New cationic amphiphilic compounds as potential antibacterial agents

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6.1 | Introduction

The use of fluorous techniques[1] for the separation of reaction mixtures has found wide attraction in synthetic organic disciplines in recent years.[2] An important development comprises the so-called 'light-fluorous' strategy, developed by Curran and co-workers.[1a,3] In this strategy, compounds differing in fluorine content are separated by chromatography using a fluorous stationary phase. For this purpose, an array of fluorinated handles have become available in recent years, including fluorous versions of the Z,[4] Boc,[5] tBu,[6] Bn,[7] THP,[8] acyl-based,[9] silyl-based,[10] and alkoxyethylether[11] protecting groups.

6.2 | Fluorous Techniques in SPPS

Although synthetic peptides containing up to 50 residues have become available *via* improved solid-phase synthesis techniques in recent decades, facile HPLC purification of the desired full-length peptide (of n residues) is often hampered by the presence of incomplete sequences (*e.g.* n-1 and n-2 sequences). This is especially true for longer peptides as the chromatographic behavior of full-length and n-1 and n-2 sequences becomes less distinguishable with increasing number of residues, resulting in tedious and lengthy HPLC purification procedures along with unavoidable handling losses. This Chapter describes two approaches for application of fluorous strategies in the purification of synthetic peptides.

6.2.1 | Fluorous chromatography

Introduction of long, polyfluorinated alkyl groups increases both hydrophobicity and fluorophilicity of molecules. To verify that the 'light-fluorous' strategy relies on fluorophilic rather than hydrophobic properties of compounds, a test peptide GEPKPAG was equipped with two handles differing from each other only in fluorine content. Thus, in order to examine their influence on the chromatographic behaviour of the peptide, the peptide was equipped with either a lipophilic *n*-undecanoyl ($C_8H_{17}(CH_2)_2COR$) moiety or a fluorinated alkanoyl handle ($C_8F_{17}(CH_2)_2COR$) were introduced.

The three peptides GEPKPAG, **L**-GEPKPAG (lipophilic) and **F**-GEPKPAG (fluorophilic) were synthesized via standard Fmoc-based SPPS procedures and evaluated for their affinity towards a FluoPhase LC column, the stationary phase of which is coated with polyfluorinated alkyl chains. Elution of the three peptides occurred with a gradient of 20→100% TFE in 0.1% aq. TFA in 18min. GEPKPAG, a peptide that is neither lipophilic nor fluorophilic, was expected to have minimal retention on the FluoPhase column; indeed, it elutes in the injection peak (Rt < 2min, not shown). Peptide **L**-GEPKPAG shows a distinct retention time (Rt 7.21min, Figure 1, trace A), indicating there is a significant hydrophobic interaction with the FluoPhase column. Fluorophilic peptide **F**-GEPKPAG however displays a significantly larger retention time than **L**-GEPKPAG (Figure 1, trace B), which is indicative of additional effects and confirms the proposed concepts of 'light-fluorous' chromatography techniques.

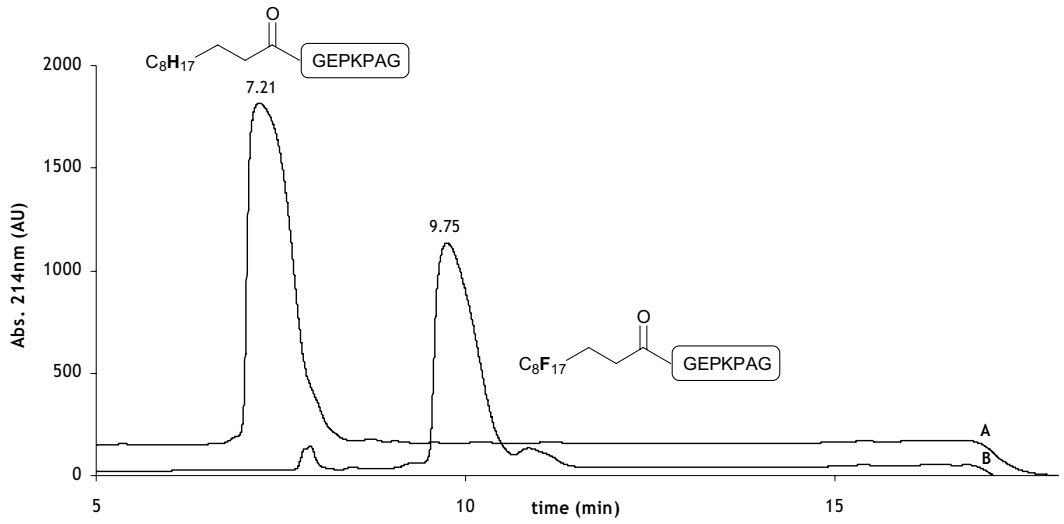
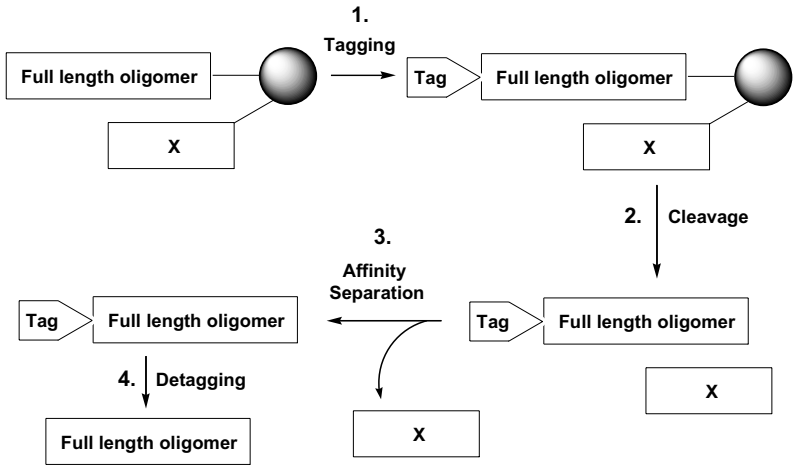


FIGURE 1 | Chromatograms (5-18min) of peptides **L**-GEPKPAG (trace A) and **F**-GEPKPAG (trace B) on a FluoPhase C_{18} RP column, applying a gradient of 20→100% TFE/H_2O + 0.05% TFA in 13min, with UV detection at 214nm.

6.2.2 | Fluorous Strategies in SPPS

The application of fluorous handles promises to be a valuable asset in stepwise solid-phase synthesis procedures. Target molecules, of which the purification from final product mixtures is hampered by the occurrence of closely eluting impurities, can be effectively isolated with the aid of fluorous-based separation.



SCHEME 1 | Schematic representation of tagging strategy. On-resin tagging of the desired product (1) yields a mixture after cleavage (2) from which the desired product can be selectively obtained through affinity separation (3). X = capped incomplete sequences.

Two different approaches can be distinguished, that is, attachment of an appropriate fluorous protecting group to either the final products (*tagging*, Scheme 1) or to the intermediate unreacted species (*capping*). The success of fluorous capping in solid-phase chemistry was demonstrated by Seeberger *et al.*[10b,c] who were able to separate the desired products from the fluorous impurities after the solid-phase assembly of oligosaccharides.

6.3 | Fluorous Tag: Design & Synthesis

Recently, the first example of the use of a fluorous tag (a fluorinated derivative of the Z-protecting group, *i.e.* FZ tag, Figure 2) in the purification of peptides obtained through solid-phase peptide synthesis was disclosed.[4a]

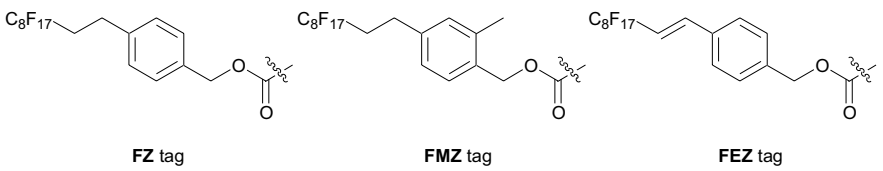


FIGURE 2 | Structures of the designed FZ tag and derivatives for tuning of acid lability.

Although the acid-lability of the FZ tag could be tuned by the introduction of additional substituents (FMZ and FEZ tags), the application of the FZ tag in Fmoc-based SPPS proved to be restricted. It requires the use of amino acids with highly acid-labile side-chain protecting groups and a highly acid-labile linker. It was reasoned that a base-labile fluorous protecting group would be an attractive alternative tag for standard Fmoc-based SPPS with acid-labile side-chain protections and standard linker/resin systems such as Wang and Rink amide resins. To this end, a fluorous version of the Msc (methylsulfonylethoxycarbonyl, Figure 3)[12] protecting group for amines, introduced by Tesser and co-workers nearly 30 years ago,[13] was designed and synthesized. The new fluorous protecting group was named FMsc (**1**), in analogy with FZ.

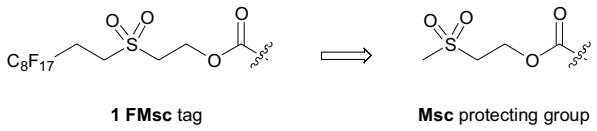
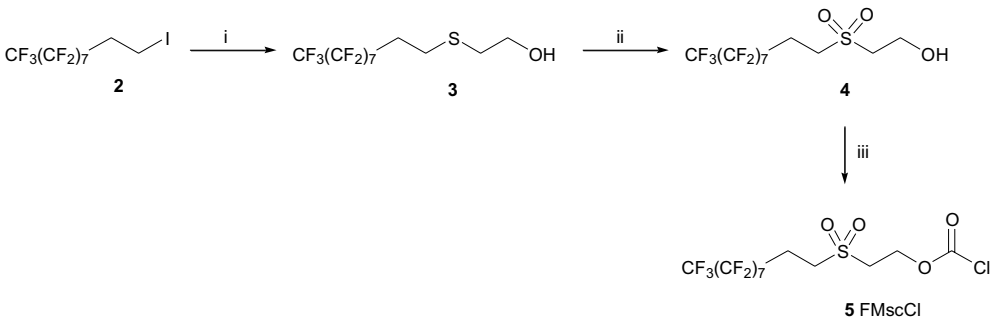


FIGURE 3 | Structures of the Msc protecting group and the derived tag **1**.

The FMscCl tagging reagent **5** was readily synthesised as follows (Scheme 2). In the first step, commercially available [1H,1H,2H,2H]-perfluorodecyl iodide **2** was substituted with 2-mercaptoethanol to give **3**. After oxidation of thioether **3** to the corresponding sulfone **4** (30% AcOOH/AcOH), target FMscCl **5** was obtained by chloroformylation of **4**, in an excellent overall yield of 88%.



SCHEME 2 | *Reagents and conditions:* (i) 2-mercaptoethanol, NaOH, tBuOH, reflux (91%); (ii) AcOOH (30% in AcOH), H_2O, 97%; (iii) phosgene (20% in toluene), THF (quant.).

6.4 | Application of the FMsc Tag

With reagent **5** in hand, the ease and efficiency of introduction and removal of the FMsc tag **1** was explored. Model peptide **6** (Table 1) was treated with FMscCl **5** (5eq.) and DiPEA (10eq.) in DMF to give the corresponding tagged peptide FMsc-**6**, as corroborated by LCMS (Figure 4, trace A). Treatment of FMsc-**6** with 2% aq. NH_3 for 15min furnished starting peptide **6** (Figure 4, trace B).

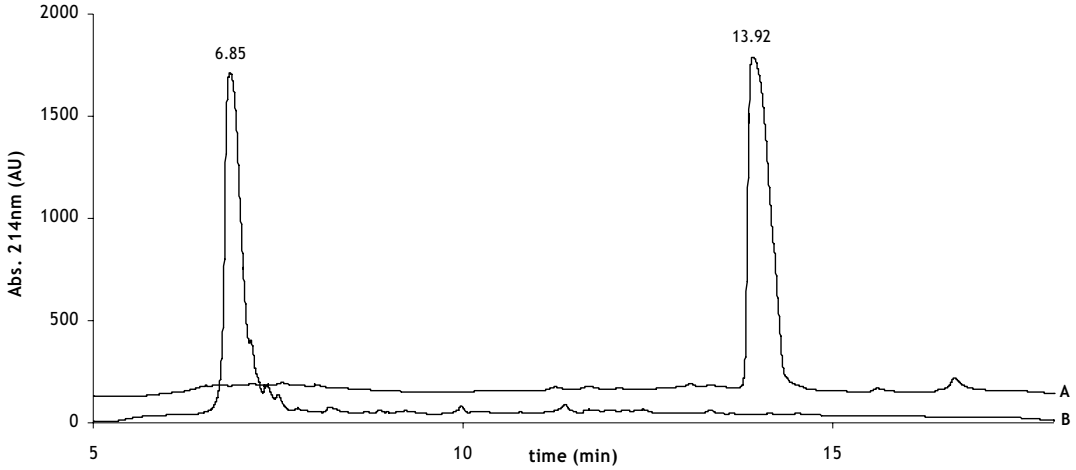


FIGURE 4 | LC chromatograms (5-18min, C18 RP column, 5→90% MeCN/0.1% aq. TFA, detection at 214nm) of peptide GEPKPAG; **A.** after on-resin tagging (FMsc-**6**); **B.** after detagging (**6**).

6.4.1 | Fluorous HPLC (FHPLC) purification

On the basis of these results, we turned our attention to the application of the FMsc tag in the purification of peptides **7**-**9** (Table 1) obtained through SPPS. These peptides are difficult to isolate in pure form using reversed-phase HPLC.[14,15] For instance, the assembly of 35-mer **9** by a standard SPPS protocol suffered from incomplete couplings leading to a tedious isolation procedure, as shown by the HPLC pattern of crude **9** (Figure 5E).

TABLE 1 | Synthetic peptides used in this study.

Peptide	Sequence
6	GEPKP AGa
7	GCCSL PPCAL NNPDYa
8	RQIKI WFQNR RMKWK Ka
9	LSELD DRADA LQAGF SQFES SAAKL KRKYW WKNLK

a = C-terminal carboxamide

Peptides **7**-**9** were synthesised using standard Fmoc-based SPPS with either HCTU (**7** and **8**) or BOP/HOBt (**9**) as condensing agents, starting from Rink amide (**7**, **8**) and Wang resins (**9**). In the latter case, the first amino acid was condensed with the resin using DIC and DMAP. Each condensation step was followed by capping (Ac_2O, DiPEA, HOBt) of the residual unreacted amines. After completion of the synthesis of the respective oligopeptide sequences and removal of the final Fmoc group, the resin-bound peptides were tagged with FMscCl/DiPEA (5 and 10eq. respectively) in DMF. The peptides were then cleaved from the solid support with concomitant liberation of the side-chain functionalities using TFA/TIS/H_2O (95/2.5/2.5, v/v/v).

At this stage, we turned our attention to the purification of tagged 35-mer **9**. As can be seen (Figure 5F), full length, FMsc-tagged **9** eluted significantly later from a fluorous HPLC (FHPLC) column than non-tagged impurities. Semi-preparative FHPLC with an aq. TFE gradient afforded FMsc-**9** in 99% purity (Figure 5G). Detagging (2% aq. NH_3, Figure 5H) finally afforded target peptide **9** in 21% overall yield (based on initial loading of the resin). Tagged peptides **7** (Figure 5) and **8** were purified with FHPLC and detagged with equal efficiency (Table 2).

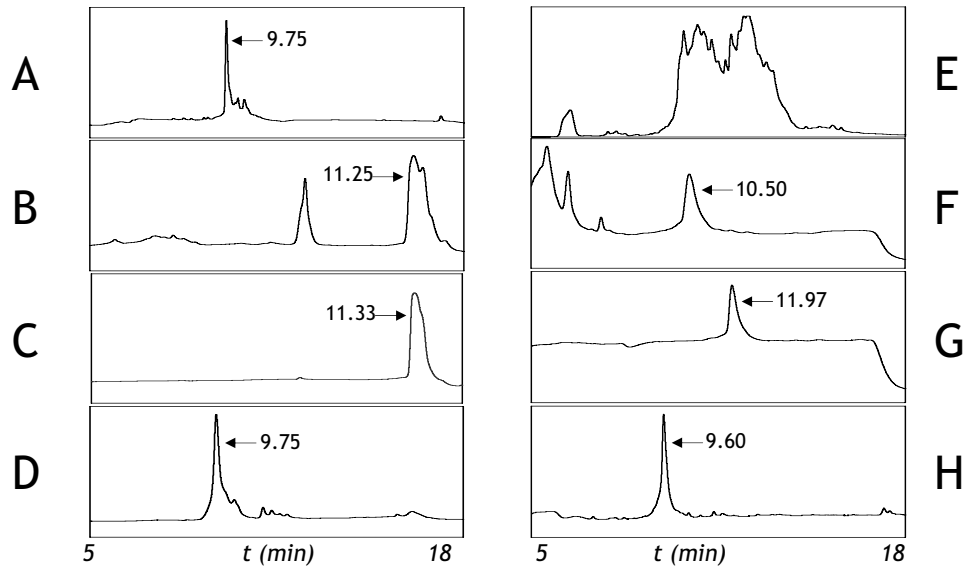


FIGURE 5 | Chromatograms (5-18min except **B** and **C**, 5-12min), UV detection at 214nm) of peptides purified by FHPLC. *Left:* peptide **7**: **A.** Crude **7**; **B**. Crude-FMsc-**7**, the apparent double peak is due to overloading of the column; **C**. FHPLC-purified FMsc-**7**; **D**. Pure **7** after detagging; shoulder is caused by S-S oxidation. *Right:* 35-mer **9**: **E**. Crude **9**; **F**. Crude FMsc-**9**; **G**. FHPLC-purified FMsc-**9**; **H**. Pure **9** after detagging. HPLC methods (see Experimental section): **A,D,E,H** method ***X***; **F**,**G** method ***Y***. **B**,**C** method ***Z***. Peaks with correct mass are indicated with arrows. Shift of retention time is due to different sample composition.

TABLE 2 | Data on detagged peptides purified by FHPLC.

Peptide	Yield (%)[A]	Purity (%)[D]	ESI-MS Tagged	ESI-MS Detagged
7	37	98	2252.0	1669.0
8	10	94	2828.8	2246.6
9	21	99	2343.0[B]	4099.121[C]

[A] Based on loading of the first amino acid; [B] $[M+2H]^{2+}$; [C] HRMS (calcd. 4099.128); [D] purity determined by integration of LC peaks.

6.4.2 | Fluorous SPE (FSPE) purification

In an alternative approach, it was investigated whether FMsc-tagged peptides **7**-**9** could be purified by fluorous silica gel extraction.[16] As an example, crude FMsc-**7** was applied to a fluorous solid-phase extraction (FSPE) cartridge. With the aim of removing non-tagged impurities, the cartridge was eluted first with 0.1% aq. TFA and then with 0.1% TFA in MeOH/H_2O 1/1 (v/v). Subsequent elution of the cartridge with 0.1% TFA/MeOH afforded FMsc-**7** in a significant enhanced purity after detagging (59% yield, 91% purity, Table 3). Although the purity of the final product was somewhat lower than was observed after FHPLC, the ease of execution and potential scale (~50mg crude FMsc-**7** could be applied to the FSPE cartridge in one single run) makes this purification procedure an attractive alternative. In a similar way, FMsc-tagged **8** was purified and detagged (Table 3). In this case, the use of a mixture of 0.1% TFA in TFE/H_2O 1/1 (v/v) proved to be more effective for elution of the fluorous peptide. In contrast, purification of FMsc-**9** on a FSPE cartridge failed, arguably due to the relative low fluorine content (only 7% by weight compared to 14% in **7** and 11% in **8**).

TABLE 3 | Data on detagged peptides purified by FSPE.

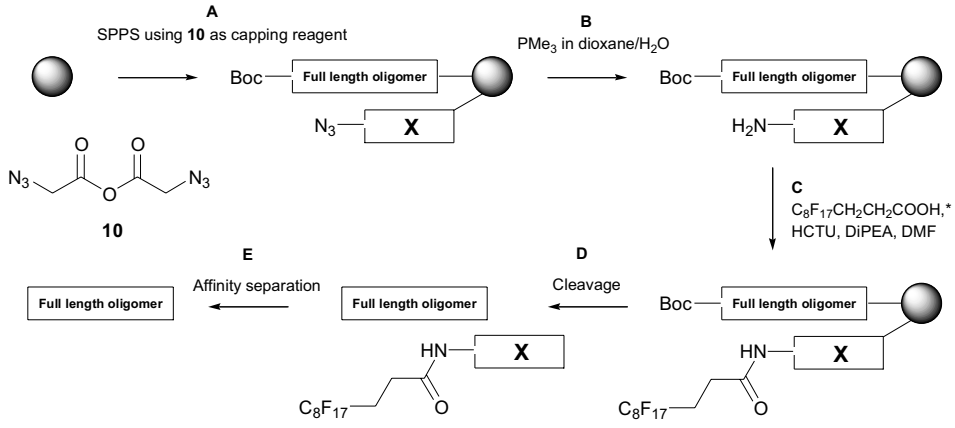
Peptide	Crude Material (mg)	Detagged Yield (%)[A]	Purity (%)[B]	Eluents[C]
7	45.9	59	91	MeOH/H_2O
8	17.2	7	72	TFE/H_2O

[A] Based on loading of the first amino acid; [B] purity determined through integration of LC peaks;
[C] containing 0.1% TFA.

6.5 | Fluorous Capping

The described fluorous methods (FZ, FMsc) based on *tagging* of the desired product, discussed in the previous paragraphs, have the common disadvantage that the fluorous product obtained after purification by either FHPLC or FSPE must be subjected to one final detagging step, which leads to inevitable handling losses. Attachment of a fluorous handle to all incomplete sequences, *i.e.* the use of fluorous *capping* reagents,[10b,c] overcomes this last reaction step. However, substitution of commonly used Ac_2O for capping in SPPS with a fluorous anhydride (*e.g.* [1H,1H,2H,2H]-perfluoroundecanoic acid anhydride) brings about some practical issues: [1H,1H,2H,2H]-perfluoroalkanoic anhydrides with sufficient fluorine content are not commercially available, and the precursor acids are rather expensive for large-scale use. Besides, the reactivity of the fluorous capping reagent is unknown and might well differ from Ac_2O.

To circumvent these issues, the following two-step fluorous capping strategy was designed. As outlined in Scheme 3, Ac_2O in standard SPPS procedures (step **A**) has been replaced by azidoacetic acid anhydride (**10**) or azidoacetic acid/HCTU. After completion of the SPPS (**A**), in which the N-terminal residue is incorporated as its *N*α-Boc-protected derivative, the on-resin azides are reduced by phosphine treatment (**B**) and subsequently capped by reaction with a perfluoroalkyl acid or its anhydride (step **C**). As the last two steps are performed after automated synthesis, their progress can be easily followed by TNBS or Kaiser tests.



SCHEME 3 | Outline of the two-step fluorous capping strategy. * or the corresponding anhydride/DiPEA if desired; the azidoacetic acid/HCTU/DiPEA conditions can be applied instead of compound **10** as well.

After cleavage from the resin (**D**), fluorous affinity purification (FHPLC/FSPE, **E**) can be applied to obtain the desired product as the first, non-fluorous, peptide eluting. This way, the fluorous acylation step **C** is applied only once (after completion of the SPPS) as in the tagging approach, rather than after every coupling step as in the original capping approach.

In a preliminary experiment, the peptide GEPKPAG was synthesized following the two-step fluorous capping strategy. During automated Fmoc-based SPPS, 0.85eq. of amino acid was used to ensure incomplete sequences. After each coupling, the resin was treated with azidoacetic acid/HCTU/DiPEA. After completion of the synthesis, treatment with PMe_3/1,4-dioxane/H_2O yielded the free amines, as verified by TNBS test. Subsequent acylation with [1H,1H,2H,2H]-perfluoroundecanoic acid/HCTU/DiPEA resulted in fluorous capped incomplete sequences. After cleavage from the resin, the crude material (14.2mg) was purified in a single run using FSPE. The FSPE cartridge, containing 0.7mL fluorous silica, was eluted with 4x5mL 10% aq. TFA, and the desired product was found primarily in the first 5mL fraction, with very low amounts in the other fractions. Importantly, no fluorous incomplete sequences were detected by LCMS in these fractions. The yield of the desired peptide was 28% of the theoretical maximum yield. Although this method does need optimization, it might eventually lead to a simple and time-effective synthesis/purification procedure for peptides.

6.6 | Summary

In summary, the development and application of a new fluorous FMsc protecting group **1** was disclosed. Application of FMscCl **5** in Fmoc-based SPPS allows the generation of full-length peptides equipped with a base-labile fluorous tag. These peptides were purified by either FHPLC or FSPE, after which the tag is easily and quantitatively removed. Furthermore, a *two-step fluorous capping* approach towards a capping strategy was discussed along with preliminary results. In conclusion, fluorous strategies can be valuable tools enabling isolation of desired products from complex crude synthetic peptide mixtures.

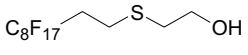
6.7 | Experimental Section

6.7.1 | HPLC methods

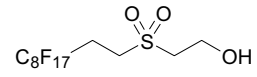
HPLC methods: **X**. Alltech Alltima C18 column (150x4.6mm), 5→90% MeCN/H2O+0.05% TFA in 17min at 1.0mL/min; **Y**. Keystone Scientific Operations FluoPhase WP column (100x4.6mm), 20→100% TFE/H_2O + 0.05% TFA in 18min at 1.5mL/min; **Z**. Identical to method **Y** but gradient elution time is 12min. UV detection in all methods at 214nm.

6.7.2 | Syntheses

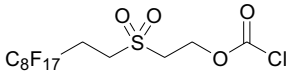
Acylated GEPKPAG dervatives
GEPKPAG was synthesized using automated Fmoc-based SPPS procedures using Rink amide resin and HCTU/DiPEA as coupling reagents. After completion of the synthesis, the last Fmoc group of GEPKPAG was removed and the resin was split into three batches (6.5µmol each), two of which were treated with undecanoic acid/DiPEA (5 and 10eq. respectively) or [1H,1H,2H,2H]-perfluoroundecanoic acid/DiPEA (5 and 10eq. respectively) in DMF for 2h. After acidic cleavage of all batches from the resins, three peptides were obtained; GEPKPAG, **F**-GEPKPAG and **L**-GEPKPAG. These peptides were analyzed by FHPLC (method **Y**): GEPKPAG Rt < 2min, **L**-GEPKPAG Rt 7.21min, **F**-GEPKPAG Rt 9.75min.



([1H,1H,2H,2H]-perfluorodecyl)sulfidylethanol (3). Under nitrogen atmosphere, NaOH (0.50g, 12.5mmol, 1.5eq.) and 2-mercaptoethanol (1.41mL, 20mmol, 2.45eq.) were refluxed in tBuOH (30mL) for 30min. Next, [1H,1H,2H,2H]-perfluorodecyl iodide **2** (4.71g, 8.15mmol) was added and the mixture was stirred under reflux conditions for an additional 3h. After evaporation of all volatiles, the crude product was subjected to silica gel column purification, yielding 3.88g (7.41mmol, 91%) of pure **3**. TLC (PE/EtOAc 1/1 (v/v)): R_f 0.70. ^{1}H NMR ($CDCl_3$): δ 3.75 (s, 1H, OH), 3.75-3.68 (m, 2H, H1), 2.71-2.42 (m, 4H, H2, H3), 2.40-2.09 (m, 2H, H4). ^{13}C NMR ($CDCl_3$): δ 123.1-104.7 (CF), 59.5 (C1), 24.6 (C2), 32.3, 31.9, 31.4 (C4), 22.4 (C3); ^{19}F NMR ($CDCl_3$): δ -5.03 (CF_3), -38.1, -45.6, -46.7, -47.0, -50.1 (CF_2). ESI-MS: 547.1 $[M+Na]^+$.



([1H,1H,2H,2H]-perfluorodecyl)sulfonylethanol (4). To a solution of **3** (3.12g, 5.95mmol) in ice-cooled AcOH (1.6mL), 32% AcOOH in AcOH (3.26mL, 1.2eq) and water (1.1mL, 10eq) were added. If gel formation occurred, EtOAc (5mL) was added to dilute the reaction mixture. After 2h, TLC showed total conversion of the starting material and $NaHCO_3$ (s) was carefully added to neutralize the mixture. Next, the mixture was extracted using a large excess of EtOAc (3x) and dried over $MgSO_4$. After filtration and removal of solvents *in vacuo*, the crude product was purified by silica column chromatography. Compound **4** was obtained as a white solid (yield: 5.77mmol, 97%). TLC (EtOAc/PE 1/1 (v/v)): R_f 0.49. ^{1}H NMR (MeOD): δ 3.90 (t, 2H, H1, $J_{1,2}$ = 5.1Hz), 3.60-3.33 (m, 2H, H3, $J_{3,4}$ = 8.0Hz), 3.25 (t, 2H, H2), 2.84-2.48 (m, 2H, H4). ^{13}C NMR (MeOD): δ 56.8 (C1), 47.5 (C2, C3), 25.9, 25.4, 25.0 (C4). ^{19}F NMR (MeOD): δ -1.17 (CF_3), -33.5, -41.6, -42.5, -43.0, -46.1 (CF_2). ESI-MS: 579.1 $[M+Na]^+$.



([1H,1H,2H,2H]-perfluorodecyl)sulfonylethoxycarbonyl chloride (5). Compound **4** (5.77mmol) was dissolved in freshly distilled THF (50mL). To this solution, phosgene (20% in toluene, 24mL, 9eq.) was added. After 16h, TLC indicated total conversion of the starting material. Phosgene and solvents were carefully removed *in vacuo* in a fumehood. The residue was coevaporated with toluene (2x), yielding 3.56g (5.75mmol, quant.) as a pinkish white solid. TLC (EtOAc/PE 1/1 (v/v)): R_f 0.85. ^{1}H NMR ($CDCl_3$): δ 4.82-4.71 (m, 2H, H1), 3.51-3.41 (m, 2H, H2), 3.41-3.26 (m, 2H, H3), 2.86-2.53 (m, 2H, H4). ^{13}C NMR (acetone-d_6): δ 120.3-110.6 (CF), 65.4 (C1), 51.8 (C2), 45.9 (C3), 24.8, 24.4, 24.0 (C4). ^{19}F NMR ($CDCl_3$): δ -3.5 (CF_3), -36.3, -44.6, -45.2, -45.8, -48.8 (CF_2). A sample of **5** was solvolyzed in MeOH to give the methyl carbonate, ESI-MS: 637.1 $[M+Na]^+$.

Loading of Wang Resin
A flask was silylated using 20% TMSCl in $CHCl_3$. Therein, the Wang resin was coevaporated with DCE, suspended in CH_2Cl_2 and treated with the first amino acid (3eq.), DIC (3eq.) and DMAP (0.1eq.) for 2h. The resin was filtered off, rinsed with CH_2Cl_2, MeOH and Et_2O and air-dried. Loading of the resin was determined by measuring the UV absorbance at 300nm of released Fmoc-chromophore upon treatment of a resin sample with 20% piperidine/DMF for 10min.

6.7.3 | FMsc Tagging Procedure

After SPPS, the free N-terminus of the full-length peptide was tagged by suspending the resin in DMF or NMP (1.5mL per 50µmol on-resin peptide), and adding FMscCl (**5**, 5eq.) and DiPEA (10eq.) and shaking at RT for 1h. The resin was filtered off and washed with DMF or NMP, MeOH and CH_2Cl_2. If TNBS or Kaiser test was positive, procedure was repeated.

6.7.4 | FSPE Procedure

Crude peptide mixture was dissolved in the smallest amount of H_2O possible, and AcOH was added to aid dissolution if necessary. This mixture was loaded onto a 5mL syringe that contained 0.7-2.0mL fluorous silica material (FTI, Pittsburg, USA) with a filter on top of the silica layer. The cartridge was subsequently eluted with volumes of 0.1% aq. TFA, 0.1% aq. TFA/MeOH 1/1 (v/v) and 0.1% TFA/MeOH and fractions (1-5mL) were collected. If LCMS analysis required the use of TFE, TFE instead of MeOH was used in the FSPE purification of these particular peptides.

6.8 | Notes & References

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CHAPTER 7 | Summary & Future Prospects