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Lanesoic Acid: A Cytotoxic Zwitterion from *Theonella* sp.

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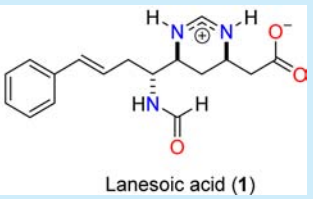
Supporting Information

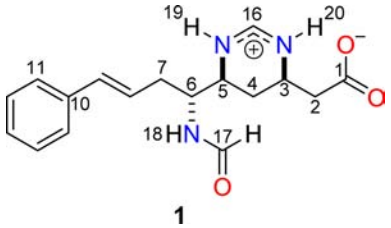
ABSTRACT: Lanesoic acid (**1**) was isolated and characterized from *Theonella* sp. during PharmaMar's ongoing program to study cytotoxic substances from marine sources. Its planar structure, elucidated by spectral analysis (NMR, IR, UV, and MS), possesses an unusual skeleton containing a tetrahydropyrimidine cation that is stabilized as a zwitterion by an internal carboxylate counterion. The stereostructure of **1** was deduced from ROESY-NOESY, *J*-based configurational analysis (JBCA), and density functional theory (DFT) computational calculations fitted using the recently published DP4+ parameter. Compound **1** was moderately active and selective against pancreas PSN1 cells (IC_{50} = 8.9 μg/mL) and inactive against colon HT-29, breast MD-MB-23, and NSCLC lung tumor cells.



Lanesoic acid (**1**)

Sponges of the genus *Theonella* are a rich and diverse source of secondary metabolites with interesting biological activities,[1] with *Theonella swinhoei* the most studied among them. Although most of them are peptides,[2] it is not unusual to find macrocycles,[3] alkaloids,[4] and steroids.[5] Recent studies indicate that this diversity could be due to symbiotic microorganisms.[6,7]

As part of the PharmaMar antitumor screening program of marine organisms, a sponge belonging to the *Theonella* genus collected at Lanes in Indo-Pacific[8] was selected due to the cytotoxic activity against human tumor cell lines shown by its organic extracts. The active CH_2Cl_2−CH_3OH extract was submitted to bioassay-guided fractionation by solvent partition RP-18 VLC chromatography followed by reversed-phase HPLC, affording three known compounds (microsclerodermin C,[9] cupolamide A,[10] and kumusine[11]/trachycladine A[12]) along with a new alkaloid containing an uncommon 1,4,5,6-tetrahydropyrimidine cation which is stabilized as a zwitterion by an internal carboxylate counterion.



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The molecular formula of compound **1** was established by HRESTOFMS as $C_{17}H_{21}N_3O_3$ based on the molecular ion parent at *m/z* 316.1657 $[M + H]^+$ (calcd for $C_{17}H_{22}N_3O_3$ *m/z* 316.1656). The analysis of ^1H and ^{13}C NMR spectral data in combination with HSQC data obtained in CD_3OD (Table 1) revealed the existence of two carbonyl groups, a carboxylic acid (δ_C 173.6) and a formamide (δ_C/δ_H 164.4/8.19) group, along with a methine (δ_C/δ_H 153.0/7.88), a monosubstituted benzene

Table 1. NMR Data of Compound 1 at 500 (^1H) and 125 (^{13}C) MHz

no.	δ_H, mult, *J* (Hz)[a]	δ_H[b]	δ_C[a]
1			173.6
2	2.71 dd, 17.2, 5.5	2.71 m	38.6
	2.62 dd, 7.9, 17.2		
3	3.94 m	3.91 m	47.6
4eq	2.33 ddd, 13.4, 3.8, 3.8	2.22 m	28.9
4ax	1.60 ddd, 13.5, 1.2, 11.2	1.57 m	
5	3.73 ddd, 11.1, 6.6, 3.8	3.74 m	53.3
6	4.19 ddd, 8.6, 6.6, 4.9	4.24 m	51.3
7	2.56, ddd, 13.9, 5.5, 4.9	2.56 m	34.8
	2.40, ddd, 13.9, 8.6, 8.6		
8	6.14 ddd, 15.8, 8.6, 5.5	6.19 ddd, 15.9, 7.1, 7.1	125.5
9	6.49, d, 15.8	6.52 d 15.9	134.9
10			138.5
11	7.31, d, 7.6	7.39 d 7.0	127.2
12	7.23, t, 7.6	7.32 t 7.0	129.6
13	7.15, t, 7.6	7.15 t 7.0	128.5
14	7.23, t, 7.6	7.32 t 7.0	129.6
15	7.31, d, 7.6	7.39 d 7.0	127.2
16	7.88, s	7.88 s	153.0
17	8.19, d, 2.7	8.16 s	164.4
18 NH	8.33, d, 9.2[c]	7.52, d, 9.4	
19 NH	9.62, bs[c]	9.84 bs	
20 NH		8.75 bs	

[a]In CD_3OD. [b]In CD_3CN. [c]In CD_3OH.

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ring [δ_C 127.2 (C11/C15), 128.5 (C13), 129.6 (C12/C14), and 138.5 (C10), δ_H 7.15 (H13), 7.23 (H12/H14) and 7.31 (H11/H15)], one disubstituted double bond (δ_C/δ_H 125.5/6.14, 134.9/6.49), three methines bearing heteroatoms (δ_C/δ_H 47.6/3.94, 53.3/3.73, 51.3/4.19), and three methylenes (δ_C/δ_H 38.6/2.71 and 2.62, 28.9/2.33 and 1.60, 34.8/2.56 and 2.40). Two more signals corresponding to interchangeable protons (δ_H 8.33 and 9.62) were observed when the ^{1}H NMR spectrum was recorded in CD_3OH and three (9.84, 8.75 and 7.52) in CD_3CN instead of CD_3OD.

Analysis of COSY and TOCSY spectra of **1** showed the presence of two spin systems (from C2 to C9 and from C11 to C15), which were connected through HMBC correlations for H8, H9, and H12/H14 to the quaternary aromatic carbon C10 and between H11/H15 and the olefinic methine carbon C9 (Figure 1). The carboxylic acid and formamide groups were

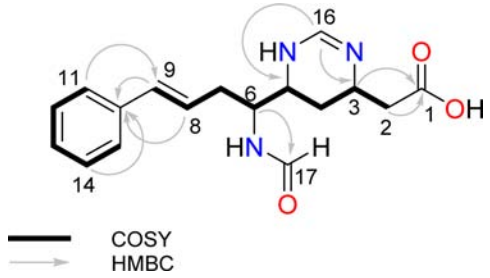

Figure 1. Important correlations observed in the NMR data of **1** in CD_3OD.

linked to the main framework by cross-peaks observed in the HMBC spectrum for H2 and H3 to carbonyl carbon C1 and between H6 and carbonyl carbon C17. Finally, the presence of a tetrahydropyrimidine ring was suggested by the chemical shifts in CD_3CN at 7.88/153.0 ppm (C16) characteristic of a methine surrounding by two nitrogen atoms, along with the HMBC correlations for H16 to the methine carbons C3 and C5 and the COSY cross peaks between H-16 and NH at 9.84 ppm.

The *E* configuration of the $\Delta^{8,9}$ double bond was established on the basis of the large coupling constant (J = 15.8 Hz) between the olefinic protons H8 and H9. The relative stereochemistry of the tetrahydropyrimidine ring was defined by a combination of ^{1}H−^{1}H coupling constant analysis, conformational studies by molecular mechanics calculations (Figure 2),

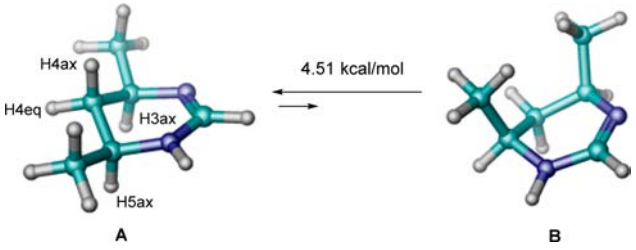

Figure 2. Conformational study of *syn*-1,4-dimethyl-1,4,5,6-tetrahydropyrimidine.

and the correlations observed in a ROESY experiment (Figure 3). Thus, the large coupling constants of H4ax with H3 (J = 13.4 Hz) and with H5 (J = 10.7 Hz), suggesting a pseudoaxial orientation for all three protons, along with a ROE correlation observed between H3 and H5, were indicative of a *cis* relative stereochemistry between those protons in the tetrahydropyrimidine ring.

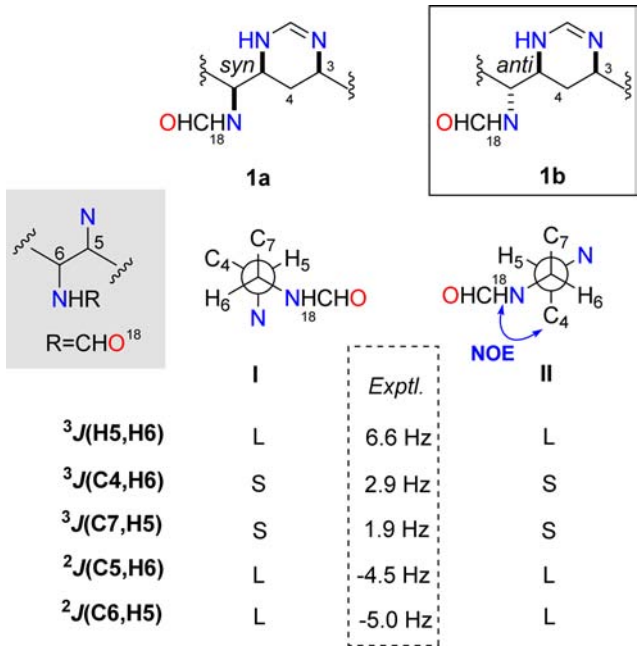

Figure 3. Conformers around C5−C6 bond compatible with the JBCA.

This proposal was corroborated by *J*-based configurational analysis (JBCA). The expected large values for the $^3J(H_3H_{4ax})$ and $^3J(H_{4ax}H_5)$ coupling constants and the set of values for $^3J(C_2H_{4ax})$, $^3J(C_2H_{4eq})$, $^3J(C_5H_3)$, $^2J(C_3H_{4ax})$, $^2J(C_3H_{4eq})$, $^3J(C_3H_5)$, $^3J(C_6H_{4ax})$, $^3J(C_6H_{4eq})$, $^2J(C_5H_{4ax})$, and $^2J(C_5H_{4eq})$ also support the *syn* orientation for these carbons. Furthermore, a conformational study for the *syn*-1,4-dimethyl-1,4,5,6-tetrahydropyrimidine showed the presence of a half-chair conformer that proved to be 4.51 kcal/mol more stable than a twisted-chair conformer in which the two substituents are in pseudoaxial positions (see Figure 2). All of these data allowed us to define the relative configuration as 3*R**,5*S** for these positions.

The most intriguing feature of the structural elucidation of the new compound was to establish the relative stereochemistry around C5−C6. The presence of the *anti* diastereoisomer **1b** instead of *syn*-**1a** (Figure 3) was deduced by a combination of JBCA, NOE correlations, and DFT-NMR calculations.

The large value for the homonuclear coupling constant $^3J(H_5H_6)$ suggested the presence of a single rotamer with both protons in an antiperiplanar arrangement. The small value of $^3J(C_7H_5)$ indicated that C7 and H5 are positioned in a synclinal disposition, while the large values of $^2J(C_5H_6)$ and $^2J(C_6H_5)$ suggested that the nitrogen atoms attached to C5 and C6 also have a synclinal disposition to H6 and H5, respectively.

Finally, the small $^3J(C_4H_6)$ value completed a set of values that matches with conformer I corresponding to the *syn* diastereoisomer **1a** and conformer II of the *anti* diastereoisomer **1b** (Figure 3). The observed NOE correlations between H6/H4 and H18/H4 only present in conformer II, where the protons of C4, H18, and H5 are close in space, allowed us to establish the *anti* relative configuration around the C5−C6 bond.

Taking into account that the flexibility of the formamide group at C6 could give an NOE interaction between neighboring groups in the space of possible conformers of the *syn* configuration of **1a** (see the Supporting Information), we wanted to confirm the proposed *anti* relative stereochemistry

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around the C5−C6 bond by using a DFT-NMR approach. Both possible diastereoisomers (**1a** and **1b**) were first submitted to a conformational search with the Macromodel program using the protocol developed by Hoye et al.[13] Having reached 68 conformers for **1a** and 52 for **1b** in a 3.0 kcal/mol window, the DFT calculations were performed using the combination B3LYP/6-31+G(d,p) for energy/frequencies and MPW1PW91/6-311+G(2d,p) for chemical shifts.

The sets of ^{1}H and ^{13}C NMR chemical shifts were compared by mean absolute error (MAE), R^2 of $\delta_{calcd}/\delta_{expt}$, by the linear regression of calculated (δ_{scaled})[14] and by the statistical DP4 parameter developed by Goodman and co-workers.[15] A 100% probability DP4 value in favor to **1b** was concordant with the *anti* (**1b**) relative stereochemistry of the new compound. Moreover, comparison of the computed calculated proton and carbon data for **1a** and **1b** with the experimental data obtained using the improved modified probability DP4+ parameter, recently published by Sarotti and co-workers,[16] also gave a 99% probability for the disposition *anti* as **1b**.

A zwitterion structure was proposed for **1** from the NMR data taken in CD_3CN (Table 1). The ^{1}H NMR spectrum in this deuterated solvent showed three extra signals that resonated as broad singlets at δ_H 7.52 (NH formamide), 8.75 (NH-20), and 9.84 (NH-19). These assignments were based on $^{1}H-^{1}H$ COSY and ROESY correlations. Thus, a $^{1}H-^{1}H$ COSY experiment revealed that δ_H 9.84 (NH-19) and 8.75 (NH-20) signals showed cross peaks to H16 (δ_H 7.88, brs). Additionally, the ROESY experiment displayed NOE correlations from H16 at 7.88 ppm (brs) to 9.84 ppm (NH-19) and 8.75 ppm (NH-20) signals, between δ_H 9.84 and H5 at 3.74 ppm, and between NH formamido at δ_H 7.52 and 8.16 ppm (H17) signals.

A $^{15}N-^{1}H$ HSQC experiment in CD_3CN clearly demonstrated the zwitterionic nature of compound **1**. The chemical shifts for N20 and N19 showed similar values (δ_N 122.3/ δ_H 8.75 and δ_N 119.4/ δ_H 9.84 ppm) and therefore a similar hybridization. $^{15}N-^{1}H$ HMBC correlations specifically acquired with a *J* value of 10 Hz, showed correlations between both N20, N19, and H16 and between N20 and H2 protons.

All these observations, along with the strong ion peak observed in the (+)-ESIMS spectrum, are compatible with a zwitterion structure with a positive charge delocalized between the two nitrogen atoms of the tetrahydropyrimide ring and the negative charge located on the carboxylate moiety at C1 (see Figure 4).[17]

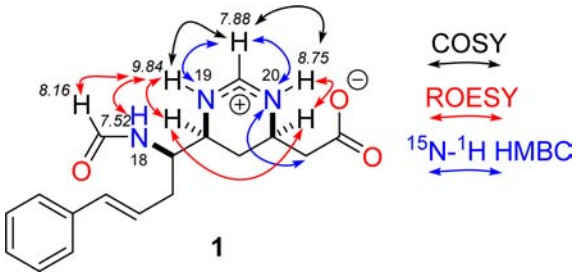

Figure 4. $^{1}H-^{1}H$ COSY, $^{1}H-^{1}H$ ROESY, and $^{15}N-^{1}H$ HMBC key correlations in CD_3CN of lanesoic acid (**1**).

In the solid state, lanesoic acid (**1**) appears to exist in the zwitterionic form, shown by an IR absorption at 1677 cm^{-1} consistent with a carboxylate or an imino moiety. Despite a 1,4,5,6-tetrahydropyrimidine ring being reported in a natural product for the first time in ectoine, obtained from a species of the bacterial genus *Ectothiorhodospira*,[18] and 5-hydroxyectoine produced by *Streptomyces parvulus*,[19] and later, in manzacidins, isolated from the sponge *Hymeniacidon* sp.,[20] a zwitterion structure as a β-amino acid was never observed in any of these compounds. We propose that the presence of the 2-acetyl moiety in **1**, instead of the carboxylic acid found at the same position in the heterocycle ring of the manzacidins and ectoine derivatives, promotes formation of a six-membered hydrogen-bonded structure that favors the zwitterion structure.

Biosynthetically, the tetrahydropyrimidine ring and the formamido moiety in lanesoic acid (**1**) might have been generated from two formic acid units and a 3,5,6-triamino ω-phenylnon-8-enoic acid which in turn could be originated from transmination of an ω-phenylalkenoid acid derivative. The biosynthetic formation of tetrahydropyrimidine ring through the condensation of a diamino compound with formic acid was first proposed in manzacidins A−C that bear the same heterocycle ring.[20] On the other hand, ω-phenylalkanoid acids, aromatic fatty acids with a phenyl unit on the terminal carbon of the acyl chain, are scarcely found in the literature. For example, they have been previously isolated from seed lipids of various genera of the subfamily Araceae of Aracea,[21] from butter fat samples,[22] as well as from a bacterium (*Vibrio alginolyticus*) associated with the marine alga *Cladophora coelothrix*.[23] The biosynthetic origin of aromatic fatty acids has been subjected to several studies.[24]

Lanesoic acid (**1**) was tested against pancreas PSN1, colon HT-29, breast MD-MB-231, and NSCLC lung tumor cells A549. The results showed that compound **1** has moderate cytotoxicity against pancreas tumor cells with an IC_{50} value of 8.9 μg/mL but that it is inactive against the other three tumor lines. This highlights the interesting selective antitumor activity displayed by **1**.

In summary, a new zwitterionic alkaloid, lanesoic acid (**1**), containing an unusual 1,4,5,6-tetrahydropyrimidine cation was isolated from a sponge belonging to *Theonella* genus colleted in Indonesia. Its structure, including its relative configuration, was established by an extensive spectral analysis along with *J*-based configurational analysis and DFT computational calculations. Compound **1** showed selective, moderate cytotoxic activity against pancreas tumor cells in a panel of different tumor cell lines.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b02832.

^{1}H NMR, ^{13}C NMR, HSQC, HMBC, $^{1}H-^{1}H$ COSY, $^{1}H-^{1}H$-NOESY, $^{1}H-^{1}H$ ROESY, HSQC-HECADE, $^{15}N-^{1}H$ HSQC, and HMBC spectra and MS of **1** (PDF)

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Notes

The authors declare no competing financial interest.

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