Supporting information

Accelerated multi phosphorylated peptide synthesis
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1. Materials and methods

1.1 Materials

All Fmoc-amino acids were obtained from GL Biochem. (Shanghai), or Matrix Innovation (Quebec City, Canada), with the following side chain protecting groups: Arg(Pbf), Asp(OtBu), Asn(Trt), Glu(OtBu), Gln (Trt), His(Trt), Ser(tBu), Thr(tBu), Cys(Trt), Lys(Boc), Tyr(tBu). (Pbf = 2,2,4,6,7- pentamethyl-2,3-dihydrobenzofuran-5-sulfonyl). Rink Amide resin (0.48 mmol/g) was purchased from Matrix Innovation. 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU), was purchased from Luxembourg Biotechnologies Ltd. (Rehovot, Israel). 1,8-Diazabicyclo(5.4.0)undec-7-ene (DBU) was purchased from Merck (Rehovot, Israel). Triisopropylsilane (TIPS), 98% was purchased from Alfa Aesar. Piperazine and Morpholine, 99% extra pure, were purchased from Acros Organics (Holland Moran Israel). Solvents: N,N-dimethylformamide (DMF), dichloromethane (DCM), acetonitrile (ACN), N,N-diisopropylethyl amine (DIEA), Trifluoroacetic acid (TFA), piperidine (Pip), methanol (MeOH), and diethyl ether were purchased from BioLab. (Jerusalem, Israel) and were peptide synthesis or HPLC-grade.

1.2 Instruments

Semi-preparative RP-HPLC were performed on a Waters 150Q LC system using a C18 column (Phenomenex Luna 5 µm, 100 Å 21.2 x 250 mm) at a flow rate of 15 mL/min and recorded at 220 nm Analytical RP-HPLC were performed on Merck Hitachi HPLC with a reverse-phase C18 analytical column. (Merck Purospher STAR RP-18 endcapped LiChroCART® 250-4.6; 5 µm, or Zorbax RX-C18 150-4.6; 5 µm) with flow rate of 1 mL/min and recorded at 220 nm Linear gradients of ACN (with 0.1 % TFA, buffer B) in water (with 0.1 % TFA, Buffer A) were used for all systems to elute bound peptides.

ESI-MS was performed on LCQ Fleet Ion Trap mass spectrometer (Thermo Scientific). Peptide masses were calculated from the experimental mass to charge (m/z) ratios from the observed multiply-charged species of a peptide.

Deconvolution of the experimental MS data was performed with the help of MagTran v1.03 software.
2. Preparation of Reagents

2.1 Reagents for coupling

Amino acid solutions were prepared by adding a solution of 3 equiv. Fmoc-protected amino acids in 1 mL DMF. An activator solution was prepared by dissolving 2.9 equiv. of HATU in 1 mL of DMF per coupling. A base solution was prepared by dissolving 8 equiv. of DIEA in 1 mL DMF per coupling.

2.2 Bases for Fmoc deprotection

Solutions of 0.5 and 5% and 20% (V/V) piperidine in DMF were prepared. 
A solution of 1% of piperazine (W/V) in DMF,
A solution of 10% of Morpholine (V/V) in DMF

3. HPLC analysis of base screening chromatograms

![HPLC Chromatograms](image-url)
Figure S1. Analytical HPLC chromatograms of the Fmoc deprotection from peptide Fmoc-\textit{a} using different bases and concentrations. \textit{a}= pSLGLGLG peptide. Fmoc-\textit{a} = Fmoc-pSLGLGLG, \textit{b}= dephosphorylated \(\beta\)-elimination product or its 3-(1-piperidinyl alanine) adduct. *residual DBU eluted with the crude peptide as reported previously.\textsuperscript{3}

4. Syntheses conditions and cycles

4.1 Conditions

Table 1S: MW-SPPS of \textbf{B2R-5p} via liberty blue synthesizer (CEM)

| Reaction          | Conditions                                                                 |
|-------------------|-----------------------------------------------------------------------------|
| Fmoc deprotection | 6 mL of 20% piperidine/DMF 10 min                                            |
| Wash              | 5 X 6 mL DMF                                                                |
| Coupling          | The resin was incubated with 5 mL of DMF solution containing: 5 equiv. AA, 5 equiv. HATU, and 10 equiv. DIEA for 5 min. Mixing: \(\text{N}_2\) bubbling 2 s on 3 s off |
| Wash              | 5 X 6 mL DMF                                                                |

Table 2S: AMPS of \textbf{B2R-5p}

| Reaction          | Conditions                                                                 |
|-------------------|-----------------------------------------------------------------------------|
| Fmoc deprotection | 3 mL of 0.5% DBU/DMF 10 s                                                   |
| Wash              | 2 X 3 mL DMF. Total 15 s                                                   |
| Coupling          | The resin was incubated with 3 mL of DMF solution containing: 3 equiv. AA, 2.9 equiv. HATU, and 8 equiv. DIEA for 1 min. Mixing: 1200 rpm |
| Wash              | 2 X 3 mL DMF. Total 15 s                                                   |
4.2 HPLC analyses for B2R-5p synthesis via MW-SPPS and AMPS

**Figure S2.** Chromatogram of crude B2R-5p synthesized via MW-SPPS. A peak associated with B2R-5p was not detected.

**Figure S3.** Chromatogram of crude (left) and pure (right) B2R-5p synthesized via AMPS. ESI-MS (bottom) of the isolated peptide.
4.3. Chromatograms and ESI-MS data of MPP library

**Figure S4.** Chromatogram of crude (left) and pure (right) V2R-5p synthesized via AMPS. ESI-MS (bottom) of the isolated peptide.

Calculated mass: 1380.47 Da
Observed mass: 1379.66 Da
Figure S5. Chromatogram of crude (left) and pure (right) **APC-4p** synthesized via AMPS. ESI-MS (bottom) of the isolated peptide.
**Figure S6.** Chromatogram of crude (left) and pure (right) P53-5p synthesized via AMPS. ESI-MS (bottom) of the isolated peptide.
**Figure S7.** Chromatogram of crude (left) and pure (right) **Vim-4p** synthesized via AMPS. ESI-MS (bottom) of the isolated peptide.
Figure S8. Chromatogram of crude (left) and pure (right) Tau-6p synthesized via AMPS. ESI-MS (bottom) of the isolated peptide.
Figure S9. Chromatogram of crude (left) and pure (right) pLam-4p synthesized via AMPS. ESI-MS (bottom) of the isolated peptide.
Figure S10. Chromatogram of crude (left) and pure (right) FFAR4-5p synthesized via AMPS. ESI-MS (bottom) of the isolated peptide.
5. Computational details: Modeling Fmoc deprotection and β-elimination processes using piperidine base

Fmoc deprotection and β-elimination reactions were also modeled using piperidine as the base. As done with DBU, the Fmoc deprotection reaction was studied from a model system consisting of one Fmoc-Ser/(HPO$_3$Bzl)-OH and one piperidine molecule. We found similar mechanisms with piperidine to those observed with DBU. The Fmoc deprotection is a two-step process, shown in Figure S11(i) and S12 (i). The first step is a proton transfer from the Fmoc group in Fmoc-Ser/(HPO$_3$Bzl)-OH to the piperidine (structure i-a), which results in a bond-breaking leading to [CO$_2$-Ser/(HPO$_3$Bzl)-OH]$^-$ and piperidineH$^+$ (i-c). The barrier for this step is calculated to be 13.8 kcal/mol. In the second step, a piperidineH$^+$ molecule transfers a proton to the N of the Ser, resulting in CO$_2$ departure and formation of pSer (i-f). The energy barrier for this step is 0.7 kcal/mol. To model the β-elimination reaction with piperidine, we used Ser/(HPO$_3$Bzl)-OH with one piperidine as a model system; the first step (not shown) is a barrier-less proton transfer from the (HPO$_3$Bzl)-OH group to the piperidine base, which results in [Ser/(PO$_3$Bzl)-OH]$^-$ and piperidineH$^+$. As in the DBU case, an additional piperidine molecule is needed to model the next reaction step. Thus, our starting point is [Ser/(PO$_3$Bzl)-OH]$^-$, piperidine and piperidineH$^+$ to maintain the neutrality of the system, as shown in Figure S11(ii-a). The second step consists of a proton being
transferred from the Cα of the [Ser/(PO₃Bzl)-OH]⁻ to the piperidine resulting in the dephosphorylation of the Ser (ii-c). The energy barrier for this step is 16.6 kcal/mol.

Figure S11. PES representing Fmoc deprotection (i) and β-elimination (ii) processes with piperidine. Above each bar, the associated chemical structure is presented. Black bars represent minima on the PES, and purple/red bars represent transition states. Δ𝐸 values are calculated with respect to the initial structures (i-a/ii-a). In the case of i-d), the Δ𝐸 value is omitted as, when going from i-c) to i-d), we have removed from the model system Fmoc-Ser/(HPO₃Bzl)-OH the molecular fragment that has dissociated. The Δ𝐸 for i-e) and i-f) are relative to the energy of i-d).

Figure S12. PES representing Fmoc deprotection (i) and β-elimination (ii) processes with DBU. Above each bar, the associated chemical structure is presented. Black bars represent minima on the PES, and purple/red bars represent transition states. Δ𝐸 values are calculated with respect to the initial structures (i-a/ii-a). In the case of i-d), the Δ𝐸 value is omitted as, when going from i-c) to i-d), we have removed from the model system Fmoc-Ser/(HPO₃Bzl)-OH the molecular fragment that has dissociated. The Δ𝐸 for i-e) and i-f) are relative to the energy of i-d).
References

(1) Attard, T. J.; O’Brien-Simpson, N. M.; Reynolds, E. C. Identification and Suppression of β-Elimination Byproducts Arising from the Use of Fmoc-Ser(PO3Bzl,H)-OH in Peptide Synthesis. Int J Pept Res Ther 2009, 15, 69–79. https://doi.org/10.1007/s10989-008-9165-9.