CHEMBIOCHEM

Supporting Information

CHEMBIOCHEM

Supporting Information

for

Enhanced Complexity and Catalytic Efficiency in the Hydrolysis of Phosphate Diesters by Rationally Designed Helix-Loop-Helix Motifs

Jesus Razkin,* Johan Lindgren, Helena Nilsson, and Lars Baltzer*

Preparation of substrates.

The synthesis of uridine 3'-(2,2,2-trichloroethylphosphate)[1] (**1**), HPNP[2] (2-hydroxypropyl-p-nitrophenyl phosphate) (**2**), and 2-hydroxypropyl-2,2,2-trichloroethylphosphate[3] (**3**) has been described previously. 4-Nitrophenyl phosphate, 4-nitrophenyl acetate (**8**), 4-nitrophenyl butyrate (**9**) and 4-nitrophenyl octanoate (**10**) are commercially available.

2,4-dinitrophenyl phosphate, Na salt. The method of Bunton and Farber[4] is used to prepare the phosphate diester and the procedure of Rawji and Milburn[5] to semihydrolyze it. Commercial 2,4-nitrophenol is recrystallized from ethanol and 5.52 g (30 mmol) are dissolved in 60 mL of acetonitrile, together with 5.1 mL (63 mmol) of dry pyridine, and cooled in an ice-water bath. $POCl_3$ 0.93 mL (10 mmol) is added and the mixture stirred for 20 min. at 0 °C and for 20 min. at r.t. It is then poured over 450 mL of ice-water. The precipitate is separated by filtration, washed thoroughly with ice-water to remove pyridine and recrystallized from acetone. The pure pyridinium bis-(2,4-dinitrophenyl) phosphate, 3.86 g (76%), is isolated as white crystals. The pyridinium ion is exchanged for Na^+ using a cation-exchange resin (Amberlite IR-120 in the Na^+ form), 75 g of resin for 6.3 g of pyridinium salt, using MeOH as solvent. The

bright yellowish sodium salt is recrystallized from ethanol. The sodium bis-(2,4-dinitrophenyl) phosphate 1 g (2.21 mmol) is hydrolyzed by rapidly dissolving it in 50 mL of 0.3 M NaOH and stirring at 25 °C for 1 h (pH 13.5). Then, 5 M HCl, 15 mL, is added (pH 0), the suspension cooled in an ice-water bath and the solid 2,4-dinitrophenol removed by filtration. The filtrate is concentrated in rotary evaporator (35 °C) to an oil and dry diethyl ether is added to precipitate the NaCl, which is removed by filtration. The ethereal filtrate is then stirred while 2,6-lutidine is added dropwise until the solution gets a permanent bright yellow color. The solid formed is filtered, washed with ether and recrystallized from ethanol to obtain the pure 2,6-lutidinium 2,4-dinitrophenyl phosphate, 461 mgr (56%), as white-yellowish solid. The 2,6-lutidinium ion can be exchanged for Na^+ using a cation-exchange resin (Amberlite IR-120 in the Na^+ form) just before using it. ^{1}H NMR: (D_2O) δ 2.73 (s, 6H), 7.64 (d, 2H), 7.76 (d, 1H), 8.28 (t, 1H), 8.54 (dd, 1H), 8.89 (d, 1H); ^{31}P NMR: (D_2O) δ -4.25. NMR data for the 2,6-lutidinium salt.

Sodium 4-nitrophenoxide.[6] 10 mL of a 10 N solution of sodium hydroxide are added over a stirred dissolution of 10.43 g (75 mmol) of 4-nitrophenol in 60 mL of boiling water. After 10 min, another portion of 10 mL of a 10 N solution of sodium hydroxide is added and the mixture rapidly cooled. The solid is separated by filtration, washed with ice-water (3x10 mL) and dried thoroughly at 110 °C to obtain 11.03 g (91%) of pure sodium 4-nitrophenoxide, as a red powder.[6]

Sodium 4-chloro-2-nitrophenoxide. Following the same procedure described before for the sodium 4-nitrophenoxide, from 5 + 5 mL of a 10 N solution of sodium hydroxide and 5.21 g (30 mmol) of 4-chloro-2-nitrophenol in 40 mL of boiling water, 5.05 g (86%) of pure sodium 4-chloro-2-nitrophenoxide are obtained as a red-brown powder.

Ethyl 4-nitrophenyl phosphate, Na salt (**5**). Cebrian's method[7] for the phosphorodichloridate and Hendry's method[8] for the final phosphate diester are modified in this procedure. Dried sodium 4-nitrophenoxide 3.22 g (20 mmol), is added in small portions over a period of 2 h to 19.6 mL (210 mmol) of $POCl_3$ in a flask equipped with reflux condenser. The vigorous reaction is moderated with cold or ice-water and the red color of the salt rapidly disappears. When the addition is completed, the mixture is stirred for 60 min. and the excess $POCl_3$ removed under high vacuum. Diethyl ether is then added to the residue and the precipitated sodium chloride removed by

filtration trough celite. Evaporation of the ether yields the crude 4-nitrophenyl phosphorodichloridate as a yellowish oil. To the crude product dissolved in dry diethyl ether (30 mL), a solution of dry pyridine 1.13 mL (14 mmol) in 8 mL of diethyl ether is added. Nitrogen is passed over the surface of the mixture and ethanol 0.81 mL (14 mmol) in 10 mL of diethyl ether is added dropwise over 15 min. The reaction is stirred for 1 h and then pyridine 1.13 mL (14 mmol) in 25 mL of water is added. After 15 min, an excess (30 mL) of 5 M HCl solution is added to the mixture and it is extracted with diethyl ether (3 x 30 mL). The ethereal phases are collected, dried (Na_2SO_4) and evaporated. The residue is dissolved in dry diethyl ether (125 mL) and a solution of cyclohexylamine in dry diethyl ether is added until the mixture gets a permanent yellowish color. After cooling, the precipitated solid is separated by filtration, washed with diethyl ether and purified by silicagel column chromatography (solvent: acetone/MeOH 8:1 to 2/1 v/v) to get the pure dicyclohexylamonium salt of ethyl 4-nitrophenyl phosphate as a yellow solid. The dicyclohexylamonium salt is transformed in the sodium salt by passing down a column of Na interchange resin, Amberlite IR-120 (Na^+) (50 g), obtaining 2.48 g (46%) of the sodium salt of ethyl 4-nitrophenyl phosphate as a white solid. ^{1}H NMR: (D_2O) δ 1.28 (t, 3H), 4.07 (q, 2H), 7.34 (d, 2H), 8.24 (d, 2H); ^{31}P NMR: (D_2O) δ -3.79.

Butyl 4-nitrophenyl phosphate, Na salt (**6**). Following the same procedure described before for **5**, from 3.22 g (20 mmol) of dried sodium 4-nitrophenoxide, 19.6 mL (210 mmol) of $POCl_3$ and 1.28 mL (14 mmol) of 1-butanol, pure sodium salt of butyl 4-nitrophenyl phosphate as a white solid is obtained, 2.87 g (48%). ^{1}H NMR: (D_2O) δ 0.87 (t, 3H), 1.35 (m, 2H), 1.62 (q, 2H), 4.01 (q, 2H), 7.35 (d, 2H), 8.25 (d, 2H); ^{31}P NMR: (D_2O) δ -3.70.

Heptyl 4-nitrophenyl phosphate, Na salt (**7**). In the same way previously described for **5**, from 0.99 g (6.15 mmol) of dried sodium 4-nitrophenoxide, 6.34 mL (68 mmol) of $POCl_3$ and 0.61 mL (4.3 mmol) of 1-heptanol, pure sodium salt of heptyl 4-nitrophenyl phosphate as a yellowish solid is obtained, 882 mgr (42%). ^{1}H NMR: (D_2O) δ 0.83 (t, 3H), 1.20 (m, 8H), 1.61 (q, 2H), 3.99 (q, 2H), 7.38 (d, 2H), 8.29 (d, 2H); ^{31}P NMR: (D_2O) δ -3.75.

Butyl 4-chloro-2-nitrophenyl phosphate, Na salt (**4**). Using the method showed before for **5**, from 5.0 g (25.5 mmol) of dried sodium 4-chloro-2-nitrophenoxide, 26 mL (280 mmol) of $POCl_3$ and 1.63 mL (17.8 mmol) of 1-butanol, pure sodium salt of

butyl 4-chloro-2-nitrophenyl phosphate as a white solid is obtained, 3.98 g (47%). ^{1}H NMR: (D_2O) δ 0.89 (t, 3H), 1.36 (m, 2H), 1.62 (q, 2H), 4.00 (q, 2H), 7.49 (dd, 1H), 7.70 (dd, 1H), 8.04 (d, 1H); ^{31}P NMR: (D_2O) δ -3.58.

References

[1] S. Mikkola, E. Stenman, K. Nurmi, E. Yousefi-Salakdeh, R. Strömberg, H. Lönnberg, *J. Chem. Soc., Perkin Trans. 2*, **1999**, 1619-1625.

[2] D. M. Brown, D. A. Usher, *J. Chem. Soc.* **1965,** 6558-6564.

[3] J. Razkin, H. Nilsson, L. Baltzer, *J. Am. Chem. Soc*. **2007**, *129*, 14752-14758.

[4] C. A. Bunton, S. J. Farber, *J. Org. Chem.* **1969**, *34*, 767-772.

[5] G. Rawji, R. Milburn, *J. Org. Chem.* **1981**, *46*, 1205-1206.

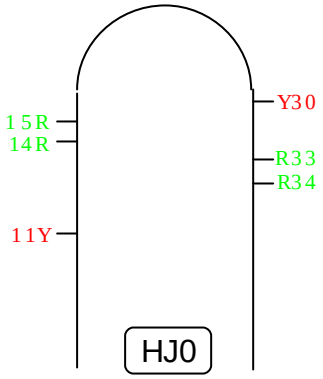
[6] J. G. Moffat, H. G. Khorana, J. Am. Chem. Soc. **1957**, *79*, 3741-3746.

[7] a) G. R. Cebrian, *Anales. real soc. espan. fis. y quim.* (Madrid), **1951**, *47B*, 841; b) A. F. Turner, H. G. Khorana, *J. Am. Chem. Soc.* **1959**, *81*, 4651-4656.

[8] P. Hendry, A. M. Sargeson, *J. Am. Chem. Soc.* **1989**, *111*, 2521-2527.

Figure S1. MALDI-TOF Mass Spectrometry spectra of peptides.

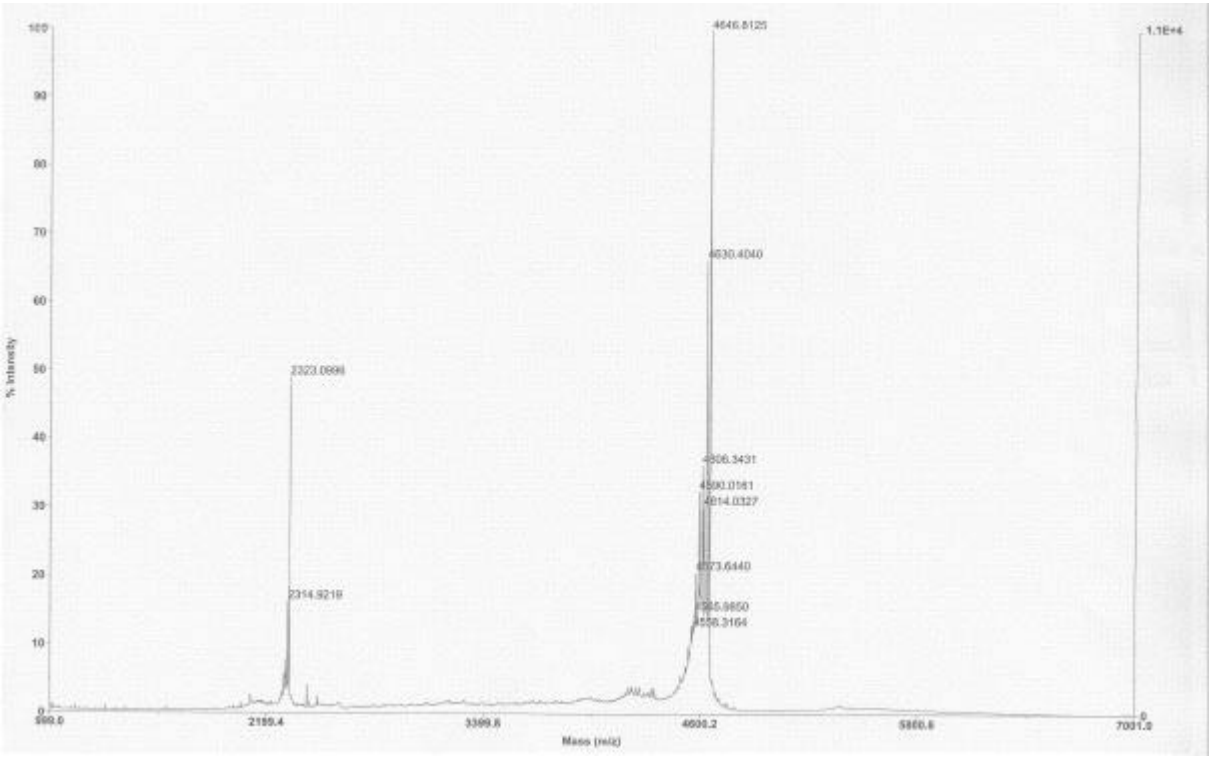
Peptide: HJ0



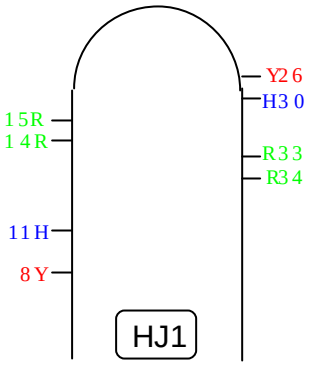
Ac-N-A-A-D-Nle-E-A-A-I-K-Y^{11}-L-A-R^{14}-R^{15}-Nle-A-A-K-
G-P-V-D-
H_2N-G-A-R-A-F-A-E-F-R^{34}-R^{33}-A-L-Y^{30}-E-A-Nle-Gln-A-A-

Calculated Molecular Weight: 4646.324

Obtained MALDI-TOF MS mass: 4646.812



Peptide: HJ1

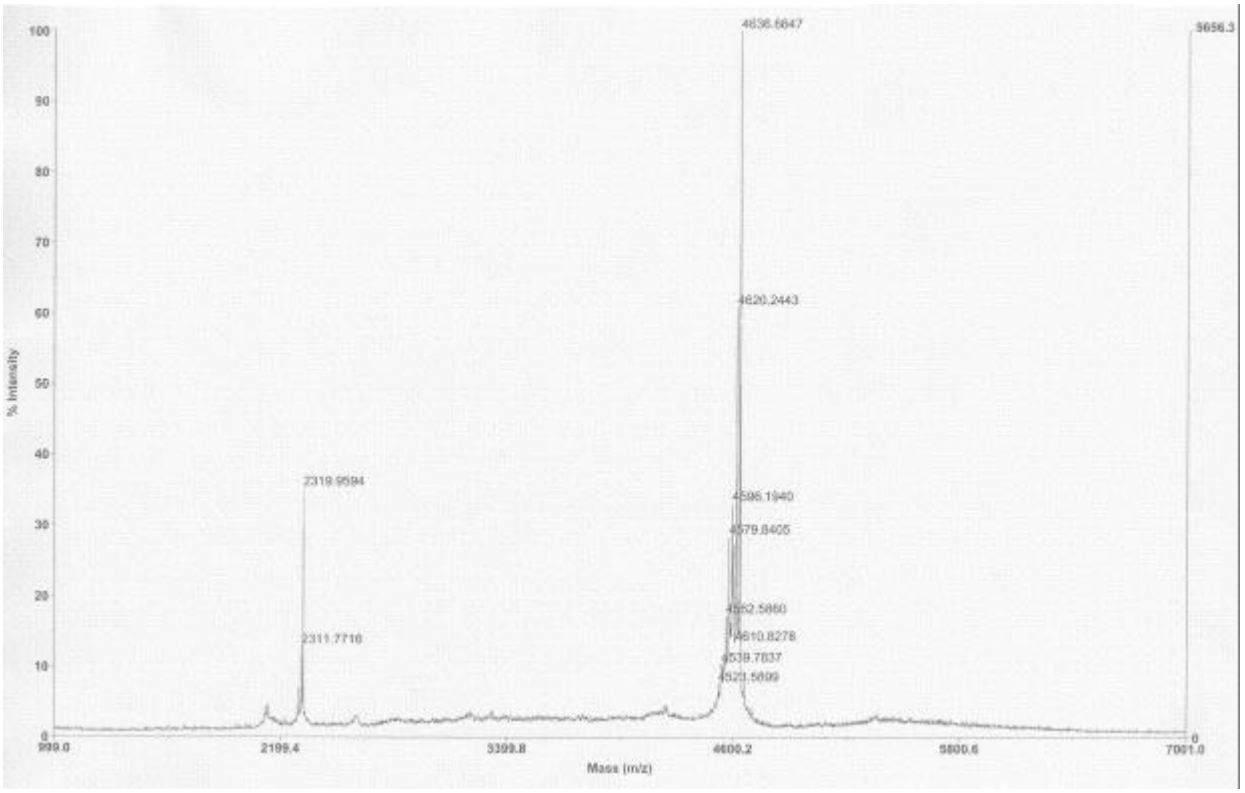


Ac-N-A-A-D-Nle-E-A-Y[8]-I-K-H[11]-L-A-R[14]-R[15]-Nle-A-A-K-G-P-V-D-

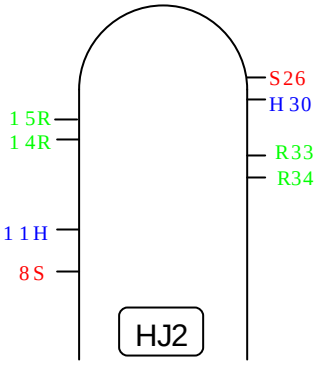
H_2N-G-A-R-A-F-A-E-F-R[34]-R[33]-A-L-H[30]-E-A-Nle-Y[26]-A-A-

Calculated Molecular Weight: 4636.294

Obtained MALDI-TOF MS mass: 4636.665



Peptide: HJ2

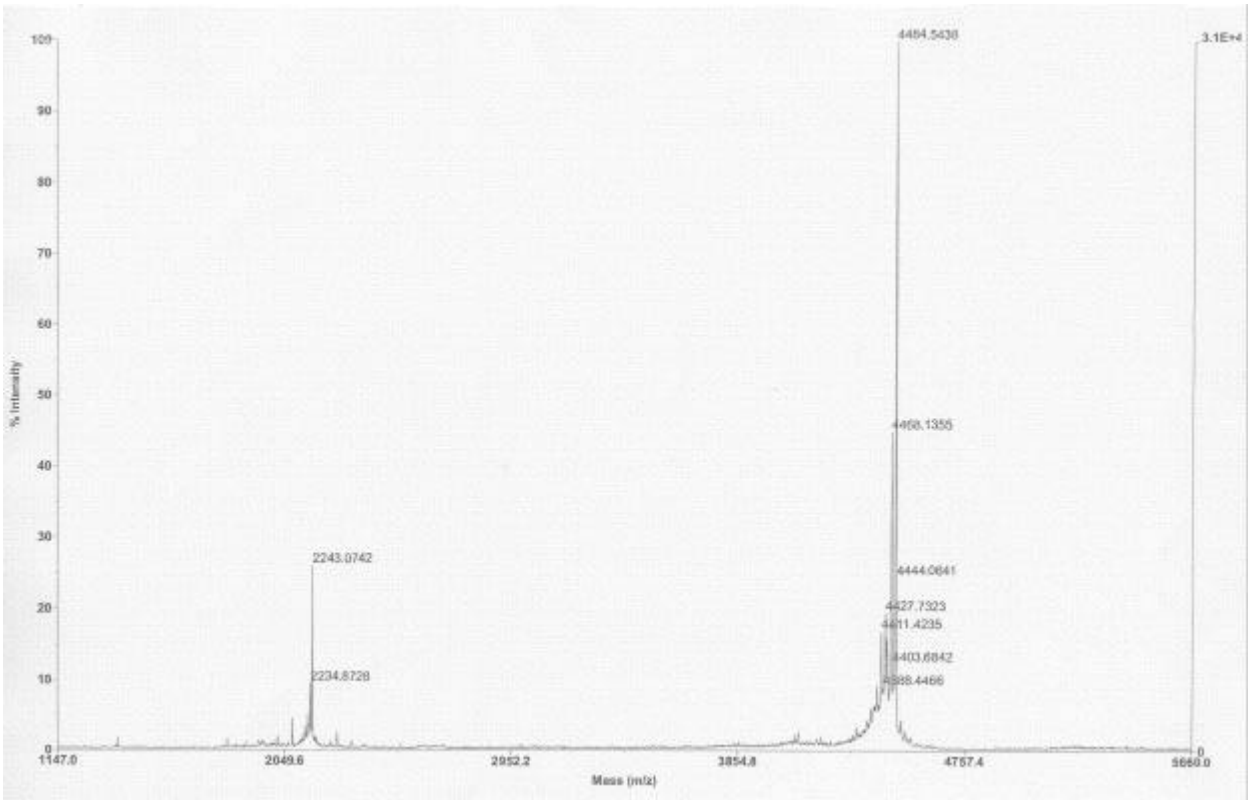


Ac-N-A-A-D-Nle-E-A-S[8]-I-K-H[11]-L-A-R[14]-R[15]-Nle-A-A-K-G-P-V-D-

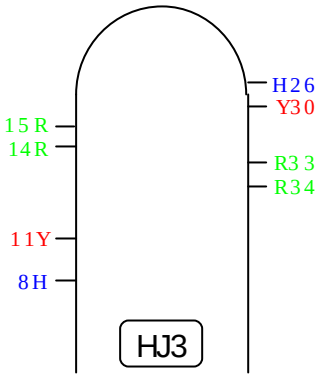
H_2N-G-A-R-A-F-A-E-F-R[34]-R[33]-A-L-H[30]-E-A-Nle-S[26]-A-A-

Calculated Molecular Weight: 4484.094

Obtained MALDI-TOF MS Mass:4484.544



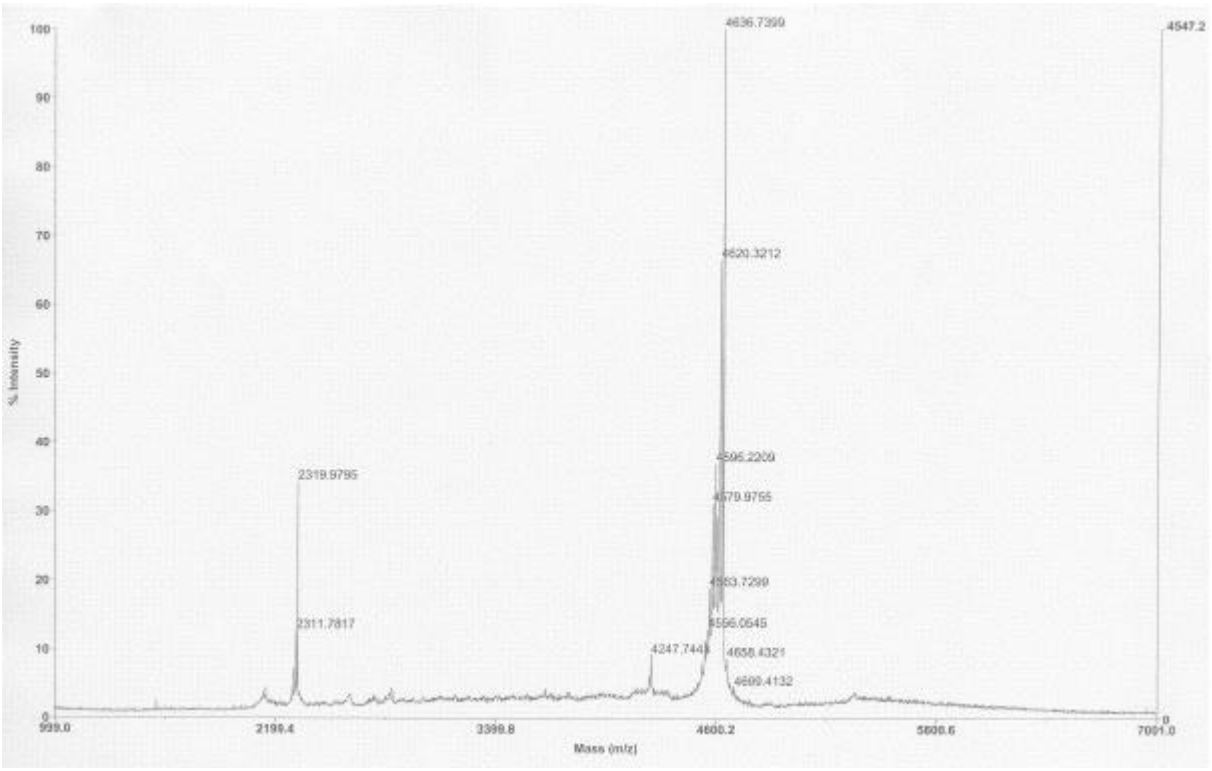
Peptide: HJ3



Ac-N-A-A-D-Nle-E-A-H[8]-I-K-Y[11]-L-A-R[14]-R[15]-Nle-A-A-K-
G-P-V-D-
H_2N-G-A-R-A-F-A-E-F-R[34]-R[33]-A-L-Y[30]-E-A-Nle-H[26]-A-A-

Calculated Molecular Weight: 4636.294

Obtained MALDI-TOF MS Mass: 4636.740



MALDI-TOF Mass Spectrometry was performed on an Applied Biosystems Voyager DE-STR mass spectrometer in a 1:10 sample: matrix relation, using α-cyano-4-hydroxycinnamic acid as matrix. Reflector mode, positive polarity and 25000 V of accelerating voltage are used.

The amino acid sequence and the schematic representation of helix-loop-helix structure is also shown. The one letter code for the amino acids is used (A is alanine, D is aspartic acid, E is glutamic acid, F is phenylalanine, G is glycine, H is histidine, I is isoleucine, K is lysine, L is leucine, N is asparagine, P is proline, Q is glutamine, R is arginine, V is valine, Y is tyrosine and Nle is norleucine). The C-terminal is amidated and the N-terminal is acetylated. Only the residues designed for catalytic site and the amino acids incorporated in the new sequences are shown in the schematic representations.

Figure S2. CD spectrum of HJ1 peptide: CD spectrum was recorded at pH 7.0 and room temperature on an ISA Jobin Yvon-Spex CD6 spectrometer, routinely calibrated with (+)-camphor-10-sulfonic acid, preparing the samples in buffer solution containing NaCl (0.15 M). The mean residue ellipticity, Q_{222}, of HJ1 at 222 nm and 0.226 mM is -22970 deg cm^2 dmol^{-1}.

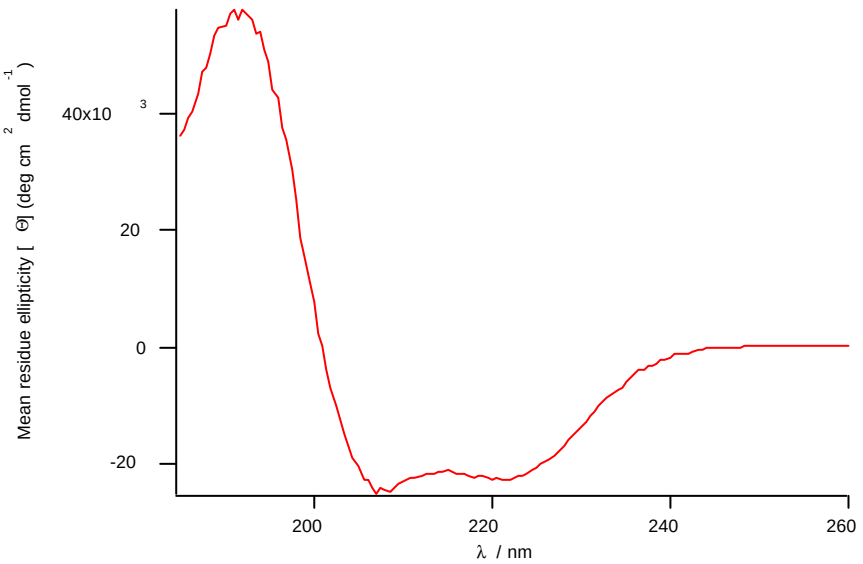
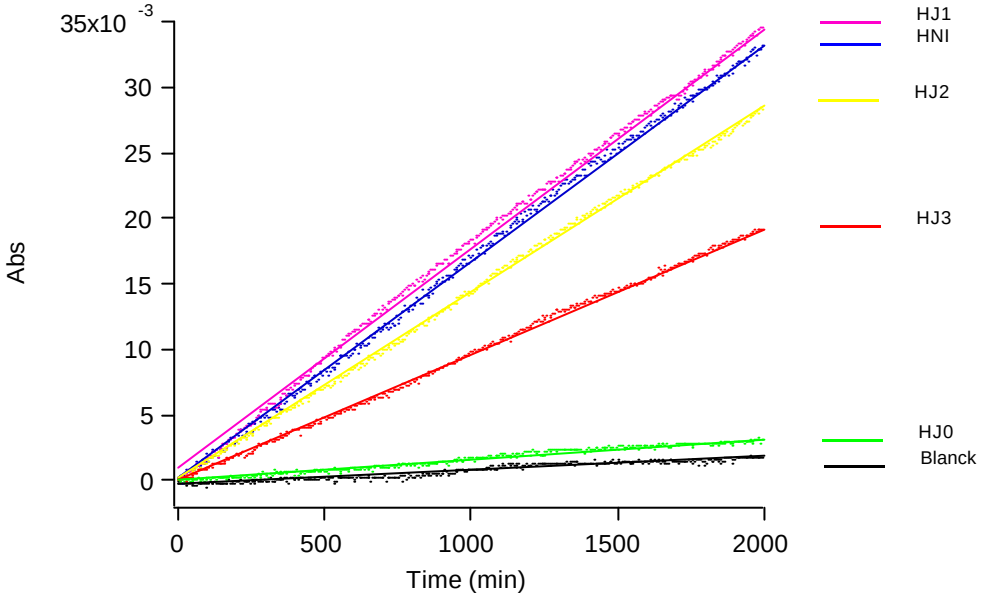
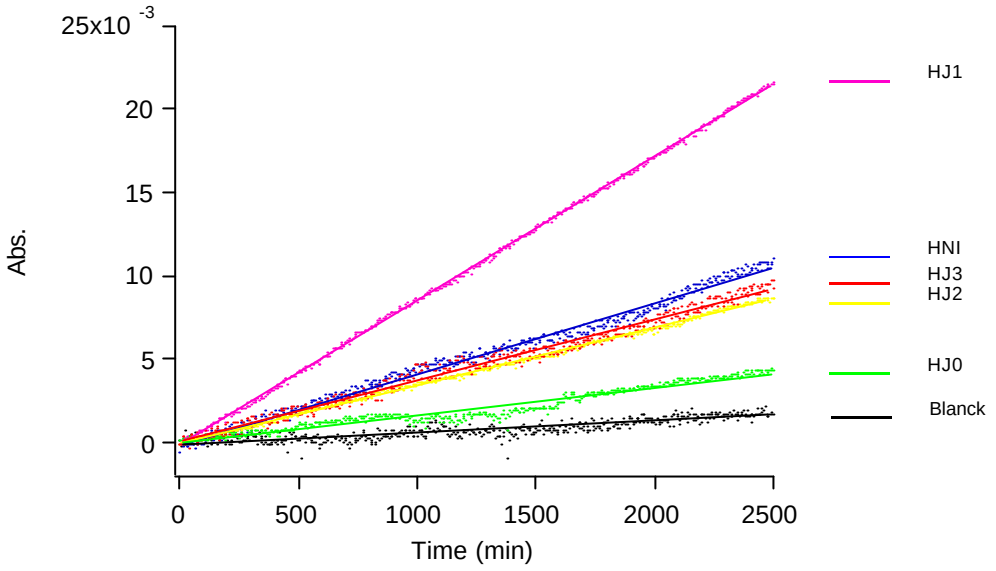


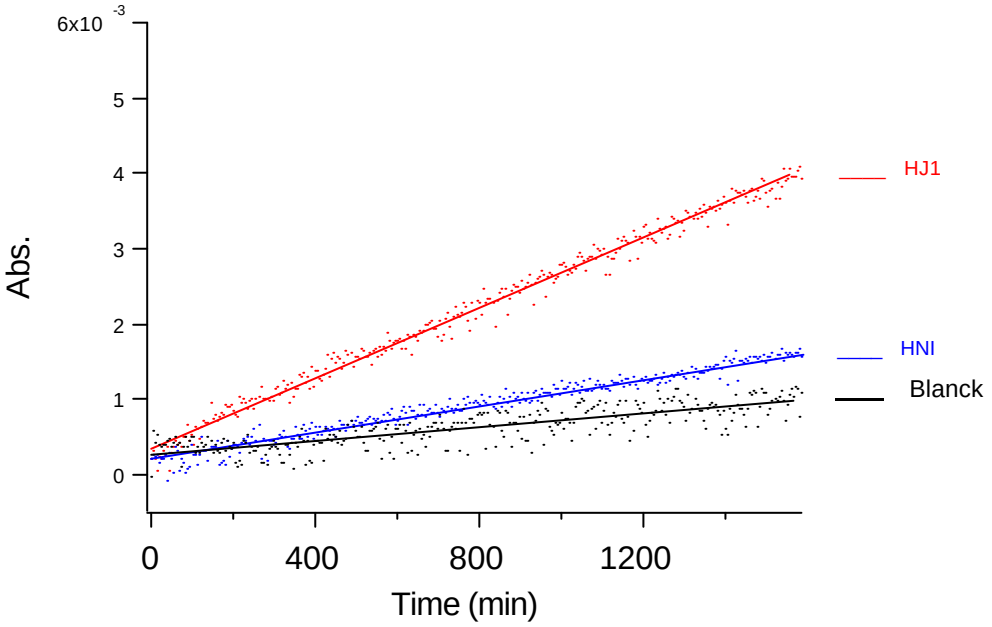
Figure S3. Kinetic profiles for the cleavage of substrates **4**, **5**, **6** and **7**. All the experiments were carried out at pH 7.0 (50 mM HEPES buffer) and 313 K, using 1 mM peptide concentration and 1-2.21 mM substrate concentration in final solutions. The evolution of the reaction is followed by UV spectroscopy, monitoring the increase in absorbance due to the formation of the 4-nitrophenolate or 4-chloro-2-nitrophenolate ions. Extinction coefficients: 7785 M^{-1} cm^{-1} for 4-nitrophenyl derivatives (at 405 nm) and 2384 M^{-1} cm^{-1} for 4-chloro-2-nitrophenyl derivatives (at 424 nm). Absorbance data points are adjusted to lines (which are also shown in graphics) by linear regression (Igor Pro, Wavemetrics Inc.).



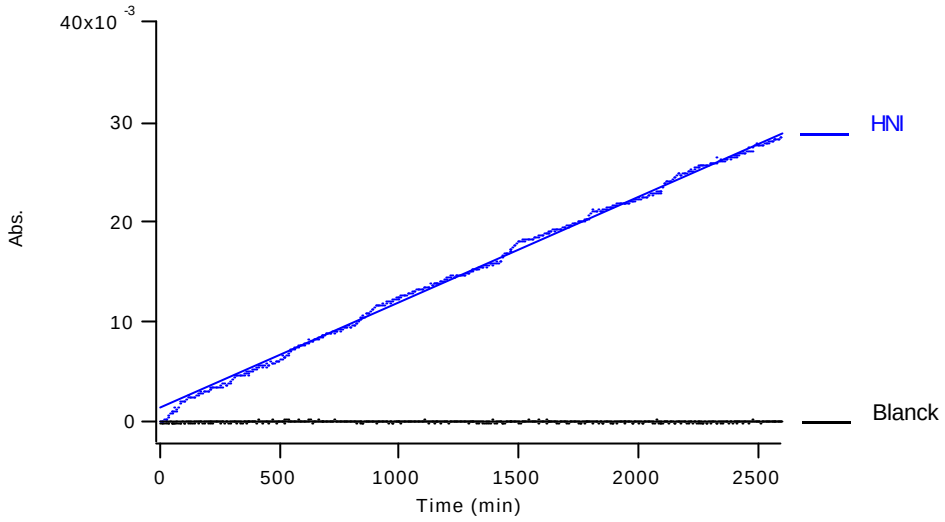
Initial kinetic profile for the cleavage of butyl 4-chloro-2-nitrophenyl phosphate (4) catalyzed by HNI (blue line/"HNI"), HJ0 (green line/"HJ0"), HJ1 (pink line/"HJ1"), HJ2 (yellow line/"HJ2") and HJ3 (red line/"HJ3") peptides. The blank experiment (black line/"blank") is also shown. Substrate concentration was 2 mM.



Initial kinetic profile for the cleavage of **butyl 4-nitrophenyl phosphate** (**6**) catalyzed by HNI (blue line/"HNI"), HJ0 (green line/"HJ0"), HJ1 (pink line/"HJ1"), HJ2 (yellow line/"HJ2") and HJ3 (red line/"HJ3") peptides. The blank experiment (black line/"blank") is also shown. Substrate concentration was 2 mM.

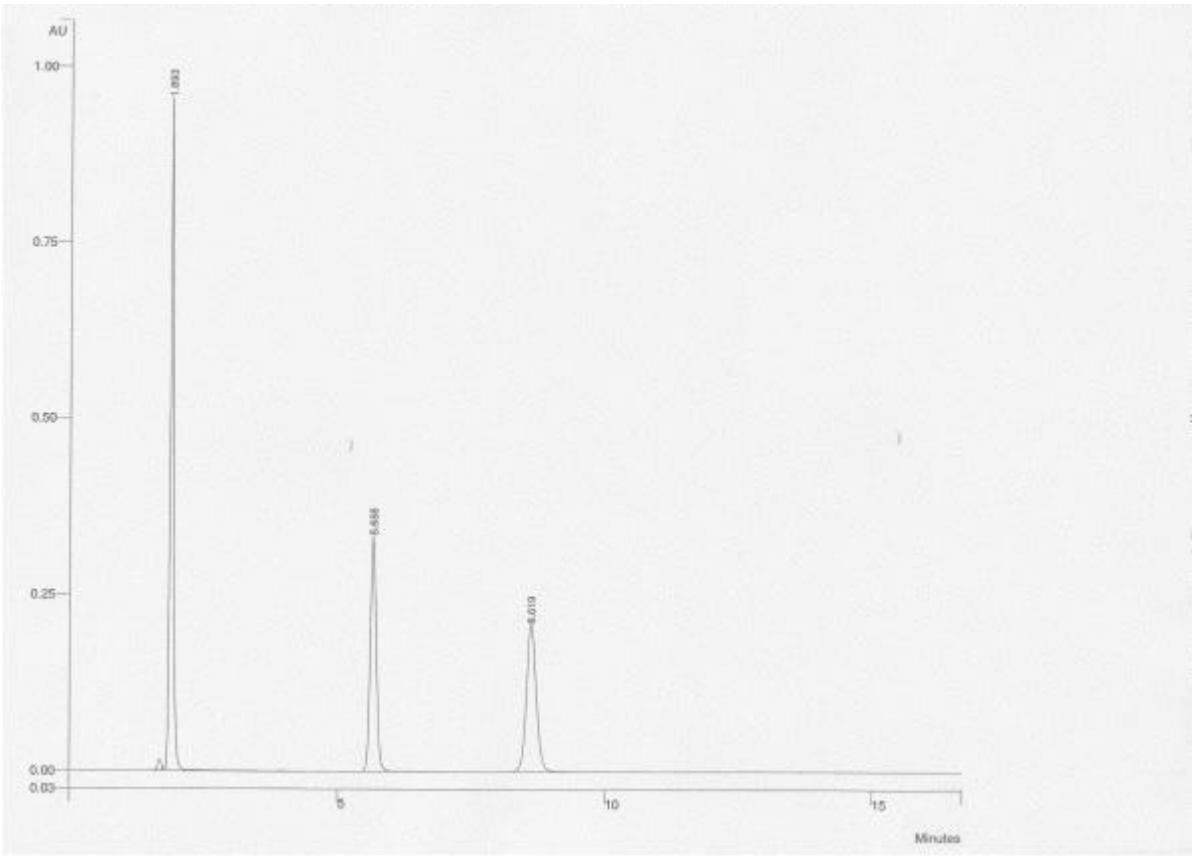


Initial kinetic profile for the cleavage of **ethyl 4-nitrophenyl phosphate** (**5**) catalyzed by HJ1 (red line/"HJ1") or by HNI (blue line/"HNI") peptide. The blank experiment (black line/"blank") is also shown. Substrate concentration: 2.21 mM for HNI and 1 mM for HJ1.



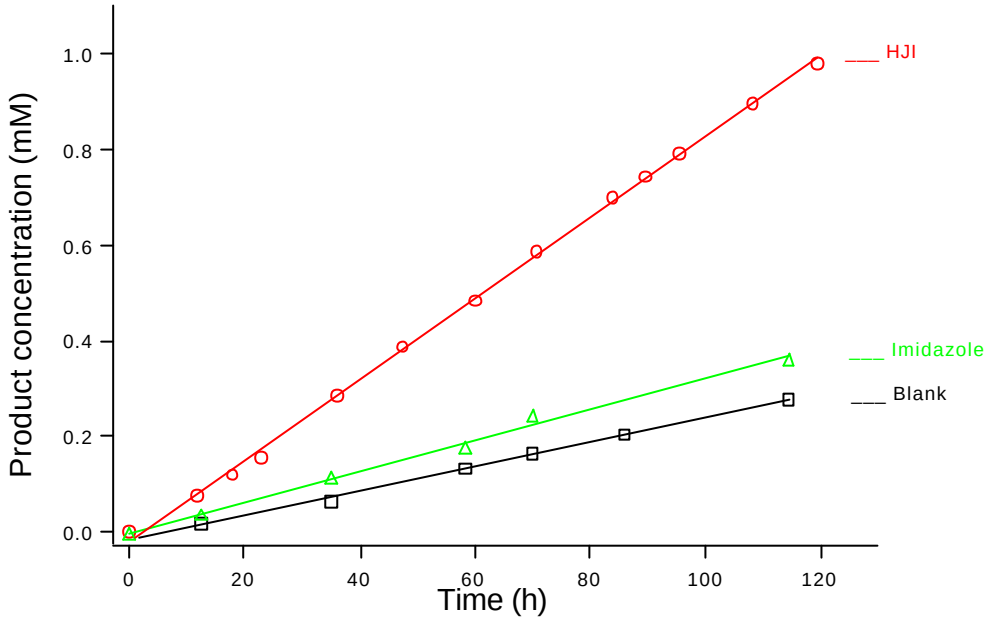
Initial kinetic profile for the cleavage of **heptyl 4-nitrophenyl phosphate** (**7**) by 1 mM HNI peptide (blue line/"HNI") together with the blank experiment (black line/ "blank"). Substrate concentration was 2.21 mM. The kinetics of substrate **7** cleavage catalyzed by HJ1 peptide and by imidazole are shown in the paper.

Figure S4. Representative HPLC chromatogram of uridine 3'-(2,2,2-trichloroethyl phosphate) (substrate 1) kinetics.



RP-HPLC. Column: Highchrom KR-100-C8-5 (250x4.6 mm, 5 µm particle size). Internal standard: 3-nitrobenzenesulfonic acid, sodium salt, 1 mM. Isocratic elution with 13% acetonitrile in sodium acetate buffer (25 mM, pH 4.3, containing 0.1 M NH_4Cl) as eluent in a 1.5 mL/min flux. Detector at 260 nm. Retention times: product 1.89 min, internal standard 5.66 min and substrate 8.62 min.

Figure S5. Initial kinetic profile for the cleavage of substrate **1**.



Initial kinetic profile for the cleavage of uridine 3'-(2,2,2-trichloroethyl phosphate) (substrate **1)** at pH 7.0 and 313K by 1 mM HJ1 peptide (red line/circles) or by 50 mM imidazole (green line/triangles), together with the blank experiment (black line/squares). Data points are adjusted to lines by linear regression. Substrate concentration was 2 mM in 50 mM buffer solution (HEPES) and reactions were followed by RP-HPLC.