# The chemical ligation of selectively *S*-acylated cysteine peptides to form native peptides *via* 5-, 11- and 14-membered cyclic transition states†

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**Cysteine and *C*-terminal cysteine peptides are selectively *S*-acylated at 0–20 °C by *N*-(Pg-α-aminoacyl)benzotriazoles to give *N*-Pg-*S*-acyl-isodi-, -isotri-, and -isotetra-peptides isolated in good yields. *N*-Fmoc-*S*-acyl-isopeptides are Fmoc deprotected to afford free *S*-acyl-isopeptides isolated in high yields. *S*-Acyl-isodi-, *S*-acyl-isotetra-, and *S*-acyl-isopenta-peptides undergo chemical ligation; migration of the cysteine *S*-acyl groups to the N-terminal amino acids *via* 5-, 11-, and 14-membered transition states giving the corresponding native di-, tetra-, and penta-peptides. By contrast, the *S*-acyl-isotripeptide prefers intermolecular acylation from one molecule to another over an 8-membered intramolecular transition state. The developed methodology allows convenient isolation of stable, unprotected *S*-acyl cysteine peptides including the first isolation of *S*-acyl-isopeptides, which should facilitate the investigation of ligation by physical organic chemistry techniques.**

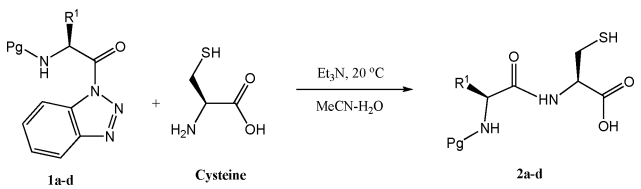
Peptides and proteins play vital roles in almost all biological and physiological processes in living organisms.[1] In native chemical ligation (NCL), chemo selective reactions of a C-terminal thioester and an N-terminal cysteine residue form a native peptide bond at the ligation site, a powerful technique for the synthesis of peptides and proteins.[2,3] Contemporary syntheses of peptides and proteins now incorporate chemo selective peptide ligation in an ever expanding manner.[4] The classical NCL method needs an N-terminal cysteine residue on a peptide or glycopeptide fragment. Thiol auxiliary ligation[5] and sugar-assisted ligation[6] can overcome this limitation, but need auxiliaries and can sterically hinder the ligation.

*S*-Acyl peptides have helped elucidate biophysical, structural and other properties of *S*-acylated proteins of mammalian cells.[7] While peptides lacking free hydroxyl or amino groups can be efficiently *S*-acylated by acyl chlorides;[8] specific *S*-acylation of cysteine-containing peptides has remained a synthetic challenge, given the lability of the thioester bond.[7] We demonstrated previously that peptide coupling of *N*-(Pg-α-aminoacyl)benzotriazoles with unprotected amino acids in MeCN–$H_2O$ occurs with preservation of chirality.[9]

We now report the following novel findings (i) selective acylation by *N*-acylbenzotriazoles under mild conditions of cysteine-containing peptides into isolated *S*-acyl isopeptides, including examples containing free hydroxyl and/or carboxyl groups; (ii) solution phase Fmoc group deprotection of *N*-Fmoc-*S*-acyl isotri-, isotetra-, and isopenta-peptides possessing free carboxyl groups; (iii) microwave-assisted chemical ligations (1 h, 50 °C) of these *S*-acyl isopeptides to form native peptides *via* 11- and 14-membered ligation transition states without use of auxiliaries.

## Synthesis of cysteine dipeptides

*N*-(Protected-α-aminoacyl)-benzotriazoles **1a–d** (see Table 1) were prepared in 75–90% yield from the corresponding *N*-protected amino acids following our published one-step procedure.[9a] *N*-Protected cysteine dipeptides **2a–d** were synthesized in 74–98% yields by peptide coupling reactions of *N*-(Pg-α-aminoacyl)-benzotriazoles **1a–d** with L-cysteine in aqueous acetonitrile (MeCN–$H_2O$, 7 : 3) in the presence of $Et_3N$ for one hour at 20 °C (Scheme 1, Table 1).[9a] Novel dipeptides **2a–d** were characterized by $^1H$, and $^{13}C$-NMR spectroscopy and elemental analysis.




**Scheme 1** Preparation of *N*-Pg-dipeptides **2a–d** from *N*-protected (α-aminoacyl)benzotriazoles **1a–d** and L-cysteine.

## Synthesis of *S*-acyl isopeptides

*S*-Acyl isodipeptides **3a**,**b** were synthesized by peptide coupling reactions between *N*-(Pg-α-aminoacyl)benzotriazoles **1a**,**b** and L-cysteine hydrochloride in aqueous acetonitrile (MeCN–$H_2O$, 10 : 1) in the presence of one equivalent of $Et_3N$ for one hour at RT in 35–65% yields (Scheme 2).[9a] Novel *S*-acyl isodipeptide hydrochloride salts **3a**,**b** were characterized by $^1H$, $^{13}C$-NMR

**Table 1** Preparation of *N*-Pg-dipeptides **2a–d**

| Reactant **1** | $R^1$ | Product **2** | Yield of **2** (%)[a] |
|---|---|---|---|
| **1a** | $CH_2Ph$ | *N*-Fmoc-L-Phe-L-Cys-OH (**2a**) | 98 |
| **1b** | $CH_2SMe$ | *N*-Fmoc-L-Met-L-Cys-OH (**2b**) | 85 |
| **1c** | H | *N*-Fmoc-L-Gly-L-Cys-OH (**2c**) | 84 |
| **1d** | CH(OH)Me | *N*-Z-L-Thr-L-Cys-OH (**2d**) | 74 |

[a] Isolated yield.

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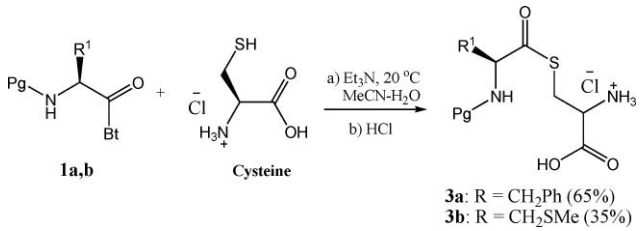
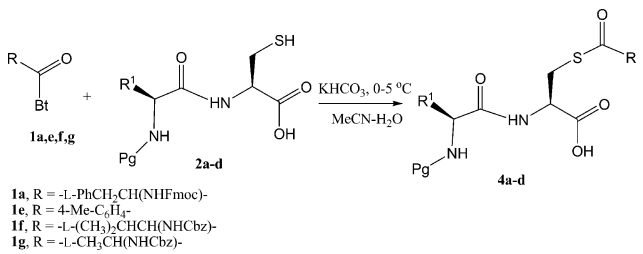



**Scheme 2** Preparation of *S*-acyl isodipeptides **3a**,**b**.

spectroscopy and elemental analysis. This is the first isolation of hydrochloride salts of *S*-(α-aminoacyl)cysteines, the unstable intermediates formed after transthioesterification during native chemical ligation.[10]

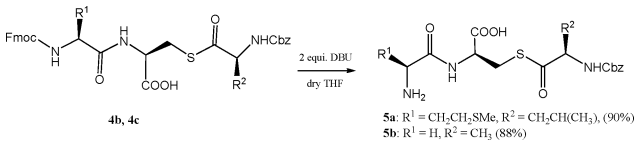
*S*-Acylation of cysteine-containing dipeptides **2a–d** (each containing a free carboxyl group) by treatment with *N*-acylbenzotriazoles **1a**,**e**,**g**,**h** at 0–5 °C in the presence of $KHCO_3$ afforded *S*-acyl isotripeptides **4a–d** in 72–87% yields (Scheme 3, Table 2). Peptide **2d**, containing a threonine residue with an unprotected hydroxyl group was thus selectively *S*-acylated to **4d**.




**Scheme 3** *S*-Acylation of dipeptides **2a–d** to form isotripeptides **4a–d**.

Classical methods for *N*-Fmoc-deprotection utilize a mild base such as 20% piperidine,[11] required in excess to deprotonate the fluorene and scavenge liberated dibenzofulvene (DBF). This method is not suitable for solution phase peptide synthesis due to low volatility of the solvent used and the solvent dependant reversible reaction of dibenzofulvene with piperidine.[12] Sheppeck II *et al.*, used thiols as dibenzofulvene scavengers in the deprotection of *N*-Fmoc-protected amines.[13]

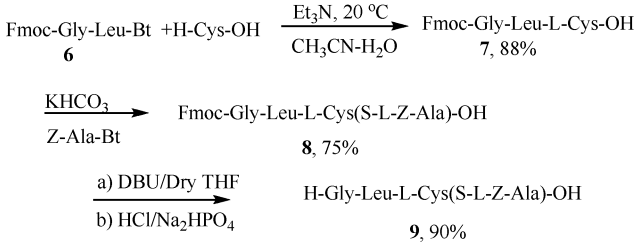
We now report a new facile and efficient procedure for Fmoc deprotection of *S*-acyl isopeptides possessing a free carboxylic group and a labile thioester functionality. When *N*-Fmoc-*S*-acyl isopeptides **4b**,**4c** in dry THF were treated with two equivalents of DBU, DBF liberated out, and peptide precipitated as a di-DBU salt of carbamate, carboxylate. DBU carbamate salts are known.[14,15] After decantation, acidification of the precipitate with 2 N HCl caused evolution of carbon dioxide. Adjustment of the pH to 5 precipitated the unprotected *S*-acyl isopeptides **5a**,**5b** in 88–90% yields (Scheme 4). Protection of carboxylic group in peptides




**Scheme 4** Fmoc deprotection of *S*-acyl isotripeptides **4b**,**4c**.

is not necessary in this procedure.[16] The *S*-acyl isotripeptides **5a** and **5b** with a free amino group are needed for the chemical ligation studies. The *S*-acyl isotripeptides **5a**,**b** were characterized by $^1$H, and $^{13}$C-NMR, and elemental analysis.

*N*-(*N*-Fmoc-Glycyl-leucyl)benzotriazole **6**, prepared by our published procedure,[9c] reacted with cysteine in acetonitrile–water (3 : 1) in the presence of one equivalent of triethylamine to give Fmoc-Gly-L-Leu-L-Cys-OH **7** in 88% yield. The *S*-acylation of **7** by Z-Ala-Bt **1g** in acetonitrile–water in the presence of one equivalent of $KHCO_3$ gave *S*-acyl isotetrapeptide **8** in 75% yield. Fmoc deprotection of **8** was achieved using DBU in dry THF to give amino-unprotected *S*-acylated isotetrapetide **9** in 90% yield (Scheme 5). Compounds **6–9** were each characterized by $^1$H, and $^{13}$C-NMR spectroscopy and by elemental analysis.




**Scheme 5** Preparation of mono free amino *S*-acyl-isotetrapeptide **9**.

Treatment of Gly-L-Cys(Z-L-Ala)-OH (**5b**) with *N*-(*N*-Fmoc-glycyl-leucyl)benzotriazole **6** afforded *S*-acyl isopentapeptide **11** as shown in Scheme 6. $^1$H-NMR and HPLC-MS confirmed compound **11** and isolated in 80% yield. Fmoc deprotection of **11** was achieved using DBU in dry THF to give mono free amino *S*-acyl isopentapetide **12** characterized by $^1$H-NMR spectroscopy and HPLC-MS (Scheme 6, ESI).

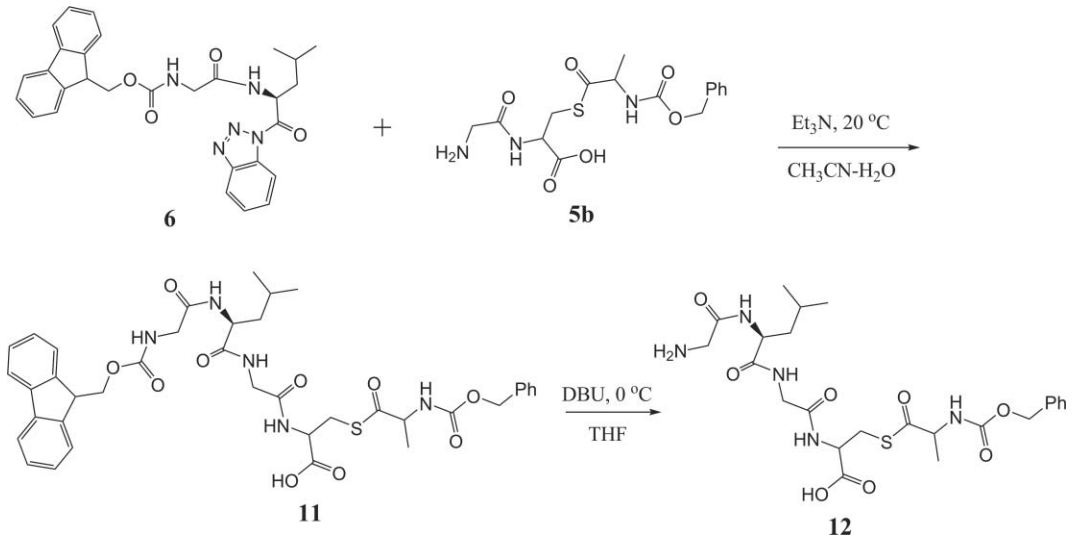
## Chemical ligations

*S*-Acyl isodipeptides **3a**,**b** underwent classical S to N acyl transfer at 20 °C in acetonitrile–water (3 : 1), in the presence of triethylamine as expected to give native cysteine dipeptides **2a**,**b** in 80–89% yields (Scheme 7). These S to N acyl transfers involve a 5-membered transition state due to proximity of the amino group in **3a**,**b** to the thioester functionality. Native cysteine dipeptides

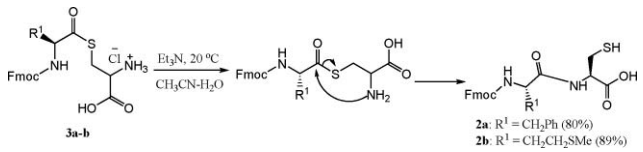
**Table 2** Preparation of *S*-acyl isotripeptides **4a–d**

| Dipeptide **2** | RCOBt used | Product **4** | Yield of **4** (%)[a] |
|---|---|---|---|
| **2a** | **1e** | Fmoc-L-Phe-L-Cys(4-Me-Ph)-OH (**4a**) | 78 |
| **2b** | **1f** | Fmoc-L-Met-L-Cys(Z-L-Leu)-OH (**4b**) | 83 |
| **2c** | **1g** | Fmoc-L-Gly-L-Cys(Z-L-Ala)-OH (**4c**) | 87 |
| **2d** | **1a** | Z-L-Thr-L-Cys(Fmoc-L-Phe)-OH (**4d**) | 72 |

[a] Isolated yield.




**Scheme 6** Preparation of mono free amino *S*-acyl isopentapeptide **12**.

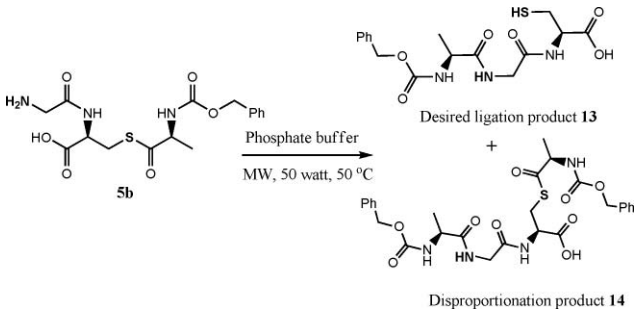



**Scheme 7** Native chemical ligation of *S*-acyl isodipeptides **3a**,**b** to give native dipeptides **2a**,**b**.

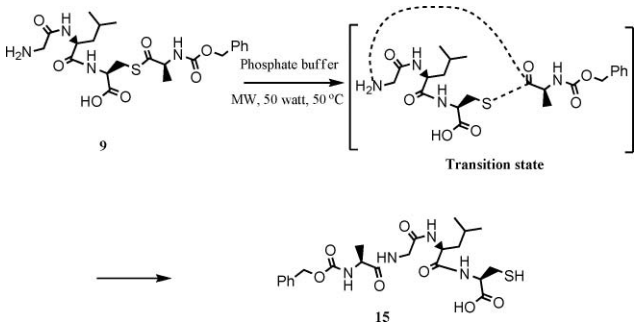
**2a**,**b** were characterized by $^{1}$H, and $^{13}$C-NMR spectroscopy and elemental analysis.

We next investigated long range chemical ligations which require eight-, eleven- and fourteen-membered ring transition states for intramolecular nucleophilic attack. Literature peptide ligations *via* direct aminolysis of thioesters are usually accomplished at 37 °C, during more than 48 h,[5] we now show that reaction was significantly accelerated by microwave irradiation. When *S*-acyl isotripeptide **5b** was suspended in $NaH_2PO_4/Na_2HPO_4$ at pH 7.8 and irradiated with microwave at 50 watts and 50 °C for one hour, the product was shown by HPLC-MS analysis (ESI) to consist mainly of the disproportionation product **14** (>85%) together with a minor amount of ligation product **13** (3%) (Scheme 8, Table 3). This indicates that intermolecular aminolysis of thioester is faster than 8-membered ring intramolecular attack.

By contrast, when *S*-acyl isotetrapeptide **9** was suspended in $NaH_2PO_4/Na_2HPO_4$ at pH 7.8 and irradiated with microwave at 50 watts and 50 °C for one hour, subsequent HPLC-MS analysis (ESI) confirmed the major product to be **15** formed by the desired ligation (Scheme 9, Table 3). In this case the intramolecular




**Scheme 8** Chemical ligation of mono free amino isotripeptide **5b**.




**Scheme 9** Chemical ligation of mono free amino isotetrapeptide **9**.

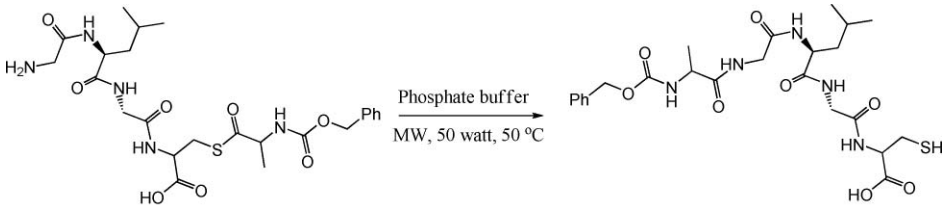
11-membered ring nucleophilic attack by the amino group on the thioester was evidently faster than the intermolecular attack.

**Table 3** Ligated native peptides formed by chemical ligation

| Entry | Ligated peptide | Retention time/min | $[M + H]^+$ found[a] | Isolated | |
|---|---|---|---|---|---|
| | | | | Yield (%)[b] | Purity (%)[c] |
| 1 | Z-Ala-Gly-Cys-OH, **13** | 27.91 | 384.1 | nd[d] | nd[d] |
| 2 | Z-Ala-Gly-Leu-Cys-OH, **15** | 21.75 | 497.1 | 95 | 67 |
| 3 | Z-Ala-Gly-Leu-Gly-Cys-OH, **16** | 24.19 | 554.2 | 90 | 61 |

[a] HPLC-MS (ESI), [b] crude yield, [c] based on $^{1}$H-NMR, [d] not determined.




12 16

**Scheme 10** Chemical ligation of mono free amino isopentapeptide **12**.



Again, when *S*-acyl isopentapeptide **12** was suspended in $NaH_2PO_4$–$Na_2HPO_4$ at pH 7.8 and irradiated with microwave at 50 watts and 50 °C for one hour. HPLC-MS analysis (ESI) confirmed the major product to be **16**, produced by the desired ligation (Scheme 10, Table 3). This result indicates that intramolecular 14-membered ring nucleophilic attack by the amino group on the thioester in **12** was also faster than intermolecular attack by one of molecule of **12** on another.

Haase and Seitz recently demonstrated that an internal cysteine residue can accelerate thioester based peptide ligation up to 25 fold providing indirect evidence for S- to N-acyl transfer *via* 8, 11, 14, 17, 20, or 23-membered transition states to form native peptides (in 3–56% hplc yields).[17] Our results show that 11- and 14-membered transition states allow easy intramolecular ligation to form native peptides, but an 8-membered transition state reaction is disfavoured.

In conclusion, we have demonstrated (i) selective *S*-acylation, in good yields and under mild conditions, of cysteine peptides having free hydroxyl and/or carboxyl groups; (ii) solution phase Fmoc group deprotection of *N*-Fmoc-*S*-acyl isotri-, isotetra- and isopenta-peptides having free carboxyl groups in 15 min with DBU and (iii) microwave assisted chemical ligation involving the migration of cysteine *S*-acyl groups in **9** and **12** to N-terminal amino acids in these peptides *via* 11- and 14-membered transition states.

As in native chemical ligation, these chemical ligations take place utilizing a single cysteine, without the use of auxiliaries. *S*-Acyl isopentapeptide **12** was converted into native pentapeptide **16** as major product (Table 3). Isotetrapeptide **9** gave native tetrapeptide **15** as major product (Table 3) and characterized by HPLC-MS. However, isotripeptide **5b** gave a mixture of the desired ligated product **13** (3%) together with disproportionation product **14** (>85%) of intermolecular *trans*-acylation. This indicates that, intermolecular *trans*-acylation is favoured over an 8-membered cyclic transition state, whereas intramolecular chemical ligation reactions are preferred when they are produced *via* 5-, 11- and 14-membered transition states.

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