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THE 6*a* OXYGEN OF THE PTEROCARPAN GLYCINOL IS DERIVED FROM MOLECULAR OXYGEN

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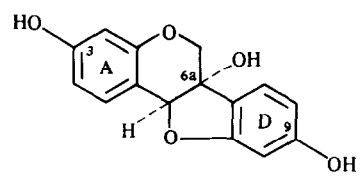
Key Word Index—*Glycine max*; Leguminosae; soybean; biosynthesis; phytoalexin; pterocarpan; isoflavonoid; oxygenase; tandem mass spectrometry.

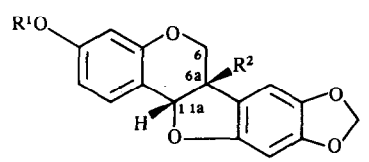
Abstract—Previous studies on the biosynthesis of pterocarpan phytoalexins in soybean, *Glycine max*, suggest that one of the late steps in the pathway is aliphatic hydroxylation of 3,9-dihydroxypterocarpan to 3,6*a*,9-trihydroxypterocarpan (glycinol). We have confirmed this hypothesis by ^{18}O labelling and tandem mass spectrometry. Glycinol synthesized by UV-irradiated cotyledons in the presence of $^{18}O_2$ contained up to three labelled oxygen atoms per molecule; $H_2{}^{18}O$ labelled the remaining two oxygens. One of the atoms labelled by $^{18}O_2$ was the 6*a* oxygen. The pathway for biosynthesis of glycinol in soybean thus differs significantly from that for the 6*a*-hydroxypterocarpan pisatin in pea.

INTRODUCTION

The 6*a*-hydroxypterocarpan phytoalexins pisatin, from *Pisum sativum* L., and glyceollins I to III, from *Glycine max* L., are efficiently labelled when phytoalexin biosynthesis is performed in the presence of the corresponding radiolabelled 6*a*-unsubstituted pterocarpan precursors, maackiain or 3,9-dihydroxypterocarpan respectively [1, 2]. Therefore these pathways were initially believed to involve direct hydroxylation at 6*a*, most likely by an oxygenase. However in the case of pisatin an ^{18}O labelling study showed that the 6*a* oxygen was labelled by $H_2{}^{18}O$ rather than $^{18}O_2$ [3], calling this assumption into question. We now report than O_2 is the source of the 6*a* oxygen of glycinol, a soybean phytoalexin that is an immediate precursor of the glyceollins [2]. The two legumes thus differ in their method of 6*a*-hydroxypterocarpan biosynthesis.



(–) Glycinol



(+) Pisatin R^1 = Me, R^2 = OH
(+) Maackiain R^1 = H, R^2 = H

RESULTS AND DISCUSSION

Cotyledons from soybean cv Harosoy 63 seedlings were irradiated with UV light and incubated with $^{18}O_2$ or $H_2{}^{18}O$ for 2 days. Glycinol was isolated and analysed by mass spectrometry.

Glycinol labelled with $^{18}O_2$ included molecules containing 0, 1, 2 and 3 atoms of ^{18}O, *m/z* 272 to 278, the triply labelled molecules representing 9% of the total (Fig. 1). A small signal at *m/z* 278 was also present in the $H_2{}^{18}O$-labelled sample, but this appeared to be a contaminant as its intensity profile during heating of the sample probe did not match the profiles of the molecular ion signals at *m/z* 272, 274 and 276. That only two atoms were labelled by $H_2{}^{18}O$ and three by $^{18}O_2$ immediately suggests that the 6*a* oxygen was one of the latter. The two oxygen substituents of ring A, derived from malonyl-CoA, are expected to be labelled by $H_2{}^{18}O$, whereas two of the $^{18}O_2$-labelled oxygens should be the ring D substituents, incorporated by direct aromatic hydroxylation. These assignments are in accord with known features of the flavonoid biosynthetic pathway, and were confirmed experimentally by the ^{18}O labelling pattern of pisatin [3]. The remaining O_2-derived atom should therefore be the one at 6*a*.

Additional evidence was obtained by examining the loss of ^{18}O label in fragments that have specifically lost the 6*a* oxygen, namely the $[M-H_2O]^+$ (base *m/z* 254), $[M-H_2O, H]^+$ (253), and $[M-CO]^+$ (244) ions. That the oxygen atom lost in these fragmentations is the one at 6*a* was demonstrated previously by the MS of 6*a*-[6*a*-^{18}O]-hydroxymaackiain [3]. To determine whether the 6*a* oxygen of glycinol was ^{18}O labelled, the samples were analysed by tandem MS. For each glycinol sample, the labelled molecular ions (*m/z* 272 to 278) were selected

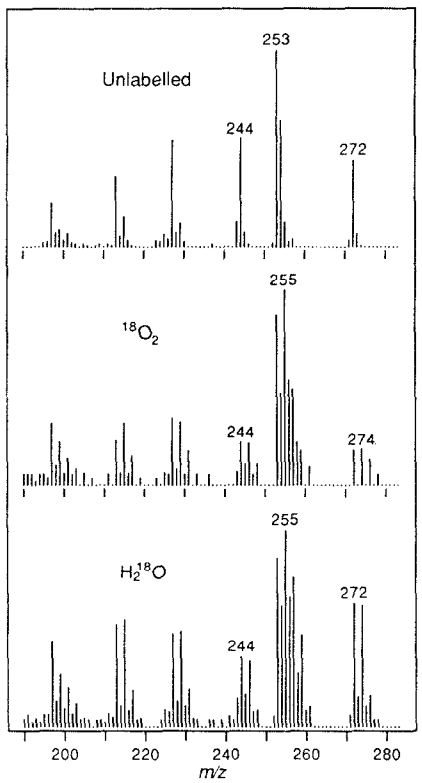


Fig. 1. Mass spectra of unlabelled and ^{18}O-labelled glycinol.

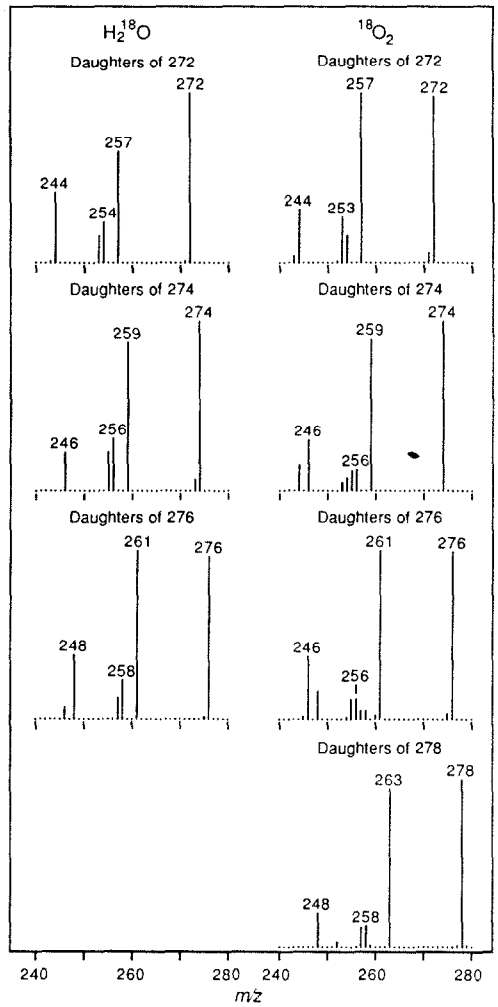


Fig. 2. Daughter mass spectra of fragments obtained from each of the molecular ions of glycinol, containing 0 to 3 atoms of ^{18}O derived from $H_2{}^{18}O$ or $^{18}O_2$.

individually by the primary mass spectrometer, fragmented by collisionally activated dissociation with argon, and the daughter fragments analysed in the second spectrometer. The daughter spectra are illustrated in Fig. 2.

The most prominent ions in the daughter spectra were the molecular ion, the three fragments of interest and a $[M-15]^+$ fragment (base *m/z* 257) corresponding to loss of methyl. The latter fragmentation apparently involves rearrangement of a methylene and a proton. It is commonly found also in EIMS of glycinol [6], and of 6*a*-hydroxymaackiain which also lacks a methyl substituent [3], though it happens to be very weak in the spectra illustrated in Fig. 1. It is of little interest here, except as an example of the behaviour of a fragment that does not involve loss of ^{18}O label.

When $H_2{}^{18}O$ was the precursor, the fragments produced by loss of the 6*a* oxygen retained all of the ^{18}O label. For example, the $[M-H_2O]^+$ fragment derived from the doubly labelled molecular ion (*m/z* 276) was also doubly labelled (*m/z* 258). The only anomaly was the presence in the daughters of *m/z* 276 of a small signal at *m/z* 246, where singly labelled $[M-CO]^+$ fragments would appear. All three of the relevant fragments were singly labelled in the daughters of the singly labelled molecular ion. Thus the 6*a* oxygen of glycinol is not derived from or exchangeable with H_2O.

When $^{18}O_2$ was the source of the label, a proportion of these fragmentations did result in loss of ^{18}O label, the proportion depending on the number of labelled atoms in the molecular ion. For the triply labelled molecules, *m/z* 278, the 6*a* oxygen was 100% labelled: the $[M-H_2O]^+$ fragmentation for example yielded only *m/z* 258 corresponding to loss of labelled H_2O, and no *m/z* 260 was detectable. The $[M-H_2O,H]^+$ and $[M-CO]^+$ fragments behaved in the same way. Approximately one-third of the singly labelled molecules gave rise to unlabelled fragments, and two-thirds of the doubly labelled molecules produced singly labelled fragments, as would be predicted if the 6*a* position is one of three positions labelled with equal efficiency.

We conclude that molecular oxygen is the source of the 6*a* oxygen of glycinol synthesized by soybean, and presumably also the 6*a* oxygen of the prenylated glycinol derivatives glyceollins I, II and III.

This result is consistent with the demonstration [4] of a soybean enzyme that hydroxylates 3,9-dihydroxypterocarpan to glycinol, a reaction dependent on NADPH and O_2. It should be pointed out, however, that pea almost certainly possesses an analogous 6*a*-hydroxylase for maackiain, despite the fact that during the biosynthesis of pisatin the 6*a* oxygen was not labelled by $^{18}O_2$ [3]. The evidence for an enzyme that can directly hydroxylate maackiain is that [6, 11*a*-$^2H_2$] maackiain is incorporated

into pisatin *in vivo* without loss of ^2H [5]. Therefore no intermediates in this conversion can be unsaturated at either of these positions, as would seem to be required if H_2O were the source of the 6*a* oxygen. One interpretation of our findings would be that this 6*a*-hydroxylase does not participate in the normal biosynthetic pathway to pisatin via endogenous intermediates, but only in the metabolism of exogenous maackiain. Alternatively, maackiain may be 6*a*-hydroxylated by some unusual mechanism that does not involve molecular oxygen directly. In any case, the present results demonstrate that the biosynthetic steps producing 6*a*-hydroxypterocarpans proceed via different enzymatic mechanisms in soybean vs pea.

EXPERIMENTAL

Materials. Soybean seed was a gift of Dr Jack Paxton. $H_2{}^{18}O$ was purchased from Amersham and $^{18}O_2$ from Monsanto Research Corp. TLC plates, silica gel GHLF, were obtained from Analtech.

^{18}O *labelling of glycinol.* Soybean cv Harosoy 63 was grown in vermiculite at 28° in continuous fluorescent light for 6 days. Cotyledons were excised and the convex abaxial surface sliced off with a scalpel. The cotyledons, cut surface upward, were irradiated with a Sylvania G15T8 germicidal lamp at a distance of 40 cm for 30 min.

Seven cotyledons, 1.4 g fr. wt, were used for $H_2{}^{18}O$ labelling. They were placed in a petri dish and a total of 1 ml H_2O, 98 atom % ^{18}O, was applied to the cut surfaces. The dish was covered and incubated in the dark at room temperature for 2 days.

Twenty cotyledons, 3.3 g fr. wt, were used for $^{18}O_2$ labelling. They were placed in a 125-ml Erlenmeyer flask containing a moistened paper filter. The flask was covered loosely and incubated in the dark at 16° for 12 hr. The flask was then sealed with a rubber stopper through which two hypodermic needles had been inserted. Using the needles as gas inlet and outlet ports, the flask was flushed with a slow stream of N_2. It was then connected to a 1-l bulb of O_2, 98 atom % ^{18}O, and 200 ml of the gas was displaced with water from the bulb into the flask. The needle ports were closed with plastic Luer-lock stopcocks and the flask was incubated as before. Two more 200-ml aliquots of $^{18}O_2$ were administered at 12-hr intervals, and the incubation was ended 12 hr after the last addition.

Isolation of glycinol. The procedure was based on that of Weinstein *et al.* [6]. Cotyledons were shaken for 2 hr in 40% EtOH, 30 ml/g fr. wt. EtOH was removed by rotary evaporation at 40°, and glycinol was extracted from the aq. soln with 1 vol. EtOAc × 2. The extract was refrigerated overnight and filtered through Whatman 1PS to remove small amounts of H_2O, and evapd to dryness at 30°. Glycinol was isolated from the residue by TLC in hexane–EtOAc (3:7, R_f 0.55) and toluene–$CHCl_3$–Me_2CO (8:5:10, R_f 0.50). The (weakly) fluorescence-quenching zones were eluted with 20–40 ml MeOH and filtered through Whatman 1. Glycinol was recognized by its sharp UV absorbance peaks at 287 and 282 nm [6] and very deep trough at 253 nm (less than 15% of A_{287}). Yield, based on the 287 nm molar extinction coefficient, 5800 [6], was *ca* 200 μg/g fr. wt of cotyledons. In preparation for mass spectrometry the soln was evapd to dryness and the residue extracted with EtOAc, leaving behind some silica that had been solubilized by the MeOH.

Mass spectrometry. EIMS was performed with a Finnigan 3300 quadrupole instrument with an ionizing voltage of 70 eV, at a probe temperature of *ca* 180°. EI/CAD tandem MS was performed with a Finnigan MAT TSQ46. Ionizing voltage was 70 eV, collision energy was −14.8 V, and the collision gas was Ar at 0.5 mtorr.

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