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Maremycin A and B, New Diketopiperazines from a Marine *Streptomyces* sp.

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In the culture broth of the marine *Streptomyces* sp. B 9173 the two novel diketopiperazines maremycin A (**1**) and B (**2**) were detected by chemical screening. Their structures were elucidated and confirmed by the synthesis of the oxindole derivative **4**.

In the course of our screening of marine bacteria for secondary metabolites, in the culture broth of the marine *Streptomyces* strain B 9173 two closely related, polar compounds, maremycin A (**1**) and B (**2**), were detected by their intense blue color reaction with anisaldehyde/sulfuric acid. They belong to the group of diketopiperazines which are biosynthetically built up by amino acids. This paper deals with the description of the producing organism, isolation and purification procedure, structure elucidation by NMR methods, the stereochemistry of **1/2**, and synthesis of a substructure **4** of the molecule.

The *Actinomycete* strain B 9173 was isolated from a sediment collected at the Pacific coast of Chile using medium containing 50% of natural seawater with incubation at 25°C[1]. Fermentation was carried out in a medium containing malt extract (1%), yeast extract (0.4%) D-glucose (0.4%), and 50% synthetic seawater in shaking flasks or fermenters at 28°C for 72 h. Extraction of the filtered culture broth with ethyl acetate and subsequent chromatography on silica gel gave maremycin A (**1**) in a yield of about 2 mg/l and maremycin B (**2**) in a yield of about 1.5 mg/l. Both compounds are well soluble in methanol, dimethyl sulfoxide and in hot acetone and slightly in chloroform, dichloromethane and ethyl acetate; both gave a blue coloration in TLC on spraying with anisaldehyde/sulfuric acid and heating. The color changed to red at room temperature.

The molecular formula $C_{17}H_{21}N_3O_4S$ was established by HR-EI mass spectra (*m/z* = 363) and is identical for both Maremycins. The UV spectrum (methanol) exhibits two absorption bands at λ = 210 and 258 nm (broad) with only small changes under basic or acidic conditions suggesting a simple aromatic system. In addition to the typical aromatic C–C valence vibration at $\tilde{\nu}$ = 1610 cm^{-1}, a hydroxy or imino absorption appears at $\tilde{\nu}$ = 3420 cm^{-1} in the IR spectrum (KBr), and carbonyl absorption bands are observed at $\tilde{\nu}$ = 1720–1730 and 1670–1680 cm^{-1}, indicating tentatively a lactam structure.

The ^{1}H-NMR and ^{13}C-NMR spectra ([D$_6$DMSO) of both compounds show the same number of signals with slightly different chemical shifts, revealing that maremycin A (**1**) and B (**2**) are closely related compounds. The ^{1}H-NMR spectrum of **2** reveals the presence of three exchangeable protons at δ = 8.42, 7.67, and 6.88, two of them with small coupling constants of *J* = 2 and 1.5 Hz. The coupling pattern of the four aromatic protons between δ = 7.4 and 6.9 points to an *ortho*-substituted ring, which is confirmed by the out-of-plane bending in the IR spectrum at $\tilde{\nu}$ = 760 cm^{-1}. The ^{13}C-NMR spectrum shows quarternary C atoms of three amide or ester groups at δ = 176.2, 167.3, and 166.1. The ^{1}H, ^{1}H-COSY spectrum reveales only a C_2 and a C_3 fragment (see Figure 1) with a small ^5J coupling between 3-H and 6-H. Long-range couplings to a quarternary atom are of diagnostic value in the HMBC spectrum. Its position could be assigned as C-3′ by ^3J couplings with the methyl group (δ = 0.83), the atoms 1″-H, 3-H, an aromatic proton and a hydroxy group at δ = 6.88. The latter shows a long-range coupling to the carbonyl C-2′. The lactam structure was assigned by couplings from the *N*-methyl group to C-2′ and C-7′ belonging to the benzenoid ring.

Of further importance were long-range couplings from terminal carbon atoms of the two chains to C-2 and C-5. ^2J- and ^3J-couplings from 3-H, 1″-H and 4-NH to C-2 were found, as well as long-range coupling from 6-CH_2, 3-H and 1-NH to C-5. This led to the conclusion that both amide moieties must be embedded in a ring system as in diketopiperazines. The methyl group at δ = 2.12 showed no long-range coupling due to the adjacent voluminous S atom.

To further confirm the structures of maremycin A and B, *N*-methylisatin (**3**) was converted in a Grignard reaction with ethylmagnesium bromide to 3-ethyl-3-hydroxy-1-

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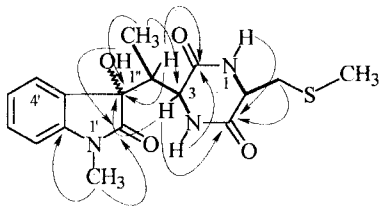
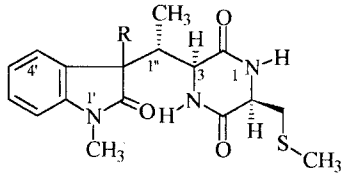
Figure 1. $^3J_{C,H}$ long range couplings observed in maremycins A (**1**) and B (**2**) by HMBC pulse sequences at 300 MHz and structures of both compounds



1/2



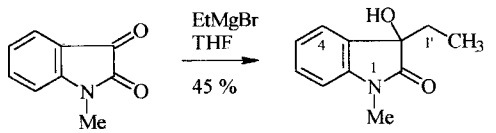
1: R = ·····OH
2: R = —OH

methyl-1,3-dihydroindol-2-one (**4**). As expected, the ^{13}C-NMR data of **4** show a close similarity to the data of the oxindole part of the maremycins, as is shown for **1** and **2** in Table 1.

Table 1. ^{13}C-NMR (50.3 MHz) data of the indole part of maremycin A (**1**), B (**2**), and 3-ethyl-3-hydroxy-1-methyl-1,3-dihydroindol-2-one (**4**)

Carbonatom	**1**[a]	**2**[a]	Carbonatom	**4**[b]
2	167.9	167.3		
3	54.2	55.5		
5	165.6	166.1		
6	53.6	54.3		
6-CH_2	36.4	36.9		
SCH_2	16.3	16.1		
2′	177.9	176.2	2	178.5
3′	76.4	77.2	3	77.2
3a′	130.6	131.4	3a	129.9
4′	124.9	123.7	4	123.8
5′	121.7	122.3	5	123.1
6′	129.0	129.2	6	129.5
7′	108.5	108.5	7	108.3
7a′	143.0	142.9	7a	143.5
NCH_3	25.8	25.7	NCH_3	26.1
1″	43.0	43.5	1′	31.5
1″-CH_3	8.3	9.8	1′-CH_3	7.6

[a] ([D_6]DMSO, 50.3 MHz). – [b] ($CDCl_3$, 50.3 MHz).

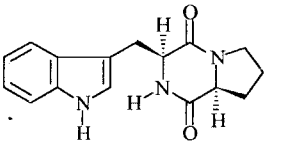


3 **4**

The configuration of the *S*-methylcysteine residue in both maremycins was determined by GC analysis on a chiral Lipodex E column to be L: hydrolysis of both isomers, trituration with methanol/HCl and subsequently with trifluoroacetic anhydride gave derivatives which showed the same retention time as an L-configurated authentic sample[2].

Nearly all diketopiperazines isolated from microbiological sources occur in a boat conformation with alkyl residues being in equatorial position and are built up of amino acids of identical configuration[3]. If this is valid also for **1**/**2**, then their diketopiperazine part should be *S,S*-configurated as in brevianamide F(**5**). The ^{1}H-NMR spectra of both maremycins differ mainly by the chemical shifts of their 3′-hydroxy signals suggesting that **1** and **2** are diastereomeric at C-3′. This is confirmed by the CD spectra of **1** and **2** which are mirror-inverted to each other but slightly different in their intensities.

Maremycin A (**1**) shows a 3-H, 1″-H coupling of less than 2 Hz whereas for **2** a value of J = 5.5 Hz was found. This is consistend only with a (1″*S*) configuration: Force-field calculations[4] revealed, that both (3′*R*/*S*,1″*R*,3*S*,6*S*) isomers should predominate in a 1″-H, 3-H *s-trans* conformation with expected coupling constants of 10–12 Hz, whilst the two (3′*R*/*S*,1″*S*,3*S*,6*S*) stereoisomers are more stable in the case of a 1″-H, 3-H *cis* orientation (ca. 60°) and have calculated coupling constants between 1.6 Hz (1″*S*,3′*S*,3*S*,6*S*) and 3 Hz (1″*S*,3′*R*,3*S*,6*S*). Therefore, the absolute configurations of **1** and **2** were tentatively assigned as depicted in the formulas.



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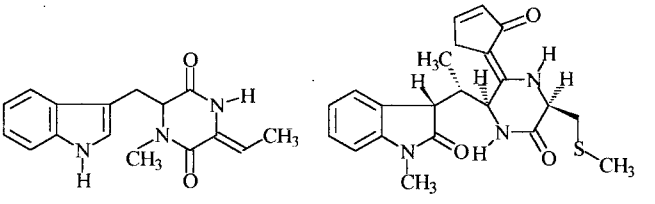
Maremycin A (**1**) and B (**2**) are the first sulfur-containing diketoperiperazines isolated from marine sources, but closely resemble two diketopiperazine derivatives obtained from terrestrial *Streptomycetes*. Tryptophan dehydrobutyrine diketopiperazine (TDD, **6**) was found by Rinehart et al.[5] in 1974, but the inhibitory effect on glutathione S-transferase activity was not reported before 1992[6]. The glutathione S-transferase isoenzyme family is one of most important drug-detoxifying enzymes in the liver. FR-900452 (**7**) isolated from *Streptomyces phaeofaciens* no. 7739[7] is a specific inhibitor of rabbit platelet aggregation induced by platelet activating factor (PAF) in vitro[8]. Both maremycins do not show any activity against fungi, Gram-positive and Gram-negative bacteria and yeasts; further activities were not tested.



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Experimental

IR (KBr): Perkin Elmer 297. – MS: Varian MAT 731 (70 mV, direct insert, high resolution with perfluorokerosene as standard). – NMR experiments: Varian VXR-500 and Bruker WM 300 instruments; TMS as internal standard. – Optical rotation: Perkin-Elmer, model 241, thermostated at 20°C.

Characterization of Streptomycete B 9173: The pure culture was maintained on yeast extract/malt extract medium[9]. The vegetative mycelium is brown-yellow, the strain forms grey aerial mycelium with spiral (Spirales) spore chains. The surface of the spores is smooth. Melanin pigment is formed on peptone-yeast extract iron agar as well as on tyrosin agar[10]. The temperature optimum is at about 30°C, the temperature maximum is below 45°C. Starch and casein are degraded, chitin, gelatin, and esculin are weakly hydrolyzed. The strain is catalase and nitrate reductase positive. The cell wall peptidoglycan of the strain contains major amounts of L-diaminopimelic acid (L-DAP) but no diagnostic sugars (cell wall chemotype I). Due to its chemical and morphological features the strain B 9173 can be assigned to the suprageneric group *Streptomycetes*[11].

Fermentation: The marine *Streptomycete* B 9173 was cultivated in 1000 ml Erlenmeyer flasks containing 160 ml of production medium (1% malt extract, 0.4% yeast extract, 0.4% D-glucose and 50% synthetic sea water, pH = 8.2 before sterilization) at 28°C for 50 h. 2 l of this culture was used as inoculum for a 20-l fermenter (Fa. Meredos, Göttingen, FRG) with the same medium as used for production; starting pH 7.3. The fermentation was stopped after 72 h at pH 7.4.

Isolation: After addition of 2% Hyflo Super-cel (Celite France S. A.), the culture broth (20 l) was filtered and the filtrate extracted five times with EtOAc. The combined extracts were concentrated in vacuo to yield 2.8 g of a brown solid which was dissolved in 250 ml of methanol and extracted twice with 100 ml of hexane. The methanolic solution was concentrated and the residue subjected to chromatography on silica gel (column 30 × 6 cm, dichloromethane/methanol, 19:1) to afford 157 mg of crude diketopiperazines. Further chromatography on silica gel (column 30 × 2 cm, ethyl acetate) gave 40 mg and 26 mg of the diastereomeric maremycins A (**1**) and B (**2**), respectively. Both isomers were separable by column chromatography with ethyl acetate, but not with more polar solvents at higher R_f value.

Maremycin A (**1**): R_f = 0.06 (ethyl acetate), blue color reaction with anisaldehyde reagent on heating; color changed to red at room temp. – M.p. 229°C. – $[\alpha]_D^{20}$ = −120.95 (*c* = 0.21; methanol). – CD (MeOH, 9.44 · 10^{-5} M): λ_{max} (Θ) = 210.4 (−107500), 239.0 (76950), 263.4 nm (−35200). – UV (methanol): λ_{max} (lg ε) = 209 (4.53), 257 nm (3.57); +HCl: λ_{max} (lg ε) = 208 (4.55), 257 nm (3.75); +NaOH: λ_{max} (lg ε) = 212 (4.44), 248 nm (3.81). – IR (KBr): $\tilde{\nu}$ = 3450−2800, 1710, 1670, 1611, 1468, 1372, 1350, 1213, 1124, 1089, 1022, 973, 850, 758, 692 cm^{-1}. – ^1H NMR (500 MHz, [D_6]DMSO): δ = 1.11 (d $J_{Me,1''}$ = 7 Hz, 3H, 1″-Me), 2.05 (m, 1H, 1″-H), 2.08 (s, 3H, SCH_3), 2.83 (*A*BX, $J_{6\text{-}CHa,b}$ = 14 Hz, $J_{6,6\text{-}CHa}$ = 4 Hz, 1H, 6-CH_a), 2.96 (A*B*X, $J_{6,6\text{-}CHb}$ = 4 Hz, 1H, 6-CH_b), 3.09 (s, 3H, NCH_3), 4.25 (AB*X*, 1H, 6-H), 4.88 (s, br., 1H, 3-H), 6.99 (d, $J_{7',6'}$ = 7.5 Hz, 1H, 7′-H), 7.01 (dd, br., $J_{5',4'}$ = $J_{5',6'}$ = 7.5 Hz, 1H, 5′-H), 7.37 (m, 2H, 4′-, 6′-H), 7.53 (s, D_2O exchange, 1H, OH), 7.88 (s, br., D_2O exchange, 1H, NH), 8.60 (s, br., D_2O exchange, 1H, NH). – EI MS (70 eV): *m/z* (%) = 363.1260[12] (5) [M^+ calcd. for $C_{17}H_{21}N_3O_4S$; 363.1254], 201 (15), 174 (66), 163 (100), 90 (48). – $C_{17}H_{21}N_3O_4S$ (363.4): calcd. C 56.18, H 5.82; found C 56.07, H 5.88.

Maremycin B (**2**): R_f = 0.06 (slightly slower moving than **1** in ethyl acetate), blue color reaction with anisaldehyde reagent on heating; color changed to red at room temp. – M.p. 216°C. – $[\alpha]_D^{20}$ = +2.94 (*c* = 0.21; methanol). – CD (MeOH, 9.44 · 10^{-5} M): λ_{max} (Θ) = 264.8 (25600), 237.2 (−50300), 210.4 nm (68000). – UV (methanol): λ_{max} (lg ε) = 209 (4.47), 258 nm (3.63; +HCl: λ_{max} (lg ε) = 208 (4.50), 258 nm (3.63); +NaOH: λ_{max} (lg ε) = 212 (4.41), 252 nm (3.90). – IR (KBr): $\tilde{\nu}$ = 3420, 3190, 3050, 2960, 1730, 1708, 1678, 1659, 1609, 1470, 1455, 1382, 1345, 1260, 1120, 1090, 986, 850, 760 cm^{-1}. – ^1H NMR (500 MHz, [D_6]DMSO): δ = 0.83 (d, $J_{Me,1''}$ = 7 Hz, 3H, 1″-Me), 2.12 (s, 3h, SCH_3), 2.33 (qd, $J_{1'',2''}$ = 7 Hz, $J_{1'',3}$ = 5.5 Hz, 1H, 1″-H), 2.85 (*A*BX, $J_{6\text{-}CHa/b}$ = 13.5 Hz, $J_{6,6\text{-}CHa}$ = 4.2 Hz, 1H, 6-CH_a), 2.96 (A*B*X, $J_{6,6\text{-}CHb}$ = 5.5 Hz, 1H, 6-CH_b), 3.10 (s, 3H, NCH_3), 4.15 (AB*X*, 1H, 6-H), 4.52 (ddd, $J_{3,1''}$ = 5.5 Hz, $J_{3,N}$ = 1.5 Hz, $J_{3,6}$ = 1.5 Hz, 1H, 3-H), 6.88 (s, 1H, D_2O exchange, OH), 7.01 (dd, $J_{7',6'}$ = 7.5, $J_{7',5'}$ = 1.0 Hz, 1H, 7′-H), 7.07 (ddd, $J_{5',4'}$ = $J_{5',6'}$ = 7.5 Hz, $J_{5',7'}$ = 1.0 Hz, 1H, 5′-H), 7.31 (dd, $J_{4',5'}$ = 7.5 Hz, $J_{4',5'}$ = 1.0 Hz, 1H, 4′-H), 7.34 (ddd, $J_{6',5'}$ = 7.5 Hz, $J_{6',7'}$ = 7.5 Hz, $J_{6',4'}$ = 1.0 Hz, 1H, 6′-H), 7.67 (d, br., $J_{N,3}$ = 1.5 Hz, 1H, D_2O exchange, NH), 8.42 (d, br., $J_{n,6}$ = 2 Hz, 1H, D_2O exchange, NH). – EI MS (70 eV): *m/z* (%) = 363.1260[12] (6) [M^+ calcd. for $C_{17}H_{21}N_3O_4S$: 363.1254], 201 (18), 174 (30), 163 (100), 82 (73).

Absolute Configuration of S-Methylcysteine in **1/2**: 1 mg of **1** and **2**, respectively, was kept for 6 h at 100°C in a sealed tube containing 1 ml of 6 N HCl. The mixture was evaporated to dryness and the residue dissolved in 1 ml of MeOH. The solution was triturated with HCl gas for 10 min at 50°C and then evaporated to dryness again. The residue was dissolved in 200 µl trifluoroacetic anhydride and the solution, after 30 min at 20°C, kept in an ice bath in vacuo (15 Torr) for 2 min to evaporate excess anhydride. The residue was dissolved in 200 µl of benzene. Authentic samples[2] of (D,L)-*S*-methylcysteine and (L)-*S*-methylcysteine were treated in the same manner. GC separation on Lipodex E (50% in OV 1701; 25 m capillary column, 130°C) gave peaks at *t* = 9.3 min for D-methylcysteine and *t* = 11.7 min for L-methylcysteine. Hydrolyzates of both maremycins gave exclusively signals as observed for authentical L-methylcysteine.

3-Ethyl-3-hydroxy-1-methyl-1,3-dihydroindol-2-one (**4**): To a stirred solution of 150 mg of *N*-methylisatin (**3**) in dry THF a freshly prepared solution of ethylmagnesium bromide (0.02 mol) in THF was added dropwise. After stirring for 1/2 h under reflux, the mixture was hydrolyzed by the addition of some pieces of ice and 20 ml of a concentrated ammonium chloride solution. The mixture was extracted twice with THF. The combined organic extracts were dried with Na_2SO_4, filtered and the solvent was evaporated. The residue was purified by chromatography on silica gel (column 40 × 2 cm, CH_2Cl_2/MeOH, 95:5) to yield 87 mg (45%) of pure **4** as faint yellow lancette-shaped crystals with m.p. 179−82°C. – R_f = 0.3 (ethyl acetate). – ^1H NMR (200 MHz, $CDCl_3$): δ = 0.75 (t, $J_{1',2'}$ = 8 Hz, 3H, NCH_3), 2.00 (qd, $J_{1',2'}$ = 8 Hz, *J* = 3 Hz, 2H, 1′-CH_2), 3.20 (s, 3H, NCH_3), 6.85 (d, *J* = 8 Hz, 1H, Ar-H),7.10 (t, *J* = 7 Hz, 1H, Ar-H), 7.40−7.20 (m, 2H, Ar-H). – EI MS (70 eV): *m/z* (%) = 191.0946[12] (28; M^+, as calcd. for $C_{11}H_{13}NO_2$), 162 (100; M − C_2H_5), 134 (38), 77 (25). – $C_{11}H_{13}NO_2$ (191.2): calcd. C 69.09, H 6.85, N 7.32; found C 68.97, H 6.95.

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[11] The strain B 9173 is deposited in the culture collection of marine actinomycetes of the Alfred-Wegner Institute for Polar and Marine Research in Bremerhaven.
[12] The molecular weights were checked by measurement of the calculated precise masses [±2.0 ppm; $R \approx 10000$].

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