New Indole Alkaloids from the North Sea Bacterium *Vibrio parahaemolyticus* Bio249[1]

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Several bis- and tris-indole derivatives were isolated from a North Sea bacterium that was closely related to *Vibrio parahaemolyticus* (98% homology). 1,1,3-Tris(3-indolyl)butane (**3**) is a new compound, and 3,3-bis(3-indolyl)butane-2-one (**1a**), arundine (**1b**), and 1,1,1-tris(3-indolyl)methane (**2a**) were isolated from a microorganism for the first time here. Additionally, many other known compounds were obtained from the ethyl acetate extract of the culture. Their structures were established on the basis of various spectral data, and their origin is discussed. All compounds were inactive against a range of bacteria and fungi.

More than 1000 alkaloids with the indole skeleton have been reported from microorganisms.[2,3] One-third of these compounds are peptides with masses beyond 600 Da where the indole is tryptophan-derived. The structural variety of the remaining two-thirds is higher, and their biological activity seems to cover a broader range, including antimicrobial, antiviral, cytotoxic, insecticidal, antithrombotic, or enzyme inhibitory activity to list only some of them.[3] In contrast, most of the peptides have mainly antibiotic activity. In our search for new bioactive molecules from marine microorganisms,[4,5] we isolated a new indole alkaloid, namely, 1,1,3-tris(3-indolyl)butane (**3**). Additionally, 3,3-bis(3-indolyl)butane-2-one (**1a**), arundine[6] (**1b**), 1,1,1-tris(3-indolyl)methane[7] (**2a**), and several other metabolites have been found, which have previously been obtained by synthesis or were isolated from other sources. We report here one of the cases where the differentiation between a biosynthetic and an abiotic origin of the metabolites is difficult.

The producing strain Bio249 was isolated from a biofilm grown on a glass plate in the North Sea and taxonomically classified as closely related to *Vibrio parahaemolyticus* on the basis of the 16S rRNA (98% homology). On a casein medium, the strain did not produce antibiotic activities; however several UV-absorbing compounds were formed, which were tentatively identified as indoles due to their color reaction with Ehrlich's reagent. The ethyl acetate extract of a bulk culture of the microorganism was subjected to column chromatography on silica gel and Sephadex LH-20, which afforded a high yield of unsubstituted indole along with indole-3-carboxylic acid,[8] indole-3-carbaldehyde,[8] arundine[6] (**1b**), vibrindole A[9] (**1c**), trisindoline[10] (**4a**), 2,2-di(3-indolyl)-3-indolone[9] (**5**), pharacine,[11] *p*-hydroxyphenylethanol,[12] phenyl acetamide,[13] and thymine. The indoles **1c** and **5** had been isolated from another strain of *V. parahaemolyticus* previously,[9] and **4a** was known as a metabolite from a *Vibrio* sp. isolated from a sponge.[10] This is the first reported occurrence, however, of the plant metabolite arundine (**1b**) and of the synthetic indoles **1a** and **2a** in a microorganism. The structures of all metabolites were identified by comparison with the reported spectral data (^{1}H, ^{13}C and MS) using AntiBase.[3]

Compound **1a** was obtained as a pale yellow solid, which gave a colorless spot on TLC. Its EI mass spectrum gave a molecular ion at 302 [$M^{+\bullet}$]. The HREIMS data (302.1419) suggested that the molecular formula is $C_{20}H_{18}N_2O$, and the IR spectrum showed characteristic bands at 3400 (NH) and 1694 (C=O) cm^{-1}. Its ^{1}H NMR spectrum in $CDCl_3$ showed two broadened *ortho*-coupled doublets at δ 7.40 and 7.38 (both J = 7.8 Hz), and two triplets at 7.17 and 7.02 (each J = 7.2 Hz) indicated the presence of a 1,2-disubstituted benzene ring. Another proton in the aromatic region at δ 6.94 (d, J = 2.2 Hz) together with the positive Ehrlich reaction on TLC indicated a 3-substituted indole ring. Furthermore its ^{1}H NMR spectrum showed the presence of two methyl singlets at δ 2.08 and 2.16. All the aromatic signals related to the indole system were found to be doubled in their intensity with respect to the methyl signals. The ^{13}C NMR and APT data indicated the presence of only 12 carbon signals of which five were due to quaternary carbon atoms, five methine carbons, and two methyl groups. The ^{1}H NMR spectrum, the MS fragmentation pattern, and high-resolution MS confirmed the presence of two indolyl moieties in a symmetrical orientation. From the foregoing spectral data the structure of the compound **1a** was established as 3,3-bis(3-indolyl)butane-2-one, which was obtained previously by synthesis, but not fully characterized.[14]

Compound **2a** was isolated as a strongly UV-absorbing solid with a high-resolution mass (361.1579), indicating the molecular formula $C_{25}H_{19}N_3$. The doublets at δ 7.48, 7.36, and 6.78 and two triplets at δ 7.18 and 6.98 in the ^{1}H NMR spectrum revealed the presence of the same 3-substituted indolyl system as found in the previous compound **1a**. Further, its ^{1}H NMR spectrum displayed a singlet at δ 6.18. All the aromatic signals were found to be tripled in their intensity relative to the higher field signal at δ 6.18. The ^{13}C and APT experiments indicated a total of only nine carbon signals which included six methine and three quaternary carbon atoms in the skeleton. Except for the methine signal at δ 32.7, all the remaining signals were found to be due to the 3-substituted indolyl system. The low number of carbon signals together with the high mass suggested the structure to be highly symmetrical. According to the EI mass fragmentation pattern with a signal at δ 117 typical of 3-substituted indoles and an M − 118 ion

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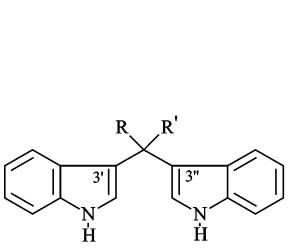
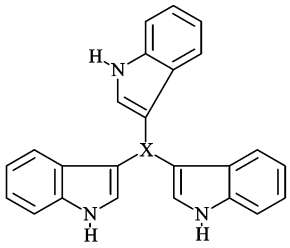
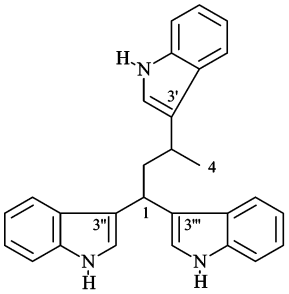
at 243, the structure of the compound was found to be 1,1,1-tris(3-indolyl)methane (**2a**), a synthetically known compound.[7]



1a: R = Ac, R' = Me
1b: R = H, R' = H
1c: R = H R' = Me



2a: X = CH
2b: X = C^+
2c: X = C^+, 1 Phe instead of indole



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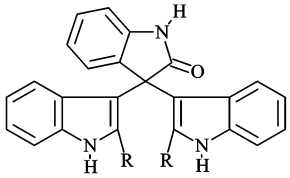
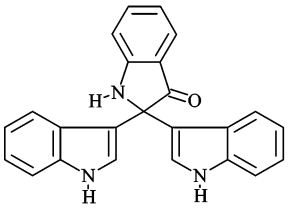


4a: R = H
4b: R = Me



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The trimeric indole **2a** has not been reported so far from natural sources, but a closely related compound, turbomycin A (**2b**), has been published recently as a natural product: Gillespie et al.[15] isolated DNA fragments from soil samples and expressed the genes in *E. coli*. One of the clones produced the orange **2b**, which had been synthesized previously,[7] and the related turbomycin B (**2c**). Both turbomycins exhibited broad spectrum antibiotic activity against both Gram-positive and Gram-negative bacteria, whereas the compound **2a** was found to be inactive against *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Streptomyces viridochromogenes*, *Mucor miehei*, and *Candida albicans*. The didehydro derivative of **2a**, bis(3-indolyl)-3*H*-indolylidenemethane, was formed by *Saccharomyces cerevisiae* under the influence of light.[16]

Compound **3** gave a high-resolution EI mass spectrum (403.2030) suggesting the molecular formula $C_{28}H_{25}N_3$. The ^{1}H NMR spectrum showed typical aromatic signals indicating again the presence of the indolyl skeleton as observed in the previous compounds. From the ^{1}H,^{1}H COSY, ^{13}C, and HMQC data it was derived that the compound contained three indolyl fragments substituted at position 3. Further, its ^{1}H NMR spectrum showed two methine proton signals at δ 4.50 (t, J = 7.8 Hz) and 3.00 (m), a methylene signal at δ 2.40 (m) and 2.60 (m), and a methyl signal at δ 1.38 (d, J = 7.0 Hz). From the coupling patterns the partial structures CH−CH_3 and CH−CH_2−CH were obtained, and

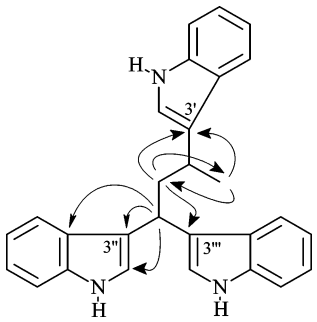

Figure 1. Selected HMBC correlations of 1,1,3-tris(3-indolyl)butane (**3**).

finally the COSY correlations indicated the presence of a trisubstituted butane fragment. From the HMQC and HMBC experiments the three indolyl fragments must be attached to the positions 1, 1, 3 of the butane backbone. In the HMBC spectrum, the methine proton at δ 4.50 showed correlations with the carbon atom 2 of the butane chain and the atoms 2″, 3″, 3a″, 2‴, 3‴, and 3a‴ of two indole moieties. Further important correlations are reported in Figure 1. The foregoing spectral data in addition to the EI mass fragmentation revealed the structure of compound **3** as 1,1,3-tris(3-indolyl)butane.

Although there are many reports on the antibiotic activities even of simple indole derivatives, we could not find any antibiotic or antifungal properties of the indole derivatives described here. 3-Hydroxymethylindole, bis(3-indolyl)methane (**1b**), and many related compounds show, however, besides other effects pronounced antiproliferative properties[17] by a G1 cell cycle arrest[18] and were therefore suggested for the chemoprevention and treatment of cancer,[19] for the treatment of premenstrual syndrome,[20] or as antiinflammatory agents, to list some of their activities.[21] The new indoles from *V. parahaemolyticus* might have related properties and will be investigated in this respect.

As indole reacts smoothly with carbonyl compounds affording the corresponding bis-arylated products, the bisindolylmethane (**1b**) can be obtained easily by reaction of indole with formaldehyde under slightly acidic conditions.[22] In a similar manner, the reaction of indole-3-carbaldehyde with indole, both isolated from *V. parahaemolyticus*, should yield **2a**, and also all other bis- and tris-indoles isolated here may be derived by Pictet−Spengler type reactions from indole and the respective carbonyl compound. This pointed to the possibility that at least some of the metabolites might have been formed artificially from indole in a reaction catalyzed by traces of acetic acid liberated by hydrolysis of the moist ethyl acetate during workup. Natural products with chiral elements are mostly found as optically active isomers, not as racemic mixtures. The compounds **1a** and **3**, however, did not show any CD effect and were racemic indeed, which supported the assumption of a nonenzymatic origin.

As we did not find a way to isolate the potential low-molecular carbonyl precursors (formaldehyde, acetaldehyde, crotonaldehyde, diacetyl, isatin) directly from the crude extract, we added 2-methylindole before work up of the culture broth to induce eventually the formation of the homologous methyl indole derivatives, e.g., **4b**. Indeed, the labeling experiment afforded additional HPLC signals in the crude extract at slightly higher retention times, which showed mass differences of m/z = 14 and 28 with respect to the original indole derivatives. In the case of the trisindoline (**4a**), MSMS of the molecular ion at m/z 363 gave only a M − 117 fragment corresponding to the loss of

one indole unit. In the labeling experiment, a satellite signal with a slightly higher retention time and the mass of 377 showed fragment ions at 245 and 259, corresponding to the loss of either indole or methylindole as expected for a monomethylated trisindoline, thus confirming that at least a part of **4a** was post-biosynthetically formed.

In an additional experiment, the bacterial cell mass was scraped off directly from the agar plate (pH 8.8), extracted by trituration with ethyl acetate, and immediately analyzed by HPLC/MS. Although the concentration of the bis- and tris-indoles found was lower with respect to the indole concentration in the previous batch fermentation, arundine (**1b**) and the indoles **2a**, **4a**, and **5** could be detected unequivocally.

Formaldehyde, acetaldehyde, and crotonaldehyde are common microbial metabolites. Isatin was isolated from a marine *Alteromonas* sp.,[23] and diacetyl is one of the odor components of the fungus *Zostera marina*.[24] From bacteria, diacetyl has not been described so far; however, extracts of marine Streptomycetes possessed sometimes a very characteristic buttery smell, pointing to diacetyl, isolate B7064 being a recent example in our group.[25] Although we were not able to prove the presence of this ketone by spectroscopic methods, the typical odor was a strong hint that diacetyl is also a bacterial metabolite. It seems clear therefore that a part of the condensation products is already formed during the life cycle of *Vibrio parahaemolyticus* Bio249; however, workup conditions are also strongly involved in the formation of the different indole oligomers.

Experimental Section

General Experimental Procedures. IR spectra were recorded on a Perkin-Elmer 1600 Series FTIR in KBr pellets. CD spectra were measured on a Jasco J-810 spectropolarimeter in CH_2Cl_2. NMR spectra were measured on Varian Unity 300 (300.145 MHz) and a Varian Inova 500 (499.876 MHz) spectrometer in $CDCl_3$ with TMS as internal standard. EIMS was recorded on a Finnigan MAT95 (70 eV), and perfluorokerosene was used as reference substance in HREIMS. HPLC/ESIMS was recorded on a Finnigan LCQ with a Rheos 4000 (Flux Instrument) quaternary pump and an EC 125/2, 100-5, C 18 nucleosil column (Macherey-Nagel & Co., Düren, Germany). Flash chromatography was carried out on silica gel (30−60 mm, J. T. Baker). R_f values were measured on Polygram SIL G/UV_{254} (Macherey-Nagel & Co., Düren, Germany) with cyclohexane/ethyl acetate, 7:3. Size exclusion chromatography was done on Sephadex LH-20 (Pharmacia). The strain *Vibrio parahaemolyticus* isolate Bio249 is deposited in the culture collection of the Department of Microbiology at the National Research Institute for Biotechnology (GBF) in Braunschweig, Germany.

Casein Medium. Casein (10 g), yeast extract (5 g), NaCl (10 g), and glucose (5 g) were dissolved in 1 L of tap water, and the medium was adjusted to pH 7.8 with 2 N NaOH and sterilized for 33 min at 121 °C. After sterilization, an end pH of 6.5 of the medium was obtained.

M_2 Medium. Malt extract (10 g), yeast extract (4 g), and glucose (4 g) were dissolved in artificial seawater (0.5 L) and tap water (0.5 L). Before sterilization, the pH was adjusted to 7.8 by addition of 2 N NaOH.

Fermentation and Isolation. A 1 cm^2 piece of agar from a 7-day-old culture of *V. parahaemolyticus* isolate Bio249 grown on M_2 medium was used to inoculate 250 mL of casein medium in a 1 L Erlenmeyer flask. One hundred of these cultures were incubated on a rotary shaker (180 rpm) at 30 °C for 4 days. The culture broth was mixed with diatomaceous earth (1 kg) and filtered through a pressure filter. The culture filtrate and the mycelium were separately extracted each with ethyl acetate. The mycelium was additionally extracted with acetone. The combined organic phases were evaporated to dryness to obtain 3.1 g of crude extract. The thus obtained extract was subjected to column chromatography on silica gel with a stepwise CH_2Cl_2/MeOH gradient (0.5 L of CH_2Cl_2, 0.5 L of CH_2Cl_2/2% MeOH, 0.5 L of CH_2Cl_2/5% MeOH, 0.5 L CH_2Cl_2/7.5% MeOH, 0.5 L CH_2Cl_2/10% MeOH, 0.5 L of CH_2Cl_2/15% MeOH, 0.3 L of CH_2Cl_2/20% MeOH, and 0.3 L of CH_2Cl_2/50% MeOH) to deliver eight fractions. Further purification of fraction 1 on Sephadex LH-20 (3 × 60 cm, MeOH) yielded 200 mg of indole (R_f = 0.75), 55 mg of 1,1,3-tris(3-indolyl)butane (**3**, R_f = 0.3), and 3 mg of pharacine[11] (R_f = 0.45). Fraction 2 yielded 6 mg of 2,2-di(3-indolyl)-3-indolone[9] (**5**, R_f = 0.55), 4 mg of vibrindole A[9] (**1c**, R_f = 0.5), 6 mg of tris(3-indolyl)-methane[7] (**2a**, R_f = 0.38), and 5 mg of trisindoline[10] (**4a**, R_f = 0.2). Fractions 3 and 4 yielded 9 mg of 3,3-bis(3-indolyl)butane-2-one (**1a**, R_f = 0.35) and 4 mg of bis(3-indolyl)methane[6] (arundine, **1b**, R_f = 0.48), respectively. Fractions 5 and 6 yielded 10 mg of indole-3-carboxylic acid,[8] 12 mg of indole-3-carbaldehyde,[8] 22 mg of *p*-hydroxyphenylethanol,[12] 13 mg of phenylacetamide,[13] and 40 mg of thymine.

3,3-Bis(3-indolyl)butan-2-one (1a): slightly yellow solid; UV (MeOH) λ_{max} (log ϵ) 274 sh (2.91), 281 (2.94), 289 (2.89); IR (KBr) ν_{max} 3423, 1694, 1636, 1455, 1422, 1339, 1245, 1200, 1111, 745 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 8.15 (br s, 2 NH, H-1′,1″), 7.40 (2 d, J = 7.8 Hz; 2 H, H-4′,4″), 7.38 (2 d, J = 7.8 Hz; 2 H, H-7′,7″), 7.17 (td, J = 7.2, J' = 1.0 Hz; 2 H, H-5′,5″), 7.02 (td, J = 7.2, J' = 1.0 Hz; 2 H, H-6′,6″), 6.94 (d, J = 2.2 Hz; 2 H, H-2′,2″), 2.16 (s, 3 H, COMe), 2.08 (s, 3 H, Me); ^{13}C NMR ($CDCl_3$, 150.8 MHz) δ 209.6 (1-$COCH_3$), 136.8 (C_q-7′a,7″a), 126.1 (C_q-3′a,3″a), 123.0 (CH-2′,2″), 122.0 (CH-6′,6″), 121.4 (CH-4′,4″), 119.5 (CH-5′,5″), 118.8 (C_q-3′,3″), 111.3 (CH-7′,7″), 52.7 (C_q-1), 26.6 ($COCH_3$), 24.8 (1-CH_3); EIMS (70 eV) m/z (%) 302 (2), 259 (100), 142 (8), 115 (6), 77 (4); (−)-ESIMS m/z 301.8 [M − H^+], 325 [M + Na^+]; HREIMS m/z 302.1421 (calcd 302.14191 for $C_{20}H_{18}N_2O$).

1,1,1-Tris(3-indolyl)methane (2a): yellowish solid; UV (MeOH) λ_{max} (log ϵ) 248 sh (3.56), 283 (3.32), 388 (3.65); IR (KBr) ν_{max} 3425, 2924, 2855, 1620, 1482, 1412, 1216, 1127, 747 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.90 (br s, 3 H, H-1′,1″,1‴), 7.48 (d, J = 8.0 Hz, 3 H, H-4′,4″,4‴), 7.36 (d, J = 8.0 Hz, 3 H, H-7′,7″,7‴), 7.18 (td, J = 7.6, J' = 1.0 Hz, 3 H, H-6′,6″,6‴), 6.98 (td, J = 7.6, J' = 0.8 Hz, 3 H, H-5′,5″,5‴), 6.76 (d, J = 2.2 Hz, 3 H, H-2′,2″,2‴), 6.18 (s, 1 H, H-1); ^{13}C NMR ($CDCl_3$, 150.8 MHz) δ 138.5 (C_q-7′a), 128.6 (C_q-3′a), 124.5 (CH-2′), 121.9 (CH-6′), 120.6 (CH-4′), 120.2 (CH-3′), 119.2 (CH-5′), 112.1 (CH-7′), 32.7 (CH-1); all primed atoms are of 3-fold intensities; EIMS (70 eV) m/z (%) 361 (100), 277 (10), 243 (50), 216 (15), 180 (10), 117 (18); HREIMS m/z (%) 361.1577 (calcd 361.15789 for $C_{25}H_{19}N_3$).

1,1,3-Tris(3-indolyl)butane (3): yellowish solid; UV (MeOH) λ_{max} (log ϵ) 276 sh (4.00), 282 (4.03), 290 (3.99); IR (KBr) ν_{max} 3411, 3051, 2924, 1620, 1456, 1419, 1337, 1219, 1094, 1011, 742, 582, 423 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.60−7.43 (m, 4 H, H-1′,1″,1‴, H-4′), 7.43 (d, J = 7.8 Hz, 1 H, H-4″), 7.41 (d, J = 7.8 Hz, 1 H, H-4‴), 7.25−7.05 (m, 6 Ar−H), 7.04−6.90 (m, 3 H, Ar−H), 6.76 (d, J = 2.0 Hz, 1 H, H-2‴), 6.70 (d, J = 2.0 Hz, 1 H, H-2′), 6.65 (d, J = 2.0 Hz, 1 H, H-2″), 4.50 (t, J = 7.8 Hz, 1 H, H-1), 3.00 (m, 1 H, H_3-3), 2.60 (m, 1 H-2_a,2_b), 2.40 (m, 1 H, H_b-2), 1.38 (d, J = 7.0 Hz, 3 H, H_3-4); ^{13}C NMR ($CDCl_3$, 50.3 MHz) δ 136.5, 136.39, 136.37 (C_q-7a′,7a″,7a‴), 126.9 (C_q-3a″3a‴), 126.6 (C_q-3a′), 122.3 (C_q-3′), 121.64, 121.62, 121.58 (CH-6′,6″6‴), 121.48 (CH-2″,2‴), 120.2 (CH-2′), 120.0 (C_q-3‴), 120.0 (C_q-3″), 119.7 (CH-4′), 119.6 (CH-4″, 4‴), 118.9 (CH-5″5‴), 118.8 (CH-5′), 111.13 (CH-7‴), 111.10 (CH-7″), 111.06 (CH-7′), 43.6 (CH_2-2), 31.8 (CH-1), 28.8 (CH-3), 21.8 (CH_3-4); EIMS (70 eV) m/z (%) 403 (40), 286 (10), 258 (25), 245 (100), 217 (10), 145 (40), 117 (15); (−)-ESIMS m/z 402.8 [M − H^+]; (+)-ESIMS m/z 426.7 [M + Na^+]; HREIMS m/z 403.2030 (calcd 403.20484 for $C_{28}H_{25}N_3$).

Biological Tests. All tests were performed as described previously using the agar diffusion method with bacteria (*Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, and *Streptomyces viridochromogenes*) and fungi (*Candida albicans*, *Mucor miehei*) as test organisms.[4]

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