Solution Synthesis of Human Peptide YY (hPYY)[1)]

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Human peptide YY (hPYY) was synthesized in a conventional manner by assembling six peptide fragments followed by deprotection with 1 M trimethylsilyl trifluoromethanesulfonate (TMSOTf)–thioanisole in trifluoroacetic acid (TFA). After purification by gel-filtration on Sephadex G-25, followed by reversed-phase high-performance liquid chromatography, a highly purified sample of synthetic hPYY was obtained. When administered in dogs, synthetic hPYY was as active as synthetic porcine PYY in terms of the effects on systemic arterial blood pressure, and splanchnic blood flow.

Keywords human peptide YY (hPYY) synthesis; thioanisole-mediated deprotection; trimethylsilyl trifluoromethanesulfonate deprotection; hPYY activity *in vivo*

Peptide YY (PYY) was isolated from the porcine duodenum by Tatemoto *et al.* in 1980.[2)] This peptide consists of 36 amino acid residues with Tyr–NH_2 at the C-terminus and exhibits a distinctive structural similarity with neuropeptide Y (NPY)[3)] and pancreatic polypeptide (PP).[4)] Although PYY-immunoreactivity has been observed in various animals, only the primary structures of porcine PYY and rat PYY[5)] were known, until Tatemoto *et al.* recently purified human PYY (hPYY) from human colonic extracts using a chemical assay method.[6)] The sequence of hPYY is different from that of porcine and rat PYY in the replacement of two amino acid residues at positions 3 and 18: Ala and Ser in porcine (rat) PYY are substituted by Ile and Asn in hPYY, respectively. Its structure was confirmed by an Fmoc-based solid-phase synthesis[7)] by the same authors.[6)]

Following our synthesis of porcine PYY (pPYY)[8)] and structurally related porcine and human NPYs,[9,10)] we wish to report herein the solution synthesis of hPYY employing the 1 M trimethylsilyl trifluoromethanesulfonate (TMSOTf)–thioanisole/trifluoroacetic acid (TFA) system[11)] as a final deprotecting reagent.

The synthetic strategy to hPYY was almost the same as for the previously reported pPYY synthesis[8)] and the synthetic route is outlined in Fig. 1. Of six fragments used in the present synthesis, fragment [1] is the intermediate tetradecapeptide amide used for the pPYY synthesis and fragments [3], [4], [5] are identical with those employed for the pPYY synthesis. The other two fragments which cover the areas of species variation were newly prepared.

Fragment [2], Z(OMe)–Asn–Arg(Mts)–Tyr–Tyr–Ala–$NHNH_2$, was prepared by the Np[12)] condensation of Z(OMe)–Asn–OH with a TFA-treated sample of Z(OMe)–Arg(Mts)–Tyr–Tyr–Ala–OMe,[8)] used for the former pPYY synthesis, followed by the usual hydrazine treatment. Fragment [6], Z(OMe)–Tyr–Pro–Ile–$NHNH_2$, was prepared in a stepwise manner starting with H–Ile–OMe. Boc–Pro–OH was introduced by the mixed anhydride (MA)[13)] method and Z(OMe)–Tyr–$NHNH_2$ by the azide[14)] method. The resulting protected tripeptide ester was converted to [6] by the usual hydrazine treatment.

The six fragments thus obtained were then condensed successively by the azide method (Fig. 1) to minimize racemization. The amount of the acyl component was increased from 1.5 to 3 eq as chain elongation progressed to secure complete condensation. All products, including the protected hPYY, were purified by precipitation from DMF with AcOEt or by column chromatography on silica gel or by gel-filtration on Sephadex LH-60. Throughout this synthesis, Val was selected as a diagnostic amino acid in acid hydrolysis. The results of acid hydrolysis of the protected intermediates and protected hPYY are listed in Table I.

The protected hPYY thus obtained was treated with 1 M TMSOTf–thioanisole in TFA in the presence of *m*-cresol to remove all the protecting groups employed. The

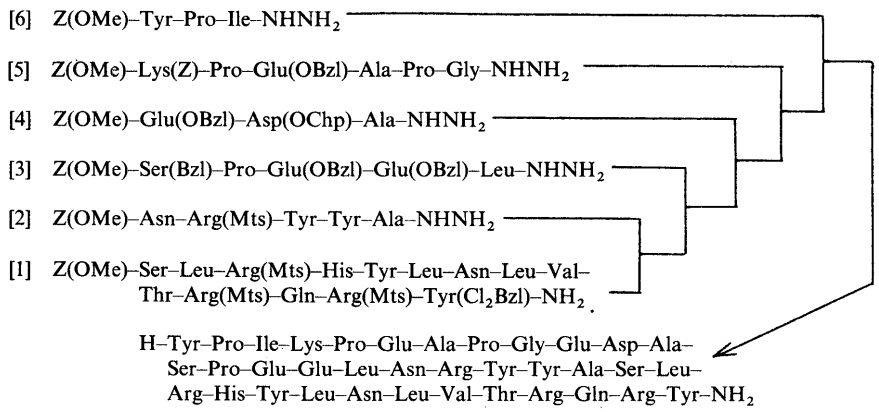

Fig. 1. Synthetic Route to Human Peptide YY (hPYY)

TABLE I. Amino Acid Ratios in 6N HCL Hydrolysates of Synthetic hPYY and Its Intermediates

	Protected peptides					Syn. hPYY	Residue
	18—36	13—36	10—36	4—36	1—36		
Asp	1.90	2.14	3.30	3.08	2.88	2.90	(3)
Thr	0.99	0.96	0.98	0.94	0.91	0.96	(1)
Ser	0.89	1.97	1.92	1.81	1.73	1.97	(2)
Glu	1.05	3.23	4.62	5.45	4.80	5.25	(5)
Pro		1.16	1.13	3.40	4.07	4.23	(4)
Gly				1.20	1.04	1.11	(1)
Ala	0.96	1.00	2.25	3.32	2.94	3.22	(3)
Val	1.00	1.00	1.00	1.00	1.00	1.00	(1)
Ile					1.00	0.84	(1)
Leu	2.87	3.90	4.16	4.00	3.76	4.25	(4)
Tyr	3.74	3.57	3.83	3.71	4.95	5.25	(5)
Lys				1.21	0.96	1.04	(1)
His	1.04	0.93	0.95	0.94	0.88	1.07	(1)
Arg	4.41	3.76	4.08	4.01	3.66	4.57	(4)
Recovery (%)	82	75	81	85	87	90	

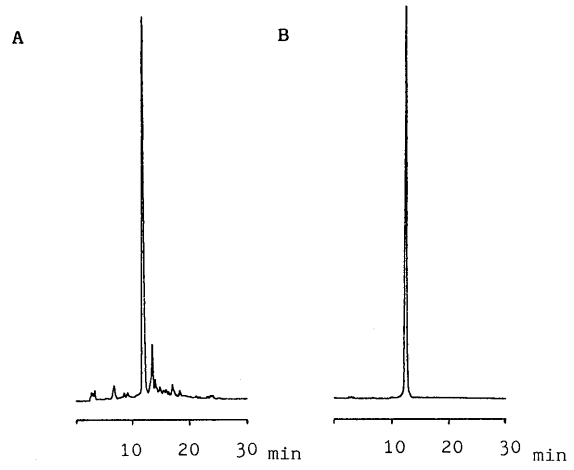

Fig. 2. HPLC Profiles of the Deprotected Sample and Purified hPYY

A. Gel-filtered sample after final deprotection with 1 M TMSOTf–thioanisole/TFA. B. Finally purified hPYY. HPLC was performed on a YMC AM-312 column (6.0 × 150 mm) using linear gradient elution from (A) to (B) (in 30 min) at a flow rate of 1 ml/min. (A): 30% CH_3CN (0.1% TFA). (B): 45% CH_3CN (0.1% TFA).

deprotected product was treated with diluted ammonia (pH 8.0) containing 5 N NH_4F to reverse a possible N→O shift[15] and to hydrolyze attached trimethylsilyl groups. The lyophilized sample was then purified by gel-filtration on Sephadex G-25, followed by high-performance liquid chromatography (HPLC). The purity of synthetic hPYY was ascertained by thin-layer chromatography (TLC), analytical HPLC (Fig. 2-B), and amino acid analyses after acid hydrolysis and enzymatic digestion. The five fragments resulting from tryptic hydrolysis at the four Arg residues were well separated and identified by HPLC (Fig. 3).

Biological effects of the synthetic hPYY on systemic arterial blood pressure and splanchnic blood flow were examined in a male mongrel dog (weighing 10 kg). Synthetic hPYY was administered intravenously as a bolus. A slight increase in systemic mean blood pressure was observed at doses of 1, and 2 μg/kg weight (106.3% and 106.1%, respectively; $n=2$). At doses of 0.1, 0.2, 0.5, 1, and 2 μg/kg, hPYY caused a decrease of superior mesenteric artery (SMA) blood flow (63.5%, 60.0%, 50.0%, 43.7%, and 23.8% of the basal flow expressed as 100%, respectively; $n=2$), and a decrease of celiac artery (CA) blood flow (71.4%, 69.0%, 63.2%, 67.6%, and 65.6% of the basal flow, respectively; $n=2$). These biological effects are comparable to those of the porcine PYY formerly synthesized in this laboratory. The details of the biological effects will be reported elsewhere.

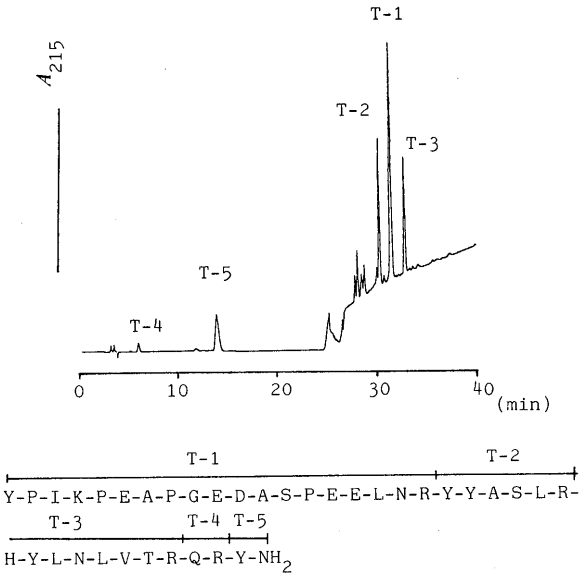

Fig. 3. HPLC Separation of Tryptic hPYY Fragments (T-1 to T-5)

HPLC was performed on a μBondapak C_{18} column (3.9 × 300 mm) using linear gradient elution from (A) to (B) (in 40 min) at a flow rate of 1 ml/min. (A): 0.1% TFA. (B): 50% CH_3CN (0.1% TFA).

Experimental

General experimental procedures employed here are essentially the same as described for the porcine PYY synthesis.[8] *Rf* values in TLC refer to the following solvent systems: Rf_1 = $CHCl_3$–MeOH–H_2O (8:3:1), Rf_2 = *n*-BuOH–AcOH–pyridine–H_2O (4:1:1:2), Rf_3 = *n*-BuOH–AcOH–AcOEt–H_2O (1:1:1:1). Leucine aminopeptidase (LAP) (lot 87F-8045) and trypsin (lot 84F-8045) were purchased from Sigma Chemical Co.

Products were purified by one of the following procedures. Procedure A: For purification of a product soluble in AcOEt, the extract was washed with 5% citric acid, 5% Na_2CO_3 and brine, then dried over Na_2SO_4 and concentrated. The residue was recrystallized or precipitated from appropriate solvents. Procedure B: For purification of a peptide less soluble in AcOEt, the crude product was triturated with ether and 5% citric acid. The resulting power was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O, and recrystallized or precipitated from appropriate solvents.

Z(OMe)–Asn–Arg(Mts)–Tyr–Tyr–Ala–OMe A mixture of Z(OMe)–Asn–ONp (2.50 g, 6.0 mmol), Et_3N (0.70 ml, 5.0 mmol), NMM (0.55 ml, 5.0 mmol), and a TFA-treated sample of Z(OMe)–Arg(Mts)–Tyr–Tyr–Ala–OMe[8] (4.66 g, 5.0 mmol) in DMF (50 ml) was stirred for 48 h at 4 °C. The DMF was removed by evaporation *in vacuo* and the product was purified by procedure B, followed by recrystallization from MeOH twice, yield 3.75 g (72%). mp 192—196 °C, $[\alpha]_D^{25}$ −37.8° ($c=1$, DMF). Rf_1 0.51. *Anal.* Calcd for $C_{50}H_{63}N_9O_{14}S$: C, 56.43; H, 6.16; N, 11.85. Found: C, 56.13; H, 6.48; N, 12.27.

Z(OMe)–Asn–Arg(Mts)–Tyr–Tyr–Ala–NHNH$_2$ [2] The above prepared pentapeptide ester (5.60 g, 5.4 mmol) in DMF (50 ml) was treated with hydrazine hydrate (2.70 ml, 54 mmol) for 48 h. The DMF was removed by evaporation *in vacuo* and the residue was triturated with EtOH. The resulting powder was collected and washed with cold EtOH, yield 4.73 g (85%). mp 223—226 °C, $[\alpha]_D^{25}$ −32.5° ($c=1$, DMF). Rf_1 0.30. *Anal.* Calcd for $C_{49}H_{63}N_{11}O_{13}S \cdot 1/2H_2O$: C, 55.77; H, 6.11; N, 14.60. Found: C, 55.70; H, 6.13; N, 14.68. Amino acid ratios in a 6N HCl hydrolysate: Asp 1.04, Ala 1.00, Tyr 2.06, Arg 1.10 (recovery of Ala, 83%).

Boc–Pro–Ile–OMe The title compound was prepared by the mixed anhydride method and purified by procedure A. The oily product was used for the next coupling reaction, yield 80%, Rf_1 0.37.

Z(OMe)–Tyr–Pro–Ile–OMe The azide [prepared from Z(OMe)–

TABLE II. Characterization of the Protected hPYY and Its Intermediates

	Purification[a] method (recryst.)	Yield (%)	mp (°C)	$[\alpha]_D^{25}$ ($c=1$, DMF)	Rf_1	Formula	Analysis (%) Calcd (Found) C	H	N
Z(OMe)–(18—36)-NH_2	B (DMF–AcOEt)	73	170—173	+13.2°	0.31	$C_{164}H_{225}Cl_2N_{37}O_{39}S_4 \cdot 6H_2O$	54.02 (53.95)	6.55 6.64	14.22 13.99)
Z(OMe)–(13—36)-NH_2	C	53	156—159	+21.3°	0.34	$C_{209}H_{280}Cl_2N_{42}O_{49}S_4 \cdot 6H_2O$	56.13 (56.37)	6.58 6.44	13.16 12.87)
Z(OMe)–(10—36)-NH_2	B (DMF–AcOEt)	81	158—160	+24.3°	0.41	$C_{235}H_{315}Cl_2N_{45}O_{56}S_4 \cdot 7H_2O$	56.54 (56.53)	6.64 6.43	12.63 12.22)
Z(OMe)–(4—36)-NH_2	D	68	159—161	+4.0°	0.41	$C_{276}H_{368}Cl_2N_{52}O_{66}S_4 \cdot 7H_2O$	57.20 (57.13)	6.64 6.48	12.57 12.30)
Z(OMe)–(1—36)-NH_2	D	75	155—159	+1.0°	0.46	$C_{296}H_{395}Cl_2N_{55}O_{70}S_4 \cdot 7H_2O$	57.63 (57.42)	6.68 6.36	12.49 12.39)

a) B, reprecipitation; C, silica-gel column chromatography; D, gel-filtration on Sephadex LH-60.

Tyr–$NHNH_2$ (6.54 g, 18.2 mmol)] and Et_3N (2.55 ml, 18.2 mmol) were combined with a TFA-treated sample of Boc–Pro–Ile–OMe (5.65 g, 16.5 mmol) in DMF (50 ml) containing Et_3N (2.31 ml, 16.5 mmol). The reaction mixture was stirred for 24 h at 4 °C, and the DMF was removed by evaporation *in vacuo*. The residue was purified by procedure A, followed by recrystallization from AcOEt with ether, yield 7.05 g (75%). mp 168—171 °C, $[\alpha]_D^{25}$ −49.2° ($c=1$, MeOH). Rf_1 0.74 *Anal.* Calcd for $C_{30}H_{39}N_3O_8$: C, 63.25; H, 6.90; N, 7.38. Found: C, 62.78; H, 7.01; N, 7.29.

Z(OMe)–Tyr–Pro–Ile–$NHNH_2$ [6] The above prepared tripeptide ester (5.00 g, 8.8 mmol) in MeOH (100 ml) was treated with hydrazine hydrate (8.17 ml, 132 mmol) for 48 h. The solvent was removed by evaporation and the residue was triturated with H_2O. The resulting powder was collected and washed well with H_2O, yield 4.80 g (96%). mp 149—151 °C, $[\alpha]_D^{25}$ −36.8° ($c=1$, DMF). Rf_1 0.56. *Anal.* Calcd for $C_{29}H_{39}N_5O_7 \cdot 1/2H_2O$: C, 60.19; H, 6.97; N, 12.10. Found: C, 59.78; H, 6.75; N, 11.96. Amino acid ratios in a 6 N HCl hydrolysate: Pro 1.09, Ile 1.00, Tyr 1.05 (recovery of Ile, 90%).

Synthesis of Protected hPYY Successive azide condensations of the six peptide fragments were carried out according to the indicated route (Fig. 1). Prior to condensation, the Z(OMe) group was removed from the respective amino component by treatment with TFA (*ca.* 0.5 ml per 0.1 g of the peptide) in the presence of anisole (*ca.* 10 eq) in an ice-bath for 60 min. The TFA-treated sample was precipitated with dry ether, dried over KOH pellets *in vacuo* for 2 h and dissolved in DMF containing Et_3N (1 eq). The corresponding azide (the amount was increased from 1.5 to 3 eq as the chain elongation progressed) in DMF and Et_3N (1 eq) were added to the above ice-chilled solution and the mixture was stirred at −4 °C until the solution became negative to the ninhydrin test. The DMF was evaporated off *in vacuo* and the residue was triturated with ether to afford a solid, which was purified by procedure B, followed by reprecipitation from DMF with AcOEt or ether. For the purification of Z(OMe)–(13—36)-NH_2, column chromatography on silica gel was employed using $CHCl_3$: MeOH : H_2O (8 : 3 : 1) as an eluant (procedure C). For the purification of Z(OMe)–(4—36)-NH_2 and Z(OMe)–(1—36)-NH_2, gel-filtration on Sephadex LH-60 (3.5 × 110 cm) was employed using DMF as an eluant. In this case, eluates (10 g each) were examined by measuring the ultraviolet (UV) absorption at 280 nm and the fractions corresponding to the first main peak were combined. The DMF was removed by evaporation *in vacuo* and the residue was treated with ether to give a powder (procedure D). Purification procedures, yields, physical constants and elemental analysis of the protected hPYY and its protected intermediates are listed in Table II.

H–Tyr–Pro–Ile–Lys–Pro–Glu–Ala–Pro–Gly–Glu–Asp–Ala–Ser–Pro–Glu–Glu–Leu–Asn–Arg–Tyr–Tyr–Ala–Ser–Leu–Arg–His–Tyr–Leu–Asn–Leu–Val–Thr–Arg–Gln–Arg–Tyr–NH_2 (hPYY) The above obtained fully protected 36-peptide amide (50 mg, 8.30 μmol) was treated with 1 M TMSOTf–thioanisole/TFA (2.25 ml) in the presence of *m*-cresol (110 μl) in an ice-bath for 3 h, then dry ether was added. The resulting precipitate was collected by centrifugation, dried over KOH pellets *in vacuo* for 2 h and dissolved in H_2O (10 ml). The pH of the solution was adjusted to 8.0 with 5% NH_4OH, and 5 N NH_4F (50 μl) was added. After 30 min, the pH of the solution was adjusted to 5.5 with 1 N AcOH and the solution was lyophilized. This sample was dissolved in 5% AcOH (3 ml) and applied to a column of Sephadex G-25 (2.5 × 110 cm), which was eluted with 5% AcOH. The fractions (8 ml each) corresponding to the main peak (tube Nos. 24—30, monitored by UV measurement at 275 nm) were combined and the solvent was removed by lyophilization to give a fluffy powder, yield 34.2 mg (96%).

Subsequent purification was performed by reversed-phase HPLC on a YMC AM-323 column (10 × 250 mm). A part of the above crude sample (*ca.* 2 mg each) was applied to the column, which was eluted with a linear gradient of CH_3CN (30—45% in 30 min) in 0.1% aqueous TFA at a flow rate of 2.5 ml/min. The eluate corresponding to the main peak (retention time 12.4 min, minotored by UV measurement at 275 nm) was collected and the solvent was removed by lyophilization to give a white fluffy powder, yield 12.2 mg (34.2% from the protected hPYY) $[\alpha]_D^{25}$ −53.0° ($c=0.1$, 1 N AcOH). Rf_2 0.10, Rf_3 0.39; Retention time, 11.7 min in HPLC on an analytical YMC AM-312 column (Fig. 2-B). Amino acid ratios in a 6 N HCl hydrolysate are listed in Table I. Amino acid ratios in an LAP digest (numbers in parentheses are theoretical); Asp (1) 0.89, Thr (1) 1.75, Ser (2) 1.75, Glu (4) 3.58, Pro (4) 3.08, Gly (1) 0.83, Ala (3) 2.72, Val (1) 1.15, Ile (1) 0.95, Leu (4) 4.00, Tyr (5) 5.04, Lys (1) 0.92, His (1) 1.05, Arg (4) 4.05; Asn (2) and Gln (1) were not determined (recovery of Leu = 65%).

Separation of Tryptic hPYY Fragments on HPLC Synthetic hPYY (560 μg) was dissolved in 1% NH_4HCO_3 (400 μl) and 16 μl of trypsin solution (2 mg/ml) was added. The mixture was incubated for 4 h at room temperature, then for 6 min at 100 °C. After lyophilization, the residue was dissolved in distilled H_2O (250 μl) and subjected to HPLC. Conditions for HPLC were as described in the legend to Fig. 3. Respective fraction was lyophilized and hydrolyzed in 6 N HCl in the usual manner. Amino acid ratios of tryptic fragments (numbers in parentheses are theoretical values): T-1 (positions 1—19); Asp 1.97 (2), Ser 1.01 (1), Glu 3.95 (4), Pro 4.52 (4), Gly 1.00 (1), Ala 1.87 (2), Ile 0.69 (1), Leu 1.00 (1), Tyr 0.96 (1), Lys 0.87 (1), Arg 1.09 (1). T-2 (positions 20—25); Ser 0.97 (1), Ala 0.96 (1), Leu 1.00 (1), Tyr 1.80 (2), Arg 1.06 (1). T-3 (positions 26—33); Asp 1.14 (1), Thr 1.00 (1), Val 1.00 (1), Leu 2.05 (2), Tyr 1.00 (1), His 0.89 (1), Arg 1.03 (1). T-4 (positions 34—35); Glu 1.00 (1), Arg 0.98 (1). T-5 (position 36); Tyr 1.00 (1). Amino acid ratios in an LAP digest of T-1, T-3, and T-4 fragments (numbers in parentheses are theoretical values): T-1; Asp 1.06 (1), Ser 0.86 (1), Glu 4.04 (4), Pro 3.20 (4), Gly 1.00 (1), Ala 1.86 (2), Ile 1.30 (1), Leu 0.98 (1), Tyr 0.84 (1), Lys 0.91 (1), Arg 0.75 (1), Asn (1) was not determined. T-3; Thr 1.25 (1), Val 1.04 (1), Leu 2.00 (2), Tyr 1.06 (1), His 1.04 (1), Arg 0.85 (1), Asn (1) was not determined and Asp was not detected. T-4; Arg 1.00 (1), Gln (1) was eluted at the position of Thr and not determined.

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References and Notes

1) Amino acids, peptides and their derivatives in this paper are of the L-configuration. Abbreviations used are those recommended by the I.U.P.A.C.-I.U.B. Commission on Biochemical Nomenclature; *J. Biol. Chem.*, **247**, 977 (1972). Z = benzyloxycarbonyl, Z(OMe) = *p*-methoxybenzyloxycarbonyl, Fmoc = 9-fluorenylmethyloxycarbonyl, Bzl = benzyl, Mts = mesitylenesulfonyl, Chp = β-cycloheptyl, Cl_2Bzl = 2,6-dichlorobenzyl, Np = *p*-nitrophenyl, Su = *N*-hydroxysuccinimidyl, TFA = trifluoroacetic acid, TMSOTf = trimethylsilyl trifluoromethanesulfonate, Et_3N = triethylamine, NMM = *N*-methylmorpholine, DMF = dimethylformamide, MeOH = methanol, EtOH

=ethanol, AcOEt=ethyl acetate, AcOH=Acetic acid.
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