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Regioselectivity and Enantioselectivity in an Antibody Catalyzed Hetero Diels–Alder Reaction

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Abstract—The Diels–Alder cycloadditions of *trans*- and *cis*-piperylene (**1** and **2**) to 4-nitroso-*N*-propylbenzamide (**3**) were selected as target reactions for the development of catalytic antibodies with regioselective and enantioselective properties (Meekel, A. A. P. Ph.D. Thesis, University of Amsterdam, 1995). The bicyclic systems **10a–c** were designed as transition state analogues and employed for the immunization of mice and the generation of monoclonal antibodies. Three of the antibodies, each obtained from immunization with a different hapten, were selected for further characterization of their catalytic activities. Among these, antibody 309–1G7, raised against the protein conjugate of **10c**, showed the best rate enhancement ($k_{cat}/k_{uncat}=2618$) in the reaction of *cis*-piperylene (**2**) with nitroso dienophile **3**. Data obtained from regioselectivity and enantioselectivity analyses demonstrated that antibody 309–1G7 favors the formation of the targeted regioisomer (>95%), with an ee of 82%. Copyright © 1996 Elsevier Science Ltd

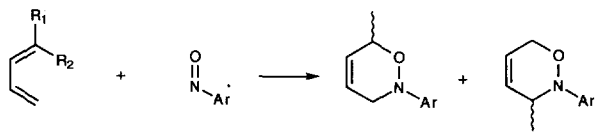
Introduction

The Diels–Alder reaction is one of the most powerful methodologies for the preparation of six-membered ring compounds. In many synthetic sequences, control of product formation, in particular in terms of regio-, diastereo-, and enantioselectivity, has been a major issue in the application of the Diels–Alder cycloaddition.[2] This consideration has generated active interest in the development of catalysts with regio- and stereoselective properties that would specifically favor the formation of the desired product. In this context it is noteworthy that Diels–Alderases have been conspicuously absent from the list of enzymes, although very recently experimental evidence has been presented indicating catalytic activity in an intramolecular Diels–Alder reaction by a crude cell-free extract from the fungus *Alternaria solani*.[3]

Within the past decade, the concept of using suitable transition-state analogues to generate catalytic antibodies has been explored and applied to a variety of chemical transformations.[4] It has been shown that the binding pocket of the antibody possesses structural characteristics, which are capable of exercising an effective stereocontrol on the reaction taking place within it. Successful examples of antibody-mediated catalysis addressing the carbocyclic version of the Diels–Alder reaction have been reported in the last few years.[5]

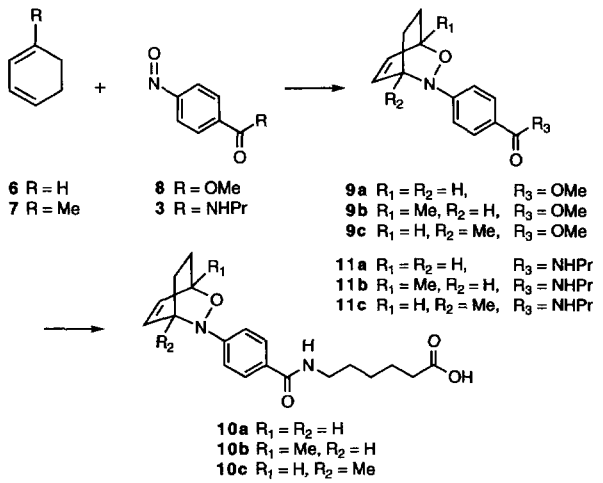
Work in our laboratory has focused attention on developing antibody catalysts which can influence the regio- and stereochemical course of a hetero Diels–Alder reaction.[6] The selected reaction was the cycloaddition of *trans*-piperylene (**1**) or *cis*-piperylene (**2**) to 4-nitroso-*N*-propyl-benzamide (**3**) (Scheme 1); it should be emphasized that the use of either diene leads to the formation of the same two regioisomeric products, namely **4** and **5**, as racemic mixtures. The presence of the hetero atoms in the dienophile avoids the generation of *endo* and *exo* adducts, while the methyl group originating from the diene serves as a diagnostic substituent for the analysis of the regioselectivity and enantioselectivity of the reaction under the influence of the antibody.

The aim of our work was to generate antibodies that would not only enhance the rate of the reaction, but would also steer the reaction towards the enantioselective formation of a targeted regioisomeric product. As haptens for the generation of the antibodies we envisaged the compounds **10a–c** (Scheme 2). Analogous bimolecular systems have been previously demonstrated to serve as efficient transition-state analogues of the Diels–Alder reaction. Haptens **10b** and **10c** were designed to produce antibodies that would influence the formation of the corresponding regioisomers. Comparison of the activity of the generated antibodies would allow the study of the effect of the methyl group on the specificity of the active site of the catalyst.



1 R_1 = Me, R_2 = H **2** R_1 = H, R_2 = Me — **3** — **4** — **5**

Scheme 1. Ar = *p*-$C_6H_4CONHPr$.



Scheme 2.

Results and Discussion

Reactions of dienes **6** and **7** with dienophile **8** afforded the bicyclic adducts **9a–c**, respectively, which were converted into the haptens **10a–c**. These three haptens, it should be noted, differ in the presence and in the location of the methyl substituent. The synthesis of the haptens is outlined in Scheme 2.

The haptens were conjugated to carrier proteins and the conjugates were used to generate antibodies in mice. Spleen lymphocytes were fused with myeloma cells and the resulting hybridomas were screened for the production of hapten-specific antibodies by inhibition ELISA, and subsequently recloned and amplified to give monoclonal antibodies following standard procedures.

After a preliminary activity study of the reaction between diene **1** and dienophile **3**, three clones were selected for further characterization. These were, namely, antibody 285-11D6 raised against hapten **10a**, antibody 290-4B10 raised against hapten **10b**, and antibody 309-1G7 raised against hapten **10c**.

Kinetic data were obtained spectrophotometrically by monitoring the decrease in absorbance of the dienophile **3** at 340 nm, a wavelength at which there is no relevant absorbance due to the dienes or the products. All experiments were carried out under pseudo-first-order conditions, with an excess of diene held at a fixed concentration. The pseudo-first order rate constants (k_{uncat}) for the background reactions (i.e., in the absence of antibodies) were found to be $3.1 \pm 0.3 \times 10^{-4}$ s^{-1} for the cycloaddition of **1** to **3**, and 7.0×10^{-5} s^{-1} for the cycloaddition of **2** to **3**.

Reaction velocities were determined by measuring the difference in initial rates between the catalysed and the background reactions, as a function of the dienophile (**3**) concentration. The data obtained from the cycloaddition between diene **1** and dienophile **3** were used to construct a double reciprocal plot of initial velocities versus the concentration of **3**. The following kinetic parameters were obtained. For antibody 285–11D6, used at a concentration of 4.3 μM, the Michaelis constant (K_M) was 1.85 mM and the apparent catalytic rate constant ($k_{cat(app)}$) 1.27×10^{-1} s^{-1}, which gave a k_{cat}/k_{uncat} value of 373. In the case of 290–4B10, used at a concentration of 14.6 μM, values for K_M of 2.94 mM and for $k_{cat(app)}$ of 9.41×10^{-2} s^{-1} were obtained, which afforded a k_{cat}/k_{uncat} ratio of 277. When reactions between *cis*-diene **2** and dienophile **3** were performed in the presence of 285-11D6 or 290-4B10; no acceleration above the background rate was observed. Antibody 309–1G7, raised against hapten **10c**, was the only one to exhibit a rate enhancement in the reactions of both dienes **1** and **2** with dienophile **3**.

Reaction of *trans*-diene **1** with dienophile **3** was carried out using 9.4 μM of antibody and, after correction for the background reaction, the kinetic data were employed to construct a Lineweaver–Burk plot that gave the following parameters: the Michaelis constant was 3.11 mM and the apparent catalytic rate constant was found to be 3.37×10^{-1} s^{-1}. In the reaction of *cis*-diene **2** with **3**, in the presence of 9.5 μM of 309–1G7, the acceleration was higher. Michaelis–Menten analysis afforded a K_M value of 3.94 mM and the $k_{cat(app)}$ was 1.83×10^{-1} s^{-1}. Compared with the corresponding rates for the background reactions, antibody 309–1G7 afforded a value of k_{cat}/k_{uncat} of 1208 in the reaction between **1** and **3**, and a value of 2618 in the reaction between **2** and **3**. The relevant kinetic data are summarized in Table 2.

The difference between the observed results can be best explained on the basis of the orientations of the methyl group of the diene in the transition states of these two reactions. In the transition state corresponding to the *trans*-diene, the methyl substituent is *pseudo*-equatorial, while in the transition state arising from the *cis*-diene, the methyl group is in a pseudo-axial position. The structure of hapten **10c** is presumably more closely related to the transition state of the reaction of **3** with *cis*-diene **2** than with *trans*-diene **1**.

Experiments were performed to determine the dissociation constants (K_D) of the antigen–antibody equilibria in solution according to a previously described protocol.[7] The monoclonal antibodies, at constant concentrations, were incubated in solution with different inhibitors and subsequently the amount of antibody which remained unsaturated at equilibrium was determined by ELISA. All three antibodies, 285–11D6, 290–4B10, and 309–1G7 were examined using hapten analogues **11a–c** as inhibitors. The data obtained were used to construct Scatchard plots and the resulting K_D values, related to the lowest value obtained, are presented in Table 1. While the K_D values obtained from these plots are not accurate and

Table 1. Normalized values of K_D

	K_D **11a**	K_D **11a**	K_D **11c**
285-11D6	4	8	25
290-4B10	57	5	87
309-1G7	21	2	1

need to be verified by other methods, they do, however, indicate clearly that each antibody displays a marked difference in affinity for the three haptens, with the highest affinity for the hapten that was used to generate it. This implies that the presence of the methyl group and its position clearly influences the binding of the hapten to the active site of the antibody catalyst, thereby demonstrating that even a minor variation in the structure of the hapten can induce specificity in the generated antibody-binding site.

In order to study the distribution of the regioisomers, the reaction mixtures were analyzed by reversed-phase HPLC. In the uncatalysed cycloadditions, the ratios of products **4**:**5** were found to be 58:42 for diene **1** to dienophile **3** and 48:52 in the case of diene **2** to **3**. In the reactions carried out in the presence of 285–11D6 and 290–4B10, the ratio of the two cycloadducts was the same as in the background reaction. When the reactions were performed under the influence of antibody 309–1G7 with either *trans*- or *cis*-piperylene, the regioisomer ratio shifted in favor of product **5**, which carries the methyl group in a position analogous to that in hapten **10c**. The data showed ratios of 51:49 for *trans*-diene **1** and 32:68 for *cis*-diene **2**. As observed in the kinetic analysis, the effect of antibody 309–1G7 is more pronounced in the reaction with the *cis*-diene than it is in the reaction with the *trans*-diene.

In order to avoid product inhibition and to minimize the effect of the background cycloaddition, the reactions of *trans*-diene **1** and *cis*-diene **2** with nitroso dienophile **3** were performed by using stoichiometric amounts of dienophile **3** and antibody 309–1G7. The reaction mixtures were analyzed by HPLC, whereupon it was found that for the *trans*-diene **1** the ratio **4**:**5** shifted further towards a higher amount of product **5** (47:53), whereas for the *cis*-diene **2** the product mixture constituted mainly of product **5** (**4**:**5** = 5:95). It is obvious that with a lower catalytic effect of the antibody the product ratio will be dominated by the high rate of the background reaction. The latter results are consistent with the picture that under the conditions employed, almost the entire reaction takes place within the active site of the antibody. Figure 1 shows the HPLC traces of the product mixtures of the reaction between *cis*-diene **2** and dienophile **3**, in the absence and in the presence of an equimolar amount of antibody 309-1G7 (see Table 2).

The enantioselectivity of antibody 309-1G7 in the reaction between *cis*-diene **2** and dienophile **3** was investigated. Efforts to analyze the reaction mixture by means of chiral HPLC failed to provide a simple procedure for determining the enantiomeric excess. A satisfactory resolution was, however, achieved by capillary electrophoretic analysis. A set of conditions was developed under which the separation of the two enantiomers of **5** was effectively accomplished, while **4** displayed only a single peak.

Examination of the products of the uncatalysed reaction exhibited the two enantiomers of **5** as two peaks in a ratio of 1:1. When the reaction was carried out in the presence of 10% of antibody 309–1G7 (compared with [**3**]), the chromatogram showed a distinct augmentation in the area of one of the enantiomers of **5**, which corresponds to an ee value of 20%, uncorrected for the background reaction, which occurs concurrently. When the same experiment was performed in the presence of an equimolar amount of antibody 309–1G7, analysis showed a very pronounced increment in the peak corresponding to the previously described enantiomer. The conditions employed did not suppress the background reaction completely. Since the HPLC analysis showed that only product **5** is formed in the antibody active site, it was possible to adjust the data for the background reaction. The corrected data gave an ee of 82% of the specific enantiomer. The absolute configuration of the latter is as yet undefined. In Figure 1 the capillary electrophoretic chromatograms of the reaction between *cis*-diene **2** and dienophile **3** in the absence and in the presence of 1 equivalent 309-1G7 are depicted.

To examine the fate of antibody 309-1G7 during the reaction, the mixtures derived from the aforementioned experiments were pooled and dialyzed exhaustively against PBS. Comparison of the recovered antibody with the initially prepared sample, in an ELISA, showed no significant differences. Kinetic experiments with the recovered antibody did not

Table 2. Kinetic and regioselectivity data collected

Antibody	[Ab]/[3]	Diene	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/k_{uncat}	**4**:**5**
None		**1**				58:42
285-11D6	0.1	**1**	1.85	1.27×10^{-1}	373	58:42
290-4B10	0.1	**1**	2.94	9.41×10^{-2}	277	58:42
309-1G7	0.1	**1**	3.11	3.37×10^{-1}	1208	58:42
309-1G7	1.0	**1**				58:42
None		**2**	3.94		2618	58:42
309-1G7	0.1	**2**		1.83×10^{-1}		58:42
309-1G7	1.0	**2**				< 5: > 95

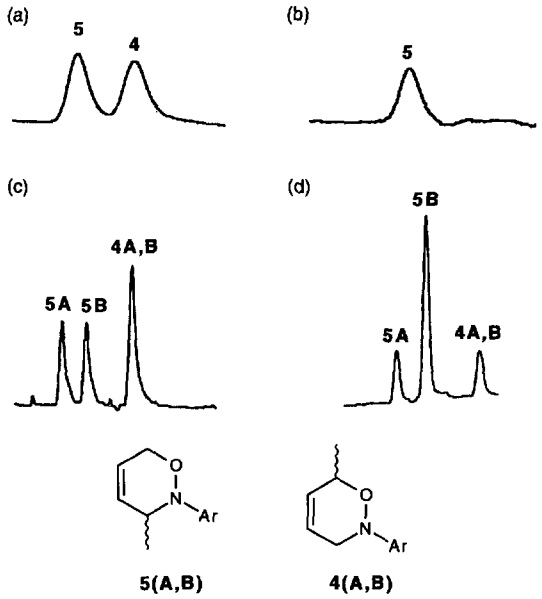


Figure 1. HPLC (a,b) and capillary electrophoretic (c,d) chromatograms of the reaction between *cis*-piperylene (**2**) and dienophile **3** in the absence (a and c) and in the presence (b and d) of 1 equiv of antibody 309-1G7.

exhibit a loss of either catalytic activity or regio- or enantioselectivity.

Inhibition experiments were carried out by studying the kinetics after incubating the antibodies with the corresponding inhibitors **11a–c**. In each case the rate of the reaction dropped to the uncatalysed value. The distribution of the regioisomers and the enantiomers in the mixtures did not differ from that observed in the absence of the antibodies. Control experiments were also performed using nonrelevant proteins such as BSA and normal mouse IgG. These showed no effect of the added proteins on the rates of reactions of **1** and **2** with **3**.

We have demonstrated the preparation of an antibody which can enhance the rate of a hetero Diels–Alder reaction and exert regio- and enantioselectivity in the formation of the product. The designed haptens proved to be adequate transition state analogues for the selected reaction. The binding sites of the generated antibodies proved to be capable of recognizing small structural changes. The results demonstrate the feasibility of rationally constructing catalysts that can govern the course of a reaction according to a specific pathway.

Experimental

4-(6-Methyl-3,6-dihydro-[1,2]oxazin-2-yl)-*N*-propylbenzamide (4) and 4-(3-methyl-3,6-dihydro-[1,2]oxazin-2-yl)-*N*-propylbenzamide (5). To a solution of nitroso dienophile **3**[8] (5 mg, 0.026 mmol) in PBS/5% DMSO v/v (10 mL) *trans*-piperylene (25 μL, 17 mg, 0.25 mmol) was added. The mixture was stirred for 4 h at room temperature and then evaporated to dryness, the residue was then dissolved in dichloromethane, filtered and evaporated to give a 1.4:1 mixture of the two regioisomers **4** and **5** as a yellow oil (6.8 mg, quant). The same procedure using *cis*-piperylene gave the same products in the ratio 1:1.1. Reactions using dichloromethane as the solvent afforded identical products. **4**: ^{1}H NMR ($CDCl_3$; 400 MHz): δ 0.97 (t, J=7.3 Hz, 3H), 1.35 (d, J=6.7 Hz, 3H), 1.62 (m, J=7.3 Hz, 2H), 3.39 (dt, J=6.8, 6.7 Hz, 2H), 3.77 (1/2 AB, J=15.7 Hz, 1H), 3.93 (1/2 AB, J=15.7 Hz, 1H), 4.70–4.74 (m, 1H), 5.86–5.91 (m, 2H), 6.08 (bs, 1H), 7.09 (d, J=8.7 Hz, 2H), 7.71 (d, J=8.7 Hz, 2H). (**5**); ^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.97 (t, J=7.3 Hz, 3H), 1.14 (d, J=6.5 Hz, 3H), 1.62 (m, J=7.3 Hz, 2H), 3.39 (dt, J=6.8, 6.7 Hz, 2H), 4.22 (bs, 1H), 4.35 (1/2 AB, J=15.6 Hz, 1H), 4.63 (1/2 AB, J=15.6 Hz, 1H), 5.87–5.90 (m, 1H), 5.93–5.96 (m, 1H), 6.12 (bs, 1H), 7.02 (d, J=8.7 Hz, 2H), 7.70 (d, J=8.7 Hz, 2H). MS (EI): exact mass calcd for $C_{15}H_{20}N_2O_2$: 260.1525. Found: 260.1528.

General procedure for the synthesis of 9a–c and 11a–c

To a solution of nitroso dienophile **3** or **8**[8] in CH_2Cl_2 (0.25 M), 1.1 equiv of diene **6** or **7**[9] was added at 30 °C and stirred for 30 min. The solvent was removed under reduced pressure to give a quantitative yield of products. Compounds **9b** and **c** and compounds **11b** and **c** were separated by chromatography over silicagel, eluting with pentane:EtOAc, 3:1.

Methyl 4-(2-oxa-3-azabicyclo[2,2,2]oct-5-en-3-yl)benzoate (9a). White crystals after recrystallization from EtOAc:hexane, mp 131–132 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.30–1.41 (m, 1H), 1.49–1.60 (m, 1H), 2.14–2.28 (m, 2H), 3.83 (s, 3H), 4.54 (m, 1H), 4.71 (m, 1H), 6.16 (dt, J=7.0, 1.3 Hz, 1H), 6.51 (dt, J=7.1, 1.6 Hz, 1H), 6.99 (d, J=8.9 Hz, 2H), 7.87 (d, J=8.9 Hz, 2H); Anal. calcd for $C_{14}H_{15}NO_3$: C, 68.55; H, 6.16; N, 5.71. Found: C, 68.59; H, 6.21; N, 5.79. MS (EI): exact mass calcd for $C_{14}H_{15}NO_3$: 245.1052. Found: 245.1069.

Methyl 4-(1-methyl-2-oxa-3-azabicyclo[2,2,2]oct-5-en-3-yl)benzoate (9b). Yield 90% in mixture; R_f 0.22; white crystals after recrystallization from EtOAc:hexane, mp 116–118 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.37–1.46 (m, 1H), 1.54–1.66 (m, 1H), 1.54 (s, 3H), 1.96–2.04 (m, 1H), 2.30 (m, 1H), 3.85 (s, 3H), 4.56 (m, 1H), 6.15 (dd, J=8.2, 5.7 Hz, 1H), 6.30 (dd, J=8.2, 1.3 Hz, 1H), 7.00 (d, J=8.8 Hz, 2H), 7.88 (d, J=8.8 Hz, 2H); Anal. calcd for $C_{15}H_{17}NO_3$: C, 69.48; H, 6.61; N, 5.40. Found: C, 69.55; H, 6.68; N, 5.47. MS (EI): exact mass calcd for $C_{15}H_{17}NO_3$: 259.1208. Found: 259.1194.

Methyl 4-(4-methyl-2-oxa-3-azabicyclo[2,2,2]oct-5-en-3-yl)benzoate (9c). Yield 10% in mixture; R_f 0.20; yellow oil. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.36 (s, 3H), 1.41–1.53 (m, 2H), 2.02–2.11 (m, 1H), 2.25–2.34 (m, 1H), 3.86 (s, 3H), 4.73 (m, 1H), 5.79 (d, J=8.2 Hz, 1H), 6.71 (dd, J=8.2, 6.0 Hz, 1H), 7.10 (d, J=8.6 Hz,

2H), 7.86 (d, J=8.6 Hz, 2H); MS (EI): exact mass calcd for $C_{15}H_{17}NO_3$: 259.1208. Found: 259.1198.

4-(2-Oxa-3-azabicyclo[2,2,2]oct-5-en-3-yl)-*N*-propylbenzamide (11a). Yellow oil. ^{1}H NMR ($CDCl_3$; 400 MHz): δ 0.95 (t, J=7.4 Hz, 3H), 1.34–1.40 (m, 1H), 1.60 (m, J=7.3 Hz, 2H), 1.53–1.64 (m, 1H), 2.18–2.30 (m, 2H), 3.37 (dt, J=7.0, 6.6 Hz, 2H), 4.50–4.52 (m, 1H), 4.71–4.73 (m, 1H), 6.00 (bs, 1H), 6.13–6.17 (m, 1H), 6.52–6.56 (m, 1H), 7.00 (d, J=8.7 Hz, 2H), 7.62 (d, J=8.7 Hz, 2H). MS (EI): exact mass calcd for $C_{16}H_{20}N_2O_2$: 272.1525. Found: 272.1519.

4-(1-Methyl-2-oxa-3-azabicyclo[2,2,2]oct-5-en-3-yl)-*N*-propylbenzamide (11b). Yield 85% in mixture; R_f 0.12; colorless oil. ^{1}H NMR ($CDCl_3$; 400 MHz): δ 0.97 (t, J=7.4 Hz, 3H), 1.38–1.45 (m, 1H), 1.54 (s, 3H), 1.61 (m, J=7.3 Hz, 2H), 1.57–1.66 (m, 1H), 1.97–2.04 (m, 1H), 2.29–2.38 (m, 1H), 3.39 (dt, J=7.2, 6.5 Hz, 2H), 4.50–4.53 (m, 1H), 6.03 (bs, 1H), 6.13 (dd, J=8.2 J=5.7, 1H), 6.31 (dd, J=8.2, 1.4 Hz, 1H), 7.02 (d, J=8.8 Hz, 2H), 7.62 (d, J=8.8 Hz). MS (EI): exact mass calcd for $C_{17}H_{20}N_2O_2$: 286.1681. Found: 286.1676.

4-(4-Methyl-2-oxa-3-aza-bicyclo[2,2,2]oct-5-en-3-yl)-*N*-propylbenzamide (11c). Yield 15% in mixture; R_f 0.10; yellow oil. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.98 (t, J=7.4 Hz, 3H), 1.34 (s, 3H) 1.41–1.50 (m, 2H), 1.62 (m, J=7.3 Hz, 2H), 2.04–2.11 (m, 1H), 2.28–2.34 (m, 2H), 3.40 (dt, J=7.3 Hz, 6.7 Hz, 2H), 4.72–4.74 (m, 1H), 6.02 (bs, 1H), 5.81 (d, J=8.2 Hz, 1H), 6.73 (dd, J=8.2, 5.9 Hz, 1H), 7.12 (d, J=8.7 Hz, 2H), 7.60 (d, J=8.7 Hz, 2H). MS (EI): exact mass calcd for $C_{17}H_{20}N_2O_2$: 286.1681. Found: 286.1669.

General procedure for the synthesis of haptens 10a–c

The ester **9a** or **b** or **c** was dissolved in THF/2 M LiOH solution in water v/v=2:1 (0.5 M). After stirring for 48 h at room temperature, the reaction mixture was acidified with 1 M HCl solution (pH 1), extracted with EtOAc, dried ($MgSO_4$) and evaporated under reduced pressure to give the acid. To a solution of this acid and 2.0 equiv of *N*-hydroxysuccinimide in DMF (0.2 M) 1.05 equiv of DCC was added and the reaction mixture was stirred overnight at room temperature. The mixture was filtered over florisil and 5 equiv of 6-aminohexanoic acid and 10 equiv of Et_3N were added. After stirring overnight at room temperature, EtOAc was added, the reaction mixture was washed with 0.1 M HCl solution, dried (Na_2SO_4) and evaporated under reduced pressure. Chromatography over silica gel, eluting with CH_2Cl_2:EtOAc:MeOH, 20:5:3 gave the pure haptens.

6-[4-(2-Oxa-3-azabicyclo[2,2,2]oct-5-en-3-yl)-benzoylamino]hexanoic acid (10a). Yield 61%; R_f 0.1; yellow oil. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.35–1.45 (m, 3H), 1.57–1.71 (m, 5H), 2.18–2.31 (m, 2H), 2.35 (t, J=7.3 Hz, 2H), 3.42 (dt, J=6.7, 6.4 Hz, 2H), 4.52–4.53 (m, 1H), 4.72–4.73 (m, 1H), 6.14–6.17 (m, 2H), 6.53–6.57 (m, 1H), 7.01 (d, J=8.6 Hz, 2H), 7.62 (d, J=8.6 Hz, 2H). MS (FAB): 345 (MH^+).

6-[4-(1-Methyl-2-oxa-3-azabicyclo[2,2,2]oct-5-en-3-yl)benzoylamino]hexanoic acid (10b). Yield 45%; R_f 0.1; yellow oil. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.36–1.45 (m, 3H), 1.50–1.71 (m, 5H), 1.54 (s, 3H), 1.94–2.03 (m, 1H), 2.26–2.34 (m, 1H), 2.34 (t, J=7.2 Hz, 2H), 3.40 (dt, J=6.7, 6.4 Hz, 2H), 4.50–4.52 (m, 1H), 6.11 (dd, J=8.2, 5.2 Hz, 1H), 6.27 (bs, 1H), 6.30 (d, J=8.1 Hz, 1H), 7.00 (d, J=8.7 Hz, 2H), 7.63 (d, J=8.7 Hz, 2H); MS (FAB): 359 (MH^+).

6-[4-(4-Methyl-2-oxa-3-azabicyclo[2,2,2]oct-5-en-3-yl)benzoylamino]hexanoic acid (10c). Yield 41%; R_f 0.1; yellow oil. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.34 (s, 3H), 1.38–1.47 (m, 2H), 1.53–1.70 (m, 6H), 2.05–2.12 (m, 1H), 2.28–2.33 (m, 1H), 2.36 (t, J=7.2 Hz, 2H), 3.42 (dt, J=6.7, 6.4 Hz, 2H), 4.71–4.74 (m, 1H), 5.81 (d, J=8.2 Hz, 1H), 6.27 (bs, 1H), 6.72 (dd, J=8.1, 6.0 Hz, 1H), 7.11 (d, J=8.5 Hz, 2H), 7.60 (d, J=8.5 Hz, 2H); MS (FAB): 359 (MH^+).

General procedure for the preparation of hapten–protein conjugates

A solution of hapten (100 equiv) dissolved in 200 μL of DMF was added to a solution of Chicken Gamma Globulins (CγG; 5 mg) or cationized bovine serum albumin (cBSA; 5 mg) in dilute aqueous HCl (1 mL, pH 5.0). A solution of EDC (100 equiv) in dilute aq HCl (200 μL, pH 5.0) was then added and the mixture was stirred overnight at 4 °C. The solution was dialyzed exhaustively against phosphate buffered saline (PBS, 10 mM phosphate buffer, 140 mM NaCl, pH 7.3). Protein concentrations of the conjugates were determined by either the Lowry[10] or the bicinchoninic acid (BCA) method (Pierce no. 23225).[11]

Production of monoclonal antibodies

Conjugates of haptens with carrier proteins (cBSA-**10a**, CγG-**10b** and CγG-**10c**) were used to immunize (ip) Balb/c mice, following standard hyperimmunization protocols.[12] Spleen lymphocytes (5.0×10^6 per fusion) and SP2/O$^+$ myeloma cells (5.0×10^6 per fusion) were submitted to electrofusion.[13] A standard procedure[12] was used to isolate the hybridoma cell lines producing antibodies specific for the corresponding haptens. Selection of the high binding antibodies was performed by indirect ELISA. The selected clones were amplified in 1 L roller bottles and the produced antibodies were purified by affinity chromatography on a GammaBind Plus Sepharose column (Pharmacia), following the manufacturer's procedure. The homogeneity of the purified antibodies was evaluated by SDS PAGE (Pharmacia PhastSystem), with Coomassie blue staining, which under reducing conditions showed only heavy and light chains. Antibodies were isotyped using a MonoAb Screen ID kit (Zymed n. 97–6550) which identified all three antibodies as IgG1 (κ light chain). Antibody concentrations were determined by UV

absorbance at 280 nm using a molecular weight of 150,000 and $\varepsilon 280 = 202{,}500$ L mol^{-1} cm^{-1}.

Kinetic analysis

The rates of the reactions were determined with UV spectroscopy, using a HP 8154A instrument, by measuring the decrease in absorbance at 340 nm of dienophile **3**. The concentration of dienes **1** and **2** was held fixed at 5 mM, while the concentration of dienophile **3** was varied between 0.1 mM and 0.6 mM; higher concentrations of **3** were not analyzed due to low solubility of the substrate in the reaction conditions. In the concentration range of dienophile **3** analyzed, saturation conditions were not reached. However, since the data obtained did show an enhancement of the reaction rate and this enhancement could be completely inhibited by the addition of free hapten, the use of Michaelis–Menten analysis is vindicated. Experiments using fixed concentrations of antibody and dienophile **3** and increasing concentrations, up to 30 mM, of diene **1** did not exhibit a change in the kinetic parameters, demonstrating that a concentration of 5 mM is saturating for the diene.

Experiments were carried out at room temperature by adding aliquots (1–10 μL) from a stock solution of dienophile **3** in DMSO (27.8 mM) to 380 μL of antibody solution in PBS, pH 7.3 (for each antibody the final concentration varied) and brought to 390 μL with DMSO; reactions were initiated by addition of 10 μL of a stock solution of either diene **1** or diene **2** in DMSO (200 mM), giving a final amount of organic solvent of 5%. Background reactions in the absence of antibody were run simultaneously to ensure constant experimental conditions.

Regioselectivity and enantioselectivity studies

The ratio of the regioisomers was determined by analyzing the reaction mixtures with analytical HPLC, using a Polygosil 60 C18 reversed-phase column (250 × 4 mm), employing isocratic conditions (MeOH: H_2O, 4:6, containing 0.1% TFA, flow 1.5 mL/min) with UV detection at 282 nm. The retention times were for dienophile **3**: 9.4 min, for product **5**: 31 min, and for product **4**: 33.5 min.

Studies on the enantioselectivity were conducted by using cyclodextrin-modified micellar electrokinetic chromatography (CD-MEKC).[14] Conditions: capillary 90 cm (60 cm to the detector), 50 μm internal diameter; separation solution: 50 mM sodium deoxycholate in 6 mM borate buffer pH 9 containing 35 mM α-cyclodextrin, applied voltage 25 kV; UV detection at 282 nm. In order of elution the retention times for the enantiomers of product **5** were 28.7 and 29.6 min, product **4** eluted at 31.1 min. Under these conditions only the separation of the two enantiomers of product **5** was achieved. Prior to CD-MEKC analysis, the mixture of products was isolated by adding equal volumes of acetonitrile to the reaction mixtures to denature the antibodies; the samples were centrifuged, the supernatant was evaporated, the residue was dissolved in dichloromethane, filtered and evaporated.

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