectively. 11′-H_3 had correlations to C-2′, C-3′ and C4′ establishing the connectivities between CH_3-11′ and C-3′ and between $\Delta^{3',4'}$ and CH_2-2′. The linkage between carbonyl atom C-5′ and C-4′ was proven by a correlation between 4′-H and C-5′. Finally, a $(CH_3)_2C=CH$–CO-fragment could be delineated by correlations between both 10′-H_3 and 12′-H_3 and C-7′, C-8′ and C-9′, and 8′-H and both C-7′ and C-9′. Still remaining to be assigned were three nitrogen, one carbon and three hydrogen atoms. The characteristic ^{13}C NMR chemical shift of C-6′ ($\delta_C = 160.8$)[17] evidenced the presence of a guanidine moiety which was placed between C-5′ and C-7′ due to the ^{13}C NMR chemical shifts ($\delta_C = 175.8$ and $\delta_C = 175.5$, respectively) of these carbon atoms, thus completing the planar structure of compound **1**.

The geometry of the double bond $\Delta^{3',4'}$ was assigned on the basis of a correlation in the NOESY spectrum between 2′-H_2 and 4′-H to be *E*. To establish the absolute configuration of **1** an ozonolysis was conducted resulting in diketone **2** (Figure 3). The absolute configuration of **2** was determined to be *S* by comparing the CD spectrum of **2** (positive Cotton effect at 292 nm; see Supporting Information Figure S9) with literature data,[18] also indicating the absolute configuration of **1** to be *S*. We propose the trivial name dotofide for compound **1**.
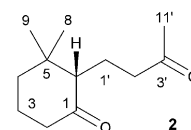
Figure 3. Structure of ozonolysis product **2**.

Chemical Investigation of *Nemertesia antennina*

The food source of *D. pinnatifida*, the hydrozoan *N. antennina* was extracted in the same way. The polar fractions obtained by VLC contained compounds such as the known metabolites homarine and trigonelline as found in *D. pinnatifida*. However, the new terpenoid **1** isolated from *D. pinnatifida* was not detectable via NMR- and LC-MS spectroscopical analyses in the crude extract or in any of the VLC fractions.

Discussion

Cladobranchia are marine slugs which have completely lost their protective shell during evolution, suggesting that they developed other strategies of defence. Only a very few members of the Cladobranchia, particularly specimens of those families which forage on octocorals, have been studied in detail concerning their secondary metabolites (e.g., Tritoniidae, Tethydidae, Facelinidae). A recent review by Putz et al.[7] summarizes all results on these few groups that usu-

ally sequester compounds from their prey. However, members of the cladobranch family Tethydidae have been shown to produce deterrent compounds de novo, i.e. *Melibe leonina* was reported to acquire an odoriferous substance, 2,6-dimethyl-5-heptenal via de novo biosynthesis.[19,20] Chemical studies of the Mediterranean *Tethys fimbria* (Tethydidae) led to the characterization of many prostaglandin-1,15-lactones which also have been shown to be produced de novo.[21–24]

During the current study, aiming to decipher defence strategies within Cladobranchia and to understand the evolutionary success (i.e. radiation) of subgroups within this taxon, the unprecedented terpenoid **1** was obtained from the Mediterranean slug *D. pinnatifida*, belonging to the hitherto not studied family Dotidae. The structure of dotofide (**1**) has a novel architecture, consisting of a sesquiterpenoid part including a substituted hexane ring similar to the one present in carotenoids (i.e. β- and α-ionon ring) and a hemiterpene unit, with both parts being interrupted by a guanidine moiety.

No traces of the new *D. pinnatifida*-derived terpenoid **1** were detectable in the prey of this marine slug, *Nemertesia antennina*. The latter, a member of the hydrozoan family Aglaopheniidae is reliably reported as the exclusive food source of *D. pinnatifida*. Likewise, the slugs investigated here were collected directly from this hydrozoan. Aglaopheniidae are very well protected by large nematocysts (stinging cells) which render them rather painful even for human skin. Nevertheless, *D. pinnatifida* is able to consume the polyps of *N. antennina* without any adverse reaction. But in contrast to many other cladobranch relatives, which sequester these stinging cells and use them for their defence,[6,16] *D. pinnatifida* is not able to do so,[13,16] probably because the animal relies on chemical defence.

Distantly related compounds are known from dorid nudibranchs (Anthobranchia), some of which display feeding deterrent or ichthyotoxic properties. Thus, a structure showing similarities with respect to the terpene part of compound **1** is the monocyclofarnesic acid glyceride **3** which was isolated from the dorid nudibranch *Archidoris montereyensis* (Anthobranchia, Doridina) (Figure 4).[25] Several dorid nudibranchs, amongst others *A. montereyensis*, have been shown to be capable of de novo biosynthesis of terpenoic acid glycerides.[25,26] Various structurally similar terpenoid metabolites, e.g. **4** and **5** (Figure 4), known from green alga of the genus *Caulerpa*[27] are assumed to be sequestered by the sacoglossan *Tridachia crispata* as defensive agents.[28] Other compounds resemble the new compound **1** with respect to the guanidine moiety. Triophamine **6** (Figure 5) which contains a rare diacylguanidine functionality was derived from the bryozoan eating dorid *Triopha catalinae* (Anthobranchia, Doridina).[29,30] Graziani and Andersen[31] could show that the acyl residues of triophamine (**6**) are formed de novo from five intact acetate units. Unfortunately, feeding deterrency or toxicity of the compound has not been tested. The structurally similar compound limaciamine **7** (Figure 5) was later found in a related species, *Limacia clavigera* (Doridina). Due to its structural similar-

ity to triophamine **6**, the authors concluded that limaciamine **7** likewise is produced de novo by *L. clavigera*.[32]
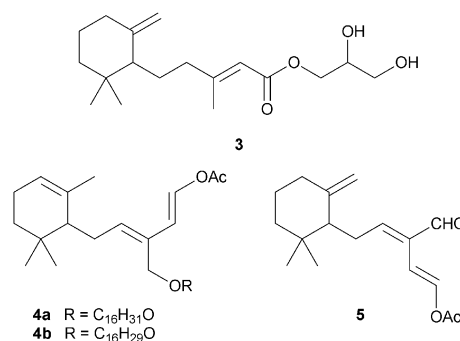

Figure 4. Structure of monocyclofarnesic acid glyceride **3** obtained from the dorid nudibranch *Archidoris montereyensi*, and terpenoid metabolites **4** and **5** known from green alga of the genus *Caulerpa*.[25–28]
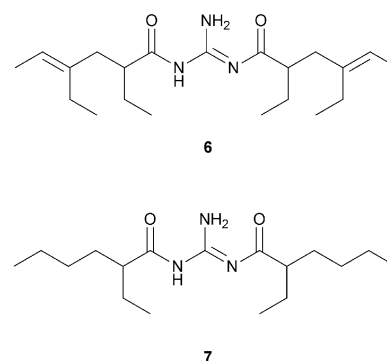

Figure 5. Structure of triophamine **6** and limaciamine **7** derived from the dorid nudibranchs *Triopha catalinae* and *Limacia clavigera*, respectively.[29–32]

Conclusions

The new terpenoid dotofide (**1**) was not detectable in the slug's food source *N. antennina*. Based on the structural similarity of the new terpenoid to compounds known from animals of the sea slug clade Doridina, which have been proven to synthesize toxic compounds de novo (e.g.[31]), it is tempting to speculate that *D. pinnatifida* produces the new terpenoid de novo as well. Other examples also illustrate that nudibranchs have well developed biosynthetic capabilities for generating terpenoids.[9,26] The lack of adequate compounds for sequestration in the prey *N. antennina* could potentially have forced the slug to produce toxic compounds itself for protection against predators. Dotidae, however are not closely related with Doridina, hence there is no evidence that the ability for de novo synthesis of these related structures may have evolved only once in a common stemline. Rather, the structural similarities of the compounds (see Figures 1, 4, and 5) may be due to constraints relating to the metabolic availability of precursors for biosynthesis. Thus, terpenoid units – in the case of **1** farnesyl- and dimethylallyl-pyrophosphate – as well as a guanidine

moiety may be available through the general terpenoid and amino acid (i.e. arginine) metabolism. It could also be envisaged that dotofide is produced by the oxidative cleavage of a precursor terpenoid, e.g. a carotenoid-type compound as known for retinal (Vit. A aldehyde). Further studies will address the biosynthetic origin and the ecological role of dotofide.

Experimental Section

General Experimental Procedures: Optical rotation was measured at room temperature in MeOH on a Jasco DIP 140 polarimeter. UV and IR spectra were obtained employing a Perkin–Elmer Spectrum BX instrument. CD spectra were recorded in CHCl$_3$ at room temperature using a JASCO J-810–150S spectropolarimeter. All NMR spectra were recorded in MeOD employing a Bruker Avance 300 DPX spectrometer. Spectra were referenced to residual solvent signals with resonances at $\delta_{H/C}$ 3.35/49.0. LC-ESIMS was performed using an Agilent 1100 system with an API 2000 Triple Quadrupole LC/MS/MS with ESI source (Applied Biosystems/MDS Sciex) and a DAD. HRESIMS were recorded on a Finnigan MAT 95 spectrometer.

Sample Collection: Samples of both *Doto pinnatifida* and its exclusive food source, the hydrozoan *Nemertesia antennina*, were sampled in Ferrol, Spain in April 2009. Specimen were collected via SCUBA diving and subsequently stored in 70% ethanol. Extraction and purification of the crude extract was performed in the laboratory in Bonn.

Extraction and Purification: The ethanol in which *Doto pinnatifida* samples were stored was evaporated and gave the first extract. The remaining material was extracted exhaustively in methanol overnight. Extraction was repeated twice with fresh methanol for 1 h

each on the following day. All together, 64 *D. pinnatifida* individuals equalling 1.13 g wet weight yielded 365 mg of extract. The *D. pinnatifida* extract was submitted to vacuum liquid chromatography (VLC; RP18 5×3 cm, water/methanol). The new terpenoid **1** was found in the non-polar fraction that was eluted with 100% methanol. This fraction was further purified by liquid chromatography on Sephadex LH-20 medium (column 1.5×11 cm) with 100% methanol as eluent to yield 2.1 mg of the pure terpenoid **1**.

The same extraction procedure was applied to the slug's food, *N. antennina*. The material (2.28 g wet weight) was extracted as described above for the *D. pinnatifida* samples. A total of 877 mg of extract was obtained. 432 mg of this extract was separated on VLC under the same conditions as for the *D. pinnatifida* extract.

Compound 1: Yellowish oil (2.1 mg; 0.19%). $[a]_D^{24} = -3.6$ ($c = 0.14$, MeOH). UV (MeOH): $\lambda_{max} = 241$ ($\varepsilon = 16609$), 276 nm ($\varepsilon = 25951$). IR (ATR) $\tilde{v}_{max} = 3334$, 2927, 2863, 1689, 1620, 1523, 1441, 1358, 1267, 1220, 1137, 1087 cm^{-1}. ^{1}H and ^{13}C NMR spectroscopic data see Table 1. LR-ESI-MS: *m/z* 358.3 (M – H)$^-$. HR-ESI-MS: calcd. for C$_{21}$H$_{34}$N$_3$O$_2$ [M + H]$^+$ *m/z* 360.2646, found 360.2659.

Compound 2: Colourless film (0.2 mg). CD (c = 0.2 mg/mL, CHCl$_3$): λ ($\Delta\varepsilon$) = 292 (+1.23). ^{1}H NMR (CDCl$_3$): δ = 2.12 (11'-H$_3$, s), 0.78 (9-H$_3$, s), 1.09 (8'-H$_3$, s).

Ozonolysis: Compound **1** (1.5 mg) was dissolved in 2 mL of CH$_3$OH and allowed to react with O$_3$ for 20 min at –78 °C. Dimethyl sulfide (5 drops) was then added and the solution was stirred for additional 45 min. The solvent was evaporated under reduced pressure to obtain 0.7 mg of a crude product which was purified by chromatography over a 3 mL silica solid-phase extraction column (J. T. Baker) yielding **2** as a colourless film (0.2 mg).

Supporting Information (see footnote on the first page of this article): Spectroscopic data and other relevant information are included for compounds **1** and **2**. This material is available on the WWW under http://www.eurjoc.org or from the author.

Table 1. NMR spectroscopic data obtained for the new terpenoid **1**.

	δ_C, mult.[a,b,e]	δ_H[a,b] (*J* in Hz)	COSY[a,c]	HMBC[a,d]	NOESY[a,c]
1	150.3, C				
2	33.3, CH$_2$	2.10, m	3, 4a, 4b, 7b	1, 3, 4, 7	
3	24.8, CH$_2$	1.59, m	2, 4a, 4b	1, 4, 5	
4	37.1, CH$_2$	a: 1.28, m	a: 2, 3, 4b, 6	a: 5, 6, 8	a: 4b, 8
		b: 1.58, m	b: 2, 3, 4a	b: 2, 3, 6, 8, 9	b: 4a, 9
5	35.8, C				
6	54.9, CH	1.80, dd (3.0, 11.4)	4a, 7a, 1', 2'	1, 4, 5, 7, 1', 2'	2, 8, 9
7	110.1, CH$_2$	a: 4.63, d (2.4)	a: 6, 7b	a: 1, 2, 6	a: 6, 7b, 11'
		b: 4.85, br. s	b: 2, 7a	b: 2, 6	b: 2, 7a,
8	28.8, CH$_3$	0.98, s		4, 5, 6, 9	
9	26.9, CH$_3$	0.91, s		4, 5, 6, 8	
1'	25.6, CH$_2$	a: 1.65, m	6, 2'	4, 5, 6, 2'	
		b: 1.72, m			
2'	40.9, CH$_2$	a: 1.99, m	6, 1'	3', 4', 11'	4'
		b: 2.17, m			
3'	161.1, C				
4'	121.8, CH	5.87, br. s	11'	2', 3', 5', 11'	2'
5'	175.8, C				
6'	160.8, C				
7'	175.5, C				
8'	121.4, CH	5.87, br. s	10', 12'	7', 10', 12'	10', 12' (w)
9'	157.1, C				
10'	27.7, CH$_3$	1.95, d (0.9)	8', 12'	7', 8', 9', 12'	8', 12'
11'	19.3, CH$_3$	2.21, d (1.0)	2', 4'	2', 3', 4', 5'	
12'	20.4, CH$_3$	2.23, d (1.0)	8', 10'	7', 8', 9', 10'	8' (w), 10'

[a] [D$_4$]MeOH, 300/75.5 MHz. [b] Assignments are based on extensive 1D and 2D NMR experiments (HMBC, HSQC, COSY). [c] Numbers refer to proton resonances. [d] Numbers refer to carbon resonances. [e] Implied multiplicities determined by DEPT.

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