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METABOLISM OF ^{14}C-INDOLE-3-ACETALDOXIME BY HYPOCOTYLS OF CHINESE CABBAGE

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Key Word Index—*Brassica campestris* sub sp. *pekinensis*; Cruciferae; Chinese cabbage; indole-3-acetaldoxime; indole-3-acetonitrile; indole-3-methylglucosinolate; IAA biosynthesis.

Abstract—The metabolism of ^{14}C-indole-3-acetaldoxime by Chinese cabbage hypocotyls was investigated using labelled tracer and incubation times less than 1 hr. Indole-3-acetonitrile, indole-3-methylglucosinolate and desulpho-indole-3-methylglucosinolate were the major metabolites, while IAA or other IAA precursors were not detected. The kinetics of the conversion of the aldoxime to the three metabolites was different under continuous feeding and pulse feeding conditions. The apparent K_m for the conversion of the aldoxime to the nitrile and the glucosinolate were 3.3 and 5.0 μM, respectively. Tissues of *Isatis tinctoria*, *Helianthus annuus* and *Zea mays* also formed significant amounts of the nitrile and *Zea mays* formed small amounts of indole-3-acetaldehyde.

INTRODUCTION

A possible role of indole-3-acetaldoxime for the biosynthesis of IAA in plants has been claimed by several authors [1–4]. Plant tissues may convert the aldoxime to indole-3-acetaldehyde, indole-3-ethanol, indole-3-acetonitrile and IAA [1–6]. Furthermore, indole-3-acetaldoxime is a precursor of the indole-3-methylglucosinolates which are secondary plant products of the crucifers [3, 7–11]. These observations and the formation of ^{14}C-indole-3-acetaldoxime from ^{14}C-L-tryptophan as well as its extraction from cabbage tissue followed by MS identification strongly support the occurrence of indole-3-acetaldoxime as an endogenous plant metabolite [12]. Other amino acid derived aldoximes play a role in the formation of several glucosinolates and cyanogenic glucosides [9–11, 13, 14]. However, the concentration of extractable indole-3-acetaldoxime in cruciferous tissues as determined by HPLC seems to be rather small [$\leqslant$ 50 pmol/g fr. wt; J. Helmlinger, unpublished] which is possibly a consequence of its rapid conversion to indole-3-methylglucosinolate via a channeled multi-enzyme catalysed reaction analogous to the biosynthesis of cyanogenic glucosides [13, 14]. In the investigations cited above the concentration of applied indole-3-acetaldoxime was varied from 3 mM [2] to 100 μM [15] mainly because of the low specific activity of the labelled aldoxime used. Under such conditions nonspecific reactions due to a low affinity of endogenous enzymes towards the aldoxime fed might result in nonspecific metabolic conversions. Therefore, in an attempt to follow the metabolic routes of ^{14}C-indole-3-acetaldoxime under conditions close to the endogenous concentrations, we investigated the metabolism of ^{14}C-indole-3-acetaldoxime at a concentration of 0.5 μM. To minimize nonspecific wound reactions, the incubation times never exceeded 1 hr. Furthermore, we have compared the concentration dependence of the *in vivo* conversions of ^{14}C-indole-3-acetaldoxime to labelled indole-3-acetonitrile and indole-3-methylglucosinolate to establish whether independent formation of these compounds does indeed occur in crucifers [1–3, 5].

RESULTS

Reaction products of the metabolism of ^{14}C-indole-3-acetaldoxime by Chinese cabbage seedlings

The metabolism of ^{14}C-indole-3-acetaldoxime when fed to hypocotyl tissue of dark grown Chinese cabbage seedlings for time intervals not exceeding 1 hr revealed three major metabolites: indole-3-acetonitrile (Fig. 1), indole-3-methylglucosinolate (Fig. 2) and desulpho indole-3-methylglucosinolate (Fig. 2). Growing the seedlings under permanent light did not influence the results

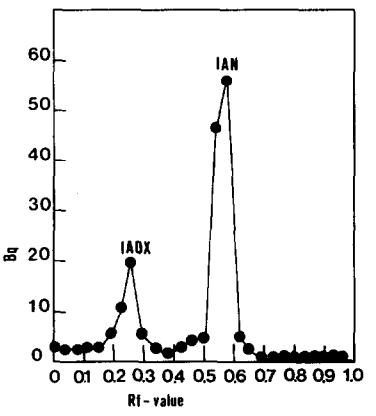

Fig. 1. Metabolism of ^{14}C-indole-3-acetaldoxime to benzene soluble products, extracted at pH 7. Distribution of radioactivity after TLC on a silica gel plate, solvent CH_3Cl–MeOH (96:4). After a 5 min pulse with 1 kBq labelled aldoxime the tissue (1 g fr. wt) was incubated for 1 hr in substrate-free buffer.

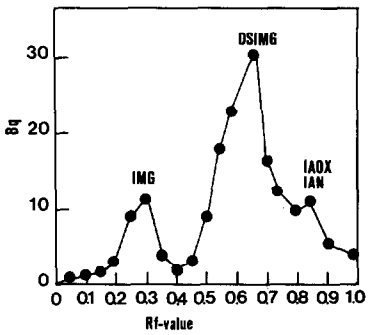

Fig. 2. Metabolism of ^{14}C-indole-3-acetaldoxime to polar H_2O soluble products under the conditions described in Fig. 1. Distribution of radioactivity after TLC on a cellulose plate, solvent *n*-BuOH–HOAc–H_2O (4:2:1).

(data not shown). These metabolites together with the nonmetabolized aldoxime accounted for 85–90% of the total label extracted from the tissue. The recovery in the methanolic extract was about 90% of the radioactivity taken up from the feeding solution.

The identification of indole-3-acetonitrile was confirmed by HPLC (data not shown). The HPLC analysis of the non-metabolized aldoxime revealed only the *Z*- and *E*-isomer [16]. There was no contamination with indole-3-ethanol, which, in the TLC system used, almost co-migrates (R_f 0.20) with the aldoxime (data not shown). The radioactivity profile of the aqueous phase after the benzene extraction, when chromatographed on cellulose plates without further column purification, revealed indole-3-methylglucosinolate and its desulpho derivative as the major metabolites with some radioactivity due to incomplete extraction of the aldoxime and the nitrile (Fig. 2). The identity of the labelled glucosinolate was confirmed by co-chromatography with an unlabelled standard on a Sephadex G 10 column and localization on cellulose TLC plates by spraying with *p*-dimethylaminocinnamaldehyde or dimethylaminobenzaldehyde. However, up to 10% of the ^{14}C-indole-3-methylglucosinolate peak on the TLC plate overlapped with the 1-methoxy-indole-3-methylglucosinolate zone (R_f 0.35). Thus a small percentage of the radioactivity may be contributed by the latter compound. The indole-3-methylglucosinolate fraction was not further analysed by HPLC. Therefore, it is possible that other indole-3-methylglucosinolate derivatives, like the 4- or 5-hydroxy- and methoxy-compounds, became equally labelled [17–19]. The identity of the desulpho compound (Fig. 2) was confirmed by co-chromatography with a ^{14}C-labelled standard derived from ^{14}C-indole-3-methylglucosinolate [20]. Here again the presence of the corresponding 4- or 5-hydroxy and methoxy derivatives cannot be excluded. All attempts to detect IAA or indole-3-acetaldehyde as significant metabolites of ^{14}C-indole-3-methylglucosinolate failed (data not shown).

Reaction kinetics of the metabolism of ^{14}C-indole-3-acetaldoxime

When 1 kBq ^{14}C-labelled aldoxime (0.55 μM) was fed to 1 g fr. wt hypocotyl tissue of Chinese cabbage seedlings in a continuous feeding experiment about 50% of the aldoxime was rapidly taken up in the initial 20 min. During this time there was a continuous incorporation of radioactivity into indole-3-methylglucosinolate, its desulpho derivative and indole-3-acetonitrile but later the accumulation of radioactivity into these compounds levelled off (Fig. 3). The glucosinolate and the nitrile accumulated label to a similar extent but the incorporation into the desulpho compound was considerably lower. For comparison, pulse experiments were performed by transferring the tissue after 5 min to substrate-free buffer. Under these conditions incorporation of label into indole-3-acetonitrile increased for up to 50 min while the glucosinolate accumulated considerably less label than the nitrile, showing only little further increase after the initial pulse (Fig. 4). Surprisingly, in pulse experiments the desulpho compound showed a far greater incorporation of label than the glucosinolate.

In an attempt to differentiate kinetically between the conversion of the aldoxime to the nitrile and the glucosinolate, the concentration dependence of both conversions were compared (Figs 5 and 6) by varying the aldoxime concentration between 0.1 and 5 μM. The

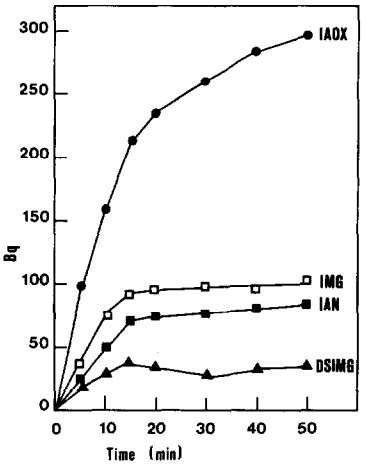

Fig. 3. Time course of ^{14}C-indole-3-acetaldoxime metabolism to labelled products. The concentration of the aldoxime was 0.55 μM (1 kBq/ml).

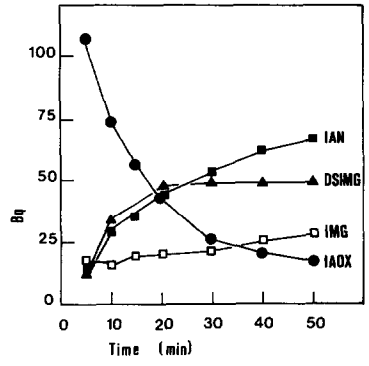

Fig. 4. Pulse feeding of ^{14}C-indole-3-acetaldoxime. The plant material was incubated for 5 min at 1 kBq/ml (concentration 0.55 μM) aldoxime and then transferred to substrate-free buffer.

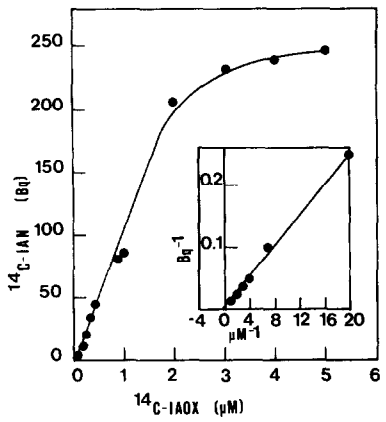

Fig. 5. Concentration dependence of ^{14}C-indole-3-acetaldoxime conversion to labelled indole-3-acetonitrile. Incubation time was 10 min. Inset: Data transformed according to Lineweaver–Burk.

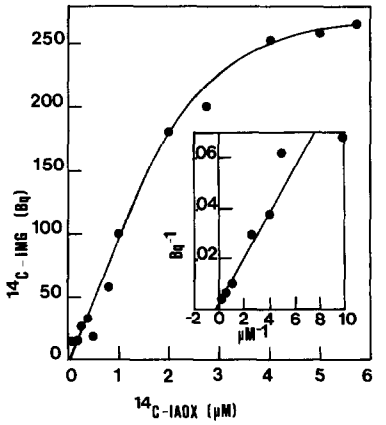

Fig. 6. Concentration dependence of ^{14}C-indole-3-acetaldoxime conversion to labelled indole-3-methylglucosinolate. Incubation time was 10 min. Inset: Data transformed according to Lineweaver–Burk.

incubation time was 10 min to assure a linear relationship between time and product formation (for comparison see Fig. 3). The results showed an apparent substrate saturation for both conversions, with apparent K_m values of 3.3 μM for indole-3-acetonitrile formation and 5.0 μM for indole-3-methylglucosinolate formation.

Conversion of ^{14}C-indole-3-acetaldoxime to ^{14}C-indole-3-acetonitrile by other plant species

Tissues of *Isatis tinctoria*, *Helianthus annuus* and *Zea mays* were incubated with labelled aldoxime under the same experimental conditions. All of these plants formed significant amounts of the nitrile (Table 1). *Zea mays* tissue showed some formation of indole-3-acetaldehyde, but in the extracts of the other plant species no aldehyde was detected (Fig. 7). *Isatis tinctoria* tissue incorporated label into indole-3-methylglucosinolate and its desulpho derivative to a similar extent as Chinese cabbage (data not shown).

DISCUSSION

Surprisingly, in cruciferous tissue indole-3-acetonitrile is the only significant benzene soluble metabolite of ^{14}C-

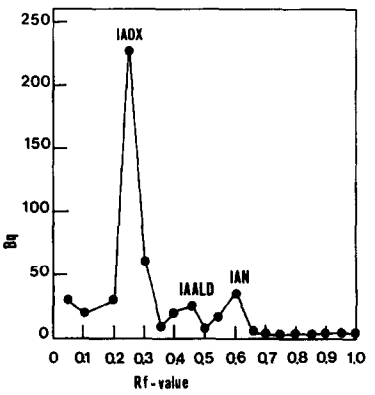

Fig. 7. Metabolism of ^{14}C-indole-3-acetaldoxime to benzene soluble products extracted at pH 7.0 in *Zea mays* mesocotyl. Conditions as described in Fig. 1.

Table 1. Formation of ^{14}C-indole-3-acetonitrile from ^{14}C-indole-3-acetaldoxime in different plant species

Plant material	Uptake of ^{14}C-aldoxime (Bq)	^{14}C-labelled nitrile formed	
		Bq	% of aldoxime taken up
Brassica camp. sub sp. *pekinensis* var. Granat (hypocotyl)	320	48	15.1
Isatis tinctoria (hypocotyl)	427	62	14.5
Zea mays (mesocotyl)	470	61	13.0
Helianthus annuus (hypocotyl)	435	36	8.1

Labelled aldoxime was fed at 0.55 μM (1.85 MBq/μmol) for 10 min to 1 g fr. wt plant material at 25°. Incorporation of label into the nitrile was calculated from the TLC radioactivity profiles of the neutral benzene soluble fraction. Values are means of two independent experiments.

indole-3-acetaldoxime (Fig. 1). In contrast to earlier reports [1, 2, 5, 6] there is no indication of indole-3-acetaldehyde, indole-3-ethanol or IAA formation. This discrepancy is probably due to the different experimental conditions, as in earlier studies 2 to 3 orders of magnitude greater aldoxime concentrations were used [2, 5, 6, 15]. The formation of labelled indole-3-methylglucosinolate and its desulpho derivative from ^{14}C-indole-3-acetaldoxime (Figs 2–4) is in agreement with earlier work [7, 8, 10, 11, 15]. However, when ^{14}C-tryptophan is fed at the same concentration under similar experimental conditions no accumulation of the labelled desulpho compound is observed [21–23; J. Helmlinger, unpublished]. This difference may be explained in two ways: either the aldoxime inhibits the conversion of the desulpho compound to indole-3-methylglucosinolate, or the exogenous aldoxime leads to such a large formation of the desulpho compound that the sulphation step becomes rate limiting. Support for an inhibition of the sulphation reaction is given by the comparison of the different feeding conditions (Figs 3 and 4). The accumulation of aldoxime in the continuous feeding experiments apparently inhibited the conversion to indole derivatives after only 15 min.

The formation of large amounts of labelled indole-3-acetonitrile via thioglucoside glucohydrolase (EC 3.2.3.1, myrosinase) catalysed breakdown of indole-3-methylglucosinolate seems to be excluded for several reasons: (1) When ^{14}C-tryptophan is fed to cruciferous tissues the label found in the nitrile rarely exceeds 5% of the label found in the glucosinolate and is often less than 1% [24; J. Helmlinger, unpublished]. (2) The *in vitro* degradation of ^{14}C-indole-3-methylglucosinolate under various experimental conditions leads to rather slow rates of nitrile formation, even at acidic pH [25, 26]. (3) The same results are obtained when the incubation is stopped, either by immersion into liquid nitrogen, or by boiling the tissue in methanol. Furthermore, *in vivo* formation of the nitrile from the glucosinolate to the extent observed in this study is rather improbable as the compartmentalization of indole-3-methylglucosinolate, thioglucoside glucohydrolase and its activator ascorbic acid are all well documented [24, 27, 28].

Other possible routes of nitrile formation could either be the postulated indole-3-acetaldoxime hydrolase reaction [5, 29] or the chemical and/or enzymatic degradation of indole-3-methylglucosinolate precursors like the thiohydroxamate [8] or the desulpho glucosinolate [7]. The pulse feeding experiments would give some support to the latter assumption when comparing the time course of the indole-3-acetonitrile and desulpho glucosinolate formation (Fig. 4). However, the conversion of the latter compound remains speculative as it has not been investigated in detail [7]. The comparison of the concentration dependence of the conversion of the aldoxime to the nitrile and the glucosinolate reveals very close yet not identical apparent K_m values (Figs 5 and 6). Thus the results suggest that the two events are somehow related, but the mechanism is still unknown.

It is highly probable that the glucosinolate biosynthesis is a channeled process, similar to the synthesis of the cyanogenic glucosides [13, 14]. In the latter, some intermediates are preferentially metabolized when compared with the same substrate added exogenously [13]. The aldoxime is an exception in this respect as it is equally well accepted when fed externally [13]. Therefore, it may be speculated that in glucosinolate biosynthesis the aldoxime is rapidly equilibrating with the catalytic site of the presumed multi-enzyme sequence.

The formation of indole-3-acetonitrile from the aldoxime by the other plant species tested is of similar magnitude when compared with the crucifers (Table 1). As the formation of aldoximes via *N*-hydroxylation of amino acids seems to be a widespread phenomenon in plants the dehydration could equally be a general capacity of many plants, with the presence of a specific nitrilase (small K_m-value) deciding the possibility of IAA formation. However, so far plant nitrilases have not been shown to be very specific for indole-3-acetonitrile as substrate [30–33]. Furthermore, the present experiments suggest that IAN formed from indole-3-acetaldoxime *in vivo* is not available for hydrolysis by nitrilase, as exogenously fed ^{14}C-indole-3-acetonitrile becomes rapidly metabolized to IAA [21; J. Helmlinger, unpublished].

EXPERIMENTAL

Chemicals. ^{14}C-Indole-3-acetaldoxime was prepared from ^{14}C-DL-tryptophan (Amersham) by a two-step procedure as described in ref. [34]. Indole-3-acetonitrile, indole-3-acetaldehyde, indole-3-ethanol and IAA were purchased from Sigma. Unlabelled indole-3-acetaldoxime was synthesized from indole-3-acetaldehyde $NaHSO_3$ (Sigma) as described [16]. All other chemicals were of the highest purity available.

Plant material. Seeds of Chinese cabbage (*Brassica campestris* subsp. *pekinensis* cv. Granat), sunflower (*Helianthus annuus* L., var. Hohes Sonnengold), maize (*Zea mays* L., Inrakorn, Kategorie 2 a of the Deutsche Saatgut AG), and woad (*Isatis tinctoria* L., wild type) were surface sterilized with 0.2% $HgCl_2$ and cultivated in Petri dishes on filter paper soaked in 10% Knop's nutrient soln in the dark or permanent light (2 fluorescent lamps Philips TL 55 20 W and TL 32 de Luxe W; 6×10^{-4} W/cm^2) at 25° for 5 days.

Feeding of ^{14}C-indole-3-acetaldoxime. The appropriate amount of ^{14}C-indole-3-acetaldoxime (sp. act. 1.85 MBq/μmol) in C_6H_6 was added to 1 ml of 100 mM MES buffer, adjusted to pH 6 with 1 M KOH. The mixture was evacuated to evaporate the C_6H_6. Hypocotyls of 5-day-old seedlings were cut with a razor blade into 2 mm segments and washed thoroughly in 100 ml/g fr. wt substrate-free buffer. After blotting the tissue dry on filter paper, 1 g of plant material was added to the feeding soln. Incubation temp. was 25°. At times depending on the type of expt, the tissue was rapidly filtered and washed with 10 ml of substrate-free buffer. The tissue segments were immediately immersed in liquid N_2 and freeze dried. For pulse expts the segments were transferred to substrate-free buffer for various times.

Extraction of labelled metabolites. The freeze dried material was homogenized in a CO_2-cooled ball mill (Model L. Bernard—Type 1241) and extracted with 80% MeOH at 4° for 30 min at a ratio of 10 ml/g dry wt. With this procedure inactivation of myrosinase proved to be unnecessary. The homogenate was centrifuged at 5000 *g* for 10 min and the supernatant concentrated *in vacuo* at 30°. The aq. residue was immediately adjusted to pH 7 with 1 M KOH and extracted twice with 3 vols of C_6H_6. The combined C_6H_6 phases were dried with Na_2SO_4 and after concentration, used directly for TLC and/or HPLC analysis. The distribution of radioactivity between the aq. and the organic phase as well as the total recovery in the MeOH extract were determined by taking appropriate aliquots and determining their radioactivity.

Identification of neutral C_6H_6 soluble metabolites. The C_6H_6 soluble fraction was chromatographed on silica gel F_{254} plates (alufoil; Merck) with $CHCl_3$–MeOH (96:4) as solvent. The

following standards were cochromatographed: IAA (R_f 0.10), indole-3-acetaldoxime (R_f 0.25), indole-3-acetaldehyde (R_f 0.35), and indole-3-acetonitrile (R_f 0.55). The standards were localized by fluorescense quenching at 254 nm. The chromatogram was then divided horizontally into 0.5 cm zones and the silica gel was scraped off with a micro spatula. The radioactivities of the different zones were determined after vigorously stirring the silica gel powder in 1 ml EtOH and subsequent addition of 3 ml of scintillant (Quickszint, Zinsser Analytical). The samples were counted in a liquid scintillation counter. Quench correction was performed according to the double channel ratio method. Individual samples were allowed to accumulate $\geqslant 10\,000$ counts. For further identification by HPLC, either the respective zones from the TLC plates were eluted with an appropriate vol. of MeOH or the total C_6H_6 fraction was evaporated to dryness and taken up in 100 μl 35% MeOH. Aliquots of 20 μl were submitted to analysis by HPLC Biotronik using a reverse phase column (Hyperchrome C_{18}-5 μ, Bischoff) equipped with a precolumn (Guard-Pak, C_{18}, precolumn module, Waters) was used with 35% aq. MeOH as solvent. Flow rate was 1 ml/min and UV-detection at 280 nm. This isocratic system allowed the separation of various lipophilic indole derivatives as described elsewhere [16, 35]. The eluate was collected in 1 ml fractions which were mixed with 3 ml of scintillant and their radioactivities were determined.

Identification of H_2O *soluble metabolites.* After C_6H_6 extraction the aq. phase was passed through a 5 ml ion exchange column (DE 52, DEAE cellulose, Whatman). The column was washed with 15 ml deionized H_2O to elute the non-bound fraction. The eluate was concentrated *in vacuo* to an appropriate vol. for TLC on cellulose-F_{254} plates (alufoil; Merck) with *n*-BuOH–HOAc–H_2O (4:1:2) as solvent. Desulpho-indole-3-methylglucosinolate (R_f 0.65) was identified by co-chromatography with a standard prepared from labelled indole-3-methylglucosinolate according to ref. [19], which involved treatment of ^{14}C-labelled glucosinolate bound to an ion exchange column with sulphatase from *Helix pomatia* (EC 3.1.6.1), and elution of the desulpho compound with deionized H_2O. The distribution of radioactivity on TLC plates was determined, as already described.

The fraction bound to DEAE-cellulose was eluted with 5 ml 2% aq. Na_2SO_4. A 4 ml aliquot was loaded on a Sephadex G10 column (20 × 2 cm). The column was eluted with deionized H_2O (flow rate 2 ml/min) and the indole-3-methylglucosinolate fraction (K_{av} 0.65) was collected. After reducing its vol. *in vacuo* the fraction was submitted to TLC on cellulose-F_{254} plates as already described. Identification of labelled indole-3-methylglucosinolate (R_f 0.25) was by co-chromatography with a standard prepared according to ref. [36]. Indole-3-methylglucosinolate was localized by colour reactions with *p*-diaminocinnamaldehyde and *p*-dimethylaminobenzaldehyde [37, 38]. The distribution of radioactivity on the TLC plate was determined as described above.

Identification of acidic CH_2Cl_2 *soluble metabolites.* After the extraction of neutral metabolites the aq. phase was adjusted to pH 3 with 1 M HCl and extracted twice with 3-fold vol. of CH_2Cl_2 (when indole-3-methylglucosinolate and its desulpho derivative were determined in the same expt, the aq. phase was divided into 2 equal portions). The organic phases were pooled and dried with Na_2SO_4. After reduction to an appropriate vol. for TLC *in vacuo* the acidic fraction was chromatographed on cellulose-F_{254} plates with EtOAc–MeCN–25% NH_4OH–H_2O (40:10:5:4) as solvent. Identification of IAA (R_f 0.31) was by co-chromatography with an authentic standard. For analysis by HPLC the respective zone from the TLC plate, or, alternatively, the total acidic fraction was used after complete evaporation of the organic solvent and taken up in 100 μl HPLC solvent. Conditions for HPLC were as already described except for the solvent which was MeOH–20 mM NH_4OAc (30:70, adjusted to pH 3.5 with 1 M HCl).

Statistical treatment of the data. All expts were repeated × 3–5. Incorporation rates for individual expts were determined in duplicates. Although the absolute radioactivities varied from expt to expt the relative incorporation of radioactivity into the different compounds proved to be reproducible. Therefore, the arithmetic means of duplicates of representative expts are presented.

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