Combination of a New Amide-precursor Reagent and Trimethylsilyl Bromide Deprotection for the Fmoc-based Solid Phase Synthesis of Human Pancreastatin and One of its Fragments (Fmoc = Fluoren-9-ylmethoxycarbonyl)

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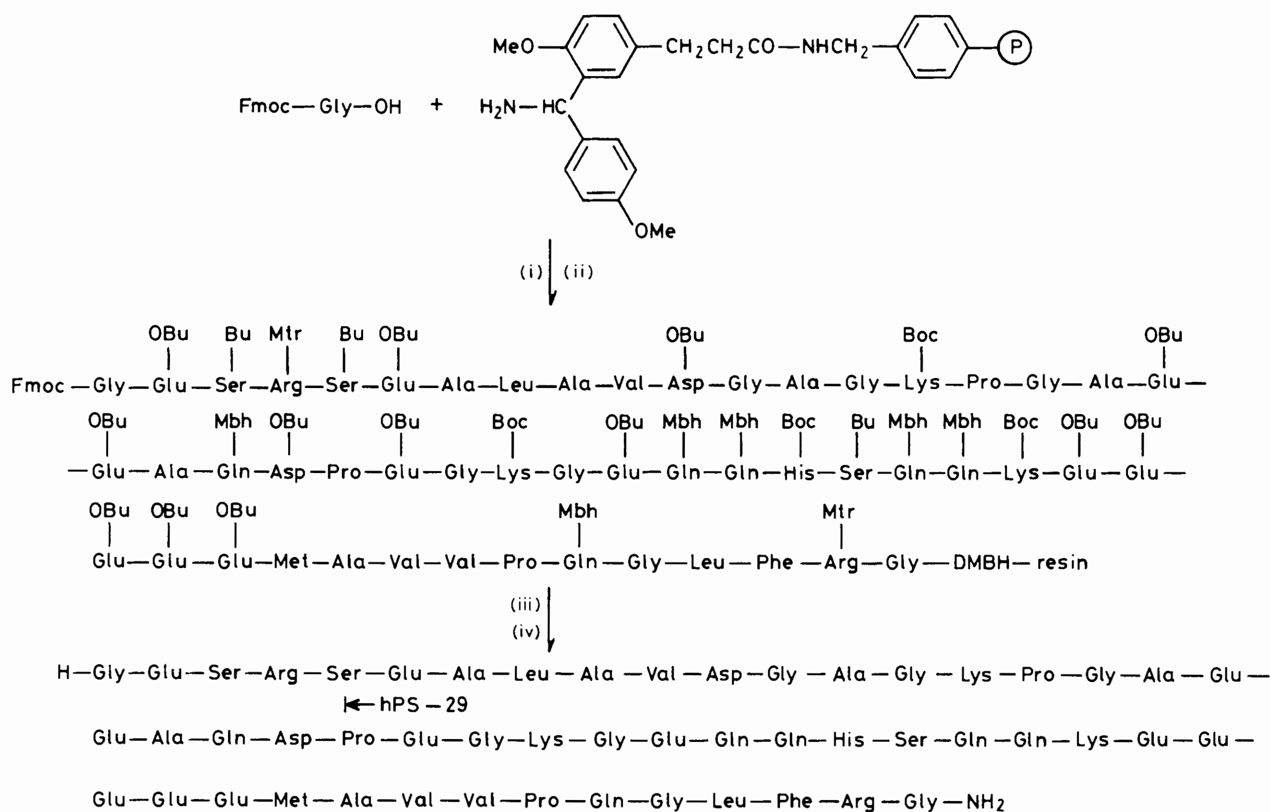
A 52- and a 29-residue peptide amide corresponding to human pancreastatin and one of its fragments were synthesized by the Fmoc-based solid phase techniques (Fmoc = fluoren-9-ylmethoxycarbonyl), and a new amide precursor reagent, 3-(α-Fmoc-amino-4-methoxybenzyl)-4-methoxyphenylpropionic acid, was employed in combination with thioanisole-mediated trimethylsilyl bromide deprotection; the synthetic peptides significantly inhibited protein output, but not juice flow or bicarbonate output from rat pancreas.

Pancreastatin, a new 49-residue peptide amide, was first isolated from a porcine pancreas by Tatemoto *et al.*[1] in 1986 using an analytical tool specific for the C-terminal amide. This peptide has been shown to inhibit glucose-induced insulin release from the isolated perfused pancreas[2] and therefore may play an important role in the regulation of insulin secretion.

In 1987, Konecki *et al.*[3] proposed the amino acid sequence of human pancreastatin (hPS), a 52-residue peptide amide (hPS-52), deduced from the gene structure of human chromogranin A since this gene contained a sequence (positions 250—301) homologus to porcine pancreastatin. Later, Sekiya *et al.*[4] reported the isolation of a 29-residue peptide amide from a pancreatic glucagonoma. This peptide, named hPS-29, corresponds to the C-terminal portion (positions 24—52) of hPS that was proposed by Konecki *et al.* We report the



Scheme 1. Fmoc-based solid phase syntheses of human pancreastatin 29 and 52; Fmoc = fluoren-9-ylmethoxycarbonyl, Boc = t-butoxycarbonyl, Bu = t-butyl; Mtr = 4-methyl-2,3,6-trimethoxybenzenesulphonyl, Mbh = 4,4′-dimethoxybenzhydryl, DIPCD = diisopropyl carbodiimide, HOBt = 1-hydroxybenzotriazole, NMP = N-methylpyrrolidone, DMF = dimethylformamide.

(i) Condensation by DIPCD and HOBt in NMP; (ii) Deprotection by 20% piperidine/DMF; (iii) 20% piperidine/DMF; (iv) 1 M TMSBr-anisole/TFA and EDT.

Table 1. Increments of fluid, bicarbonate, and protein outputs stimulated by CCK-8 with and without hPS-29 and hPS-52.

	Control (without pancreastatin)	Pancreastatin (24—52)	(200 pmol/kg/h) (1—52)
Fluid output (ml/3 h)	0.37 ± 0.055	0.21 ± 0.064	0.29 ± 0.082
Bicarbonate output (μ equiv./ 3 h)	3.66 ± 1.03	3.80 ± 1.32	1.31 ± 3.23
Protein output (mg/3 h)	47.11 ± 2.80	27.65 ± 4.38	28.30 ± 4.43

Fmoc-based solid phase syntheses of both hPS-52 and 29 using a newly introduced amide precursor reagent and a new deprotecting reagent.

Recently we introduced a new amide precursor reagent,[5] 3-(α-Fmoc-amino-4-methoxybenzyl)-4-methoxyphenylpropionic acid (Fmoc-DMBH-$CH_2CH_2CO_2H$), for linkage to resin supports.[6] This acid-sensitive modified dimethoxybenzhydrylamine type reagent can be prepared easily and introduced smoothly by means of dicyclohexylcarbodiimide (DCC)[7] onto an aminomethyl polystyrene resin through the propionic acid side chain. We also introduced recently a new deprotecting procedure involving trimethylsilyl bromide (TMSBr) in trifluoroacetic acid (TFA).[8] This reagent has the ability to cleave readily benzyl, t-butyl, and phenylsulphonyl type protecting groups in the presence of a soft nucleophile such as thioanisole. Because TMSBr is a volatile reagent, it is particularly useful in solid phase peptide synthesis, since it allows easier separation of deprotected peptides from the resin in comparison with non-volatile deprotecting reagents, such as trifluoromethanesulphonic acid (TFMSA)[9] or trimethylsilyl trifluoromethanesulphonate (TMSOTf).[10] By the use of Fmoc-amino acids,[11] routine manipulation during solid phase syntheses are simpler than for Boc-based procedures.[12] The syntheses were carried out manually according to the principle of Sheppard *et al.*,[6] using the side-chain protected following Fmoc-amino acids; Arg(Mtr),[13] Lys(Boc), His(Boc), Glu(OBu^t), Asp(O^{t}Bu), and Ser(Bu^t). In addition Gln(Mbh)[14] was used to suppress base-catalysed pyrrolidone formation.[15] First, Fmoc-DMBH-resin (172 mg, amine-content 0.58 mmol/g, polystyrene resin cross-linked with 1% divinylbenzene) was treated twice (5 and 15 min) with 20% piperidine in dimethylformamide (DMF) to remove the Fmoc group, and then each Fmoc amino acid (2.5 equiv.) in NMP was condensed successively by means of di-isopropylcarbodi-imide–1-hydroxybenzotriazole (DIPCD-HOBt) (2.5 equiv. each)[16] (Scheme 1). The container was shaken until the resin showed a negative Kaiser test[17] (usually 1 h). For introduction of Arg(Mtr) and Gln(Mbh), double couplings were necessary to complete the reaction.

At the stage of 29 cycles, a part of the protected peptide resin (100 mg) was treated with 1 M TMSBr–thioanisole in TFA in the presence of EDT (ethanedithiol) and *m*-cresol at 0 °C for 2 h to cleave the peptide amide from the resin and at the same time to remove all protecting groups. The deprotected 29-residue peptide amide (Sekiya's sequence) was purified

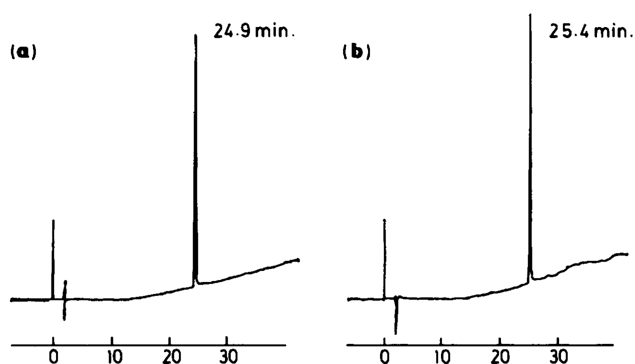


Figure 1. H.p.l.c. of synthetic hPS-29(a) and hPS-52(b). column: Cosmosil 5PhT-300 (4.6 × 150 mm); flow rate: 1 ml/min; elution: Gradient elution with MeCN (10—60%, 50 min) in aqueous TFA (0.1%); detection: 222 nm.

to homogeneity by gel-filtration on Sephadex G-15 using NH_4HCO_3 (0.1 M), followed by h.p.l.c. on an Ultrapore RPSC column which was eluted by a gradient of MeCN (15—25%, 60 min) in aqueous 0.1% TFA; yield 25.7 mg (54%, based on the Gly loaded on the resin). Since the desired peptide was obtained in good yield, the peptide chain was further elongated to the stage of the 52-peptide. The protected peptide resin (100 mg) was deprotected and cleaved from the resin as described above to afford the 52-residue peptide amide (Konecki's sequence). The deprotected peptide was purified to homogeneity by gel-filtration on Sephadex G-15 using NH_4HCO_3 (0.1 M), followed by h.p.l.c. on an Ultrapore RPSC column in essentially the same manner as described above; yield 4.9 mg (9.0%, based on the Gly loaded on the resin). The purity of synthetic hPS-29 and hPS-52 thus obtained was ascertained by amino acid analysis, after hydrolysis with 6 M HCl and analytical h.p.l.c. on a Cosmosil 5PhT-300 column [retention time 24.9 and 25.4 min respectively, by gradient elution with MeCN (10—60%, 50 min) in aqueous TFA (0.1%)] (Figure).

The effects of hPS-29 and hPS-52 on pancreatic secretion by stimulated exogenous CCK-8 (100 pmol/kg/h) (cholecystokinin-8, purchased from Peptide Institute, Inc., Osaka, Japan) were examined in rats ($n = 5 \sim 9$).[18] Both synthetic peptides (200 pmol/kg/h each) significantly inhibited pancreatic protein output, but not juice flow or bicarbonate output by one-way analysis of variance (Table 1).

This work shows that relatively complex peptide amides containing Arg can be synthesized by Fmoc-based solid phase synthesis with the aid of our newly introduced amide precursor reagent and the thioanisole-mediated TMSBr deprotection technique. HPS-29 was obtained in good yield (54%); however as chain elongation progressed, the yield was considerably lowered. Our new techniques offer useful insights regarding the scope and limitation of current protocols for Fmoc-based solid phase syntheses.

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