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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION AND SOME PROPERTIES OF (*E*)- AND (*Z*)-3-INDOLEACETALDOXIME

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SUMMARY

An isocratic reversed-phase high-performance liquid chromatographic (HPLC) system for the separation of (*E*)- and (*Z*)-3-indoleacetaldoxime was developed. The indentities of the isomers were confirmed by NMR spectrometry. Both isomers could be converted into 3-indoleacetonitrile with acetic anhydride. UV irradiation of both isomers in ethanolic solution led to rapid photolysis. For the identification of the degradation products an isocratic silica gel HPLC system was developed, which showed that 3-methylindole was one major product whereas neither 3-indoleacetonitrile nor 3-indoleacetamide was formed in significant amounts. Conversely, in acetic acid both isomers were relatively stable to UV light. The implications of the results for studies of the role of 3-indoleacetaldoxime as a natural compound in higher plants are discussed.

INTRODUCTION

An essential role of 3-indoleacetaldoxime in the biosynthesis of the plant hormone 3-indoleacetic acid (IAA) has been implicated by several workers[1,2]. It has been reported that plant tissues may convert 3-indoleacetaldoxime into the corresponding aldehyde and nitrile[2], both of which are possible precursors of IAA, and to the glucosinolate glucobrassicin (3-indolemethylglucosinolate), a natural product in some plant families[3–5]. The presence of endogenous 3-indoleacetaldoxime in higher plants has been demonstrated by mass spectrometry[6]. However, no attention has been paid to the occurrence of stereochemical isomers of 3-indoleacetaldoxime, and in metabolic studies the stereospecificity was not investigated. As the glucosinolate glucobrassicin occurs exclusively in the *E* conformation[7], it may be postulated that the endogenous aldoxime appears only as one isomer in plants.

It was the aim of this study to demonstrate the chemical synthesis of (*E*)- and (*Z*)-3-indoleacetaldoxime (Fig. 1) from the corresponding aldehyde, the separation of the isomers by high-performance liquid chromatography (HPLC) and some of their chemical properties to allow an experimental reappraisal of their different biochemical functions.

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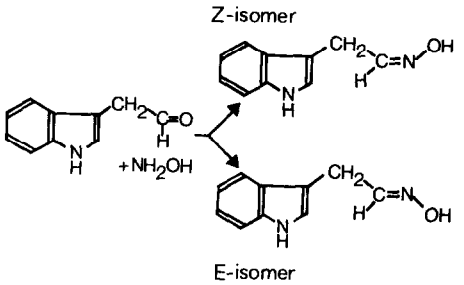

Fig. 1. Synthesis of (*E*)- and (*Z*)-3-indoleacetaldoxime.

EXPERIMENTAL

Materials

Compounds used were 3-indoleacetaldehyde · $NaHSO_3$ (Sigma), [side-chain-3-^{14}C]DL-tryptophan, specific activity 1.85 MBq μmol^{-1} (Amersham International), 5% NaOCl solution (Merck), 3-indoleacetamide and 3-indoleacetonitrile (Sigma), 3-methylindole (Fluka) and 3,3'-diindolylmethane, synthesized from 3-hydroxymethylindole (Sigma) with NaOH according to Leete and Marion[8].

Preparation of 3-indoleacetaldoxime. 3-Indoleacetaldoxime was synthesized from 3-indoleacetaldehyde · $NaHSO_3$ according to Ahmad *et al.*[9]. The free aldehyde was released by addition of 1 ml of 1 *M* Na_2CO_3 to 1 ml of 0.1 *M* 3-indoleacetaldehyde · $NaHSO_3$ and subsequent thorough mixing with 10 ml of benzene for 5 min. The benzene phase was transferred into 2 ml of 0.1 *M* hydroxylamine (pH 7.0) and the two layers were stirred vigorously for 1 h at 25°C. The benzene phase was pipetted off and dried with sodium sulphate. The concentration of 3-indoleacetaldoxime was determined by the method of Ahmad *et al.*[9].

For the preparation of crystalline 3-indoleacetaldoxime, the dried benzene phase was evaporated to dryness by rotary film evaporation and the aldoxime resolved in a small volume of acetone. Crystalline 3-indoleacetaldoxime was precipitated by adding distilled water.

[2-^{14}C]Indoleacetaldoxime. This was prepared by a microscale method according to Hofmann *et al.*[10].

Tryptophan. [Side-chain-3-^{14}C]-DL-tryptophan was dissolved in 1 ml of Na_2CO_3 (pH 10.0), overlayered with benzene and stirred vigorously at 45°C. [^{14}C]Tryptophan was converted into [2-^{14}C]indoleacetaldehyde by adding 0.2% NaOCl solution at 4 μl min^{-1} for 1 h. The benzene phase was transferred into 1 *M* hydroxylamine (pH 7.0) and further processed as described above. The radiochemical purity was determined by thin-layer chromatography (TLC) and HPLC to be >95%.

TLC and HPLC procedures

TLC was performed on silica gel 60 F_{254} plates (Merck) with chloroform–methanol (96:4) as the solvent. The identity of all compounds was confirmed by co-chromatography with authentic standards, by fluorescence quenching at 254 nm and by colour reaction with dimethylaminocinnamaldehyde. R_F values: 3-indoleacetic acid (0.10), 3-indoleacetamide (0.12), 3-indoleethanol (0.23), 3-indoleacetal-

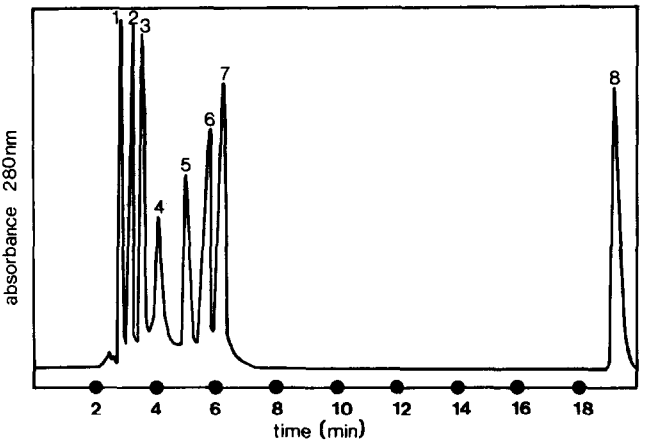

Fig. 2. HPLC separation of 3-substituted indoles: (1) 3-methylindole; (2) 3,3′-diindolylmethane; (3) 3-indoleacetonitrile; (4) 3-indoleacetaldehyde; (5) 3-indoleacetaldoxime; (6) 3-indoleacetic acid; (7) 3-indoleethanol; (8) 3-indoleacetamide. Column, LiChrosorb Si 60, 5 μm; solvent system, chloroform–methanol–acetic acid (98:1.5:0.5); flow-rate, 1 ml min^{-1}; detection, absorbance at 280 nm.

doxime [(*Z*)- and (*E*)-isomer 0.25], 3-indoleacetaldehyde (0.34), 3-indoleacetonitrile (0.48), 3-methylindole (0.54), 3-3′-diindolylmethane (0.58).

HPLC was performed with a Biotronik BT 3020 HPLC pump, a BT 3021 column unit and a BT 3030 UV detector. All compounds were detected by their absorbance at 280 nm. For the separation of (*E*)- and (*Z*)-3-indoleacetaldoxime, a reversed-phase Si C_{18} column was used (Hyperchrome NC; Bischoff Analysentechnik und -geräte GmbH) with either 46% or 35% aqueous methanol as the solvent at a flow-rate of 0.7 ml min·−1. A general separation of a wide range of indolic compounds (which, however, did not separate the aldoxime isomers) was achieved on a silica gel column (LiChrosorb Si 60, 5 μm; Merck) with chloroform–methanol–acetic acid (98:1.5:0.5) as the solvent at a flow-rate of 1 ml min^{-1} (Fig. 2). This system was used for the identification of indolic degradation products after UV irradiation of the individual aldoxime isomers.

Conversion of 3-indoleacetaldoxime to 3-indoleacetonitrile

Acetic anhydride (10 mmol) was added to 0.1 mmol of either (*E*)- or (*Z*)-3-indoleacetaldoxime dissolved in 10 ml of benzene. The reaction mixture was stirred at 25°C for 20 min, then unreacted acetic anhydride and the acetic acid formed during the reaction were eliminated by stirring the benzene phase with 10 ml of water and adding dropwise 1 *N* NaOH until the aqueous phase reached a constant pH of 8.5. The benzene phase was dried with sodium sulphate and reduced to a convenient volume for preparative TLC. Aliquots were evaporated to dryness and resolved in ethanol for direct application to the HPLC system. The identity of the 3-indoleacetonitrile formed was confirmed by co-chromatography with an authentic standard.

Alternatively, the aldoximes were spotted on a silica gel 60 F_{254} plate and acetic anhydride was applied directly as spots covering the areas of the aldoximes. After 10 min the plate was dried in a stream of cold air until all the excess of acetic

anhydride is evaporated. TLC revealed one major compound co-chromatographing with authentic 3-indoleacetonitrile in the TLC and HPLC systems described above.

Photolysis of (E)- and (Z)-3-indoleacetaldoxime

The isomers were, separated by HPLC. The solvent was evaporated by rotary film evaporation at 35°C and the aldoxime was taken up in ethanol at a final concentration of 0.1 m*M*. The solution was irradiated in a Desaga multi-purpose instrument for UV at 254 and 366 nm simultaneously. The temperature was either 4 or 25°C. Aliquots of 20 µl were analysed directly by HPLC and the peak areas were quantified by automatic integration with a Spectra Physics SP 4270 integrator.

Physical spectra of 3-indoleacetaldoxime

UV spectra of (*E*)- and (*Z*)-3-indoleacetaldoxime were obtained on a diode-array detector (Hewlett-Packard 1040 A) coupled to a plotter (HP 85 A) for the range 190–600 nm. NMR spectra (270 MHz) were obtained on a Bruker WH 270 NMR spectrometer with C^2HCl_3 as solvent. IR spectra were obtained using potassium bromide pills on a Perkin-Elmer 521 IR spectrophotometer. The spectra were in agreement with the data published by Ahmad *et al.*[9].

RESULTS AND DISCUSSION

Synthesis of (E)- and (Z)-indoleacetaldoxime

(*E*)- and (*Z*)-3-indoleacetaldoxime were synthesized from 3-indoleacetaldehyde and separated by HPLC on a reversed-phase column (Fig. 3). The *E*/*Z* ratio was 1.5 at 25°C (Fig. 4). The UV spectra determined with a diode-array detector were identical (Fig. 5) and so were their IR spectra (data not shown).

The identification of the *E*- and *Z* isomers was achieved by comparison of the NMR spectra. The doublet signal of the methylene-H for the *Z* isomer appears at σ 3.88 ppm and the corresponding signal for the *E* isomer is shifted to σ 3.68 ppm.

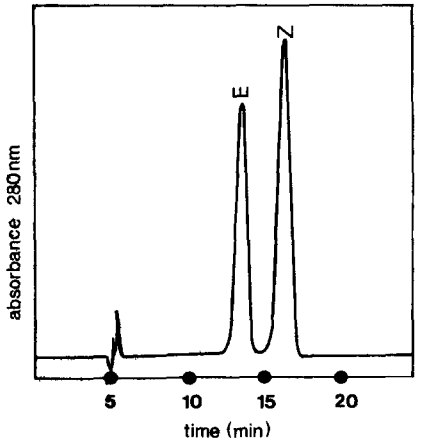

Fig. 3. HPLC profile of (*E*)- and (*Z*)-3-indoleacetaldoxime. Column, reversed-phase Si C_{18}; solvent, 46% aqueous methanol; flow-rate, 0.7 ml min^{-1}; detection, absorbance at 280 nm.

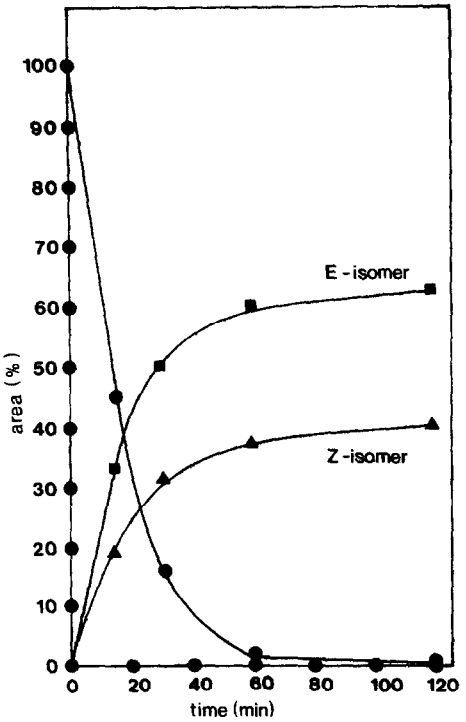

Fig. 4. Time course of conversion of 3-indoleacetaldehyde (●) into (*E*)- and (*Z*)-3-indoleacetaldoxime. Initial concentrations: 3-indoleacetaldehyde 0.1 m*M*, hydroxylamine 0.1 *M*. Reaction temperature, 25°C; pH, 7.0.

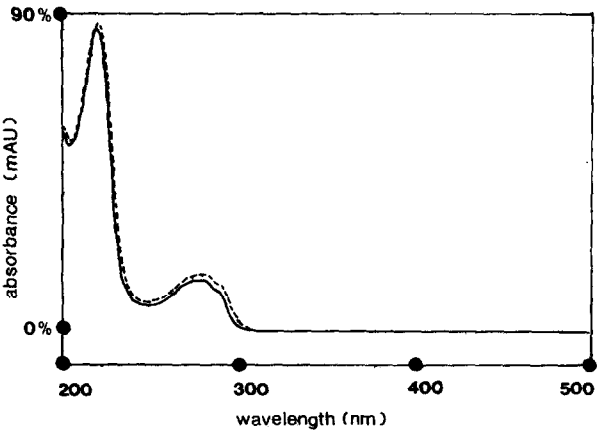

Fig. 5. UV spectra of (*E*)- and (*Z*)-3-indoleacetaldoxime obtained with a diode-array detector after HPLC separation (for conditions see Fig. 3). Continuous line, spectrum at 13.54 min; broken line, spectrum at 16.46 min.

It is well known that the methine proton of the (*E*)-aldoximes is shifted to high field compared with the *Z* isomer[11]. Crystallization led to the almost exclusive precipitation of the *Z* isomer under the conditions described under Experimental.

As in higher plants the biological activities of the two isomers could be completely different in their respective functions as the precursor of indoleglucosinolates and/or the precursor of 3-indoleacetonitrile (which is readily converted into the plant hormone 3-indoleacetic acid), the HPLC separation of the isomers provides a means of assessing several still unsettled questions[1]. With other plant hormones such as abscisic acid and some cytokinins, the introduction of HPLC systems to separate the stereoisomers has already contributed substantially to our understanding of their biological functions[12,13].

Properties of (E)- and (Z)-3-indoleacetaldoxime

Both aldoxime isomers were converted into 3-indoleacetonitrile with acetic anhydride (Fig. 6). The reaction was fairly selective when appropriate precautions were taken (complete exclusion of water, prior separation from any contaminants).

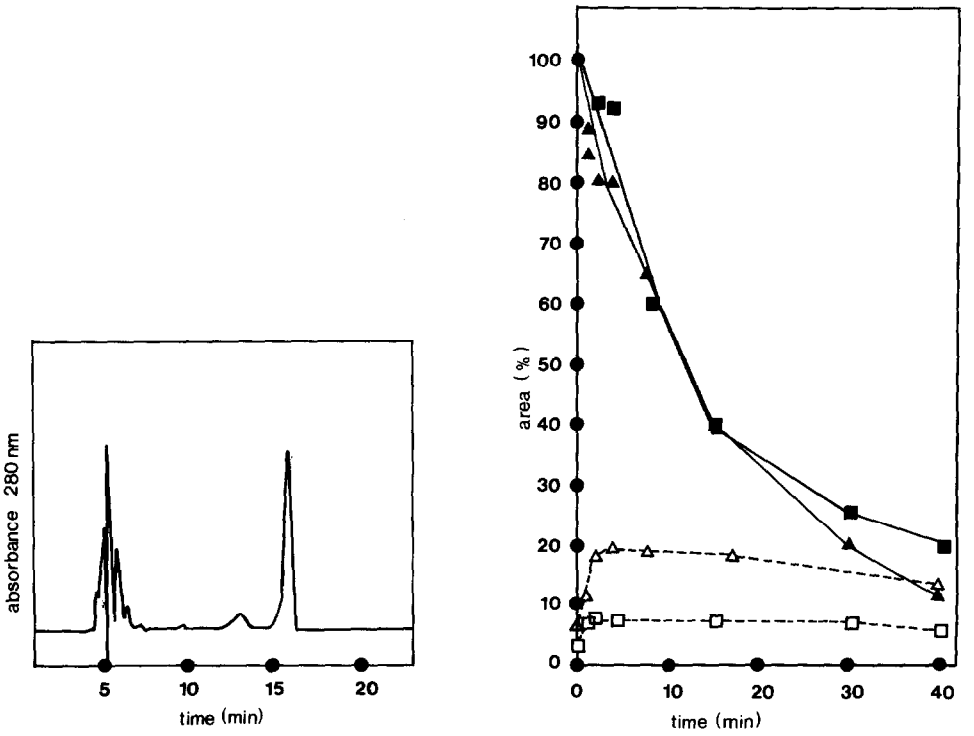

Fig. 6. HPLC separation of product after reaction of (*E*)-3-indoleacetaldoxime with acetic anhydride. HPLC profile after prior purification of 3-indoleacetonitrile formed by TLC. Retention time for authentic 3-indoleacetonitrile, 16.4 min. Conditions as in Fig. 3. The corresponding profile after reaction of (*Z*)-3-indoleacetaldoxime is identical (data not shown).

Fig. 7. Time course of UV photolysis of (*E*)-3-indoleacetaldoxime (▲) and (*Z*)-3-indoleacetaldoxime (■). △, *Z* Isomer formed during photolysis of *E* isomer; □, *E* isomer formed during photolysis of *Z* isomer. HPLC conditions as in Fig. 3.

When performed directly on the TLC plate the reaction will be a useful tool for identifying 3-indoleacetaldoxime in plant extracts when both the presumed aldoxime and the nitrile thus formed are subsequently submitted to HPLC and mass spectrometry.

Irradiation of the separated isomers with UV light (simultaneously at 254 and 356 nm) at 25 or 4°C led to only limited isomerization (Fig. 7) but, as expected, the conversion of the *E* to the *Z* isomer was significantly higher than the reverse isomerization. However, a rapid photolysis was observed for both isomers (Fig. 7). 3-Methylindole was identified as a major product by TLC and HPLC (see Fig. 2). For comparison, 3-indoleacetonitrile was irradiated under the same conditions; only 18% degradation was observed after 40 min, making the nitrile an unlikely intermediate in the photolysis of the aldoxime. However, with acetic acid as the solvent both aldoxime isomers were stable under UV light, but in this instance no significant isomerization was observed. All analytical procedures were confirmed by using [2-^{14}C]indoleacetaldoxime isomers.

In the literature it has been suggested that some ketoximes and several arylaldoximes may be converted into the corresponding amides by UV irradiation via a photo-Beckmann rearrangement[14–17]. As the occurrence of 3-indoleacetamide as a possible intermediate in 3-indoleacetic acid biosynthesis in higher plants has recently gained new interest[18], we investigated the possibility of its formation during the photolysis of 3-indoleacetaldoxime. However, neither 3-indoleacetamide nor 3-indoleacetonitrile was found in the reaction medium after 5 or 30 min.

We conclude that future investigations on 3-indoleacetaldoxime in higher plants will have to take account of the occurrence of stereoisomers. UV light should be avoided, especially when handling alcoholic plant extracts. Chemical conversion of the aldoxime to the amide or the nitrile during conventional extraction procedures for plant hormone analysis seems unlikely, according to our results.

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