4-Bromobutyryl-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2·TFA (5). The protected peptide resin, bromobutyryl-Tyr(Bzl)-Phe-Gln-Asn-Cys-Pro-Arg(Tos)-Gly-PAM resin was prepared by coupling 4-bromobutyryl chloride to the resin, H-Tyr(Bzl)-Phe-Gln-Asn-Cys-Pro-Arg(Tos)-Gly-PAM resin, until it gave a negative Kaiser test. Peptide 5 was deprotected, cleaved from the resin, and purified by RPHPLC as described before. Yield: 75 mg, 8% from Boc-Gly-PAM resin (1.2 g, 0.68 mequiv/g). FABMS: $(M + H)^+$ calcd for $C_{47}H_{67}BrN_{14}O_{12}S$ m/e 1133, measured 1133. The peptide was used in the next step without further characterization.

δ[1-Carba,Gly^9]vasopressin (6). Liquid ammonia (50 mL) was condensed into a 250-mL 3-neck round bottom flask equipped with a dry ice condenser and cooled with a dry ice–2-propanol bath under an atmosphere of argon. A small piece of sodium was added, and the solution turned blue. A crystal of ammonium acetate hydrate was then added to decolorize the solution. A suspension of peptide 5 (10 mg) in degassed absolute ethanol was added into the stirred liquid ammonia at −78 °C under argon. The cooling bath was removed after the completion of addition, and the reaction mixture was allowed to reflux for 6 h. The ammonia was evaporated under a slow stream of argon overnight. Ethanol and residual ammonia were removed in vacuo. The solid residue was dissolved in degassed 0.2% TFA in water (7 mL), the pH was adjusted to 2 with TFA, and the residue was purified with RPHPLC. Removal of solvents by lyophilization gave peptide 6 as a white powder, 6.5 mg, in 65% yield. High-resolution FABMS (using deoxygenated ethanol): $(M + H)^+$ calcd for $C_{47}H_{67}N_{14}O_{12}S$ m/e 1051.4783, measured 1051.4780. Amino acid Anal.: Tyr, 0.98; Phe, 1.05; Glx, 1.08; Asx, 1.10; Cys-X, 0.90; Pro, 1.05; Arg, 1.05; Gly, 0.94.

Registry No. 1, 132126-26-2; **2a**, 132126-28-4; **2b**, 132126-30-8; **2c**, 132126-32-0; 3, 132155-35-2; 4, 132126-34-2; 5, 132126-36-4; 6, 64156-79-2; BOC-Ser(Bzl)-OH, 23680-31-1; BOC-Lys(Cl-Z)-OH, 54613-99-9; BOC-Cys(MeOBzl)-OH, 18942-46-6; $ClCH_2COOH$, 79-11-8; CH_2=CHCOOMe, 96-33-3; $PhCH_2Br$, 100-39-0; $Br(CH_2)_3COCl$, 927-58-2.

Peptide Synthesis Catalyzed by Modified α-Chymotrypsin in Low-Water Organic Media

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Enzyme-catalyzed synthesis of peptide bonds in organic solvents has been investigated by using α-chymotrypsin either modified with poly(ethylene glycol) or immobilized on different supports, in order to find out the importance of water content in the reaction. High yields of peptide synthesis were obtained whatever the type of enzyme derivative used. By varying the type of support, a modification in the enzyme environment was observed and resulted in a significant increase in the reaction yield when nucleophiles with poor affinity for the enzyme were used. Since organic solvents also affected substrate specificity with respect to the donor ester, a general methodology was proposed for the enzymatic synthesis of peptides in low-water organic media.

Introduction

The enzymatic synthesis of active peptides such as Leu- and Met-enkephalins (H_2N-Tyr-Gly-Gly-Phe-Leu(Met)-COOH) may be considered as an interesting example of the synthetic applicability of proteases to the preparation of naturally occurring peptides.[1-3] Other reactions such as esterification,[4] one-pot tripeptide synthesis,[5] and synthesis of isopeptides[6] or peptides containing D-amino acids[7] have been described. Two strategies are generally used in protease-catalyzed peptide formation: kinetics and equilibrium-controlled synthesis.[8] The enzymatic synthesis of peptides has the advantage of taking place under mild experimental conditions, and epimerization of chiral carbons is not observed. However, in many cases, the addition of water-soluble organic solvents to increase substrate solubility is accompanied by dramatic changes in the enzyme catalytic properties. A high percentage of organic solvent would be advantageous in suppressing competitive hydrolysis of the acyl-enzyme intermediate as well as secondary hydrolysis of the newly synthesized peptides. Such processes have been investigated in biphasic systems,[9] reversed micelles,[10] but in recent years catalysis in low-water organic media has been given increasing attention.[11] The enzyme can be directly suspended in the organic media, although it is preferable to stabilize enzymes through their immobilization on inert supports when water-miscible solvents are used. The enzyme can also be derivatized with poly(ethylene glycol) (PEG), an amphiphilic polymer, in order to make it soluble in organic solvents with most if not all of its original activity.[12] Modified enzymes have been used in the synthesis of peptides in benzene or 1,1,1-trichloroethane.[13,14] In the

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(1) Kullmann, W. *J. Biol. Chem.* **1980**, *255*, 8324.
(2) Kullmann, W. *Enzymatic Peptide Synthesis*; CRC Press: Boca Raton, FL, 1987; pp 61–82.
(3) Wan Yu Feng, D. M.; Xu, J. *Acta Chem. Sin.* **1985**, *3*, 236.
(4) Kise, H.; Shirato, H. *Tetrahedron Lett.* **1985**, *26*, 6081.
(5) Barbas, C. F.; Wong, C. H. L. *Tetrahedron Lett.* **1988**, *29*, 2907.
(6) Kitaguchi, H.; Tai, D. F.; Klibanov, A. M. *Tetrahedron Lett.* **1988**, *29*, 5487.
(7) West, J. B.; Wong, C.-H. *J. Org. Chem.* **1986**, *51*, 2728.
(8) Jakubke, H. D.; Kuhl, P.; Konnecke, A. *Angew. Chem., Int. Ed. Engl.* **1985**, *24*, 85.
(9) Khmelnitski, Y. L.; Dien, F. K.; Semenov, A. N.; Martinek, K. *Tetrahedron* **1984**, *40*, 4425.
(10) Luthi, P.; Luisi, P. L. *J. Am. Chem. Soc.* **1984**, *106*, 7285.
(11) Zaks, A.; Klibanov, A. M. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 3192.
(12) Matsushima, A.; Okada, M.; Inada, Y. *FEBS Lett.* **1984**, *178*, 275.
(13) Gaertner, H. F.; Puigserver, A. J. *Protein: Struct. Funct. Genet.* **1988**, *3*, 130.
(14) Gaertner, H. F.; Puigserver, A. J. *Eur. J. Biochem.* **1989**, *181*, 207.

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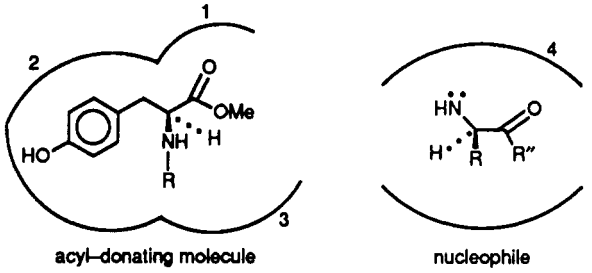
present paper, we show that the methodology of protease-catalyzed peptide synthesis in low-water organic media can be generalized, the enzyme being either soluble or immobilized. Several biocatalysts have been used for this purpose: α-chymotrypsin modified with cyanuric chloride activated methoxypoly(ethylene glycol) (PEG-Chti); α-chymotrypsin adsorbed on Celite (Chti-Celite) or pore glass (Chti-glass beads); α-chymotrypsin immobilized on Sepharose activated with either *p*-nitrophenyl chloroformate (Chti-carbonate), aldehyde (Chti-aldehyde), or tosyl chloride (Chti-tosyl).

α-Chymotrypsin is a well-known serine protease that catalyzes the synthesis of peptides in a two-step reaction, the acyl-enzyme intermediate being deacylated by either water or any added nucleophile.[15] N-acylated amino acid esters or peptide esters are used as acyl donors (the final carboxyl group position being occupied preferentialy by L-Phe, L-Trp, or L-Tyr) and amino acid derivatives as nucleophiles. The acyl donor is adsorbed on to a site where three subsites (1, 2, and 3) exclusively allow the native



enzyme to accept molecules with the specific configuration of *N*-acyl-L-amino acid.[7] Since subsite 2 is planar and strongly lipophilic, aromatic amino acids such as L-Phe, L-Trp, and L-Tyr can only be recognized as acyl donors. The nucleophile is adsorbed in subsite 4, a quite flexible region that will accommodate water, alcohols, and both L- and D-amino acids. Nevertheless, all these subsites in the enzyme active site can be constrained during the immobilization process so that selectivity could be changed when isosters of *N*-benzoyl-L-phenylalanine ethyl ester are used.[16]

In the present paper several samples of modified and immobilized α-chymotrypsin were investigated in the synthesis of peptide bonds in organic solvents with low percentages of water in order to point out the advantages of this methodology over catalysis in water–organic solvent media. The hydrophobic substrates such as Phe-NH_2 appeared as the most efficient acceptor nucleophiles.

Results and Discussion

High reaction yields were obtained in a typical experiment, which was carried out in 1,1,1-trichloroethane and involved Bz-Tyr-OEt as an acyl donor and Phe-NH_2 as a nucleophile, regardless of the type of enzyme derivative used (Figure 1). As already mentioned, a minimal amount of water was essential for enzymatic catalysis to proceed, and in all cases the reaction yield was dependent on the amount of water added to the reaction medium. Furthermore, each support could be characterized by an optimal percentage of water that reflects its capacity to adsorb water. Our results are in agreement with aquaphilicity determination of the support, as developed by Reslow et al.[17] The highest extent of synthesis was obtained with

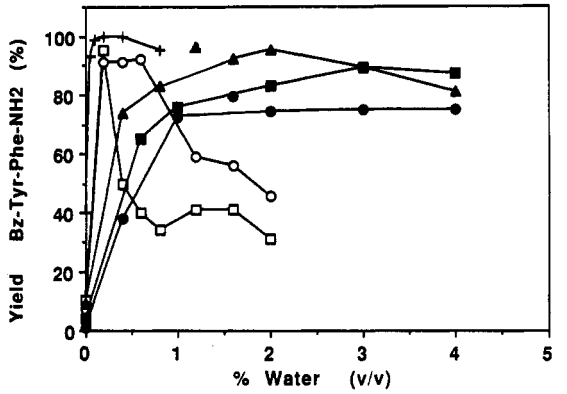


Figure 1. Influence of water on peptide synthesis catalyzed by different α-chymotrypsin derivatives in 1,1,1-trichloroethane. The reaction mixture (0.5 mL) containing 5 mM Bz-Tyr-OEt, 20 mM Phe-NH_2 0.8% MeOH, and DIPEA (v/v) and chymotrypsin modified with PEG (+), adsorbed on Celite (□) or glass beads (○) and immobilized on tosyl (■), aldehyde (▲), or carbonate (●) activated Sepharose was incubated at 37 °C for 20 h.

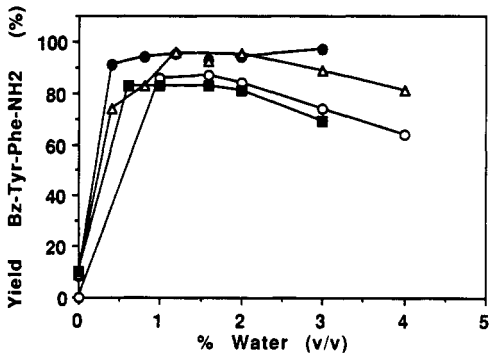


Figure 2. Influence of water on peptide synthesis catalyzed by chymotrypsin immobilized on aldehyde-activated Sepharose in different organic solvents. Reaction conditions were the same as in Figure 1 except that all reagents were solubilized in benzene (●), 1,1,1 trichloroethane (△), acetonitrile (○), or *tert*-amyl alcohol (□).

0.1% added water and PEG-Chti or with 0.2% added water and Chti adsorbed on Celite or glass beads, while a 1% water concentration at least was necessary in the case of Sepharose-immobilized Chti. Moreover, the plateau observed over a large range of water concentrations (1–4%) was further evidence of the ability of the polysaccharidic support to adsorb high amounts of water. On the other hand, Chti-Celite showed maximal activity over a narrow range of water concentrations (less than 0.4%), and for a higher water content, large amounts of the initial substrate were left in the reaction mixture. So it could be expected that increasing water concentration in samples containing chymotrypsin adsorbed on Celite or glass beads resulted in a high tendency of the enzyme to dissolve in the reaction medium, with a dramatic effect on stability.

Since about the same enzyme activity was used in all experiments, the catalytic activity of the different derivatives in 1,1,1-trichloroethane could be compared. Solubilization of the enzyme in the organic medium, enhancing internal diffusion and ensuring high conformational flexibility, may account for the high rates of synthesis observed with PEG-Chti. In the same way, higher activity of Chti-aldehyde when compared to other Sepharose-immobilized enzymes may be assigned to the presence of a somewhat longer arm between the support and the enzyme

(15) Blow, D. M. *Enzymes*; Academic Press: New York, 1971; Vol. III, pp 185–246.
(16) Cabezas, M. J.; Campo, C.; Llama, E.; Sinisterra, J. V. *J. Mol. Catal.*, in press.
(17) Reslow, M.; Adlercreutz, P.; Mattiasson, B. *Eur. J. Biochem.* **1988**, *172*, 573.

Table I. Influence of the Amino Acid Derivative Used as Nucleophile on Peptide Synthesis Catalyzed by Chymotrypsin in 1,1,1-Trichloroethane[a]

acceptor nucleophile	product (elution time, min)[b]	yield (%) PEG-Chti	yield (%) Chti/agarose
Phe-NH_2	Bz-Tyr-Phe-NH_2 (15.1)	95–98	75
Tyr-NH_2	Bz-Tyr-Tyr-NH_2 (11.4)	91	74
Thr-NH_2	Bz-Tyr-Thr-NH_2 (8.7)	94	94
Ser-NH_2	Bz-Tyr-Ser-NH_2 (7.8)	89	89
Gly-NH_2	Bz-Tyr-Gly-NH_2 (8.3)	69	64
Arg-NH_2	Bz-Tyr-Arg-NH_2 (6.1)	8	68
Lys-NH_2	Bz-Tyr-Lys-NH_2 (5.3)	4	67
Glu-NH_2	Bz-Tyr-Glu-NH_2 (8.7)	2	25

[a] Reaction was carried out as described in Figure 1 or 2 with a 4 M excess of nucleophile over Bz-Tyr-OEt, in the presence of 0.09% added water and PEG-Chti or 1% (v/v) 50 mM sodium phosphate buffer (pH 7.8) and Chti immobilized on carbonate-activated Sepharose. In all cases, except with Phe-NH_2, the amino acid amide was in suspension in the reaction mixture. [b] A linear gradient from 20 to 60% acetonitrile in 25 mM triethylammonium phosphate (pH 3.0) over 20 min with a flow rate of 1.0 mL/min was used.

Table II. Effect of the Donor Ester on PEG-Chymotrypsin-Catalyzed Peptide Synthesis in Benzene[a]

donor ester	peptide (elution time, min)[b]	yield (%)
Bz-Tyr-OEt	Bz-Tyr-Phe-NH_2 (15.1)	95–98
Ac-Phe-OEt	Ac-Phe-Phe-NH_2 (5.7)[c]	85
Ac-Trp-OEt	Ac-Trp-Phe-NH_2 (5.8)[c]	95–98
Bz-Arg-OEt	Bz-Arg-Phe-NH_2 (9.7)	95–98
Bz-Lys-OMe	Bz-Lys-Phe-NH_2 (9.2)	95–98
Bz-Ala-OMe	Bz-Ala-Phe-NH_2	0
Z-Ser-OMe	Z-Ser-Phe-NH_2	0
Z-Glu-OBzl	Z-Glu-Phe-NH_2	0
Bz-Asp-OMe	Bz-Asp-Phe-NH_2	0

[a] The reaction mixture in benzene was comparable to that used in Table I. [b] A linear gradient from 20 to 60% acetonitrile in triethylammonium phosphate over 20 min with a flow rate of 1.0 mL/min was used. [c] A linear gradient from 40 to 80% acetonitrile in triethylammonium phosphate over 20 min with a flow rate of 1.0 mL/min was used.

that readily permits conformational changes in the protein.

The organic solvent may directly interact with the essential water in the vicinity of the enzyme and thus affect catalysis. While PEG-Chti was totally inactivated in water-miscible organic solvents, immobilized chymotrypsin could be used in various organic–water media and in hydrophobic solvents. As shown in Figure 2, high yields of peptide synthesis were obtained in benzene, 1,1,1-trichloroethane, acetonitrile, and *tert*-amyl alcohol as well as in toluene or THF (results not shown). The more hydrophobic the solvent, the higher the maximum yield.

In order to confirm the general use of this methodology for peptide synthesis, various amino acid derivatives were investigated as nucleophiles or acyl donors under the above-mentioned conditions (Tables I and II). Since nucleophile specificity in protease-catalyzed peptide synthesis is known to reflect the P'_1 specificity[18] in the corresponding hydrolytic reaction,[19] high yields of peptide bond formation could be expected with hydrophobic or bulky amino acid amides (Phe-NH_2, Tyr-NH_2). High yields were still obtained with hydrophilic amino acids (Ser-NH_2, Gly-NH_2). Hydrophobic PEG-Chti gave low yields with charged acceptor nucleophiles such as Arg-NH_2 or Lys-NH_2. Nevertheless, high yields were obtained with these nucleophiles using Chti/agarose. It may therefore be assumed that in the case of Chti/agarose the presence of an hydrophilic polymer increased significantly the water concentration in the enzyme vicinity as well as local concentration of the charged nucleophile near the enzyme active site. Thus, it appeared that using the appropriate enzyme derivative resulted in a significant increase in the variety of peptides that can be synthesized by the same enzyme in organic media. Increasing the solubility of polar nucleophile substrates by operating in polar solvents (acetonitrile or THF) did not improve the reaction yield but facilitated the separation of the soluble products from the immobilized enzyme in the case of large-scale synthesis. Moreover, the fact that ester derivatives of aromatic as well as basic amino acids were efficient acyl donors strengthened the potential of the methodology used throughout this study. The modification of substrate specificity of chymotrypsin in organic media has already been discussed.[14,20] These results confirm the observed high specificity of the enzyme for hydroxylated aromatic amino acid esters in organic media[21] and show a sharp improvement of the reaction yield when compared to those obtained in aqueous organic phase.[22,23] At high water concentrations, the high yields reported so far were obtained with hydrophobic products precipitating in the reaction medium, while more hydrophilic substrates lowered the yield of peptide.[19,24]

Several peptides were synthesized on a large scale with either PEG-Chti or Sepharose-immobilized Chti as a catalyst. Since the crystallized peptides were pure, additional purification was not necessary. Their elemental analyses are shown in Table III. Besides activity, stability is one of the most important properties of biocatalysts when they have to be used in synthetic processes. Accordingly, some experiments of reuse were carried out with PEG-Chti in the synthesis of Bz-Tyr-Phe-NH_2 (Table IV). Since Bz-Tyr-Phe-NH_2 precipitated in the reaction medium containing soluble PEG-Chti, the peptide could be removed by filtration and synthesis was repeated by adding reagents to the mixture. Appearance of some product of hydrolysis may result from some difficult control of the water content in the reaction medium. Peptide synthesis decreased with the number of cycles. However, a nearly 80% yield in the fifth cycle of synthesis following a total period of incubation of more than 60 h supports a high stability of PEG-Chti in organic media, especially because some enzyme was lost at each separation step. Thus, these biocatalysts are interesting for their potential applications in organic synthesis, particularly in the cases of Tyr-Phe, Phe-Phe and Trp-Phe for which yields >90% are not accessible by other enzymatic methodologies.

Experimental Section

Materials. Chymotrypsin was from Worthington Biochemical Co. (Freehold, NJ). Monomethoxypoly(ethylene glycol) (M_r 5000), cyanuric chloride, *p*-nitrophenyl chloroformate, and controlled-pore glass (200–400 mesh, surface area 8.2 m^2/g, mean pore diameter 208 nm) were supplied by Sigma Chemical Co. (St. Louis, MO). Celite (30–80 mesh) and Sepharose CL 6B were obtained from BDH. Ac-Tyr-OEt, Bz-Tyr-OEt, Ac-Phe-OEt, Bz-Lys-OMe, Bz-Arg-OEt, Bz-Ala-OMe, Z-Ser-OMe, Z-Glu-OBzl, Bz-Asp-OMe, Phe-NH_2, Tyr-NH_2, Ser-NH_2, Thr-NH_2, and Lys-NH_2 were from Bachem (Bubendorf, Switzerland). Anhydrous solvents (puran grade) containing less than 100 ppm water were supplied by SDS

(18) Schechter, I.; Berger, A. *Biochem. Biophys. Res. Commun.* **1967**, *27*, 157.
(19) Morihara, K.; Oka, T. *Biochem. J.* **1977**, *163*, 531.
(20) Zaks, A.; Klibanov, A. M. *J. Am. Chem. Soc.* **1986**, *108*, 2767.
(21) Ricca, J. M.; Crout, D. H. G. *J. Chem. Soc., Perkin Trans. 1* **1989**, 2126.
(22) Kise, H.; Fujimoto, K.; Noritomi, H. *J. Biotechnol.* **1988**, *8*, 279.
(23) Salvador, D.; Sinisterra, J. V.; Guisan, J. M. *J. Mol. Catal.*, in press.
(24) Oka, T.; Morihara, K. *J. Biochem.* **1977**, *82*, 1055.

Table III. Characterization of Peptides Synthesized on a Large Scale

peptide (mp, °C)	empirical formula (MW)	elemental anal.: found (calcd) C	H	N
Bz-Tyr-Phe-NH_2 (268–270)[a]	$C_{25}H_{25}N_3O_4$ (431)	69.4 (69.6)	5.7 (5.8)	9.4 (9.7)
Bz-Tyr-Tyr-NH_2 (271–272)	$C_{22}H_{24}N_3O_5$ (447)	67.0 (67.1)	5.6 (5.6)	9.2 (9.4)
Bz-Tyr-Thr-NH_2 (247–248)	$C_{20}H_{23}N_3O_5$ (385)	62.0 (62.3)	6.1 (6.0)	10.9 (10.9)
Bz-Tyr-Gly-NH_2 (218)	$C_{18}H_{19}N_3O_4$ (341)	63.6 (63.3)	5.7 (5.6)	12.2 (12.3)
Bz-Tyr-Ser-NH_2 (210–212)	$C_{19}H_{21}N_3O_5$ (371)	61.1 (61.5)	5.8 (5.7)	11.0 (11.3)
Bz-Tyr-Lys-NH_2 (250)[a]	$C_{22}H_{28}N_4O_4$ (412)	64.2 (64.1)	6.9 (6.8)	13.7 (13.6)
Bz-Lys-Phe-NH_2 (223–224)	$C_{22}H_{28}N_4O_3$ (396)	66.5 (66.7)	7.0 (7.1)	14.2 (14.1)

[a] Decomposition of the product.

Table IV. Reutilization of PEG-Chymotrypsin in the Synthesis of Bz-Tyr-Phe-NH_2

cycle no.	yield (%) peptide	Bz-Tyr-OH	total reactn time (h)
1	100	0	3
2	100	0	17
3	72	6	19
4	60	2	46
5	78	5	66

(Peypin, France) and were further dried by storage over 0.4-nm molecular sieve.

Enzyme Derivatives. Modification of Chymotrypsin with Cyanuric Chloride Activated Methoxypoly(ethylene glycol). In the first step, monomethoxypoly(ethylene glycol) (M_r = 5000, 4 mmol) was activated with cyanuric chloride (2 mmol). Both compounds were dissolved in 100 mL of benzene containing 6.5 g of anhydrous sodium carbonate, and the mixture was stirred for 40 h at 80 °C. The resulting solution was then centrifuged to discard the precipitate and subsequently poured into 200 mL of petroleum ether to precipitate the cyanuric chloride activated methoxypoly(ethylene glycol). The precipitation step was repeated six times in order to completely remove unreacted cyanuric chloride. Finally, the precipitate was dried in vacuo and stored at −20 °C.

Chymotrypsin was modified by adding a 10 M excess of activated poly(ethylene glycol) over amino groups to an enzyme solution (2 mg/mL) in 40 mM sodium tetraborate buffer at pH 10.0 The resulting mixture was gently stirred at 20 °C for 1 h, further dialyzed at 4 °C against water in an ultrafiltration cell fitted with an Amicon PM 30 membrane (cutoff M_r 30 000), and finally lyophilized.

Preparation of Chymotrypsin Adsorbed on a Support. Chymotrypsin (30 mg) dissolved in 15 mL of a 50 mM sodium phosphate buffer (pH 7.8) was thoroughly mixed with 0.6 g of Celite or pore glass and finally freeze-dried.

Immobilization of Chymotrypsin on *p*-Nitrophenyl Chloroformate Activated Sepharose CL 6B. Activation of Sepharose CL-6B in anhydrous acetone was carried out as described by Wilchek and Miron.[25] The number of active groups present on *p*-nitrophenyl choroformate activated Sepharose was estimated at 40 μmol/g of wet gel by spectrophotometric determination of *p*-nitrophenol released after hydrolysis with 0.2 N NaOH for 15 min at room temperature.

Chymotrypsin (160 mg) dissolved in 16 mL of a 0.1 M sodium phosphate buffer (pH 7.5) was added to 4 g of activated gel, prewashed with cold water, and the solution was stirred for 48 h at 4 °C. The gel was then washed with the coupling buffer, incubated for a few minutes with 0.1 M ethanolamine at pH 8.0, further washed with cold water, and finally lyophilized.

Immobilization of Chymotrypsin on Tosyl Chloride Activated Sepharose CL 6B. The activation of Sepharose CL 6B was carried out according to the method previously described by Sinisterra et al.[26,27] for activation of polysaccharides. Chymotrypsin (5 mg) dissolved in 5 mL of a 0.1 M sodium phosphate buffer (pH 7.8) was added to 1 g of activated Sepharose and the resultant mixture stirred for 3 h at 4 °C. Then the solid was washed with 2 × 10 mL of bidistilled water and lyophilized.

Immobilization of Chymotrypsin on Aldehyde Sepharose CL 6B. Sepharose CL 6B was activated in the presence of glycidol according to the procedure proposed by Shainoff.[28] Chymotrypsin (1 mg) dissolved in 5 mL of a 5 mM sodium phosphate buffer (pH 8.0) was mixed with 0.5 g of aldehyde-activated Sepharose and the solution stirred for 72 h at 4 °C. The Sepharose derivative was then filtered and washed with 10 mL of a 5 mM sodium phosphate buffer (pH 8.0). The imine bonds were then reduced by $NaBH_4$ (1 mg/mL of gel) at pH 8.0. After 30-min incubation at 25 °C, the enzymatic derivative was washed with 2 × 10 mL sodium phosphate buffer (pH 8.0) and water and finally lyophilized.

Enzymatic Reactions. Enzymatic Activity of PEG-Modified or Immobilized Chymotrypsin. Enzymatic activity was determined by titrimetry at pH 7.9 and 25 °C in a 5 mM Tris-HCl buffer containing 40 mM NaCl and 10 mM Ac-Tyr-OEt as a substrate with the aid of a Radiometer recording pH-stat. Esterase activity of PEG-chymotrypsin was 35.8 U/mg while the protein concentration was assayed by the Hartree method. Activities of chymotrypsin adsorbed on Celite or glass beads and immobilized on either aldehyde-, tosyl-, or chlorocarbonate-activated Sepharose were 10.6, 6.9, 5.5, 0.6, and 6.9 U/mg of lyophilized material, respectively.

Enzyme-Catalyzed Peptide Synthesis in Organic Solvents. The standard reaction mixture in the investigated organic solvents (0.5 mL) consisted of 5 mM donor ester, 20 mM acceptor nucleophile with 0–4% added water (or 50 mM sodium phosphate buffer (pH 7.8)), the modified or immobilized enzyme, 0.8% methanol, and diisopropylethylamine (DIPEA) to solubilize the starting materials. Roughly comparable amounts of enzyme with respect to activity in water were used in all experiments, i.e., PEG-Chti (0.4 mg/mL), Chti adsorbed on Celite or glass beads or immobilized on aldehyde- or carbonate-activated Sepharose (2 mg/mL), or Chti immobilized on tosyl-Sepharose (20 mg/mL). Reaction was performed at 37 °C and stopped by addition of 100 μL of glacial acetic acid. The reaction mixture was then evaporated to dryness under reduced pressure and finally dissolved in 1 mL of the starting HPLC eluent system. The reaction products were separated by reversed-phase HPLC (Waters) on a Lichrosorb RP18 column (8 × 250 mm, particle size 5 μm; Merck) and quantitatively analyzed against standard synthetic dipeptides (see Preparative Enzymatic Peptide Synthesis) or amino acid composition of the collected material following hydrolysis in 5.6 N HCl at 110 °C for 24 h.

(25) Wilchek, M.; Miron, T. *Biochem. Int.* **1982**, *4*, 629.

(26) Ballesteros, A.; Sanchez-Montero, J. M.; Sinisterra, J. V. *J. Mol. Catal.* **1986**, *38*, 227.

(27) (a) Sanchez-Montero, J. M.; Sinisterra, J. V.; Ballesteros, A. Spanish Patent 8803769, 1988. (b) *Appl. Biochem. Biotechnol.* **1989**, *22*, 205.

(28) Shainoff, J. R. *Biochem. Biophys. Res. Commun.* **1980**, *95*, 690.

Preparative Enzymatic Peptide Synthesis. In a typical synthesis, the ester donor Bz-Tyr-OEt (125 mg, 0.4 mmol) and the nucleophile acceptor (1.6 mmol) were incubated at 37 °C in 1,1,1-trichloroethane containing 0.8% MeOH and DIPEA and 0.09% or 1% water depending on whether the enzymatic catalyst was PEG-Chti or Chti-carbonate. The enzyme/substrate ratio was comparable to that used for small-scale experiments. The reaction was monitored by HPLC and stopped when all the ester substrate was used.

In the cases of Bz-Tyr-Phe-NH_2 and Bz-Lys-Phe-NH_2, the precipitated peptides were filtered, washed with benzene, and dried. Bz-Tyr-Phe-NH_2 was further washed with water and dried under vacuum. Bz-Lys-Phe-NH_2 was recrystallized from methanol/ether (3/1, v/v). Bz-Tyr-Tyr-NH_2 and Bz-Thr-Thr-NH_2 were washed with 1 mM HCl and crystallized from methanol/ether. Crystallization of Bz-Tyr-Ser-NH_2 under the same conditions was preceded by acetone precipitation of the excess of Ser-NH_2. In the cases of Bz-Tyr-Gly-NH_2 and Bz-Tyr-Lys-NH_2, the reaction mixture was dried and redissolved in MeOH. The excess nucleophile was discarded by chloroform precipitation, and the product was further purified by flash chromatography ($CHCl_3$/MeOH (3/1) for Bz-Tyr-Gly-NH_2 and $CHCl_3$/MeOH/CH_3COOH (32%) (10/3.5/1) for Bz-Tyr-Lys-NH_2).

Reutilization of the Biocatalyst. The reaction mixture in benzene (4 mL) consisted of 5 mM Bz-Tyr-OEt, 20 mM Phe-NH_2, about 0.1% (v/v) water, 1 μM PEG-Chti, 0.8% (v/v) MeOH, and DIPEA to solubilize the starting material and was incubated at 37 °C. The reaction was monitored by HPLC. When a plateau was reached, the peptide was separated by filtration and benzene, water, and reagents were added so that the volume, water content, and the ester/nucleophile ratio were maintained constant throughout the experiment.

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Registry No. Chti, 9004-07-3; PEG, 25322-68-3; Bz-Tyr-OEt, 3483-82-7; Phe-NH_2, 5241-58-7; Tyr-NH_2, 4985-46-0; Thr-NH_2, 49705-99-9; Ser-NH_2, 6791-49-7; Gly-NH_2, 598-41-4; Arg-NH_2, 2788-83-2; Lys-NH_2, 32388-19-5; Glu-NH_2, 636-65-7; Bz-Tyr-Phe-NH_2, 119153-83-2; Bz-Tyr-Tyr-NH_2, 119153-84-3; Bz-Tyr-Thr-NH_2, 129678-28-0; Bz-Tyr-Ser-NH_2, 129678-29-1; Bz-Tyr-Gly-NH_2, 7369-86-0; Bz-Tyr-Arg-NH_2, 131684-49-6; Bz-Tyr-Lys-NH_2, 131684-50-9; Bz-Tyr-Glu-NH_2, 131704-13-7; Ac-Phe-OEt, 2361-96-8; Ac-Trp-OEt, 2382-80-1; Bz-Arg-OEt, 971-21-1; Bz-Lys-OMe, 17039-40-6; Bz-Ala-OMe, 7244-67-9; Z-Ser-OMe, 1676-81-9; Z-Glu-OBzl, 3705-42-8; Bz-Asp-OMe, 82933-21-9; Ac-Phe-Phe-NH_2, 24809-25-4; Ac-Trp-Phe-NH_2, 119206-29-0; Bz-Arg-Phe-NH_2, 66127-60-4; Bz-Lys-Phe-NH_2, 119237-54-6; Bz-Ala-Phe-NH_2, 71448-22-1; Z-Ser-Phe-NH_2, 25529-46-8; Z-Glu-Phe-NH_2, 130970-34-2; Bz-Asp-Phe-NH_2, 131684-51-0; agarose, 9012-36-6.

Fuscosides A–D: Antiinflammatory Diterpenoid Glycosides of New Structural Classes from the Caribbean Gorgonian *Eunicea fusca*

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Four new diterpenoid arabinose glycosides, fuscosides A–D (**1**, **3**, **5**, and **6**), have been isolated from the Caribbean gorgonian *Eunicea fusca* Duchassaing and Michelotti. Fuscoside A (**1**) is a diterpenoid 2′-*O*-acetylarabinose glycoside possessing a new bicyclic carbon skeleton related in biosynthetic origin to the eremophilane class of sesquiterpenoids. The structures of these new compounds were assigned on the basis of chemical and spectral studies. Fuscosides A and B (**1**, **3**) are effective topical antiinflammatory agents with potencies equivalent to indomethacin and manoalide. Fuscoside B (**3**) selectively inhibits the synthesis of leukotrienes LTB_4 and LTC_4, but not PGE_2, in the mouse peritoneal macrophage, suggesting it is a selective inhibitor of leukotriene synthesis.

Marine octocorals of the order Gorgonacea, the sea whips and sea fans (phylum Cnidaria) are abundant, chemically-rich invertebrates found in the reef and and shallow-water habitats of the Caribbean Sea.[1,2] As part of our interest in this interesting group of invertebrates,[3,4] we have focused our attention on the antiinflammatory metabolites produced by Caribbean gorgonians of the genera *Pseudopterogorgia* and *Eunicea*.[5-7] We recently described the pseudopterosins and secopseudopterosins, novel antiinflammatory and analgesic glycosides isolated from several species of *Pseudopterogorgia*.[8] Other gorgonian-derived glycosides include the muricins, aminogalactose glycosides possessing truncated steroidal aglycones, isolated from the pacific gorgonian *Muricea fruticosa*,[9] and moritoside, an aromatic glycoside isolated from a Japanese gorgonian of the genus *Euplexaura*.[10]

In this paper, we report the structures of four new diterpenoid arabinose glycosides, fuscosides A–D (**1**, **3**, **5**, and **6**), isolated from the Caribbean gorgonian *Eunicea fusca* Duchassaing and Michelotti. Fuscoside A (**1**) possesses a novel carbon skeleton that has a bicyclic component related to the eremophilane class of sesquiterpenoids. To the best of our knowledge, fuscoside A (**1**) is the first metabolite possessing this rearranged diterpenoid carbon

(1) Bayer, F. M. *The Shallow Water Octocorallia of the West Indian Region*; Nijhoff: The Hague, 1961; 373 pp.
(2) Faulkner, D. J. *Nat. Prod. Rep.* 1984, *1*, 251; *Ibid.* 1984, *1*, 551; *Ibid.* 1986, *1*, 3; *Ibid.* 1987, *4*, 539; *Ibid.* 1988, *5*, 613.
(3) Pawlik, J. R.; Burch, M. T.; Fenical, W. *J. Exp. Mar. Biol. Ecol.* 1987, *108*, 55.
(4) Fenical, W. *J. Nat. Prod.* 1987, *50*, 1001.
(5) Shin, J.; Fenical, W. *J. Org. Chem.* 1988, *53*, 3271.
(6) Shin, J.; Fenical, W. *Tetrahedron Lett.* 1989, 6821.
(7) (a) Burch, M. T. M. S. Thesis, University of California, San Diego, 1986, 93 pp. (b) Shin, J. Ph.D. Dissertation, University of California, San Diego, 1989, 222 pp.
(8) (a) Look, S. A.; Fenical, W.; Jacobs, R. S.; Clardy, J. *Proc. Natl. Acad. Sci. U.S.A.* 1986, *92*, 1786. (b) Look, S. A.; Fenical, W.; Matsumoto, G. K.; Clardy, J. *J. Org. Chem.* 1986, *51*, 5140. (c) Look, S. A.; Fenical, W. *Tetrahedron* 1987, *43*, 3363.
(9) Bandurraga, M. M.; Fenical, W. *Tetrahedron* 1987, *43*, 1057.
(10) Fusetani, N.; Yasukawa, K.; Matsunaga, S.; Hashimoto, K. *Tetrahedron Lett.* 1985, 6449.

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