Phenazine and Phenoxazinone Biosynthesis in *Brevibacterium iodinum*

R. B. Herbert and J. Mann

Department of Organic Chemistry, The University, Leeds LS2 9JT, England

A. Römer

Institut für Organische Chemie der Universität, Greinstr. 4, D-5000 Köln 41, Bundesrepublik Deutschland

The source of nitrogen in the phenazine, iodinin **5**, and in 2-aminophenoxazinone **7** in *Brevibacterium iodinum* cultures is shown to be primarily from the amide nitrogen of glutamine in experiments with (S)-[CO^{15}NH$_2$]glutamine, [^{15}N]ammonium sulphate, and (S)-[^{15}N]glutamic acid. The biosynthesis of **5** and **7** is closely related; biosynthesis of **7** like that of **5** diverts from the shikimic acid pathway at a stage prior to anthranilic acid **8**.

Shikimic acid **1** is an early key intermediate in the biosynthesis of microbial phenazines, *e.g.* iodinin **5** [1, 2]. In the particular case of iodinin **5**, in *Brevibacterium iodinum*, it has been proved that the phenazine nucleus is formed from two molecules of shikimic acid **1** and the carbon atoms in **5** to which hydroxy-groups are attached correspond to the atom which bears a carboxy-group in **1** [2]. It appears that phenazine-1,6-dicarboxylic acid **4** is the first phenazine to be formed and is the one from which all the others derive [3, 16]. Evidence obtained with bacterial mutants indicates that biosynthesis along the shikimic acid pathway leading to tryptophan probably diverts to phenazine production between chorismic acid **2** and anthranilic acid **8** [4]. Anthranilic acid **8**, a further intermediate on the biosynthetic route to tryptophan and an attractive possible source of the phenazine ring system, is not a phenazine precursor [1], nor is 3-aminobenzoic acid [5]. It follows from the evidence obtained that some aminated derivative related to shikimic acid **1** or chorismic acid **2** is probably involved in the formation of these heterocycles. A clue to the nature of this intermediate is provided by the natural occurrence of **9** [6], oryzoxymycin **10** [7] (2S,3S)-2,3-dihydro-3-hydroxyanthranilic acid **11** [8], and isochorismic acid **12** [9]. Isochorismic acid **12** is known to derive from chorismic acid **2** [9] and it seems probable that **9**, **10**, and **11** are formed by amination of chorismic acid

with **3** as a likely common intermediate. This hypothetical intermediate could also give rise on the one hand to anthranilic acid [10, 11] and on the other to phenazines. (2S,3S)-2,3-Dihydro-3-hydroxyanthranilic acid **11** itself has been found not to act as a phenazine precursor [1]. To throw light on the nature of the aminated intermediate we have investigated the source of the two nitrogen atoms in iodinin **5** biosynthesis in *B. iodinum*.

[^{14}C]Shikimic acid **1** is very efficiently incorporated (up to 75% in experiments reported here) if it is administered to *B. iodinum* cultures during late log-phase growth commencing when phenazine production has just begun. Clearly during the feeding period shikimic acid is used primarily for phenazine production. Other precursors should be used similarly.

Incorporation of (S)-[CO^{15}NH$_2$]glutamine into iodinin **5** was observed to occur at a level similar to that of [^{14}C]shikimic acid (Table I; Experiments I, VII, VIII and IX). Further, (S)-[CO^{15}NH$_2$]glutamine was a significantly better source of phenazine nitrogen than was [^{15}N]ammonium sulphate; label from (S)-[^{15}N]glutamic acid was incorporated at a level lower than that of [^{15}N]ammonium sulphate (Table I). Significant dilabelling of iodinin by (S)-[CO^{15}NH$_2$]glutamine proves that both phenazine nitrogen atoms can derive from the amide nitrogen of this amino-acid.

A striking, and consistent, feature of our results was that (S)-[CO^{15}NH$_2$]glutamine gave rise to a greater than statistical amount of dilabelled iodinin
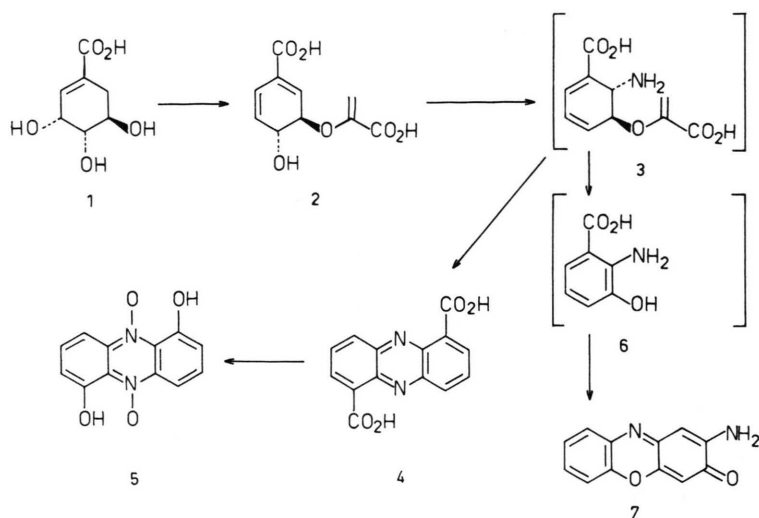
species: roughly half as much as monolabelled species (Table I). In contrast [15N]ammonium sulphate gave rise to iodinin with a very low abundance of dilabelled species even when the amount of monolabel present in the iodinin was similar to that derived from [15N]glutamine. From the differing results obtained with the two precursors it may be concluded that the 15N-label from the glutamine fed, in contrast to that from ammonium sulphate, must for some time swamp the endogenous nitrogen source, or sources, immediately available for phenazine biosynthesis. Arguably the differing results could arise if there are two immediate sources both derived from glutamine but only one derived from ammonium sulphate, or if glutamine is more efficiently transferred than ammonium sulphate to the site of biosynthesis and more rapidly utilized. Further ex-

periments are in hand which are designed to discover why similar levels of monolabelled species could be observed with both precursors.

The manner of iodinin labelling by [15N]-labelled glutamine and ammonium sulphate, and the ob-

Table I. Incorporation of [15N]-labelled precursors into iodinin (5). Average values of several experiments.

Precursor	Excess atom [%]	15N$_1$ [%]	15N$_2$ [%]
[15N]Glutamine	10.8	11.7	5.0
[15N]Glutamine + Ammonium sulphate	10.8	11.6	5.0
[15N]Ammonium sulphate	5.8	11.0	0.3
[15N]Ammonium sulphate + Glutamine	4.6	9.0	0
[15N]Glutamic acid	2.8	5.5	0.1
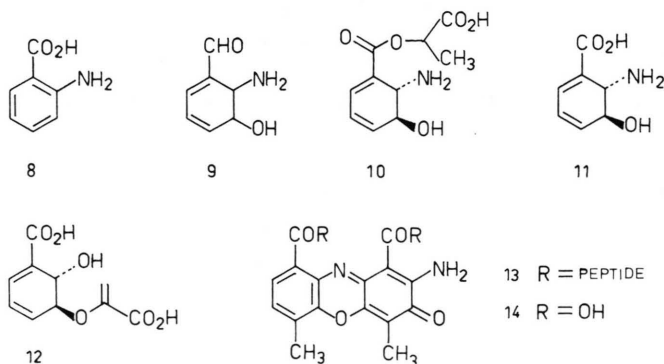
servation that glutamine is a better iodinin precursor than is ammonium sulphate, indicates clearly that glutamine is the primary source of nitrogen in phenazine biosynthesis. In support we found that added unlabelled glutamine reduced the extent of [^{15}N]ammonium sulphate incorporation slightly but unlabelled ammonium sulphate had essentially no effect on [CO^{15}NH$_2$]glutamine incorporation (Table I; Experiments III through VI).

Anthranilic acid **8** is biosynthesized from chorismic acid **2** with the amide nitrogen of glutamine providing the amino group of **8**. The reaction is mediated by anthranilate synthase (EC 4.1.3.27). This enzyme isolated from several bacteria has been investigated in detail [11]. In general, it is an aggregate of at least two non-identical protein chains designated AS I (anthranilate synthase component I) and AS II (anthranilate synthase component II); other enzymes of the pathway from chorismic acid to tryptophan may also be part of the aggregate. AS II binds glutamine and delivers nitrogen to AS I which binds chorismic acid **2** and carries out the reaction: chorismic acid **2** + NH$_3$ → anthranilic acid **8** + pyruvic acid. Ammonia may substitute *in vitro* for glutamine, being operative with AS I alone for the synthesis of anthranilic acid. Our results for iodinin biosynthesis from [^{15}N]-labelled glutamine and ammonium sulphate are consistent with a similar biosynthetic pathway involving enzymes related to AS I and AS II. It appears from the competition experiments with glutamine and ammonium sulphate, although these are complicated by the more rapid use of glutamine relative to ammonium sulphate, that there is some independence in the incorporation of the two nitrogen sources. Independent use of ammonium ion could occur directly with the analogue of AS I.

We conclude tentatively that the enzymes involved in the amination of chorismic acid **2** (or related compound) leading to the secondary metabolites, the phenazines, and the enzymes, AS I and AS II, involved in the biosynthesis of the primary metabolite, anthranilic acid **8**, are similar but distinct. Analogy for this conclusion may be found in the detailed observations on the biosynthesis of fatty acids and of the secondary metabolite, 6-methylsalicylic acid, in *Penicillium patulum* [12]: The multienzyme complex responsible for 6-methylsalicylic acid biosynthesis is similar to, but distinct from, that involved in fatty acid biosynthesis and the same substrates and co-

factors are used. We note the observations in some bacteria that the amidotransferase (AS II) of anthranilate synthase is also part of the enzyme complex involved in the amination of chorismic acid **2** which yields the primary metabolite, *p*-aminobenzoic acid [13].

B. iodinum cultures produce, in addition to iodinin, very small amounts of 2-aminophenoxazinone **7** [14]. The biosynthesis of the structurally related metabolite, actinomycin **13**, has been studied in *Streptomyces antibioticus* [15]. Actinomycin **13** has been found to derive *via* tryptophan. A later intermediate is 3-hydroxy-4-methylanthranilic acid (as **6**), oxidative dimerization of which gives actinocinin **14**. We find that 2-aminophenoxazinone **7** does not derive in *B. iodinum* from tryptophan. In an experiment with (S)-[5-^{3}H]tryptophan and D-[2,3,4,5(*n*)-^{14}C]shikimic acid, the latter was found to be a much better, if not exclusive, precursor for **7** (Experiments VII and VIII). Moreover the incorporation of [^{14}C]shikimic acid was not decreased in the presence of a large excess of (R,S)-tryptophan (Experiment IX). [^{3}H]Anthranilic acid was not incorporated (Experiment X). It follows that 2-aminophenoxazinone **7** derives from the shikimic acid pathway diverting from it at a point prior to anthranilic acid. The level (excess atom %) of 2-aminophenoxazinone **7** labelling from the [^{15}N]-labelled precursors was notably similar to that of iodinin **5** (Table II). This suggests that there is a close biosynthetic relationship in *B. iodinum* between phenazine and phenoxazinone biosynthesis and that there is possibly a common intermediate in the biosynthesis of both metabolites, which could be **3** referred to above as a likely intermediate in the biosynthesis of several metabolites.

Table II. Incorporation of [^{15}N]-labelled precursors into 2-aminophenoxazinone (**7**). Average values of several experiments. Location of ^{15}N is not known.

Precursor	Excess atom [%]	^{15}N$_1$ [%]	^{15}N$_2$ [%]
[^{15}N]Glutamine	12.9	23.1	1.4
[^{15}N]Glutamine + ammonium sulphate	12.7	21.5	2.0
[^{15}N]Ammonium sulphate	5.1	9.4	0.4
[^{15}N]Ammonium sulphate + Glutamine[a]	~6.5	~8.0	~1.7
[^{15}N]Glutamic acid[a]	~6.8	~5.9	~3.8

[a] Uncertain values due to tiny amounts of **7** being produced.

Possible biosynthetic pathways to phenazine-1,6-dicarboxylic acid **4** and iodinin **5**, and to 2-aminophenoxazinone **7** consistent with available data are illustrated in the Scheme.

The 2-aminophenoxazinone **7** formed in the experiment with [$CO^{15}NH_2$]glutamine contained more monolabelled and less dilabelled species than the iodinin, which may be attributed to mixing of labelled with unlabelled species at a stage prior to phenoxazinone formation. Under the conditions of the experiments no exchange of nitrogen occurs in **7**.

Experimental

General

Brevibacterium iodinum was cultured as described previously [1a]. D-[2,3,4,5(n)-^{14}C]Shikimic acid (84 mCi mmol^{-1}) and (S)-[5-^{3}H]tryptophan (24 mCi mmol^{-1}) were purchased from the Radiochemical Centre, Amersham, England. [^{3}H]Anthranilic acid was as used previously [1a]. Radioactivity was measured with a Packard 300 CD scintillation counter. ^{15}N labelled compounds with an isotopic enrichment of 95% were purchased from Rohstoffeinfuhr, Düsseldorf, Germany.

Administration of precursors

All compounds, unless otherwise stated, were added to the growing cultures of *B. iodinum* in aqueous solutions in four to five batches over a six to nine hour period commencing when iodinin production was just apparent (CHCl$_3$ extract). Metabolites were isolated after a further 24 h.

Isolation of metabolites

The cultures were extracted with an equal volume of chloroform. Drying of this extract (MgSO$_4$) and evaporation to low volume gave crystalline iodinin (approx. 50 mg l^{-1}) which was collected and recrystallized several times (CHCl$_3$) before analysis; radioactive samples were crystallized to constant activity.

The mother liquors of the above extract were subjected to preparative t.l.c. (Merck precoated plate, Kieselgel 60 F 254, 0.25 mm, 20 cm by 20 cm; thrice eluted with 3% MeOH in CHCl$_3$ with 5 drops HCO$_2$H per 100 ml). The 2-aminophenoxazinone was isolated and further purified by h.p.l.c. (Varian

5000, Micropak Si 10 column, 30−50% EtOAc in C$_6$H$_{14}$ and containing 0.2% isopropanol, detection by UV). The amount of 2-aminophenoxazinone was estimated from the h.p.l.c. peak area (approx. 8 µg l^{-1}).

Estimation of ^{15}N incorporation

Mass spectra were obtained using a Varian MAT 212 and an SS 200 data system. Measurements were carried out with the following settings throughout: emission 1 mA, filter 3000, and electron multiplier 1 · 7 KV. The amount of ^{15}N in iodinin and 2-aminophenoxazinone were measured mass spectrometrically by determination of the peak intensities of the molecular ions (iodinin m/e 244 to 246 and 2-aminophenoxazinone m/e 212 to 214). The mass ranges from m/e 220−250 and m/e 190−220 were chosen, respectively, when scanning over the peak groups. The average of the intensities of 24 scans were used. Unlabelled iodinin and 2-aminophenoxazinone isolated from *B. iodinum* cultures were used as standards. In a test experiment the amount of ^{15}N-labelled iodinin was determined using high resolution mass spectrometry (Varian MAT 731). At a resolution of 30 000 the peak intensities of C$_{12}$H$_8$^{14}N^{15}NO$_4$ and C$_{11}$^{13}CH$_8$^{14}N$_2$O$_4$ were separately determined. Both methods gave the same results.

Results of feeding experiments with B. iodinum

The results given under the same Roman numeral were obtained with the same batch of cultures.

Estimation of the weight of 2-aminophenoxazinone produced was not reliable enough for the calculation of label recovery or specific activity.

In the following, Gln = glutamine, NH$_4$ = (NH$_4$)$_2$SO$_4$, Glu = glutamic acid, [^{15}N]Gln = [$CO^{15}NH_2$]glutamine, SA = shikimic acid, Trp = tryptophan.

Experiment I: [^{15}N]Gln (86 µg atom ^{15}N) plus [^{14}C]SA (5 µCi): **5** (18 mg), 28.8% ^{15}N recovered, 16.9 excess atom %, 16.1% ^{15}N$_1$, 8.9% ^{15}N$_2$, 74.5% ^{14}C incorporation.

Experiment III: A, [^{15}N]NH$_4$ (289 µg atom ^{15}N) plus Gln (487 µmol): **5** (24 mg), 3.2% ^{15}N recovered, 4.7 excess atom %, 9.3% ^{15}N$_1$, 0% ^{15}N$_2$; B, [^{15}N]Gln (89 µg atom ^{15}N): **5** (26 mg), 14.3% ^{15}N recovered, 6.0 excess atom %, 6.4% ^{15}N$_1$, 2.8% ^{15}N$_2$; C, [^{15}N]NH$_4$ (281 µg atom ^{15}N) plus [^{14}C]SA (1 µCi): **5** (23 mg), 3.4% ^{15}N recovered, 5.1 excess atom %, 9.3% ^{15}N$_1$, 0.4% ^{15}N$_2$, 13.9% ^{14}C incorporation.

Experiment IV: A, [^{15}N]NH$_4$ (284 µg atom ^{15}N) plus [^{14}C]SA (1 µCi): **5** (14.5 mg), 3.0% ^{15}N recovered, 7.2 excess atom %, 13.9% ^{15}N$_1$, 0.3% ^{15}N$_2$, 59.2% ^{14}C incorporation; **7**, 4.8 excess atom %, 9.1% ^{15}N$_1$, 0.2% ^{15}N$_2$; B, [^{15}N]NH$_4$ (284 µg atom ^{15}N) plus Gln (479 µmol, added at the beginning of the experiment): **5** (15 mg), 1.2% ^{15}N recovered, 2.7 excess atom %, 5.3% ^{15}N$_1$, 0% ^{15}N$_2$; **7**, approx. 7% ^{15}N$_1$; C, [^{15}N]Gln (97 µg atom ^{15}N): **5** (14 mg), 12.9% ^{15}N recovered, 11.0 excess atom %, 12.1% ^{15}N$_1$, 5.0% ^{15}N$_2$; **7**, approx. 7.5% ^{15}N$_1$.

Experiment V: A, [^{15}N]NH$_4$ (340 µg atom ^{15}N) plus Gln (538 µmol): **5** (16 mg), 1.8% ^{15}N recovered, 4.7 excess atom %, 9.4% ^{15}N$_1$, 0% ^{15}N$_2$; **7**, approx. 7.4% ^{15}N$_1$; B, [^{15}N]NH$_4$ (339 µg atom ^{15}N): **5** (18 mg), 3.4% ^{15}N recovered, 7.8 excess atom %, 14.9% ^{15}N$_1$, 0.4% ^{15}N$_2$; **7**, 6.9 excess atom %, 13.3% ^{15}N$_1$, 0.3% ^{15}N$_2$; C, [^{15}N]NH$_4$ (355 µg atom ^{15}N) plus Gln (536 µmol, added at the beginning of the experiment): **5** (11 mg), 1.6% ^{15}N recovered, 6.2 excess atom %, 12.1% ^{15}N$_1$, 0.1% ^{15}N$_2$; **7**, 6.5 excess atom %, 9.7% ^{15}N$_1$, 1.7% ^{15}N$_2$.

Experiment VI: A, [^{15}N]Gln (92 µg atom ^{15}N): **5** (16 mg), 14.1% ^{15}N recovered, 10.0 excess atom %, 11.1% ^{15}N$_1$, 4.5% ^{15}N$_2$; **7**, 11.1 excess atom %, 19.4% ^{15}N$_1$, 1.4% ^{15}N$_2$; B, [^{15}N]Gln (91 µg atom ^{15}N) plus NH$_4$ (5 000 µmol, added at the beginning of the experiment): **5** (14 mg), 13.1% ^{15}N recovered, 10.5 excess atom %, 11.4% ^{15}N$_1$, 4.8% ^{15}N$_2$; **7**, 12.1 excess atom %, 20.7% ^{15}N$_1$, 1.7% ^{15}N$_2$; C, [^{15}N]Gln (96 µg atom ^{15}N) plus NH$_4$ (5 000 µmol): **5** (14 mg), 13.0% ^{15}N recovered, 11.0 excess atom %, 11.7% ^{15}N$_1$, 5.2% ^{15}N$_2$; **7**, 13.3 excess atom %, 22.2% ^{15}N$_1$, 2.2% ^{15}N$_2$.

Experiment VII: A, [^{15}N]Gln (88 µg atom ^{15}N) plus [^{14}C]SA (1 µCi) plus R,S-Trp (490 µmol): **5** (21 mg), 21.9% ^{15}N recovered, 11.3 excess atom %, 12.0% ^{15}N$_1$,

5.3% ^{15}N$_2$; **7**, 14.7 excess atom %, 26.7% ^{15}N$_1$, 1.4% ^{15}N$_2$, 29.6% ^{14}C incorporation; B, [^{3}H]Trp (20 µCi): **5** (19.5 mg) and **7**, 0% ^{3}H incorporation.

Experiment VIII: A, [^{15}N]Gln (177 µg atom ^{15}N) plus [^{14}C]SA (5 µCi): **5** (23.5 mg), 10.9% ^{15}N recovered, 10.1 excess atom %, 12.1% ^{15}N$_1$, 4.1% ^{15}N$_2$, 16.2% ^{14}C incorporation; **7**, 0.1% ^{14}C incorporation; B, [^{15}N]NH$_4$ (94 µg atom ^{15}N): **5** (6 mg), 1.7% ^{15}N recovered, 3.2 excess atom %, 6.0% ^{15}N$_1$, 0.1% ^{15}N$_2$; **7** approx. 3.6 excess atom %, approx. 5.9% ^{15}N$_1$, approx. 0.6% ^{15}N$_2$; C, [^{15}N]Glu (99 µg atom ^{15}N): **5** (8.5 mg), 1.9% ^{15}N recovered, 2.7 excess atom %, 5.1% ^{15}N$_1$, 0.2% ^{15}N$_2$; **7**, approx. 6.8 excess atom %, approx. 5.9% ^{15}N$_1$, approx. 3.8% ^{15}N$_2$; D, [^{3}H]Trp (100 µCi): **5** (24.5 mg), 0.05% incorporation; **7**, 0.001% incorporation.

Experiment IX: A, [^{15}N]Gln (174 µg atom ^{15}N) plus [^{14}C]SA (5 µCi): **5** (11 mg), 5.2% ^{15}N recovered, 10.2 excess atom %, 11.9% ^{15}N$_1$, 4.3% ^{15}N$_2$, 8.6% ^{14}C incorporation; **7**, 0.01% ^{14}C incorporation; B, [^{15}N]Glu (109 µg atom ^{15}N): **5** (4 mg), 0.9% ^{15}N recovered, 2.9 excess atom %, 5.9% ^{15}N$_1$, 0% ^{15}N$_2$; C, [^{14}C]SA (5 µCi) plus R,S-Trp (490 µmol): **5** (5 mg), 4.7% ^{14}C incorporation; **7**, 0.01% incorporation.

Experiment X: [^{3}H]Anthranilic acid (3.9 mCi): **5** (4.0 mg) and **7**, 0% incorporation.

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