Enzymatic Activity Catalysing *Exo*-selective Diels–Alder Reaction in Solanapyrone Biosynthesis

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The crude enzyme from *Alternaria solani* is able to catalyse the [4+2] cycloaddition of prosolanapyrone III **6** to the *exo* adduct solanapyrone A **1** whose optical purity is estimated as 92 ± 8% e.e. by HPLC analysis monitored using a CD spectrometer; this enzyme also catalyses the oxidation and [4 + 2] cycloaddition of prosolanapyrone II **5** to **1** with 99 ± 4% e.e.

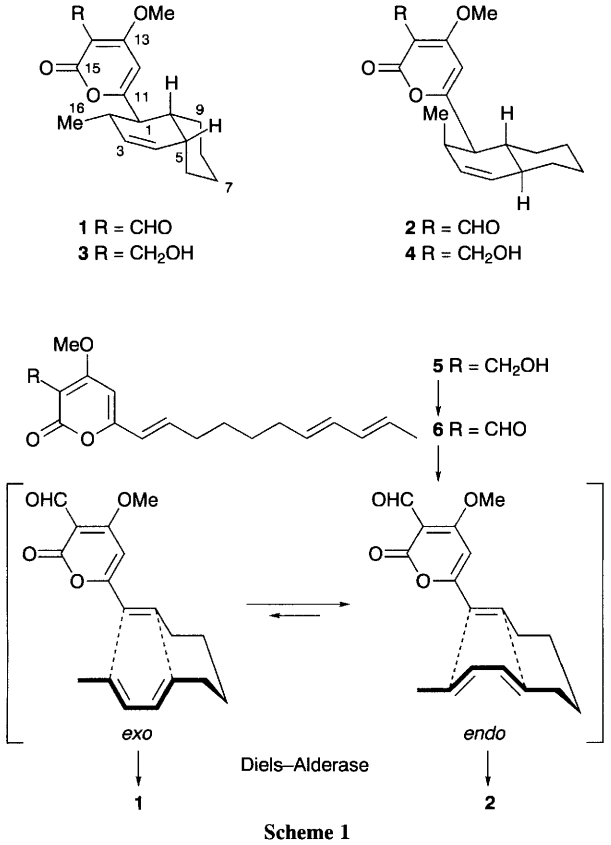
The Diels–Alder reaction is one of the most important reactions in organic synthesis owing to its versatility and remarkable stereoselectivity.[1] In response to the increasing demand for catalytic Diels–Alder reactions, a number of both chemical and biological methods have been developed.[2,3] On the other hand, the involvement of Diels–Alder reactions in biosynthesis has frequently been indicated in the literature,[4] and a number of apparent Diels–Alder adducts including polyketides, terpenoids and alkaloids have been isolated from a variety of sources, *i.e.* microorganisms, plants and marine animals.[4] To date, however, there has been no report of the isolation and characterization of the enzyme, Diels-Alderase, catalysing a Diels–Alder reaction, although numerous efforts have been made.[5]

Recently, we unambiguously established that the phytotoxins solanapyrones **1**–**4**[6] produced by the pathogenic fungus *Alternaria solani* have been biosynthesized *via* [4 + 2] cycloaddition by incorporation experiments with multiply labelled precursors (Scheme 1).[7] According to the results from feeding experiments, this unprecedented enzyme reaction proceeds with high *exo* selectivity, which cannot be achieved *via* chemical synthesis.[1,3b,6b] Here we report our preliminary result on the enzyme which catalyses the decalin formation in solanapyrones.

In our earlier work, we proposed that prosolanapyrone II **5** is first oxidized to prosolanapyrone III **6** which is then cyclized *via* a Diels–Alder reaction in the solanapyrone biosynthesis.[7] Thus, we first used **6** as a substrate for the enzymatic Diels–Alder reaction. Before studying the enzymatic activity, the reactivities of **5** and **6** were examined since we were aware that a significant level of the cycloaddition occurred in an aqueous solution.[7] Under standard conditions (30 °C, 10 min), 15% of **6** was converted to give the *exo* adduct solanapyrone A **1** and the *endo* adduct solanapyrone D **2** in a 3 : 97 ratio, with the remaining 85% of the substrate unchanged. For Diels–Alder reactions in aqueous media, a similar predominant formation of *endo* adducts has been reported.[8] On the other hand, no cycloadduct was detected in the case of prosolanapyrone II **5** under the same conditions.

After a number of attempts, we found the enzymatic activity in a cell-free extract of *A. solani*. This extract converted **6** to **1** and **2** in an *exo*-selective manner. Throughout our study, this observation was reliably used for the differentiation between enzymatic and non-enzymatic reaction. The cell-free extract was partially purified and used in this study.†

Incubation (30 °C, 10 min) of **6** with the crude enzyme resulted in the consumption of 25% of the substrate to give the corresponding amount of the cycloadducts **1** and **2** in a 53 : 47 ratio, with the rest of the substrate intact.‡ A control experiment with denatured enzyme showed that 10% of **6** was converted to **1** and **2** in a 3 : 97 ratio. These results show that actual enzymatic consumption of **6** is 15% and that the *exo* : *endo* ratio for the enzymatic reaction products is calculated as 87 : 13. With four times more concentrated enzyme solution than normal, the reaction with the enzyme proceeded 4.1 times faster than the non-enzymatic one. In order to estimate the optical purity of the products, HPLC analysis detected continuously by CD spectrometer was undertaken.§ On the basis of UV and CD absorption of enantiomerically pure natural solanapyrones,§ the optical purity of **1** was calculated as 92 ± 8% e.e. from a negative CD absorption at 300 nm (Fig. 1). On the basis of acceleration of the reaction rate and the remarkable enantioselectivity, it is concluded that the cycloaddition of prosolanapyrone III **6** to solanapyrone A **1** is catalysed by a specific enzyme.



1 R = CHO
3 R = CH_2OH

2 R = CHO
4 R = CH_2OH

Scheme 1

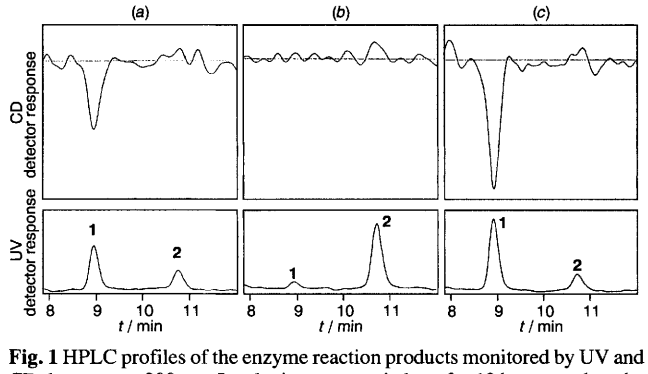


Fig. 1 HPLC profiles of the enzyme reaction products monitored by UV and CD detectors at 300 nm. Incubation was carried out for 12 h to complete the reactions. (*a*) With prosolanapyrone III **6**; (*b*) control with **6**; (*c*) with prosolanapyrone II **5**.

With this crude enzyme, prosolanapyrone II **5** was also converted to **1** and **2** *via* a [4 + 2] cycloaddition reaction. Under the same conditions, incubation of **5** resulted in consumption of 25% of the substrate to give 19% of cycloadducts **1** and **2** in a 85 : 15 ratio, along with 6% of **6**. The observed *exo* : *endo* ratio was similar to the ratio (**1** : **2** = *ca.* 83 : 17) found in solanapyrones[6c] isolated from the culture broth. The reaction products **1**, **2** and **6** were characterized by ^{1}H NMR and mass spectra. According to the HPLC analysis as described above, the optical purity of the obtained **1** was calculated as 99 ± 4% e.e. (Fig. 1). This enantioselectivity is exactly reflected in the optical purity of natural solanapyrones. In this incubation, a two-step reaction consisting of oxidation and cycloaddition is apparently involved. The sequence of the reactions is unambiguously confirmed as the oxidation from **5** to **6** and followed by the cycloaddition, based on the following observations: (*a*) the cycloaddition was completely suppressed under conditions excluding oxygen (argon atmosphere); (*b*) a small amount of the intermediate prosolanapyrone III **6** was detected in the presence of oxygen; (*c*) neither **3** nor **4** were detected in the reaction under both ordinary and argon atmospheres. Furthermore, consumption of equimolar molecular oxygen monitored by oxygen electrode against total concentration of the oxidation products **1**, **2** and **6** suggests that the conversion of **5** to **6** is not a dehydrogenation but an oxidation.

Comparing the enzymatic reactions of **5** and **6**, some losses of the *exo* : *endo* ratio and the enantioselectivity in the reaction of **6** were observed (Fig. 1). This was readily explained by the concomitant non-enzymatic reaction. Thus, it is speculated that a single enzyme which catalyses a two-step reaction is responsible for producing optically pure solanapyrones in the *exo* : *endo* ratio found in natural solanapyrones. Detection of the small amount of the intermediate **6** in the incubation of **5** could be explained by leakage from the denatured enzyme which retains the activity for oxidation but not that for the Diels-Alder reaction.

Whether the two-step conversion is catalysed by a single or two enzyme(s) is not currently settled. In order to answer this question and the reaction mechanism, we are working on further purification and characterization of the Diels-Alderase.

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Footnotes

† The crude enzyme was prepared as follows. The mycelium of *A. solani* grown for two weeks was disrupted with a Polytron homogenizer in 50 mmol dm^{-3} potassium phosphate buffer, pH 7.0, containing 0.25 mmol dm^{-3} PMSF and was further broken in a French press at 1350 kg cm^{-2}. Cell debris was removed by centrifugation (41, 170 g, 60 min) and the supernatant was dialysed overnight against 10 mmol dm^{-3} potassium phosphate buffer, pH 7.0. The cell extract was then subjected to ion-exchange chromatography on DEAE-Sepharose in the above buffer, eluting with a linear gradient of NaCl (0–0.3 mol dm^{-3}). The protein concentration of the crude enzyme was 0.26 mg ml^{-1}.

‡ Reactions were carried out at 30 °C for 10 min or 12 h in mixtures containing 50 mmol dm^{-3} potassium phosphate buffer, pH 7.0, a crude enzyme (0.1 ml) and a substrate in MeOH (20 μl, final concentration 50 μmol dm^{-3}) in a total volume of 1 ml. Denatured enzyme (100 °C, 5 min) was used as a control. The *exo* : *endo* ratio found in the reaction products varied in each preparation. HPLC analysis of reaction products was performed with a Silica gel column (Wakosil 5sil, EtOAc–$CHCl_3$ 3 : 2, 1 ml min^{-1}, UV 300 or 320 nm). Product formation was quantified against an internal standard (coumarin).

§ Enantiomeric purity of natural solanapyrones was examined in ref. 7. UV [λ_{max}/nm (EtOH; ε/dm^3 mol^{-1} cm^{-1}]: **1**; (11,700), **2**; 328 (11,800). CD: **1**; λ_{max}/nm (EtOH) 326 (Δε-3730); **2**; 313 (Δε-1770). The low Δε value of **2** made detection of the signal by CD detector difficult. All efforts to separate racemic **1** and **2** by chiral HPLC columns were unsuccessful.

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