The Biosynthesis of Valanimycin. Further Evidence for the Intermediacy of a Hydroxylamine in N–N Bond Formation

Ronald J. Parry* and Wenying Li

Department of Chemistry, Rice University, Houston, Texas 77251, USA

New evidence supporting the hypothesis that the biosynthesis of the N–N bond of the antibiotic valanimycin involves the reaction of a hydroxylamine with an amine to give a hydrazine is obtained by partial purification of a hydroxylamine-forming enzyme and by successful incorporation experiments with a postulated hydrazine intermediate.

Compounds containing N–N bonds constitute a widespread and diverse group of natural products.[1] The naturally occurring azoxy compounds represent a growing class of compounds of this type. The antibiotic valanimycin **1** (Scheme 1) is an azoxy compound produced by *Streptomyces viridifaciens* MG456-hF10. In addition to antibacterial activity, valanimycin exhibits potent antitumour activity against L1210 and P388 mouse leukaemia cells.[2] Natural products related to valanimycin include the cycad toxins macrozamin and cycasin,[3] the carcinogen elaiomycin,[4] the antifungal agents LL-BH872α[5] and maniwamycins A and B[6] and the nematocidal compounds jietacins A and B.[7]

Recent investigations of valanimycin biosynthesis have established that the primary precursors of this antibiotic are serine and valine, with the β-nitrogen atom of the antibiotic being derived from serine, and the α-nitrogen atom being derived from valine *via* the intermediacy of isobutylamine.[8] Precursor incorporation experiments with labelled isobutylhydroxylamine also supported the hypothesis[9] that N–N bond formation involves the reaction of a hydroxylamine with an amine to yield a hydrazine. We now present evidence that provides additional support for this hypothesis.

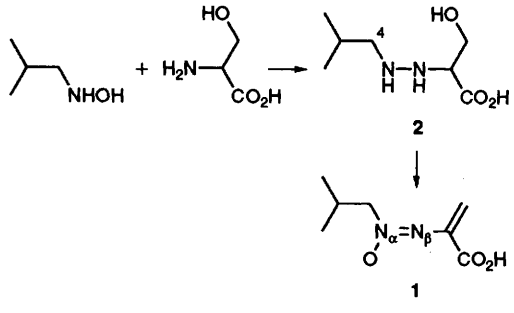
Cell-free extracts were prepared by sonication of 24 h old cultures of *S. viridifaciens* in phosphate buffer A,† nucleic acids were removed with polymin P, and the proteins fractionally precipitated with ammonium sulfate. The proteins precipitating at 40–70% ammonium sulfate saturation were redissolved in phosphate buffer, dialysed, and chromatographed on phenylagarose. The fraction eluting with phosphate buffer B‡ was concentrated, dialysed, and then incubated at 30 °C with isobutylamine and NADH and FAD. At the end of the incubation period, proteins were precipitated with trichloroacetic acid and the supernatant analysed for the presence of isobutylhydroxylamine using the spectrophotometric assay for hydroxylamines developed by Belanger *et al.*[10] The formation of isobutylhydroxylamine was dependent on the presence of *both* FAD and NADH, while incubations with NADPH and FAD displayed approximately half the activity observed with NADH. No isobutylhydroxylamine formation was detected in incubations lacking isobutylamine or in incubations in which the protein had been removed by filtration through a filter with a 10000 mass cutoff. The formation of isobutylhydroxylamine in the active extracts was confirmed by a high-resolution MS analysis of the residue obtained by evaporation of the deproteinized extract (calc. 89.0841. Found M, 89.0842). The substrate specificity of the hydroxylamine forming enzyme was examined using the spectrophotometric assay. The results (Table 1) indicate that isobutylamine is the best substrate of those examined: amines with short carbon chains are poor substrates (entries 2 and 3), while amines with sidechains larger than isobutylamine show diminished activity (entries 8–10). The fact that isobutylamine is the most active substrate suggests that the observed enzymatic activity is very likely to be associated with the valanimycin biosynthetic pathway. The NADH and FAD dependence of the activity suggests that the enzyme may be related to other flavoprotein monooxygenases, such as lysine N^6-hydroxylase.[11]

If the biosynthesis of valanimycin proceeds by the reaction of isobutylhydroxylamine with serine to yield a hydrazine, then the hydrazino acid **2** would be expected to be an intermediate (Scheme 1). Evidence for the intermediacy of **2** was obtained by precursor incorporation experiments. The unlabelled hydrazino acid was synthesized in racemic form from ethyl 2,3-dibromopropionate *via* hydrazinoserine by the route shown in Scheme 2.[12] The synthesis was then modified to yield two labelled forms of **2**. Reduction of (1-^{13}C)isobutyronitrile[8] with diisobutylaluminium hydride gave (1-^{13}C)isobutyraldehyde which was then converted into (4-^{13}C)-**2**. The use of ($^{15}N_2$)hydrazine in the synthesis yielded a sample

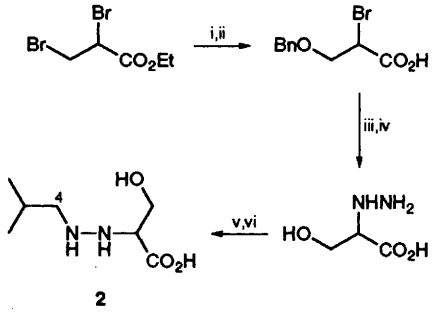
Table 1 Relative activity of aliphatic amines with hydroxylamine-forming enzyme[a]

Entry	Substrate	Relative activity[b]
1	Isobutylamine	1.0
2	Methylamine	0.0
3	Ethylamine	0.20
4	Propylamine	0.89
5	Isopropylamine	0.33
6	(*R,S*)-*sec*-Butylamine	0.75
7	Butylamine	0.70
8	Pentylamine	0.37
9	(*R,S*)-2-Methylbutylamine	0.46
10	Isopentylamine	0.37

[a] Incubations were carried out with shaking for two hours at 30 °C in 200 μl of 100 mmol dm^{-3} sodium phosphate buffer, pH 7.6, containing NADH (2 mmol dm^{-3}), FAD 5 μmol dm^{-3}), amine (10 mmol dm^{-3}), and enzyme (50 μl). Hydroxylamine concentration was determined by the method of Belanger *et al.*[10] [b] The relative activities are an average of three determinations.



Scheme 1 Hypothetical pathway to valanimycin



Scheme 2 *Reagents and conditions*: i, NaOBn; ii, NaOH; iii, N_2H_4; iv, H_2, Pd/C; v, Me_2CHCHO; vi, $NaBH_3CN$

of **2** containing a double ^{15}N-label. Each of these forms of labelled **2** was then administered to washed cells[8] of *S. viridifaciens* and the resulting valanimycin isolated as its ammonia adduct.[8] The ammonia adduct of the valanimycin produced from (4-^{13}C)-**2** exhibited *ca.* 1.1% ^{13}C enrichment at C-4, suggesting that this precursor had been specifically incorporated. Additional evidence in support of this conclusion was provided by administration of ^{15}N-labelled **2**. The valanimycin ammonia adduct obtained from valanimycin formed from ($^{15}N_2$)-**2** exhibited two doublets in the ^{15}N NMR spectrum at δ 345.5 and 347.9 (J_{NN} 14 Hz), with the ^{15}N enrichment at each position being *ca.* 0.7%. The labelling pattern from ^{15}N-labelled **2** indicates that this compound has been incorporated into valanimycin without cleavage of the N–N bond, and, in combination with the results obtained with (4-^{13}C)-**2**, provides strong evidence for the intact incorporation of **2** into valanimycin.§

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Footnotes

† Buffer A consists of sodium phosphate (50 mmol dm^{-3}) H_4edta (1 mmol dm^{-3}) benzamidine (1 mmol dm^{-3}), phenylmethylsulfonyl fluoride (PMSF) (1 mmol dm^{-3}), and 15% glycerol, pH 7.0.

‡ Buffer B has the same composition as buffer A, but lacks PMSF.

§ The fact that **2** is a poorer precursor of valanimycin than either isobutylamine or isobutylhydroxylamine (see ref. 8) may in part be due to the fact that **2** is racemic. Another factor, which may be involved, is less efficient transport of **2** to the site of valanimycin biosynthesis.

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