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Tetrahedron Letters 44 (2003) 4515–4518

TETRAHEDRON LETTERS

Chemoselective coupling of peptide fragments using the Staudinger ligation

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Received 14 March 2003; revised 10 April 2003; accepted 17 April 2003

Abstract—Here we report the first Staudinger ligations which yield tetra- and pentapeptides starting from *N-terminal α-azido peptides* and *C-terminal peptide o*-(diphenylphosphine)phenyl esters. Mass spectrometric analysis of the reaction mixture provided a better insight into the mechanism of the Staudinger ligation and has been used to explain the observed intermediates and to optimize the ligation reaction. As a result, the optimized reaction enables the chemoselective coupling of peptides containing amino acids other than glycine at the ligation site. © 2003 Elsevier Science Ltd. All rights reserved.

Among the existing methodologies for the chemical synthesis of large polypeptides or mini-proteins, the native chemical ligation[1] as developed by Kent and co-workers has the highest potential.

Despite several reported successful syntheses[2] the native chemical ligation methodology is restricted in its use by the requirement of an N-terminal cysteine residue or a cysteine-mimicking auxiliary of the C-terminal peptide segment.[3]

Recently, a novel chemoselective ligation reaction has been described, based on a modified Staudinger reaction developed earlier for the modification of cell-surface glycans,[4] which uses C-terminal phosphine (thio)esters and N-terminal azides.[5] This so-called Staudinger ligation should be independent of the nature of the amino acid side chain at the N-terminus of the C-terminal peptide fragment. However, so far it has only been used for acetyl transfer to an azido acid[5b] or for the synthesis of dipeptides in which at least one glycine residue was present.[5a,c,6]

To explore the possibilities of the Staudinger ligation for the total synthesis of proteins, we studied the ligation process of *peptide* fragments by mass spectrometry

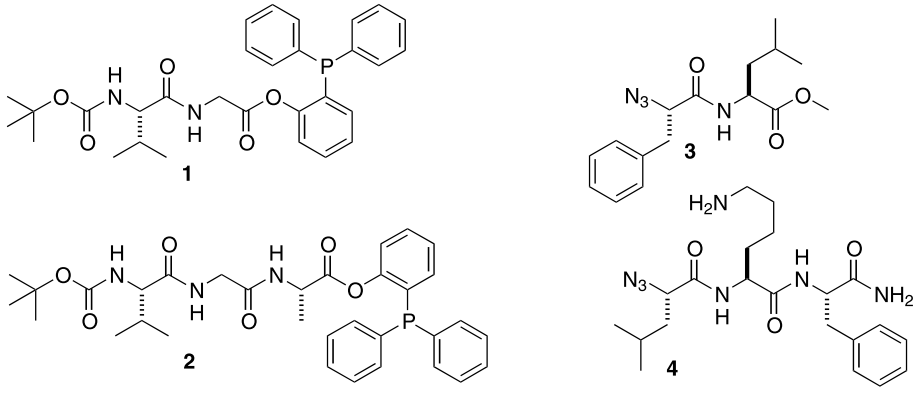

Figure 1. Phosphines and azido peptides used in this study.

Keywords: amide-forming ligation; azido peptides; peptide *o*-(diphenylphosphine)phenyl esters; Staudinger ligation.
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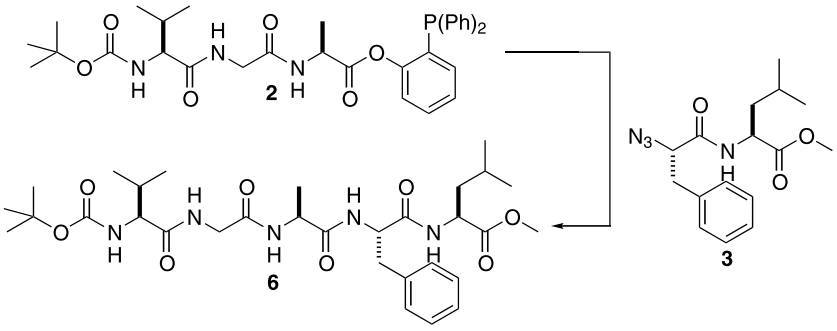
0040-4039/03/$ - see front matter
doi:10.1016/S0040-4039(03)01014-1

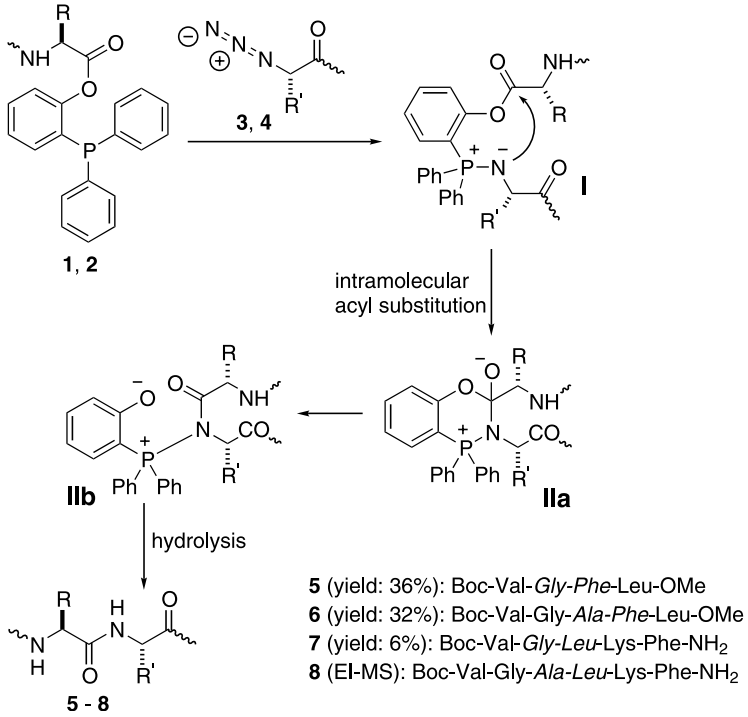
in order to monitor the formation of the ligation product, the intermediates and by-products. This resulted in optimized reaction conditions, with respect to the presence of reagents and temperature, for carrying out a Staudinger ligation. Thus, a chemoselective ligation of peptide fragments was found to be possible affording *penta*- or *hexa*peptides and which is independent of the presence of a glycine residue at the ligation site (Fig. 1 and Scheme 1).

Although thioesters are for obvious reasons popular in chemical ligation strategies, we envisioned (oxy)esters as promising alternatives. In fact protein synthesis in Nature involves the use of (oxy)esters of ribose in aa-tRNA substrates.[7] Thus, by proper tuning of the reactivity of an ester and/or the reaction conditions, it should be possible to use ultimately (oxy)esters in chemical ligation strategies for efficient synthesis of proteins. In addition, thioesters are not always synthetically easy to access. Thus, it was decided to use (oxy)esters, e.g. the *o*-(diphenylphosphine)phenol auxiliary,[5b] to transfer a peptidyl fragment to an azido peptide in a Staudinger ligation.

Ligation of phosphine **1**[8] and azide **3**[9] was carried out at 50 mM in THF/H_2O 3/1 v/v as originally described by Bertozzi and co-workers.[4a,5b] The reaction was monitored by TLC and EI-MS. It was found that the iminophosphorane (intermediate **I** in Scheme 2) was formed almost instantaneously. An intramolecular *O*→*N*-acyl shift may result in the formation of amidophosphonium salt **IIa** followed by collapse to **IIb**. However, **I** and **IIa**/**b** have identical molecular mass and therefore it is difficult to distinguish between these intermediates. Despite its presumed rapid hydrolysis, **I**/**II** was found to be very stable and **5** (Boc-Val-Gly-Phe-Leu-OMe) was formed in minimal amounts and by-products



Scheme 1. The Staudinger ligation affording a pentapeptide which is independent of the presence of a glycine residue at the ligation site.



Scheme 2. Proposed reaction mechanism of the Staudinger ligation.[14] This mechanism is based on mass spectroscopic analysis of the reaction mixture. The amino acid residues in italics indicate the site of ligation.

resulting from, e.g. the hydrolysis of the ester bond and oxidation of the phosphine were found as major products instead.

In order to attempt to reduce the by-product formation and/or to increase the concentration of possible intermediate **IIb**, it was decided to run the Staudinger ligation with only one equivalent or in the absence of water. After hydrolysis, the equilibrium mixture should result in the preferred formation of ligation product **5**. Indeed, TLC and EI-MS analysis showed only the formation of **I**/**II**, and **5** was isolated in 25% yield. Apparently, the absence of excess water favored the intramolecular acyl substitution. The next step in the optimization process was to increase the temperature, as was mentioned in the literature,[4b] 47°C should be ideal. Running the Staudinger ligation at this temperature resulted in the formation of **5** in an increased yield of 36%. NMR analysis showed that the phenylalanine retained its chiral integrity since no diastereomers could be detected which is in agreement with the data of Raines and co-workers.[6]

These optimized reaction conditions[10] were used for the ligation of peptides **2** and **3** in which glycine residues are absent at the ligation site and Boc-Val-Gly-Ala-Phe-Leu-OMe **6** was obtained in a satisfactory yield of 32%.

Since one of the major goals of chemical ligation strategies of peptides is the ligation of *unprotected* peptides, azido peptide **4**[11] was used as a model of an unprotected peptide. This model peptide was also chosen to study any non-specific aminolysis of the phosphine by the nucleophilic ε-amino group of the lysine residue. Ligation of **1** and **4** resulted in formation of Boc-Val-Gly-Leu-Lys-Phe-NH_2 **7** in a somewhat disappointing yield of 6%. NMR studies showed the presence of an amide-NH–Leu-αCH correlation, however, an amide-NH–Lys-εCH_2 correlation was also found,[12] hence a non-specific aminolysis could not be ruled out.[13] Finally, ligation of peptides **2** and **4** resulted in the formation of Boc-Val-Gly-Ala-Leu-Lys-Phe-NH_2 **8**, which could only be detected by EI-MS. It should be mentioned, however, that the formation of the intermediate **I**/**II** resulting from **2** and **4** was rapid and unambiguously detected by EI-MS and was stable for at least 2 h in aqueous THF. The apparent stability of said intermediates may have resulted in a slow product formation and a low yield.

An explanation for the slow formation of the ligation product might be the increased steric congestion in the intermediates due to the presence of the amino acid side chains or due to the increased length of the peptide fragments. This may interfere with formation of the optimal conformation of the six-membered transition-state which is necessary for the intramolecular nucleophilic attack of the iminophosphorane resulting in the $O \rightarrow N$ acyl shift.

In conclusion, we have shown that N-terminal *peptidyl* azides and C-terminal *peptide o*-(diphenylphosphine)phenyl (oxy)esters are suitable synthons for the synthesis of, up to now, pentapeptides featuring the Staudinger ligation.[14] Moreover, we have also shown that chemical ligation of peptides is independent of the presence of glycine residues at the ligation site. Under present investigation is further optimization of the conditions of the Staudinger ligation and although there may still lay a considerable way ahead, the possibility of now having relatively hindered amino acids at the ligation site (Ala, Leu, Phe) gives confidence that this will be successful and that also large peptides can be synthesized by chemical ligation independent of their sequence.

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8. The phosphines **1**, **2** were synthesized via a carbodiimide-mediated esterification of the peptide acid with *o*-(diphenylphosphine)phenol. The latter compound was synthesized according to: (a) Herd, O.; Hessler, A.; Hingst, M.; Machnitzki, P.; Tepper, M.; Stelzer, O. *Catal. Today* **1998**, *42*, 413–420; (b) Herd, O.; Hessler, A.; Hingst, M.; Tepper, M.; Stelzer, O. *J. Organomet. Chem.* **1998**, *552*, 69–76.
9. Compound **3** was synthesized by diazotransfer as described in the literature: (a) Alper, P. B.; Hung, S.-C.; Wong, C.-H. *Tetrahedron Lett.* **1996**, *37*, 6029–6032; (b) Lundquist, J. T., IV; Pelletier, J. C. *Org. Lett.* **2001**, *3*, 781–783.
10. General procedure for the Staudinger ligations: An equimolar quantity of the phosphinyl ester (**1** or **2**) and azido peptide (**3** or **4**) were dissolved in dry and oxygen-free THF at a final concentration of 50 mM and stirred for 16 h at 47°C. Subsequently, an excess of H_2O was added and stirring was continued for 2 h. The reaction mixtures yielding the ligation products Boc-Val-Gly-Phe-Leu-OMe **5** and Boc-Val-Gly-Ala-Phe-Leu-OMe **6** were evaporated to dryness and redissolved in EtOAc (20 mL). Subsequently, the EtOAc layer was washed with 1N $KHSO_4$ (15 mL), brine (15 mL), 5% $NaHCO_3$ (15 mL) and brine (15 mL), dried (Na_2SO_4) and evaporated in vacuo. The residue was purified by column chromatography. The ligation reaction of **1** and **4** was worked-up differently, the quenched reaction mixture was diluted with cold MTBE/hexane 1/1 v/v to precipitate Boc-Val-Gly-Leu-Lys-Phe-NH_2 **7**. The peptide was collected by centrifugation and the pellet was resuspended in MTBE/hexane and centrifuged again. The pellet thus obtained was dissolved in *tert*-BuOH/H_2O 1/1 v/v and lyophilized. Its identity was checked with EI-MS and NMR.
11. Compound **4** was synthesized on Fmoc-Rink-ArgoGel using standard Fmoc/*t*Bu SPPS protocols. The α-amino group of leucine was converted into the corresponding azido functionality by a diazo transfer on the solid phase: Rijkers, D. T. S.; van Vugt, H. R. R.; Jacobs, H. J. F.; Liskamp, R. M. J. *Tetrahedron Lett.* **2002**, *43*, 3657–3660.
12. Compound **7** contains at least two ligation products with equal molecular mass and HPLC retention time. The NH–Leu-αCH correlation was found at 8.2/4.4 ppm and the NH–Lys-εCH_2 correlation was found at 7.9/3.3 ppm. **7** was dissolved in 450 μL H_2O/D_2O 9/1 v/v at pH 3.72 at 278.1 K on a Varian INOVA-500 NMR spectrometer using a TOCSY pulse-sequence. Based on amide NH integration signals the NH–Leu-αCH ligation product was estimated to be present in 23–33% of the isolated peptides.
13. Although (oxy)esters are less sensitive towards nucleophilic attack than the intrinsically reactive thioesters, non-specific aminolysis was observed which is in contrast to the results of Raines et al.[5a]
14. Boc-Val-Gly-Phe-Leu-OMe **5**: was obtained in 36% yield (37 mg) after purification by column chromatography (EtOAc). R_f (EtOAc): 0.53; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.32$ (broad s, 1H), 7.27 (m, 7H), 5.41 (d, 1H), 4.88 (dd, 1H), 4.57 (m, 1H), 4.13 (m, 3H), 3.70 (s, 3H), 3.15 (m, 2H), 2.08 (m, 1H), 1.65 (m, 3H), 1.44 (s, 9H), 0.95 (m, 12H); EI-MS (50 eV) calcd for $C_{28}H_{44}N_4O_7$ (548.69): m/z (%): 549.45 (100) $(M+H)^+$. Boc-Val-Gly-Ala-Phe-Leu-OMe **6**: was obtained in 32% yield (33 mg) after purification by column chromatography (EtOAc→EtOAc/MeOH 98/2 v/v). R_f (EtOAc): 0.51; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.70$–6.91 (m, 9H), 5.44 (d, 1H), 4.94 (m, 1H), 4.63 (m, 2H), 4.16 (m, 3H), 3.71 (s, 3H), 3.29 (m, 2H), 2.18 (m, 1H), 1.74–1.49 (m, 3H), 1.46 (d, 3H), 1.25 (s, 9H), 0.92 (m, 12H); EI-MS (50 eV) calcd for $C_{31}H_{49}N_5O_8$ (619.36): m/z (%): 620.55 (100) $(M+H)^+$. Boc-Val-Gly-Leu-Lys-Phe-NH_2 **7**: HPLC purity: 90% (R_t: 15.37 min on an Adsorbosphere XL C8, 90 Å, 5 μm, 250×4.6 mm, in a linear gradient of 100% buffer A (0.1% TFA in H_2O) to 100% buffer B (0.085% TFA in CH_3CN/H_2O 95/5 v/v) in 20 min at 1 mL/min); EI-MS (50 eV): m/z: calcd $(M+H)^+$ 662.42, found $(M+H)^+$, 662.80. Boc-Val-Gly-Ala-Leu-Lys-Phe-NH_2 **8**: EI-MS (50 eV): m/z: calcd $(M+H)^+$ 733.46, found $(M+H)^+$, 733.80.