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Aryldithioethyloxycarbonyl (Ardec): A New Family of Amine Protecting Groups Removable under Mild Reducing Conditions and Their Applications to Peptide Synthesis**

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Abstract: The development of phenyldithioethyloxycarbonyl (Phdec) and 2-pyridyldithioethyloxycarbonyl (Pydec) protecting groups, which are thiol-labile urethanes, is described. These new disulfide-based protecting groups were introduced onto the ε-amino group of L-lysine; the resulting amino acid derivatives were easily converted into N^{α}-Fmoc building blocks suitable for both solid- and solution-phase peptide synthesis. Model dipeptide-(Ardec)s were prepared by using classical peptide couplings followed by standard deprotection protocols. They were used to optimize the conditions for complete thiolytic removal of the Ardec groups both in aqueous and organic media. Phdec and Pydec were found to be cleaved within 15 to 30 min under mild reducing conditions: i) by treatment with dithiothreitol or β-mercaptoethanol in Tris·HCl buffer (pH 8.5–9.0) for deprotection in water and ii) by treatment with β-mercaptoethanol and 1,8-diazobicyclo-[5.4.0]undec-7-ene (DBU) in *N*-methylpyrrolidinone for deprotection in an organic medium. Successful solid-phase synthesis of hexapeptides Ac-Lys-Asp-Glu-Val-Asp-Lys(Ardec)-NH_2 has clearly demonstrated the full orthogonality of these new amino protecting groups with Fmoc and Boc protections. The utility of the Ardec orthogonal deprotection strategy for site-specific chemical modification of peptides bearing several amino groups was illustrated firstly by the preparation of a fluorogenic substrate for caspase-3 protease containing the cyanine dyes Cy3.0 and Cy5.0 as FRET donor/acceptor pair, and by solid-phase synthesis of an hexapeptide bearing a single biotin reporter group.

Keywords: enzymes · FRET (fluorescence resonance energy transfer) · fluorescent probes · peptides · protecting groups · solid-phase synthesis

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[**] Applications of Ardec chemistry to oligonucleotide and peptide synthesis were first described in a patent entitled "Trace-less cleavable cross-linker reagents and use thereof", filled on February 12, 2004 at the European Patent Office: A. Romieu, G. Turcatti, EP 04100542.2. This patent is based on preliminary experiments done at Manteia Predictive Medicine SA, a Swiss-based private company (spin-out from Serono) developing an ultra high-throughput DNA sequencing technology. This technology was jointly acquired by Lynx Therapeutics Inc. (www.lynxgen.com) and Solexa Ltd. (www.solexa.com) in March 2004. Both companies completed a merger in March 2005 and became Solexa Inc.

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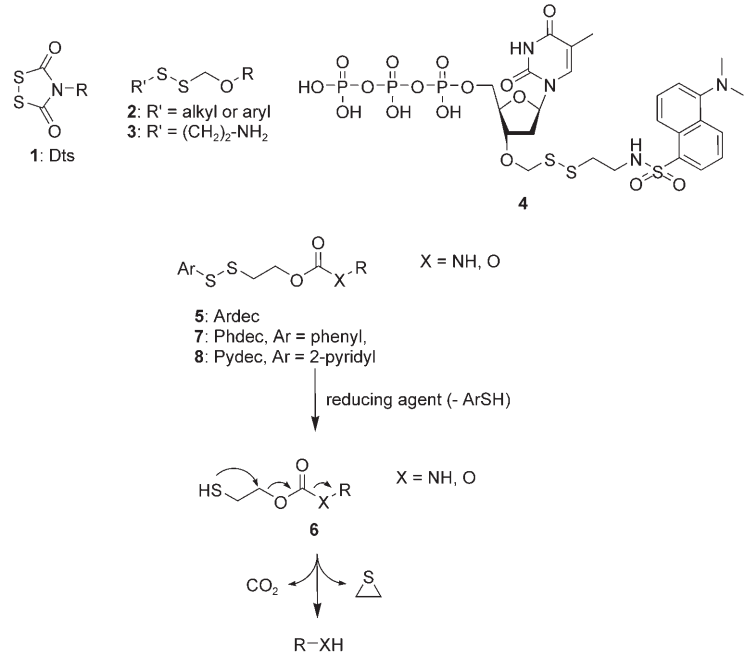
Introduction

Selective temporary protection or inactivation of a chemically reactive functionality in biological compounds is an important tool in the field of organic and bioorganic chemistry especially for the controlled synthesis of biopolymers (i.e., oligonucleotides, oligosaccharides, peptides, and conjugates thereof).[1–6] The majority of the known protecting groups are acid or base labile; the conditions for their removal are often too harsh and can be incompatible with complex biomolecules. Consequently, numerous sophisticated protecting groups have been developed which involve mild deblocking reagents, for instance the use of enzymes, fluoride ion sources, noble metals or UV light.[7–10] Surprisingly, few efforts have been devoted to the design of disulfide bridge containing protecting groups although they can undergo highly selective reductive cleavage by mild treatment with a thiol or a trialkylphosphine in various organic solvents or weakly basic aqueous buffers.[11] The dithiasuccinoyl group **1** has been developed as an amino protecting group for solid-phase synthesis of modified peptides and protected peptide nucleic acids (Scheme 1).[12,13] This group is stable under strongly acidic conditions and photolysis but it is rapidly and specifically removed under mild conditions by thiolysis. Until the recent publication of Barany and Merrifield about the efficient synthesis of 1,2,4-dithiazolidine-3,5-diones,[14] the lack of a simple and robust method to prepare Dts-amines in high yields prevented the widespread use of this heterocycle as an orthogonal amino protecting group. Kwiatkowski suggested the protection of hydroxyl groups of biological compounds with an alkyldithiomethyl moiety **2**.[15] The aminoethyl derivative **3** was used as a 3′-OH blocking group of nucleotides (e.g., 3′-*O*-(2-*N*-dansylethyldithiomethyl) derivative of 5′-triphosphate of thymidine **4**) to get reversible fluorescent terminators suitable for DNA sequencing by synthesis.[16] However, the multi-step synthetic procedure required for the transformation of alcohols to their corresponding alkyldithiomethyl ethers is not suitable for a routine use of such protecting groups (namely: Pummerer reaction to generate the methylthiomethyl ether, halogenation and subsequent nucleophilic substitution to generate the corresponding alkyl- or arylthiosulfonate derivative and finally a disufide exchange reaction with an alkyl- or an arylthiol). Extension of this protection strategy to a wide range of polyfunctional (bio)molecules thus appears tricky. With the goal in mind to develop a new cleavable disulfide-based protecting group applied to the biological chemistry of peptides meeting both the requirement of an easy and straightforward introduction onto the peptide side chains and a specific and mild thiolytic deprotection, we have examined the chemistry of the aryldithioethyloxycarbonyl functionality. Indeed, we thought that the cleavage of the disulfide bond of **5** with thiols (or other reducing agents) should generate the unstable 2-thioethyl carbonate (or carbamate) **6** which might decompose spontaneously into the corresponding alcohol (or amine), carbon dioxide and ethylene episulfide. This kind of intramolecular elimination reaction has already been reported in the literature, especially for the removal of various S-acyl and disulfide derivatives of the 2-thioethyl moiety from phosphate esters.[17]

Herein we describe the development of the phenyldithioethyloxycarbonyl and the 2-pyridyldithioethyloxycarbonyl protecting groups **7** and **8**. The novel thiol-labile urethanes were introduced as lysine side chain protecting groups both in solution- and solid-phase peptide synthesis, and used for the preparation of atypical peptides.



Scheme 1. Disulfide bridge containing protecting groups already reported in literature and principle of aryldithioethyloxycarbonyl protecting groups.

Results and Discussion

Preparation of N$^{\alpha}$-Ardec-protected lysine monomers: Orthogonal protection of the ε-amino group of L-lysine with the Ardec groups firstly requires deactivation of the α-amino and α-carboxy groups by formation of a copper(II) complex and subsequent *N*-alkoxycarbonylation with an active carbonate derived from the corresponding (2-aryldithio)ethanol. Chemical methods for the introduction of various alkoxycarbonyl moieties as amino protecting groups are well documented.[18] To avoid

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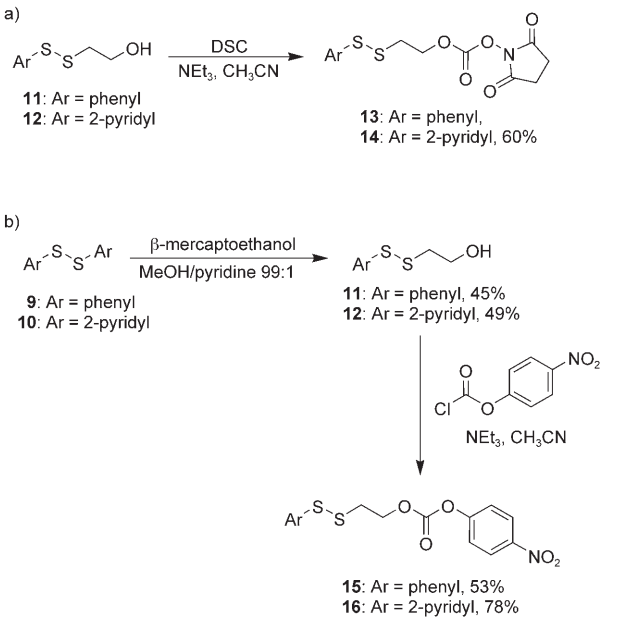
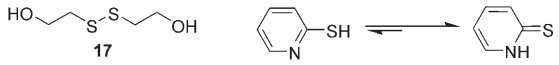
the use of unstable and toxic haloformates, several alkoxycarbonylating reagents having either heterocyclic (e.g., imidazolyl, *N*-hydroxysuccinimidyl, 1-hydroxybenzotriazolyl) or electron-withdrawing group substituted phenol (e.g., 4-nitrophenyl, pentafluorophenyl) leaving groups have been developed. In a first approach, we used the succinimidyl carbonates **13** and **14** (Scheme 2a). Active carbonate **14** was easily



Scheme 2. Structure and synthesis of the activated reagents suitable for the Ardec protecting groups introduction. a) Succinimidyl carbonates, only **14** was prepared. b) 4-Nitrophenyl carbonates (Ardec-ONp).

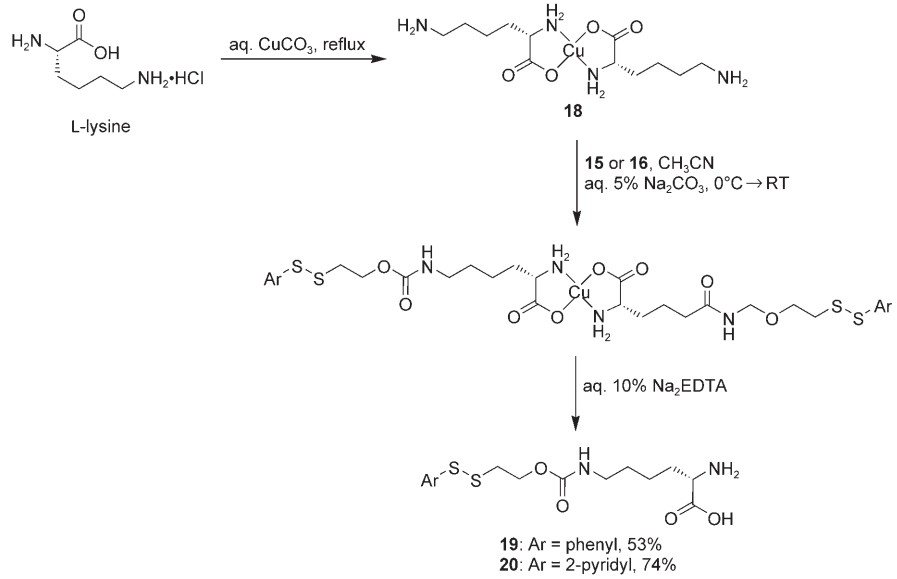
prepared from (2-(2′-pyridyl)dithio)ethanol **12** and DSC. This compound was found to be very stable especially in aqueous buffers but showed poor acylating reactivity towards amino groups of amino acids and nucleosides. Consequently, we turned towards the synthesis of active carbonates **15** and **16** derived from 4-nitrophenol (4NP).[19] These intermediates were found to be more reactive than the corresponding *N*-hydroxysuccinimide (NHS) derivatives. The reagents, (2-phenyldithio)ethyl 4-nitrophenyl carbonate (**15**) and (2-(2′-pyridyl)dithio)ethyl 4-nitrophenyl carbonate (**16**), were prepared from the commercially available diaryl disulfide **9** and **10** in two steps (Scheme 2b). Disulfide exchange between β-mercaptoethanol and diaryl disulfide **9** or **10** in methanol in the presence of pyridine gave **11** and **12** in 45 and 49% yield, respectively.[20] As previously reported for some unsymmetrical disulfides, **11** was found to be susceptible to disproportionation to the corresponding symmetrical disulfides **9** and **17** (Scheme 3) both in pure form and in solution in various organic solvents.[21] This disproportionation reaction was not



Scheme 3. Chemical structure of disulfide **17** and 2-pyridinethiol/2-pyridinethione tautomeric equilibrium.

observed with (2-(2′-pyridyl)dithio)ethanol (**12**) because the 2-pyridinethiol released is in equilibrium with the 2-pyridinethione which is not able to react with the unsymmetrical disulfide (Scheme 3).[22] Consequently, (2-phenyldithio)ethanol (**11**) had to be used immediately after its purification by silica gel chromatography. Treatment of **11** and **12** with 4-nitrophenyl chloroformate in dry acetonitrile in the presence of triethylamine enabled their clean conversion into the *N*-alkoxycarbonylation reagents **15** and **16**. These activated carbonates are stable compounds which were easily purified by silica gel chromatography. Compound **15** was obtained in a pure form as a white powder whereas **16** was found to be contaminated with small amounts (~5%) of 4NP. Interestingly, these reagents could be stored at 4°C for several months without significant degradation.

Selective N^{ε}-acylation of the copper(II) complex of L-lysine **18** with Ardec-ONp **15** or **16** was achieved by using the method reported by Rosowsky and Wright for the introduction of the Teoc protecting group (Scheme 4).[23] Accordingly, removal of the metal ion with ethylenediamine tetraacetic acid disodium salt gave the targeted N^{ε}-Ardec-protected lysine monomers **19** and **20** which were isolated in good yields by simple filtration; they were obtained in over



Scheme 4. Introduction of the Ardec protecting groups onto the ε-amino group of lysine.

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95% purity (checked by RP-HPLC) and so they did not require further purification. Their structures were confirmed by detailed measurements, including ESI mass spectrometry and NMR analyses.

Preparation of N^{α}-Fmoc-N^{ε}-Ardec-lysine building blocks and their use in solution-phase peptide synthesis: The N^{ε}-Ardec-protected monomers H-Lys(Phdec)-OH (**19**) and H-Lys(Pydec)-OH (**20**) were converted into the corresponding N^{α}-Fmoc building blocks **21** and **22** by using *N*-hydroxysuccinimidyl 9-fluorenylmethyl carbonate under the standard conditions reported by Lapatsanis et al. (Scheme 5).[24] The compounds were isolated by silica gel chromatography and characterized on the basis of elemental and MS analyses and consistent IR and ^{1}H and ^{13}C NMR spectral data.

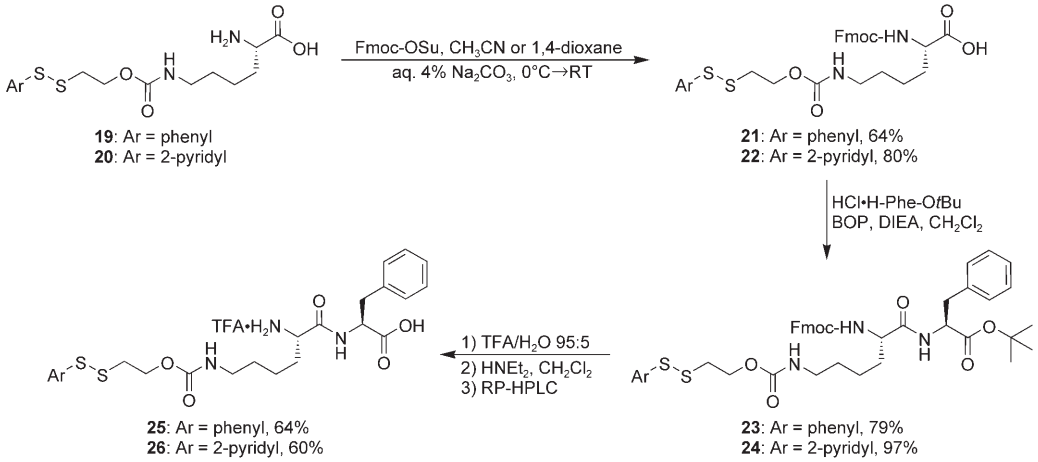
To evaluate the suitability of the Phdec and Pydec groups for peptide chemistry, dipeptides H-Lys(Ardec)-Phe-OH **25** and **26** were synthesized by coupling either Fmoc-Lys-(Phdec)-OH (**21**) or Fmoc-Lys(Pydec)-OH (**22**) with H-Phe-O*t*Bu and subsequent removal of the Fmoc and *t*Bu protecting groups. Coupling reaction was achieved with BOP reagent in the presence of DIEA in dry dichloromethane.[25] The fully-protected dipeptides Fmoc-Lys(Ardec)-Phe-O*t*Bu **23** and **24** were isolated by silica gel chromatography in 79 and 97% yields. Thereafter, sequential treatment of **23** and **24** with TFA/H_2O (95:5) and diethylamine provided the targeted dipeptides **25** and **26** which were purified by RP-HPLC. Their structures were confirmed by detailed measurements, including MALDI-TOF mass spectrometry and NMR analyses. Furthermore, RP-HPLC analyses of the crude reaction mixtures at each step of this peptide assembly process have clearly shown that no cleavage modification of Lys(Ardec) residues occurred. Indeed, no trace of dipeptides Fmoc-Lys-Phe-O*t*Bu, Fmoc-Lys-Phe-OH and H-Lys-Phe-OH were detected after the coupling, *t*Bu and Fmoc removal steps, respectively. Thus, the Phdec and Pydec protecting groups are completely stable under usual conditions applied in peptide synthesis.

Stability studies of the Ardec protecting groups under the cleavage conditions used for other protecting groups: To assess the orthogonality and compatibility between Ardec and other protecting groups currently used in peptide chemistry (e.g., allyl, Aloc, Boc, Dde, Fmoc, silyl and photolabile protecting groups), stability studies were performed in solution assays with dipeptides H-Lys(Ardec)-Phe-OH **25** and **26**. It was found that Ardec groups could be readily cleaved from the lysine side chain only under aqueous basic conditions (Table 1, entries 4–5 and 10–11), returning the unprotected dipeptide H-Lys-Phe-OH (**28**). The Ardec moieties were found to be stable to all of the other conditions investigated, but RP-HPLC analysis showed that the reaction with a large excess of hydrazine caused removal of

Table 1. Stability of the Ardec protecting groups to a variety of conditions.[a]

	Cleavage conditions (equiv)	Phdec	Pydec
1	95% TFA/H_2O (215)	stable	stable
2	10% HCl/H_2O (3600)	stable	stable
3	1 M Et_2NH/CH_2Cl_2 (30)	stable	stable
4	2 M LiOH/H_2O (2200)	cleaved	cleaved
5	2 M LiOH/H_2O (10)	cleaved	partially cleaved (21%)
6	5% DBU/NMP (180)	stable	stable
7	5% N_2H_4/NMP (190)	stable	cleaved
8	5% N_2H_4/NMP (10)	stable	partially cleaved (20%)
9	$[Pd(PPh_3)_4]$ in $CHCl_3$/AcOH/NMM 37:2:1 (3)	stable	stable
10	1 M TBAF in THF (185)[b]	cleaved	cleaved
11	1 M TBAF in THF (10)[b]	cleaved	cleaved
12	TEA·3HF (25)	stable	stable
13	UV light (350 nm)/NMP	stable	stable

[a] Stability of the Ardec protecting groups was evaluated by solution assays with dipeptides H-Lys(Ardec)-Phe-OH **25** and **26**. The crude reaction mixtures were analyzed by RP-HPLC (after 1 h of incubation at room temperature, system E) to detect and quantify the recovered Ardec-protected dipeptide **25** or **26** and unprotected dipeptide **28** (for more details, see Experimental Section). [b] Presence of water (solution of TBAF in THF is highly hygroscopic) may responsible of the cleavage.



Scheme 5. Preparation of N^{α}-Fmoc-N^{ε}-Ardec-lysine building blocks and their use in the synthesis of model dipeptides H-Lys(Ardec)-Phe-OH.

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Pydec (entry 7). Thus, deprotection conditions for the Dde (or its hindered variant ivDde) amine protecting group should be carefully checked (i.e., equivalents N_2H_4) to be completely orthogonal to Ardec.

Selective removal of Ardec protecting groups in solution: We postulated that the Ardec groups are removed in reductive conditions by thiols or trialkylphosphines through a 2-thioethyl carbamate intermediate, which spontaneously decomposes to give the free amino function (Scheme 1). The reaction is driven to completion by loss of one equivalent of ethylene episulfide and one equivalent of gaseous carbon dioxide. Furthermore, an important aspect of this deprotection strategy must be the ability to efficiently remove the Ardec group under mild reducing conditions and in aqueous media compatible with the stability of peptides (or proteins). To confirm the mechanism and to define an optimized protocol for quantitative thiolytic removal of the Ardec groups at the peptide level, deprotection efficiency was evaluated by solution assays with dipeptides H-Lys(Ardec)-Phe-OH **25** and **26**.

Dipeptide H-Lys(Pydec)-Phe-OH (**26**) was treated with increasing amounts (i.e., 5, 10, 20, 40 and 80 equiv) of DTT or β-mercaptoethanol in Tris·HCl buffer, at four different pH values (i.e., 7.5, 8.0, 8.5 and 9.0) for 30 min. The crude deprotection reactions were analyzed by RP-HPLC to detect and quantify the recovered Pydec-protected dipeptide **26**, the 2-thioethyl carbamate intermediate **27** and dipeptide H-Lys-Phe-OH (**28**) in order to evaluate the rates of disulfide reduction and intramolecular elimination. As expected, the clean and quantitative conversion of **26** into the unprotected dipeptide **28** occurred either using DTT or β-mercaptoethanol (Figure 1).[26] The identification of H-Lys-Phe-OH ($t_R = 12.8$ min) was confirmed by co-injection with a standard independently prepared from Fmoc-Lys(Boc)-OH and H-Phe-O*t*Bu. When Tris·HCl buffer at pH 7.5 was used, the quantitative yield of the disulfide reduction step was not affected but the major product from deprotection was the 2-thioethyl carbamate intermediate **27** ($t_R = 18.5$ min). These results clearly show that a pH value over 8.0 is required for the decomposition of 2-thioethyl carbamate derivatives This suggest that the fragmentation reaction of **27** which initially involves an internal nucleophilic attack of the thiol group on the α carbon atom of the 2-thioethyl moiety is the key step of this reductive deprotection mechanism and may be favoured by the formation of a thiolate anion, a better nucleophile than the corresponding thiol. As the pK_a value of the thiol group of **27** is close to 9.0,[27] the limiting step for the reaction is this acid–base equilibrium leading to the formation of thiolate anion intermediate. Similar results were obtained with dipeptide H-Lys(Phdec)-Phe-OH (**25**) which suggests that structural features of the aryl disulfide bridge have little influence on the rate of Ardec removal. The use of tri-*n*-butylphosphine as the deblocking reagent was also explored but the deprotection rates were found to be significantly lower than with DTT and β-mercaptoethanol (data not shown). A possible explanation can be found in the heterogeneity of the deprotection mixture (a suspension of immiscible PBu_3 was observed) despite the use of propan-1-ol as organic cosolvent.

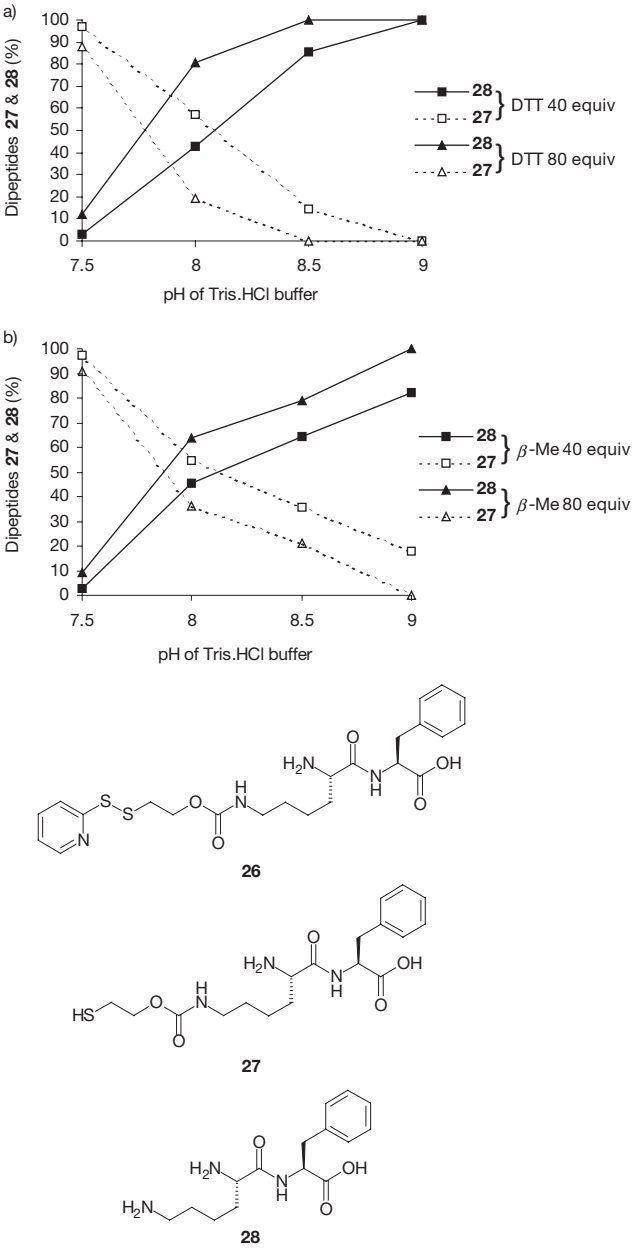

Figure 1. Rates of reductive deprotection of H-Lys(Pydec)-Phe-OH (**26**) in Tris·HCl buffer as a function of pH. a) reductive deprotection mediated by DTT. b) Reductive deprotection mediated by β-mercaptoethanol. Ardec removal was performed with reducing agent/Tris·HCl buffer (25 mM), 30 min at room temperature. Under these conditions, starting dipeptide H-Lys(Pydec)-Phe-OH (**26**) was never detected. Percentages of **27** and **28** were determined by RP-HPLC as reported in Supporting Information.

This reductive deprotection was also investigated in organic media. Thus, dipeptide H-Lys(Pydec)-Phe-OH (**26**) was treated with increasing amounts (i.e., 10, 20, 40 and 80 equiv) of β-mercaptoethanol in NMP for 15 min in the absence or presence of a base such as DIEA, piperidine,

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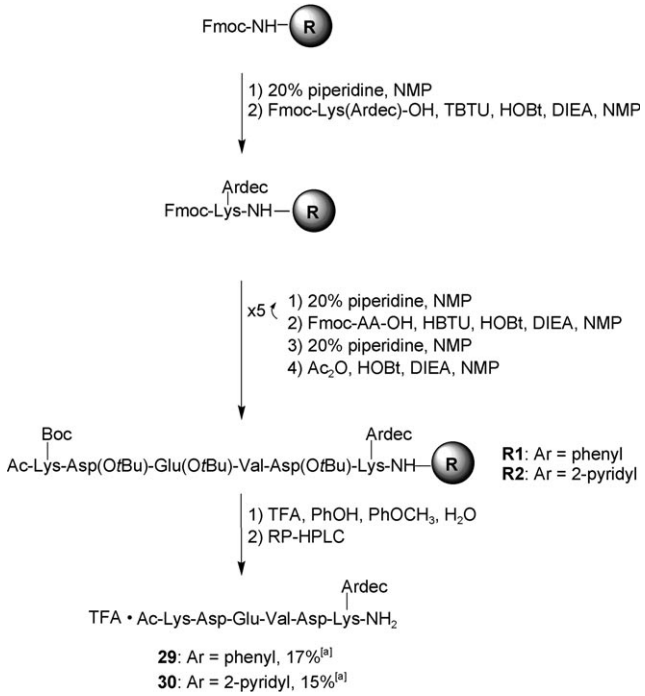
triethylamine and DBU. Preliminary experiments have shown that the thiolytic removal of the Ardec amino protecting groups works with a wide range of organic solvents (i.e., CH_2Cl_2, CH_3CN, DMF, NMP and pyridine). We have chosen NMP in order to directly transfer these reducing conditions to solid-phase deprotection. HPLC analyses of the crude deprotection reactions and subsequent quantification of 2-thioethyl carbamate intermediate **27** and dipeptide H-Lys-Phe-OH (**28**) have clearly shown that only the addition of DBU resulted in complete thiolytic removal of the Pydec group. Indeed, in the absence of base or with piperidine, triethylamine or DIEA, only the 2-thioethyl carbamate intermediate **27** was detected. The unexpected inefficacy of secondary and tertiary amines to induce decomposition of **27** into ethylene episulfide and CO_2 is in agreement with a higher pK_a value of thiol group in polar aprotic solvents than in water. The reaction time and amount of DBU were varied in order to optimize the deprotection conditions. Quantitative removal of the Pydec group from the lysine side chain was found to be: β-mercaptoethanol (80 equiv)/DBU (160 equiv) in NMP for 15 min. When submitted to the same anhydrous reducing conditions, dipeptide H-Lys-(Phdec)-Phe-OH (**25**) gave similar deprotection rates.

N^{α}-Fmoc-N^{ε}-Ardec-lysine building blocks in solid-phase peptide synthesis: To check the full orthogonality of the Ardec groups with Fmoc and Boc protection in solid-phase peptide synthesis and to demonstrate its utility in the preparation of highly functionalized peptides, a model hexapeptide bearing two lysine residues substituted with two different chemical modifications was prepared by using either Fmoc-Lys(Phdec)-OH (**21**) or Fmoc-Lys(Pydec)-OH (**22**). Hexapeptides Ac-Lys-Asp-Glu-Val-Asp-Lys(Ardec)-NH_2 **29** and **30** contain the Asp-Glu-Val-Asp motif which is a specific substrate for caspase-3.[28] The cleavage site is located after the aspartic acid residue from the C-terminal side. Caspases belong to the aspartate-specific cysteinyl proteases that play a critical role as mediators of apoptotic cell death;[29] in particular caspase-3 has been identified as being a key mediator of apoptosis of mammalian cells.[30]

Activation of caspase-3 indicates that the apoptotic pathway has progressed to an irreversible stage and for this reason there is a growing interest both in identifying caspase inhibitors to minimize cell death in pathological conditions and for inducing caspase activation in cancer cells.[31] In addition, caspase-3 is widely used for monitoring apoptosis induction for general cytotoxicity screening. This interest for caspase-3 resulted in the development of several assays using a variety of formats and amenable to high-throughput screening.[32–37] Peptides **29** and **30** have been chosen in order to study the selective chemical modification of each one of the lysine N^{ε}-side-chain amines with two different fluorescent markers. This will lead to the development of a novel strategy for the fluorescent labeling of peptides[38] and the preparation of a novel fluorogenic substrate useful for detecting apoptosis in whole cells and for cell-based high-throughput screening of apoptosis inhibitors or inducers.[39] Indeed, double-labeled peptides are currently used as fluorescent probes based on the principle of fluorescence resonance energy transfer to detect various enzyme activities.[40,41]

Starting from a Fmoc Rink amide MBHA resin, peptide chain assembly was carried out by using standard Fmoc/*t*Bu chemistry (Scheme 6).[42] All amino acids were activated by



Scheme 6. Solid-phase synthesis of peptides(Ardec) **29**, **30**; [a] overall yields from Rink amide MBHA resin.

using HBTU or TBTU/HOBt/DIEA in NMP.[25] The conductimetric analyses of the Fmoc-deprotection steps have revealed that the coupling of Fmoc-Lys(Phdec)-OH (**21**) and Fmoc-Lys(Pydec)-OH (**22**) proceeded with the same efficiency as the standard Fmoc-protected amino acids. Final acetylation of the N-terminal side was achieved by treatment with Ac_2O/HOBt/DIEA in NMP. The hexapeptides were simultaneously cleaved from the resin and side chain deprotected by treatment with TFA in the presence of phenol, anisole and water. The crude Ardec-protected peptides were isolated by precipitation in cold *tert*-butyl methyl ether and analyzed by RP-HPLC and MALDI-TOF mass spectrometry. Ac-Lys-Asp-Glu-Val-Asp-Lys(Phdec)-NH_2 (**29**) and Ac-Lys-Asp-Glu-Val-Asp-Lys(Pydec)-NH_2 (**30**) were identified as the major products of the SPPS. However, a common by-product was observed on the RP-HPLC elution profiles of the deprotection mixtures and accounted for 25 and 5% of the crude peptide, respectively. The material was isolated and analyzed by MALDI-TOF mass spectrometry from which the homodimer structure **31** was proposed (m/z 1753.92 $[M+H]^+$, calcd for: 1752.75). Formation of **31** may be explained by a disproportionation reaction of the di-

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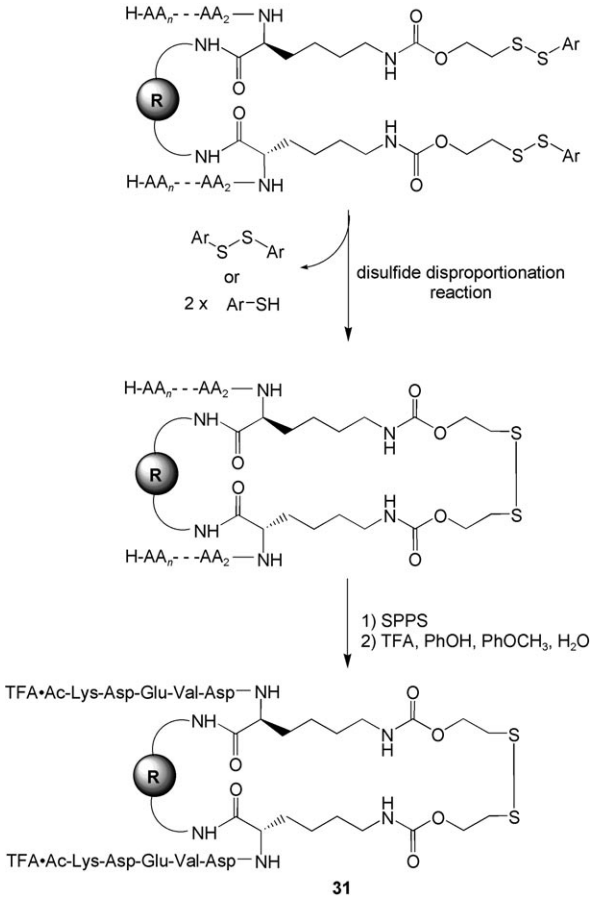
sulfide bridges of two Ardec-protected peptide resin bound units, with subsequent release of a diaryl disulfide (Scheme 7). The tendency of homodimerization is probably



Scheme 7. Proposed mechanism for the formation of peptide dimer **31**.

favoured by the high effective concentration of reactive sites at the resin surface (resin loading) because this type of disulfide homodimer was not detected during the solution-phase synthesis of dipeptides **25** and **26**. However, this side reaction significantly occurs only during the synthesis of Phdec-protected peptides because only the unsymmetrical phenyl disulfide derivatives are prone to disproportionation to the corresponding symmetrical disulfides. Further experiments are in progress in order to improve the solid-phase synthesis, as use of a resin of a lower loading. RP-HPLC purification of Ac-Lys-Asp-Glu-Val-Asp-Lys(Phdec)-NH_2 (**29**) and Ac-Lys-Asp-Glu-Val-Asp-Lys(Pydec)-NH_2 (**30**) provided the pure products (purity $>$ 95%) in overall yields from Fmoc Rink amide MBHA resin of 17 and 15%, respectively. They displayed the expected molecular weights of the Ardec-protected peptides *m*/*z* 986.73 ($[M+H]^+$, calcd for: 985.38) and *m*/*z* 987.52 ($[M+H]^+$, calcd for: 986.38), as indicated by MALDI-TOF mass spectrometry analyses.

Fluorescent labeling of Ardec-protected peptides: The utility of the Ardec orthogonal deprotection strategy for the selective labeling of peptides bearing several amino groups was illustrated by the preparation of **34**, a FRET substrate of caspase-3 protease (Scheme 8). The hexapeptide Ac-Lys-Asp-Glu-Val-Asp-Lys-NH_2 was doubly modified with cyanine dyes Cy3.0 and Cy5.0 which proves their utility in a wide range of bioanalytical applications due to their high extinction coefficients, high fluorescence quantum yields and resistance to photobleaching.[43,44] The fluorescent donor Cy3.0 dye was coupled to the side chain of the free lysine of Ac-Lys-Asp-Glu-Val-Asp-Lys(Pydec)-NH_2 (**30**) using the corresponding *N*-hydroxysuccinimidyl ester in dry NMP in the presence of DIEA. Purification by RP-HPLC provided the Cy3.0 labeled peptide **32** in 60% yield. The clean removal of the Pydec protecting group from the lysine residue of **32** was achieved by treatment with an excess of DTT (50 equiv) in Tris·HCl buffer (pH 9.0). The resulting unprotected hexapeptide **33** was isolated by RP-HPLC in quantitative yield. Finally, the fluorescent acceptor Cy5.0 dye was attached to the free amino group of **33** by treatment with the corresponding *N*-hydroxysuccinimidyl ester in a mixture of sodium bicarbonate buffer (pH 8.5) and NMP. The fluorogenic caspase-3 substrate was isolated by RP-HPLC in a moderate 30% yield and its structure was confirmed by MALDI-TOF mass spectrometry.[45] When we measured the fluorescence spectrum of this probe after excitation of the donor Cy3.0 at 540 nm, a major emission at 665 nm corresponding to the Cy5.0 fluorescence as well as a minor emission at 562 nm corresponding to the remaining untransferred Cy3.0 fluorescence were observed (Figure 2). The emission

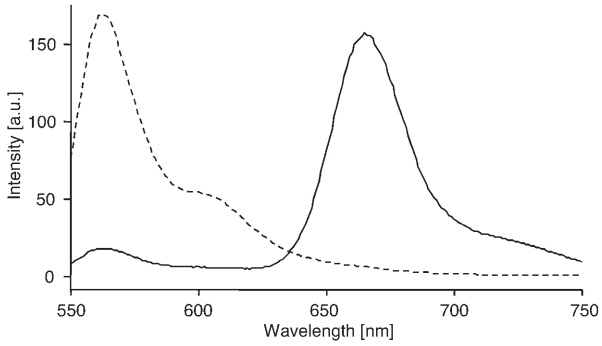


Figure 2. Fluorescence emission spectra (excitation at 540 nm) of peptides **33** labeled with Cy3.0 only (——) and **34** labeled with Cy3.0 and Cy5.0 (-----) in HPLC grade water at 25°C, concentration 0.53 μM.

of Cy5.0 (acceptor) resulting from the excitation of Cy3.0 (donor) occurred via the non-radiative transfer from the donor to the acceptor according to the theory of Förster.[46] The efficiency of this energy transfer (E) is typically measured using the relative fluorescence intensity of the donor (Cy3.0), in the absence (F_D) and presence (F_{DA}) of acceptor (Cy5.0): $E = 1-(F_{DA}/F_D)$. For our fluorescent probe, this was accomplished by comparing the Cy3.0 emission for peptides **33** and **34**. As depicted in Figure 2, the value of F_{DA}/F_D is close to 0.10 so that the transfer efficiency is approximately 90% ($E=0.89$). The FRET efficiency E depends on the

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inverse-sixth-power of the distance between the donor and acceptor: $E=1/[1+(R/R_0)^6]$ where R_0 is the distance at which half of the energy is transferred (i.e., Förster distance), and depends on the spectral characteristics of the dyes and their relative dipole orientation.[47] Thus, the FRET results allow to estimate the distance between the two cyanine dye molecules within peptide **34**. If one assumes an R_0 value of 53 Å for the Cy3.0/Cy5.0 FRET pair (previously determined by Ishii et al.)[48] and a random dipole–dipole orientation value of 2/3 for the orientation factor κ^2, a distance of 37 ± 1.0 Å is obtained. In general, FRET is better suited for detecting changes in distances rather than absolute distances because of the dependence on the relative orientation of the dyes, the κ^2 factor, which is often poorly understood. The efficiency of the energy transfer (donor quenching) obtained with the doubly labeled substrate **34** is high enough to use this read-out for monitoring caspase-3 activity. Enzymatic action will result from dequenching of donor fluorescence upon separation of the two dyes as a consequence of enzymatic peptide bond cleavage. As expected, incubation of peptide **34** with recombinant human caspase-3 has resulted in an appreciable increase of the fluorescence emission of Cy3.0 (Figure 3a,b). This dequenching effect of the donor is concomitant with a decrease of Cy5.0 acceptor emission due to the cancellation of FRET resulting from the protease cleavage. The donor fluorescence change is in agreement with an approximate efficiency of transfer of 90% suggesting that the peptide bond cleavage reaction induced by caspase-3 went to completion. Direct excitation of the acceptor at 640 nm also resulted in a significant increase of the fluorescence emission. Furthermore, a control reaction in which peptide **34** was incubated only with the caspase-3 buffer, did not give non-specific cleavage of this probe.[45]

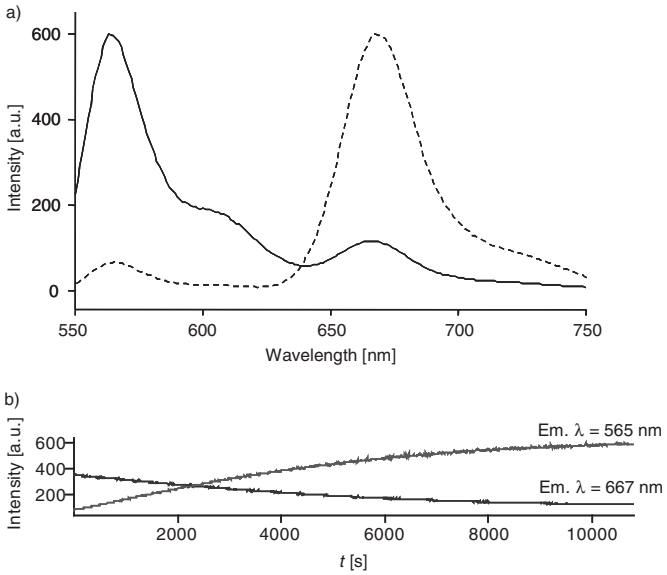
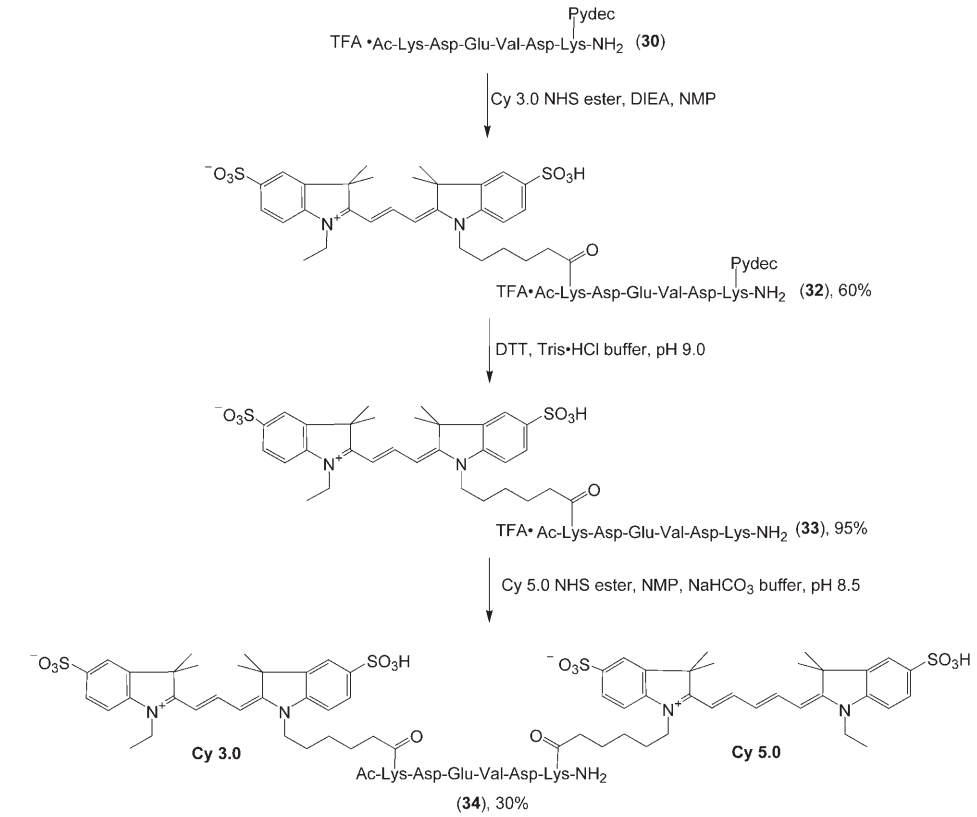


Figure 3. a) Fluorescence emission spectra (excitation at 540 nm) of peptide **34** in caspase-3 buffer at 25 °C (concentration 0.53 μM) with (——) or without (-----) incubation of recombinant human caspase-3 (0.008 U, incubation time 3 h). b) Fluorescence emission time course (excitation at 540 nm) of peptide **34** with recombinant human caspase-3 at 565 and 667 nm.

Selective removal of Ardec protecting groups on solid-phase: As a further proof for the compatibility of the Ardec groups with Fmoc/*t*Bu strategy, solid-supported deprotection and subsequent chemical solid-supported derivatization of a distinct lysine residue within the protected hexapeptide resin Ac-Lys(Boc)-Asp(O*t*Bu)-Glu(O*t*Bu)-Val-Asp(O*t*Bu)-Lys(Phdec)-Rink amide MBHA **R1** was performed (Scheme 9).

Suitable conditions for quantitative removal of the Ardec-amino protecting groups on the solid-phase, estimated on the basis of the results of solution deprotection assays, were β-mercaptoethanol (0.5 M)/DBU (1.0 M) in NMP for 30 min. Under these re-



Scheme 8. Preparation of the energy-transfer substrate of caspase-3 enzyme **34**.

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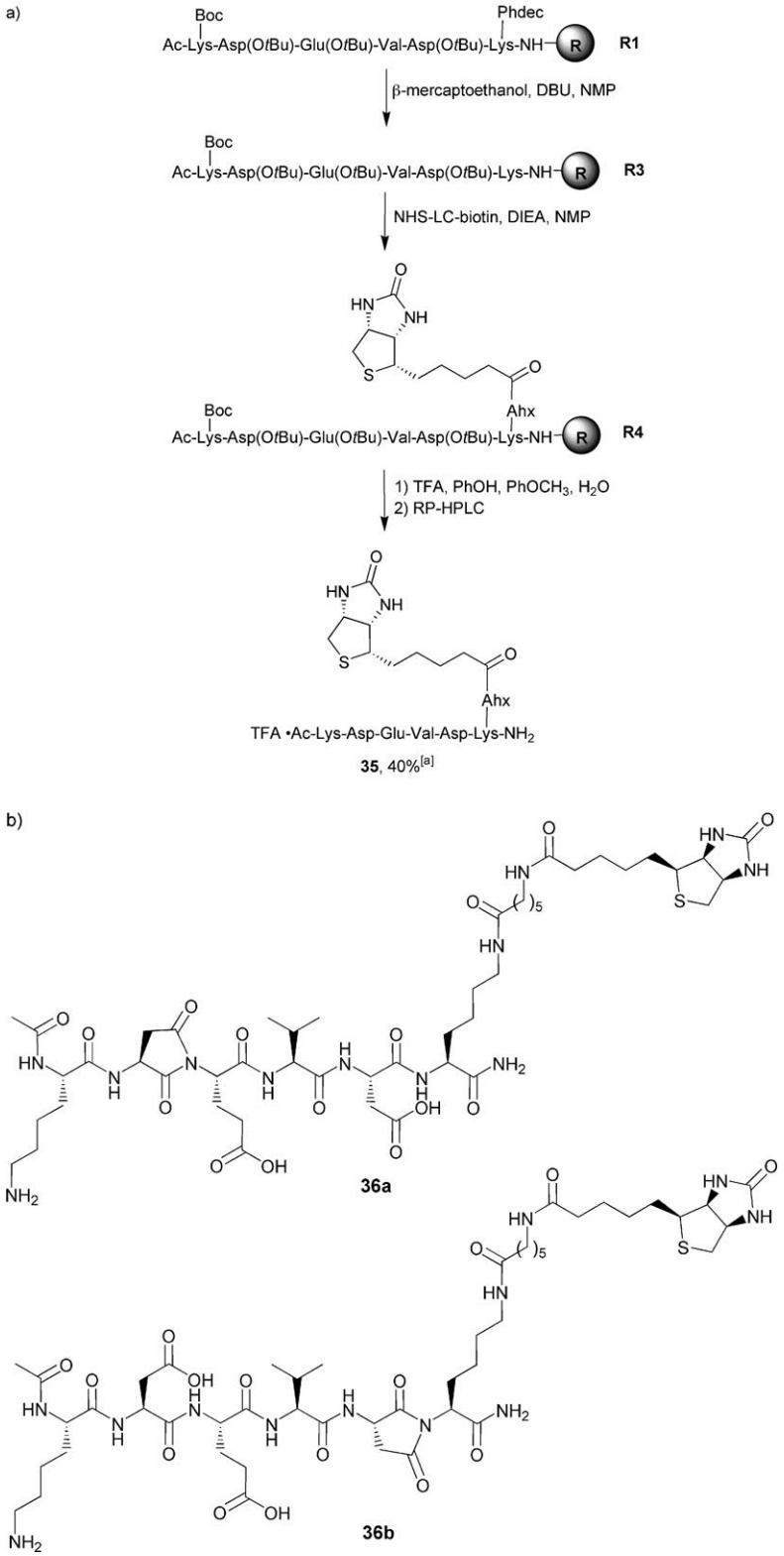

Scheme 9. a) Solid-phase synthesis of biotinylated peptide **35**. b) Structure of aspartimide side products **36a** and **36b**; [a] overall yield from Rink amide MBHA resin.

ductive deprotection conditions, the peptide remains still bound to the resin and all acid-labile protecting groups (including the Boc of lysine residue from the N-terminal side) remain intact. The selective deprotection of the Phdec group was followed by acylation of the resulting amino group with a biotinylation reagent (EZ-Link NHS-LC-biotin) in dry NMP in the presence of DIEA. Deprotection of all the other side chains and simultaneous cleavage from the resin yielded, after precipitation in cold TBME, the crude biotinylated peptide **35** which was finally purified by RP-HPLC (yield: 40%). For this latter chromatographic purification, it was essential to use a slow linear gradient of CH_3CN (0.25% min^{-1}) in aqueous TFA to remove a side product which accounted for 10% of the crude biotinylated peptide and identified as an aspartimide derivative **36a** or **36b** (Scheme 9b, m/z 1095.65 $[M+H]^+$, calcd for: 1094.54). The aspartimide formation is one the best documented side reactions in peptide synthesis.[49] This sequence-dependent cyclization is catalyzed both by acids and bases and frequently occurs during the solid-phase synthesis of peptides bearing the Asp–Gly motif. Furthermore, it was shown that the use of harsh Fmoc cleavage conditions (i.e., use of DBU instead of piperidine) promoted the aspartimide formation even for Asp–AA motifs where Gly is replaced by more sterically hindered amino acids (e.g., Ala, His(Trt), Tyr(*t*Bu)). Therefore, it is likely that our reductive deprotection conditions partly affected the integrity of the Asp-(O*t*Bu)–Glu(O*t*Bu) or Asp-(O*t*Bu)-L-ys(Ardec) patterns. As suggested by Mergler et al.,[49b] this side reaction could have been suppressed by incorporation of Asp residues bearing a β-carboxylic acid protecting group bulkier than *t*Bu ester (3-methyl-pent-3-yl ester). Analysis of the conjugate **35** using MALDI-TOF mass spectrometry indicated that the biotin derivative was attached only once to the peptide (Figure 4).

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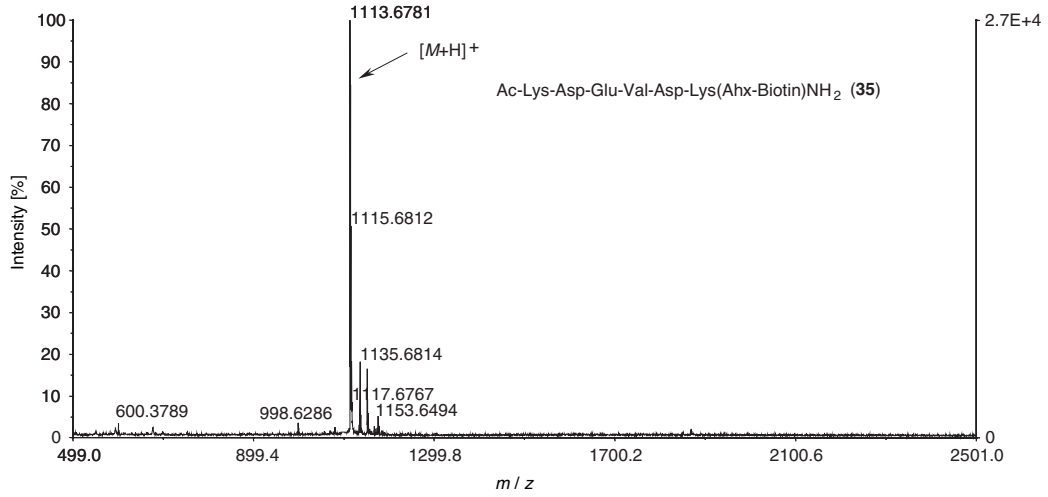

Figure 4. MALDI-TOF mass spectrum of the biotinylated peptide **35**, in the positive mode, α-cyano-4-hydroxycinnamic acid was used as matrix, $[M+H]^+$: m/z: calcd for: 1113.56, found 1113.67.

Since removal of the Ardec protecting group was catalyzed by a strong base such as DBU it was necessary to check if racemization of some amino acids occurred during the deprotection process.[50] HPLC and ^{1}H NMR analyses were considered suitable for this purpose. On the RP-HPLC elution profiles of the biotinylated peptide **35** before and after purification, a single sharp peak was observed reflecting the lack of diastereoisomeric peptide mixture.[45] Furthermore, this was unambiguously confirmed by 1D and 2D ^{1}H NMR spectra which are consistent with the presence of a single diastereomer (Figure 5). Assignment of the proton NMR spectrum of biotinylated peptide **35** in pure methanol at 20 °C was carried out by the sequential assignment strategy proposed by Wüthrich.[51] The first step involved analysis of coupling based two dimensional NMR spectra ^{1}H,^{1}H COSY and ^{1}H,^{1}H TOCSY experiments to identify spin systems characteristic of particular amino acids. Then, the spin system residues were assigned to specific locations in their sequence by observation in the ^{1}H,^{1}H NOESY spectrum of NOE signals between resonances of sequentially adjacent residues. All resonance assignments are summarized in Table 2 (see Experimental Section). From these experiments, we concluded that despite the use of a strong base such as DBU, the Ardec deprotection process did not cause detectable racemization of amino acids in the biotinylated peptide **35**. This is one of the most important requirements which must be fulfilled by a new temporary or semipermanent protecting group designed for a routine use in peptide synthesis.

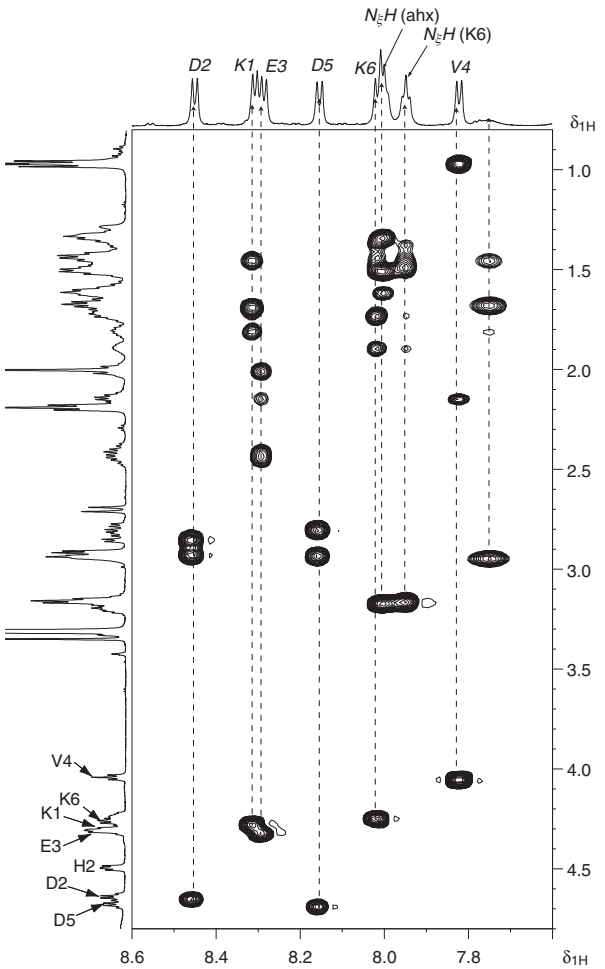

Figure 5. Part of the TOCSY spectrum of biotinylated peptide **35**, recorded in MeOH at 20 °C with a spin lock time of 80 ms. In this spectrum, are shown the connectivities among the amide proton (top) and its aliphatic protons (side chain protons, left), indicated by the residue number.

Conclusion and Perspectives

In summary, new disulfide-containing protecting groups for amines are described. The aryldithioethyloxycarbonyl protecting groups are easily removed by thiols under mild basic conditions. They are stable to a wide variety of conditions,

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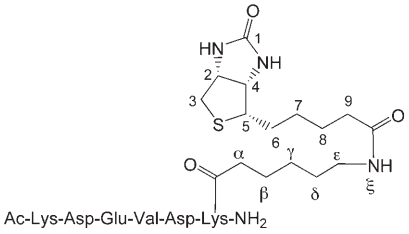
Table 2. Resonance assignments (spectra recorded in MeOH at 20 °C).



AA residue	NH	Hα	Hβ	Hγ	Hδ	Hε	Others
							CH_3: 2.00 (Ac)
Lys (K1)	8.31	4.28	1.80/1.70	1.46	1.67	2.95	NH_3^+: 7.74
Asp (D2)	8.44	4.65	2.93/2.85				
Glu (E3)	8.28	4.32	2.15/2.01	2.44			
Val (V4)	7.82	4.06	2.15	0.97			
Asp (D5)	8.14	4.69	2.93	2.80			
Lys (K6)	8.01	4.25	1.74	1.89	1.50/1.38	3.16	NH: 7.94 NH_2: 7.40/7.15 ($CONH_2$)

Ahx	NHξ	2×Hα	2×Hβ	2×Hγ	2×Hδ	2×Hε
	8.00	2.17	1.62	1.33	1.51	3.17

biotin	2×NH	H-2	2×H-3	H-4	H-5	2×H-6	2×H-7	2×H-8	2×H-9
	_[a]	4.5	2.94/2.7	4.30	3.22	1.75/1.63	1.43	1.60	2.17

[a] Not observed due to exchange with residual water of MeOH.

including many that are used for the removal of other protecting groups (allyl, Aloc, Boc, Dde, Fmoc, silyl and photolabile protecting groups). The utility of the Ardec moiety as protecting group of the lysine side chain is shown with examples from peptide synthesis in solution and on solid support. Since the Pydec moiety is not prone to the disulfide disproportionation reaction leading to the formation of peptide dimers, this protecting group is a more valuable tool than Phdec especially for the synthesis of large peptides intended for post-synthetic modifications in solution. Furthermore, the use of mild reducing conditions has enabled the selective deprotection of lysine side chain and its subsequent chemical modification with molecular tags such as biotin and cyanine dyes. Thus, the Ardec groups may be a useful alternative to the well-known Dde (or its hindered variant ivDde) and Mtt groups which are removed by treatment with bivalent nucleophiles (hydrazine and hydroxylamine) and by treatment with TFA (1% in CH_2Cl_2), respectively, especially for the preparation of cyclic and branched peptides.[52,53] The extension of the Ardec chemistry to amino acids bearing an hydroxyl group within their side chains (serine, threonine, tyrosine) is currently under way and will be reported in due course. It should allow the synthesis of fully functional and sensitive post-translationally site-specific modified proteins (glyco- and lipoproteins) which play important roles in numerous biological processes.[54] Furthermore, in order to get functionalized biopolymers suitable for chemoselective ligation reactions,[55,56] the Ardec-based protection strategy might be extended to other chemical functional groups such as aldehydes and ketones, for which few protecting groups removable under mild conditions are available.[57]

Experimental Section

Abbreviations: Ahx: aminohexanoic acid, Aloc: allyloxycarbonyl, Ardec: aryldithioethyloxycarbonyl, Ardec-ONp: aryldithioethyloxycarbonyl 4-nitrophenyl carbonate, β-Me: β-mercaptoethanol, BOP: benzotriazol-1-yl-*N*-oxytris(dimethylamino)phosphonium hexafluorophosphate, CHAPS: 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, CHCA: α-cyano-4-hydroxycinnamic acid, Cy3.0 dye: 3*H*-indolium-1-(5-carboxypentyl)-2-[(1*E*,3*E*)-3-(1-ethyl-1,3-dihydro-3,3-dimethyl-5-sulfo-2*H*-indol-2-yl-idene)-1-propenyl]-3,3-dimethyl-5-sulfo-, inner salt, Cy5.0 dye: 3*H*-indolium, 2-[(1*E*,3*E*,5*E*)-5-[1-(5-carboxypentyl)-1,3-dihydro-3,3-dimethyl-5-sulfo-2*H*-indol-2-ylidene]-1,3-pentadienyl]-1-ethyl-3,3-dimethyl-5-sulfo-, inner salt, DBU: 1,8-diazobicyclo-[5.4.0]undec-7-ene, Dde: 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl, DIEA: *N*,*N*-diisopropylethylamine, DSC: *N*,*N*′-disuccinimidyl carbonate, Dts: dithiasuccinoyl, DTT: dithiothreitol, EZ-Link NHS-LC-biotin: *N*-hydroxysuccinimidyl-6-(biotinamido)hexanoate, FRET: fluorescence resonance energy transfer, HBTU: 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, HEPES: 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid, HOBt: *N*-hydroxybenzotriazole, NHS: *N*-hydroxysuccinimide, ivDde: 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl, Mpe: 3-methylpent-3-yl, Mtt: 4-methyltrityl, NHS: *N*-hydroxysuccinimide, NMM: *N*-methylmorpholine, NMP: *N*-methylpyrrolidinone, 4NP: 4-nitrophenol, Phdec: phenyldithioethyloxycarbonyl, Pydec: 2-pyridyldithioethyloxycarbonyl, TBTU: 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate, TEAA: triethylammonium acetate, Teoc: (2-trimethylsilyl)ethyloxycarbonyl, Tris·HCl: tris(hydroxymethyl)aminomethane hydrochloride, TSTU: *O*-(*N*-succinimidyl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate.

General methods: Column chromatographic purifications were performed on silica gel (40–63 μm) from SdS. TLC were carried out on Merck DC Kieselgel 60 F-254 aluminium sheets. Compounds were visualized by one or more of the following methods: 1) fluorescence quenching, 2) spray with a 0.2% (w/v) ninhydrin solution in absolute ethanol, 3) spray with a 3.5% (w/v) phosphomolybdic acid solution in absolute ethanol. Acetonitrile was freshly distilled over CaH_2 under an argon atmosphere prior to use. Dichloromethane was dried by distillation over P_2O_5. DIEA and triethylamine were distilled from CaH_2 and stored over BaO. Sulfocyanine dyes Cy3.0 and Cy5.0 were prepared by using literature procedures.[44] EZ-Link NHS-LC-biotin was purchased from Pierce. Recombinant human caspase-3 enzyme (5.52 U mg^{-1}) was purchased from Sigma. The HPLC grade solvents (CH_3CN and MeOH) were obtained from Acros. Aqueous buffers for HPLC were prepared using water purified with a Milli-Q system. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DPX 300 or AMX-400 spectrometer (Bruker, Wissembourg, France). Chemical shifts are expressed in parts per million (ppm) from CD_3CN (δ_H=1.96, δ_C = 1.32 (CH_3), 118.26 (CN)), CD_2Cl_2 (δ_H=5.30, δ_C=53.52), $CDCl_3$ (δ_H=7.26, δ_C=77.36), [D_6]DMSO (δ_H=2.54, δ_C=40.45) or D_2O (δ_H=4.79).[58] *J* values are given in Hz. ^{13}C substitution was determined with a JMOD pulse sequence, differentiating signals of methyl and methine carbons pointing "down" (−) from methylene and quaternary carbons pointing "up" (+). NMR experiments related to biotinylated peptide **35** were achieved at 20 °C on a Bruker DMX 600 spectrometer (Bruker, Wissembourg, France) equipped with a 5 mm triple

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resonance (^{1}H, ^{13}C, ^{15}N) Cryoprobe including shielded z gradients. Bruker XWINNMR software was used to acquire and process the 1D and 2D spectra. The one and two-dimensional spectra were collected with carrier frequency in the middle of the spectrum coinciding with the water resonance which was suppressed by either presaturation using continuous irradiation during relaxation delay or by using a gradient pulse Watergate sequence.[59] TOCSY spectra was acquired with a 80 ms spin-lock time and NOESY experiment was performed using mixing time of 300 ms. ^{1}H chemical shifts were measured relative to the residual signal of the methyl group of the methanol whose shift relative to tetramethylsilane was arbitrarily chosen at 3.31 ppm. Optical rotations were measured by using a Perkin Elmer 341 polarimeter. Infrared (IR) spectra were recorded as thin-film on sodium chloride plates or KBr pellets using a Perkin Elmer FT-IR Paragon 500 spectrometer with frequencies given in reciprocal centimeters (cm^{-1}). UV-visible spectra were obtained on a Varian Cary 50 scan spectrophotometer. Fluorescence spectroscopic studies were performed with a Varian Cary Eclipse spectrophotometer. Mass spectra were obtained with a Micromass Quattro Micro QAA118 spectrometer or a Finnigan LCQ-ion trap apparatus equipped with an electrospray source except for the volatile compounds **11** and **12** which were analyzed with a Thermoquest Finnigan Trace GC coupled to an Auto Mass Multi III analyzer using electron impact ionization. The purified peptides were characterized by MALDI-TOF mass spectrometry on a Voyager DE PRO in the reflector mode with CHCA as a matrix.

Gas chromatography separations: Compounds **11** and **12** were analyzed with the following chromatographic system: J&W Scientific DB5-MS capillary column (0.25 mm × 30 m) coated with a 0.25 μm film of methylsiloxane substituted by 5% phenylsiloxane. The constant pressure was 100 kPa. The injection was performed in the split mode with the temperature of the injection port set at 250 °C. The temperature of the GC oven was maintained at 50 °C for 2 min and then raised to 250 °C at a rate of 25 °C min^{-1} and finally left at that latter temperature for 35 min.

HPLC separations: Several chromatographic systems were used for the analytical experiments and the purification steps. System A: RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 μm, 4.6 × 150 mm) with CH_3CN and triethylammonium acetate buffer (TEAA, 0.1 M, pH 7.0) as eluents [100% TEAA (2 min), linear gradient from 0 to 80% of CH_3CN (40 min)] at a flow rate of 1.0 mL min^{-1}. Dual UV detection was achieved at 254 and 285 nm. System B: RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 μm, 4.6 × 150 mm) with CH_3CN and 0.1% aqueous trifluoroacetic acid (aq. TFA, 0.1%, v/v, pH 2.0) as eluents [100% aq. TFA (5 min), linear gradient from 0 to 60% of CH_3CN (30 min)] at a flow rate of 1.0 mL min^{-1}. Triple UV detection was achieved at 210, 260 and 285 nm. System C: RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 μm, 4.6 × 150 mm) with CH_3CN and aq. TFA as the eluents [80% aq. TFA (5 min), linear gradient from 20 to 90% of CH_3CN (35 min)] at a flow rate of 1.0 mL min^{-1}. UV detection was achieved at 254 nm. System D: RP-HPLC (Interchrom Nucleosil C_{18} column, 5 μm, 10.0 × 250) with CH_3CN and aq. TFA as the eluents [100% aq. TFA (5 min), linear gradient from 0 to 50% of CH_3CN (50 min)] at a flow rate of 4.0 mL min^{-1}. UV detection was achieved at 260 nm. System E: RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 μm, 4.6 × 150 mm) with CH_3CN and aq. TFA as the eluents [100% aq. TFA (2 min), linear gradient from 0 to 60% of CH_3CN (32 min)] at a flow rate of 1.0 mL min^{-1}. Triple UV detection was achieved at 260, 285 and 340 nm. System F: RP-HPLC (Vydac C_{18} column, 5 μm, 22.0 × 250) with CH_3CN/TFA (99.9:0.1) and aq. TFA as the eluents [90% aq. TFA (5 min), linear gradient from 10 to 40% of CH_3CN (40 min)] at a flow rate of 10.0 mL min^{-1}. Dual UV detection was achieved at 215 and 280 nm. System G: RP-HPLC (Vydac C_{18} column, 5 μm, 4.6 × 250) with CH_3CN/TFA (99.9:0.1) and aq. TFA as eluents [90% aq. TFA (5 min), linear gradient from 10 to 90% of CH_3CN (40 min)] at a flow rate of 1.0 mL min^{-1}. Dual UV detection was achieved at 215 and 280 nm. System H: RP-HPLC (Waters XTerra MS C_{18} column, 5 μm, 7.8 × 100 mm) with CH_3CN and aq. formic acid (0.1%, v/v) as eluents [100% aq. formic acid (5 min), linear gradient from 0 to 20% (20 min) and 20 to 80% (80 min) of CH_3CN] at a flow rate of 2.5 mL min^{-1}. UV detection was achieved at 260 nm. System I: RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 μm, 4.6 × 150 mm) with CH_3CN and aq. TFA as eluents [100% aq. TFA (5 min), linear gradient from 0 to 60% of CH_3CN (40 min)] at a flow rate of 1.0 mL min^{-1}. Dual UV detection was achieved at 260 and 550 nm. System J: RP-HPLC (Waters XTerra MS C_{18} column, 5 μm, 7.8 × 100 mm) with CH_3CN and aq. formic acid as the eluents [100% aq. formic acid (5 min), linear gradient from 0 to 20% (20 min) and 20 to 50% (60 min) of CH_3CN] at a flow rate of 2.5 mL min^{-1}. UV detection was achieved at 260 nm. System K: RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 μm, 4.6 × 150 mm) with CH_3CN and aq. TFA as eluents [100% aq. TFA (5 min), linear gradient from 0 to 15% (10 min) and 15 to 60% (90 min) of CH_3CN] at a flow rate of 1.0 mL min^{-1}. Triple UV detection was achieved at 260, 550 and 650 nm. System L: RP-HPLC (Waters XTerra MS C_{18} column, 5 μm, 7.8 × 100 mm) with CH_3CN and aq. TFA as eluents [100% aq. TFA (5 min), linear gradient from 0 to 10% (10 min) and 10 to 50% (160 min) of CH_3CN (40 min)] at a flow rate of 2.5 mL min^{-1}. UV detection was achieved at 218 nm.

2-(Phenyldisulfanyl)ethanol (11): Diphenyl disulfide (7.0 g, 31.8 mmol) was dissolved in a mixture of pyridine and MeOH (300 mL, 1:99). β-Mercaptoethanol (2.1 mL, 30 mmol) was added dropwise to the stirred solution and the mixture was left to stir at room temperature overnight. The reaction was checked for completion by TLC (cyclohexane/ethyl acetate 1:1) and the mixture was evaporated to dryness. The resulting residue was purified by chromatography on a silica gel column (350 g) with a step gradient of ethyl acetate (0→50%) in cyclohexane as the mobile phase, giving **11** as a colorless oil (2.51 g, 13.5 mmol, 45%). $R_f = 0.62$ (cyclohexane/ethyl acetate 1:1); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.80$ (br s, 1H, OH), 2.83 (t, $^{3}J(H,H) = 5.9$ Hz, 2H), 3.79 (m, 2H), 7.15–7.20 (m, 3H), 7.24–7.29 ppm (m, 2H); ^{13}C NMR (75.5 MHz, $CDCl_3$): $\delta = 41.6$, 60.2, 127.5, 128.3 (2C), 129.5 (2C), 137.3 ppm; IR (neat): $\nu_{max} = 1021$, 1067, 1154, 1218, 1296, 1437, 1474, 1577, 2873, 2924, 3355 cm^{-1} (broad); HPLC (system A): $t_R = 24.1$ min, purity 96%; UV/Vis (recorded during the HPLC analysis): $\lambda_{max} = 244$ nm; GC-MS: $t_R = 12.8$ min; m/z: calcd for $C_8H_{10}OS_2$: 186.30; found: 186.0 [$M^{+\bullet}$], 142.0 [$PhSS^{+\bullet}$], 110.0 [PhS^{+}]; elemental anlysis calcd (%) for $C_8H_{10}OS_2$: C 51.58, H 5.41, S 34.42; found: C 51.69, H 5.61, S 34.38.

2-(Pyridin-2-yldisulfanyl)ethanol (12): Bis(2-pyridyl) disulfide (2.2 g, 10.0 mmol) was dissolved in a mixture of pyridine and MeOH (100 mL, 1:99). β-Mercaptoethanol (0.7 mL, 10 mmol) was added dropwise to the stirred solution and the mixture was left to stand at room temperature overnight. The reaction was checked for completion by TLC (CH_2Cl_2/ethyl acetate 8:2) and the mixture was evaporated to dryness. The resulting residue was purified by chromatography on a silica gel column (100 g) with a step gradient of ethyl acetate (0→5%) in dichloromethane as the mobile phase, giving **12** as a colorless oil (0.92 g, 4.9 mmol, 49%). $R_f = 0.59$ (CH_2Cl_2/ethyl acetate 8:2); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 2.93$ (t, $^{3}J(H,H) = 5.1$ Hz, 2H), 3.79 (m, 2H), 5.76 (br s, 1H, OH), 7.12–7.60 (m, 3H), 8.48–8.50 ppm (m, 1H); ^{13}C NMR (75.5 MHz, $CDCl_3$): $\delta = 43.0$, 58.5, 121.9, 122.3, 137.2, 150.2, 159.4 ppm; IR (neat): $\nu_{max} = 1045$, 1063, 1117, 1147, 1285, 1417, 1455, 1558, 1574, 2865, 2923, 3047, 3332 cm^{-1} (broad); HPLC (system A): $t_R = 17.8$ min, purity 91%; UV/Vis (recorded during the HPLC analysis): $\lambda_{max} = 237$ nm, 281 nm; MS (ESI, positive mode): m/z: calcd for $C_7H_9NOS_2$: 187.28; found: 187.46 [M+H]$^{+}$; GC-MS: $t_R = 9.6$ min, m/z: 187.0 [$M^{+\bullet}$], 111.0 [$PyS^{+\bullet}$]; elemental analysis calcd (%) for $C_7H_9NOS_2$: C 44.89, H 4.84, N 7.48, S 34.24; found: C 44.64, H 4.63, N 7.88, S 34.21.

2-(Pyridin-2-yldisulfanyl)ethyl *N*-hydroxysuccinimidyl carbonate (14): 2-(Pyridin-2-yldisulfanyl)ethanol (**12**) (0.38 g, 2.0 mmol) was dissolved in dry CH_3CN (15 mL). Triethylamine (0.55 mL, 4.0 mmol) and DSC (0.62 g, 2.4 mmol) were added and the reaction mixture was stirred at room temperature overnight under an argon atmosphere. The reaction was checked for completion by TLC (CH_2Cl_2/ethyl acetate 8:2) and the mixture was evaporated to dryness. The resulting residue was purified by chromatography on a silica gel column (30 g) with a step gradient of ethyl acetate (0→5%) in dichloromethane as the mobile phase, giving activated carbonate **14** as a pale yellow oil (0.39 g, 1.2 mmol, 60%). $R_f = 0.69$ (CH_2Cl_2/ethyl acetate 8:2); ^{1}H NMR (400 MHz, CD_2Cl_2): $\delta = 2.74$ (s, 4H), 3.08 (t, $^{3}J(H,H) = 8.0$ Hz, 2H), 4.49 (t, $^{3}J(H,H) = 8.0$ Hz, 2H), 7.09 (m, 1H), 7.65 (m, 2H), 8.39 ppm (br d, $^{3}J(H,H) = 4.0$ Hz, 1H); ^{13}C NMR (100 MHz, CD_2Cl_2): $\delta = 25.9$ (2C), 37.2, 68.9, 120.5, 121.6, 138.1, 149.6,

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151.7, 159.2, 169.0 ppm (2C); UV/Vis (water): λ_{max} (ε) = 290 (8188), 371 (20702 $mol^{-1} dm^3 cm^{-1}$); HRMS (ESI, positive mode): m/z: calcd for $C_{12}H_{13}N_2O_5S_2$: 329.0265, found 329.0269 $[M+H]^+$.

2-(Phenyldisulfanyl)ethyl 4-nitrophenyl carbonate (15): 2-(Phenyldisulfanyl)ethanol (**11**) (2.34 g, 12.6 mmol) was dissolved in dry CH_3CN (35 mL). Triethylamine (2.1 mL, 15.1 mmol) was added and the resulting mixture was cooled at −20 °C. Then, 4-nitrophenylchloroformate (3.0 g, 14.9 mmol) was added and the reaction mixture was stirred at room temperature overnight under N_2 atmosphere. The reaction was checked for completion by TLC (cyclohexane/ethyl acetate 7:3) and the mixture was evaporated to dryness. The resulting residue was purified by chromatography on a silica gel column (200 g) with a step gradient of ethyl acetate (0→20 %) in cyclohexane as the mobile phase, giving activated carbonate **15** as a white powder (2.3 g, 6.5 mmol, 53 %). R_f = 0.53 (cyclohexane/ethyl acetate 7:3); m.p. 52 °C (decomp); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.05 (t, ^3J(H,H) = 6.7 Hz, 2H), 4.52 (t, ^3J(H,H) = 6.7 Hz, 2H), 7.23–7.58 (m, 7H), 8.25–8.31 ppm (m, 2H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 36.7, 66.8, 122.1 (2C), 125.7 (2C), 127.7, 128.4 (2C), 129.5 (2C), 136.8, 145.8, 152.5, 155.7 ppm; IR (KBr): ν_{max} = 1075, 1110, 1164, 1218 (broad), 1297, 1347, 1380, 1439, 1477, 1492, 1520, 1594, 1616, 1769, 2859, 2959, 3057, 3081, 3117 cm^{-1}; HPLC (system A): t_R = 37.7 min, purity 90 %; UV/Vis (recorded during the HPLC analysis): λ_{max} = 241, 266 nm; MS (ESI, positive mode): m/z: calcd for $C_{15}H_{13}NO_5S_2$: 351.40; found: 391.50 $[M+K]^+$; elemental analysis calcd (%) for $C_{15}H_{13}NO_5S_2$: C 51.27, H 3.73, N 3.99, S 18.25; found: C 51.25, H 3.69, N 3.93, S 18.31.

2-(Pyridin-2-yldisulfanyl)ethyl 4-nitrophenyl carbonate (16): 2-(Pyridin-2-yldisulfanyl)ethanol (**12**) (0.85 g, 4.5 mmol) was dissolved in dry CH_3CN (13 mL). Triethylamine (0.70 mL, 5.0 mmol) was added and the resulting mixture was cooled at −20 °C. Then, 4-nitrophenylchloroformate (1.05 g, 5.2 mmol) was added and the reaction mixture was stirred at room temperature overnight under N_2 atmosphere. The reaction was checked for completion by TLC (cyclohexane/ethyl acetate 7:3) and the mixture was evaporated to dryness. The resulting residue was purified by chromatography on a silica gel column (50 g) with a step gradient of ethyl acetate (0→30 %) in cyclohexane as the mobile phase, giving activated carbonate **16** as a colorless oil (1.27 g, 3.6 mmol, 78 %). R_f = 0.43 (cyclohexane/ethyl acetate 7:3); ^{1}H NMR (400 MHz, CD_2Cl_2): δ = 3.08 (t, ^3J(H,H) = 6.3 Hz, 2H), 4.46 (t, ^3J(H,H) = 6.3 Hz, 2H), 7.05 (m, 1H), 7.28–7.64 (m, 4H), 8.18 (m, 2H), 8.37 ppm (br d, ^3J(H,H) = 4.5 Hz, 1H); ^{13}C NMR (100 MHz, CD_2Cl_2): δ = 37.2, 67.1, 120.3, 121.5, 122.2 (2C), 125.6 (2C), 137.6, 145.8, 150.1, 152.6, 155.8, 159.6 ppm; IR (neat): ν_{max} = 664, 718, 761, 858, 985, 1082, 1112, 1214 (broad), 1346, 1419, 1448, 1522, 1593, 1617, 1764 (broad), 2958, 3081, 3116 cm^{-1}; HPLC (system A): t_R = 31.6 min, purity 91 %; UV/Vis (recorded during the HPLC analysis): λ_{max} = 243, 272 nm; MS (ESI, positive mode): m/z: calcd for $C_{14}H_{12}N_2O_5S_2$: 352.39; found: 353.63 $[M+H]^+$; elemental analysis calcd (%) for $C_{14}H_{12}N_2O_5S_2$ (contaminated with 6 % of 4NP): C 47.96, H 3.44, N 8.07, S 17.11; found: C 47.83, H 3.49, N 8.06, S 17.02.

N^{ε}-(2-Phenyldisulfanylethyloxycarbonyl)-L-lysine (19): L-Lysine monohydrochloride (0.37 g, 2.0 mmol) was dissolved in distilled water (3 mL) and basic $CuCO_3$ (i.e., $CuCO_3{\cdot}Cu(OH)_2{\cdot}H_2O$, 0.31 g, 1.3 mmol) was added. The resulting mixture was refluxed for 2 h and then the hot suspension was filtered over Celite 545 and washed with distilled water (~20 mL). After cooling to 4 °C, the reaction mixture was basified to pH 9–10 with Na_2CO_3 (1.08 g, 10.2 mmol) and activated carbonate **15** (0.60 g, 1.7 mmol) dissolved in CH_3CN (11 mL) was added. After stirring at room temperature overnight, a bulky blue precipitate was formed. The reaction mixture was acidified to pH ~7 with glacial acetic acid and CH_3CN was removed under reduced pressure. The blue solid was collected by filtration and washed with cold distilled water (2×10 mL) and diethyl ether (2×10 mL). The copper complex was added to a saturated EDTA disodium salt solution (5 g in 50 mL of distilled water) and the resulting suspension was vigorously stirred at room temperature overnight. The initial blue color disappeared gradually and a white solid was separated. After filtering and washings with cold distilled water (2×10 mL) and pentane (2×20 mL), residual water was removed by lyophilization to give N^{ε}-Phdec-lysine **19** as a white solid (0.45 g, 1.25 mmol, 74 %). M.p. > 260 °C; $[\alpha]_D^{20}$ = +10.2° (c = 1 in AcOH 80 %); ^{1}H NMR (300 MHz, [D$_6$]DMSO + 5 % [D]TFA): δ = 1.34–1.43 (m, 4H), 1.78 (m, 2H), 3.01 (m, 4H), 3.93 (m, 1H), 4.18 (t, ^3J(H,H) = 6.2 Hz, 2H), 7.30–7.60 (m, 5H), 8.30 ppm (br s, 1H, NH_3^+); ^{13}C NMR (75.5 MHz, [D$_6$]DMSO + 5 % [D]TFA): δ = 22.6, 29.8, 30.5, 38.2, 40.5, 52.7, 62.3, 128.2 (3C), 130.3 (2C), 137.3, 156.8, 172.0 ppm; IR (KBr): ν_{max} = 687, 736, 1029, 1140, 1240, 1251, 1273, 1326, 1415, 1534, 1579, 1688, 2868, 2948, 3049, 3342 cm^{-1}; HPLC (system B): t_R = 26.4 min, purity 94 %; UV/Vis (recorded during the HPLC analysis): λ_{max} = 234 nm; MS (ESI, positive mode): m/z: calcd for $C_{15}H_{22}N_2O_4S_2$: 358.48; found: 359.09 $[M+H]^+$, 716.99 (dimer) $[2M+H]^+$; elemental analysis calcd (%) for $C_{15}H_{22}N_2O_4S_2$: C 50.26, H 6.19, N 7.81, S 17.89; found: C 49.48, H 6.23, N 7.74, S 16.81.

N^{ε}-(2-(Pyridin-2-yldisulfanyl)ethyloxycarbonyl)-L-lysine (20): L-Lysine monohydrochloride (0.38 g, 2.1 mmol) was dissolved in distilled water (3 mL) and basic $CuCO_3$ (i.e., $CuCO_3{\cdot}Cu(OH)_2{\cdot}H_2O$, 0.30 g, 1.25 mmol) was added. The resulting mixture was heated under reflux for 2 h and then the hot suspension was filtered over Celite 545 and washed with distilled water (~20 mL). After cooling to 4 °C, the reaction mixture was basified to pH 9–10 with Na_2CO_3 (1.1 g, 10.5 mmol) and activated carbonate **16** (0.62 g, 1.8 mmol) dissolved in CH_3CN (12 mL) was added. After stirring at room temperature overnight, a green precipitate was formed. The reaction mixture was acidified to pH ~7 with glacial acetic acid and CH_3CN was removed under reduce pressure. The green solid was collected by filtration and washed with cold distilled water (2×10 mL) and diethyl ether (2×10 mL). The copper complex was added to a saturated EDTA disodium salt solution (5 g in 50 mL of distilled water) and the resulting suspension was vigorously stirred at room temperature overnight. The initial green color disappeared gradually and a yellow solid was separated. After filtering, washings with cold distilled water (2×10 mL) and pentane (2×20 mL) and drying under vacuum N^{ε}-Pydec-lysine (**20**) was obtained as a yellow solid (0.34 g, 0.95 mmol, 53 %). M.p. > 260 °C; $[\alpha]_D^{20}$ = +12.4° (c = 1 in 80 % AcOH); ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 1.38 (m, 4H), 1.60–1.72 (m, 2H), 2.97 (m, 3H), 3.10 (t, ^3J(H,H) = 5.9 Hz, 2H), 4.18 (t, ^3J(H,H) = 5.9 Hz, 2H), 7.29 (m, 2H), 7.85 (m, 2H), 8.50 ppm (d, ^3J(H,H) = 4.1 Hz, 1H, NH_2); ^{13}C NMR (75.5 MHz, [D$_6$]DMSO + 5 % [D]TFA): δ = 22.6, 29.8, 30.5, 38.4, 40.5, 52.7, 62.4, 120.6, 122.4, 139.1, 150.4, 156.8, 159.2, 172.0 ppm; IR (KBr): ν_{max} = 756, 1144, 1274, 1326, 1419, 1447, 1534, 1576, 1686, 2947, 3342 cm^{-1}; HPLC (system B): t_R = 19.2 min, purity 95 %; UV/Vis (recorded during the HPLC analysis): λ_{max} = 262 nm; MS (ESI, positive mode): m/z: calcd for $C_{14}H_{21}N_3O_4S_2$: 359.47; found: 360.12 $[M+H]^+$, 382.19 $[M+Na]^+$; elemental analysis calcd (%) for $C_{14}H_{21}N_3O_4S_2$: C 46.78, H 5.89, N 11.69, S 17.84; found: C 46.54, H 5.98, N 11.55, S 17.53.

N^{α}-(9-Fluorenylmethyloxycarbonyl)-N^{ε}-(2-phenyldisulfanyl-ethyloxycarbonyl)-L-lysine (21): N^{ε}-Phdec-lysine **19** (0.37 g, 1.0 mmol) was dissolved in 4 % sodium carbonate solution (6 mL) and cooled to 4 °C. A solution of Fmoc-OSu (0.28 g, 0.83 mmol) in 1,4-dioxane (6 mL) was added in one portion to the stirred solution and the mixture was left at room temperature overnight. The reaction was checked for completion by TLC (CH_2Cl_2/MeOH/AcOH 95:4:1) and the mixture was acidified to pH ~7 with glacial acetic acid. After partial removal of 1,4-dioxane under reduced pressure, the reaction mixture was lyophilized. The resulting residue was purified by chromatography on a silica gel column (30 g) with a step gradient of MeOH (0→5 %) in dichloromethane as the mobile phase. After drying under vacuum, N^{α}-Fmoc-N^{ε}-Phdec-lysine (**21**) was obtained as a pale yellow foam (0.30 g, 0.52 mmol, 64 %). R_f = 0.60 (CH_2Cl_2/MeOH/AcOH 95:4:1); $[\alpha]_D^{20}$ = +4.9° (c = 1 in CH_2Cl_2); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.25–1.85 (m, 6H), 2.86–3.13 (m, 4H), 4.19–4.80 (m, 6H), 5.67–5.74 (m, NH), 6.32 (br s, NH), 7.18–7.75 ppm (m, 13H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 29.6, 31.9, 37.9, 40.7, 47.5, 53.8 (2C), 62.9, 67.4, 120.3 (2C), 125.4, 127.4 (2C), 128.1 (6C), 129.4 (2C), 137.3, 141.6 (4C), 144.0, 144.2, 156.7 ppm; IR (KBr): ν_{max} = 739, 1066, 1250, 1449, 1526, 1706 (broad), 2939, 3324 cm^{-1}; HPLC (system C): t_R = 30.2 min, purity 92 %; UV/Vis (recorded during the HPLC analysis): λ_{max} = 262 nm; MS (ESI, positive mode): m/z: calcd for $C_{30}H_{32}N_2O_6S_2$: 580.73; found: 581.11 $[M+H]^+$, 1161.04 (dimer) $[2M+H]^+$; elemental analysis calcd (%) for $C_{30}H_{32}N_2O_6S_2{\cdot}CH_4O$ (MeOH): C 60.76, H 5.92, N 4.57, S 10.47; found: C 60.98, H 5.47, N 4.78, S 10.24.

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N^{α}-(9-Fluorenylmethyloxycarbonyl)-N^{ε}-(2-(pyridin-2-yldisulfanyl)ethyloxycarbonyl)-L-lysine (22): N^{ε}-Pydec-lysine **20** (0.31 g, 0.90 mmol) was dissolved in 4% sodium carbonate solution (5 mL) and cooled to 4°C. A solution of Fmoc-OSu (0.25 g, 0.73 mmol) in a mixture of CH_3CN/1,4-dioxane 5:1 (6 mL) was added in one portion to the stirred solution and the mixture was left at room temperature overnight. The reaction was checked for completion by TLC (CH_2Cl_2/MeOH/AcOH 90:8:2) and the mixture was acidified to pH ~7 with glacial acetic acid. After removal of CH_3CN under reduced pressure, the reaction mixture was lyophilized. The resulting residue was purified by chromatography on a silica gel column (40 g, dry loading) with a step gradient of MeOH (0→10%) in dichloromethane as the mobile phase. After drying under vacuum, N^{α}-Fmoc-N^{ε}-Pydec-lysine (**22**) was obtained as a yellow foam (0.34 g, 0.58 mmol, 80%). R_f = 0.54 (CH_2Cl_2/MeOH/AcOH 90:8:2); $[\alpha]_D^{20} = -3.3°$ (c = 1 in CH_2Cl_2); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.22–1.52 (m, 4H), 1.88 (m, 2H), 2.99 (t, ^3J(H,H) = 5.6 Hz, 2H), 3.19 (m, 2H), 4.22 (t, ^3J(H,H) = 6.9 Hz, 1H), 4.33–4.42 (m, 4H), 4.86 (m, 1H), 5.70 (br d, ^3J(H,H) = 7.7 Hz, NH), 7.13–7.76 (m, 11H), 8.48 ppm (br d, ^3J(H,H) = 3.8 Hz, 1H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 22.1, 29.4, 32.1, 38.3, 40.6, 47.5, 53.9, 63.5, 67.3, 120.3 (2C), 120.6, 121.4, 125.5 (2C), 127.4 (2C), 128.0 (2C), 138.1, 141.6 (4C), 144.1 (2C), 149.3, 156.5, 160.2 ppm; IR (KBr): ν_{max} = 741, 760, 1046, 1082, 1119, 1250, 1418, 1449, 1531, 1715 (broad), 2945, 3065, 3324 cm^{-1}; HPLC (system C): t_R = 24.9 min, purity 94%; UV/Vis (recorded during the HPLC analysis): λ_{max} = 262 nm; MS (MALDI-TOF, positive mode): m/z: calcd for $C_{29}H_{31}N_3O_6S_2$: 581.71; 582.3 $[M+H]^+$, 604.2 $[M+Na]^+$, 620.2 $[M+K]^+$ (CHCA matrix); elemental analysis calcd (%) for $C_{29}H_{31}N_3O_6S_2$: C 59.88, H 5.37, N 7.22, S 11.02; found: C 59.36, H 5.47, N 7.15, S 10.64.

General procedure for the coupling of Fmoc-Lys(Ardec)-OH to L-phenylalanine *tert*-butyl ester: DIEA (90 µL, 0.51 mmol) was added to a solution of N^{α}-Fmoc-N^{ε}-Ardec-lysine **21** or **22** (0.1 g, 0.17 mmol) and L-phenylalanine *tert*-butyl ester hydrochloride (37 mg, 0.17 mmol) dissolved in dry CH_2Cl_2 (1–2 mL), followed by BOP coupling reagent (75 mg, 0.17 mmol). The resulting reaction mixture was stirred at room temperature overnight under N_2 atmosphere. The reaction was checked for completion by TLC (CH_2Cl_2/MeOH 99:1 or 97:3) and the mixture was evaporated to dryness. The resulting residue was purified by chromatography on a silica gel column (10–15 g) with a step gradient of MeOH (0→3%) in dichloromethane as the mobile phase. The appropriate fractions were pooled and then concentrated to dryness, giving the full-protected dipeptide **23** or **24** as white solids.

N^{α}-(9-Fluorenylmethyloxycarbonyl)-N^{ε}-(2-(2-(phenyldisulfanyl)ethyloxycarbonyl)-L-lysyl-L-phenylalanine *tert*-butyl ester (23): Yield: 80%; R_f = 0.42 (CH_2Cl_2/MeOH 99:1); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.4 (s, 9H), 1.41–1.85 (m, 4H), 2.87–3.13 (m, 4H), 3.43–3.3.49 (m, 1H), 4.08–4.44 (m, 6H), 4.68–4.77 (m, 2H, α-CH Lys and Phe), 5.39 (br d, ^3J(H,H) = 6.0 Hz, NH), 6.30 (br d, ^3J(H,H) = 6.4 Hz, 1H), 7.11–7.77 (m, 18H), 8.44 ppm (d, ^3J(H,H) = 4.9 Hz, 1H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 29.0 (3C), 29.6, 30.9, 37.0, 37.7, 40.9, 47.5, 48.0, 53.5, 63.2, 67.5, 71.4, 120.5 (2C), 125.6 (2C), 127.9 (6C), 128.4 (2C), 128.9 (4C), 129.2 (2C), 132.5, 136.5 (2C), 140.2, 141.9 (2C), 145.5 (2C), 172.0, 174.7 ppm; IR (KBr): ν_{max} = 739, 1263, 1537, 1693 (broad), 2939, 3062, 3312 cm^{-1}; HPLC (system C): t_R = 34.1 min, purity 95%; MS (MALDI-TOF, positive mode): m/z: calcd for $C_{43}H_{49}N_3O_7S_2$: 784.01; found: 806.57 $[M+Na]^+$ (CHCA matrix); elemental analysis calcd (%)for $C_{43}H_{49}N_3O_7S_2{\cdot}CH_4O$ (MeOH): C 64.76, H 6.55, N 5.15, S 7.86; found: C 64.51, H 6.57, N 5.16, S 7.85.

N^{α}-(9-Fluorenylmethyloxycarbonyl)-N^{ε}-(2-(2-(pyridin-2-yldisulfanyl)-ethyloxycarbonyl)-L-lysyl-L-phenylalanine *tert*-butyl ester (24): Yield: 95%; R_f = 0.26 (CH_2Cl_2/MeOH 97:3); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.40 (s, 9H), 1.41–1.85 (m, 4H), 2.97–3.22 (m, 4H), 3.66–3.77 (m, 1H), 4.11–4.43 (m, 6H), 4.71 (q, ^3J(H,H) = 6.4 Hz, ^3J(H,H) = 13.9 Hz, 1H, α-CH Lys), 4.87 (m, 1H, α-CH Phe), 5.52 (br d, ^3J(H,H) = 8.2 Hz, NH), 6.37 (br d, ^3J(H,H) = 7.4 Hz, 1H), 7.05–7.77 (m, 16H), 8.44 ppm (d, ^3J(H,H) = 4.9 Hz, 1H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 22.3, 29.1 (3C), 29.4, 32.1, 37.1, 38.4, 40.8, 47.6, 54.0, 55.5, 63.5, 67.4, 67.5, 118.6, 121.6, 125.7, 126.5 (2C), 127.8 (6C), 128.4 (2C), 128.9 (2C), 136.5 (2C), 136.7, 140.2, 141.9 (2C), 149.8, 157.5 (2C), 171.7, 174.3 ppm; IR (KBr): ν_{max} = 739, 760, 845, 1152, 1253, 1447, 1522, 1654, 1718 (broad), 2935, 2976, 3312, 3411 cm^{-1}; HPLC (system C): t_R = 33.3 min, purity 93%; MS (ESI, positive mode): m/z: calcd for $C_{42}H_{48}N_4O_7S_2$: 785.00; found: 785.3 $[M+H]^+$; elemental analysis calcd (%) for $C_{42}H_{49}N_4O_7S_2{\cdot}CH_4O$ (MeOH): C 63.21, H 6.42, N 6.86, S 7.85; found: C 63.25, H 6.52, N 6.83, S 7.77.

General procedure for the removal of Fmoc-protecting group and *tert*-butyl ester: Fmoc-Lys(Ardec)-Phe-O*t*Bu **23** or **24** (0.1 g, 0.12 mmol) was dissolved in CH_2Cl_2 (2 mL) and cooled to 4°C. TFA (1.9 mL) and deionized water (0.1 mL) were added and the resulting reaction mixture was warmed to room temperature and stirred for 2 h. The reaction was checked for completion by TLC (CH_2Cl_2/MeOH 95:5) and the mixture was evaporated to dryness. TFA was removed by azeotropic distillation with cyclohexane (3 × 10 mL). Finally, the product was isolated by chromatography on a silica gel column (10–15 g) with a step gradient of MeOH (0→5%) in dichloromethane as the mobile phase, giving the dipeptide Fmoc-Lys(Ardec)-Phe-OH as a white solid (yield 85–95%). Fmoc-Lys(Ardec)-Phe-OH (40 mg, 0.055 mmol) was dissolved in CH_2Cl_2 (2 mL). Et_2NH (0.17 mL, 1.64 mmol) was added and the resulting reaction mixture was stirred at room temperature overnight. The reaction was checked for completion by TLC (CH_2Cl_2/MeOH 95:5) and the mixture was evaporated to dryness. The resulting residue was taken up in a mixture of aq. TFA and CH_3CN 4:1 (5 mL) and purified by semi-preparative RP-HPLC (system D, 2 injections). The appropriate fractions were lyophilized, giving the TFA salt of dipeptide H-Lys(Ardec)-Phe-OH as a white amorphous powder.

[N^{ε}-(2-Phenyldisulfanylethyloxycarbonyl]-L-lysyl-L-phenylalanine (25): Yield after RP-HPLC purification: 64%; ^{1}H NMR (300 MHz, CD_3CN): δ = 1.29–1.46 (m, 4H), 1.81 (m, 2H), 2.95–3.23 (m, 6H), 3.92 (m, 1H, α-CH Lys), 4.20 (t, ^3J(H,H) = 6.0 Hz, 2H), 4.63 (m, 1H, α-CH Phe), 5.73 (br s, 1H, NH Phdec), 7.24–7.60 ppm (m, 12H); ^{13}C NMR (75.5 MHz, CD_3CN + 10% H_2O): δ = 21.7, 29.5, 31.2, 37.1, 38.1, 40.6, 53.7, 54.8, 62.7, 118.3, 127.4, 127.9, 128.2 (2C), 129.1 (2C), 129.9 (4C), 137.5, 157.4, 169.4, 173.2 ppm (weak signal); IR (KBr): ν_{max} = 722, 740, 800, 839, 1139, 1203, 1524, 1679 (broad), 2951, 3061 cm^{-1} (broad); HPLC (system E): t_R = 25.9 min, purity 94%; UV/Vis (CH_3CN): λ_{max} (ε) = 237 (6635), 260 nm (1467 mol^{-1} dm^3 cm^{-1}); MS (MALDI-TOF, positive mode): m/z: calcd for $C_{24}H_{31}N_3O_5S_2$: 505.66; found: 506.4 $[M+H]^+$, 528.4 $[M+Na]^+$, 544.4 $[M+K]^+$ (CHCA matrix); too hygroscopic for elemental analysis.

[N^{ε}-(2-(Pyridin-2-yldisulfanyl)ethyloxycarbonyl]-L-lysyl-L-phenylalanine (26): Yield after RP-HPLC purification: 60%; ^{1}H NMR (300 MHz, D_2O): δ = 1.07–1.55 (m, 4H), 1.79 (m, 2H), 2.91–3.26 (m, 6H), 3.89 (t, ^3J(H,H) = 6.4 Hz, 1H, α-CH Lys), 4.19–4.29 (m, 2H), 4.63 (q, ^3J(H,H) = 8.7 Hz, ^3J(H,H) = 6.0 Hz, 1H, α-CH Phe), 7.24–7.37 (m, 5H), 7.59 (t, ^3J(H,H) = 6.8 Hz, 1H), 8.06–8.19 (m, 2H), 8.51 ppm (br d, ^3J(H,H) = 5.7 Hz, 1H); ^{13}C NMR (75.5 MHz, D_2O): δ = 23.2, 30.7, 32.7, 38.5, 39.9, 42.0, 55.1, 56.7, 64.5, 125.9, 127.1, 129.5, 131.0 (2C), 131.4 (2C), 138.7, 145.6, 146.8, 158.7, 160.2, 171.8, 176.6 ppm; IR (KBr): ν_{max} = 702, 723, 764, 800, 839, 1138, 1204, 1261, 1420, 1454, 1538, 1679 (broad), 2944, 3065 cm^{-1} (broad); HPLC (system E): t_R = 20.8 min, purity 97%; UV/Vis (water): λ_{max} (ε) = 282 (3591), 260 nm (2250 mol^{-1} dm^3 cm^{-1}); MS (MALDI-TOF, positive mode): m/z: calcd for $C_{23}H_{30}N_4O_5S_2$: 506.65; found: 507.6 $[M+H]^+$, 569.6 $[M+K]^+$ (CHCA matrix); too hygroscopic for elemental analysis.

L-Lysyl-L-phenylalanine (28): This compound was prepared from N^{α}-Fmoc-N^{ε}-Boc-lysine and L-phenylalanine *tert*-butyl ester hydrochloride using the classical peptide coupling and deprotection protocols described for dipeptides **25**, **26**. Yield after RP-HPLC purification: 40%; ^{1}H NMR (300 MHz, D_2O): δ = 1.28–1.88 (m, 6H), 2.93 (t, ^3J(H,H) = 7.5 Hz, 2H), 3.00–3.25 (m, 2H), 3.91 (t, ^3J(H,H) = 6.4 Hz, 1H, α-CH Lys), 4.62 (q, ^3J(H,H) = 8.8 Hz, ^3J(H,H) = 6.0 Hz, 1H, α-CH Phe), 7.27–7.38 ppm (m, 5H); ^{13}C NMR (75.5 MHz, D_2O): δ = 21.2, 26.6, 30.6, 36.6, 39.3, 53.0, 54.9, 127.5, 129.1 (2C), 129.4 (2C), 136.9, 169.7, 175.2 ppm; IR (KBr): ν_{max} = 724, 802, 841, 1140, 1204, 1437, 1457, 1546, 1679 (broad), 3066 cm^{-1} (broad); HPLC (system E): t_R = 12.8 min, purity 97%; UV/Vis (water): λ_{max} (ε) = 260 nm (210 mol^{-1} dm^3 cm^{-1}); MS (MALDI-TOF, positive mode): m/z: calcd for $C_{15}H_{23}N_3O_3$ 293.37; found: 294.4 $[M+H]^+$ (CHCA matrix); too hygroscopic for elemental analysis.

Stability studies of the Ardec groups at the dipeptide level: Solutions of dipeptide(Ardec) **25** or **26** in dry NMP (1 mg mL^{-1}, 1.6 mM for **25**, 1.4 mM

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for **26** or 10 mg mL^{-1}, 16 mM for **25** and 14 mM for **26**) were prepared. Aliquots of the solution (20 μL) were incubated with different amounts of aq. or organic solutions corresponding to the cleavage conditions reported in Table 1 (entries 1–12), for 1 h at room temperature. Thereafter, aliquots were quenched with a 1 M TFA/NMP solution, diluted with aq. TFA and analyzed by RP-HPLC (system E). For the stability towards UV light, 45 μL of the solutions (1 mg mL^{-1}) were transferred into a quartz fluorescence cell. UV irradiation was performed with a fluorescence spectrophotometer by continuous excitation at 350 nm for 1 h at 25 °C. Thereafter, aliquots were diluted with aq. TFA and anlyzed by RP-HPLC (system E). Quantification of **25** (or **26)** and **28** was achieved by measurement of the corresponding peak areas at $t_R = 25.9$ (or 21.0) and 12.8 min and by using the following extinction coefficients at 260 nm: $\varepsilon =$ 1467 (**25**), 2250 (**26**) and 210 mol^{-1} dm^3 cm^{-1} (**28**).

Studies on removal of the Ardec groups at the dipeptide level: *Deprotection in water*: Solutions of dipeptide(Ardec) **25** or **26** in deionized water (1 mg mL^{-1}, 1.6 mM for **25**, 1.4 mM for **26**) were prepared. Aliquots of the solution (10 μL) were incubated with different amounts (5, 10, 20, 40 and 80 equiv) of reducing agent (DTT or β-mercaptoethanol) in solution (25 mM) in Tris·HCl buffer (0.1 M) at pH 7.5, 8.0, 8.5 and 9.0, for 30 min. Thereafter, aliquots were quenched with aq. TFA and analyzed by RP-HPLC (system E).

Deprotection in organic medium: Solutions of dipeptide(Ardec) **25** or **26** in dry NMP (1 mg mL^{-1}, 1.6 mM for **25**, 1.4 mM for **26**) were prepared. Aliquots of the solution (10 μL) were incubated with different amounts (10, 20, 40 and 80 equiv) of a (1:1, mol per mol) β-mercaptoethanol/base (DBU, DIEA, piperidine and TEA) mixture (25 mM) in dry NMP for 15 min. Thereafter, aliquots were quenched with a solution of TFA in dry NMP (0.25 M), diluted with aq. TFA and analyzed by RP-HPLC (system E). For further deprotection studies with DBU, aliquots of the solution were incubated with 80 equiv of β-mercaptoethanol and different amounts of base (160, 240 and 320 equiv) in solution in dry NMP for 15 min.

Solid-phase synthesis of hexapeptides Ac-Lys-Asp-Glu-Val-Asp-Lys-(Ardec)-NH$_2$ (29, 30): Fmoc Rink amide MBHA resin (0.19 g, loading 0.52 mmol g^{-1}) was treated with a 20% piperidine solution in NMP (10 mL) for 20 min. Thereafter, the resin was washed with NMP (2 × 10 mL) and CH_2Cl_2 (2 × 10 mL). The completion of the Fmoc removal was checked by the Kaiser ninhydrin test (positive after this treatment).[60] Fmoc-Lys(Ardec)-OH **21** or **22** (0.29 g, 0.5 mmol) was coupled to the resin with TBTU (0.16 g, 0.5 mmol), HOBt (67 mg, 0.5 mmol) and DIEA (0.26 mL, 1.5 mmol) in NMP (5 mL) for 2 h (Kaiser ninhydrin test negative after this time). After washings with NMP (2 × 10 mL) and 2-propanol (2 × 10 mL), the resin was swollen in NMP (1 mL) and transferred into a reaction vessel which was placed in the ABI 433 A peptide synthesizer. Automated synthesis was carried out according to a general Fmoc strategy. Fmoc-Asp(O*t*Bu)-OH (0.41 g, 1.0 mmol), Fmoc-Glu-(O*t*Bu)-OH (0.44 g, 1.0 mmol), Fmoc-Val-OH (0.34 g, 1.0 mmol) and Fmoc-Lys(Boc)-OH (0.47 g, 1.0 mmol) were successively coupled with HBTU (1.0 mmol), HOBt (1.0 mmol) and DIEA (3.7 mmol). Deprotection of Fmoc groups was carried out with a 20% piperidine solution in NMP (3 × 1 min). Acetylation of the free amino group from the N terminal side was achieved by treatment with a mixture of Ac_2O (0.43 M), HOBt (0.015 M) and DIEA (0.13 M) in NMP (5 min). After completion of the synthetic reaction, the peptide resin Ac-Lys(Boc)-Asp(O*t*Bu)-Glu-(O*t*Bu)-Val-Asp(O*t*Bu)-Lys(Ardec)-Rink amide MBHA was washed with NMP and CH_2Cl_2 and dried under vacuum (0.25 and 0.2 g, of peptidyl resins **R1** and **R2** respectively). Two thirds of the resin was treated with TFA/PhOH/$PhOCH_3$/H_2O 85.8:5.6:4.3:4.3 (10 mL) at room temperature for 2 h. The filtrate from the cleavage reaction was precipitated with cold TBME (40 mL) and the peptide was collected by centrifugation. The white pellet was washed with TBME (2 × 15 mL) and dried under vacuum (25 and 38 mg of crude peptides **29** and **30**; respectively). The crude peptide was purified by semi-preparative RP-HPLC (system F, 2 injections). The appropriate fractions were lyophilized, giving the TFA salt of hexapeptide Ac-Lys-Asp-Glu-Val-Asp-Lys(Ardec)-NH_2 as a white amorphous powder.

Ac-Lys-Asp-Glu-Val-Asp-Lys(Phdec)-NH$_2$ (29): Overall yield after RP-HPLC purification: 17%; HPLC (system G): $t_R = 21.5$ min, purity 95%; MS (MALDI-TOF, positive mode): *m*/*z*: calcd for $C_{41}H_{63}N_9O_{15}S_2$: 985.38; found: 986.8 $[M+H]^+$, 1010.8 $[M+Na]^+$, 1024.8 $[M+K]^+$ (CHCA matrix).

Ac-Lys-Asp-Glu-Val-Asp-Lys(Pydec)-NH$_2$ (30): Overall yield after RP-HPLC purification: 15%; HPLC (system G): $t_R = 16.1$ min, purity 93%; MS (MALDI-TOF, positive mode): *m*/*z*: calcd for $C_{40}H_{62}N_{10}O_{15}S_2$: 986.38; found: 987.4 $[M+H]^+$, 1009.4 $[M+Na]^+$, 1025.3 $[M+K]^+$ (CHCA matrix).

Peptide dimer 31: Overall yield after RP-HPLC purification: 4%; HPLC (system G): $t_R = 15.9$ min, purity 95%; MS (MALDI-TOF, positive mode): *m*/*z*: calcd for $C_{70}H_{116}N_{18}O_{30}S_2$: 1752.75, 1753.9 $[M+H]^+$, 1775.8 $[M+Na]^+$, 1791.8 $[M+K]^+$ (CHCA matrix).

Synthesis and characterization of fluorogenic caspase-3 substrate Ac-Lys-(Cy 3.0)-Asp-Glu-Val-Asp-Lys(Cy 5.0)-NH$_2$ (34)

Cy 3.0 carboxy succinimidyl ester: Cy 3.0 carboxylic acid (3 mg, 4.75 μmol) was introduced into a Reacti-Vial (Pierce, No. 13222) and dissolved in dry NMP (75 μL). A solution (75 μL) of TSTU reagent in dry NMP (1.4 mg, 4.75 μmol) and DIEA (1.65 μL, 9.5 μmol) were added and the resulting reaction mixture was protected from the light and stirred at room temperature for 1 h.[61]

Ac-Lys(Cy 3.0)-Asp-Glu-Val-Asp-Lys(Pydec)-NH$_2$ (32): Peptide Ac-Lys-Asp-Glu-Val-Asp-Lys(Pydec)-NH_2 (**30**) (6.50 mg, 5.35 μmol, weighted in a 1.0 mL Eppendorf tube) was dissolved in dry NMP (130 μL) and DIEA (4.1 μL, 23.7 μmol) was added. After complete solubilization by vortexing, the resulting solution was added to the crude reaction mixture containing the succinimidyl ester of Cy 3.0 dye. The reaction mixture was protected from the light and stirred at room temperature for 1 h. Finally, the reaction mixture was quenched by dilution with aq. formic acid (2 mL) and purified by RP-HPLC (system H, 2 injections). The product-containing fractions were lyophilized to give the peptide-Cy 3.0 conjugate **32** as a pink amorphous powder. Quantification was achieved by UV/Vis measurements at $\lambda_{max} = 549$ nm of the Cy 3.0 dye by using the $\varepsilon = 150000$ value (yield after RP-HPLC purification: 60%); HPLC (system I): $t_R =$ 26.2 min, purity 95%; UV/Vis (recorded during the HPLC analysis): $\lambda_{max} = 549$ nm; MS (MALDI-TOF, positive mode): *m*/*z*: calcd for $C_{71}H_{98}N_{12}O_{22}S_4$: 1598.58; found: 1600.8 $[M+H]^+$, 1621.8 $[M+Na]^+$, 1637.7 $[M+K]^+$ (CHCA matrix).

Removal of the Pydec group: Peptide Ac-Lys(Cy 3.0)-Asp-Glu-Val-Asp-Lys(Pydec)-NH_2 (**32**) (5.5 mg, 3.2 μmol, weighed in a 2.0 mL Eppendorf tube) was dissolved in 2.5 mL of Tris·HCl buffer (0.1 M, pH 9.0) and a solution of DTT (25 mg in 1 mL, 160 μmol) in Tris·HCl buffer (0.1 M, pH 9.0) was added. The reaction mixture was protected from the light and stirred at room temperature for 9 h. The deprotection was checked for completion by RP-HPLC (system I) and the reaction mixture was quenched by dilution with aq. formic acid (2 mL). Purification was achieved by RP-HPLC (system H, 2 injections). The product-containing fractions were lyophilized to give the peptide Ac-Lys(Cy 3.0)-Asp-Glu-Val-Asp-Lys-NH_2 (**33**) as a pink amorphous powder. Quantification was achieved by UV/Vis measurements at $\lambda_{max} = 549$ nm of the Cy 3.0 dye by using the $\varepsilon = 150000$ value (yield after RP-HPLC purification: 95%); HPLC (system K): $t_R = 27.2$ min, purity 98%; UV/Vis (recorded during the HPLC analysis): $\lambda_{max} = 549$ nm; MS (MALDI-TOF, positive mode): *m*/*z*: calcd for $C_{63}H_{91}N_{11}O_{20}S_2$: 1385.58; found 1386.4 $[M+H]^+$, 1408.4 $[M+Na]^+$, 1424.4 $[M+K]^+$ (CHCA matrix).

Cy 5.0 carboxy succinimidyl ester: Cy 5.0 carboxylic acid (1 mg, 1.5 μmol) was converted into the corresponding succinimidyl ester using the procedure previously described for Cy 3.0 dye.

Ac-Lys(Cy 3.0)-Asp-Glu-Val-Asp-Lys(Cy 5.0)-NH$_2$ (34): Peptide Ac-Lys-(Cy 3.0)-Asp-Glu-Val-Asp-Lys-NH_2 **33** (3 mg, 2.0 μmol, weighed in a 1.0 mL Eppendorf tube) was dissolved in sodium bicarbonate buffer (300 μL, 0.1 M, pH 8.5). After complete solubilization by vortexing, the resulting solution was added to the crude reaction mixture containing the succinimidyl ester of Cy 5.0 dye. The reaction mixture was protected from the light and stirred at room temperature for 4 h. Finally, the reaction mixture was quenched by dilution with aq. formic acid (2 mL) and

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purified by RP-HPLC (system J, 2 injections). The product-containing fractions were lyophilized to give the peptide Ac-Lys(Cy3.0)-Asp-Glu-Val-Asp-Lys(Cy5.0)-NH_2 **34** as a blue-purple amorphous powder. Stock solution of fluorogenic caspase-3 substrate **34** was prepared in HPLC grade water and UV/Vis quantification was achieved at $\lambda_{max}=643$ nm of the Cy5.0 dye by using the $\varepsilon=250000$ value (yield after RP-HPLC purification: 30%); HPLC (system K): $t_R=36.0$ min, purity 95%; UV/Vis (recorded during the HPLC analysis): $\lambda_{max}=549$, 643 nm; MS (MALDI-TOF, positive mode): m/z: calcd for $C_{96}H_{129}N_{13}O_{27}S_4$: 2023.80; found: 2025.7 $[M+H]^+$, 2063.6 $[M+K]^+$ (CHCA matrix).

In vitro peptide cleavage by recombinant human caspase-3: A 0.53 μM solution of peptide **34** was prepared in caspase-3 buffer (500 μL, 100 mM NaCl, 40 mM HEPES, 10 mM DTT, 1 mM EDTA, 10% (w/v) sucrose and 0.1% (w/v) CHAPS, pH 7.2) and transferred into a quartz fluorescence cell. Human recombinant caspase-3 (5 μL, 0.008 U) was added and the resulting mixture was incubated at 25°C. After excitation at 540 nm, fluorescence at 565 nm and 667 nm were simultaneously monitored over time with measurements recorded every 5 s.

Solid-phase deprotection of the Ardec groups

Application to the synthesis of Ac-Lys-Asp-Glu-Val-Asp-Lys(Ahx-biotin)-NH_2 (35): The peptide resin Ac-Lys(Boc)-Asp(O*t*Bu)-Glu(O*t*Bu)-Val-Asp(O*t*Bu)-Lys(Phdec)-Rink amide MBHA (**R1**) (54 mg, 0.02 mmol) was swollen in dry NMP and treated with a freshly prepared solution of β-mercaptoethanol (0.5 M) and DBU (1.0 M) in dry NMP (4.25 mL) for 1 h. Thereafter, the resin was washed with NMP (2×5 mL) and CH_2Cl_2 (2×5 mL). The completion of the Phdec removal was checked by the Kaiser ninhydrin test (positive after this treatment). EZ-Link NHS-LC-biotin (55 mg, 0.10 mmol) was reacted to the free amino group of the peptide resin **R3**, in the presence of DIEA (41 μL, 0.23 mmol), in dry NMP (1.8 mL) overnight (Kaiser ninhydrin test negative after this time). After washings with NMP (2×5 mL) and CH_2Cl_2 (2×5 mL) drying under vacuum, the resin was treated with TFA/PhOH/$PhOCH_3$/H_2O 85.8:5.6:4.3:4.3 (5 mL) at room temperature for 2 h. The filtrate from the cleavage reaction was precipitated with cold TBME (15 mL) and the peptide was collected by centrifugation. The white pellet was washed with TBME (2×5 mL) and dried under vacuum. The crude peptide was purified by semi-preparative RP-HPLC (system L, 2 injections). The appropriate fractions were lyophilized, giving the TFA salt of hexapeptide Ac-Lys-Asp-Glu-Val-Asp-Lys(Ahx-biotin)-NH_2 (**35**) as a white amorphous powder. Yield after RP-HPLC purification: 40%; HPLC (system B): $t_R=18.0$ min, purity > 95%; MS (MALDI-TOF, positive mode): m/z: calcd for $C_{48}H_{80}N_{12}O_{16}S$; 1112.55; found: 1113.7 $[M+H]^+$, 1135.7 $[M+Na]^+$, 1151.7 $[M+K]^+$ (CHCA matrix).

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