Table I. Kinetic Isotope Effects for the Thermolysin-Catalyzed Hydrolysis of N-(3-[2-Furyl]acryloyl)-Gly-LeuNH$_2$[a]

$[S]_0$ (10^{-6} M)	$^{H}k_E/^{\beta D}k_E$	$^{H_2O}k_E/^{D_2O}k_E$
50	0.956 ± 0.017	0.78 ± 0.02
80	0.967 ± 0.018	0.81 ± 0.01
100	0.960 ± 0.021	0.79 ± 0.03
	0.961 ± 0.016	0.80 ± 0.02

[a] Kinetic experiments were conducted at 24.8 ± 0.1 °C and in a pH 7.48 buffer containing 0.10 M HEPES, 0.01 M $CaCl_2$, and 1.7% DMSO. The enzyme was prepared as a 3 mg/mL stock solution in 0.10 M HEPES, 0.01 M $CaCl_2$, and 2.5 M NaBr and buffered at pH 7.5. Final enzyme concentration in reaction solutions was approximately 0.2 μM. Reaction progress curves for the hydrolysis of the Gly-Leu bond were monitored at 322 nm ($\Delta\epsilon_{322}$ = −2300). Since in these experiments $[S]_0 \ll K_m > 10$ mM, the reactions were first-order in substrate and could be fit, by nonlinear least squares, to a simple exponential rate law. β-DIE[15,16] and SIE[20] were determined as detailed in previous publications.

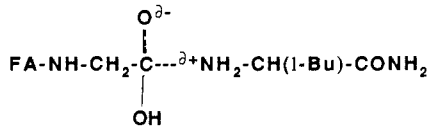
dependence of (i) k_E on the concentration of FA-Gly-LeuNH$_2$ (k_E = 20 100 ± 700 M^{-1} s^{-1}, 30 < $[S]_0$ < 150 mM); (ii) k_E on the concentration of FA-Gly(d_2)-LeuNH$_2$ (k_E = 21 000 ± 500 M^{-1} s^{-1}, 30 < $[S]_0$ < 150 uM); and, (iii) the β-DIE on substrate concentration (see Table I).

The β-DIE for k_E is 0.961 ± 0.016 (Table I) and reflects the loss of hyperconjugation that occurs as the sp^2-hybridization of the substrate in its ground state changes to the partial sp^3-hybridization of the substrate in the tetrahedral intermediate-like transition state.[14,15] The transition state for the thermolysin-catalyzed hydrolysis of FA-Gly-LeuNH$_2$ must therefore resemble the tetrahedral intermediate that occurs along the reaction pathway during amide bond hydrolysis.

The SIE for k_E is 0.80 ± 0.02 (Table I) and stands in marked contrast to solvent isotope effects for serine protease-catalyzed reactions, which typically range from 2.5–3.5.[16] These latter effects reflect the general acid/general base catalysis that promotes acyl transfer to and from the active site serine. The isotopic silence observed here for thermolysin suggests an absence of protolytic catalysis in the transition state for k_E.[17]

We see then, that the rate-limiting step for the thermolysin-catalyzed hydrolysis of FA-Gly-LeuNH$_2$ must involve heavy atom rearrangement ($^{H}k_E/^{\beta D}k_E$ = 0.96) unaccompanied by proton transfer ($^{H_2O}k_E/^{D_2O}k_E \sim 1$). In the context of the current view of thermolysin catalysis,[2-6,11] this combination of isotope effects allows several potential rate-limiting steps to be excluded immediately: (i) formation of the initial encounter complex; (ii) conformational isomerization of this complex; (iii) general-base catalyzed attack of water on the substrate to form a tetrahedral intermediate; (iv) general-acid catalyzed expulsion of the amine leaving group during decomposition of the tetrahedral intermediate; and (v) product release. Steps (i), (ii), and (v) need not be considered as potential rate-limiting steps since these processes do not involve heavy atom rearrangement and would not be expected to generate a β-DIE. Likewise, steps (iii) and (iv) cannot

Chart I



be rate-determining since both of these processes are subject to protolytic catalysis and would generate significant normal SIE.

The reaction step that is most consistent with the experimenal results of this study is collapse of a zwitterionic tetrahedral intermediate to yield FA-NH-CH$_2$-COOH and H$_2$N-CH(*i*-Bu)-CONH$_2$. In the transition state for this step (see Chart I), the α-carbon of the scissle bond has sp^3-character since it resembles, to some degree, the tetrahedral intermediate that preceeds it on the reaction path. The sp^3-character of this transition state reduces the hyperconjugation of the glycine β-hydrogens relative to the reactant state of substrate free in solution. This situation generates the observed β-DIE of 0.96. Also in this transition state, the α-nitrogen of the departing amino amide exists in a protonated, partially cationic state.[18] Since protonation of the α-nitrogen is complete before the substrate and enzyme enter into the transition state, this situation is equivalent to specific-acid catalysis. Thus, the product of transition state fractionation factors can be taken as unity, and we can propose that the observed SIE of 0.80 corresponds to the ground state fractionation factor of the active site Zn^{2+}–H_2O.[17]

(13) Isotope effects on k_E are sensitive to impurities in either of the labeled reactants if these impurities can act as competitive inhibitors or activators of the enzyme under study. Since such materials are anticipated to be present at levels below HPLC or TLC detection limits, one must take advantage of their kinetic signatures to detect them. To assume kinetic purity, one must demonstrate that the first-order rate constant determined at low substrate concentration is independent of substrate concentration for both the deuteriated and nondeuteriated materials and that the isotope effect is independent of substrate concentration.

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(18) The mechanism advanced in this paper for tetrahedral intermediate decomposition, involving specific but not general acid catalysis of amine expulsion, has been discussed previously for chymotrypsin-catalyzed reactions[20] and is based on general mechanistic considerations of amide hydrolysis.

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Direct Observation of Multiple Environments for the H_δ but Not the H_ϵ Proton of a Histidine Residue in Staphylococcal Nuclease

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The ^{1}H NMR spectral properties of the H_ϵ protons of histidines are frequently used to study the structural properties of proteins. For example, Dobson and Fox recently demonstrated by ^{1}H NMR spectroscopy of the well-resolved H_ϵ (or C2H) protons of the four histidine residues in Staphylococcal nuclease (SNase) that SNase can exist as an equilibrium mixture of two folded and two unfolded conformations; this conformational heterogeneity was attributed to cis–trans isomerism of Pro 117.[1,2] We have deuteriated the aromatic rings of the phenylalanine, tyrosine, and tryptophan residues of SNase such that the obscured H_δ (or C4H) protons of the four histidine residues can also be directly observed by ^{1}H

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NMR and used as another determinant of conformational properties. Our observations under conditions that are thought to eliminate the conformational heterogeneity caused by isomerism of Pro 117 demonstrate that the folded state of SNase still exists as a mixture of conformations. In particular, the H_δ but not the H_ϵ proton of His 121 exists in at least two slowly exchanging environments since two resonances are observed for this H_δ proton. We attribute the enhanced sensitivity of the H_δ proton to conformational heterogeneity to the hydrogen-bonding asymmetry of the imidazole functional group and the resulting nonequivalence of the rotamers about the β-γ side chain bond.

The studies of Fox and Dobson were based solely on observations of the resonances associated with the H_ϵ protons since there are reasonably well resolved from the other aromatic protons when the pH is 6 or less. We hypothesized that additional information might be obtained from observation of the resonances associated with the H_δ protons, but these resonances are obscured by the aromatic resonances associated with the three phenylalanine, seven tyrosine, and single tryptophan residues in SNase. The spectral properties of the H_δ proton should be of special interest in investigations of conformational heterogeneity detected by the environments of the histidine residues since they can be anticipated to be more sensitive to rotation about the β-γ bond than those of the H_ϵ proton. The reason for this sensitivity is that rotation about this bond will substantially alter the position of the H_δ proton, whereas that of the H_ϵ proton will be minimally altered; the angle subtended by C_δ upon bond rotation is 130°, whereas that subtended by C_ϵ is only 15°.[3] Solid-state NMR studies have shown that the ortho and meta protons of phenylalanine, for example, are much more sensitive to rotation about the β-γ bond than the para protons for precisely this reason.[4] However, unlike phenylalanine and tyrosine, rotamers about the β-γ bond of histidine are likely to be energetically nonequivalent due to the chemical asymmetry of the heterocyclic ring. To the best of our knowledge, no comparison of the sensitivities of the H_δ and H_ϵ protons to conformational heterogeneity has been made since spectral congestion usually obscures the resonances for the H_δ protons.

A sample of SNase in which all of the aromatic ring protons of the phenylalanine, tyrosine, and tryptophan residues were replaced with deuterons was prepared with methods described elsewhere.[5,6]

The aromatic regions of the ^1H NMR spectra of the deuteriated SNase recorded at pH 7.8 in the absence and presence of Ca^{2+} and thymidine 3′,5′-bisphosphate (active site ligands) are compared with the spectrum of protiated SNase in the presence of ligands in Figure 1. Fox and Dobson observed that the presence of active site ligands eliminated the conformational heterogeneity that was attributed to the isomerism of Pro 117.[1] The assignments of the resonances given in the figure legend are based on data obtained on mutant enzymes (data not shown) as well as interresidue NOEs and are in agreement with those proposed recently.[2,7] The spectrum of the deuteriated SNase obtained in the absence of ligands has seven sharp resonances and two less intense, broad resonances at 6.9 ppm, a chemical shift characteristic of H_δ protons and assigned to the H_δ proton of His 121. Several additional minor resonances can be observed with chemical shifts characteristic of H_δ and H_ϵ protons, and these are not due entirely to residual phenylalanine, tyrosine, or tryptophan protons (data not shown). This spectrum provides direct and unrefutable evidence for at least two major forms of SNase present in solution since at least two resonances are observed for the H_δ (but not the H_ϵ) proton of His 121.

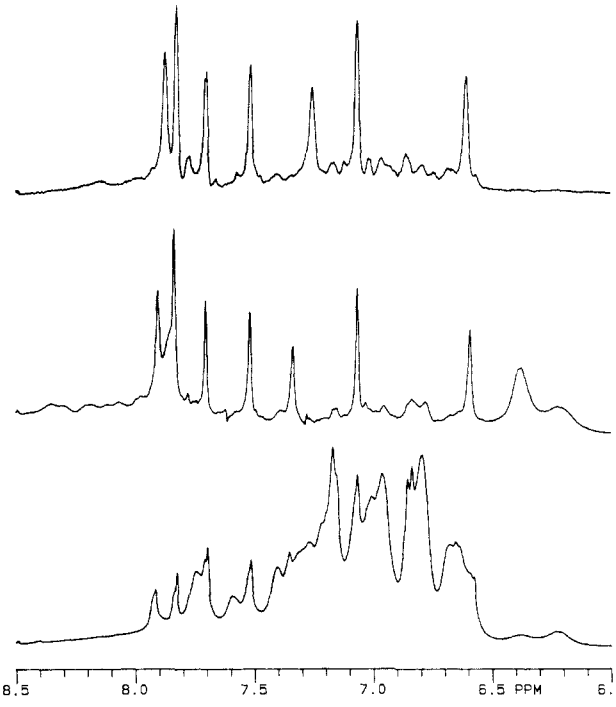

Figure 1. Aromatic regions of the proton NMR spectra at 400 MHz of SNase deuteriated in the phenylalanine, tyrosine, and tryptophan residues. Bottom, spectrum of protiated SNase in the presence of active site ligands at pH 7.8 and 30 °C; middle, spectrum of deuteriated SNase in the presence of active site ligands at pH 7.8 and 30 °C; top, spectrum of deuteriated SNase in the absence of active site ligands at pH 7.8 and 30 °C. In the middle and top spectra the assignments of the four sharp resonances associated with H_ϵ (7.5 to 8.1 ppm) from upfield to downfield are 121, 124, 8, and 46, respectively. The assignments of three sharp resonances associated with the H_δ protons (6.6 to 7.4 ppm) from upfield to downfield are 124, 8, and 46; the resonances associated with the H_δ proton of 121 are centered at 6.9 ppm. Signals from $H_{1'}$ of bound and free thymidine 3′,5′-bisphosphate appear between 6.3 and 6.5 ppm, and those from H_6 appear at 6.9 and 7.9 ppm.

The presence of the active site ligands does not induce major changes in the resonances of any of the histidine protons (Figure 1). The resonances assigned to His 46 undergo chemical shift changes which result from a change in the pK_a of this residue upon binding of the ligands in the adjacent active sites. The two resonances associated with H_δ of His 121 are not altered by the binding of ligands, demonstrating that cis–trans isomerism of Pro 117 is not the only cause for conformational heterogeneity in SNase.

Two resonances are observed for H_δ of His 121 over the temperature range of 5–40 °C in the absence of active site ligands (data not shown). One- and two-dimensional magnetization transfer experiments have been performed both in the absence and presence of ligands at 30 °C, and these data (not shown) indicate that the rate of interconversion of the His 121 H_δ resonances is approximately 10 s^{-1}.

The conformational heterogeneity described by Fox and Dobson at pH 5.5 in the absence of active site ligands is likely to be the result of cis–trans isomerism of Pro 117. However, in the presence of active site ligands we have detected at least two resonances for the H_δ but not H_ϵ protons of His 121 (as well as minor resonances for H_δ protons whose number and intensity can be modulated by pH.[8] It is plausible that two resonances are observed for H_δ of His 121 since the histidine ring may slowly flip between two

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(8) We have also studied the pH dependence of the aromatic region of the spectrum of the deuteriated SNase in the presence of active site ligands. Over the pH range from 5 to 8.5, the H_δ of His 121 appears to remain in multiple environments as judged by the absence of an eighth sharp resonance. At both extremes of pH, minor resonances in the chemical shift range for the H_δ protons are observed. At intermediate pH values the minor peaks observed for the H_δ protons increase in both number and intensity. The details of these studies will be reported elsewhere.

orientations about the β-γ bond; in one orientation N_δ (or N3) of His 121 could be a hydrogen bond acceptor, and in the other orientation N_ϵ (or N1) can act as a hydrogen bond donor to neighboring residues such as Glu 75, Tyr 91, and/or Tyr 93.[9]

The direct observation of the H_δ protons of histidine residues has allowed heterogeneity to be detected under conditions where it would have been missed if only the H_ϵ protons had been studied. For example, our isotopic labeling revealed heterogeneity in addition to that caused by proline isomerism[1,2] and allowed direct observation of the H_δ resonance of His 121 that could not be located in studies recently reported by Markley's laboratory.[7] We expect that the ability to detect conformational heterogeneity in SNase via both the H_δ and H_ϵ protons will prove useful in our studies of the effect of active site mutations on the conformations of the mutant proteins.[5,6]

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A Practical and Enantioselective Synthesis of Glycosphingolipids and Related Compounds. Total Synthesis of Globotriaosylceramide (Gb_3)

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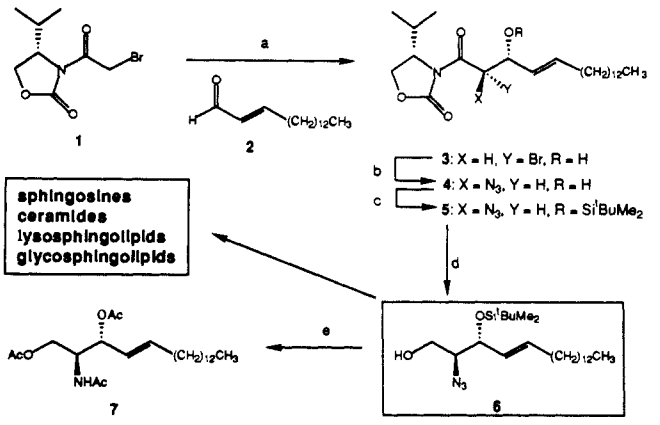
Glycosphingolipids are a class of naturally occurring bioactive compounds usually embedded in the membrane of all animal cells and in some plant cells.[1] The clinically important blood group antigens[2] and the immunologically relevant tumor-associated oligosaccharides[3] are examples of glycosphingolipids. The "intelligent" roles attributed to these biomolecules include mediation of cell-cell recognition and communication, growth regulation, and antibody interactions.[4] Due to their increasingly recognized importance in biomedical research, these molecules have attracted considerable attention from the isolation[5] and synthetic[6] points of view. Despite these efforts, however, these molecules remain relatively inaccessible, particularly in homogeneous form. In this communication, we report (1) a practical, short, and enantioselective route to glycosphingolipids which can also deliver enantiomerically pure sphingosine, ceramides, lysosphingolipids, and other related derivatives and (2) a total synthesis of globotriaosylceramide (Gb_3, **8**) and confirmation of its structure.

Scheme I[a]



[a] Reagents and conditions: (a) 1.2 equiv of $(nBu)_2BOTf$, 1.4 equiv of Et_3N, -78 °C, 30 min, then 20 °C for 2 h, added 0.75 equiv of **2** at 0 °C, 2 h, then H_2O_2/MeOH/ether, 0 °C, 1 h, 72%; (b) 2.0 equiv of NaN_3, DMSO, 25 °C, 12 h, 92%; (c) 1.5 equiv of t-$BuMe_2SiOTf$, 2.0 equiv of 2,6-lutidine, CH_2Cl_2, 0 °C, 2.5 h, 97%; (d) 3.0 equiv of $LiBH_4$, THF 0 °C, 3 h, 81%; (e) (i) 1.5 equiv of nBu_4NF, THF, 25 °C, 1 h, 90%, (ii) 10 equiv of $HS(CH_2)_3SH$, 10 equiv of Et_3N, MeOH, 25 °C, 24 h, (iii) 4.0 equiv of Ac_2O, 4.0 equiv of DMAP, CH_2Cl_2, 0 °C, 90%.

The strategy for the present synthesis of glycosphingolipids focuses on the asymmetric construction of the sphingosine equivalent **6** (Scheme I) following the principles advanced by Evans et al.[7] and Pridgen et al.[8] and its efficient and stereospecific coupling to carbohydrate fragments with the two-stage activation procedure for glycosidation recently reported from these laboratories[9] (Scheme II). The details for the synthesis of the sphingosine pregenitor **6** are shown in Scheme I. Thus, the oxazolidinone derivative **1** was converted to its boron enolate and condensed with the α,β-unsaturated aldehyde **2** to afford derivative **3**[10] in 72% yield.[11] Displacement of the bromide in **3** with NaN_3 led to the azide **4** in 92% yield with complete inversion of stere-

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(10) All new compounds exhibited satisfactory spectral and analytical and/or exact mass data. Yields refer to spectroscopically and chromatographically homogeneous materials.

(11) In addition to compound **3**, a second product, presumed to be the other syn diastereoisomer of **3**, was obtained in ca. 5% yield.

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