Occurrence of 3,4-Diacetoxybutinylbithiophene in *Tagetes patula* and Its Enzymatic Conversion

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3,4-Diacetoxybutinylbithiophene was found to occur in considerable amounts in roots and hypocotyls of young *Tagetes* seedlings. This compound was converted enzymatically to 3,4-dihydroxybutinylbithiophene by crude extracts from this plant. Experiments performed with enzyme preparations from plants of different stages of development supported the existence of two position-specific, subsequently working dithiophene acetate esterases. It is suggested that 3-hydroxy-4-acetoxybutinylbithiophene is the intermediate product, formed by an highly specific 3,4-diacetoxybutinylbithiophene: 4-acetate esterase.

Introduction

Thiophene derivatives are chemotaxonomical markers for some tribes of the Asteraceae (Compositae) family [1, 2]. Six tribes are reported to contain dithiophenes, and the Heliantheae, Helenieae, Arctotideae and Cynareae, particularly, are characterized by high accumulation of these compounds.

Substitution patterns, most frequently, encountered in these thiophene derivatives, are acetoxy- or hydroxy groups on the side-chain. Examples are 3,4-diacetoxybutinylbithiophene, 3-hydroxy-4-acetoxybutinylbithiophene and 3,4-dihydroxybutinylbithiophene which occur in fourteen *Echinops* (Cynareae) species, and in seven Arctotideae species [2, 3]. The existence of vicinal acetoxy- and/or hydroxy groups in the side-chains was also recently reported for monothiophenes from *Leuzea* (Cynareae) roots [4].

Several hypotheses concerning biosynthetic and/or metabolic conversions of these compounds are based on tracer experiments (*e.g.* [2, 5]). However, the enzymatic conversion of thiophenes or, of acetylesters has only been reported in one study [6]. This dealed with the existence of 4-hydroxybutinylbithiophene [2, 7–9] and of an highly specific hydrolase in adult *Tagetes* plants. We now report the occurrence of 3,4-diacetoxybutinylbithiophene in *Tagetes* seedlings and its conversion by a two-step reaction using crude enzyme preparations isolated from this material.

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Material and Methods

Plant material

Seedlings of *Tagetes patula* L. cv. "Zitronenzwerg" were grown in moist Vermiculite under illumination conditions as previously described [8]. Plant growth stages are abbreviated as follows: "6D2L", for example means, plants were grown for 6 days in the dark, then for 2 days under illumination (15 h light period/day).

Chemicals

CH_3CN and H_2O used for HPLC were of HPLC-grade (Baker Chem., Deventer, Netherlands; Roth, Karlsruhe, F.R.G.). All other reagents used were of analytical grade.

3,4-Diacetoxybutinylbithiophene was prepared from roots and from hypocotyls of young *Tagetes* seedlings by semi-preparative HPLC (see below). For reference purposes, it was compared with a sample of authentic material kindly supplied by Prof. J. Lam (Aarhus, Denmark). 3,4-Dihydroxybutinylbithiophene was obtained by alkaline hydrolysis of the diacetate (satd. ethanolic KOH, 5 min incubation at room temp.). From previous work [6, 8], samples of 4-hydroxybutinylbithiophene, 4-acetoxybutinylbithiophene, dithienylbutinene and terthienyl were available as reference substances.

Isolation and purification of 3,4-diacetoxybutinylbithiophene

Tagetes roots and/or hypocotyls were homogenized in EtOH and extracted with petrol, as

previously described [8]. After evaporation to dryness, the residue was dissolved in abs. EtOH. This solution was subjected to semi-preparative HPLC (col. 8 × 250 mm; LiChrosorb RP18, 5 µ, Merck, Darmstadt, F.R.G.; gradient: linear 75% CH_3CN – 25% H_2O to 100% CH_3CN (15 min), then 100% CH_3CN for 15 min, flow 2.5 ml/min; detection spectrophotometrically at 333 nm). Fractions containing the diacetate (R_t = 9.2 min) or, after alakline hydrolysis, the dialcohol (R_t = 4.9 min) were collected and immediately subjected to freeze drying. The residue was taken up in ethyleneglycolmonomethylether and stored at – 20 °.

Isolation of partially purified enzyme extracts

Plant material was homogenized and insoluble residues were removed by filtration and by centrifugation; protein containing fractions were collected after gel filtration on Sephadex G-25. These steps were previously described [6]. Protein concentration was performed by ultrafiltration on an Amicon PM30 membrane. These protein concentrates were used as enzyme source.

Enzyme assay

Enzyme assays were carried out at 30 ° and precautions were taken to avoid UV or direct daylight. The mixture (100 µl) contained 3 nmol of the respective substrate and 0.1 mg of bovine albumin in 0.1 mM KPi buffer, pH 8.0. The reaction was stopped by immediate injection of the mixture to an HPLC (4 × 250 mm column, Spherisorb ODS2, 5 µ (Phase Separations, Queensferry, U.K.); solvent: 75% CH_3CN – 25% H_2O, isocratic; detection spectrophotometrically at 333 nm). Peak integration was performed by a SP 4100 computing integrator. Enzyme activity was calculated by the decrease in substrate as well as by the amount of product formed expressed as arbitrary units (A.U.). At incubation time 0 or when incubated with boiled enzyme preparations, no enzyme activity was detectable in controls.

Protein estimation

Protein content was measured following the method of Ohnishi and Barr [10].

Results and Discussion

1. The occurrence of 3,4-diacetoxybutinylbithiophene and other natural products in Tagetes patula seedlings

Fig. 1 shows the typical semi-preparative HPLC fractionation of an EtOH-PE extract from roots of *Tagetes* seedlings, grown for six days in the dark and then illuminated for two days (stage "6D2L"). Four of these UV-A absorbing compounds were identified as 4-hydroxybutinylbithiophene (**1**), 4-acetoxybutinylbithiophene (**6**), dithienylbutinene (**11**) and α-tertiophene (**13**) by their spectral and chromatographic properties [2, 6–9] and by comparison with authentic reference substances. Compound **2** as well as compound **7** are benzofuran derivatives [11]; the structures of compounds **8, 9, 10** and **14** have not been elucidated. None of these compounds exhibits the spectral properties of the two acetylenic compounds which were isolated from leaves of adult plants [7]. **3** Showed the spectral and chromatographic properties of 3,4-diacetoxybutinylbithiophene as described in the literature [3] and cochromatographs with authentic material. This is

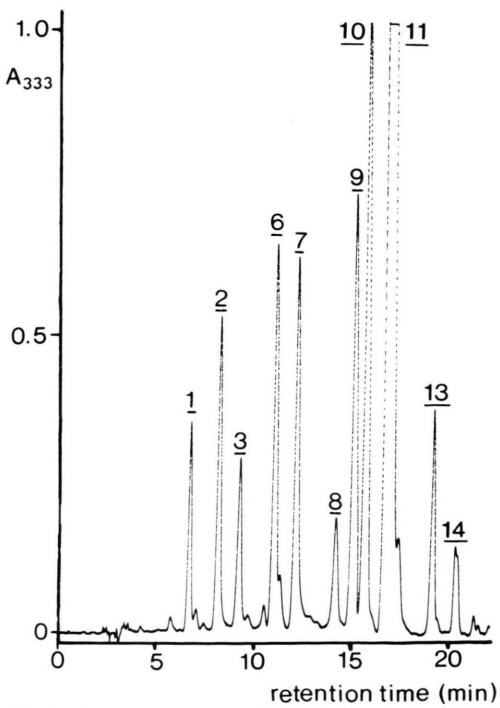

Fig. 1. Semi-preparative HPLC of a crude EtOH-PE extract from roots of *Tagetes* seedlings, stage "6D2L". Seedlings cultivation, isolation and separation techniques are described under "Material and Methods".

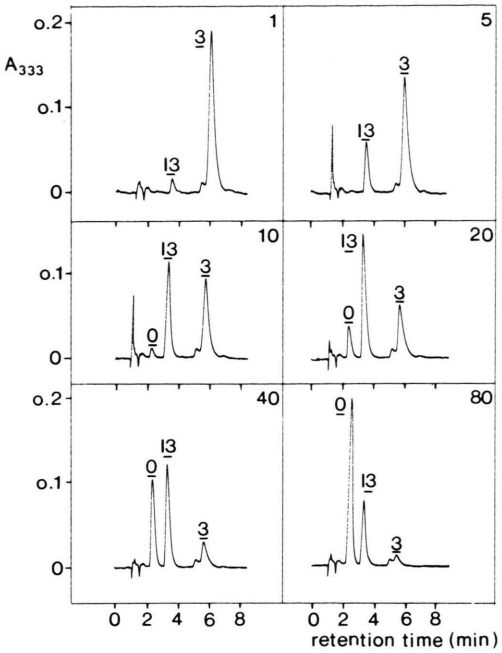

Fig. 2. Changes in levels of substrate (3,4-diacetoxybutinylbithiophene **3**), intermediate product (**I3**) and end product (3,4-dihydroxybutinylbithiophene **0**) during "E3" assay incubation determined by analytical HPLC. The numbers in the upper right corner represent the time of incubation. Separation conditions are described under "Material and Methods". Enzyme source: Crude extracts from roots, stage "2D7L".

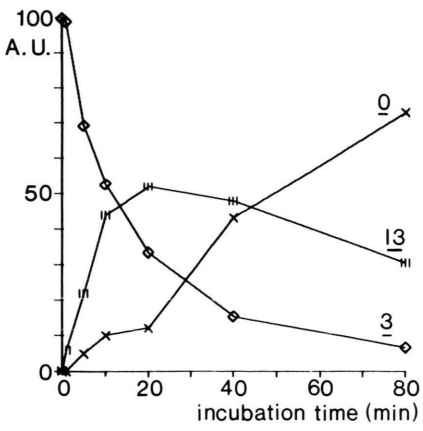

Fig. 3. Kinetics of substrate (**3**), intermediate (**I3**) and end product (**0**) formation in the "E3" assay. Conditions are shown in Fig. 2. A.U. = Arbitrary Units from peak integration.

the first report concerning the occurrence of a dithiophenyl diacetate in a species of *Tagetes*.

2. Enzymatic conversion of 3,4-diacetoxybutinylbithiophene

The hydrolysis of 3,4-diacetoxybutinylbithiophene **3** by crude enzyme extracts was observed over an 80 min period (Fig. 2). During the first 20 min of incubation, the diacetate **3** is converted to an intermediate product, **I3.** After approximately 10 min of incubation, a second product, **0** appears; by comparison of substrate disappearance with formation of **I3** and **0** it can clearly be seen that compound **0** is a product of the enzymatic conversion of **I3.** Samples of **0** were collected and analyzed; its identity with 3,4-dihydroxybutinylbithiophene was established by spectral data [3] and by cochromatography with authentic material. The kinetics involved in this reaction are shown in Fig. 3. The sigmoidal course of the formation of **0** points to a cooperation of two enzymes in the conversion of the diacetate. The enzymic hydrolysis of 3,4-diacetoxybutinylbithiophene will be referred to as the "E3"-reaction.

3. Activity of esterases in hypocotyls and roots of different developmental stages

In these experiments, the diacetate **3** as well as the monoacetate **6** were used as substrates because it was expected that the 4-acetoxybutinylbithiophene: 4-acetate esterase which was recently described [6] would participate in the conversion of **3**. As far as we are aware, this enzyme acts specifically on the 4-position of dithiophene acetate esters. In the following, the enzymic hydrolysis of **6** will be called "E6"-reaction.

Comparative analyses using crude enzyme extracts from plants illuminated from 2 days (stage "2D2L") to 10 days (stage "2D10L") showed remarkable differences in the activities of "E3" and of "E6". Also, the values obtained from roots and from hypocotyls of the same developmental stage as enzyme source differed significantly (Table I). The formation of **I3** and of the dialcohol, **0** in "E3" assays, incubated for 10 min, concurs very well with the enzymatic activity of "E3" and of "E6", respectively; a high ratio of "E3"/"E6" leads to an exclusive formation of **I3** (*e.g.*, hypocotyls, stages "2D2L", "2D5L", "2D10L") whereas a low "E3"/

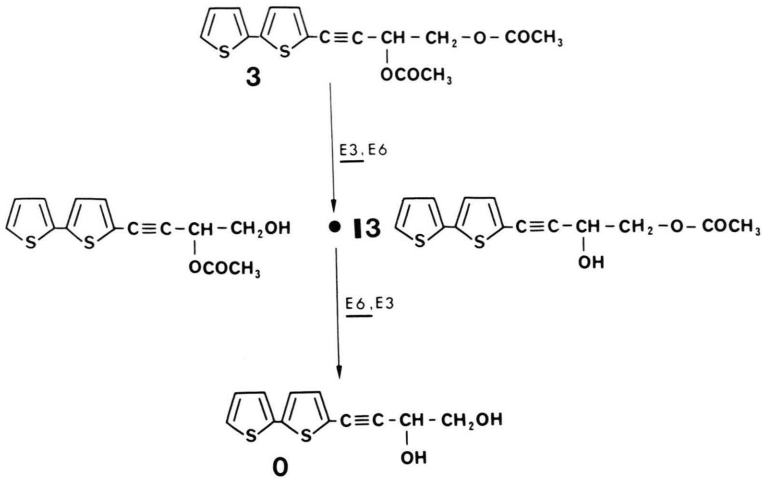

Fig. 4. Hypothetical scheme for the conversion of 3,4-diacetoxybutinylbithiophene (**3**), catalyzed by two sequently working, position specific dithiophene: acetate esterases, "E3" and "E6". Of the two possible structures for the intermediate **13**, results obtained here favour the one shown on the right hand of the scheme.

Table I. Influence of the developmental stage and of the enzyme source on the activity of "E3" and of "E6" and dependence of the formation of the intermediate **13** and of the end product **0** from the "E3"/"E6" ratio.

Developmental stage[a]	Enzyme source	Spec. enzyme activity[b]		Ratio	Formation[c] of	
		"E3"	"E6"	"E3"/"E6"	**13**	**0**
2D2L	roots	24	33	0.73	±	±
	hypocotyls	100	17	5.88	++	−
2D5L	roots	24	43	0.56	−	++
	hypocotyls	88	22	4.00	++	−
2D7L	roots	21	57	0.37	−	++
	hypocotyls	23	30	0.77	±	±
2D10L	roots	20	27	0.74	±	±
	hypocotyls	33	12	2.75	++	−

[a] For definition of stages, see under "Material and Methods".
[b] Arbitrary Units from peak integration/mg protein · min.
[c] HPLC-recognizable product formation in "E3" assays after 10 min of incubation. − = This product not detectable; ± = both **13** and **0** detectable; ++ = only this product detectable.

"E6" ratio promotes the formation of **0** (*e.g.*, roots, stages "2D5L", "2D7L"). Both the products appear in stages with a medium "E3"/"E6" ratio (*e.g.*, hypocotyls, stages "2D7L" and roots, "2D10L"). These results strongly suggest that "E6" is responsible for the conversion of **13** to **0**.

Conclusions

The results shown here confirm that 3,4-diacetoxybutinylbithiophene **3** is hydrolyzed to 3,4-dihydroxybutinylbithiophene **0** by two highly position specific enzymes in a sequential manner. Because of the specificity of "E6" to hydrolyze 4-acetoxybutinylbitiophene **6** [6] it is supposed that the first step is an hydrolysis in the 3-position to give **13**. Therefore, **13** is, most probably, 3-hydroxy-4-acetoxybutinylbithiophene (Fig. 4). Thus, hydrolysis of the diacetate is catalyzed by a new enzyme, most probably, a 3,4-diacetoxybutinylbithiophene: 4-acetate esterase. Further attempts to obtain sufficient amounts of **13** for chemical analysis are in progress. Preliminary experiments to separate "E3" from "E6" showed very close biochemical characteristics for both the enzymes. More experimental work on their purification has to be done.

The product of the enzymic conversion of 4-acetoxybutinylbithiophene **6**, 4-hydroxybutinylbithio-

phene **1**, accumulates in significant amounts in distinct organs and/or particular stages of development of *Tagetes* seedlings [8]. In contrast, neither **13** or **0** appears in large amounts in the system. Presumably, these derivatives represent extremely short-lived intermediates in the dithiophene metabolism.

Acknowledgements

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