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DD-Diketopiperazines: Antibiotics Active against *Vibrio anguillarum* Isolated from Marine Bacteria Associated with Cultures of *Pecten maximus*

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Abstract: Bacterial strains CF-20 (CECT5719) and C-148 (CECT5718), isolated from cultures of larvae of molluscs, are shown to produce substances **1**−**5** with strong antibiotic activity against *Vibrio anguillarum* (MIC: 0.03−0.07 μg/mL) and identified as the DD-diketopiperazines cyclo(D)-Pro-(D)-Phe (**1**), cyclo(D)-Pro-(D)-Leu (**2**), cyclo(D)-Pro-(D)-Val (**3**), cyclo(D)-Pro-(D)-Ile (**4**), and cyclo-*trans*-4-OH-(D)-Pro-(D)-Phe (**5**). Comparison with other stereoisomers indicates that inhibition of *V. anguillarum* is associated with the presence of at least one D-amino acid in the diketopiperazine system. This is the first time a series of DD-diketopiperazines has been isolated from a single natural source and their inhibitory activity against *V. anguillarum* described.

Among the pathogenic bacteria present in the marine environment, the *Vibrios* occupy a prominent place due to the great economical losses associated with the infections it produces. Particularly important in this respect is *V. anguillarum*, which has been identified as responsible for the Vibriosis suffered by wild and farmed bivalves,[1] crustaceans,[2] and fishes[3] all around the world. The mortality is especially high when the animals are in the larval stage, and this is a very important limitation for the development of aquaculture.

In the course of a program directed to the isolation from marine sources of substances with antibiotic activity that could provide protection to aquaculture, we observed that on some occasions the cultures used in aquaculture showed higher survival of marine larvae and an associated decrease in the concentration of *V. anguillarum*. Thus, we reasoned that this might be due to the presence of an antibiotic-producing bacteria that inhibits the growth of *Vibrio* and therefore allowed the larvae to grow unaffected. Accordingly, we decided to study the bacteria living in the cultures of different bivalve molluscs and evaluate their ability to inhibit the growth of *Vibrio*.

In this paper we describe the isolation of the inhibitors of *V. anguillarum* **1**−**5** produced by the bacterial strains CF-20 (CECT5719) (compounds **1**−**5**) and C-148 (CECT5718) (compounds **1**−**4**) and their identification as the DD-diketopiperazines **1**−**5**. Comparison of the inhibitory activity of **1**−**5** with that of their stereoisomers prepared by synthesis allowed a relationship to be established between the stereochemistry of the diketopiperazines and their inhibitory activity. The taxonomy and fermentation procedure of strains CF-20 and C-148, together with the use of the diketopiperazines for the protection of cultured larvae and other applications in aquaculture, has been the subject of a recent patent application.[4]

The bacterial strains isolated from pectinid larvae of different marine organisms cultured in Galicia (NW Spain) were cultured and the broth and the pellet screened against *V. anguillarum*. As a result, we found that two marine bacterial strains named C-148 (CECT5718) and CF-20 (CECT5719), collected from *Pecten maximus* cultures, produced strong inhibition to *V. anguillarum*.

Selective extraction of the broth and the pellet obtained from co-cultures of these bacterial strains with *V. anguillarum* afforded fractions with high inhibition activity against *V. anguillarum*. These were submitted to column chromatography on silica gel, eluting with hexanes−EtOAc mixtures, and the active fractions (all of the separations were guided by bioassay with *V. anguillarum*) were then submitted to normal-phase HPLC, affording the pure bioactive compounds **1**−**5**. These were identified by their spectroscopic properties (^{13}C and ^{1}H NMR and (+)-FABMS) and confirmed by synthesis as the diketopiperazines **1**

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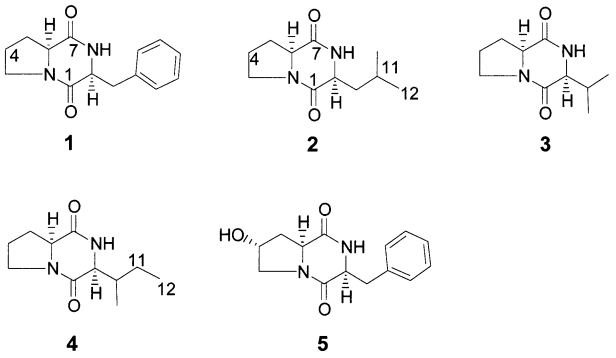

Figure 1.

Table 1. MIC Values Obtained for **1**−**5**, Their Stereoisomers, and Other Diketopiperazines

compound	MIC (μg/mL)
cyclo(D)-Pro-(D)-Phe (**1**)	0.03
cyclo(D)-Pro-(D)-Val (**2**)	0.05
cyclo(D)-Pro-(D)-Ile (**3**)	0.05
cyclo(D)-Pro-(D)-Leu (**4**)	0.04
cyclo(D)-*trans*-4-OH−Pro-(D)-Phe (**5**)	0.07
cyclo(D)-Val-(D)-Leu	0.05
cyclo(D)-Pro-(L)-Phe	0.10
cyclo(D)-Pro-(L)-Val	0.11
cyclo(D)-Pro-(L)-Ile	0.12
cyclo(D)-Pro-(L)-Leu	0.13
cyclo(L)-Pro-(D)-Phe	0.13
cyclo(L)-Pro-(D)-Val	0.14
cyclo(L)-Pro-(D)-Ile	0.11
cyclo(L)-Pro-(D)-Leu	0.12
cyclo(D)-Leu-(D)-His	0.07

(cyclo(D)-Pro-(D)-Phe), **2** (cyclo(D)-Pro-(D)-Leu), **3** (cyclo(D)-Pro-(D)-Val), **4** (cyclo(D)-Pro-(D)-Ile), and **5** (cyclo-*trans*-4-OH-(D)-Pro-(D)-Phe) (Figure 1). These compounds showed a very strong inhibitory activity against *V. anguillarum* with MIC values in the range 0.03−0.07 μg/mL, 10 times lower than that of some of the antibiotics actually in use in aquaculture such as oxytetracycline (MIC 0.5 μg/mL).

The identification of diketopiperazines **1**−**5** was straightforward from the MS fragmentations and the presence of characteristic ^{13}C NMR chemical shifts for the amide carbonyl groups (δ_C 165−170) and the ^{1}H NMR signals of the amino acids. That proline was one of the components of **1**−**4** that was easily deduced from the presence of three broad methylene multiplets in those compounds (δ_H 1.8−3.7), while in **5** this amino acid was replaced by *trans*-hydroxyproline. The NMR spectra clearly indicated that valine, leucine, isoleucine, and phenylalanine were the other amino acid residues in compounds **1**−**5**. Comparison of these data (Tables 1 and 2) with those reported in the literature for the LL isomers of **1**−**5** confirmed their planar structures, but interestingly, when their optical rotations were measured, the signs were the opposite,[5,6] indicating that **1**−**5** were in fact the less common DD enantiomers.

This was further confirmed by comparison with authentic samples of the DD, LL, DL, and LD isomers, prepared by synthesis. In this way, the DD and the LL isomers of **1**−**5** were prepared by cyclization in formic acid of the corresponding t-boc-dipeptide methyl esters,[7,8] while their LD and DL isomers were prepared by epimerization with base of the corresponding LL and DD compounds.[9−11]

The synthetic DD isomers showed MIC values equivalent to those of the natural samples **1**−**5** (Table 1), while the LL enantiomers were shown to be completely devoid of inhibitory activity against *V. anguillarum*. This constitutes an additional and independent proof of the DD stereochemistry of **1**−**5**. When only one of the asymmetric carbons of the diketopiperazines **1**−**5** is inverted, as in the LD and DL isomers, the activity is not completely lost and the MIC values are 2−3 times higher than those of the DD isomers.

Table 2. ^{13}C NMR Data ($CDCl_3$, 300 MHz) of Compounds **1**−**5**

carbon	**1**	**2**	**3**	**4**	**5**
1	169.4	170.2	170.1	170.3	169.7
3	45.2	45.0	45.4	45.0	54.3
4	22.2	22.2	22.7	22.6	68.0
5	28.3	28.4	28.3	28.0	37.5
6	58.9	58.7	58.9	59.1	56.0
7	164.9	164.9	165.0	166.4	165.1
9	56.1	60.3	60.4	53.2	54.3
10	36.6	28.4	35.3	38.2	36.5
11		19.0	24.5	24.4	
12		15.9	12.0	23.1	
13			15.7	21.1	
Ar					
1'	135.8				135.7
2'	128.9				129.1
3'	127.3				129.0
4'	129.1				127.4

Other diketopiperazines containing amino acids different from the components of **1**−**5** have also been tested (Table 1). Our results indicate that the presence of proline or hydroxyproline is not necessary for the activity. For example, cyclo(D)-Val-(D)-Leu shows an MIC of 0.05 μg/mL, similar to those of **1**−**5.**

Many terrestrial yeast, lichens, and fungi are known to produce diketopiperazines in culture.[12] Their presence in a few marine bacterial culture broths has also been reported. For example, the LL enantiomers of **1**−**4** have been isolated from a cyanobacterium isolated from the sponge *Calyx* cf. *podatypa*,[10] from *Pseudomonas aeruginosa*,[5] from roasted coffee,[13] and from other sources,[9,14−16] while the LL enantiomer of **5** was reported in a *Jaspidae* sponge[6] and in the marine bacterial strain A108.[17] Some LL-diketopiperazines have recently been identified as quorum-sensing bacterial sensors.[18,19] These signaling compounds are used by Gram-negative bacteria for cell-to-cell communication and allow the bacteria to regulate gene expression in response to population density.

In the literature there is only one report of DD enantiomers of diketopiperazines as natural compounds.[10] In that paper, the isolation of DD cyclo Pro-Phe (**1**) and DD cyclo Pro-Leu (**2**) from a *Calyx* sponge is mentioned. Unfortunately, the ^{13}C NMR data reported for DD cyclo Pro-Leu do not match that of our synthetic sample of **2**, nor the report by Young et al.,[9] and seem to correspond more probably with the LL isomer.

Therefore, compounds **2**−**5** are reported here for the first time as natural compounds. The presence of a series of DD-diketopiperazines in these two marine bacterial strains and their potent inhibitory activity against the pathogen *V. anguillarum*, clearly superior to that of some of the antibiotics actually in use in aquaculture such as oxytetracycline (MIC 0.5 μg/mL), are noteworthy. Their use for the protection of cultured larvae in aquaculture and other applications has been registered.[4]

Experimental Section

General Experimental Procedures. Optical rotations were measured in EtOH and MeOH on a JASCO DIP 360 polarimeter using a 1 dm path length cell. ^{1}H NMR and ^{13}C NMR were recorded at 300 MHz in d-chloroform. EIHRMS and FABHRMS were measured in a FISONS VG mass spectrometer. Column chromatography was carried out on silica gel 60 (230−400 mesh). For semipreparative HPLC purifications a

Table 3. ^{1}H NMR Data ($CDCl_3$, 300 MHz) of Compounds **1**−**5**

proton	1	2	3	4	5
3	3.69−3.61 (1H, m)	3.55 (1H, dt)	3.61−3.48 (2H, m)	3.59−3.49 (2H, m)	3.85 (d)
	3.61−3.50 (1H, m)	3.63 (1H, m)			
4	1.92−1.81 (2H, m)	2.02−1.99 (1H, m)	2.01−1.90 (1H, m)	1.94−1.86 (1H, m)	4.71 (t)
		1.93−1.88 (1H, m)	1.90−1.81 (1H, m)	2.02−1.99 (1H, m)	
5	2.38−2.28 (1H, m)	2.40−2.30 (1H, m)	2.31−2.22 (1H, m)	2.13 (1H, m)	2.31 (ddd)
	2.10−2.00 (1H, m)	2.11−2.01 (1H, m)	2.11−2.02 (1H, m)		
6	4.08 (1H, t)	4.09 (1H, dt)	4.07 (1H, t)	4.12 (1H, t)	4.65 (dd)
N-H	5.62 (1H, brs)	5.72 (1H, dd)	5.99 (1H, brs)	5.91 (1H, brs)	6.12 (S)
9	4.27 (1H, dd)	3.94 (1H, brs)	3.96 (1H, brs)	4.01 (1H, dd)	4.45 (dd)
10	3.59−3.45 (1H, m)	2.64 (1H, m)	2.42−2.31 (1H, m)	2.01 (1H, ddd)	2.85 (dd)
	2.77 (1H, dd)			1.76−1.71 (1H, m)	
11		0.91 (3H, d)	1.52−1.39 (1H, m)	1.70−1.67 (1H, m)	
			1.32−1.11 (1H, m)		
12			0.92 (3H, t)	0.94 (3H, d)	
Me-11				1.00 (3H, d)	
Me-10		1.06 (3H, d)	1.05 (3H, d)		
Ar	7.41−7.18 (5H)				7.35 (br, m)

μ-Porasil (7.8 × 300 mm) column for normal-phase and a μ-Bondapack C_{18} (7.8 × 300 mm) for reversed-phase separations were used.

For the bioassays, the pure compounds or fractions were dissolved in ethanol, placed on 96-well microplates, and incubated with *V. anguillarum* for 24−48 h before measurement of the inhibition with a microplate reader. The results are expressed as diameter of inhibition. MIC values are the minimum inhibitory concentration of substance necessary to produce an inhibition of the *V. anguillarum* growth and are expressed as μg/mL.

Isolation of Metabolites. The culture broth (45 L) of CF-20 (CECT5719) and *V. anguillarum* (R82) strain was centrifuged at 2724*g*, the supernatant extracted with CH_2Cl_2−MeOH (3:1), and the extract concentrated in vacuo. CH_2Cl_2 was added to the residue, the mixture was extracted with water, and the organic layer was evaporated in vacuo to give 700 mg of active extract. This residue was applied to a silica gel column and eluted with CH_2Cl_2−EtOAc mixtures (progressively from 2:1 to 1:1), affording 39 mg of a fraction active against *V. anguillarum*, which was submitted to normal-phase HPLC on a μ-Porasil column eluting with 2:3 hexane−EtOAc (at 3 mL/min) to yield 3.5 mg of **4** (t_R 22 min), 4 mg of **3** (t_R 29 min), 3 mg of **2** (t_R 37 min), and 5 mg of **1** (t_R 44 min).

Similarly, 45 L of supernatant co-culture of C-148 with *V. anguillarum* was extracted and chromatographed on a silica gel column as before. Reversed-phase HPLC of the active fractions, on a μ-Bondapak C-18 column and eluting with 95:5 MeOH−H_2O (at 2 mL/min), afforded 3 mg of pure **5** (t_R 17 min) and a mixture of **1**−**4**, which were separated by normal-phase HPLC (μ-Porasil; hexane−EtOAc (2:3); flow 3 mL/min) as before, giving 4 mg of **4**, 3.5 mg of **3**, 3 mg of **2**, and 2.5 mg of **1**.

Cyclo(D-Pro-D-Phe) [1]: $[\alpha]_D$ +88.7° (*c* 0.22, EtOH); NMR data, Tables 1 and 2; EIMS *m*/*z* 244, 215, 194, 153, 125, 120, 91, and 70.

Cyclo(D-Pro-D-Val) [2]: $[\alpha]_D$ +120.1° (*c* 0.10, EtOH); NMR data, Tables 1 and 2; EIMS *m*/*z* 196, 154, 125, 91, and 70.

Cyclo(D-Pro-D-Ile) [3]: $[\alpha]_D$ +168.1° (*c* 0.13, EtOH); NMR data, Tables 1 and 2; EIMS *m*/*z* 210, 154, 125, 86, and 70.

Cyclo(D-Pro-D-Leu) [4]: $[\alpha]_D$ +128.3° (*c* 0.11, EtOH); NMR data, Tables 1 and 2; EIMS *m*/*z* 210, 194, 154, 125, 86, and 70.

Cyclo(*trans*-4-OH-D-Pro-D-Phe) [5]: $[\alpha]_D$ +7.3° (*c* 0.02 MeOH); NMR data, Tables 1 and 2; EIMS *m*/*z* 261, 170, 141, 120, 91, and 68.

Preparation of Diketopiperazines 1−5 and Their LL Enantiomers. Diketopiperazines **1**−**4** and their LL enantiomers were synthesized by cyclization of the corresponding dipeptide methyl ester as described by Nitecki et al.[8] Diketopiperazine **5** and its LL enantiomer were prepared by coupling *N*-carbobenzoxy-*trans*-4-OH-proline with phenylalanine methyl ester followed by catalytic hydrogenation to give **5**.[20]

Preparation of LD and DL 1−5 by Epimerization. The LD and DL isomers of **1**−**5** were prepared by epimerization with base of the corresponding LL and DD **1**−**5** as described by Adamczeski[10] and Ott.[11]

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