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Note

Synthesis of a modified peptide fragment analog Val-Tyr (*P*)-Val-Ala-Ala-OH of cAMP protein kinase regulatory sub unit type II employing Fmoc chemistry

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The synthesis of new peptide fragment analog (Val-Tyr(*P*)-Val-Ala-Ala-OH) incorporating Tyr into the phosphorylation site of the cAMP protein kinase regulatory sub unit type II in place of Ser (Val-Ser(*P*)-Val-Ala-Ala-OH) has been described. The phosphopentapeptide fragment is prepared by Fmoc chemistry employing the global phosphorylation method. The yield (68%) as well as purity of the final peptide is satisfactory. The peptide is fully characterized by HPLC, NMR and mass spectral data.

Keywords: Phosphorylation, cAMP protein kinase, phosphatases, Fmoc-chemistry, bis-TMS method

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Phosphorylation and dephosphorylation of proteins, a dynamic event mediated by protein kinases and phosphatases, the final common pathway of all signal transduction mechanisms plays an important role in cellular function including the regulation of cell cycle[1,2]. Phosphorylation of proteins, in effect, can alter interaction between proteins and with other molecules, protein stability, function and subcellular localization[3]. The aberrations in the dynamics of protein phosphorylation are often associated with disease processes such as cancer[4], diabetes[5,16] and neurodegenerative disorders[6]. Protein kinases and phosphatases, in view of their role in the patho-physiology of disease, have attracted the attention of investigators as the target molecules for the new drug discovery[7]. One of the main constraints in the study of the protein kinases and phosphatases are the difficulty of employing substrate(s) of endogenous in nature[8]. In view of the difficulties associated with the purification of endogenous substrates, in sufficient quantities from natural sources, investigators are often constrained to employ synthetic compounds that are not peptidic in nature[9]. In particular, the assay of protein phosphatases is often based on the use of non-peptidyl phospho-compounds though phospho-peptides are the preferred substrates[10]. Nevertheless, the use of phosphopeptide, phosphorylation site with respect to amino acid sequence same as that of endogenous substrates, could be an ideal substrate to monitor the activity of a given protein phosphatase activity[11]. In this context, the earlier investigators have used the synthetic peptide sequence, analog to the peptide sequence of protein kinase A (PKA) regulatory subunit type II (RII) (H-Asp-Leu-Asp-Val-Pro-Gly-Arg-Phe-Asp-Arg-Arg-Val-Ser(P)-Val-Ala-Ala-Glu-OH), as the substrate for the protein phosphatase 2B (PP 2B)[12,13]. The change of phosphoamino acid residue of this peptide, wherein Ser (P) is replaced by Tyr(*P*), i.e., H-Val-Tyr(*P*)-Val-Ala-Ala-OH, finds its application in the assay of tyrosine phosphatase activity of calcineurin[14].

The chemical synthesis of phosphopeptides can be achieved by using either protected phosphoamino acid as a building block during the assembly/construction of peptide chain or alternatively phosphorylation of free alcohol or phenol side chains after complete synthesis of peptide, a protocol referred to as global phosphorylation[11]. Several reagents such as phospho-chloridates[16-17] and phosphoromidates[18], are being extensively used to phosphorylate the peptides. The present communication deals with solution phase phosphopeptide synthesis taking analog (Val-Tyr(*P*)-Val-Ala-Ala-OH) incorporating Tyr into the phosphorylation site of the cAMP protein kinase employing Fmoc chemistry.

Results and Discussion

In order to synthesize the phosphopentapeptide, we employed the global phosphorylation route employing di-*tert*-butyl *N*,*N*-diethylphosphoramidite for phosphorylation. The designed phosphopeptide fragment was synthesized by solution phase employing convergent strategy, which involves the separate synthesis of two peptide fragments (Fmoc-Val-Tyr-

OH and Fmoc-Val-Ala-Ala-OH). The dipeptide acids Fmoc-Val-Ala-OH **1**, Fmoc-Val-Tyr-OH **3** and tripeptide acid Fmoc-Val-Ala-Ala-OH **2** are synthesized *via* mixed anhydride method employing the bis- and tris-amino acids using the reported procedures[19]. These peptides were recrystallized using MeOH and water to obtain white crystalline solids in 85-90 % yield.

The Fmoc group from the tripeptide **2** was deprotected using diethyl amine (DEA) at r.t. to obtain Val-Ala-Ala-OH **4**. The deprotection was found to be complete in 45 min. After the complete deprotection of the Fmoc group, as monitored by TLC as well as IR analysis by the absence of characteristic stretching vibrational frequency around 1700 cm^{-1} for carbonyl group, the resulting solution was concentrated under reduced pressure. The residue was recrystallized using diethyl ether. The free tripeptide **4** was then dissolved in DCM and refluxed with a mixture of TEA and TMS-Cl in dry DCM to generate Bis-TMS-Val-Ala-Ala **5** (**Scheme I**).

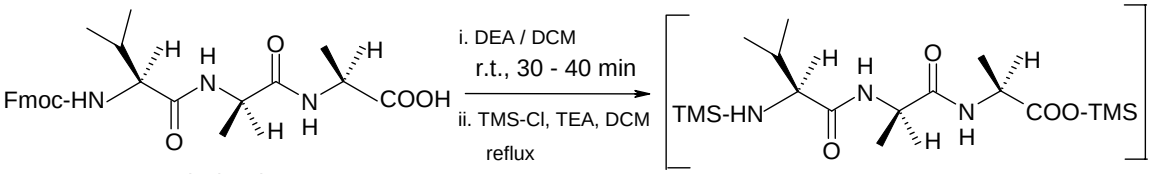
On the other hand, the Fmoc-Val-Tyr-OH **1** was converted to its mixed anhydride using EtOCOCl in presence of NMM at 0°C and coupled with freshly prepared bis-TMS-Val-Ala-Ala **5** (**Scheme II**) to yield the Fmoc-protected pentapeptide, Fmoc-Val-Tyr-Val-Ala-Ala-OH **6**, as a white crystalline solid.

The Fmoc-protected pentapeptide was phosphorylated using di-*tert*-butyl *N*,*N*-diethylphosphoramidite. In a typical reaction, the Fmoc-pentapeptide **6** was treated with TBDMS-Cl in dry DMF in presence of *N*, *N*-di-Isopropyl ethyl amine (DIEA) to protect the carboxyl group. The 1*H*-tetrazole was added as an activator for the phosphorylation reagent. It was followed by the addition of di-*tert*-butyl *N*,*N*-diethylphosphoramidite at r.t. The *in situ* generated Fmoc-pentapeptide di-*tert*-butylydiphosphoramidite triester Fmoc-Val-Tyr[$P(OBu^t)_2$]-Val-Ala-Ala-OTBDMS **7** was cooled to –10 ºC and oxidized. Oxidation of **7** employing *m*-chloroperoxybenzoic acid (MCPBA), proceeds rapidly resulting in corresponding Fmoc-pentapeptide di-*tert*-butylphosphate Fmoc-Val-Tyr[$P(OBu^t)_2O$]-Val-Ala-Ala-OH **8**. After the work-up, the product was isolated as a white crystalline solid and the crude peptide was directly used without any further purification in the next step.

The Fmoc-pentapeptide di-*tert*-butylphosphate, Fmoc-Val-Tyr[$P(OBu^t)_2O$]-Val-Ala-Ala-OH **8** was deprotected in two steps to obtain the free phosphopentapeptide Val-Tyr[$P(OH)_2O$]-Val-Ala-Ala-OH **10.** In the first step, the deprotection of the Fmoc group was carried out by treating **8** with DEA in dry DCM at r.t. The reaction mixture was then concentrated under reduced pressure and the residue was recrystallized using diethyl ether to obtain Val-Tyr[$P(OBu^t)_2O$]-Val-Ala-Ala-OH **9**. During this recrystallization step, the side product dibenzofulvene adduct and residual DEA were completely removed as a soluble impurity. In the second step, **9** was treated with 50% TFA in DCM to cleave the two *tert*-butyl groups of the phosphate group deprotection completed within 20 min as per TLC. The solvent and the residual TFA were removed by adding more DCM and concentrating under reduced pressure. The resulting residue was recrystallized using diethyl ether and the free phosphopentapeptide TFA salt ($F_3CCO_2^-$ H_3N^+-Val-Tyr(*P*)-Val-Ala-Ala-OH) **10** was collected by filtration followed by dry ether wash in 68% yield. The HPLC analysis of tyrosyl peptide (obtained by deprotection of Fmoc group from **6** and phosphotyrosyl peptide **11** are compared. The retention time for tyrosyl and phosphotyrosyl peptides were found to be 14.5 and 8.6 min with purity of 78% and 98% respectively (**Figure 1A** and **1B**). The free phosphotyrosylpentapeptide **11** was fully characterized by ^1H NMR, ^{13}C NMR and mass spectrum.

Experimental Section

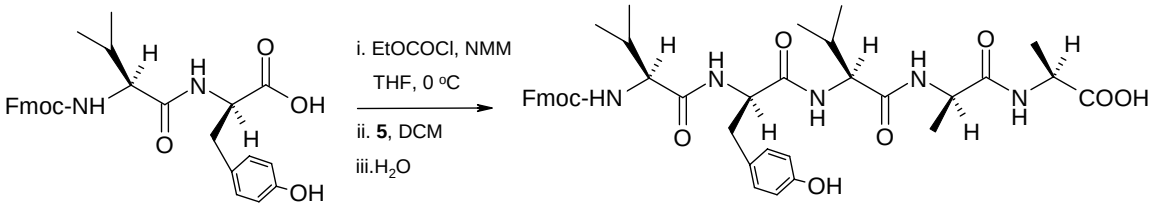
Melting points were determined using open capillary method and are uncorrected. IR spectrum was recorded on a Nicolet model impact 400D FT-IR spectrometer (KBr pellets, 3 cm^{-1} resolution). TLC analysis was carried out on precoated silica gel G plates using the solvent systems ethyl acetate:*n*-hexane (35:65, v/v) and chloroform:methanol:acetic acid (40:2:1, v/v/v). ^1H and ^{13}C NMR spectra were recorded on a Bruker AMX 400 MHz spectrometer. Mass spectrum was recorded on a MALDI-TOF (KRATOS). Amino acids were purchased from Sigma Aldrich Co., U.S.A. The free peptide **11** was subjected for HPLC to check the purity[20]. In brief, the peptide solution (25 µl ~25 µg), after injecting through the guard column (1cm, 5 µ) and C-18 separation column (1mm id, 5 µ particle size and 25 cm length, maintained at constant temperature of 40 °C) was eluted with acetate buffer (0.05 *M*, *p*H 6.8) and methanol with a linear gradient of 10-100 over a period of 50 min with a constant flow rate 1ml/min. The elution of tyrosyl peptide was monitored at 278 nm over an extended period of 60 min to see if any additional peaks (impurities) appear.



Fmoc-Val-Ala-Ala-OH **2**

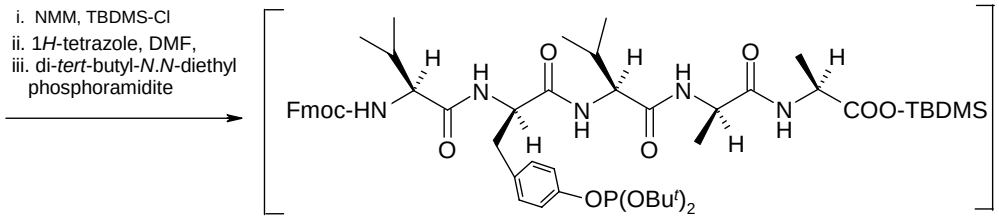
Bis-TMS-Val-Ala-Ala-OH **5**

Scheme I

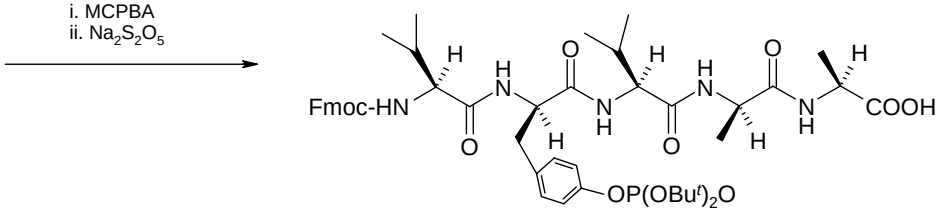


Fmoc-Val-Tyr-OH **3**

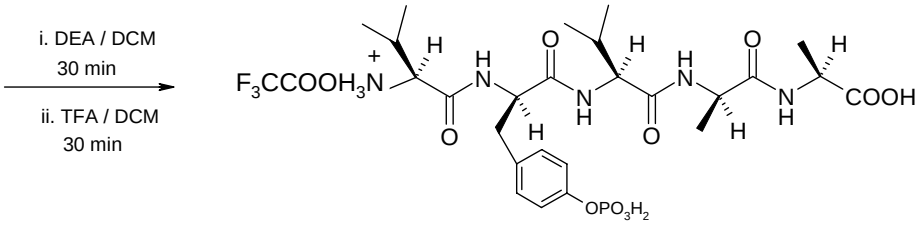
Fmoc-Val-Tyr-Val-Ala-Ala-OH **6**



Fmoc-Val-Tyr[P(OBu^t)$_2$]-Val-Ala-Ala-OTBDMS **7**



Fmoc-Val-Tyr[P(OBu^t)$_2$O]-Val-Ala-Ala-OH **8**



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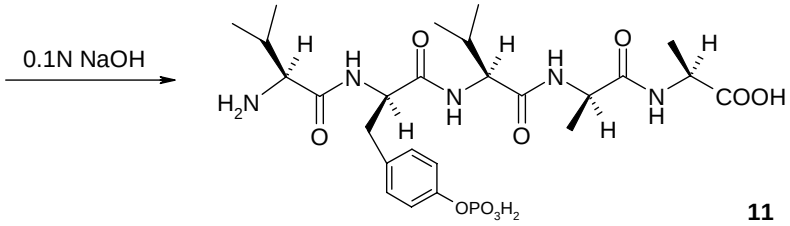

11

Scheme II

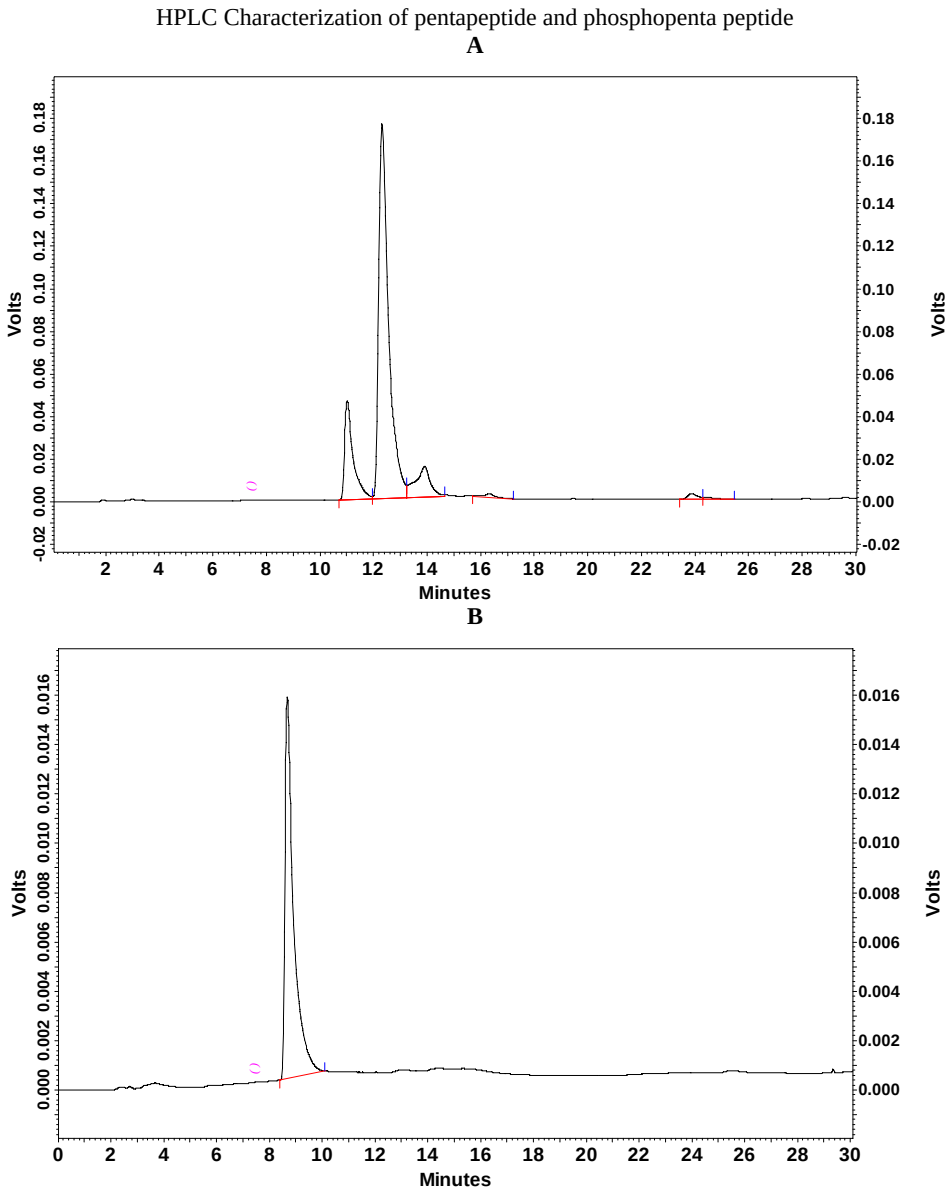

Figure 1—HPLC profile of free tyrosyl peptide and phosphotyrosyl peptide
A. Val-Tyr-Val-Ala-Ala-OH
B. Val-Tyr (*P*)-Val-Ala-Ala-OH

General procedure for the synthesis of bis-TMS-amino acids. To a suspension of amino acid (1 mmole) in DCM (5 mL) was added 0.264 mL (2.2 mmoles) of TMS-Cl and 0.3 mL (2.2 mmoles) of TEA and refluxed for 2 hr. The resulting solution was cooled to r.t. and used for the coupling reaction directly.

General procedure for the synthesis of peptide acids. A clear solution of Fmoc-amino acid or peptide acid (1 mmole) in 5 mL of dry THF was cooled to 0°C. A 0.11 mL (1 mmole) of NMM and 0.1 mL (1 mmole) of EtOCOCl were added and stirring was continued at the same temperature. After 10 min. freshly prepared *bis*-TMS-amino acid or peptide acid [prepared from 1.2 mmoles of amino acid or peptide under reflux using a mixture of TMS-Cl (0.264 mL, 2.2 mmoles), TEA (0.3 mL, 2.2 mmoles) in 10 mL of dry DCM] was added and the resulting mixture was stirred until completion of the reaction. The completion of reaction was monitored by TLC analysis. After the completion of reaction, it was evaporated under reduced pressure and the residue was dissolved in 10 mL of 10 % Na_2CO_3 solution and then washed with diethyl ether (10 mL × 3). The

aqueous layer was acidified with dilute HCl (until *p*H = 2) and the precipitate as a white solid.

In the synthesis of dipeptide Fmoc-Val-Tyr-OH, the Tris-TMS-Tyr was prepared using a mixture of 3.5 equivalents of TMS-Cl and TEA.

Fmoc-Val-Ala-OH 1. A 0.34 g (1 mmole) of Fmoc-Val-OH was coupled with freshly prepared Bis-TMS-Ala [0.107 g (1.2 mmoles) of Ala-OH was refluxed with TMS-Cl (0.264 mL, 2.2 mmoles), TEA (0.3 mL, 2.2 mmoles) in 10 mL of dry DCM] following the above general procedure for coupling. Finally, the crude peptide acid was recrystallized using methanol and water to obtain the pure compound **1**. Yield, 0.37 g (90%), m.p. 205-208°C, ^{1}H NMR (δ, DMSO-d_6): 0.85 (6H, m), 1.2 (3H, d), 1.85 (1H, m), 3.85 (1H, t), 4.05 - 4.35 (4H, m), 5.1 (1H, d), 6.7 (1H, d), 7.25 – 7.8 (8H, m); ^{13}C NMR (δ, DMSO-d_6): 17.2, 18.6, 19.5, 29.1, 47.6, 49.0, 58.5, 66.7, 120.0, 124.9, 127.1, 127.6, 154.9, 168.9, 171.3; ES-MS : [M+Na] 432.4, [M+K] 448.5.

Fmoc-Val-Ala-Ala-OH 2. A 0.41 g (1 mmole) of Fmoc-Val-Ala-OH **1** was coupled with freshly prepared bis-TMS-Ala using the above general procedure for coupling. The crude tripeptide **2** was recrystallized using MeOH and water to obtain the analytically pure tripeptide Fmoc-Val-Ala-Ala-OH **2** as a white solid. Yield, 0.41 g (85%), m.p. 220 - 22°C, ^{1}H NMR (δ, DMSO-d_6): 0.85 - 0.9 (6H, m), 1.2 - 1.25 (6H, m), 1.85 (1H, m), 3.85 - 4.4 (6H, m), 5.12 (1H, d), 6.65 – 6.85 (2H, m), 7.25 - 7.75 (8H, m); ^{13}C NMR (δ, DMSO-d_6) 17.2, 17.3, 18.6, 19.5, 29.2, 47.3, 48.8, 48.9, 58.5, 66.7, 120.0, 125.0, 127.1, 127.6, 155.1, 169.2, 171.8; ES-MS: [M+Na] 503.3, [M+K] 519.4.

Fmoc-Val-Tyr-OH 3. A 0.34 g (1 mmole) of Fmoc-Val-OH was coupled with freshly prepared Tris-TMS-Tyr (prepared from 0.181 g (1 mmole) of Tyr refluxed with 0.43 mL (3.5 mmoles) of TMS-Cl and 0.5 mL (3.6 mmoles) of TEA in 15 mL of DCM for 2 hr) using the above general procedure for coupling. The resulting crude dipeptide **3** was purified by column chromatography using the solvent system MeOH and $CHCl_3$ with varying concentrations from 5 to 10% of MeOH in $CHCl_3$. Yield, 0.452 g (90%), m.p. 149 - 50°C, ^{1}H NMR (δ, DMSO-d_6): 0.9 (6H, m, 1.85 (1H, m), 2.87 (2H, d), 3.85 - 3.95 (2H, m), 4.1 - 4.35 (3H, m), 5.25 (1H, d), 6.65 (1H, d), 7.15 - 7.85 (12H, m); ^{13}C NMR (δ, DMSO-d_6): 18.6, 19.5, 29.1, 36.5, 47.3, 54.1, 58.5, 66.7, 120.0, 124.3, 125.0, 127.2, 127.6, 129.7, 132.4, 141.2, 144.3, 154.2, 156.4, 168.6, 173.1; ES-MS: [M+Na] 519.5, [M+K] 540.5.

Val-Ala-Ala-OH 4. A 0.481 g (1 mmole) of Fmoc-Val-Ala-Ala-OH **2** was treated with 5 mL (5 mmoles) of DEA in 5 mL of dry DCM while stirring at r.t. for 45 min. The resulting clear solution was concentrated under reduced pressure and the residue was recrystallized using diethyl ether. The obtained solid was pure enough and was used as such in the next step for the generation of bis-TMS-Val-Ala-Ala **5**.

Fmoc-Val-Tyr-Val-Ala-Ala-OH 6. A 0.502 g (1 mmole) of Fmoc-Val-Tyr-OH was coupled with freshly prepared *bis*-TMS-Val-Ala-Ala [0.259 g (1 mmole) of Val-Ala-Ala-OH **4** in 15 mL DCM was refluxed in presence of 0.264 mL (2.2 mmoles) of TMS-Cl and 0.3 mL of (2.2 mmoles) of TEA] using the above general procedure for coupling. After the reaction, the solvent was removed under reduced pressure and the residue was dissolved in 10 mL of 10 % Na_2CO_3 solution and then washed with ether (10 mL×3). The separated solid was filtered and acidified with dilute HCl (until *p*H = 2). The separated solid Fmoc-protected pentapeptide **6** was collected by filtration. It was purified by recrystallization using DMF and water to obtain the white crystalline solid. Yield 0.64 g, (86%), m.p. 228-29°C, ^{1}H NMR (δ, DMSO-d_6): 0.7 - 0.9 (12H, m), 1.2 - 1.3 (6H, m), 1.9 2H, m), 2.82 (2H, m), 3.85 (2H, t), 4.05 - 4.42 (5H, m), 6.76 (1H, d), 6.92 (1H, d), 7.3 - 8.1 (15H, m); ^{13}C NMR (δ, DMSO-d_6): 17.34, 18.02, 19.11, 30.39, 30.59, 36.62, 46.75, 47.64, 47.86, 53.92, 57.54, 60.34, 65.67, 114.8, 120.04, 120.55, 125.29, 127.06, 127.61, 130.04, 140.71, 143.73, 143.98, 155.75, 155.97, 170.23, 170.95, 171.60, 173.99; ES-MS: [M+Na] 544.310, [M+K] 560.296.

Fmoc-Val-Tyr(P(OBu^t)$_2$O)-Val-Ala-Ala-OH 8. To a solution of 0.743 g (1 mmole) of Fmoc-Val-Tyr-Val-Ala-Ala-OH **6** in dry DMF (10 mL), 0.11 mL (1 mmole) of NMM and 0.149 g (0.95 mmole) of TBDMS-Cl were added at r.t. After 5 min, 0.326 g, (5 mmoles) of 1*H*-tetrazole was added in one lot followed by the addition of 0.5 g (2 mmoles) of di-*tert*-butyl diethylphosphoramidite in dry DMF (2 mL) at r.t. After 45 min, the solution was cooled to –10°C and 0.52 mg (3 mmoles) of MCPBA was added. The resulting mixture was stirred at 5°C for 45 min. A solution of aqueous 10% aq. $Na_2S_2O_5$ (5 mL) was added at 0°C and the mixture was stirred rapidly at r.t. for 30 min. Then, the reaction mixture was extracted with diethyl ether (10 mL × 3) and the combined organic phase was washed using aqueous $Na_2S_2O_5$ solution (10 mL × 3) and extracted with 10% aqueous Na_2CO_3 (20 mL × 3). The combined aqueous layer

was acidified using 30% citric acid to *p*H 3.5 – 4.0 and extracted with EtOAc (3 × 10 mL). The EtOAc layer was evaporated under reduced pressure at r.t. and the residue **8** was recrystallized using EtOAc and hexane to obtain the pure white crystalline powder. Yield 0.637 g (68%), m.p. 200-02°C, ^{1}H NMR (δ, DMSO-d_6): 0.7-1.15 (30 H, m), 1.25-1.3 (6H, m), 1.9 (2H, m), 2.82 (2H, m), 3.85 (2H, m), 4.05 - 4.42 (5H, m), 6.78 (1H,d), 6.94 (1H, d), 7.12 - 8.1 (15H, m) ; ^{13}C NMR (δ, DMSO-d_6): 17.34, 18.02, 19.11, 28.6, 30.39, 30.59, 36.62, 46.75, 47.64, 47.86, 53.92, 57.54, 60.34, 65.67, 78.1, 114.80, 120.04, 120.55, 125.29, 127.06, 127.61, 130.04, 140.71, 143.73, 143.98, 155.75, 155.97, 170.23, 170.95, 171.60, 173.99 ; ES-MS : [M+Na] 544.310, [M+K] 560.296.

Val-Tyr(*P*)-Val-Ala-Ala-OH 11. A 0.937 g (1 mmoles) of Fmoc-Val-Tyr(P(OBu^t)$_2$O)-Val-Ala-Ala-OH **8** was treated with DEA (5 mL, 5.0mmoles) in dry DCM (5 mL) at r.t. while stirring for about 30-45 min. The resulting clear solution was evaporated under reduced pressure followed by the addition of dry diethyl ether to obtain 0.643 g (90%) of the crude di-*tert*-butylphosphate derivative as a foamy solid. Further, it was treated with TFA (3 mL) in dry DCM (3 mL) at r.t. while stirring for 20 – 30 min. After the complete deprotection, as monitored by TLC, the evaporation under reduced pressure, followed by the addition of DCM and reevaporation gave the solid free of excess of TFA. The TFA salt of the Val-Tyr (*p*)-Val-Ala-Ala-OH **10** was washed thoroughly with diethyl ether to remove residual solvents. The TFA salt **10** was then dissolved in methanol (5 mL) and the *p*H of the solution was adjusted to 6.5 by adding 0.1*N* NaOH. The solvent was then evaporated *in vacuuo* and the residue was recrystallized with methanol:dry ether to give the crystalline powder **11**. Yield, 0.485 g (68%), m.p. 190-93°C, ^{1}H NMR (δ, DMSO-d_6) 0.7-0.9 (12H, m), 1.2-1.3 (6H, m), 1.9 (2H, m), 2.78 - 2.82 (2H, m), 3.85 (2H, m), 3.95 - 4.15 (4H, m), 6.7 (1H, m), 7.15 - 8.1 (16H, m); ^{13}C NMR (δ, DMSO-d_6): 17.34, 18.02, 19.11, 30.39, 30.59, 36.62, 46.75, 47.86, 53.92, 57.54, 60.34, 65.67, 114.80, 120.55, 130.04, 140.71, 143.73, 143.98, 155.75, 170.23, 170.95, 171.60, 173.99; ES-MS: [M+Na] 544.310, [M+K] 560.296.

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