Journal of Peptide Science
J. Peptide Sci. **7**: 115–120 (2001)

$Cu(OBt)_2$ and $Cu(OAt)_2$, Copper(II)-based Racemization Suppressors Ready for Use in Fully Automated Solid-phase Peptide Synthesis

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Received 11 August 2000
Accepted 3 September 2000

Abstract: The complexes $Cu(OBt)_2$ and $Cu(OAt)_2$, which are derived from copper(II) and HOBt and HOAt, respectively, are shown to be more effective in suppressing racemization during solid-phase peptide synthesis (SPPS) than are those compounds currently being used for this purpose. These compounds can readily be used in conjunction with the commonly applied coupling reagents in fully automated systems for solid-phase peptide chemistry. Copyright © 2001 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: solid phase peptide synthesis; racemization; chirality; Cu(II)-based complexes; epimerization

INTRODUCTION

Stereochemical control is a prerequisite for the successful assembly of peptides by both solution and solid-phase methods. The loss of configuration of an amino acid residue is one of the most important side-reactions that occurs in peptide synthesis [1] and gives rise to considerable problems with regard to purification and evaluation of the desired compounds. The use of potent activation reagents to perform the coupling steps often increases the occurrence of these racemic species. These activation reagents convert the carboxylic acid group of the amino acid residue into an ester function that bears a good leaving group. This transformation tends to increase the acidity of the α-proton and favours the formation of 5(4*H*)-oxazolone, both of which may lead to racemization [2,3]. This phenomenon is more likely to occur when residues like His, Ser and Cys are introduced into the peptide sequence [4–8].

The present paper introduces the use of Cu(II)-based complexes as potent suppressors of racemization in solid-phase synthesis. These compounds, when applied in the presence of the commonly used coupling reagents in peptide synthesis, avoid the use of tertiary bases (like DIEA and NMM) that are often required for coupling. The utility of these new compounds will be demonstrated and evaluated by examining a series of different, known models that are frequently applied in the literature and are known to be particularly prone to racemization. The specific Cu complexes investigated were $Cu(OBt)_2$ and $Cu(OAt)_2$.

Cu(II)-based complexes have recently been developed by Blodgett *et al.* [9] ($Cu(OBt)_2$, $Cu(OAt)_2$, $Cu(OOBt)_2$, $Cu(OSu)_2$ and $Cu(OpNp)_2$ were used in this work) and have been proven to reduce racemization in solution peptide segment coupling

Abbreviations: Abbreviations used for amino acids and the designations of peptides follow the rules of the IUPAC–IUB Commission of Biochemical Nomenclature in *J. Biol. Chem.* 1972; **247**: 977–983; AAA, amino acid analysis; BOP, benzotriazol-1-yl-*N*-oxy-tris-(dimethylamino)phosphonium hexafluorophosphate; Et3SiH, triethylsilane; Fmoc-AM linker, *p*-[(*R*,*S*)-α-[1-(9*H*-Fluoren-9-yl)methoxyformamido]-2,4-dimethoxybenzyl]phenoxyacetic acid; HOOBt, 1-oxo-2-hydroxydihydrobenzotriazine; HO*p*Np, *p*-nitrophenol; HOSu, *N*-hydroxysuccinimide; MBHA, *p*-methylbenzhydrylamine.

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involving DIPCDI. Furthermore, $Cu(OBt)_2$ has proven to be very efficient in the suppression of racemization in solid-phase peptide assembly in the reverse $N \rightarrow C$ direction using a Cl-Trityl resin and an allyl ester as temporary protecting groups [10] Synthesis of these compounds is straightforward and can be performed easily (J.K. Blodgett, pers. comm.).

EXPERIMENTAL PART

MBHA resin (0.65 mmol/g), linker Fmoc-AM, and Fmoc-protected amino acids were obtained from Calbiochem-Novabiochem (Läufelfingen, Switzerland); Fmoc-Phe-Ser(Bu^t)-OH was purchased from Bachem Feinchemikalien AG (Bubendorf, Switzerland); DIPCDI and BOP were obtained from Albatross Chem Inc. (Montreal, Quebec, Canada); $Cu(OBt)_2$, $Cu(OAt)_2$, HBTU and TBTU were obtained from Luxembourg Industries Ltd. (Tel Aviv, Israel). Solvents for peptide synthesis (DMF and CH_2Cl_2) were obtained from SDS (Peypin, France). Nitrogen was bubbled through DMF prior to use in order to remove volatile contaminants and was then stored over activated 4-Å molecular sieves. HPLC supplies (CH_3CN and Nucleosil C_{18} reversed-phase columns, 4×250 mm, 10 μm) were obtained from Scharlau (Barcelona, Spain). Peptide resin samples were hydrolysed with 12 N aqueous HCl/propionic acid (1:1) at 155°C for 1–3 h. Subsequent amino acid analyses were performed on a Beckman System 6300 autoanalyzer. Peptide synthesis transformations and washes were performed at 25°C unless indicated otherwise.

Solid-phase Peptide Synthesis (SPPS)

Syntheses were performed manually in polypropylene syringes (5 ml) fitted with a polyethylene porous disc on a 50-μmol scale (100 mg of amino acyl resin). The solvent for all reactions and washings was DMF unless indicated otherwise. Removal of Fmoc was achieved with piperidine/DMF (1:4, v:v) (3×1 min + 2×5 min). Couplings without preactivation: Fmoc-protected amino acids or dipeptides (1.5, 3 or 4.5 equiv.) and the coupling reagent (1.5, 3 or 4.5 equiv.) were dissolved in the appropriate solvent (0.5 ml). After the addition of base (3, 6 or 9 equiv.), the mixture was added to the peptide resin and the coupling was left for the time indicated in each case. Following incorporation of the His, Cys and Ser residues, an acetylation step was performed in order to allow the determination of the yields by amino acid analysis (AAA).

Cleavage of peptides from the resin was performed with TFA:H_2O:Et_3SiH (92:3:5) for 2 h. Peptides were precipitated by the addition of cold Bu^t methyl ether. The solution was decanted off and the solid was triturated with cold Bu^t methyl ether, which was again decanted off. This process was repeated twice.

Analytical HPLC

The separation of crude diastereoisomers was performed on a Shimadzu instrument comprising two solvent delivery pumps (model LC-6A), an automatic injector (model SIL-6B), a variable wavelength detector (model SPD-6A), a system controller (model SCL-6B) and a plotter (model C-R6A).

H-L-Phe-L/D-Ser-L-Pro-NH$_2$. Linear gradient over 20 min of 0.036% TFA in CH_3CN and 0.045% aqueous TFA from 1:19 to 1:4, flow rate 1.0 ml/min, with UV detection at 220 nm. *Rt*: 13.4 and 15.0 min for H-L-Phe-L-Ser-L-Pro-NH_2 and H-L-Phe-L-Ser-L-Pro-NH_2, respectively.

H-Gly-L/D-His-L-Phe-NH$_2$. Linear gradient over 30 min of 0.036% TFA in CH_3CN and 0.045% aqueous TFA from 0:1 to 3:17, flow rate 1.0 ml/min, with UV detection at 220 nm.

H-Gly-L/D-Cys-L-Phe-NH$_2$. Linear gradient over 30 min of 0.036% TFA in CH_3CN and 0.045% aqueous TFA from 1:19 to 1:1, flow rate 1.0 ml/min, with UV detection at 220 nm.

H-Gly-L/D-Ser-L-Phe-NH$_2$. Linear gradient over 30 min of 0.036% TFA in CH_3CN and 0.045% aqueous TFA from 1:19 to 3:7, flow rate 1.0 ml/min, with UV detection at 220 nm.

RESULTS AND DISCUSSION

The first model studied involved the 2-h coupling of Fmoc-Phe-Ser(Bu^t)-OH onto H-Pro-AM-Ile-MBHA resin [11] under various conditions. First, a comparative study was performed using different common coupling reagents that require the use of a base. These reagents were then employed in conjunction with the racemization suppressors HOBt and HOAt. The results obtained without the Cu complexes are shown in Table 1 and indicate that a significant amount of the epimeric tripeptide was detected in all cases. The use of a larger excess of protected dipeptide resulted in higher coupling

Table 1 Coupling of Fmoc-Phe-Ser(Bu^t)-OH onto H-Pro-AM-Ile-MBHA Resin: Effect of Coupling Reagents and Racemization Suppressors (HOBt and HOAt)

Entry	Coupling method[a]	Equiv.[b]	LDL-isomer[c] (%)	Coupling yield[d] (%)
1	HBTU–DIEA	1.5	53	79
2	HATU–DIEA	1.5	43	76
3	BOP–DIEA	1.5	50	76
4	TBTU–DIEA	3	50	100
5	TBTU–DIEA	4.5	30	95
6	TBTU–HOBt–DIEA	1.5	47	57
7	TBTU–HOAt–DIEA	1.5	46	57
8	BOP–HOBt–DIEA	1.5	54	87
9	BOP–HOAt–DIEA	1.5	49	64

[a] Couplings were performed without preactivation for 2 h (see 'Experimental Part' section); [b] Equiv. excess each of Fmoc-dipeptide, coupling reagent, base and racemization suppressors (where applicable) with respect to the H-Pro-AM-Ile-MBHA resin; [c] percentage with respect to LLL-isomer calculated by integration of peak areas in RP-HPLC (see 'Experimental Part' section); [d] calculated by AAA of acid hydrolysed peptide resins (see 'Experimental Part' section).

yields and a similar degree of racemization was observed (entries 4 and 5). The addition of HOBt and HOAt did not result in any improvement in the extent of racemization and, in fact, resulted in lower yields of the crude products (entries 6–9).

The data shown in Table 2 clearly show the effectiveness of introducing the new Cu(II)-based reagents for the suppression of racemization. In Table 2, the results are displayed relative to TBTU/HBTU (entries 1, 2) and BOP (entry 13). It can clearly be seen that a decrease in racemization is achieved in all cases. However, one drawback is the lower yields obtained following introduction of the Cu(II)-based reagents. The use of a larger excess of racemization suppressor did not result in any significant change in the level of racemization

Table 2 Coupling of Fmoc-Phe-Ser(Bu^t)-OH onto H-Pro-AM-Ile-MBHA Resin: Effect of Racemization Suppressors [$Cu(OBt)_2$ and $Cu(OAt)_2$] on TBTU- and BOP-mediated Couplings

Entry	Coupling method[a]	Equiv.[b]	LDL-isomer[c] (%)	Coupling yield[d] (%)
1	TBTU–DIEA	4.5	30	95
2	HBTU–DIEA	1.5	43	79
3	TBTU–$Cu(OBt)_2$	4.5:9	14	38
4	TBTU–$Cu(OBt)_2$	1.5:3	12	45
5	TBTU–$Cu(OBt)_2$	1.5:1.5	16	34
6	TBTU–$Cu(OBt)_2$	1.5:0.75	15	41
7	TBTU–$Cu(OBt)_2$	1.5:0.5	17	38
8	TBTU–$Cu(OAt)_2$	4.5:9	11	55
9	TBTU–$Cu(OAt)_2$	1.5:3	16	53
10	TBTU–$Cu(OAt)_2$	1.5:1.5	9	54
11	TBTU–$Cu(OAt)_2$	1.5:0.75	14	48
12	TBTU–$Cu(OAt)_2$	1.5:0.5	16	51
13	BOP–DIEA	1.5	50	76
14	BOP–$Cu(OBt)_2$	1.5:3	16	42
15	BOP–$Cu(OBt)_2$	1.5:1.5	14	40
16	BOP–$Cu(OAt)_2$	1.5:3	13	51
17	BOP–$Cu(OAt)_2$	1.5:1.5	16	46

[a–d] See Table 1.

observed, nor did it lead to any improvement in the yields. The results obtained with $Cu(OAt)_2$ were slightly better than those with $Cu(OBt)_2$.

The next stage in the study involved the evaluation of these Cu(II)-based reagents in conjunction with DIPCDI, a reagent that does not require the use of an additional base (Table 3). In these cases, there was no detectable evidence for a decrease in racemization and, as in the examples discussed above, the yields were reduced significantly. Once again, $Cu(OAt)_2$ performed better than $Cu(OBt)_2$.

Due to its general use in segment coupling processes, DMF was used as the solvent in all cases reported here. The replacement of DMF with a less polar solvent (such as CH_2Cl_2, CH_3CN or THF) led to similar racemization levels and yields (Table 4).

Finally, the effect of adding a base was studied. From the results obtained it can be concluded that the absence of a base is clearly a prerequisite when applying the Cu(II)-based reagents as racemization suppressors (Table 5).

A second model studied involved the stepwise solid-phase synthesis of the tripeptides H-Gly-X-Phe-NH_2 (where X = His, Cys and Ser) [12], in which the risk of racemization is greater when residues such as His, Cys and Ser are incorporated into a peptide sequence.

In the first study, the amino acid residues were incorporated without preactivation (minimizing racemization) and the coupling time was set at 45 min. The results are given in Table 6 and clearly demonstrate the advantage of applying the Cu(II)-based reagents as racemization suppressors. However, it must be pointed out that in the case of His the Cu(II) can be complexed by the imidazole ring of the His residue, a situation that favours

Table 3 Coupling of Fmoc-Phe-Ser(Bu^t)-OH onto H-Pro-AM-Ile-MBHA Resin: Effect of Racemization Suppressors [$Cu(OBt)_2$ and $Cu(OAt)_2$] on DIPCDI Couplings

Entry	Coupling method[a]	Equiv.[b]	LDL-isomer[c] (%)	Coupling yield[d] (%)
1	DIPCDI–HOBt	1.5	30	93
2	DIPCDI–HOAt	1.5	24	87
3	DIPCDI–$Cu(OBt)_2$	1.5	40	36
4	DIPCDI–$Cu(OAt)_2$	1.5	23	67

[a–d] See Table 1.

Table 4 Coupling of Fmoc-Phe-Ser(Bu^t)-OH onto H-Pro-AM-Ile-MBHA resin: Solvent Effects on Racemization and Yields

Entry	Coupling method[a]	Equiv.[b]	Solvent	LDL-isomer[c] (%)	Coupling yield[d] (%)
1	TBTU–$Cu(OBt)_2$	1.5:3	DMF	12	45
2	TBTU–$Cu(OBt)_2$	1.5:3	CH_3CN	19	43
3	TBTU–$Cu(OBt)_2$	1.5:3	CH_2Cl_2	19	34
4	TBTU–$Cu(OBt)_2$	1.5:3	THF	18	50

[a–d] See Table 1.

Table 5 Coupling of Fmoc-Phe-Ser(Bu^t)-OH onto H-Pro-AM-Ile-MBHA Resin: Effect of DIEA on Racemization and Yields

Entry	Coupling method[a]	Equiv.[b]	LDL-isomer[c] (%)	Coupling yield[d] (%)
1	TBTU–$Cu(OBt)_2$	1.5:3	12	45
2	TBTU–$Cu(OBt)_2$–DIEA	1.5:1.5:3	52	45

[a–d] See Table 1.

Table 6 Racemization Studies on the Synthesis of H-Gly-X-Phe-NH2 (where X = His, Cys or Ser) by Coupling of Fmoc-X-OH onto H-Phe-AM-Ile-MBHA Resin, and Subsequent Coupling of Fmoc-Gly-OH

Entry	Coupling method[a]	Equiv.[b]	DL-isomer[c] (%)	Coupling yield[d] (%)
1	Fmoc-His(Trt)-OH–TBTU–NMM	5:5:10	3	100
2	Fmoc-His(Trt)-OH–TBTU–$Cu(OBt)_2$	5:5:10	3	87
3	Fmoc-His(Trt)-OH–TBTU–$Cu(OAt)_2$	5:5:10	3	97
4	Fmoc-Cys(Trt)-OH–TBTU–NMM	5:5:10	6	100
5	Fmoc-Cys(Trt)-OH–TBTU–$Cu(OBt)_2$	5:5:10	1	75
6	Fmoc-Cys(Trt)-OH–TBTU–$Cu(OAt)_2$	5:5:10	1	71
7	Fmoc-Ser(Bu^t)-OH–TBTU–NMM	5:5:10	1	100
8	Fmoc-Ser(Bu^t)-OH–TBTU–$Cu(OBt)_2$	5:5:10	$\ll 1$	70
9	Fmoc-Ser(Bu^t)-OH–TBTU–$Cu(OAt)_2$	5:5:10	$\ll 1$	74

[a] Couplings were performed without preactivation for 45 min (see 'Experimental Part' section); [b] Equiv. excess of Fmoc-X-OH, coupling reagent, racemization suppressors (where applicable) and base (where applicable) with respect to the H-Phe-AM-Ile-MBHA resin; [c] percentage with respect to LL-isomer calculated by integration of peak areas in RP-HPLC (see 'Experimental Part' section); [d] calculated by AAA of acid hydrolysed peptide resins (see 'Experimental Part' section).

racemization, as described previously in solution synthesis (J.K. Blodgett, pers. comm.). The yields were slightly lower than those obtained using conventional conditions.

The next step of the investigation focused on the influence of performing a preactivation step, a very important stage in the operation of most automated synthesizers. In these experiments, the amino acid residues in the presence of TBTU and $Cu(OBt)_2$ were dissolved in DMF. After 5 min, the mixture was added to the peptide resin. The results in Table 7 show that the level of racemization decreases from almost complete racemization to values below 1%. The coupling yields are, however, slightly lower when Cu(II)-based racemization suppressors are employed.

Table 8 shows the results obtained when double couplings with preactivation were performed. These conditions are the same as those applied in fully automated peptide synthesis. From these results we can conclude that the level of racemization does not increase from the levels observed earlier and that coupling yields increase to become almost quantitative.

CONCLUSIONS

It has been clearly demonstrated that the new Cu(II)-based racemization suppressors $Cu(OBt)_2$ and $Cu(OAt)_2$ are more effective in reducing racemization than the compounds currently used being for this purpose. However, as far as the coupling efficiency is concerned, the new Cu complexes seem to result in lower yields than the commonly used reagents. This disadvantage can be easily overcome by performing double coupling protocols. These reagents show great promise for application in fully automated peptide synthesis together with phosphonium and aminium coupling reagents, such as

Table 7 Racemization Studies on the Synthesis of H-Gly-Cys-Phe-NH_2 by Coupling of Fmoc-Cys-OH onto H-Phe-AM-Ile-MBHA Resin, and Subsequent Coupling of Fmoc-Gly-OH: Effect of Preactivation (5 min)

Entry	Coupling method[a]	Equiv.[b]	DL-isomer[c] (%)	Coupling yield[d] (%)
1	Fmoc-Cys(Trt)-OH–TBTU–NMM	5:5:10	42	100
2	Fmoc-Cys(Trt)-OH–TBTU–$Cu(OBt)_2$	5:5:10	<1	87
3	Fmoc-Cys(Trt)-OH–TBTU–$Cu(OAt)_2$	5:5:10	<1	81

[a–d] See Table 6.

Table 8 Racemization Studies on the Synthesis of H-Gly-X-Phe-NH_2 (where X = His, Cys or Ser) by Coupling of Fmoc-X-OH onto H-Phe-AM-Ile-MBHA Resin, and Subsequent Coupling of Fmoc-Gly-OH: Effect of Double Couplings with Preactivation (5 min)

Entry	Coupling method[a]	Equiv.[b]	DL-isomer[c] (%)	Coupling yield[d] (%)
1	Fmoc-Cys(Trt)-OH–TBTU–$Cu(OBt)_2$	5:5:10	1	100
2	Fmoc-His(Trt)-OH–TBTU–$Cu(OBt)_2$	5:5:10	ND[e]	91
3	Fmoc-Ser(Bu^t)-OH–TBTU–$Cu(OBt)_2$	5:5:10	1	95

[a] The mixture of Fmoc–amino acid, coupling reagent, and $Cu(OBt)_2$ was added to the resin after 5 min; [b] Equiv. excess of Fmoc-X-OH, coupling reagent and racemization suppressors with respect to the H-Phe-AM-Ile-MBHA resin; [c] percentage with respect to LL-isomer calculated by integration of peak areas in RP-HPLC (see 'Experimental Part' section); [d] calculated by AAA of acid hydrolysed peptide resins (see 'Experimental Part' section); [e] ND = not determined.

HBTU, HATU and BOP, for the synthesis of peptides containing residues such as Cys and Ser, which are particularly prone to racemization. The advantage in these cases is due to the fact that, even during the long preactivation times used in automated instruments, the degree of racemization is kept very low. The complex $Cu(OAt)_2$ was usually found to perform slightly better than $Cu(OBt)_2$.

Acknowledgements

We would like to acknowledge Drs Nathalie Thieriet and James K. Blodgett for their helpful discussions and Yoav Luxembourg for supporting this work, which was further supported by CICYT (PB96-1490), and Generalitat de Catalunya [Grup Consolidat (1995SGR 494) and Centre de Referència en Biotecnologia].

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