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Chemoselective peptide bond formation using formyl-substituted nitrophenylthio ester

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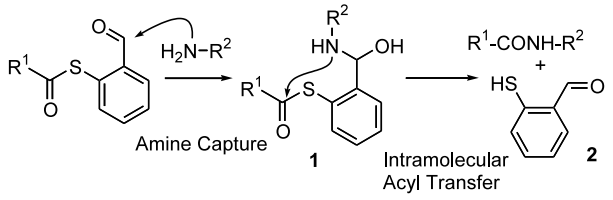
Abstract—A novel method for peptide bond formation utilizing amino acid 2-formyl-4-nitrophenylthio ester has been developed. The reaction can be performed in water-containing media and is compatible with various types of amino acid side-chain functional groups. Use of *N*-methylmaleinimide as an additive is essential for the reaction to proceed with high efficiency. It captures liberated formyl-substituted thiophenol through 1,4-addition followed by aldol cyclization.

Thioester-mediated peptide bond formation has recently attracted attention and been shown to be particularly suitable for block condensation of large peptide fragments. In particular, Aimoto's pioneering work demonstrated that Ag^+-aided thioester activation is highly efficient.[1] Kent et al. also developed an ingenious method called native chemical ligation, which offers exceptional chemoselectivity.[2] Kent's approach allows for the use of completely unprotected peptide for fragment condensation, although application of the method is restricted by the inherent limitation of the requirement of a cystein residue at the ligation site of the C-terminal fragment. Taking this limitation into account, further innovative approaches including Staudinger ligation,[3] phenylmercaptoethylamine-assisted ligation,[4] and mercaptobenzylamine-assisted ligation[5] have been reported, although the generality of these recent proposals has yet to be scrutinized.

In this paper, the authors report a novel entry to thioester-based peptide bond formation, proceeding by amine capture followed by acyl transfer (Scheme 1). The technique is shown to be highly efficient, chemoselective, and compatible with aqueous media.

Specifically, a 2-formyl-4-nitrophenylthio ester (**3**) was designed based on the following considerations. A formyl group placed at the *ortho* position can be expected to capture amine,[6] proceeding to the transient generation of hemiaminal (**1**), which should be facile for acyl transfer to create peptide linkage. Although amide bond formation through hemiaminal via N–O acyl transfer has been reported by Kemp et al.,[7] their approach seems suitable only for reactive amine due to the competing imine formation. However, since thioester has higher reactivity toward aminolysis, acyl transfer is expected to predominate over imine formation. A nitro group at the *para* position enhances the activity of the leaving group of the thiol component.[8] Initial concern was focused on the non-productive interaction of liberated aldehyde (**2**) with amine, which may retard peptide bond formation. This interaction was fortuitously eliminated by the use of *N*-methylmaleimide as a scavenger (vide infra).

Fmoc-valine-derived **3** was used as the thioester, which in turn was prepared as depicted in Scheme 2. Specifically, commercially available 2-chloro-5-nitrobenzaldehyde† was converted to thiolate, which was



Scheme 1. Plausible pathway of the reaction.

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† Purchased from Tokyo Kasei Kogyo Co. (¥ 6,750/25 g).

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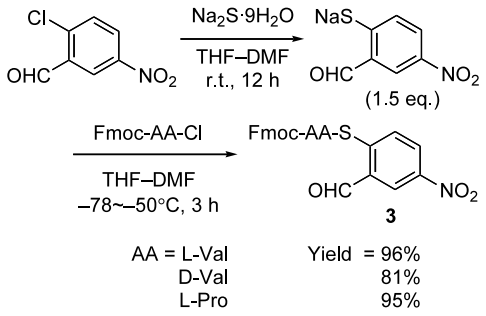
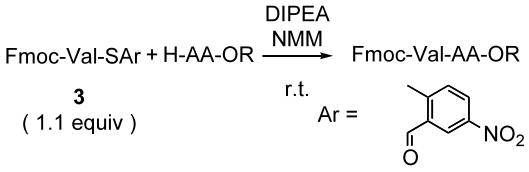


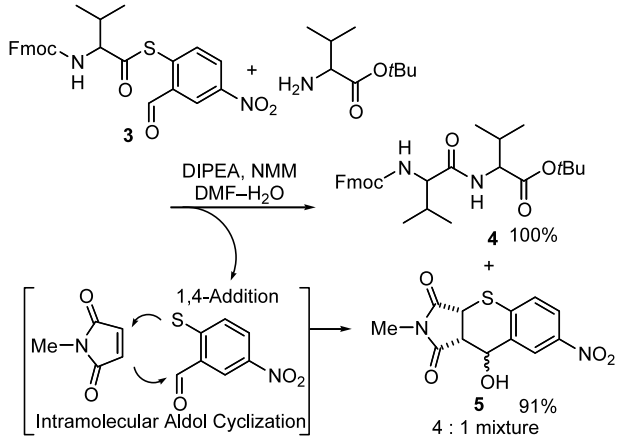
Scheme 2. Synthesis of formyl-substituted nitrophenylthio ester.

Table 1. Reaction of **3** under various conditions[a]



Entry	H-AA-OR	Solvent	Time	Yield (%)
1[b]	AcOH·H-Gly-O*t*Bu	DMF–H_2O (9:1)	3 days	90
2		DMF–H_2O (9:1)	2.5 h	91
3	HCl·H-Val-O*t*Bu	DMF–H_2O (9:1)	2.5 h	100
4		DMSO–H_2O (9:1)	2.5 h	84
5		DMF	2.5 h	83
6		CH_2Cl_2	2.5 h	99
7[c]		DMF–H_2O (9:1)	2.5 h	32

[a] DIPEA (1.1 equiv.) and NMM (1.5 equiv.).
[b] Without DIPEA.
[c] Without NMM.



Scheme 3. Trapping of resulting formyl-substituted nitrothiophenol by NMM.

then reacted with Fmoc-Val-Cl[9] to give **3** in high yield.‡

Reactions with glycine or valine ester were examined initially, the results of which are summarized in Table 1. In the majority of cases, diisopropylethylamine (DIPEA) was added to liberate free amine from the corresponding hydrochloride or acetate. In the absence of DIPEA, the reaction proceeded with impractically slow kinetics (entry 1). Use of *N*-methylmaleimide (NMM) was found to be essential for attaining a clean result, and afforded much higher yield than without (entry 3 and 7).§ The loss of 2-formyl-4-nitrobenzenethiol was accounted for by the formation of tricyclic product **5**, which was isolated in high yield as a 4:1 mixture of diastereomers and dipeptide **4** (Scheme 3).¶ Thus, NMM appeared to be functionally driving the reaction, not only by capturing thiol, but also by

‡ Preparation of Fmoc-valine 2-formyl-4-nitrophenyl-thioester **3**: To a solution of 2-chloro-4-nitrobenzaldehyde (2.38 g, 12.8 mmol) in THF (45 mL) and DMF (4.5 mL) was added $Na_2S·9H_2O$ (3.08 g, 12.8 mmol) in one portion at room temperature, and the reaction mixture was stirred at room temperature for 12 h. After cooling down to −78°C, a solution of Fmoc-Val-Cl (3.00 g, 8.55 mmol) in THF (5 mL) was added to the mixture, the reaction mixture was stirred for 3 h during which time the reaction temperature was slowly going up to −50°C. Then the reaction was quenched by sat. $KHSO_4$ aq. and THF was removed in vacuo from the resulted mixture. Product was extracted with ethyl acetate and combined organic layer was washed with H_2O, sat. $NaHCO_3$ aq., H_2O, and brine, then dried over Na_2SO_4, and concentration in vacuo. The product mixture was purified by flash column chromatography using a gradient solvent system (hexane/ethyl acetate = 10/1 to 5/1 to 2/1 to 1/1 to 1/2) to give title compound **3** (4.15 g, 96%): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.93 (3H, d, J = 6.8 Hz, $\underline{Me}_2CH$), 1.00 (3H, d, J = 6.8 Hz, $\underline{Me}_2CH$), 2.21–2.41 (1H, m, $Me_2C\underline{H}$), 4.24 (1H, dd, J = 6.8, 6.4 Hz, Fmoc), 4.44 (1H, dd, J = 8.4, 4.4 Hz, Val-α-C$\underline{H}$), 4.51 (1H, dd, J = 10.8, 6.8 Hz, Fmoc), 4.65 (1H, dd, J = 10.8, 6.4 Hz, Fmoc), 5.26 (1H, d, J = 8.4 Hz, N$\underline{H}$), 7.24–7.41 (4H, m, Fmoc), 7.59–7.61 (2H, m, Fmoc), 7.64 (1H, dd, J = 10.8 Hz, Ar), 7.71–7.77 (2H, m, Fmoc), 8.39 (1H, dd, J = 8.4, 2.4 Hz, Ar), 8.81 (1H, d, J = 2.4 Hz, Ar), 10.08 (1H, s, CHO); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 17.30, 19.38, 30.75, 47.32, 66.51, 67.11, 119.98, 120.01, 123.78, 124.77, 124.86, 127.03, 127.48, 127.71, 127.75, 137.49, 137.88, 137.99, 141.30, 141.33, 143.38, 143.43, 148.49, 156.03, 187.97, 196.46; MALDI-TOF MS calcd for $C_{27}H_{24}N_2NaO_6S$ ([M+Na]$^+$) 527.1, found 527.1.

§ A typical experimental procedure for peptide bond formation: Preparation of Fmoc-Val-Val-O-*t*Bu **4a**: To a solution of **3** (50.0 mg, 99.1 μmol), valine *t*-butyl ester hydrochloride (18.9 mg, 90.1 μmol) and *N*-methylmaleinimide (15.0 mg, 135 μmol) in 1.0 mL of DMF–H_2O (9:1) was added 1.0 M solution of diisopropylethylamine (100 μL, 0.10 mmol) in dioxane at room temperature. The mixture was stirred for 2.5 h at room temperature and concentrated in vacuo. The product mixture was purified by flash column chromatography using a gradient solvent system (hexane/ethyl acetate = 20/1 to 10/1 to 5/1 to 2/1 to 1/1) to give title compound **4a** (44.6 mg, 100%).

¶ Spectroscopic data of the tricyclic product **5** (major diastereomer): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.11 (3H, s, $\underline{Me}$N), 3.87 (1H, d, J = 2.4 Hz, OH), 3.96 (1H, dd, J = 9.6, 8.8 Hz, C$\underline{H}$CH(OH)), 4.31 (1H, d, J = 9.6 Hz, SC$\underline{H}$), 4.92 (1H, dd, J = 8.8, 2.4 Hz, C$\underline{H}$OH), 7.59 (1H, d, J = 8.4 Hz, Ar), 8.15 (1H, dd, J = 8.4, 2.0 Hz, Ar), 8.56 (1H, d, J = 2.0 Hz, Ar); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 25.69, 41.68, 49.79, 69.33, 120.70, 123.02, 129.10, 130.17, 140.08, 173.41, 176.91; FAB MS 295 ([M+H]$^+$).

blocking the non-productive interaction of aldehyde with amine. When conducted in DMF, reactions were substantially more efficient in the presence of H_2O (entry 3 and 5), presumably by governing hemiaminal↔imine equilibrium in favor of the former. The use of aq. DMSO (entry 4) was also successful, yet at somewhat lower efficiency. CH_2Cl_2 afforded high yield under anhydrous conditions (entry 6).

The reaction course was monitored by ^1H NMR (400 MHz, DMF-d_6–D_2O, 9:1) to confirm the mechanistic supposition, revealing the formation of transient species that could be assigned to hemiaminal (δ 6.2, s),[‖] which was disappeared at the end of the reaction. Under identical conditions, regioisomeric 2-nitro-4-formylphenylthio ester**,[10] gave corresponding peptide in substantially reduced yield (74%). These results indicated that the fomyl group at *ortho*-position is obviously effective to give high yielding reaction, and that hemiaminal is a major reactive intermediate in the case of 2-formyl derivative **3**. Energy minimization of the hemiaminal, as calculated by MOPAC-PM_3, showed that its hydroxyl group forms an intramolecular hydrogen bond with the carbonyl oxygen of the thioester, and that a nitrogen atom orients toward the carbonyl carbon to form a favorable configuration for nucleophilic attack (Fig. 1).

Peptide bond formation in aq. DMF was also examined using other amino acid esters (Table 2), including side-chain functionalized (entries 3 and 6), cyclic (entry 4) and sterically hindered (entry 7) esters. In most cases, just 1.1 equivalent of thioester was sufficient to give products in high yield.[††] No racemization was detectable by HPLC analysis of Fmoc-L-Val-L-Val-O*t*Bu and Fmoc-D-Val-L-Val-O*t*Bu, both of which were formed in a mutually exclusive manner (entries 1 and 2). Mechanistic considerations, as well as the observed compatibility with aqueous reaction environments, strongly suggested that chemoselective amide formation might well be possible using amino acids with unprotected hydroxy group. In fact, the reaction of serine methyl ester (entry 8) gave desired dipeptide in 82% yield and tyrosine *t*-butyl ester (entry 9) gave Fmoc-Val-Tyr(OH)-O*t*Bu in nearly quantitative yield.

The suitability of this method for glycopeptide synthesis was verified by reaction using a glycosylated asparagine derivative with unprotected sugar moiety (Scheme 4). Fmoc-Asn(*N*-acetylglucosamine)-O*t*Bu (**6**)[11] was subjected to Fmoc deprotection, and the resultant amine was reacted with thioester **3** (1.1 equiv.) in aq. DMF. The desired acylated product (**4i**) was obtained in high yield.[‡‡]

[‖] The NMR experiment was performed by using thioester **3** and glycine *t*-butyl ester HCl salt (1:1) in the presence of an equimolar amount of DIPEA.

[**] Half-life of **3** and regioisomeric 2-nitro-4-formylphenylthio ester in DMF-d_6–pH 9.0 phosphate buffer (10:1) at ~25°C were ca. 7 h and over 2 days, respectively.

[††] The reaction of Fmoc-Pro-SAr with Val-O*t*Bu also gave corresponding peptide in 98% yield.

[‡‡] Details of the synthesis of glycopeptide will be reported in a separate paper.

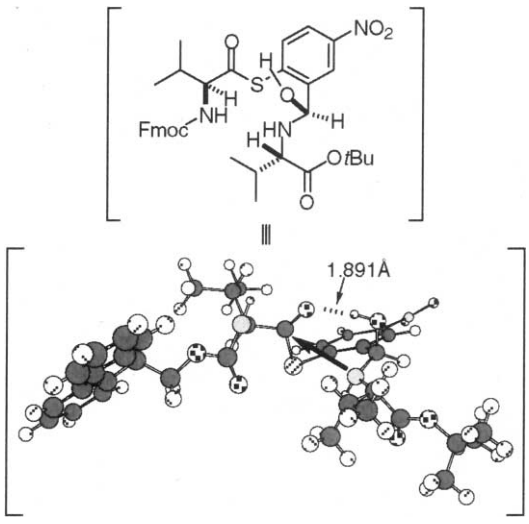
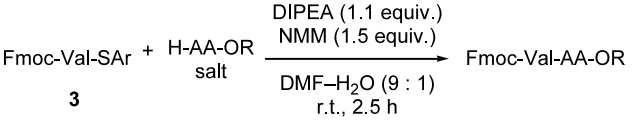


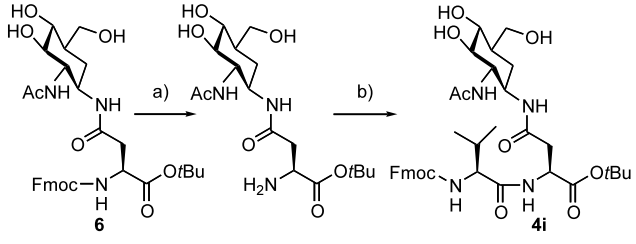
Figure 1. The energy-minimized structure of the hemi-aminal intermediate as calculated by MOPAC-PM_3. The dotted line shows the intramolecular hydrogen bond between the hydroxyl group of the hemi-aminal and the carbonyl oxygen of the thioester (1.891 Å). The bold arrow indicates the direction of nucleophilic attack of a nitrogen atom of the hemi-aminal toward the carbonyl carbon.

Table 2. Synthesis of various peptides



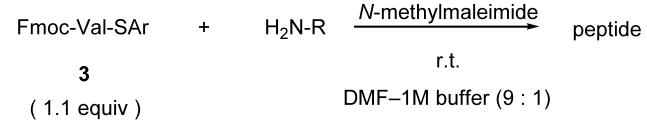
Entry	Fmoc-Val-AA-OR	Yield (%)
1	Fmoc-Val-Val-O*t*Bu (**4a**)	100
2	Fmoc-D-Val-Val-O*t*Bu (**4a′**)	91
3	Fmoc-Val-Lys(Z)-O*t*Bu (**4b**)	97
4	Fmoc-Val-Pro-O*t*Bu (**4c**)	100
5	Fmoc-Val-Gly-O*t*Bu (**4d**)	91
6	Fmoc-Val-Thr(O*t*Bu)-O*t*Bu (**4e**)	95
7	Fmoc-Val-Aib-OBn (**4f**)	72[a]
8	Fmoc-Val-Ser(OH)-OMe (**4g**)	82
9	Fmoc-Val-Tyr(OH)-O*t*Bu (**4h**)	98

[a] 2.2 equiv. of thioester.



Scheme 4. Synthesis of glycopeptide **4i**. *Reagents and conditions*: (a) 20% piperidine–DMF; (b) Fmoc-Val-SAr (**3**) (1.1 equiv.), NMM (1.5 equiv.), DMF–H_2O (9:1), 98% in two steps.

Table 3. Synthesis of peptides under buffered media



Entry	Time (h)	Buffer or base	Product	Yield (%)[a]
1	2.5	Phosphate (pH 8.0)	Fmoc-Val-Val-O*t*Bu (**4a**)	93 (100)
2	2.5	Phosphate (pH 6.8)	Fmoc-Val-Val-O*t*Bu (**4a**)	71
3	2.5	$NaHCO_3$	Fmoc-Val-Val-O*t*Bu (**4a**)	97
4	2.5	KOAc	Fmoc-Val-Val-O*t*Bu (**4a**)	56
5	2.5	Phosphate (pH 8.0)	Fmoc-Val-Gly-O*t*Bu (**4d**)	100 (93)
6	8	Phosphate (pH 8.0)	Fmoc-Val-Aib-O*t*Bu (**4j**)	75 (61[b])
7	8	Phosphate (pH 8.0)	Fmoc-Val-Asp-O*t*Bu (**4k**)	92 (57)
8	2.5	Phosphate (pH 8.0)	Fmoc-Val-Gly-Gly-OH (**4l**)	77 (33)
9	2.5	$NaHCO_3$	Fmoc-Val-Gly-Gly-OH (**4l**)	53
10	6	Phosphate (pH 8.0)	Fmoc-Val-Trp-O*t*Bu (**4m**)	89 (77)
11	6	Phosphate (pH 8.0)	Fmoc-Val-Arg-OEt (**4n**)	81 (42)

[a] Yields in parenthesis obtained using DIPEA (1.1 equiv.).
[b] 2.2 equiv. of thioester.

Being aware of the limited stability of the thioester linkage under basic conditions, buffered media were examined in place of DIPEA in aq. DMF. As shown in Table 3, phosphate buffer (pH 8.0) was highly effective[§§] and proved to be particularly suitable for substrates having ionizable functionalities (entries 7, 8, and 11) and Trp residue (entry 10). Also the reaction with highly hindered amino acid (**4j**) was quite successful (entry 6). A system containing 1 M $NaHCO_3$ also gave excellent results for Val-O*t*Bu (entry 3), while more basic conditions (entry 4) and slightly acidic buffer (entry 2) were less satisfactory.

In conclusion, a novel peptide formation reaction using Fmoc-amino acid 2-formyl-5-nitrophenylthio ester has been developed. The reaction was demonstrated to be highly chemoselective[12] and compatible with unprotected carbohydrate as well as a negatively or positively charged amino acid side chain.

Acknowledgements

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[§§] Half-life of **3** in DMF–pH 8.0 phosphate buffer (10:1) was ca. 20 h at ~25°C.