Biosynthesis of the Marine Antibiotic Pentabromopseudilin. 2. The Pyrrole Ring

Jörg D. Peschke, [1] Ulf Hanefeld,[1,2*] and Hartmut Laatsch[1]

[1] *Institut für Organische und Biomolekulare Chemie der Georg-August-Universität, Tammannstr. 2, D-37077 Göttingen, Germany.*

[2] *Department of Chemistry, Campus Box 351700, University of Washington, Seattle, Washington 98195-1700, USA.*

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Dedicated to Prof. H.G. Floss on the occasion of his 70th birthday

The biosynthesis of the potent marine antibiotic, pentabromopseudilin (**1**), was investigated. Feeding studies with *Alteromonas luteoviolaceus* were performed on a defined media. D,L-[5-^{13}C]proline was incorporated symmetrically, demonstrating that the pyrrole ring of pentabromopseudilin derives from proline.

Key words: Pentabromopseudilin, *Alteromonas luteoviolaceus*, biosynthesis, D,L-[5-^{13}C]proline.

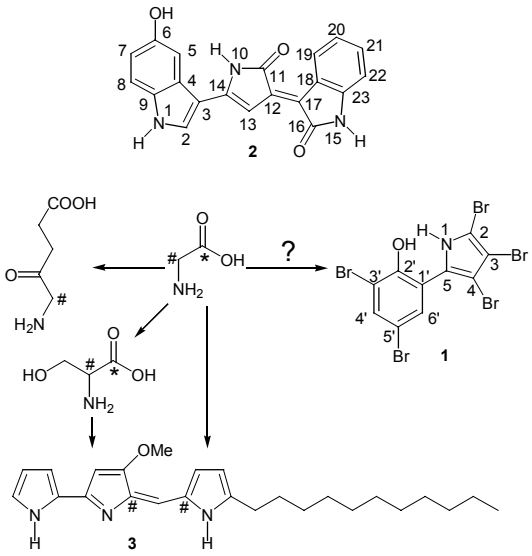
Nature has devised many different biosynthetic pathways towards pyrrole rings. Next to the well-studied aminolaevulinic acid based biosynthesis of the porphyrins, it was shown that in the prodigiosins (**3**) each of the pyrrole rings is build in a different way, starting from pro[1]line, serine, glycine and acetate.[1)] Proline is also the pyrrole precursor in streptopyrrole,[2)] however, the carbonyl group undergoes an unusual rearrangement, similar to the one observed in the biosynthesis of pyralomicin 1a.[3)] Verrucarin E is build up entirely from acetate[4)] while in the case of glycerinopyrin the heterocycle is formed from leucine.[5)] In the biosynthesis of pyrrolnitrin a chemically daunting rearrangement of tryptophan is performed.[6)] Pentabromopseudilin (**1**), a powerful pyrrole based marine antibiotic,[7)] was isolated from *Pseudomonas bromoutilis* and, together with violacein (**2**) from *Chromobacteria* and *Alteromonas luteoviolaceus* (Scheme 1).[8)] Its structure was elucidated by X-ray crystallography and confirmed by three independent syntheses.[9-11)] The biosynthesis of violacein (**2**) has been elucidated, and it was shown, that it is derives from tryptophan.[12)]

Two structural features of **1** are unusual: the straightforward carbon framework and its extremely high content of bromine (over 70%) combined with a high biological activity. Although the carbon framework is simple, there is no obvious biosynthetic route that should lead to this compound. In previous studies with *Alteromonas luteoviolaceus* it was shown that the benzene moiety of **1** derives from the shikimic acid pathway and that *p*-hydroxy benzoic acid (**4**) is its direct precursor.[13)] In those studies it was also found that neither [U-^{13}C]glucose, labelled acetate, tryptophan or glycerol labelled the pyrrole ring, thereby ruling out a biosynthesis similar to the one of verrucarin

[1] *Corresponding author: Current address: Gebouw voor Scheikunde, Technische Universiteit Delft, Julianalaan 136, 2628 BL Delft, The Netherlands. Fax: +31 15 278 4289; Tel: +31 15 278 9304; E-mail: u.hanefeld@tnw.tudelft.nl. [1]

E or to that of pyrrolnitrin. Thus the pyrrole ring of **1** is posing a formidable challenge for biosynthetic research.

Based on the fact that glycine is a biosynthetic precursor of amino-laevulinic acid and of the prodigiosins, both directly



Scheme 1: Pentabromopseudilin and violacein; proposed glycine based biosynthesis of pentabromopseudilin.

and *via* its conversion to serine, a feeding experiment with [1,2-^{13}C]glycine was performed (Scheme 1). Unexpectedly, C1', C2' and C3' as well as C5' of **1** showed enrichments of 1-2%. Furthermore a coupling between C1' and C2' (^{1}J = 68 Hz) indicated an intact conversion of glycine *via* serine and phosphoenolpyruvate into the sugar metabolism and shikimate. The conversion of glycine into serine was also confirmed by the labelling (approx. 1 %) of C11 and C14 of **2**, however, no labelling of the pyrrole ring of **1** was detected.

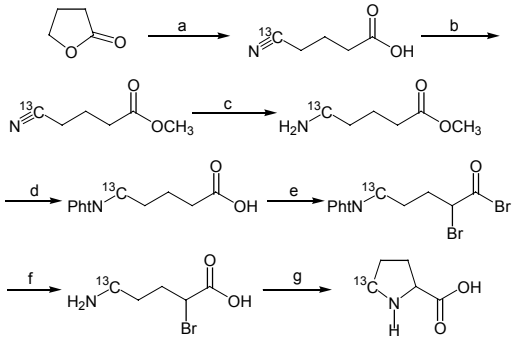
While L-[U-^{14}C]proline afforded an incorporation of 4.9 %, L-[U-^{13}C]proline was, surprisingly, not incorporated into the pyrrole ring of **1**. Instead, C4' and C6' showed significant enrichments and both showed a coupling as did C5' ($^{1}J_{C4'-C5'}$ = 63 Hz; $^{1}J_{C5'-C6'}$ = 64 Hz; no ^{2}J visible). A fatty acid that was also isolated clearly showed the labelling pattern derived from [1,2-^{13}C]acetate. This strongly suggests that proline is degraded and reused *via* the primary metabolism. Similarly, neither D,L-[5-^{13}C]ornithine[14] nor [1,4-^{13}C] putrescine[15,16] were utilised for the formation of the pyrrole ring of **1**. In order to rule out any disturbances from the complex medium an approach based on limited media was developed.

A mixture of L-[U-^{13}C]amino acids[17] as the only carbon source afforded a pentabromopseudilin (**1**) where strong C,C couplings indicated the incorporation of an intact C_4 unit into the pyrrole ring. On a yeast extract/bacto peptone medium this mixture of uniformly ^{13}C labelled amino acids was incorporated only into the benzene ring (up to 9 % with the typical coupling patterns of a symmetrical shikimic acid derivative, namely **4**) but not into the pyrrole ring of **1**. Obviously the complex medium contained a late precursor of the biosynthesis of the pyrrole ring.

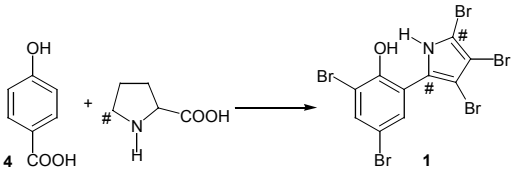
Alteromonas luteoviolaceus also grew well on a defined medium, consisting solely of glycine and glucose. Previous feeding experiments had shown that neither compound is a direct precursor of the pyrrole ring of **1**, so it was not surprising that under these conditions no antibiotic was formed. Upon addition of proline or 4-hydroxyproline to this second medium, however, **1** was formed again. Proline was therefore reconsidered as a possible precursor of the pyrrole ring. In order to trace the incorporation of proline D,L-[5-^{13}C]proline was synthesised based on a modified literature procedure.[18] By integrating several steps (a and b; e,f and g; Scheme 2) it could be improved significantly. Starting from γ-butyrolactone and [^{13}C]-cyanide crude γ-cyanobutyrate was obtained and directly converted into its methyl ester. Subsequently the nitrile group was reduced catalytically. The amino group was protected with phthalic anhydride, while saponifying the ester at the same time, again improving on the original synthesis. Bromination in the α-position, followed by deprotection of the amino group and base catalysed cyclisation gave the desired D,L-[5-^{13}C]proline in seven steps with an overall yield of 55 %.

In a feeding study with 2 l of a defined medium consisting of D,L-[5-^{13}C]proline, L-tyrosine, L-histidine, L-ornithine, glycine and KBr 16 mg of pentabromopseudilin **1** were obtained after

shaking the *Alteromonas luteoviolaceus* cultures at 24 ºC for 72 h. **1** was indeed labelled in the pyrrole ring at C2 and C5, with 30% ^{13}C each (Scheme 3).[19] The overall enrichment of **1** with 60 % demonstrates that proline is converted almost directly into the pyrrole ring. The fact that two carbon atoms, C2 and C5, are equally ^{13}C enriched clearly indicates that at least one intermediate on the biosynthetic pathway from proline to **1** must be symmetrical. This is not the case for the proline-based pyrrole biosyntheses described earlier. It therefore indicates that the proline based formation of the pyrrole ring of **1** proceeds *via* a unique pathway.



Scheme 2: Synthesis of D,L-[5-^{13}C]proline.
Reagents and conditions: a,b) $K^{13}CN$, 185 ºC, 55 min, then reflux in ether, filter off, redissolve in acetone, add MeI, reflux 19 h, 90 % of the ester over 2 steps; c) H_2, Pd/C, MeOH, conc. HCl, 24 h, 78 %; d) Phthalic acid anhydride, 190 ºC, 5 h, 92 %; e,f,g) Br_2, PBr_3 65 ºC, 11 h, filter off, then add conc. HCl and reflux 5 h, cool, filter off, add 20 % aqueous KOH, reflux, 85 % D,L-[5-^{13}C]proline over 3 steps.



Scheme 3: Biosynthesis of pentabromopseudilin from **4** and proline.

As the bacto peptone was shown to contain high concentrations of 4-hydroxyproline (explaining the unsuccessful feeding experiments on complex medium) and bacteria are known to be able to transform proline *via* 4-hydroxyproline into pyrrole-2-carboxylic acid, this, too, was fed. However, neither pyrrole-2-carboxylic acid nor pyrrole itself could induce the formation of **1** by *Alteromonas luteoviolaceus* on the glycine/glucose medium. This suggests that although 4-hydroxyproline is very close to the final, symmetrical biosynthetic precursor of **1,** the aromaticity might only be introduced once the carbon framework of **1** is assembled. Further details are still under investigation.

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