Title: Azapeptide Synthesis Fully Integrated into Standard Solid-Phase Peptide Synthesis (SPPS): Case Study with Azapeptide-GLP-1

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Abstract: Therapeutic peptides could benefit significantly from the well-established azapeptide (substitution of backbone alpha atom(s) with N, producing an enzyme resistant semicarbazide bond), yet their broader application remains limited due to the challenges associated with their synthesis, particularly in generating diverse libraries for systematic functional evaluation. Traditional azapeptide synthesis methods rely on the activation of hydrazine derivatives using carbonyl-donating reagents such as phosgene, chloroformates, or *in situ* activation of thiocarbazates. Despite advances in peptide chemistry, no fully automated system for azapeptide synthesis has been available until now. Herein, we present a fully automated solid-phase azapeptide synthesis platform, employing Fmoc-protected benzotriazole esters as bench-stable, pre-activated aza-amino acid building blocks. This protocol enables the rapid and efficient synthesis of azapeptide libraries, considerably reducing reaction times while improving both crude purity and isolated yields. To rigorously test this platform, we synthesized azapeptide analogues of the 31-residue peptide hormone, glucagon-like peptide-1 (GLP-1), modifying the proteolytic enzyme-sensitive sites. This fully automated azapeptide synthesis platform marks a significant milestone in peptide chemistry, addressing key barriers to library generation and offering a scalable and efficient tool for the development of azapeptide-based therapeutics.

Graphic for Table of Contents

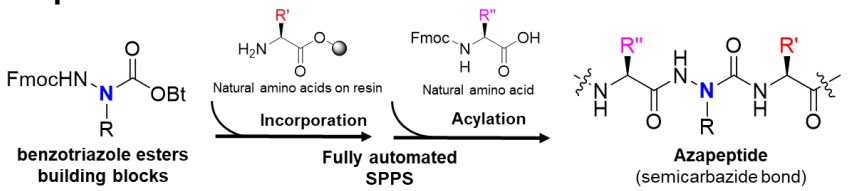

Table of Contents: Bench-Stable, Orthogonal, Fully Automated Solid Phase Azapeptide Synthesis System. Benzotriazole ester building blocks can be incorporated into a peptide sequence using solid-phase peptide synthesis (SPPS) in a rapid and fully automated manner to construct libraries of azapeptide-substituted candidates for biological screening. R, R', R'': natural amino acid side chains (372 character)

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Azapeptides[1] – peptides comprising nitrogen substitution of alpha carbon atoms in the backbone – are promising class of peptidomimetics that address the inherent instability of peptides. This structural modification imparts a rigid trigonal geometry and enhances the resistance to enzymatic hydrolysis. Despite their therapeutic potential, highlighted by the clinical success of Goserelin[2] (Zoladex®) for prostate and breast cancer and Atazanavir[3] (Reyataz®) for HIV infection, azapeptide development has been constrained by synthetic challenges. Conventional synthetic routes[1b, 4] typically rely on the activation of hydrazine derivatives with carbonyl-donating reagents, such as phosgene or chloroformates, which are often unstable, and cumbersome to process (Figure 1a). Although alternative strategies, such as sub monomer azapeptide synthesis[5] and customized solid-phase peptide synthesis (SPPS)[1b], have enriched the field, they frequently suffer from low yields and harsh reaction conditions. In our previous work, we introduced a semi-automated synthetic platform using 16 thiocarbazate building blocks as stable but easily activated precursors[6] (Figure 1b). This discovery enabled the generation of azapeptide libraries with enhanced stability and biological activity, as demonstrated in two model peptides, FSSE and bradykinin.

Herein, we report the development of a fully automated solid phase azapeptide synthesis platform (Figure 1c) that employs a fully automated synthetic strategy using Fmoc-protected benzotriazole esters as bench stable, pre-activated aza-amino acid building blocks. This platform enables the rapid and efficient synthesis of azapeptide libraries – significantly reducing reaction times, minimizing by-products, and enhancing both crude purity and ultimate isolated yields. Using our methodology, we edit a dipeptidyl peptidase 4 (DPP-4) cleavage site in the 31-amino acid hormone, glucagon-like peptide-1 (GLP-1)[7]; four aza-amino acid-substituted GLP-1 analogues are strategically engineered for subsequent biological testing.

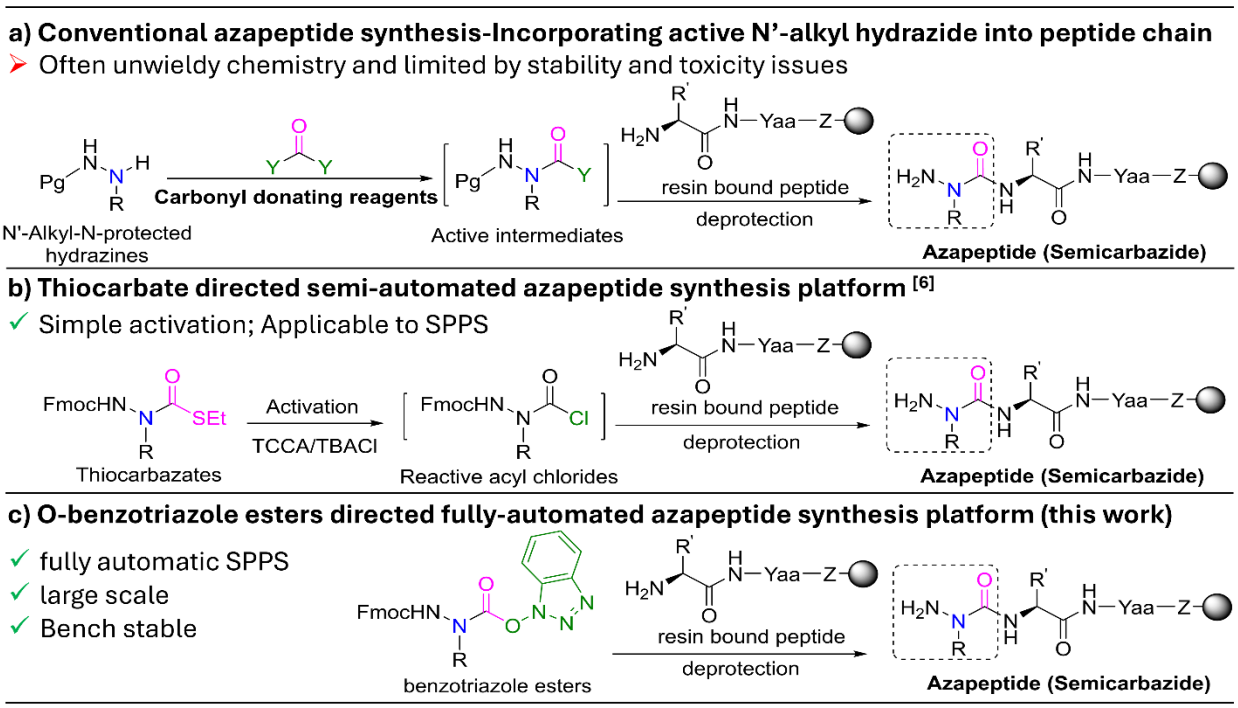


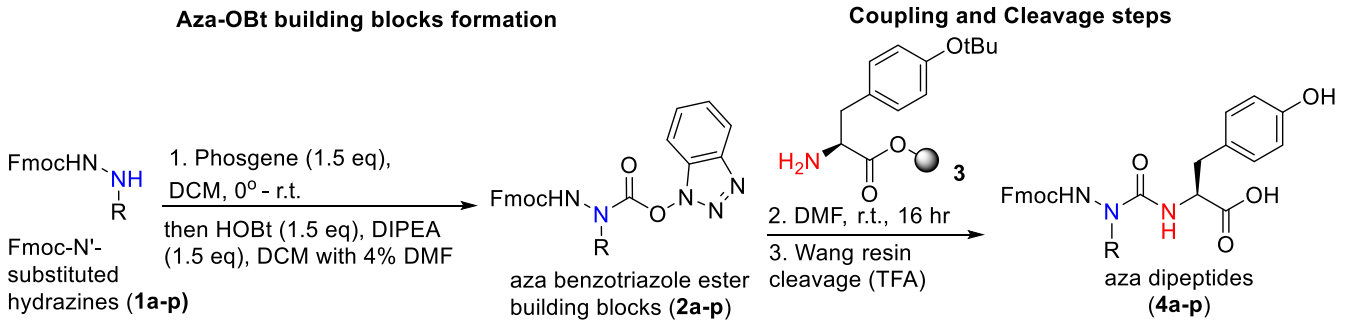
Figure 1. Current azapeptide synthetic strategies. a) conventional azapeptide synthesis-activation of N'-alkylated hydrazine derivatives with carbonyl donating reagents (such as chloroformates, p-nitrophenyl chloroformate, bis(2,4-dinitrophenyl) carbonate, carbonyldiimidazole (CDI), 1,1′-Carbonyl-di-(1,2,4-triazole) (CDT), bis(pentafluorophenyl) carbonate, and N,N'-disuccinimidyl carbonate (DSC); b) thiocarbazate-based azapeptide synthesis-simple activation (5-10 mins at r.t.) of shelf-stable thiocarbazate building block[6], compatible with solid phase chemistry; c) O-benzotriazole esters-based azapeptide synthesis-bench stable, pre-activated aza precursors, providing fully automation strategy in solid phase azapeptide synthesis[8]. Abbreviations – Pg: Boc, Ddz, Phth and Fmoc; R, R': H, amino acids side chain; Yaa: amino acids; Z: O (Wang resin), NH (Rink amide resin)

Unlike natural amino acids, aza-amino acids exhibit inherent instability and require alternative activation strategies[4a, 9]. Inspired by the work by Gibson et al.[10], which proposed an active azasarcosine benzotriazole ester formed *in situ* for azapeptide coupling, we investigated whether 1-hydroxybenzotriazole (HOBt) could react with aza-amino acyl chloride to form stable carbazates suitable for isolation, storage, and subsequent coupling to natural amino acid without activation. To achieve this, sixteen Fmoc-azaAA-benzotriazole ester (Fmoc-azaAA-OBt) building blocks (**2a-2p**) were synthesized by treating N-Fmoc-N'-substituted hydrazines (**1a-1p**)[4] with 1.5 equivalents of phosgene to form transient acyl chlorides, followed by immediately trapping with 1.5 equivalents of HOBt (pre-dissolved in DMF). As anticipated, these transformations typically proceeded well and produced bench stable benzotriazole building blocks in high yields (Table 1, **2a-2p**, 73.3%-96.1%, Supplementary Table S1 and Supplementary Information). These benzotriazole building blocks maintain long-term stability in the solid state at -20°C and remain stable for at least six months at room temperature. Additional stability tests demonstrated that these building blocks remain intact in DMF at room temperature for 24 hours, with recovery rates

exceeding 95%. Notable exceptions included glycine (azaglycine carbazate undergoes intramolecular cyclization to form oxadiazalone[11]), serine, threonine, and cysteine (inherently incompatible with azapeptide chemistry, therefore, not investigated).

To demonstrate the utility of these aza-building blocks, incorporating aza-amino acids to the tyrosine pre-loaded Wang resin was employed as a model system for automated solid phase azapeptide synthesis (Table 1). The syntheses were conducted on 0.1 mmol of amino acid loaded Wang resin; a representative SPPS cycle includes swelling, Fmoc deprotection (20% piperidine in DMF), successive washes (DMF and DCM), and semicarbazide formation. Following standard Fmoc/tBu SPPS protocols, 5 equivalents of Fmoc-aza benzotriazole building blocks (**2a-p**) was auto-dissolved in DMF and coupled to tyrosine pre-loaded Wang resin (**3**) and the resin suspension was shaken for 16 hours at room temperature. Our data have showed that over 80% of aza-amino acid was integrated into a Wang or Rink amide resin within one hour (see supporting information, section 2.5), and optimal conversion was achieved after 16 hours of shaking. All sixteen benzotriazole esters were successfully coupled to tyrosine pre-loaded Wang resin with excellent conversion (85.4%-100%, Table 1, **4a-p**). After cleavage from the resin, the resulting azaAA-Y dipeptides (**4a-p**) were obtained in 62.7%-95.1% crude purity. The resulting azadipeptides were characterized by HPLC, HRMS and NMR analyses (see supporting information, section 2.3). However, specific challenges were noted for dipeptides containing azatyrosine and azatryptophan, which previously revealed side-chain cleavage during standard TFA treatment[4b, 6]. To address this, we optimized cleavage conditions by adjusting TFA concentration and reaction temperature. Under optimized cleavage conditions, azaWY-OH (**4f**) and azaYY-OH (**4o**) were successfully collected in 62.7% and 83.2% crude purity respectively. In sum, we have established a robust, fully automated strategy to incorporate aza-amino acid residue into peptides using conventional solid phase peptide synthesizer.

Table 1. Automated incorporation of aza-moieties: synthesis of azadipeptides using 16 benzotriazole ester building blocks via standard SPPS. Reagents and conditions: (1) Synthesis of benzotriazole esters building blocks: Phosgene (1.5 equiv), DCM, 0° C – r.t., 0.5 h then HOBt (1.5 equiv), DIPEA (1.5 equiv), 4% DMF in DCM, r.t., 0.5 h; (2) Integration of the aza-amino acids in SPPS: NH_2-Tyr(tBu)-Wang Resin (1 equiv), aza-amino acid OBt (5 equiv, 0.2 M), DMF, r.t., 16 h; (3) Cleavage step: TFA/H_2O/TIPS (95:2.5:2.5. v/v/v), r.t., 2 h, 10-25ml/g of resin. Synthesis and characterization of these aza-amino acid building blocks and azadipeptides are detailed in Supplementary Information.



Entry	aza benzotriazole building blocks (**2a-p**)			aza dipeptides (**4a-p**)					
	R: amino acid side chain	Isolated Yield		Sequence	Conversion rate[a]		Crude purity (254 nm)	HRMS (cal. H^+)	HRMS (obs. H^+)
1	Fmoc-azaArg(*bis*-Boc)-(OBt)[b]	**2a**	83.0%	Fmoc-azaRY-OH	**4a**	98.5%	95.1%	561.2456	561.2454
2	Fmoc-azaHis(Trt)-(OBt)	**2b**	78.0%	Fmoc-azaHY-OH	**4b**	99.4%	90.3%	542.2034	542.2032
3	Fmoc- azaLys(Boc)-(OBt)	**2c**	82.7%	Fmoc- azaKY-OH	**4c**	96.5%	88.8%	533.2395	533.2392
4	Fmoc-azaAsp(*t*Bu)-(OBt)	**2d**	93.0%	Fmoc-azaDY OH	**4d**	95.3%	85.5%	520.1714	520.1713
5	Fmoc-azaGlu(*t*Bu)-(OBt)	**2e**	86.8%	Fmoc-azaEY-OH	**4e**	99.8%	90.1%	534.1871	534.1892
6	Fmoc-azaTrp(Boc)-(OBt)	**2f**	92.4%	Fmoc-azaWY-OH[c]	**4f**	98.4%	62.7%	591.2165	591.2163
7	Fmoc-azaGln(Trt)-(OBt)	**2g**	85.0%	Fmoc-azaQY-OH	**4g**	85.4%	83.4%	533.2031	533.2056
8	Fmoc-azaAsn(Trt)-(OBt)	**2h**	73.3%	Fmoc-azaNY-OH	**4h**	95.9%	85.9%	519.1801	519.1803
9	Fmoc-azaAla-(OBt)	**2i**	87.4%	Fmoc-azaAY-OH	**4i**	99.1%	86.7%	476.1816	476.1837
10	Fmoc-azaVal-(OBt)	**2j**	85.0%	Fmoc-azaVY-OH	**4j**	92.0%	88.3%	504.2129	504.2130
11	Fmoc-azaIle-(OBt)	**2k**	82.5%	Fmoc-azaIY-OH	**4k**	91.1%	85.9%	518.2286	518.2306
12	Fmoc-azaLeu-(OBt)	**2l**	89.2%	Fmoc-azaLY-OH	**4l**	93.3%	90.9%	518.2286	518.2283
13	Fmoc- azaMet-(OBt)	**2m**	93.3%	Fmoc- azaMY-OH	**4m**	96.2%	77.6%	536.1850	536.1848
14	Fmoc-azaPro-(OBt)	**2n**	96.1%	Fmoc-azaPY-OH	**4n**	99.0%	82.7%	502.1973	502.1987
15	Fmoc-azaTyr(*t*Bu)-(OBt)	**2o**	91.0%	Fmoc-azaYY-OH[d]	**4o**	88.9%	83.2%	568.2078	568.2089
16	Fmoc-azaPhe-(OBt)	**2p**	92.1%	Fmoc-azaFY-OH	**4p**	100%	90.3%	552.2129	552.2127

[a] Conversion rates were based on recovered starting material (brsm). [b] For Fmoc-azaArg(bis-Boc)-(OBt) (**2a**), requiring 2.5 equiv HOBt & 2.0 equiv DIPEA for 4 h; [c] Fmoc-azaWY-OH (entry 6, **4f**) was cleaved using a solution of TFA/DCM (50: 50 v/v) at -15°C, 2 h, followed by lyophilization from 0.1% AcOH; [d] Fmoc-azaYY-OH (entry 15, **4o**) was cleaved using a solution of TFA/H_2O (95: 5 v/v) at 0°C, 2 h.

Upon removing the Fmoc protection group of the azapeptide, the resulting semicarbazide (Figure 1) is ready to be acylated to continue the sequence (Table 2). Due to the decreased nucleophilicity of the terminal nitrogen atom, the coupling of the subsequent amino acid to the aza-amino acid residue is always challenging in azapeptide synthesis[4a, 12]. In the past decade, various traditional peptide coupling conditions have been used to mitigate this difficult coupling.[5b] Recently, Ploom et.al[4a] found that the rate of azapeptide bond formation depends on the bulkiness of the following amino acid side group (R) and the hindrance of the aza-amino acid. To explore the optimization strategy for effective acylation of the semicarbazide moiety, the azaF^{2}I-dipeptide (**6**) was selected as a model aza-amino acid residue, it was synthesized by coupling Fmoc-azaPhe benzotriazole ester (**2p**) to isoleucine preloaded Wang resin (**5**) with a crude purity of 96%. Next, a series of natural amino acids with structurally representative side chains (Table 2a, entry 1-9) and various coupling reagents and conditions (Table 2a, entry 9-14) were systematically evaluated for azatripeptide (**8a-i**, table 2a) formation. (2-(6-chloro-1-H-benzotriazol-1-yl)-1,1,3,3-tetramethylaminium) hexafluorophosphate (HCTU) was selected as the initial coupling reagent due to its cost-effectiveness and reliable performance in peptide synthesis. At room temperature, treated with 10 equivalents of HCTU, subsequent amino acid (**7a-i**) was coupled to azaF^{2}I loaded Wang resin. With extended coupling cycles (2- 5 cycles), moderate to high conversion rates were achieved for amino acids with flexible side chains (Table 2a, entry 1-7, 76.3%-91.5%), the resulting AA-azaF^{2}I-tripeptides (**8a–g**) were successfully isolated in 50.0-79.5% yields, approaching the range observed for their corresponding natural tripeptides (49.5%-95.0%, see supporting information, supplementary Table S2). While effective for less bulky amino acids, HCTU showed limitations with highly sterically hindered amino acids such as valine and isoleucine (Table 2a, entry 8 and 9), with conversion rates of only 17.4% and 35.4%, respectively.

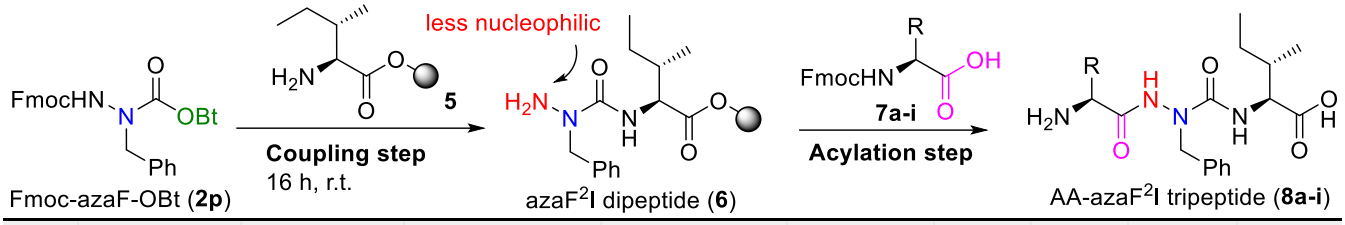
To address these issues, alternative coupling reagents, including N,N-diisopropylcarbodiimide (DIC), 1-[(Dimethylamino)-(morpholino)methylene]-1H-[1,2,3]triazolo[4,5-b]pyridine-1-ium 3-oxide hexafluorophosphate (HDMA), propylphosphonic anhydride (T3P®) and (1-Cyano-2-ethoxy-2 oxoethylidenaminooxy) dimethylamino-morpholino-carbenium hexafluorophosphate (COMU), were evaluated for the acylation of isoleucine to azaF^{2}I resin-bound residue **6** (Table 2a, entry 10-13). However, these studies resulted in low conversion rate, required multiple coupling cycles and prolonged reaction times, indicating that the steric hindrance significantly restricted the efficiency of the acylation step for valine and isoleucine (Table 2a, entry 8-13). Additionally, bulky, branched side chains continued to hinder the coupling efficiency of bulky aza-amino acid acylation (i.e., azaVal). We and others[13] encountered low conversion of the desired azaVal-tripeptide (see supporting information, supplementary Table S3). Although various groups[13a] have made progress in overcoming these obstacles triphosgene (BTC), Ghosez reagent (1-Chloro-N,N,2-trimethyl-1-propenylamine) and Chloro-N,N,N',N'-tetramethylformamidinium hexafluorophosphate (TCFH), these halogenation reagents are not fully compatible with automated SPPS. Therefore, a new approach is required to address these limitations. To achieve an efficient post-aza coupling process, we investigated the effect of elevated temperature. Using a Liberty Blue microwave peptide synthesizer, acylation of isoleucine to resin-linked azadipeptide **6** (Table 2a, entry 14) was performed with 10 equivalents of DIC and 5 equivalents of Oxyma Pure as activators under microwave conditions at 60°C for four coupling cycles. Remarkably, microwave-assisted acylation achieved a conversion rate of 88.3% (a 20% increase), resulting in an isolated yield of 63.0%, while reducing reaction time by 50% (4 hours). These results underscore the importance of addressing steric hindrance in semicarbazide acylation. While HCTU was effective adequately for less hindered amino acids, COMU showed limited performance for bulky branched residues under conventional methods; but the microwave-assisted synthesis represented a significant breakthrough, enabled faster and more efficient azapeptide bond formation.

Based on these findings, we identified a protocol for fully automated solid phase azapeptide synthesis using microwave irradiation as follows: (1) The peptide was synthesized on a 0.1 mmol scale on Wang or Rink Amide resin (0.2-0.3 mmol/g substitution). All natural amino acid cycles used 1 min/90ºC deprotection and a single 2 min/90ºC coupling with DIC/Oxyma pure (10eq/5eq). (2) All aza-amino acids incorporation cycles used 60 min/60°C coupling with aza-OBt building blocks (5 eq). (3) All acylation of semicarbazide used double 60 min/60°C coupling for unhindered amino acids with DIC/Oxyma, 4 cycles for bulky amino acids residues (60 min/cycle). To validate the robustness of this protocol, a 10-amino acid glucagon like peptide-1 (GLP-1) fragment[14] (**12**) was automatically synthesized under optimized SPPS conditions. As shown in Table 2b, microwave irradiation significantly improved the efficiency of azaPhe-benzotriazole ester-directed azapeptide synthesis. Incorporation of azaPhe into octapeptide (**9**) was significantly reduced from 16 hours at room temperature to 1 hour at 60°C, while acylation of Fmoc-glutamic acid (**11**) to semicarbazide (**10**) required just 2 hours under microwave conditions compared to 8 hours at room temperature. The use of DIC and Oxyma Pure further improved crude purity by minimizing side-product formation. These results highlight microwave-assisted synthesis as a more efficient and reliable method for azapeptide synthesis, providing strong support for the synthesis of much longer azapeptides using our platform.

Table 2. Automated acylation of resin-linked azapeptides: model reactions of coupling natural amino acid to aza-amino acid under traditional and microwave-assisted SPPS conditions. Reagents and conditions: a. Synthesis of azaPhe tripeptide **8a-i**. 1) Coupling step, NH_2-Ile-Wang Resin (**5**, 1 equiv), Fmoc-azaF-OBt (**2p**, 5 equiv), DMF, r.t., 16 h; 2) Fmoc removal, 20% piperidine solution in DMF, 2.5 min; 3) Acylation step (entry 1-13), Fmoc-amino acids **7a-i** (5 equiv), coupling reagents (10 equiv) with 12 equiv of NMM in DMF under traditional solid phase peptide synthesizer; entry 14, Fmoc-L-Ile-OH (**7i**, 5 equiv), DIC (10 equiv)/OxymaPure® (5 equiv) in DMF using microwave assisted peptide synthesizer. 4) Fmoc removal, 20% piperidine solution in DMF, 2.5 min; b. Synthesis of azaF-decapeptide **12** using optimized conditions. Traditional peptide synthesizer: 1) Coupling step, NH_2-IAKVVEGG-Rink amide Resin (**9**, 1 equiv), Fmoc-azaF-OBt (**2p**, 5 equiv), DMF, r.t., 16 h; 2) Fmoc removal, 20% piperidine solution in DMF, 2.5 min; 3) Acylation step, Fmoc-L-Glu (OtBu)-OH **11** (5 equiv), COMU (10

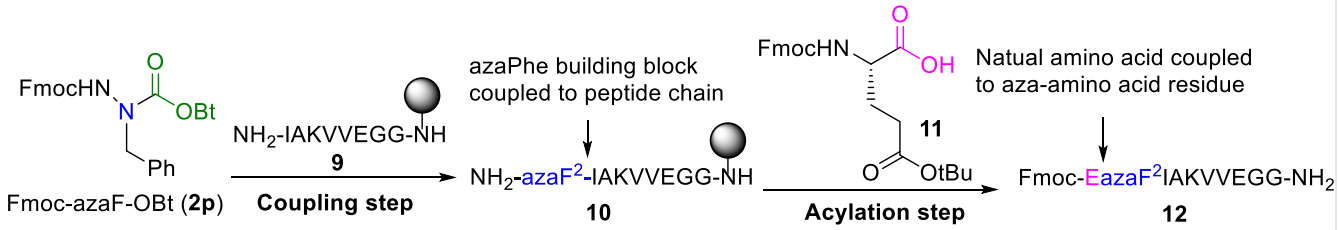
equiv) with 12 equiv of NMM in DMF, 4 coupling cycles, 8 h; Microwave Peptide Synthesizer: 1) Coupling step, NH_2-IAKVVEGG-Rink amide Resin (**8**, 1 equiv), Fmoc-azaF-OBt (**2p**, 5 equiv), DMF, 60°C MW, 1 h; 2) Fmoc removal, 10% piperidine solution in DMF, 1 min at 90°C MW.; 3) Acylation step, Fmoc-L-Glu (OtBu) **11** (5 equiv), DIC (10 eq) /OxymaPure® (5 eq) in DMF, 2 coupling cycles, 2 h at 60°C MW. Synthesis and characterization of these azatripeptides are detailed in Supplementary Information.

a. Optimized acylation conditions for model AA-azaFI-tripeptide synthesis



Entry	azaPhe-tripeptides	R (natural amino acid residues)	Coupling reagent	Rxn time/Temp.	% of Conversion	Crude purity (215 nm)	Crude yields	Isolated yields	Purity (215 nm)
1	**H**azaF^{2}I-OH **(8a)**	Histidine	HCTU (10 eq)	2 h / coupling cycle 2 cycles, 4 h, r.t.	90.0%	72.7% (3:1)*	83.1%	50.0%	99.1%* (3:1)
2	**R**azaF^{2}I-OH **(8b)**	Arginine	HCTU (10 eq)	2 h / coupling cycle 5 cycles, 10 h, r.t.	76.3%	76.7%	70.5%	62.1%	98.4%
3	**Y**azaF^{2}I-OH **(8c)**	Tyrosine	HCTU (10 eq)	2 h / coupling cycle 4 cycles, 8 h, r.t.	88.1%	76.3%	83.2%	79.5%	90.3%
4	**W**azaF^{2}I-OH **(8d)**	Tryptophan	HCTU (10 eq)	2 h / coupling cycle 3 cycles, 6 h, r.t.	82.7%	80.6%	77.8%	53.6%	98.6%
5	**P**azaF^{2}I-OH **(8e)**	Proline	HCTU (10 eq)	2 h / coupling cycle 4 cycles, 8 h, r.t.	87.1%	88.7%	96.7%	69.1%	97.6%
6	**M**azaF^{2}I-OH **(8f)**	Methionine	HCTU (10 eq)	2 h / coupling cycle 5 cycles, 10 h, r.t.	86.7%	67.0%	78.4%	61.0%	96.1% (12:1)
7	**A**azaF^{2}I-OH **(8g)**	Alanine	HCTU (10 eq)	2 h / coupling cycle 3 cycles, 6 h, r.t.	91.5%	70.5%	90.9%	77.4%	97.0%
8	**V**azaF^{2}I-OH **(8h)**	Valine	HCTU (10 eq)	2 h / coupling cycle 4 cycles, 8 h, r.t.	17.4%	N.A.	N.A.	N.A.	N.A.
9	**I**azaF^{2}I-OH **(8i)**	Isoleucine	HCTU (10 eq)	2 h / coupling cycle 4 cycles, 8 h, r.t.	35.4%	N.A.	N.A.	N.A.	N.A.
10			DIC (10 eq)		18.0%	N.A.	N.A.	N.A.	N.A.
11			T3P® (10 eq)		28.3%	N.A.	N.A.	N.A.	N.A.
12			HDMA (10 eq)		44.3%	N.A.	N.A.	N.A.	N.A.
13			**COMU** (10 eq)		**68.8%**	66.0%	75.8%	**40.8%**	95.0%
14			**DIC (10 eq)/ OxymaPure® (5 eq)**	1 h /coupling cycle 4 cycles, 4 h, **60 °C MW.**	**88.3%**	61.2%	90.8%	**63.0%**	98.7%

b. Fully automated solid phase azapeptide synthesis for model azaF-decapeptide



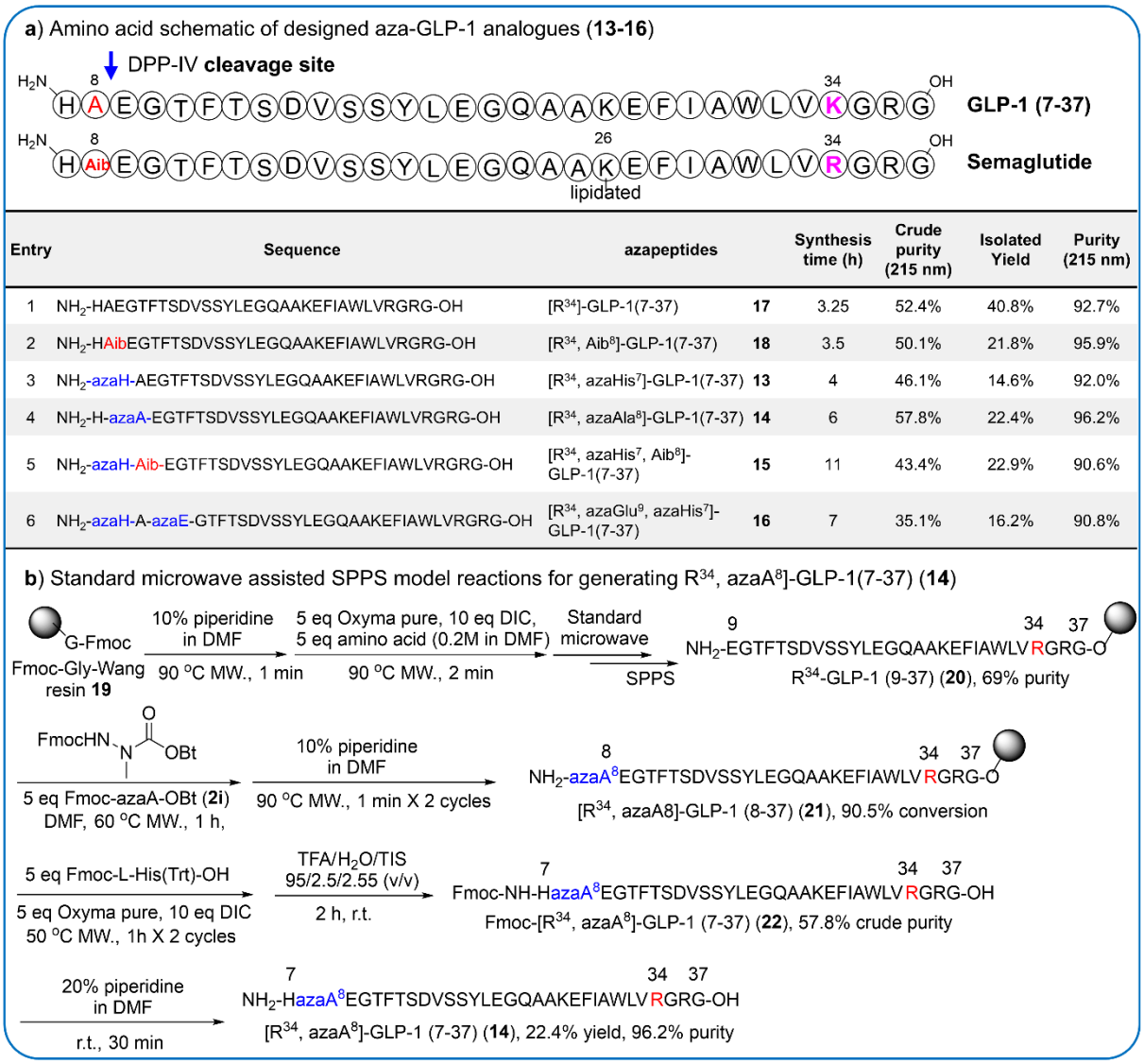
SPPS	Rxn temperature	azaPhe-semicarbazide (**10**)			azaPhe-azadecapeptide (**12**)				
		Rxn time	% of Conversion	Crude purity	Coupling reagent	Rxn time	% of Conversion	Crude purity	Side product
Traditional Peptide Synthesizer	r.t. (25 °C)	16 h	99.7%	94.4%	COMU (10 eq)	2 h / coupling cycle 4 cycles, 8 h, r.t.	85.6%	69.3%	7.5%
Microwave Peptide Synthesizer	60 °C MW.	1 h	99.9%	95.1%	DIC (10 eq)/ OxymaPure® (5 eq)	1 h / coupling cycle 2 cycles, 2 h	95.9%	83.8%	No

Encouraged by the successful synthesis of the 10-amino acid GLP-1 fragment, we applied our fully automated solid-phase azapeptide synthesis platform to construct GLP-1 azapeptide analogues. GLP-1, a 30- or 31-residue peptide hormone, plays important roles in promoting glucose-dependent insulin secretion and inhibiting glucagon release. Its therapeutic potential has been harnessed in diabetes and obesity treatments[7], leading to several FDA-approved analogues. Semaglutide[15], the first FDA-approved oral GLP-1 treatment for type 2 diabetes, replaces alanine at position 8 with alpha-aminoisobutyric acid (Aib), an unnatural amino acid, to prevent DPP-IV degradation and adds a lipid chain to lysine at position 26 to optimize receptor binding (Scheme 1a). Recently, Kumar et al.[16] demonstrated that replacing key residues with aza-amino acids stabilizes GLP-1 (7-36) amide, Semaglutide and GIP analogues by preventing DPP4-catalyzed hydrolysis and inactivation. While their results were promising, the reported synthetic methods utilized less robust protocols – including multiple carbonyl donors such as triphosgene, disuccinimidyl carbonate (DSC) and 4-nitrophenyl

chloroformate, requiring prolong reaction times (>36 hours), non-SPPS-compatible conditions (-10°C), and labor-intensive steps – limiting the reproducibility and efficiency aza-GLP-1 synthesis.

By applying aza-alanine, aza-histidine and aza-glutamic acid benzotriazole esters, four R^{34} aza-GLP-1 (7-37) derivatives targeting DPP-IV were successfully generated with 35.1–57.8% crude purities and 14.6–22.9% yields (Scheme 1a, entry 3-7, **13-16**). Each aza-GLP-1 analogue incorporated single or multiple aza-residues around the DPP-4 cleave site, improving enzymatic stability while maintaining functionality. For example, [R^{34}, azaAla^{8}]-GLP-1(7-37) (**14**) was highlighted as a representative analogue by integrating aza-alanine at position 8 using our optimized microwave SPPS protocol (Scheme 1b, **14**). Within 6 hours, [R^{34}, azaA^{8}]-GLP-1 (7-37) (**14**) was automatically produced and isolated in 22.4% yield, comparable to the [$R^{34,}$ Aib^{8}]-GLP-1 analogue (**18**), which yielded 21.8% in 3.5 hours. In general, the total synthesis time for single aza-modified GLP-1 analogues was 4–6 hours; for multi-aza substitutions, synthesis times were extended 7–11 hours, which is significantly improved over traditional approach. Notably, this work represents the first fully automated synthesis of GLP-1 analogues containing multiple aza-residues. By combining microwave-assisted synthesis with aza-benzotriazole ester building blocks, we have established a streamlined and reproducible approach to azapeptide GLP-1 analogues. Future studies will investigate the stability of these analogues in various conditions, such as in the presence of peptidases or in serum/whole blood, scale up syntheses of the leading GLP-1 azapeptides, in addition to in vivo studies to evaluate the efficacy and toxicity.

Overall, we developed a novel fully automatic solid phase synthetic platform for azapeptides synthesis utilizing aza-benzotriazole ester building blocks. These results highlight the robustness and reliability of our platform for synthesizing challenging peptides like GLP-1. The availability of on-demand syntheses of peptides resistant to proteolytic degradation enzymes marks a significant advancement in the pharmaceutical industry. Our approach has the potential to enable the more rapid synthesis and future development of novel azapeptide-based therapeutics, which can address unmet medical needs.



Entry	Sequence	azapeptides		Synthesis time (h)	Crude purity (215 nm)	Isolated Yield	Purity (215 nm)
1	NH_2-HAEGTFTSDVSSYLEGQAAKEFIAWLVRGRG-OH	[R^{34}]-GLP-1(7-37)	**17**	3.25	52.4%	40.8%	92.7%
2	NH_2-HAibEGTFTSDVSSYLEGQAAKEFIAWLVRGRG-OH	[R^{34}, Aib^{8}]-GLP-1(7-37)	**18**	3.5	50.1%	21.8%	95.9%
3	NH_2-azaH-AEGTFTSDVSSYLEGQAAKEFIAWLVRGRG-OH	[R^{34}, azaHis^{7}]-GLP-1(7-37)	**13**	4	46.1%	14.6%	92.0%
4	NH_2-H-azaA-EGTFTSDVSSYLEGQAAKEFIAWLVRGRG-OH	[R^{34}, azaAla^{8}]-GLP-1(7-37)	**14**	6	57.8%	22.4%	96.2%
5	NH_2-azaH-Aib-EGTFTSDVSSYLEGQAAKEFIAWLVRGRG-OH	[R^{34}, azaHis^{7}, Aib^{8}]-GLP-1(7-37)	**15**	11	43.4%	22.9%	90.6%
6	NH_2-azaH-A-azaE-GTFTSDVSSYLEGQAAKEFIAWLVRGRG-OH	[R^{34}, azaGlu^{9}, azaHis^{7}]-GLP-1(7-37)	**16**	7	35.1%	16.2%	90.8%

Scheme 1. Fully automatic synthesis of aza-Glucagon-like peptide-1 (GLP-1) analogues: trial application of optimized azapeptide synthesis platform. a) Amino acid schematic of designed aza-GLP-1 analogues showing aza-amino acid substitutions around DPP-IV cleavage sites; summary of GLP-1 and GLP-1-based azapeptide analogues (**13-16**); b) Standard microwave assisted SPPS for generating [R^{34}, $azaA^{8}$]-GLP-1(7-37) (**14**) was presented as model reactions. Synthesis and HPLC/HRMS of aza-Glucagon-like peptide-1 (GLP-1) analogues are detailed in Supplementary Information (section 2.6).

Supporting Information

The authors have cited additional references within the Supporting Information.[4, 6, 17]

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Keywords: azapeptide • solid phase peptide synthesis • GLP-1

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