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Effect of Copper Salts on Peptide Bond Formation Using Peptide Thioesters

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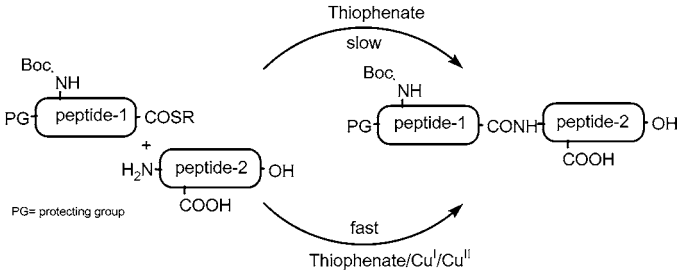
ABSTRACT



In the present paper, systematic studies revealed that Cu(I) salts in general and Cu(II) salts under certain circumstances promote effective reaction between peptide thiol esters and the N-terminal amino function of a second peptide segment to give the native amide bond for both solution- and solid-phase syntheses. Chiral integrity was retained. Reaction conditions were optimized and applied to the synthesis of a small protein, the identity of which was confirmed by NMR analysis.

Thiol esters of carboxylic acids were introduced as useful acylating reagents for amines many years ago.[1] However, their application as protected amino acid thioesters for the stepwise synthesis of peptides is only of limited use due to their relatively low reactivity. In contrast, the use of peptide thioesters as key intermediates for ligation and segment condensation processes has become increasingly important. Besides the well-established process of native chemical ligation[2] (NCL), a convergent peptide synthesis method[3a,b] (CPS) as introduced by Blake[3a] and later improved by Aimoto[4] applies partially protected peptide thioesters as building blocks to give native amide bonds, thus allowing the assembly of long peptides and proteins not previously readily accessible by conventional peptide synthesis methods.[5] Whereas the NCL method depends on the presence of Cys residues at certain junction points, the latter method takes advantage of partially protected peptide thioesters which can be reacted directly with any primary amino group of a second partially protected peptide segment. Since its invention, this method has been continuously improved by the introduction of various additives to increase the reactivity of the peptide thioesters.

In particular, the combined use of silver ion in the presence of HOOBt was demonstrated to be effective due to the in situ transformation of the thioester into the more reactive OOBt-ester.[6]

The present paper describes an extension of this method to the use of copper salts as additives to accelerate the reaction between peptide thiol esters and a second peptide segment to give the native amide bond for both solution- and solid-phase syntheses.

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The use of copper was primarily initiated by the goal to use $Cu(OBt)_2$ according to the "transesterification protocol" described by Aimoto[6] transferring the less reactive thioester into a more reactive active ester and to take advantage of the ability of $Cu(OBt)_2$ to maintain the chiral integrity of the activated C-terminal amino acid when used for active ester based segment condensation either in solution[7] or on solid support.[8]

Initially, a simple model peptide thioester (T1: Msc-*Leu-Tyr-Arg-Ala-Gly*-SR) was synthesized according to previous Fmoc-based protocols,[9] purified, and subsequently coupled to another resin bound peptide (N1: *H-Phe-Tyr*(*OtBu*)-*Gly-Lys*(*Boc*)-*Ala-resin*) in the presence of $Cu(OBt)_2$/TMP (Table 1, entry g). In parallel, control experiments were performed

Table 1. Comparison of Acylation Yields for Different Additives on Solid Support[a]

entry	additive	yield (%)
a		0
b	TMP	0
c	thiophenate	25
d	thiophenate/TMP	25
e	$CuCl_2$/TMP	0
f	$CuCl_2$/Thiophenate/TMP	25
g	$Cu(OBt)_2$/TMP	90
h	$CuCl_2$/HOBt/TMP	10
i	CuCl/TMP	45
j	thiophenate/CuCl/TMP	85
k	CuCl/HOBt/TMP	80

[a] Key: thioester peptide (T1), Msc-Leu-Tyr-Arg-Ala-Gly-S$(CH_2)_2$COOEt; 5 equiv of T1 in DMF, 0.2 M final concentration, 5 equiv of base, 5 equiv of additives, 40 °C, 16 h.

in which the peptide thioester was reacted with the resin bound C-terminal peptide without any additives or only in the presence of a base (Table 1, entries a and b). In addition the reaction was conducted with sodium thiophenate added to the aliphatic thioester to increase reactivity via conversion to the more reactive aromatic thioester. While the reactions carried out without any additive or only with collidine (TMP) as base gave no target material after 16 h, the addition of sodium thiophenate yielded 25% of the target peptide (Table 1, entry c), which is obviously due to direct reaction of the more reactive thioester with the resin bound peptide.

Interestingly, it was found that addition of $Cu(OBt)_2$/TMP significantly accelerated the speed of reaction resulting in a reaction yield of >80% after 16 h (Table 1, entry g).

To elucidate whether the effect is related to a possible transesterifcation process (thioester → active ester), the condensation reaction was repeated using $CuCl_2$/TMP and mixtures of $CuCl_2$/TMP with thiophenate or HOBt. It was found that addition of either $CuCl_2$ or mixtures involving thiophenate or HOBt gave no significant acceleration compared to control experiments (Table 1, entries e and h).

Additional experiments were performed to examine the influence of the oxidation state of copper. Thus, in contrast to the use of $CuCl_2$/TMP which gave none of the desired product it was found that the addition of CuCl gave product in the range of 35−45%.

These yields could be noticeably increased (>88% yield) by prior addition of thiophenate to preactivate the alkyl thioester (Table 1, entry j). On the other hand, use of the commercially available Cu^I−thiophenolate salt as additive gave significantly lower amounts of product, thus suggesting that formation of a more reactive thioester prior to addition of the Cu^I additive is advantageous with regard to the speed of reaction. Furthermore, the combined use of CuCl and HOBt (Table 1, entry k) showed a synergistic effect with regard to yields of the target peptide when compared to the use of CuCl/TMP (Table 1, entry I, 45%) or TMP/HOBt (yield: 0%) alone.

Since the first system examined involved a relatively unhindered coupling (Gly/Phe; T1/N1), the most effective additive combinations used [CuCl/thiophenate/TMP and $Cu(OBt)_2$/TMP] and were also examined in the case of more hindered couplings, e.g., Ala/Phe (T2/N1) and Leu/Phe (T3/N1). It was found that in these cases high yields of coupling products could also be obtained (Table 2). In the case of the most hindered system (Leu/Phe), the time course for the reaction (Figure 1) showed that reaction was slower at first (ca. 10% after 4 h) but eventually reached a high level.

Table 2. Comparison of Acylation Yields and Configurational Loss for Different Ligation Points Using Different Methods on Solid Support[a]

	T2 (T3)−N1			
entry	thioester	additive	yield (%)	DL-isomer (%)
a	T2	$Cu(OBt)_2$/TMP	80	nd
b	T3	$Cu(OBt)_2$/TMP	80	2.6
c	T2	Thiophe/CuCl/TMP	85	nd
d	T3	Thiophe/CuCl/TMP	80	2

[a] Key: thioester peptides (T2), Msc-Leu-Tyr-Arg-Ala-Ala-S$(CH_2)_2$COOEt; (T3), Msc-Leu-Tyr-Arg-Ala-LeuS$(CH_2)_2$COOEt; resin-bound peptide N1, H-Phe-Tyr(tBu)-Gly-Lys(Boc)-Ala-resin; reaction time 16 h; nd, not determined.

To extend these results to reactions carried out in solution, peptide thioesters T1 and T3 were used for reaction with the amino function of C-terminal peptide N2 in DMF. The reaction conditions varied compared to the solid-phase approach with regard to the reaction concentration (T1:10 mM; N2 3.3 mM) used.

It was found that after 24 h reaction yields were high in all cases (Table 3). However, for the Leu-Phe ligation site, when CuCl/thiophenate was used, the yield was only 50%, most likely because of steric hindrance at the coupling site.

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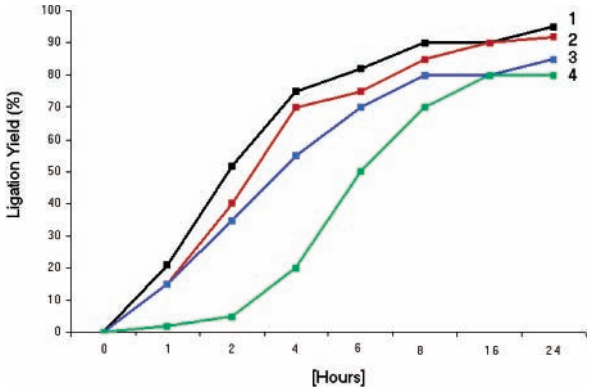

Figure 1. Time course for reaction of peptide thioesters with resin-bound peptide N1. Curve 1: T1 in the presence of Cu(OBt)2/TMP. Curve 2: T1 in the presence of CuCl/thiophenate. Curve 3: T3 in the presence of Cu(OBt)2/TMP. Curve 4: T3 in the presence of CuCl/thiophenate/TMP.

Time course experiments showed results similar to those observed for the solid-phase reactions.

It was also found that the use of copper salts accelerated the hydrolysis of thioesters (1−5%) under the reaction conditions used. This effect was more pronounced in cases of more hindered junctions such as Leu-Phe or Glu-Ala (10−30%) where the ligation times were prolonged.

Since it is well-known that epimerization at the activated C-terminal amino acid of the N-terminal segment is one of the main drawbacks for segment condensation processes the question of chiral integrity also needed to be addressed for the new procedure.

To determine levels of epimerization during the condensation reaction, the thioester peptide T3 was synthesized having either L-Leu or D-Leu at the C-terminal end so as to allow preparation of the corresponding stereoisomeric standards for HPLC comparison of the full length peptides after reaction with N1 (Table 2, solid phase) and with N2 (Table 3, solution). Relatively low values of configurational loss were detected. Since the addition of copper salts during coupling processes has been reported to suppress epimerization significantly,[8c,10] the extent of loss of configuration found in our experiments might at least in part be related to this effect.

Table 3. Comparison of Acylation Yields and Configurational Loss for Gly-Phe and Leu-Phe Ligation Points for Different Methods in Solution[a]

	T1 (T3)−N2			
entry	thioester	additive	yield (%)	DL-isomer (%)
a	T1	thiophene/CuCl/TMP	75	
b	T3	thiophene/CuCl/TMP	50	4
c	T1	$Cu(OBt)_2$/TMP	75	
d	T3	$Cu(OBt)_2$/TMP	75	7

[a] Key: (T1), Msc-Leu-Tyr-Arg-Ala-Gly-S$(CH_2)_2$COOEt; (T3), Msc-Leu-Tyr-Arg-Ala-Leu-S$(CH_2)_2$COOEt; (N2), H-Phe-Tyr-Gly-Ser-Ala-NH_2; reaction time 24 h.

Finally, to determine whether the thioester/copper approach would also be useful for longer peptide segments or even small proteins the Z38-sequence[11] of the B-domain of protein A was synthesized. Segments 1−16 and 1−17 were chosen as thioester components to be reacted with segments (17−38) and (18−38) as C-terminal units, respectively. The peptide thioester segments[9] and the C-terminal segment were prepared and purified according to known Fmoc-based procedures and reprotected using Boc-OSu in solution as reported.[4,6b]

Again the two methods [(thiophenate/CuCl/collidine and $Cu(OBt)_2$] shown to be most effective during the model studies were applied in solution for the ligation. In these cases yields were lower but still adequate[12] (Table 4, entries 1−4).

Table 4. Z38 Domain Preparation by Copper-Mediated Ligation in Solution[a]

	Z38				
	N-terminal segment	C-terminal segment	ligation point	additive	yield (%)
1	[1−17]	[18−39]	Ala/Leu	$Cu(OBt)_2$	40
2	[1−17]	[18−39]	Ala/Leu	CuCl Thioph/TMP	40
3	[1−16]	[17−39]	Glu/Ala	$Cu(OBt)_2$	65
4	[1−16]	[17−39]	Glu/Ala	CuCl Thioph/TMP	45

[a] Key: 10 mM final concentration in DMF; 60 h reaction time; Z38 sequence: AVAQSFNMQQQRRFYEALHDPNLNEEQRNAKIKSIRDD.

The product obtained from reaction with $Cu(OBt)_2$ was purified via GPC and preparative HPLC and analyzed using MS and NMR. In addition to the expected MS (MS calcd 4563.1 $[M + H]^+$, found 1141.4 $[M + 4H]^{4+}$) it was found that the Z38 mini-domain of protein A synthesized via ligation using $Cu(OBt)_2$ showed the same NMR structure as a sample synthesized via stepwise approaches.[11,12]

In conclusion, it has been shown that amide bond formation between a peptide thioester and a primary amine in the form of a partially protected peptide can be accelerated by addition of either CuCl or $Cu(OBt)_2$. A systematic study showed that the method was useful for different peptide thioesters independent of their C-terminal nature. Also, it was shown that the rate-enhancing effect of these additives was evident for both solid- and solution-phase reactions. In addition to the increased reaction rate, relatively low levels

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of epimerization were observed. The methodology could be successfully extended to the reaction of longer segments as demonstrated by application to a 38-mer protein domain which was assembled in a reasonable yield.[12]

Whereas for the copper(I) salt the cuprous ion itself seems to be the key component for the accelerating effect for the copper(II) salt [$Cu(OBt)_2$] transesterification (thioester → active ester) must be considered as a key step since the rate-enhancing effect was much less pronounced in the case of $CuCl_2$ vs CuCl. Although various copper species in general were recently shown to have rate-accelerating effects for a variety of reactions[7,8,13] such as those of amines with aryl halides[14] their influence on amide bond formation by means of peptide thioesters has not yet been described. While it seems to be evident that cuprous ion is involved in the transition complexes which facilitate the final amide bond formation, mechanistic details on the reaction must await further studies.

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Supporting Information Available: Complete protocols for the synthesis, purification, and characterization of all peptides used in this work, including ESI, HPLC, and NMR characterization of the Z domain. This material is available free of charge via the Internet at http://pubs.acs.org.

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