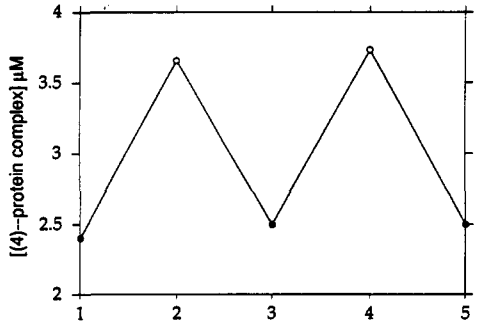
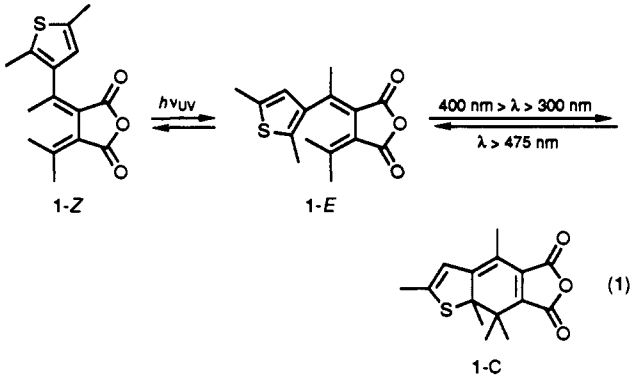


Figure 2. Reversible photoregulated complexation of **2**-modified Con A to **4**. Points 2 and 4 (○) correspond to complexation states of the substrate when **2**-modified Con A is in the *C* form. Points 1, 3, and 5 (●) correspond to decomplexation states when **2**-modified Con A is in the *E* form.

are anticipated to affect its binding properties toward these pyranoses.

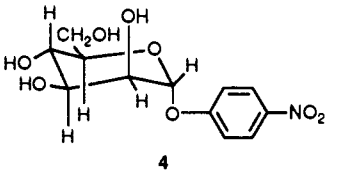
Fulgide dyes are well established photochromic materials.[13] Thiophenefulgide (**1**)[14] undergoes three photostimulated isomerization processes[15] (eq 1) where UV irradiation of **1**-*Z* results in **1**-*E* that undergoes subsequent conrotatory electrocyclization to **1**-*C*. Reversible isomerization of **1**-*C* to **1**-*E* is photoinduced



by filtered light, $\lambda > 475$ nm.[16] Con A (Sigma) was modified by thiophenefulgimide (**2**).[17] The modification process was carried out by reacting 50 mg of Con A in 6 mL of an aqueous solution that contained 250 mg $NaHCO_3$ with 5-15 mg of **2** dissolved in 200 μL of THF at 0 °C for 24-48 h (Scheme I). This modification leads to functionalization of Con A lysine residues, and the loading degree is controlled by the amount of added **2** and the reaction time. The resulting modified protein, **3**, exhibits photochromic properties (Figure 1). Illumination of **3**-*E*, 300 nm $< \lambda <$ 400 nm, results in isomerization to **3**-*C*. Further illumination of **3**-*C*, $\lambda > 475$ nm, restores the original absorption spectrum of **3**-*E*. The loading degrees of Con A by **2** are determined by following the absorbance of **3**-*C* at $\lambda = 532$ nm ($\epsilon = 2200\ M^{-1}\ cm^{-1}$) and determining the protein content or by comparing the fluorescence intensities resulting from the interaction of fluorescamine[18] with **3**-*E* and unmodified Con A. Both methods give excellent agreement (±5%).

Photoregulated association of 4-nitrophenyl α-D-mannopyranoside (**4**) to **3**-*E* and **3**-*C* has been followed by determination of the association constants of **4** to the photochromic protein.

Table I summarizes the association constants of **4** to Con A in its two photochromic states as a function of loading degree. It is evident that, as the loading degree of Con A increases, its



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affinity for **4** declines and the association constant decreases in its value. It is also concluded that, up to a loading degree of 9, the affinity of **3**-*C* is higher than that of **3**-*E* toward binding of **4**. At a loading degree of 12, where all lysine residues of Con A are modified by **2**, a substantial decrease in the binding constant of **4** is observed, and no difference between states *E* and *C* is detected. The largest difference in binding constants of **3**-*E* and **3**-*C* to **4** is observed at a loading degree of 9. This difference in the binding constants allows us to reversibly photoswitch the association and dissociation of **4** to **2**-modified Con A (Figure 2). We see that in the presence of **3**-*E*, only 2.4×10^{-6} M of **4** is bound. Upon illumination, $\lambda = 300$-400 nm, and isomerization to **3**-*C*, enhanced binding of **4** is observed. Further illumination, $\lambda > 475$ nm, regenerates **3**-*E* and the intermolecular complex is dissociated.

We thus conclude that chemical modification of Con A by thiophenefulgimide (**2**) allows the photoinduced binding of 4-nitrophenyl α-D-mannopyranoside to the protein. Photoswitchable association and dissociation of the protein-substrate assembly is induced by the two photochromic states of the protein. Further studies on photoregulation of the binding of substrates to other proteins using this approach are in progress in our laboratory.

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Registry No. Thiophenefulgide, 118408-54-1; α-D-mannopyranose, 7296-15-3.

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Reaction of (6*R*)-6-F-EPSP with Recombinant *Escherichia coli* Chorismate Synthase Generates a Stable Flavin Mononucleotide Semiquinone Radical

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Chorismate synthase (EC 4.6.1.4), the seventh enzyme in the shikimate pathway,[1] catalyzes the conversion of 5-enolpyruvylshikimate 3-phosphate (**1**, EPSP) to chorismate **2**.[2] The reaction involves the removal of the C-6 *pro-R* hydrogen and loss of

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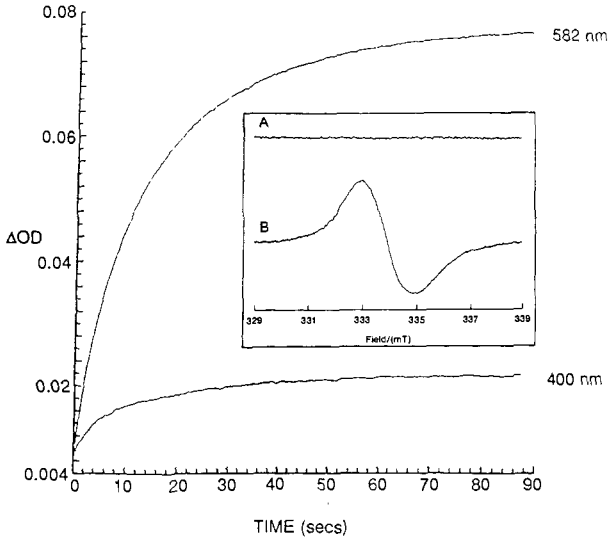
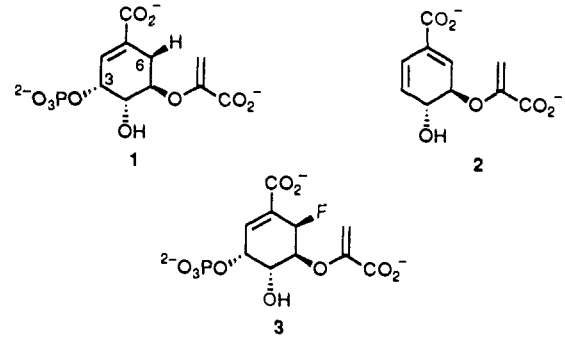


Figure 1. Absorbance changes occurring at 400 and 582 nm when chorismate synthase (40 μM) was mixed with (6*R*)-6-F-EPSP (100 μM) in an anaerobic stopped-flow spectrophotometer at 25 °C, pH 7.0. Both syringes contained $FMNH_2$ (60 μM) and $Na_2S_2O_4$ (1 mM) in phosphate buffer (50 mM). Insert A: EPR spectrum of chorismate synthase (50 μM), $FMNH_2$ (50 μM), and $Na_2S_2O_4$ (500 μM) in potassium phosphate buffer (50 mM) at pH 7.0. Insert B: same as for A except (6*R*)-6-F-EPSP (100 μM) was reacted for ca. 60 min before the sample was frozen. Spectra were recorded on a Bruker ER 300 SH spectrometer at 120 K with 200 μW of microwave power at 9.3667 GHz using 0.5 mT field modulation at 100 KHz.

phosphate in what is formally a trans 1,4-elimination to generate a diene.[3-5]



Although the reaction does not involve an overall oxidation or reduction, all chorismate synthases require a reduced flavin ($FMNH_2$) for activity (1 equiv of $FMNH_2$ bound per 39K subunit for the *Escherichia coli* enzyme).[6-10] Transient absorbance changes during single turnover experiments with EPSP (in the presence of $Na_2S_2O_4$ required to reduce FMN to $FMNH_2$) showed a direct involvement of flavin in the catalytic cycle.[10,11] The difference spectrum ($\Delta\epsilon_{max,400nm}$ = 1.3 mM^{-1} cm^{-1}) calculated from the amplitudes of the stopped-flow traces recorded at several

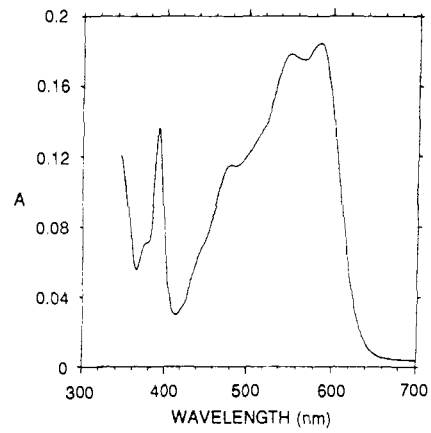


Figure 2. UV/visible difference spectrum for the products and reactants under the conditions of Figure 1 (inserts A and B). Spectra were recorded ca. 30 min after reaction in a 1-cm path length cell at 25 °C. The spectra were not recorded below 340 nm due to the strong absorbance of $Na_2S_2O_4$.

wavelengths suggests that either a charge-transfer complex or, more likely, a flavin–C4 adduct is an intermediate. After exhaustion of substrate (EPSP), the flavin returns to the hydroquinone state. We now report that, with the substrate analogue (6*R*)-6-F-EPSP ((6*R*)-6-fluoro-5-enolpyruvylshikimate 3-phosphate, (**3**), a competitive inhibitor of *Neurospora crassa* chorismate synthase, K_i = 3 μM[12]), the $FMNH_2$ cofactor of *E. coli* chorismate synthase is quantitatively oxidized to yield a stable flavin semiquinone radical that is not reduced in the presence of the dithionite ion (500 μM).

Figure 1 shows the absorbance changes occurring at 400 and 582 nm when enzyme, pre-incubated with $FMNH_2$ and $Na_2S_2O_4$, is mixed in the stopped-flow spectrophotometer with (6*R*)-6-F-EPSP under strictly anaerobic conditions. Unlike the rapid transient effects (millisecond time scale) observed with EPSP as substrate (see Figure 1 of ref 10), only a relatively slow increase in absorbance, which is greater at 582 than at 400 nm, occurs (Figure 1). The stability of the reaction product (no apparent change in the purple color of the solution after 6 h in the presence of $Na_2S_2O_4$, 500 μM) allowed the EPR spectrum (insert B, Figure 1) and the UV/visible difference spectrum (Figure 2) to be recorded. Both spectra are characteristic of a protein-bound, FMN semiquinone (FMN_{SQ}) free radical present as a mixture of neutral and anionic forms.[13,14] The FMN_{SQ} must be bound to the protein since FMN_{SQ} free in solution rapidly conproportionates to oxidized FMN and $FMNH_2$,[15] and the oxidized FMN would be subsequently reduced back to $FMNH_2$ by the excess $Na_2S_2O_4$ present. The value $\Delta\epsilon_{582nm}$ = 3.7 mM^{-1} cm^{-1}, calculated from the enzyme concentration (50 μM), or 4.4 ± 0.5 mM^{-1} cm^{-1}, calculated from the EPR signal spin integration (see below), is similar to that for *Klebsiella pneumoniae* flavodoxin in the semiquinone and hydroquinone states ($\Delta\epsilon_{580nm}$ = 4.1 mM^{-1} cm^{-1}).[16] The EPR signal has a 19 G line width and integrates to 42 ± 5 μM spins on the basis of *K. pneumoniae* flavodoxin semiquinone and CuEDTA standards. The oxidation of the reduced flavin to generate the N-5 radical must be accompanied by an associated reduction. However, there is no evidence in the EPR spectrum for another radical derived either from (6*R*)-6-F-EPSP or an aromatic side chain on the protein (cf. Trp 191 in cytochrome c peroxidase[17]). Using plasma emission spectroscopy, we fail to detect any Mn, Fe, Co, Ni, Cu, Zn, or Mo in homogeneous enzyme (detection limit 0.05 g-atom of metal ion per mole of enzyme monomer),

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and so electron transfer to a transition metal appears unlikely. It is possible that the missing electron has been transferred to (bi)sulfite in the solution formed as a result of dithionite oxidation. This would require the enzyme-bound $FMNH_2/FMN_{SQ}$ couple to have an unprecedentedly low E_m of less than -550 mV (NHE).[18] It would also require a >100 mV decrease in the $FMNH_2/FMN_{SQ}$ E_m value when (6*R*)-6-F-EPSP binds since, in the absence of this substrate analogue, no FMN_{SQ} (<2%) is detected by EPR (insert A, Figure 1) or UV/visible spectroscopy (data not shown).

The observation of the stoichiometric formation of a stable N-5 flavin radical upon binding (6*R*)-6-F-EPSP to chorismate synthase was unexpected, as this was clearly not the same intermediate previously detected spectrophotometrically with the natural substrate EPSP.[10] However, a free radical could be a transient intermediate in the enzyme reaction with EPSP which does not accumulate when rapid removal of a hydrogen radical from C-6 is possible.[19]

Acknowledgment. M.N.R. and S.B. are grateful to the Science and Engineering Research Council and ICI Agrochemicals for support under the CASE awards scheme for postgraduate studies.

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An (η^2-Tetrafluoroethylene)ruthenium Complex with a Metallacyclopropane Structure but with a Low Barrier to Propellor Rotation

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The nature of the transition metal-olefin bond has been the subject of significant experimental and theoretical work over the past four decades. Various levels of theory concur that the two-component interaction originally proposed by Dewar, Chatt, and Duncanson[2] (Figure 1) is still the best way of picturing this interaction.[3] Within a continuum of possible combinations of these two interactions, an increase in the contribution of the metal-d to olefin-π^* backbonding component is thought to be responsible for increased pyramidalization at the bound carbon atoms, leading to a more metallacyclopropane structure. The activation barriers to propellor rotation of the olefin about the metal-olefin bond axis have also been the subject of considerable theoretical and experimental attention.[3,4] The rhodium complex

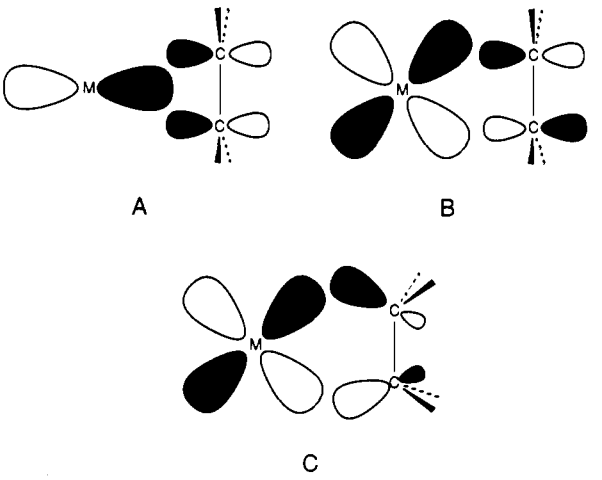

Figure 1. (A) The olefin-metal donor and (B) olefin-metal acceptor components of the olefin-metal bond. (C) Effect of pyramidalization at carbon on the acceptor component. See ref 3 for more comprehensive treatments.

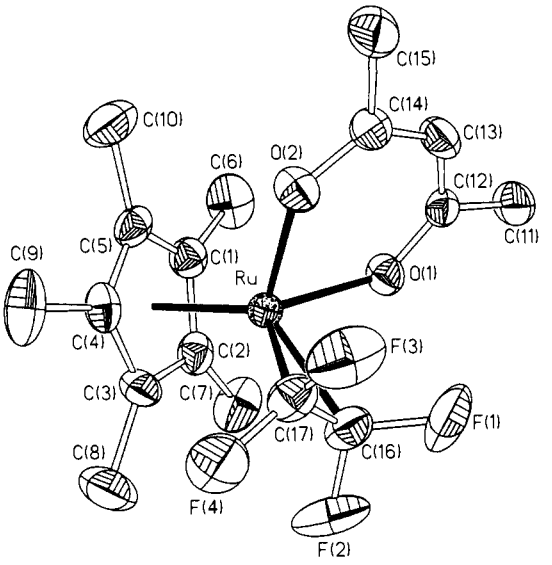

Figure 2. ORTEP representation of **3a**.

1 is often used as a paradigm; its structure shows a more metallacyclopropane interaction for C_2F_4 than for C_2H_4. However, the C_2H_4 rotates fast on the NMR time scale yet the C_2F_4 is stereochemically rigid.[5,6] Indeed, high barriers to propellor rotation are the norm for C_2F_4 complexes,[7,8] and none has been

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