A Convenient Synthesis of Aspartame

Gunnar Lindeberg[1]
Institute of Biochemistry, University of Uppsala, Box 576, S-751 23 Uppsala, Sweden

Peptide synthesis, despite its growing field of application and recently acknowledged importance,[2] usually is a neglected subject in chemistry/biochemistry courses. One can think of several reasons for this lack of popularity: the chemical synthesis of peptides, as it is generally exercised, is a time-consuming and laborious undertaking. Already the preparation of a small peptide is a multi-step procedure where the total time required cannot be substantially reduced since reactions and purifications cannot be speeded up beyond certain limits. Although the synthesis of a simple di- or tripeptide demonstrates the use of blocking groups and coupling methods, the peptide as such is rarely of particular interest and can be characterized by physical and chemical methods only (NMR, IR, end-group determination, chromatographic and electrophoretic behavior, etc.). Small peptides with easily measurable biological effects are few. Larger peptides can be synthesized by the solid-phase procedure (*1*) in what may be judged a reasonably short period of time. There are several biologically active peptides in the decapeptide range, for example, hormones; yet considerably more time and effort is needed for their synthesis and purification than is the case for di- and tripeptides. In addition, one is faced with the problem of setting up rather sophisticated assay systems, and there is a risk that the final emphasis of the experiment will lie in physiology rather than chemistry. Other factors, such as the rather high cost of amino acid derivatives and other reagents, the lack of suitable equipment for organic chemistry in many biochemistry laboratories—not to mention special equipment for solid-phase synthesis—and the handling of aggressive or hazardous chemicals by inexperienced persons also need consideration.

The submitted experiment, an enzymatic synthesis of the dipeptide sweetener aspartame (*2*) that has found widespread use as a sucrose substitute, is an attempt to circumvent most of the objections mentioned above. The application of modern concepts in peptide chemistry, such as enzyme-catalyzed condensation and catalytic transfer hydrogenation, makes the procedure simple and straightforward (Fig. 1). The one-tube synthesis of aspartame can be accomplished easily in a couple of days and requires a minimum of equipment. The testing of biological activity is also simple: the threshold value, that is, the lowest concentration at which the (sweet) taste can still be detected, is determined with a dilution series and related to that of a reference substance (sucrose). The simple design of the experiment also makes it possible to include one or more analogues in the synthesis, which allows some conclusions to be drawn about structural requirements for the biological effect. The progress of the reactions and the purity of the intermediates can be conveniently monitored by TLC (Fig. 2) or by HPLC (Fig. 3) if such equipment is available.

Methods

The use of proteolytic enzymes as catalysts for synthetic reactions has found increasing applications in the last few years, particularly in semi-synthesis of proteins. The elegant trypsin-mediated conversion of porcine insulin into the hu-

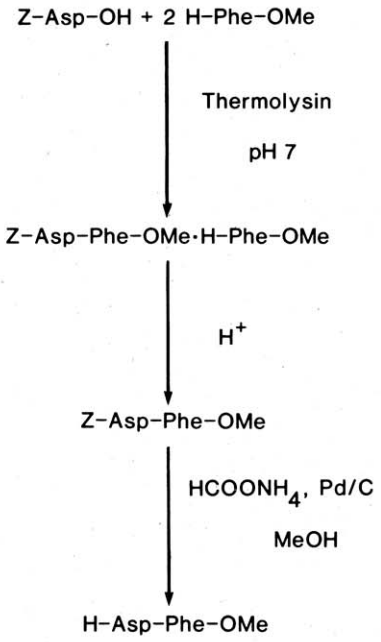

Figure 1. Synthesis of aspartame.

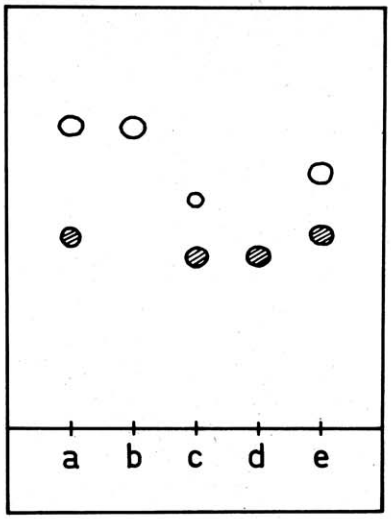

Figure 2. TLC of aspartame and intermediates. (a) Z-Asp-Phe-OMe·H-Phe-OMe, (b) Z-Asp-Phe-OMe, (c) crude, (d) purified H-Asp-Phe-OMe, (e) a mixture of Z-Asp-OH and H-Phe-OMe. Silica gel in *n*-BuOH:AcOH:H_2O (3:1:1). Visualization by UV at 254 nm and ninhydrin (hatched).

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[1] Present address: Institute of Immunology, University of Uppsala, Box 582, S-751 23 Uppsala, Sweden.

[2] The 1984 Nobel Prize in Chemistry was awarded to R. B. Merrifield for the development of solid-phase peptide synthesis.

man form (*3*, *4*) can be mentioned as an example of that approach. However, small or medium-sized peptides have also been prepared by a combination of enzymatic and chemical methods. The reader is referred to one of the recent reviews in this field, such as the one by Jakubke (*5*), for further details. Not surprisingly, aspartame has also been the subject of enzymatic approaches, and so the first step of this experiment is patterned on the procedure of Isowa et al. (*6*): Z–Asp–OH[3] is condensed with H–Phe–OMe in the presence of thermolysin to yield the partially protected dipeptide Z–Asp–Phe–OMe. In the presence of excess amine component a sparingly soluble salt is formed that precipitates from the reaction mixture. Consequently, the equilibrium is shifted toward synthesis, and the reaction is driven almost to completion. The salt is washed free from enzyme and unreacted material, and treated with aqueous HCl. The amine component, which is acid soluble, is then removed by repeated centrifugations and washings. The β-carboxyl in the Asp residue need not be protected, a fact that considerably facilitates the preparation of starting materials. The peptide bond is selectively formed between the α-carboxyl of the aspartic acid and the amino group of the phenylalanine ester. As a matter of fact, the specificity of the enzyme can be further exploited: even if one or both of the amino acids is used as the less expensive DL-derivative, Z–L-Asp–L-Phe–OMe is still the sole product of the condensation reaction, and the yield remains essentially unchanged. The dipeptide salt (Fig. 1) is preferentially formed with H–D-Phe–OMe if both stereoisomers of that component are present.

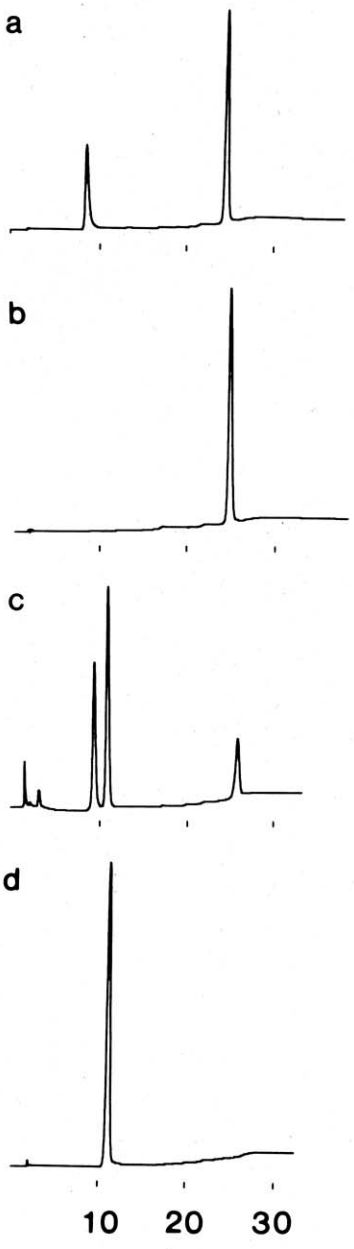

Figure 3. HPLC of aspartame and intermediates. Notations as in Figure 2. Support: Spherisorb S10 ODS, 4.6 × 150 mm. Buffer A: 0.1 M sodium phosphate, pH 3.0. Buffer B: 0.1 M sodium phosphate, pH 3.0, in 50% EtOH. Gradient: 5–90% B in 20 min, then isocratic. Flow rate: 1 mL/min. Detection: UV at 210 nm.

Removal of the benzyloxycarbonyl group, the remaining step of the synthesis, is usually accomplished by acidolysis (HBr/AcOH) or catalytic hydrogenation. Treatment with HBr/AcOH must be done under anhydrous conditions in order to avoid hydrolysis of peptide and ester bonds and would in this case require an additional drying step. Catalytic hydrogenation usually proceeds smoothly but sometimes needs considerable time for complete reaction. Since gaseous hydrogen is used, the deprotection has to be carried out in a well-ventilated hood, a fact that, together with the problems of hydrogen supply, makes the procedure unsuited for use in a student laboratory. However, transfer hydrogenation, where the reducing ability is supplied by a hydrogen donor rather than molecular hydrogen, provides a rapid and experimentally simple means for removal of benzyl-type protecting groups. A comprehensive review of catalytic transfer hydrogenation was recently published by Johnstone et al. (*7*). With the use of two equivalents of ammonium formate as hydrogen donor (*8*) and 0.1–0.2 parts by weight (theoretical values) of 5% Pd/C as catalyst, the deblocking of Z–Asp–Phe–OMe in MeOH is complete in less than 5 min. The use of the 5% catalyst is recommended because of its lower tendency for spontaneous ignition once it has been activated. The rate of deprotection is not significantly increased with the 10% catalyst originally suggested (*8*). The crude product, after removal of catalyst and solvent, is redissolved in water and lyophilized in order to reduce the amount of volatile salts.

Solutions of aspartame have limited stability, especially outside the pH region 3–5 and/or at elevated temperature. Primary decomposition products are the free peptide, which is formed by hydrolysis of the ester bond at low pH values, and the diketopiperazine obtained by ring closure mainly at neutral or alkaline conditions. The physiological effects of the latter compound in particular, but also of aspartame itself, have been questioned on several occasions and, for some years, caused the withdrawal of aspartame as a food additive. However, extensive investigations have not confirmed the suspicion that aspartame would be unsuitable for human consumption. A joint FAO/WHO expert committee established acceptable daily intake levels of 40.0 and 7.5 mg/kg body weight for aspartame and diketopiperazine, respectively (*9*). In the present experiment diketopiperazine and other negatively charged impurities, if present, are removed by ion-exchange chromatography immediately before dilution and tasting.

A large number of analogues to aspartame have been synthesized in the search for still sweeter substances and with the aim to explain taste properties in terms of chemical structure. The effect of some structural modifications may be studied also in this experiment: the influence of the size of the ester group is illustrated by substitution of H-Phe–OEt for H–Phe–OMe, a change that reduces sweetness potency to less than half the original value. Elongation of the side-chain carrying the free carboxyl group, accomplished by exchanging Asp by Glu, yields an inactive (tasteless) prod-

[3] Z = benzyloxycarbonyl (carbobenzoxy).

uct, whereas deletion of the side-chain carboxyl, that is, replacement of Asp with Ala, brings about a conversion from sweet to bitter taste. The analogues are synthesized essentially by the same methods as the parent compound. Z–Glu–Phe–OEt, due to its higher solubility, is not isolated by centrifugation but is extracted into EtOAc after acidification. The simple chromatographic procedure used for aspartame and the other analogues cannot be applied for H–Ala–Phe–OMe because the diketopiperazine in this case is uncharged. Crystallization from water is also not feasible, since rapid cyclization is observed when the solution is heated. However, a homogeneous product is recovered by extraction with ice water followed by lyophilization in order to remove volatile salts.

Experimental Procedure

Z–Asp–OH, Z–Ala–OH, Z–Glu–OH, H–Phe–OMe·HCl and H–Phe–OEt·HCl were prepared according to published procedures but are also commercially available. Thermolysin (~40 U/mg) was obtained from Boehringer/Mannheim, QAE–Sephadex from Pharmacia, and 5% palladium on charcoal from Fluka. TLC was run on precoated silica gel plates (Merck) in *n*-BuOH:AcOH:H_2O (3:1:1). A solvent migration of 5–10 cm beyond the application line was adequate.

Z–Asp–Phe–OMe–H–Phe–OMe

Z–Asp–OH (267 mg, 1 mmol) and H–Phe–OMe·HCl (431 mg, 2 mmol) were weighed into a centrifuge tube of ~10 mL capacity and suspended in H_2O (3 mL). The pH was adjusted to 7 (indicator paper) by portion-wise addition of 4 M NaOH (~700 μL) while the mixture was being stirred on a vortex mixer. Thermolysin (2 mg in 200 μL H_2O) was added to the resulting clear solution, which was then kept in a water bath at 40 °C overnight. Occasionally, the product precipitated as an oil but this could be brought to crystallize by repeated cooling and stirring. The precipitate was broken up with a glass rod, centrifuged and washed by centrifugation with 5 × 4 mL H_2O. A sample was collected for TLC.

Z–Asp–Phe–OMe

The peptide salt was decomposed by trituration in an ice bath with a mixture of H_2O (3 mL) and 1 M HCl (1 mL). A glass rod rather than a vortex mixer was used for stirring, since the product at this stage tended to stick to the walls of the tube. After ~10 min the precipitate had changed appearance and gave the impression of being more compact. It was centrifuged and washed with 5 × 4 mL ice water. A sample was saved for TLC.

H–Asp–Phe–OMe, Aspartame

Z–Asp–Phe–OMe (still moist product above, <1 mmol) and $HCOONH_4$ (126 mg, 2 mmol) were dissolved in MeOH (2mL). A sample of the solution was applied onto a TLC plate. The catalyst, 5% Pd/C (50 mg), was added and washed down with MeOH (0.5 mL). The suspension was kept at room temperature in the open test tube until gas evolution had ceased (~20 min). Samples were withdrawn and spotted onto the TLC plate after 2, 5, and 10 min. The catalyst was then removed by centrifugation and filtration of the supernate through a tightly packed plug (5–10 mm) of cotton wool in a Pasteur pipet. Catalyst and filter were washed with MeOH (0.5 mL). The filtrate was collected in a small beaker and taken to dryness in a stream of nitrogen with gentle heating (hair dryer). The residue was dissolved in a small amount of warm water (3–5 mL) and lyophilized.

H–Asp–Phe–OEt

This substance was prepared according to the above procedure starting with Z–Asp–OH (267 mg, 1 mmol) and H–Phe–OEt·HCl (460 mg, 2 mmol).

H–Glu–Phe–OEt

Z–Glu–OH (281 mg, 1 mmol) and H–Phe–OEt·HCl (460 mg, 2 mmol) were condensed as described above. EtOAc (2.5 mL) and 1 M HCl (2 mL) were then added to the reaction mixture, which was shaken vigorously in order to dissolve the precipitate. The phases were separated (centrifugation), and the aqueous phase was transferred to a second test tube with the aid of a disposable syringe fitted with a short length of polyethylene tubing, and extracted in the same manner with more EtOAc (2 × 2 mL). The combined organic extracts were washed with H_2O (5 × 2 mL) and sat. NaCL (2 mL) and then evaporated to dryness in a stream of nitrogen with gentle heating. The residue was dissolved in MeOH and deprotected as described above.

H–Ala–Phe–OMe

Z–Ala–OH (446 mg, 2 mmol) and H–Phe–OMe·HCl (431 mg, 2 mmol) were condensed in the presence of thermolysin (2 mg) after neutralization with 4 M NaOH (700 μL). The product was collected by centrifugation and washed with H_2O (5 × 4 mL). Deprotection was performed in MeOH (4 mL) as described above, but using 252 mg (4 mmol) of $HCOONH_4$. The residue obtained after evaporation of the filtrate was extracted in the cold with ice water (2 × 2 mL, centrifugation). The extract was filtered, lyophilized, and used without further purification in the sweetness evaluation.

Purification and Testing

An aqueous solution of crude lyophilized aspartame (50 mg dissolved in 5 mL of H_2O) was neutralized with 2 M NaOH and passed through a small column (5 × 40 mm, Pasteur pipet) of QAE–Sephadex A-25 (acetate form, packed in H_2O). The product was eluted with H_2O (2 mL), and its concentration in the eluate was determined from the UV absorbance (ϵ_{257} = 200). The solution was diluted to cover the range 0.003–0.3% and tasted, starting with the lowest concentration, in order to establish the threshold value. Dilution series of analogues and sucrose (0.2–4%) were treated accordingly. The sweetness was expressed as the quotient,

$$S = \frac{\text{threshold value (sucrose)}}{\text{threshold value (peptide)}}$$

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