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Biosynthesis of the Marine Antibiotic Pentabromopseudilin. 1. The Benzene Ring

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The biosynthesis of the potent marine antibiotic, pentabromopseudilin (**1**), was investigated in *Alteromonas luteoviolaceus* grown in a complex medium by feeding experiments with ^{13}C-acetate, various amino acids, and differently labeled glucoses. The results show that the benzene ring of **1** originates from the shikimate pathway *via* *p*-hydroxybenzoic acid as a direct precursor. No incorporation of ^{13}C into the pyrrole ring was observed in any of these experiments.

Introduction

The potent marine antibiotic, pentabromopseudilin (**1**), was isolated from *Pseudomonas bromoutilis*[1] and, together with violacein (**2**), from *Chromobacteria*[2] and *Alteromonas luteoviolaceus*.[3,4] Its structure was determined by X-ray crystallography[5] and confirmed by two different syntheses.[3,6] **1** is a stronger antibiotic than penicillin, shows antitumor activity, and is highly phytotoxic. Its mechanism of action was elucidated by structure–activity studies.[7]

The interesting biological properties and the high bromine content (over 70%) combined with a straightforward carbon skeleton for which no obvious precursor exists made **1** a challenging object for biosynthetic investigations. By addition of bromide to the usual culture medium, the production of **1** by *Alteromonas luteoviolaceus* was increased more than 10-fold to 15–25 mg/L, making these studies possible.

Results and Discussion

Due to the few hydrogen atoms present in **1**, the exact assignment of the ^{13}C NMR spectrum was difficult. By an inverse gated spectrum (see supplementary material) the signals of C-3′ and C-5′ could be distinguished, showing a doublet (^2J = 3.3 Hz) for C-3′ (δ = 112.7) and a triplet (^2J = 4.1 Hz) for C-5′ (δ = 110.3). The signal at δ = 151.5 under these conditions appears as a triplet (^3J = 7.8 Hz) with a significantly larger coupling constant. This and the chemical shift identifies it as the signal of C-2′. The three signals in the range of δ = 98–101 belong to C-2–C-4. Only C-3 and C-4 couple with the N*H* (^3J is larger than ^2J), therefore the signal at δ = 100.9 was assigned to C-2. A COLOC spectrum confirmed this result. All other signals could only be distinguished by analysis of spectra of labeled samples of **1**. The signals of C-1′ (δ = 122.0), C4′ (δ = 134.9), C6′ (δ = 133.0), and C-5 (δ = 126.5) were identified by a 2D INADEQUATE spectrum of **1** obtained after feeding of [U-^{13}C]glucose to *Alteromonas luteoviolaceus*. The signals of C-3 and C-4 could only be differentiated with the help of a sample of **1** in which C-1′ and C-3′ were highly labeled (≈85%) by [3,5-$^{13}C_2$]-*p*-hydroxybenzoic acid (**10**). In this sample the pyrrole signal at δ = 100.3 shows a coupling constant of 3.3 Hz, the other at δ = 98.4, one of 2.3 Hz. Therefore the former was assigned tentatively to C-3 and the latter to C-4. With the aid of the ^{13}C NMR spectrum, the ^1H signals could be assigned by a ^1H–^{13}C HETCOR spectrum. The signal for H-4′ is that at δ = 7.78 and the signal for H-6′ that at δ = 7.38.

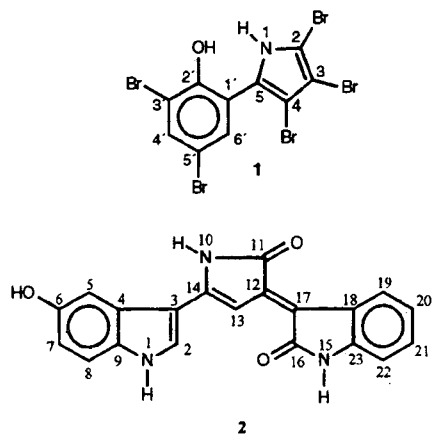

Figure 1.

Feeding Experiments with General Precursors. For many phenols and for the pyrrole verrucarin E,[8] an acetate origin has been established. It was therefore conceivable that **1** is acetate-derived. Feeding of [1-^{13}C]- and [2-^{13}C]acetate yielded no detectable incorporation. [1,2-$^{13}C_2$]Acetate gave insignificant labeling of the benzene ring with unexplainable coupling patterns. A fatty acid isolated was highly labeled, showing that the low enrichment in **1** was not due to a lack of precursor uptake. Therefore an acetate origin of **1** was excluded.

The 3-phenylpyrrole, pyrrolnitrin (**4**), is known to be derived from tryptophan[9] *via* a rearrangement (Scheme 1); **1** could also be formed from tryptophan by an additional

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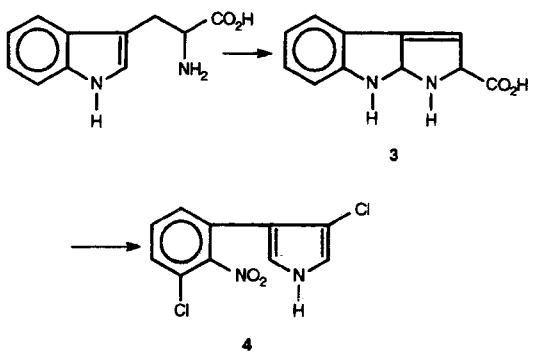
Table 1. Results of the Feeding Experiments for Pentabromopseudilin (1)

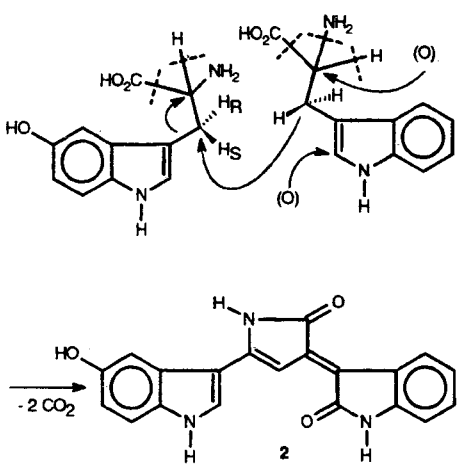
no.	precursor fed[14] (mg)	vol of medium, mL	carbon atoms labeled[a] in 1 (%; calcd accord. to lit.[15]) C-1′	C-2′	C-3′	C-4′	C-5′	C-6′
1	[1-^{13}C]Na-acetate 90% ^{13}C (31 mg + 35 mg after 23.5 h, 27 mg after 44 h)	1000	–	–	–	–	–	–
2	[2-^{13}C]Na-acetate 99% ^{13}C (58 mg + 58 mg after 18 h)	1000	–	–	–	–	–	–
3	[1,2-$^{13}C_2$]Na-acetate 90% ^{13}C (58 mg + 59 mg after 21.5 h)	1000	–	–	–	–	–	–
4	DL-[3-^{13}C]tryptophan 61% ^{13}C (41 mg)	600	–	–	–	–	–	–
5	[1,3-$^{13}C_2$]glycerol 99% ^{13}C (61 mg + 75 mg after 20 h)	1000	–	–	–	–	–	–
6	[*carboxy*-^{13}C]benzoic acid 99% ^{13}C (59 mg + 61 mg after 22 h)	1000	–	–	–	–	–	–
7	[U-$^{13}C_6$]glucose 99% ^{13}C (147 mg)	1000	12.4	10.4	11.5	6.7	2.4	6.7
8	[1-^{13}C]glucose 99% ^{13}C (99 mg)	1000	–	–	–	–	–	–
9	[2-^{13}C]glucose 99% ^{13}C (95 mg)	1000	2.2	–	2.2	–	–	–
10	[1,2-$^{13}C_2$]glucose 99% ^{13}C (100 mg)	1000	2.4	–	2.2	–	–	–
11	[3-^{13}C]glucose 99% ^{13}C (99 mg)	1000	8.9	–	8.8	–	–	–
12	[6-^{13}C]glucose 99% ^{13}C (103 mg)	1000	–	–	–	2.8	–	2.9
13	[2-^{13}C]shikimic acid 99% ^{13}C (49 mg + 49 mg after 20 h)	1000	–	–	–	–	–	–
14	[2-^{13}C]shikimic acid methyl ester 99% ^{13}C (108 mg)	1000	–	–	–	–	–	–
15	*p*-hydroxy-[2,3,5,6-D_4]benzoic acid 99% D (51 mg + 51 mg after 18 h)[b]	1000						
16	*p*-hydroxy-[3,5-$^{13}C_2$]benzoic acid 99% ^{13}C (30 mg + 30 mg after 17.5 h)	800	83.6	–	86.9	–	–	–

[a] The pyrrole ring was always unlabeled. [b] D NMR (DMSO, 46 MHz): δ = 7.35 (s, br, 1 D, D–6′), 7.70 (s, br, 1 D, D-4′).

Scheme 1



Scheme 2



α,β-shift during the biosynthesis. As a similar 1,2-shift occurs during the formation of violacein (**2**)—also produced by *Alteromonas luteoviolaceus*—this hypothesis had to be tested (Scheme 2). Feeding of [3-^{13}C]tryptophan yielded unlabeled **1** and labeled **2**. The labeling and coupling pattern in **2** is as described by Hoshino and Ogasawara,[10] i.e. ^{13}C at C-12 and C-13 (^1J = 54.8 Hz). Therefore tryptophan could be excluded as a precursor of **1**.

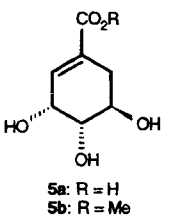


Figure 2.

A feeding experiment with the very general precursor glycerol (fed as [1,3-$^{13}C_2$]glycerol) to obtain information about the proximate precursor(s) of **1** indirectly, by coupling analysis, was unsuccessful, as were those with [*carboxy*-^{13}C]benzoic acid, a possible precursor of the benzene ring and C-5 of the pyrrole ring (Table 1). However, when [U-^{14}C]glucose was fed, a specific incorporation rate of 5.7% into **1** was observed.

Feeding of ^{13}C-Labeled Glucoses and Shikimic Acid (5a). As there is no straightforward possibility to degrade pentabromopseudilin (**1**) in a defined manner, the glucose feeding experiment was repeated with [U-$^{13}C_6$]-glucose. The benzene ring was thereby highly enriched while the pyrrole ring, surprisingly, showed no labeling at all (supplementary material available). The formation of the pyrrole ring obviously is not linked to that of the benzene ring. The labeling of the benzene ring is difficult to interpret. While C-1′ and C-3′ show very high enrichments (12.4 and 11.5%) and are mainly labeled as single carbons (singlets), the also highly labeled C-2′ (10.4%) appears as a strong double doublet, suggesting that it is the centerpiece of a carbon chain. In contrast to this, C-4′ and C-6′ are labeled significantly less (each 6.7%). Both display a strong doublet and should therefore be at the end of a carbon chain. C-5′ would be expected to be the centerpiece of this chain but with an incorporation rate of 2.4% and the presence of only a weak doublet is seems more likely to be derived from glucose as a single carbon. These results show that the benzene ring is carbohydrate-derived but it was not immediately obvious in which way.

Although this labeling pattern does not necessarily implicate the sugar metabolite shikimic acid (**5a**) as a precursor, it was chosen to be fed, as it is a very common precursor of aromatic compounds. But neither [2-^{13}C]-

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Scheme 3

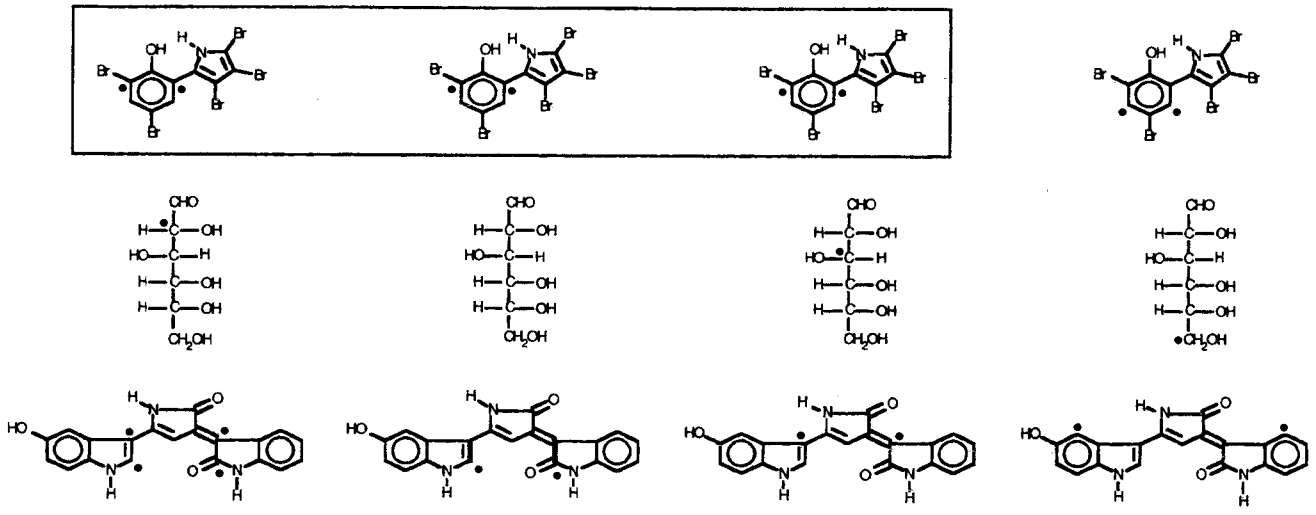


Table 2. Results of the Feeding Experiments for Violacein (2)

	no. of the feeding experiment (as in Table 1)							
	4	8	9	10	11	12	13	14
C-atoms labeled (coupling, Hz)	12, 13 (54.8)	–	2, 3, 16, 17	2, 3 (65.4); 16, 17 (60.0)	3, 8, 17, 22	5, 19	–	–

[a] **2** was not isolated in experiments nos. 1–3, 5–7, 15, and 16.

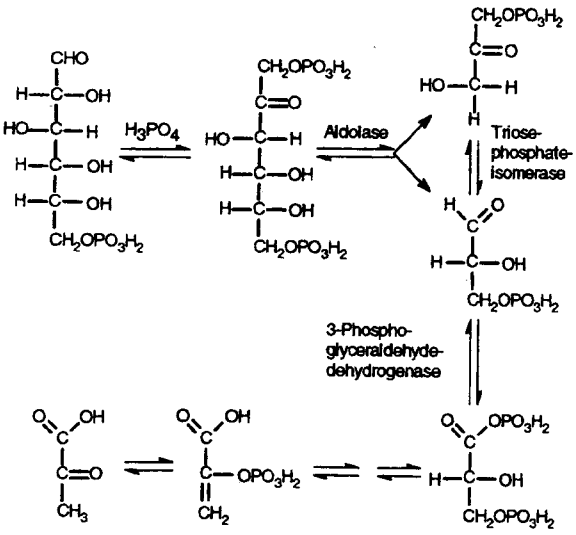
shikimc acid (**5a**) nor its methyl ester (**5b**) were incorporated into **1**. Since violacein (**2**), which in both cases was also isolated, showed no labeling either, it had to be concluded that both the acid and its ester were not transported into the cells. Therefore no conclusions can be drawn from these experiments.

A feeding experiment with [1-^{13}C]glucose showed that C-1 of glucose was, surprisingly, not incorporated into **1** or **2**. This was unexpected as C-1 of glucose is normally converted into C-3 of phosphoenolpyruvate and then into aromatic precursors. The feeding of [2-^{13}C]glucose also gave an unexpected result (Scheme 3). It labeled **1** in *two* positions, at C-1′ and C-3′ (both 2.2%) and **2** at C-2, C-3, C-16, and C-17. The labels at C-2 and C-16 of **2** derive from [1-^{13}C]ribose and those at C-3 and C-17 from [2-^{13}C]-ribose. Both isotopomers of ribose can be formed from [2-^{13}C]glucose. To gain additional information, [1,2-$^{13}C_2$]-glucose was fed giving the same result for **1** (C-1′ 2.4%; C-3′ 2.2%) as the feeding of [2-^{13}C]glucose. [1,2-$^{13}C_2$]-glucose was incorporated into **2** by the same pathways as [2-^{13}C]glucose, this time *via* [1-^{13}C]ribose and [1,2-$^{13}C_2$]-ribose (Table 2). The anthranilic acid-derived parts of **2** were unlabeled in the [1-^{13}C]-, [2-^{13}C]-, and [1,2-$^{13}C_2$]-glucose feeding experiments, suggesting misleadingly that glucose does not label the shikimic acid (**5a**) formed by *A. luteoviolaceus*.

Feeding of [3-^{13}C]glucose gave another unexpected result. Again, **1** was labeled at C-1′ (8.9%) and C-3′ (8.8%) as in the experiments with [2-^{13}C]- and [1,2-$^{13}C_2$]glucose. **2** shows incorporation at C-3, C-8, C-17, and C-22; C-3 and C-17 are labeled *via* [2-^{13}C]ribose while the labels at C-8 and C-22 must derive from [5-^{13}C]shikimic acid and thus from [1-^{13}C]erythrose 4-phosphate (Scheme 4). This is the first labeling result that definitely must be due to incorporation *via* shikimate.

The fact that the parts of violacein (**2**) derived from **5a** were only labeled *via* [1-^{13}C]erythrose 4-phosphate and never *via* phosphoenolpyruvate led to the hypothesis that *A. luteoviolaceus* may lack the enzyme triosephosphate isomerase (TIM). Therefore, in glycolysis it cannot convert

Scheme 4

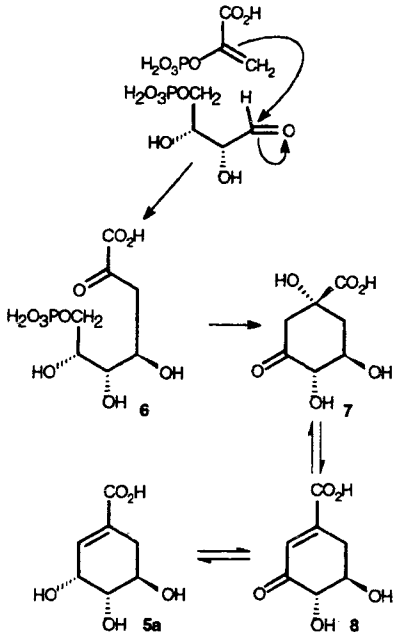


dihydroxyacetone phosphate into glyceraldehyde 3-phosphate and thus not into phosphoenolpyruvate (Scheme 4). If pentabromopseudilin (**1**) is shikimate-derived its labeling *via* [2-^{13}C]-, [1,2-$^{13}C_2$]-, and [3-^{13}C]glucose must, according to this hypothesis, also have occurred *via* erythrose 4-phosphate (Scheme 5).

As all labels in **1** are C_2-symmetrical to an axis through C-2′ and C-5′, the benzene ring should derive from an intermediate also symmetrical to this axis. According to this postulate a single-labeled precursor of the benzene ring would label **1** in two positions. If *A. luteoviolaceus* lacks TIM and if **1** is biosynthesized from a symmetrical shikimate-derived precursor, the labeling in the [2-^{13}C]-, [1,2-$^{13}C_2$]-, and [3-^{13}C]glucose feeding experiments must all be due to [1-^{13}C]erythose 4-phosphate. Indeed, these differently labeled glucoses can all be converted into [1-^{13}C]-erythrose 4-phosphate.

To test this hypothesis [6-^{13}C]glucose was fed. This should be converted into [4-^{13}C]erythrose 4-phosphate and

Scheme 5



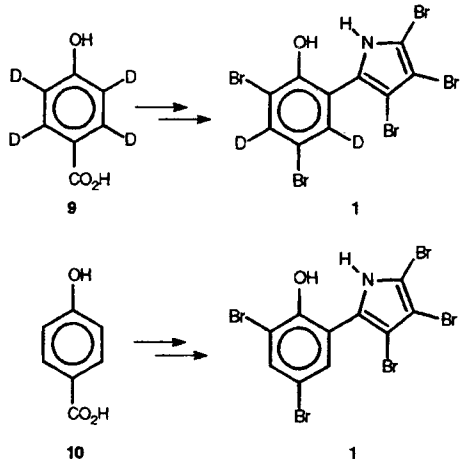
then into [2-^{13}C]shikimic acid (**5a**). **5a** should label **2** at C-5 and C-19 and, *via* symmetrical intermediates, should label C-4′ and C-6′ in **1**. The results were as expected indicating that **1** is indeed shikimate-derived: C-4′ (2.8%) and C-6′ (2.9%) were labeled, again in a symmetrical manner.

Shikimic acid (**5a**) can be converted into several compounds that have the symmetry implicated for the precursor of the benzene ring of pentabromopseudilin (**1**). On mechanistic grounds, *p*-hydroxybenzoic acid is the most plausible candidate. It can be easily transformed into the benzene ring of **1** by decarboxylation, and its phenolic hydroxyl group provides activation for the further substitutions.

A feeding experiment with *p*-hydroxy-[2,3,5,6-D_4]-benzoic acid (**9**) showed that it is efficiently incorporated into the benzene ring of **1**. In the deuterium NMR spectrum broad peaks at $\delta = 7.70$ (D-4′) and $\delta = 7.35$ (D-6′) were visible. The incorporation rate could not be determined as there are no other protons in **1** that could have been used as reference. To lend additional support to this result *p*-hydroxy-[3,5-$^{13}C_2$]benzoic acid (**10**) was isolated from a mixture with [1,3-$^{13}C_2$]salicylic acid obtained by a Kolbe–Schmitt reaction from [2,6-$^{13}C_2$]-phenol. [2,6-$^{13}C_2$]Phenol was prepared from [2,6-$^{13}C_2$]*p*-nitrophenol.[11]

p-Hydroxy-[3,5-$^{13}C_2$]benzoic acid (**10**) was incorporated into **1** in the expected way (Scheme 6) labeling C-1′ (84.6%) and C-3′ (87%). From this result it is obvious that *p*-hydroxybenzoic acid is an immediate precursor of pentabromopseudilin (**1**). These results also explain the coupling patterns observed in the [U-$^{13}C_6$]glucose feeding experiment. C-1′ and C-3′ are mainly labeled as single carbons (singlets), which in both cases is due to the formation of [1-^{13}C]erythrose 4-phosphate. The doublet signal of C-2′ derives from [2,3,4-$^{13}C_3$]erythrose 4-phosphate while its double doublet signal has its origin in [U-$^{13}C_4$]erythrose 4-phosphate. Formation and incorporation of both types of highly labeled erythrose is also

Scheme 6



evident from the double doublet/doublet signal of C-1′ and C-3′ as well as the doublet signals of C-4′ and C-6′. C-5′ derives from C-2 of phosphoenolpyruvate and therefore can only couple with either C-4′ or C-6′; consequently its signal just shows a doublet. The fact that C-4′, C-5′, and C-6′ are considerably less enriched than C-1′, C-2′, and C-3′ must be due the lack of TIM, providing less phosphoenolpyruvate than erythrose from the [U-$^{13}C_6$]glucose.

Conclusions

The above feeding experiments with differently ^{13}C-labeled glucose samples showed that the benzene ring of pentabromopseudilin (**1**) is derived from carbohydrate metabolism. The results for violacein (**2**) and **1** in these experiments indicate that *A. luteoviolaceus* lacks triosephosphate isomerase (TIM) in its glycolysis pathway. Furthermore, they show that shikimic acid (**5a**) is converted *via* a symmetrical intermediate into the benzene ring of **1**. By feeding [2,3,5,6-D_4]- (**9**) and *p*-hydroxy-[3,5-$^{13}C_2$]benzoic acid (**10**) it was demonstrated that *p*-hydroxybenzoic acid is the direct precursor for this moiety (incorporation rate ≈ 85%). The pyrrole ring of **1** was not labeled in any of these experiments and also not in experiments with labeled acetate, tryptophan, benzoic acid, and gycerol.

Experimental Section

***p*-Hydroxy-[3,5-$^{13}C_2$]benzoic Acid (10) and [1,3-$^{13}C_2$]-Salicylic Acid.** A solution of 1.09 g (11.35 mmol) [2,6-$^{13}C_2$]-phenol (99% ^{13}C)[11] and 0.67 g (11.92 mmol) KOH (1.05 equiv) in water was evaporated to dryness and the obtained potassium phenolate powdered. After drying (15 h/0.01 Torr), the salt was heated for 24 h at 190 °C under 5 bar of CO_2. The cooled reaction mixture was dissolved in water, and the acids were precipitated with 3 N HCl and extracted with ether (4 × 100 mL). The combined crude extracts were chromatographed (silica gel; petroleum ether/*tert*-butyl methyl ether, 2:1 + 1% glacial acetic acid) to yield 0.79 g (49.7%) of [1,3-$^{13}C_2$]salicylic acid (99% ^{13}C), mp 159 °C (lit.[12] 158 °C), and 0.16 g (11.3%) of **10** (99% ^{13}C), mp 204 °C (lit.[12] 214–215 °C). **10**: ^{1}H-NMR ([D_6]-DMSO, 300 MHz) $\delta = 6.82$ (ddm), 7.79 (dm), 11.1 (s br); ^{13}C-NMR ([D_6]DMSO, 50 MHz) $\delta = 115.0$ (s, 99% ^{13}C), 121.4 (s), 131.5 (dd), 161.6 (t), 167.2 (s). **[1,3-$^{13}C_2$]Salicylic acid**: ^{1}H-NMR ([D_6]DMSO, 300 MHz) $\delta = 6.92$ (tddd), 6.94 (dddd), 7.50 (ddddd), 7.79 (ddd), 12.20 (s br); ^{13}C-NMR ([D_6]DMSO, 50 MHz)

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δ = 112.9 (s), 117.0 (s), 119.0 (s), 130.4 (dd), 135.6 (dd), 161.4 (t), 172.1 (d).

Culture. *A. luteoviolaceus* NCMB 1893 (National Collections of Industrial and Marine Bacteria Ltd, Aberdeen, Scotland) was kept on agar petri dishes (5 g of Bacto-peptone (Difco), 3 g of yeast-extract (Difco), and 15 g of bacto-agar (Difco) in 750 mL of synthetic seawater[13] and 250 mL of tapwater, pH = 8, autoclaved 15 min at 121 °C) and transferred on a new dish every 3 days.

Fermention/Feeding Experiments. One liter of fermentation medium (5 g of Bacto-peptone (Difco), 3 g of yeast extract (Difco), and 1 g of KBr in 750 mL of synthetic seawater[13] and 250 mL of tapwater, pH = 8, autoclaved for 15 min at 121 °C) in five 500-mL baffeled flasks was inoculated with one agar petri-dish of a 1-d old preculture of *A. luteoviolaceus* and shaken at 185 rpm and 24 °C in the dark. If not stated otherwise, the labeled compounds were added directly after inoculation (dissolved in 10–20 mL of water; added to the solutions of **5a**, **9**, and **10** NaOH, pH = 8). The cultures were harvested after 65–74 h by extraction with ethyl acetate until the organic layer remained colorless. After drying over $MgSO_4$, the solvent was evaporated and the residue adsorbed on 3 g of silica gel. Crude **1** was washed off with 300–450 mL dichloromethane and rechromatographed (silica gel; hexane/ether, 4:1); yield 12–29 mg/L. **2** was then eluted with acetone and rechromatographed on acidic alumina (acetone/methanol, 95:5) to separate it from less-polar deoxyviolacein, dissolved in ethyl acetate, washed with 0.5 N HCl (3 × 100 mL), and then adsorbed on silica gel. The adsorbed **2** was washed with 500 mL of methylene chloride + 0.5% methanol, eluted with acetone, dissolved in ethyl acetate, and washed with water (3 × 100 mL); yield 10–20 mg/L.

NMR Experiments. ^{13}C-NMR spectra were recorded at 75 MHz in [D_6]DMSO. The spectra of **2** were recorded with a standard ^{13}C-program; all spectra of labeled samples of **1** were recorded with a delay of 6.00 s.

Pentabrompseudilin (1): ^{13}C-NMR δ = 151.5 (C-2′), 134.8 (C-4′), 133.0 (C-6′), 126.5 (C-5), 122.0 (C-1′), 112.7 (C-3′), 110.3 (C-5′), 100.8 (C-2), 100.3 (C-3*), 98.4 (C-4*); ^{1}H-NMR δ = 7.38 (d), 7.78 (d), 9.80 (s), 12.60 (s); (*) assignment may be exchanged.

Violacein (**2**): ^{13}C-NMR δ = 96.9, 104.6, 105.7, 108.9, 113.1, 113.3, 118.7, 120.8, 122.4, 125.6, 126.3, 129.3, 129.6, 131.5, 136.9, 141.8, 147.6, 152.9, 170.2, 171.6. The signals of **2** were unequivocally assigned by Hoshino and Ogasawara.[10]

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Supplementary Material Available: ^{13}C-NMR spectrum of pentabromoopseudilin (**1**) (2 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

(13) Synthetic seawater contains the following: 1. Trace-element solution: 0.611 g of H_3BO_3; 0.389 g of $MnCl_2 \cdot 4H_2O$; 0.056 g of $CuSO_4 \cdot 5H_2O$; 0.056 g of $ZnSO_4 \cdot 7H_2O$; 0.119 g of $AlK(SO_4)_2 \cdot 12H_2O$; 0.059 g of $NiSO_4 \cdot 6H_2O$; 0.059 g of $Co(NO_3)_3 \cdot 6H_2O$; 0.059 g of TiO_2; 0.059 g of $(NH_4)_6(Mo_7O_2)_4 \cdot 4H_2O$; 0.028 g of LiCl; 0.028 g of $SnCl_2$; 0.028 g of KI; water *ad* 1 liter. 2. Stock-solution: 55.00 g of KCl; 16.00 g of $NaHCO_3$; 8.00 g of KBr; 3.40 g of $SrCl \cdot 6H_2O$ (dissolve separately); 2.20 g of H_3BO_3; 0.24 g of NaF; 0.16 g of NH_4NO_3; water *ad* 1 liter. 3. Synthetic seawater: 1.000 g of iron ammonium citrate; 194.500 g of NaCl; 88.000 g of $MgCl_2 \cdot 6H_2O$; 32.40 g of Na_2SO_4; 18.00 g of $CaCl_2$; 0.08 g of Na_2HPO_4; 0.075 g of SiO_2; 10 mL of Stock-solution; 100 mL of Trace-element solution; water *ad* 10 liter.

(14) The labeled compounds were obtained from: Isotec Inc. (Miamisburg, Ohio), Cambridge Isotope Laboratory (Cambridge), Omicron Inc. or Sigma (St. Louis); [2-^{13}C]shikimic acid methylester was synthesized according to Meier, R. M.; Tamm, C., *Helv. Chim. Acta* **1991**, *74*, 807 and Falsone, G.; Peters, W. *Liebigs Ann. Chem.* **1978**, 1905.

(15) Scott, A. I.; Townsend, C. A.; Okada, K.; Kajiwara, M.; Cushley, R. J.; Whitman, P. J. *J. Am. Chem. Soc.* **1974**, *96*, 8069. The ^{13}C-signal-intensities of unlabeled **1** shows great variations, therefore the incorporation rates might not be exactly reproducible.