Protocol

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'Sulfo-click' for ligation as well as for site-specific conjugation with peptides, fluorophores, and metal chelators

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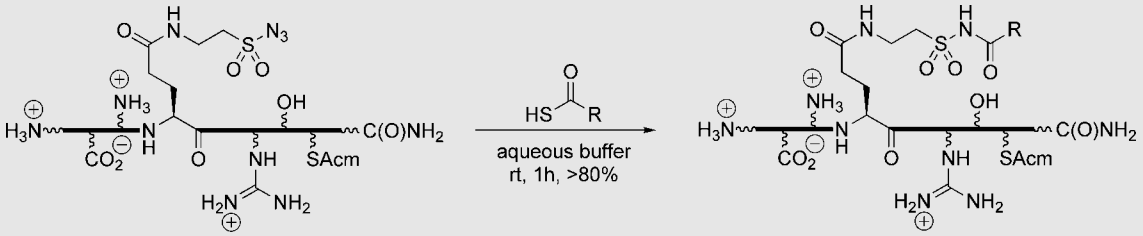
The 'sulfo-click' reaction, which is a chemoselective amidation reaction involving the reaction of an aminoethane sulfonyl azide with a thio acid, encompasses a new approach for ligation and conjugation. Detailed protocols are provided for decorating biologically active peptides or dendrimers with biophysical tags, fluorescent probes, metal chelators, and small peptides by using this reaction as a novel, metal-free 'sulfo-click' approach. Copyright © 2009 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: amidation reactions; bioconjugation; chemoselectivity; 'sulfo-click'; thio acids/sulfonyl azides

REACTION SCHEME



GENERAL OPTIMIZED PROCEDURE

Thio acid/sulfonyl azide amidation reaction between Boc-Val-Gly-SH and H-Gly-Arg-Arg-Arg-Arg-Ser-Val-Glu(Gly-Ψ[$CH_2SO_2N_3$])-Trp-Cys(Acm)-Ala-NH_2. To a solution of H-Gly-Arg-Arg-Arg-Arg-Ser-Val-Glu(Gly-Ψ[$CH_2SO_2N_3$])-Trp-Cys(Acm)-Ala-NH_2 (10 mg, 6 µmol) in an aqueous HEPES buffer (25 mM, pH 7.4; 2 ml), Boc-Val-Gly-SH (4 mg, 14 µmol) was added as a single portion. After 1 h of stirring at room temperature, the reaction was complete, according to HPLC analysis (Alltech Prosphere C18, eluted with a linear gradient of 5-95% of CH_3CN in H_2O, both phases containing TFA (0.1%)), and characterized by mass spectrometry: calcd. for $C_{73}H_{126}N_{30}O_{20}S_2$: *m/z* $[M + H]^+_{ave}$: 1809.10, found: $[M + 2H]^{2+}_{ave}$: 905.47.

Scope and Comments

The search for novel ligation and/or bioconjugation reactions is a very active research area, as these reactions not only provide important tools for studying peptide–protein and protein–protein interactions in general but are also crucial for attaching a variety of ligands for imaging and targeting purposes. Versatile approaches for the chemoselective introduction of biophysical probes into peptides/proteins include the thiolene coupling [1], thiol-disulfide exchange [2], native chemical ligation [3,4], the modified

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Staudinger ligation [5–8], oxime/hydrazone ligation [9], and the Cu(I)-catalyzed azide-alkyne 1,3-dipolar cycloaddition [10,11]. We have applied the chemoselective reaction of a thio acid and a sulfonyl azide developed by Williams and coworkers [12–14] to peptide-based thio acids and amino acid–derived sulfonyl azides as a novel chemical ligation reaction to provide an entry toward large densely functionalized peptide mimic systems with a high degree of chemical diversity [15] (**3**, Scheme 1A). In addition, we have explored its use as a novel approach for the preparation of the *N*-acyl sulfonamide linker **5** (Kenner's safety catch linker [16]), as shown in Scheme 1B [17].

The thio acid/sulfonyl azide amidation reaction, which we have denoted as 'sulfo-click', requires a sulfonyl azide (e.g. HCl.H-Gly-Ψ[CH_2SO_2]-N_3 **6** or its DOTA-conjugated derivative **7**) and a suitable thio acid (e.g. dipeptide thio acid **2**, coumarin thio acid **9** as fluorescent label, and biotin thio acid derivative **10**) or a protected precursor (e.g. **8**) as building blocks (Figure 1). The sulfonyl azides **1** and **6** were prepared from their *N*-protected amino acid derivatives as previously described [18]. The thio acids could be prepared via the corresponding succinimidyl ester followed by reaction with NaHS [19], via a BOP-mediated coupling with NaHS [17], or via the *S*-(2,4,6-trimethoxybenzyl) ester of the desired acid followed by acidolysis with TFA [20]. In a typical experiment, the sulfonyl azide was mixed with 2,6-lutidine in a suitable solvent (DMF, $CHCl_3$, aqueous buffer) and the thio acid derivative was added. The thio acid and sulfonyl azide reacted smoothly at room temperature and the reaction was complete within 15 min. The resulting acyl sulfonamides could be isolated in good to excellent yield. Also, in more complex systems [21,22] with unprotected peptide fragments (i.e. **12** and **14**), the thio acid/sulfonyl azide amidation reaction allowed the chemoselective and site-specific incorporation of suitably labeled chemical entities (peptides, fluorophores and metal chelators) as shown in Schemes 2 and 3.

The thio acid/sulfonyl azide amidation reaction was found to be extremely versatile as a metal-free click reaction for the incorporation of metal chelators like 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA)-derivative **7** to obtain DOTA-conjugated peptides (**16**) for tumor imaging and targeting [23].

Experimental Procedure

Example 1 (Scheme 1A)

To a mixture of Cbz-Phe-Ψ[CH_2SO_2]-N_3 **1** (64 mg, 0.17 mmol) and 2,6-lutidine (26 µl, 0.22 mmol, 1.3 equiv) in $CHCl_3$ (2.5 ml), Boc-Val-Gly-SH **2** (64 mg, 0.22 mmol) was added as a single portion. The reaction mixture was stirred for 15 min at room temperature, after which it was evaporated to dryness. The residue was purified by crystallization from EtOAc/hexane to give the peptidyl sulfonamide **3** as a white solid in nearly quantitative yield (102 mg, 0.17 mmol). R_f 0.48 (Merck silica gel 60-F_{254} plate, EtOAc/hexane/HOAc, 49.5 : 49.5 : 1 v/v/v); R_t 17.08 min [Alltech Adsorbosphere XL C8, 90 Å, 5 µm, 250 × 4.6 mm, using a linear gradient of 100% buffer A (0.1% TFA in H_2O) to 100% buffer B (0.1% TFA in CH_3CN/H_2O 95 : 5 v/v) in 20 min at 1 ml/min]; Mp 191 °C; $[\alpha]_D^{23}$ −29.9 (*c* 0.1 DMF); ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.89 (s, 1H), 8.16 (m, 1H), 7.53 (d, J = 9.1 Hz, 1H), 7.34–7.17 (m, 10H) 6.75 (d, J = 9.1 Hz, 1H), 4.98 (m, 2H), 4.18 (m, 1H), 3.84 (m, 3H), 3.59 (m, 2H), 2.90 (m, 1H), 2.78 (m, 1H), 1.99 (m, 1H), 1.38, (s, 9H), 0.86 (m, 6H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 172.1, 169.2, 155.7, 155.4, 137.9, 137.3, 129.4, 128.5, 127.9, 127.6, 127.2, 126.6, 78.2, 65.3, 60.0, 59.7, 56.1, 48.4, 42.3, 30.6, 28.4, 19.4, 18.3; ESMS calcd. for $C_{29}H_{40}N_4O_8S$: 604.26, found: *m/z* 605.55 $[M + H]^+$, 627.50 $[M + Na]^+$ (All ESMS data as reported in this protocol were measured on a Shimadzu LCMS-QP8000 single quadrupole bench-top mass spectrometer operating in a positive ionization mode). Elemental analysis: calcd for $C_{29}H_{40}N_4O_8S$: C, 57.60; H, 6.67; N, 9.27, found C, 57.52; H, 6.65; N, 9.21.

Example 2 (Scheme 1B)

A portion of Tentagel™ S NH_2 amino methyl resin (3 g, 0.8 mmol) was allowed to swell in NMP for 1 h. Subsequently, the resin was washed with 50% HOAc in NMP (10 ml; 2 × 10 min), NMP (10 ml; 2 × 2 min), *i*-PrOH (10 ml; 1 × 2 min), NMP (10 ml; 2 × 2 min), 20% D*i*PEA in NMP (10 ml; 1 × 2 min), NMP (10 ml; 3 × 2 min), *i*-PrOH (10 ml; 1 × 2 min) and finally NMP (10 ml; 3 × 2 min). A solution of succinic anhydride (801 mg, 8 mmol, 10 equiv) and D*i*PEA (1.4 ml, 8 mmol, 10 equiv) in NMP (10 ml) was added to the resin and the resulting mixture was shaken for 1 h before the resin was filtered and washed with NMP (10 ml; 3 × 2 min), *i*-PrOH (10 ml; 1 × 2 min), and NMP (10 ml; 3 × 2 min). Completion of the reaction was confirmed by a negative Kaiser test [24] and a positive Malachite green test [25] for the acid-functionalized resin. Then, a solution of HCl.H-Gly-Ψ[CH_2SO_2]-N_3 **6** (597 mg, 3.2 mmol, 4 equiv), BOP (1.4 g, 3.2 mmol, 4 equiv), and D*i*PEA (1.1 ml, 6.4 mmol, 8 equiv) in NMP (10 ml) was added to the resin, and the obtained reaction mixture was shaken for 16 h. Finally, the resin was filtered and washed with NMP (10 ml; 3 × 2 min), *i*-PrOH (10 ml; 1 × 2 min), and CH_2Cl_2 (10 ml; 3 × 2 min) and dried under vacuum. A portion of dried sulfonyl azide functionalized resin **4** (185 mg, 50 µmol, 1 equiv) was allowed to swell in DMF (1 ml) for 1 h before the solvent was drained. To the swollen resin, 2,6-lutidine (465 µl, 4 mmol, 80 equiv) and the N^{α}-protected amino thio acid (4 equiv) in DMF (800 µl) were added and the obtained reaction mixture was gently shaken for 3–6 h. The resin was subsequently washed with DMF (1 ml; 3 × 2 min), *i*-PrOH (1 ml; 1 × 2 min), and CH_2Cl_2 (1 ml; 4 × 2 min) and dried to give resin **5**. See reference 17 for more specific details.

Example 3 (Scheme 2)

To a solution of H-Gly-Arg-Arg-Arg-Arg-Ser-Val-Glu(Gly-Ψ[$CH_2SO_2N_3$])-Trp-Cys(Acm)-Ala-NH_2 **12** (10 mg, 6 µmol) (analysis data: R_t 26.35 min [Alltech Prosphere C18, 300 Å, 5 µm, 250 × 4.6 mm, using a linear gradient of 100% buffer A (0.1% TFA in H_2O/CH_3CN 95 : 5 v/v) to 30% buffer B (0.1% TFA in CH_3CN/H_2O 95 : 5 v/v) in 40 min at 1 ml/min]; ESMS calcd for $C_{61}H_{104}N_{30}O_{16}S_2$: 1578.80, found: *m/z* 791.38 $[M + 2H]^{2+}_{ave}$) in an aqueous 25 mM HEPES buffer (2 ml, pH 7.4), Boc-Val-Gly-SH (4 mg, 14 µmol) was added as a single portion. After 1 h of stirring at room temperature, the reaction was complete, according to HPLC analysis. The reaction mixture was loaded onto a preparative HPLC [Alltech Adsorbosphere C8, 90 Å, 10 µm, 250 × 22 mm, using a linear gradient of 100% buffer A (0.1% TFA in H_2O/CH_3CN 95 : 5 v/v) to 100% buffer B (0.1% TFA in CH_3CN/H_2O 95 : 5 v/v) in 40 min at 5 ml/min] and peptide **13** was isolated in 80% yield (8.7 mg, 4.8 µmol) and characterized by mass spectrometry. R_t 35.45 min [Alltech Prosphere C18, 300 Å, 5 µm, 250 × 4.6 mm, using a linear gradient of 100% buffer A (0.1% TFA in H_2O/CH_3CN 95 : 5 v/v) to 30% buffer B (0.1% TFA in CH_3CN/H_2O 95 : 5 v/v) in 40 min at 1 ml/min]; ESMS calcd for $C_{73}H_{126}N_{30}O_{20}S_2$: 1809.10, found: *m/z* 905.47 $[M + 2H]^{2+}_{ave}$.

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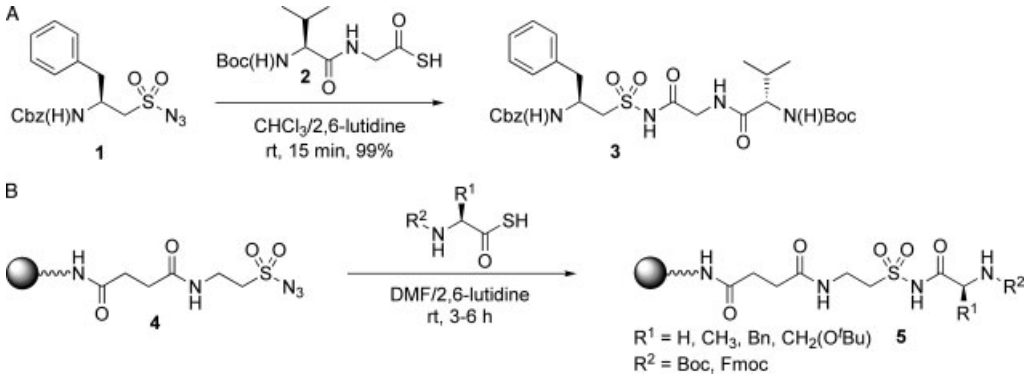


Scheme 1. A. Synthesis of peptidyl sulfonamide **3** [15]. **B.** Preparation of the *N*-acyl sulfonylamide linker **5** [17].

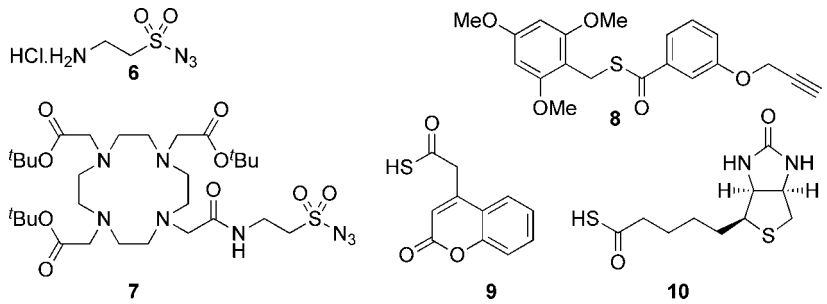
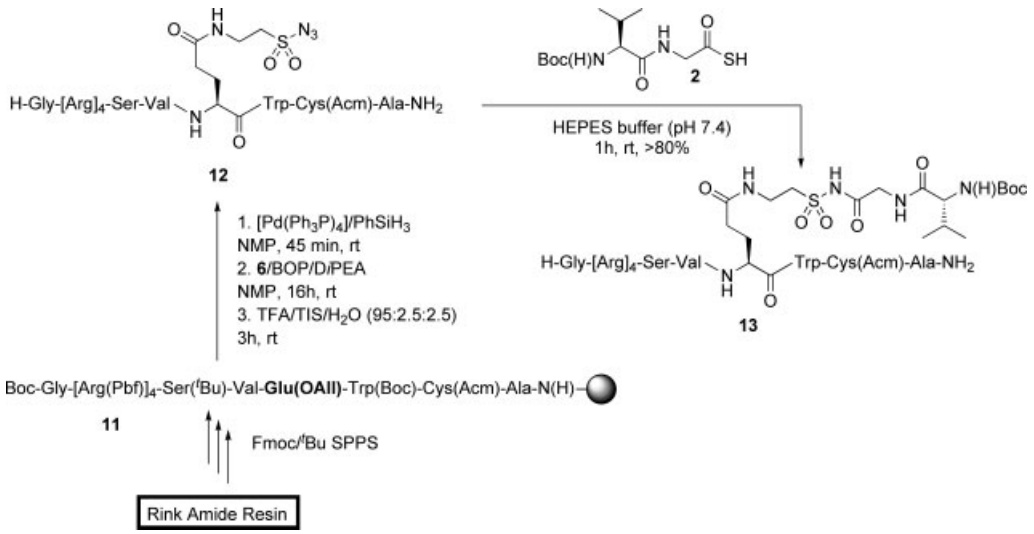
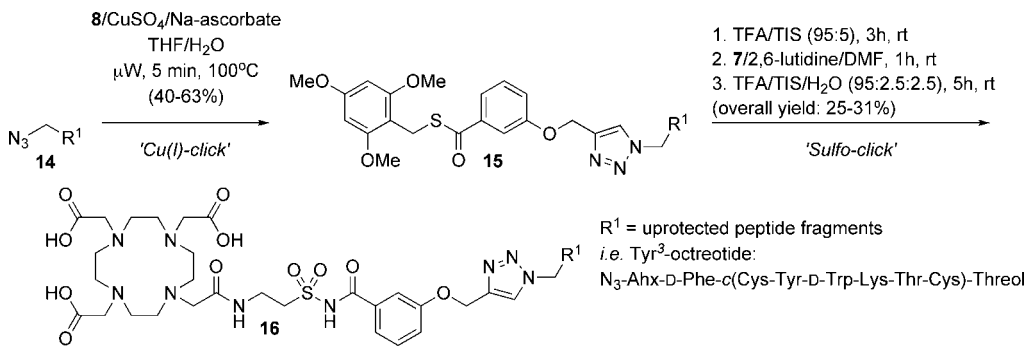


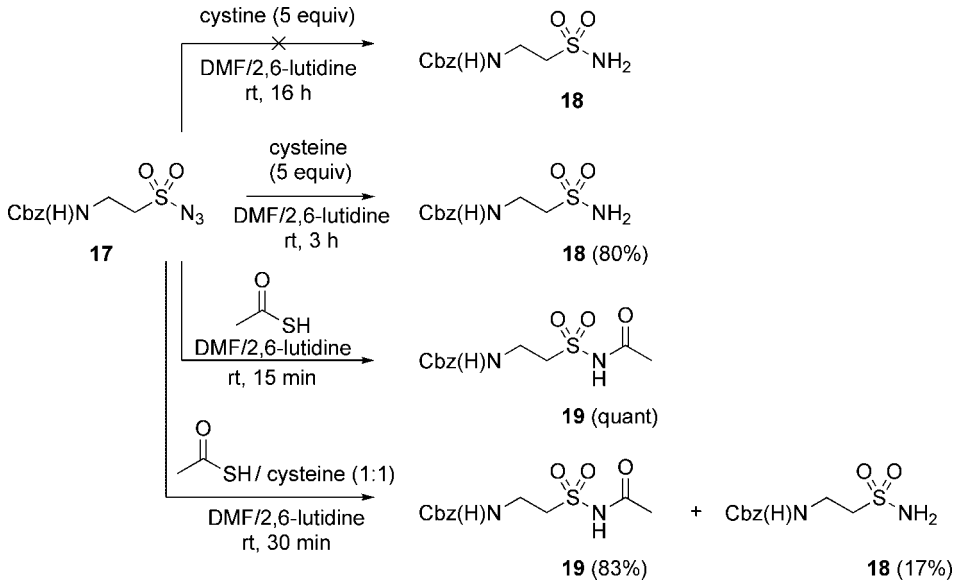
Figure 1. Building blocks for the thio acid/sulfonyl azides amidation reaction.



Scheme 2. The site-specific modification of a semiprotected human lactoferrin (hLF) peptide sequence.



Scheme 3. The 'sulfo-click' reaction as a versatile metal-free conjugation reaction.



Scheme 4. Scope and selectivity of the thio acid/sulfonyl azide amidation reaction in the presence of (free) thiol functionalities.

Example 4 (Scheme 3)

Alkyne **8** (3.0 mg, 7.9 µmol) and N_3-Ahx-D-Phe-*cyclo*[Cys-Tyr-D-Trp-Lys-Thr-Cys]-Threol **14** (9.3 mg, 7.9 µmol, 1.0 equiv) were dissolved in THF/H_2O (0.4 ml; 1 : 1 v/v). To this solution, aqueous 0.1 M $CuSO_4$ (16 µl, 1.6 µmol, 0.2 equiv) and 0.5 M Na-ascorbate (16 µl, 7.9 µmol, 1.0 equiv) were added and the reaction mixture was heated at 100 °C for 5 min under microwave irradiation. Then, the solvents were removed under reduced pressure and the residue was dissolved in CH_3CN/H_2O (2 ml; 1 : 1 v/v) and lyophilized. Following semipreparative HPLC [Alltech Alltima C8, 90 Å, 10 µm, 250 × 10 mm, using a linear gradient of 100% buffer A (0.1% TFA in H_2O/CH_3CN 95 : 5 v/v) to 100% buffer B (0.1% TFA in CH_3CN/H_2O 95 : 5 v/v) for 120 min at 4.8 ml/min], the trimethoxybenzyl-protected intermediate **15** was obtained in 47% yield (5.8 mg, 3.8 µmol). R_t 22.6 min [Alltech Alltima C8, 90 Å, 5 µm, 250 × 4.6 mm, using a linear gradient of 100% buffer A (0.1% TFA in H_2O/CH_3CN 95 : 5 v/v) to 100% buffer B (0.1% TFA in CH_3CN/H_2O 95 : 5 v/v) in 20 min at 1 ml/min]; ESMS calcd for $C_{75}H_{95}N_{13}O_{17}S_3$: 1545.61, found: *m/z* 1546.90 $[M + H]^+$, 1569.30 $[M + Na]^+$. The intermediate **15** (2.5 mg, 1.6 µmol) was treated with TFA/TIS (150 µl; 95 : 5 v/v) for 3 h at room temperature to obtain the thio acid. After concentration *in vacuo*, the residue was dissolved in dry DMF (120 µl), and DOTA-conjugated sulfonyl azide **7** (1.1 mg, 1.6 µmol, 1.0 equiv) was added. To this clear yellow solution, 2,6-lutidine (1.0 µl, 8.5 µmol, 5.2 equiv) was added, and the reaction mixture was stirred for 1 h at room temperature. The solvent was removed by evaporation and the residue was treated with TFA/TIS/H_2O (120 µl; 95 : 2.5 : 2.5 v/v/v) for 5 h at room temperature to remove the *tert*-butyl moieties. The volatiles were removed under reduced pressure and compound **16** was obtained in 25% yield (0.76 mg, 0.4 µmol) after semipreparative HPLC [Alltech Alltima C8, 90 Å, 10 µm, 250 × 10 mm, using a linear gradient of 100% buffer A (0.1% TFA in H_2O/CH_3CN 95 : 5 v/v) to 100% buffer B (0.1% TFA in CH_3CN/H_2O 95 : 5 v/v) in 120 min at 4.8 ml/min]. R_t 18.28 min [Alltech Alltima C8, 90 Å, 5 µm, 250 × 4.6 mm, using a linear gradient of 100% buffer A (0.1% TFA in H_2O/CH_3CN 95 : 5 v/v) to 100% buffer B (0.1% TFA in CH_3CN/H_2O 95 : 5 v/v) in 20 min at 1 ml/min]; ESMS calcd for $C_{83}H_{115}N_{19}O_{23}S_3$: 1841.76, found: *m/z* 922.33 $[M + 2H]^{2+}$, 615.23 $[M + 3H]^{3+}$.

Limitations

The 'sulfo-click' reaction between a thio acid/sulfonyl azide can be performed in nearly all organic and aqueous solvents/buffers and is not affected by unprotected functional groups. The presence of free sulfhydryl moieties, however, should be avoided to prevent reduction of the sulfonyl azide into the corresponding amide.

On the basis of model experiments (Scheme 4), it was found that an excess of cysteine reduced the sulfonyl azide **17** into sulfonamide **18** (80% yield within 3 h); however, an equimolar mixture of Cbz-Gly-Ψ[CH_2SO_2]-N_3 (**17**) cysteine and thioacetic acid in the presence of 2,6-lutidine/DMF resulted in the formation of Cbz-Gly-Ψ[CH_2SO_2]-NHAc **19** (83%) and Cbz-Gly-Ψ[CH_2SO_2]-NH_2 **18** (17%), an indication that the preferred coupling was faster than the concurrent reduction.

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