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Antibody-Catalyzed Rearrangement of a Peptide Bond: Mechanistic and Kinetic Investigations

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Abstract: Catalysis of the deamidation of asparagine residues may provide a powerful method for the deactivation of proteins. Catalytic antibodies (Gibbs et al. *Science* **1992**, *258*, 803) have been induced that catalyze the deamidation of a model dipeptide through an intermediate succinimide. Investigations of the mechanistic characteristics of two such antibodies, RG2-23C7 and RG2-2E4, revealed their ability to accelerate the hydrolysis of either the *R*- or *S*-enantiomers of the succinimide by factors of 10−500-fold to yield differing ratios of the aspartate and isoaspartate products. The mixed product ratios imply that two tetrahedral binding sites of unequal effectiveness were induced in response to the tetrahedral mimics (a phosphinate or secondary hydroxyl) within the hapten structure. The antibody RG2-2E4 also catalyzes the deamidation of either the D- or L-asparagine within the dipeptide through the intermediate cyclic imide, resulting in a multistep reaction sequence featuring a series of tetrahedral transition states. pH−rate profiles do not implicate functional groups within the antibodies' combining sites for either the deamidation or hydrolytic reactions. The strategy of bifunctional or higher order transition state mimics should provide a route to developing catalytic antibodies for reactions requiring multistep processing.

A principal goal of catalytic antibody research has been the development of antibodies that are capable of inactivating peptides and proteins.[1] One approach would involve the generation of antibodies that cleave the peptide bond by hydrolytic means. However, uncatalyzed peptide bond hydrolysis is extremely slow at physiological pH and temperature ($t_{1/2}$ of approximately 9 years).[2] For an antibody to exhibit a significant k_{cat}, it must accelerate this reaction by more than 10^8-fold, whereas most existing catalytic antibodies exhibit more modest rate accelerations ($k_{cat}/k_{uncat} \sim 10^2-10^5$).[1] Thus, there has been only a single report of peptide bond cleavage by a catalytic antibody which was not substrate specific and relied on metal catalysis.[3]

In addition to peptide bond cleavage, enzyme inactivation may be achieved through modification of side chain residues or through rearrangement of the main peptide chain. One example of this type of process is the deamidation of asparagine residues and the associated β-aspartyl shift (Scheme 1).[4] Spontaneous deamidation through this cyclization mechanism has been implicated in the inactivation of a number of enzymes.[5] In contrast to the long half-life for peptide bond hydrolysis, the deamidation reaction has a much shorter half-life (varying from days to weeks depending upon the sequence).[4] Therefore, antibodies for the catalysis of the deamidation reaction would have to exhibit only moderate rate accelerations for detectable peptide deamidation.

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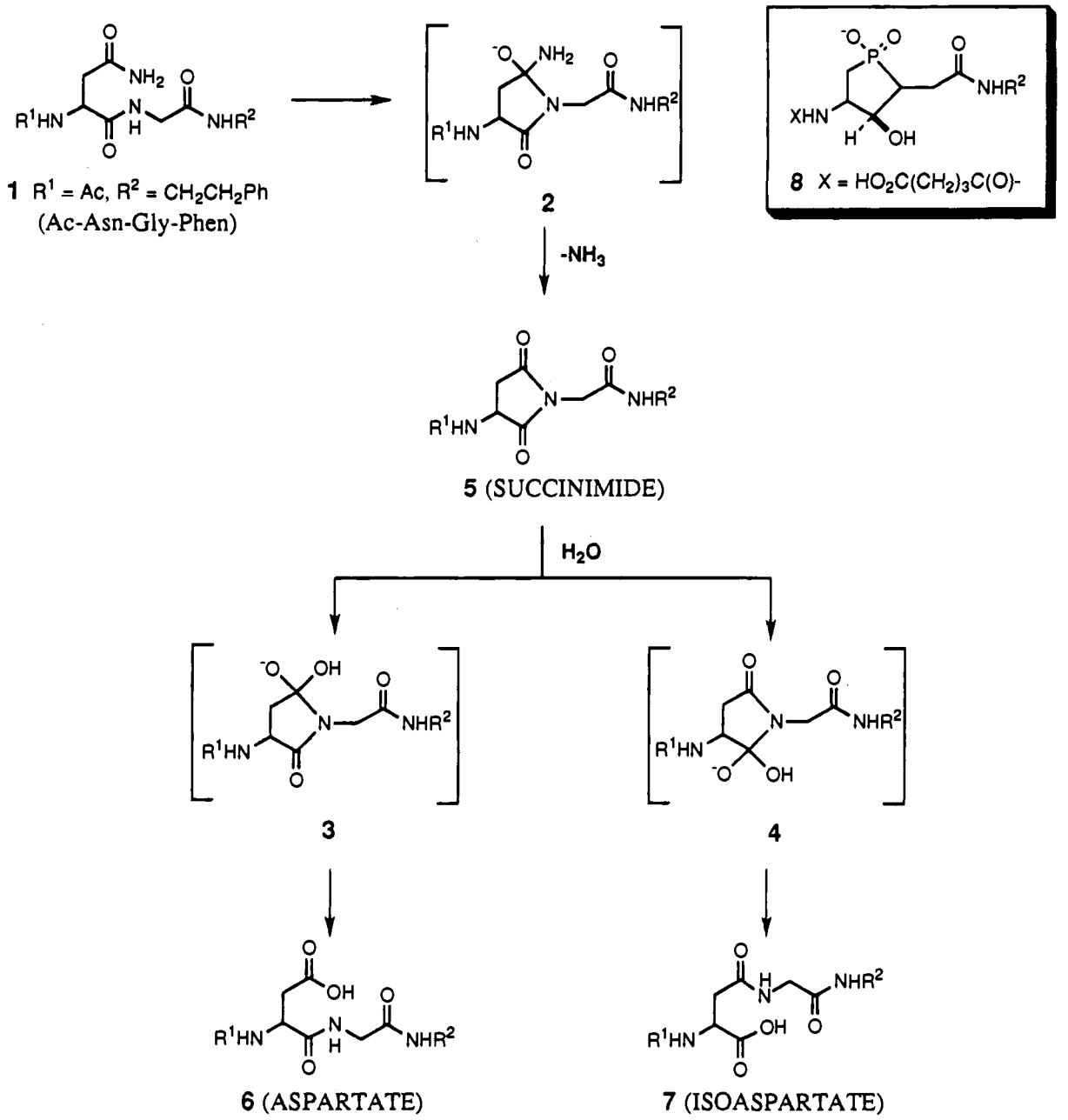
Scheme 1



We have recently reported antibodies that catalyze the deamidation and β-aspartyl shift of modified asparaginyl-glycyl dipeptides (**1**, Scheme 1).[6] These antibodies were induced by cyclic phosphinate transition state analog **8**, which mimics intermediates **2**–**4** (Scheme 1) and their associated transition states. Two classes of antibodies were generated: (1) those that catalyze only the hydrolysis of succinimide **5** and (2) those that catalyze both the rate-limiting deamidation and the subsequent succinimide hydrolysis. Preliminary kinetic measurements on one antibody from each class (RG2-23C7 and RG2-2E4) suggested that they possess two tetrahedral recognition sites that were generated in response to the two tetrahedral mimics, the phosphinate and the secondary alcohol, in **8**. As a consequence these antibodies control the isoaspartate to aspartate product ratio.[6] A model was proposed (Figure 1), based on kinetic and thermodynamic measurements, to explain the observed product distributions.[6b] We describe here the outcome of studies on these catalytic antibodies to determine their substrate specificities, the nature of their reaction pathways, the bifunctionality of their active sites, and the source of the rate acceleration. In addition, detailed procedures for the synthesis of hapten **8** and the various substrates are presented in the Experimental Section.

Results and Discussion

Succinimide Hydrolysis. Earlier we demonstrated that RG2-23C7 catalyzed the hydrolysis of the enantiomeric forms of succinimide **5** via attack at either carbonyl.[6] A detailed kinetic analysis[6b] was performed by following the reaction course at pH 8.35 and fitting the data to the second part of Scheme 2 using computer simulations of the reaction time course (KINSIM).[7] This analysis indicated that RG2-23C7 binds **5-D** roughly four times better than it binds **5-L** (Table 1, k_{-7}/k_7).

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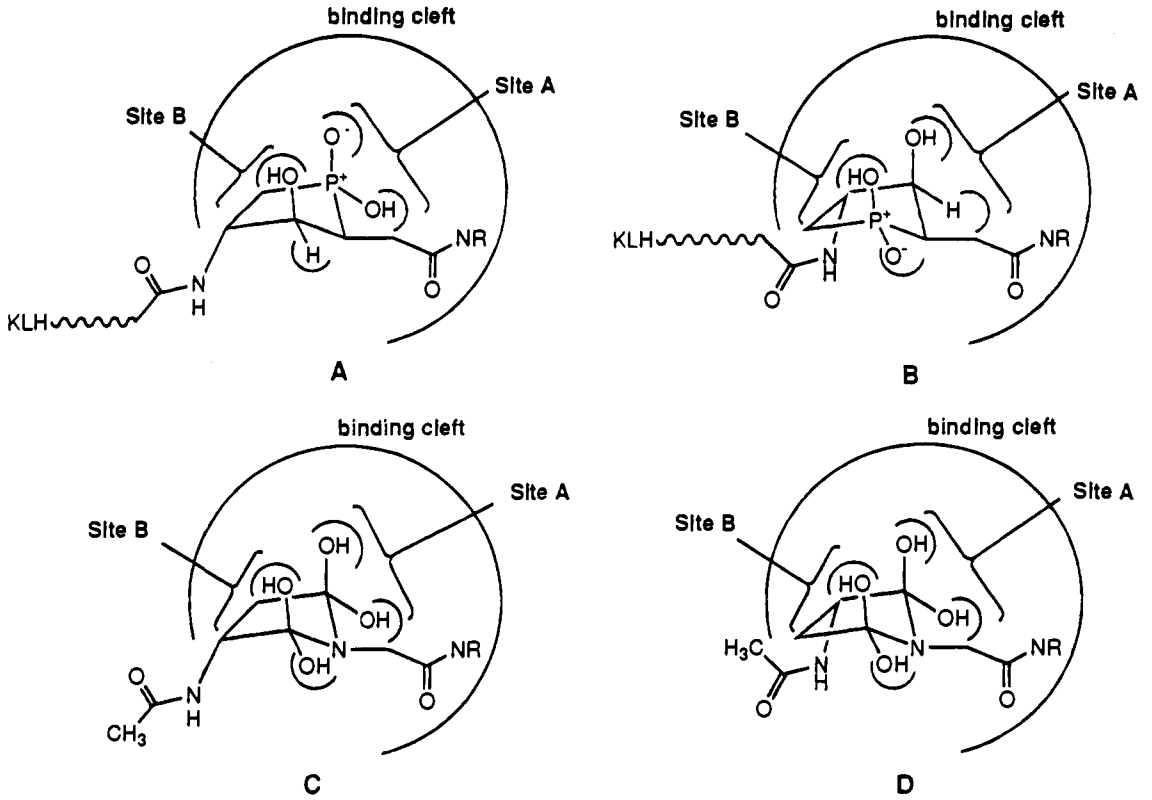

Figure 1. (A) Binding of the D-isomer of **8** in the antibody active site. (B) Binding of the L-isomer of **8** in the antibody active site. (C) Binding of intermediates in the antibody active site for aspartate and isoaspartate formation for the hydrolysis of **5-D**. (D) Binding of intermediates in the antibody active site for aspartate and isoaspartate formation for the hydrolysis of 5-L.

Scheme 2

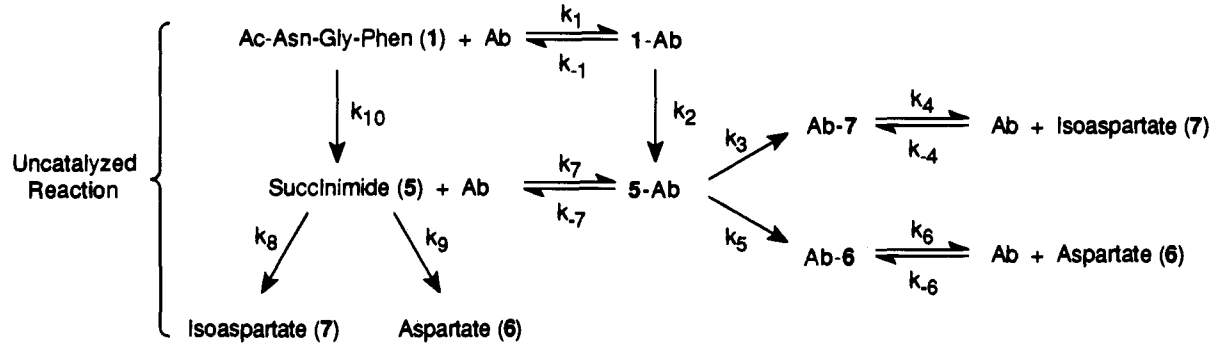

Table 1. Kinetic Constants[a,b] for the Hydrolysis of D- and L-Succinimides (**5-D** and **5-L**) by Antibody RG2-23C7 at pH 8.35

	5-D	5-L
k_{-7}/k_7	0.24 μM	0.83 μM
k_3 (isoasp)	0.52 min^{-1}	3.5 min^{-1}
k_5 (asp)	0.56 min^{-1}	0.12 min^{-1}
k_4/k_{-4} (isoasp)	0.24 μM	0.25 μM
k_6/k_{-6} (asp)	0.14 μM	0.10 μM
k_3/k_8	70.3	486
k_5/k_9	287	61.5

[a] From ref 6b. [b] ±10% from the simulation of the kinetic parameters.

Table 2. Kinetic Constants[a] for the Deamidation of D- and L-Ac-Asn-Gly-Phen (**1-D** and **1-L**) and the Hydrolysis of D- and L-Succinimides (**5-D** and **5-L**) by Antibody RG2-2E4 at pH 8.35

	D-isomer	L-isomer
k_{-1}/k_1	18.3 μM	1.7 μM
k_2	0.063 h^{-1}	0.0096 h^{-1}
k_{-7}/k_7	28.0 μM	66.7 μM
k_3 (isoasp)	0.23 min^{-1}	0.12 min^{-1}
k_5 (asp)	0.036 min^{-1}	0.10 min^{-1}
k_4/k_{-4} (isoasp)	3.3 μM	8.8 μM
k_6/k_{-6} (asp)	1.9 μM	2.3 μM
k_2/k_{10}	14	2.1
k_3/k_8	32	17
k_5/k_9	18	51

[a] ±10% from the simulation of the kinetic parameters.

Although a racemic mixture of the transition state analog **8** was used to generate the RG2 antibodies, these results suggested that the RG2-23C7 antibody may have been induced by the D-isomer of **8**. In contrast to the uncatalyzed reaction, which has an isoaspartate to aspartate product ratio of 3.6, this ratio was ~1 in the antibody-catalyzed reaction with substrate **5-D** and ~30 with substrate **5-L** (Table 1, k_3/k_5). These results were explained on the basis of three assumptions: (1) RG2-23C7 possesses two tetrahedral binding sites, one that was induced in response to the phosphinate moiety of **8** (site A, Figure 1) and one in response to the secondary alcohol of **8** (site B, Figure 1);[8a,b] (2) the site induced by the phosphinate is more effective catalytically; and (3) the *N*-acetyl group of the succinimide protrudes from one face of the binding site so that it has a small effect on binding.[8c-e] Thus, when **5-D** is the substrate (Figure

Table 3. Kinetic Constants[a] for the Demethoxylation of D-Ac-Asp(OMe)-Gly-Phen **12-D** by Antibody RG2-2E4

k_{-1}/k_1	1.0 μM
k_2	0.28 min^{-1}
k_{-7}/k_7	28.0 μM
k_3 (isoasp)	0.23 min^{-1}
k_5 (asp)	0.036 min^{-1}
k_4/k_{-4} (isoasp)	3.3 μM
k_6/k_{-6} (asp)	1.9 μM
k_2/k_{10}	1
k_3/k_8	32
k_5/k_9	18

[a] ±10% from the simulation of the kinetic parameters.

1C), attack at the carbonyl in site A to form the intermediate leading to the aspartate product is preferred since site A is more active than site B. However, this preference is offset by the intrinsically 4-fold greater rate of isoaspartate formation owing to the electronic effect of the *N*-acetyl substituent,[9] and therefore the asp and isoasp products are formed in approximately the same amount. On the other hand, with **5-L**, the strong preference for the formation of the isoasp product is a result of attack at the preferred site A reinforced by the electronic effect of the *N*-acetyl substituent (Figure 1D).

Our preliminary investigations[6a] indicated that 2E4 is also capable of catalyzing the hydrolysis of both enantiomeric forms of succinimide **5**. This was inferred since the ratio of isoaspartate (**7**) to aspartate (**6**) products for the antibody-catalyzed reaction differed from that of the uncatalyzed reaction: 4.7 with substrate **1-D** and 2.4 with **1-L**. Therefore, like RG2-23C7, RG2-2E4 catalyzes the hydrolysis of both enantiomeric forms of **5**, but with a product distribution opposite to that of RG2-23C7. To determine the rate at which RG2-2E4 catalyzes the hydrolysis of **5** and whether the model proposed for RG2-23C7 (Figure 1) might apply to RG2-2E4, this antibody was subjected to further kinetic analysis.

Enantiomerically pure **6** and **7** were prepared, and their dissociation constants (k_6/k_{-6} for **6** and k_4/k_{-4} for **7**) were determined by fluorescence titration.[10] The on-rate constants (k_7, k_{-4}, and k_{-6}) were all assumed to be diffusion controlled. Background rate constants (k_8 and k_9) were measured in the absence of antibody. The succinimide dissociation constant (k_{-7}/k_7) and the catalytic rate constants (k_3 and k_5) were then determined from computer-generated fits of the reaction time course to the second part of Scheme 2 (KINSIM).[7] As was the case with RG2-23C7, the reaction was completely inhibited by the presence of an amount of the racemic hapten **8** equal to four times the antibody concentration (one L- or D-hapten per binding site). This confirmed that the reaction was a result of the antibody and not a contaminant. However, we were unable to obtain an accurate binding constant for **8** by fluorescent titration owing to its very tight affinity and attendant small fluorescence changes observed upon titration of the binding site. We also examined whether the antibody was capable of catalyzing the isomerization of the two products (**6** and **7**). The small amount of isomerization observed in the presence of antibody (1−2% after 10 days) was no greater than the background rate, indicating that the product ratio observed in the presence of antibody was strictly a result of antibody-directed hydrolysis of the succinimide.

The kinetic constants for the hydrolyses of **5-D** and **5-L** by RG2-2E4 are given in Table 2. RG2-2E4 hydrolyzes both enantiomeric forms of **5** to **6** and **7** more slowly than RG2-23C7. Antibody RG2-2E4 binds **5-D** more tightly than **5-L** (k_{-7}/k_7). However, both succinimide stereoisomers bind much less tightly to RG2-2E4 than to RG2-23C7. Once the D-isomer is bound, hydrolysis to isoasp **7** is favored 6-fold over hydrolysis to asp **6** (k_3/k_5), in contrast to RG2-23C7, where the rates of hydrolysis at both carbonyls are equivalent. On the other hand, with **5-L**, the rate constants for formation of **6** (k_5) and **7** (k_3) are almost the same in contrast to RG2-23C7, which strongly favors formation of **7**. The reduced affinity of RG2-2E4 for both of the stereoisomers of **5** obscures the origin of the antibody's binding site with respect to the stereochemistry of the immunizing hapten. If the antibody was produced in response to the L-hapten then the partitioning of **5** in the hydrolytic reaction is consistent with the model in Figure 1. A second explanation is that the antibody is indeed elicited by the D-hapten; however, the access of solvolytic water to site A is now impaired in RG2-2E4 so that the intrinsic activity of the two sites is reversed from that seen with RG2-23C7. This rationale is supported by the substrate selectivity of RG2-2E4 in the deamidation reaction (*vide infra*).

Although the model proposed in Figure 1 provides an explanation for the data obtained from succinimide hydrolysis by 23C7 and 2E4, the question arises as to whether the antibody could process both succinimide enantiomers through a single active site (presumably one induced by the phosphinate moiety in hapten **8**). In the case of a single site, the conversion of either the D- or L-succinimide to the two hydrolysis products (**6** and **7**) would require the antibodies to accommodate the reacting carbonyl at a single locus. To accomplish productive binding in this case, the antibody must be able to accommodate the AcNH moiety in all four possible geometric positions. The easiest solution would have the AcNH moiety not binding within the active site cavity. Apart from the conflict of this proposal with the apparently significant difference in the binding of the two enantiomers of the hapten **8** to the antibodies (*vide supra*), there is a kinetic argument against the one site model. As a consequence of the lack of AcNH binding, one would predict that the processing of the D- or L-isomer of **5** to either product (asp **6** or isoasp **7**) would occur with the same rate constant. For 23C7, the rates of formation of isoaspartate from the D- and L-succinimides differ by a factor of 7; for the formation of aspartate the ratio is 5. For 2E4, a similar analysis reveals a ratio smaller but significantly different from 1. For the two-site model, one must only postulate that one face of the succinimide ring is open to solvent to accomodate both D- and L-succinimide isomers. Consequently, the rate of conversion of, for example, the D-succinimide to isoasp versus the L-succinimide to asp should be in the ratio of 4 (the difference in chemical reactivity). This relationship appears to be well approximated for 23C7 (range 4−6) and for 2E4 (range 2−4). Further support for the two-site model is the fact that a secondary hydroxyl group in a hapten can induce a hydrolytic site in an antibody.[8a,b] For these reasons, we favor the concept that two sites of unequal effectiveness are processing the two carbonyl centers of the succinimides.

The model proposed in Figure 1 for antibody catalysis of succinimide hydrolysis also implies that the rate acceleration is purely a result of the antibody providing complementarity to the tetrahedral transition states. However, it is possible that catalysis may also be a result of a specific residue in the antibody

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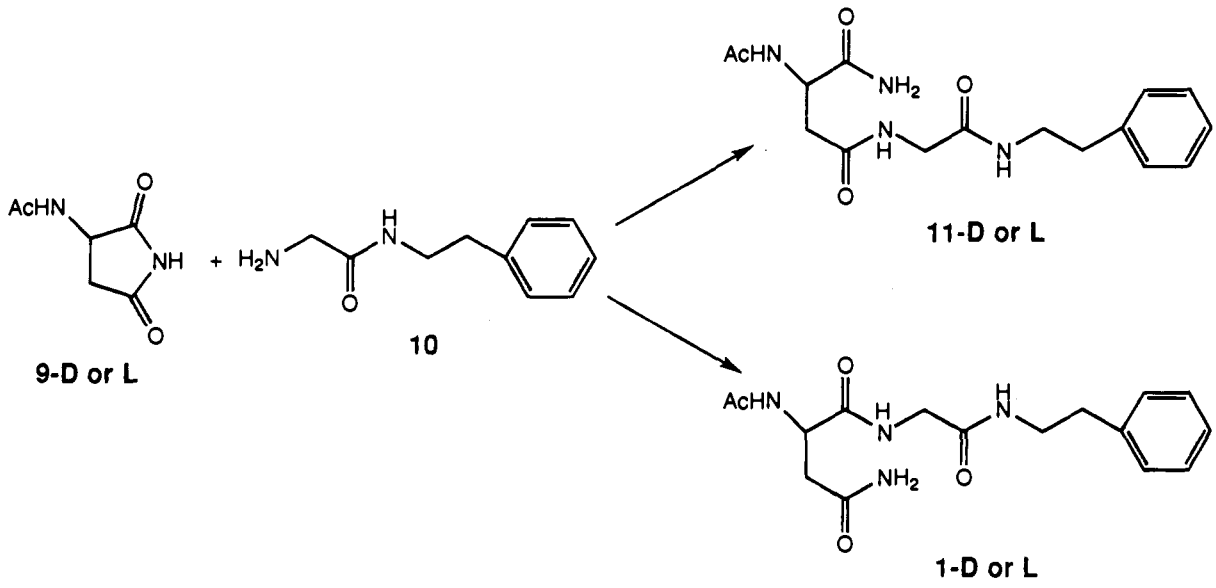
Scheme 3



active site acting as a general acid or base. To determine if such a pH-sensitive residue is in the antibody active site, we determined the rate of hydrolysis of **5** in the presence of antibody RG2-23C7 at pH 7.60, 8.35, and 9.00 in 100 mM bicine/5% DMSO (μ = 0.1). The reaction was followed by HPLC, and the data were fit, using KINSIM, to a modified version of Scheme 2 in which the hydrolytic rate constants (k_3, k_5, k_8, and k_9) were first order in hydroxide. The rates of hydrolysis of **5** to **6** and **7** (k_9 and k_8) in the absence of antibody were found to be proportional to the hydroxide ion but not buffer concentration between pH 7.60 and 9.00. The resulting simulations for hydrolysis of **5** by RG2-23C7 clearly showed direct proportionality to hydroxide concentration, indicating that no catalytic group with a pK_a between 7.60 and 9.00 was involved in the catalysis.

Reaction of Succinimide 9 with *N*-Phenethylglycinamide (10) Catalyzed by RG2-23C7. It is reasonable to propose that RG2-23C7 catalyzes the hydrolysis of succinimide **5** by stabilizing the tetrahedral transition states and substrate recognition involves tight binding of the *N*-phenethylglycinamide moiety. A logical prediction would then be that RG2-23C7 would catalyze the reaction between the free amine of **10** and one of the succinimide carbonyls of **9**. Such a reaction would yield either **1** or (*N*-acetylisoasparaginyl)-*N*-phenethylglycinamide (**11**) (Scheme 3).

Assays were performed under the usual conditions[6b] using 3.45 μM RG2-23C7 (6.9 μM binding sites), 25 μM unsubstituted succinimide **9**, and 500 μM *N*-phenethylglycinamide (**10**). The relative amounts of asparagine product **1** and isoasparagine product **11** could not be distinguished since they had identical retention times on the HPLC. The rapid spontaneous hydrolysis of **9** interfered with the accurate determination of the rate acceleration for the amide bond formation. However, in the presence of antibody, in the first 24 h, the reaction to couple **9** and **10** was approximately 30 times faster than spontaneous peptide formation with both the D- and L-succinimides (**9-D** and **9-L**). The modest catalysis for amide bond formation is not surprising, since stabilization of the tetrahedral transition state for amide formation and for hydrolysis would be similar.

RG2-2E4-Catalyzed Deamidation. A significant feature of RG2-2E4 is its ability to catalyze the deamidation of **1-D** to succinimide **5-D**.[6a] This property of RG2-2E4 to catalyze both the deamidation and the subsequent succinimide hydrolysis resulted in a complex multistep reaction scheme (Scheme 2). The kinetic constants obtained for the RG2-2E4-catalyzed succinimide hydrolysis at pH 8.35 left as unknowns only the substrate dissociation constant (k_{-1}/k_1), the deamidation catalytic constant (k_2), and the uncatalyzed deamidation constant (k_{10}). The D- and L-isomers of **1** were incubated with antibody in 100 mM bicine/5% DMSO (pH 8.35, μ = 0.1), and their disappearance was monitored. The dissociation constant of **1** (k_{-1}/k_1) and the catalytic deamidation constant (k_2) were determined by fitting the data to Scheme 2 using KINSIM. The kinetic constants for the deamidation of both the L- and D-peptides thus obtained are given in Table 2. The $t_{1/2}$ of ca. 100 h for deamidation of **1-L** precluded its detection in our earlier reports.[6]

RG2-2E4 binds **1-L** one order of magnitude tighter than it binds **1-D** (k_{-1}/k_1 = 1.7 and 18 μM, respectively). This is opposite to the result observed for the succinimides **5-D** and **5-L**, where the D-isomer binds tighter. On the other hand, the antibody binds the asp products of the reaction, **6-D** and **6-L** (k_6/k_{-6}), also with micromolar affinity but does not significantly discriminate between them. Note that **6-D** and **6-L** are carboxylate analogs of the amide substrates **1-D** and **1-L**. Consequently, the binding constants of the Asn-Gly substrates are probably not reliable indicators as to the stereochemistry of the eliciting hapten. In addition, the tightness of substrate binding is not directly related to catalytic ability. At pH 8.35, the antibody-catalyzed deamidation of **1-D** is 14 times faster than the uncatalyzed rate whereas the deamidation of **1-L** is only two times faster. RG2-2E4's ability to more efficiently catalyze the deamidation of **1-D** and bind the D-isomer of the succinimide is consistent with the model presented (Figure 1A) since the cyclic imide formed during the reaction probably more closely resembles the cyclic phosphinate hapten. Therefore, the antibodies' discrimination between the different isomeric forms of **5-D** and **5-L** is probably a better indicator of the enantiomeric form of the hapten against which the antibody was elicited. Presuming that RG2-2E4 was generated against the D-isomer of **8** (Figure 1A), fitting **1-D** into the binding cleft places the tetrahedral intermediate for deamidation in the more active phosphinate site (site A, Figure 2A). In contrast, the tetrahedral intermediate of **1-L** would be placed in the less active secondary alcohol site (site B, Figure 2B). The inaccessibility of the

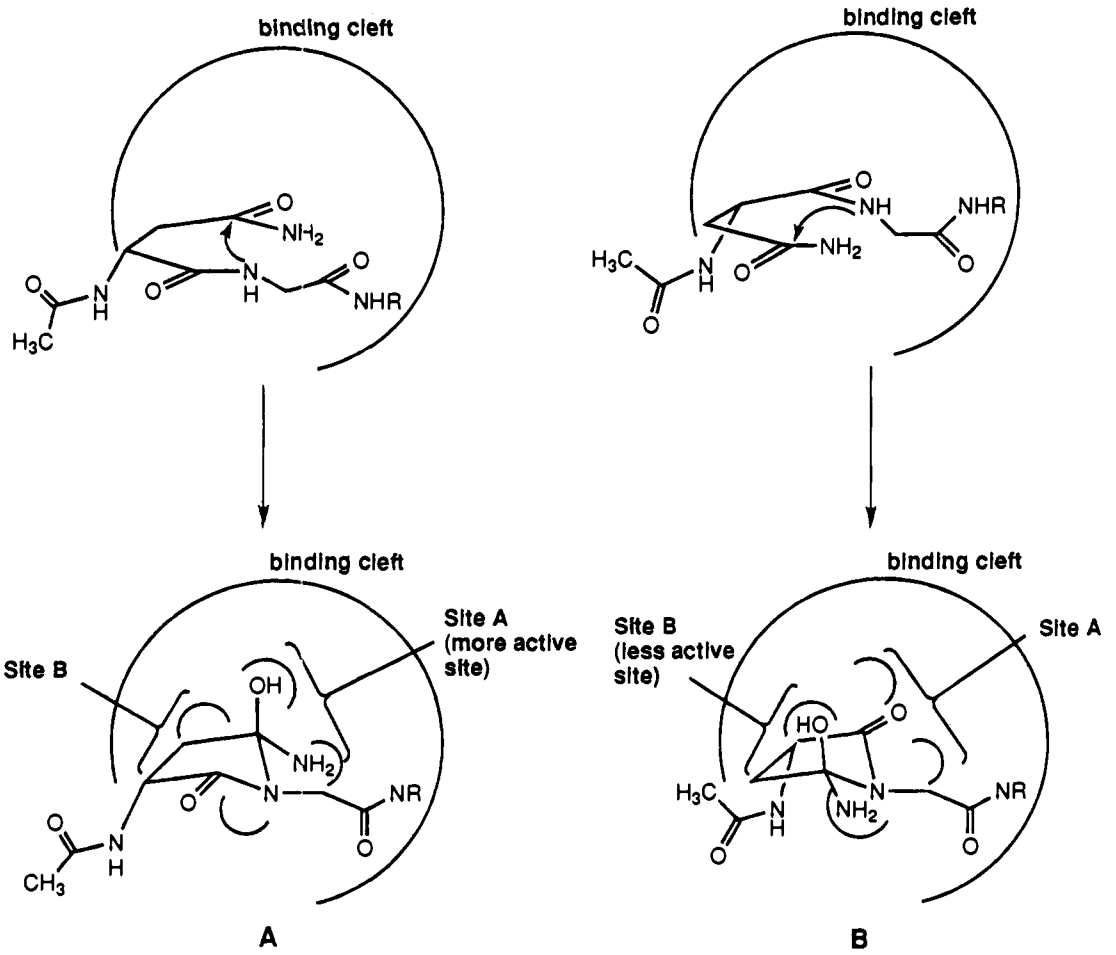


Figure 2. (A) Binding of D-Ac-Asn-Gly-Phen (**1-D**) in the antibody active site and formation of the tetrahedral intermediate for deamidation. (B) Binding of L-Ac-Asn-Gly-Phen (**1-L**) in the antibody active site and formation of the tetrahedral intermediate for deamidation.

phosphinate site to water solvent (*vide supra*) should not hamper catalysis since water is not directly involved in the cyclization reaction.

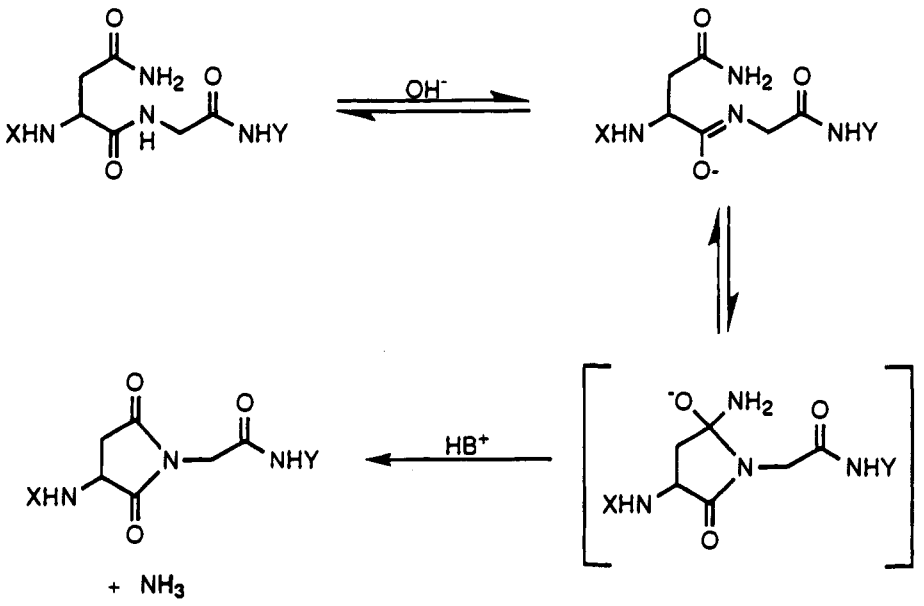
The proposed mechanism for the spontaneous deamidation of asparagine residues in model peptides containing Asn-Gly sequences involves deprotonation of the peptide nitrogen, followed by attack of the resulting anion on the asparagine side chain carbonyl to form the tetrahedral intermediate (Scheme 4).[11a-e] Loss of ammonia from this intermediate yields the succinimide. Studies on the cyclization of Ac-Gly-Asn-Gly-Gly-NHMe have shown that the reaction is subject to buffer catalysis acting to protonate the nitrogen atom of the leaving group, thus facilitating its removal.[11a] We wished to determine whether there is a residue in the antibody active site with a pK_a between 7.60 and 9.00 acting as a general acid or base catalyst. The rate of deamidation of **1-D** was thus determined in the presence of RG2-2E4 at pH 7.60 and 9.00. After determining the background deamidation rate constant (k_{10}), the substrate dissociation constant (k_{-1}/k_1) and the catalytic rate constant (k_2) (Scheme 2) were obtained from computer fits to data collected by HPLC.

The rate constants, uncorrected for buffer catalysis, for the spontaneous deamidation of **1** (k_{10}) were 2.9×10^{-5}, 7.7×10^{-5}, and 12.0×10^{-5} min^{-1} at pH's 7.60, 8.35, and 9.00, respectively. The antibody-catalyzed deamidation rate constants (k_2) were 2.5×10^{-4}, 10.5×10^{-4}, and 40.0×10^{-4} min^{-1} at pH 7.60, 8.35, and 9.00, resulting in k_2/k_{10} values of 8.6, 13.6, and 33.3, respectively. The substrate dissociation constant (k_{-1}/k_1) remained constant throughout the pH range studied. Thus, the rate of deamidation for the antibody-catalyzed reaction increases proportionally with hydroxide ion concentration so that no catalytic group with a pK_a between 7.60 and 9.00 appears in the antibody-catalyzed deamidation. The increase in rate with hydroxide concentration is probably a result of the increased concentration of the anionic nitrogen nucleophile.

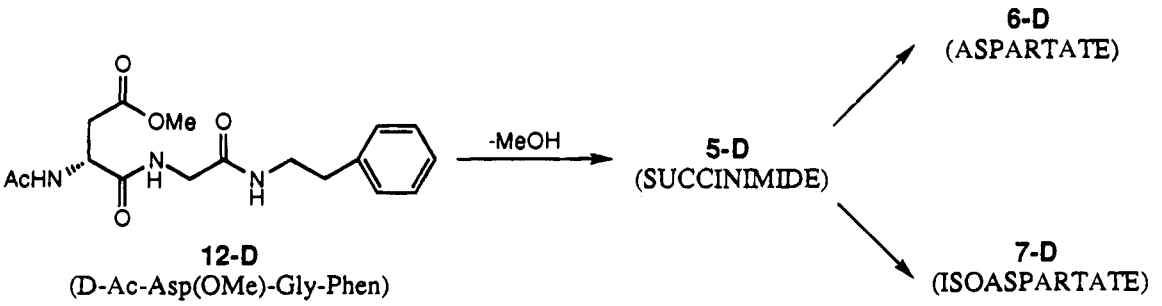
Methyl Ester Cleavage by RG2-2E4. It is reasonable to propose that RG2-2E4-catalyzed deamidation of **1-D** derives from a favorable ground state conformation for formation of intermediate **2** and a binding site that complements its associated transition state. Thus it was presumed that the antibody would be capable of catalyzing the demethoxylation of the methyl ester analog of the dipeptide substrate, **12-D** (Scheme 5). To test this hypothesis, the D-methyl ester was synthesized and the kinetic analysis described above for deamidation (Scheme 2) of **1-D** was repeated at pH 8.35. The same isoaspartate, **7**, and aspartate, **6**, products were observed along with a measurable buildup of succinimide **5**. The binding of **12-D** to RG2-2E4 was characterized by its strong competitive inhibition ($K_i = 1.0$ μM) of antibody-catalyzed hydrolysis of the succinimide. Without antibody, **12-D** cyclizes to form the succinimide relatively rapidly, and this process is approximately 270 times faster than deamidation of **1-D**. Surprisingly, the rate for the demethoxylation was identical in the presence and absence of RG2-2E4 (k_2/k_{10}, Table 3). The lack of catalysis observed with **12-D** suggests that the deamidation of **1-D** by the antibody is

(11) (a) Capasso, S.; Mazzarella, L.; Sica, F.; Zagari, A.; Salvadori, S. *J. Chem. Soc., Perkin Trans. 2* **1993**, 679. (b) Capasso, S.; Mazzarella, L.; Sica, F.; Zagari, A. *Pept. Res.* **1989**, *2*, 195 (c) Patel, K.; Borchardt, R. T. *Pharmacol. Res.* **1990**, *264*, 54. (d) Capasso, S.; Mazzarella, L.; Zagari, A. *Pept. Res.* **1991**, *4*, 234. (e) Klotz, A. V.; Thomas, B. A. *J. Org. Chem.* **1993**, *58*, 6985.

Scheme 4



Scheme 5



not merely a result of the antibody providing a favorable ground state conformation and stabilization of the transition state for formation of intermediate **2**. Studies[12] on the mechanism of imide formation of various β-esters of aspartyl-containing dipeptides in mildly basic solution suggest that the reaction involves deprotonation of the amide nitrogen followed by rate-limiting attack of the resulting anion on the ester carbonyl to form the tetrahedral intermediate. Rapid expulsion of the alkoxide leaving group yields the succinimide. If breakdown of intermediate **2** (Scheme 1) by expulsion of ammonia is rate limiting in the antibody-catalyzed reaction, then RG2-2E4 should have little effect on the demethoxylation of **12-D** where formation of the tetrahedral intermediate is rate determining. Furthermore, the absence of any rate acceleration in the cyclization step suggests this substrate is not bound in a more reactive conformation. Rate-determining expulsion of ammonia in the antibody-catalyzed reaction is consistent with studies on the spontaneous deamidation of model peptides in which loss of ammonia is rate limiting and involves general acid catalysis.[11a] It is possible that there is a residue in the antibody active site with a pK_a greater than 9.0 that assists in the expulsion of ammonia from the tetrahedral intermediate.

Conclusion

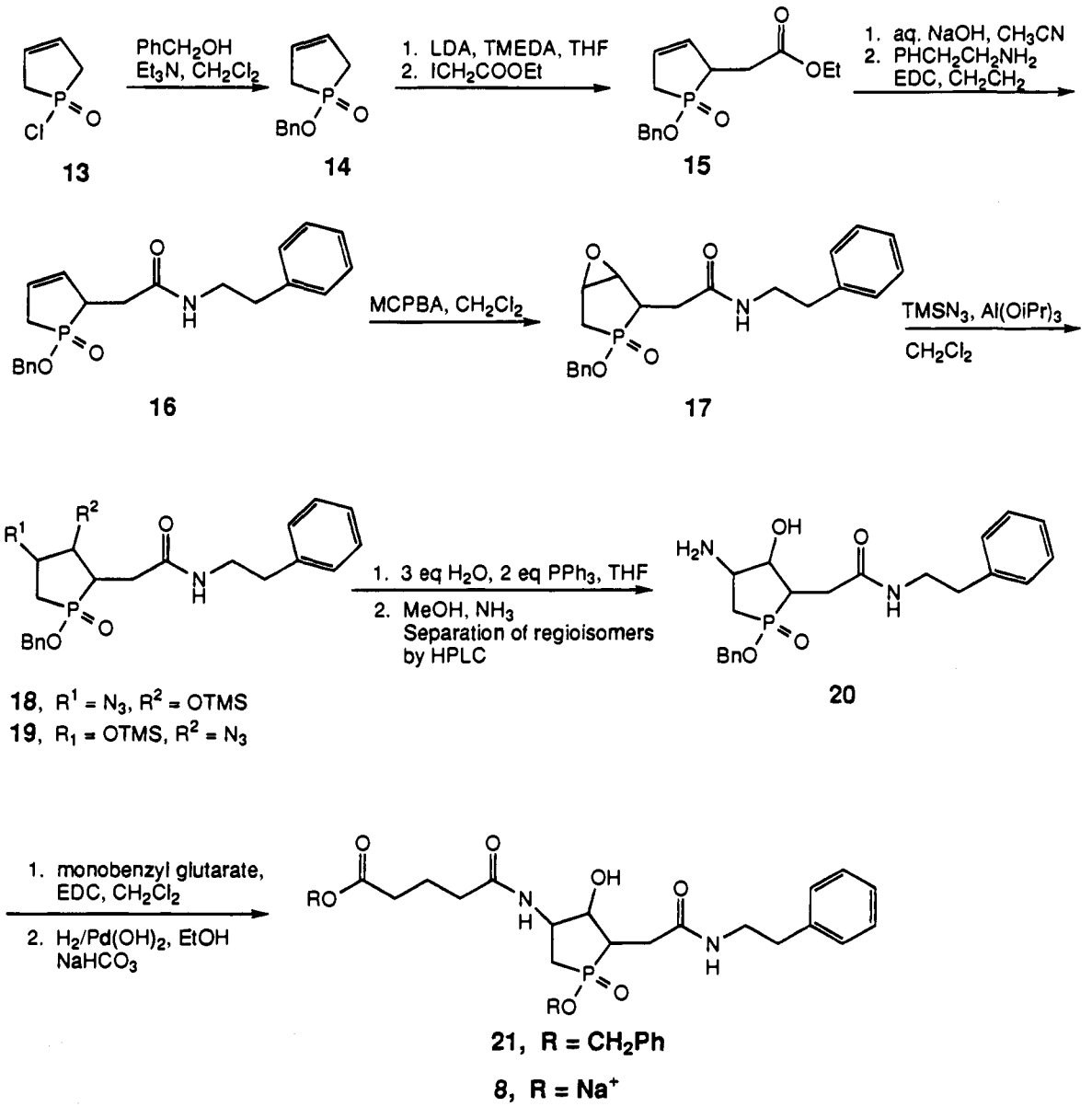
We have demonstrated that a bifunctional transition state analog can be used to elicit antibodies capable of catalyzing a two-step process that minimally involves four transition states flanking two transient tetrahedral intermediates. The initial cyclization reaction for the amide may benefit from the template effect of the antibody combining site that constrains the substrate into a more reactive conformation. Hydrolysis of the resulting imide is promoted by transition state stabilization, although the presence of general acid–base catalysis is not revealed in the pH–rate profile. The most likely rate-limiting step in the cyclization reaction is decomposition of the tetrahedral intermediate, with expulsion of ammonia, as evidenced by the absence of antibody catalysis for the cyclization of the methyl ester. The phosphinate mimic of the tetrahedral species is 7-fold more effective in inducing an operative active site than the secondary hydroxyl moiety, based upon succinimide hydrolysis. However, other factors such as access to solvent may intervene. The presence of general acid–base catalysis is not implicated in the pH–rate profile. The strategy of bifunctional or higher order transition state mimics should provide a route to developing catalytic antibodies that can effect reactions requiring multistep processing.

Experimental Section

General Methods (Synthesis). Except where specified, reactions were carried out in flame-dried glassware under an atmosphere of dry argon. Liquid transfers were made using oven-dried syringes and needles. Tetrahydrofuran and diethyl ether were freshly distilled from benzophenone and sodium. Enantiomerically pure amino acid starting materials were purchased from BaChem, California, unless stated

(12) Bernhard, S. A.; Berger, A.; Carter, J. H.; Katchalski, E.; Sela, M.; Shalitin, Y. *J. Am. Chem. Soc.* **1962**, *84*, 2421.

Scheme 6



otherwise. All other reagents were obtained from Aldrich and used as purchased, unless otherwise stated. Melting points were obtained on a Laboratory Devices Mel-Temp II melting point apparatus. All ^{1}H-NMR spectra were obtained at ambient temperature on either a Bruker WM-360 or a Bruker 200 spectrometer using $CDCl_3$ as the solvent unless otherwise stated. Chemical shifts are reported in parts per million relative to the solvents' residual protons ($CDCl_3$, δ 7.24; DMSO-d_6, δ 2.49 for the central peak of the quintet; CD_3OD, δ 3.30 for the central peak of the quintet or δ 4.78 for the singlet). ^{31}P-NMR spectra were obtained on a Bruker WM-360 using $CDCl_3$ as the solvent unless otherwise stated. Chemical shifts are reported in parts per million relative to 85% phosphoric acid (external). ^{13}C-NMR spectra were likewise obtained on a Bruker WM-360 using $CDCl_3$ as the solvent unless otherwise stated. Chemical shifts are reported in parts per million relative to the solvents' residual carbons ($CDCl_3$, δ 77.0 for the central peak; DMSO-d_6, δ 39.5 for the central peak; CD_3OD, δ 49.0 for the central peak). Electron impact mass spectra (EI-MS) were obtained on a Kratos MS-50, chemical ionization mass spectra (CI-MS) were obtained on a Kratos MS-25, and fast atom bombardment mass spectra (Fab-MS) were collected on a Kratos MS9-50. All high-resolution mass spectra (HRMS) were obtained by electron ionization.

1-(Benzyloxy)phosphol-3-ene 1-Oxide (14). A thick-walled glass tube (Ace Glass Co., catalog number 8648-83, ~120 mL capacity) equipped with a stirbar was cooled to ~−30 °C and charged with PCl_3 (17.5 mL, 27.5 g, 0.20 mol), $P(OCH_2CH_2Cl)_3$ (20.3 mL, 27.0 g, 0.10 mol), 2,6-di-*tert*-butyl-*p*-cresol (0.22 g, 0.001 mol), and liquid butadiene (condensed from gaseous C_4H_6 at −70 °C; 27 mL, 17 g, 0.32 mol). The glass tube was sealed with a screw cap and heated to 105 °C using an oil bath (**CAUTION**: this procedure should only be performed behind a safety shield in a hood). After 19 h, the tube was removed from the oil bath and allowed to cool to room temperature. Filtration of the cloudy yellow solution afforded a mixture of 1,2-dichloroethane and the desired 1-chlorophosphol-3-ene 1-oxide (**13**) along with a small amount of the 1-hydroxyphosphol-3-ene 1-oxide. In contrast to the original report,[13] none of the isomeric 1-chlorophosphol-2-ene 1-oxide was obtained. ^{1}H-NMR: δ 6.0 (2H, H_3 and H_4, d), 3.1−2.8 (4H, $2H_2$ and 2 H_5, m). ^{13}C-NMR: δ 126.2 (d, J_{C-P} ~ 17 Hz), 36.5 (d, J_{C-P} ~ 76 Hz). ^{31}P-NMR: δ 80.9. To a rapidly stirred solution of benzyl alcohol (4.6 mL, 44.3 mmol) in Et_3N (20 mL) and CH_2Cl_2 (45 mL) at 0 °C was added a portion of the crude phospholene/1,2 dichloroethane mixture (~40 mmol) dropwise slowly via syringe. The resulting suspension was stirred for 12 h at room temperature and concentrated (rotovap), and the white solid was taken up in CH_2Cl_2 (~200 mL). The solution was then washed with saturated $NaHCO_3$ (2 × 50 mL), dried ($MgSO_4$), filtered, and concentrated. Purification by flash chromatography (5 × 15 cm silica gel, 80% EtOAc/hexanes) afforded 6.8 g (81%) of **14** as a pale yellow oil. ^{1}H-NMR: δ 7.5−7.3 (5H, aromatic H, m), 5.9 (2H, H_3 and H_4, d), 5.1 (2H, OCH_2Ph, d), 3.0−2.8

(13) Modritzer, K. *Synth. React. Inorg. Met.-Org. Chem.* **1975**, *5*, 45.

(4H, $2H_2$ and $2H_5$, m). ^{13}C-NMR: δ 136.2 (d, $J_{C-P} \sim 5$ Hz), 128.7, 128.5, 128.0, 126.9 (d, $J_{C-P} \sim 16$ Hz), 66.3 (d, $J_{C-P} \sim 5$ Hz), 29.4 (d, $J_{C-P} \sim 91$ Hz). ^{31}P-NMR: δ 73.9. EI-MS: *m/z* 208 (M, 23%), 102 (M − 106, 25%), 91 (M − 117, 100%), 65 (M − 143, 16%). HRMS: 208.0654 (calcd for $C_{11}H_{13}PO_2$ 208.0653).

Ethyl (1′-(Benzyloxy)-1′-oxophosphol-3′-en-2′-yl)acetate (15).[14] To a rapidly stirred solution of benzyl phosphinate **14** (2.70 g, 13.0 mmol) in TMEDA (9.7 mL) and THF (16.2 mL) at −78 °C under Ar was added LDA (2.0 M in heptane/THF/ethylbenzene, 7.1 mL, 14.3 mmol) dropwise over a period of 5 min. The orange red solution was stirred for 10 min, and then ethyl iodoacetate (2.3 mL, 19.5 mmol) was added neat via syringe. After 1 h, the reaction was quenched with EtOH/H_2O (2:1, 2 mL), allowed to warm slowly to −20 °C, added to saturated NH_4Cl (75 mL), and then extracted with CH_2Cl_2 (3 × 100 mL). The combined organic layers were then dried ($MgSO_4$), filtered, and concentrated. Purification by flash chromatography (4 × 15 cm silica gel, ethyl acetate) afforded 800 mg (21%) of **15** as a pale yellow oil. ^{1}H-NMR: δ 7.5−7.2 (5H, aromatic, m), 6.0−5.8 (2H, H_3· and H_4·, m), 5.2−5.1 (2H, OCH_2Ph, d), 4.15 (2H, OCH_2CH$_3$, q), 2.95 (1H, H_2·, m), 2.8 (1H, one −CH_2CO$_2$Et, ddd), 2.55, (1H, one −CH_2CO$_2$Et, m), 2.5−2.4 (2H, $2H_5$·, m), 1.25 (3H, OCH$_2$$CH_3$, t); these assignments are consistent with decoupling experiments. ^{13}C-NMR: δ 171.8 (d, $J_{C-P} \sim 9$ Hz), 136.3 (d, $J_{C-P} \sim 7$ Hz), 131.5 (d, $J_{C-P} \sim 19$ Hz), 128.5, 128.3, 127.9, 126.4 (d, $J_{C-P} \sim 15$ Hz), 66.5 (d, $J_{C-P} \sim 7$ Hz), 60.7, 35.4 (d, $J_{C-P} \sim 92$ Hz), 33.4, 29.7 (d, $J_{C-P} \sim 92$ Hz), 14.1. ^{31}P-NMR: δ 71.3. EI-MS: *m/z* 294 (M, 4%), 208 (M − 86, 10%), 102 (M − 192, 13%), 91 (M − 203, 100%), 65 (M − 229, 10%). HRMS: 294.1029 (calcd for $C_{15}H_{19}PO_4$ 294.1021).

***N*-Phenethyl(1′-(benzyloxy)-1′-oxophosphol-3′-en-2′-yl)acetamide (16).** To a well-stirred solution of **15** (1.25 g, 4.25 mmol) in CH_3CN (16.3 mL) was added NaOH (0.25 M, 16.3 mL, 4.25 mmol) dropwise via syringe. After 1 h at room temperature the reaction was acidified to a final pH of ~3 by dropwise addition of 1 M HCl. The resulting suspension was extracted with $CHCl_3$ (3 × 75 mL), and the combined organic layers were dried ($MgSO_4$) filtered and concentrated. In this case the crude product was used directly in the next reaction; however, (1′-(benzyloxy)-1′-oxophosphol-3′-en-2′-yl)acetic acid could be purified by flash chromatography (silica, 10% MeOH/$CHCl_3$). ^{1}H-NMR: δ 7.5−7.2 (5H, aromatic, m), 6.0−5.7 (2H, H_3· and H_4·, m), 5.2−5.1 (2H, OCH_2Ph, d), 3.0 (1H, m), 2.8−2.3 (4H, m). ^{31}P-NMR: δ 74.3. CI-MS: *m/z* (relative intensity) 267 (MH^+, 30%), 199 (MH^+ − 68, 5%), 159 (MH^+ − 108, 100%), 91 (MH^+ − 176, 37%).

To a solution of the crude acid in CH_2Cl_2 (15 mL) was added phenethyl amine (453 mg, 3.61 mmol, 0.85 equiv) and EDC (1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride, 692 mg, 3.61 mmol, 0.85 equiv). The resulting mixture was stirred at room temperature for 2 h and then taken up in additional CH_2Cl_2 (~100 mL). The organic layer was washed with 5% HCl (3 × 25 mL), dried ($MgSO_4$), filtered, and concentrated. Purification by flash chromatography (4 × 15 cm, silica gel, EtOAc) afforded 827 mg (53%) of **16** as a white solid. ^{1}H-NMR: δ 7.5−7.1 (10H, aromatic, m), 6.5 (1H N-H, br s), 6.0−5.7 (2H, H_3· and H_4·, m), 5.2−5.0 (2H, OCH_2Ph, m), 3.7−3.4 (2H, −NHCH_2CH$_2$Ph, m), 3.0 (1H, H_2·, m), 2.85 (2H, −NHCH$_2$$CH_2$Ph, t), 2.6−2.3 (4H, m). ^{13}C-NMR: δ 170.6 (d, $J_{C-P} \sim 7$ Hz), 138.8, 136.5, 136.1, 132.2 (d, $J_{C-P} \sim 20$ Hz), 128.7, 128.6, 128.55, 128.5, 128.0, 126.4, 126.0 (d, $J_{C-P} \sim 14$ Hz), 66.7 (d, $J_{C-P} \sim 6$ Hz), 40.8, 36.4 (d, $J_{C-P} \sim 90$ Hz), 35.9, 35.6, 29.7 (d, $J_{C-P} \sim 90$ Hz). ^{31}P-NMR: δ 73.1. EI-MS: *m/z* 370 (M + 1, 2.5%), 369 (M, 11%), 249 (M − 120, 12%), 105 (M − 264, 11%), 104 (M − 265, 15%), 91 (M − 287, 100%). HRMS: 369.1467 (calcd for $C_{21}H_{24}NO_3P$ 369.1494).

***N*-Phenethyl(1′-(benzyloxy)-3′,4′-epoxy-1′-oxophosphol-2′-yl)acetamide (17).**[15] A solution of **16** (227 mg, 0.615 mmol) and MCPBA (72%, 206 mg 0.86 mmol) in CH_2Cl_2 (2.5 mL) was refluxed under Ar for 24 h, stirred for 12 h at room temperature, and then stirred for 1 h with saturated $NaHCO_3$ (~2.5 mL). The mixture was then taken up in additional CH_2Cl_2 (~100 mL) and washed with saturated $NaHCO_3$ (3 × 25 mL), and the combined aqueous layers were back-extracted with CH_2Cl_2 (1 × 25 mL). The combined organic layers were dried ($MgSO_4$), filtered, and concentrated. Purification by flash chromatography (2 × 15 cm, silica gel, 3% MeOH/$CHCl_3$) afforded 136 mg (57%) of the epoxide **17**. ^{1}H-NMR: δ 7.5−7.1 (10H, aromatic, m), 6.9 (1H, N-H, br s), 5.1−5.0 (2H, OCH_2Ph, m), 3.7−3.5 (2H, H_3· and H_4·, m), 3.5−3.35 (2H, −NHCH_2CH$_2$Ph, m), 2.9−2.8 (1H, H_2·, m), 2.85 (2H, −NHCH$_2$$CH_2$Ph, t), 2.7−2.5 (2H, m), 2.2−2.0 (2H, m). ^{13}C-NMR: δ 170.5 (d, $J_{C-P} \sim 10.5$ Hz), 138.9, 135.9, 128.6, 128.56, 128.5, 128.4, 128.1, 126.2, 66.5 (d, $J_{C-P} \sim 6.5$ Hz), 57.4 (d, $J_{C-P} \sim 10.5$ Hz), 52.4 (d, $J_{C-P} \sim 5$ Hz), 40.7, 35.5, 34.7 (d, $J_{C-P} \sim 90$ Hz) 31.7, 28.8 (d, $J_{C-P} \sim 90$ Hz). ^{31}P-NMR: δ 70.8. EI-MS: *m/z* 385 (M, 5%), 294 (M − 101, 5%), 265 (M − 120, 9%), 120 (M − 265, 7%), 104 (M − 289, 11%), 91 (M − 293, 100%). HRMS: 385.1426 (calcd for $C_{21}H_{24}NO_4P$ 385.1443).

***N*-Phenethyl(1′-(benzyloxy)-3′-(trimethylsiloxy)-4′-azido-1′-oxophosphol-2′-yl)acetamide (18) and *N*-Phenethyl(1′-(benzyloxy)-3′-azido-4′-(trimethylsiloxy)-1′-oxophosphol-2′-yl)acetamide (19).**[16] Azidotrimethylsilane (0.410 mL, 3.09 mmol), Al(OiPr)$_3$ (65 mg, 0.32 mmol), and CH_2Cl_2 (1 mL) were stirred together under Ar for 2 h at room temperature. Epoxide **17** (398 mg, 1.03 mmol) was dissolved in CH_2Cl_2 (2 × 0.5 mL wash) and added via syringe. After 3 days, the mixture was taken up in CH_2Cl_2 (20 mL) and filtered through Celite. The solvent was removed and the crude oil purified by flash chromatography (2 × 15 cm, silica gel, EtOAc) to afford 280 mg (54%) of **18** and **19** in an approximately 50:50 ratio. The two isomers were not separated at this point but were characterized as the mixture and carried onto the next step. ^{1}H-NMR: δ 7.5−7.1 (10H, aromatic, m), 6.4−6.3 (1H, N-H, two br t), 5.15−4.95 (2H, OCH_2Ph, m), 4.2−3.4 (2H, H_3· and H_4·, three m), 3.5−3.35 (2H, −NHCH_2CH$_2$Ph, m), 2.8 (2H, −NHCH$_2$$CH_2$Ph, t), 2.7−2.1 (4H, three m), 1.9−1.7 (1H, m). ^{13}C-NMR: δ 170.8 (d, $J_{C-P} \sim 7$ Hz), 170.0 (d, $J_{C-P} \sim 4$ Hz) 138.8, 138.76, 136.0 (d, $J_{C-P} \sim 5.3$ Hz), 135.9 (d, $J_{C-P} \sim 5.2$ Hz), 128.6, 128.55, 128.53, 128.49, 128.19, 128.03, 126.4, 74.9 (d, $J_{C-P} \sim 15.4$ Hz), 72.1 (d, $J_{C-P} \sim 16.9$ Hz), 69.7 (d, $J_{C-P} \sim 21.2$ Hz), 67.1 (d, $J_{C-P} \sim 6.4$ Hz), 66.9 (d, $J_{C-P} \sim 6.6$ Hz), 62.2 (d, $J_{C-P} \sim 13$ Hz), 40.81, 40.78, 38.8 (d, $J_{C-P} \sim 82.7$ Hz), 36.4 (d, $J_{C-P} \sim 86.7$ Hz), 35.61, 35.54, 34.7 (d, $J_{C-P} \sim 82.9$ Hz), 34.0, 29.9, 28.3 (d, $J_{C-P} \sim 86.2$ Hz), −0.19, −0.26. ^{31}P-NMR: δ 66.8, 56.1. IR (CCl_4): ν 2120 (N=N=N, s). El-MS: *m/z* 500 (M, 5%), 409 (M − 91, 6%), 380 (M − 120, 9%), 120 (M − 380, 6%), 105 (M − 395, 13%), 104 (M − 396, 13%), 91 (M − 400, 100%), 73 (M − 427, 14%). HRMS: 500.1965 (calcd for $C_{24}H_{33}N_4O_4PSi$ 500.2009).

***N*-Phenethyl(1′-(benzyloxy)-3′-hydroxy-4′-amino-1′-oxophosphol-2′-yl)acetamide (20).**[17] A mixture of azides **18** and **19** (70 mg, 0.14 mmol) and water (7.6 μL, 0.42 mmol) was dissolved in THF (1.2 mL). Triphenylphosphine (73 mg, 0.28 mmol) was added, and the reaction was stirred for 14 h at room temperature. The mixture was then concentrated and purified by flash chromatography (2 × 15 cm, silica gel, 20% MeOH/$CHCl_3$) to afford ~100 mg (~100%) of a mixture of *N*-phenethyl(1′-(benzyloxy)-3′-hydroxy-4′-(triphenylphosphazinyl)-1′-oxophosphol-2′-yl)acetamide and *N*-phenethyl(1′-(benzyloxy)-3′-(triphenylphosphazinyl)-4′-hydroxy-1′-oxophosphol-2′-yl)acetamide. Positive ion FAB mass spectrum: 663. A solution of the two phosphazines (~100 mg) in MeOH (15 mL) was cooled to 0 °C and saturated with gaseous ammonia. The stoppered flask was allowed to come to room temperature (**CAUTION**: this procedure should only be performed behind a safety shield in a hood). After 20 h, the solvent was removed

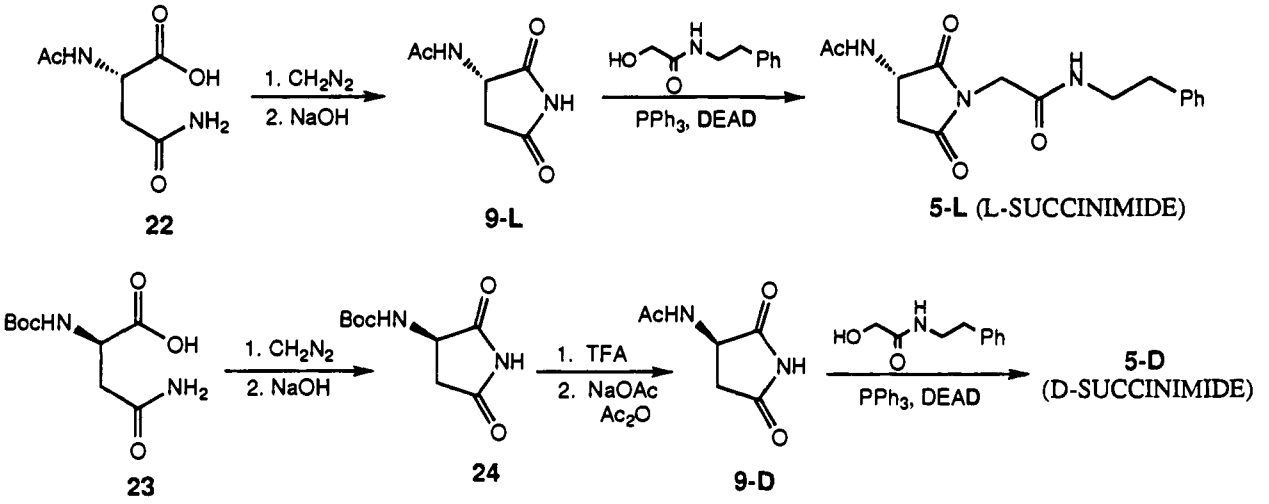
(14) This procedure is based on that of Lampin et al.: Lampin, J. P.; Mathey, F.; Bartet, B. *Bull. Soc. Chim. Fr.* **1971**, 317. The reason for the poor yield of **15** is unclear; less than 10% of **14** was recovered. Use of butyllithium as a base as described by Lampin was slightly more effective than LDA on a small scale (28% yield on a 1 mmol scale) but worked very poorly on a larger scale.

(15) Epoxidation of other phospholenes: Quin, L. D.; Symmes, C., Jr.; Middlemas, E.D.; Lawson, H. F. *J. Org. Chem.* **1980**, *45*, 4688.

(16) The procedure used was that developed by Emziane et al.: Emziane, M.; Lhoste, P.; Sinou, D. *Synthesis* **1988**, 541.

(17) Reduction of azides to amines with triphenylphosphine: (a) Baum, K.; Berkowitz, P. T.; Grakauskas, V.; Archibald, T. G. *J. Org. Chem.* **1983**, *48*, 2953. (b) Mungall, W. S.; Green, G. L.; Heavner, G. A.; Letsinger, R. L. *J. Org. Chem.* **1975**, *40*, 1659. (c) Knouzi, N.; Vaultier, M.; Carrie, R. *Bull. Soc. Chim. Fr.* **1985**, 815. Catalytic hydrogenation of **18** and **19**, even under the very mild conditions of Corey et al.,[17d] led to both reduction of the azide and cleavage of the benzyl protecting group. The resulting aminophosphinic acids were extremely difficult to isolate and purify. (d) Corey, E. J.; Nicolaou, K. C.; Balanson, R. D.; Machida, Y. *Synthesis* **1975**, 590.

Scheme 7



and the residue chromatographed (2 × 15 cm, silica gel, 20% MeOH/$CHCl_3$) to afford 30 mg (53%) of amine **20** and its regioisomer *N*-phenethyl(1′-(benzyloxy)-3′-amino-4′-hydroxy-1′-oxophosphol-2′-yl)-acetamide in a 1:1 ratio. The desired product, **20** (11 mg, 18%), was obtained using HPLC equipped with a Whatman Partisil ODS-3 Magnum 9 semipreparative column (25% CH_3CN/75% 0.1% TFA in H_2O, retention time of **20** = 28.2 min). After HPLC, the pooled fractions containing **20** were concentrated and passed through a small silica column (1 × 15 cm, 20% MeOH/80% $CHCl_3$) to remove C-18 silica that had leached off the HPLC column. ^{1}H-NMR (CD_3OD): δ 7.5−7.1 (10H, aromatic, m), 5.15−5.05 (2H, OCH_2Ph, m), 4.02−3.93 (1H, H_3', dt), 3.5−3.3 (3H, H_4', −NHCH_2CH_2Ph, m), 2.86−2.74 (3H, H_2', −NHCH$_2CH_2$Ph, m), 2.65−2.37 (2H, H_2', −CH_2CO, m), 2.25−2.12 (1H, H_5', td), 1.8−1.68 (1H, H_5', td) (assignments are consistent with decoupling experiments). ^{31}P-NMR: (CD_3OD) δ 77.6. Positive ion FAB MS: *m/z* 403 (MH^+, 100%), 304 (M − 99, 17%), 278 (M − 125, 30%), 263 (M − 140, 25%).

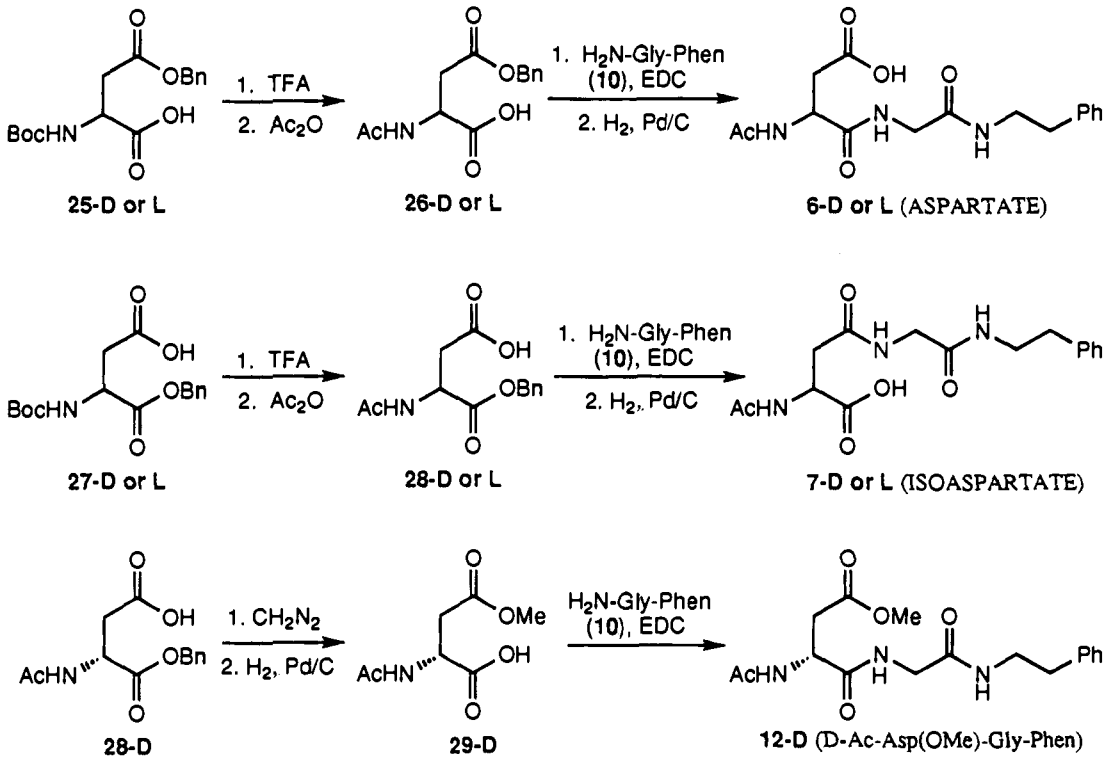
***N*-Phenethyl(1′,3′-dihydroxy-4′-((5-hydroxy-5-oxopentanoyl)amino)-1′-oxophosphol-2′-yl)acetamide Disodium Salt (8).** A solution of **20** (11 mg, 0.027 mmol), monobenzyl glutarate (6.68 mg, 0.30 mmol, 1.1 equiv), and EDC (5.76 mg, 0.030 mmol, 1.1 equiv) in CH_2Cl_2 (1 mL) was stirred for 16 h at room temperature. The reaction mixture was concentrated and purified by flash chromatography (1 × 15 cm silica gel, 5% MeOH/95% $CHCl_3$) to afford 7.1 mg (44%) of *N*-phenethyl-(1′-(benzyloxy)-3′-hydroxy-4′-((5-(benzyloxy)-5-oxopentanoyl)amino)-1′-oxophosphol-2′-yl)acetamide (**21**). ^{1}H-NMR: δ 7.4−7.1 (15H, aromatic, m), 6.30−6.24 (1H, PhCH$_2$CH$_2$N*H*CO−, broad t), 5.69−5.63 (1H, CHN*H*CO−, d), 5.09 (2H, PhCH_2OCO−, s), 4.98−4.92 (2H, PhCH_2OPO−, d), 4.39 (1H, OH, s), 4.22−4.11 (1H, H_4', m), 4.14−4.02 (1H, H_3', m), 3.51−3.33 (2H, PhCH$_2CH_2$NH−, m), 2.77−2.71 (2H, PhCH_2CH$_2$−, t), 2.53−2.29 (4H, H_2', CH_2CONH−, H_5', m), 2.32−2.27 (2H, CH$_2CH_2$COOCH$_2$Ph, t), 2.06−1.99 (2H, NHCOCH_2CH$_2$-CH$_2$−, t), 1.87−1.77 (2H, −CH$_2CH_2$CH$_2$−, quint), 1.63−1.52 (1H, H_5', m) (assignments are consistent with decoupling experiments). ^{31}P-NMR (CD_3OD): δ 70.3. Positive ion FAB MS: *m/z* 607 (MH^+, 100%), 517 (M − 90, 85%), 461 (M − 146, 23%), 427 (M − 180, 18%), 403 (M − 204, 10%). A solution of **21** (7.1 mg, 0.0117 mmol), $NaHCO_3$ (0.5 M in H_2O, 46.8 μL, 0.0234 mmol, 2 equiv), and Pd(OH)$_2$/C (~2.5 mg) in EtOH (3 mL) was stirred under H_2 for 16 h at room temperature and atmospheric pressure. The reaction was filtered through a plug of Celite and glass wool and concentrated to afford 4.5 mg of **8**. The sample was 95% pure by ^{31}P- and ^{1}H-NMR. ^{1}H-NMR (CD_3OD): δ 7.4−7.1 (5H, aromatic, m), 4.19−4.04 (2H, H_3', H_4', m), 3.41−3.33 (2H, PhCH$_2CH_2$NH−, m), 2.82−2.75 (2H, PhCH_2CH$_2$−, t), 2.56−2.40 (2H, H_2', CH_2CONH−, H_5', m), 2.40−2.25 (1H, H_2', m), 2.25−2.15 (4H, NHCO$CH_2CH_2CH_2$COO$^-$, m), 2.15−2.04 (1H, H_5', td), 1.94−1.82 (2H, −CH$_2CH_2$CH$_2$−, quint), 1.53−1.40 (1H, H_5', td) (assignments are consistent with decoupling experiments). ^{31}P NMR (CD_3OD): δ 51.8. Negative ion FAB MS: *m/z* 425 (M − H, 100%), 407 (M − 18, 40%), 389 (M − 36, 35%), 379 (M − 46, 17%), 349 (M − 76, 28%), 335 (M − 90, 18%), 303 (M − 122, 43%).

3-(*N*-Acetyl-L-amino)succinimide (9-L). *N*-Acetyl-L-asparagine **22** (Fluka, 1.0 g, 5.75 mmol) was suspended in methanol (10 mL) in an Erlenmeyer flask. The suspension was stirred at room temperature behind a blast shield as an ethereal solution of diazomethane was slowly added. The diazomethane solution was added until a yellow color persisted for 10 min and nitrogen gas no longer evolved. Excess diazomethane was quenched with a drop of acetic acid. The desired methyl ester, which precipitated from solution as white solid, was collected by filtration and dried in vacuo. No further purification was necessary. Evaporating the filtrate to dryness and purifying the resulting crude solid by repetitive recrystallizations from methanol/ether yielded additional pure product. The combined yield of *N*-acetyl-L-asparagine α-methyl ester was 918 mg (85% yield). To a suspension of this methyl ester (400 mg, 2.12 mmol) in H_2O (5.1 mL) was added 1.0 M NaOH (2.04 mL, 2.04 mmol). The mixture was stirred at room temperature for 7 min. The solution was filtered and then acidified with 1.2 M HCl (pH ≈ 2). The mixture was evaporated to dryness and the residue suspended in methanol and filtered through Celite. The filtrate was evaporated to dryness, and the residue was recrystallized from hot ethyl acetate with petroleum ether. The combination of three crops from the recrystallization resulted in 200 mg of **9-L** as a white solid (60% yield).

3-(*N*-^{t}Boc-D-amino)succinimide (24). *N*-^{t}Boc-D-asparagine (**23**, 1.334 g, 5.75 mmol) was suspended in methanol (5 mL) in an Erlenmeyer flask. The suspension was stirred at room temperature behind a blast shield as an ethereal solution of diazomethane was slowly added. The diazomethane solution was added until a yellow color persisted for 10 min and nitrogen gas was no longer evolved. Excess diazomethane was quenched with a drop of acetic acid. The solvent was removed under vacuum resulting in a gummy white solid. Multiple recrystallizations from diethyl ether yielded 1.1 g (78% yield) of *N*-^{t}Boc-D-asparagine α-methyl ester as a white solid. To a suspension of this methyl ester (720 mg, 2.93 mmol) in H_2O (7 mL) was added 1.0 M NaOH (2.82 mL, 2.82 mmol). The mixture was stirred at room temperature for 7 min. The solution was filtered and then acidified with 1.2 M HCl (pH ≈ 2). The mixture was cooled on ice for 30 min, during which time the desired aminosuccinimide, **24**, crystallized from solution as a white solid (365 mg). Evaporation of the mother liquor followed by titration into ethyl acetate and recrystallization from ethyl acetate/petroleum ether resulted in an additional 100 mg of **24** (total yield of 90%).

3-(*N*-Acetyl-D-amino)succinimide (9-D). Trifluoroacetic acid (8.0 mL) was added to a solution of **24** (365 mg, 1.71 mmol) in CH_2Cl_2 (24 mL). The reaction was stirred at room temperature for 1.2 h. The solvent was removed in vacuo. The resulting oil was dissolved in H_2O (20 mL) and again evaporated to dryness. The residue was dissolved in H_2O (4.2 mL). The solution was stirred at room temperature while acetic anhydride (208 μL, 2.04 mmol) was added immediately followed by 2.6 M sodium acetate (1 mL). After the mixture was stirred for 3 min at room temperature, second portions of acetic anhydride (208 μL, 2.04 mmol) and 2.6 M NaOAc (1 mL) were added to the mixture. The

Scheme 8



reaction mixture was filtered and then evaporated to dryness. The residue was dissolved in methanol and then re-evaporated. The resulting oil was dissolved in ethyl acetate and oiled out with petroleum ether. The oil was washed with diethyl ether. The washed oil was redissolved in methanol and evaporated to an off-white powder. Pure **9-D** was obtained by recrystallization from methanol/diethyl ether/petroleum ether (120 mg, white crystals). The mother liquors from the recrystallizations were combined, evaporated to dryness, and chromatographed through silica (6:1 CH_2Cl_2/CH_3OH, $R_f = 0.5$). The resulting white solid was recrystallized as above to yield an additional 100 mg of **9-D** (total yield of 83%).

3-(*N*-Acetylamino)-1-((phenylethyl)glyoxalamido)succinimide (5-L and 5-D). Succinimide **5-L** was synthesized as follows: **9-L** (43.5 mg, 0.279 mmol) and *N*-phenethylglycolamide (50 mg, 0.279 mmol, prepared by the condensation of glycolic acid and phenethylamine with EDC) were dissolved in THF (2 mL). To this solution was added triphenylphosphine (73.2 mg, 0.279 mmol) followed by diethyl diazodicarboxylate (45 mL, 0.286 mmol). The flask was wrapped in foil to protect from light and the mixture stirred at room temperature under argon for 15 h. The mixture was evaporated to dryness. The residue was chromatographed through silica (19:1 CH_2Cl_2/MeOH R_f = 0.25) to afford 70 mg of **5-L** (79% yield). ^{1}H-NMR: δ 7.48 (1H, CH_3CONH-, d, $J \sim 6.8$ Hz), 7.25 (1H, $-CH_2CONH-$, t, $J \sim 5.0$ Hz), 7.21−7.06 (5H, aromatics, m), 4.19−4.04 (3H, $-NCH_2CO-$, $CH_3CONHCH-$, m), 3.38 (2H, $-NHCH_2CH_2-$, q, $J \sim 7.4$ Hz), 2.96 (1H, $-CHCH_2CO-$ dd, $J \sim 9.4$, 18.2 Hz), 2.74−2.67 (3H, $-CHCH_2CO-$, $-CH_2CH_2Ar$, m), 1.89 (3H, CH_3CO-). EI-MS: *m/z* 317 (M^+, 4%), 226 (M − 91, 13%), 197 (M − 120, 22%), 169 (M − 148, 39%), 141 (M − 176, 25%), 112 (M − 195, 15%), 104 (M − 213, 100%), 99 (M − 218, 26%), 91 (M − 226, 32%). **5-D** was synthesized in an identical manner by starting with **9-D** and had a ^{1}H-NMR and mass spectrum identical to those of **5-L**.

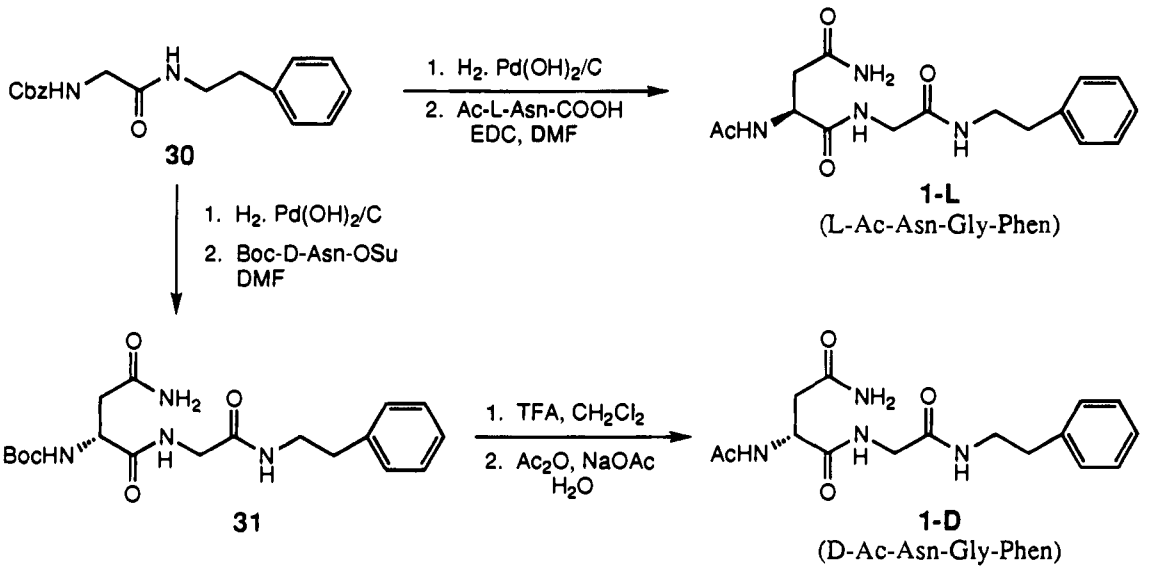
β-Benzyl *N*-Acetylaspartate (26-L or 26-D). β-Benzyl *N*-^{t}Boc-(L- or D)-aspartate **25-L** or **25-D** (646 mg, 2.0 mmol) was dissolved in 25% trifluoroacetic acid in CH_2Cl_2 (32 mL). The reaction was stirred at room temperature for 2 h and then evaporated to dryness. The residue was twice dissolved in H_2O (5 mL) and again evaporated to dryness. The residue was dissolved in H_2O (5 mL) and heated to 55 °C. Acetic anhydride (480 μL) was added followed immediately by a NaOAc (400 mg) solution in H_2O (1 mL). After being stirred for 3 min at 55 °C, the reaction mixture was cooled on ice. The solution was diluted with water (15 mL), filtered, and extracted into ethyl acetate (4 × 20 mL). Evaporation yielded **26-L** or **26-D**; the yields for both isomers were approximately 80% (~420 mg).

(*N*-Acetylaspartyl)-*N*-phenethylglycinamide (6-L and 6-D). β-Benzyl *N*-acetylaspartate (**26-L** or **26-D**; 212 mg, 0.8 mmol) was dissolved in THF (2.5 mL). EDC (192 mg, 1 mmol) was added to the solution. The reaction was stirred for 3 min at room temperature, and *N*-phenethylglycinamide **10** (160 mg, 0.9 mmol) was added as a solution in THF (2 mL). After 1 h at room temperature the THF was removed in vacuo. The residue was dissolved in CH_2Cl_2 (25 mL) and washed with dilute HCl. The aqueous layer was subsequently extracted with CH_2Cl_2 (2 × 25 mL). The CH_2Cl_2 layers were combined, dried over Na_2SO_4, filtered, and evaporated to dryness, giving a yellow oil. The oil was purified by chromatography on silica using 9:1 CH_2Cl_2/CH_3OH ($R_f = 0.45$) to yield ~200 mg (~60% yield) of pure L- or D-(*N*-acetyl-β-benzylaspartyl)-*N*-phenethylglycinamide. The purified benzyl ester was debenzylated by treating it in an ethanol solution with H_2 in the presence of 10% Pd on carbon for 1.5 h. After being filtered to remove the catalyst, the mixture was evaporated to dryness. The residue was dissolved in water (5 mL) and washed with CH_2Cl_2 (4 × 5 mL). The aqueous layer was evaporated, yielding the desired compound **6-L** or **6-D** as a white solid (~110 mg, ~69% for both **6-L** and **6-D**). ^{1}H-NMR (CD_3OD): δ 7.28−7.15 (5H, aromatics, m), 4.62 (1H, $CH_3CONHCH-$, t, $J \sim 6.2$ Hz), 3.80 (2H, $-NHCH_2CONH-$, ABq, $J \sim 16.9$ Hz), 3.40 (2H, $-CONHCH_2CH_2-$, m), 2.88−2.74 (4H, $-CHCH_2COOH$, $-CH_2CH_2Ar$, m), 1.99 (3H, CH_3CONH-, s). CI-MS *m/z* 318 (M + 1 − 18, 100%).

(*N*-Acetylisoaspartyl)-*N*-phenethylglycinamide (7-L and 7-D). The isoaspartate peptides **7-L** and **7-D** were synthesized as described above for the aspartate peptides **6-L** and **6-D** except starting with *N*-^{t}Boc-α-benzyl-(L- or D)-aspartate **27-L** or **27-D** (646 mg, 2.0 mmol). Overall yields of **7-L** and **7-D** were similar to those obtained for compound **6**. ^{1}H-NMR (acetone-d_6; acetone-d_5 = 2.04 ppm): δ 7.28−7.15 (5H, aromatics, m), 4.74 (1H, $CH_3CONHCH-$, bs), 3.80 (2H, $-NHCH_2CONH-$, ABq, $J \sim 16.7$ Hz), 3.39 (2H, $-CONHCH_2CH_2-$, m), 2.83−2.75 (4H, $-CHCH_2COOH$, $-CH_2CH_2Ar$, m), 1.92 (3H, CH_3CONH-, s). CI-MS: *m/z* 318 (M + 1 − 18, 100%).

β-Methyl *N*-Acetyl-D-aspartate (29-D). α-Benzyl *N*-acetyl-D-aspartate (**28-D**; 243 mg, 0.918 mmol) was suspended in methanol (5 mL) in an Erlenmeyer flask. The suspension was stirred at room temperature behind a blast shield as an ethereal solution of diazomethane

Scheme 9



was slowly added. The diazomethane solution was added until a yellow color persisted for 10 min and nitrogen gas was no longer evolved. Excess diazomethane was evaporated by blowing argon over the solution. The mixture was evaporated to a yellow oil, which was chromatographed through silica using 190:10:1 $CH_2Cl_2/CH_3CN/CH_3OH$ ($R_f = 0.13$), resulting in α-benzyl β-methyl *N*-acetyl-D-aspartate (128 mg, 50%). This benzyl/methyl diester was debenzylated by catalytic hydrogenation in ethanol (3 mL) with 10% Pd/C (30 mg). The flask was flushed with argon then stirred under a hydrogen atmosphere at room temperature for 2 h. The catalyst was removed by filtration through Celite and rinsed with methanol (5 mL). The filtrate and rinse were evaporated to dryness resulting in 86 mg of **29-D**, in 100% yield, which was not characterized but taken directly on to **12-D**.

(***N*-Acetyl-β-methyl-D-asparty**l)-***N*-phenethylglycinamide (12-D).** EDC (88 mg, 0.46 mmol) was added to a suspension of β-methyl *N*-acetyl-D-aspartate (**29-D**) (86 mg, 0.46 mmol) in THF (2 mL). After being stirred for 3 min at room temperature, *N*-phenethylglycinamide **10** (82 mg, 0.46 mmol) was added to the reaction. The reaction was stirred for an additional 1 h at room temperature and then evaporated to dryness. The residue was dissolved in CH_2Cl_2 (20 mL). The CH_2Cl_2 solution was washed with dilute HCl (15 mL). The aqueous layer was subsequently extracted with CH_2Cl_2 (2 × 10 mL). All the CH_2Cl_2 was combined, dried over Na_2SO_4, filtered, and evaporated to dryness. The resulting residue was recrystallized from ethyl acetate/petroleum ether to yield 80 mg (50%) of **12-D** as a white solid. ^{1}H-NMR (CD_3OD): δ 7.28−7.15 (5H, aromatics, m), 4.64 (1H, $CH_3CONHCH-$, t, $J \sim 6.1$ Hz), 3.80 (2H, $-NHCH_2CONH-$, ABq, $J \sim 16.9$ Hz), 3.67 (3H, $-COOCH_3$, s), 3.40 (2H, $-CONHCH_2CH_2-$, m), 2.90−2.74 (4H, $-CHCH_2COOCH_3$, $-CH_2CH_2Ar$, m), 1.98 (3H, CH_3CONH-, s).

***N*-CBz-*N*-phenethylglycinamide (30).** *N*-CBz-glycine (1.05 g, 50 mmol), phenethylamine (1.25 mL, 100 mmol), and EDC (0.96 g, 50 mmol) were dissolved in CH_2Cl_2 (20 mL) and stirred at room temperature for 48 h. Dichloromethane (20 mL) was added and the mixture washed with 1 N HCl (4 × 20 mL), dried ($MgSO_4$), filtered, and concentrated. The yellow solid was purified by flash chromatography (silica gel, 5% MeOH/$CHCl_3$, 4.0 × 15 cm) to afford 970 mg (62%) of the title compound as a white solid. ^{1}H-NMR: 7.4−7.1 (10 H, aromatic, m), 6.1−6.0 (1H, $-CH_2CONH-$, broad s), 5.45−5.35 (1H, $-OCONHCH_2-$, broad s), 3.85−3.8 (2H, $-NHCH_2CO-$, d, $J \sim 5.8$ Hz), 3.59−3.49 (2H, $-NHCH_2CH_2-$, m), 2.85−2.75 (2H, $-CH_2CH_2Ph$, t, $J \sim 7.6$ Hz). The free amine, *N*-phenethylglycinamide (**10**), was prepared by catalytic hydrogenation of the title compound at room temperature and atmospheric pressure.

(***N*-Boc-D-asparaginyl)-*N*-phenethylglycinamide (31).** Boc-D-asparagine (255 mg, 1.1 mmol), *N*-hydroxysuccinimide (138 mg, 1.2 mmol) and EDC (138 mg, 1.1 mmol) were dissolved in DMF (10 mL) and stirred for 16 h at room temperature to form *N*-Boc-D-asparaginyl *N*-hydroxysuccinimidyl ester. In a separate reaction vessel, **10** was produced by catalytic hydrogenation of 312 mg (1.0 mmol) of **30**, which was dissolved in 10 mL of EtOH (along with $Pd(OH)_2/C$ (~5 mg)) and stirred under H_2 for 16 h at atmospheric pressure. The solution was concentrated by rotary evaporation and the crude residue taken up in DMF (10 mL) and added to the crude solution of *N*-Boc-D-asparaginyl *N*-hydroxysuccinimidyl ester. After 3 h, the solution was concentrated by rotary evaporation and the resulting crude oil was purified by flash chromatography (silica gel, 5% MeOH/$CHCl_3$, 4.0 × 15 cm) to afford 220 mg (56%) of **31** as a white solid. ^{1}H-NMR (DMSO-d_6): δ 8.21−8.15 (1H, $-CONHCH_2CO-$, t, $J \sim 5.6$ Hz), 7.86−7.80 (1H, $-CONHCH_2CH_2-$, t, $J \sim 5.5$ Hz), 7.40 (1H, $-CONH_2$, s), 7.3−7.15 (5 H, aromatic, m), 7.0−6.95 (2H, $-CONHCH-$, $-CONH_2$, m), 4.2−4.1 (1H, $-NHCHCO-$, q, $J \sim 6.8$ Hz), 3.63−3.6 (2H, $-NHCH_2CO-$, d, $J \sim 5.8$ Hz), 3.3−3.2 (2H, $-NHCH_2CH_2Ph$, m), 2.74−2.65 (2H, $-CH_2CH_2Ph$, t, $J \sim 7.6$ Hz), 2.45−2.35 (2H, $-CHCH_2CO-$, m), 1.35 (9H, $(CH_3)_3C-$, s). ^{13}C NMR (DMSO-d_6): δ 171.7, 171.6, 168.5, 155.2, 139.2, 128.5, 128.2, 126.0, 78.4, 51.3, 42.3, 37.1, 35.1, 28.1. Positive ion FAB MS: *m/z* 393 (M + H, 40%), 337 (M − 56, 20%), 320 (M − 73, 20%), 293 (M − 100, 100%), 234 (M − 159, 20%), 179 (M − 200, 98%).

(***N*-Acetyl-D-asparaginyl)-*N*-phenethylglycinamide (1-D).** The protected dipeptide **31** (502 mg, 1.28 mmol) was added to CH_2Cl_2 (30 mL) containing trifluoroacetic acid (10.5 mL) and stirred under Ar for 1 h. The reaction mixture was concentrated by rotary evaporation, the residue was taken up in H_2O (30 mL), and this process was performed two more times. The residue was dissolved in 3 mL of H_2O, and acetic anhydride (0.144 mL, 1.28 mmol) was added immediately followed by a 2.4 M aqueous solution of sodium acetate (0.640 mL, 1.56 mmol, 1.2 equiv). This was stirred vigorously at room temperature for 10 min and then cooled in an ice bath, which resulted in the formation of white crystals. The crystals were obtained by vacuum filtration and washed with cold H_2O. Acetic anhydride (0.077 mL, 0.80 mmol) was added to the filtrate immediately followed by a 2.4 M aqueous solution of sodium acetate (0.320 mL, 0.78 mmol). The solution was stirred for 10 min and then cooled on ice, which resulted in the formation of white crystals. The crystals were obtained by vacuum filtration, combined with the first batch of crystals, and purified by flash chromatography (silica gel, 20% MeOH/$CHCl_3$, 4 × 15 cm) to yield 300 mg (70%) of **1-D** as a white solid. ^{1}H-NMR (DMSO-d_6): δ 8.21−8.16 (1H, $-CONHCH_2CO-$, t, $J \sim 5.6$ Hz), 8.14−8.08 (1H, CH_3CONH-, d, $J \sim 7.6$ Hz), 7.86−7.80 (1H, $-CONHCH_2CH_2-$, t, $J \sim 5.5$ Hz), 7.40 (1H, $-CONH_2$, s), 7.3−7.15 (5 H, aromatic, m), 7.0−6.95 (1H, $-CONH_2$, s), 4.5−4.4 (1H, $-NHCHCO-$, q, $J \sim 6.8$ Hz), 3.67−3.51 (2H, $-NHCH_2CO-$, m), 3.3−3.2 (2H, $-NHCH_2CH_2Ph$, m), 2.74−2.65 (2H, $-CH_2CH_2Ph$, t, $J \sim 7.6$ Hz), 2.45−2.35 (2H, $-CHCH_2CO-$, m), 1.88 (3H, $(CH_3CO-$, s). ^{13}C NMR (DMSO-d_6):

δ 171.7, 171.3, 169.6, 168.6, 139.4, 128.5, 128.3, 126.0, 49.9, 42.4, 37.1, 35.1, 28.6. Positive ion FAB MS: *m/z* 335 (M + H, 100%), 317 (M − 18, 35%), 300 (M − 35, 10%).

(*N*-Acetyl-D-asparaginyl)-*N*-phenethylglycinamide (1-L). The amine **10** was produced by catalytic hydrogenation of **30** (1.0 g, 3.21 mmol), which was dissolved in 25 mL of EtOH (along with $Pd(OH)_2/C$ (~40 mg)) and stirred overnight for 16 h at atmospheric pressure. The EtOH was removed by rotary evaporation and then the residue was dried under high vacuum for 6 h. The residue was dissolved in DMF (40 mL), and to this solution was added *N*-acetyl-L-asparagine (**22**; Fluka, 614 mg, 3.53 mmol, 1.1 equiv) and EDC (676 mg, 3.53 mmol, 1.1 equiv). After 48 h at room temperature, the solvent was removed by rotary evaporation and the desired product purified by flash chromatography (silica gel, 20% MeOH/$CHCl_3$, 4 × 15 cm) to yield 470 mg (46%) of **1-L** as a white solid. ^{1}H-NMR (DMSO-d_6): δ 8.21−8.16 (1H, −CON*H*CH_2CO−, t, $J \sim 5.6$ Hz), 8.14−8.08 (1H, CH_3-CONH−, d, $J \sim 7.6$ Hz), 7.86−7.80 (1H, −CON*H*CH_2CH_2−, t, $J \sim 5.5$ Hz), 7.40 (1H, −CON*H*$_2$, s), 7.3−7.15 (5 H, aromatic, m), 7.0−6.95 (1H, −CON*H*$_2$, s), 4.5−4.4 (1H, −NHC*H*CO−, q, $J \sim 6.8$ Hz), 3.67−3.51 (2H, −NH*CH*$_2$CO−, m), 3.3−3.2 (2H, −NH*CH*$_2$$CH_2$Ph, m), 2.74−2.65 (2H, −$CH_2$*CH*$_2$Ph, t, $J \sim 7.6$ Hz), 2.45−2.35 (2H, −CH*CH*$_2$CO−, m), 1.88 (3H, (*CH*$_3$CO−, s). ^{13}C NMR (DMSO-d_6): δ 171.7, 171.3, 169.6, 168.6, 139.4, 128.5, 128.3, 126.0, 49.9, 42.4, 37.1, 35.1, 28.6. Positive ion FAB MS: *m/z* 335 (M + H, 100%), 317 (M − 18, 35%), 300 (M − 35, 10%).

General Methods (Biochemical). All measurements were carried out at 25 °C in 0.1 M bicine buffer (Sigma), 5% DMSO, $\mu = 0.10$ (NaCl), at the pH's indicated. Prior to use, stock antibody solutions were dialyzed into bicine buffer of appropriate pH. HPLC analyses were performed on a Waters 600E system using a Vydac C18, 0.46 × 25 cm column. The samples were run on a gradient consisting of CH_3-CN and H_2O (0.1% TFA). The effluent was monitored at 254 nm by a Waters 484 detector. All fluorescence studies were performed using a SLM Instruments 8000 fluorimeter. Plot fitting was performed using the RS/1 program from BBN Software Products Corp. Kinetic simulations were carried out using KINSIM[7] on a MicroVax II/GPX computer.

Antibody RG2-23C7 Concentration. Antibody RG2-23C7 (75 μL) was diluted to 3 mL with bicine at pH 8.35 in a fluorescence cuvette. The antibody solution was excited at 280 nm and monitored at 334 nm as a 50 μM solution of L-isoaspartate compound **7-L** was added in 2 μL aliquots. Variations in the instrument were corrected for by simultaneously titrating a 21 μM solution of tryptophan in pH 8.35 buffer. This tryptophan solution gave the identical starting fluorescence as the antibody solution. After correction for random variations, the antibody fluorescence data were plotted versus the concentration of L-isoaspartate compound **7-L**. The break in the titration occurred at 0.95 μM, indicating that the stock antibody solution was 19.3 μM (38.6 μM binding sites).

Antibody RG2-2E4 Concentration. Antibody RG2-2E4 (100 μL) was diluted to 1 mL with bicine at pH 8.35 in a fluorescence cuvette. The antibody solution was excited at 280 nm and monitored at 327 nm as a 1.0 mM solution of D-aspartate compound **6-D** was added in 1 μL aliquots. Variations in the instrument were corrected for by simultaneously titrating a 49 μM solution of tryptophan in pH 8.35 buffer. This tryptophan solution gave an identical starting fluorescence as the antibody solution. After correction for random variations, the antibody fluorescence data were plotted versus the concentration of D-aspartate compound **6-D**. The break in the titration occurred at 13.0 μM, indicating that the stock antibody solution was 65 μM (130 μM binding sites).

Determination of Dissociation Constants (k_4/k_{-4} and k_6/k_{-6}). All product dissociation constants were determined by fluorescence titrations in which the antibody concentrations were less than the corresponding dissociation constants. Titrations were done in triplicate, and an average dissociation constant was determined. A sample titration for RG2-23C7 with L-isoaspartate product **7-L** at pH 8.35 is described below. The stock solution of RG2-23C7 (1.8 μL) was diluted with pH 8.35 bicine buffer (3 mL). The resulting solution was excited at 280 nm and monitored at 334 nm while the concentration of L-isoaspartate **7-L** was increased (0.05, 0.10, 0.20, 0.30, 0.50, 0.69, 0.94, 1.18, 1.60, 2.58, 4.05, and 6.49 μM) by the addition of 1−7.5 μL aliquots of either a 0.10 mM or a 1.00 mM solution. Variations in the instrument were corrected for by simultaneously titrating a 0.82 μM solution of tryptophan in pH 8.35 buffer. This tryptophan solution gave a starting fluorescence identical to that of the antibody solution. The solutions were stirred and allowed to thermally equilibrate for 3 min before a reading was taken. After correction for random variations, the antibody fluorescence data were plotted versus the concentration of L-isoaspartate compound **7-L**. The plotted data were fit to the quadratic equation for equilibrium binding: $K_D = F_i - (DF/2[Ab])-(0.5)\{k_D + 2[Ab] + [\mathbf{7\text{-}L}] - ((K_D + 2[Ab] + [\mathbf{7\text{-}L}])^2 - (4)2[Ab]-[\mathbf{7\text{-}L}])^{1/2}\}$, where K_D = dissociation constant, F_i = initial fluorescence, DF = total change in fluorescence, 2[Ab] = concentration of binding sites, and [**7-L**] = concentration of **7-L** added.

Succinimide Hydrolysis. Antibody solutions, dialyzed into the appropriate pH buffers, were equilibrated at 25 °C, and 500 μM succinimide **5-D** or **5-L** in DMSO was added. At various times, an aliquot was withdrawn and quenched with perchloric acid to a final pH of approximately 2.5. The quenched solution was injected onto a C_{18} reverse-phase column, and the amounts of starting succinimide and hydrolysis products were determined by peak integration. The total concentrations of the starting succinimide **5** and the aspartate **6** and isoaspartate **7** products were normalized to 500 μM, and the normalized data were fit to the reaction scheme using KINSIM.[7]

Deamidation of 1 and Demethoxylation of 12-D. The protocol for the succinimide hydrolysis was used with 500 μM of **1-D** or **1-L** or **12-D** substituted for **5**.

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Supplementary Material Available: Figures including NMR spectral data for the decoupling experiments performed on compounds **20**, **21**, and **8** and computer generated fits to the HPLC data (36 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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