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Facile Hydrolysis and Formation of Amide Bonds by N-Hydroxyethylation of α-Amino Acids

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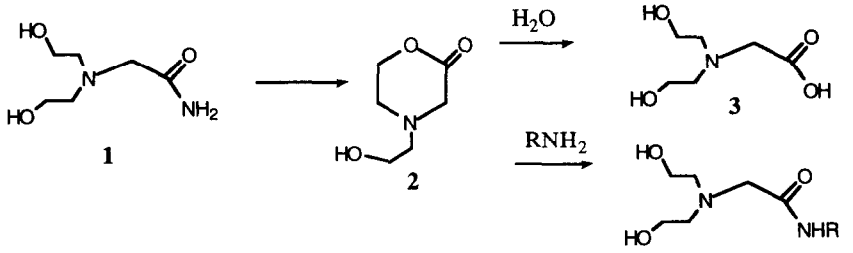
Abstract: The C-terminal amides of α-amino acids are readily hydrolyzed at 25° and pH 7 when the N-terminus is N-hydroxyethylated, with one or two hydroxyethyl groups. The reaction proceeds via cyclization to a morpholinolactone (**2**) which is rapidly hydrolyzed by water. In the presence of equimolar amounts of amines or amino acid derivatives, **2** reacts in H_2O without condensing agents to form a new peptide bond.

Amide bonds are highly resistant to hydrolysis. Recently, catalytic antibodies[1] and catalytic RNA molecules[2] were prepared which hydrolyze amides. But in general, the substrates of such catalysts have been amides which were activated by strain or electronic factors. Smaller organic molecules of precisely designed structures also have, on occasion, exhibited high rates of hydrolysis, and attachment of certain metal binding sites can facilitate amide hydrolysis.[3-6] However, most classes of amides are completely stable in water. We were thus surprised to observe, during the synthesis of some molecules designed to act as linkers, that hydroxyethylation of the N-terminus of α-amino acid amides (specifically the glycine derivative **1** $(HOCH_2CH_2)_2NCH_2CONH_2$) led to rapid rates of amide hydrolysis (half lives of a few hours at 25°, pH 7, in water) for this unstrained and electronically unactivated type of amide. Furthermore, transamidation was promoted, which resulted in the facile synthesis of peptide bonds from simple amide derivatives in water without the need of condensing agents.

^1H NMR spectroscopy was used to monitor the progress of the hydrolysis of bis N-2-hydroxyethylgylcineamide (**1**). The rate of hydrolysis of **1** was followed in 0.1 M phosphate buffer (pD 7.0) and at various temperatures. The k_{obs} for the disappearance of this glycine amide derivative was determined to be 6.40×10^{-5} sec $^{-1}$ at 24 °C, corresponding to a half life of 3 hours. Tenfold increases or decreases in buffer concentration had only a modest (less than twofold change) on the rate of amide hydrolysis. This rate is significantly faster that the hydrolysis of glycine amide,[7] which under similar conditions has been found to have a k_{obs} of 3×10^{-9} sec^{-1}, corresponding to a half life of approximately 7 years, or the hydrolysis of 4-hydroxybutyramide,[8] which gives a k_{obs} of 1.1×10^{-5} sec^{-1} at 100°C. The hydrolysis of **1** proceeds through a

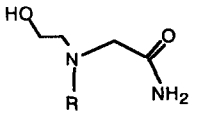
lactone intermediate **2**, which could be detected in the NMR spectrum of **1** during the hydrolysis experiments.[9]



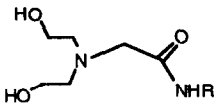
Opening of the lactone by water results in the acid **3** (bicine). Further support for this mechanism comes from experiments with an authentic sample of **2** that could be synthesized independently (by heating solid bicine to 195 °C for 10 min, then cooling under vacuum). The lactone is hydrolyzed very easily in aqueous solutions (k_{obs} $8.07x10^{-5}$ sec^{-1} in 0.1M phosphate buffer, pD 7.0) or in humid air and reacts with all substrates at rates consistent with it being an intermediate in these reactions.[10] A range of other N-hydroxyethyl amide derivatives was investigated at 25°, pD 7.0 (Table 1).[11] The putrescine derivative **4**, the dipeptides **5-7** and the tripeptide **8** all showed high rates of hydrolysis, although they hydrolyzed more slowly than **1**.

Table 1. Structures of Hydrolysis Substrates and Peptide Products (Phosphate buffer, pD 7.0, 25°)

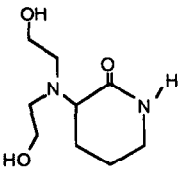
Substrate	k_{obs}		
1 R = H	$6.4x10^{-5}$ sec^{-1}	**10** R = H	$2.2x10^{-6}$ sec^{-1}
4 R = $-(CH_2)_4NH_2$	$1.3x10^{-5}$ sec^{-1}	**11** R = $CH_2CH_2OSi(CH_3)_2{}^tBu$	$1.4x10^{-6}$ sec^{-1}
5 R = $-CH_2CONH_2$	$1.7x10^{-6}$ sec^{-1}		
6 R = $-CH(Ph)CONH_2$	$7.5x10^{-7}$ sec^{-1}		
7 R = $-CH(CH_2OH)CONH_2$	$2.7x10^{-6}$ sec^{-1}		
8 R = $-CH_2CONHCH_2CONH_2$	$3.5x10^{-6}$ sec^{-1}		



10, 11



1, 4-8



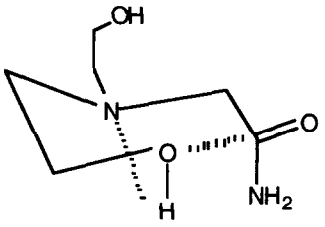
9

The ornithine derived amide, **9**, did not undergo enhanced hydrolysis. Compounds with a single hydroxyethyl group, **10** or **11**, underwent hydrolysis more slowly than would be predicted by statistical factors alone, suggesting that both hydroxyethyl groups are involved in hydrolysis.

It has been reported that 2-amino alcohols and related compounds catalyze hydrolysis of activated esters and activated amides.[12] In order to investigate the possibility of comparable chemistry in this system, we allowed **1** to react with glycine amide, and monitored the reaction for the generation of glycine at pD's 7 and 9, 25°C. We did indeed observe the formation of a new product when **1** and glycine amide were allowed to react

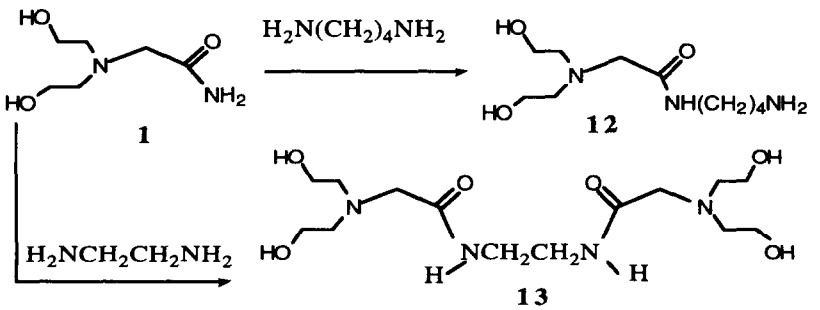
under these conditions. However, this new product was the dipeptide **5**, the result of condensation of **1** and glycine amide. This reaction represents the synthesis of a dipeptide from unactivated amides in aqueous solution without a condensing agent. Other nucleophiles, including phenylalanine amide, glycylglycine and serine amide also gave condensation products (**5-8**, Table 1), in yields of 40-60% for equimolar amounts of reactants. Of course, these peptides were metastable, eventually undergoing hydrolysis to release acid **3**.

Unlike standard unactivated amides, which show a minimum in the rate of hydrolysis near neutral pH,[13,14] and maxima at extremes of pH, **1**'s rate of hydrolysis increases only slightly with pH from pH 4 to pH 8 (3.2 to 7.3×10^{-5} sec^{-1}). The recently elucidated mechanism for penicillin acylase[15] suggests one possible source of rate accleration in our system. For the acylase, the N-terminal serine hydroxyl is activated by hydrogen bonding to the protein's terminal NH_2. A similar conformation for **1** can be constructed as shown below. For a structure minimized using the CACHE forcefield and the constraint that one hydroxyethyl group



oxygen should be within 3 Å of the amide carbonyl, the amine nitrogen is positioned 2.99 Å from the oxygen. In the crystal structure of the serine protease α-chymotrypsin, the nitrogen of the catalytic triad's His 57 is positioned 2.72Å from the oxygen of Ser 195.[16] Thus, we view **1** as a very simple serine protease mimic. The second hydroxyethyl group is positioned either to help stabilize the tetrahedral intermediate, or aid in the departure of the amine. Such a compact transition state may explain why amide **9** did not exhibit enhanced reactivity. Alternatively, one could achieve catalysis through amine protonation, with the protonated tertiary amine serving as a hydrogen bond donor to the amide carbonyl, activating the amide toward attack by one of the hydroxyethyl groups.

We have noticed several aspects of this reaction that remain to be explained. When **1** is treated with an excess of most diamines, such as putrescine, the expected transamidation product **12** is formed in excellent yield. However, when a large excess of ethylenediamine reacts with **1**, the only product which can be isolated is the dimer **13**. This dimer shows no enhanced hydrolysis. The crystal structures of both the glycine derivative **1** and compound **13** were determined by single crystal X-ray diffraction. Both compounds show the normal hydrogen bonding patterns seen for amides, with no close interactions between the hydroxyl groups and the amide carbonyls.



The N-terminus of proteins, in particular the N-terminal valine of hemoglobin, has been known for some time to react with ethylene oxide to generate N-hydroxyethyl residues.[17] This chemical modification has been used as a dosimeter for ethylene oxide exposure *in vivo*.[18] The lability of the N-hydroxyethyl amides and peptides reported herein raises the possibility that at least some modified proteins can lose N-terminal residues after exposure to ethylene oxide. If enhanced hydrolysis is seen with terminal N-hydroxyethylated hemoglobin, it could affect estimates of human exposure to the carcinogen ethylene oxide made using that marker.

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