Bioorganic & Medicinal Chemistry 18 (2010) 3679–3686

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Acidic and basic deprotection strategies of borane-protected phosphinothioesters for the traceless Staudinger ligation

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ARTICLE INFO

Article history:
Received 18 January 2010
Revised 1 April 2010
Accepted 6 April 2010
Available online 9 April 2010

Keywords:
Traceless Staudinger ligation
Peptide ligation
Chemoselectivity
Azides
Phosphines
Thioester
Glycosyl amides

ABSTRACT

The traceless Staudinger ligation has recently found various applications in the field of peptide synthesis and modification, including immobilization and cyclization strategies. In this report, we utilize the traceless Staudinger ligation in the formation of amide bonds, which allows the acquisition of acylated aminosugars and peptides as well as the cyclization of peptides. A key element in these synthetic procedures is the use of a borane-protected phosphinomethanethiol, which is demonstrated to be prone towards oxidation in its unprotected form, during the synthesis of phosphinothioesters. In combination with acidic and basic deprotection strategies for the borane-protected phosphinothioesters, amide bonds can be formed in the presence of azides in moderate to good overall yields.

1. Introduction

In recent years, the importance of chemoselective ligation and modification strategies for protein synthesis has increased immensely.[1] These relatively new and often semi-synthetic methods, including enzymatic protein ligation and native chemical ligation (NCL), enabled the synthesis or modification of biologically important peptides and proteins that were impossible to be accessed alone by chemical methods, namely solid-phase peptide synthesis (SPPS), or biological expression.[2] The acquisition of homogenous protein material is particularly important to study protein–protein interactions, which requires minimal manipulations within the proteins' primary structure[3] or post-translational modification with small molecules, such as sugars[4] or fatty acids,[5] to investigate possible interactions in detail. Protein semi-synthesis is often accomplished by synthesis of the non-natural peptide fragment by SPPS followed by chemoselective ligation to a second recombinant fragment, yielding the desired manipulated protein.[1,6] To date, the most common ligation strategy is the NCL,[7] which uses a C-terminal peptide thioester and an N-terminal cysteine peptide to form a natural amide bond by a capture/rearrangement strategy comprised of a reversible trans-thioesterification and a subsequent S→N shift.[8] Although, this ligation method is widely used, it is limited due to its reliance on cysteine, which is the second least common residue.

In 2000, the Staudinger ligation was introduced as a new chemoselective ligation strategy that allows the formation of amide bonds starting from an azide and a phosphine, in which the latter is covalently linked to an ester group.[9] The aza-ylide intermediate rearranges intramolecularly to produce an amide linkage and a phosphine oxide, in which the two products are still covalently bound to each other. The ligation is bio-orthogonal, which implies a high selectivity of the two reaction partners azide and phosphine. Consequently, high reaction rates of the intramolecular acylation step enable execution of the reaction among biomolecules in an aqueous medium and in vivo applications.[10]

In order to make the reaction applicable for peptide ligations, a traceless version of the Staudinger ligation was developed (Scheme 1A).[11,12] The phosphine is positioned in a thioester **1**, which first forms an intermediate iminophosphorane **3** by reaction with an azide **2**.[13] An S→N shift leads then to the formation of the amide **4** and liberation of the oxidized phosphine linker. Raines and co-workers have developed several phosphine linkers to form different thioesters **1** for the traceless Staudinger ligation with azides **2**.[14] Since its discovery, the traceless Staudinger ligation has been advanced to peptide couplings (Scheme 1A, R = amino acid or small

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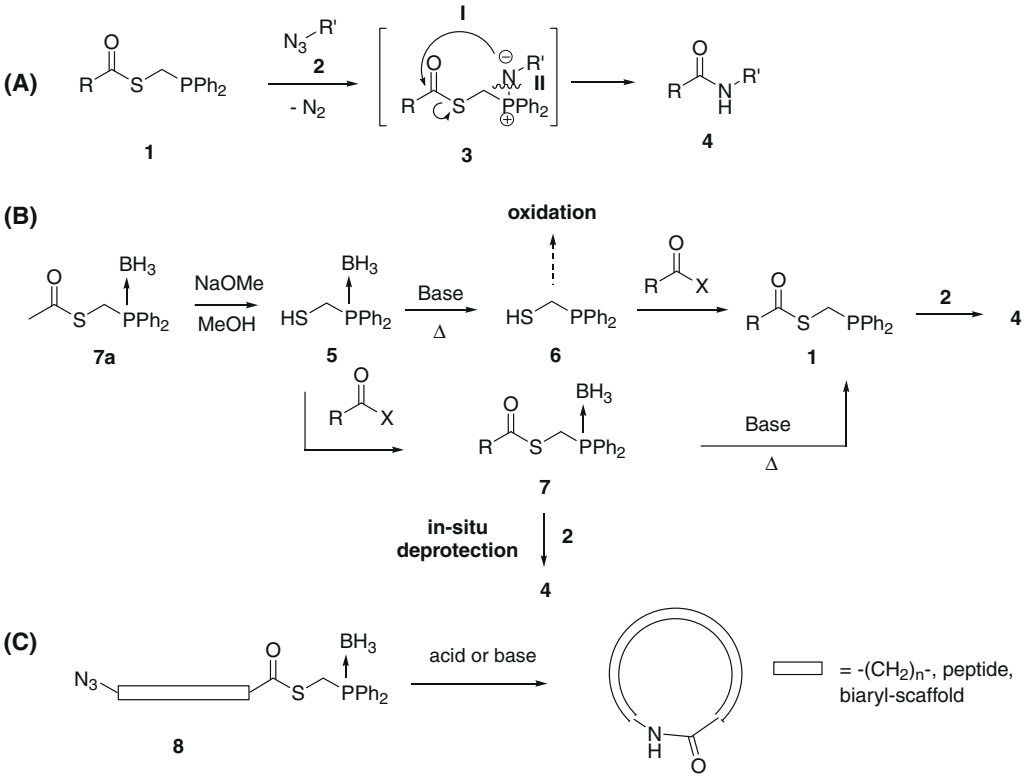
doi:10.1016/j.bmc.2010.04.015



Scheme 1. (A) Traceless Staudinger Ligation. (B) Phosphinothioester synthesis. (C) Acid or basic deprotection strategies for the cyclization of small molecules and peptides by the traceless Staudinger ligation.

peptides)[15] including a water-soluble version,[16] and detailed mechanistic investigations,[17] and various applications were performed. Those studies included for instance the site-specific immobilization of peptides on surfaces[18] or the ligation of protected (*glyco*-) peptide fragments.[19] One of the most promising phosphine linkers is the frequently applied diphenylphosphinomethanethiol in **1**. During the reaction pathway a favourable five-membered transition state is formed in the final S→N shift to furnish the amide bond. Thioesters **1** are usually synthesized by reaction of activated carboxylic acids with free phosphinothiol **6**, which can be obtained by deacetylation of the commercially available acetyl thioester **7a** to borane-protected diphenylphosphinomethanethiol **5** and subsequent borane deprotection under basic conditions (Scheme 1B).[12,14,16,18a,19a,b]

Alternatively but less frequently, **5** can be used directly in the condensation to form a borane-protected thioester before borane deprotection to **1** is initiated, again by basic treatment, which is typically isolated before the traceless Staudinger ligation is induced by azide addition.[20,21]

In addition to the intermolecular traceless Staudinger ligation, also an intramolecular variant has been investigated. In this, the borane-protecting group of the phosphine in phosphinothioesters, which is known to prevent a Staudinger reaction with azides,[22] is utilized to install a phosphinothioester in an azidopeptide to ensure a traceless Staudinger ligation and thereby cyclization of bis-functionalized azido-phosphinothioesters only upon deprotection with bases (Scheme 1C).[21] This protocol has been applied to the synthesis of lactams as well as biaryl-containing natural product analogues.[21] Building on these investigations, our group has recently developed a TFA deprotection strategy for medium-sized azidopeptide phosphinothioesters that simultaneously releases the borane and the peptide side chain protecting groups by TFA addition and thereby allows a chemoselective intramolecular cyclization of unprotected peptides in the presence of several peptide functionalities.[23]

In conjunction with these investigations, we now present data showing that the use of protected phosphinothiols in the synthesis of (peptide) phosphinothioesters has a considerable practical advantage, since the unprotected diphenylphosphinomethanethiol **6** is very sensitive towards oxidation. Consequently, we show that these derivatives can be employed in a straightforward manner in subsequent traceless Staudinger ligations in combination with both deprotection strategies to peptide and carbohydrate substrates.

2. Results and discussion

2.1. Stability studies of phosphinothiol linkers

Our first goal was to investigate the stability of the different protected and unprotected phosphinothiol derivatives **5**, **6** and **7a**. In these studies, which were performed by ^{1}H and ^{31}P NMR measurements over the course of 15 days, it was found that the borane-protected acetyl thioester **7a** as well as the de-acetylated derivative **5** showed excellent stability upon air exposure, since no oxidation products were determined within the detection limit. In contrast, phosphinothiol **6** that was obtained by deprotection of **5** with DABCO in DMF over 4 h at 40 °C showed a fast oxidation when left in an open flask. After 5 h about two third and after 15 h more than 90% of the phosphine **6** was oxidized already (Scheme 1B and Fig. 1). As a result, although synthetically feasible as demonstrated in previous publications,[14–19] undesired oxidation of **6** before or during thioester formation could limit the overall yield especially for thioesters **1** when it is difficult to ensure strictly inert conditions, for example, during protein conjugation in aqueous buffers or lysates.[20]

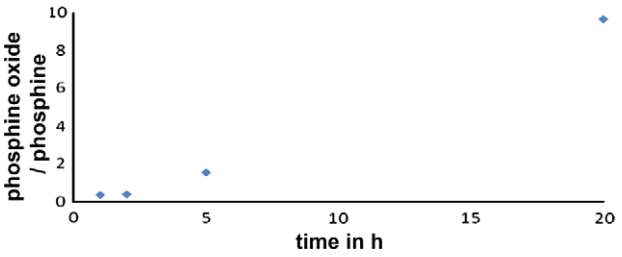


Figure 1. Oxidation of phosphinothiol **6** in air.

2.2. Basic and acidic deprotection strategies for azide transformations by the traceless Staudinger ligation

Building upon these results, we intended to employ borane-protected thioester **7a** to the formation of simple acetylated amino acids by the traceless Staudinger ligation with azidoglycine **9** as model substrate under in situ-deprotection conditions. First, we screened the best conditions for borane deprotection of **7a** under basic conditions to minimize reaction temperature and the required amount of base, in particular to allow also a later applicability in polypeptide ligations. Deprotection studies of **7a** revealed complete borane deprotection after 20 min at 70 °C or after 4 h at 40 °C with 3 equiv DABCO (1,4-diazabicyclo[2.2.2]octane) in DMSO to yield thioester **1a** (see Section 4, Table 1). Lowering the amount of base or the use of DIPEA (diisopropylethylamine) only led to incomplete deprotection. Transfer of the deprotection conditions at 40 °C to intermolecular model reactions for the base induced traceless Staudinger ligation of **7a** with **9** furnished the corresponding *N*-acetylglycine **10** in 78% conversion after 12 h in DMSO-d_6 or in 84% conversion after 12 h in DMF-d_7 (Scheme 2A), which supported the use of DMF for further investigations.

Next, we probed the use of acidic deprotection conditions for the removal of the borane-protecting group in analogous in situ traceless Staudinger ligations.[23] To exclude thioester cleavage, a short exposure of **7a** to TFA, usually between 1 and 2 h, turned out to be essential, in which absence of water in the cleavage cocktail minimized oxidation of the free phosphine.[24] Again, azidoglycine **9** was used as a substrate for acetylation, which proceeded quantitatively to **10** starting from 1.1 equiv **7a** in DMF at 40 °C within 12 h. During these studies it became evident that an excess of base is necessary to avoid the formation of amine by-products, since acidic conditions enhance the electrophilicity of the intermediate iminophosphorane and thus lead to hydrolysis by Staudinger reduction (data not shown). In order to further compare the two different deprotection strategies, acetylated β-GlcNAc azide **11** was used in an analogous reaction with **7a** to yield a glycosyl amide **12** (Scheme 2B).[25] TFA deprotection of **7a** and subsequent traceless Staudinger ligation gave **12** in 62% yield. Alternatively, basic deprotection with DABCO furnished **12** in a comparable yield of 60%.

In addition, the phosphorylated azidopeptide **13**, which contains residues 433–437 of the intrinsically unstructured Tau-protein,[26] was chosen as another peptide substrate for an acetylation by the traceless Staudinger ligation to compare the acidic and basic deprotection strategies (Scheme 3). Since initial studies indicated a slower conversion of azidopeptides when compared to azidoglycine **9**, 10 equiv of **7a** were used to counteract potential oxidation. As described before, **7a** was first deprotected and then directly employed for the traceless Staudinger ligation with **13**. Basic deprotection with DABCO was performed in situ in presence of **13** and both reactions were performed in DMF at 40 °C. After 16 h LC–MS analysis revealed that only under the acidic deprotection conditions a full conversion to **14** was observed, whereas under basic conditions the reaction was finished after 40 h. To further enhance the reaction rate, the basic route was repeated at 70 °C to accelerate borane deprotection, as evident from the previous deprotection study (see Table 1). Now full conversion of **13** was achieved after 16 h, however, the dehydroalanine peptide **15** was additionally observed as by-product. These results imply that the traceless Staudinger ligation with **13** proceeds slower under the basic deprotection conditions and moreover, elevated temperatures lead to elimination to the dehydroalanine. Therefore, the basic deprotection conditions should be avoided for phosphorylated peptide, which favors the acidic deprotection strategy for this substrate.

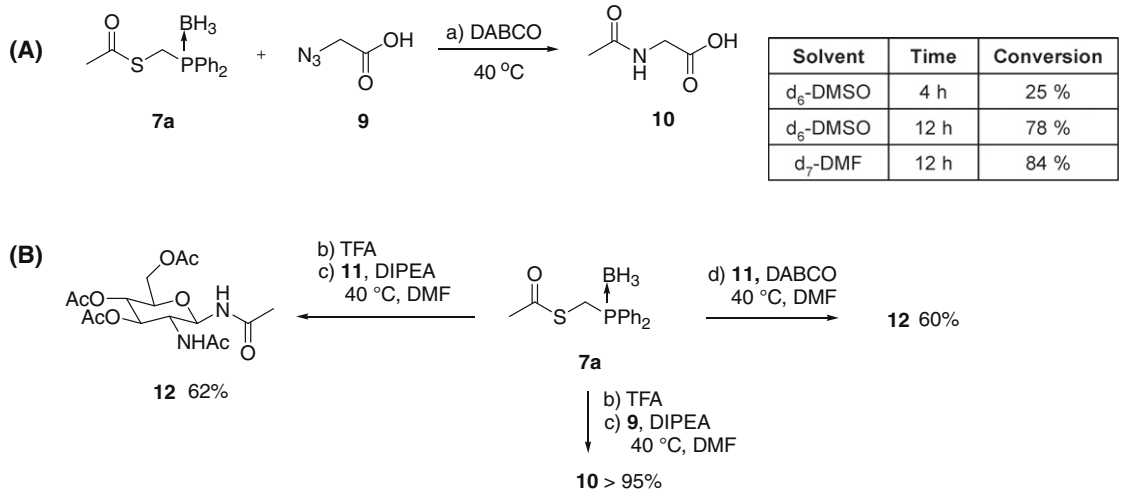
2.3. Borane-protected phosphinothiols in the synthesis and cyclization of peptide phosphinothioesters

In a subsequent investigation, we turned our attention to the synthesis of a more challenging C-terminal peptide-phosphinothioester by using borane-protected phosphinothiol **5**. Although, the basic deprotection could theoretically be applied on a synthetically accessible borane-protected peptide-phosphinothioester **16** to allow a subsequent Staudinger ligation, an additional acidic treatment is required to release the acid-labile side chain protecting groups of the peptide (Scheme 4A). In addition to this practical reason, the removal of the side chain protecting groups prior to the formation of an amide bond by the traceless Staudinger ligation has several advantages: First, side chain protecting groups could sterically hinder peptide ligation or cyclization; second, the solubility in polar solvents is increased which might prevent aggregation and finally, a chemoselective peptide ligation or cyclization is possible, which in the case of peptide cyclization could lead to preorganization of the peptide chain and thus enhance intramolecular amide bond formation.

First, we investigated the use of the borane-protected phosphinothiol **5** in the synthesis of the peptide-phosphinothioester **17**. A model peptide comprising of 7 amino acids (AspAspPheGlnAspPheGly) was synthesized on an acid-lable TGT resin, which was cleaved from the resin with 0.5% TFA in CH_2Cl_2. The C-terminally deprotected peptide was converted into the thioester **16** by activation with DIC. TFA/TIS-treatment rendered the fully depro-

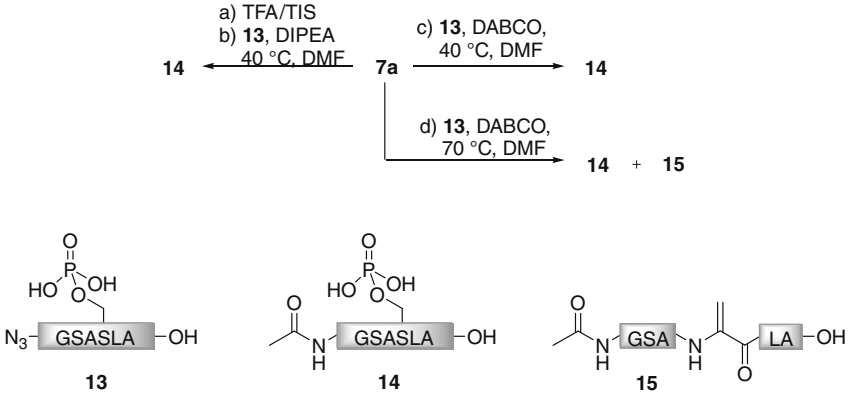
Table 1
Borane-deprotection under basic conditions for **7a** and in situ formation of **10**: Temperature measurements after 1 h unless stated otherwise (r = ratio of deprotection/protection according to –S*CH*$_2$P– chemical shifts; '—' = no deprotection observed; 'fd' = full deprotection). See also Section 4.2.2

Entry	Temperature (*r* value)	25 °C (*r*)	40 °C (*r*)	55 °C (*r*)	65 °C (*r*)	70 °C (*r*)	75 °C (*r*)
1	Heating only; without base	—	—	—	0.09:1	0.17:1	0.22:1
2	1 equiv DIPEA	—	—	—	0.25:1	0.43:1	0.91:1
3	2 equvi DIPEA	—	—	0.17:1	0.31:1	1.03:1	1.45:1
4	3 equiv DIPEA	—				*20* min: 1.25:1	
5						*60* min: 8.44:1	
6	2 equiv DABCO	—	0.20:1	0.39:1	1.15:1	1.88:1	4.68:1
7	3 equiv DABCO					*20* min: fd	
8	3 equiv DABCO		*1* h: 2.2:1				
9			*4* h: fd				



Solvent	Time	Conversion
d_6-DMSO	4 h	25 %
d_6-DMSO	12 h	78 %
d_7-DMF	12 h	84 %

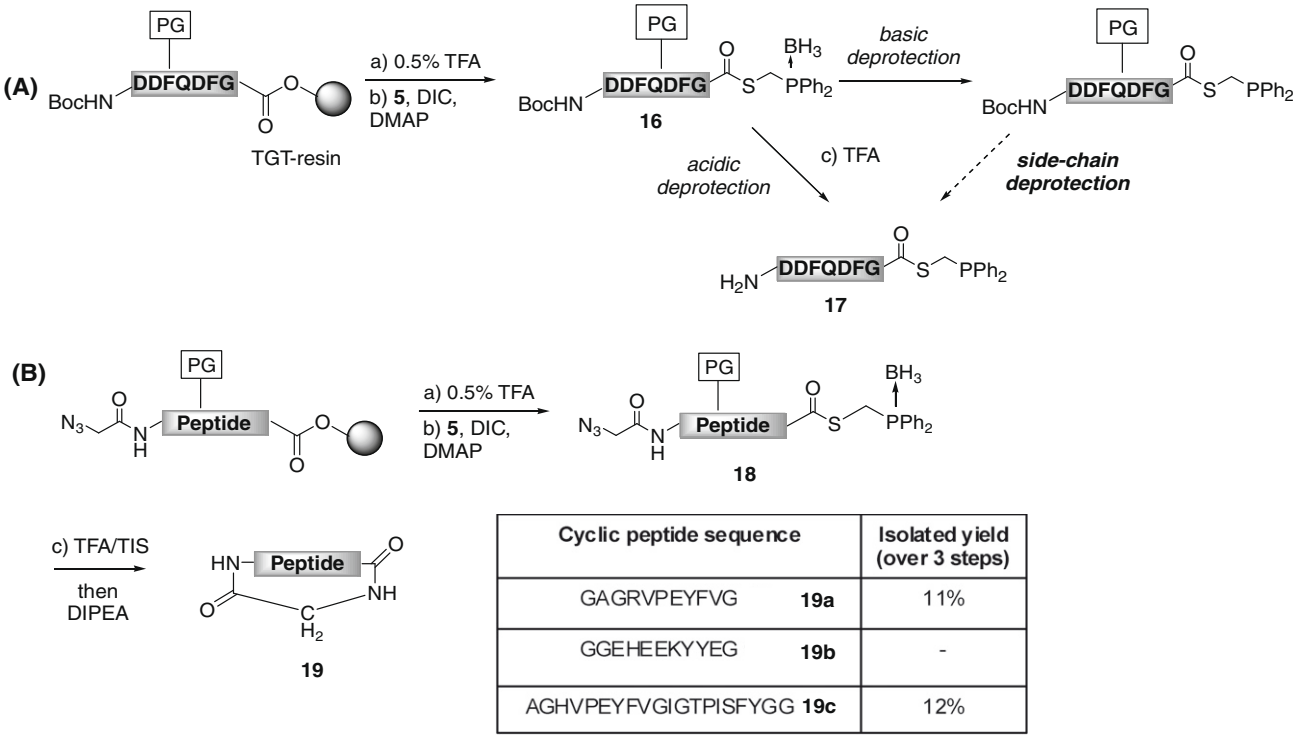
Scheme 2. Model studies for the intermolecular Staudinger ligation by basic (A) and acidic (B) deprotection of protected phosphinothioesters. Reagents and conditions: (a) DABCO (3 equiv), deuterated solvents, 40 °C; (b) 99% TFA, 1 h, then TFA removal under high vacuum; (c) **9** or **11**, DIPEA, DMF, 40 °C; (d) **11**, DABCO, DMF, 40 °C. For exact conditions see Section 4. TFA = trifluoroacetic acid, DIPEA = diisopropylethylamine, DABCO = 1,4-diazabicyclo[2.2.2]octane, DMF = dimethylformamide, DMSO = dimethylsulfoxide.



Scheme 3. Acidic and basic deprotection of borane-protected phosphinothioester for traceless Staudinger ligation with azido phospho-serine peptide **15**. Reagents and conditions: (a) 97.5% TFA (2.5% TIS), 1 h, then TFA removal under high vacuum; (b) **13**, DIPEA, DMF, 40 °C, 16 h; (c) **13**, DABCO, DMF, 40 °C, 40 h; (d) **13**, DABCO, DMF, 40 °C, 16 h. For exact conditions see Section 4.

tected peptide-phosphinothioester **17** in an excellent conversion with only minimal amounts of oxidized peptide as a side product, thereby demonstrating the benefits of the acid deprotection strategy for unprotected peptide-phosphinothioesters (see Section 4).

Finally, we subsequently subjected this protocol to the synthesis of cyclic peptides. Previous studies in our lab have demonstrated that medium sized peptides can be yielded by such a traceless Staudinger cyclization strategy in yields between 20% and 36% over three synthetic steps.[23] In these examples, we have shown that the reaction proceeds in the presence of amino acid side chain functional groups including a carboxylic acid, alcohol and phenol. Here, we intended to probe the performance of unprotected nucleophilic peptide side chains such as amines and guanidines in Arg and Lys for interference with the S→N shift during the traceless Staudinger ligation (Scheme 4B). Two azido-peptide thioesters **18a** and **18b** were synthesized including either an arginine or a lysine residue (see Scheme 4 for sequence). Deprotection with TFA and cyclization in DMF were performed by previously established conditions. In both cases side products were detected which were identified as oxidized thioesters and non-cyclized peptides with an N-terminal amine. Preparative separation delivered the Arg-containing cyclic peptide **19a** in overall 11% yield over three steps, which included the phosphinothioester synthesis, the global TFA deprotection and the traceless Staudinger cyclization. In contrast, the purification of the Lys-containing cyclic peptide **19b**, which was detected in the crude reaction mixture by LC–MS analysis, turned out to be impossible, due to significant amount of the Lys-side chain cyclized product. The formation of this unwanted cyclization product can be attributed to the deprotonation of the Lys side chain with an excess of DIPEA present, which attacks the labile thioester. Several attempts to optimize this reaction by varying the equivalents of DIPEA used to scavenge residual TFA led to no significant improvement for the formation of **19b**. Despite this limitation in the synthesis of cyclic peptide containing Lys residues, we chose a larger head-to-tail cyclic variant of the circular bacterial protein Microcin J25 as the final synthetic target.[27] This 21 amino acid comprising polypeptide has previously been synthesized in a head-to-tail variant by an intramolecular NCL followed by heterogeneous desulfurization conditions, which delivered the cyclic peptide **19c** in 25% yield over two steps starting from the peptide thioester.[28] Our analogous strategy by the traceless Staudinger ligation yielded head-to-tail cyclic **19c** in 12% overall yield, which how-



Cyclic peptide sequence		Isolated yield (over 3 steps)
GAGRVPEYFVG	**19a**	11%
GGEHEEKYYEG	**19b**	-
AGHVPEYFVGIGTPISFYGG	**19c**	12%

Scheme 4. (A) Peptide-phosphinothioester synthesis by acid deprotection. (B) Cyclization of peptides by the traceless Staudinger ligation. Reagents and conditions: (a) 0.5% TFA, 2.5% TIS, CH_2Cl_2, 2 h; (b) **5** (1.5 equiv), DIC (3 equiv), DMAP (cat.), CH_2Cl_2, 8 h; (c) 97.5% TFA, 2.5% TIS, 1 h, then precipitation from dry ethyl ether. For further information see Section 4.

ever included the peptide-phosphinothioester synthesis in contrast to the previously mentioned study.

3. Conclusion

In conclusion, the presented results support the use of the borane-protected diphenylphosphinomethanethiol (**5**) as an expedient phosphine linker for the traceless Staudinger ligation of small molecules as well as carbohydrates and larger peptides. The borane group preserves phosphine stability against oxidation and enables triggering of the traceless Staudinger ligation with azides, which is essential for cyclization strategies. So far, acidic and basic deprotection conditions have been established for borane deprotection of phosphinothioesters. The basic deprotection strategy proceeds well in organic solvents and gives good yields in subsequent reactions. Nevertheless, the rapid oxidation of the diphenylphosphinomethanethiol may limit the application of the traceless Staudinger ligration in biological systems, since the evaluated deprotection conditions are too harsh in this environment. The acidic deprotection with TFA can be performed at room temperature, thus delivering free phosphine and simultaneously removing side chain protecting groups in peptides. Hence, the chemoselectivity of the traceless Staudinger ligation can be addressed which broadens the scope of this amide bond forming reaction. One potential drawback in the use of the acidic deprotection strategy is the final addition of bases, such as DIPEA, to scavenge residual TFA to avoid the competing Staudinger reduction of the azide to an amine. This procedure may limit the chemoselectivity of the traceless Staudinger ligation, since under these basic conditions Lys side chains can form an amide bond with the phosphinothioester as demonstrated in cyclization studies in small peptides. Nevertheless, the presented results show that the application of the borane-protected phosphinomethanethiol in the traceless Staudinger ligation presents a promising strategy to other amide bond formation reactions.

4. Experimental section

4.1. Materials and methods

4.1.1. Solvents and chemicals

All reactions were performed under argon unless stated otherwise. Solvents such as ethyl acetate, hexane and CH_2Cl_2, were purchased as p.a. grade and distilled once prior to use. Reagents—including deuterated as well as dry solvents and acetonitrile—are commercially available as reagent grade and did not require further purification. The resins as well as Fmoc-protected natural L-amino acids were purchased from Novabiochem. Flash column and thin layer chromatography were performed with Merck Silica Gel 60. TLC analysis was carried out under ultraviolet light (λ = 254 nm) or via additional staining with *p*-anisaldehyde. Azido-glycine (**9**),[29] 2-Acetamido-2,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl azide (**11**)[30] and borane-protected diphenylphosphinomethanethiol acetate (**7a**)[31] were synthesized by published protocols.

4.1.2. Analytical methods

An Agilent 6210 ToF LC/MS system (Agilent Technologies, Santa Clara, CA, USA) with ESI source was used for mass detection of the peptides. H_2O and MeCN (both including 1% acetic acid) were used as eluent. The flow rate was 0.5 ml/min. Conversion of peptide reactions was determined by LC/MS analysis (C_{18}-column, constant flow of 0.5 ml/min: 3 min at 3% MeCN (with 1% AcOH), gradient 3–100% MeCN (with 1% AcOH) over 19 min (Gradient A)). ^1H, ^{13}C and ^{31}P NMR spectra were recorded on a *Jeol ECX-400* 400 MHz spectrometer at ambient temperature.

4.1.3. Preparative HPLC

HPLC purification of the peptides was performed on a JASCO LC-2000 Plus system using a reversed phase C18 column (5 μm, 25 × 250 mm, constant flow of 18.9 ml/min: 5 min at 7% MeCN (with 0.1% TFA), gradient 7–95% MeCN (with 0.1% TFA) over 30 min (Gradient B)), consisting of a Smartline Manager 5000 with interface module, two Smartline Pump 1000 HPLC pumps, a 6-port-3-channel injection valve with 2.5 mL loop, a UV detector (UV-2077) and a high pressure gradient mixer.

4.1.4. Peptide synthesis

Peptides were synthesised with ABI 433A Peptide Synthesizer from Applied Biosystems via standard Fmoc-based conditions (Fast-moc protocol with HOBt/HBTU conditions) on a preloaded TGT resin (0.28 mmol/g).

4.2. General synthesis

4.2.1. Stability studies of diphenylphosphinomethanethiols 5, 6 and 7a

The borane-protected phosphine species **5** (1 equiv) was dissolved in dry DMF (1 mL) under argon. DABCO (3 equiv) was added, and the mixture was heated at 40 °C for 4 h. Afterwards, the solvent was removed under reduced pressure and the residue was left in an open flask and monitored over a time period of 1–24 h by ^{1}H NMR and ^{31}P NMR. Figure 1 depicts the oxidation of phosphine **6** based on ^{1}H NMR analysis after 1, 2, 5 and 20 h (the integral ratio (phosphine oxide/phosphine) is shown): ^{31}P NMR (121 MHz, $CDCl_3$): 26.7 (P═O), −7.5 (P).

Borane-protected phosphines **5** and **7a** were left in an open flask in air and monitored over a time period of 15 days by ^{1}H NMR and ^{31}P NMR. ^{31}P NMR (121 MHz, $CDCl_3$): δ = 19.5 (d, P-BH_3, **7a**); 22.5 (d, P-BH_3, **5**).

4.2.2. Borane-deprotection under basic conditions for 7a and in situ formation of 10

The optimal basic deprotection conditions of **7a** were carried out with DIPEA and DABCO as bases. **7a** was dissolved in an NMR tube using DMSO-d_6 deuterated solvent. Deprotection was monitored out at different temperatures, starting from rt up to 75 °C, and different equivalents of DIPEA were added (1, 2 and 3 equiv). For comparative purposes, the same tests were then carried out using DABCO as a base (see Table 1). The second model test was carried out to optimize the basic deprotection conditions. Here, thioester **7a** was dissolved in DMSO-d_6, to which the azido-glycine **9** and 3 equiv of DABCO were added and the reaction was carried out at 40 °C. Again these tests were done in NMR tubes. Progress was checked on an hourly basis until the desired product **10** was obtained in the absence of the starting materials.

Subsequently, **7a** (0.32 mmol) was dissolved in 0.7 ml deuterated solvent (DMSO-d_6 or DMF-d_7) in an NMR tube to which 108 mg DABCO (0.96 mmol, 3 equiv) and 34 mg azidoglycine (**9**) (0.32 mmol, 1 equiv) were added. The reaction was carried out at 40 °C and the conversion to *N*-acetylglycine (**10**) was determined by ^{1}H NMR analysis (see Scheme 3A). The analytical data was in accordance with the commercially available *N*-acetylglycine (Aldrich): ^{1}H NMR (500 MHz, DMSO-d_6/DMF-d_7): δ = 8.58 (br s, 1H), 7.73–7.77 (m, 1H), 3.48–3.52 (m, 2H), 1.81 (s, 3H).

4.2.3. Acidic deprotection of 7a and application in the traceless Staudinger ligation with azidoglycine 9

Acetyl phosphinothioester **7a** (0.176 mmol, 1.1 equiv) was treated with 0.5 ml 99% TFA. After stirring for 1 h the mixture was concentrated, dissolved in 0.35 ml DMF, 16.2 mg azidoglycine (**9**) (0.160 mmol, 1 equiv) and 0.34 ml DIPEA (1.92 mmol, 12 equiv) were added. The reaction was carried out at room temperature and the conversion to *N*-acetylglycine (**10**) was determined by ^{1}H NMR analysis after 12 h. The analytical data was in accordance with the commercially available *N*-acetylglycine (Aldrich): ^{1}H NMR (400 MHz, DMF-d_7): δ = 8.58 (br s, 1H), 7.73–7.77 (m, 1H), 3.63–3.67 (m, 2H), 1.95 (s, 3H).

4.2.4. Synthesis of *N*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl) acetamide (12)

Route 1 (acidic deprotection conditions): 20 mg (0.069 mmol) phosphinothioester **7a** was treated with 1 ml 99% TFA for 1 h and the volatiles were removed under high vacuum. The residual phosphonium salt was dissolved without further purification in 1 ml dry DMF and the solution was degassed by three cycles of vacuum and argon. DIPEA (12 equiv) as well as 1 equiv of glycosyl azide **11** were added to this solution and the resulting reaction mixture was stirred for 18 h at 40 °C before the reaction mixture was concentrated under high vacuum. The residue was chromatographed on a silica gel column with chloroform–methanol (19:1). Pure acetylated GlcNAc (**12**) was obtained in 62% yield (16.7 mg, 0.043 mmol).

Route 2 (basic deprotection conditions): 20 mg (0.069 mmol) phosphinothiol **7a** was dissolved in 1 ml dry DMF and the solution was degassed by three cycles of vacuum and argon. 3 equiv of DABCO as well as 1 equiv of glycosyl azide **11** were added. The temperature was elevated to 40 °C and the reaction mixture was stirred for 18 h. The residue was chromatographed on a silica gel column with chloroform–methanol (19:1). acetylated GlcNAc (**12**) was obtained in 60% yield (14 mg, 0.031 mmol): ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.09 (d, 1H), 6.07 (d, 1H), 5.23 (t, 1H), 5.10–5.25 (m, 2H), 4.39 (dd, 1H) 4.16–4.25 (m, 2H), 3.85 (m, 1H), 2.19, 2.17, 2.14, 2.08, 2.06 (5s, 15H, Ac); HRMS (ESI-ToF): *m/z* = 389.159 $[M+H]^+$ (calcd: *m/z* = 389.156), 411.137 $[M+Na]^+$ (calcd: *m/z* = 411.137). Further analytical analysis is in accordance with reported results.[32]

4.2.5. Synthesis of *N*-acetyl phospho-serine peptide 14

Peptide **13** was synthesized on a preloaded Wang-resin and azido glycine **9** was coupled manually using 5 equiv amino acid, HOBt, HBTU and DIPEA in DMF. The peptide was then purified by preparative HPLC and analysed by HRMS (ESI-ToF): *m/z* = 611.223 $[M+H]^+$ (calcd: *m/z* = 611.219).

Route 1 (acidic deprotection conditions): 9.5 mg (32.8 μmol, 10 equiv) Phosphinothioester **7a** was treated with 1 ml 97.5% TFA (2.5% TIS) for 1 h and the volatiles were removed under high vacuum. The residual phosphonium salt was dissolved without further purification in 1 ml dry DMF and the solution was degassed by three cycles of vacuum and argon. 5 equiv of DIPEA as well as 1 equiv of azido peptide **13** were added to this solution and the resulting reaction mixture was stirred for 16 h at 40 °C. Conversion was checked by LCMS (ESI-ToF): *m/z* = 627.242 $[M+H]^+$ (calcd: *m/z* = 627.239). The product was eluted at 12.8 min (constant flow: 5% CH_3CN (with 1.0% AcOH), gradient: 5–65% CH_3CN (with 1.0% AcOH) over 25 min; 65–100% CH_3CN (with 1.0% AcOH) over 1 min).

Route 2 (basic deprotection conditions): 9.5 mg (0.069 mmol) phosphinothiol **7a** was dissolved in 1 ml dry DMF. The solution was degassed by three cycles of vacuum and argon. 3 equiv of DABCO as well as 1 equiv of azido peptide **13** were added. The temperature was elevated to 40 °C (a) or 70 °C (b), respectively. Conversion was determined by LCMS after 16 and 40 h: HRMS (ESI-TOF): (a) after 40 h: *m/z* = 627.242 $[M+H]^+$ (calcd: *m/z* = 627.239); (b) after 16 h: *m/z* = 627.245 $[M+H]^+$ (calcd: *m/z* = 627.239); *m/z* = 529.267 $[M-H_3PO_4+H]^+$ (calcd: *m/z* = 529.262).

4.2.6. Synthesis of peptide phosphinothioester 17

The peptide was synthesized on an ABI 433a peptide synthesizer on a TGT-resin (Novabiochem) with the first amino acid

(Gly) already attached to the resin. The last amino acid was introduced with an N-terminal Boc-protection. The peptide was cleaved from the resin with 0.5% TFA in CH_2Cl_2 (including 2.5% TIS) for 2 h. The resin was filtered off and washed with CH_2Cl_2. The filtrate and the washing solution were combined, and the solvent was removed under high vacuum. The C-terminally deprotected peptide was then dissolved in dry CH_2Cl_2 (5 ml, 0.033 mmol). After the addition of 3 equiv DIC and catalytic amounts of DMAP, borane-protected diphenylphosphinomethane-thiol **5** (5 equiv) was added and the reaction mixture was stirred for 12 h. The crude reaction mixture containing the protected phopshinothioester **16** was treated with a solution containing 97.5% TFA and 2.5% TIS (0.5 ml, 0.033 mmol) for 1 h. The globally deprotected peptide thioester **17** was precipitated from 10 ml dry ethyl ether and characterized by LC–MS.

Analytical HPLC and HRMS (ESI-TOF): m/z = 1057.3638 $[M+H]^+$ (calcd: m/z = 1057.3525) peptide **17** eluted at 15.84 min (Gradient A).

4.2.7. Peptide cyclization by the traceless Staudinger ligation

The azidopeptide was synthesized on an ABI 433a peptide synthesizer on a TGT-resin (Novabiochem) with the first amino acid (Gly) already attached to the resin. The peptide was cleaved from the resin with 0.5% TFA in CH_2Cl_2 (including 2.5% TIS) for 2 h. The resin was filtered off and washed with CH_2Cl_2. The filtrate and the washing solution were combined, and the solvent was removed under high vacuum. The C-terminally deprotected peptide was dissolved in dry CH_2Cl_2 (5 ml, 0.033 mmol). After the addition of 3 equiv DIC and catalytic amounts of DMAP, borane-protected diphenylphosphinomethane-thiol **5** (1.5 equiv) was added and the reaction mixture was stirred for 12 h. The crude reaction mixture containing the protected phosphinothioester **18** was treated with a solution containing 97.5% TFA and 2.5% TIS (0.5 ml, 0.033 mmol) for 1 h. The globally deprotected thioester was precipitated from 10 ml dry ethyl ether, characterized by LC–MS to verify conversion to **18** and re-dissolved in dry DMF (5 ml, 0.033 mmol). For the cyclization 20 equiv DIPEA were added to the reaction mixture and the reaction mixture was stirred for 12 h. The cyclized peptides **19** were purified by preparative HPLC (Gradient B). For yields see Scheme 4B. LC/HRMS analysis was carried out to confirm the identity of the final products and the peptide intermediates.

Analytical HPLC and HRMS (ESI-TOF): **19a:** m/z = 1133.5745 $[M+H]^+$ (calcd: m/z = 1133.5848); **19c:** m/z = 2107.0337 $[M+H]^+$ (calcd: m/z = 2107.0393), 1054.0136 $[M+2H]^{2+}$ (calcd: m/z = 1054.0236). The peptide **19a** eluted at 13.67 min and **19c** eluted at 16.09 min (Gradient A).

Acknowledgements

The authors acknowledge financial support from the German Science Foundation (DFG) within the Emmy-Noether program (HA 4468/2-1), the Sonderforschungsbereich 765 'Multivalency' and the Fonds der Chemischen Industrie (FCI, doctoral scholarship to M.M.) The Royal Scientific Society of Jordan is acknowledged for a Scholarship to D.M.M.J.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.04.015.

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