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Cleaving protected peptides from 2-chlorotrityl chloride resin. Moving away from dichloromethane

Mahama Alhassan, Othman Al Musaimi,
Jonathan M. Collins, Fernando Albericio* and
Beatriz G. de la Torre*

Anisole and 1,3-dimethoxybenzene are excellent alternatives to DCM for cleaving protected peptides.

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PAPER

Cleaving protected peptides from 2-chlorotrityl chloride resin. Moving away from dichloromethane†

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In recent years, the work of various research groups has allowed the substitution of the hazardous solvents most widely used in solid-phase peptide synthesis, namely DMF, NMP, DCM, DEE, among others, by several much less hazardous solvents. Indeed, greener alternatives have been found for almost all steps of the process, with the exception of the cleavage of protected peptides from 2-chlorotrityl chloride resin. Here, after careful screening of several of the so-called green solvents, we propose 2% TFA in either anisole or 1,3-dimethoxybenzene as optimal for the cleavage step. The higher boiling point of these solvents compared with the DCM allows the preparation of protected peptides with less risk of premature removal of the most labile protecting groups, such as the Trt of His. Our findings once again evidence the value/versatility of green solvents in strict chemical terms.

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Introduction

Peptides are key molecules in both biomedical research and drug development. In this regard, in 2019, the US Food and Drug Administration approved six new drugs which are either peptides or which partly comprise peptides.^{1,2} Although, peptides approved this year by the FDA could be considered of medium size, the great boom of peptides in the drug arena is for the treatment of metabolic diseases.³ These peptides are of large size (>30 mer) and prepared by coupling of protected peptides.

Medium and large size peptides, in both research and also in drug manufacturing, are prepared by solid-phase peptide synthesis (SPPS).^{4,5} From a sustainability perspective, SPPS also has various pros and cons.⁶ The biggest drawback is that this technique calls for the use of large amounts of solvent. This feature is due to the fact that intermediates are not iso-

lated in SPPS and the excess of reagents and soluble side products are removed by washings. In this regard, *N,N*-dimethylformamide (DMF), *N*-methylpyrrolidone (NMP), and dichloromethane (DCM) are the three solvents most widely used in SPPS. However, all these compounds have been declared hazardous by the corresponding agencies.^{7,8}

In this context, in recent years, our group and others have been working on the substitution of these solvents for green ones.^{6,9} Thus, greener alternatives have been proposed for most of the SPPS steps. As a summary, γ -valerolactone (GVL) and 2-methyltetrahydrofuran (2-MeTHF) have been proposed for the incorporation of the first amino acid on a Wang-type resin,^{10,11} 2-MeTHF for the same purpose but on chlorotrityl chloride (CTC)-resin,¹¹ *N*-butyl pyrrolidinone (NBP),¹² GVL,¹³ 2-MeTHF,¹⁴ propylene carbonate-EtOAc (1:9),¹⁵ Anisole-dimethyl carbonate (7:3),¹⁶ and NBP-EtOAc (1:1)¹⁷ for peptide bond formation; and cyclopentyl methyl ether (CPME) and 2-MeTHF for the precipitation of the unprotected peptide after global deprotection.^{11,18} However, one step that has received little attention is the cleavage of protected peptides from CTC-resin. This step is convenient for the preparation of protected peptides ready to be used in a convergent approach for the construction of large peptides.^{19,20} To the best of our knowledge, there is only one report in the literature by the Polypeptide Group. In this regard, they cleaved the very simple Fmoc-Gly-Gly-OH from the CTC-resin using 1% trifluoroacetic acid (TFA) in a green binary mixture of CH₃CN-EtOAc (1:1) (cleavage yield: 96%).¹⁷ The same report indicates that 2-MeTHF as solvent yields poor release of the peptide (cleavage

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yield: 8%), which reflects the difficulty of this cleavage step. In the present study, after screening mixtures containing 2% of TFA in green solvents, we propose two distinct cleavage cocktails. The performance of each one has been demonstrated for the cleavage of several protected peptides from CTC-resin.

Results and discussion

First, a selection of ten solvents-including the mixture EtOAc-CH₃CN (1 : 1) previously described¹⁷ was screened for the cleavage of a model peptide from CTC-resin. Although, this cleavage is normally carried out with several consecutive short treatments with 1–2% TFA in DCM, it was decided to perform just one treatment with 2% TFA in the different solvents for 30 min –instead of many short treatments– with a double goal: to investigate a friendly protocol and to overcome problems due to the higher viscosity of the majority of the solvents when compared with DCM. The high viscosity can hamper a fast diffusion of the solvent inside the resin and therefore penalize the cleavage process, mostly if several treatments for a short period of time are carried out. From the beginning, 2% was preferred *vs.* 1%, because it was thought that the presence of the ether in the solvent can slightly buffer the acidity of the TFA. The study was carried out taking as model Fmoc-Leu-enkephalin-CTC-resin (Scheme 1).

To be consistent with the green principles, the elongation of the model peptide has been carried out using green solvents previously described by our group.^{11,21} The protected peptide resin was divided into equal aliquots and treated separately with TFA–green solvent (2 : 98), including DCM only as reference. The filtrates were collected over CH₃CN to avoid precipitation and then analysed by reversed phase HPLC. Detection was recorded at 220 and 300 nm (Fmoc absorbance), the later to avoid interference with some of the solvent peaks (Fig. S1–S11†). For evaluation of the extension of cleavage with 2% TFA, the same resin was treated with TFA–triisopropylsilane (TIS)–H₂O (95 : 2.5 : 2.5) to determine if peptide remained in the solid support after the first cleavage. This Fmoc-peptide was analysed similarly by reversed phase HPLC. Comparison of the analysis from both experiments rendered the cleavage yield of

the first protected peptide with 2% TFA (Table 1) (Fig. S12–S18†).

The results shown in Table 1 support previous findings regarding the difficulty of this cleavage step.¹⁷ Using a slightly larger peptide (5 amino acids) than this group (2 amino acids), the yields were already lower: 28% *vs.* 96% in CH₃CN–EtOAc (1 : 1) (#2) and 5.3% *vs.* 8% in 2-MeTHF (#3). GVL also gave a low cleavage yield (#7) and NBP (#10), cyrene, and isoamyl/iso-butyl acetates (#5, 8, 9) did not lead to any cleavage. In contrast, the three aromatic solvents, anisole (#4), 1,3-dimethoxybenzene (#6), and toluene (#11) showed results comparable to those achieved by DCM (#1). Of note, Fmoc-Tyr-Gly-Gly-Phe-Leu-OH was not detected in any case, thereby indicating the stability of the *t*Bu of the Tyr under these conditions.

Anisole appears as one of the greenest solvents in all guidelines,^{7,22,23} and although 1,3-dimethoxybenzene has not been extensively used as a green solvent, our first analysis in this regard indicates that it could be considered one. It is used in the food industry as a flavoring agent and is classified in the Substances Added to Food inventory (formerly Everything Added to Foods in the United States (EAFUS)).²⁴ It was also reported in Roquefort cheese.²⁵ In contrast, toluene, which appears as a green solvent in some guidelines, has been ques-

Table 1 Screening of green solvents for cleavage of protected peptides from CTC-resin

#	Solvent ^a	Cleavage yield (%)
1	DCM	98
2	EtOAc–CH ₃ CN (1 : 1)	28
3	2-MeTHF	5.3
4	Anisole	100
5	Cyrene	0
6	1,3-Dimethoxybenzene	94
7	GVL	8.2
8	Isoamyl acetate	0
9	Isobutyl acetate	0
10	NBP	0
11	Toluene	100

^a Abbreviations: 2-MeTHF: 2-methyltetrahydrofuran; Cyrene: dihydrolevoglucosenone; GVL: γ -valerolactone; NBP: *N*-butyl pyrrolidinone (TamiSolve).

Scheme 1 Protocol used for the initial screening of 2% TFA-green solvents.

tioned with respect to its effects on the environment (harmful to aquatic life and therefore its release into the environment must be avoided).²⁶ Given these considerations, we decided to continue the study with anisole and 1,3-dimethoxybenzene.

In addition to the other green features of these two solvents (the higher boiling point of both (153 °C for anisole and 85–87 °C for 1,3-dimethoxybenzene) is also a great advantage over DCM (40 °C) for the cleavage of protected peptides with a low content of TFA. Thus, for protected peptides containing highly labile protecting groups such as the Trt of the side-chain of His, the high volatility of DCM could be detrimental during the work-up because its premature evaporation can be translated into an increase in the amount of TFA in the cleavage cocktail, with the subsequent removal of the protecting group. In this regard, both anisole and 1,3-dimethoxybenzene could be considered much safer solvents than DCM as they conserve the chemical structure of the protected peptides. Then and also using model peptides, we examined the stability of Trt protecting groups of the side-chain of His and Asn to the two TFA mixtures. Thus, the two protected peptides Fmoc-Leu-His(Trt)/Asn(Trt)-Leu-O-CTC-resin were prepared and treated separately with 2% TFA in anisole/1,3-dimethoxybenzene/DCM (the latter as reference) for 30 min. The cleaved peptides were analysed by HPLC (Fig. S19–S24†). While Asn(Trt) was fully stable in the three solvents, the His(Trt) only showed full stability in anisole and 1,3-dimethoxybenzene, since 12% of deprotected His was found in case of DCM.

Finally, the performance of the cleavage mixtures based on anisole/1,3-dimethoxybenzene were evaluated using longer protected peptides related to Enfuvirtide (T-20), which is an envelope fusion inhibitor used to treat HIV-infected patients.²⁷ T-20 is a peptide comprising 36 amino acids, produced on a multikg scale by the coupling of protected peptides.¹⁹ This synthesis has been considered a change of paradigm in the peptide production field.²⁸ Thus, the sequence of three peptides (Fig. 1), two 10 mers (A, B) and one 13 mer (C), were elongated on CTC-resin following a full green SPPS approach developed in our laboratory: 2-MeTHF for the incorporation of the first residue to the CTC-resin and GVL for the peptide elongation, coupling and Fmoc removal.^{11,21}

First of all, the quality of the synthetic processes was analysed by HPLC and LC-MS (Fig. S25–S27,† top chromatograms) after global deprotection of the peptide from the resin with TFA–TIS–H₂O (95 : 2.5 : 2.5). Then, the protected peptide CTC-resins were treated with cleavage mixtures [2% TFA in anisole/

1,3-dimethoxybenzene/DCM (the latter as reference)] for 30 min. Then, the cleavage solutions were filtered off and collected over cold H₂O to facilitate the precipitation. After centrifugation and decantation, the solids were washed twice with H₂O. The solids and the supernatants were analysed by HPLC. While the HPLC of the solids (Fig. 2) showed in the three cases the presence of the protected peptide, the HPLC corresponding to the supernatants showed a flat profile (Fig. S28 and S29†). This indicates a fully precipitation of the protected peptide in H₂O. Furthermore, the remaining resins, after the 2% TFA cleavages, were treated with TFA–TIS–H₂O (95 : 2.5 : 2.5) and the corresponding HPLC analysis show a total absence of the unprotected peptide reaffirming the full cleavage of the protected peptides during the 2% TFA treatment in the three solvents (Fig. S30–S38†).

Conclusions

After careful screening of different green solvents, anisole and 1,3-dimethoxybenzene have emerged as an excellent replacement for the hazardous DCM in the cleavage of protected peptides from CTC-resins. The higher boiling point of the two green solvents in comparison with DCM allows a more convenient manipulation of the cleavage solution. Although similar results are obtained when the cleavage is carried out by several short treatments or by just a 30 min treatment, the latter is recommended because is a friendlier manipulation. In this regard, this work has shown once again that the use of green solvents instead of being detrimental is beneficial from a strictly chemical point of view. Thus, using anisole or 1,3-dimethoxybenzene as solvents for the cleavage of His-containing protected peptides from CTC-resins is much safer for preserving the integrity of the Trt side chain protection of His than DCM. Of note, the price, which is one of the main criticisms associated to the use of some green solvents, does not apply to the either anisole or 1,3-dimethoxybenzene, which are both very costly affordable.

In this context, it is envisaged that either anisole or 1,3-dimethoxybenzene will replace DCM for this key step in the industrial preparation of protected peptides required for the synthesis of larger peptides *via* coupling of protected peptides.

As a final remark, Fig. 3 shows the efforts made so far to greening the solid-phase synthesis of unprotected and protected peptides. Besides of fining tuning those steps, research-

Fragment 17-26 (A)

Fmoc-Glu(tBu)-Lys(Boc)-Asn(Trt)-Glu(tBu)-Asn(Trt)-Glu(tBu)-Leu-Leu-Glu(tBu)-Leu-OH

Fragment 27-36 (B)

Fmoc-Asp(tBu)-Lys(Boc)-Trp(Boc)-Ala-Ser(tBu)-Leu-Trp(Boc)-Asn(Trt)-Trp(Boc)-Phe-OH

Fragment 24-36 (C)

Fmoc-Leu-Glu(tBu)-Leu-Asp(tBu)-Lys(Boc)-Trp(Boc)-Ala-Ser(tBu)-Leu-Trp(Boc)-Asn(Trt)-Trp(Boc)-Phe-OH

Fig. 1 Sequences of the protected peptides used in this study.

Fig. 2 Cleaved protected peptides A, B, and C. * is due to solvent peak. Phenomenex Luna 5 μm C₅, (50 $\times$ 4.6) mm, 100 Å column at 300 nm. Mobile phase A: 0.1% TFA in H₂O; mobile phase B: 0.1% TFA in CH₃CN. Gradient elution: 60–100% (B) in 10 min and then extra 10 min at 100%.

SPPS Process Flow Chart

Fig. 3 Efforts carried for greening the SPPS process.

ers working in this area with a green approach should concentrate their work in recycling processes, in identifying the best solvents for coupling of protected peptides, and in downstream process.

Experimental part

Materials and methods

PuroSynth CTC-resin was used for all syntheses. All reagents and solvents were obtained from commercial suppliers and were used without further purification unless otherwise stated. Analytical HPLC was performed on an Agilent 1100 system using Chemstation software for data processing. Column: Phenomenex Luna 5 μm C₅, (50 $\times$ 4.6) mm, 100 Å column, with flow rate of 1.0 mL min^{−1}, 30 °C column oven, and UV detection at 300 nm. Mobile phase A: 0.1% TFA in H₂O; mobile phase B: 0.1% TFA in CH₃CN. 5–95% in 15 min gradient

elution was considered in all peptides. However, for long peptides (10 AAs and more), the eluting gradient was 60–100% in 10 min, and continued at 100% of mobile phase B for 10 more min. LC-MS was performed on Ultimate™ 3000, Aeris™ 3.6 µm-wide pore column, Phenomenex C₁₈ (150 × 4.6 mm) column. Buffer A: 0.1% formic acid in H₂O; buffer B: 0.1% formic acid in CH₃CN.

Peptide synthesis

CTC-resin was swelled in 2-MeTHF for about 10 min. The Fmoc-amino acids (2 equiv.) were dissolved in 2-MeTHF (0.5 mL per 100 mg resin) and sonicated for 10 min. *N,N*-Diisopropylethylamine (DIEA) (4 equiv.) was then added to the solution, which in turn was added to the previously swollen resin and allowed to react for 1 h under mechanical shaking.¹¹ After this, MeOH (80 µL per 100 mg of resin) was added to endcap any unreacted chloride of the CTC-resin. Next, peptides were synthesized manually following a protocol developed in our laboratory based on Fmoc/*t*Bu-AA-OH, *N,N*-diisopropylcarbodiimide (DIC), OxymaPure (5 equiv. each) in GVL, and shaking for 30 min (syntheses were not optimized). Fmoc was removed using 20% freshly prepared 4-methylpiperidine in GVL. For characterization of the model peptides included in this study see Fig. S39–S41.†

Peptide cleavage protocol

Peptide resin was placed in a syringe fitted with porous polyethylene disc. It was then swelled with the corresponding green solvent for 10 min. The solvent was then filtered off, the cleavage solution (600 µL, 2% TFA in the green solvent) was added per 30 mg of the peptide resin, and the syringe was closed with a cap and was shaken for 30 min at rt. Finally, the filtrate was collected over CH₃CN (4.0 mL), and the cleaved resin was washed 4 times with CH₃CN (1 mL each time). The filtrates were again collected in the same tube. An aliquot (5 µL) was then injected into the HPLC system. Finally, to check the efficiency of the cleavage protocol, the resin was dried and treated with TFA-TIS-H₂O (95:2.5:2.5) (600 µL). The same work-up was followed, and the resulting solution was monitored by HPLC.

Peptide precipitation protocol

The same cleavage protocol was followed, but the filtrate was collected over cold water to precipitate the peptide directly. The sample was allowed to settle in the fridge until the peptide had completely settled down. It was then centrifuged. Next, the supernatant was removed, and the resulting peptide was washed twice with water and then dissolved in CH₃CN-H₂O (1:99). Finally, freeze-drying was carried out accordingly.

Conflicts of interest

There are no conflicts to declare.

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