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ORIGINAL ARTICLE

Heating and microwave assisted SPPS of C-terminal acid peptides on trityl resin: the truth behind the yield

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Abstract Despite correct purity of crude peptides prepared on trityl resin by Fmoc/tBu microwave assisted solid phase peptide synthesis, surprisingly, lower yields than those expected were obtained while preparing C-terminal acid peptides. This could be explained by cyclization/cleavage through diketopiperazine formation during the second amino acid deprotection and third amino acid coupling. However, we provide here evidence that this is not the case and that this yield loss was due to high temperature promoted hydrolysis of the 2-chlorotrityl ester, yielding premature cleavage of the C-terminal acid peptides.

Keywords Peptide synthesis · Microwave heating · Diketopiperazine · Fmoc/tBu strategy · 2-Chloro chlorotrityl resin

Introduction

Peptides are attracting increasing attention as therapeutics due to their role as mediators of key biological functions associated with their low toxicity and high specificity. In 2011, more than 60 peptide drugs were on the market, and nearly 600 are still in clinical development. Part of the success in developing peptides as drug candidates is due to the easy automation of solid phase peptide synthesis (SPPS). Automated SPPS improves the speed of synthesis and increases the crude purity using excess of reactants that can be easily removed by filtration. The Fmoc strategy is preferred over the Boc strategy for routine SPPS as it does not require harsh acidic treatment to release the peptide from the support at the end of the synthesis (Amblard et al. 2006, *Fmoc Solid Phase Synthesis: A Practical Approach,* Ed. W. Chan, P. White, Oxford Press 2000). Consequently, most of the peptide synthesizers operate in Fmoc/tBu SPPS. Along with automation, many improvements were made to develop more efficient methods for SPPS involving new solid supports (García-Ramos et al. 2010), linkers, coupling reagents (El-Faham et al. 2009; Subiros-Funosas et al. 2009), anti-aggregation agents (Nicolas et al. 1997) and novel procedures (Higa et al. 2010; Yoshiya et al. 2010). First used in organic synthesis, microwave (MW) heating also impacted the peptide chemistry field, in particular since the introduction of the first dedicated peptides MW-assisted synthesizer (Collins and Collins 2003). A lot of examples of MW-assisted SPPS can now be found in literature (Pedersen et al. 2012). Despite several speculations about a putative direct MW effect on the growing peptide chain, it is now widely admitted that the significant improvement in speed and purity of the synthesis is only due to thermal effects (Bacsa et al. 2008a, b; Kappe 2004). Coupling and deprotection are generally performed around 70 °C leading to an overall 50-fold increase of kinetics. In these conditions, special care should be taken to avoid intramolecular side reactions, which are favored

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at higher temperature. The main concern is probably epimerization of activated amino acids, which proceeds mainly via oxazolone formation. Fortunately, epimerization is still maintained at low levels (<0.2 %) for standard MW-mediated coupling steps (i.e., 70 °C heating for 10 min). However, His, Cys and Asp residues, which are more sensitive to epimerization, have to be coupled at lower (50 °C) or even room temperature (Palasek et al. 2007). Indeed, loss of chiral integrity proceeds also through side chain-dependent mechanisms, i.e., imidazole N-π proton abstraction, enol form stabilization and epimerized aspartimide hydrolysis, respectively (Amblard et al. 2006). In the later case, aspartimide and related side products (α and β piperidimide, β-branched peptide, epimerized side products) appear during Fmoc removal cycles. It has been shown that replacing piperidine by piperazine lowers the proportion of undesired compounds (Palasek et al. 2007).

Surprisingly, although the crude purity of peptides obtained by MW-assisted SPPS is usually reported (Pedersen et al. 2012), yield data are difficult to find. The recovery of a sufficient amount of peptide after RP-purification to undergo further studies is often the only concern of chemists. However, we noticed that SPPS yields are sometimes alarmingly low despite a rather good crude purity. This paradox can sometimes be explained by an incomplete cleavage of the linker or a non-covalent entrapment of the cleaved materials within the polymer matrix. In this case, extensive washings and/or prolonged cleavage procedure may solve the issue. Unfortunately, we observed that isolation with good yields of C-terminal carboxylic acid peptides synthesized by Fmoc/tBu SPPS on trityl linker under elevated temperatures was still troublesome. One plausible explanation could be the diketopiperazine (DKP) formation, a well-known side reaction happening at the stage of Fmoc removal from the dipeptidyl-resin. The intramolecular attack of the free amino group on the C-terminus ester bond with the linker leads to the cyclization-cleavage of the 6-membered heterocycle and the release of the resin linker as alcohol counterpart. This results in a significant decrease of the yield (Gisin and Merrifield 1972). One cannot exclude the possibility of hydrolysis of the trityl ester bond, well studied for trityl acetate esters in aqueous solution at RT which leads to low but detectable amount of trityl carbocation formation through an S_N1 mechanism. Nevertheless, nothing was reported on solid phase synthesis at elevated temperature. Herein, we investigated the impact of heating on 2-Cl trityl resin, examining the quantity, the nature and the mechanism leading to the release of growing peptide chains during the synthesis of C-terminal carboxylic acid peptides.

Materials and methods

The following abbreviations were used: DCM dichloromethane, DIEA diisopropylethylamine, DBF dibenzofulvene, DKP diketopiperazine, DMF *N,N*-dimethylformamide, MSA methane sulfonic acid, MW microwave, HBTU *O*-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, HFIP hexafluoroisopropanol, HOBt hydroxybenzotriazole, pip piperidine, NMP *N*-methyl pyrrolidone, Pbf 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl, TFA trifluoroacetic acid, TFE trifluoroethanol. Other abbreviations used are recommended by the IUPAC-IUB Commission.

DMF, anhydrous DMF, amine-free DMF were obtained from Sigma–Aldrich with the following specifications:

DMF	Assay spec ≥99 %; impurity ≤0.1 % (H_2O)
Anhydrous DMF	Assay spec ≥99.8 %; impurity ≤0.005 % (H_2O)
Amine-free DMF	Assay spec ≥99.9 %; free amine as dimethyl amine <8 ppm; acid ≤0.005 meq/g; base ≤0.0003 meq/g; evaporation residue <0.0005 %

NMP was obtained from SDS with the following specifications: assay spec ≥99 %; impurity ≤0.5 % (H_2O); evaporation residue = 0.5 %; acid (CH_3COOH) = 0.06 %.

All other solvents were obtained from Carlo Erba and were used without purification. Protected amino acids, resins and HBTU were purchased from Iris Biotech GmbH. Other reagents were purchased from Aldrich and Lancaster.

Peptide synthesis

Microwave-assisted peptide syntheses were performed using Fmoc/tBu SPPS strategy on a Liberty™ Microwave Peptide Synthesizer (CEM Corporation, Matthews, NC) providing MW irradiation at 2,450 MHz. Temperature consign was set up at 70 °C.

First amino acid anchoring on 2-chloro chlorotrityl PS resin, example of Fmoc-Ala-OH

The 2-chloro chlorotrityl PS resin (1.00 g, 1.22 mmol, initial theoretical loading = 1.22 mmol/g) was conditioned for 15 min in DCM. Then, a 10 mL DMF solution of Fmoc-Ala-OH (3 eq., 3.66 mmol, 1.14 g) and DIEA (5 eq., 6.1 mmol, 788 mg, 1,063 mL) was poured onto the resin and stirred overnight. The resin was filtered and then washed with DMF (2×), DCM/MeOH 1/1 v/v (2×), DMF (2×), DCM (2×) and dried overnight in vacuo.

Coupling

On a 0.1 mmol resin scale, according to resin initial loading, coupling reactions were performed with 5 eq. of amino acid (0.5 mmol, 2.5 mL of 0.2 M solution in DMF), 5 eq. of HBTU (0.5 mmol, 2.5 mL of 0.2 M solution in DMF), and 10 eq. of DIEA (1 mmol, 0.5 mL of 2 M NMP solution). The resin was stirred for 90 min at room temperature or heated at 70 °C under MW irradiation (50 W). The resin was filtered and then washed with DMF (2×) and DCM (2×).

Fmoc deprotection

Fmoc removal was carried out in DMF/pip 8/2 v/v solution for 3 min under stirring, at room temperature. The resin was filtered and a DMF/pip solution was added again and stirred for 20 min at room temperature or heated (50 W, 70 °C). In both cases, the resin was filtered and then washed with DMF (2×) and DCM (2×).

Determination of resin loading by titration of dibenzofulvene released by Fmoc deprotection

1–2 mg of dried resin were weighted precisely and poured into 4 mL piperidine/DMF (2/8, v/v) solution, which was stirred for 1 h. The solution was then filtered and optical density of dibenzofulvene-piperidine adduct was measured at 299 nm in 1 cm wide quartz glass chamber (WPA Biowave II spectrometer). Piperidine/DMF (2/8, v/v) solution was used as reference. The chamber was emptied and cleaned and measurements repeated twice with fresh solutions. The loading was calculated with the average value of three readings into the following equation.

$$\text{Loading}(\text{mmol/g})_{299\,\text{nm}} = A \times v / (\varepsilon \times l \times m)$$

with ε (molar extinction coefficient) = 6,234 M^{-1} cm^{-1}, l (quartz glass cuvette length) = 1 cm, v (volume) = 4.10^{-3} L, m (weight of resin) = 1–2 mg, A = average value of the 3 readings of absorbance.

Maximal theoretical loading (noted 'loading theo. Max.' in Fig. 2) mathematically decreased while peptide weight increased on the solid support. It was calculated on the basis of molecular weight increment according to the following formula:

$$\text{Maximal theoretical loading} = \text{initial loading} / [1 + (\text{initial loading (mmol/g)} \times \text{molecular weight increment (g/mmol)})]$$

Cleavage of 2-Chloro-chlorotrityl-peptidyl resin to yield fully deprotected peptides

Resin (0.1 mmol scale, 228 mg) was placed in 10 mL of cleavage cocktail (TFA/water/triisopropylsilane 95/2.5/2.5 v/v/v) and stirred for 2 h. After removal of the resin by filtration, cleavage cocktail was concentrated using a vacuum centrifugator EZ2+ genevac. The deprotected peptide was precipitated three times in diethyl ether, dissolved in an acetonitrile/water 50/50 v/v solution containing 0.1 % TFA and freeze dried.

Results and discussion

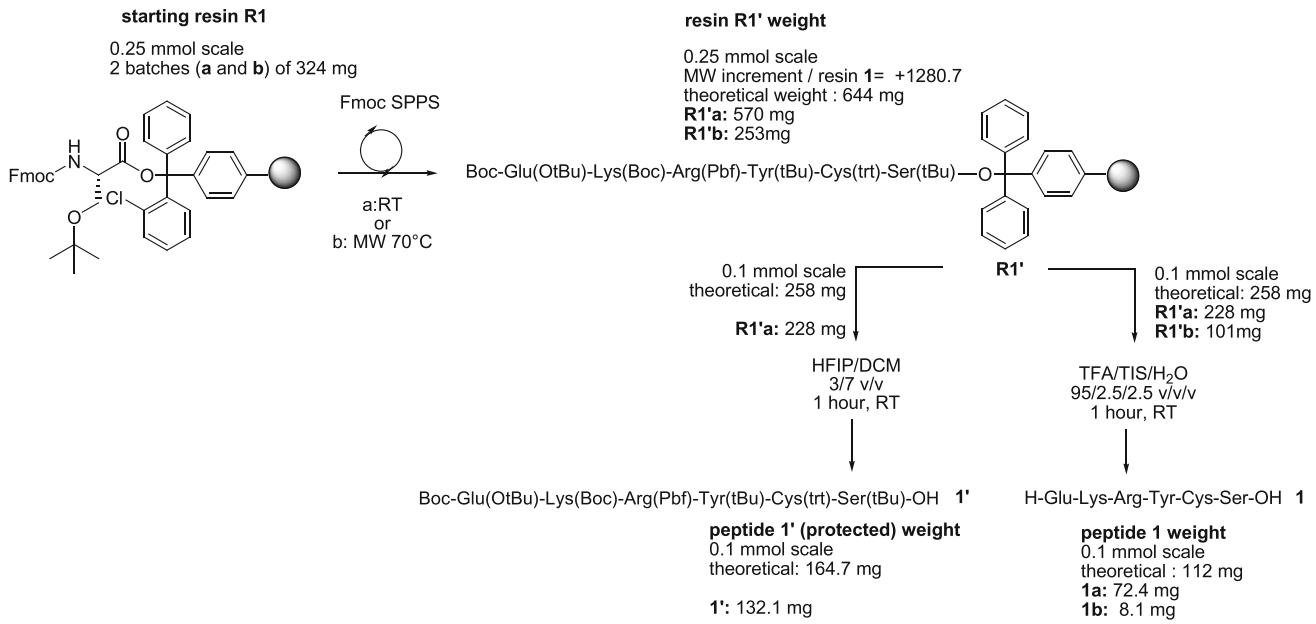
Fmoc-SPPS on 2-chloro chlorotrityl PS resin: yield comparison at RT and at 70 °C

Three peptides were selected for a comparative study of yields: a hexapeptide of sequence H-Glu-Lys-Arg-Tyr-Cys-Ser-OH (**1**); the 11-mer peptide β-amyloid 25−35 (**2**) and the 13-mer factor α (**3**). Identical batches of preloaded 2-chloro chlorotrityl PS resin were used for Fmoc-SPPS with or without MW heating (70 °C). Identical protocols were used in both cases and the whole synthesis was performed with the same automated synthesizer CEM liberty. For more clarity, compounds and resins obtained at room temperature without MW heating were noted with an '**a**' suffix, whereas materials submitted to MW irradiation are noted with a '**b**' suffix.

We first performed the synthesis of peptide **1**: Fmoc-Ser(tBu)O-2-chlorotrityl PS resin **R1** (0.25 mmol, 324 mg, 0.722 mmol/g) was used for two syntheses with or without MW heating; resulting in peptidyl resins **R1′b** and **R1′a,** respectively (Scheme 1).

Before TFA-mediated cleavage, the two batches of resin **R1′a** and **R1′b** [Boc-Glu(OtBu)-Lys(Boc)-Arg(Pbf)-Tyr(tBu)-Cys(trt)-Ser(tBu)-O-2-chlorotrityl-PS] were lyophilized and carefully weighted. Calculated on the basis of molecular weight increment, the maximal theoretical weight of resin was 644 mg. The first evidence was that the weight obtained for resin **R1′b** (253 mg) was significantly lower than **R1′a** (570 mg) and very close to the weight of the totally cleaved trityl resin, which should be 228 mg.

These results were confirmed by weighting the peptide cleaved from the resin. Samples of resin (101 mg of **R1′b** and 228 mg of **R1′a**) corresponding to a theoretical scale of 0.1 mmol were cleaved with TFA/TIS/water solution (95/2.5/2.5 v/v/v). After freeze drying, only 8.1 mg of peptide (**1b** calc. yield = 7.2 %) were obtained versus 72.4 mg for **1a** (calc. yield = 64.6 %). As expected,



Scheme 1 Synthesis of peptide **1** H-Glu-Lys-Arg-Tyr-Cys-Ser-OH on 2-chloro chlorotrityl resin with/without heating (**1b/1a**) and mild cleavage to yield protected peptide **1′**

Table 1 Model peptides and corresponding yields synthesized on 2-chloro chlorotrityl PS resin with/without heating (a/b)

Cmpd #	Sequence	MW (g/mol)	Yield (%) (RT)	Yield (%) (70 °C)
1	H-EKRYCS-OH	784.8	**1a**, 64.6	**1b**, 7.2
2	H-GSNKGAIIGLM-OH	1,060.3	**2a**, 95	**2b**, −/2.6*
3	H-WHWLQLKPGQPMY-OH	1,684.0	**3a**, 91	**3b**, −/0.5*

* No peptide was recovered with coupling/deprotection time of 90 min/20 min. Yields presented are obtained with 45 min/10 min coupling/deprotection cycles

the crude peptides analyzed by RP-HPLC (Fig. 1) showed very similar patterns with 66 % purity (determined by peak integration at 214 nm).

The same weightings and calculations were performed during the synthesis of peptides **2** and **3**, (0.17 mmol scale). The results were even more striking with these two longer peptides. Indeed, while satisfactory yields were obtained for **2a** and **3a** (95 and 91 %, respectively), no peptide was recovered after cleavage from resins **R2′b** and **R3′b**. The syntheses were performed again, halving the duration of coupling and deprotection times at 70 °C (10 min deprotection and 45 min coupling steps). In this case, expected peptides were obtained in very low yields (**2b** 2.6 %, **3b** 0.5 %) (Table 1).

The very low yield obtained after MW-SPPS could not be explained by a failure of the synthesis as the HPLC profiles were similar (Supporting Information) and did not display any truncated peptides. The phenomenon could only be explained by an undesired premature cleavage of the peptide from the trityl resin, happening during the synthesis and related to the overall heating duration as demonstrated by the yield improvement when the reaction times were reduced by 50 %. Thus, we speculated that the trityl ester bond was not totally stable at 70 °C, resulting in a continuous undesired thermal cleavage during the course of the synthesis. To afford the proof and to investigate the mechanism of cleavage, we worked on a simple tripeptide model Fmoc-Leu-Phe-Ala-OH.

Stepwise thermal cleavage experiments on the model tripeptide Fmoc-Leu-Phe-Ala-OH

We needed to investigate if the loading loss was happening in a continuous manner during the course of the synthesis or if it was stressed at a particular step. Fmoc-Phe-Ala-OH **5′**, Fmoc-Leu-Phe-Ala-OH **7′** and H-Leu-Phe-Ala-OH **7″** were synthesized as reference compounds. Solutions of **5′**, **7′**, **7″**, DBF and commercial Fmoc-Ala-OH **4′** at different concentrations (ranging from 5 µM to 50 mM) were prepared and analyzed by RP-HPLC. Peak areas were recorded and calibration curves were drawn to determine the molar absorption coefficient of each compound (Supporting Information). In addition, DKP c[Ala-Phe] **8** was also prepared in solution and purified (see Supporting Information for the synthesis). c[Ala-Phe] DKP **8** coeluted with the DMF peak in RP-HPLC. As a consequence, we planned to use LC/MS ESI + SIR analyses to quantify c[Ala-Phe] DKP in DMF solutions during second deprotection and third coupling steps.

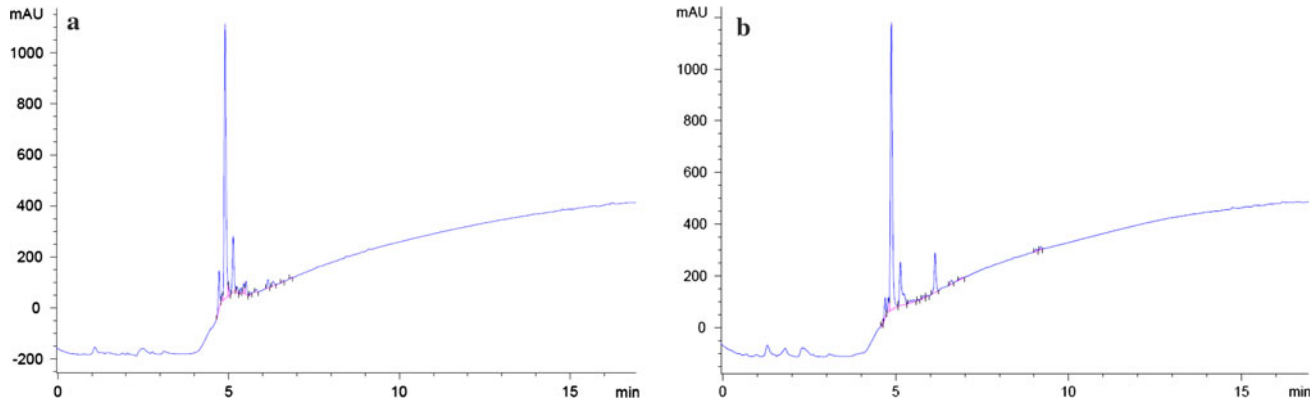


Fig. 1 Chromatograms of **a** (*right*) and **b** (*left*) recorded at λ = 214 nm in conditions B (see Supporting Information)

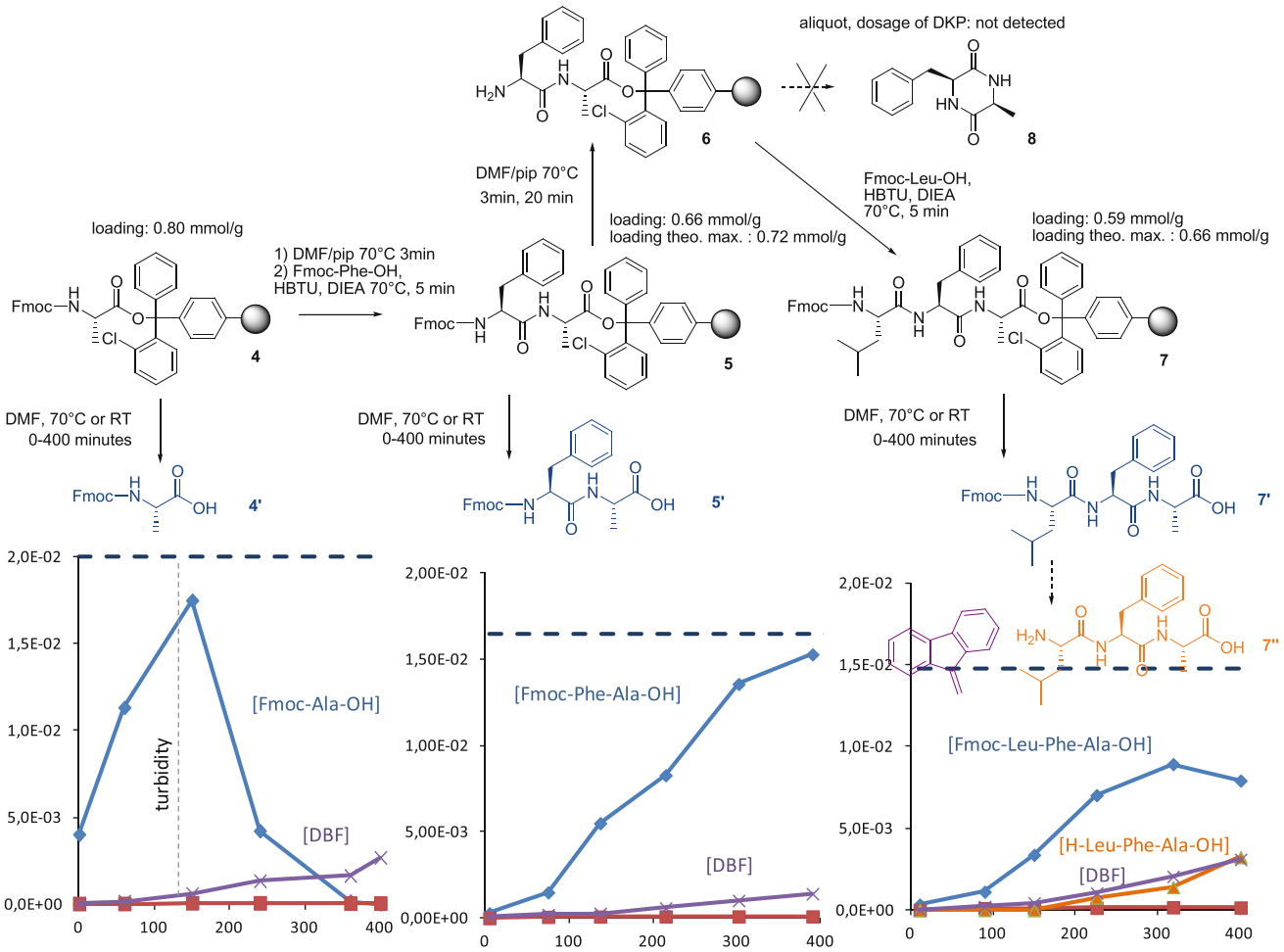


Fig. 2 Thermal cleavage experiments in DMF. Synthesis of Fmoc-Leu-Phe-Ala-O-2-chlorotrityl PS resin. *filleddiamond* [Fmoc-peptide] 70 °C; *filledsquare* [Fmoc-peptide] RT; × [DBF] 70 °C; *filledtriangle* [H-Leu-Phe-Ala-OH] 70 °C; *dashed line* maximal concentration calculated from resin loading

Different solutions of **8** with various concentrations (5 µM to 50 mM) were prepared and integration of the peak area expressed in number of ions at m/z = 219.2 allowed to draw a calibration curve. A good correlation was obtained ($R^2 = 0.9992$). With all these references in hand, we planned several experiments (Fig. 2).

Starting from resin **4**, Fmoc-Ala-O-2-chlorotrityl PS (1 g, 0.80 mmol/g, 0.8 mmol), tripeptide **7** was prepared using standard MW-SPPS conditions ("Materials and methods"). Before each deprotection step, a small amount of resin was freeze dried and weighted to perform an UV DBF-pip adduct titration (see "Materials and methods"). Compared to theoretical maximum loading, the experimental values were lower (0.66 vs. 0.72 mmol/g and 0.59 vs. 0.66 mmol/g at the stage of di and tripeptides, respectively). These measurements were in accordance with our hypothesis of loading loss. Along with this titration, two aliquots of 100 mg of resin were collected and poured into 4 mL of DMF. Samples were heated at 70 °C or left at room temperature on the bench for 400 min. Thanks to calibration curves, compounds eventually released in solution from the resin beads were detected and quantified.

In the samples left at RT (Fig. 2, *filledsquare*, red curves), very low concentrations of peptides were detected after 400 min. A maximum of 1.4×10^{-4} M was measured, which corresponded to less than 1 % cleavage (curves presented in Supporting Information). On the contrary, increasing concentrations of Fmoc-protected peptides were detected at 70 °C (Fig. 2, *filleddiamond*). The thermal cleavage phenomenon was significant enough to lead to a loss of half the loading within about 220 min (50 % after 215 min for dipeptide **5′** and 48 % after 225 min for tripeptide **7′**). This cleavage was even faster in the case of resin **4** for which 87 % of Fmoc-Ala-OH **4′** was released after 150 min. Noteworthy, after 2 h heating, cleavage of the peptide from the resin was concomitant with some extend of Fmoc removal, which led to increase the DBF concentration in the sample (Fig. 2, ×, purple curves). In sample containing resin **4**, a white turbidity and a slight precipitate appeared after two hours. In the case of resin **7**, a decrease of protected peptide concentration was detected after 300 min heating along with increasing concentrations of unprotected tripeptide **7″** (Fig. 2, *filledtriangle*).

Complementary experiments were also performed with higher DMF qualities (anhydrous DMF, amine-free DMF) and N-methyl pyrrolidone as well (See "Materials and methods"). No significant differences were observed compared to standard DMF (data not shown).

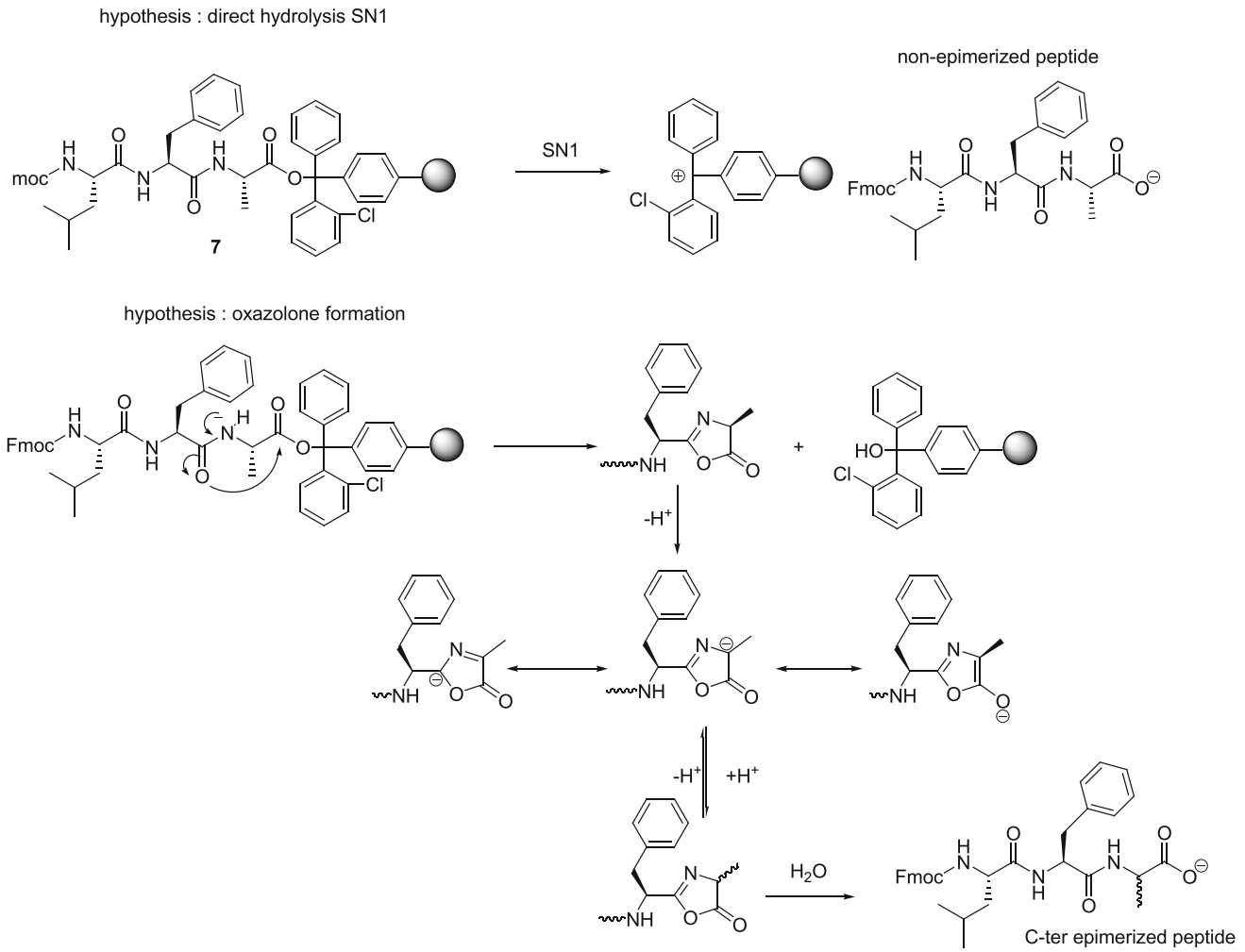
We also checked that this cleavage was specific of the 2-Chlorotrityl linker. For that purpose, Fmoc-Ala-OH was anchored on hydroxymethylphenoxy PS (Wang) resin (0.75 mmol/g) and Rink amide aminomethyl PS (0.96 mmol/g). Resin samples were submitted to the same treatment as resin **4**. Even after 400 min, the release of Fmoc-Ala-OH (or Fmoc-Ala-NH_2 in the case of Rink amide linker) was not detected (data not shown).

To complete this study, we investigated the extent of a putative cyclization-release of the peptide through the intermolecular DKP formation. DKP cyclization happens in basic conditions at the stage of Fmoc removal from the dipeptidyl-resin or during the subsequent coupling step. The intramolecular attack of the free amino group on the C-terminus ester bond leads to the cyclization-cleavage of the DKP and the release of the resin linker as the alcohol counterpart (Gisin and Merrifield 1972). For structural reasons, DKP is favored by Gly/Pro containing sequences, N-alkyl amino acids and D/L alternation. Benzyl ester related linkers such as hydroxymethylphenoxy, and particularly electron withdrawing hydroxymethylbenzamido, also favor this side reaction. Bulkier 2-Chlorotrityl linkers have been designed to prevent this side reaction and gave satisfactory protection against this premature cleavage. But we cannot exclude that heating, which favors intramolecular reaction, could lead to DKP formation on trityl linker. This was verified by stopping the MW synthesizer at the stage of resin **6**, after 3 min of DMF/pip (8/2, v/v) solution treatment (10 mL), which removed the Fmoc protecting group at the N-terminus of phenylalanine residue. An aliquot (20 µl) of the solution was taken and the reactor was emptied. Then, a second deprotection cycle of 20 min was carried out and an aliquot was also taken. A complete DKP formation on the remaining resin in the reactor would have resulted in a 48 mM concentration of c[Ala-Phe] when a 1 % DKP formation would have corresponded to 480 µM concentration in the sample. The two LC/MS analyses did not reveal any significant amount of c[Ala-Phe] in any DMF/pip solution (<0.1 %). This indicated that the 2-Clorotrityl linker was probably bulky enough to prevent DKP formation at 70 °C on a dipeptide sequence composed of amino acids with the same stereochemistry.

To the best of our knowledge, neither the effect of temperature on hydrolysis of trityl esters nor the study of peptidyl trityl ester on solid support has been investigated. However, the mechanism of trityl acetate hydrolysis in aqueous solutions has been determined (Bunton and Konasiewicz 1955). Bunton et al. used ^{18}O as isotopic tracer in aqueous dioxane to highlight the fission of the alkyl-oxygen bond leading to the stabilized trityl carbocation. In addition, solvolysis rates of trityl acetate were determined in MeOH, EtOH, acetone and acetic acid (Swain et al. 1960) and more recently in water/acetonitrile (1/1 v/v) at 25° C (Horn and Mayr 2010a, b). These studies demonstrated that trityl ester hydrolysis occurred as a classical S_N1 reaction: carbocations are formed as short-lived intermediates, immediately trapped by the solvent.

Even if we could reasonably propose that the same mechanism operates on peptidyl trityl ester bonds on solid support, we cannot exclude that the peptide cleavage proceeds through oxazolone formation (Scheme 2).

We could hypothesize that basic conditions during coupling and deprotection cycles promote oxazolone



Scheme 2 Hypotheses of trityl ester hydrolysis: SN1 or oxazolone formation?

formation. This well-known intramolecular reaction occurs upon acid carboxylic activation and is responsible of epimerization at the C-terminal amino acid of a peptide sequence (Amblard et al. 2006; Bergmann and Zervas 1928). Often observed during peptide fragment coupling, epimerization is initiated by abstraction of the proton on the Cα, favored by resonance forms of oxazolone anion (Neuberger 1948). The case studied in this paper is very different from what happens classically during activation of a C-terminus peptide: trityl is neither electro withdrawing nor a good leaving group. However, we cannot exclude that temperature could promote this reaction. Thus, if oxazolone formation is responsible of trityl ester bond cleavage, epimerization should be detected. To that purpose, **7′(D)** (Fmoc-Leu-Ala-(D)Phe-OH), a diastereoisomer of **7′**, was synthesized as reference compound. We first checked that diastereoisomers **7′** and **7′(D)** were separated by C_{18} RP-HPLC using slow gradient conditions (Supporting Information). Upon heating of resin **7**, only non-epimerized (L) peptide **7** was detected, as demonstrated by the co-injection of reference **7′(D)** and heated solution from resin **7**.

Taken all together, these experiments demonstrated clearly that the trityl ester bond was not stable enough upon heating, undergoing a S_N1 hydrolysis during MW-assisted SPPS. Of course, typical reaction times for deprotection (3 min) and coupling (7–10 min) using MW irradiation were much shorter than reaction times reported in Fig. 2. However, during the synthesis of a 20-mer for example, the cumulative heating time will easily reach 200 min, the average duration for which ~50 % cleavage was observed for short model peptides **4′**, **5′** and **7′**.

At last, we decided to evaluate the possibility to take profit of this thermal instability of the trityl linker ester bond to synthesize fully protected peptides C-terminal carboxylic acid peptides avoiding acidic treatment.

Non-acidic cleavage of 2-Chlorotrityl resin to yield C-terminal carboxylic protected peptides

Trityl linkers are particularly attractive for convergent synthesis of larger peptides (Lloydwilliams et al. 1993; Benz 1994). 2-Chloro chlorotrityl is the linker of choice to yield C-terminal carboxylic acid peptide fragments, which can be activated to be coupled with the free N terminus of another fully protected fragment. Hyper acid sensitive linkers such as 2-chloro chlorotrityl are usually used in Fmoc/tBu SPPS but instead of using concentrated TFA solution to cleave the side chain protecting groups along with the ester bond of the linker, milder acidic conditions such as 1 % TFA in DCM, 10 % AcOH (Barlos et al. 1991), 10 % TFE (Hoekstra 2001), or HFIP (Bollhagen et al. 1994) are used. As an alternative to such acidic treatment, we propose that heating a 2-chlorotrityl resin would release the protected peptide in neutral conditions. Peptide **1′** [Boc-Glu(OtBu)-Lys(Boc)-Arg(Pbf)-Tyr(tBu)-Cys(trt)-Ser(tBu)-OH, MW 1,646.8 g/mol, Fig. 3] is the protected analogue of peptide **1**. It was chosen for this study as it displays most of the common protecting groups used in Fmoc/tBu strategy, which should be kept intact on the peptide chain during cleavage. 228 mg of resin **R1′a** were treated with a HFIP/DCM 3/7 v/v solution at RT for 1 h. After concentration in vacuo of the cleavage cocktail, 132.1 mg of the protected peptide **1′** were obtained (86 % purity, 80 % yield).

Protected peptide **1′** was purified by preparative RP-HPLC (61.7 mg, 37 % overall yield) and different solutions of pure peptide were prepared (10 µM to 1.5 mM) and analyzed by RP-HPLC at $\lambda = 214$ nm (conditions B, Supporting Information). Peak areas were recorded and a calibration curve was drawn to determine the molar absorbance coefficient of **1′** (Supporting Information).

Another aliquot of 100 mg of resin **R1′a** in 4 mL DMF was placed in a MW reactor and heated at 70 °C for 21 cycles of 1 h. Between each cycle, 10 µl were taken from the reactor and analyzed by HPLC to monitor the thermal cleavage of peptide **1′**. First of all, despite careful examination and ESI LC/MS SIR analyses, no partially deprotected peptide was detected, indicating as expected, that side chain protecting groups such as pbf, trityl thioether, terButyl, Boc were stable at 70 °C, even for prolonged periods of time (21 h). More importantly, the protected peptide **1′** was released in a time-dependent manner at 70 °C in DMF without the use of any other reagent (Fig. 3). According to HFIP treatment, cleavage of 100 mg of resin **R1′a** yielded 57 mg of the protected peptide **1′** in the 4 mL reactor, which corresponded to a maximal concentration of 8.7 mM. Noteworthy, the release was slower than what was observed with di and tripeptides. Indeed, a concentration of 3.4 mM was measured after 21 cycles,

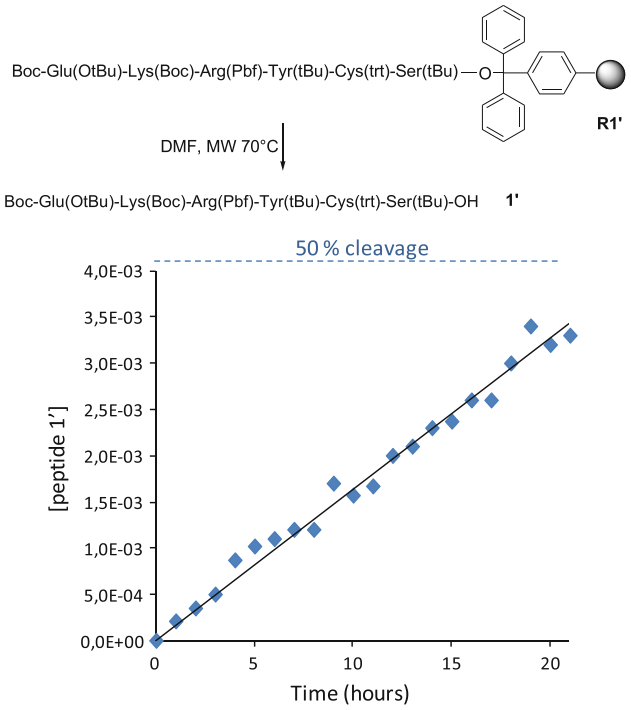


Fig. 3 Thermal cleavage of protected peptide **1′** from 2-chlorotrityl peptidyl resin **R1′a** in DMF at 70 °C

which corresponded to 39 % cleavage. Heating was extended to 72 h and a concentration of 8.4 mM was detected in the reactor, showing that it was possible to quantitatively cleave peptide **1′** from the trityl resin. However, cleavage solutions turned slightly yellow and the presence of trityl (<10 %) removed from the side chain of the cysteine residue was also detected.

Conclusion

With no doubt, since its introduction in SPPS, MW heating has proved to be a useful tool for peptide chemists, at least for speeding up the synthesis, reducing the synthesis of a 20-mer from a couple of days to a handful of hours. In most cases, cleavage yield is not an issue for the chemist as far as the crude purity is good enough to recover the desired amount of peptide suitable for biological screenings. Indeed, the scale of the syntheses performed in research laboratories (and in most of automated synthesizer reactors) fulfills perfectly the needs of in vitro or preliminary in vivo assays (generally ~0.1 mmol). However, the use of 2-chlorotrityl resins for the synthesis of C-terminal carboxylic acid peptides in MW-assisted SPPS is accompanied by particularly low yields, which come from a direct thermal hydrolysis of the trityl ester bond throughout the course of the synthesis, releasing amount of the growing peptide chain in solution. For the shortest model

peptides without side chain protecting groups described in this study, total cleavage was achieved in less than 6 h. For protected hexapeptide **1′**, complete cleavage required 48 h. On the light of these results, correct yields could not be expected with trityl resin when prolonged heating times are required (e.g., long peptides, double coupling procedures). When possible, hydroxymethylphenoxy linker (HMPA or Wang resin in the case of PS matrixes) has to be privileged. We demonstrated that the benzyl ester bond connecting the C-terminus of peptides and the linker was stable at 70 °C at least over 24 h. Of course, when fully protected peptides (for convergent synthesis or head to tail cyclization) or peptides particularly prone to DKP formation have to be prepared, hydroxymethylphenoxy linkers cannot be chosen and the synthesis has to be performed on trityl linker at RT.

It is possible to get advantage of the thermal instability of trityl-peptidyl resin to cleave in neutral conditions a fully protected peptide. However, due to long reaction times that could be required (>48 h), this method cannot replace systematically mild acidic cleavage (e.g., 1 % TFA) and a careful monitoring of the released peptide has to be set up to ensure that side chain protecting groups are not partially removed.

Conflict of interest The authors declare that they have no conflict of interest.

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