nounced structural congruence of the electrophilic sites (arrows in **2** and **3**) of reductively activated mitomycin C (mitosene **2**) and oxidatively activated pyrrolizidine esters (dehydropyrrolizidine alkaloids, **3**) suggests that deoxyguanosine residues of 5′-d(CG) might likewise be cross-linkable by activated pyrrolizidine alkaloids.[20] Dehydroretronecine diacetate (**4**) was incubated with a radiolabeled DNA duplex containing a single 5′-d(CG) sequence.[21] Processing of the least electrophoretically mobile product as above afforded short, radiolabeled fragments for cleavage from the radiolabeled end through G11 (Figure 3). This is consistent with cross-linkage predominantly at the deoxyguanosine of 5′-d(CG).

These experiments demonstrate conclusively that sequence-random cleavage of cross-linked DNAs can be used to define sites of interstrand cross-linkage in DNA at single-nucleotide resolution.[22]

Acknowledgment. We thank the Sloan Foundation and the National Institutes of Health (AG00417, GM35466, GM32681) and National Science Foundation (DIR-8220099) for financial support, Susan Ribeiro for synthetic DNA, Ug-Sung Kim and Gary Anderson for technical assistance, Dr. John C. Amedio, Jr., and Professor James D. White for the generous gift of dehydroretronecine, and Professor Stanley Raucher for helpful discussions.

(20) (a) Culvenor, C. C. J.; Downing, D. T.; Edgar, J. A.; Jago, M. V. *Ann. N.Y. Acad. Sci.* **1969**, *163*, 837. (b) Mattocks, A. R. *J. Chem. Soc. C* **1969**, 1155.

(21) Labeled DNA (1.7 mM base pairs) in 50 μL of 50 mM sodium citrate buffer (pH 5.0), 5 mM NaCl, 5 mM $MgCl_2$ was vortexed at 25 °C with 0.4 mL of a $CDCl_3$ solution of dehydroretronecine diacetate. After 35 min, the DNA solution was ethanol precipitated and subjected to 25% denaturing PAGE. Cross-linked material was identified by comparison to the mitomycin cross-linked DNA of the same sequence. The specific activity of the cross-linked DNA was enhanced by reexposure to Klenow fragment and [α-^{32}P]-dATP prior to fragmentation.

(22) This chemical-based method complements existing methods involving inhibition of enzymatic reactions (polymerase,[23] restriction endonuclease,[3b] exonuclease[3c,24]).

(23) Piette, J. G.; Hearst, J. E. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 5540.

(24) (a) Sage, E.; Moustacchi, E. *Biochemistry* **1987**, *26*, 3307. (b) Boyer, V.; Moustacchi, E.; Sage, E. *Biochemistry* **1988**, *27*, 3011. (c) Kochel, T. J.; Sinden, R. R. *J. Mol. Biol* **1989**, *205*, 91.

Enzymatic Peptide Synthesis via Segment Condensation in the Presence of Water Mimics

Hiroshi Kitaguchi† and Alexander M. Klibanov*

Department of Chemistry
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

Received August 28, 1989

Convergent condensation of peptide segments (e.g., prepared by the stepwise solid-phase methodology[1]) is a powerful strategy for the synthesis of biologically active polypeptides, in particular if many homologous sequences are needed.[2] Enzymatic methods, being mild and selective, have proven attractive for peptide synthesis.[3] However, the use of enzymes as catalysts of peptide segment coupling has been rare[4] due to their propensity to concomitantly hydrolyze the growing polypeptide chain.[3] Thus the versatile protease thermolysin[5] has been widely used for the synthesis of dipeptides (including the commercial production of the sweetener aspartame[6]) but not polypeptides.[7]

Table I. Initial Rates of the Reaction between Z-Gly-Gly-Phe and Phe-NH_2 Catalyzed by Thermolysin in *tert*-Amyl Alcohol Containing Various Cosolvents[a]

cosolvent, % (v/v)	reactn rate,[b] μM h^{-1} (mg of enzyme)$^{-1}$
none	0
1% water[c]	19
4% water[c]	3500
1% water[c] + 9% formamide[d]	3800
1% water[c] + 9% ethylene glycol[d]	1500
1% water[c] + 9% glycerol	820
1% water[c] + 9% ethylene glycol monomethyl ether	140
1% water[c] + 9% methanol	130
1% water[c] + 9% ethylene glycol dimethyl ether	60
1% water[c] + 9% dimethylformamide	51
1% water[c] + 9% tetrahydrofuran	25

[a] Conditions: 10 mM Z-Gly-Gly-Phe, 25 mM Phe-NH_2, 0.5 mg/mL thermolysin,[5] 45 °C, shaking at 300 rpm. The enzyme (Sigma) was prepared by lyophilization of a 5 mg/mL aqueous solution, pH 7.2, containing 10 mM $Ca(CH_3COO)_2$; the resultant powder was placed in a substrate solution and sonicated for 5 s. No peptide-bond formation was detected without thermolysin. [b] The initial rate was measured by HPLC (Waters' μBondapak C_{18} column, $CH_3CN/H_2O/CF_3COOH$ (45:55:0.1) as a mobile phase) by following the formation of the tetrapeptide product. [c] In all cases, the term "water" refers to an aqueous solution, pH 7.2, containing 10 mM $Ca(CH_3COO)_2$. [d] No reaction was observed when *all* of the water was replaced with formamide or ethylene glycol.

Many of these thermolysin-catalyzed reactions have been carried out in aqueous–organic mixtures in order to shift the thermodynamic equilibrium toward peptide-bond formation.[3] This reaction medium should also diminish the unwanted secondary proteolytic cleavage. Attempts to maximize these benefits by completely replacing water with organic solvents,[8] however, result in the loss of thermolysin activity: e.g., as one can see in the first line of Table I, the enzyme fails to form a (favored in water[9]) Phe–Phe peptide bond in anhydrous *tert*-amyl alcohol.[10] This enzymatic reaction becomes noticeable at 1% of water and very fast at 4% (lines 2 and 3, respectively, in Table I). Unfortunately, the efficient synthesis of the Phe–Phe peptide bond catalyzed by thermolysis in *tert*-amyl alcohol containing 4% of water is accompanied by a substantial secondary hydrolysis: e.g., after 6

† Present address: Ashigara Research Laboratories, Fuji Photo Film Co., Ltd., Minamiashigara, Kanagawa 250-01, Japan.

(1) Merrifield, R. B. *Angew. Chem., Int. Ed. Engl.* **1985**, *24*, 799.

(2) Kaiser, E. T.; Mihara, H.; Laforet, G. A.; Kelly, J. W.; Walters, L.; Findeis, M. A.; Sasaki, T. *Science* **1989**, *243*, 187.

(3) Jakubke, H.-D.; Kuhl, P.; Konnecke, A. *Angew. Chem., Int. Ed. Engl.* **1985**, *24*, 85. Kullmann, W. *Enzymatic Peptide Synthesis*; CRC Press: Boca Raton, FL, 1987.

(4) For a recent clever example, see: Nakatsuka, T.; Sasaki, T.; Kaiser, E. T. *J. Am. Chem. Soc.* **1987**, *109*, 3808.

(5) Endopeptidase (EC 3.4.24.4) from *Bacillus thermoproteolyticus*. For a recent review, see: Matthews, B. W. *Acc. Chem. Res.* **1988**, *21*, 333.

(6) Oyama, K.; Kihara, K. *CHEMTECH* **1984**, *14*, 100.

(7) Isowa, Y.; Ohmori, M.; Ichikawa, T.; Kurita, H.; Sato, M.; Mori, K. *Bull. Chem. Soc. Jpn.* **1977**, *50*, 2762. Isowa, Y.; Ichikawa, T.; Ohmori, M. *Ibid.* **1978**, *51*, 271. Isowa, Y.; Ichikawa, T. *Ibid.* **1979**, *52*, 796. Isowa, Y., et al. *Tetrahedron Lett.* **1979**, 2611. Oyama, K.; Nishimura, S.; Nonaka, Y.; Kihara, K.; Hashimoto, T. *J. Org. Chem.* **1981**, *46*, 5241. Oyama, K.; Kihara, K.; Nonaka, Y. *J. Chem. Soc., Perkin Trans. 2* **1981**, 356. Nakanishi, K.; Kamikubo, T.; Matsuno, R. *Bio/Technology* **1985**, *3*, 459. Ooshima, H.; Mori, H.; Harano, Y. *Biotechnol. Lett.* **1985**, *7*, 789. DeMiranda, M. T. M.; Cheng, E.; Muradian, J.; Seidel, W. F.; Tominaga, M. *Bioorg. Chem.* **1986**, *14*, 182. Riechman, L.; Kasche, V. *Biochim. Biophys. Acta* **1986**, *872*, 269. Nakanishi, K.; Matsuno, R. *Eur. J. Biochem.* **1986**, *161*, 533. Kamihira, M.; Taniguchi, M.; Kobayashi, T. *Agric. Biol. Chem.* **1987**, *51*, 3427. Cassells, J. M.; Halling, P. J. *Enzyme Microb. Technol.* **1988**, *10*, 486. Sakina, K.; Kawazura, K.; Morihara, K.; Yajima, H. *Chem. Pharm. Bull.* **1988**, *36*, 3915 and 4345. Ferjancic, A.; Puigserver, A.; Gaertner, H. *Biotechnol. Lett.* **1988**, *10*, 101. Cheng, E.; De Miranda, M. T. M.; Tominaga, M. *Int. J. Peptide Protein Res.* **1988**, *31*, 116. Cassells, J. M.; Halling, P. J. *Biotechnol. Bioeng.* **1989**, *33*, 1489.

(8) Klibanov, A. M. *Trends Biochem. Sci.* **1989**, *14*, 141.

(9) Oka, T.; Morihara, K. *J. Biochem.* **1980**, *88*, 807.

(10) This solvent has been successfully used for subtilisin-catalyzed peptide synthesis: (a) Margolin, A. L.; Tai, D.-F.; Klibanov, A. M. *J. Am. Chem. Soc.* **1987**, *109*, 7885. (b) Kitaguchi, H.; Tai, D.-F.; Klibanov, A. M. *Tetrahedron Lett.* **1988**, *29*, 5487. (c) Chinsky, N.; Margolin, A. L.; Klibanov, A. M. *J. Am. Chem. Soc.* **1989**, *111*, 386.

0002-7863/89/1511-9272$01.50/0 © 1989 American Chemical Society

Table II. Preparative Synthesis of Oligopeptides via Segment Condensation Catalyzed by Thermolysin in *tert*-Amyl Alcohol Containing 1% of Water and 9% of a Water Mimic[a]

COOH donor	NH_2 donor	reactn product[b]	isolated yield, %
Z-Gly-Gly-Phe	Phe-NH_2	Z-Gly-Gly-Phe-Phe-NH_2[c]	76
Z-Gly-Gly-Phe	Phe-Phe-NH_2	Z-Gly-Gly-Phe-Phe-Phe-NH_2[d]	72
Z-Gly-Pro-Phe-Pro-Leu	Leu-NH_2	Z-Gly-Pro-Phe-Pro-Leu-Leu-NH_2[e]	73
Z-Gly-Pro-Gly-Gly-Pro-Ala	Leu-Leu-Phe-NH_2	Z-Gly-Pro-Gly-Gly-Pro-Ala-Leu-Leu-Phe-NH_2[f]	67

[a] Conditions: 3 mg/mL thermolysin (prepared as described in footnote *a* to Table I) was used as a catalyst at 45 °C; water mimics were ethylene glycol in the fourth entry and formamide in all others; suspensions containing the enzyme and substrates were shaken at 300 rpm. The COOH and NH_2 donor substrate concentrations were, respectively, 150 and 200 mM (first entry), 40 and 50 mM (second entry), 100 and 200 mM (third entry), and 40 and 80 mM (fourth entry). The reaction times were (top to bottom) 17, 37, 30, and 96 h. The peptide synthesis reactions were stopped by evaporating the solvent under vacuum; the residues formed were washed with 1 N HCl, 0.5 M $NaHCO_3$, and water, followed by drying and recrystallization/reprecipitation. See footnote *c* to Table I for the meaning of "1% of water" here. Note that, apart from their enzyme activating effect, ethylene glycol and formamide greatly improve the solubility of peptides in *tert*-amyl alcohol. [b] Product compositions were confirmed by amino acid analysis. [c] The crystalline product (64 mg) had mp 201-202 °C and $[\alpha]^{25}_D$ -24.0° (*c* 0.2, DMF). [d] The amorphous product (61 mg) had $[\alpha]^{25}_D$ -18.0° (*c* 0.2, DMF). [e] The amorphous product (56 mg) had $[\alpha]^{25}_D$ -77.5° (*c* 0.2, DMF). [f] The amorphous product (65 mg) had $[\alpha]^{25}_D$ -59.5° (*c* 0.2, DMF).

h, when 82% of Z-Gly-Gly-Phe has reacted, almost one-third of the product is the dipeptide Z-Gly-Gly (with the rest being the desired tetrapeptide).[11]

In a quest to reconcile the opposing effects of water on the desired product yield and enzymatic reaction rate, we have addressed the latter phenomenon mechanistically. It seems likely that water activates thermolysin by enhancing the enzyme's conformational flexibility.[8,12] Since water's role as a molecular lubricant in proteins[13] is due to its ability to form multiple hydrogen bonds, other solvents mimicking water in this respect may, at least partially, substitute for it without promoting the hydrolytic side reactions. This hypothesis has been experimentally confirmed with several hydrogen bond forming solvents:[14] as seen in Table I, when three-quarters of the 4% of water in *tert*-amyl alcohol are replaced with 9% of formamide, the high level of thermolysin activity is retained, exceeding the rate observed when water is omitted by 200-fold; with two other water mimics,[14] ethylene glycol and glycerol, the reaction rates are not as high but still far greater than without them. Indicatively, the lesser the solvent's ability to form multiple hydrogen bonds, the lower its activating action on thermolysin (Table I).

Encouraged by the vigorous peptide synthesis catalyzed by thermolysin in *tert*-amyl alcohol containing 1% of water and 9% of formamide, we have utilized this solvent for the preparative enzymatic synthesis of Z-Gly-Gly-Phe-Phe-NH_2. As shown in the first line of Table II, the tetrapeptide has been prepared with a good yield; significantly, no formation of byproducts has been detected, in contrast to the situation observed at a 4% of water content.

The substrate specificity of thermolysin in *tert*-amyl alcohol containing either 1% of water and 9% of formamide or 4% of water is similar to that in water:[9] L-Phe and L-Ala are favored as carboxyl and L-Phe and L-Leu as amino group donors.[15] When thermolysin was presented with *N*-Ac-Phe and Phe-Lys-*O*-*tert*-Bu as substrates, only the natural Phe-Phe (as opposed to the unnatural Phe-ε-Lys) linkage was formed,[16] pointing to thermolysin's high fidelity even under these extreme conditions.

Table II depicts the results of the preparative segment condensation catalyzed by thermolysin in *tert*-amyl alcohol containing 1% of water and 9% of formamide or ethylene glycol. Four tetra- to nonapeptides were prepared in one step, with good isolated yields and with no appreciable secondary cleavage. Thus partial replacement of water with water-mimicking cosolvents may be beneficial for enzymatic peptide segment coupling by combining high reaction rates and the absence of side reactions. This approach should be applicable to other water-sensitive enzymatic processes in nonaqueous media.

Registry No. $CH_3CH_2CMe_2OH$, 75-85-4; Z-Gly-Gly-Phe, 13171-93-2; Phe-NH_2, 5241-58-7; Z-Gly-Pro-Phe-Pro-Leu-OH, 61867-13-8; Z-Gly-Pro-Gly-Gly-Pro-Ala-OH, 13075-38-2; Phe-Phe-NH_2, 15893-46-6; Leu-NH_2, 687-51-4; Leu-Leu-Phe-NH_2, 108370-29-2; Z-Gly-Gly-Phe-Phe-NH_2, 123963-61-1; Z-Gly-Gly-Phe-Phe-Phe-NH_2, 123963-62-2; Z-Gly-Pro-Phe-Pro-Leu-Leu-NH_2, 123992-45-0; Z-Gly-Pro-Gly-Gly-Pro-Ala-Leu-Leu-Phe-NH_2, 123963-63-3; H_2NCHO, 75-12-7; $HOCH_2CH_2OH$, 107-21-1; $(CH_2OH)_2CHOH$, 56-81-5; $MeOCH_2CH_2OH$, 109-86-4; MeOH, 67-56-1; $MeOCH_2CH_2OMe$, 110-71-4; Me_2NCHO, 68-12-2; thermolysin, 9073-78-3; tetrahydrofuran, 109-99-9.

(11) Conditions: 20 mM Z-Gly-Gly-Phe, 50 mM Phe-NH_2, and 1 mg/mL thermolysin; for other conditions, see Table I. The enzymatic reaction was followed by HPLC precalibrated with the authentic peptides.

(12) Rupley, J. A.; Gratton, E.; Careri, G. *Trends Biochem. Sci.* **1983**, *8*, 18. Zaks, A.; Klibanov, A. M. *J. Biol. Chem.* **1988**, *263*, 8017.
(13) Finney, J. L.; Poole, P. L. *Comments Mol. Cell. Biophys.* **1984**, *2*, 129.
(14) Ray, A. *Nature* **1971**, *231*, 313.

(15) Reaction conditions were similar to those described in Table I.
(16) Determined by HPLC precalibrated with both isomers synthesized by the methods described in ref 10b and confirmed by ^1H NMR.

(17) This work was financially supported by NIH Grant GM39794.

Substituent Effects on the Gas-Phase Acidity of Silane

Mark S. Gordon* and David E. Volk

Department of Chemistry
North Dakota State University
Fargo, North Dakota 58105

David R. Gano

Department of Chemistry, Minot State University
Minot, North Dakota 58201

Received August 21, 1989

In a previous paper,[1] the gas-phase acidities of XH_n compounds (X = C, N, O, F, Si, P, S, Cl) were predicted with ab initio wave functions. At the MP4[2] level of theory with extended basis sets [6-311++G(3df,2pd)[3] for second-period atoms and 6-31++G-(3df,2pd)[4] for third-period atoms], the calculated gas-phase acidities for these species were determined to be within 2 kcal/mol of experimental values. Similar results for the second period were obtained by DeFrees and McLean.[5]

In the present work, with 6-31G(d) geometries and full MP4/MC-311++G[6](3df,2pd) energies, the effects of CH_3, NH_2,

(1) Gordon, M. S.; Davis, L. P.; Burggraf, L. W.; Damrauer, R. *J. Am. Chem. Soc.* **1986**, *108*, 7889. It was demonstrated in this and related works that the 6-31G(d) basis set is reasonable for the prediction of anion geometries, even though prediction of energetics requires diffuse functions in the basis set.

(2) Krishnan, R.; Frisch, M. J.; Pople, J. A. *J. Chem. Phys.* **1980**, *72*, 4244.
(3) (a) Frisch, M. J.; Pople, J. A.; Binkley, J. S. *J. Chem. Phys.* **1984**, *80*, 3265. (b) Spitznagel, G. W.; Clark, T.; Schleyer, P. v. R.; Hehre, W. J. *J. Comput. Chem.* **1987**, *8*, 1109.
(4) (a) Hariharan, P. C.; Pople, J. A. *Theor. Chim. Acta* **1973**, *28*, 213. (b) Gordon, M. S. *Chem. Phys. Lett.* **1980**, *76*, 163.
(5) DeFrees, D. J.; McLean, A. D. *J. Comput. Chem.* **1986**, *7*, 321.

0002-7863/89/1511-9273$01.50/0 © 1989 American Chemical Society