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ARTICLE *in* EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY · MARCH 2015

Impact Factor: 3.45 · DOI: 10.1016/j.ejmech.2015.03.028 · Source: PubMed

Accepted Manuscript

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PII: S0223-5234(15)00195-6

DOI: 10.1016/j.ejmech.2015.03.028

Reference: EJMECH 7773

To appear in: *European Journal of Medicinal Chemistry*

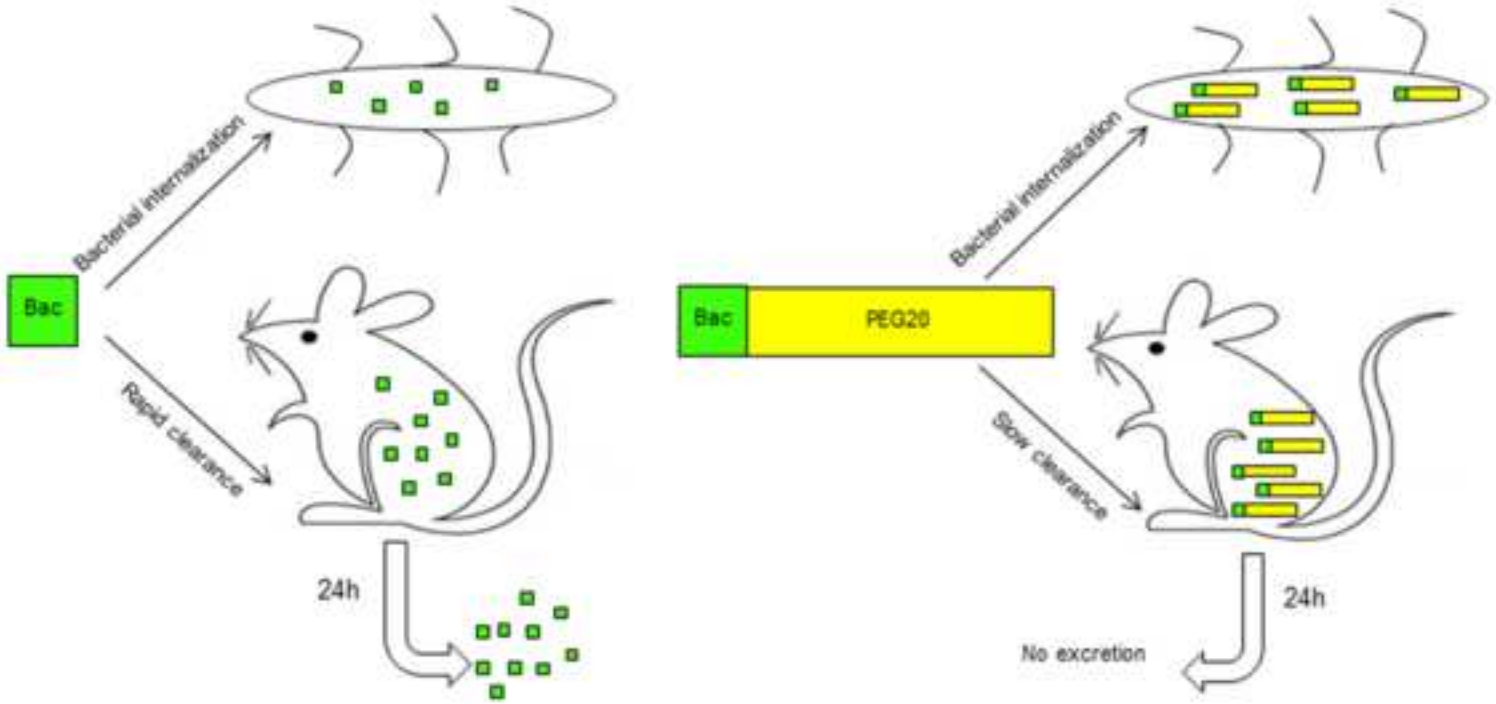
Received Date: 13 January 2015

Revised Date: 11 March 2015

Accepted Date: 13 March 2015

Please cite this article as: M. Benincasa, S. Zahariev, C. Pelillo, A. Milan, R. Gennaro, M. Scocchi, PEGylation of the peptide Bac7(1-35) reduces renal clearance while retaining antibacterial activity and bacterial cell penetration capacity, *European Journal of Medicinal Chemistry* (2015), doi: 10.1016/j.ejmech.2015.03.028.

Graphical Abstract



Highlights

Internally acting antibacterial peptide Bac7(1-35) was PEGylated in two forms

PEGylation does not hamper the capacity of Bac7(1-35) to penetrate into target cells

A releasable conjugate is more active than a stably linked one

PEGylation increases peptide distribution and reduces clearance in a mouse model

PEGylation of the peptide Bac7(1-35) reduces renal clearance while retaining antibacterial activity and bacterial cell penetration capacity

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Abstract

The proline-rich antibacterial peptide Bac7(1-35) protects mice against *Salmonella typhimurium* infection, despite its rapid clearance. To overcome this problem the peptide was linked to a polyethylene glycol (PEG) molecule either via a cleavable ester bond or via a non-hydrolysable amide bond. Both the PEGylated conjugates retained most of the *in vitro* activity against *S. typhimurium.* In addition, the ester bond was cleaved in human serum or plasma, releasing a carboxymethyl derivative of Bac7(1-35) which accounts for a higher activity of this peptide with relative to the other, non-hydrolysable form. Both PEGylated peptides maintained the capacity of the unconjugated form to kill bacteria without permeabilizing the bacterial membranes, by penetrating into cells. They exploited the same transporter as unmodified Bac7(1-35), suggesting it has the capacity to internalize quite sizeable cargo if this is linked to Bac7 fragment. PEGylation allows the peptide to have a wide distribution in mice, and a slow renal clearance, indicating that this strategy would improve the bioavailability of Bac7, and in principle of other antimicrobial peptides. This can be an equally important issue to reducing cytotoxicity for therapeutic use of these antibacterials.

Short title: Characterization of PEGylated antimicrobial peptide Bac7(1-35)

Keywords: antimicrobial peptide; proline-rich; PEGylation; antibacterial activity; cell penetrating; optical imaging

1. Introduction

Antibiotic resistance has become a major public health problem within the lifetime of most people living today. The number of bacterial species and strains no longer susceptible to antibiotics is growing up day by day. To counteract this situation new antimicrobials with novel mechanism of action are greatly needed. Antimicrobial peptides (AMPs) from innate immunity of animals are among the most interesting candidates for this role [1, 2].

Proline-Rich Antimicrobial peptides (PR-AMPs) are a widespread group of antimicrobial peptides (AMPs) present in mammalian neutrophils, as peptides belonging to the cathelicidin family, and in the hemolymph of several invertebrate species [3, 4]. Despite their different origin and evolution, they have common hallmarks, such as *i)* a high content of arginine and proline residues, *ii)* activity

directed mainly against Gram-negative bacteria, *iii)* a remarkably low cytotoxicity towards eukaryotic cells, *iv)* the lack of extensive membrane damaging effects, *v)* and reduced activity of the all-*D* enantiomers, consistent with a mode of action based on recognition of stereospecific molecular targets and/or transporters [4, 5].

Studies on the bovine PR-AMPs Bac7 have revealed important aspects of the mode of action and the antimicrobial activity of this peptide [6, 7]. The 1-35 fragment has *in vitro* antimicrobial activity against Gram-negative *Escherichia coli, Salmonella typhimurium, Klebsiella pneumonia* and *Enterobacter cloacae* at concentrations ranging from 1 to 10 μM, comparable to that of the natural peptide. Furthermore, it remains active against antibiotic-multi-resistant clinical isolates due to a mechanism of action that is different to those of currently used antibiotics [6].

Bac7(1-35) has the capacity to translocate into both bacteria [5, 7] and eukaryotic cells without cell damage [8, 9]. Uptake into *E. coli* and other Gram–negative bacteria is mediated, at least in part, by the SbmA transporter [5, 10], a dimeric inner membrane protein involved in the transport of different types of peptides, in cooperation with the cognate outer membrane protein YaiW [11, 12], and has been shown to target proteins involved with protein production (the chaperone DnaK and ribosomal subunits [3, 4, 13]).

The therapeutic potential of Bac7(1-35) has been assessed in mice infected with *S. typhimurium*, resembling a model of typhoid fever infection [14]. No toxic effects were observed when the peptide was administered to the mice i.p. up to 75 mg/kg and it significantly increased the survival rates and reduced the bacterial load in liver and spleen of infected animals [14]. However, its circulating lifetime was low as it was easily removed by murine kidneys due to its small size and/or was degraded. The peptide reached kidneys and bladder by 1 and 3 hours, respectively, after injection and was totally excreted within 24 hours [14].

Among the several antimicrobial peptides that are currently undergoing clinical trials, most are being tested for topical use [15]. Very few are being considered for systemic therapy because of the many hurdles that must be overcome [16]. First, peptide drugs have short circulating half-lives, due to proteolytic digestion and rapid kidney clearance, and are often antigenic [17, 18]. They also tend to show low therapeutic indices *in vivo* [16, 19] in part due to a reduced activity in the presence of serum and plasma components [20, 21].

The polyethyleneglycol (PEG) moiety is frequently attached to peptide and protein drugs (PEGylation) in order to improve the *in vivo* efficacies of these drugs, by reducing their cytotoxicity and immunogenicity or by prolonging the *in vivo* half-life [17, 22, 23]. It has also been successfully used to modify AMPs [24-27]. For example, a 5 kDa PEG linked to the N-terminus of tachyplesin resulted in a compound with reduced cytotoxicity and sensitivity to serum inhibition [26]. The cytotoxicity of the peptide magainin 2 was also significantly reduced after PEGylation [25]. N-terminally PEGylated AMPs consisting of fragments belonging to human LL-37 and insect cecropin A also showed reduced toxicity towards lung epithelial primary cell cultures [28]. However, to the best of our knowledge, there are no studies relating to its use simply to improve the pharmacokinetics of an internally acting AMP without altering its activity.

Here, we describe the modification of Bac7(1-35) by linking it to a long 20-kDa PEG chain to reduce the clearance rate of the peptide. The different derivatives, one hydrolyzable and the other stable, were tested for their *in vitro* activity against *S. typhimurium* cells, and the effects of human serum and plasma on the kinetics of peptide release and its stability were investigated. Our linkage strategy also allowed preparation of a fluorescent variant of the PEGylated peptide, for use in optical imaging analysis to monitor its biodistribution and permanence in the body of mice.

2. Materials and Methods

2.1 Materials

20 kDa mPEG-OH was purchased from Nektar therapeutics (Huntsville, AL, USA, Lot. 307360) or from Sunbio Chemicals Co., Ltd. (South Korea, Lot. C1OH-020-09135) and was dried before use by azeotrope distillation from toluene. As indicated in the technical specifications the polydispersivity is 452 ± 36 residues.

Activating reagents, HCTU and PyBOP, were from Calbiochem-Novabiochem AG (Switzerland). Anhydrous DMF, NMP and PIP were from Biosolve Ltd (The Netherlands); DIPEA, TFA, TA, TIPS from Fluka Chemie AG (Switzerland). All other reagents and solvents were reagent grade and were purchased from Fluka, Sigma-Aldrich (USA), Biosolve Ltd (Netherlands), Alexis (USA) and Advanced ChemTech (USA). Fmoc-protected amino acids were obtained from Novabiochem (Switzerland), Inbios (Italy), Iris Biotech GmbH (Germany) or Bachem AG (Switzerland).

2.2 Purification methods

Analytical RP-HPLC was carried out on a Gilson HPLC System. Samples were eluted with a linear gradient from A = 0.1% TFA in water to B = 0.1% TFA in MeCN.

Preparative RP-HPLC was performed on a Waters RCM with PrepPak Cartridge Delta-Pak 300 15RP18 (100 x 25 mm I.D.) at a flow rate of 7 ml/min or on a Waters Prep LC universal base module with a PrepPak Cartridge Delta-Pak 300 15RP18 (100 x 40 mm I.D) column at a flow rate of 18 ml/min. Samples were injected manually and eluted from the column with a gradient slope from 0.6% to 0.8% B/min. Pure fractions, according to analytical RP-HPLC or ESI-MS analysis, were pooled and freeze-dried. TFA was removed after lyophilizing three times with 10 mM HCl solution.

IEX-HPLC was carried out on AKTA Basic 10 (Amersham Pharmacia, Sweden) using one or two column in series, HiTrap™ SP HP (5 mL, Pharmacia) equilibrated in 20 mM Na phosphate buffer pH 6.5. Elution was carried out with a NaCl gradient.

The eluent from analytical RP-HPLC or preparative RP-HPLC was collected as fractions and aliquots analyzed on Applied Biosystems Sciex API 150EX or ion trap mass spectrometer (Amazon SL, Bruker) for the correct peptide conjugate.

2.3 Peptide synthesis

All the peptides used in this study are shown in Scheme 1, Scheme 2 and Table 1. The synthesis of Bac7(1-35) [Bac, in Table 1] and its further conjugation to BODIPY® FL N-(2-aminoethyl) maleimide fluorophore [Bac-BY in Table 1] are also described in the Supplementary data. Small portions of the crude peptide resins were cleaved, deprotected with a modification of the procedure as described in the Supplementary data and purified by RP-HPLC to furnish Bac7(1-35) (**1**) (Scheme 1 and Table 1). The C-terminally extended Bac-Cys(H)-OH (**2a**) could be obtained in two ways, directly synthesizing it in the solid state from Cys-substituted chlorotrityl resin**,** or in solution from **1a** via intermediate **3** (Scheme 1). In the latter case, N-terminally and side-chain protected crude peptide Boc-Bac(1-35)-OH (**1a**) was cleaved from the resin with HFIP, and Bac-arylthioester (**3**) was prepared according to the general method of Beyermann [29, 30]. The free thiol group of **2a** was than reacted with excess bromoacetic acid in the presence of base to form the S-carboxymethylated Bac-derivative (**2**) (Scheme 1 and Table 1).

2.4 PEGylation of Bac7(1-35)

Two sets of C-terminally PEGylated Bac7(1-35) were synthesized: C-terminal esters – left column on Scheme 2, and C-terminal amides – right column on Scheme 2. mPEG-$OCOCH_2Br$ (**4**) was prepared by bromoacetylation of commercial HO-PEG-OMe (mPEG-OH) with $BrCH_2COBr$/DIEA, according

to published procedures [31, 32]. NH_2-PEG-OMe (mPEG-NH_2) (**5**) was prepared in 3 steps from mPEG-OH. Bromoacetylation of **5** with the activated ester of bromoacetic acid $BrCH_2COOSu$ (Scheme 2) produced amide **6**. Coupling of **5** with N, S-protected cysteine led to H-Cys(H)-NH-PEG-OMe (**7**). Thioether ligation of H-Bac7(1-35)-Cys(*H*)-OH (**2a** - Scheme 1) with excess of bromoacetyl-PEG-ester (**4**) or bromoacetyl-PEG-amide (**6**) produce the expected C-terminal PEGylated Bac7(1-35) ester [**8**, Bac_E-PEG] or amide [**9**, Bac_A-PEG, see Table 1], with good yield.
H-Cys(H)-OC(O)-OPEG-OMe (**10**) was prepared with excellent purity and yield by activation of an excess of Boc-Cys(Trt)-OH with Boc_2O/pyridine [33], followed by coupling to mPEG-OH, deprotection and purification of the ester. Similarly, mPEG-NH_2 was coupled to Boc-Cys(Trt)-OH, activated with HCTU/DIEA to obtain H-Cys(H)-NH-PEG-OMe (**7**). An excess of H-Cys-OPEG-OMe (**10**) or H-Cys-NH-PEG-OMe (**7**) were then reacted with Bac7(1-35)-SPhNHAc (**3**) under conditions of native chemical ligation [34] and respectively formed the C-terminal PEGylated ester Bac7(1-35)-Cys-OPEG-OMe (**11**) and PEGylated amide Bac7(1-35)-Cys-NHPEG-OMe (**12**). Michael addition of Alexa Fluor 680 maleimide or BODIPY® FL N-(2-aminoethyl) maleimide to the free thiol group in **11** or **12** formed the corresponding succinimide adducts (**13**, **14**) and (**15**, **16**), according to modifications of a published procedure [14] or further explained in the Supplementary data. The free thiol in Bac7(1-35)-Cys(H)-OPEG-OMe (**11**) was reacted with an excess of bromoacetic acid in the presence of base (as already described for **2 →2a)** and formed S-carboxymethylated Bac7(1-35)-Cmc-OPEG-OMe (**17**).
A detailed description of all the reactions reported in Scheme 2 is available in the Supplementary data. The concentration of peptides was determined using three methods*: i)* by accurate weighing after lyophilisation; *ii)* using the method of Waddell [35]; *iii*) by analytical RP-HPLC using an internal standard. PEGylated peptides were also checked by acetic acid-urea PAGE for any eventual release of free peptide (see Supplementary data).

2.5 Serum and plasma preparation

Murine serum and plasma were drawn from a pool of mice. Human serum and plasma were taken from a pool of healthy donors. Human and murine plasma was obtained with 2% (v/v) Na-citrate as anticoagulant. Fluid samples were centrifuged at 13000 g for 5 min and stored at -20°C until use.

2.6 Bacterial strains and growth conditions

The strains used in this study were *Salmonella enterica* serovar Typhimurium ATCC 14028 (*S. typhimurium*), *E. coli* BW25113 and *E. coli* BW25113Δ*sbmA*, a deletion mutant for the *sbmA* gene. The inoculum was incubated overnight at 37°C with shaking. For the assays the overnight bacterial cultures were diluted 1:30 in fresh Mueller-Hinton Broth (MHB) and incubated at 37°C with shaking for approximately 2h.

2.7 Bac7 stability and its release from PEGylated forms in serum and plasma

Bac and Bac_E-PEG (120 µg) were incubated in 200 µl of 25% (v/v) murine or human serum/plasma in PBS at 37°C. At different times, aliquots were withdrawn, diluted 1:5 in sample buffer (12% SDS, 6% dithiothreitol, 40% glycerol, 0.05% bromophenol blue, 150 mM Tris-HCl, pH 7), incubated for 15 min at 60°C and analyzed on a 16% Tricine/SDS gel. Proteins were then blotted onto nitrocellulose membrane and incubated overnight with shaking in 40 mM Tris-HCl, pH 7.5, 5% non-fat milk, 0.1% Tween 20, 200 mM NaCl (Blocking Solution, BS) at 4°C. Samples were incubated at room temperature for 2h with rabbit anti-Bac7(1-35) IgG diluted 1:1000 in BS, followed by a HRP-conjugated anti-rabbit IgG (1h incubation). The detection of the chemiluminescence was performed using the commercially available kit ECL Plus Western Blotting Detection Reagent (Pierce).

2.8 *In vivo* biodistribution by time-domain optical imaging

In vivo biodistribution studies have been performed as previously described [14]. Two mice were injected intraperitoneally with 200 µl of Bac_E-PEG-Alexa680 in PBS corresponding to 6.9 nmol AlexaFluor® 680, one monitored in the abdominal region and the other in the renal region for 24 hours. A blank image was acquired before treatment of each animal and this was subtracted from the images of the treated animal. The experiment was repeated twice.

Two-dimensional scanning regions of interest (ROI) were selected and the laser power, integration time and scan step were optimized according to the signal emitted. The data were recorded as temporal point-spread functions, and the images were reconstructed as fluorescence intensity and lifetime. All the experimental procedures were performed according to the guidelines of the European (86/609/ EEC) and the Italian (D.L.116/92 and subsequent addenda) laws and approved by the Italian Ministry of University and Research as well as by the Animal Experimentation Committee of the University Animal House.

2.9 Minimum inhibitory concentration

Broth microdilution susceptibility assay was performed as previously described [6]. Briefly, two-fold serial dilutions of peptides were prepared in 96-well microplates in MHB with the addition of human or murine serum or plasma to reach the final concentration of 25% after bacteria addition, and 100% MHB as control. To study the effect of the preincubation on Bac_E-PEG activity, bacteria were added to each well containing peptide in 25% serum or plasma after 24h at 37°C. The MIC (Minimum Inhibitory Concentration) was taken as the lowest concentration of antimicrobial peptide resulting in the complete inhibition of visible growth after 24h of incubation at 37°C.

2.10 Growth kinetics assay

Bacterial growth inhibition tests were performed using mid-log phase bacterial cultures diluted in MHB to 1 x 10^6 CFU/ml, in presence or absence of different concentrations of peptides. Bacterial growth was followed at 37°C with intermittent shaking for 4h by measuring every 10 min the absorbance at 620 nm with a microplate reader (Tecan Trading AG, Switzerland). To evaluate if the culture medium causes the release of free peptide, Bac_E-PEG was incubated in MHB at 37°C for different times up to 4h. At the end of incubation, 10 µl of each sample were loaded on a 16% Tricine/SDS gel. As control, Bac in water or MHB was loaded.

The gel was stained by Coomassie blue and iodine staining for protein bands and PEG species, respectively. For iodine staining, the gel was stained with a 5% Barium chloride solution in milliQ water (5% w/v $BaCl_2$ in 100 ml milliQ water, ~240 mM HCl) for 15 min, washed in distilled water for 30 min, floated in 0.1 M iodine solution (2% w/v KI, 1.27% w/v I_2 in milliQ water) for 15 min and washed in distilled water again [36].

2.11 Flow cytometric analysis

Integrity of the cell membrane in *Salmonella* cells was assessed by measuring the PI uptake by flow cytometry, as previously described [7]. For the uptake evaluation, we used a cytofluorimetric method described in Benincasa et al. [37]. Data analysis was performed with the FCS Express3 software (De Novo Software, Los Angeles, CA).

2.12 Confocal scanning laser microscopy (CSLM)

CSLM analysis were performed by using a Nikon C1-SI confocal microscope. An oil immersion objective lens was used. The optimum photomultiplier setting was determined in preliminary

experiments performed *ad hoc*, and then the same setting was used for all samples. *Salmonella typhimurium* ATCC 14028 cells treated for 2h with 0.25 µM Bac-BY, 1 µM Bac_E-PEG-BY and 4 µM Bac_A-PEG-BY, or for 10 min with 0.25 µM Polymyxin B, were prepared following the same protocol used for the flow cytometric uptake assay without any fixation. Ten µl of each bacterial suspension were placed between two cover-glasses to obtain an unmovable monolayer of cells. The image stacks collected by CSLM were analyzed with the EZ-C1 Free Viewer (Nikon Corporation) and the Image J 1.40g (Wayne Resband, National Institutes of Health, USA) software.

3. Results

3.1 Preparation of PEGylated forms of Bac7(1-35)

Bac7(1-35) (hereafter named Bac) was conjugated to 20 kDa poly(ethylene glycol) (PEG) using two different strategies (Scheme 1). In one case the peptide was linked to the polymer through an amide bond (Bac_A-PEG) (compound **9** in the Scheme), and in the other via an ester bond (Bac_E-PEG) (compound **8**) that in principle allows the peptide to be released by blood esterases producing a carboxymethyl derivative of Bac7(1-35) (Bac_{CMC}) (compound **2**). Corresponding fluorescent derivatives of Bac_A-PEG and Bac_E-PEG have also been prepared to allow detection of the PEGylated peptides in mice or when internalized into bacterial cells (Scheme 1). All the compounds and their names are listed in Table 1.

3.2 Antimicrobial activity of PEGylated Bac

Both amide- and ester-linked forms of the PEGylated peptide showed a good antibacterial activity against *S. typhimurium* (Table 2). In particular, when assayed in Mueller-Hinton broth (MHB), Bac_E-PEG showed MIC values of 4-8 µM and Bac_A-PEG of 8-16 µM, respectively 4- and 8-fold less active than that of the unconjugated peptide. The anti-salmonellae activity was evaluated also by growth inhibition assays. At 2 µM, both Bac_E-PEG and Bac_A-PEG reduced bacterial growth by 75% after 4h (Figure 1A and 1B) while unmodified Bac showed a similar growth inhibition at 0.5 µM (Figure 1C), confirming a difference in activity in line with the MIC. The rapid antibacterial effect of the modified peptides, which initiated without delay, suggested that the PEGylated forms *per se* were at least in part responsible for the antibacterial activity in spite of the steric hindrance due to the presence of the PEG group. To further support this hypothesis, Bac_E-PEG was incubated in MHB for 4h, and the products analyzed by SDS-PAGE. No free peptide was detected (data not shown), confirming that short-term antimicrobial activity was due to the conjugated forms.

3.3 Release of Bac in human body fluids and stability of the native peptide

The kinetics of release of Bac from Bac_E-PEG in the presence of human serum and plasma is shown in Figure 2. A peptide with the expected size was detected using an anti-Bac7 antibody after 8h of incubation with either plasma or serum and release was nearly complete within 72h (Figure 2A and 2B). On the other hand Bac_A-PEG did not release free Bac within the same time period (data not shown). To verify if Bac is stable and not degraded in the body fluids after being released from PEG, a Western blot analysis of unmodified Bac incubated with human serum or plasma was carried out (Figure 2C). Results indicated that the Bac was not degraded in both body fluids up to at least 72h incubation.

3.4 Effects of serum and plasma on the antibacterial activity of PEGylated Bac

Bacterial susceptibility testing was performed in the presence of human serum or plasma to evaluate their effects on the antibacterial activity of all the peptides (Table 2). Different results were obtained depending on the type of body fluid. The addition of 25% human serum to the medium did not modify the anti-salmonellae activity of Bac_A-PEG (MIC =8-16 μM) or of unconjugated Bac (MIC =0.5-1 μM). Conversely, it resulted in an appreciable increase in the activity of Bac_E-PEG (MIC =2-4 μM) likely due to the partial release of the peptide (Table 2). In addition, when the assay was carried out using an aliquot of Bac_E-PEG preincubated for 24h with 25% human serum, the activity showed a further 2 fold increase, reaching a MIC of 1 μM. This value is similar to that measured for Bac_{CMC}, the carboxymethyl peptide that would be obtained by ester bond hydrolysis of Bac_E-PEG (0.5-1 μM) (Table 2). Overall these results show that in human serum an active peptide is released from the polymer explaining the somewhat higher activity of Bac_E-PEG than Bac_A-PEG.

Interestingly, in the presence of plasma (25%) the activity of all the peptides against *S. typhimurium* was increased 4-8 fold (Table 2). It is significant that the increase in activity occurred to the same extent for both PEGylated and non PEGylated peptides and we are currently unable to propose a reason for this effect, so that further investigations are required to clarify it.

As *in vivo* experiments were carried out in a mouse model, the effects of murine serum and plasma were also tested. In contrast to what was observed in the presence of human body fluids, the antibacterial activity of unconjugated Bac and of both the pegylated forms was scarce in the presence of murine serum or plasma (Table 2).

3.5 Internalization into *S. typhimurium* cells

In order to evaluate whether the PEGylated derivatives retained the same mechanism of action as the native peptide, we monitored the cellular uptake using the fluorescent derivatives Bac_A-PEG-BY and Bac_E-PEG-BY. First, however, we performed a permeabilization assay to exclude that the PEGylated forms could damage the bacterial membranes. The percentage of propidium iodide (PI)-positive bacterial cells treated with the PEGylated peptides at their MIC, or slightly above MIC values, did not increase when compared to the free peptide (< 2% of PI positive cells, data not shown), suggesting membrane permeabilization does not occur.

When Bac_A-PEG-BY and Bac_E-PEG-BY were incubated with *S. typhimurium* at 1 μM concentrations, the detected fluorescent signal indicated that they had been internalized into the cells, albeit at a lower level than unPEGylated Bac-BY (Figure 3A). Furthermore, Bac_E-PEG-BY exhibited a higher level of internalization than Bac_A-PEG-BY. Uptake assays were also carried out using Bac-BY, Bac_E-PEG-BY and Bac_A-PEG-BY at a concentration corresponding to ¼ the MIC values. Under this condition a similar amount of each peptide was found inside the bacterial cells and with an analogous time-dependence (Figure 3B).

The localization of the peptides in *S. typhimurium* cells was examined by confocal scanning laser microscopy (CSLM) (Figure 3C). A homogeneous distribution of Bac and of both the PEGylated derivatives in the cell cytoplasm was observed. In contrast, the membrane-acting antibiotic polymyxin B was visible mainly on the cellular surfaces, as expected.

To evaluate whether the internalization of the PEGylated compounds involves the same inner membrane protein transporter SbmA responsible for Bac translocation, we repeated the uptake assays using the *E. coli* BW25113Δ*sbmA* mutant strain with deleted *sbmA* gene. The susceptibility of this mutant strain was significantly decreased to both the PEGylated forms with respect to the wild-type strain (Figure 4A and 4B), and corresponded to a parallel reduction in the level of uptake of both Bac_A-PEG-BY and Bac_E-PEG-BY by the mutated strain (Figure 4C). This confirms the involvement of the SbmA protein also in the internalisation of the 20kDa-PEGylated form of the peptide.

3.6 *In vivo* biodistribution

In vivo biodistribution analyses and evaluation of the permanence of the peptide in mice were performed using another fluorescent derivative of Bac_E-PEG. For this purpose, the maleimide group of Alexa Fluor680© was linked to the free thiol group of the cysteine residue located between the peptide and the PEG tail to give Bac_E-PEG-Alexa. Biodistribution analyses were performed on healthy mice by monitoring the availability of the labeled, PEGylated peptide up to 24h after its intraperitoneal administration and performing *ex vivo* analysis at 24h. Results showed that the 24 kDa PEGylated form of Bac had a much wider distribution than the unPEGylated peptide and was detected in different organs (kidney, liver and peritoneal cavity) (Figure 5A and 5B). Furthermore, it was still present at 24h post-injection, suggesting a slower renal clearance compared to the unmodified peptide [14] and clearly due to the increased molecular mass. *Ex vivo* analysis showed a significant concentration of the PEGylated Bac in liver even at 24h after injection, as well as in kidney (Figure 5C), indicating a prolonged *in vivo* half-time for the peptide after its conjugation to PEG.

4. Discussion

AMPs are a promising class of antimicrobial biomolecules that originated millions of years ago as part of innate immunity [38]. As pharmaceuticals, however, AMPs have several disadvantages including toxic side effects observed at higher doses (especially for lytic AMPs targeting the membranes), and short circulation time in blood, due to their degradation by blood-borne proteases and/or a rapid clearance through the urinary tract. We have previously shown that Bac is not toxic for eukaryotic cells [8] and well tolerated in a mouse toxicity model [14] but rapidly cleared by the kidney when injected intraperitoneally into mice.

In this work we have demonstrated that PEGylated derivatives of Bac [i.e. Bac7(1-35)] retain an appreciable antimicrobial activity and have substantially the same mode of action as the unmodified peptide, while exhibiting a lower clearance rate. This chemical modification could therefore considerably enhance its *in vivo* efficacy. It is a further example of the usefulness of covalent attachment to poly(ethyleneglycol) (PEG) chains of potential therapeutic agents as a leading approach for overcoming limits, such as physical and chemical instability, immunogenicity and rapid body clearance [39].

In selecting the best strategy for linking PEG to Bac we considered the particular mode of action of this peptide. Bac has to penetrate into bacterial cells to kill them [7] and the modification of amino acids at its N-terminus markedly reduces its activity [40]. For this reason we preferred to link the PEG tail to the C-terminus. Furthermore, we decided to use an ester bond in order to obtain a conjugate which was susceptible to blood esterases. As expected, while stable in MH medium, the unPEGylated peptide was progressively released by Bac_E-PEG on a time-scale of several hours in the presence of human serum or plasma. The released Bac peptide, being very rich in proline, then itself remains stable in serum for at least 72 hours, and showed MIC values comparable to those of the native peptide (Table 2).

The mechanism of action of unmodified Bac involves transport by the SbmA membrane protein, so it was quite encouraging that the PEGylated conjugates should continue to have an appreciable anti-salmonella activity, and somewhat surprising for the Bac_A-PEG conjugate which is linked via a stable amide bond. This suggested that even in the ~24 kDa PEGylated form they could exert some antimicrobial activity. In the case of Bac_E-PEG it is likely that the this activity was due to both the PEGylated form and free released peptide. The difference in anti-salmonella activity between Bac_E-

PEG and Bac_A-PEG is effectively only observed in presence of serum and plasma, but not medium, and thus likely due to the partial release of the peptide. The contribution to the antibacterial activity of the intact form is suggested by that of Bac_A-PEG, showing an 8-fold reduction of activity against *S. typhimurium* relative to Bac (Table 2).

Both PEGylated peptides exhibited loss of activity when assayed in murine plasma and serum with respect to human fluids. This result is in agreement with previous observations showing significantly reduced *in vitro* and *in vivo* antibacterial activity in presence of serum and plasma of mice, also due to peptide degradation [14]. The different influence of human and murine biological fluids on the antibacterial activity of Bac could find an explanation in the different composition of blood enzymes and/or proteins in the two species. A number of proteases and carboxy esterases have in fact been identified in murine blood that seem to be absent in human blood [41].

Despite an at least five-fold increased in size, both Bac_E-PEG and Bac_A-PEG conserve a mechanism of action mediated by their uptake into target cells. The detection of cytoplasmic fluorescence in salmonellae cells, particularly after incubation with the esterase-stable Bac_A-PEG-BY, indicates that the PEGylated forms can still translocate across the bacterial membranes. Internalization of the PEGylated peptides was understandably lower than that of unconjugated Bac, in agreement with a reduced, but not abrogated, activity. The fluorophore in the labelled derivatives was linked to the peptide and not to the PEG molecule (Scheme 1), so for Bac_E-PEG-BY it could not be excluded that the fluorescence inside the bacterial cells was due only to peptide released from PEG on the surface of the target cells and subsequently internalized, but this is quite unlikely for a stable derivative such as Bac_A-PEG-BY.

We have conclusively shown that the inner membrane protein SbmA, a secondary transporter with low-specificity for substrates previously shown to translocate Bac, is also involved in the uptake of Bac_E-PEG and Bac_A-PEG [5, 10, 12]. This protein is required for the uptake of structurally quite different molecules including proline-rich peptides, bleomycin, bacterial mycrocins and peptide nucleic acids [42]. Our results suggest that SbmA's transport capacity extends to the uptake of even quite large molecules (≈24 kDa), albeit with a somewhat reduced efficiency, making it a potentially useful for transporting cargo into bacteria that express it.

The reduced excretion of the peptide is one of the most important effects of Bac7 PEGylation. A fluorescent derivative of Bac_E-PEG was still detectable at 24h post-injection while the unmodified peptide disappeared within 3h after injection [14]. Despite the molecular weight cutoff for glomerular filtration is thought to be 30–50 kDa [43], the increased mass of Bac7(1-35) to ≈ 24 kDa in the PEGylated form strongly delays the excretion of the peptide. The slower renal clearance increases peptide biodistribution compared with the unPEGylated form, which included the liver and peritoneal cavity other than kidney in agreement with previous observation. This result supports the possibility of increasing the therapeutic potential of Bac7.

In conclusion we have demonstrated that the addition of a 20kDa tail of PEG to Bac is a workable strategy to enhance the *in vivo* properties of the peptide, improving its pharmacokinetics, while only marginally affecting its antibacterial activity and not substantially modifying its mode of action. PEG has already been approved for use in the formulation of many drugs and cosmetics [44] and the number of PEGylated products on the market is continuously increasing [39]. For this reason this kind of modification is promising for the future therapeutic application of anti-infective agents based on Bac7, and may be applied also to a vast repertoire of other AMPs with relative ease.

ACKNOWLEDGMENTS

We thank Dr. S. Drioli and prof. G.M. Bonora for help in the initial stages of the project, and Dr. N. Antcheva for help with the conjugation of BODIPY. We thank prof. A. Tossi for reading the manuscript and for useful advice. The authors declare no competing financial interest**.**

AUTHOR CONTRIBUTIONS

Monica Benincasa performed most of the experiments on the antibacterial activities of the peptides and wrote the initial draft of the manuscript; Sotir Zahariev designed, synthesized and purified all the compounds some of which with the help of Chiara Pelillo; Chiara Pelillo and Annalisa Milan performed part of the *in vitro* assays and data analysis; Renato Gennaro contributed to conceptual advices and to the writing of the manuscript; Marco Scocchi supervised the experiments and edited the manuscript with the contribution of all the other authors. All authors have given approval to the final version of the manuscript.

FUNDING

This study was supported by the Regione Friuli Venezia Giulia grant under the LR 26/2005, art. 23, for the R3A2 network.

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Figure captions

Figure 1 Growth kinetics of *Salmonella typhimurium* in the presence of BacE-PEG, BacA-PEG and Bac. Bacterial growth in MHB was monitored at 620 nm. The dotted line indicates the growth rate of the untreated bacterial cells, the continuous lines indicate the growth rate of bacterial culture in the presence of the indicated concentrations of BacE-PEG (**A**), BacA-PEG (**B**) and Bac (**C**). Growth kinetics are representative of three independent experiments with identical results.

Figure 2 Evaluation of Bac release from PEG conjugates in the presence of human biological fluids and Bac7 stability in human blood fractions. Western blot analyses of BacE-PEG in human plasma (**A**) and human serum (**B**). BacE-PEG was incubated in 25% human plasma or serum up to 72 h at 37°C. Samples were then loaded on the gel to allow peptide detection with a specific anti-peptide antibody. Western blot analysis of Bac incubated for different times at 37°C in 25% human serum or plasma (**C**). Lane 1: 0.5 µg Bac; lanes 2-6: Bac after incubation with human or murine serum and plasma for the indicated times; lane 7: serum or plasma alone.

Figure 3 Uptake studies of PEGylated forms of Bac. (**A**) Fluorescence of *S. typhimurium* cells exposed for 10 min to 1µM Bac-BY, BacA-PEG-BY and BacE-PEG-BY, and analyzed by flow cytometry with (grey histograms) or without (empty histograms) incubation with Trypan Blue (TB) quencher for 10 min at 37°C. (**B**) Fluorescence of *S. typhimurium* cells exposed for the indicated times to 0.25µM Bac-BY, 4 µM BacA-PEG-BY and 1 µM BacE-PEG-BY (¼ the respective MIC values), and analyzed by flow cytometry after incubation with Trypan Blue (TB) quencher for 10 min at 37°C. For both plots, the Mean Fluorescence Intensity (MFI) value, with SD as error bars, is reported in the y axis. (**C**) Confocal microscopy images of *S. typhimurium* treated with Bac-BY, Polymyxin B, BacE-PEG-BY and BacA-PEG-BY. All images are representative sections from the middle of the bacterial cell. Many fields were examined and, for each experiment, over 95% of the cells displayed the pattern of the respective representative cell shown here.

Figure 4 The involvement of the membrane protein SbmA in the activity/uptake of the PEGylated peptides in *E. coli*. Growth kinetics of *E. coli* BW25113 and *E. coli* BW25113ΔsbmA in the presence of BacE-PEG-BY (**A**) and BacA-PEG-BY (**B**) at their MIC value. In the figure the continuous line indicates the wild-type *E. coli* BW25113 strain, and the dotted line indicates the mutant *E. coli* BW25113ΔsbmA. Growth kinetics are representative of three independent experiments with identical results. (**C**) Uptake of 4 µM BacA-PEG-BY and 2 µM BacE-PEG-BY into *E. coli* BW25113 and BW25113ΔsbmA strains. The Mean Fluorescence Intensity (MFI) value, with SD as error bars, is reported in the y axis.

Figure 5 Biodistribution of BacE-PEG-Alexa in healthy mice after i.p. injection. The animals were placed in a prone position (**A**) and supine position (**B**); fluorescence emission in the regions of interest encompassing respectively the kidneys, the thorax and abdomen, were acquired at the indicated times. Panel B on the right shows the fluorescence emission of the mouse in supine position at 24h post injection and normalized with respect to the prescan acquired before peptide injection. (**C**) Ex vivo images: post-mortem images of mice at 24h after i.p. injection of PEGylated BacE-PEG-Alexa. Imaging of the mice was performed immediately after sacrifice, after removal of the

abdominal wall, and normalized. A specific signal was detected in kidney, liver and the peritoneal cavity (panel on the left); ex vivo images of organs at 24h after i.p. injection of PEGylated BacE-PEG-Alexa. Imaging of the organs was performed immediately after sacrifice and normalized. The images are representative of one of three mice that showed comparable results.

Table 1 List of peptides used in this study

Compound	Name used	Number*
Bac7(1-35)	Bac	1
Bac7(1-35)-Cys(BODIPY-maleimide)	Bac-BY	-
Bac7(1-35)-Cys(H)-NH-PEG-OMe	Bac_A-PEG	9
Bac7(1-35)-Cys(BODIPY-maleimide)-NH-PEG-OMe	Bac_A-PEG-BY	15
Bac7(1-35)-Cys(-CH_2CO-OPEG-OMe)-OMe	Bac_E-PEG	8
Bac7(1-35)-Cys(-CH_2COOH)	Bac_{CMC}	2
Bac7(1-35)-Cys(BODIPY-maleimide-)-O-CH_2CO-OPEG-OMe	Bac_E-PEG-BY	13
Bac7(1-35)-Cys(ALEXAFluorC2-maleimide-)-O-CH_2CO-OPEG-OMe	Bac_E-PEG-Alexa	14

*Numbers indicate the corresponding compounds described in Scheme 1.

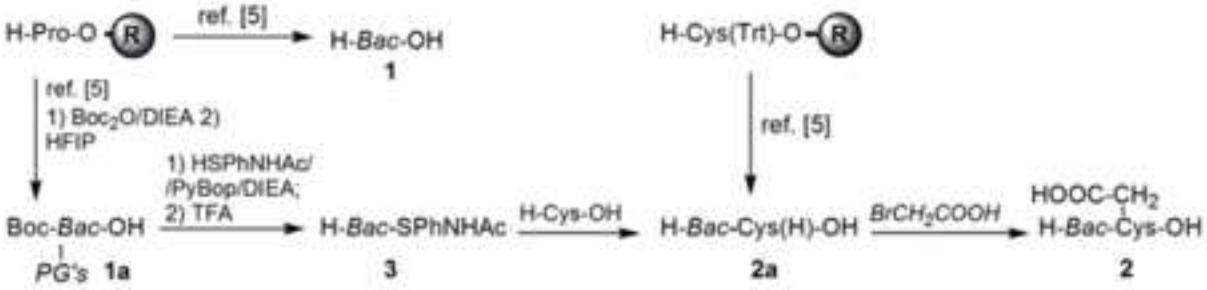
Table 2 Microdilution susceptibility assay of Bac7(1-35) and its PEGylated forms against *Salmonella typhimurium*

Peptide	MIC (μM)				
	Mueller-Hinton	Human		Murine	
		Serum	Plasma	Serum	Plasma
Bac	1-2	0.5-1	0.06-0.12	32	≥32
Bac_A-PEG	8-16	8-16	2	>32	>32
Bac_E-PEG	4-8	2-4	1-2	64	16
Bac_E-PEG (24h)*	2	1	0.25	nd	nd
Bac_{CMC}	0.5-1	0.5	0.03-0.06	nd	nd

MIC values are representative of three independent experiments with comparable results.

* Peptide was incubated for 24h in the indicated media before the addition of bacteria (see Experimental Section).

Scheme 1. Preparation of peptide precursors

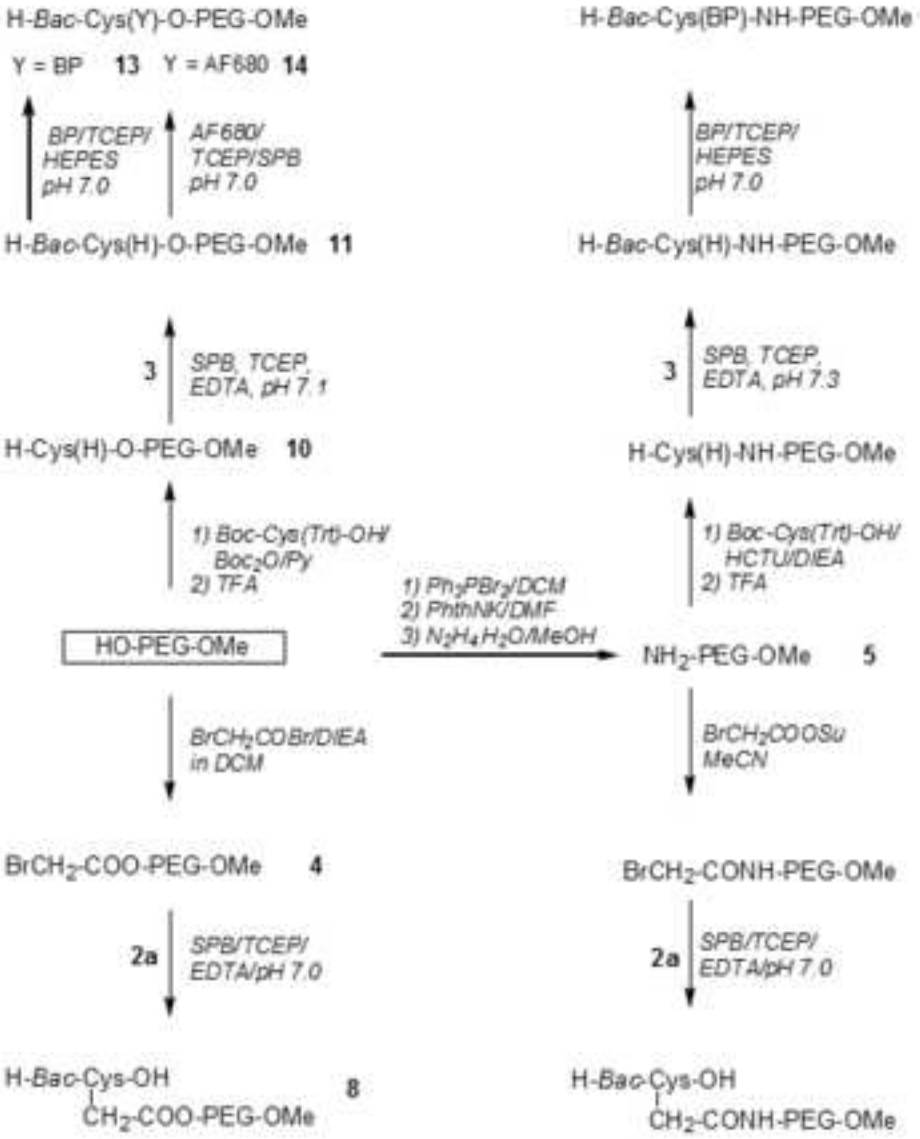


Bac: H-R'RIRPRPPRLPRPRPRPLPFPRPGPRPIPRPLPFP[35]-OH;

R: trityl type resin; **PG's**: acid cleavable protecting groups

Scheme 2

Scheme 2. Amide or ester bond C-terminal PEGylation of Bac7(1-35)



AF680: Alexa Fluor 680 Maleimide (Cys-succinimide adduct); **BP**: BODIPY® FL N-(2aminoethyl)maleimide (Cys-succinimide adduct); **HO-PEG-OMe** = HO-CH_2CH_2-$(OCH_2CH_2)n_{av}$-OCH_2CH_2OMe, where n_{av} = 452≈ 36

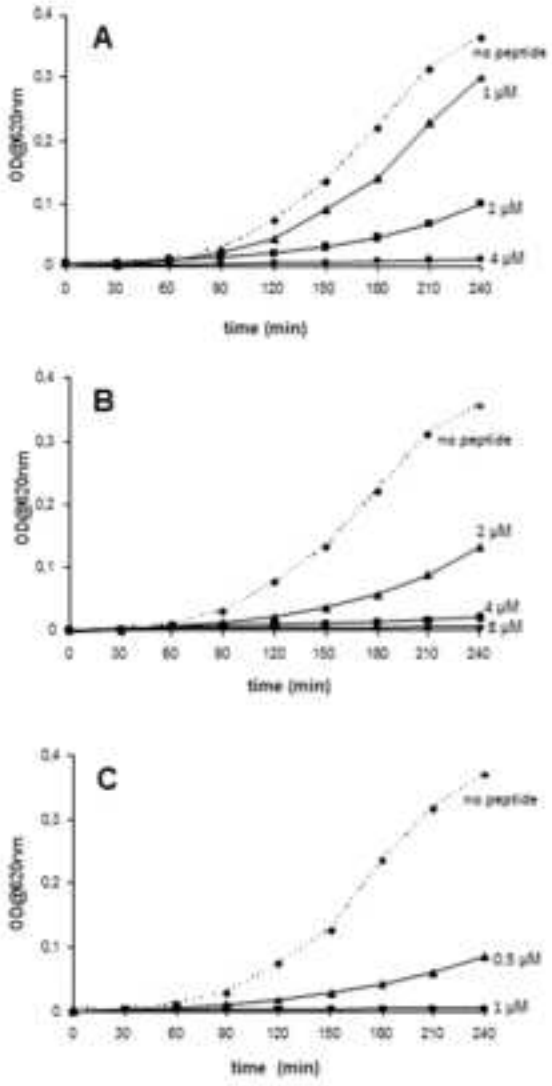


Figure 1

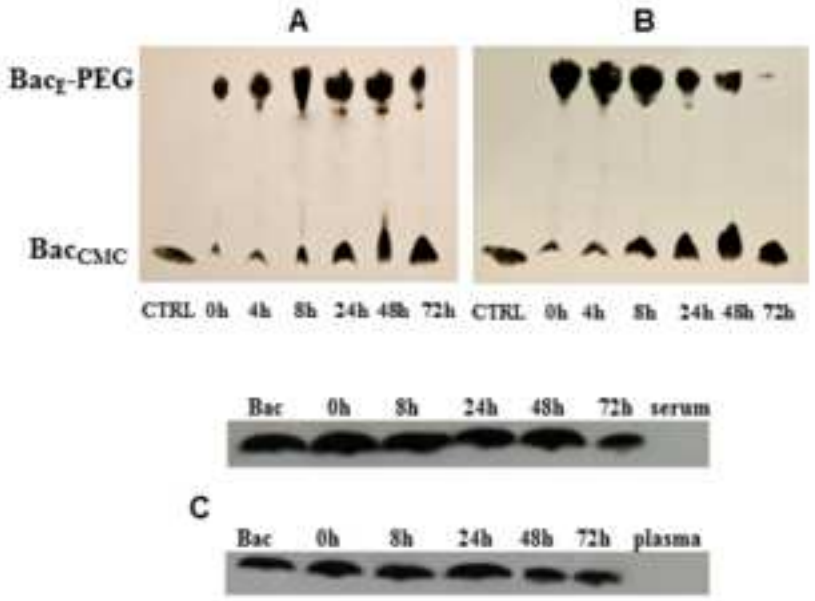


Figure 2

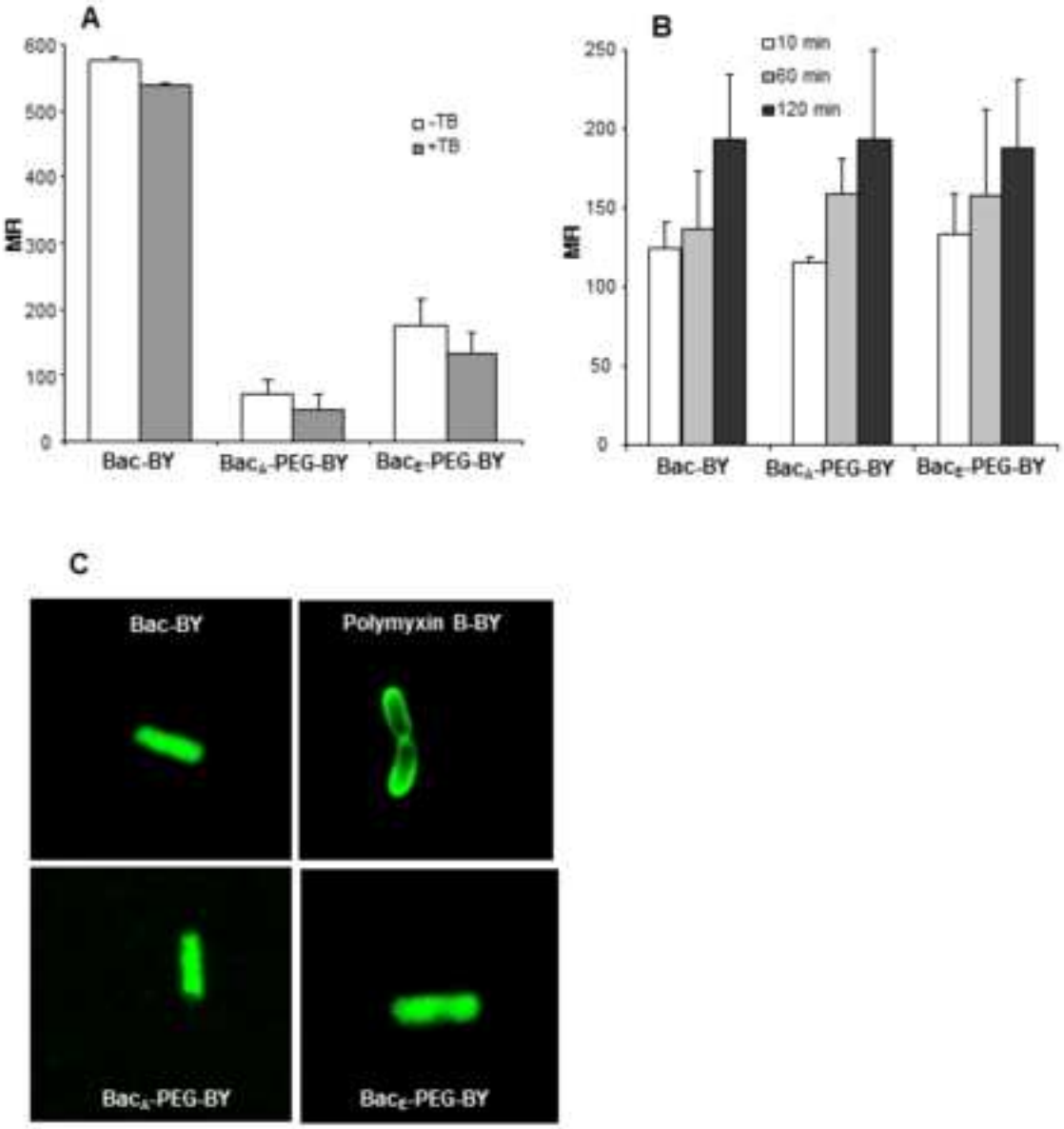


Figure 3

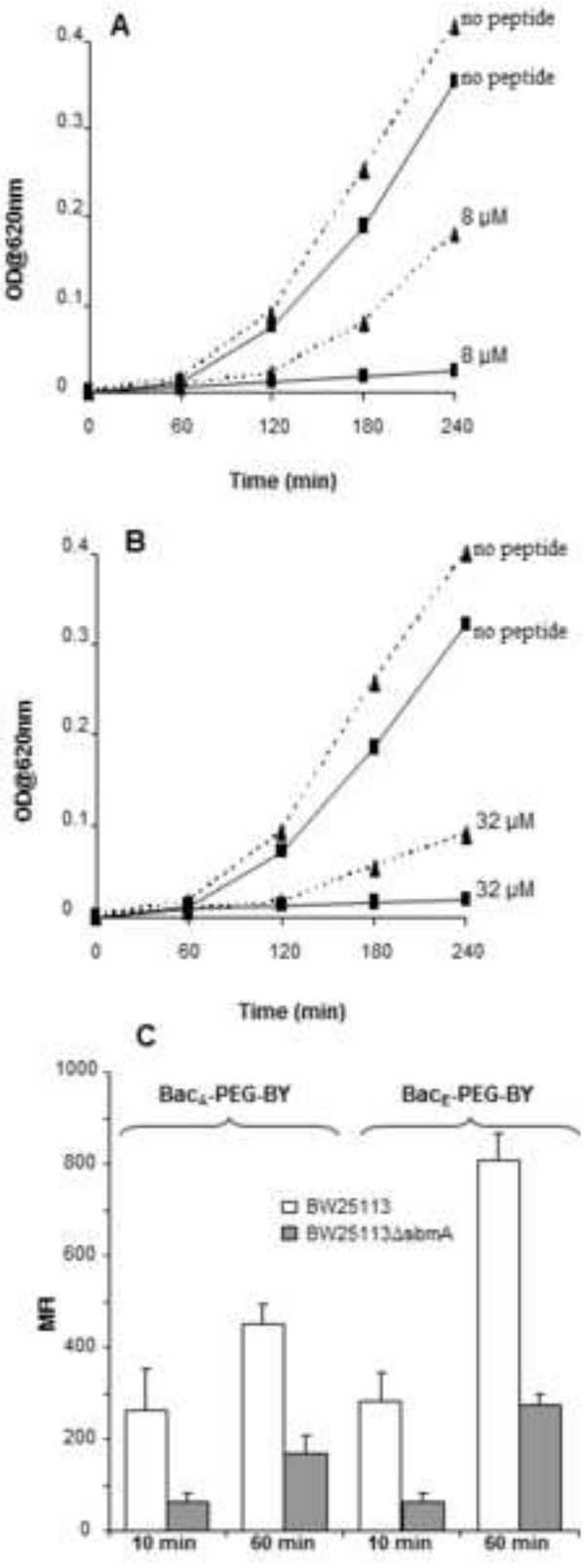


Figure 4

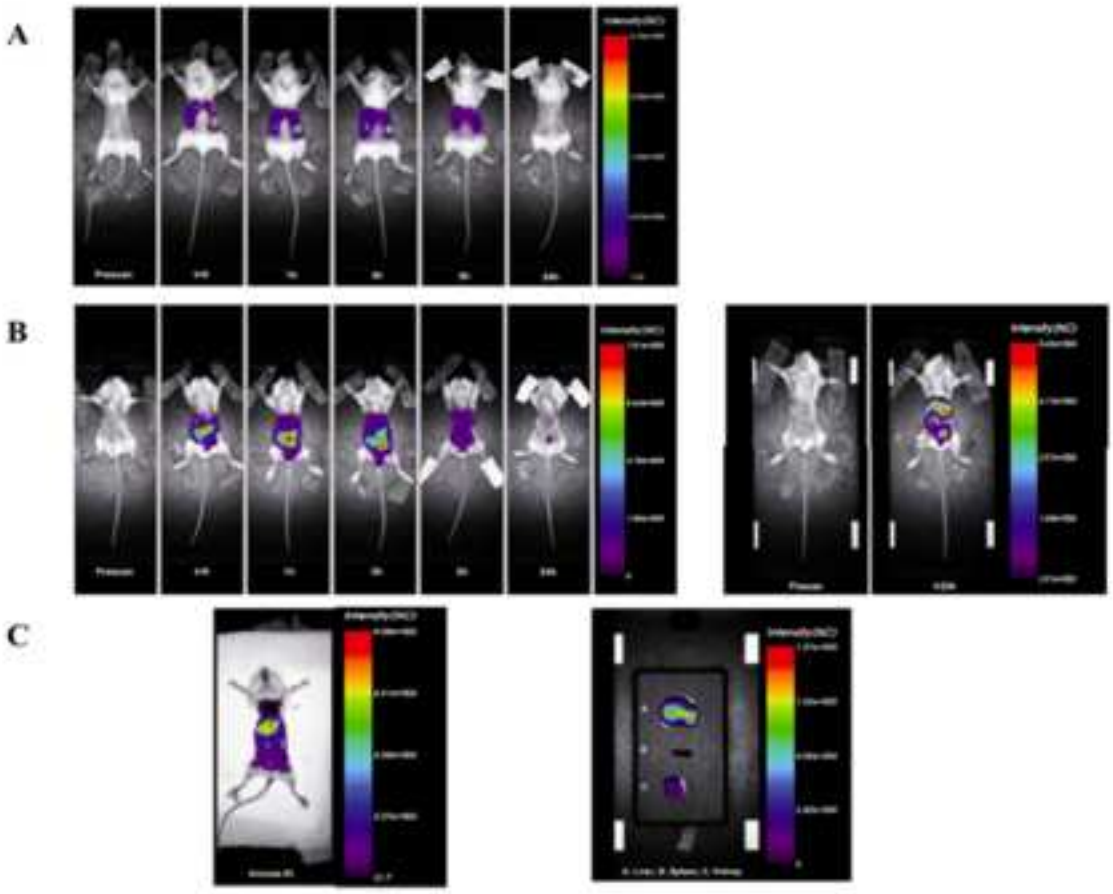


Figure 5

Supplementary data

PEGylation of the peptide Bac7(1-35) reduces renal clearance while retaining antibacterial activity and bacterial cell penetration capacity

Monica Benincasa, Sotir Zahariev, Chiara Pelillo, Annalisa Milan, Renato Gennaro, Marco Scocchi

Table of contents

page

Synthesis of Bac7(1-35), Bac, 1

SPPS of peptide **1**, *(H-Bac-OH)* was performed on a CEM Liberty Automated Microwave Peptide Synthesizer using Fmoc chemistry in 0.1 and 0.2 mmol scales on 2-chlorotrityl resin. The resin was manually loaded with 4-fold molar excess of Fmoc-Pro-OH and 4 eq. DIPEA in DCM (Fmoc-substitution 0.22 mmol/g). For successive couplings, 5-fold excess of Fmoc- and side-chain protected amino acids, PyBop activator and DIPEA were added using NMP as solvent. The coupling temperature was set to 45°C. The Fmoc deprotection was effected using by 20% piperidine and 0.1 M HOBt in DMF.
Peptide was cleaved from the resin using a cocktail of TFA/thioanisole/water/3,6-dioxa-1,8-octane-dihiol (DODT)/TIPS (85/3/2/8/2, v/v), precipitated in methyl tert-butyl ether (-20°C) and then purified by RP-HPLC on a Phenomenex preparative column (Jupiter™, C_{18},10 µm, 90 Å, 250x21,20 mm) using a 10-40% CH_3CN in 60 min gradient with a 8 ml/min flow. The correct sequence was verified by ESI-MS [Esquire 4000 Brucker Daltonics]. After lyophilisation, the concentration of resuspended peptide stock solutions was determinated *i*) from the weight of resuspended peptide, *ii*) by spectrophotometric determination of the peptide bonds with ε_{214} calculated as described by Kuipers and Gruppen , *ii*) by spectrophotometric determination of Tyr (ε_{280} = 1200 M^{-1} cm^{-1}) by Waddell method.

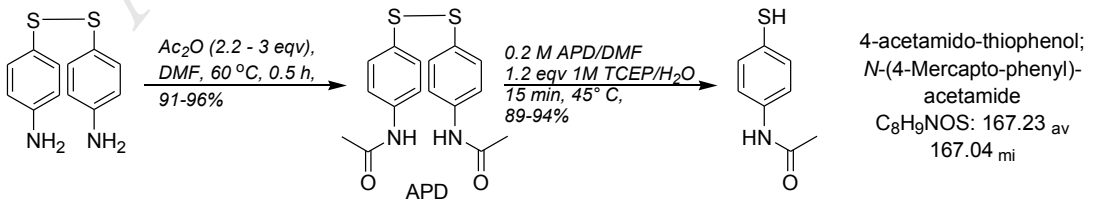
Synthesis of fluorescent dye labelled H-Bac-Cys(BP)-OH

H-Bac-Cys(H)-OH **2a** (Scheme 1, main text) was assembled on H-Cys(Trt)- 2-chlorotrityl resin by SPPS, as above described. After cleavage thiol group of C-terminal cysteine of crude peptide was reacted with fluorescent dye BODIPY®FL [N-(2-aminoethyl)maleimide] (1 equiv/5 equiv) in 10 mM SPB pH 7.4, containing 30% MeCN. The reaction was performed under nitrogen bubbling with stirring for 3 h at room temperature and subsequently overnight at 4°C. The reaction was monitored periodically by analytical RP-HPLC and ESI-MS. Upon completion (about 24 h) 10-fold excess of cysteine was added to reaction mixture to quench unreacted dye. After 60 min the reaction mixture was diluted with 0.05% TFA in water to a final concentration of 10% CH_3CN and pH 2.5. The labelled peptide was purified by RP-HPLC on a on a Phenomenex semi-preparative column (Jupiter™, C_{18}, 5 µm, 300 Å, 100x10 mm) with a linear gradient from 5% to 30% of CH_3CN in 40 min and 2 ml/min flow. The labelled peptide purity was confirmed by ESI-MS.

Preparation of starting products and reagents

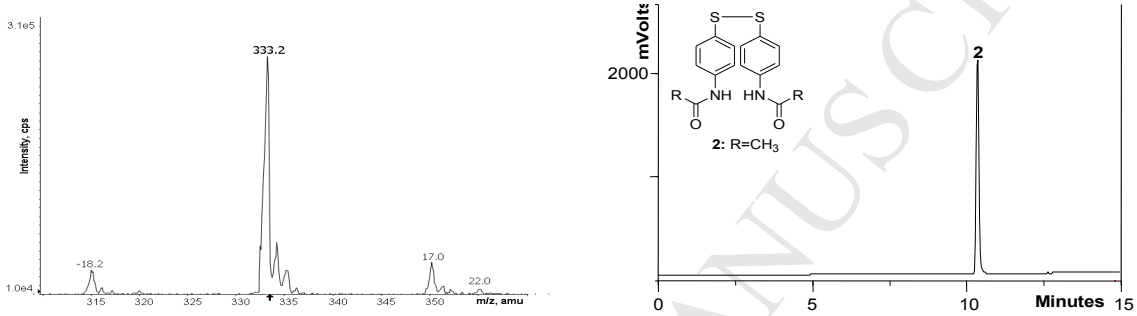
Preparation of 4-acetamidophenol (HS-Ph-NHAc, N-(4-Sulfanylphenyl)acetamide).

The synthesis of starting product for preparation *of* **3** arylthiol *HS-Ph-NHAc* was performed in two steps on 40-100 mmol scale, starting from commercial (4-aminophenyl) disulfide, compare to ref. .



Step 1: Acetylation of (4-aminophenyl)disulfide to (4-acetamido-phenyl)disulfide (APD).

10.21 g (100 mmol) acetic anhydride, 24 ml DMF and 4 ml pyridine were added at 60° C to 9.93 g (40 mmol) dry (4-aminophenyl)disulfide (Aldrich, 98%). Exothermic reaction occurs and product was completely dissolved (~ 1 min). After first 5 min the reaction was completed in 95 % (by RP-HPLC). The mixture was stirred 0.5h at 60° C. Ethanol (70 ml) was added, the mixture was cooled in ice bath (10 min), Et_2O (30 ml) were added, cooled for 0.5h at 4° C and product was filtered, washed first with Et_2O (3 times 20 ml, each one) and then with petroleum ether (fraction 40-60° C, 2 times, 20 ml each one). The product was dried under positive pressure of nitrogen and then in vacuum (1-2 mbar, overnight) to constant weight. Yield, crude 12.77 g (96%), purity (HPLC) > 98.5%; $C_{16}H_{16}N_2O_2S_2$ (332.07); $[M+H]^+$ (found) 333.2



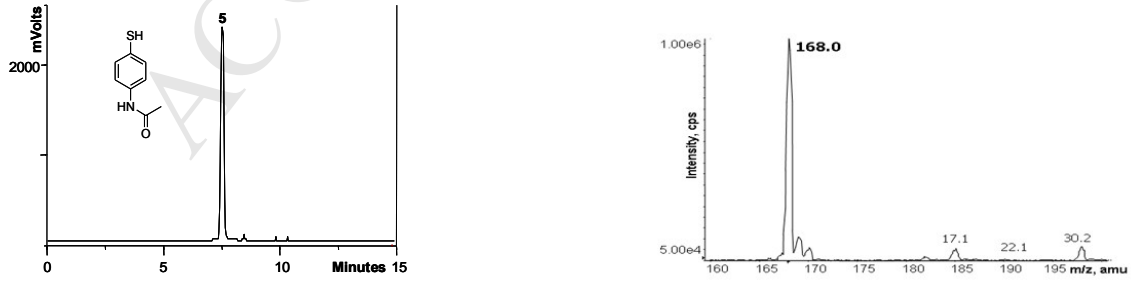
Step 2. TCEP reduction of APD to Acetamido-thiophenol HS-Ph-NHAc.

To 0.2 M solution of (4-acetamido-phenyl)disulfide in DMF were added 1.2 equivalent 1M water solution of TCEP.HCl. The temperature rise to ~ 45° C and the yellowish color of the solution disappears. The mixture was stirred for 15 min, filtered (if necessary), washed on the filter with DMF/H_2O (1/1, v/v; 1 ml/mmol disulfide) and to stirred solution was dropped water to beginning of crystallization (~ one volume), cooled in ice bath and additional one volume water was added. After 0.5h in ice bath, the crystals were filtered under positive nitrogen pressure, washed with water, with petroleum ether and dried (under nitrogen).

4-Acetamido-thiophenol: 10 mmol scale, yield 94%, retention time: 7.56 min (purity 97.5%), C_8H_9NOS 167.04; $[M+H]^+$ (found) 168.0

Note: For reduction of technical grade 4-Acetamido-thiophenol (Aldrich, Cat.: A820-1 (purity ~ 90%, contains ~ 7-10% (4-acetamido-phenyl)disulfide), only 0.06-0.15 equivalents TCEP.HCl were sufficient.

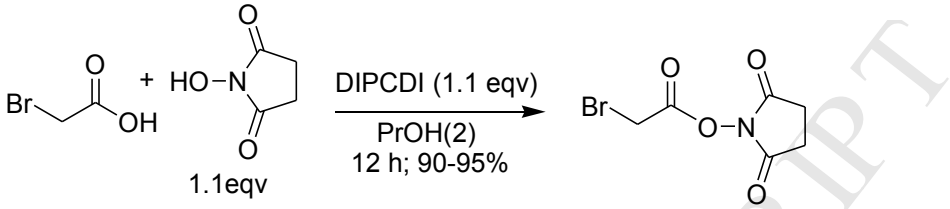
4-acetamidothiophol from crude 4-acetamidothiopho (Aldrich catalog number A820-1, 2.1 g (12.55 mmol) scale, yield 1.12 g, white needle crystals (53,4 %). Purity (HPLC) > 99%, disulfide contains < 0.2%.



Detector traces (214 nm, left panel) from RP HPLC purification of 4-Acetamido-thiophenol and its ESI+ spectra

Preparation of $BrCH_2COOSu$

Bromoacetic acid N-hydroxysuccinimide ester (Bromoacetic acid 2,5-dioxo-pyrrolidin-1-yl ester) was prepared follows the procedure, described for Boc-βAla-OSu, of Inman et al. .



To 10 mmol $BrCH_2COOH$ and 11 mmol HOSu in 15 ml dry PrOH(2) was added 10.5 mmol N,N'-Diisopropylcarbodiimide (DIPCDI). Exothermic reaction occurs and after 30 min the product starts to crystallize. The mixture was stirred for 1h at room temperature and overnight at 4° C. The product was filtered, washed with 2x 5 ml PrOH(2), PA (20 ml) and dried in vacuo. Yield 2.12 - 2.24 g (90 - 95 %) in respect to $BrCH_2COOH$, purity > 98%, melting point 115-116° C.

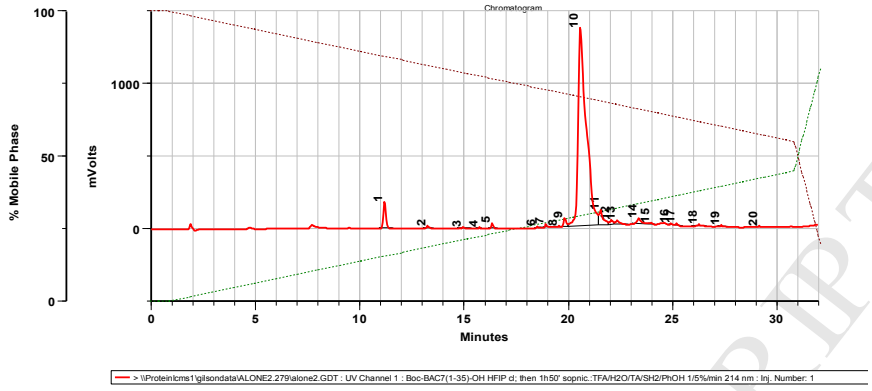
Peptide Synthesis (see Scheme 1 and 2 in main text)

1. Preparation of protected peptide **1a**

1.1. N-terminus Boc protection of resin bound Bac (Boc-Bac-(PG,s)-O-2Cl Trt-resin).

Resin bound side chain protected H-Bac-(1-35)-O-2Cl Trt-resin (0.78 g, starting resin substitution, 0.33 mmol/g) was pre-swollen in DCM, washed with DMF. The filtered resin was shaken with 1.5 mmol (0.327 g, 5 equivalent) Boc_2O and 0.3 mmol (0.039 g) DIEA in 2 ml DMF for 2h at room temperature, filtered and protection procedure was repeated one more time. The resin was filtered, washed with DMF (2x 10 ml); PrOH(2): 2x 10 ml and DCM (4x 5 ml) and dried overnight at 3 mm Hg residual vacuo.

Control experiment: Analysis of starting peptide-resin product: (preparation of **1**)
10 mg N- and side-chain protected peptide-resin (from step 1.1) was cleaved/deprotected/isolated according published procedure and analyzed by RP-HPLC.



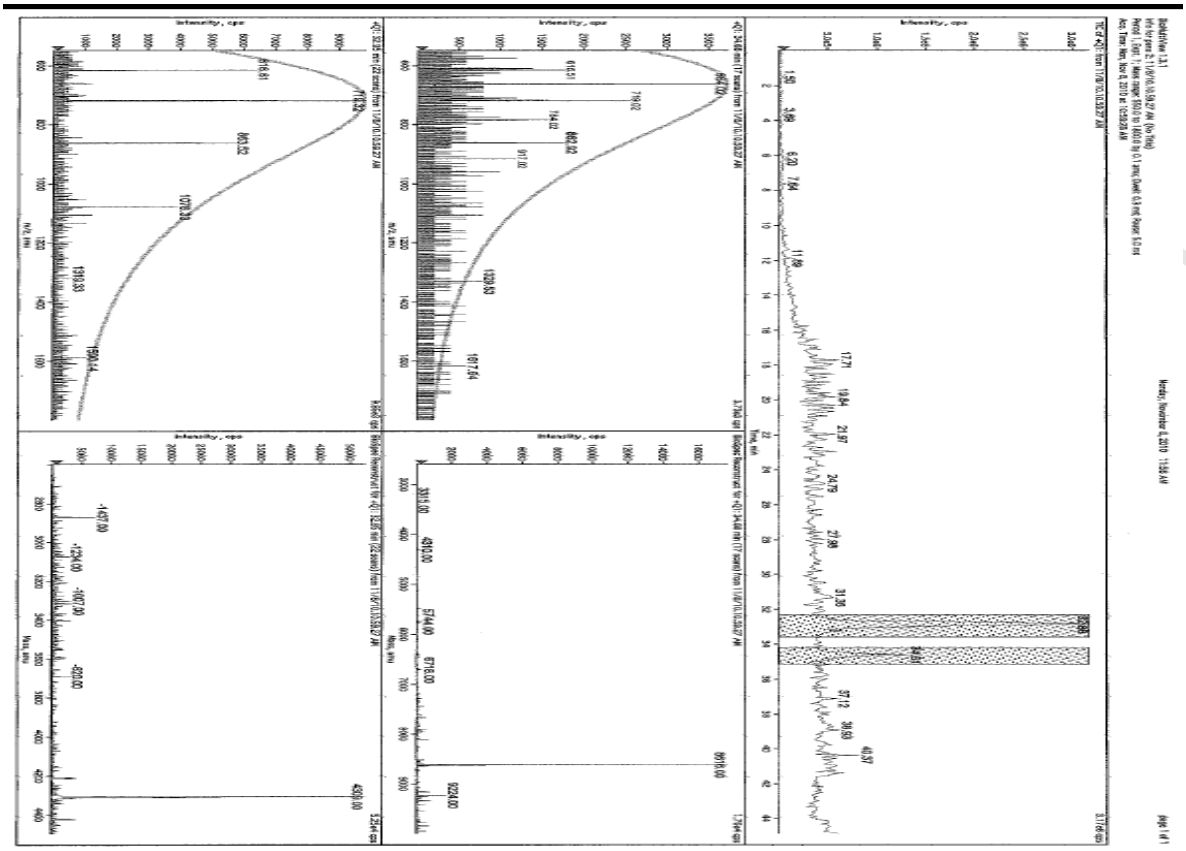
Detector traces (214 nm) from RP-HPLC separation of crude H-Bac-OH **1**, *prepared from* **1a.**
Separation conditions: Column Gemini 5RP18 (150X4.60 mm, Phenomenex); Gradient: 0 - 1 min (0% B, isocratic); 1-31 (0-45% B; gradient slop 1.5%/min); flow rate 1 ml/min. For main peak 10, centered at 20.57 min (~85.7% from total area, ESI-MS found 4208.0, calculated $C_{196}H_{322}N_{68}O_{36}$ *(MW 4207.2)*

1.2. Cleavage from the resin of protected peptide **1a**

Peptide-resin from step 1.1. was mixed with 40% HFIP in DCM (3 ml each one, 6 times for 5 min each one) and collected filtrates were carefully evaporated in vacuo at room temperature. The rest (oil) was mixed with 12 ml Et_2O/PA (1/2), stirred (Vortex mixer), the pellets were separated (centrifuged), washed with petroleum ether (2x 6 ml), dried under steam of N_2 followed in vacuo at 40° C (overnight). Yield 0.64 g.

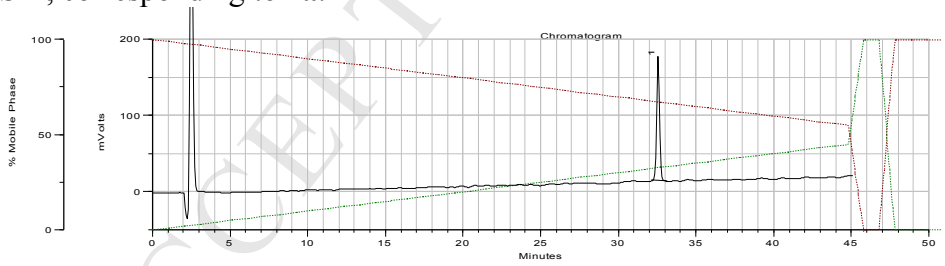
2. Preparation of H-Bac-Cys(H)-OH, **2a** *and H-Bac-Cmc-OH,* **2**.

Automated SPPS of H-Bac7(1-35)-Cys(H)-OH **2a**, isolation and purification was performed according procedure above described. RP HPLC-ESI-MS analysis of purified freeze-dried **2a** demonstrate formation of two peaks, one more hydrophilic: first, ESI+ found 4309.0, which correspond to MW of **2a** (MW calculated for $C_{199}H_{327}N_{69}O_{37}S$ (MW monoisotopic 4307.55; MW average 4310.33) and second hydrophobic, ESI+ found 8616.0, corresponding to its disulfide linked dimer.



LC-ESI-MS of Bac7(1-35)-Cys(H)-OH before TCEP.HCL treatment: peak at 32.5 min (MW found 4309.0)correspond to MW of Bac7(1-35)-Cys(H)-OH (calc. 4310.33); peak at 34.6 min (MW found 8616.0) correspond to MW of oxidized, -S-S- dimer: [Bac7(1-35)-Cys(-)-OH]$_2$

RP HPLC analysis of peptide after TCEP treatment (area > 99% from total area, *see below*) demonstrate absence of peak at 34.6 min corresponding to disulfide linked dimmer {*[Bac7(1-35)-Cys(-)-OH]$_2$*} and correct ESI+, corresponding to **2a**.



Detector traces from RP HPLC separation of Bac7(1-35)-Cys(H)-OH after TCEP.HCL treatment:
Separation conditions: Column Jupiter 5RPC4 4.6x150mm (Phenomenex); Flow rate: 1ml/min; Detection: UV$_{214}$ and ELSD (Alltech); Gradient: 0-45%B for 45min (gradient slop 1%/min); For peak with at 32.55 min (area 100% from total area): MW found 4309.0 (ESI-MS); MW calculated for $C_{199}H_{327}N_{69}O_{37}S$ (MW mono-isotopic 4307.55; MW average 4310.33)

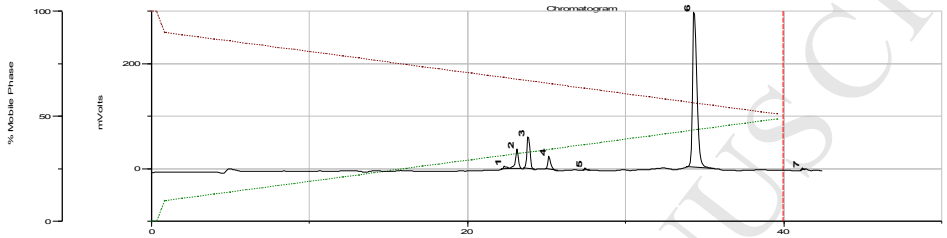
2.2. Preparation of H-Bac-Cys(CH$_2$COOH)-OH ≡ H-Bac-Cmc-OH **2**, (Scheme 1, main text).

Reaction scheme:

H-Bac-Cys(*H*)-OH + Br*CH$_2$COOH* → H-Bac-Cys(*CH$_2$COOH*)-OH ≡ H-Bac-Cmc-OH, **2**

1.1 mg **2a**, corresponding to 0.1856 µmol pure peptide (peptide contains ~ 69%, purity, according RP-HPLC-ESI-MS ~ 92% and ~ 7% -SS-dimer) was mixed for 10 min at room temperature with 100 µL freshly prepared 1M SPB pH 7.3 containing 10 mM TCEP. The reduced peptide was mixed for 1h at room temperature with 100 µL (15 equivalents) freshly prepared solution of 4.8 mg/ml $BrCH_2COOH$ in water and then quenched with 9 µL 25% TFA. The mixture was diluted with water to 1 ml and product was purified by semi-preparative RP-HPLC (Jupiter 5RP18 (250x10; Phenomenex) gradient 0-48% MeCN for 60 min at 2.5 ml/min, detection at 214. The collected pure fractions (according ESI-MS, MW found 4368; calculated for $C_{201}H_{329}N_{69}O_{39}S1$ (4368.37), delta mass from **2a** was + 58 Da) were pulled and freeze-dried. Yield ~ 0.7 mg, 86%.

The synthesis of **2** was repeated one more time starting from 11 mg Bac7(1-35)-Cys(H)-OH, yield purified **(2)** was 6.6 mg (81%).



RP-HPLC analysis detector traces (214 nm) of reaction mixture from preparation of **2**
Reaction conditions: Column: 5RP18 (4.6X 150 mm, Phenomenex); flow rate 1 ml/min; Gradient 0-10% B in 1 min; then 10-50% B in 40 min.

Peak Name	*R. Time*	*Area %*	*Sample Descrip.*
6	*34.35*	*76.36*	*H-Bac7(1-35)-Cmc-OH,* **2**

For peak 6: calculated for $C_{201}H_{329}N_{69}O_{39}S_1$: 4365.56, mono-isotopic, 4368.37, average;.ESI+ found, 4364,0

3. Preparation of H-Bac7(1-35)-COSPhNHAc, **3**

3.1. Preparation of Boc-Bac7(1-35)-COSPhNHAc

212.5 mg (0.03 mmol) crude Boc-Bac7(1-35)-OH (**1a**, procedure 1.2.) and 25 mg (0.15 mmol, 5 equivalents) HS-Ph-NHAc were dissolved in 1 ml DCM/DMF, (1/1, v/v) and 31.2 mg (0.24 mmol) DIEA were added. The solution was cooled in ice bath (15 min), when 62 mg (0.12 mmol) solid PyBop were added, mixed 10 min at 4° C and 35 min at room temperature. DCM was evaporated (under stream of N_2). To vigorously stirred reaction mixture were added 9 ml ice cooled Et_2O/PA (1/2, v/v). The oil formed was separated by centrifugation, washed with Et_2O/PA (1/2, v/v; 2 times 5 ml, each one), with PA (5 ml) and dried in vacuo. The yield of Boc-Bac7(1-35)-SPhNHAc was 256 mg (118%); product contains Ac-NH-Ph-SS-Ph-NHA and side product from reaction between PyBop and HSPhNHAc.

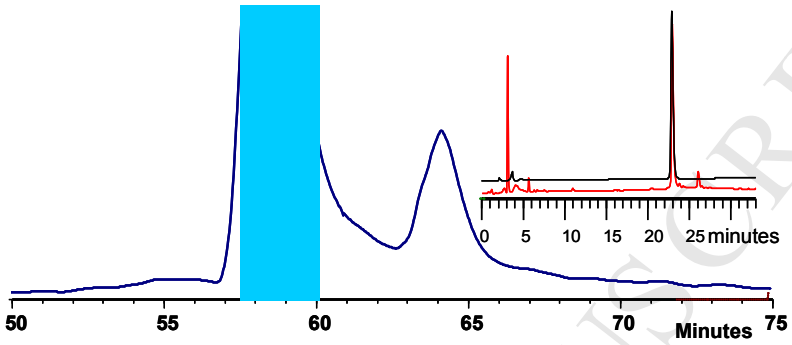
Boc-Bac7(1-35)-COSPhNHAc was prepared also in 0.015 mmol scale, yield 91 mg (105%).

3.2. Deprotection of H-Bac-SPhNHAc, isolation and purification of **3**

100 mg crude product (from 3.1.) was mixed at 4 °C with 10 ml TFA/TA/TIPS/PhOH/H_2O = 90/4/2/2/2/ and the sonicated for 1.5h at 4 °C (1 h reaction, complete deprotection by RP-HPLC/MS analysis; main peak ESI+ found 4356.0; for impurity ESI+ found 407.5, *see below*). Volatile solvents were evaporated in vacuo (10 min) at room temperature, 15 ml water was added, extracted with Et_2O, upper phases were discarded and H_2O-phases were re-extracted five additional times and H_2O-phases were freeze-dried.

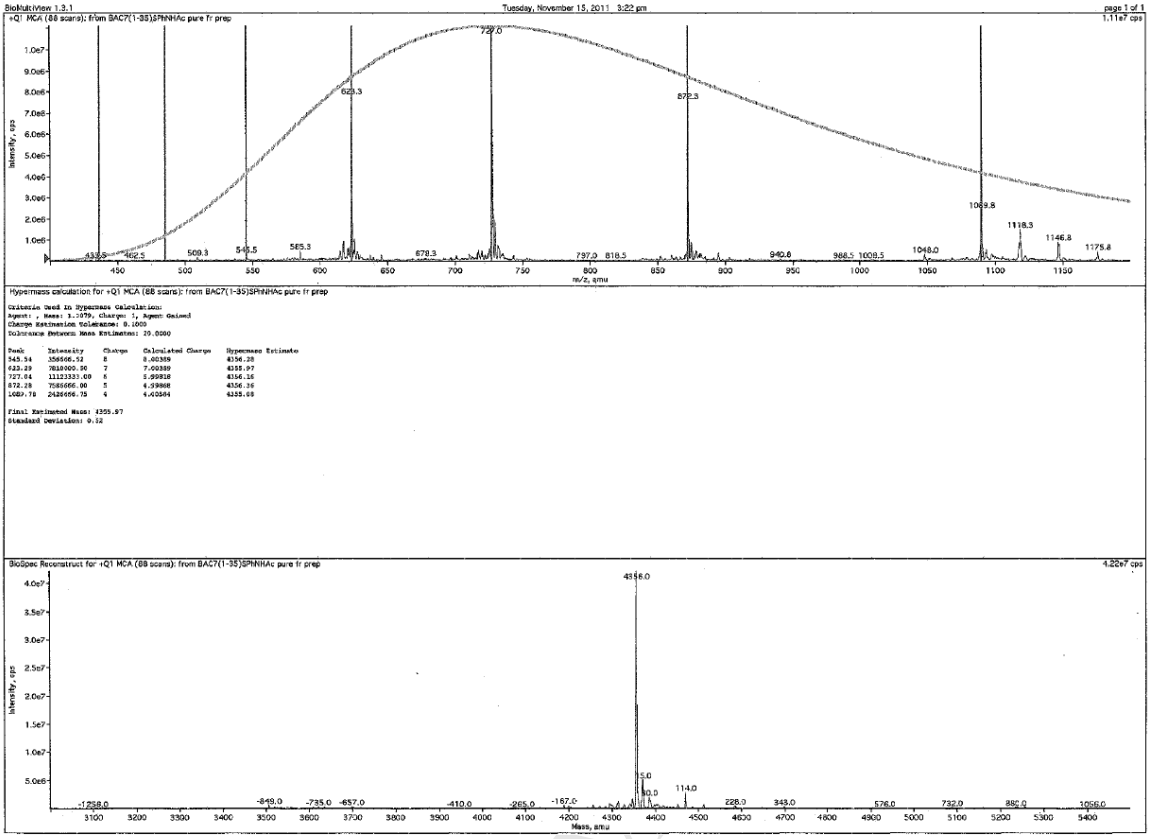
For purification of **3**, where used 3 methods:
Method 1) for preparative (~ 100 mg scale) by RP-HPLC or by
Method 2) combination of IE and RP-HPLC and
Method 3) *small scale* (3- 5 mg) by semi-preparative RP-HPLC.

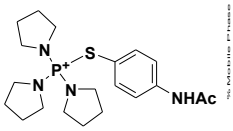
Method 1): preparative RP-HPLC with analysis of collected fraction by ESI-MS



Detector traces from preparative RP-HPLC separation of Bac7(1-35)-SPhNHAc (two separate runs ~ 45 mg each one). Inserts: detector traces from analytical RP-HPLC of crude (red line) and purified (black line) product
Separation conditions preparative RP-HPLC: PrepPak ® Cartridge Delta-Pak™ 15RP18 (40X100 mm, Waters; Buffers A=0.1% TFA in H_2O; B=0.1% TFA in MeCN, Elution: 0 - 1 min: isocratic 0%B (flow 9ml/min), then gradient from 1 min (0%B, 9 ml/min) to 71min (50%B at 12 ml/min), detection at 225 nm. Collected pure (according ESI-MS, see below) fractions from 57.5-60.2 min (shown with turquoise rectangle, were freeze-dried. Isolated yield (from two separation) 42 mg (47%)
Inserts: Separation conditions: column Phenomenex C18 1-31(gradient: 10-40B) flow 1 ml/min , detection at 214 nm; For peak at 23.17 min, ESI+ found 4355.97 ± 0.52[629.8(7+);727.3(6+); 872.2(5+); 1089.8 (4+)], calculated 4356.40 (separate run for H-Bac7(1-35)-OH, peak at 20.55 min, ESI-MS found 4207.2); The peak with retention time at 26.3 min (after main one) is S-(4-acetamidophenylthio) tripyrrolidino-phosphonium (ESI-MS found 407.0).

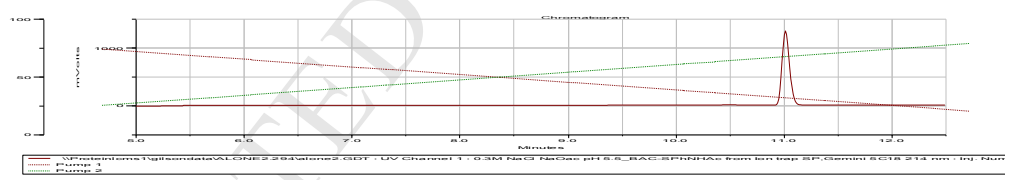
Method 2): combination of ion exchange chromatography on HiTrap™ SP HP (5 ml, Pharmacia) Product ~ 10 mg was loaded 0.1 M NaOAc buffer pH 5.5 and column was washed with 15 ml 0.1 M NaOAc buffer pH 5.5; impurities were eluted with 15 ml 0.1 M NaOAc containing 0.2 M NaCl ; then the product was eluted with 1.3-1.5 M Na+ (1 M NaOAc+ 0.3-0.5 M NaCl).
Product containing fractions were desalted/re-purified by two semi-preparative RPHPLC runs on Zorbax 5RP18 9.4X250 mm, flow 3 ml/min; gradient 0-90 %B (45 min); collected fractions 23.0 -25.1 min were freeze-dried. Yield 8.6 mg (86%).



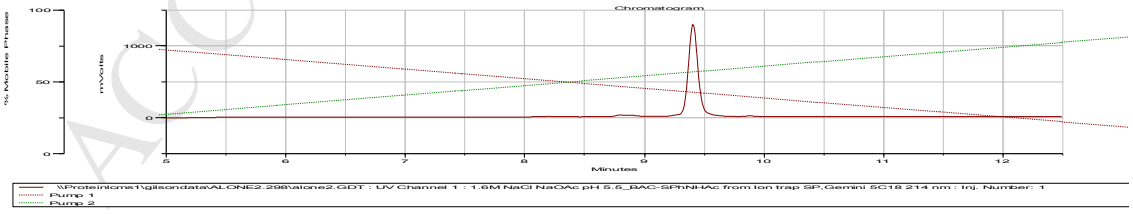


$C_{20}H_{32}N_4OPS^+$

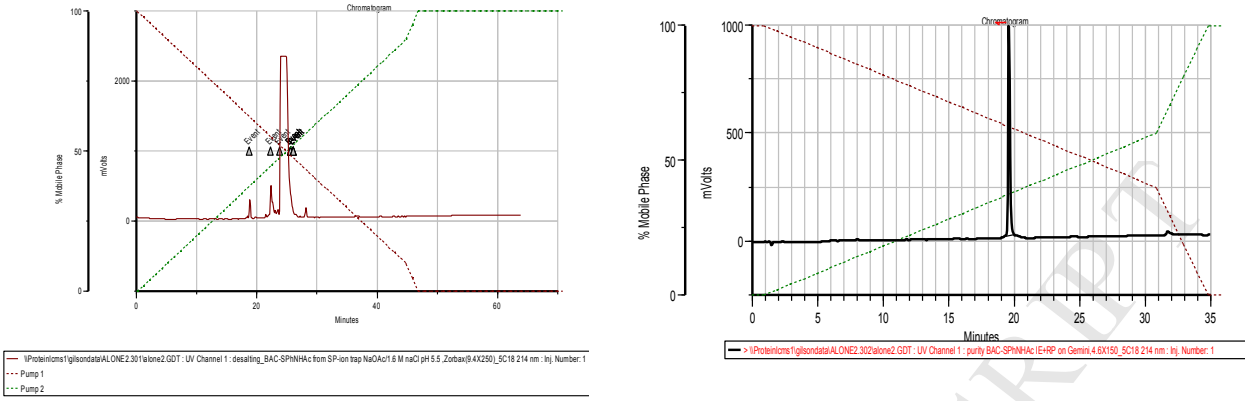
Exact Mass: 407.20



Detector traces from RP-HPLC separation of fraction eluted from at ~ 0.3 M Na^+Cl/OAc, pH 5.5; ESI-MS of peak centered at ~ 11.2 min, ESI+ found 407.26 calculated 407.20.



Detector traces(214 nm) from RP-HPLC separation of **3** *(fraction eluted from HiTrap™ SP HP (5 ml, Pharmacia) ~ 1.3-1.6 M NaCl, pH 5.5). ESI+ for peak centered at 9.4 min was found 4356.0, which corresponding to calculated for product* **3,** *ESI+ found MW 4356.0.*



Left panel: detector traces (214 nm) from preparative RP HPLC purification/desalting of **3** *and analytical RPHPLC separation (Gemini, 4.6X150_5C18, 214 nm) of purified* **3** *(gradient (0-50%B, 1-31'); for main peak at 19.59 min, ESI-MS found* MW_{av} *4356.0*

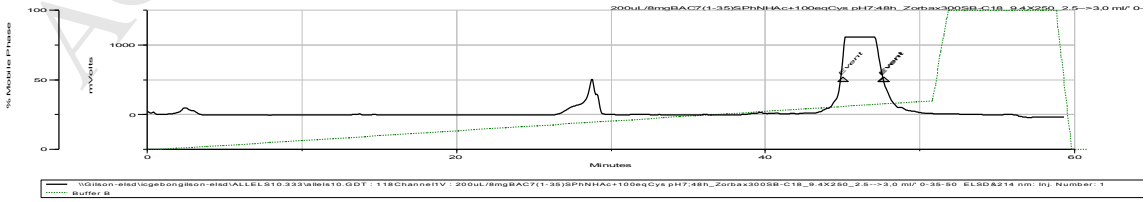
Peak Name	*R. Time*	*Area*	*Sample Descrip.*
1.	*19.59*	*100.00*	*purity BAC-SPhNHAc* **3** *(IE+RP-HPLC purified)*

Method 3) Efficient purification (injection size 3-5 mg/run) of crude **3**, was performed on semi-preparative RP-HPLC 5RP4 or 5RPCN (250X 100 mm) Phenomenex columns (note: Zorbax columns were inefficient).

3.3. Preparation of **2a** *by NCL of 3 and excess of cysteine.*

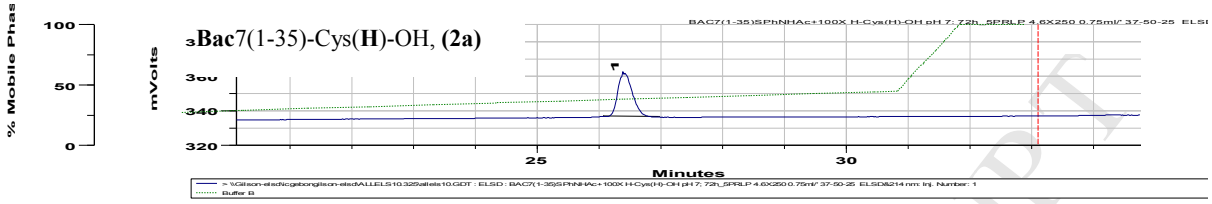
Reaction scheme:
H-Bac-SPhNHAc, **3** + H-Cys(H)-OH/buffer pH 7→H-Bac7(1-35)-Cys(H)-OH, **2**

5 mg cysteine (free base, 42 µmol, 30 equivalents) in 0.090 ml 0.4 M NaOAc/ 30mmol TCEP/10 mmol EDTA buffer pH 7.0, was added (under N_2) to 8.0 mg (1.397 µmol) dry powder of **2a** and gentile mixed for 48 h (*for RP-HPLCand ESI-MS analysis, see below*). Reaction mixture was diluted with 0.3 ml 30 mmol TCEP.HCl pH 2 and 0.6 ml H_2O and purified (3 runs: each one 0,33 ml, diluted to 1 ml with H_2O) on 5RP18 Zorbax 9.4X250 column, gradient 0 – 35% MeCN (1-51 min), flow 2.5 at beginning to 3 ml/min at the end of the gradient. Collected pure (ESI-MS) fractions from 3 runes were freeze-dried. Yield 5.2 mg (65 %) or 4.37 mg (56 %) based on free SH-groups in the product (*note: unexpected high yield for NCL with C-terminal proline)*



Detector traces from semi-preparative RP-HPLC of **2a** *(from NCL of* **3** *and Cysteine*

Analysis of reaction mixture was performed at 6 h, 18 h, 24 h and 48 h (peaks area of **2a** was not changed after 48 h). Small portions (~ 30 µg) was mixed with 5 equivalent TCEP, separated by RP-HPLC (gradient 1-45% MeCN for 30 min)

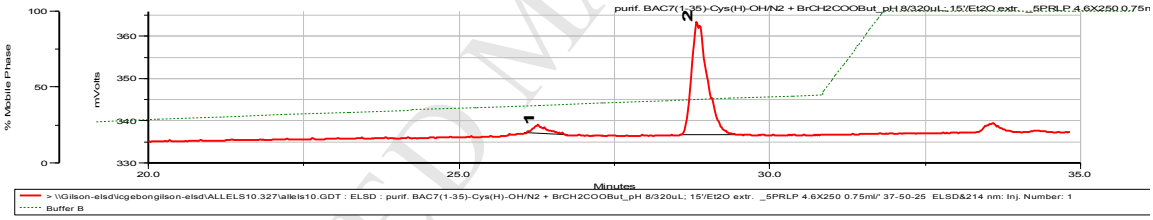


Detector traces RP-HPLC separation of purified **3** *(from NCL of* **2a** *and Cysteine, peak 1 area > 99%); ESI-MS of peak 1: calculated for* $C_{199}H_{327}N_{69}O_{37}S_1$ *(4310.3*$_{av}$ *or 4307.6*$_{mi}$*), ESI+ found 4311.0.*

3.3.1. Analysis of **2a** *for presence of free SH by thioether ligation*

2a + $BrCH_2COOC(CH_3)_3$ → *H-Bac-Cys[*$CH_2COOC(CH_3)_3$*]-OH*

Collected pure fraction **2a** (peak 26.15 – 26.82 min) was mixed with 60 µL 0.8 M SPB pH 8.0 and 0.2 µL $BrCH_2COOtBu$, sonicated for 15 min at 20° C, diluted with 0.5 ml H_2O, extracted with 3X 0.5 ml Et_2O, flushed/bubbled with N_2 for 1 min and again separated by RP HPLC for the same conditions.



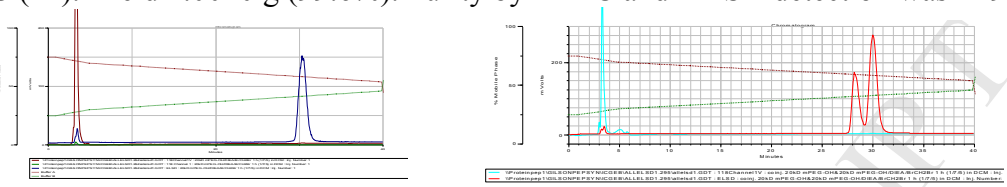
Detector traces RP-HPLC separation of reaction mixture between **3** *and excess* $BrCH_2COOtBu$ *to H-Bac-Cys(*$CH_2COOtBu$*)-OH (thioether ligation, main peak 2*, area > 84% *)*

4. Preparation of $BrCH_2CO$*-O-PEG-OMe* **4** *by Bromoacetylation of mPEG-OH, modified procedure, ref. .*

Reaction scheme:
mPEG-OH+$BrCH_2COBr$/DIPEA → mPEG-$OC(O)CH_2Br$, **4** + DIPEA.HBr.

2 g (0.1 mmol) mPEG-OH was dissolved in 30 ml dry toluene at 80° C and solvent was evaporated in vacuo (6 mm Hg residual pressure) to dryness. The rest (oil) was dissolved in 9 ml dry DCM containing 2.5 mmol (0.323 g, 0.413 ml, 25 equivalents) DIPEA, cooled to -10° C and then to vigorously stirred solution at temperature bellow -5° C were dropped 30 equivalents (3 mmol, 0.413 ml, 0.606 g) $BrCH_2CO$-Br in 2 ml DCM (5 min), stirred at -5-10° C for additional 15 min and then 1h at room temperature. 15 ml PrOH(2) were added to the dense brown mixture and DCM was evaporated in vacuo at room temperature. 10 ml Et_2O were dropped (5 min) to ice cooled partially crystallized product, stirred and cooled for additional 1h, filtered, washed with 2x 5 ml ice cooled PrOH(2), with PrOH(2)Et_2O (1/1, 5 ml), with Et_2O (2x 5 ml) and dried in vacuo. The product was re-dissolved in MeCN (7 ml), 7 ml Et_2O were

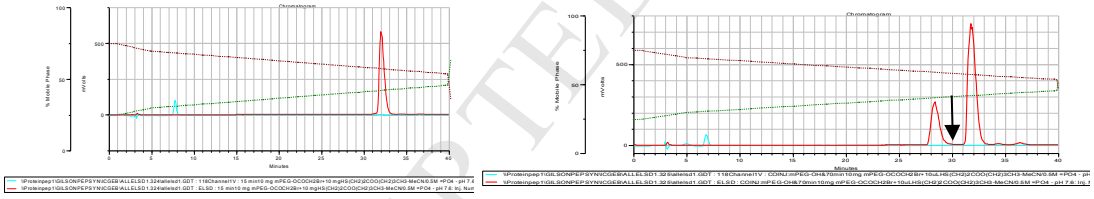
added, filtered, the rest on the filter was washed with 2 ml Et_2O/MeCN (1/1). Collected filtrates were mixed with ice cooled and Et_2O to beginning of opalescence, cooled for additional 30 min at 0° C and Et_2O (6 volumes) were added for ~ 1 min . After 1h at 0° C the product was filtered, washed with MeCN/Et_2O 1/8 (10 ml), then with Et_2O (10 ml), PA (10 ml) and dried under stream of dry N_2 and then in vacuo at 40° C (4h). Yield 2.0040 g (99.6%). Purity by HPLC and ELSD detection was > 99%.



Detector traces from RP-HPLC separation (Column 5 µm, 300 Å PLRP-S (4.6X250 mm), flow 0.75 ml/min) of **4***, (left panel, ELSD detection, > 99% purity) and co-injection of HO-PEG-OMe and* **4** *(right panel, ELSD detection – in red and 214 nm – in turquoise.) Peak centered at ~ 28.16 min: HO-PEG-Me; peak centered at ~ 30.00 min: $BrCH_2C(O)$-O-PEG-OMe* **4,**

Model reaction (thioether ligation): analysis of reactivity of **4** *with $HS(CH_2)_3COO(CH)_3CH_3$*

2 mg **4**, 2 mg $HS(CH_2)_3COO(CH)_3CH_3$ and 0.06 ml 0.5 M SPB pH 7.6 were sonicated for 15 min at room temperature. Formation of thioether MeO-PEG-O-C(O)-CH2-S$(CH_2)_3COO(CH)_3CH_3$ (RP-HPLC analysis, left panel) was near to quantitative (new product with retention time ~ 32 min, >99% was formed).
After 70 min ~ 60 µg from reaction mixture (in respect to **4**) were co-injected with mPEG-OH as a "internal standard" and the result (see RP-HPLC analysis, right panel) demonstrate again complete reaction.



Left panel, detector traces (ELSD) from RP-HPLC separation (Column 5 µm, 300 Å PLRP-S (4.6X250 mm), flow 0.75 ml/min) of reaction mixture from 15 min thioether ligation of $HS(CH_2)_3COO(CH)_3CH_3$ and ***(4)****, (area main peak , > 99%: MeO-PEG-O-C(O)-CH_2-S$(CH_2)_3COO(CH)_3CH_3$) and right panel, co-injection of HO-PEG-OMe and reaction mixture 70 min ligation, ELSD detection – in red and 214 nm – in turquoise; peak centered at ~ 28.16 min, mPEG-OH; peak at ~ 30.00 min mPEG-OC(O)CH_2Br, not present.*
Peaks identification: 1. $HSCH_2CH_2COO(CH_2)_3CH_3$ ~ 7.8 min; 2. mPEG-OH ~ 28-16-28.30 min; 3. mPEG-OC(O)CH_2-$SCH_2CH_2COO(CH_2)_3CH_3$ ~31.8 min, ligation product 4. mPEG-OC(O)CH_2Br, r.t.~ 30 min.

5. Preparation of 20kDa Methoxypolyethylene glycol amine, H_2N-PEG-OMe, **5**.

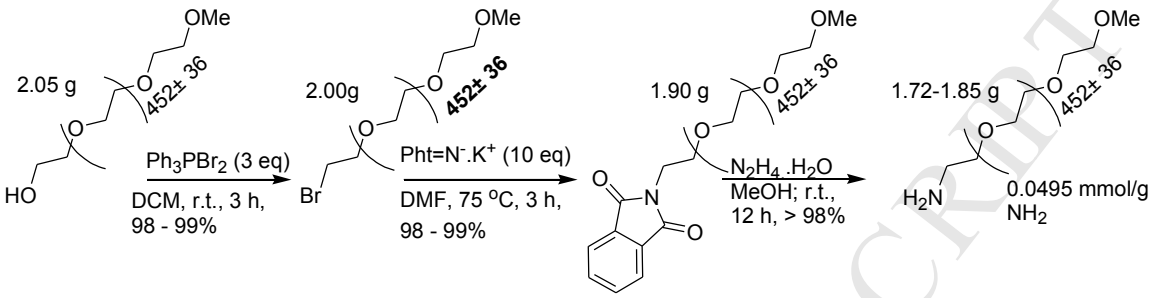
Two methods A and B were applie for preparation of **5***:*

A) treatment of homemade mPEG-OTos with ammonia/ammonium chloride according, Kwang Nho, Orinda (Korea, Republic of); Changmin Hyun, Junghun Lee, Kyonggi-do and Youngkyoung

Pak, United States Patent 6,828,401, priority May 7, 2003). Product **5**, prepared by method **A**, contains 14-18% product of hydrolysis mPEG-OH and was abandoned.

B) Gabriel synthesis (in 3 steps) according modified procedures of Pillai et al. and So et al. .

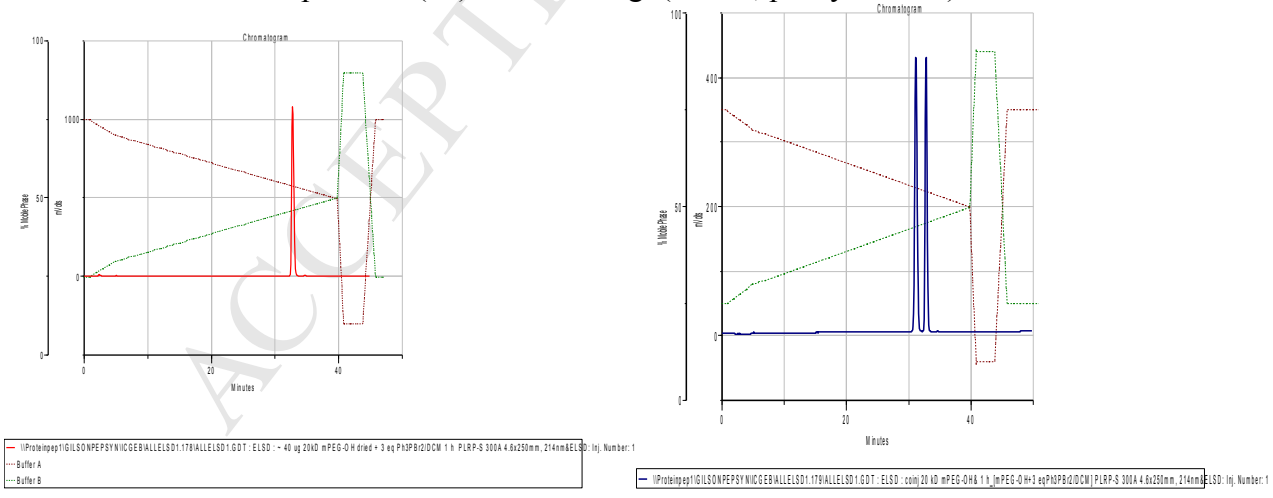
Reaction scheme:



5.1. Preparation of mPEG-Br from mPEG-OH.

mPEG-Br was prepared according modified procedure of Ngu et al. .

To 0.5 L single-neck round bottom flask equipped with a magnetic stir bar was placed 40 g (2 mmol) 20 kD mPEG-OH. The product was dissolved in 80 ml DCM and 100 ml toluene at 60° C and solvents were evaporated at 80° C in vacuo. The rest (oil) was dissolved (under N_2) in 100 ml dry DCM, cooled in ice bath (15 min), stirred for 1 min with Ph_3P (0.1 g) and then with (6 mmol, 3 equivalents) Ph_3PBr_2 dissolved in 70 ml dry DCM was added, stirred 10 min in ice bath and then at room temperature (in total 3 h). The reaction mixture was evaporated to half its volume and product was precipitated with 6-8 volumes Et_2O, cooled for 0.5 h in ice bath, filtered, washed with Et_2O and dried in stream of dry N_2. The product was transferred back to the flack, the filter was washed with DCM and the product was dissolved in total 150 ml DCM. 300 ml PrOH(2) were added and DCM was evaporated. Ethyl ether was added to beginning of crystals formation (~ 100 ml), the mixture was cooled 1h at 4° C, filtered, washed with 100 ml cold PrOH(2)/Et_2O (1/1), with Et_2O (100 ml) and PA (100 ml), dried under N_2 and then at 45° C and 3 mbar residual pressure (3h). Yield 39.4 g (98.5%, purity > 99 %).



Left panel, detector traces (ELSD) from RP-HPLC separation of mPEG-Br (1 h reaction, area main peak > 98.5%); right panel co-injection and peaks identification: mPEG-OH (31.14 min) and purified mPEG-Br (32.78 min). Column 5 µm, 300 Å PLRP-S (4.6X250 mm), flow 0.75 ml/min;

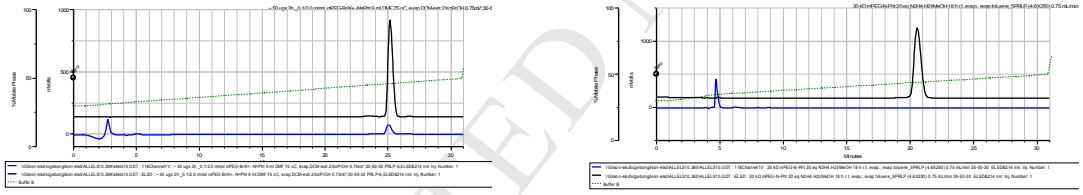
5.2. Preparation of Phth=N-PEG-OMe

mPEG-Br (0.1 mol, 2 g from step 5.1.) and 178 mg (1 mmol, 10 eq K-phthalimide in 9 ml dry DMF was heated at 75° C (external oil bath) for 2.5h. The solvent was evaporated at 75° C in vacuo, the rest re-evaporated two timed with 10 ml toluene (each one) , and the rest was extracted from 18 ml DCM and 30 ml 0.1 M K_2CO_3. Water phases were re-extracted 8 times with 6 ml DCM (each one) and collected DCM layers were dried (Na_2SO_4), filtered and DCM evaporated. The rest crystallized from two times from 30 – 40 ml ml PrOH(2), washed with Et_2O, with PA and dried, Yield 1.8 g (90%), Purity: UV_{214} > 96%, retention time 25.05 min; purity ELSD 98.2 %.

5.3. Hydrazinolysis of Phth=N-PEG-OMe to NH_2-PEG-OMe, **5**.

Phth=N-PEG-OMe (1.9 g, 0.095 mmol) in 15 ml MeOH and 0.1 g (2 mmol) hydrazine hydrate $N_2H_4.H_2O$ in 3 ml MeOH were stirred overnight at room temperature. The solvents were evaporated in vacuo at 60° C and re-evaporated two times (15 ml each one) with toluene. The rest was dissolved in ~ 25 ml DCM and 30 ml PrOH(2) was added. DCM was evaporated in vacuo. At the end of evaporation the crystallized product was diluted with 1 volume Et_2O, cooled in ice bath for 0.5h, filtered, washed with Et_2O (20 ml) , PA (20 ml) and dried. The yield of **5** was 1.72 g (95.5% or 86% from 3 steps), purity > 99 %; retention time 20.57 min.

The procedure was repeated one more time, starting from 2 g (0.1 mmol) mPEG-OH. The yield (second batch) of **5** was 1.85 g (92.5% from 3 steps or 97.3% per step, purity > 99.85%.



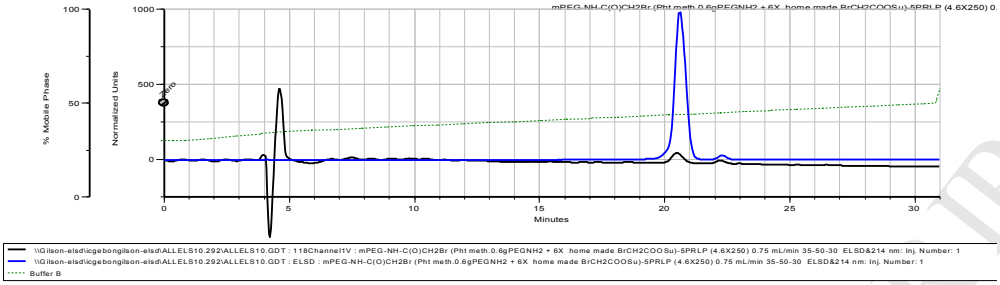
Left panel, detector traces (ELSD&UV_{214}) from RP-HPLC separation of Phth=N-PEG-OMe (step 5.2.); right panel detector traces (ELSD&UV_{214}) from RPHPLC separation of NH_2-PEG-OMe, **5**. *Column 5 µm, 300 Å PLRP-S (4.6X250 mm), flow 0.75 ml/min;*

6. Preparation of BrCH2CO-NH-PEG-OMe, **6**.

Moderate yields in bromoacetylation of mono-disperse (low molecular weight) amino-PEG's were recently published .

400 mg **5** (0.02 mmol) and 28 mg (0.12 mmol) $BrCH_2COOSu$ (6 equivalents) were dissolved in 1.2 ml dry MeCN under argon, 5 mg (0.06 mmol) dry powdered $NaHCO_3$ was added and then mixed for 3.5 h at room temperature. Water (0.05 ml) was added, stirred for additional 0.5 h, cooled to room temperature, 10 ml Et_2O was added and stirred for additional 15 min in ice bath. The solvents were separated from the precipitated reaction product, the rest was dissolved in 3 ml PrOH(2) at 65° C, cooled to room temperature and 3 ml Et_2O was added. The mixture was cooled for 1 h in ice bath and the crystals were separated from the solvents, washed with Et_2O (two times, 5 ml each one), with PA (10 ml) and dried at room temperature in vacuo, yield 384 mg (96%).

This procedure was repeated one more time starting from 0.6 g (0.03 mmol) mPEG-NH_2 . Isolated yield was 576 mg (96%).



Detector traces (ELSD&UV_{214}) from RP-HPLC separation of BrCH2CO-NH-PEG-OMe **6***: retention time 20.61 min (97.92%, ELSD); impurity - identifies as a Phth=N-PEG-OMe: 22.31min (2.08%); Column 5 µm, 300 Å PLRP-S (4.6X250 mm), flow 0.75 ml/min.*

Note: HO-PEG-OMe (not reacted in first step of preparation of **6** *or formed during the reaction steps as a result of hydrolysis) can not react with $BrCH_2COOSu$ (NHS-esters can be safely crystallized from dry alcohols even in the presence of base like DIPEA) and only amide* **6**, *but not ester* **4** *can be formed.*

7. Preparation of H-Cys(H)-CONH-PEG-OMe, **7**.

7.1. Preparation of Boc-Cys(Trt)-CO-NH-PEG-OMe, ref.

1 g (0.05 mmol) **5**, 232 mg Boc-Cys(Trt)-OH (0.5 mmol, 10 equivalents) and 207 mg HCTU (5 mmol, 10 equivalents) were partially dissolved in 2.5 ml DCM. 130 mg DIPEA (1 mmol, 20 equivalents) in 0.7 ml DMF were added, sonicated for 2 min (tan colored solution formed), mixed for 15 min at room temperature, and the product was precipitated (Et_2O, 30 ml), filtered, washed on the filter with Et_2O (3x 5 ml), re-dissolved in PrOH(2) (15 ml) and precipitated Et_2O (15 ml) at 4° C (0.5 h), filtered, washed with Et_2O (3x 5 ml), PA (5 ml) and the solid dried. Yield 1.02 g (> 99.7%).

7.2. Deprotection of Boc-Cys(Trt)-CO-NH-PEG-OMe to **7** *(as a TFA salt)*

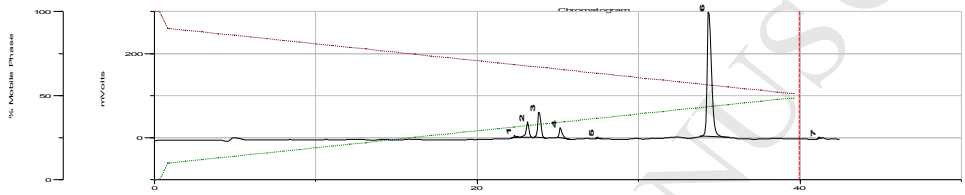
To 0.4 g Boc-Cys(Trt)-CO-NH-PEG-OMe (from step 7.1.) were added TIPS/H_2O/DODT = 0.125/0.125/0.250 ml, ice cooled TFA (1 ml) and after 5 min additional 3.5 ml ice cooled TFA, stirred 30 min at room temperature, TFA was evaporated under N_2 and the product was precipitated with Et_2O (40 ml), stirred at 4° C (15 min), crystals were separated from solvents by filtration, washed on the filter with Et_2O (3x 5 ml) and dried. Yield 0.39 g (98.7%). For analysis 50 mg (7)-TFA-salt were dissolved in 0.5 ml PrOH(2) at 65° C, cooled to 4° C, and then slowly were added 0.3 ml Et_2O. The product was centrifuged, the supernatant was eliminated, the solid was washed with 3x 1 ml Et_2O, PA (1 ml) and dried at 40° C /3 mm Hg overnight and analysed:

No	Name	R. time (min)	Purity Area (%)	Method	HPLC run No
7.1	Boc-Cys(Trt)-HN-PEG-OMe	43.4	91.7	this paper & [2]	allelsd6.058
7	H-Cys(H)-HN-PEG-OMe	26.5	98.7	this paper	allelsd6.059

RP HPLC conditions: sample size injected, 30-60 µg, Loop, 1 ml; Column PLRP-S, 300Å, 4.6X250 mm; flow 0.75 ml/min; Buffers, A=0.1% TFA/H_2O; B=0.1%TFA/MeCN; Isocratic; 30% B for1 min, Gradient: 30-35%B (1-4 min); 35-47%B (4-40 min); 47-90%B (40-41 min); isocratic 90%B for 3 min.

8. Preparation of ester bond PEGylated Bac, **8** *(Bac_E-PEG) by thioether ligation of* **2a** *and* **4**.

2a 10 mg (1.76 µmol) was dissolved in 0.6 ml 0.3 M SPB pH 7.3, containing 10 mM TCEP.HCl; 30 min later, 40 mg **4** (dry powder) were added under N_2, mixed for 6 h (**8** ~ 60% from total area by RP HPLC) and then new 40 mg $BrCH_2COOPEG$-OMe (in total 80 mg, 4 µmol) were added; pH was adjusted to ~ 7.3 (if necessary) and mixed overnight (total time 21 h). The reaction mixture was diluted with water to 30 ml and quenched/acidified with 10% TFA to pH ~4-5. The purity of crude product (RP-HPLC: peak 6, absorbance at 214 nm) was ~ 76% from total area.



Detector traces (214 nm) from RP-HPLC separation of **8**, *crude reaction mixture*

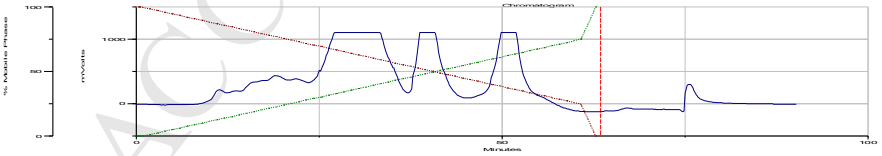
Peak Name	*R. Time*	*Area %*	*Sample Descrip.*
6	*34.35*	*76.36*	*crude* **8**, *21 h reaction time*

Separation conditions: Column 5RP4 (4.6X 150 mm, Phenomenex) flow 1 ml/min; gradient 10-50%B (1-41 min).

The product was purified in two steps by combination of IE and RP-HPLC chromatography [as described for **3**]:

a) *first step: IE-separation chromatography,* was performed on strong cationic exchanger of Hi-Trap™ SP HP (5 ml, Pharmacia). The excess of **4** was not retained in the column. 3 runs were performed: first 5 ml from total 30 ml (16.7% from total amount) were used to find/improve the separation conditions.

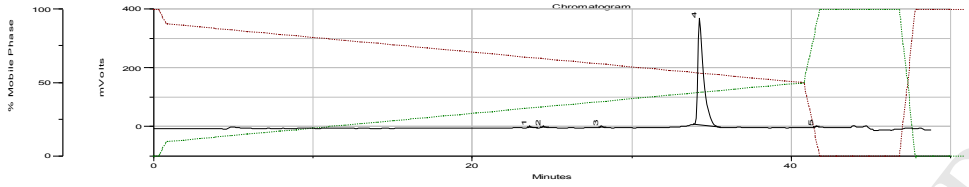
Separation conditions for 2nd and 3rd runs: after injection (12.5 ml each one), the column (HiTrap™ SP HP (5 ml, Pharmacia) was washed with 25 ml 50 mmol SPB pH 6.0 at flow rate 1 ml/min. The product was eluted with gradient from 50 mmol SPB pH 6.0 and 1 ml/min to 100 mmol SPB pH 6.0 containing 1.5 M NaCl and flow rate 1.5 ml/min. Collected main peak, centered at ~ 22.0 – 36.0 min (~ 0.6 M Na Cl) was identified (by analytical RP-HPLC on 5RP4 column, see below) as a reaction product **8**.



Detector traces (220 nm) from IE separation of crude **8** *(first 5 ml) on HiTrap™ SP HP (5 ml, Pharmacia), prepared from 10 mg* **2a** *and 80 mg $BrCH_2COOPEGOMe$* ***(4):*** *for details see text. First (main) peak (25.8-35.0 min, ~ 12 ml), identified (see below) as a reaction product* **8**, *was pooled and desalted/purified on semi-preparative RP HPLC (C4-columns)*

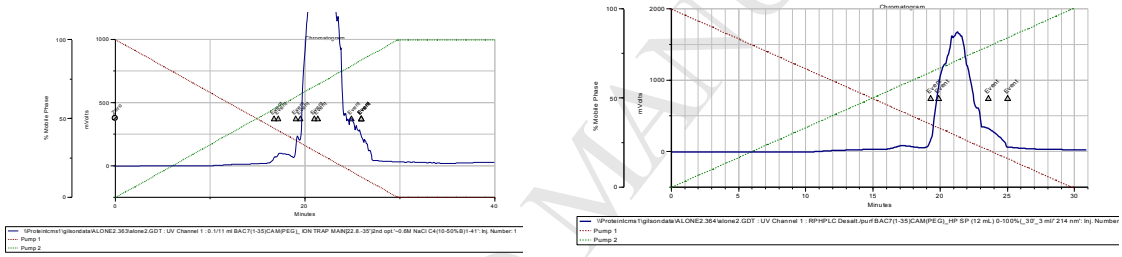
Second run (12.5 ml), main peak 22.8 – 35.0 min was identified as a product **8**:

a) by retention time at RPHPLC (absorbance at 214 nm) and increased signals of ELSD detectors for **8** in respect to UV absorbance of **2a** and *b)* by absence of peak with retention time corresponding to BrAc-PEG-derivative **4**.



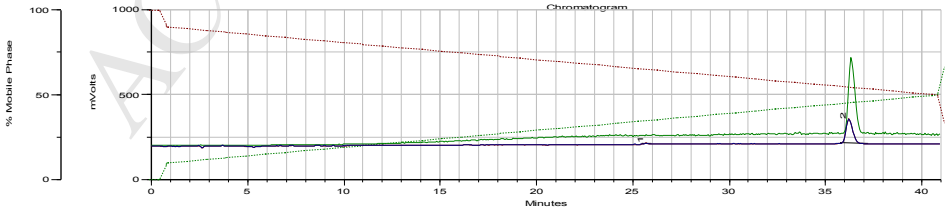
Detector traces from RP HPLC separation of 12 µL fraction from main peak, 2nd IE purification (peak at 25.8-35.0 min): Separation conditions: Column 300 Å Jupiter 5RP4 (150X4.6, Phenomenex), flow 1 ml/min.; gradient (0.0-0.5 min)-isocratic 0%B , (0.5 to 1.0 min), gradient 0.0-10%B; (1.0 to 41.0 min,) gradient 10-50%B = gradient slop 1%/min; detection UV at 214 nm and ELSD): main peak 4, identified as a **8** *(purity, ~ 94.5 %).*

b) *Second step:* RP-HPLC (purification and desalting, two runs) were performed on semi-preparative column 5RP4 (10x 250 mm, Phenomenex); Collected desalted fraction, absorbance cut-off 250-300 mVolts (20.1-24.1 min) were pooled and freeze-dried and again analyzed.



Detector traces from semi-preparative RP-HPLC separation/desalting of crude **8** *(from step1).*
left panel: injected fraction from step1 [1st (18.9-26.5 min) and 3rd (20.1-24.1 min), total 21 ml]
right panel: injected fraction from step1 [2nd IE separation (25.8-35.0 min, ~ 12 ml)
Separation conditions: Column 300 Å Jupiter 5RP4 (10X250 mm), Phenomenex), gradient 0-100% MeCN, containing 0.1%TFA (30 min), flow 2 ml/min at 0 min to 3 ml/min at 30 min, detection at 214 nm.
Fractions with absorbance > 250 mVolt (both runs) were pooled and freeze-dried and then analyzed.
Fractions with absorbance < 250 mVolt (for-peak and tail) were pooled and freeze-dried separately.

Fractions (18.9-20.1 + 24.1-26.5 min from above purification steps) were pooled separately and freeze-dried. Yield isolated purified **8**, 18.9 mg (41.8%) with purity > 98% (see below)
Additionally from "for-peaks" and "tails" was isolated 5.2 mg **8** with purity ~ 90% (in total recalculated yield 52.16%)



Detector traces (blue line UV_{214}, green line ELSD detection) from RPHPLC separation of freeze-dried and purified **8**.
Separation conditions: Injection size: ~ 40 µg from total 18.9 mg **8***; Column 300 Å Jupiter 5RP4 (150X4.6, Phenomenex), flow 1 ml/min.; gradient (0.0-0.5 min)-isocratic 0%B , then 0.5 to 1.0 min, gradient 0.0-10%B; and 1.0 to 41.0 min, gradient*

10-50%B (gradient slop 1%/min) main peak, detection 214 nm was peak centered at 36.22 min (98.2%), main peak ELSD detector, peak centered at 36.38 min, purity > 99.0%.

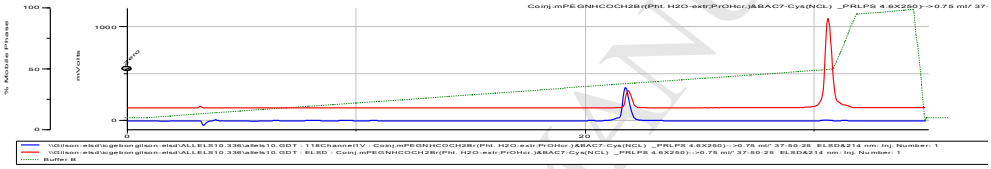
Peak Name	*R. Time*	*Area %*	*Sample Descrip.*
1	*25.72*	*1.80*	*impurity*
2	*36.22*	*98.20*	*Purity* **8,** *18.9 mg (214 nm).*

9. Preparation of amide bond PEGylated Bac **9***, (Bac$_A$-PEG) by thioether ligation of* **2a** *and* **6***.*

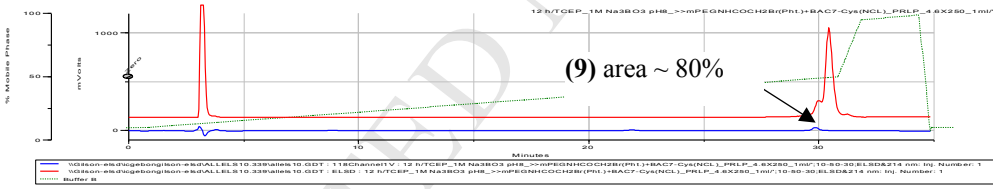
Preparation of **9,** was performed essentially as described for preparation of **8**.

10 mg **2a** was dissolved in 0.6 ml 1M borate buffer pH 8 containing 0.1 mg TCEP.HCl. 30 min later under N_2 were added 50 mg **6** as a dry powder, mixed for 1 h (RP-HPLC, **9** ~ 60% from total area) and then new 50 mg $BrCH_2COOPEG$-OMe (in total 100 mg, 5 µmol,) were added, mixed overnight (total time 12 h). Isolated **9**, yield 15.2 mg (33.6%) with RP-HPLC purity > 99 % (by area of the peak UV_{214} and ELSD detection).

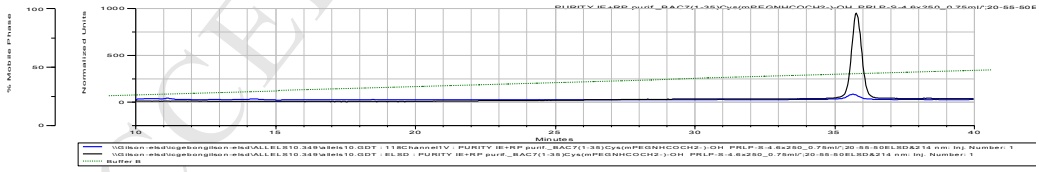
Analysis, 2 steps purification of **9** are shown below:



ligation time 0: Detector traces (ELSD and UV-214nm) from RP-HPLC separation of co-injected **2a** & **6**.



ligation time of **2a** *&* **6**, *12h: Detector traces (ELSD and UV-214nm) from RP-HPLC separation of reaction mixture from preparation of* **9**; *Column PRLP-S 4.6X250 1ml/min;gradient 10-50%B in 30 min*

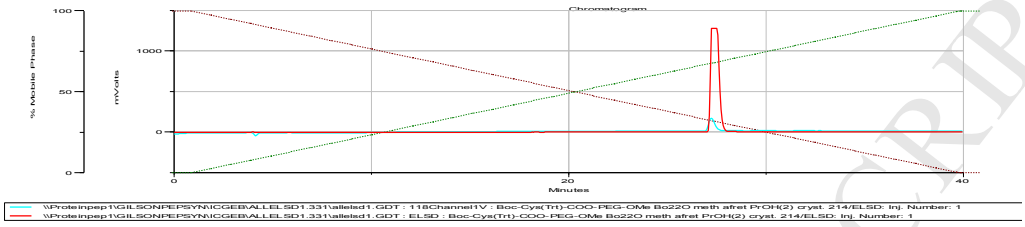


Detector traces (blue line, UV214 nm and black line, ELSD) from RP-HPLC separation of H-Bac-Cys(-CH$_2$CO-NH-PEG-OMe)-OH, **9** *purified by IE (HiTrap™ SP HP 5 ml, Pharmacia, 1.2 M Na+) and desalted/re-purified by semi-preparative RP-HPLC. Purity: UV, peak centered at 35.76 min, > 99%; ELSD, peak centered at 35.65 min, > 99.3%*

10. Preparation of H-Cys(H)-CO-O-PEG-OMe, **10,** *ref.*

10.1. Preparation of Boc-Cys(Trt)-O-PEG-OMe (protected PEG-derivative of **10***)*

1 mmol (10 equivalents) Boc-Cys(Trt)-OH was dissolved in 1.4 ml dry DMF and 0.1 ml dry Pyridine; BOC_2O (0.25 g) were added. The mixture was stirred/sonicated 10 min at room temperature, added to 2 g (0.1 mmol) dry mPEG-OH (Nektar) dissolved in 8 ml dry DCM and shaken overnight. The product was precipitated with Et_2O (8 volumes), filtered, washed with Et_2O (3X5 ml), dried in vacuo at room temperature, re-dissolved in 5 ml DCM, 15 ml PrOH(2) were added, followed of 30 ml Et_2O and cooled for crystallization. The crystals formed were filtered, washed with 20 ml Et_2O, with 20 ml PA and dried in vacuo. Yield 1.9 g (92.8%), purity > 99.9%.

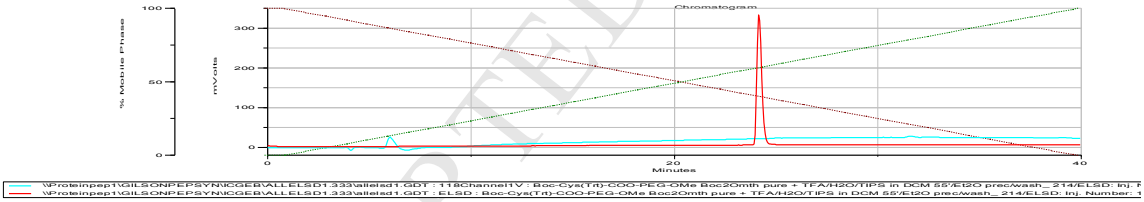


Detector traces (ELSD, red line and UV-214 nm turquoise line) from separation of Boc-Cys(Trt)-CO-O-PEG-OMe (12 h reaction, crystallized from PrOH(2); area peak centered at ~ 27.4 min, > 99.9%); Column 5 µm, 300 Å PLRP-S (4.6X250 mm), flow 0.75 ml/min; gradient 1-41 min (0 to 100 % B).

Peak Name	*R. Time*	*Area %*	*Sample Descrip.*
1	*27.4*	*100.00*	*Boc-Cys(Trt)-COO-PEG-OMe*

10.2. Deprotection of Boc-Cys(Trt)-O-PEG-OMe from 10.1. to **10**

0.205 g Boc-Cys(Trt)-O-PEG-OMe (0.01 mmol) (from Step 10.1) was dissolved in 0.5 ml DCM and 1.5 ml TFA/TIPS/H_2O=(90/5/5). After 1 h the solvent was evaporated under N_2, the rest was mixed with 5 ml Et_2O, precipitated product was separated and washed with Et_2O (2x5 ml), separated by centrifugation, washed with PA (5 ml) and dried in vacuo. Yield 1.98 g. Purity > 99.9%.

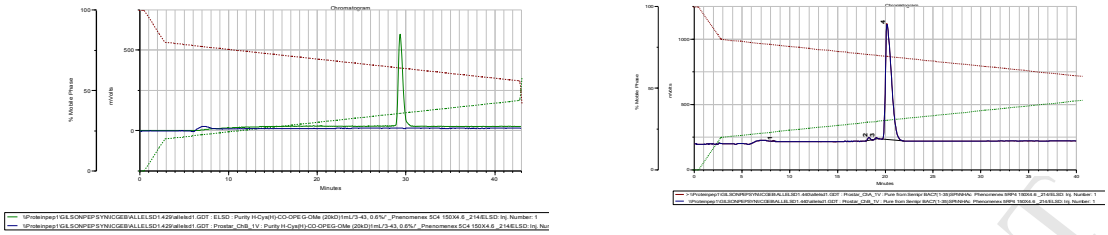


Detector traces (ELSD, red line and UV-214 nm turquoise line) from separation of Boc-Cys(Trt)-CO-O-PEG-OMe (1 h reaction of deprotection). Column 5 µm, 300 Å PLRP-S (4.6X250 mm), flow 0.75 ml/min; gradient 1-41 min (0 to 100 % B).

Peak Name	*R. Time*	*Area %*	*Sample Descrip.*
6	*24.14*	*100.00*	*H-Cys(H)-COO-PEG-OMe,* **10**

11. Preparation of ester H-Bac-Cys(H)-O-PEG-OMe **11** *by NCL of* **3** *and* **10**.

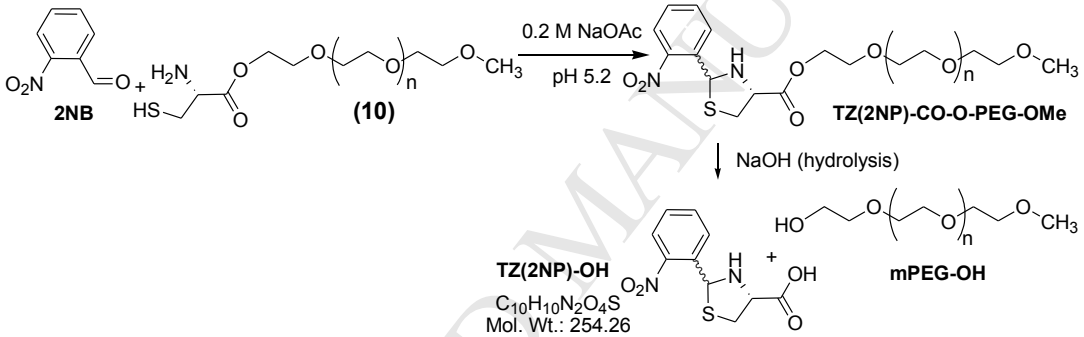
11.1. Purity of starting products **3** *and* **10**.



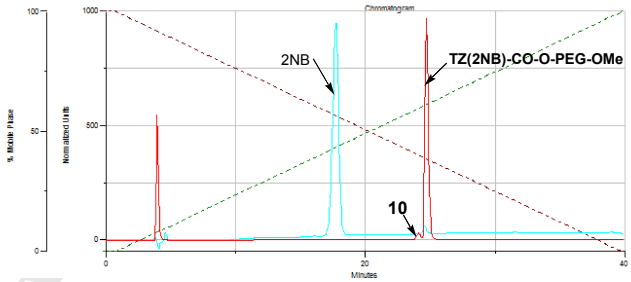
Left panel, detector traces (ELSD) from RP-HPLC separation of **10** *(area > 99%); Right panel, detector traces (ELSD) from RP HPLC separation of* **3** *(area peak 4 > 98.2 %)*
Separation conditions: column 5RP4 (150X 4.6 mm, Phenomenex), flow 0.75 ml/min; gradient 15-47 % B (3-43 min; gradient slop 0.6%/min)

11.2. Thiazolidine ligation of 10 *and 2-nitro-benzaldehyde (2NB).*

(11.2. is model reaction for quantitation and demonstration of presence of free H_2N- and HS- in **10** *by reaction with 2-nitro-benzaldehyde (thiazolidine ligation).*



1 mg (6.6 µmol, 33 equivalents) 2-nitrobenzaldehyde, dissolved in 15 µL MeCN was mixed for 15 min at room temperature with 4.1 mg (0.2 µmmol) H-Cys(H)-O-OPEG-OMe **10** in 85 µL 0.5 M NaOAc pH 5.2. 2 µL from reaction mixture were diluted with 28 µL 1% TFA in water and 15 µL were separated by RP-HPLC (see below).



Detector traces (ELSD, red line and 214 nm, turquoise line) of reaction mixture (thiazolidine ligation, 11.2) of **10** *and 2-nitro-benzaldehyde. Separation conditions: column 5RP4 (150X 4.6 mm, Phenomenex), flow 0.75 ml/min; gradient 0-1 min (0% B, isocratic); gradient 1-100 % B (1-40 min); The peak, centered at 24.8 min was identified as a PEG-ester of 2-(2-Nitro-phenyl)-thiazolidine-4-carboxylate, absorbs at 214 nm, area of the peak (ELSD detection)was >98% from total area.*

Collected main peak at 28.4 min, mPEG-ester of 2-(2-Nitro-phenyl)-thiazolidine-4-carboxylate was freeze-dried, re-dissolved in 9 µL 0.2 M NaOH (pH ~12), 15 min later acidified with 5 µL H_3PO_4 and again separated by RPHPLC to recover starting HO-PEG-OMe and 2-(2-Nitro-phenyl)-thiazolidine-

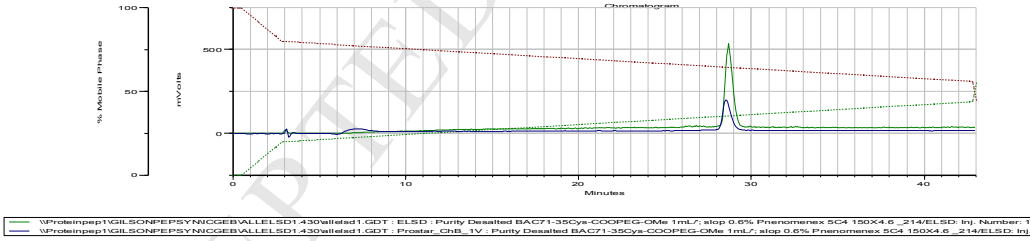
4-carboxylate, TZ(2NP)-OH. Analytical RP-HPLC revealed that TZ(2NP)-OH coeluted with authentic sample and expected ESI+, found 255.0.

11.3. Preparation of **11** *from* **10** *and* **3** *(small scale)*

0.4 ml 1M SPB pH 7.3, containing 10 mmol EDTA, 5 mM TCEP.HCl in an eppendorg tube of 1.5 ml was flushed with N_2 (2 min) and then added to 31.5 mg (~ 3 equivalents) freshly prepared H-Cys(H)-CO-OPEG-OMe **10.** After 30 min this solution was added to 3 mg (~ 0.50 µmol) solid dry powder BAC7(1-35)-SPhNHAc **3**. Two times 100 µL water (each one) were used for quantitative transfer of **10**, the tube was clossed (under N_2) and mixed 16 h at room temperature.
The reaction was quenched by adding 1.2 mg (10 µmol, 20 equivalents) solid cysteine-free base and 100 µL 0.5M SPB, containing 30 mM TCEP and pH was adjusted (if necessary) to pH 7.0-7.3 (universal pH paper), mixed for addition 1 h, diluted with 20 volumes of water, acidified to pH 4-5 with 10% H_3PO_4 and purified in two steps:

Step 1: by IE chromatography (HiTrap™ SP HP (5 ml, Pharmacia) as described for **3** & **8**, briefly:
The product was loaded/washed on Ion Trap HP SP (5 ml, Pharmacia) with 50 mmol Na-phosphate buffer pH 6 for 60 min at flow 1 ml/min, detection at 214 nm. The product was eluted from the column with gradient from 0-1.5 M NaCl in 50 mM SPB pH 6 for 60 min. Collected fractions (27.5 – 34.1 min, ~ 8 ml) corresponding to 0.66 – 0.8 M NaCl *(entrance in the column)* were acidified with 10% H_3PO_4 to pH 3 and re-purified/ desalted in step 2.

Step 2: Collected fractions from step 1 were re-purified/desalted on semi-preparative RP-HPLC column 5RPC4 (250X10 mm), Phenomenex, (as described for **3** & **8**) and collected fractions of purification of purified **11** were freeze-dried. Yield ~ 4.2 mg (33%).



Detector traces (ELSD-green line; 214 nm – blue line) from RP-HPLC separation of **11**, *product from NCL of 3 mg* **3** *and excess of* **10**. *Area of the main peak at 28.7 min (purity of* **11**), *was >99% (both ELSD and 214 nm);*
For Separation condition compare 11.1, where: retention time of **3** *was 20.3 and of* **10** *was 29.7 min.*

11.3. 1. Preparation of **11** *from* **10** *and* **3** *(preparative scale)*

11 was prepared (two times) starting from 10 mg **3**,each one:

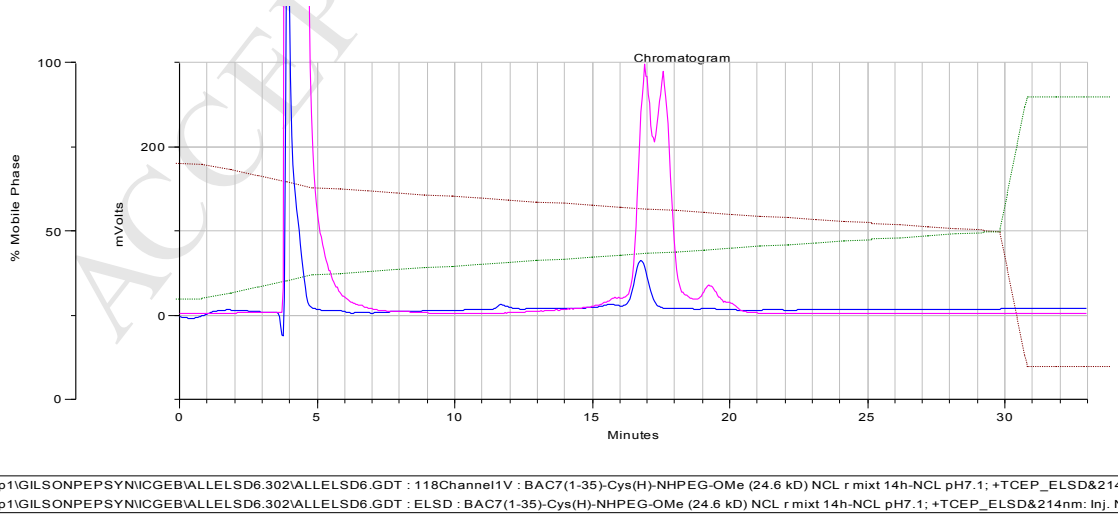
10 mg 1.75 µmol **3** and 80 mg **10** in 1 ml buffer for NCL (see 11.3.) were mixed for 28 h under N_2. Reaction was quenched with 3.6 mg cysteine and 1 mg TCEP at pH 7.0 – 7.3, mixed for additional 2 h, diluted to 20 ml water, filtered, washed on the filter with two portions of 5 ml water, acidified with 10% H_3PO_4 to pH 3.5 and loaded on HiTrap 5 ml SP at 1.5 ml min. The column was washed with in total 30 ml H_2O [For analysis, see bellow, 100 µL (from total ~ 60 ml flow-through fraction) were separated by analytical RP-HPLC: no absorbance at 214 nm, only excess of **10** was eluted; the product **11**, which was

fully retained in the column was eluted with salt gradient (see 13.1, step 1) and fractions which absorbed at 214 nm fraction were collected and re-purified by 3 consecutive column, (~33% from collected fraction per run, see 13.1., step 2) by semi-preparative RP-HPLC. Collected pure fractions (from 3 runs) were freeze-dried.
The yield of **11** from 1st 10 mg **3** was ~ 21 mg (46%).
The yield of **11** from 2nd10 mg **3** was ~ 24 mg (53%).

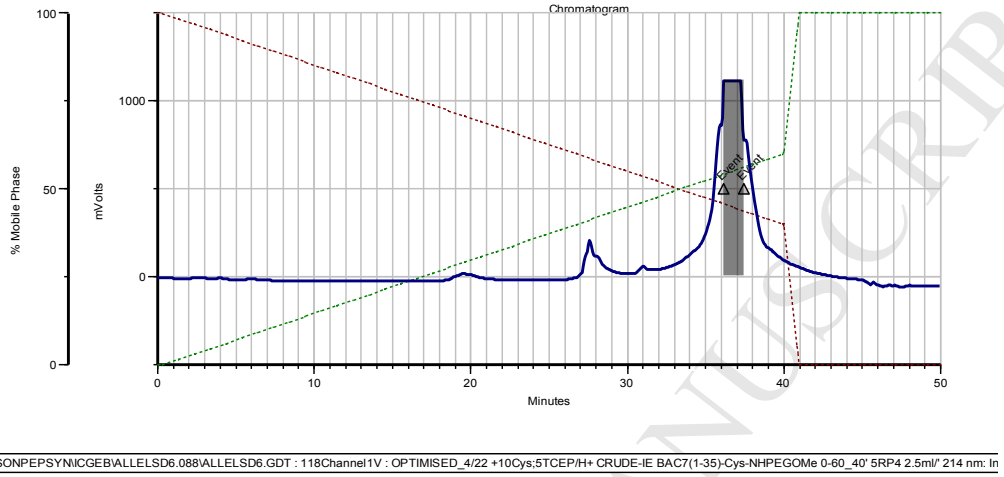
12. Preparation of amide H-Bac-Cys(H)-NH-PEG-OMe, **12** *by NCL of* **3** *and* **7**.

(See preparation of **11**): 9.8 mg (1.66 µmol) **3** and 80 mg (4 µmol ~ 2.4 equivalents) **7** TFA salt in 0.7 ml 0.50 M SPB pH 7.1, containing 0.02 M EDTA and 0.02 M TCEP.HCl were gentile stirred overnight (18h) and then analyzed *(both UV and ELSD detection):*
Analysis 1) ~ 1 µL, corresponding to ~ 130 µg PEGylate product from reaction mixture was mixed (quenched) with 20 µl 10% TFA and 7-8 µL were analysed by RPHPLC; both the product **7** and "thiolactone" H-Cys[Bac7(1-35)]-NH-PEG-OMe were detected.
Analysis 2) separately 1 µL from reaction mixture was mixed with 2 µL 1M SPB pH 7, containing 0.2 M cysteine and 0.04 M TCEP; after 60 min the mixture was diluted with 17 µL 5% TFA and again 7-8 µL were injected in order to eliminate interference of "thiolacton": aas a result was detected only **7**.
After in total 20 h, the reaction mixture was diluted with water to 20 ml, pH was adjusted to 6 with H_3PO_4 and purified from excess of H-Cys-PEG-OMe on 5 ml HiTrap SP HP (Pharmacia), pre-equilibrated in 0.05 M phosphate buffer pH 6, washed with equilibration buffer (25 ml at 3 ml/min) and the product was eluted with 1.5 M NaCL in 0.1 M phosphate buffer pH 7.2 (25 ml). Collected fraction of crude product (~25 ml) were purified/desalted (6 runs each 4.0 ml) on 5RP4 10X250 Phenomenex (2.5 ml/min, gradient 0-60% MeCN containing 0.1% TFA in 40 min at 2.5 ml/min. Pure fractions (both UV/ELSD absorbed) were pooled and freeze-dried. Yield of **12** was 12 mg (1.66 µmol) 28%.

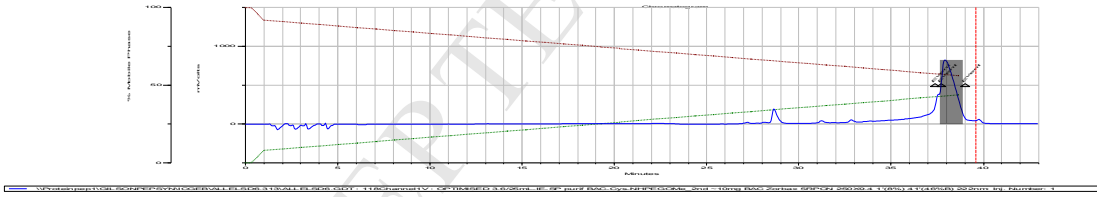
12 was prepared again in 1.66 µmol scale by NCL from **7** and **12** with exception that **12** was purified/desalted by semi-preparative RPHPLC on Zorbax 5RPCN 10X250 Phenomenex (optimized separation conditions: without memory effect and tailing/fronting: for conditions (7 runs each 3.6 ml),. Pure fractions (UV-detection, see below) were pooled and freeze-dried. The yield of **12** was 28 mg, 65.7%.



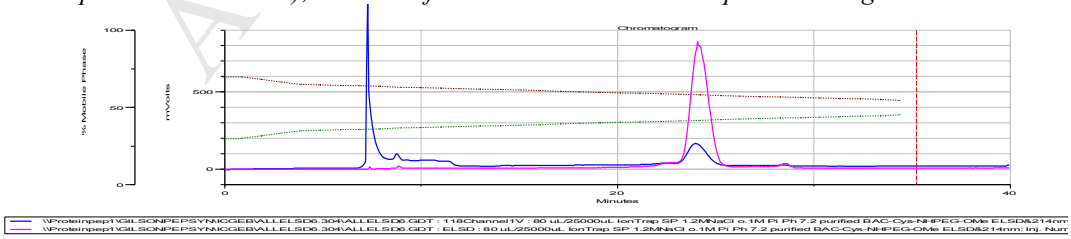
Detector traces (214 nm, blue line; ELSD, pink line) from RP-HPLC separation of reaction mixture from 14h NCL of ***(3)*** *and* ***(7)****. Separation conditions: column PLRP column, gradient 1 min isocratic 30% B; 1-5 min (30-37%B); 5-30 min (37-50 %B), flow 1 ml/min, detection UV 214 (blue line)&ELSD (pink line)*
Peak identification: peak centered 11.7 min, not identified (area < 5% at 214 nm).
Peak centered 15,5 min: "Thiolactone": H-Cys(H-Bac)-NHPEG-OMe
Peak centered at 16.80 min, ligation product **12***: H-BAC-Cys(H)-NH-PEG-OMe;*
Peak centered at 17.70 min, the excess of **7***: H-Cys(H)-NH-PEG-OMe.*



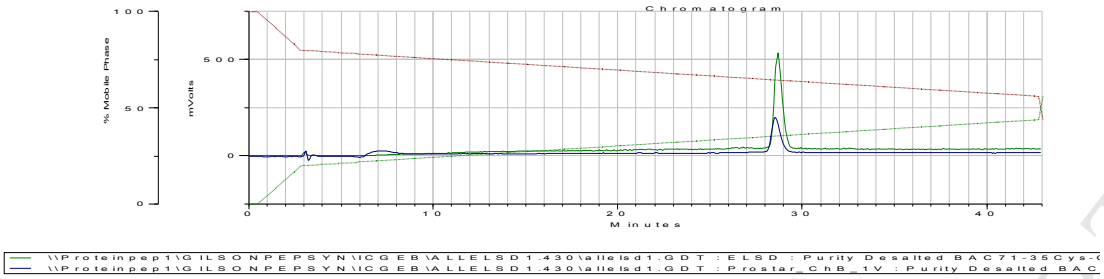
Detector traces (214 nm) from semi-preparative RP-HPLC of 4 ml from total 25 ml fraction from IE separation of **12***, (first 1,66 µmol scale). Separation conditions: column Jupiter 5RP4 (250X10 mm) Phenomenex, not optimized column; 2.5 ml/min gradient slop 1.5%/min MeCN; collected fraction are shown with turquoise rectangle*
(Note: Column 5RP4 is not optimal for separation of **12** *(both fronting and tailing were observed), better separation was performed on semi-preparative 5RPCN-column, see and compare bellow)*



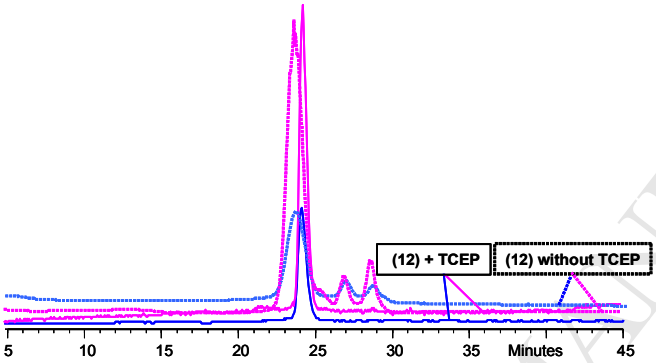
Optimized semi-preparative RP-HPLC (desalting /purification) of 3.6 ml (from total 25 ml fraction from IE separation) of **12***. (second 1,66 µmol scale: where isolated yield of* **12** *was 28 mg, 65.7%)*
Separation conditions: column Zorbax 5RPCN (250X9.4 mm); Gradient conditions : 0.0-0.5 min (0%B), 0.5-1.0 min(0-8%B);1.0-41.0 min (8-46%B); Flow:2.5 ml/min from 0.0 to 1.0 min; then gradient from 2.5 ml/min to 3.0 ml/min at 41.0 min (gradient slop 0.95%/min MeCN); Collected fraction are shown with turquoise rectangle.



Detector traces (blue line- 214 nm; red line ELSD) from RP-HPLC separation of collected fractions from purified **12** *by IE (optimized conditions)*



Detector traces (blue line, 214 nm; green line, ELSD) from RP-HPLC separation of purified & desalted **12**. *The area of the peak centered at 28.7 min was > 98%. The product is free of* **1** *(from hydrolysis of* **3**, *from H-Bac-SPhNHAc* **3** *and H-Cys(H)-NH-PEG-OMe* **7.** *Absorbance ratio ELSD/UV_{214} for* **12** *is 3.36; for* **3** *is 0.34.*



Detector traces (blue line - 214 nm; pink line - ELSD) from RP-HPLC separation of one year old purified **12**: *dashed lines, represent* **12** *not treated with TCEP; plain line, for* **12** *treated with TCEP. Separation conditions: Sample size ~ 80 ug; Column, PLRP-S, 300Å, (4.6X250 mm, Polymer Lab), flow 0.75 ml/min; gradient: 30% MeCN, isocratic for 1min; gradient 30-35% MeCN from 1 to 4 min; gradient 35-47% MeCN from 4-40 min*

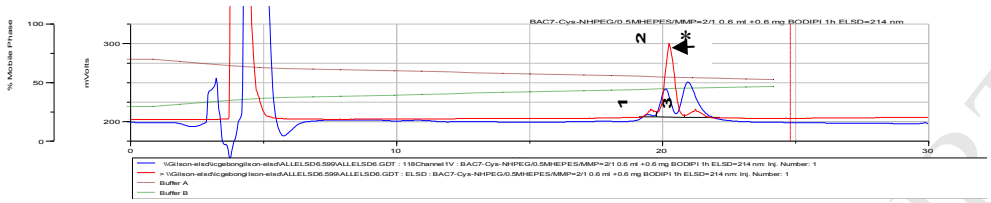
13. Preparation of **13** *by addition of BODIPY maleimide to Bac-ester* **11**; *Preparation of* **15** *by addition of BODIPY maleimide to Bac-amide 12..*

Preliminary experiments (BODIPY stability): BODIPY-reagent was dissolved in MeCN or DMSO and then mixed with SPB buffers dissolved **11** *or* **12**, *containing EDTA and TCEP, in both cases were observed precipitation and/or degradation of BODIPY-reagent.*

13.1. <u>General procedure</u> for preparation of **13** *or* **15** *(according modified and adapted procedure ref. .*

One equivalent freshly purified **11** or **12** in 0.3 – 0.5 M HEPES buffer, containing 5 mM EDTA and 5 mM TCEP.HCl, pH 7.0-7.5 was mixed under N_2 in a dark with 3 equivalents of BODIPY FL N-(2-aminoethyl) maleimide in 1 volume NMP/or DMF (in respect to HEPES buffer) and stirred 1 – 12 h at room temperature. The reaction was quenched with 25-30 equivalents solid cysteine (free base), stirred for additional 0.5 h and pH was adjusted to ~ 3 (H_3PO_4). The product was purified by RP-HPLC.
Preparation of **15,** according the *<u>general procedure</u>*: **12**, 15 mg (0.58 µmol), in 0.4 ml 0.5 M HEPES buffer pH 7.2 was mixed with 0.6 mg BODIPY-FL-maleimide in 0.2 ml NMP. Portions from reaction mixture [~ 2 µL, which correspond to 50-80 µg **12**] were diluted with 30 µL HPLC buffer A and analyzed. Reaction mixture (3h reaction time) was allowed to reacts with cysteine (according the general procedure), diluted/acidified with TFA and then purified by semi-preparative RP-HPLC 5RPCN col-

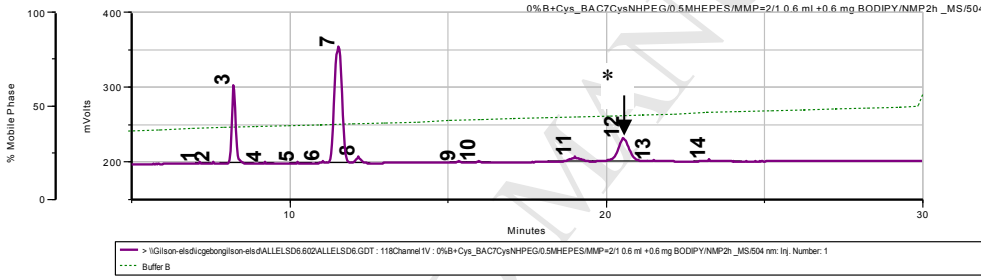
umn (4 runs, shown below), detection at 504 nm. Collected fractions (4 runs) were freeze-dried. Yield of **15** was 6.4 mg (42%).



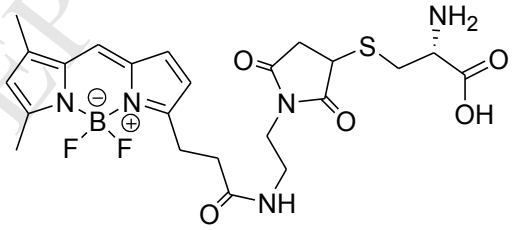
Detector traces (214 nm, blue line and ELSD, red line) from RP-HPLC separation of reaction mixture (1h reaction) of **12** *and 3 equivalents BODIPY NL maleimide. (Note*: difficult separation of peaks 1,2 and 3)*
Peaks identification (ELSD, red traces):

1.	**12**, *Bac7(1-35)-Cys(H)-NHPEG-OMe*	*7.15%*
2	**15**, *Bac7(1-35)-Cys(BP)-NHPEG-OMe at 20.25 min*	*82.15%*
3.	*BODIPY® FL N-(2-aminoethyl) maleimide*	*10.70 %*

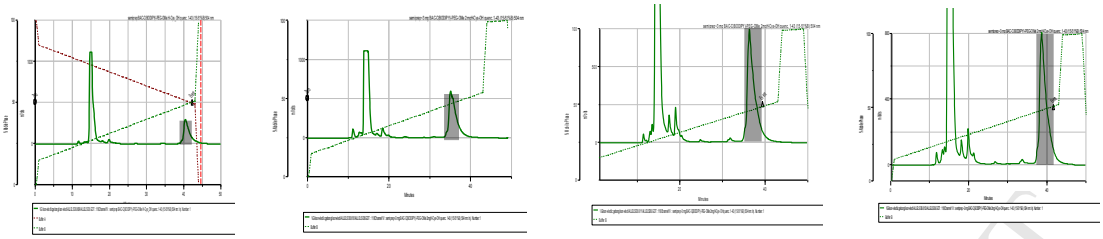
After 2h, 2 µL from reaction mixture was mixed with ~ 12 µg cysteine and 10 min later acidified with H_3PO_4 and analyzed by HPLC:



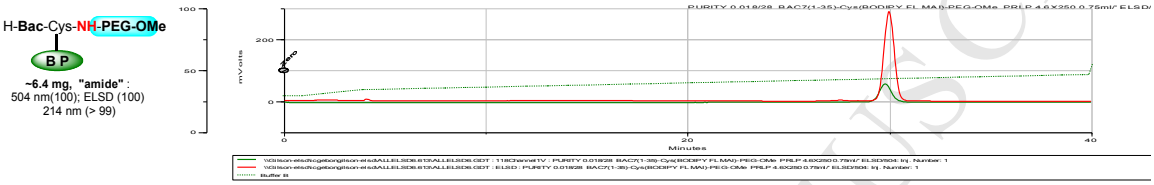
Detector traces (214 ml, violet line) from RP-HPLC separation of reaction mixture (2h reaction; cysteine quenched; H_3PO_4) of **12** *and 3 equivalents BODIPY NL maleimide*
Peak 7 at 11.54 min (55.31%) was identified by ESI-MS as a BODIPY FL N-(2-aminoethyl)maleimide cysteine adduct : MH+ found 536.3 calculated for $C_{23}H_{28}BF_2N_5O_5S$ (535.19-monoisopic); peak 11/12, as a **15***; Area peaks 11+12 (21% from total area); Note*: Product* **15** *(corresponding to peak 12) was easily separated from BODIPY- cysteine adduct, peak 7.*



BODIPI® FL N-(2-aminoethyl) maleimide
cysteine adduct $C_{23}H_{28}BF_2N_5O_5S$ (535.19)



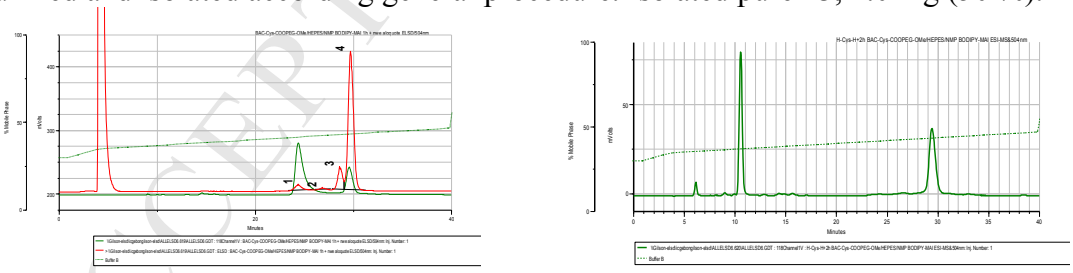
Detector traces (504 nm) from semi-preparative RP-HPLC separation of maleimide adduct of H-Bac-Cys(BP)-NH-PEG-OMe **15**. *Cysteine quenched, acidified reaction mixture was diluted to ~ 3 ml and separated on Zorbax 5RPCN (250X 9.4 mm) column (4 runs); After injection, first washed at flow 2 ml/min (~ 8 min), gradient 0.3-1.0 min(0-15% B) at flow 2.4 ml/min followed of gradient 15 – 52% B from 1.0 min (2.4 ml/min) to 43.0 min (2.7 ml/min. Collected fraction where freeze-dried and are shown with yellow rectangle.*



Detector traces (ELSD, red traces and 504 nm-green trace) from separation of purified **15**.
(main peak at 29.75 min (504 nm), area 100%; at 29.93 min (ELSD), area 99.9%)
Separation conditions: Column 5 µm, 300 Å PLRP-S (4.6X250 mm), flow 0.75 ml/min;

13.1.1. Preparation of **13** *according the general procedure*

7.8 mg (0.379 µmol) **11**, in 0.2 ml 0.5M HEPES buffer, containing 5 mM EDTA and 5 mM TCEP.HCl, pH 7.2, was mixed with 0.4 mg BODIPY-FL-maleimide in 0.1 ml NMP. Portions of from reaction mixture [~ 2 µL, corresponding to ~ 35 µg **11**] were diluted with 30 µL HPLC buffer A and analyzed. After 4 h, **13** was purified and isolated according general procedure. Isolated pure **13**, 4.0 mg (50 %).

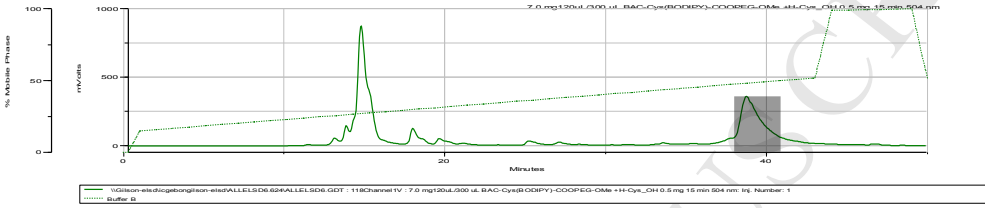


<u>Left panel</u>, detector traces (504 nm, green line and ELSD, red line) from RP-HPLC separation of reaction mixture (2h reaction) of ***(11)*** *and 4 equivalents BODIPY® FL N-(2-aminoethyl) maleimide. <u>Right panel</u>, detector traces (504 nm) after reaction with cysteine*
Separation conditions: Column 5 µm, 300 Å PLRP-S (4.6X250 mm), flow 0.75 ml/min;

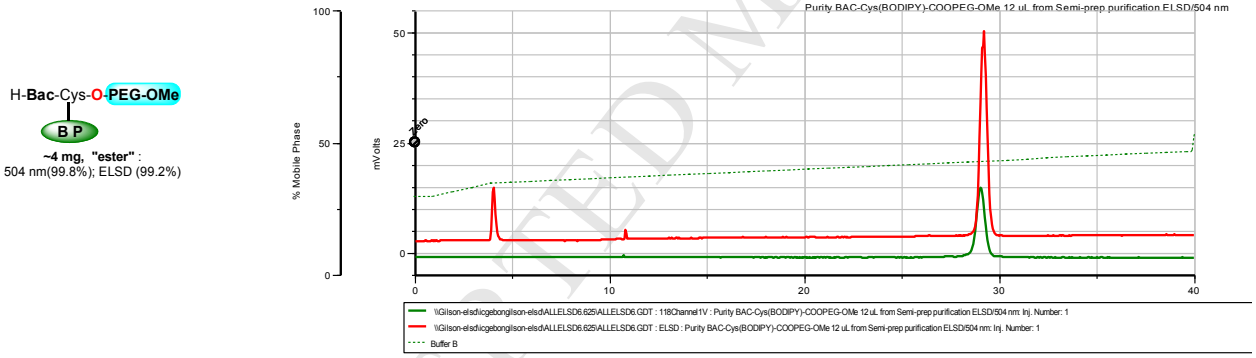
Peak Name	*R. Time*	*Area %*	*Sample Descrip.*
1	*24.27*	*4.64*	*BODIPY® FL N-(2-aminoethyl) maleimide*
2	*26.83*	*1.05*	*?*
3	*28.58*	*11.95*	*?*
4	*29.72*	*82.36*	**13** *Bac-Cys(BODIPY® adduct)-O-PEG-OMe*

Note: ESI-MS was not able to demonstrate MW of the product **13**, *but the peak* ***4*** *at ~ 29.7 min (> 82% from total area) contains PEG (high ELSD signal) and in the same time absorbed both at 214 nm (peptide) and 504 nm (BODIPY).*
When cysteine added to reaction mixture, the peak ***1*** *(left panel) at ~ 24.3, which correspond to BODIPY® FL N-(2-aminoethyl) maleimide, was shifted to ~ 11.5 min (right panel): ESI+ for this was found 536.3; calculated for* $C_{23}H_{28}BF_2N_5O_5S$ *(535.19-monoisopic), correspond again to MW of BODIPY® FL N-(2-aminoethyl) maleimide cysteine adduct.*

After 4.5h the reaction mixture was quenched with 0.5 mg (50-80 equivalents) cysteine for 15 min (pH 7) and separated by RP-HPLC (3 portions (1st ~ 30 µl and two portions of 150 µL, each one). Each one of the 3 portions was diluted to 2 ml with 5% TFA (pH ~ 2.5-3.0) and applied to semi-preparative RP-HPLC separation.

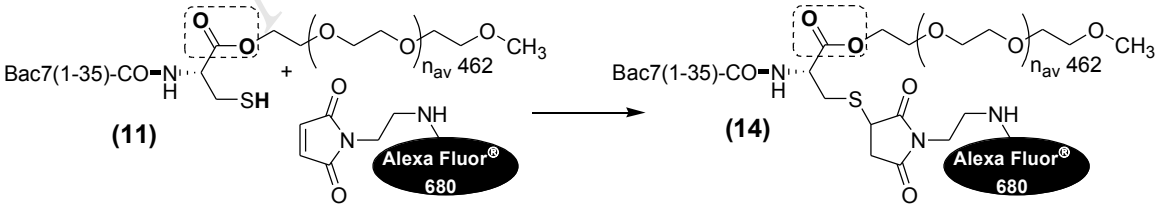


Detector traces (504 nm) from semi-preparative RP-HPLC separation of maleimide adduct of H-Bac-Cys(BP)-O-PEG-OMe **13**. *150 from total 330 µL of reaction mixture reacted for 0.25 h with 0.5 mg cysteine and then acidified with TFA, diluted to ~ 2 ml and separated on Zorbax 5RPCN (250X 9.4 mm) column, washed at flow 2 ml/min (~ 21 min), then eluted with gradient 0.0-0.3 min (0% B – isocratic); gradient 0.3-1.0 min(0-15% B) at flow 2.4 ml/min gradient 15 – 52% B (1.0 – 43.0 min, slop 0.88%/min, Collected fraction (shown with orange rectangle where freeze-dried.*

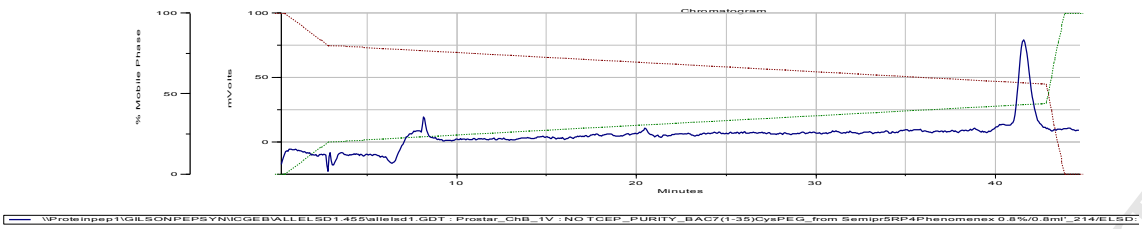


Detector traces (ELSD, red traces and 504 nm-green trace) from separation of purified **13**.
Separation conditions: Column 5 µm, 300 Å PLRP-S (4.6X250 mm), flow 0.75 ml/min;
Purity **13**: *main peak at centered at 29.00 min (504 nm), area 99.8%; at 29.18 min (ELSD), area 99.2%.*

14. *Synthesis of H-Bac-Cys(AF)-CO-OPEG-OMe* **14** *by thioether ligation (Michael addition) of Alexa Fluor® 680 C2-maleimide to H-Bac-Cys(H)-CO-OPEG-OMe,* **11**.



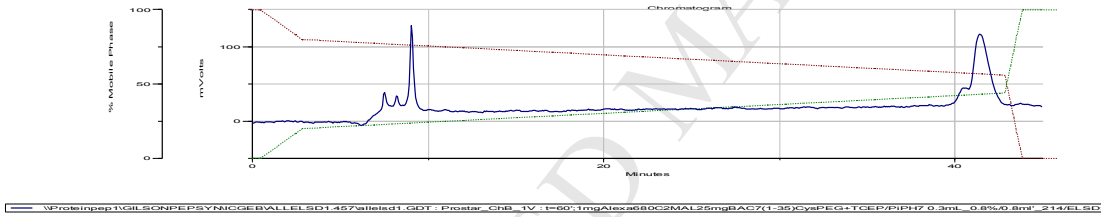
14.1. Analysis of H-Bac7(1-35)-Cys(H)-O-PEG-OMe **11**



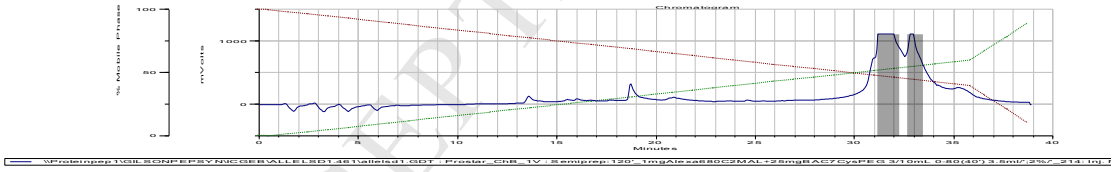
Detector traces (214 nm) from RP-HPLC separation of **11**. *(Note: Without TCEP).*
Separation conditions. Column 5RP4 (4.6X 150 mm, Phenomenex) flow 0.8 ml/min; 0-0.5 min (0& B, isocratic), gradient 0-15% B (0.5 – 3 min); gradient 15 - 47% B (slop 3-43min, gradient slop 0.8%/min . Injection size ~ 30 µg.

14.2. Preparation of H-Bac-Cys(AF)-OPEG-OMe, **14**

1 mg Alexa Fluor® 680 C2-maleimide was mixed for 2 h at room temperature with 25 mg **11** (*see 14.1*), dissolved in 0.4 ml, freshly prepared, N_2 flushed 5 mM TCEP, 5 mmol EDTA, 0.4 M SPB pH 7.0 (green solution formed). Portions (0.2 – 0.3 µL, which correspond to ~ 50 ug **11**) were taken at time 0,1h and 2h, mixed with 20 µL 5% H_3PO_4 (reaction quenched) and analyzed by RPHPLC (no difference in detector traces between 1 and 2 h). After 3 h, the mixture was quenched (pH ~ 3.5 – 4.0) with 5% H_3PO_4, diluted to 21 ml, and separated (3 runs, injected size 7 ml each one) on semi-preparative 5RP4 column 250X10 mm Phenomenex (3 runs: injected for separation, 7 ml each one), gradient ~ 2%/min



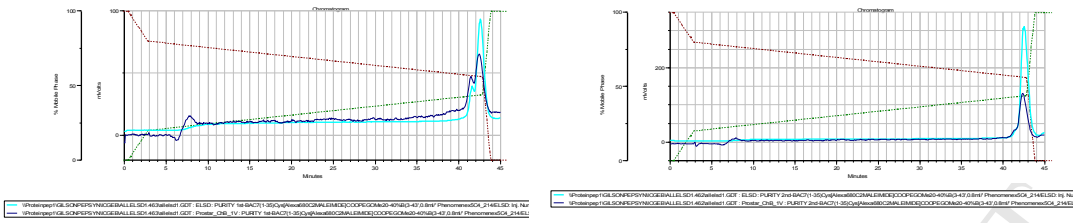
Detector traces (214 nm) from analytical RP-HPLC separation of reaction mixture at 1h reaction – preparation **14**.
Reaction condition: see text. Separation conditions: see conditions for 14.1. & 14.2.



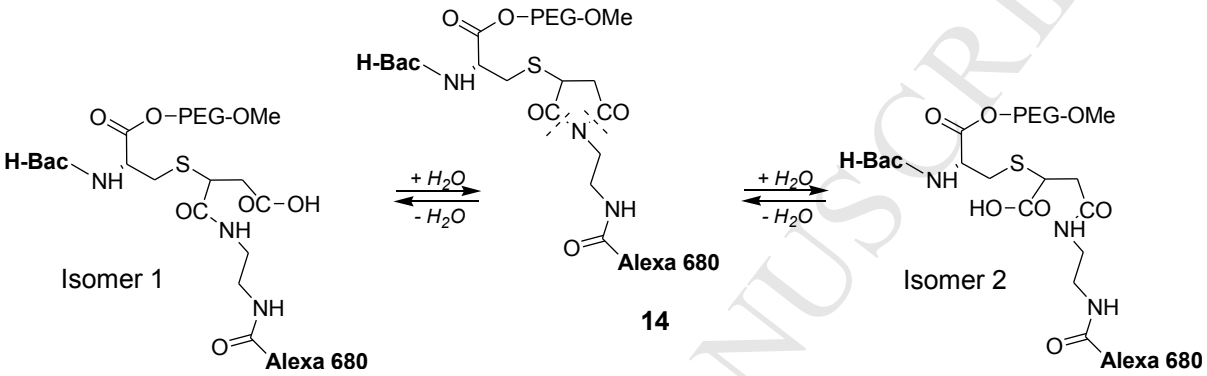
Detector traces (214 nm) from semi-preparative separation of reaction mixture (3 h reaction) from preparation of **14**.
Separation conditions: column 5RP4 (250X 10 mm), Phenomenex, gradient 0-72% B for 36 min (gradient slop 2 %/min); Injection size: 7 ml. Collected fraction from 3 runs (31.2 – 32.0 min, named peak 1, shown as a orange rectangle) and (32.6 – 33.5 min, named peak 2, shown as a blue rectangle) were freeze-dried separately (both freeze-dried products appears as a violet powders). Yields: for peak 1 was 3.0 mg; for peak 2, 5.7 mg.

The procedure 14.2 was repeated one more time. Isolated yield was 7.6 mg.

Portions from isolated and freeze-dried **14** corresponding to collected fractions named peaks 1 & peak 2 were separated by analytical RP-HPLC:



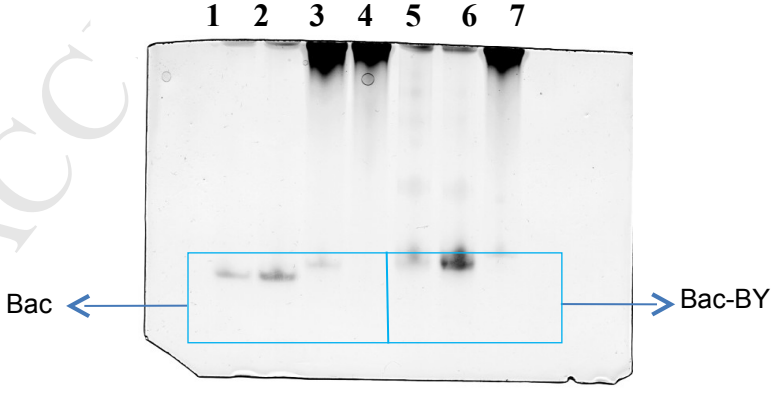
Detector traces (ELSD, turquoise line and UV-214 nm, blue line) from analytical RP-HPLC separation of purified **14.** *Left plot,* **14** *purity peak 1 (3 mg, ~ 80%) and right plot,* **14** *purity peak 2 (5.7 mg, > 93%)*



14 *and its two maleamic acids isomers (plausible explanation of RPHPLC separated two peaks, see above)*

Acetic acid-urea-PAGE

Stacking gel and the Resolving gel o acetic acid-urea-PAGE were prepared respectively at 4% and 16% of acrylamide in Solvent running (4M urea, 0.45N CH_3COOH). Once prepared the gel, it has been set on a vertical electrophoresis system and immerse in the Running buffer (5% CH_3COOH in milliQ water). A pre-running at 200V was necessary until the stabilization of the amperage. Samples were prepared with the addition of Loading Buffer 2x (40% glycerol, 100mM DTT melt in solvent running) and loaded 10 µl. The running was setting at a constant voltage of 200V for 75 min. The gel was stained overnight by Coomassie blue and destained with 10% acetic acid solution.



Acetic acid-urea-PAGE of the pegylated forms of Bac7(1-35). Lane: Bac 1 µg (1) or 2 µg (2), 4 µg of BacE-PEG (3) and BacA-PEG (4), Bac-BY 1 µg (5) or 2 µg (6), 4 µg BacE-PEG -BY (7).

After the purification by high-performance liquid chromatography (HPLC) and analysis by mass spectrometry, the purity and the completeness of the reaction of the four pegylated Bac7 peptides have been also checked by acetic acid-urea-PAGE to avoid the basic conditions of the Tricine-SDS-PAGE which making unstable the ester bond.

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