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# 1-NITRO-2-PHENYLETHANE, A POSSIBLE INTERMEDIATE IN THE BIOSYNTHESIS OF BENZYLGLUCOSINOLATE*†

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**Abstract**—Results have been obtained consistent with the hypothesis that *aci* tautomers of nitro compounds are precursors of glucosinolates. When DL-[3-$^{14}$C]phenylalanine and [$^{14}$C]1-nitro-2-phenylethane were fed to shoots of *Tropaeolum majus* L., the incorporation of tracer from each compound into benzylglucosinolate was found to be similar. Conversion of $^{14}$C from 1-nitro-2-phenylethane into the aglycone moiety of benzylglucosinolate was specific. The natural occurrence of 1-nitro-2-phenylethane in *T. majus* and its formation in this plant from [1-$^{14}$C]phenylacetaldoxime were demonstrated by gas chromatography and by means of a trapping experiment.

## INTRODUCTION

INVESTIGATIONS of glucosinolate biosynthesis in higher plants have established phenylacetaldoxime (I in Fig. 1)[1,2] and phenylacetothiohydroximate (VII)[3,4] as precursors of

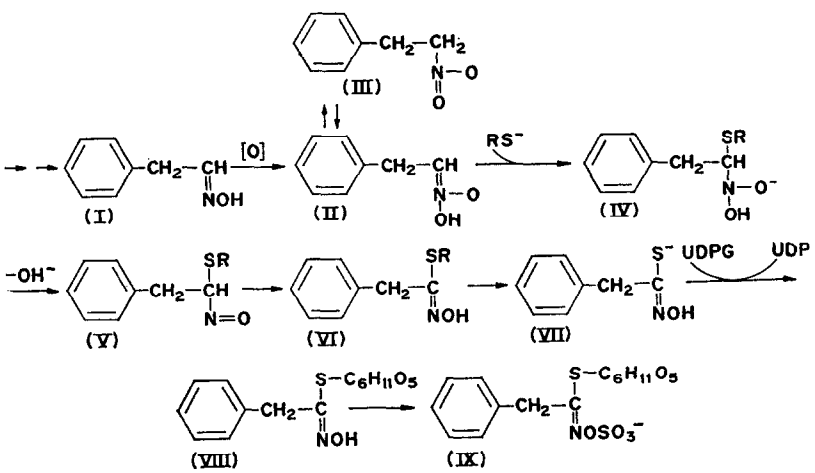


FIG. 1. PROPOSED MECHANISM FOR THE BIOSYNTHESIS OF BENZYLGLUCOSINOLATE FROM PHENYLACETALDOXIME.

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[1] B. A. TAPPER and G. W. BUTLER, *Arch. Biochem. Biophys.* **120,** 719 (1967).
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[4] M. MATSUO and E. W. UNDERHILL, *Biochem. Biophys. Res. Commun.* **36,** 18 (1969).

benzylglucosinolate (IX) and both of these precursors have been found to occur naturally in *Tropaeolum majus*. It has been demonstrated in *T. majus*[4] that phenylacetothiohydroximate is derived from phenylacetaldoxime and the latter from phenylalanine.[2] Ettlinger and Kjaer[5] have suggested 1-nitro-2-phenylethane (III) as its *aci* tautomer (II) (2-phenylethanenitronic acid) as a possible intermediate between the aldoxime and the thiohydroximate. They note thiohydroximic acids may be formed by base catalyzed reactions of thiols and primary nitro compounds.[6]

In this communication we report the confirmation of this hypothesis by means of plant feeding experiments.

## RESULTS

Table 1 compares the conversion of $^{14}C$ from DL-[3-$^{14}C$]phenylalanine and from [$^{14}C$]1-nitro-2-phenylethane into benzylglucosinolate. The data were obtained from one of

TABLE 1. COMPARISON OF LABELED COMPOUNDS AS PRECURSORS OF BENZYLGLUCOSINOLATE

| Compound fed* | | | Benzylglucosinolate isolated† | | | |
|---|---|---|---|---|---|---|
| | ($\mu$c) | ($\mu$c/mmole) | Wt.‡ (mg) | ($\mu$c/mmole) | % $^{14}C$ converted§ | dilution‖ |
| DL-[3-$^{14}C$]Phenylalanine | 10·65 | 207 | 129 | 1·65 | 4·1 | 125 |
| [$^{14}C$]1-Nitro-2-phenylethane | 3·83 | 82 | 99 | 0·63 | 3·4 | 130 |

* Labeled compounds were fed to *T. majus* (67 g fr. wt.).
† Isolated as the tetramethylammonium salt.
‡ Weight of glucosinolate extracted was determined by GLC assay.

§ $\%\ ^{14}C \text{ converted} = \dfrac{\text{Specific activity of glucosinolate} \times \text{mmole extracted} \times 100.}{\text{Activity taken up by plant}}$.

‖ $\text{Dilution} = \dfrac{\text{Specific activity of compound fed}}{\text{Specific activity of benzylglucosinolate}}$.

several similar plant feeding experiments. Approximately 50 $\mu$moles of each compound were fed to *T. majus* shoots which were allowed to metabolize under continuous light for 24 hr. Although a slightly lower incorporation of $^{14}C$ into benzylglucosinolate was observed when [$^{14}C$]1-nitro-2-phenylethane was fed as compared to [3-$^{14}C$]phenylalanine, both compounds may be considered efficient precursors of the glucoside. It is possible that the lower conversion of $^{14}C$ from 1-nitro-2-phenylethane may reflect a difference in precursor solubility, rate of transport, or availability at the site of glucosinolate biosynthesis rather than a difference in efficiency of biochemical conversion.

All of the activity present in benzylglucosinolate (sp. act. 0·63 $\mu$c/mmole), derived from the nitro precursor, was recovered in the glucoside aglycone, isolated as benzylthiourea (sp. act. 0·63 $\mu$c/mmole). Degradation of benzylthiourea[7] confirmed that the incorporation

[5] M. G. ETTLINGER and A. KJAER, in *Recent Advances in Phytochemistry* (edited by T. J. MABRY, R. E. ALSTON and V. C. RUNECKLES), Vol. 1, p. 59. Appleton–Century–Crofts, New York (1968).
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[7] E. W. UNDERHILL, M. D. CHISHOLM and L. R. WETTER, *Can. J. Biochem. Physiol.* **40**, 1505 (1962).

of tracer was specific: the ratio of $^{14}C$ in the benzyl carbons to that in the isothiocyanate or thiourea carbon was 1:3·67, practically the same ratio as was originally present in the precursor fed (1:3·76).

Data which demonstrate 1-nitro-2-phenylethane is derived from phenylacetaldoxime in *T. majus* were obtained from a trapping experiment in which $^{14}C$-labeled aldoxime and non-radioactive nitro compound were fed to a plant shoot. 1-Nitro-2-phenylethane recovered from the plant by steam distillation and purified by Silica Gel column chromatography and GLC (UC-W98 column) had a specific activity of 38·8 μc/mmole. It was established that the radioactivity of this fraction was not due to traces of radioactive benzyl cyanide or benzyl isothiocyanate by adding 5 μl each of these to an aliquot (6·5 μl) of the fraction containing 1-nitro-2-phenylethane and resolving the mixture by GLC (UC-W98). The specific activity of the recovered nitro compound remained unchanged (38·0 μc/mmole) and no activity was demonstrable in the isothiocyanate and nitrile fractions. The remaining 1-nitro-2-phenylethane fraction was next collected from a QF-1 column and its specific activity was found to be essentially unchanged (37·5 μc/mmole). Further GLC fractionation on an FFAP column afforded 1-nitro-2-phenylethane with a specific activity of 32·1 μc/mmole. This last fraction was reduced to phenethylamine and its benzenesulfonamide derivative formed. The specific activity of phenethylamine benzenesulfonamide when recrystallized first from benzene–petrol and then from ethanol–water remained constant at 31·6 μc/mmole.

The specific activity of benzyl isothiocyanate recovered from the first GLC fractionation (UC-W98) in the trapping experiment was 2·74 μc/mmole which represents a dilution (see Table 1 for definition) of 180.

1-Nitro-2-phenylethane was identified by GLC as a component present in the steam distillate of *T. majus*. The relative retention time (RRT) of synthetic 1-nitro-2-phenylethane to 2-phenylethyl isothiocyanate was the same as the RRT of the plant-derived component on each of the three columns employed (RRT = 0·50, 0·90, 0·48, on UC-W98, QF-1 and OV-17, respectively). The quantity of 1-nitro-2-phenylethane derived from the plant (100 g fr. wt.) was estimated by GLC to be 50 μg.

## DISCUSSION

Our plant feeding experiments have demonstrated the efficient and specific incorporation of $^{14}C$ from labeled 1-nitro-2-phenylethane into the aglycone moiety of benzylglucosinolate and have confirmed the suggestion of Ettlinger and Kjaer[5] that *aci* tautomers of nitro compounds may be precursors of glucosinolates. A trapping experiment was also carried out where labeled phenylacetaldoxime and unlabeled 1-nitro-2-phenylethane were fed to *T. majus* shoots. In this experiment we recovered 1-nitro-2-phenylethane which possessed a constant specific activity after GLC and recrystallization of the benzenesulfonamide derivative of phenethylamine formed from the nitro compound. These results clearly demonstrate the formation of 1-nitro-2-phenylethane from the aldoxime. Detection of this nitro compound in the steam distillate of *T. majus* further demonstrated its natural occurrence in the plant. However, it seems probable that the active species in the biosynthetic reaction is the *aci* tautomer (nitronic acid) rather than either the parent nitro compound or its nitronate anion.

1-Nitro-2-phenylethane has been found in two species of Lauraceae[8] and in fruits of

[8] O. R. Gottlieb and M. T. Magalhães, *J. Org. Chem.* **24**, 2070 (1959).

*Dennettia tripetala* G Baker.[9] Gottlieb *et al.*[10] have suggested this $C_6$–$C_2$ nitro compound is formed from phenylalanine. Since phenylacetaldoxime has been previously demonstrated to be derived in *T. majus* from phenylalanine,[2] the results presented here both confirm and extend their hypothesis.

We are unaware of any reports of the biological oxidation of oximes to nitro compounds. However, the chemical oxidation of oximes to nitro compounds is readily accomplished.[11,12] In this reaction the *aci*-nitro analogue is presumably formed first and the nitro compound derived by a prototropic shift. Isolation and study of the enzyme(s) involved in the formation of 1-nitro-2-phenylethane from the aldoxime in *T. majus* may provide useful information basic to the understanding of biological oxidation of organic nitrogen.

Ettlinger and Kjaer[5] have suggested that *S*-substituted thiohydroximic acids (VI in Fig. 1) may be formed by the addition of a mercaptide to an *aci*-nitro tautomer (II) by the mechanism II → IV → V → VI. Any speculation of the possible intermediates between the *aci* tautomer of 1-nitro-2-phenylethane and desulfobenzylglucosinolate (VIII) should take into account the studies by Wetter and Chisholm[13] who have demonstrated that cysteine is a very efficient source of the thiohydroximate sulfur. In addition, the formation of desulfobenzylglucosinolate by a glucosyltransferase enzyme isolated from *T. majus*[4,14] has been shown to involve UDP glucose and sodium phenylacetothiohydroximate (VII). Hence, if one assumes the substituted thiohydroximic acid VI is derived from cysteine (R = $CH_2CH(NH_2)COOH$), its conversion to VII may possibly be mediated by a C-S lyase such as cystathionase or related enzymes.[15–17]

## EXPERIMENTAL

*Labeled compounds.* DL-[3-$^{14}$C]Phenylalanine and [1-$^{14}$C]phenylacetic acid were obtained from commercial sources; [1-$^{14}$C]phenylacetaldoxime was prepared previously.[2] [$^{14}$C]1-Nitro-2-phenylethane was synthesized by the following sequence of reactions: [1-$^{14}$C]methyl phenylacetate (from [$^{14}$C]phenylacetic acid-1, 1 mc, 10 mmoles) in 20 ml of anhydrous $Et_2O$ was added dropwise with stirring to $LiAlH_4$ (30 mmoles) in 20 ml of $Et_2O$ and the mixture refluxed 2 hr. $Et_2O$ extraction of the acidified reaction mixture gave 1·1 g of crude [$^{14}$C]2-phenylethanol. The alcohol was converted to [$^{14}$C]1-iodo-2-phenylethane by heating at 150° for 5 hr with red P (100 mg) and $I_2$ (1·3 g).[18] The mixture was extracted with 15 ml $Et_2O$ and the ether successively washed with 5 ml each of $H_2O$, 5% NaOH and $H_2O$. The ether was removed and the residue distilled to give [$^{14}$C]1-iodo-2-phenylethane (1·33 g, b.p.$_{11}$ 107°). A mixture of [$^{14}$C]1-iodo-2 phenylethane and $AgNO_2$ (1·3 g) in 5 ml of ether was shaken for 3 days in the dark.[19] Distillation of the product afforded [$^{14}$C]1-nitro-2-phenylethane (b.p.$_{13}$ 127°, 400 mg, specific activity 82 μc/mmole). A single radioactive spot was observed for this material on thin-layer chromatography (benzene–petrol, 5:1; Silica Gel G).

The distribution of $^{14}$C in 1-nitro-2-phenylethane was determined by permanganate oxidation of the product. Unexpectedly, the recovered benzoic acid was found to be radioactive, possessing a constant specific activity of 17·2 μc/mmole after recrystallization and sublimation. Hence, the distribution of $^{14}$C in 1-nitro-2-phenylethane was $C_6H_5CH_2$—$CH_2NO_2$ = 1:3·76.


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*Precursor feeding experiments.* The methods used for the administration of labeled compounds to *T. majus* shoots, isolation of benzylglucosinolate, formation and degradation of benzyl isothiocyanate and $^{14}C$ counting have been described previously.[3,7] Quantitation of benzylglucosinolate in aqueous plant extracts was achieved by an adaptation of a GLC method[20] for aliphatic glucosinolates. Benzylglucosinolate was hydrolyzed by myrosinase at pH 7 and the liberated benzyl isothiocyanate was extracted into methylene chloride containing a known weight of 2-phenylethyl isothiocyanate. Enzymic hydrolysis of the glucosinolate and extraction of the liberated aglycone into methylene chloride were complete using the described conditions.[20] The weight of benzyl isothiocyanate was determined (internal standard method) from the relative peak areas of the two isothiocyanates obtained by GLC using a 20% FFAP column operated at 210° (see below).

*Gas chromatography.* The gas chromatograph was a Hewlett–Packard model 5754, equipped with dual flame ionization and thermal conductivity detectors. Peak areas were determined by an Infotronics model CRS-104 digital integrator. The system was operated using helium carrier gas (30 ml/min), hydrogen (20 ml/min) and air (250 ml/min) supplied to the flame detector, the injector and detectors maintained at 240°. Benzyl isothiocyanate was quantitatively determined using a 1·8m × 3 mm o.d. column of 20% FFAP on 60–70 mesh acid-washed and DMCS-treated Chromosorb W operated at 210°. The preceding column (operated at 200°) and the following two columns were used for the purification and isolation of 1-nitro-2-phenylethane recovered in the trapping experiment: 10% UC-W98 on 80–100 mesh Diatoport S (Hewlett–Packard) 1·8 m × 3 mm o.d. (170°) and 16% QF-1 on 60–80 mesh acid-washed and DMCS-treated Chromosorb W, 1·8 m × 6 mm o.d. (180°). Components were collected by condensation in small glass tubes inserted in the thermal conductivity detector exit port. A 1·8 m × 3 mm o.d. column containing 6% OV-17 on 80–100 mesh Diatoport S (170°) was employed in addition to those above for detection of 1-nitro-2-phenylethane.

*Trapping experiment.* [1-$^{14}C$]Phenylacetaldoxime (5·66 mg, 20·7 $\mu$c) and non-radioactive 1-nitro-2-phenylethane (7·01 mg) were fed to *T. majus* (56 g fr. wt.) and the shoot allowed to metabolize for 6 hr. The plant was ground in a Waring blendor with 100 ml of 0·05 M potassium biphthalate buffer pH 4 and 200 mg of carrier 1-nitro-2-phenylethane. The mixture was steam distilled and the distillate (150 ml) was extracted 3 times with methylene chloride. The extracts were combined, dried over $Na_2SO_4$ and the solvent removed. The residue was applied to a Silica Gel column (100 ml, 0·05–0·20 mm) and the column eluted with $CHCl_3$. The 1-nitro-2-phenylethane fraction also contained benzyl isothiocyanate and a trace of benzyl cyanide. This fraction was resolved by GLC into individual components using a UC-W98 column. The RRT for benzyl cyanide, 1-nitro-2-phenylethane and benzyl isothiocyanate were 0·49, 1·00 and 1·35 respectively. 1-Nitro-2-phenylethane was further purified on QF-1 and FFAP columns. The nitro compound isolated from the last column (110 mg) was reduced[21] to phenethylamine in the presence of Adams catalyst and hydrogen at room temperature and atmospheric pressure. The amine was converted to the sulfonamide with benzenesulfonyl chloride and the derivative was recrystallized to constant specific activity from benzene-Skellysolve B and from aq. EtOH.

*Detection of 1-nitro-2-phenylethane.* Shoots of *T. majus* (100 g fr. wt.) were cut directly into 100 ml of hot (90°) 0·05 M potassium biphthalate buffer pH 4 to inactivate the myrosinase activity. The mixture was ground in a Waring blendor, steam distilled and the distillate (100 ml) continuously extracted overnight with ether. The dried ($Na_2SO_4$) ether extract was concentrated to approximately 1 ml and a known weight of 2-phenylethyl isothiocyanate (internal standard) was added. Aliquots (1 $\mu$l) of the ether extract were chromatographed on UC-W98, QF-1 and OV-17 columns. The retention times of synthetic 1-nitro-2-phenylethane and the plant-derived component relative to 2-phenylethyl isothiocyanate were 0·50, 0·90 and 0·48, respectively, on these columns. The amount of 1-nitro-2-phenylethane present in the ether extract was estimated by the internal standard method using the UC-W98 and OV-17 columns.

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*Key Word Index—Tropaeolum majus;* Tropaeolaceae; glucosinolates; benzylglucosinolate; biosynthesis; 1-nitro-2-phenylethane.