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O–N-Acyl migration in N-terminal serine-containing peptides: mass spectrometric elucidation and subsequent development of site-directed acylation protocols

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Abstract—The synthesis of a modified pentapeptide involving the palmitoylation of the hydroxyl group of a serine residue present at the N-terminal position is presented. An O–N-acyl shift was observed by LC/MS/MS, the two isobaric molecules exhibiting upon collisional activation dissociation (CAD) different fragmentation behaviours. The synthetic pathway was thereafter modified to control the palmitoylation site (O or N). The method was validated with another serine acylation (octanoylation). The evidenced mass spectrometric criteria could serve to decipher peptide post-translational modifications in proteomics.

Among post-translational modifications in peptides, acylation of hydroxylated amino acid residues, particularly serine residues, is an important phenomenon. As an example, the peptide hormone ghrelin is octanoylated on a serine residue and this modification is crucial for the biological activity.[1] In order to study in more detail modifications affecting the serine residue in peptides, we synthesized on solid support a series of pentapeptides having a serine at the N-terminus. This serine was then acylated on the resin and the compounds were analysed by mass spectrometry.

The first strategy used to synthesize the O-Palm pentapeptide is described below (Scheme 1, path a). The pentapeptide **1** was prepared according to standard Fmoc SPPS on a Rink amide polystyrene resin.[2] Fmoc-Ser(Trt)-OH was used at the last coupling cycle of the synthesis. At the end of the chain elongation, the trityl group masking the serine hydroxyl function was selectively removed under mild acidic conditions.[3] On-resin esterification of the free alcohol function by palmitic acid chloride was then performed to yield the anchored peptide **2**.[4] After Fmoc deprotection, the expected modified peptide H-Ser(Palm)-Phe-Leu-Leu-Arg-NH_2 was released from the resin upon treatment with a solution of TFA/H_2O/triisopropylsilane (95/2.5/2.5) for 4 h. Analysis of the crude mixture by LC/UV/ESI-MS[5] indicated the presence of two isobaric compounds of the expected mass, $(M+H)^+ = 872.7$ Th. As an illustration, the extracted ion chromatogram corresponding to the detection of the protonated molecule (mass 872.7 Th) is reproduced in Figure 1a.

On the other hand, the N-Palm pentapeptide analogue was prepared starting from resin-tethered pentapeptide **1** (Scheme 1, path b). After Fmoc deprotection and N-acylation with palmitic acid chloride, the expected modified peptide Palm-Ser-Phe-Leu-Leu-Arg-NH_2 was released from the resin and fully deprotected upon treatment with TFA/TIS/H_2O (95/2.5/2.5) for 4 h. As for the first experiment, two isobaric compounds were detected by LC/UV/ESI-MS (Fig. 1b).

The presence of two isomers can be explained by the migration of the palmitoyl substituent. O–N-Acyl shift of β-aminoalcohols is a well-known reversible side reaction in peptide synthesis (Scheme 2). This intramolecular rearrangement leads to the transfer of the peptide backbone onto the hydroxyl groups of serine residues under acidic conditions.[6] In contrast, under basic conditions such as Fmoc removal using piperidine solution, nucleophilic attack of the free ε-amino group of O-acylated serine induces an O–N-acyl transfer.[7] In

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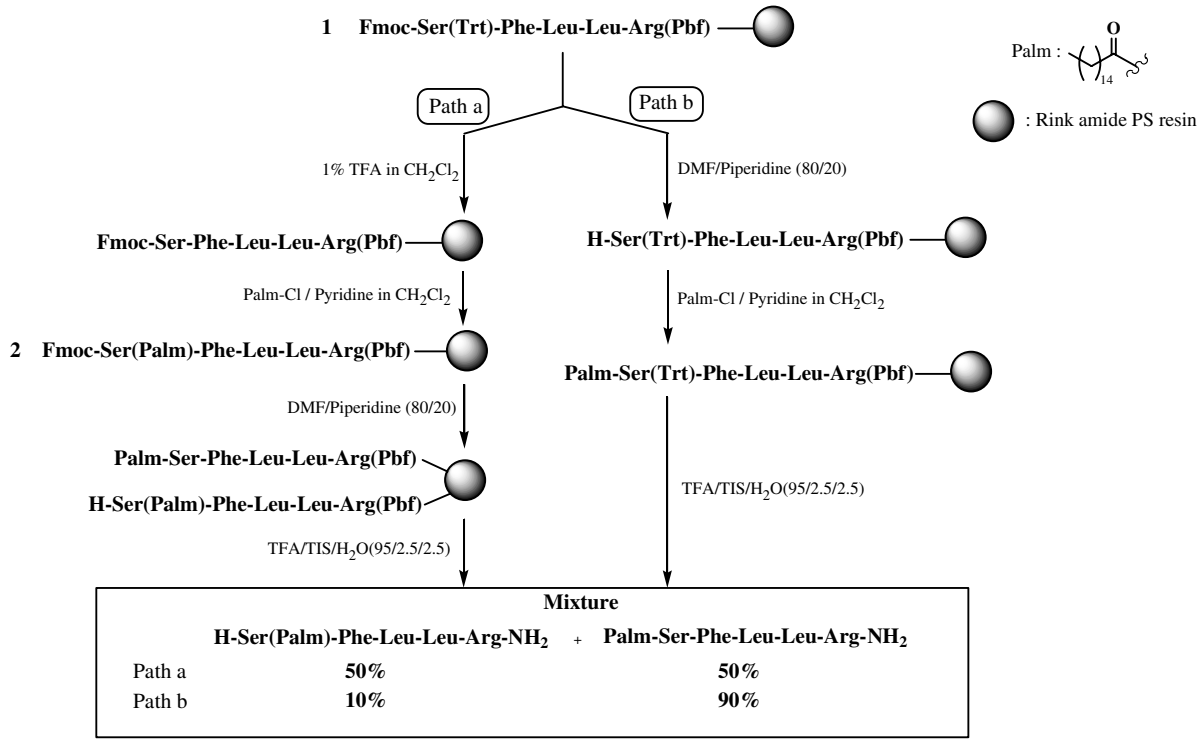


Scheme 1. Non-optimized SPPS syntheses of O- and N-Palm N-terminal serine-containing peptides.

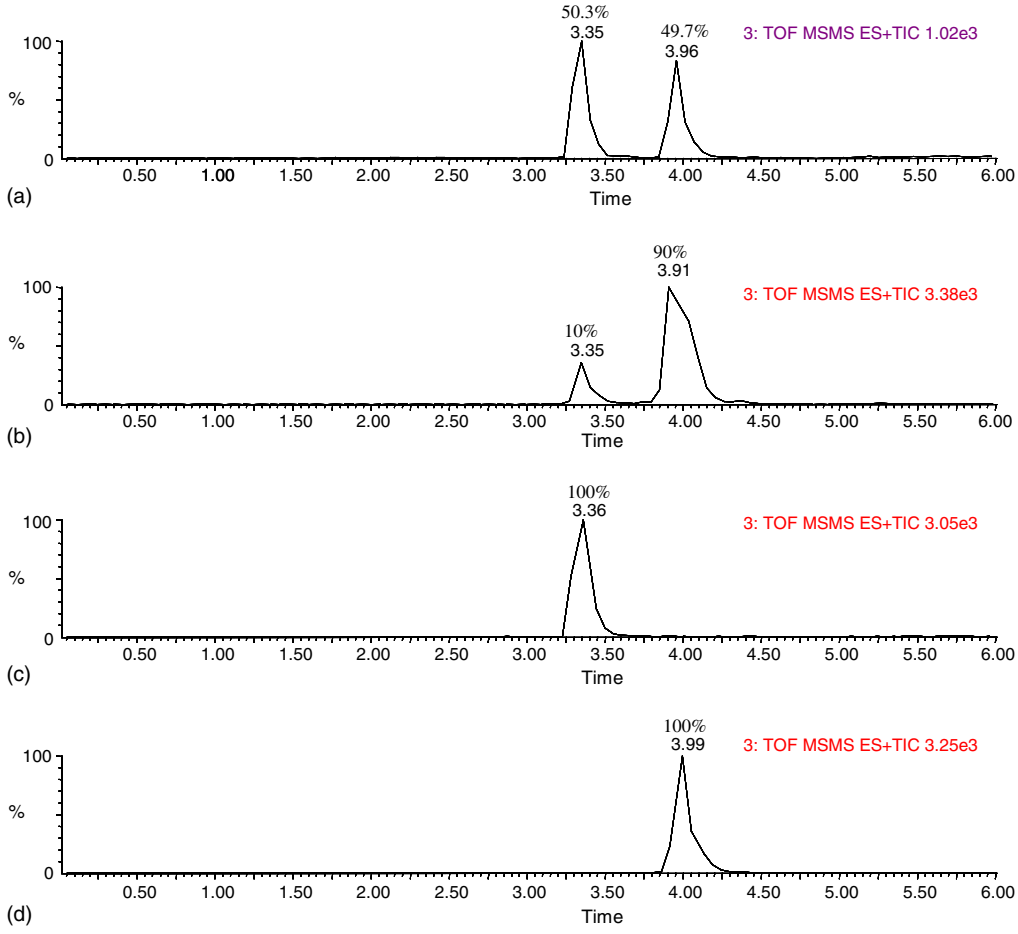
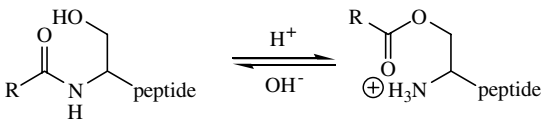


Figure 1. Extracted ion chromatogram (mass 872.7 Th) of the crude mixture: (a) from Scheme 1, path a; (b) from Scheme 1, path b; (c) from Scheme 4, path a, and (d) from Scheme 4, path b.



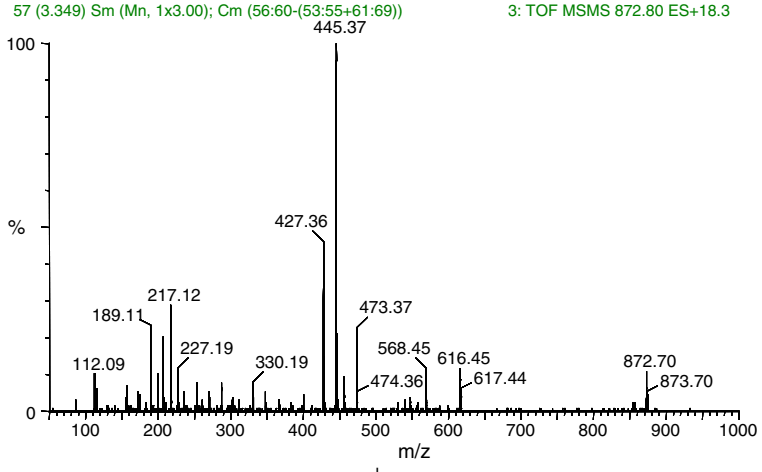
Scheme 2. Reversible O–N-acyl shift.

this study, the acyl migrating group is not a peptide chain and the rearrangement is probably favoured.

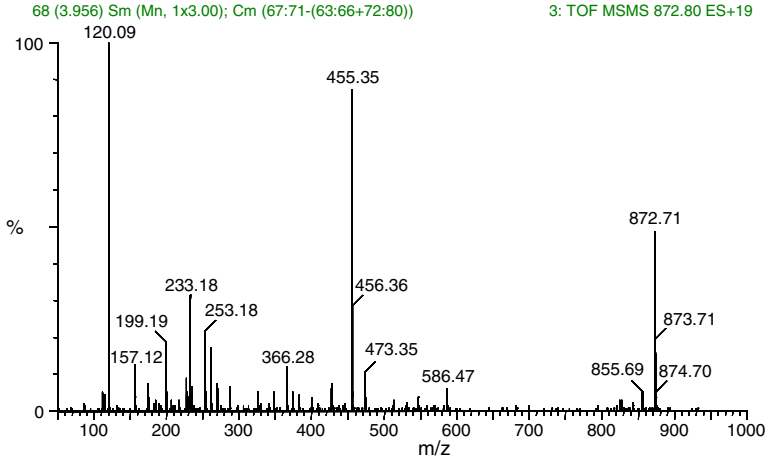
LC/MS/MS experiments were conducted to sequence the modified peptides and thus validate this hypothesis. Differences between the two isomer CAD mass spectra were clearly seen as shown in Figure 2a and b (analysis of the crude mixture recovered from Scheme 1, path a). Under the same CAD conditions each isomer exhibited a specific fragmentation pathway from the protonated molecules at 872.7 Th:

- loss of palmitic acid (256 Da) leading to the ion at 616 Th for the more polar compound (Rt = 3.35 min),
- no loss of palmitic acid for the less polar compound (Rt = 3.96 min).

To explain this loss of palmitic acid from the protonated molecule of O-Palm peptide, one should consider the sites of protonation available on the peptide: either an oxygen atom, especially due to the presence of a serine residue, or a nitrogen atom (amine, amide and guanidine functions). Protonation of nitrogen leads to relatively stable protonated molecules whereas protonation of oxygen could promote extensive dehydration.[8] Protonation of the oxygen of the serine side chain should thus give rise to the loss of a neutral molecule: either water when the hydroxyl group is free or a carboxylic acid molecule when the hydroxyl group has been acylated. Therefore, the observed loss of palmitic acid described in Scheme 3 indicated that the palmitoyl substituent was located on the side chain. The position of the palmitoyl group was further confirmed by the relative retention times of the two detected compounds. The most polar compound eluted at 3.35 min should correspond to the expected peptide with a free N-terminal amino group, H-Ser(Palm)-Phe-Leu-Leu-Arg-NH_2, whereas the less polar structure at 3.96 min should refer to the *N*-palmitoyl peptide, Palm-Ser-Phe-Leu-Leu-Arg-NH_2. So, the loss of the neutral molecule of palmitic acid only occurred in the case of O-palmitoylation.

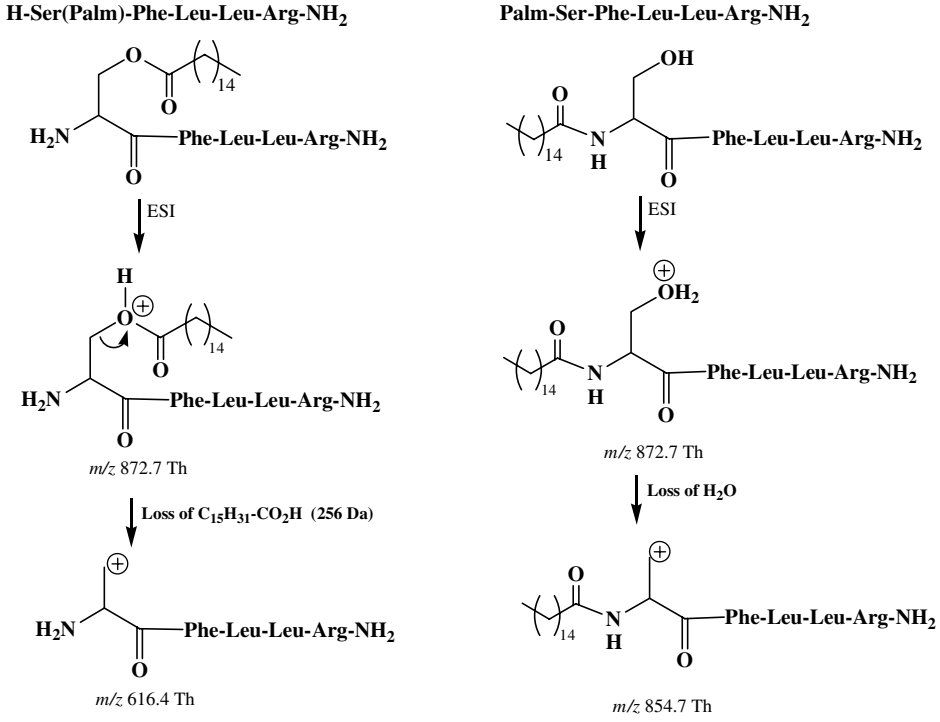


(a) CAD of MH^+ of *O*-Palm peptide



(b) CAD of MH^+ of *N*-Palm peptide

Figure 2. CAD mass spectra of the O- and N-palmitoylated peptides obtained via Scheme 1, path a.



Scheme 3. O- and N-Palmitoylated pentapeptides.

Among the detected sequence fragment ions, it should be noted that dehydration was observed for N-acylated molecules as expected. The loss of water was particularly abundant in the CAD mass spectra of the protonated molecules but was greatly dependent on the value of the collision energy. Since the loss of palmitic acid was never observed for N-acylated molecules whatever the CAD tuning, the presence of the ion at 616 Th constituted the relevant criterion to distinguish between the two isomers.

These mass spectrometric criteria were used to develop a new synthetic protocol aimed at directing the site of acylation and thus produce solely *O*-acyl or *N*-acyl peptides.

To generate the O-acylated peptide, basic conditions have to be avoided while deprotecting the N-terminal serine residue. Thus, the base-labile N-terminal Fmoc protecting group was replaced, on resin, by the acid-labile Boc protecting group as described in Scheme 4, path a. Afterwards, selective trityl removal in mild acidic solution followed by the acylation step were performed. Simultaneous N-terminal Boc deprotection and peptide cleavage from the resin was achieved in acidic medium, preventing the palmitoyl migration, the free amine being quenched as a trifluoroacetate salt. LC/MS/MS analysis showed a single compound (99%) with a retention time of 3.36 min corresponding to the more polar compound (Fig. 1c). In the CAD mass spectrum of the protonated molecule, this compound exhibited the expected loss of palmitic acid, yielding the ion at 616 Th.

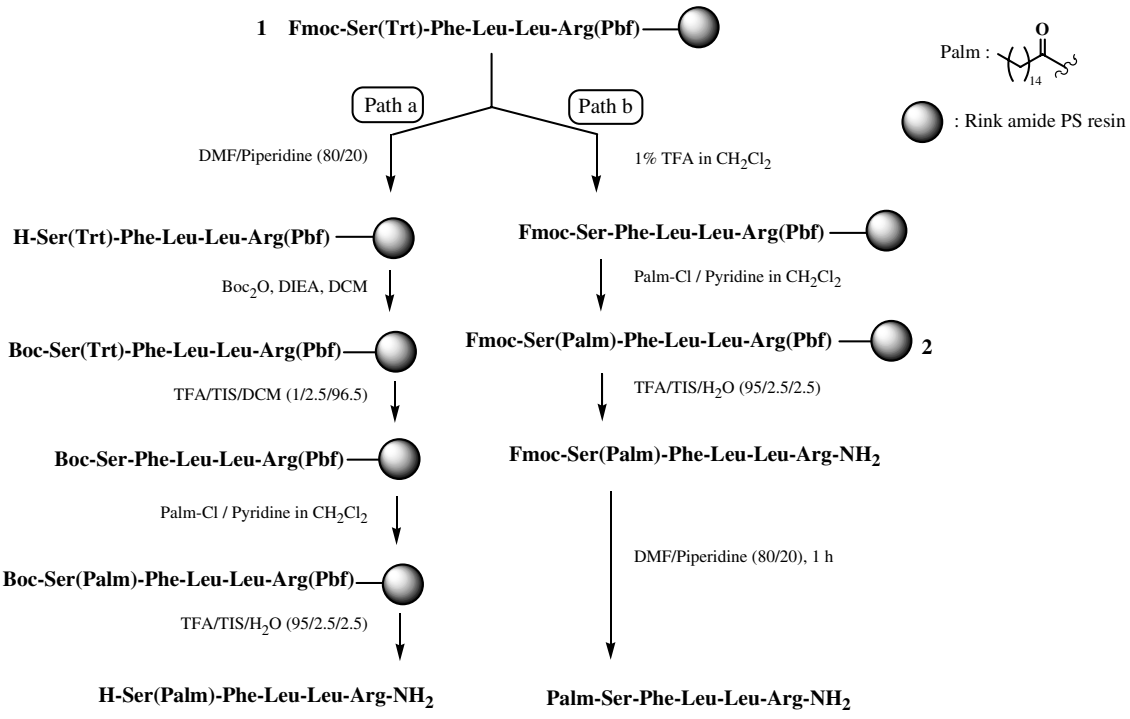
To synthesise selectively the N-Palm peptide analogue, we decided to take advantage of the O–N-acyl shift rearrangement (Scheme 4, path b). Resin-bound Fmoc-protected O-palmitoylated pentapeptide **2** was first released from the resin by TFA treatment. The Fmoc protecting group was then removed in solution. In this case, only the less polar compound was observed (Fig. 1d). As basic treatment was the last step of the synthesis, we hypothesized that the less polar compound ($Rt = 3.99$ min) was the N-Palm pentapeptide. This hypothesis was validated by CAD analysis.

In summary, two synthetic protocols have been developed to prepare selectively O- or N-palmitoylated peptides:

- O-palmitoylation: use of N-terminal Boc protection prior to side chain serine deprotection and esterification (Scheme 4, path a),
- N-palmitoylation: release in solution of the N-terminal Fmoc-protected peptide and deprotection of the Fmoc group in basic medium to achieve complete transfer of the palmitoyl group from the serine side chain onto the N-terminal position (Scheme 4, path b).

Finally, the influence of the nature of the acyl group as well as the position of the serine in the peptide chain was studied. Esterification by *n*-octanoic acid instead of palmitic acid was performed. Hexapeptides bearing an extra alanine residue in the N-terminal position were produced and submitted to selective O- or N-acylation. Compounds (listed in Table 1) were prepared specifically using the methods previously described.

O–N-Acyl migration was observed in N-terminal serine-containing peptides whatever the nature of the acyl



Scheme 4. Selective synthesis of O- or N-palmitoylated N-terminal serine-containing peptide.

Table 1

Compound[a]	$(M+H)^+$ (Th)	Mass of R–COOH (Da)	Fragment ion issued by the loss of RCOOH (Th)
H-Ser(Palm)-Phe-Leu-Leu-Arg-NH_2	872.7	256	616.4
Palm-Ser-Phe-Leu-Leu-Arg-NH_2	872.7	256	—
H-Ser(*n*-Oct)-Phe-Leu-Leu-Arg-NH_2	760.5	144	616.4
n-Oct-Ser-Phe-Leu-Leu-Arg-NH_2	760.5	144	—
H-Ala-Ser(Palm)-Phe-Leu-Leu-Arg-NH_2	943.8	256	687.5
Palm-Ala-Ser-Phe-Leu-Leu-Arg-NH_2	943.8	256	—
H-Ala-Ser(*n*-Oct)-Phe-Leu-Leu-Arg-NH_2	831.6	144	687.5
n-Oct-Ala-Ser-Phe-Leu-Leu-Arg-NH_2	831.6	144	—

[a] Palm and *n*-Oct represents $C_{15}H_{31}$–CO and C_7H_{15}–CO, respectively.

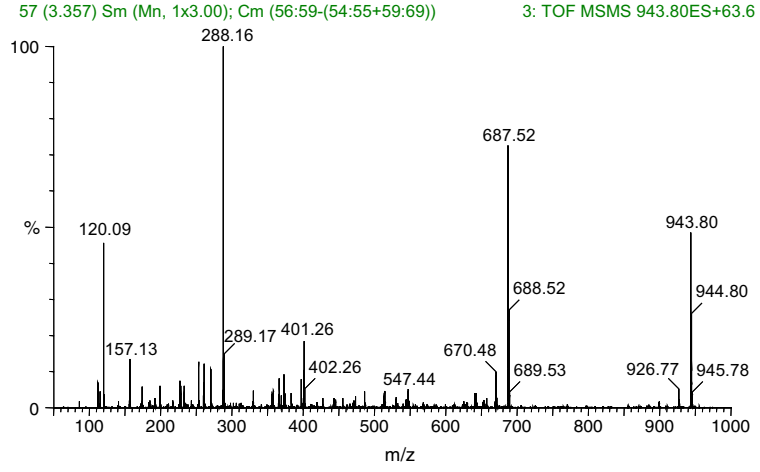


Figure 3. CAD mass spectrum of the protonated molecule H-Ala-Ser(Palm)-Phe-Leu-Leu-Arg-NH_2.

group (*n*-Oct and Palm). Moreover, such isomerisation was only observed when the serine residue was located at the N-terminal position.[9] In all cases, the mass spectrometric criteria described were verified. The loss of

palmitic acid (256 Da) or *n*-octanoic acid (144 Da) was exclusively observed when the acyl group was present on the serine side chain (O-acylation) whatever the position of this residue in the peptide chain. For instance, the CAD mass spectrum of the hexapeptide, H-Ala-Ser(Palm)-Phe-Leu-Leu-Arg-NH_2, is reproduced in Figure 3. No neutral loss was detected for any of the N-acylated peptides.

In a single run, the LC/MS/MS analysis allowed the detection of the presence of two isobaric compounds and the assignment of the acylation site. Palmitoylation or *n*-octanoylation of serine residues may occur during protein post-translational modification. In proteomics such side chain acylation can be evidenced by the loss of the corresponding neutral carboxylic acid.

References and notes

1. Benarek, M. A.; Feighner, S. D.; Pong, S. S.; McKee, K. K.; Hreniuk, D. L.; Silva, M. V.; Warren, V. A.; Howard, A. D.; Van Der Ploeg, L. H.; Heck, J. V. *J. Med. Chem.* **2000**, *43*, 4370–4376.
2. Solid phase synthesis was performed on an automated synthesiser ACT496 Ω. The serine side chain was protected with a trityl group (Trt) and the arginine side chain was protected with a Pbf group (pentamethyldihydrobenzofuran-5-sulfonyl). Each well was filled with 150 mg of PS-Rink amide resin (Novabiochem). For the coupling step, three solutions were successively added to the reaction vessels: 400 μL of a 0.5 M solution of *N*-Fmoc protected amino acid (Senn Chemicals) in *N*-methyl pyrrolidone; 400 μL of a 0.5 M solution of *N*-methylmorpholine in DMF and 400 μL of a 0.5 M solution of HBTU in DMF. The coupling time was 90 min and deprotection (20 min) was carried out using 1.2 mL of DMF/piperidine 80/20 (v/v) solution. Five washes of 1.5 mL were used between steps including DMF (×2), DCM (×1), methanol (×1) and DMF (×1). Side chain deprotection and cleavage of peptides was performed for 2 h in the robot's 96-reactor block using 1.5 mL of cleavage cocktail (trifluoroacetic acid/water/triisopropylsilane 95/2.5/2.5, v/v/v) per well. After removal of the resin by filtration, peptides were precipitated in diethyl ether and pelleted by centrifugation. The ether was decanted off and the crude peptide was dissolved in acetonitrile/water (50/50, v/v) containing 0.1% TFA.
3. On-resin trityl deprotection was performed by adding TFA/TIS/DCM (1/2.5/96.5, v/v/v) solution to the resin beads. After 30 min, the resin was washed three times with DCM and the deprotection solution was added for a further 30 min. This deprotection cycle was repeated until trityl removal was complete, as monitored by HPLC analysis of the deprotection filtrate solution.
4. On-resin palmitoylation was performed using pyridine (30 equiv, 1 M) and palmitoyl chloride (15 equiv, 0.5 M) in DCM for 90 min. The resin was then washed with (DCM (2×), DMF (1×), MeOH (1×) and finally DCM (1×)).
5. ESI mass spectra were recorded on a QTof I mass spectrometer (Waters-Micromass, Manchester, UK) fitted with a Z-spray ionisation source. Calibration was performed with an aqueous solution of phosphoric acid (0.1%) in the mass range of 50–1000 Da. Nitrogen was used as both nebulising and drying gas. The temperature of the source and of the drying gas was set at 80 and 150 °C, respectively. The capillary voltage was set at 2900 V whereas the cone voltage was fixed at 30 V. Argon was chosen as the collision gas in MS/MS experiments. The collision energy was optimised for each sample. A Waters 2790 HPLC module equipped with an autosampler and a diode array UV detector PDA 996 (Waters-Micromass, Manchester, UK) was coupled to the mass spectrometer. Elution was performed in the HPLC module on a C-18 reversed phase column (Symmetry Shield, 50 × 2.1 mm, 3.5 μm) at a flow rate of 0.6 mL/min under gradient conditions from 100% solvent A (water 0.1% formic acid) to 100% solvent B (acetonitrile 0.1% formic acid) in 5 min. The flow was split prior to the mass spectrometer entrance allowing roughly 1/10 of the eluent in the ESI source. The MassLynx 4.0 software (Waters-Micromass, Manchester, UK) was used to control the equipment. All samples were dissolved in a solution composed of water/acetonitrile/formic acid (49/49/2, v/v) and 5 μL were injected for each experiment.
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9. Special care must be taken during purification and storage. After purification by RP-preparative HPLC using acidic solvents, compounds separated must be quickly frozen and freeze-dried to avoid O–N-acyl transfer. This isomerisation was promptly observed in solution in the case of N-terminal serine-containing peptides but occurred very slowly if the serine was located in the peptide chain.