**Electronic Supplementary Information**

# *In situ* thioester formation for protein ligation using α-methyl cysteine

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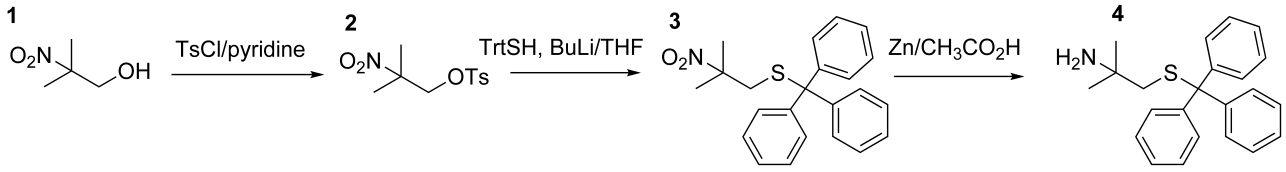
## Abbreviations

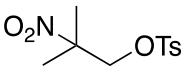
ACN: acetonitrile. BPTI: bovine pancreatic trypsin inhibitor. CHCA: α-cyano-4-hydroxycinnamic acid. DCM: dichloromethane. DIC: 1,3-diisopropylcarbodiimide. DIEA: *N,N*-diisopropylethylamine. DMF: *N,N*-dimethylformamide. EDTA: ethylenediamine tetraacetic acid. Fmoc: 9-fluorenylmethoxycarbonyl. Gdm: gem dimethyl unit (2-Amino-2-methylpropyl sulfide). HBTU, *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide. HMB: 2-hydroxy-4-methoxybenzyl. HOBt: 1-hydroxybenzotriazole. MALDI-TOF MS: matrix-assisted laser desorption ionization time-of-flight mass spectrometry. MPAA: 4-mercaptophenylacetic acid. mpaV: mercaptopropionic acid valine. murKC: Murine KC. PyBOP: benzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate. rt: room temperature. TCEP: tris(2-carboxyethyl)phosphine. TFA: trifluoroacetic acid. THF: tetrahydrofuran.

## I- Synthesis of Building Blocks:



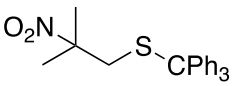

**Scheme S1.** Synthesis of 2-Amino-2-methylpropyl trityl sulphide **4**

### 2-Methyl-2-nitropropyl 4-methylbenzene sulfonate (2)



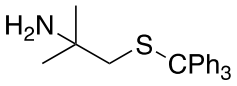
To a solution of 2-methyl-2-nitro-1-propanol (10 g, 84 mmol) in dry pyridine (50 mL) was added at 0 °C *p*-toluene sulphonyl chloride (16.20 g, 85 mmol) and the resulting mixture was stirred at room temperature for 4 h. The solvent was evaporated and the residue was dissolved in EtOAc (150 mL) and washed successively with 1 M aq. HCl (150 mL) and brine (2 x 150 mL). The organic phase was dried and evaporated to give **2** (16.53 g, 72 %) as white crystals, mp 76-77 °C (EtOAc-hexanes), lit.[1] mp 75.3-76.7 °C (aq. MeOH). $^{1}$H NMR (400 MHz, $CDCl_3$): $\delta$ 7.73 (2H, d, $J$ = 8.4 Hz, ArH), 7.35 (2H, d, $J$ = 8.4 Hz, ArH), 4.26 (2H, s, $CH_2$), 2.44 (3H, s, $ArCH_3$), 1.57 (6H, s, $C(CH_3)_2$). $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta$ 145.7 ($SO_2C_{ar}$), 132.0 ($C_{ar}CH_3$), 130.3 ($CH_{ar}$), 128.2 ($CH_{ar}$), 86.0 ($CNO_2$), 77.4 ($CH_2$), 23.3 ($C(CH_3)_2$), 22.1 ($C_{ar}CH_3$).

### 2-Methyl-2-nitropropyl trityl sulfide (3)



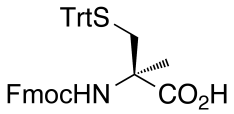
To a solution of triphenylmethane thiol (5.06 g, 18.3 mmol) in dry THF (75 mL) was added dropwise at 0 °C *n*-butyl lithium (2.5 M in hexane; 7.65 mL, 19.1 mmol). A solution of tosylate **2** (5.00 g, 18.3 mmol) in dry THF (50 mL) was added and the resulting mixture was heated under reflux for 3 h. The solvent was evaporated; the residue was diluted in ethyl acetate (150 mL) and washed with brine (3 x 100 mL). The organic phase was dried and evaporated and the residue was flash chromatographed [EtOAc–hexanes (5:95)] to give **3** (4.98 g, 72%) as white crystals, mp 103-105 °C, ($Et_2O$ - hexanes). $^{1}$H NMR (400 MHz, $CDCl_3$): $\delta$ 7.41-7.43 (6H, m, ArH), 7.20-7.31 (9H, m, ArH), 2.67 (2H, s, $CH_2$), 1.43 (6H, s, $C(CH_3)_2$). $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta$ 144.3 (Car), 188.7 ($CH_{ar}$), 128.3 ($CH_{ar}$), 127.2 ($CH_{ar}$), 87.9 ($CNO_2$), 67.6 ($SCPh_3$), 41.3 ($CH_2$), 26.1 ($C(CH_3)_2$). Calculated % for $C_{23}H_{23}NO_2S$: C, 73.18; H, 6.14; N, 3.71. Found: C, 73.45; H, 6.17; N, 3.58%.

**2-Amino-2-methylpropyl trityl sulfide (4)**




To a stirred solution of **3** (1.51 g, 4 mmol) in acetic acid (80 mL) was added freshly activated zinc dust (13.08 g, 200 mmol) and the mixture was stirred for 2 h, after which time it was filtered through a pad of Celite. The filtrate was evaporated and the residue was dissolved in EtOAc (150 mL), washed with 1 M aq. NaOH (3 x 100 mL) and brine (2 x 100 mL), dried and evaporated. The residue was flash chromatographed [EtOAc – hexanes - $Et_3N$ (3:7:0.5)] to give **4** (0.91 g, 65 %) as white crystals, mp 125-127 °C ($Et_2O$ - hexanes). $^1$H NMR (600 MHz, $CDCl_3$): $\delta$ 7.43-7.46 (6H, m, ArH), 7.25-7.30 (6H, m ArH), 7.19-7.22 (3H, m, ArH), 4.95 (2H, br s, $NH_2$), *2*.32 (2H, s, $CH_2$), 1.01 (6H, s, $C(CH_3)_2$). $^{13}$C NMR (100 MHz, $CDCl_3$): δ 145.0 (Car), 129.9 ($CH_{ar}$), 128.1 ($CH_{ar}$), 126.9 ($CH_{ar}$), 66.7 ($CNH_2$), 58.1 ($SCPh_3$), 40.5 ($CH_2$), 24.7 ($C(CH_3)_2$). HRMS (ES): Calcd for $(C_{23}H_{25}NS + H)^+$, 348.1780. Found: 348.1773.

**(*R*)-2-{[[(9*H*-Fluoren-9-yl)methoxy]carbonyl]amino}-2-methyl-3-(tritylthio)propanoic acid, (Fmoc-αMe-L-Cys(Trt)-OH) (5)**




A mixture of (*R*)-2-amino-3-mercapto-2-methylpropionic acid hydrochloride (2.0 g, 11.65 mmol) purchased from Iris Biotech (H-αMe-L-Cysteine.HCL CAS # 148766-37-4), trityl alcohol (3.34 g, 2.82 mmol) and $BF_3{\cdot}Et_2O$ (2.05 g, 14.45 mmol) in glacial acetic acid (50 mL) was heated under nitrogen to 80 ˚C for 30 min. After cooling to room temperature the solvent was evaporated under reduced pressure and re-evaporated from toluene (2 x 50 mL). The residue was dissolved in a mixture of water (40 mL) and dioxane (40 mL), cooled to 0 ˚C and treated with anhydrous solid $Na_2CO_3$ (3.09 g, 29.3 mmol). A solution of Fmoc-Cl (3.62 g, 13.98 mmol) in dioxane (20 mL) was added dropwise and the mixture was stirred at room temperature overnight. The reaction mixture was concentrated *in vacuo* to about half its original volume, diluted with water (150 mL) and basified to pH 12 with I M aq. NaOH and washed with $Et_2O$ (3 x 100 mL). The aq. phase was then acidified to pH 3 with 1 M aq. HCl and extracted with EtOAc (3 x 150 mL). The combined organic phases from the acidic solution were washed with brine, dried and evaporated to viscous oil. Flash chromatography [EtOAc - MeOH (97:3)] gave **5** (3.07 g, 44 %) as a white foam. $^1$H NMR (400 MHz, DMSO$d_6$): $\delta$ .7.90 (2H, d, $J$ = 7.8 Hz, ArH), 7.77 (2H, t, $J$ = 7.8 Hz, ArH), 7.72 (1H, s, $CO_2H$), 7.29-7.32 (20H, m, ArH and NH), 4.35 and 4.28 rotamers (2H, 2 x t, $J$ = 6.9 Hz, $OCH_2$), 4.21 and 4.18 rotamers (1H, 2 x d, $J$ = 6.9 Hz, $OCH_2CHAr$), 3.35 (1H, d, $J$ = 11.9 Hz, half $SCH_2$), 2.27 (1H, d, $J$ = 11.9 Hz, half $SCH_2$), 1.09 and 1.07 rotamers (3H, 2 x s, $CH_3$). HRMS (ES): Calcd for $(C_{38}H_{33}NO_4S + Na)^+$, 622.2028. Found: 622.2038.

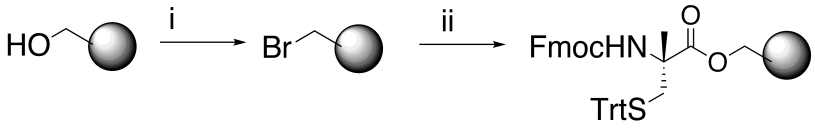
**II- Synthesis and characterization of the peptides used in the ligation reactions**

All peptides were prepared by standard automated solid-phase synthesis using DIC/HOBt or HBTU/DIEA activation for Fmoc/t-Bu chemistry (CS Bio 336 automated synthesizer). Couplings were carried out with a 5-fold excess of activated amino acid for a minimum of 45 min. Cleavage was performed by treatment of the peptide-resin with a cleavage cocktail containing TFA and as scavengers, triethylsilane, $H_2O$, ethanedithiol in the ratio 94.5:2.5:2.5:0.5 (v/v) for 1.5 h. The cleavage cocktail was filtered, the filtrate sparged ($N_2$) and the peptide precipitated ($Et_2O$, Na- dried, 4 $^o$C) and freeze-dried.

Peptides were purified by semi-preparative HPLC on a RP-C18 column (22 x 250 mm, Vydac) using linear gradients of $CH_3CN$ in 0.1 % TFA/$H_2O$ with a flow rate of 15 mL.min$^{-1}$. HPLC gradients below are given with A= 0.1% TFA in $H_2O$ and B = 0.1 % TFA, 10 % $H_2O$, 90 % $CH_3CN$. Detection was performed at 214 nm.

Peptides were characterized by MALDI-TOF MS on a BRUKER microflex using CHCA matrix (10 $mg.mL^{-1}$ in $CH_3CN/H_2O$/TFA, 50:50:0.1). The ion positive reflector mode was used for peptides with m/z < 4000, the ion positive linear mode was used for peptides with m/z > 4000.

**Resin loading with Fmoc-αMe-L-Cys(Trt)-OH**




**Scheme S2.** Loading of Wang resin with Fmoc-αMe-L-Cys(Trt)-OH.
i) $PBr_3$ /$CH_2Cl_2$ 45 min. rt, ii) Fmoc-αMe-L-Cys(Trt)-OH (**5**), DIEA (2 equiv, 0.2 M), DMF, 16 h, rt.

Resin loading was adapted from Topping et al.,[2] and performed on small batches, typically: Wang resin (2.0 g; 0.38 mmol/g, Merck Chemicals) swollen in dry $CH_2Cl_2$was stirred in $PBr_3$ (1.0 M; 5 mL) for 45 min at room temperature, drained and washed with $CH_2Cl_2$ followed by DMF. Esterification was performed with Fmoc-αMe-L-Cys(Trt)-OH (500 mg; 0.8 mmol), DIEA (200 mg; 1.6 mmol) in dry DMF, for 16 h with shaking. The resin was washed with DMF and $CH_2Cl_2$, dried, yielding 2.35 g of loaded resin. Subsequent coupling of several different Fmoc amino acids to this resin was performed under automated conditions as above. Resin was tested with 2,4,6-Trinitrobenzenesulfonic acid solution, (TNBS), all resins gave a negative TNBS test.[3]

***H*-LAPAG-gdm.** Boc-LAPAG-OH was synthesized on 2-chlorotrityl resin by standard automated solid-phase synthesis using HBTU/DIEA activation for Fmoc/t-Bu chemistry. The peptide was cleaved (TFE/DCM 1:1, 1 mL, 1 h), filtered and evaporated. The Boc protected peptide was dissolved in 10 % buffer B (B = 0.1 % TFA, 10 % $H_2O$, 90 % $CH_3CN$) and lyophilized. The Boc protected peptide (40 mg, 7.6 $10^{-5}$ moles) dissolved in 600 µL of DCM was activated with 1.1 eq. DIC (13 µL), 1 eq. DIEA (13 µL) and 1 eq. 2-amino-2-methylpropyl trityl sulphide (**4**) (26.5 mg) was added. The mixture was left for 3 h at rt. The solvent was sparged with $N_2$ and peptide precipitated in $Et_2O$. Final deprotection was performed with TFA/Triethylsilane/$H_2O$ 10:0.5:0.5, 300 µL, 2 h). The crude peptide was precipitated in $Et_2O$. HPLC purification was performed on a Vydac RP-C18 using the gradient 0 to 50 % B in 30 min (retention time of product 21 min) yielding 2 mg of peptide, 5% yield (purity > 95 %). m/z = 515.1 $[M+H]^+$ (first isotope), calc.: 515.3.

***H*-LAPAGC-*OH***, was synthesised on 2-chlorotrityl resin by standard automated solid-phase synthesis using HBTU/DIEA activation for Fmoc/t-Bu chemistry. 22 mg of peptide were obtained after HPLC purification (10 to 50 % B in 30 min), 34 % yield (purity > 98 %). m/z = 531.5 $[M+H]^+$ (first isotope), calc.: 531.2.

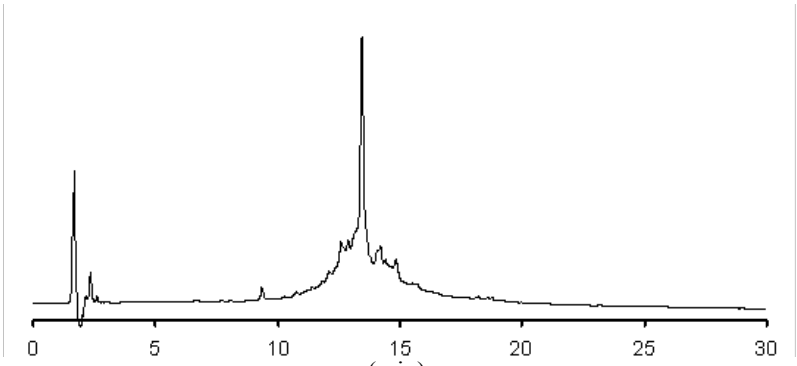
***H*-LAPAG-α(Me)C-*OH***. Fmoc-α(Me)Cys(trt)-OH (300 mg, 0.5 mmol) was loaded on Wang resin (500 mg, resin loading 0.2 mmol/g, 0.1 mmol) following the procedure by Topping *et al.* (Scheme S2) as described above.[2] The peptide was synthesized by standard automated solid-phase synthesis using DIC/HOBt activation for Fmoc/t-Bu chemistry. 21.2 mg of peptide were obtained after HPLC purification (10 to 50 % B in 30 min), 33 % yield (purity > 98 %). m/z = 545.6 $[M+H]^+$ (first isotope), calc.: 545.3.

***H*-LAPAA-α(Me)C-*OH***. The peptide was synthesized as previously described for LAPAG-α(Me)C. 19.5 mg of peptide were obtained after HPLC purification (10 to 50 % B in 30 min), 29 % yield (purity > 98 %). m/z = 559.5 $[M+H]^+$ (first isotope), calc.: 559.

***H*-CFAPRGKR-*NH2***, The peptide was synthesized on Rink resin (Polymer labs) (1 g, resin loading 0.39 mmol/g). Synthesis yielded 1.55 g of dried peptide-resin. A portion of the peptide-resin (0.54 g) was cleaved and HPLC purification (10 to 50% B in 30 min) yielded 61.4 mg of peptide, 47% yield (purity > 95 %). m/z = 933.6 $[M+H]^+$ (first isotope), calc.: 933.5.

***H*-CFAPLV-*NH*$_2$**, The peptide was synthesized on Rink resin (Polymer labs) (500 mg, resin loading 0.39 mmol/g). Synthesis yielded 650 mg of peptide-resin. HPLC purification using the gradient 15 to 45 % B in A over 30 min yielded 37 mg peptide, 24% yield (purity > 98 %). m/z = 648.0 $[M+H]^+$ (first isotope), calc.: 648.3.

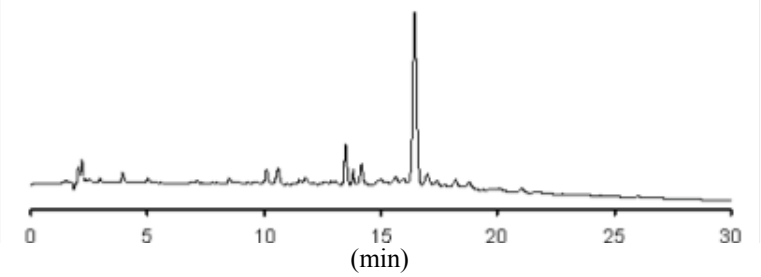
**Hmb-BPTI(1-37)-α(Me)C** =
*H*-RPDFCLEPPYTGPCKARIIRYFYNAKA(Hmb)GLCQTFVYGG-α(Me)C-*OH*
The peptide was assembled on Wang resin (290 mg, resin loading 0.2 mmol/g, 0.058 mmol) as previously described for LAPAG-α(Me)C (Scheme S2). Synthesis yielded 550 mg of peptide-resin. The peptide-resin was treated for 30 min with 10 eq. acetic anhydride and 5 eq. DIEA in DMF to acetylate the Hmb group and prevent its cleavage during TFA treatment. The peptide was cleaved (standard TFA procedure) and precipitated. Subsequently, deacetylation was performed using very dilute hydrazine hydrate (1 eq.) in phosphate buffer pH 7 with monitoring to completion by analytical HPLC and LCMS.[4] HPLC purification using the gradient 15 to 35 % B in 5 min then 35 to 50 % B over 30 min yielded 14.5 mg peptide, 5 % yield (purity > 98 %). m/z = 4470.7 $[M+H]^+$ (average isotope composition), calc.: 4470.2.




**Analytical HPLC of crude Hmb-BPTI(1-37)-α(Me)C.** HPLC analysis was performed on a RP-C18 column using a linear gradient of 20 to 60 % B over 30 min, with a flow rate of 1 mL/min.

**BPTI(38-58)** = *H*-CRAKRNNFKSAEDCMRTCGGA-*OH*. BPTI(38-58) was synthesized on Fmoc-Ala-Wang resin (200 mg, 0.47 mmol/g, polymer Labs). HPLC purification performed with a gradient of 10 to 50 % B over 30 min yielded 43 mg; 15 % yield. m/z = 2318.0 $[M+H]^+$ (first isotope), calc.: 2317.3.

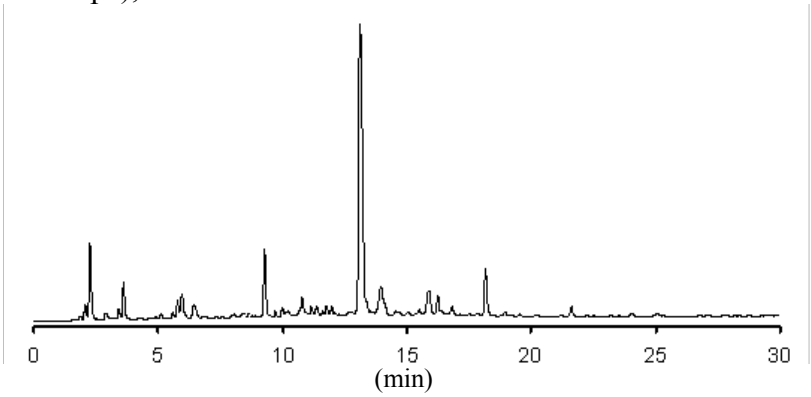
**MurKc(1-34)-α(Me)C** = *H*-APIANELRCQCLQTMAGIHLKNIQSLKVIPSGPH-α(Me)C-*OH*. The peptide was assembled on Wang resin (150 mg, resin loading 0.2 mmol/g, 0.03 mmol) as previously described for LAPAG-α(Me)C. Synthesis yielded 260 mg of dried peptide-resin. HPLC purification was performed using a linear gradient of 20 to 50 % B over 30 min and yielded 10.5 mg peptide. 8.5 % yield. m/z = 3798.4 $[M+H]^+$ (first isotope), calc.: 3800.0.




**Analytical HPLC of crude MurKc(1-34)-α(Me)C.** HPLC analysis was performed on a RP-C18 column using a linear gradient of 20 to 40 % B over 30 min, with a flow rate of 1 mL/min.

**MurKc(35-72)** = *H*-CTQTEVIATLKNGREACLDPEAPLVQKIVQKMLKGVPK-*OH*. The peptide was synthesized on Fmoc-Lys(Boc)-Novasyn resin (500 mg, resin substitution 0.07 mmol/g) (Merck Chemicals) using DIC/HOBt activation. HPLC purification using a linear gradient of 20 to 60 % B over 30 min yielded 15 mg peptide, 8.6 % yield (purity > 98 %). m/z = 4151.9 $[M+H]^+$ (first isotope), calc.: 4152.0.

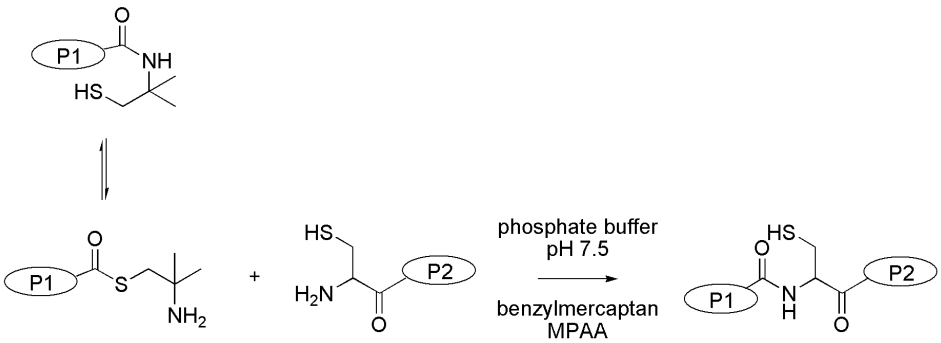
**ROP(26-37)-α(Me)C=** *H*-LNELDADEQADL-α(Me)C-*OH*. The peptide was assembled on Wang resin loaded with Fmoc-α(Me)Cys . HPLC purification yielded 14 mg peptide, 10 % yield (purity > 98 %). m/z = 1462.9 $[M+H]^+$ (first isotope), calc.: 1462.6.




**Analytical HPLC of crude ROP(26-37)-α(Me)C.** HPLC analysis was performed on a RP-C18 column using the gradient using a linear gradient of 10 to 50 % B over 30 min, with a flow rate of 1 mL/min.

**ROP(38-63)** = *H*-CESLHDHADELYRSCLARFGDDGENL-*OH*. The peptide was assembled on Fmoc-Leu-NovaSyn TGT resin (Merck Chemicals) (500 mg, resin loading 0.2 mmol/g, 0.1 mmol). *N*-α-Fmoc-*N*-α-(2-Fmoc-oxy-4-methoxybenzyl)glycine (Merck Chemicals) was used instead of Fmocglycine at Gly60 to prevent aspartimide formation.[5] Synthesis yielded 765 mg of peptide-resin. HPLC purification using the gradient 15 to 45 % B in A over 30 min yielded 80 mg peptide, 23 % yield (purity > 98 %) - m/z = 2964.1 $[M+H]^+$ (first isotope), calc.: 2965.3.

## III- Ligation with a peptide containing a *C*-terminal gdm




**Scheme S2**. P1 and P2 = unprotected peptides, MPAA = 4-mercaptophenylacetic acid.

LAPAGgdm (final concentration: 10 mM) and CFAPLV (5 mM) were dissolved in degassed 0.2 M sodium phosphate buffer containing 2 mM EDTA, 2% benzylmercaptan and 30 mM 4-mercaptophenylacetic acid, final pH 7.5. The reaction was monitored by analytical HPLC injecting aliquots of the reaction mixture on a on a RP-C4 column using the following gradient: 10 min at 0 % B then linear gradient of 0 to 30% B in 30 min, with a flow rate of 1 $mL.min^{-1}$. The ligation product LAPAGCFAPLV was isolated and characterized by MALDI-TOF MS. m/z = 1057.4 $[M+H]^+$ (first isotope), calc. 1057.6.

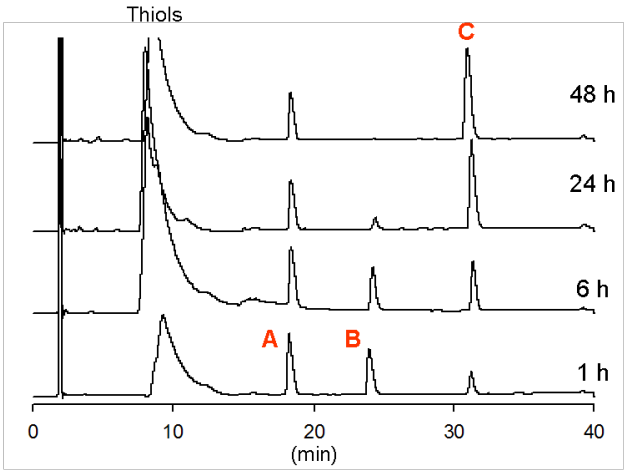
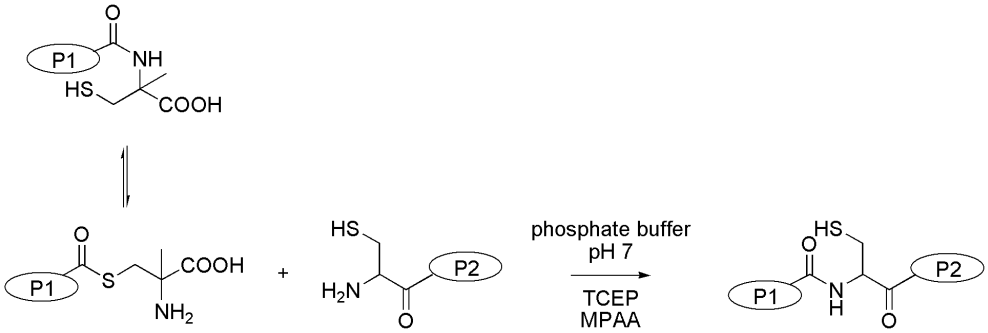



**Figure S1**. Analytical HPLC time course of the ligation reaction of LAPAG-gdm (**A**) and CFAPLV (**B**). **C** = ligation product LAPAGCFAPLV.

## IV- Ligations with peptides containing a *C*-terminal α-methyl cysteine




**Scheme S3**. P1 and P2 = unprotected peptides, MPAA = 4-mercaptophenylacetic acid.

***LAPAG-α(Me)C** (1.42 mg, 2.15 µmol; final concentration 12.5 mM) and peptide **CFAPRGKR** (2.45 mg, 1.75 µmol; 10 mM) were dissolved in 175 µL degassed 0.2 M sodium phosphate buffer, 2 mM EDTA, 50 mM TCEP, 60 mM MPAA, final pH 7. The reaction mixture was heated at 40 °C. The reaction was monitored by analytical HPLC injecting aliquots of 2 µL of the reaction mixture on a RP-C18 column (Phenomenex) using a linear gradient of 0 to 40 % B in 30 min with a flow rate of 1 mL.min$^{-1}$. The ligation product **LAPAGCFAPRGKR** was isolated by HPLC (1.15 mg, 36 % yield) and characterized by MALDI-TOF MS. m/z = 1342.8 $[M+H]^+$ (first isotope) (observed), calc.: 1342.7.

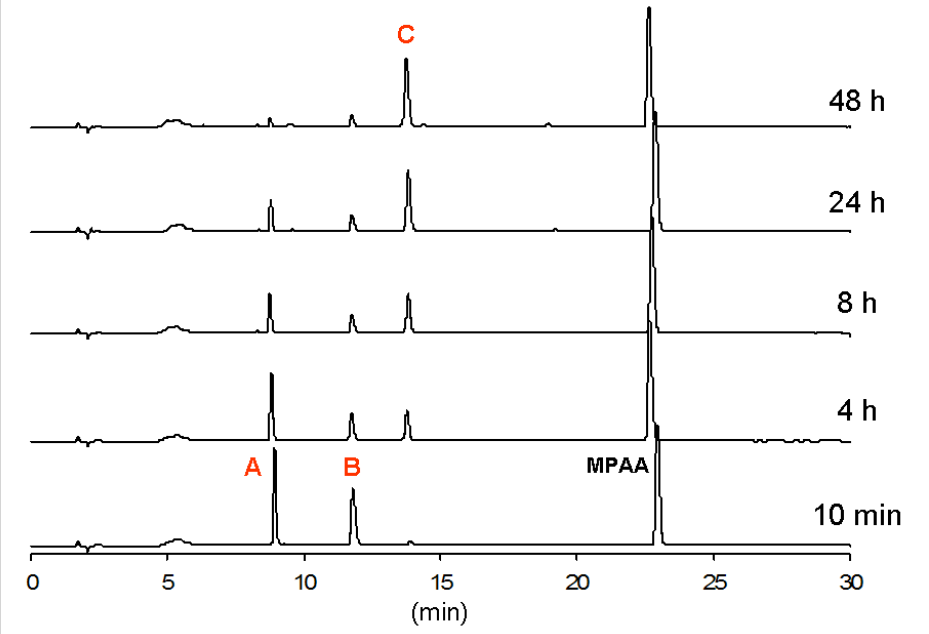



**Figure S2**. Analytical HPLC time course of the ligation reaction of CFAPRGKR (**A**) and LAPAG-α(Me)C (**B**). **C** = ligation product LAPAGCFAPRGKR, MPAA = 4-mercaptophenylacetic acid.

***LAPAA-α(Me)C** (1.06 mg, 1.58 µmol; final concentration 12.5 mM) was ligated to **CFAPRGKR** (1.74 mg, 1.25 µmol; 10 mM) using the same procedure. The ligation product (**LAPAACFAPRGKR**) was isolated and characterized by MALDI-TOF MS. m/z = 1357.0 $[M+H]^+$ (first isotope) (observed), calc.: 1356.8.

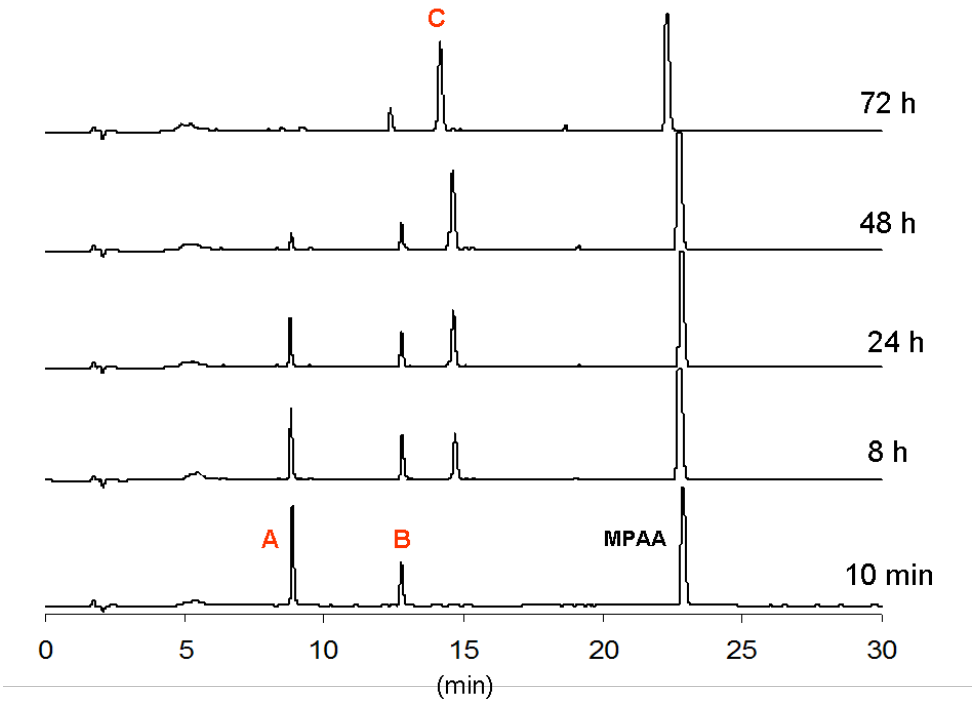



**Figure S3**. Analytical HPLC time course of the ligation reaction of CFAPRGKR (**A**) and LAPAA-α(Me)C (**B**). **C** = ligation product LAPAACFAPRGKR, MPAA = 4-mercaptophenylacetic acid.

## *Hmb-BPTI Ligation

**Hmb-BPTI(1-37)-α(Me)C** (1 mg, 0.197 µmol; final concentration 2 mM) and **BPTI(38-58)** (0.98 mg, 0.325 µmol; final concentration 3.25 mM) were dissolved in 98 µL of degassed 0.2 M sodium phosphate buffer, containing 6 M guanidine.HCl, 2 mM EDTA, 50 mM TCEP, 60 mM MPAA, final pH 7. The reaction was monitored by analytical HPLC injecting aliquots of 2 µL of the reaction mixture on a RP-C18 column (Phenomenex) using a linear gradient of 0 to 75 % B in 23 min with a flow rate of 1 $mL.min^{-1}$. The ligation product (Hmb-BPTI) was isolated (0.5 mg, 30 %) and characterized by MALDI-TOF MS. m/z= 6650 $[M+H]^+$ (average isotope composition), calc: 6650. In these conditions (pH 7, 60 mM MPAA), the reaction was completed in 48 hours (see HPLC monitoring of the ligation in the manuscript). The reaction was repeated at pH 7 with 300 mM MPAA, or pH 6 with 300 mM MPAA and the reaction was completed in 32 and 24 hours, respectively.

## *MurKc Ligation

**MurKc(1-34)-α(Me)C** (0.82 mg, 0.19 µmol; final concentration 0.44 mM) and **MurKc(35-72)** (0.89 mg, 0.18 µmol; final concentration 0.42 mM) were dissolved in 430 µL of degassed 0.2 M sodium phosphate buffer, containing 6 M guanidine.HCl, 2 mM EDTA, 50 mM TCEP, 60 mM MPAA, final pH 7. The reaction was monitored by analytical HPLC injecting aliquots of 10 µL of the reaction mixture on a RP-C18 column (Phenomenex) using a linear gradient of 20 to 50 % B in 30 min with a flow rate of 1 $mL.min^{-1}$. The ligation product was isolated and characterized by MALDI-TOF MS. m/z= 7815 $[M+H]^+$ (average isotope composition), calc: 7816. In these conditions (pH 7, 60 mM MPAA), the reaction was completed in 72 hours. When the reaction was repeated at pH 6 using 300 mM MPAA, the reaction was completed in 48 hours.

***ROP Ligation**

**ROP(26-37)-α(Me)C** (1 mg, 0.6 µmol; final concentration 3 mM) and **ROP(38-63)** (1.8 mg, 0.55 µmol; final concentration 2.7 mM) were dissolved in 200 µL of degassed 0.2 M sodium phosphate buffer, containing 6 M guanidine.HCl, 2 mM EDTA, 50 mM TCEP, 300 mM MPAA, final pH 6. The reaction was monitored by analytical HPLC injecting aliquots of 1 µL of the reaction mixture on a RP-C18 column (Phenomenex) using a linear gradient of 15 to 45 % B in 30 min with a flow rate of 1 mL.min$^{-1}$. The ligation product was isolated and characterized by MALDI-TOF MS. m/z= 4292 $[M+H]^+$ (average isotope composition), calc: 4294. In these conditions (pH 6, 300 mM MPAA), the reaction was completed in 30 hours. The reaction was repeated at different pH and MPAA concentration, see Table 1 of the manuscript.

**V- Test ligation with a peptide containing a *C*-terminal cysteine**

***LAPAGC** (0.75 mg, 1.16 µmol; final concentration 12.2 mM) and **CFAPRGKR** (1.33 mg, 0.96 µmol; 10 mM) were dissolved in 96 µL degassed 0.2 M sodium phosphate buffer, 2 mM EDTA, 50 mM TCEP, 60 mM MPAA, final pH 7. The reaction mixture was heated at 40 °C. The reaction was monitored by analytical HPLC injecting aliquots of 2 µL of the reaction mixture on a RP-C18 column (Phenomenex) using a linear gradient of 0 to 40 % B in 30 min with a flow rate of 1 mL.min$^{-1}$.

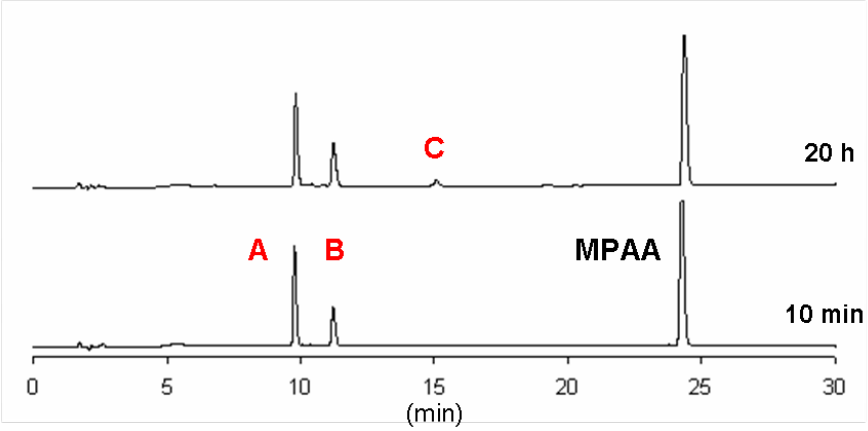



**Figure S4**. Analytical HPLC time course of the ligation reaction of CFAPRGKR (**A**) and LAPAGC (**B**) performed at final pH 7 in presence of 60 mM MPAA. **C** = ligation product LAPAGCFAPRGKR, MPAA = 4-mercaptophenylacetic acid.

*The ligation between **LAPAGC** and **CFAPRGKR** was repeated at pH 6 in the presence of 300 mM MPAA (except for that, the buffer composition and peptide concentrations were the same as above).

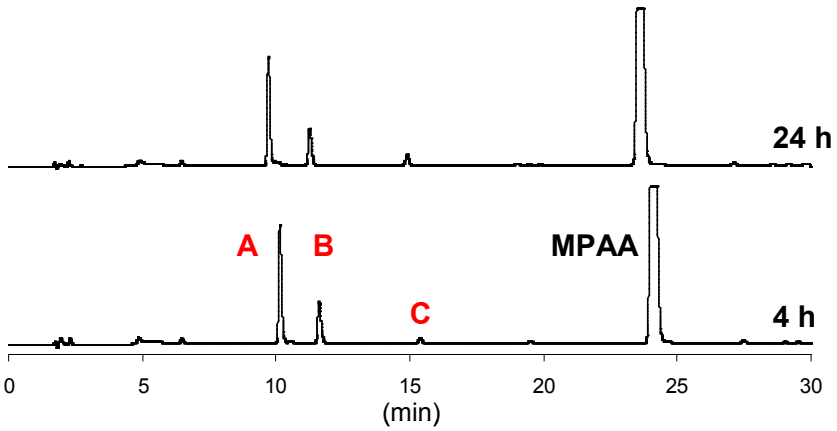



**Figure S5**. Analytical HPLC time course of the ligation reaction of CFAPRGKR (**A**) and LAPAGC (**B**) performed at final pH 6 in presence of 300 mM MPAA. **C** = ligation product LAPAGCFAPRGKR, MPAA = 4-mercaptophenylacetic acid.

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