Accumulation and Biosynthesis of Solanapyrone Phytotoxins by *Ascochyta rabiei*

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The biosynthesis of the phytotoxins solanapyrone A, B and C produced by the phytopathogenic fungus *Ascochyta rabiei* has been investigated. To optimize feeding conditons for the tracer experiments the growth of the fungus and the accumulation of the toxins in submers culture were determined. The accumulation kinetics revealed that formation of the toxins occurs in the stationary phase of the growth indicating that synthesis of solanapyrones follows a typical pattern of secondary metabolism. Incorporation experiments with sodium [1-^{14}C]- and [2-^{13}C]acetate were performed and the NMR-spectroscopically determined labelling pattern of the ^{13}C-enriched solanapyrone A compound confirmed that the carbon skeleton of this compound is formed via the polyketide pathway. The biosynthetic route to solanapyrone B is discussed.

Introduction

The necrotrophic fungus *Ascochyta rabiei* (Pass. Labrousse) is one of the major pathogens of chickpea (*Cicer arietinum* L.), which is an important crop legume in semi-arid areas of South Asia, Middle East, Mediterranean regions and North America (Nene Y. L., 1982; Kaiser W. J., 1992). The fungus induces the symptoms of "Ascochyta blight" a leaf spot disease that causes severe losses in crop yield. Fungal attack leads to death of infected plants at the end of disease development. Especially during humid and cool seasons the disease can spread epidemically. The histology of the disease development has been investigated by Höhl (1990). The histological symptoms observed during infection development are consistent with the action of phytotoxins, i.e. toxic compounds, which are synthesized by the fungus during the pathogenesis.

In liquid culture *A. rabiei* synthesizes the three toxic compounds solanapyrone A, B and C (Fig. 1) (Alam *et al.,* 1989; Höhl *et al.,* 1991), which are released in the culture medium. These toxins have first been found in the culture filtrate of *Alternaria solani*, which is the causal agent of early blight of tomato and potato (Matern *et al.*1978). The chemical structures of solanapyrones A, B and C were elucidated by NMR- spectroscopy and mass- spectrometrie (Ichihara *et al.,* 1983). In recent studies with *A. solani* solanapyrone A was investigated in the context of biological Diels-Alder reactions (Oikawa *et al.,* 1989; Oikawa *et al.,* 1994). Tracer experiments with labelled acetate and precursors containing both diene and dienophile moieties provided strong experimental support that the decalin moiety is formed via a biosynthetic intramolecular Diels-Alder reaction of a reduced polyketide.

In the course of our studies on *A. rabiei* and its toxins (Höhl *et al.,* 1991) the origin of the carbon skeleton of the solanapyrones was now determined by feeding experiments using ^{14}C- and ^{13}C-labelled sodium acetate. These studies should confirm that the solanapyrones of *A. rabiei* are also formed from acetate units in a polyketide pathway.

Because quality and quantity of the in vitro toxin production by *A. rabiei* are nutrient dependent (Höhl *et al.,* 1991; Chen and Strange, 1991; Latif *et al.,* 1993; Chen and Strange, 1994) the accumulation of the three solanapyrone compounds in the culture medium had to be determined under the growth conditions of a submers culture with a chickpea seed extract medium.

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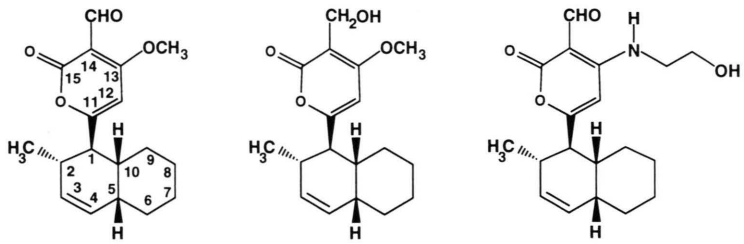


Solanapyrone A Solanapyrone B Solanapyrone C

Fig 1. Chemical structures of solanapyrone A, B and C.

Materials and Methods

Culture of Ascochyta rabiei

The strain of *A. rabiei* (Kovachevsky; CBS No. 535.65) used in these studies was obtained from the Centraalbureau voor Schimmelcultures, Baarn, Netherlands. Stock cultures of the *Ascochyta rabiei* isolate were kept on chickpea seed extract agar as previously described (Kraft and Barz, 1985). For toxin production the fungus was cultivated in submers culture on a gyrotory shaker (160 rpm; 22 °C; 300 ml Erlenmeyer flask) in chickpea seed extract medium (Kraft and Barz, 1985), which was inoculated with a defined volume of a spore suspension (500 000 spores per flask). Fungal growth was followed for up to 28 days. Mycelial dry weight was determined by lyophilization of fungal mass for 36 hours.

Analysis and purification of the solanapyrone toxins

Isolation of toxins was achieved by separating mycelium and culture filtrate through a foam rubber filter. The culture filtrate was extracted three times with chloroform and the combined organic layers were dried (Na_2SO_4), evaporated to dryness under reduced pressure to yield a high viscous, yellow oil. The solanapyrone toxins were analyzed by reversed-phase HPLC on a RP-18 column (Merk, LiCrospher 100 (5 µm, 125 mm)). The separation of the compounds was achieved using a mobile phase gradient of 50 % acetonitrile to 60 % acetonitrile in bidistilled water and a flow rate of 0.72 ml/min. Compounds were monitored with an UV-photodiode array detector at a wavelength of 326 nm. The solanapyrones were identified by their retention times and characteristic UV-spectra (Höhl *et al.*, 1991). For quantitative HPLC-analysis calibration with external standards of the toxins was performed. The reference solanapyrone compounds were purified from the fungal culture filtrate and identified by UV-, MS- and ^{13}C-NMR-data (Ichihara *et al.*, 1983). Silica gel column flash-chromatography eluted with chloroform : methanol (50:1) afforded the crude solanapyrones which were further purified by semi-preparative reversed-phase HPLC (column: Merk, LiCrosorb (7 µm, 250 mm); mobile phase: 50 % bidisitilled water and 50 % acetonitrile; flow rate: 4 ml/min.). After evaporating the solvent solanapyrones crystallized as white crystals (m.p. A 79 °C; B 58 °C; C 77 °C).

Incorporation of labelled sodium acetate precursor

For incorporation experiments aqueous solutions of [1-^{14}C]- and [2-^{13}C]sodium acetate, respectively, were added in different amounts to submers cultures of *A. rabiei*. In the first experiment 240 µCi (8.9 MBq) [1-^{14}C]acetate diluted with unlabeled acetate up to a concentration of 1 mmol was added to the culture at the 8th day of the growth. Alternatively, undiluted [2-^{13}C]acetate (altogether 166 mg) was added in 2 portions at the 8th and the 14th day. Fermentation was continued in both experiments until the 22nd day of culture.

NMR-spectroscopy

NMR-spectra of ^{13}C-labelled compounds were recorded in $CDCl_3$ without external standard at 127 MHz on a Bruker WM-300 spectrometer.

Results and Discussion

Accumulation of the toxins in submers culture

Since the ability of a fungus to produce certain compounds may vary during the course of a

growth cycle, the rate of product accumulation must be studied before biosynthetic investigations can be undertaken. Therefore fungal growth and accumulation of the solanapyrone coumpounds in the culture filtrate were investigated in several experiments to determine the optimal application time of labelled precursors. Figure 2 shows a typical growth curve of *A. rabiei.* and Fig. 3 depicts the accumulation of the three solanapyrone toxins within the fungal growth of 28 days. In all kinetics the stationary phase of fungal growth is reached at the 8th day with an average dry and fresh weigth per flask of 54 g and 2.8 g, respectively. At the beginning of the stationary growth phase toxin formation is triggered, which reaches its maximal values between the 18th and 28th day of the growth. The data in Fig. 3 also show a distinct sequence of product accumulation with solanapyrone A being the first compound followed by solanapyrone B and finally solanapyrone C. During toxin accumulation maximal average concentrations of 180 µmol/l solanapyrone A, 158 µmol/l solanapyrone B and 26 µmol/l solanapyrone C, respectively, were found.

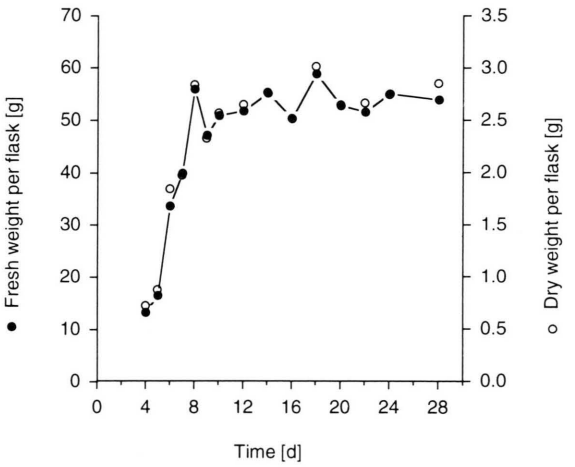


Fig. 2. Growth of *Ascochyta rabiei* in submers culture with chickpea seed extract medium as determined by fresh weight and dry weight of mycelium. For each point in this kinetic one culture flask was used.

Incorporation experiments

To determine the biosynthetic origin of the carbon skeleton of the solanapyrones, we applied, in separate experiments, sodium [1-^{14}C]acetate and [2-^{13}C]acetate to shake cultures of *A. rabiei* grown up to the stationary phase. In the first experiment with [^{14}C]acetate, an average absolute incorporation rate of 2.5 % in all three solanapyrone compounds and a specific incorporation rate of 29 % in solanapyrone A were found, indicating that acetate is significantly incorporated in the solanapyrones. This experiment was performed with three parallel fungal cultures. In view of the multiple acetate pathways, even an absolute incorporation rate of 0.1 % is considered to be significant for acetate incorporation (Franck, 1984). In these ^{14}C-experiments some 31 % of the applied radioactivity was released as $^{14}CO_2$ from the fungal cultures.

The high incorporation rate of acetate precursor which was again observed in the second experiment with [2-^{13}C]acetate allowed the exact ^{13}C-

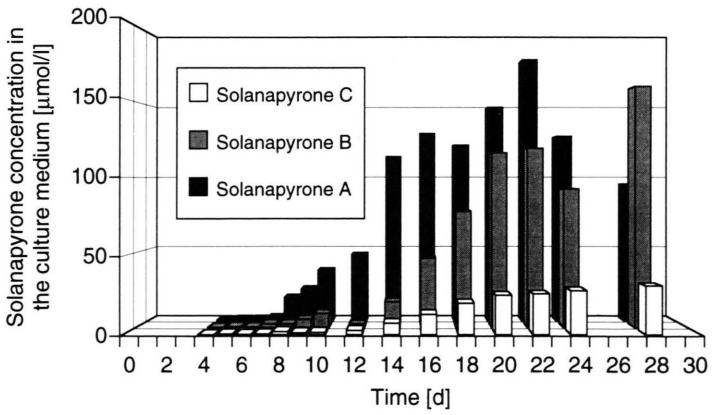
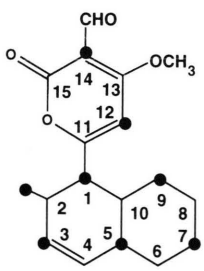


Fig. 3. Accumulation of the solanapyrone toxins in the culture medium of *Ascochyta rabiei.* Toxin concentrations were determined by reversed phase-HPLC in an error range of 9%. For each point in the accumulation kinetic the culture filtrate of one fungal culture was extracted.

NMR-spectroscopic determination of the labelling pattern in the solanapyrone A molecule. The labelling pattern (Fig. 4) was deduced from a comparison of the ^{13}C-NMR-spectrum of solanapyrone A with natural ^{13}C-abundance and the ^{13}C-enriched solanapyrone A. In Table I the assignment of the different carbon atoms is indicated. The acetate precursor typically gave 2–4 fold peak enhancement for each labelled carbon in the ^{13}C-NMR-spectra (Table I).

The incorporation rates and labelling pattern show that the solanapyrone carbon skeleton as produced by *A. rabiei* consists of a polyketide chain which is synthesized from eight acetate units. The head-to-tail arrangement of acetate units in the main chain of the polyketide, which can be deduced from the alternating labelling pattern, results from a series of condensation-reduction steps similar to fatty acid formation. Further reduction to a triene moiety leads in an intramolecular Diels-Alder reaction to the cis-decalin moiety of the solanapyrones (Oikawa *et al.* 1994). Previous tracer experiments with [S-$^{13}CH_3$]-labelled methionin in *A. solani* cultures confirmed that the formyl- and the alcohol- function, respectively, and the carbon atom of the methoxy group of the pyrone ring in solanapyrone A and B are introduced via C_1-pathway; the former functions are probably first introduced as a methyl group (Oikawa *et al.* 1989). Subsequent oxidation of this methyl-group, possibly cytochrome P_{450} dependent, leads to the pyrone moiety of solanapyrone B, which may then be converted by a NAD(P)$^+$ dependent oxidoreductase into solanapyrone A. The kinetics of the solanapyrone accumulation in Fig. 3 clearly fail to support this sequence of biosynthesis. In submers culture the intensive airation of the cultures possibly leads to a high pool of coenzyme NAD(P)$^+$ in comparison to NAD(P)H and this facilitates that solanapyrone B is rapidly oxidized to solanapyrone A. This compound will therefore accumulate earlier in the culture medium. In static surface cultures however the accumulation of solanapyrone B occurs prior to solanapyrone A (Höhl *et al.*, 1991) which might be the result from the less intensive oxygen supply in this type of culture.

In order to corroborate the solanapyrone phytotoxins as possible fungal pathogenicity factors of *A. rabiei* in pathogenesis of chickpea, the exact time point of toxin formation during infection development will have to be determined. For our fu-



Solanapyrone A

Fig. 4. Labelling pattern (carbon atoms with increased ^{13}C-content: ●) of solanapyrone A after [2-^{13}C]acetate incorporation.

Table I. ^{13}C-NMR spectral assignment of solanapyrone A (Fig. 1).

Carbon	Chemical shift δ [ppm][a]	Chemical shift δ [ppm][b]	Chemical shift δ [ppm][c]
CHO	186.56	186.60	186.68
C_{11}	176.37	176.39	176.39
C_{13}	173.64	173.65	173.55
C_{15}	162.25	162.28	162.35
C_4	131.48	131.53	131.52
C_3	130.00	130.02	**130.04** (4.1)
C_{14}	101.76	101.80	**101.83** (2.3)
C_{12}	95.80	95.86	**95.80** (2.8)
OCH_3	57.69	57.72	57.65
C_1	47.91	47.94	**47.98** (2.0)
C_5	36.73	36.77	**36.76** (2.5)
C_{10}	36.02	36.03	36.05
C_2	35.11	35.16	35.14
C_6	29.68	29.72	29.72
C_9	28.34	28.37	**28.37** (2.6)
C_7	25.81	25.86	**25.84** (2.3)
C_8	20.96	21.00	21.02
2-CH_3	20.21	20.28	**20.28** (4.2)

[a] Data of solanapyrone A with natural ^{13}C abundance.
[b] Literature data (Oikawa *et al.* 1989).
[c] Data of the ^{13}C enriched solanapyrone A isolated from *A. rabiei*. The ^{13}C-enriched positions are printed in bold letters. In brackets the ratio of carbon signal intensities from samples with enriched and natural abundance measured under identical conditions are indicated.

ture studies on solanapyrone biosynthetic enzymes the present data were a first step to elucidate the main route in the biosynthesis of the solanapyrones in *A. rabiei.*

Acknowledgement

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