The Biosynthesis of Phenazines: Incorporation of [^{2}H]Shikimic Acid †

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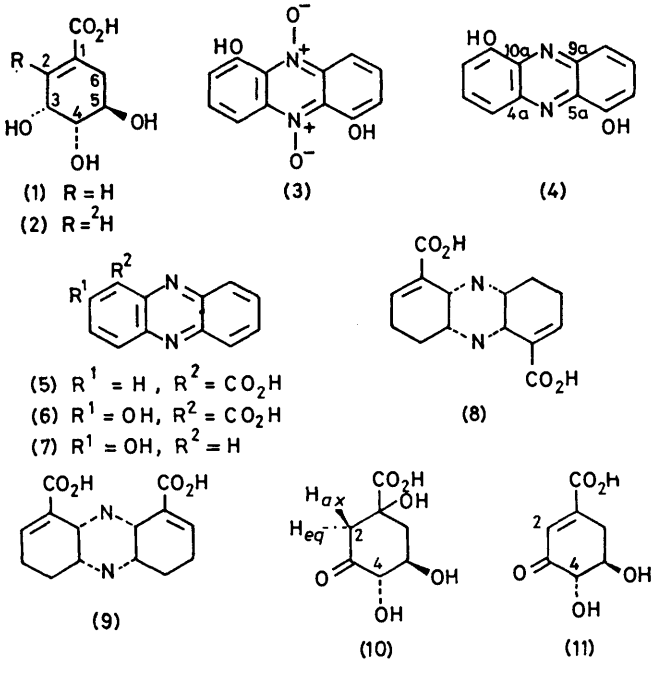
The incorporation of [2-^{2}H]shikimic acid into iodinin gives results which prove that the phenazine ring system is formed from two units of shikimic acid, and which establish the orientation of these units. The incorporation of [2,4-$^2H_2$]shikimic acid into iodinin and other phenazines occurs with partial loss of the C-4 deuteron. The synthesis of the [^{2}H]shikimic acids required modifications to existing methods.

SHIKIMIC acid (1) is clearly implicated as an intermediate in the biosynthesis of microbial phenazines, *e.g.* iodinin (3) [1,2] and phenazine-1-carboxylic acid (5),[2,3] and it can act as the sole carbon source in the formation of the phenazine skeleton.[4] The results of the experiments with ^{14}C-labelled shikimic acid [1-3] show that, if the genesis of (3) and (5) is from two molecules of (1), they are formed according to pattern (8) or (9). Definitive evidence which proves that two shikimic acid units are involved, and which distinguishes between them, has been lacking, however. (Such information is an essential preliminary to the search for the so far elusive intermediates in phenazine biosynthesis.) We considered that results from the use of [2-^{2}H]shikimic acid (2) as substrate for iodinin biosynthesis would provide a simple solution to both these problems, a solution which is only attainable with difficulty if at all with radioactive isotopes. Although in principle use of ^{13}C labelling could have provided a similar solution, we found uncertainties particularly in the assignment of resonances to C-4a, C-5a, C-9a, and C-10a in (4), which may be derived from (3). Subsequently, the ^{13}C n.m.r. spectra of several phenazines have been recorded and assignment of resonances made.[5] This has allowed the appropriate biosynthetic experiment to be carried out with more certainty, and the results obtained [6] are in support of ours.



Our first approach to the preparation of [2-^{2}H]-shikimic acid was through dehydroquinic acid (10), which has been reported [7] to exchange preferentially the C-2 axial proton at pH 7 and, at higher pH, both this proton and the one at C-4. Dehydroquinic acid is enzymically convertible into shikimic acid *via* dehydroshikimic acid (11) [8] with loss of the C-2 equatorial proton,[9] thus providing in principle a simple route to [2-^{2}H]shikimic acid. But our experience was that the C-2 axial proton of dehydroquinic acid would not undergo significant exchange except under conditions which led to the exchange of the C-4 proton as well. This allowed the preparation of [2,4-$^2H_2$]shikimic acid [as (1)] using a crude enzyme preparation [10] from *Escherichia coli* 83–24.

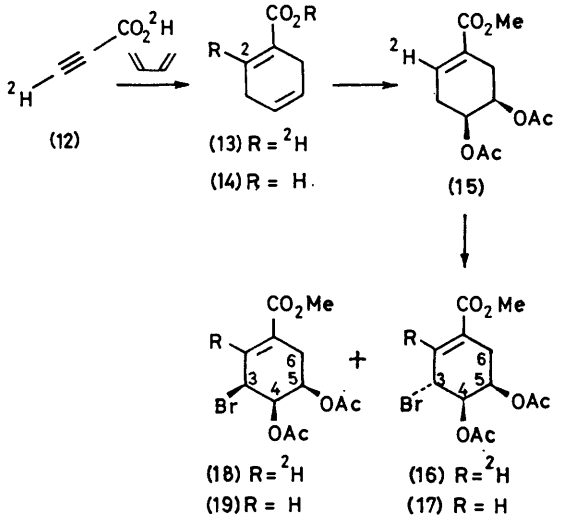
Alternative syntheses of shikimic acid branching from cyclohexa-1,4-dienecarboxylic acid (14) have been reported [11,12] which lend themselves to the preparation of material with a deuteron at C-2. The acid (13) was prepared following the published procedure,[11] but by reaction of butadiene with dideuteriated propiolic acid (12). Use of [3-^{2}H]propiolic acid also gave (13) but with a lower deuterium content at C-2, as a result of exchange with the carboxy-group during the Diels–Alder reaction; in a similar reaction using propiolic acid, butadiene, and deuterium oxide, (14) was isolated deuteriated to a considerable extent at C-2.

The more simple route [12] to shikimic acid from (13) was followed but in fact a lower overall yield (2%) was obtained than with the alternative [11] (6%), and the [2-^{2}H]shikimic acid (2) could not be freed from contaminating 4-*epi*-shikimic acid.

In the discussion which follows, although reference is made only to the deuterio-series, exactly parallel results were obtained for non-deuteriated material. Bromination of (15) gave the expected [12] mixture of bromo-compounds which was separated, since purification of (20) at the next stage could not be effected. The minor monobromo-compound (18) did not react with potassium acetate, in contrast to the major isomer (16), but did react with silver acetate to give the same product (20).

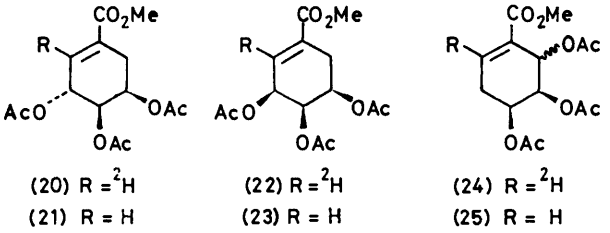
† Preliminary communication: R. B. Herbert, F. G. Holliman, and J. B. Sheridan, *Tetrahedron Letters*, 1976, 639.

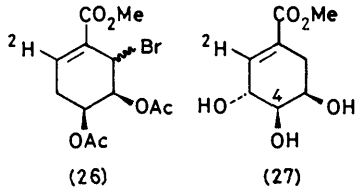
The material from (16) was contaminated with (22), and also (24) which presumably arises from (26) as an impurity in the bromo-compound (16). Hydrogen fluoride catalysed isomerization of (20) gave, after deacetylation,



methyl [2-^{2}H]shikimate which, in spite of repeated recrystallization, contained finally 11% of methyl [2-^{2}H]-4-*epi*-shikimate (27).

Comparison of the n.m.r. spectra of the two monobromo-compounds, (17) and (19), allowed deduction of their structures based on, and consistent with, approximate half-chair conformations in which the bromine is pseudoequatorial and one of the *cis*-acetoxy-groups is

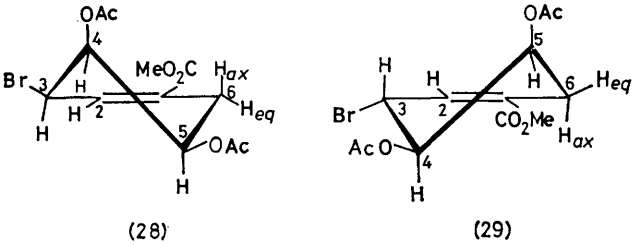




equatorial. The signals for the protons at C-2 and C-6 in each isomer were assigned simply from their chemical shifts. Decoupling experiments, involving irradiation at the frequencies corresponding to these protons, allowed assignment of the other signals and estimation of coupling constants. The minor isomer showed small values for $J_{4,5}$ (2.0 Hz) and $J_{3,4}$ (4.3 Hz); H-4 was thus equatorial. The coupling constants between H-5 and the protons on C-6 (6.0 and 7.5 Hz) indicated that H-5 was axial. The structure (28) followed. The spectrum of the major monobromo-compound showed a relatively large value for $J_{3,4}$ (5.6 Hz) indicating that H-3 was *pseudo*-axial and H-4 was axial; a small value for $J_{4,5}$ (2.0 Hz) meant that H-5 was equatorial. The structure (29) followed. Coupling constants similar to those for this compound were obtained for (21) derived from the two bromo-compounds, which in turn are similar to those for 4-*epi*-shikimic acid, which has been assigned a similar conformation.[13]

The second route [11] from (13) proceeded smoothly to give pure (±)-[2-^{2}H]shikimic acid (2) containing 91% deuterium. Samples of shikimic acid from either route could be used for the feeding experiments, however, for we found that [2-^{2}H]-4-*epi*-shikimic acid [as (27)] did not label iodinin (3) *in vivo*.

A mixture of (±)-[2-^{2}H]- and (±)-[1,6-$^{14}C_2$]-shikimic acid [as (2)] was administered to *Brevibacterium iodinum* cultures. Both isotopic labels were incorporated into iodinin (3) to the same extent (22%), thus showing that hydrogen (deuterium) is not lost from C-2 of shikimic acid during biosynthesis. The iodinin gave a mass spectrum with molecular ions corresponding to dideuteriated (7.5%), monodeuteriated (32%), and unlabelled

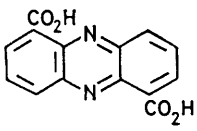


(60.5%) species. The presence of dideuteriated species proves that iodinin (3) arises from two molecules of shikimic acid.

Catalytic reduction of the labelled iodinin gave phenazine-1,6-diol [as (4)] without loss of deuterium. Exchange in boiling aqueous 0.5M-sodium hydroxide resulted in essentially complete loss of deuterium within 4 h (k 3.3 × 10^{-4} s^{-1}). Rate constants for base-catalysed exchange at the positions *ortho* and *para* to the hydroxy-groups in phenazine-1,6-diol were found to be 3.8 × 10^{-4} and 1.4 × 10^{-4} s^{-1}, using [2,4,7,9-$^2H_4$]phenazine-1,6-diol obtained by sodium deuterioxide–deuterium oxide exchange on (4). No significant exchange of *meta*-protons in (4) was observed, so the rate of exchange of *meta*-deuterons could not be determined but it must be very much less than 1.4 × 10^{-4} s^{-1}. In the phenazine-1,6-diol derived from the biosynthetic experiment, the higher exchange rate is associated with *all* the deuterons present. They must therefore be *ortho* to the hydroxy-groups since the alternative *para* location is inconsistent with the ^{14}C-labelling studies.[1,2] This demonstrates that the biosynthesis of iodinin (3) follows pattern (8) and not the alternative (9), which requires half the deuterium label to be *meta* to the hydroxy-groups.

A consideration of the structures of microbial phenazines, and their known inter-relationships.[14-16] indicates that they are all formed, like iodinin, from shikimic acid

by pattern (8). This pattern is seen at its simplest in the naturally occurring phenazine-1,6-dicarboxylic acid (30),[17] and is also apparent in other metabolites which have two aryl-C_1 units, *e.g.* lomofungin (31).[18] A simple view of phenazine biosynthesis, supported by the results of the present study, points to phenazine-1,6-dicarboxylic acid (30) as a common precursor for all microbial phenazines. But, although it has been shown to be an efficient precursor for lomofungin (31) in *Streptomyces*



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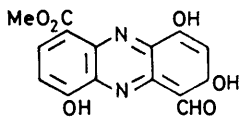

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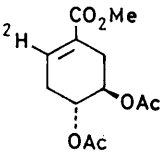

(32)

lomodensis,[19] persistent attempts to implicate (30) in the biosynthesis of metabolites like (3) and (5), produced by *Pseudomonas* and related species, have failed.[14,20] It is not yet clear whether these differing results are the consequence of different cell wall permeability in *Pseudomonas* and *Streptomyces* or whether distinction between the biosynthesis of metabolites with two aryl-C_1 units (lomofungin) and others, *e.g.* iodinin, occurs at an earlier stage. It is to be noted that phenazine-1,6-dicarboxylic acid (30) accumulates in cultures of *Pseudomonas phenazinium* mutants, the parent strain of which normally produces iodinin and related metabolites.[16]

Incorporation of (±)-[2,4-$^2H_2$]shikimic acid [as (1)] into iodinin gave material, mass spectral analysis of which indicated that it was a mixture of mono-, di-, tri-, and tetra-deuteriated species, as well as undeuteriated material. The presence of di- and tetra-deuteriated species was expected, but not mono- and tri-deuteriated material. This is explicable, though, if some of the deuterium is lost by exchange in the course of biosynthesis. It is known from the foregoing results that (±)-[1,6-$^{14}C_2$,2-^2H]shikimic acid is incorporated into iodinin without loss of deuterium, so any exchange process must occur at C-4 of shikimic acid (1). The simplest assumption is that this occurs by interconversion of shikimic acid with dehydroshikimic acid (11) *in vivo*, and loss by enolization of (11). A similar result was observed in the conversion of [2,4-$^2H_2$]shikimic acid into phenazine-1-carboxylic acid (5), 2-hydroxyphenazine-1-carboxylic acid (6), and 2-hydroxyphenazine (7) in *Pseudomonas aureofaciens* cultures, although here the loss of the C-4 proton was almost complete (for these compounds no tetradeuterio-species were observed).

EXPERIMENTAL

For general procedures see the preceding paper.[1] N.m.r. spectra were obtained almost exclusively at 90 MHz.

[2,4-$^2H_2$]*Dehydroquinic Acid.*—Dehydroquinic acid [7] (0.24 g; evaporated twice from deuterium oxide) was dissolved in deuterium oxide (20 ml). Solid anhydrous potassium carbonate was added in portions until the pH reached 9.5 (meter reading; no adjustment for deuteriated solvent). The solution was set aside at 30 °C. The pH fell slowly and was readjusted periodically to 9.4. After 8 days the deuteriated dehydroquinic acid (0.11 g) was isolated as described previously.[7] N.m.r. analysis indicated that exchange at C-4, and at C-2 for the axial proton, was essentially complete. Exchange of the C-2 equatorial proton had occurred to the extent of *ca.* 25%.

[2,4-$^2H_2$]*Shikimic Acid.*—This was adapted from a previously published enzymic method [10] for the synthesis of shikimic acid from phosphoenolpyruvate. The reaction mixture contained phosphate buffer (1M, pH 8.2, 16 ml), glucose 6-phosphate (84.6 mg), NADPH (28.2 mg), [2,4-$^2H_2$]-dehydroquinic acid (110 mg), glucose 6-phosphate dehydrogenase, and crude dialysed sonicate of *Escherichia coli* 83–24 containing dehydroshikimate reductase [21] (10 ml, 1.3 units ml^{-1}, 0.045 units per mg of protein) in a total volume of 100 ml. The mixture was incubated at 37 °C for 2.3 h; a test [22] for shikimic acid then showed a yield of 66% based on dehydroquinic acid. 3M-Perchloric acid (8.3 ml) was added and the shikimic acid isolated essentially as described previously.[10] The n.m.r. spectrum showed that the C-2 and C-4 protons were absent.

[1,3-$^2H_2$]*Propiolic Acid.*[23]—Potassium hydrogen acetylenedicarboxylate (50 g) was stirred and warmed with deuterium oxide (26 ml). The mixture was taken to dryness *in vacuo* and deuterium oxide (100 ml) added. The mixture was heated for 6 h on a steam-bath with the exclusion of water. The solution was cooled and carefully acidified with deuteriosulphuric acid (sulphur trioxide plus deuterium oxide) and extracted with ether (3 × 80 ml). The aqueous layer was saturated with sodium chloride and again extracted with ether (2 × 20 ml). The combined extracts were washed with a saturated solution of sodium chloride in deuterium oxide and dried (Na_2SO_4). The ether was removed by fractional distillation and the [1,3-$^2H_2$]-propiolic acid collected by fractionation at 49 mmHg.

Synthesis of [2-^2H]*Shikimic Acid.*—Both routes to [2-^2H]-shikimic acid began with [1,3-$^2H_2$]propiolic acid and followed the published procedures [11,12] for undeuteriated material. Observational and procedural differences are noted below.

(a) *Synthesis* via *methyl* cis-4,5-*diacetoxy*[2-^2H]*cyclohex*-1-*enecarboxylate*. Bromination of methyl *cis*-4,5-diacetoxy-[2-^2H]cyclohex-1-enecarboxylate gave a mixture of bromo-compounds which was separated on Kieselgel G (17 g of material on 1.2 kg) using chloroform as eluant: (16) (32% yield) and (18) (12%), also dibromo-material (24%). The non-deuteriated analogue (17) of (16) showed δ($CDCl_3$) 6.95 (H-2), 5.52 (H-5), 5.35 (H-4), 4.72 (H-3), 3.79 (CO_2Me), 2.95 and 2.60 (H-6*ax*, H-6*eq*), and 2.10 and 2.08 (OCO*Me* × 2); $J_{2,3}$ 3.6; $J_{3,4}$ 5.6; $J_{4,5}$ 2.0 Hz. The analogue (19) of (18) showed δ($CDCl_3$) 6.93 (H-2), 5.47 (H-4), 5.18 (H-5), 5.03 (H-3), 3.8 (CO_2Me), 2.65 (H-6*ax*, H-6*eq*), and 2.17 and 2.07 (OCO*Me* × 2); $J_{2,3}$ 3.5; $J_{3,4}$ 4.3; $J_{4,5}$ 2.0; $J_{5,6}$ 6.0 and 7.5 Hz.

The bromo-compound (16) gave (20) (46% yield) on treatment with potassium acetate in acetic acid as described (chromatographed on Kieselgel G with chloroform as eluant), the same product being obtained from (18) (1.2 g) with silver acetate (1.5 g) in moist acetic acid (25 ml; 1% water) (3 h at reflux) (almost quantitative yield); δ for (21) ($CDCl_3$) 6.78 (H-2), 5.65 (H-3), 5.43 (H-5), 5.16 (H-4), 3.79 (CO_2Me), 2.72 (H-6*ax*, H-6*eq*), 2.11, 2.10, and 2.08 (OCO*Me* × 3); $J_{2,3}$ 3.0; $J_{3,4}$ 7.0; $J_{4,5}$ 1.8; $J_{5,6}$ 4.5 Hz. Methyl [2-^2H]triacetyl-4-*epi*-shikimate (20) gave on isomerization with hydrogen fluoride, and removal of the acetyl groups, methyl [2-^2H]shikimate, which repeated recrystallization

from methanol failed to free from the 4-epimer. Hydrolysis gave [2-^{2}H]shikimic acid.

Other products isolated from the reaction of (16) with potassium acetate in acetic acid were partially deacetylated (22) (26%) [δ for (23) ($CDCl_3$) 6.63 (H-2), 5.69 (H-3), 5.55 H-4), 5.14 (H-5), 3.79 (CO_2Me), 2.80 (H-6*eq*), 2.53 (H-6*ax*), 2.10 and 2.05 (3 H and 6 H; OCO*Me*); coupling constants were derived in conjunction with a Nicolet NMRCAL programme: $J_{2.3}$ 2.4; $J_{2.4}$ 0.8; $J_{2.6eq}$ −2.2; $J_{2.6ax}$ −2.0; $J_{3.4}$ 3.8; $J_{3.6ax}$ 3.5; $J_{3.6eq}$ 0.6; $J_{4.5}$ 2.0; $J_{5.6eq}$ 7.0; $J_{5.6ax}$ 9.5; $J_{6eq,6ax}$ −17.5 Hz] and (24) (10%), derived presumably from (26) as an impurity in the starting material [δ for (25) ($CDCl_3$) 7.19 (H-2), 5.83 (H-6), 5.33 (H-4), 5.20 (H-5), 3.79 (CO_2Me), 2.66 (C-3 protons), and 2.09, 2.08, and 2.07 (OCO*Me* × 3); $J_{2.3}$ 4.2; $J_{4.5}$ 2.0; $J_{5.6}$ 4.3 Hz].

(b) *Synthesis* via *methyl* trans-4,5-*diacetoxy*[2-^{2}H]*cyclohex*-1-*enecarboxylate*.—Methyl [2-^{2}H]cyclohexa-1,4-dienecarboxylate (13) (93% deuterium) gave [2-^{2}H]shikimic acid (91% deuterium) *via* methyl *trans*-4,5-diacetoxy[2-^{2}H]-cyclohex-1-enecarboxylate (32) (used without purification) essentially as described for undeuteriated material;[11] the intermediary methyl [2-^{2}H]shikimate was purified on Kieselgel with chloroform containing 10% methanol, and hydrolysed with 0.1M-potassium hydroxide in 20% aqueous methanol;[24] the shikimic acid, with characteristic n.m.r. spectrum,[25] was isolated *via* an ion-exchange column (Amberlite IR-120; H^+ form).

Incorporation of (±)-[1,6-$^{14}C_2$,2-^{2}H]*Shikimic Acid into Iodinin*.—(±)-[1,6-$^{14}C_2$,2-^{2}H]shikimic acid (37 mg, 0.8 μCi; radioactive derivative purchased from the Commissariat a l'Énergie Atomique, Gif-sur-Yvette, France) was fed over 5 h to a *Brevibacterium iodinum* culture, which had produced 1/4 of maximum iodinin before feeding commenced. Iodinin (14 mg) was isolated and recrystallized (chloroform) (22% incorporation of both isotopes by scintillation counting and mass spectrometry). Iodinin (2.3 mg) in dioxan (7.5 ml) was hydrogenated over platinum oxide (3 mg) at atmospheric pressure and room temperature for 45 min. The near colourless solution (dihydrophenazine) was left exposed to air to give yellow phenazine-1,6-diol: deuterium contents $^2H_0$ 60.7, $^2H_1$ 31.9, $^2H_2$ 7.5% (iodinin); $^2H_0$ 62.3, $^2H_1$ 29.6, $^2H_2$ 8.0% (phenazine-1,6-diol). Exchange in refluxing aqueous 0.5M-potassium hydroxide was essentially complete in 4 h (k 3.3 × 10^{-4} s^{-1}).

[2,4,7,9-$^2H_4$]*Phenazine*-1,6-*diol*.—All operations were carried out with exclusion of moisture. Phenazine-1,6-diol (16 mg) in sodium deuterioxide (0.5M) in deuterium oxide (6 ml) was heated in a sealed tube at 140—160 °C (furnace temp.) for 16 h. Water was added followed by dilute sulphuric acid. The mixture was extracted with three portions of chloroform. The combined extracts were washed thrice with water (to remove labile deuterium), dried, and evaporated. The crude [2,4,7,9-$^2H_4$]phenazine-1,6-diol was recrystallized from benzene: yield 6 mg; $^2H_4$ 90.4% (by mass spectrometry). Exchange in refluxing aqueous 0.5M-potassium hydroxide, k_{ortho}: 3.8 × 10^{-4} s^{-1}; k_{para} 1.4 × 10^{-4} s^{-1}. [Rate constant for the disappearance of $^2H_4$ material = $2k_{ortho} + 2k_{para}$; k_{para} = rate constant for the conversion of $^2H_1$ into $^2H_0$; k_{para} is the lower of the two rate constants from the biosynthetic experiment (see discussion).]

Incorporation of [2,4-$^2H_2$]*Shikimic Acid*.—[2,4-$^2H_2$]-Shikimic acid (17.4 mg; $^2H_2$ 76, $^2H_1$ 19%; determined on trimethylsilyl derivative) was fed to *B. iodinum* cultures. The iodinin isolated (51 mg) showed $^2H_4$ 0.3, $^2H_3$ 1.5, $^2H_2$ 6, $^2H_1$ 10, $^2H_0$ 83%.

[2,4-$^2H_2$]Shikimic acid (35 mg; $^2H_2$ 64, $^2H_1$ 36%) was fed to *Pseudomonas aureofaciens* cultures. The phenazines were isolated (89 mg) and separated on Kieselgel G to give phenazine-1-carboxylic acid ($^2H_2$ 3, $^2H_1$ 11, $^2H_0$ 86%; determined on the methyl ester); 2-hydroxyphenazine-1-carboxylic acid ($^2H_1$ 6, $^2H_0$ 94%; determined on methyl 2-methoxyphenazine-1-carboxylate); and 2-hydroxyphenazine ($^2H_2$ 1, $^2H_1$ 6, $^2H_0$ 93%).

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