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Enzymatic Synthesis of Cholecystokinin-octapeptide[1)]

KIYOSHI SAKINA,*·[a] KEIKO KAWAZURA,[a] KAZUYUKI MORIHARA,[a] and HARUAKI YAJIMA[b]

Kyoto Research Laboratories, Toho Pharmaceutical Ind., Co., Ltd.,[a] Nagaokakyo-shi, Kyoto 617, Japan and Faculty of Pharmaceutical Sciences, Kyoto University,[b] Sakyo-ku, Kyoto 616, Japan

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Cholecystokinin-octapeptide was synthesized by enzymatic condensations of three fragments, without side-chain protection, except for Tyr. Fmoc–Asp–Tyr(SO_3Bal/2)–OH, prepared by the concerted action of proteases, followed by pyridinium trifluoroacetylsulfate treatment, was used as the N-terminal fragment. In the final step, the N^{α}-Fmoc group employed as a sole protecting group was easily removed by base treatment without affecting the SO_3 moiety.

Keywords——CCK-8 enzymatic synthesis; enzymatic fragment condensation; thermolysin; papain; Tyr-sulfation; pyridinium trifluoroacetylsulfate

Various proteases have been used as catalysts for practical peptide synthesis.[2-4)] In contrast to chemical peptide synthesis, the most attractive feature of the enzymatic method is that synthesis can be performed in principle without protection of amino acid side-chain functionals, since proteases show specificity for the α-carboxyl or α-amino group at the reaction site, with retention of the chiral integrity. Thus, in the course of peptide synthesis, the use of enzymes at the stage of fragment condensation seems to offer several advantageous features. Taking advantage of these attractive features of the enzymatic method, we have synthesized the cholecystokinin-octapeptide (CCK-8).[5)]

CCK-8 contains many functional amino acids, *i.e.*, sulfated Tyr at position 2, two Met, one Trp, and two Asp, and the presence of the acid-labile sulfate moiety makes the chemical synthesis of CCK-8 rather difficult. As shown in Fig. 1, three peptide fragments were selected as building blocks to construct the entire peptide backbone of CCK-8, *i.e.*, Fmoc–Asp–Tyr(SO_3Bal/2)–OH (C), H–Met–Gly–Trp–OMe (B), and H–Met–Asp–Phe–NH_2 (A). The base-labile Fmoc group[6)] was applied for protection of the N-terminal aspartic acid and the Tyr residue was sulfated before fragment condensation.

The C-terminal tripeptide amide, H–Met–Asp–Phe–NH_2 (A), was prepared in a stepwise manner as shown in Fig. 2a, by the active Np[7)] or Tcp[8)] ester procedure. The two protecting groups, Z and Bzl, were cleaved from Z–Asp(OBzl)–Phe–NH_2 by hydrogenation, and the

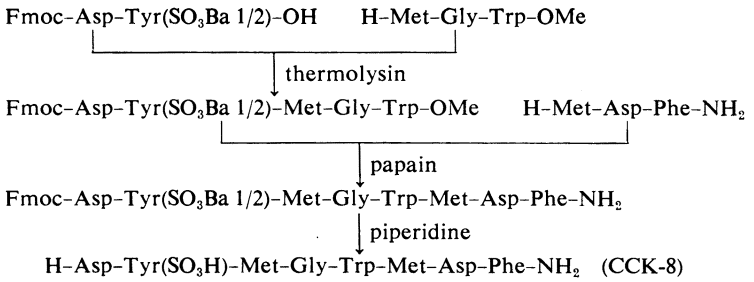

Fig. 1. Synthetic Scheme for CCK-8

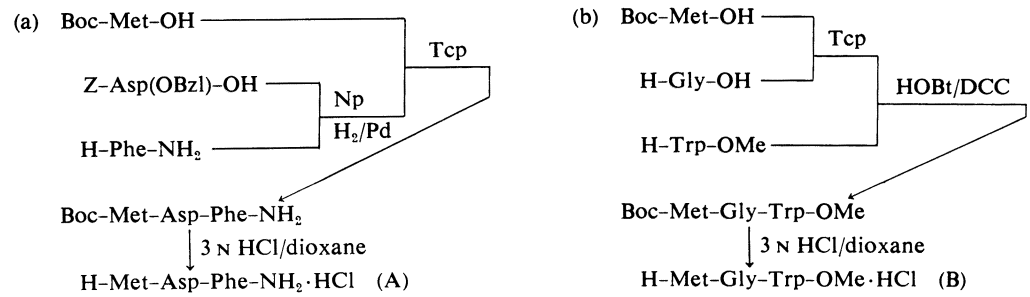

Fig. 2. Synthetic Schemes for the C-Terminal (A) and the Middle (B) Fragments

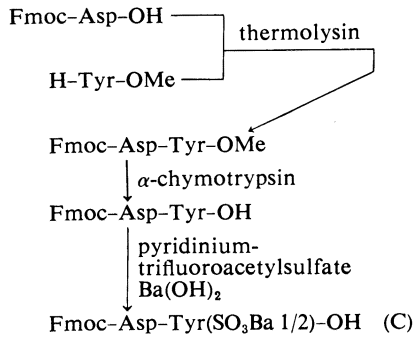

Fig. 3. Synthetic Scheme for the N-Terminal Fragment (C)

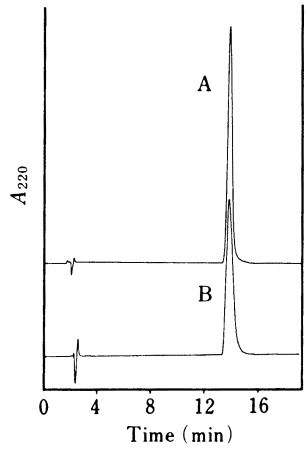

Fig. 4. HPLC Comparison of Enzymatically Synthesized CCK-8 (A) and Chemically Synthesized CCK-8 (B)

Column: Nucleosil $5C_{18}$ (4.0 × 200 mm). Solvent system: 26% CH_3CN in 0.2% TFA. Flow rate: 1.0 ml/min.

resulting dipeptide amide was allowed to react with Boc–Met–OTcp[9] to give Boc–Met–Asp–Phe–NH_2, from which the Boc group was removed by treatment with 3 N HCl in dioxane.

The middle tripeptide, H–Met–Gly–Trp–OMe (B), was synthesized as shown in Fig. 2b. Boc–Met–Gly–OH prepared from Boc–Met–OTcp and H–Gly–OH, was condensed with H–Trp–OMe by the HOBt/DCC procedure.[10] The Boc group was cleaved from Boc–Met–Gly–Trp–OMe by treatment with 3 N HCl in dioxane.

The N-terminal dipeptide, Fmoc–Asp–Tyr(SO_3Ba1/2)–OH, was prepared according to the scheme illustrated in Fig. 3. Thermolysin was useful to condense Fmoc–Asp–OH and H–Tyr–OMe, as reported in the enzymatic synthesis of a similar peptide, an aspartame derivative (Z–Asp–Phe–OMe).[11] In this case, temporal protection of the Tyr α-carboxyl group was needed, since this enzyme is an endoprotease. Removal of the methyl ester from Fmoc–Asp–Tyr–OMe was accomplished effectively by α-chymotrypsin, which shows specificity for hydrophobic amino acids. Usual saponification with base can not be applied to this peptide, because of the presence of the base-labile Fmoc group. Next, Fmoc-Asp–Tyr–OH was sulfated. First, we tried to use pyridine–SO_3 complex[12] or pyridinium acetylsulfate.[13] However, both of these reagents generated considerable amounts of by-products. Thus, this dipeptide was sulfated with pyridinium trifluoroacetylsulfate[14] in acetonitrile and the product was isolated as its Ba salt.[15] In this sulfation reaction, acetonitrile as a solvent gave fewer by-

products than the other solvents tested, *i.e.*, DMF and EtOH. This hydrophilic peptide, Fmoc–Asp–Tyr($SO_3Ba1/2$)–OH, was purified by gel-filtration on Sephadex LH-20 using 25% MeOH as an eluant. In the infrared (IR) spectrum of the product, the characteristic band ($1050\,cm^{-1}$)[16] due to a sulfate ester was observed.

Construction of the peptide backbone was then carried out according to the scheme shown in Fig. 1. The middle fragment was coupled with the N-terminal fragment by the use of thermolysin, a metallo endoprotease, which acts specifically on the imino side of hydrophobic amino acids. In order to suppress the undesired hydrolysis of the established peptide bond, the reaction was performed in a high concentration of the organic solvent (95% acetonitrile), under which sufficient enzyme activity was maintained to complete the condensation reaction. The final condensation was carried out with the aid of papain, which has a rather broad substrate specificity. As demonstrated in our enzymatic synthesis of delta sleep-inducing peptide,[17] the reaction under basic conditions (pH 8—9)[18] in the presence of mercaptoethanol was effective to suppress peptide bond hydrolysis without particular difficulty.

Removal of the N^{α}-Fmoc group from the resulting octapeptide proceeded smoothly, on treatment with 50% piperidine in DMF, and the product was purified to homogeneity by reversed-phase high-performance liquid chromatography (HPLC). The synthetic peptide exhibited a retention time identical with that of an authentic sample of CCK-8, as shown in Fig. 4.

As described above, we were able to demonstrate the usefulness of the enzymatic method for the synthesis of a Tyr(SO_3H)-containing peptide as an example.

Experimental

Thermolysin and papain (1 : 350) were obtained from Wako Pure Chemicals. α-Chymotrypsin (type II) and aminopeptidase M were from Sigma and Boehringer, respectively. CCK-8 was purchased from Peptide Institute, Inc., Osaka. IR spectra were measured with a Shimadzu IR-435 infrared spectrophotometer. HPLC was conducted with a Shimadzu LC-4A model, using a column of Nucleosil $5C_{18}$ (4.0 × 200 mm, Macherey–Nagel). Thin layer chromatography (TLC) were carried out on silica gel plates (Kieselgel 60, Merck) using the following solvents: Rf_1 $CHCl_3$–MeOH (10 : 1), Rf_2 $CHCl_3$–MeOH–H_2O (65 : 35 : 10, lower phase), Rf_3 $CHCl_3$–MeOH–AcOH (5 : 1 : 0.5), Rf_4 *n*-BuOH–AcOH–H_2O–AcOEt (3 : 1 : 1 : 5). Acid hydrolysis (6 N HCl) was carried out at 110 °C for 18 h, and amino acids were determined with a Hitachi 835 analyzer. Trp-containing peptides were hydrolyzed with 4 M methanesulfonic acid (MSA) containing 0.2% 3-(2-aminoethyl)-indole (Pierce) at 110 °C for 18 h. Aminopeptidase M digestion was performed according to Hofmann *et al.*[19] (2 units of enzyme/μmol of peptide).

N^{α}-Deprotection——The Boc group was cleaved with TFA (*ca.* 10 ml per 1 g of a peptide) in the presence of thioanisole (2 eq or more) at room temperature for 30 min, or with 3 M HCl/dioxane (*ca.* 20 ml per 1 g of peptide) at room temperature for 1 h. After evaporation of the solvent *in vacuo*, the residue was treated with dry ether. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* for 3 h and then used for the condensation reaction.

Z–Asp(OBzl)–Phe–NH_2——H–Phe–NH_2 · HCl (3.77 g, 18.8 mmol) was dissolved in DMF (50 ml), together with Et_3N (2.61 ml, 18.8 mmol) and Z–Asp(OBzl)–ONp (8.60 g, 18.0 mmol). The solution was stirred for 16 h, then concentrated and the residue was treated with ether. The resulting powder was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O, and crystallized from DMF and H_2O; yield 8.00 g (85%), mp 175—176 °C, $[\alpha]_D^{25}$ −23.2° (c=0.5, DMF), Rf_1 0.55. *Anal.* Calcd for $C_{28}H_{29}N_3O_6$: C, 66.79; H, 5.81; N, 8.34. Found: C, 66.61; H, 5.73; N, 8.38.

Boc–Met–Asp–Phe–NH_2——Z–Asp(OBzl)–Phe–NH_2 (7.80 g, 15.5 mmol) was hydrogenated over Pd black (400 mg) in DMF–H_2O (100 ml–20 ml). The solution was filtered, then the filtrate was concentrated and the residue was crystallized from DMF and AcOEt to give H–Asp–Phe–NH_2 (3.58 g, 83%). This dipeptide (3.50 g, 12.5 mmol) was dissolved in DMF (100 ml), together with *N*-methylmorpholine (1.38 ml, 12.5 mmol) and Boc–Met–OTcp (5.36 g, 12.5 mmol). The solution, after being stirred at room temperature for 40 h, was concentrated and the residue was treated with ether. The resulting powder was washed with 5% citric acid and H_2O, and crystallized from DMF and ether; yield 5.40 g (85%), mp 208—209 °C, $[\alpha]_D^{25}$ −39.2° (c=0.5, DMF), Rf_3 0.57. Amino acid ratios in a 6 N HCl hydrolysate: Asp 1.00, Met 0.95, Phe 0.98 (recovery of Asp, 100%). *Anal.* Calcd for $C_{23}H_{34}N_4O_7S$: C, 54.10; H, 6.71; N, 10.97. Found: C, 53.83; H, 6.71; N, 11.06.

Boc–Met–Gly–OH——A mixture of Boc–Met–OTcp (8.57 g, 20.0 mmol) in DMF (20 ml) and H–Gly–OH (1.80 g, 24.0 mmol) in H_2O (10 ml) containing Et_3N (3.30 ml, 24.0 mmol) was stirred at room temperature for 16 h,

then the solvent was evaporated off and the residue was dissolved in 5% NH_4OH. The aqueous phase, after being washed with AcOEt, was acidified with citric acid and extracted with AcOEt. The organic phase was washed with H_2O–NaCl, dried over Na_2SO_4 and then concentrated. The residue was crystallized from AcOEt and petroleum ether; yield 4.95 g (81%), mp 124—126 °C, $[\alpha]_D^{25}$ −16.4° ($c=0.5$, MeOH) [lit.[5] mp 125—126 °C, $[\alpha]_D^{23}$ −12° ($c=1.2$, DMF)], Rf_3 0.66. *Anal.* Calcd for $C_{12}H_{22}N_2O_5S$: C, 47.04; H, 7.24; N, 9.14. Found: C, 46.99; H, 7.20; N, 9.23.

Boc–Met–Gly–Trp–OMe——H–Trp–OMe·HCl (4.08 g, 16.0 mmol) was dissolved in DMF (20 ml) together with *N*-methylmorpholine (1.76 ml, 16.0 mmol), Boc–Met–Gly–OH (4.95 g, 16.0 mmol) and HOBt (3.25 g, 24.0 mmol). DCC (4.13 g, 20.0 mmol) was added at 0 °C, then the mixture was stirred at room temperature for 16 h and filtered. The filtrate was concentrated and the residue was extracted with AcOEt. The organic phase was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O–NaCl, dried over Na_2SO_4 and concentrated. The residue was triturated with *n*-hexane and precipitated from AcOEt with petroleum ether; yield 7.70 g (95%), mp 48—50 °C, $[\alpha]_D^{25}$ −3.6° ($c=0.5$, MeOH), Rf_1 0.53. Amino acid ratios in a 4 M MSA hydrolysate: Gly 1.00, Met 0.98, Trp 1.00 (recovery of Gly, 97%). *Anal.* Calcd for $C_{24}H_{34}N_4O_6S$: C, 56.90; H, 6.76; N, 11.06. Found: C, 56.90; H, 6.92; N, 10.95.

Fmoc–Asp–OH——Fmoc–OSu[20] (8.10 g, 24.0 mmol) in CH_3CN (25 ml) was added to a stirred solution of H–Asp–OH (3.33 g, 25.0 mmol) in H_2O (25 ml) containing Et_3N (3.67 ml, 50.0 mmol). The pH of the reaction mixture was maintained at 8.5—9.0 by addition of Et_3N for 30 min. After filtration, the filtrate was concentrated and acidified with 2 N HCl. The resulting precipitate was extracted with AcOEt. The organic phase was washed with H_2O–NaCl, dried over Na_2SO_4 and concentrated. The residue was crystallized from AcOEt and petroleum ether; yield 6.26 g (73%), mp 183—184 °C, $[\alpha]_D^{25}$ −28.4° ($c=0.5$, DMF), Rf_3 0.36. *Anal.* Calcd for $C_{19}H_{17}NO_6$: C, 64.22; H, 4.82; N, 3.94. Found: C, 64.16; H, 4.72; N, 3.98.

Fmoc–Asp–Tyr–OMe——A mixture of H–Tyr–OMe·HCl (4.87 g, 21.0 mmol) and Fmoc–Asp–OH (3.88 g, 10.5 mmol) in H_2O (7.5 ml) was adjusted to pH 6.0 with 7% NH_4OH (7.5 ml). Thermolysin (102 mg) was added and the solution was stirred at room temperature for 24 h. The solution was acidified with 1 N HCl and extracted with AcOEt. The organic phase was washed with 0.5 N HCl, 0.2 M sodium phosphate buffer (pH 7.2) and H_2O–NaCl, dried over Na_2SO_4 and then concentrated. The residue was crystallized from EtOH and petroleum ether; yield 3.19 g (57%), mp 177—178 °C, $[\alpha]_D^{25}$ −11.6° ($c=0.5$, MeOH), Rf_3 0.76. *Anal.* Calcd for $C_{29}H_{28}N_2O_8$: C, 65.41; H, 5.30; N, 5.26. Found: C, 64.85; H, 5.26; N, 5.28.

Fmoc–Asp–Tyr–OH——Fmoc–Asp–Tyr–OMe (3.19 g, 6.0 mmol) was dissolved in a mixture of MeOH (120 ml) and 80 mM Tris–HCl buffer (pH 7.8, 120 ml containing 50 mM $CaCl_2$). α-Chymotrypsin (100 mg/10 ml of 1 mM HCl) was added and the solution, after being adjusted to pH 7.8 with Tris, was stirred at room temperature for 24 h. The reaction mixture was concentrated to about half the initial volume, then acidified with 0.5 N HCl and extracted with AcOEt. The organic phase was washed with H_2O–NaCl, dried over Na_2SO_4, and concentrated. The residue was crystallized from EtOH and petroleum ether; yield 3.04 g (98%), mp 190—191 °C, $[\alpha]_D^{25}$ −7.6° ($c=0.5$, DMF), Rf_3 0.38. *Anal.* Calcd for $C_{28}H_{26}N_2O_8 \cdot 1/2H_2O$: C, 63.75; H, 5.16; N, 5.31. Found: C, 64.00; H, 5.40; N, 5.24.

Fmoc–Asp–Tyr($SO_3Ba1/2$)–OH——Pyridinium trifluoroacetylsulfate (1.05 g, 3.84 mmol) prepared according to Penke *et al.*[13] was added to an ice-chilled solution of Fmoc–Asp–Tyr–OH (500 mg, 0.96 mmol) in CH_3CN (10 ml), and the mixture was stirred at room temperature for 16 h. A suspension of $Ba(OH)_2 \cdot 8H_2O$ (2.12 g, 6.72 mmol) in H_2O (50 ml) was added, and CO_2 gas was bubbled into the solution. The precipitate was removed by centrifugation. A half of the supernatant was applied to a column of Sephadex LH-20 (4.5 × 80 cm), which was eluted with 25% MeOH. The fractions (16 ml each) corresponding to the main peak (tube No. 76—92, examined by TLC, Rf_4 0.26) were combined, then the solvent was evaporated off and the residue was treated with EtOH to afford a powder. The rest of the supernatant was similarly purified; total yield 350 mg (55%), mp >250 °C, $[\alpha]_D^{25}$ −14.0° ($c=0.2$, DMF), Rf_4 0.26, IR (KBr): 1050 cm^{-1}. Amino acid ratios in a 6 N HCl hydrolysate: Asp 1.00, Tyr 0.96 (recovery of Asp, 77%). *Anal.* Calcd for $C_{28}H_{25}N_2O_{11}SBa1/2$: C, 50.48; H, 3.78; N, 4.20. Found: C, 50.05; H, 3.69; N, 4.03.

Fmoc–Asp–Tyr($SO_3Ba1/2$)–Met–Gly–Trp–OMe——Fmoc–Asp–Tyr($SO_3Ba1/2$)–OH (400 mg, 0.60 mmol) and H–Met–Gly–Trp–OMe·HCl (1.33 g, 3.00 mmol) were dissolved in a mixture of CH_3CN (11.4 ml) and 50 mM $CaCl_2$ (0.6 ml), then the solution was adjusted to pH 5.0 with 5% NH_4OH (0.1 ml). After addition of thermolysin (40 mg), the solution was stirred at room temperature for 48 h, and then concentrated. H_2O was added. The resulting precipitate was collected by centrifugation and washed with H_2O. This product was purified by column chromatography on Al_2O_3 (neutral, 2.2 × 14 cm) using $CHCl_3$–MeOH–5% NH_4OH (65 : 35 : 10, lower phase) as an eluant. The product was finally precipitated from MeOH with ether; yield 420 mg (66%), mp 148—151 °C, $[\alpha]_D^{25}$ −13.0° ($c=0.2$, DMF), Rf_3 0.24, IR (KBr): 1050 cm^{-1}. Amino acid ratios in a 4 M MSA hydrolysate: Asp 1.04, Gly 1.00, Met 0.98, Tyr 1.00, Trp 0.95 (recovery of Gly, 96%). *Anal.* Calcd for $C_{47}H_{49}N_6O_{14}S_2Ba1/2$: C, 53.52; H, 4.68; N, 7.97. Found: C, 52.94; H, 4.70; N, 7.92.

Fmoc–Asp–Tyr($SO_3Ba1/2$)–Met–Gly–Trp–Met–Asp–Phe–NH_2——Fmoc–Asp–Tyr($SO_3Ba1/2$)–Met–Gly–Trp–OMe (95 mg, 90 μmol) and H–Met–Asp–Phe–NH_2·HCl (81 mg, 180 μmol) were dissolved in EtOH (0.54 ml) and the pH of the solution was adjusted to 8.0 with 0.2 M Na_2CO_3 (1.26 ml). After addition of 2-mercaptoethanol (13 μl) and papain (13 mg), the solution was stirred at 40 °C for 10 min, and then acidified with 5% citric acid. The resulting powder was washed with H_2O and AcOEt and precipitated from DMF with AcOEt; yield 78 mg (60%), Rf_2 0.25, IR (KBr): 1050 cm^{-1}. Amino acid ratios in a 4 M MSA hydrolysate: Asp 2.27, Gly 1.00, Met 2.13, Tyr 0.89, Phe 1.25, Trp

0.91 (recovery of Gly, 88%). This compound, contaminated with a small amount of H–Met–Asp–Phe–NH_2, was used in the next reaction without further purification.

H–Asp–Tyr(SO_3H)–Met–Gly–Trp–Met–Asp–Phe–NH_2 (CCK-8)——The above N^{α}-protected octapeptide (50 mg, 35.0 μmol) was treated with piperidine (340 μl) in DMF (340 μl) at room temperature for 15 min. Purification was performed by reversed-phase HPLC on Nucleosil $5C_{18}$ (1.0 × 25 cm). Portions of the above solution (*ca.* 100 μl each) were applied to the above column and eluted with CH_3CN (25%) in 50 mM $AcONH_4$ (pH 6.0) at a flow rate of 3.0 ml per min. The eluates corresponding to the main peak (retention time 12.3 min) were collected and lyophilized to give a powder; yield 27 mg (64%), $[\alpha]_D^{25}$ −10.0° (c = 0.1, 1 N NH_4OH), Rf_2 0.29, IR (KBr): 1050 cm^{-1}. The purity of the final product was examined by HPLC (Fig. 4). Amino acid ratios in a 4 M MSA hydrolysate: Asp 2.07, Gly 1.00, Met 1.90, Tyr 0.99, Phe 0.94, Trp 0.94 (recovery of Gly, 98%). Amino acid ratios in an aminopeptidase M digest: Asp 1.95, Gly 1.00, Met 1.89, Tyr(SO_3H) 0.90, Phe 0.97, Trp 0.94 (recovery of Gly, 90%).

References and Notes

1) Amino acids and peptide derivatives mentioned in this paper are of the L-configuration. The following abbreviations are used: Z = benzyloxycarbonyl, Boc = *tert*-butoxycarbonyl, Fmoc = 9-fluorenylmethyloxycarbonyl, Np = *p*-nitrophenyl, Tcp = trichlorophenyl, Su = *N*-hydroxysuccinimidyl, DCC = dicyclohexylcarbodiimide, HOBt = *N*-hydroxybenzotriazole, Bzl = benzyl, DMF = dimethylformamide, TFA = trifluoroacetic acid.
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