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Exploring the Scope of the 29G12 Antibody Catalyzed 1,3-Dipolar Cycloaddition Reaction

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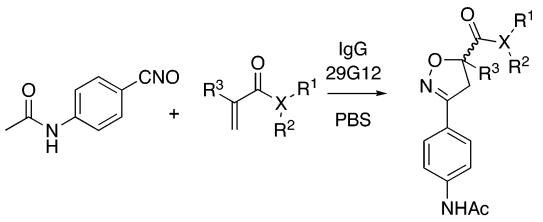

71-98 % *ee*

29G12 is a murine monoclonal antibody programmed to catalyze the regio- and enantioselective 1,3-dipolar cycloaddition reaction between 4-acetamidobenzonitrile *N*-oxide **1a** and *N*,*N*-dimethylacrylamide **2a** (Toker, J. D.; Wentworth, P., Jr.; Hu, Y.; Houk, K. N.; Janda, K. D. *J. Am. Chem. Soc.* **2000**, *122*, 3244). Given the unique nature of 29G12 as a protein biocatalyst for this chemical reaction, we have investigated both the substrate specificity and mechanistic parameters of the 29G12-catalyzed process. These studies have shown that while 29G12 is specific for its dipole substrate **1a**, the antibody is highly promiscuous with respect to the dipolarophiles it can process. 29G12 accepts a bulky hydrophobic dipolarophile cosubstrate, with rates of product formation up to 70-fold faster than with the original substrate **2a**. In all cases, the respective isoxazoline products are produced with exquisite regio- and stereochemical control (78−98% ee). Comparison between the steady-state kinetic parameters from the 29G12-catalyzed reaction of **1a** with the most efficient versus the original dipolarophile cosubstrate (**2m** and **2a**, respectively), reveals that while the effective molarities (EM)s are almost identical ($EM_{(\mathbf{2m})}$ 26 M; $EM_{(\mathbf{2a})}$ 23 M), the affinity of 29G12 for the larger dipolarophile **2m** is more than 1 order of magnitude higher than for **2a** [K_m(**2m**) 0.44 ± 0.04 mM; K_m(**2a**) 5.8 ± 0.4 mM]. Furthermore, when **2m** is the cosubstrate, the affinity of 29G12 for its dipole **1a** is also greatly improved [K_m(**1a**) 0.82 ± 0.1 mM compared to K_m(**1a**) 3.4 ± 0.4 mM when **2a** is the cosubstrate]. An analysis of the temperature dependence of the 29G12-catalyzed reaction between **1a** and **2m** reveals that catalysis is achieved via a decrease in enthalpy of activation ($\Delta\Delta H^{\ddagger}$ 4.4 kcal mol^{-1}) and involves a large increase in the entropy of activation ($\Delta\Delta S^{\ddagger}$ 10.4 eu). The improved affinity of 29G12 for the nitrile oxide **1a** in the presence of **2m**, coupled with the increase in $\Delta\Delta S^{\ddagger}$ during the 29G12-catalyzed reaction between **1a** and **2m** supports the notion of a structural reorganization of the active site to facilitate this antibody-catalyzed reaction.

Introduction

The 1,3-dipolar cycloaddition (1,3-DPC) reaction, a concerted pericyclic process between a 1,3-dipole and an alkene (or alkyne), is a classic reaction in organic chemistry.[1,2] Curtius,[3] in 1883, discovered the dipole diazoacetic acid, and 5 years later Buchner documented the condensation between diazoacetic acid and α*β*-unsaturated esters and the 1,3-DPC reaction was born.[4]

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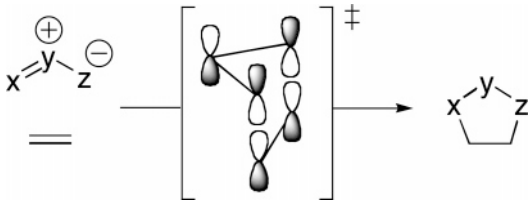
SCHEME 1



However, the influence and impact of Huisgen throughout the latter half of the 20th century to our understanding of all aspects of the 1,3-DPC reaction cannot be overstated.[1,5] In fact, it is a fair assessment that Huisgen established the field of 1,3-DPC chemistry as an independent discipline within synthetic and mechanistic organic chemistry.

The 1,3-dipole, represented by a zwitterionic octet structure, undergoes cycloadditions to the multiple bond within the dipolarophile (Scheme 1).

The 1,3-DPC reaction is a powerful carbon−carbon bond-forming reaction with tremendous synthetic utility that allows access to a wide diversity of chiral five-membered heterocycle building blocks. Catalytic asymmetric approaches to this reaction generally have utilized transition-metal salts,[6] but recently MacMillan has shown that an organocatalytic approach, utilizing imidazolidinone-based chiral amines, is also effective.[7] There is at present no confirmed naturally occurring biological catalyst for the 1,3-DPC, and therefore, as part of our ongoing efforts in the catalytic antibody field,[8−10] we are interested in probing the natural diversity of the murine protein immune system to elicit catalytic antibodies for this reaction. In this regard, we reported the generation of the first antibody catalyst of the 1,3-DPC reaction between 4-acetamidobenzonitrile *N*-oxide **1a** and *N*,*N*-dimethylacrylamide **2a** (Figure 1).[11] The antibody, 29G12, elicited to hapten **3a** catalyzes the formation of the favored 5-substituted oxazolidine **5a** as the only product, with excellent enantioselectivity (98% ee).

The nature of the transition states of these reactions was one of last century's most contentious mechanistic issues[5,12,13] but the asynchronous concerted mechanism is now accepted, with recent quantum mechanical studies providing high-level theoretical support for a planar, pericyclic transition state with aromatic character.[14,15] Frontier MO theory has been utilized to account for the regioselectivity observed in the general reaction between a nitrile oxide and dipolarophile, which, due to favorable dipole LUMO−dipolarophile HOMO overlaps, strongly favors formation of the 5-substituted isoxazoline.[13,16] The activation energy calculated for the formation of the

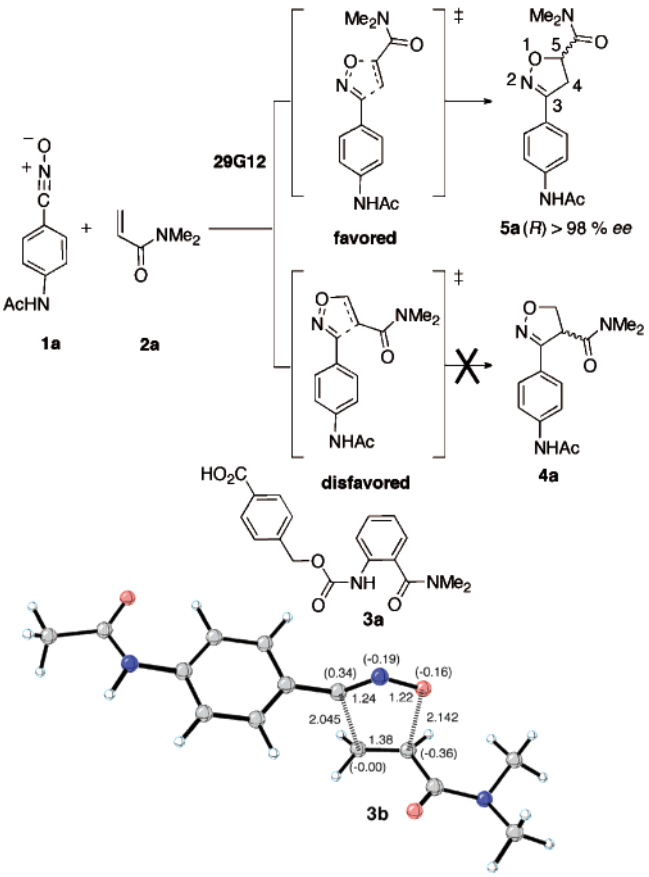


FIGURE 1. 29G12-catalyzed 1,3-dipolar cycloaddition reaction. The noncatalyzed reaction between **1a** and **2a** is kinetically controlled, passing through two plausible transition states, the lowest energy transition state being **3b**, to yield regioisomeric isoxazolines **4a** and **5a**. Antibody 29G12 elicited to hapten **3a** catalyzes the reaction between **1a** and **2a** to form the isoxazoline **5a** in >98 ee. Energies obtained with B3LYP/6-31G* calculations, using a polarized continuum model of solvation (PCM); bond lengths reported in angstroms.[11]

5-substituted product **5a** (15.5 kcal mol^{-1}) is 4.9 kcal mol^{-1} lower than for formation of the 4-isomer **4a** in water (energies obtained with B3LYP/6-31G* calculations, using a polarized continuum model of solvation).[11] By analogy with the Diels−Alder cycloaddition reaction,[17] this bimolecular process requires a significant control of translational entropy as it proceeds through an entropically disfavored, highly ordered transition state, with large activation entropies in the range −30 to −40 cal mol^{-1} K^{-1}. Hapten **3a** was built upon the "entropic trap" concept, where in principle the translational entropy of the reaction is reduced by bringing the two substrates into the correct orientation for reaction within the programmed antibody binding site.[18−21] The planar aro-

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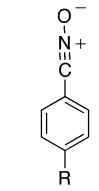


FIGURE 2. Nitrile oxides investigated as cosubstrates with antibody 29G12.

matic core of **3a** was designed to mimic the aromatic character of the calculated TS‡ **3b**.[11,14,15]

Given the unique status of 29G12 as a biocatalyst for the 1,3-DPC reaction, we have probed its substrate specificity and regio- and enantioselectivity with a range of substituted dipolarophiles (**2a**−**t**) and nitrile oxides (**1a**−**c**) (Figures 1 and 2). Having discovered that 29G12 can catalyze the1,3-dipolar cycloaddition reaction with a panel of dipolarophile substrates, we have probed the mechanism by which catalysis occurs via steady-state kinetics and temperature dependence.

Results and Discussion

Synthesis of Acrylamide and Acrylate Cosubstrates and 5-Substituted Δ²-Isoxazoline Products. The dipolarophiles utilized as cosubstrates for this investigation were obtained either commercially or by chemical synthesis. Thus, acrylamides **2d**,**f**−**i**,**l**−**n**,**p**−**t** and acrylate esters **2k** and **2o** were prepared via the reaction between their respective acryloyl chloride and corresponding amine or alcohol respectively in dichloromethane as described previously (Scheme 2).[22]

The cycloaddition products **5a**−**t** of the reaction between dipolarophiles **2a**−**t** and **1a** were all prepared as analytically pure compounds for use as HPLC standards (Scheme 2). Two synthetic approaches were used. In the first, the benzohydroximinoyl chloride **8**, prepared in two steps from aldehyde **6**,[23] was stirred with the dipolarophiles and triethylamine at room temperature (method a). The second approach employed harsher conditions; thus, the oxime **7** chloramines-T and the dipolarophile were heated under reflux in ethanol for the requisite period of time (method b).

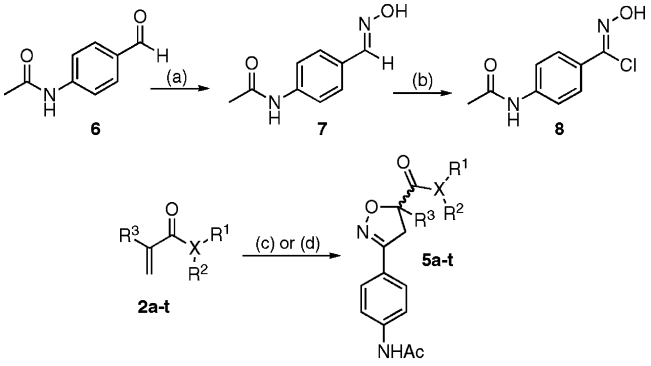
Substrate Tolerance Studies with 29G12. Two strategies were employed to probe the ability of 29G12 to tolerate modifications to its substrates **1a** and **2a**. In the first, acrylamide **2a** was held fixed and the nitrile oxide was modified. In the second, nitrile oxide **1a** was fixed and the dipolarophile was varied.

Structural modifications to nitrile oxide **1a** are poorly tolerated by 29G12. All catalytic activity of the antibody is abolished by replacement of the *p*-acetamido group with either a *p*-NO_2 **1b** or *p*-H **1c** (Figure 2).

This observation was surprising because the attachment point of hapten **3a** to the carrier protein is at the equivalent locus to where the para position of the aromatic ring of the nitrile oxide substrates is anticipated to be. Prevailing knowledge, gained from numerous structural analyses of catalytic antibodies, has suggested that the point on the hapten with least antibody recognition and hence the most tolerated structural modifications to substrates should be at this locus.[10,24] However, the low tolerance for modification of structural features at the para position of **1a** suggests that this locus is either deeply buried or is making key interactions with specific antibody residues.

The cosubstrate tolerance of the dipolarophile by antibody 29G12 was investigated at distinct points around the dipolarophile structure: R^1, R^2, and R^3 and at the attachment point to the acryloyl carbonyl, designated X (Scheme 2). The study involved a comparison of the initial rates of isoxazoline product formation (**5a**−**t**) from the respective dipolarophiles **2a**−**t** (1 mM) and **1a** (1 mM), catalyzed by 29G12 (3.4 μM) in an aqueous buffer

SCHEME 2. Synthetic Approach to Dipolarophiles 2a−t and Isoxazoline Products 5a−t Used in This Study[a]



R^1	R^2	R^3	X		
methyl	methyl	H	N	**2a**	**5a**
H	H	H	N	**2b**	**5b**
methyl	H	H	N	**2c**	**5c**
methyl	H	methyl	N	**2d**	**5d**
-	-	H	methyl	**2e**	**5e**
iso-propyl	H	H	N	**2f**	**5f**
sec-butyl	H	H	N	**2g**	**5g**
iso-butyl	H	H	N	**2h**	**5h**
tert-butyl	H	H	N	**2i**	**5i**
methyl	-	H	O	**2j**	**5j**
iso-butyl	-	H	O	**2k**	**5k**
iso-butoxymethylene	H	H	N	**2l**	**5l**
phenyl	H	H	N	**2m**	**5m**
benzyl	H	H	N	**2n**	**5n**
phenyl	-	H	O	**2o**	**5o**
benzyl	methyl	H	N	**2p**	**5p**
4-methoxyphenyl	H	H	N	**2q**	**5q**
4-nitrophenyl	H	H	N	**2r**	**5r**
cyclohexyl	H	H	N	**2s**	**5s**
napthyl	H	H	N	**2t**	**5t**

[a] Conditions: (a) NH_2OH, ethanol, 40 °C; (b) *N*-chlorosuccinimide, DMF, 35 °C; (c) **8**, dipolarophile, TEA, ethanol, rt (method a); (d) **7**, dipolarophile, chloramine-T, ethanol, reflux (method b).

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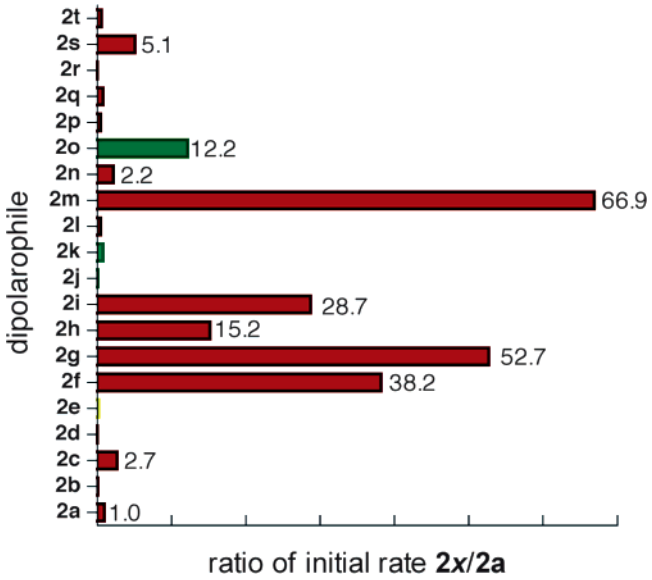

FIGURE 3. Bar graph showing the relative initial rate of product formation for the 29G12-catalyzed 1,3-DPC between dipole **1a** and dipolarophiles **2a**−**t**. Note the value for the ratio of the initial rate **2x**/**2a** for each dipolarophile (**2a**−**t**) is shown at the end of each bar. Initial rates were measured by monitoring product (**5a**−**t**) formation by HPLC at least in duplicate and were linear ($r^2 > 0.985$). The bar colors within the figure represent the chemical structure of the dipolarophile: red, acrylamides; black, acylrates; yellow, vinyl ketone.

system [morpholinoethanesulfonic acid (MES), pH 6.5, 4% v/v DMSO] at 4 °C (Figure 3).

This analysis revealed a number of subtle and dramatic aspects to the tolerance of structural modifications to its dipolarophile cosubstrate by 29G12. Deletion of one methyl group from the tertiary amide of **2a** to give the secondary amide **2c** results in an ~ 3-fold increase in the 29G12-catalyzed rate of formation of **5c** versus **5a**. However, deletion of the second methyl group to give the primary acrylamide **2b** results in a 10-fold drop in the initial rate of 29G12-catalyzed formation of **5b** relative to **5a**. Addition of a methyl group at locus R^3 as in substrate **2d** results in a complete abolishment of 29G12 catalytic activity.

Dipolarophiles where large hydrophobic components replace the methyl group of **2c** are excellent cosubstrates for 29G12. For the cosubstrates where R^1 is isopropyl (**2f**), *sec*-butyl (**2g**), isobutyl (**2h**), *tert*-butyl (**2i**), and phenyl (**2m**) (R^2 is H in all cases), the 29G12-catalyzed rate of formation of **5f**, **5g**, **5h**, **5i**, and **5m** is 38.2, 52.7, 15.2, 28.7, and 66.9-fold faster than formation of **5a**. This remarkable tolerance of 29G12 to such significant structural modifications at the R^1 locus argues that this component of the acrylamide is either highly solvent exposed or more likely buried in a flexible hydrophobic binding pocket. The critical nature of substrate structure versus catalytic activity of 29G12 was emphasized by the observation that the initial rate of 29G12-catalyzed product formation with the *N*-benzyl acrylamide cosubstrate **2n** is only ~2-fold more active than **2a**, whereas the *N*-phenyl substrate **2m** is ~67-fold more active. In addition, *p*-methoxyphenyl acrylamide **2q** is approximately as equivalent a cosubstrate as **2a.** The insertion of the methylene in **2n** being responsible for a 30-fold reduction in the catalytic efficiency of 29G12 and the addition of the *p*-methoxy group being responsible for a ~70-fold drop in catalytic efficiency of 29G12. Interestingly, *p*-nitrophenyl acrylamide **2r** is not a cosubstrate for 29G12.

TABLE 1. Enantioselectivity and Relative Rate of 29G12 with Substrates 2a, 2i, 2f, 2g, and 2m

dipolarophile	% ee[a]	k_{rel}
N,N-dimethylacrylamide (**2a**)	98	1
N-tert-butylacrylamide (**2i**)	94	28.7
N-isopropylacrylamide (**2f**)	85	38.2
N-sec-butylacrylamide (**2g**)	97	52.7
N-phenylacrylamide (**2m**)	71	66.9

[a] Measured by analytical chiral HPLC.

The nature of the group attached to the carbonyl of the acrylamide is also an important locus for 29G12 activity. When the amine is replaced with oxygen (to yield the corresponding acrylate esters), the ability of 29G12 to catalyze the reaction is impaired (cf. **2m** and **2o**; **2h** and **2k**; **2c** and **2j**), suggesting that recognition of the amide, preferentially with a free N−H is optimal for catalytic activity. Deletion of the heteroatom and replacement with a methyl group to give the cosubstrate methyl vinyl ketone **2e** completely abolishes the catalytic activity of 29G12.

Enantioselectivity with Modified Acrylamide Cosubstrates. A critical component of the 1,3-DPC is stereochemical control. With its primary substrates **1a** and **2a**, antibody 29G12 exhibits excellent enantioselectivity, generating the 5-(*R*)-acylisoxazoline enantiomeric product **5a** in up to 98% ee.[11] A series of experiments were undertaken to explore the enantioselectivity of the 29G12-catalyzed effect of the improved hydrophobic cosubstrates *N*-phenylacrylamide **2m**, *N-sec*-butylacrylamide **2g**, *N*-isopropylacrylamide **2f**, and *N-tert*-butylacrylamide **2i** with **1a** (Table 1). The enantiomeric excess, ee, was determined by chiral HPLC analysis of the enantiomeric mixture of isoxazoline products. The absolute stereochemistry of the isoxazoline **5m**, generated by the 29G12-catalyzed 1,3-DPC with the dipolarophile **2m** and dipole **1a**, was determined unambiguously from X-ray crystallographic analysis of the enantiomer generated in the antibody-catalyzed reaction (see the Supporting Information). This analysis revealed that the 29G12-catalyzed 1,3-DPC is highly enantioselective with a broad range of substrates and the absolute stereochemistry of the 29G12-catalyzed process does not change in moving from **2a** to **2m**, both giving the 5(*R*)-enantiomer of the isoxazoline in each case.

Steady-State Kinetic Investigation of 29G12 with *N*-Oxide (1a) and *N*-Phenylacrylamide (2m). Based on initial rate data, *N*-phenylacrylamide **2m** appeared to be the best cosubstrate with nitrile oxide **1a** for the 29G12-catalyzed 1,3-DPC addition reaction (Figure 3). Steady-state kinetic parameters were measured for the antibody 29G12 catalyzed reaction between **1a** and **2m** by the method of initial rates, following formation of **5m** at −5 °C. A family of Lineweaver−Burk plots was constructed at four fixed concentrations of either nitrile oxide **1a** or acrylamide **2m**, with the other cosubstrate being varied (200−1000 μM). These Lineweaver−Burk plots were a family of intersecting lines, suggesting that

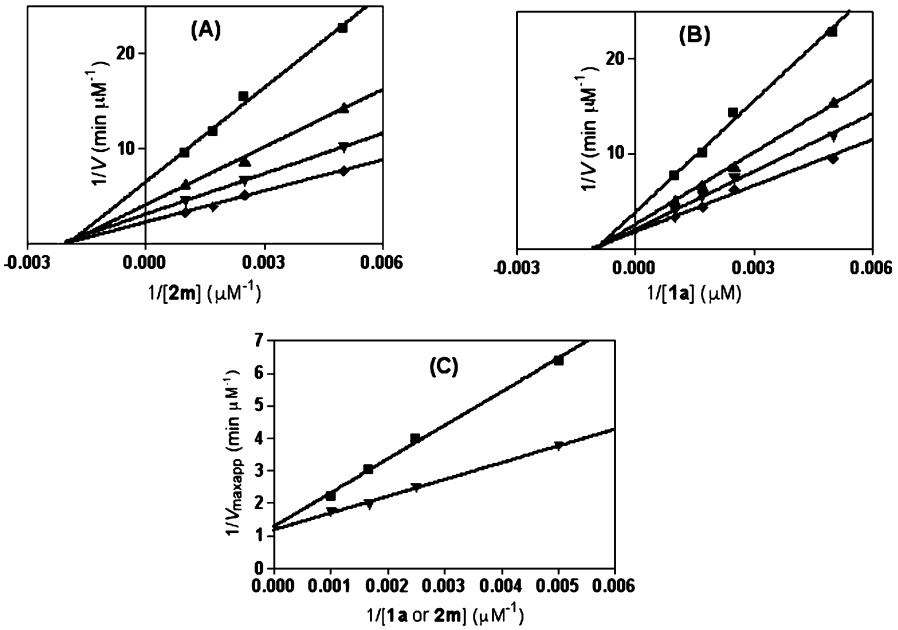

FIGURE 4. Graphical determination of the steady-state kinetic parameters of antibody 29G12. (A) Lineweaver−Burk plot of the initial velocities of cycloadduct **5m** formation in the presence of 29G12; dipole **1a** concentrations were fixed [(■ 200 μM; $y = 3313x + 6.385$, $r^2 = 0.993$), (▲ 400 μM; $y = 2031x + 3.991$, $r^2 = 0.994$), (▼ 600 μM; $y = 1421x + 3.016$, $r^2 = 1.000$), and (◆1000 μM; $y = 1096x + 2.196$, $r^2 = 0.996$)], and the concentration of dipolarophile **2m** was varied within the same range. (B) Lineweaver−Burk plot of the initial velocities of cycloadduct **5m** formation in the presence of 29G12; acrylamide **2m** concentrations were fixed [(● 200 μM; $y = 3898x + 3.748$, $r^2 = 0.993$), (▲ 400 μM; $y = 2529x + 2.477$, $r^2 = 0.998$), (▼ 600 μM; $y = 2036x + 1.948$), and (◆1000 μM; $y = 1617x + 1.729$)], and the concentration of dipolarophile **1a** was varied within the same range. (C) Replot of the V_{max}(app) obtained from the Lineweaver−Burk plots [(■ dipole (**1a**); $y = 1035x + 1.266$, $r^2 = 0.997$), (▼ dipolarophile (**2m**); $y = 516x + 1.163$, $r^2 = 0.996$)] to obtain the true V_{max} value as 1/(y intercept) and Michaelis−Menten constant (K_m) values as −1/(x intercept).

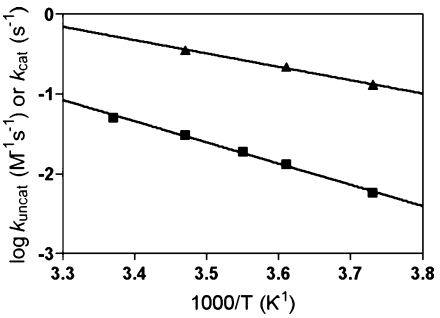

FIGURE 5. Arrhenius plot of the 29G12-catalyzed 1,3-DPC reaction between **1a** and **2m**. 29G12-catalyzed: [▲; $y = -1.674x + 5.374$, $r^2 = 0.997$], and noncatalyzed (■; $y = -2.657x - 7.699$, $r^2 = 0.995$) 1,3-DPC reaction.

the 29G12-catalyzed 1,3-DPC between **1a** and **2m** follows a completely random, sequential, *bi-uni* kinetic mechanism that proceeds via a ternary antibody−substrate complex (Figure 4A and B).[25−27]

The slopes and y-intercepts obtained from this analysis were re-plotted as a function of the nitrile oxide **1a** or *N*-phenylacrylamide **2m** concentration (Figure 5C). From this secondary plot, the kinetic parameters for the 29G12-catalyzed reaction were measured to be $k_{cat} = 0.13 \pm 0.21$ s^{-1}; K_m(**1a**) = 0.82 ± 0.12 mM, and K_m(**2m**) = 0.44 ± 0.04 mM. The noncatalyzed rate constant for the second-order reaction between **1a** and **2m** was determined by the method of initial rates and found to be $k_{uncat} = 5.67 \pm 0.5 \times 10^{-3}$ M^{-1} s^{-1} (measured at −5 °C). An analysis of the lower estimate of the enhancement ratio (k_{cat}/K_m(**1a**)/k_{uncat}) is 5.16×10^4 with an effective molarity (k_{cat}/k_{uncat}) of 23 M. Wolfenden has shown that the overall catalytic proficiency of a biocatalyst catalyzing a multisubstrate reaction can be expressed in terms of its transition-state affinity by comparison of the third-order rate constant k_{cat}/K_m(**A**)·K_m(**B**) with the second-order rate constant k_{non}.[28] Such an analysis for the 29G12-catalyzed reaction between **1a** and **2m**, $k_{cat}/[K_m$(**1a**)·K_m(**2m**)]/k_{uncat} yields a catalytic proficiency of 6.32×10^7 M^{-1}, equivalent to a transition-state dissociation constant of 15.8 nM.

Comparison of these data with our reported steady-state kinetics of 29G12 with **2a** reveals that the observed initial rate increase of the 29G12-catalyzed process with dipolarophile **2m** relative to **2a** is reflected in an almost 60-fold increase in the catalytic proficiency of 29G12, whereas the EM values are almost identical [EM (**2m**) 26 M, EM (**2a**) 23 M]. This apparent anomaly seems to be a result of increased efficiency of substrate binding, as expressed in a lower K_m, (0.44 mM for **2m** vs 5.8 mM for **2a**). The origin of this increased binding energy may well be additional contacts made between the antibody and the larger hydrophobic surface of **2m**, relative to **2a**. This hypothesis is supported by the fact that, in general, dipolarophiles with large hydrophobic groups, such as **2i**, **2f**, and **2g**, exhibit a similar increase in initial rate of catalysis with 29G12 relative to **2a** vide infra (see Figure 3).

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TABLE 2. Temperature Dependence of the 29G12-Catalyzed, k_{cat}, and Noncatalyzed, k_{uncat},1,3-DPC Reaction between 1a and 2m

T/K	k_{cat}/s^{-1}	$k_{uncat} \times 10^3/M^{-1}\ s^{-1}$
268	0.13 ± 0.02	5.67 ± 0.5
277	0.22 ± 0.02	13.3 ± 0.1
282	n.d.[a]	19.1 ± 0.2
288	0.36 ± 0.03	31.0 ± 0.1
297	n.d.[a]	50.6 ± 0.4

[a] Not determined.

More interestingly, when the larger dipolarophile **2m** is a cosubstrate with **1a**, 29G12 actually lowers the K_m of **1a** from 3.4 mM to 0.8 mM. Such an increase in substrate affinity has evolved as a regulatory mechanism for activation of enzymes in vivo but has not previously been observed with catalytic antibodies. Generally, in the case of enzymes, the increase in substrate affinity is precipitated by binding of the "activator" molecule to an allosteric site, leading to a structural reorganization of the active site as is the case with protein kinase C δ1 activation by free fatty acids binding to a peripheral site.[29] However, in the case of 29G12, it seems that a reorganization of the combining site may be occurring upon binding of **2m** leading to an increase in the affinity of 29G12 for **1a** and presumably *vice versa* because the kinetics of the reactions supports completely random, sequential, *bi-uni* kinetic mechanism. This improved binding of **1a** with **2m** suggests that "substrate-initiated binding site reorganization" may be an unrecognized approach to improving the catalytic efficiency of multisubstrate catalytic antibodies. The ability of protein receptors to be able to reorganize hydrophobic domains to facilitate binding of hydrophobic ligands is now being recognized from SAR analyses of drug−receptor interactions.[30]

Temperature Dependence of the 29G12-Catalyzed Process. To determine the relative importance of entropic and enthalpic stabilization to the catalysis of this bimolecular reaction, the temperature dependence of the 29G12-catalyzed and -uncatalyzed 1,3-DPC reactions between **1a** and **2m** were investigated. The steady-state kinetic parameters K_m(**1a**), K_m(**2m**), and k_{cat} for the 29G12-catalyzed process and the uncatalyzed rate constant k_{uncat} between −5 and 24 °C are compiled in Table 2. An Arrhenius analysis of the temperature dependence of k_{uncat} and k_{cat} reveals that the activation parameters for the uncatalyzed reaction are $\Delta H^{\ddagger} = 11.5$ kcal mol^{-1} and $\Delta S^{\ddagger} = -25.1$ eu and the antibody-catalyzed reaction are $\Delta H^{\ddagger} = 7.1$ kcal mol^{-1} and $\Delta S^{\ddagger} = -35.5$ eu in aqueous buffer [50 mM *N*-morpholinoethanesulfonic acid (MES), 150 mM NaCl, pH 6.5] (Figure 5).

Clearly, the antibody functions by lowering the enthalpy of activation ($\Delta\Delta H^{\ddagger}$ 4.4 kcal mol^{-1}) rather than the anticipated effects on entropy. This observation is in line with the observed enthalpy dependence of the 29G12-catalyzed reaction with dipolarophile **2a**[11] and supports the notion that, while the observed rate of the reaction for **2m** is significantly higher than **2a** under identical conditions, the mechanism of catalysis appears to be analogous. Thus, antibody-binding and stabilization of the lower polarity transition state relative to the ground state, programmed by hapten **3b**, coupled with the fortuitous potential for hydrogen bonding at the amide carbonyl carbon of **2m**, may well be assisting the 29G12 mechanism. These effects, both of which are enthalpic in nature, will destabilize the entropy of the process by necessitating solvent ordering and restriction of antibody binding-site residues, respectively, in the transition state.

The clear effect of enthalpic stabilization ($\Delta\Delta H^{\ddagger}$ 4.4 kcal mol^{-1}) by 29G12 as a route to catalysis, coupled with a net increase in the entropy ($\Delta\Delta S^{\ddagger}$ 10.4 eu) of activation, is clearly counterintuitive to the notion of how one might anticipate a biocatalyst evolving to catalyze a bimolecular reaction. However, 29G12 clearly exhibits a preference for hydrophobic dipolarophiles. Therefore, the catalytic process seems to involve a desolvation phenomenon, i.e., the antibody is partitioning the hydrophobic dipolarophiles from the aqueous buffer system into the favorable environment of the binding site, thus having the positive effect on enthalpy. Desolvation has previously been shown to account for the reduction in $\Delta\Delta H^{\ddagger}$ in antibody-catalyzed unimolecular elimination and decarboxylation processes.[31,32] However, in the end, the composite character of these quantities and the probable role of solvation effects in determining them exclude drawing more firm conclusions.

Conclusions. A substrate tolerance study of the 29G12-catalyzed enantioselective 1,3-dipolar cycloaddition reaction has revealed the presence of an unoptimized pocket that accepts a range of bulky hydrophobic dipolarophiles. Steady-state kinetic parameters with the most efficient cosubstrate, **2m**, revealed the remarkable phenomena that the affinity of 29G12 for the larger dipolarophile **2m** is much higher than for its native dipolarophile **2a** and that when **2m** is a cosubstrate, the affinity of 29G12 for dipole **1a** is also increased. This "substrate optimization" suggests that there is significant reorganization occurring within the active site of 29G12 during the catalytic process. A thermodynamic analysis of the 29G12-catalyzed process offers support for this assertion with there being a significant increase in the entropy of activation coupled with a decrease in enthalpy of activation. The nature of the structural reorganization of the antibody combining site will be ultimately assessed using X-ray crystallography.

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Supporting Information Available: All synthetic information for dipolarophiles (**2a**−**t**) and isoxazolines (**5a**−**t**), 29G12 antibody production and purification methods, kinetics assay methods, and X-ray structural information of *R*-**5m**. An X-ray crystallographic file (CIF). This material is available free of charge via the Internet at http://pubs.acs.org.

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