Native Chemical Ligation with Aspartic and Glutamic Acids as *C*-Terminal Residues: Scope and Limitations

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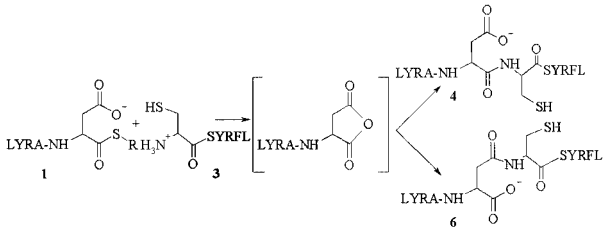
A new side reaction during Native Chemical Ligation (NCL) at aspartic and glutamic acid/cysteine ligation sites is reported. To exploit NCL at these sites, test peptides having either a *C*-terminal unprotected aspartyl or glutamyl thioester were ligated with a peptide containing an *N*-terminal free cysteine generating, in each case, two major compounds having equal masses but different retention times by HPLC analysis. Comparison of these ligation products with reference peptides of identical sequence synthesized by total SPPS (solid phase peptide synthesis) with both natural and unnatural backbones at the cysteinyl residue is in agreement with the hypothesis of migration of the thioester moiety on the side chain carboxyl group of both *C*-terminal Asp and Glu residues. Enzymatic digestion of the purified ligation products with a V8-protease further confirmed the nature of the side reaction, with a total resistance of the non-natural backbone peptide to the protease. To overcome this problem the use of a protecting group stable to both HF cleavage and ligation conditions, but removable after ligation, became a necessity. A series of side-chain protected derivatives were selected, specifically 9-fluorenylmethyl ester (OFm), (phenylsulfonyl)ethyl ester (OPse), 2,2,2-trichloroethyl ester (OTce), and phenacyl esters (OPac). For the glutamic acid, OPse gave the best results, generating the correct peptide and avoiding the isomerization at the γ-carboxyl group. In the case of the aspartic residue, all the protecting groups were removed under basic conditions, worsening the problem and generating, for example, 70% yield of the β-isomer after OFm deprotection. This prompted us to propose a protecting group derived from the phenacyl ester 1-methyl-2-oxo-2-phenylethyl ester (OMop). This protecting group showed greater stability during the ligation reaction than OPac and OTce, and was easily removed by Zn/acetic acid reduction after the ligation step.

Introduction

Native chemical ligation[1] is emerging as a powerful tool in protein chemistry to prepare proteins of small to medium size by chemical synthesis. The key aspect of this technique is the possibility to ligate two unprotected polypeptide fragments in aqueous solution when the *C*-terminal fragment possesses an *N*-terminal cysteine, and the *N*-terminal fragment is synthesized as a *C*-terminal thioester. The proposed mechanism of this reaction involves two steps, a *trans*-thioesterification between the *C*-terminal thioester moiety with the thiol of the *N*-terminal cysteine and a subsequent S-to-N internal acyl shift through a five-membered ring intermediate that generates a natural amide bond. Although an extensive study[2] showed that any of the 20 naturally occurring amino acids was compatible in a Xaa-Cys ligation, no further investigation was carried out to identify potential side reactions at the *C*-terminal thioester residue. A possible case study involves aspartic and glutamic acids, since the proximity of the β- or γ-carboxyl to the thioester could cause a migration of the thioester moiety onto the carboxylic acid side chain. In this case the consequence would be the formation of the unnatural β (for the aspartic acid) or γ (for the glutamic acid) amide bond between the Asp/Glu and the cysteine as side product. To test our hypothesis model peptide C^{α} thioesters of Asp and Glu with unprotected side chains (Scheme 1) were synthesized and ligated under standard conditions with an *N*-terminal Cys peptide.



Scheme 1

Results and Discussion

Ligation with Asp/Glu Unprotected Side-Chain Thioesters

To investigate the compatibility of the native chemical ligation strategy with Asp-Cys and Glu-Cys ligation sites, the thioester (sr) peptides, H-LYRAD-sr (**1**), H-LYRAE-sr

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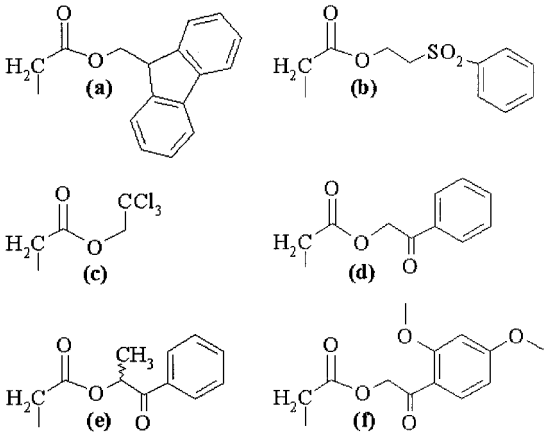

Scheme 2. Protecting groups tested for Glu or Asp side chain protection: OFm (a), OPse (b), OTce (c), OPac (d), OMop (e), and OdiMeOPac (f)

(**2**) and the *N*-terminal free Cys peptide, H-CSYRFL-OH (**3**) were synthesized. Short peptides were retained for this investigation to facilitate the detection by HPLC of side reaction products. Analysis of the thioester peptides after HF cleavage shows a single component for H-LYRAE-sr (**2**), while H-LYRAD-sr (**1**) showed about 20% of thioester hydrolysis. After purification and lyophilization, peptide **1** was found to be partially unstable, generating after eight weeks storage at +4 °C, a 30% yield of the hydrolysed product. When the aspartyl thioester peptide **1** was involved in a ligation reaction with the *C*-terminal peptide **3** under standard NCL conditions (pH 6.5, 1% thiophenol and 1% benzylmercaptan) the presence of two ligated products was observed after 2 h incubation, both compounds presenting the expected mass for the correct product (Figure 1). The

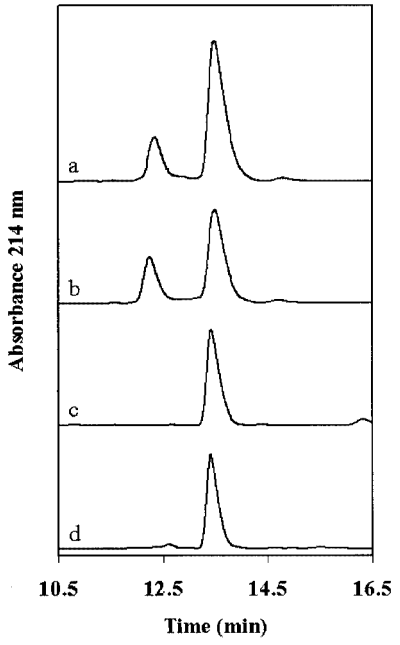

Figure 1. HPLC analysis of ligation products using different H-LYRAE(X)-sr peptides: (a) ligation between **2** and **3**; (b) ligation between substrates **2** and **3** co-injected with compound **7**; (c) ligation product between substrates **8** and **3** after basic treatment; (d) ligation product between substrates **9** and **3** after basic treatment

relative ratio between the two ligation products is about 1:2. Furthermore, during the ligation, 40% of the thioester peptide was hydrolysed. A similar result was obtained in the ligation involving the glutamyl thioester peptide, although the two peaks corresponding to the ligated material were present in a relative ratio of 1:4. Neither modification of the pH of the ligation medium between pH 6.2 and 7.0 nor reduction of the thiol concentration by lowering the concentration of thiophenol as low as 0.1% had any effect on the final ratio between the two peaks in both ligations. To correctly define the nature of the two entities with different HPLC elution times, the expected ligation products H-LYRADCSYRFL-OH (**4**), H-LYRAECSYRFL-OH (**5**), and the suspected ligation products — the unnatural isomers H-LYRAD(β)CSYRFL-OH (**6**) and H-LYRAE(γ)CSYRFL-OH (**7**) — were synthesized by SPPS. Comparing the R_t of these standards with the ligation products we were able to confirm that for both the Asp and Glu ligations the first eluted peak (the most hydrophilic compound), which is present in the lowest amount, corresponds to the unnatural backbone isomers, supporting the idea of a migration of the thioester to the side chain carboxyl (Scheme 1). This was further confirmed by V8-protease digestion of purified ligation products. This proteolytic enzyme, selective for Asp-Xxx and Glu-Xxx, readily digested the second eluted (more hydrophobic) ligation product for both Glu and Asp, while it was ineffective on the first eluted compounds. The same results were obtained with the fully synthetic products, both compounds **6** and **7** being totally resistant to the protease. These experiments together confirm that ligation at a Glu- or Asp-Cys site with the unprotected carboxyl side chain results in 20−30% isomerization of the resulting peptidic chain at the ligation site and a significant amount of hydrolysed Asp-thioester.

To clarify the mechanism of this reaction, H-LYRAD-sr was incubated for 1 h at pH 6.5 without any thiol and no *N*-terminal Cys peptide. Mass spectrometry of fractions obtained by HPLC analysis of the product mixture after 30 minutes provided evidence of the presence of 50% thioester hydrolysed product, 18% of a product hydrolysed and dehydrated consistent with the generation of succinic anhydride at the *C*-terminus, and 20% of the original thioester peptide. The presence of the succinic anhydride product could support the idea that this intermediate in the ligation reacts either with thiophenol or with the Cys-containing peptide on the β-carbonyl, generating the β-isomer. Furthermore, due to its high reactivity, the succinic anhydride intermediate is highly unstable in aqueous solutions and rapidly undergoes ring opening, generating the thioester hydrolysed product.

Ligation with Side-Chain-Protected Glutamyl Thioesters

It is clear from previous results that a protecting group strategy has to be taken into consideration for the synthesis of Asp/Glu-thioester peptides. Such a protecting group should have the following characteristics: i. be stable to HF cleavage, ii. be stable during the ligation reaction, and iii. be easily removable after the ligation reaction without jeop-

ardizing the integrity of a fully unprotected polypeptide. Most of the available protecting groups do not match the first requirement. We were focusing at this stage on commercially available Nα-Boc-Asp/Glu with side chain protecting groups, and we selected for our experiments the 9-fluorenylmethyl ester (OFm)[3] and the (phenylsulfonyl)-ethyl ester (OPse)[4] (Scheme 2). Synthesis by SPPS of H-LYRAE(OFm)-sr (**8**) and H-LYRAE(OPse)-sr (**9**) gave, in both cases, predominantly the correct peptides, but with significantly less side products with the last protecting group. The major identified side products with the OFm protecting group were the loss of the OFm group (≈ 20% of the crude material)[5] and the alkylation of the OFm group by Br-Z (≈ 25% of the crude material). Obviously this problem is limited to peptides containing a tyrosine, but this side reaction has also been reported in the case of the benzyl protecting group.[6]

When these peptides were tested in an NCL reaction at pH 6.2, both peptides generated the expected ligation product within two hours. It appears that the protected glutamic acid undergoes ligation at a similar rate to the unprotected residue, which is considered itself as a favorable site for ligation among the 20 amino acids.[2] The stability of these protecting groups under ligation conditions at different pH values was investigated. The OPse group was shown to be less stable than the OFm group, especially at higher pH values. If the OPse protecting group is employed, the ligation has to be carried out at pH 6.0 and over a short period of time, and hence with a higher excess of *N*-terminal Cys peptide in order to generate the ligation product before any significant loss of the protecting group. The OFm group is, on the other hand, completely stable in the pH range analysed.

The OPse group has the advantage of allowing the use of much milder conditions for the removal procedure, and this reaction can be carried out directly in the ligation medium in order to reduce the number of purification steps. As shown in Table 1, the OPse group required less drastic conditions giving complete removal in 0.1 M Na_2CO_3, 10% BME, pH 9 at 37 °C in 2 h, while the OFm group was cleaved in 15 minutes in the presence of 10% BME, 20% DMF, 20% piperidine at pH12−13. It is interesting to notice that β-mercaptoethanol heavily affected the rate of deprotection. When these conditions were applied to H-LYRAE(OPse)CSYRFL-OH (**12**) and H-LYRAE(OFm)CSYRFL-OH (**13**), only one compound was generated with the correct α-backbone, and there was no trace of any side reaction.

Ligation with Aspartyl Thioesters Protected with β-Removable Protecting Groups

The ligation with the aspartyl thioester peptide proved to be a more difficult case than the glutamic acid thioester. Introduction of the previously described OPse group on the Asp side chain [H-LYRAD(OPse)-sr (**14**)] for the ligation reaction results in the formation of 50% of the unprotected aspartimide derivative of the ligation product as well as the expected protected ligation product. Furthermore, deprotection of the first compound under the conditions described for Glu(OPse) results in the formation of 70% of the β-form and 30% of the natural α-product (Figure 2e). Comparable results were obtained when the protected ligation product was first isolated and then deprotected under basic conditions.

H-LYRAD(OFm)-sr (**15**), on the other hand, proved to be completely stable during the ligation reaction, generating a single product. During the deprotection reaction at pH 12, however, two isoforms were generated, with the β-form representing 70% of the final product (Figure 2, c). This cannot be related to the presence of the free thiol of the neighboring Cys since OFm deprotection of the *S*-acetamide-alkylated H-LYRAD(OFm)C(Cam)SYRFL-OH peptide results in the formation of the two isoforms in the same ratio.

Aspartic Acid Protection Removed by Reduction

It was clear at this stage that the basic conditions employed for the OFm and OPse removal were the major obstacle to obtain the native product. This led us to investigate another class of protecting groups that are stable to HF but can be promptly removed under reductive acidic conditions, which includes the phenacyl ester (OPac) [7] and the 2,2,2-trichloroethyl (OTce) esters[8] (Scheme 2). Since the Asp protected with these groups is not commercially available, these protecting groups were first incorporated into a series of test peptides with the aim of investigating the stability of such moieties in the ligation conditions (0.2 M sodium phosphate, 6 M guanidine, 0.1% thiophenol, pH 6.5 at room temperature). The Ac-D(X)YAKYAKL-OH peptide was

Table 1. Conditions tested for OFm and OPse removal

Buffer conditions	OPse extent of deprotection (%/time)	OFm extent of deprotection (%/time)
$NaHCO_3$ 0.2 M, pH = 9	50%/two days	–
Na_2CO_3 0.2 M, pH = 12	38%/two days	–
$NaHCO_3$ 0.2 M, 10% BME, pH = 9	100%/2 h	–
Na_2CO_3 0.2 M, 10% BME, pH = 12	100%/2 h	–
20% pip., 20% DMF, pH = 13	–	70%/2 h
20% pip., 20% DMF, 10% BME, pH = 13	–	100%/15 min
200 equiv. DEA, 10% BME, pH = 11	–	100%/2 h

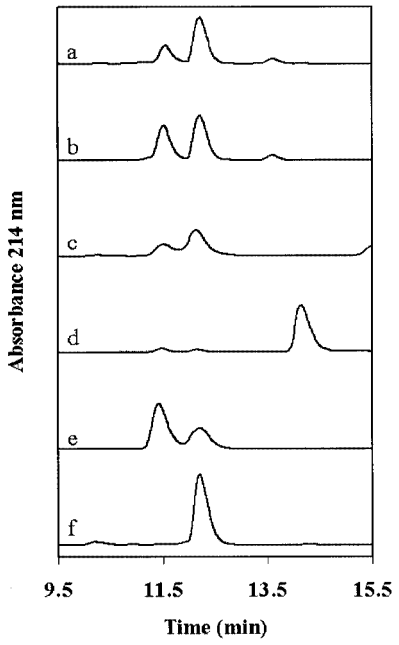

Figure 2. HPLC analysis of ligation products using different H-LYRAD(X)-sr peptides: (a) ligation between substrates **1** and **3**; (b) ligation between substrates **1** and **3** co-injected with compound **6**; (c) ligation product between substrates **15** and **3** after basic treatment; (d) ligation product between substrates **14** and **3**; (e) ligation product between substrates **14** and **3** after basic treatment; (f) ligation product between substrates **16** and **3** after Zn° reduction

derivatized with four different protecting groups: OTce, OPac, 1-methyl-2-oxo-2-phenylethyl ester (OMop) and (2′′,4′′-dimethoxy)phenacyl (OdiMeOPac). Since there have been controversial reports on OPac stability,[9,10] and with the aim of reducing potential hydrolysis, we synthesised the sterically hindered version of OPac — OMop — by introducing an extra methyl in position 1′.[9] This approach has been successfully used to increase the stability of the phenacyl group when employed as a handle for solid-phase synthesis.[15,16] Furthermore, we introduced two methoxyl groups in positions 2′′ and 4′′ of the phenyl group to verify the incidence of the phenyl ring electron donating effect, generating diMeOPac. As reported in Table 2, the OMop group presented the highest stability, with a half-life of 10 h under ligation conditions, which is sufficient for a ligation at a favorable residue site.[2] All the other compounds showed much shorter half-live.

Table 2. Stability of the different Asp protections tested at pH 6.5, 0.1% thiophenol

Protection	Hydrolysis $t_{1/2}$ (h)
OMop	9.9
OdiMeOPac	3.8
OTce	1.5
OPac	1.2

Ligation with Asp(OMop) Thioester Peptide

Synthesis of Boc-Asp(OMop)-OH was carried out with a slight modification of a procedure described for Boc-Asp(Pac)-OH.[9] The synthesis of H-LYRAD(OMop)-sr (**16**) did not give any problems. HF cleavage of the peptide was conducted at −5 °C since at 4 °C we observed the presence of 10% of the deprotected peptide. At this level it was not possible to detect the two different diastereoisomers derived from the OMop protection. Ligation with H-CSYRFL-OH was completed in 1 h at pH 6.5, with generation of a unique ligated product with the OMop protection and 5% already deprotected peptide. Zn/Acetic Acid deprotection of isolated H-LYRAD(OMop)CSYRFL-OH for 30 minutes resulted in only the H-LYRAD(α)CSYRFL-OH, confirming that the methyl group does not affect the rate of reduction (Figure 2, f).

Conclusions

Our work has proved that the ligation at Asp-Cys and Glu-Cys ligation sites, when conducted with an unprotected residue at the ligation site, results in the generation of 20−30% non-natural backbone isoforms. Characterization of this side reaction was easy on a small peptide but could be challenging on a larger fragment. To overcome this problem we investigated commercially available protecting groups for glutamic acid and proved that the use of OPse side-chain protection, although not ideal, is the best solution since OPse removal can be achieved under milder conditions than for OFm. Aspartic acid was more problematic, since the use of the available side-chain protecting groups enhanced the generation of the non-natural isoform. For this amino acid (and probably also for glutamic acid), the best solution lies in a protecting group amenable to removal by reduction with Zn in acetic acid. Unfortunately, the classical phenacyl ester proved to be unstable in the ligation reaction. A sterically hindered version — 1-methyl-2-oxo-2-phenylethyl ester — withstands longer reaction times, and generates only the expected natural α-compound at the ligation site. This study pointed out OMop protection as one of the possible ways to overcome the isomerization reaction when approaching a ligation at an aspartic acid site. Considering the advantage of avoiding basic conditions for the deprotection step, we suggest further exploration of OMop also for glutamic acid protection. A further possible development of this research could come from the exploration of novel phenacyl-based derivatives with additional electron donating groups on the phenyl ring of OMop, to enhance the ligation stability of this protecting group.

Experimental Section

Abbreviations

ACN: acetonitrile; BME: 2-mercaptoethanol; Boc: *tert*-butoxycarbonyl; COSY: correlation spectroscopy; DAE: dimethylamine; DCM: dichloromethane; DIEA: *N*,*N*-diisopropylethylamine;

DMAP: 4-dimethylaminopyridine; DMF: dimethylformamide; DMSO: dimethyl sulfoxide; HBTU: *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate; HF: hydrogen fluoride; HMBC: heteronuclear multiple bond correlation; HSQC: heteronuclear single-quantum correlation; MPAL: β-mercaptopropionic acid-leucine; NCL: native chemical ligation; NMM: *N*-methylmorpholine; OdiMeOPac: (2′′,4′′-dimethoxy)phenacyl group; OFm: 9-fluorenylmethyl ester; OMop: 1-methyl-2-oxo-2-phenylethyl ester; OPac: phenacyl esters; OPse: (phenylsulfonyl)ethyl ester; OTce: 2,2,2-trichloroethyl ester; Pam: 4-hydroxymethylphenylacetic acid; PyBOP: benzotriazolyloxytris(pyrrolidinio)phosphonium hexafluorophosphate; RP-HPLC: reversed-phase high performance liquid chromatography; R_t: retention time; SPPS: solid phase peptide synthesis; sr: β-mercaptopropionic acid-leucine; TFA: trifluoroacetic acid; TMS: tetramethylsilane; TIS: triisopropylsilane.

Reagents

Boc amino acids were used with the following side chain protection: Arg(Tos), Asp(OChx), Cys(pMeBzl), Glu(OChx), Lys(2ClZ), Ser(Bzl) and Tyr(2BrZ). Boc amino acids were obtained from Orpegen Pharma (Heidelberg, Germany). HBTU was obtained from Luxembourg Industries (Tel-Aviv, Israel). All the other chemicals were from Fluka or Aldrich (Buchs, Switzerland) and used as received. DIEA was from Applied Biosystems (Foster City, CA). Boc-Asp(OPse)-OH.DCHA and Boc-Glu(OPse)-OH.DCHA were from A&PEP Inc. (Bucheon, Korea). Boc-Asp(OFm)-OH, Boc-Asp-OChx. DCHA, Boc-Asp-O*t*Bu, Boc-Glu-OChx and Boc-Glu-(OFm)-OH were from Bachem (Bubendorf, Switzerland). V8 protease (Type XVII-B) was from Sigma (Buchs, Switzerland).

Solid Phase Peptide Synthesis

All peptides were prepared by SPPS on a 0.2 mmolar scale, using machine-assisted protocols on a custom-modified Applied Biosystems model 433A peptide synthesizer, using in situ neutralization/HBTU activation procedure for Boc chemistry as described previously.[11] *C*-terminal peptides were synthesized on the appropriate Boc-aminoacyl-Pam preloaded resin. *C*-terminal Asp or Glu thioester peptides were synthesized starting from the MPAL resin (β-mercaptopropionic acid-leucine) following a published procedure using PyBop as activator.[13,14] Boc-Asp(OPse)-OH and Boc-Glu-(OPse)-OH were desalted according to standard protocols (Novabiochem, Laüfelfingen, Switzerland) before activation with PyBop.

After chain assembly was completed, the peptides were deprotected and cleaved from the resin by treatment with anhydrous HF for 1 h at 0 °C with 5% *p*-cresol as scavenger. The peptides were precipitated with diethyl ether, dissolved in aqueous acetonitrile, lyophilized, and purified by preparative RP-HPLC on a Waters 600 HPLC module, using a C18 Waters DeltaPak preparative column. Peptide identity was confirmed by ESI-MS with a Bruker Esquire 3000 Ion Trap (Bruker Daltonics, Bremen, DE).

Analytical RP-HPLC of all the products was performed on a Waters 2690 HPLC module with 214 nm UV detection, using a Symmetry 300 C18 column, with a linear gradient of buffer B in buffer A over 30 minutes at 1 mL/min. Buffer A = 0.1% TFA in water; buffer B = 0.1% TFA in ACN. Data were recorded and analysed using the software system Millennium 32.

NMR Studies

High-resolution NMR spectra of Boc-Asp(OMop)-OH were recorded in [D_6]DMSO with a Bruker DRX-500 NMR spectrometer operating at a field of 11.74 T and equipped with a pulsed field-gradient unit [Bruker BioSpin AG, Fällanden, Switzerland]. Chemical shift data are given in ppm relative to internal TMS. Apparent scalar couplings are given in Hz. Assignment of ^{1}H and ^{13}C chemical shift data is ascertained by making use of the appropriate homonuclear or heteronuclear correlation experiments including COSY, HSQC, and HMBC (heteronuclear multiple bond correlation).[14] Boc-Asp(OMop)-OH is an inseparable diastereomeric mixture (1:1) of [2*S*,1′*R*]- and [2*S*,1′*S*]-4-(1-methyl-2-oxo-2-phenylethyl) 2-[(*tert*-butoxycarbonyl)amino]succinate.

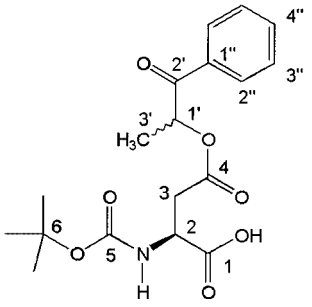

Figure 3. Structure of Boc-Asp(OMop)-OH

If the diastereomers show clearly distinct resonances, two shift values are given in the following listings. All data refers to the dominant rotamer of the *N*-Boc grouping (> 95%). ^{1}H NMR ([D_6]DMSO, 500 MHz): δ = 1.36 (br. s, *t*Bu), 1.41 [d, *J* = 7 Hz, $H_3C(3')$]; 2.65−2.90 (m, AB parts of two ABMX systems, $H_2C(3)$]; 4.28−4.35 (m, M parts of two ABMX systems, HC(2)]; 6.03/6.04 (two quart. *J* = 7 Hz, HC(1′)]; 7.08/7.10 (2d, *J* = 8.7 Hz, X-parts of two ABMX systems, HN), 7.55 (arom. *m*-H), 7.68 (arom. *p*-H), 7.98 (arom. *o*-H), 12.7 (s, br. $HO_2C(1)$] ppm. ^{13}C NMR ([D_6]DMSO, 125 MHz): δ = 16.86/16.93 [$H_3C(3')$]; 28.14/28.46 (Me_3 of *t*Bu), 35.53/35.78 [$H_2C(3)$]; 49.79/49.84 [HC(2)]; 71.70/71.78 [HC(1′)]; 78.30 [C(6)]; 128.1/128.4 (arom. *o*-C), 129.0/129.1 (arom. *m*-C), 133.7 (arom. C(1′′), 133.8/133.9 (arom. *p*-C), 155.2 [C(5)]; 168.9/169.0 [C(4)]; 172.4/172.5 [C(1)]; 196.3/196.4 [C(2′)] ppm.

Ligation Reaction

The *C*-terminal unprotected peptide (1.5 equivalents) and the *N*-terminal peptide (1 equivalent) were solubilized in freshly degassed 0.2 M sodium phosphate buffer, pH 7.5, containing 6 M guanidine at a concentration of 2.5 mM each. After addition of 1% thiophenol and 1% benzylmercaptan the pH was adjusted to 6.5 (or different thiols concentration or pH as reported in the text). Aliquots of this solution were treated with equal volumes of BME for 5 minutes and then with 10% TCEP for 10 minutes to completely hydrolyse any thiol adduct before HPLC analysis. The ligation reaction was monitored by HPLC using a gradient starting from either 10% of B with a gradient slope of 1.66% of B per minute or from 20% of B and with an increase of 0.33% B per minute when a baseline resolution of the different isomerization products was required.

Enzymatic Digestion

The peptides were solubilized in 0.1 M NH_4HCO_3, 0.2% BME at a 5 mM concentration, and V8 protease added for a final enzyme/peptide ratio of 1 unit/20 μg. Digestion was stopped after 24 h incubation at 37 °C by acidification with an equal volume of 10%

TCEP. The extent of digestion was evaluated by RP-HPLC and the fragments obtained were characterized by ESI-MS.

Synthesis of Protected Asp Peptides

Evaluation of different protecting groups for the β-carboxyl of Asp was carried out by coupling the precursor Fmoc-Asp(*t*Bu)-OH at the *N*-terminus of the peptide H-YAKYAKL-OH still on the Pam resin. The *N*-terminus was deprotected with piperidine, acetylated and the *t*Bu was then removed in order to add the different protecting groups. For OPac, OMop and OdiMeOPac the resins were reacted with the α-bromo ketones phenacyl bromide, α-bromopropiophenone and 2-bromo-2′,4′-dimethoxyacetophenone, respectively. The same protocol was used for the three products, using a 20 M solution of bromo ketone over the resin-free carboxylic acid with 1 equivalent of DIEA in DMF overnight at room temperature. For OTce the resin was treated with 2,2,2-trichloroethanol (40 equiv. of the resin free carboxylic acid), DMAP (0.1 equiv.), NMM (1 equiv.) and PyBop (1 equiv.) in DMF overnight at 37 °C. The final products were cleaved and deprotected by HF treatment. The peptides were purified by preparative RP-HPLC. OMop generated two diastereoisomers which were resolved by HPLC. Only the major product was used. The overall esterification yield in this procedure was about 50% and only the OTce derivative was obtained in a significant amount (15%).

Synthesis of Boc-Asp(OMop)-OH

The cesium salt of Boc-Asp-O*t*Bu (1.38 mmol) was prepared under standard conditions and reacted with α-bromopropiophenone (1.3 mmol) in DMF (4 mL) by incubation overnight at room temperature. The white precipitate was discarded after filtration and the DMF solution was dried under vacuum. The resulting oil was diluted with ethyl acetate, and the organic phase was washed with 1 M $NaHCO_3$ (4 times), then with saturated NaCl, dried with $MgSO_4$, and finally concentrated under vacuum.

The resulting Boc-Asp(OMop)-O*t*Bu (single spot by TLC, R_t = 0.95 chloroform/methanol/acetic acid, 90:8:2) was deprotected in TFA/Tis/H_2O (95:2.5:2.5,v/v) for 2 h. TFA was evaporated under vacuum and the remaining TFA was removed by multiple cycles of acetonitrile and ethanol evaporation. The resulting oil was solubilized in DMSO (2.5 mL), Boc_2O (2.76 mmol) and DIEA (2.76 mmol) were added and the reaction mixture was stirred overnight at room temperature. The solution was diluted with water (10 mL) and acidified to pH 3.5 with $KHSO_4$. The aqueous phase was extracted with diethyl ether, the combined organic fractions were washed with 1 N $KHSO_4$ and saturated NaCl, dried with $MgSO_4$ and finally concentrated at reduced pressure. The oily product was used without further purification for coupling to MPAL resin using PyBop as activator.

Zn/Acetic Acid OMop Removal

The purified peptide H-LYRAD(OMop)CSYRFL-OH (5 mg) obtained via ligation, was diluted in 30% aqueous acetic acid, mixed with activated zinc powder and stirred for 30 minutes. The aqueous solution was separated and the final product was obtained by a desalting step with no further purification. Zinc powder (1 g) was previously activated as follows: 1 N aqueous HCl (4 × 4 mL, 3 min), H_2O (4 × 4 mL, 1 min), and stored in H_2O until use (daily prepared).

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