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Evaluation of COMU as a coupling reagent for *in situ* neutralization Boc solid phase peptide synthesis

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Benzotriazole-based coupling reagents have dominated the last two decades of solid phase peptide synthesis. However, a growing interest in synthesizing complex peptides has stimulated the search for more efficient and low-cost coupling reagents, such as COMU which has been introduced as a nonexplosive alternative to the classic benzotriazole coupling reagents. Here, we present a comparative study of the coupling efficiency of COMU with the benzotriazole-based HBTU and HCTU for use in *in situ* neutralization Boc-SPPS. Difficult sequences, such as ACP(65–74), Jung–Redeman 10-mer, and HIV-1 PR(81–99), were used as model target peptides on polystyrene-based resins, as well as polyethylene glycol-based resins. Coupling yields obtained using fast *in situ* Boc-SPPS cycles were determined with the quantitative ninhydrin test as well as via LC-MS analysis of the crude cleavage products. Our results demonstrate that COMU coupling efficiency was less effective compared to HBTU and HCTU with HCTU ≥ HBTU > COMU, when polystyrene-based resins were employed. However, when the PEG resin was employed in combination with a safety catch amide (SCAL) linker, more comparable yields were observed for the three coupling reagents with the same ranking HCTU ≥ HBTU > COMU. Copyright © 2012 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: coupling reagents; *in situ* neutralization Boc-SPPS; COMU; HBTU; HCTU; difficult sequences; SCAL linker; ChemMatrix resin; solid phase peptide synthesis

Introduction

The success of solid phase peptide synthesis (SPPS) relies heavily on the reaction of the microenvironment determined by the properties of the solid support, the linker, the solvent, and the physical properties of the growing peptide chain [1–4]. In a given microenvironment, the coupling reagent choice is crucial for a successful amide bond formation, and efficient coupling reagents have to deliver a fast and high yielding amide bond formation. This high efficiency must be linked to high chemical selectivity and, at the same time, avoid racemization, as well as potential side reactions such as the formation of *N*-acylurea, diketopiperazine, or guanidylation [5–7]. A multitude of coupling reagents have been developed, often with the promise of improved performance over previously reported reagents [8]. Comparing the efficacy of coupling reagents is not a trivial task because of the many parameters influencing amide bond formation [8]. Nevertheless, over the past two decades, benzotriazole-based coupling reagents like HBTU[9] and HCTU[10,11] have emerged as very cost-efficient coupling reagents. These coupling reagents have found applications in automated syntheses[12–17] and in industrial peptide production processes [10,18]. However, the recent reclassification of 1-hydroxybenzotriazole as an explosive has led to a search for alternative coupling reagents that do not contain the potentially explosive benzotriazole moiety [19]. COMU [20–22] (Figure 1) has been introduced as a nonexplosive alternative to the classical benzotriazole coupling reagents. The reagent is particularly appealing for use in fully automated peptide synthesizers, as it demonstrates three times higher solubility in DMF (1.5 M) compared to HBTU and HCTU, and it is of comparable cost. The coupling efficiency of COMU has been shown to be superior to both HBTU and HCTU and, often, also to the relatively expensive gold standard HATU [9] when used in Fmoc-chemistry SPPS [20–23]. Varying solution stabilities of the novel coupling reagent COMU have been reported [20,24]. Although NMR investigations of d_7-DMF under exposure to air have shown that COMU has superior stability compared to HBTU and HCTU [20,24], similar closed vial studies delivered an opposite outcome. [20,24]

Reports of excellent coupling performance of COMU in Fmoc SPPS prompted us to investigate this new coupling reagent for application in '*in situ* neutralization' Boc-chemistry SPPS. Despite

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Abbreviations: *AM, aminomethyl; Boc, tert-butyloxycarbonyl; COMU, (1-Cyano-2-ethoxy-2-oxoethylidenaminooxy) dimethylamino-morpholino-carbenium hexafluorophosphate; DMF, N,N-dimethylformamide; DIPEA, N,N-diisopropylethylamine; ESI-MS, electrospray ionization mass spectrometry; Fmoc, 9-Fluorenylmethoxycarbonyl; Fmoc-SCAL (safety catch amide linker), 4,4′-Bis (methylsulfinyl)-2-(4-carboxybutoxy)-N-Fmoc-benzhydrylamine; HATU, 2-(1H-7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate; HBTU, 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HCTU, 2-(6-Chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate; LC-MS, liquid chromatography mass spectrometry; PEG, polyethylene glycol; p-MBHA, 4-methylbenzhydrylamine; PAM, phenylacetamidomethyl; TFA, trifluoroacetic acid.*

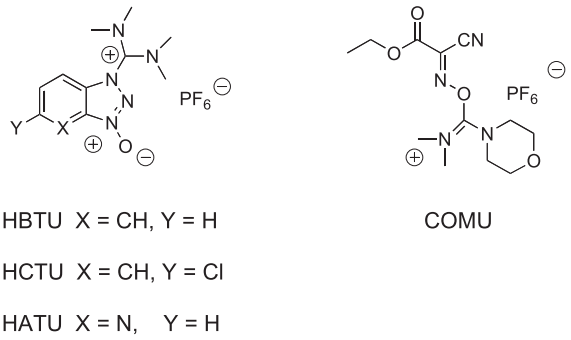

Figure 1. Structure of 1-benzotriazole-based coupling reagents (HBTU, HCTU, and HATU) compared to COMU.

the progress made in Fmoc chemistry, we continuously aim toward improving Boc-chemistry [25–27]. This is because of the fact that some difficult peptide sequences can be more efficiently assembled by employing *in situ* neutralization Boc-SPPS where coupling is performed on the protonated resin with neutralization proceeding while coupling is in progress, leading to superior coupling yields [25,26,28]. This form of Boc-SPPS is particularly suited for the synthesis of cysteine-rich toxins,[29] peptide α-thioesters for native chemical ligation chemistry[30], and selenocysteine containing peptides as stable bioisosteric analogs for drug development [31]. With the aim of further improving access to these complex peptide molecules, we were interested in investigating the performance of the novel coupling reagent COMU when used in *in situ* neutralization Boc-SPPS [26,28]. Here, we describe a comparative study of COMU with HBTU and HCTU.

Results and Discussion

To compare the coupling efficiency of COMU with the most popular coupling reagents HBTU and HCTU, we employed the commonly used quantitative ninhydrin test [32] in combination with RP-HPLC/ESI-MS. With this set of data, the coupling performance in a particular solid phase chemistry system can be effectively investigated.

As modern coupling reagents, like HBTU, HCTU, and presumably also COMU, exhibit fast reaction times, comparative studies of coupling performance are challenging. Hence, one-minute coupling times were used so that significant differences in the reactivities of the coupling reagents could be readily monitored. The *in situ* neutralization Boc-SPPS protocol [26,28] has been successfully used in our group for many years and was employed to evaluate the investigated coupling reagents. The protocol consists of an overall 4.3-minute coupling cycle (Figure 2). Amino

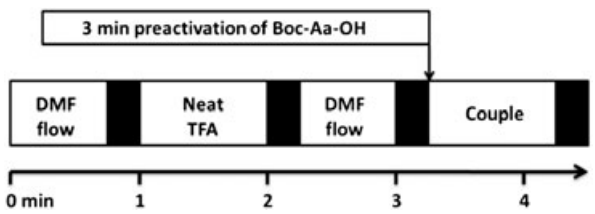

Figure 2. 4.3-minute '*in situ* neutralization' Boc-SPPS cycle. A one-minute neat TFA Boc-removal, followed by a 45-second vacuum-assisted DMF flow wash. A one-minute coupling, again, followed by a 45-second vacuum-assisted DMF flow wash completes the coupling cycle. The black regions indicate vacuum-assisted drainage steps. The amino acids were preactivated with a coupling reagent and base for 3 min prior to coupling.

acids are preactivated for 3 min with a coupling reagent and a base, while Boc-deprotection and flow washes are performed. The actual coupling of preactivated amino acid is then performed for 1 min. The coupling yields were evaluated using the quantitative ninhydrin test performed after each one-minute coupling. After completion of the synthesis, the crude peptide purity was evaluated with LC-MS, and the by-products formed because of inefficient couplings were identified.

Only freshly prepared solutions of coupling reagents were used in order to eliminate the potential influence of varying stability of coupling reagents on the coupling yields [20,24,33]. The resins used were allowed to swell overnight in DMF prior to commencing synthesis.

In order to distinguish between different high performing coupling reagents, we selected several test peptides that had previously been reported as difficult to synthesize. For our initial model peptide, we used the well-known fragment of the acyl carrier protein ACP(65–74), which has been previously used in a multitude of investigations of coupling reagent performance [1,12,34]. ACP(65–74) (sequence: VQAAIDYING) is known to be a 'difficult peptide sequence' because of the strong chain aggregation [35,36]. In swelling studies, chain aggregation was observed leading to decreased swelling of the peptidyl-resin during assembly of the N-terminal region [37]. Figure 3 shows the assembly of ACP (65–74) in its C-terminal acid and amide forms on two polystyrene-based resins, *p*-MBHA-(amide) and Boc-Gly-O-CH_2-PAM resin (acid) with the use of HBTU (three-minute pre-activation) and one-minute *in situ* neutralization coupling (Figure 2).

A decrease in coupling yield was observed in the coupling of the last four amino acids [ACP(65–68)], especially in the coupling of Gln66 and Val65 which displayed coupling yields as low as 90%. A major difference was observed between the two selected resins when Boc-Gly-O-CH_2-PAM-polystyrene resin showed much lower coupling yields compared to the *p*-MBHA resin. The major by-products formed using the *p*-MBHA resin were identified as des-Asn66 (948.8 Da) yielding a 42% crude purity with the use of high performance liquid chromatography (HPLC) analysis. In contrast, when using the Boc-Gly-O-CH_2-PAM resin, a crude purity of 20% was obtained (Figure 3). Consistent with the ninhydrin coupling yields (Figure 3A), the major by-products could be identified as des-Gln66 (935.6 Da), des-Val65 (964.6 Da), des-(Gln66, Asn73) (821.6 Da), and des-(Gln66, Val65) (836.6 Da) through LC-MS analysis of the deletion products (Figure 3A and 3B).

In addition to the polystyrene-based resins, we included the PEG-based resin, ChemMatrix®. This resin type demonstrates excellent swelling properties in a range of solvents and has been shown to improve crude purities with both Fmoc-chemistry and Boc-chemistry [38–42].

The aminomethyl ChemMatrix® resin was loaded with a triple Gly spacer (Gly_3) followed by the SCAL linker [43,44]. The Gly_3-spacer was introduced because of initial slow couplings with the aminomethyl ChemMatrix® resin, as well as to avoid the difficult handling caused by strong aggregation of resin beads to each other. After the attachment of the Gly_3-spacer, the handling of the resin improved, and it allowed easier sampling of resin for the ninhydrin test. Coupling yields following the introduction of the Gly_3-spacer were greatly improved. The final loading was determined by using the Fmoc loading test [45,46]. The SCAL linker was selected, as it is stable towards TFA and HF and may only be cleaved after activation renders it TFA labile [43,44]. This resin-linker combination has previously been used in our group for Boc-SPPS of diseleno analogs of conopeptides and has proven

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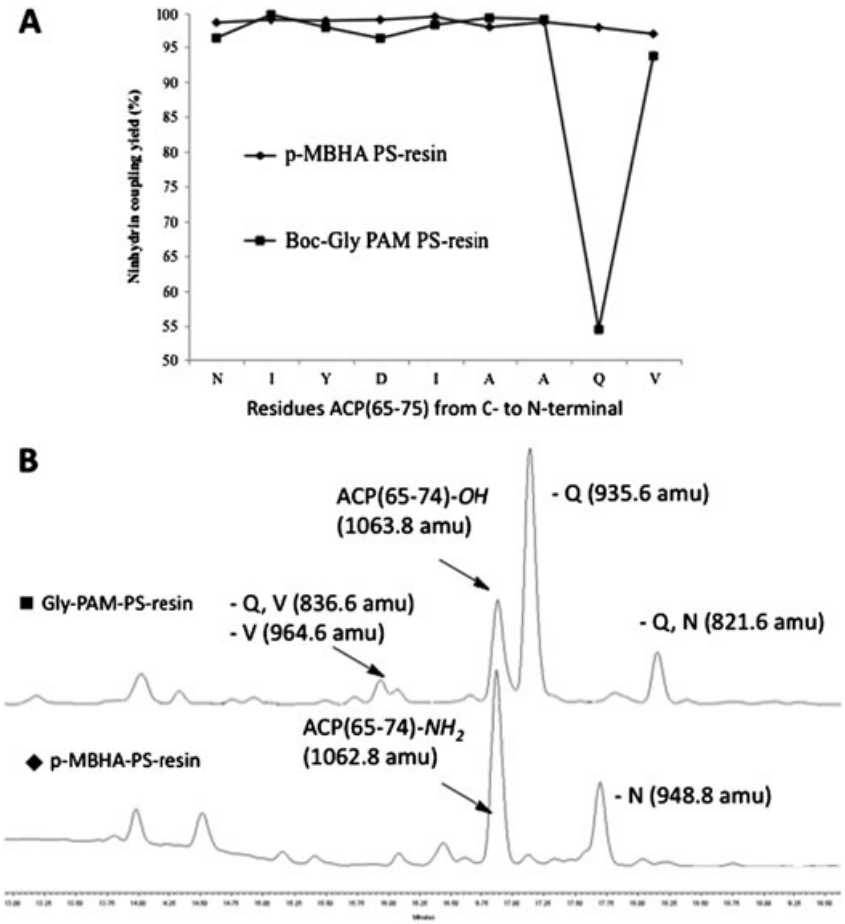

Figure 3. Boc-SPPS of ACP(65–74)-NH_2/*OH* on *p*-MBHA resin (♦) and Boc-Gly-PAM resin (■) employing a 4.3-minute '*in situ* neutralization' Boc-SPPS cycle (HBTU 3 min pre-activation, 1 min coupling).. Correlation of (A) coupling yields obtained by quantitative ninhydrin test with (B) HPLC-MS assignment of obtained crude product and related deletion products.

suitable for parallel HF cleavage of multiple peptides compartmentalized in tea bags [31].

On the basis of the initial HBTU results (Figure 3A and 3B), coupling of the last four amino acids of ACP(65–68) was selected to be compared with the COMU coupling efficiency with that of HBTU and HCTU. ACP(69–74) was pre-assembled on the two polystyrene-based resins, *p*-MBHA PS-resin and a Boc-Gly-O-CH_2-PAM PS-resin, as well as the SCAL-loaded ChemMatrix® resin. The pre-assembly delivered coupling yields >99.8%. Because of the much larger swelling volume of the ChemMatrix® SCAL resin, it was necessary to use 0.2 M HBTU solution for amino acid activation (compared to 0.5 M for the PAM/*p*-MBHA-PS-resin) to enable the complete coverage of the resin with reagent. The relative excess of amino acid compared to free amines on resin was constant in all the experiments performed. On completion of pre-assembly, the peptidyl-resins were dried down, split, and used for the comparative studies that employed HBTU, HCTU, and COMU as coupling reagents. Similar coupling yields for the full synthesis of ACP(65–74) (Figure 3) were observed for the last four couplings using HBTU; hence, drying down and reswelling the hexapeptidyl-resin, ACP(69–74), did not influence resolvation and subsequent couplings. The coupling efficiency of the last four amino acid couplings that was measured by employing the quantitative ninhydrin test and the final crude products were investigated through LC-MS analysis. Figure 4 shows the results obtained for the coupling yields obtained for ACP(65–68) on three different pre-assembled resins employing HBTU-activation, HCTU-activation, and COMU-activation in the three-minute pre-activation, one-minute coupling *in situ* neutralization Boc-SPPS protocol.

Table 1 summarizes the crude product composition and the observed deletion products as analyzed by RP-HPLC-MS. The deletion products observed (Table 1) correlate well with the coupling yields obtained from the quantitative ninhydrin test (Figure 4A), e.g. a 50% coupling yield for the Gln coupling on Boc-Gly-Pam PS-resin (HBTU) compares well with the 55% des-Gln66 product (Table 1), as analyzed by HPLC. On the *p*-MBHA resin, the observed coupling yield of 98% for the Gln coupling compares well with the formation of about 3% of the appropriate deletion product. Whereas, the observed HPLC results may be complicated by the presence of multiple deletion products, as well as other side products. In general, the HPLC results gave a reasonable correlation with the quantitative ninhydrin test data.

On all test resins, COMU provided the lowest ninhydrin coupling yields; whereas, better coupling yields were achieved when HBTU and HCTU were used (See average coupling yields in Table 1).

The use of 2 eq of base (relative to the coupling reagent) has been shown to improve coupling yields when COMU is used for Fmoc SPPS [20]. For comparison, a similar excess of base was used for the HBTU and HCTU experiments, departing from our originally reported protocol [28], but only similar or marginally improved yields are observed (Table 1) .

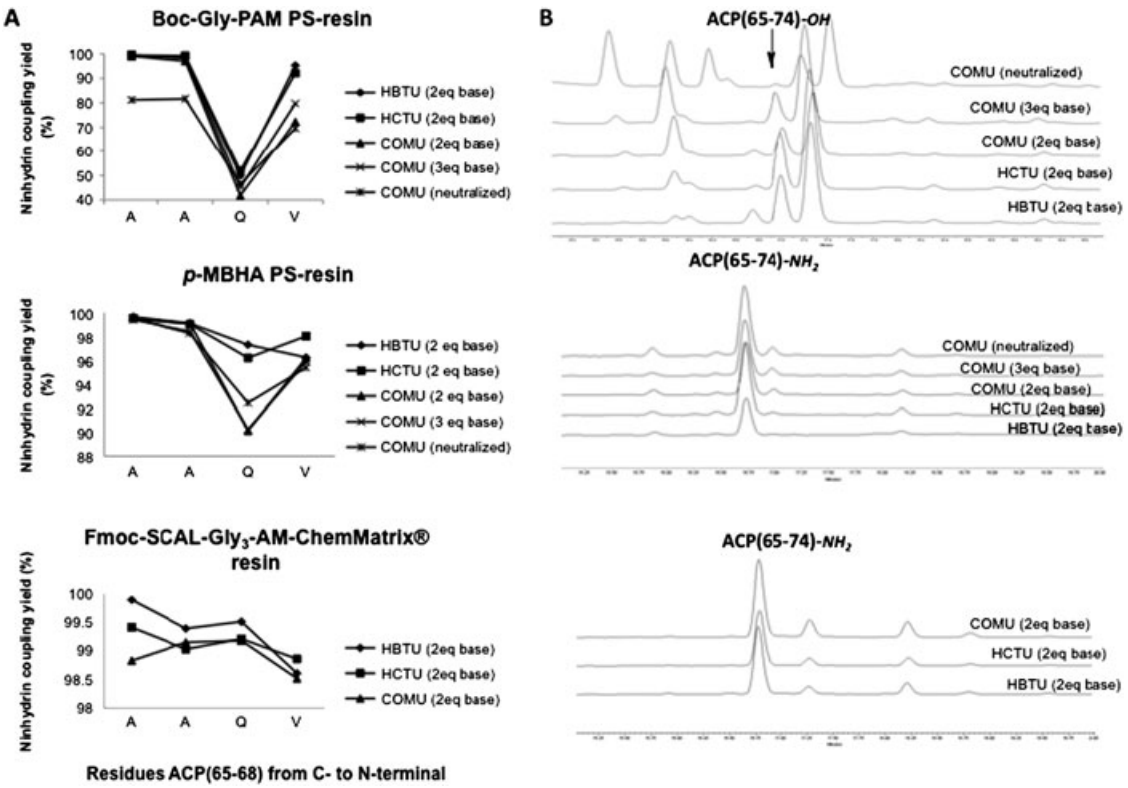

Figure 4. Boc-SPPS of ACP(65–74)-NH_2/*OH* with HBTU, HCTU, or COMU as coupling reagents. Assembly on *p*-MBHA resin, Boc-Gly-PAM resin, and Fmoc-SCAL-G_3-ChemMatrix resin employing a 4.3-minute (three-minute pre-activation/one-minute coupling) '*in situ* neutralization' Boc-SPPS cycle and ACP (69–74) pre-assembled on resin. (**A**) (left) coupling yields obtained by quantitative ninhydrin test for synthesis of peptide fragment ACP(65–68); (**B**) (right) HPLC traces of crude product ACP(65–74)-NH_2/*OH* obtained using varying coupling reagents and conditions.

Prior neutralization of the resin with the use of 10% DIPEA in DMF did not improve the coupling efficiency (Table 1), which is consistent with a combination of lower coupling efficiency and increased aggregation. However, similar or lower coupling yields, compared to HBTU (preneutralization), were observed when using COMU (data not shown). Consistently similar coupling reagent performance was seen for all three resins, HCTU ≥ HBTU COMU (Figure 4 and Table 1).

To examine if there is a kinetic difference between the three coupling reagents, we performed the ACP(65–74) assembly with the use a five-minute coupling time. Ninhydrin tests were performed after 1, 2, and 5 min. The coupling reagents showed similar kinetic profiles, and the previously observed ranking of the coupling reagents was preserved with the HCTU being equal or slightly better than the HBTU and with both being better than COMU.

During pre-activation, activated ester species that are responsible for amide bond formation are generated. While a pre-activation period of 3 min has been shown to be efficient for the benzotriazole-based coupling reagents [26,28], is it possible that for COMU, the activated species are slower to form during the conditions selected (three-minute pre-activation plus one-minute coupling)? Nevertheless, we did not investigate this possibility given that results from previous work demonstrated COMU to be a very fast coupling reagent [20], and concluded that the slow formation of the activated ester species is a very unlikely cause for the observed lower coupling yields. To investigate whether the activated ester species that were formed when using COMU decomposes during pre-activation, we repeated the same experiments with a shorter pre-activation period. Decreasing the pre-activation time to 20 s did not improve the coupling yields, as observed when using COMU.

The combination of the SCAL linker and the ChemMatrix® resin proved to be an excellent system for synthesizing ACP(65–74), where minimal deletions within the last four amino acids were detected when RP-HPLC/ESI-MS was used (Figure 4 and Table 1). Moreover, consistently good coupling yields were observed for the three coupling reagents on this SCAL linker–ChemMatrix® system with a ranking of HCTU ≥ HBTU > COMU. In order to establish whether the observed relative improved performance of COMU on the SCAL linker–ChemMatrix® system is caused by the SCAL linker itself or the ChemMatrix® resin, ACP(65–74) was assembled on a Fmoc-SCAL linker-Gly_3-AM-polystyrene resin. Comparable results to the *p*-MBHA resin were observed, and we may conclude that improved COMU performance was ascribed to the ChemMatrix® resin.

Given the above results, we were interested to see whether the performance of the coupling reagents was peptide-dependent. Thus, we selected the Jung–Redemann 10-mer (WFTTLISTIM-NH_2) as another difficult model sequence [47–50]. After initial methionine coupling with a coupling yield of >99.8%, the decapeptide was assembled on the SCAL-G_3-AM ChemMatrix® resin employing HBTU, HCTU, or COMU and applying the 4.3-minute coupling cycle (three-minute pre-activation/one-minute coupling) (Figure 5 and Table 2).

As previously reported[47], lower coupling yields were observed after the coupling of Leu5. In general, good to average coupling yields could be achieved, and very similar results were observed for all three coupling reagents. However, no improvement was observed when COMU was used compared to when

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Table 1. LC-MS analysis of the crude peptides of ACP(65–74) obtained through *in situ* neutralization Boc-SPPS synthesis on *p*-MBHA-PS-resin, Gly-PAM-PS-resin, and SCAL-G_3-AM ChemMatrix resin, comparing HBTU, HCTU, and COMU

Coupling reagent	HBTU	HCTU	COMU	COMU	COMU
(eq DIPEA used[a])	(2 eq)	(2 eq)	(2 eq)	(3 eq)	(preneutralization)
Boc-Gly-PAM PS-resin					
Average coupling yield [%]	85.7	85.7	77.5	80.6	69.7
			Crude constitution by HPLC-ESI-MS [%]		
ACP(65–74)-OH	24.1	24.0	18.1	13.8	0
des-Ala67	7.3	1.9	4.4	1.3	—
des-Val65	3.8	3.0	—	—	—
des-Gln66	55.8	56.3	42.6	46.3	—
des—Gln66,Val65	3.7	7.8	27.4[b]	28.0[1]	—
des-Gln66,Val65,Ala67	1.1	1.2	2.5	3.2	—
***p*-MBHA PS-resin**					
Average coupling yield [%}	98.0	98.2	96.3	96.0	96.4
			Crude constitution by HPLC-ESI-MS [%]		
ACP(65–74)-NH_2	70.6	75.6	72.4	72.2	65.1
des-Ala67	2.9	3.7	3.4	2.9	3.7
des-Val65	5.6	3.8	6.8	4.2	7.0
des-Gln66	2.4	3.7	8.9	10.3	8.7
SCAL-G_3-ChemMatrix® resin					
Average coupling yield [%]	99.4	99.1	98.2	—	—
			Crude constitution by HPLC-ESI-MS [%]		
ACP(65–74)-NH_2	75.7	75.8	67.5	—	—
des-Ala67	3.6	3.8	3.8	—	—
des-Val65	3.9	4.0	6.9	—	—
des-Gln66	3.2	2.6	5.0	—	—

[a]Relative to coupling reagent.
[b]des-Val is included in this peak.

benzotriazole-based coupling reagents were used. The formyl protecting group on tryptophan was not removed prior to HF-cleavage, as Trp containing peptides are known to be sensitive to the SCAL-linker cleavage conditions used [44]. Thus the Jung–Redeman 10-mer was obtained as the Trp(CHO) product. LC-MS analysis showed Trp(CHO)1 and Phe2 deletions, as well as three other peaks exhibiting the same molecular weight with the desired peptide. These products were presumed to be the D-Ser epimer and two depsipeptides arising from an N → O shift at either Ser or Thr, in accordance with previous studies [47]. As expected, the two assumed depsipeptide peaks disappeared upon treatment with dilute ammonium hydroxide because of O → N shift [47]. No further experiments were performed to fully identify the three products obtained.

Another reported difficult sequence [29], the C-terminal sequence of the human immunodeficiency virus-1 proteinase (81–99) (HIV-1 PR(81–99)), was investigated. The difficult sequence region does not arise until after the coupling of Ile (93); hence, HIV-1 PR(93–99) was pre-assembled on the SCAL-linker-G_3-AM ChemMatrix® resin. Again, coupling yields were driven to >99.8%, as established by quantitative ninhydrin test. The full peptide sequence was then completed by using the three different coupling reagents with the 4.3-minute coupling cycle (three-minute pre-activation/one-minute coupling).

According to the ninhydrin analysis, HCTU performed better than HBTU and COMU, resulting in average coupling yields of 96.9% (HCTU) > 95.0% (HBTU) > 94.6% (COMU) and significantly higher crude purity of product 31.2% (HCTU) *versus* 19% (HBTU) and 16.6% for COMU (Figure 6).

Conclusion

A comparative study between the COMU and the commonly used coupling reagents, HBTU and HCTU, was undertaken using *in situ* neutralization Boc-SPPS. Using a very fast synthesis protocol (three-minute amino acid pre-activation with coupling reagent/base followed by a one-minute single coupling, resulting in a 4.3-minute full cycle), we evaluated the coupling reagents with the use of the quantitative ninhydrin test and HPLC-MS studies of the crude cleaved products. Both sets of analytical data were consistent and confirmed a ranking of coupling efficiency as HCTU ≥ HBTU > COMU, when the *in situ* neutralization Boc-SPPS protocol on polystyrene-based resins (*p*-MBHA, PAM) was employed. Thus, the results obtained did not confirm a superior performance of COMU, as was observed when Fmoc chemistry was employed in previous studies. Similar results were obtained in *p*-MBHA and Boc-Gly-PAM resin when a range of different conditions was used.

In contrast, the combination of a SCAL linker and a PEG-based resin, ChemMatrix®, resulted in an improved microenvironment, which allowed all three coupling reagents to perform with similar coupling efficiencies for the difficult peptides, although the previously obtained ranking (HCTU ≥ HBTU > COMU) persisted.

Experimental Section

Materials and methods

All solvents and reagents were obtained commercially and were used without further purification. N^{α}-Boc-L amino acids were

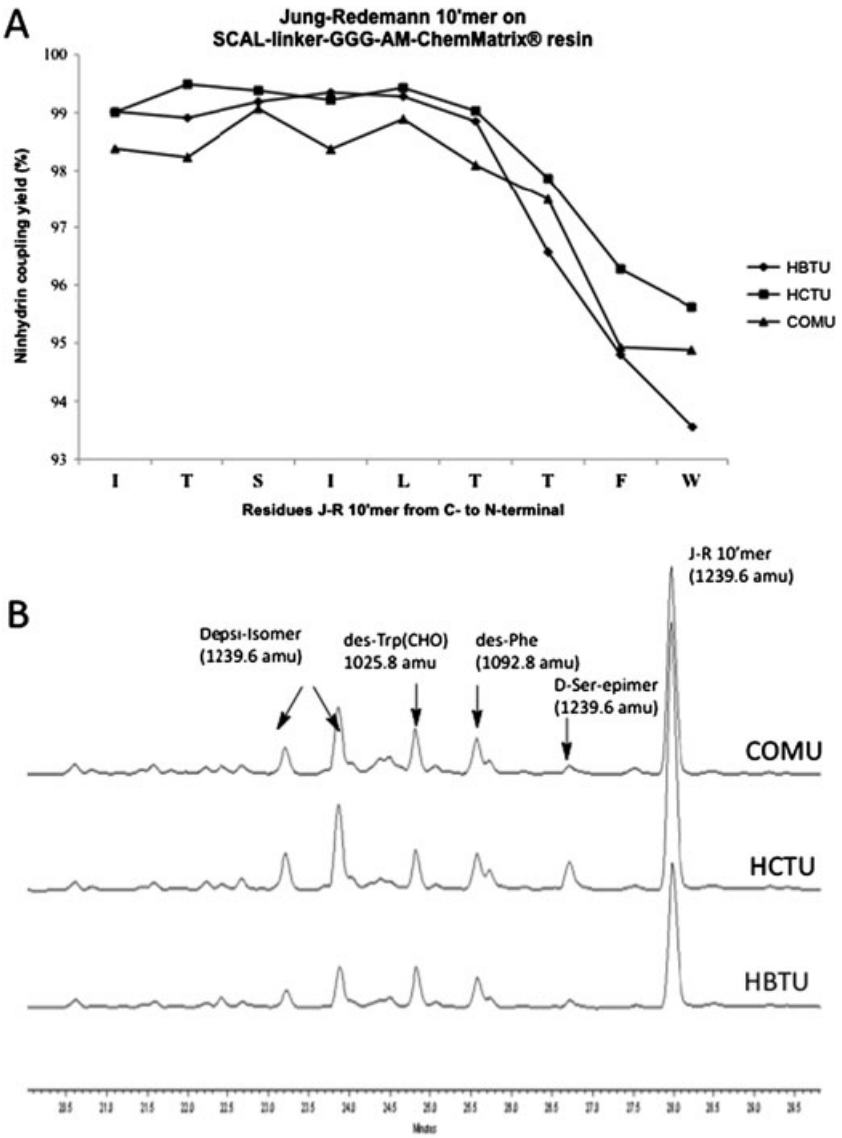

Figure 5. Boc-SPPS of Jung–Redeman 10-mer with HBTU, HCTU, or COMU as coupling reagents. Assembly on Fmoc-SCAL-Gly_3-AM ChemMatrix® resin employing a 4.3-minute (three-minute pre-activation/one-minute coupling) '*in situ* neutralization' Boc-SPPS cycle and preloaded on the resin. (**A)** coupling yields obtained with the quantitative ninhydrin test; (**B)** HPLC traces of obtained crude product J-R 10'mer with some assigned side products and related coupling reagents used.

Table 2. LC-MS analysis of crude peptides of the Jung–Redeman 10-mer obtained through *in situ* neutralization Boc-SPPS synthesis on SCAL-G_3-AM ChemMatrix resin, comparing HBTU, HCTU, and COMU

Coupling reagent	HBTU	HCTU	COMU
(eq DIPEA used[a])	(2 eq)	(2 eq)	(2 eq)
SCAL-Gly_3-ChemMatrix® resin			
Average coupling yield [%]	97.7	98.4	97.6
		Crude constitution by HPLC-ES-MS [%]	
J-R 10-mer-NH_2	44.0	45.9	43.4
des-Trp(CHO)1	11.5	6.8	8.9
des-Phe2	8.8	6.4	7.4
Depsi-1	4.9	6.4	5.2
Depsi-2	13.0	16.5	15.5
D-Ser epimer	3.0	5.6	2.4

[a]Relative to coupling reagent.

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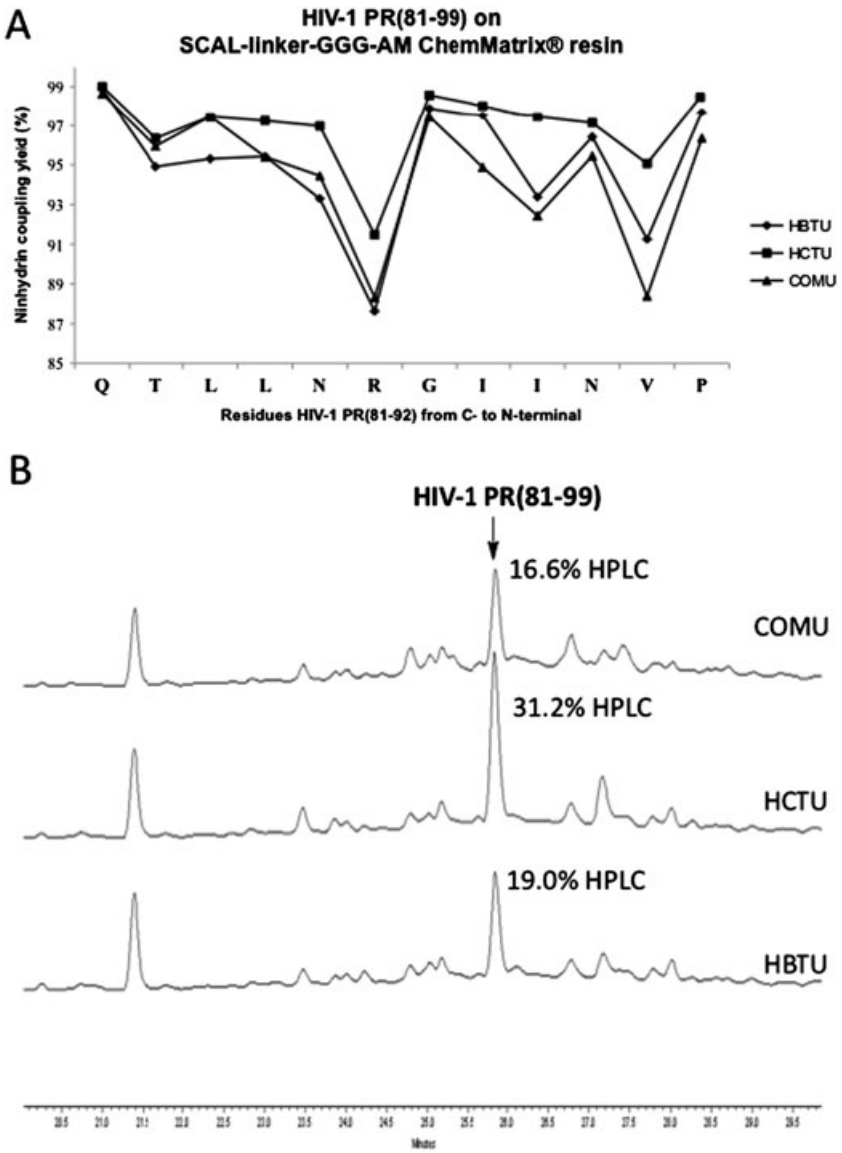

Figure 6. Boc-SPPS of HIV PR(81–99) with HBTU, HCTU, or COMU as coupling reagents. Assembly on Fmoc-SCAL-Gly_3-AM ChemMatrix® resin employing a 4.3-minute (three-minute pre-activation/one-minute coupling) '*in situ* neutralization' Boc-SPPS cycle and HIV PR(93–99) pre-assembled on resin. (**A**) coupling yields obtained with the quantitative ninhydrin test for sequence HIV PR(81–92); (**B**) HPLC traces of obtained crude product HIV-1 PR(81–99) with integration yields in relation to coupling reagent used.

purchased from Novabiochem (Merck Pty., Kilsyth, Vic., Australia). The following side chain-protected Boc-amino acids were used: Arg(Tos), Asn(Xan), Asp(Chxl), Cys(4-MeBzl), Gln(Xan), Tyr(2-BrZ), Thr(Bzl), Trp(For), Ser(Bzl), and Lys(2-ClZ). Solvents used for peptide chain assembly were of peptide synthesis grade. TFA and DMF were purchased from Auspep (Melbourne, Vic., Australia). Dichloromethane was purchased from Fisher Scientific (Scoreby, Vic., Australia). HCTU was purchased from Peptides International (Louisville, Kentucky, USA), HBTU from Fluka (Buchs, Switzerland) and COMU from Sigma-Aldrich (Sydney, NSW, Australia). Anhydrous HF gas was purchased from BOC Gases (Sydney, NSW, Australia), and scavengers *p*-cresol and *p*-thiocresol from Sigma-Aldrich. N^{α}-Boc-L-Gly-phenylacetamidomethyl resin [Boc-Gly-PAM resin, loading = 0.59 mmol/g, 100–200 mesh, 1% DVB (divinylbenzene)] was purchased from Peptides International, 4-methylbenzhydrylamine resin (*p*-MBHA-resin, loading = 0.67 mmol/g, 100–200 mesh, 1% DVB) from Novabiochem, and aminomethyl ChemMatrix® hydrochloride (loading = 0.74 mmol/g, 35–100 mesh) from Matrix Innovation (Montreal, Canada). Fmoc-SCAL was purchased from CSPS Pharmaceutical (San Diego). HPLC-grade-acetonitrile (EM Science) was supplied by Laboratory Supply (Australia).

Fmoc-SCAL-G_3-AM ChemMatrix® resin

Boc-Gly-OH (4 eq) was coupled with the AM ChemMatrix® resin (0.76 mmol/g, 35–100 mesh, Matrix Innovation) with the use of HBTU (3.9 eq, 0.5 M) and DIPEA (7.8 eq). The ninhydrin test [32] was employed to ensure a coupling yield of above 99.8% for each glycine residue attached. Deprotection with TFA was followed by double coupling of the Fmoc-SCAL linker (MW = 645.6, 1.5 eq) with the use of HBTU (1.4 eq) and DIPEA (3 eq) to yield the desired loaded resin. Final loading is 0.49 mmol/g.

Peptide Assembly

Pre-assembled peptides

Couplings were performed using HBTU (3.9 eq, 0.5 M in DMF (polystyrene) or 0.2 M in DMF (ChemMatrix®)), Boc-Aa-OH (4 eq), and DIPEA (7.8 eq relative to the resin), until a satisfying coupling yield was achieved (>99.8% by quantitative ninhydrin test [32]).

Comparative couplings

The resins were swelled overnight in DMF. Solutions of coupling reagents were freshly prepared. Unless otherwise noted, the couplings were performed using the 4.3-minute coupling cycle (three-minute pre-activation/one-minute coupling time), described in Figure 2, as well as employing coupling reagent (3.9 eq, 0.5 M for PS-resins, and 0.2 M for ChemMatrix®-resins), Boc-Aa-OH (4 eq), and DIPEA (7.8 eq compared to the resin). Ninhydrin tests [32] were performed after each coupling. The resins were dried down prior to cleavage: DMF wash followed by a CH_2Cl_2 wash and a MeOH wash. The resin was then dried under a flow of N_2.

Couplings with extra base

Same procedure was used, as described in Figure 2; however, 11.7 eq DIPEA (compared to the resin, 3 eq compared to the coupling reagent) was used.

Couplings on preneutralized peptidyl-resin

A one-minute neutralization step prior to coupling with the use of 10% DIPEA in DMF was incorporated into the standard coupling cycle.

Peptide Cleavage

*Cleavage of p-*MBHA and PAM-resins

In each case, approximately 100 mg of peptide resin was cleaved using 0.25 mL *p*-cresol and 0.25 mL *p*-thio-cresol in 10 mL HF at 0 °C for 1 h. After evaporation of the HF, the peptides were precipitated in cold Et_2O, filtered and redissolved in 50% CH_3CN (0.05% TFA) in water, and lyophilized.

Two-Step Cleavage of SCAL-Linker Resins

Sidechain deprotection: The peptidyl-resin was deprotected using 0.25 mL *p*-cresol in 10 mL HF at 0 °C for 1 h. After evaporation of the HF, the deprotected peptidyl-resin was filtered off and washed with cold Et_2O.

Reductive cleavage from the resin: Approximately 50 mg of deprotected resin was cleaved using 50 mg NH_4I and 0.1 mL Me_2S in 2 mL TFA at room temperature overnight. The TFA solution was filtered off and was precipitated from cold ether. After centrifugation, the solid residue was dissolved in 50% CH_3CN (0.05% TFA) in water and lyophilized.

Characterization

HPLC analysis and purification

Analytical HPLC runs were performed using a Shimadzu HPLC system LC10A with a dual wavelength UV detector set at 214 nm and 254 nm. A reversed-phase C-18 column (Hypersil C18, 130 Å, 5 μm, 250 mm × 4.6 mm) with a flow rate of 1 mL/min was used. Gradient elution was performed (40 °C) with the following buffer systems: (i) 0.05% TFA in water and (ii) 0.043% TFA in 90% acetonitrile in water, from 0% (ii) to 80% (ii) in 40 min. Absorbance was monitored at 214 nm and 254 nm. Crude purities are given by peak areas at 214 nm.

Electrospray mass spectrometry (ES-MS)

Electrospray mass spectra were collected inline during analytical HPLC runs on an Applied Biosystems API-150 spectrometer operating in the positive ion mode with an OR of 20, Rng of 220, and Turbospray of 350°. Masses between 300 and 2200 amu were detected (step 0.2 amu, Dwell 0.3 ms).

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