Pergamon

Tetrahedron 56 (2000) 489–495

TETRAHEDRON

On the Specificity of Reactions Catalysed by the Antibody H11

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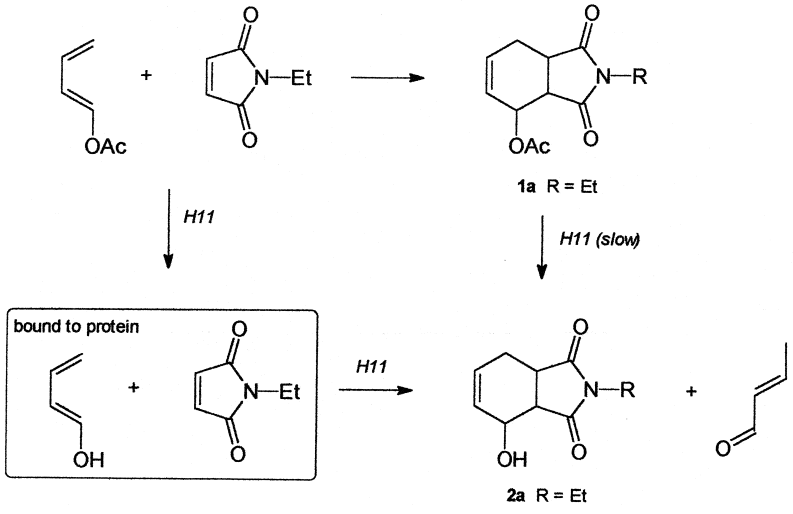
Received 26 August 1999; revised 8 November 1999; accepted 25 November 1999

Abstract—The substrate specificity and the stereochemical course of the reactions catalysed by the antibody H11 (which was raised to a protein conjugated derivative of the adduct of 1-acetoxy-buta-1,3-diene **1**) have been investigated. The antibody shows high selectivity for acetoxybutadiene which it hydrolyses to the corresponding dienol, the major diene component of the cycloaddition reactions observed. However, it tolerates a range of *N*-alkylmaleimides. The stereochemical course of cycloaddition is shown to produce a significant enantiomeric excess of the 3a*R*, 4*S*, 7a*R*-*endo*-diastereoisomer by analysis with Mosher's ester derivatives. This study also revealed that H11 is capable of slowly catalysing the hydrolysis of *N*-alkylmaleimide substrates. The implications for the mechanism of action of H11 are discussed.

In a previous paper we presented evidence consistent with the course of cycloaddition catalysed by the antibody H11 being through initial rapid hydrolysis of the substrate acetoxybutadiene followed by trapping of the dienol by an *N*-alkylmaleimide bound to the antibody (Scheme 1).[1] Information was based upon studies of chemical modification of the antibody, which implied the importance of carboxylate functional groups in the catalytic mechanism together with the intrinsic reactivity of the hydrolysis product, butan-1,3-dien-1-ol. Although the acetoxy adduct **1** was observed, the major cycloaddition product was the hydroxy adduct **2a**. It was possible to rule out cycloaddition leading directly to a hydroxy adduct **2a** away from the antibody binding site because the dienol tautomerised affording crotonaldehyde more rapidly under the reaction conditions than cycloaddition took place. Crotonaldehyde itself does not undergo cycloaddition with maleimides under these conditions. With this information to hand, a number of further questions were raised about the specificity and selectivity of reactions catalysed by H11. In this paper, we present evidence to show that 1-acetoxybuta-1,3-diene is a



Scheme 1. Reactions catalysed by H11 from previous study. [1]

Keywords: antibody H11; acetoxybutadiene; Mosher's ester.
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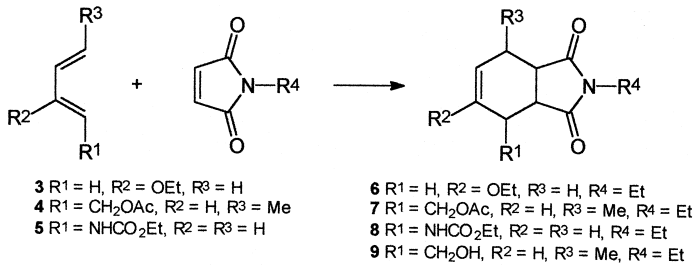
0040–4020/00/$ - see front matter
PII: S0040-4020(99)01037-6



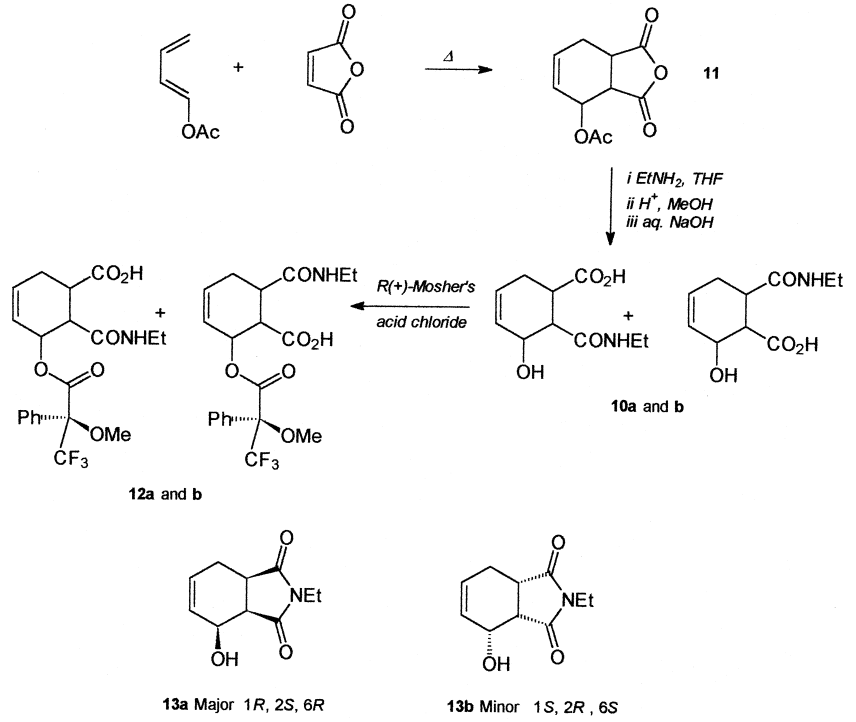
Scheme 2. Additional reactions examined in this study.

relatively specific substrate as a diene precursor whereas a variety of *N*-alkylmaleimides are accepted as dienophiles. We also show that the cycloaddition reaction occurs with significant stereoselectivity. Evidence emerged from the study of the stereochemical course of the reaction that H11 is also able to hydrolyse maleimides slowly to maleamic acids but does not hydrolyse the imide ring of cycloadducts.

Substrate Specificity

Previous studies have shown that acetoxybutadiene[1] and *N*-ethyl- and *N*-benzylmaleimide[2] are substrates for cycloaddition reactions catalysed by H11. In view of the unexpected preponderance of the hydroxy adduct **2a** in the reaction products it was of interest to establish whether any other dienes with electron donating substituents would be accepted. Accordingly, we examined 2-ethoxybuta-1,3-diene **3**, 1-acetoxyhexa-2,4-diene **4**, and 1-ethoxycarbonylaminobuta-1,3-diene **5** (Scheme 2). The last of these three has previously been used as a substrate in studies of cycloaddition catalysed by antibodies.[3]

Reaction conditions were established in which all of these compounds and H11 were soluble. Typically, millimolar concentrations of substrates were compatible with micromolar concentrations of H11 in 3:1 phosphate buffered saline (PBS)/acetonitrile. Initial investigations were carried out using UV absorption analysis at 300 nm, but this was found to be unsatisfactory for the cases in question due to the low sensitivity of the method. Reactions were therefore followed by removing samples from control reactions and reactions containing H11 at set times, freezing the solutions in liquid nitrogen, and subsequently measuring the concentration of products by HPLC. In the case of ethoxybutadiene **3**, no difference in the rate of reaction between control and H11-containing samples was observed. To study 1-acetoxyhexa-2,4-diene **4**, it was necessary to raise the reaction temperature from 25 to 37°C in order to ensure solubility of the reactants. No catalysis of cycloaddition was observed. Moreover no evidence for other products such as hydrolysis of the acetate ester was found. This result demonstrates that H11 is not a non-specific esterase. With carbamate **5** as substrate, initial experiments indicated that catalysis might be occurring. However, it was not possible to quantify this observation due to the small size of the effect. We therefore



Scheme 3. Reactions and structures of compounds used to determine the stereochemical course of reactions mediated by H11.

conclude that H11 is specific within the range of substrates tested for acetoxybutadiene presumably because of its size and the increased reactivity in cycloaddition of the dienol produced by hydrolysis (Scheme 1).

The ability of H11 to accept both *N*-ethyl- and *N*-benzylmaleimides as substrates has already been noted. It might therefore be anticipated that other *N*-alkylmaleimides would be substrates. *N*-Phenylmaleimide was shown to undergo cycloaddition with acetoxybutadiene in the presence of H11 affording both the corresponding acetoxy and hydroxy adducts (**1b** and **2b**, R=Ph). Kinetic measurements were not undertaken with this substrate combination but the formation of the hydroxy adduct **2b** is evidence that the cycloaddition takes place when bound to the antibody.[1] The *N*-alkyl substituent in the hapten used to generate H11 was *n*-butanoyl.[2] It was therefore possible that a maleimide with a longer alkyl chain than ethyl might bind more tightly to the antibody. The kinetics of the reaction between *N*-butylmaleimide and acetoxybutadiene were investigated by measuring the formation of products at 37°C using HPLC analysis of samples removed from the reaction mixture and frozen immediately. Product was quantified as the total of the respective acetoxy and hydroxy adducts **1c** and **2c** (R=*n*-Bu) minus the quantity of acetoxy adduct produced by the control reaction. Analysis of the data gave the kinetic constants K_M=6.6 mM, v_{max}= 9×10^{-6} min^{-1}. The K_M value of 6.6 mM is of the same order as that obtained for *N*-ethylmaleimide (8.3 mM) and no additional affinity can therefore be identified. It can be concluded that the *N*-alkyl substituent does not play a major role in binding during catalytically significant events mediated by H11. By comparison, the kinetic constants for acetoxybutadiene[1] (k_{cat}/k_{solv}=7000 and K_M=0.027 mM at 30°C) imply significant affinity of acetoxybutadiene for the antibody.

In the context of the scope of hydrolytic reactions, *N*-ethyl- and *N*-phenylmaleimides were tested individually as substrates for hydrolysis reactions catalysed by H11. Surprisingly there was some measurable hydrolysis leading to maleamic amides, **10a** and **10b** (Scheme 3), which were identified by HPLC in comparison with a synthetic sample. On the other hand, when any of the bicyclic imides was incubated in the presence of H11, there was no evidence for the hydrolysis of the imide ring.

Stereochemical Course of the Reactions Catalysed by H11

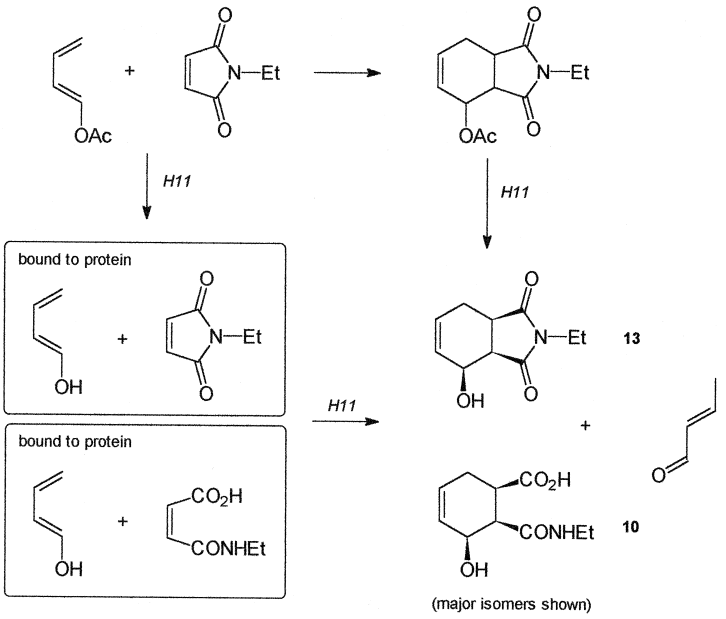
Cycloadditions between maleimides and dienes of the type effective with H11 normally afford the *endo* adduct and thus a pair of enantiomeric products at equal abundance would be expected from the reaction of *N*-ethylmaleimide and acetoxybutadiene in the absence of antibody. A change in this ratio from 1:1 would imply some degree of stereoselectivity in the reactions catalysed by H11. We were unable to separate the isomers of the hydroxy adduct **2** by chiral chromatography. The determination of the stereochemical course of the reactions catalysed by H11 therefore relied upon the preparation of a derivative of the alcohol of the hydroxy adduct **2**. It was first necessary to establish reaction conditions for hydrolysis of the acetoxy adduct and its subsequent conversion into a derivative with a chiral reagent.

The development of a suitable method for analysis was not straightforward because chemical hydrolysis of acetoxy adducts **1** was always accompanied by decomposition, presumably due to retro-aldol opening of the six-membered ring. Chiral chromatographic separations and ^{1}H NMR experiments using chiral shift reagents were also unsuccessful. Bearing in mind that the major product in reactions catalysed by H11 is in any case a hydroxy adduct (e.g. **2**), it should be possible to determine the stereochemical course of the reaction by quantitative derivatisation of the crude reaction products. In order to clearly identify the relevant products, ^{19}F NMR on the Mosher's ester derivatives was selected as the analytical method.[4] Mosher's acid was converted into the acid chloride (which contained a small amount of the symmetrical anhydride) and the crude acid chloride used to convert the hydroxy adducts into Mosher's esters. With the mixture produced by derivatising a sample of the hydroxy adduct **2** prepared in the absence of H11, two distinct ^{19}F resonances were observed (−72.09 and −72.40 ppm, Table 1). The constitution of the products of this derivatisation was confirmed by mass spectrometry.

A preparative incubation of acetoxybutadiene, *N*-ethylmaleimide, and H11 was carried out in the normal solvent mixture (PBS pH 7.4:acetonitrile, 3:1 v/v), the organic products extracted with dichloromethane and derivatised with Mosher's acid chloride. The ^{19}F NMR of this sample (Table 1) showed resonances corresponding with unreacted starting material and the bicyclic adduct **2**, the two resonances of which were present (−72.08 and −72.40 ppm). In addition there was a pair of resonances (−72.1 and −72.26 ppm) and a single resonance (−71.92 ppm). Bearing in mind the observation of hydrolysis of maleimides by H11, a reasonable possibility was that these resonances arose from derivatisation of *mono*cyclic adducts of maleamic acids (Scheme 2). In such a case, two regioisomers of the maleamic acid might be expected,

Table 1. Observed resonances from Mosher's ester derivatives

Bicyclic adduct	Monocyclic adduct	H11 Incubation	Relative intensity	Starting Mosher's acid
	−71.70	−71.70		−71.70
	−71.87?			
	−71.95* (minor)	−71.92*		
	−72.04* (minor)	Not well resolved		
−72.09″		−72.08″	2.1	
	−72.13^ (major)	−72.10^	1.0	
	−72.25^ (major)	−72.26^	5.4	
−72.40″		−72.40″	3.8	



Scheme 4. Summary of reactions catalysed by H11 from this and previous study.[1]

each of which could exist as two enantiomers assuming the normal *cis*-stereochemical course of a cycloaddition. To identify these resonances conclusively, we undertook the synthesis of the maleamic acids and their subsequent derivatisation to form Mosher's esters.

Acetoxybutadiene was reacted with maleic anhydride to form the cycloadduct **11**, the configuration of which was unambiguously assigned as *endo,cis* by X-ray crystallography.[5] Treatment of the adduct with ethylamine afforded a mixture of enantiomeric regioisomers **10a** and **10b** without cleavage of the acetate ester. The acetate was hydrolysed and the resulting mixture of maleamic acids derivatised as before with Mosher's acid chloride.

The ^{19}F resonances observed in the spectrum are shown in Table 1. The resonances assigned to the two regioisomers of the monocyclic adduct with the amide derivative of each of the two carboxylic acids are marked ∗ and ^. These resonances correspond to those observed in the derivatives of products of reactions in the presence of H11. As shown in Table 1, the bicyclic adduct showed one pair of resonances marked ″ in which the higher field resonance was also more intense. Similarly the major monocyclic adduct showed a pair of resonances in which the high field resonance was also the more intense. These data confirm the origin of the resonances observed in the derivatives of products from H11 and are consistent with the ability of H11 to act as an imide hydrolase. From measurements of the intensity of the resonances, an estimate of the enantiomeric excess of approximately 30% can be estimated for the bicyclic adduct **2** present in the mixture of products and about 70% for the major monocyclic adduct **10**. The difference between the two estimates of enantiomeric excess is consistent with the interpretation shown in Scheme 4 that once bound to H11, a substrate molecule follows only one of the possible pathways leading to product. Either the bicyclic hydroxy adduct **2** is formed or the monocyclic adduct **10** but H11 apparently does not hydrolyse **2** to give **10**. This is consistent with the observations reported in the previous section.

Mosher's ester derivatives also give the opportunity to determine the absolute configuration of a compound.[4] In this case, the environment of the ^{19}F nuclei can be assessed by molecular modelling. Using the Cache molecular modelling software (Oxford Molecular), the structures of the enantiomers of the Mosher's ester derivatives of **13a** and **13b** were built and energy minimised (Fig. 1). These calculations showed that the trifluoromethyl group of one

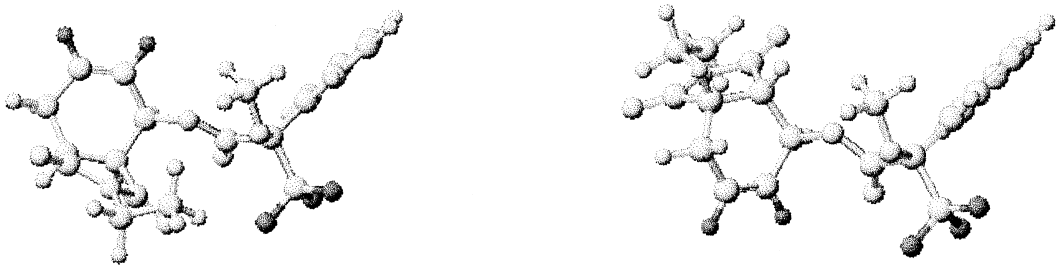


Figure 1. Calculated structures of the epimeric Mosher's ester derivatives **13a** and **13b**. The alkene hydrogen atoms and the fluorine atoms are shaded.

enantiomer was within range of the anisotropic influence of the alkene of the six-membered ring of the adduct. Having used the *R*-(+) isomer of Mosher's acid to prepare the derivatives, it can therefore be deduced that the major isomer produced by H11 has the probable configuration 3a*R*, 4*S*, 7a*R* (**13a**) and the minor isomer the 3a*S*, 4*R*, 7a*S* configuration (**13b**).

Summary of Selectivity of H11

The above results are consistent with the catalytic activity of H11 as promoting cycloaddition chiefly through its ability to act as a hydrolase. The hydrolysis products are evidently partially captured whilst still bound to the protein leading to a measurable degree of asymmetric induction. The observations presented here and in previous papers[1,2] show that hydrolysed products do not take part in cycloaddition reactions under the conditions of the H11 mediated reactions. It is ironic that H11 should turn out to be able to catalyse the formation of chiral maleamic acid derivatives directly because the formation of such products was originally one of the grounds for selecting the target molecule **1**.[2] The observation of specific hydrolysis at the surface of an antibody is well-precedented [6] even in cases where such an activity was not sought.[7] It has been pointed out that such reactions are to be expected if a reasonable degree of binding exists because of the probability that a charged amino acid side chain group will be found adjacent to the binding site on any protein[8] including antibodies.[9] Such charged groups promote general acid or general base catalysis within a water-accessible region. Both enol ether hydrolysis[10] and imide hydrolysis[11] by antibodies have been described previously. We thus summarise the principal reactions catalysed by H11 in Scheme 4. The availability of relevant catalytic functional groups implicated by this reaction course and the chemical modification studies reported previously[12] can be seen in the CDR regions identified in the sequence determined for the antibody H11.[10] The details of the determination of the sequence, the construction of a computer model of the antibody H11, and its relationship to the mechanisms of the reactions catalysed will be described in a forthcoming paper.

Experimental

In experiments with H11, PBS–acetonitrile refers to a solution of phosphate buffered saline, pH 7.4, 10 mM and acetonitrile (3:1 v/v). For all kinetic experiments, calibration curves for HPLC were obtained for the respective substrates and products.

4-Acetoxymethyl-2-ethyl-7-methyl-3a,4,7,7a-tetrahydro-isoindole-1,3-dione (7). 1-Acetoxyhexan-2,4-diene (89.6 mg, 0.8 mmol) and *N*-ethylmaleimide (115 mg, 1.0 mmol) were dissolved in acetonitrile (25 mL) and the solution heated under reflux for 24 h. After evaporation of the solvent the crude product was purified by flash chromatography on silica eluting with ethyl acetate/hexane (1:1 v/v); the required adduct was obtained as a non-crystalline solid with no distinct melting point (134 mg, 63%). δ_H ($CDCl_3$) 1.07 (3H, t, J=7.2 Hz, CH_2CH_3), 1.46 (3H, d, J=7.5 Hz, C7–CH_3), 2.09 (3H, s, CH_3CO), 2.43 (1H, q, J=7.5 Hz, H7), 2.62 (1H, m, H4), 3.05 (1H, dd, J=5.3 and 2.2 Hz, H7a), 3.22 (1H, dd, J=5.7 and 2.6 Hz, H3a), 3.31 (2H, q, J=7.2 Hz, CH_2CH_3), 4.51 (1H, dd, J=11.1 and 8.2 Hz, CH_2OAc), 4.68 (1H, dd, J=11.1 and 8.2 Hz, CH_2OAc), 5.78 (2H, bd s, CH=CH). ν_{max}/cm^{-1} [KBr] 1855, 1784, 1736, 1379, 1243, 1050. EI-MS Found: *m*/*z* 265.1309. $C_{14}H_{19}NO_4$ requires 265.1314.

Rate of hydrolysis of *N*-ethylmaleimide catalysed by H11

UV method. Solutions of *N*-ethylmaleimide in PBS–acetonitrile (3 mL) at concentrations of 1.33, 2.66, 4.0 and 5.33 mM were prepared and incubated at 25°C in a UV cuvette measuring the absorbance at 300 nm (substrate). Concentrations of substrate were determined from a calibration curve (ϵ=533, r=0.999). The reaction was followed by monitoring the loss of substrate at 300 nm. The pseudo-first order rate constant was obtained from a plot of initial rate against concentration and the rate constant obtained from the slope, $k=2.8\times10^{-4}$ min^{-1} (r=0.89).

HPLC method. Solutions of *N*-ethylmaleimide were prepared and incubated as for the UV method. Samples were removed at intervals over 3 h and immediately frozen in liquid nitrogen. Subsequently, the solutions were thawed and 10 μL samples were analysed by HPLC (ODS2, eluting with acetonitrile–water 1:1 v/v). Similar experiments were carried out with *N*-phenylmaleimide.

Investigation of reactions of diverse substrates mediated by H11

Dienes. *UV method*: Solutions of H11 (5 μM in antibody), *N*-ethylmaleimide (1.3 mM), and the appropriate diene (1.2–2.4 mM) were prepared in PBS–acetonitrile (3 ml) and the reactions followed by UV spectroscopy at 300 nm. A control reaction containing all reactants other than H11 was also followed. *HPLC method*: For the control reaction the appropriate diene (3.5 μmol) and *N*-ethylmaleimide (4 μmol) were dissolved in PBS–acetonitrile (3 mL) at 37°C and samples (20 μL) removed at intervals over 2 h. For the test reaction, H11 (0.27 mg) was also present in the solution.

***N*-Butylmaleimide.** The HPLC method was used to measure the rate of addition of 1-acetoxybuta-1,3-diene and *N*-butylmaleimide in the presence and absence of H11 in PBS–acetonitrile as solvent at 37°C. The quantities used were: diene (1.8 mg, 16 μmol); *N*-butylmaleimide (1 mg, 6.5 μmol, 2.2 mM; 0.75 mg, 4.9 μmol, 1.6 mM; 0.5 mg, 3.3 μmol, 1.1 mM); H11 (0.27 mg). The reactions were followed over a 46 min period during which samples (20 mL) were removed and immediately frozen in liquid nitrogen. The eluent for HPLC analysis was water–acetonitrile (13:7 v/v). The initial rates determined for each concentration were 2.36×10^{-6}; 1.67×10^{-6}; 1.31×10^{-6} M min^{-1}, respectively, from which the kinetic parameters $K_M=6.6\times10^{-3}$ M, and $k_{cat}=15$ min^{-1} were determined by a computational model of the Lineweaver Burk plot.

Synthesis of Mosher's ester derivatives (14a and b) of the bicyclic hydroxyadduct (2a). *R*(+)-α-methoxy-α-trifluoromethylphenylacetic acid (Mosher's acid, 0.812 g, 3.5 mmol) was dissolved in thionyl chloride and the mixture heated under reflux for 48 h. The excess thionyl chloride was evaporated under reduced pressure and the residual oil kept dry, cold, and under nitrogen until required for use. The hydroxy adduct **2** (28 mg, 0.15 mmol) and the acid chloride (38 mg, 0.15 mmol) were dissolved in dry dichloromethane (5 drops) and pyridine (5 drops) and allowed to stand under nitrogen for 12 h. The reaction mixture was then diluted with ether and the solution washed with dil. hydrochloric acid, aqueous sodium bicarbonate solution and brine. The organic solution was dried ($MgSO_4$) and the solvent evaporated to afford the required mixture of *Mosher's esters* as a colourless non-crystalline solid (41 mg, 67%). δ_H ($CDCl_3$) 0.99–1.02 (overlapping t, 3H, NCH_2CH_3), 2.36 (1H, m, H4), 2.67 (1H, m, H6), 3.06 (1H, m, H1), 3.43–3.45 (3H, 2×s, OCH_3), 3.5 (2H, m, NCH_2), 5.68–5.76 (1H, t J=5.0 Hz), 6.10–6.35 (2H, m, CH=CH), 7.38 (5H, s, C_6H_5). δ_c ($CDCl_3$) 12.88 (NCH_2CH_3), 21.93 and 22.16 (NCH_2), 33.77 and 33.88 (C5), 36.39 (C6), 43.45 and 43.55 (C1), 55.43 and 55.63 (OCH_3), 67.57 and 68.05 (C2), 121.18 ($C[CF_3]OMe$), 124.77, 125.43, 127.39 and 127.47, 128.55 and 128.60, 129.83 and 129.89, 131.28 (C_6H_5), 126.38 (C4), 134.68 (C3), 165.65 (CF_3), 174.84 (CO_2–), 178.73 and 178.83 (CON). δ_F ($CDCl_3$) −72.09 and −72.40. LREI-MS Found m/z 411, $C_{20}H_{21}F_3O_5N$ requires 411, FAB^+ found 412.1.

Synthesis of adducts and derivatives of maleamic acids

Acetic acid 1,3-dioxo-1,3,3a,4,7,7a-hexahydro-isobenzofuran-4-yl ester (11). 1-Acetoxy-1,3-butadiene (2.187 g, 19.50 mmol) was added at room temperature to a suspension of maleic anhydride (1.750 g, 17.84 mmol) in benzene (40 mL) with stirring. The stirring was continued at room temperature overnight. Solvent was removed under reduced pressure and the crude product was purified by chromatography on silica gel using ethyl acetate/*n*-hexane (1:3 v/v). The required cycloadduct was obtained as a white crystalline solid (2.135 g, 52%), mp 53–56°C. [lit.13a mp.56–57.5°C, lit.13b mp 54–55°C, lit.13c mp 56–59°C] δ_H ($CDCl_3$) 2.05 (3H, s, CH_3CO_2); 2.45–2.53 (2H, m, H-6-α and -β); 3.43–3.49 (1H, ddd, J=10, 8, and 4 Hz, H-1); 3.57–3.61 (1H, dd, J=10 and 5 Hz, H-2); 5.41–5.44 (1H, m, H-3); 6.09–6.16 (2H, m, H-4 and H-5). ν_{max}/cm^{-1} 1855, 1784, 1736.

3-Acetoxy-5(or 4)(carboxamidoethyl)cyclohex-1-ene-4(or 5)-carboxylic acid. 3-Acetoxy-cyclohex-4-ene-1,2-dicarboxylic acid anhydride (506 mg, 2.407 mmol) was dissolved in THF (20 mL, dry) to which was added ethylamine (2.5 mL, 2.0 M solution in THF) with stirring at room temperature. Stirring was continued overnight at room temperature and then the solvent was removed under reduced pressure. The crude product was dissolved in dichloromethane then extracted with sodium hydrogen carbonate. The aqueous layer was collected, cooled to 0°C and then acidified with conc. HCl. The acidified solution was extracted with dichloromethane, dried and then the solvent was removed under reduced pressure to give the crude product which was purified by column chromatography on silica using methanol/ethyl acetate 1:3 as eluant. The products were obtained as a colourless oil (242 mg, 39%). δ_H ($CDCl_3$) 1.13–1.19 (3H, t, J=5.1 Hz, CH_2CH_3); 2.04 and 2.05 (3H, 2×s, CH_3CO_2); 2.14–2.20 (1H, m); 2.86–3.18 (3H, m); 3.29–3.40 (2H, m, CH_2CH_3); 5.59–6.18 (3H, m); 6.55 (1H, s, CONH). ν_{max}/cm^{-1} 3296, 1745, 1723, 1634, 1587. HREI-MS: Found: m/z 255.11135 $C_{12}H_{17}NO_5$ requires 255.11067.

Methyl 3-hydroxy-5(or 4) (carboxamidoethyl)cyclohex-1-ene-4(or 5)-carboxylate. 3-Acetoxy-5(or 4)(carboxamidoethyl)cyclohex-1-ene-4(or 5)-carboxylic acid (204 mg, 0.800 mmol) was dissolved in methanol (10 mL) to which was added conc. HCl (300 μl) at room temperature with stirring. The stirring was continued at room temperature overnight. The solvent was removed under reduced pressure and the product purified by silica gel column chromatography eluting with methanol/ethyl acetate (1:3 v/v) to give the products as a colourless oil (181 mg, 99%). δ_H ($CDCl_3$) 1.10–1.22 (3H, t, J=5.0 Hz CH_2CH_3); 2.38–2.47 (2H, m); 2.96–2.98(1H, m); 3.27–3.38 (2H, m, CH_2CH_3); 3.72 and 3.73 (3H, 2×s, CO_2CH_3); 4.32–4.39 (2H, m); 5.77–5.97 (3H, m); 7.10 (1H, s, CONH). ν_{max}/cm^{-1} 3311, 1740, 1651, 1631, 1546. HREI-MS: Found: m/z 227.11709 $C_{11}H_{17}O_4N$ requires 227.11576.

3-Hydroxy-5(or 4)(carboxamidoethyl)cyclohex-1-ene-4(or 5)-carboxylic acid (10a and b). Methyl 3-hydroxy-5(or 4)(carboxamidoethyl)cyclohex-1-ene-4(or 5)-carboxylate (170 mg, 0.748 mmol) was suspended in aqueous sodium hydroxide (5 mL, 0.1 M) for 1 h at room temperature then extracted with dichloromethane (25 mL). The water layer was cooled to 0°C then acidified with conc. HCl, followed by extraction with ethyl acetate (2×25 mL). The organic extract was dried (Na_2SO_4), and the solvent removed under reduced pressure to give the required products as a colourless oil (40 mg, 25%). δ_H ($CDCl_3$) 1.09–1.18 (3H, t, J=5.0 Hz CH_2CH_3); 2.31–2.58 (2H, m); 2.97–3.13 (2H, m); 3.23–3.30 (2H, m, CH_2CH_3); 4.47–4.52 (1H, m); 5.76–5.88 (2H, m); 7.62–7.65 (1H, t, CONH). ν_{max}/cm^{-1} 3311, 1723, 1634, 1587. HREI-MS: Found m/z 196.09631 $C_{10}H_{14}O_3N$ requires 196.09737 [M−H].

5(or 4)(Carboxamidoethyl)cyclohex-1-ene-4(or 5)-carboxylic acid [*R*(+)-α-methoxy-α-trifluoromethyl phenylacetic acid] ester (12a and b). Mosher's acid (102 mg, 0.404 mmol) was dissolved in thionyl chloride (1 mL) and the reaction mixture heated under reflux for 48 h. Excess thionyl chloride was removed under reduced pressure at 40°C. The acid chloride was dissolved in dichloromethane (1 mL, dry) then added to 3-hydroxy-5(or 4)(carboxamidoethyl)cyclohex-1-ene-4(or 5)-carboxylic acid at room temperature. Pyridine (100 μL, dry) was added to the reaction mixture with stirring at room temperature. The reaction mixture was left stirring at room temperature for 48 h. Dilution with ether (25 mL) was then followed by addition of dilute HCl (5 mL). The mixture was extracted then washed with water, dried (Na_2SO_4) and ether was then removed under reduced pressure at room temperature to give the required products as a pale yellow oil (37 mg). δ_F see Table 1.

Determination of stereochemical course of reaction catalysed by H11

1-Acetoxybuta-1,3-diene (14.8 mg, 0.132 mmol), *N*-ethylmaleimide (8 mg, 0.064 mmol), and H11 (1.62 mg) were dissolved in PBS–acetonitrile (24 mL) and the solution stirred at 37°C for 24 h. A second experiment was run for 12 h. At the end of each reaction, the solution was frozen in liquid nitrogen and the solvent lyophilised. The organic product was extracted from the residue with dichloromethane (15.7 and 29.4 mg product) and converted into the Mosher's ester derivatives using the method described above. The products were analysed by ^{19}F NMR and the observed spectra are reported in Table 1. Evidence for the presence of the maleamic acid derivatives was obtained from the mass spectra (FAB^+) found *m*/*z* 430 ($M^+ + 1$) and 452 ($M^+ + 23$) as required by $C_{20}H_{23}F_3O_6N + Na$.

Acknowledgements

We thank the Basque Government for financial support (S.L.).

References

1. Pitt, A. R.; Stimson, W. H.; Suckling, C. J.; Tellado, J.-M.; Vazzana, C. *Isr. J. Chem.* **1996**, *36*, 171–175.
2. Suckling, C. J.; Tedford, M. C.; Bence, L.; Irvine, J.; Stimson, W. H. *J. Chem. Soc., Perkin Trans. 1* **1993**, 1925–1929.
3. Braisted, A. C.; Schultz, P. G. *J. Am. Chem. Soc.* **1990**, *112*, 7430–7431.
4. Dale, J. A.; Dull, D. L.; Mosher, H. S. *J. Org. Chem.* **1969**, *34*, 2543–2549.
5. Kennedy, A. R.; Khalf, A. I.; Suckling, C. J. *Acta Crystallogr. C55*, IUC9900072.
6. (a) Wade, H.; Scanlan, T. S. *Ann. Rev. Biophys. Biomol. Struct.* **1997**, *26*, 461–493. (b) Gigant, B.; Charbonnier, J. B.; Golinelli-pimpaneau, B.; Zemel, R. R.; Eshhar, Z.; Green, B. S.; Knossow, M. *Eur. J. Biochem.* **1997**, *246*, 471–476.
7. (a) Khalaf, A. I.; Proctor, G. R.; Suckling, C. J.; Bence, L. M.; Irvine, J. I.; Stimson, W. H. *J. Chem. Soc., Perkin Trans. 1*, **1992**, 1475–1481. (b) Stimson, W. H.; Bence, L. M.; Irvine, J.; Khalaf, A. I.; Proctor, G. R.; Tedford, M. C.; Suckling, C. J. *J. Clin. Lab. Immunol.* **1994**, *43*, 167–175.
8. Hollfelder, F.; Kirby, A. J.; Tawfik, D. S. *Nature* **1996**, *383*, 60–63.
9. Riva, S.; Mendozza, S.; Carrea, M.; Chattopadhyay, G.; Tramontano, P. *Appl. Biochem. Biotechnol.* **1998**, *75*, 33–44.
10. Brooks, L.; Suckling, C. J.; Stimson, W. H. *Biochem. Soc. Trans.* **1996**, *24*, 313S. EMBL/GENBANK L41672, L41689, L41728.
11. Redmond, J. L.; Jahangiri, G. K.; Stoudt, C.; Lerner, R. A. *J. Am. Chem. Soc.* **1993**, *115*, 3909–3917.
12. Liotta, L. J.; Benkovic, P. A.; Miller, G. P.; Benkovic, S. J. *J. Am. Chem. Soc.* **1993**, *115*, 350–351.
13. (a) McDonald, E.; Suksamran, A.; Wylie, R. D. *J. Chem. Soc., Perkin Trans. 1*, **1979**, 1893–1900. (b) Dolby, L. J. *J. Org. Chem.* **1962**, *27*, 2971–2975. (c) Ubukata, M.; Sakamura, S. *Tetrahedron Lett.* **1977**, 3473–3476.