Towards Cost-Efficient, Scalable Green SPPS

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Introduction

Over the last half a century or so the awareness of the profound impact of Industrial Revolution on Earth's ecology has been steadily increasing.[1] For example, in recent years there has been a considerable focus on use of green chemistries in manufacturing of chemicals and pharmaceuticals.[2] As the solvent consumption in these industries is enormous a specific point of emphasis has become the utilization of greener solvents[3] and various green solvent guidelines have been put forth.[4] In the field of synthetic peptides great advances have been made in the past decades while the impact of peptide chemistry on the environment has remained largely unaddressed.[5] In fact, the vast majority of amide bond formations are still carried out in DMF and CH_2Cl_2, two solvents that have been discussed as being questionable in sustainable chemical processes.[4,6-7] In the realm of synthetic peptides SPPS constitutes a prevalent methodology and several reports on greening of SPPS have appeared.[8] Nevertheless, the reported green solid-phase peptide syntheses hinge on the use of solvents that are compatible with expensive PEG resins and require use of large excesses of AA raw materials in couplings or, entirely new sets of protected amino acid derivatives.[9] We set out to investigate a green SPPS methodology which would i) only employ environmentally benign solvents ii) utilize low excesses of AA raw materials iii) work well with less expensive PS resins iv) be compatible with standard Fmoc SPPS practices.[10] At the outset of this study we considered evaluating EtOAc which is an inexpensive industrial solvent, can be sourced renewably[4] and in solution phase amidations exhibits favorable reaction kinetics.[8] Nevertheless, swelling of PS resins in EtOAc is not as good as in DMF[11] and in a recent work by Jad et al., 2-MeTHF was found better suited for green SPPS protocols than EtOAc.[9b] On the other hand, during a recent evaluation of His couplings in SPPS we found that replacing DMF with a DMF/EtOAc mixture (1:1) was beneficial, resulting in a decrease of racemization and an increase of coupling rates.[12] We reasoned that replacing DMF with a greener polar aprotic solvent could result in a sustainable synthetic methodology while maintaining the favorable reactivity attributes observed with DMF/EtOAc. Herein, we report an assessment of two cosolvents for EtOAc in green Fmoc SPPS: N,N'-Dimethyl propylene urea (DMPU) and DMSO, both of which were classified as greener alternatives for DMF.[4]

Table 1. de'Fmocing of Fmoc-RAM AMS using 5% 4-MP/solvent for 5 min[1]

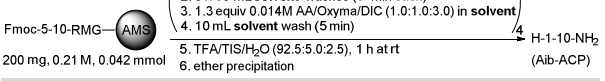
Entry	Solvent	Conversion (%)[2] 25 °C	Conversion (%)[2] 50 °C	Swelling (mL/g)[3]
1	DMF	90.5	99.3	5.8
2	EtOAc	5.9	36.7	5.6
3	2% DMPU/EtOAc	8.6	65.7	6.0
4	10% DMPU/EtOAc	18.2	84.0	6.2
5	50% DMPU/EtOAc	62.8	98.4	7.4
6	2% DMSO/EtOAc	13.7	62.0	5.2
7	10% DMSO/EtOAc	28.9	91.1	5.6
8	50% DMSO/EtOAc	91.9	99.1	4.5

[1] In red, conventional solvent (DMF); in green, green solvents; in yellow, satisfactory de'Fmocing conversions (>90%); RAM, Rink amide (Knorr) linker; AMS, aminomethyl PS/DVB(1%) resin; 0.66M Fmoc-RAM AMS resin was used throughout. [2]Conversions were obtained by determining the residual Fmoc content on the de'Fmoced resins using a literature method for Fmoc content determinations, see ref 31. [3]0.44M AMS, PS/DVB(1%) resin was used for the swelling determinations.

Results & Discussion

Before evaluating different green protocols in test peptide SPPS we examined kinetics of a model reaction on a PS resin in different solvents. To this end we opted to investigate de'Fmocing of an Fmoc-RAM AMS resin for which we used 4-methylpiperidine (4-MP)[13] as the base instead of piperidine (Pip) and to offset the slightly higher cost of the former, 4-MP was used at 5% v/v instead of 20% v/v which is customary for Pip.[14] Thus, while excellent rate of de'Fmocings was achieved with DMF (Table 1, entry 1) with EtOAc the kinetics of Fmoc removal were unsatisfactory even at elevated temperature (entry 2).[15] Nevertheless, we found that simply upon adding a polar cosolvent and/or altering the reaction temperature, suitable rates of Fmoc removal could be attained (entries 3 – 8). It is worth noting that DMSO/EtOAc (entries 6 – 8) exhibited higher rates of Fmoc removals than the higher swelling DMPU/EtOAc did (entries 3 – 5).[16] Having determined that favorable kinetics for reactions on PS resins in DMPU/EtOAc or DMSO/EtOAc could be obtained we turned to examine these solvent systems in a SPPS of a model peptide. We chose to assess the aggregation prone, hindered Aib-Aib motif containing Aib-ACP decapeptide (**1**)[17] which has been synthesized in good purity (87.1%) using a green SPPS protocol on a ChemMatrix resin but on a PS resin could only be accessed in 25.4% purity despite optimization and use of large excesses (3 equiv) of AA raw materials.[9b]

Table 2. Assessment of Val[1]-Aib[4] part of Aib-ACP SPPS[1]



Entry	Temp (°C)	Solvent: de'Fmocings (1)	Solvent: Couplings (3)	Solvent: Washes (2 & 4)	de'Fmocing time (min)	Coupling time (min)	HPLC purity (%)[2]: Aib-ACP	HPLC purity (%)[2]: des-Aib
1	40	50%DMPU/EtOAc	50%DMPU/EtOAc	DMF	15	25	37.8	46.1
2	40	20%DMPU/EtOAc	50%DMPU/EtOAc	DMF	15	25	37.1	45.7
3	40	50%DMPU/EtOAc	20%DMPU/EtOAc	DMF	15	25	51.1	32.2
4	40	20%DMPU/EtOAc	20%DMPU/EtOAc	DMF	15	25	50.1	33.1
5	50	50%DMPU/EtOAc	50%DMPU/EtOAc	DMF	15	25	45.2	38.5
6	50	20%DMPU/EtOAc	50%DMPU/EtOAc	DMF	15	25	40.9	40.9
7	50	50%DMPU/EtOAc	20%DMPU/EtOAc	DMF	15	25	55.2	26.8
8	50	20%DMPU/EtOAc	20%DMPU/EtOAc	DMF	15	25	51.9	29.6
9	55	20%DMPU/EtOAc	20%DMPU/EtOAc	DMF	2 x 15	25	73.1	6.3
10	55	20%DMPU/EtOAc	20%DMPU/EtOAc	DMF	3 x 15	25	74.7	6.1
11	55	20%DMPU/EtOAc	20%DMPU/EtOAc	DMF	2 x 15	2 x 25	77.6	1.6
12	55	20%DMPU/EtOAc	20%DMPU/EtOAc	DMF	3 x 15	2 x 25	76.4	1.9
13	50	10%DMPU/EtOAc	10%DMPU/EtOAc	DMF	2 x 15	25[2]	79.3	2.5
14	50	10%DMPU/EtOAc	10%DMPU/EtOAc	10%DMPU/EtOAc	2 x 15	25[2]	67.8	4.6
15	50	10%DMPU/EtOAc	10%DMPU/EtOAc	2%DMPU/EtOAc	2 x 15	25[2]	70.7	4.3
16	50	10%DMPU/EtOAc	10%DMPU/EtOAc	EtOAc	2 x 15	25[2]	73.3	3.3
17	55	10%DMPU/EtOAc	10%DMPU/EtOAc	2%DMPU/EtOAc	2 x 15	25[2]	69.8	4.7
18	55	10%DMPU/EtOAc	10%DMPU/EtOAc	2%DMSO/EtOAc	2 x 15	25[2]	73.2	3.1
19	55	10%DMSO/EtOAc	10%DMSO/EtOAc	2%DMPU/EtOAc	2 x 15	25[2]	78.8	2.3
20	55	10%DMSO/EtOAc	10%DMSO/EtOAc	2%DMSO/EtOAc	2 x 15	25[2]	79.6	2.3

[1] In red, conventional solvent (DMF); in green, green solvents; in yellow, satisfactory HPLC purities of the product (>75%); RMG, Ramage (tricyclic amide) linker; AMS, aminomethyl PS/DVB(1%) resin; in all couplings, 33.3% of DIC was added at the outset and the remaining 66.6% at t= 15 min; Fmoc-AA-OH derivatives were used for Aib[4]-Gln[2] couplings, Boc-Val-OH was used for Val[1] coupling; All final Boc-1-10 resins were *i*- PrOH washed (2 x 5 mL) and dried to constant weight *en vacuo* before proceeding to TFA cleavage (step 5). [2]Only Aib[4] and Aib[3] couplings were re-coupled (25 min). [3]HPLC analyses were carried out using Kinetex C18 column (30 °C, 50x4.6mm, 2.6um), TFA/H_2O (0.1:100, **A**), and TFA/MeCN (0.1:100, **B**) mobile phases, gradient of 40% B over 15 min and flow rate of 1.0 ml min^{-1}.

$$\text{H-Val}^1\text{-Gln}^2\text{-Aib}^3\text{-Aib}^4\text{-Ile}^5\text{-Asp}^6\text{-Tyr}^7\text{-Ile}^8\text{-Asn}^9\text{-Gly}^{10}\text{-NH}_2\ (\mathbf{1})$$

We set out to examine SPPS of **1** in a two-steps approach and i) carry out a series of small scale syntheses for the most difficult, Val[1]–Aib[4] part of the peptide ii) perform a large scale synthesis of the entire peptide based on the small scale experiments. Thus, our evaluation of Val[1]–Aib[4] SPPS commenced with an Fmoc-5-10 resin[18] and entailed an examination of temperature, solvents, as well as extent of de'Fmocings and couplings, respectively (Table 2). 5% 4-MP was used for all de'Fmocings and 1.3 equiv AA/Oxyma/DIC[19] was used for couplings throughout. To maximize the usage of the recyclable EtOAc solvent our aim was to find conditions in which minimal amounts of a polar aprotic cosolvent could be used without compromising the efficiency of the chemistries involved. Our strategy was to i) examine green solvents for the chemical steps while keeping DMF as the wash solvent ii) green the whole SPPS process upon identifying suitable green steps for the couplings and de'Fmocings. Thus, using EtOAc/DMPU as the solvent system we first probed the effect of temperature as well as the content of cosolvent (entries 1 – 8). While content of DMPU in de'Fmocings seemed to have only a marginal effect on the purity (entries 1, 3, 5, 7 vs 2, 4, 6, 8) decreasing the content of DMPU in couplings and increasing the temperature were both beneficial (entries 1, 2, 5, 6 vs 3, 4, 7, 8). Next, using 20% DMPU/EtOAc for all chemical steps the extent of both couplings and de'Fmocings was examined at 55°C (entries 9 – 12). While purities of the product for 2 x 15 min and 3 x 15 min de'Fmocings respectively were quite comparable (entries 9 and 11 vs 10 and 12) extending couplings from 1 x 25 min to 2 x 25 min resulted in an appreciable purity increase accompanied by a marked decrease in the content of the major des Aib impurity (entries 9 and 10 vs 11 and 12). We next tried to decrease the DMPU content even further (10%) which worked well with DMF as the wash solvent (79.3% product purity, entry 13) while resorting to green solvents for washes resulted in a purity drop to 68 – 73% (entries 14 – 16). In an attempt to improve the efficiency of the solvent washes we raised the temperature from 50 to 55°C while also examining EtOAc/DMPU vs EtOAc/DMSO as SPPS solvents (entries 17 – 20). We determined that 10% DMSO/EtOAc as the solvent for the chemistry (entries 19 and 20) worked better than the corresponding 10% DMPU/EtOAc syntheses did (entries 17 and 18). In fact, the experiment using 10% DMSO/EtOAc for chemical steps and 2% DMSO/EtOAc for solvent washes gave the highest purity of all conditions examined (entry 20, 79.6%). Based on the assessment of Val[1]–Aib[4] SPPS delineated in Table 2 we considered using the entry 20 conditions in a scale-up synthesis of the whole Aib-ACP model peptide. Nevertheless, DMSO as a SPPS solvent has been implied in the degradation of oxidation prone AAs[20] for which we set out to examine the propensity of AAs to decompose in EtOAc/DMSO before proceeding with the Aib-ACP scale-up.

Table 3. Stability of 0.1M Fmoc-Cys(Trt)-OH (Cys)[1]

Entry	Conditions	Loss of Fmoc (%)[2]: 48 h at 25 °C	Loss of Fmoc (%)[2]: 16 h at 50 °C	Formation of other byproducts (%)[2]: 48 h at 25 °C	Formation of other byproducts (%)[2]: 16 h at 50 °C
1	Cys in DMF	0.9	27.4	15.3	18.3
2	Cys/Oxyma (1:1) in DMF	< 0.5	< 0.5	13.0	7.9
3	Cys/Oxyma (1:1)+10 mol% DTT in DMF	< 0.5	< 0.5	< 0.5	< 0.5
4	Cys/Oxyma (1:1)+10 mol% DITU in DMF	< 0.5	< 0.5	< 0.5	< 0.5
5	Cys in 10%DMSO/EtOAc	< 0.5	< 0.5	< 0.5	< 0.5
6	Cys/Oxyma (1:1) in 10%DMSO/EtOAc	< 0.5	< 0.5	< 0.5	< 0.5
7	Cys/Oxyma (1:1)+10 mol% DTT in 10%DMSO/EtOAc	< 0.5	< 0.5	< 0.5	< 0.5
8	Cys/Oxyma (1:1)+10 mol% DITU in 10%DMSO/EtOAc	< 0.5	< 0.5	< 0.5	< 0.5

[1] In red, conventional conditions; in green, green conditions; in yellow, suitable Cys stability results (>99.5%); Cys degradations were determined by taking out 20 µL aliquots of reaction mixtures, diluting them with 1 mL MeCN and carrying out HPLC analyses. [2]Determined by integrating the dibenzofulvene (DBF) peak formed during the stability assessment. [3]Determined by integrating all impurities formed during the stability assessment except for those related to loss of Fmoc (DBF and H-Cys(Trt)-OH).

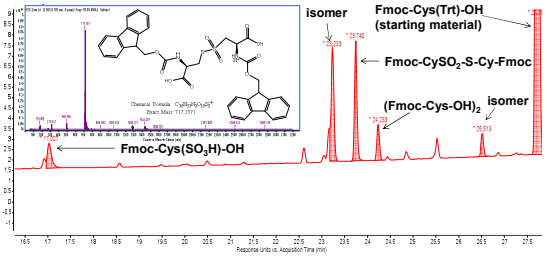
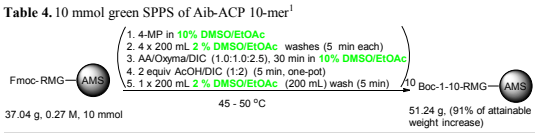


Figure 1. LC-HRMS analysis of Fmoc -Cys(Trt)-OH/Oxyma in DMF (Table 3, entry 2) after 16 h at 50 °C. Identities of impurities that were not present in unstressed Fmoc -Cys(Trt)-OH are shown. In the insert, MS spectrum and structure of Fmoc-$CySO_2$-S-Cy-Fmoc are given.

As the substrate for this stability assessment we chose Fmoc-Cys(Trt)-OH, a common building block in Fmoc SPPS of Cys containing peptides. We subjected this compound to a series of conditions to determine when the molecule breaks down and when it does not. To simulate a setting of the synthesis we examined the stability of Fmoc-Cys(Trt)-OH alone and in the presence of Oxyma. Furthermore, as Cys oxidation can be inititated by reactive oxygen species (ROS)[21] we opted to evaluate the kinetics of Cys breakdown in the presence of small amounts of sulfur containing species: these sulfurous compounds could scavenge ROS present in the reaction thereby preventing AA degradation. As possible ROS scavengers we chose to examine i) the common reducing agent DTT[22] and ii) 1,3-diisopropyl-2-thiourea (DITU).[23] We set out to test Cys stability both in EtOAc/DMSO and in the standard Fmoc SPPS solvent DMF and we analyzed all the stressed Cys samples by HPLC. In the pertinent HPLC chromatograms we integrated i) dibenzofulvene (DBF) peak indicating loss of the Fmoc group ii) peaks indicating the breakdown occurring at the thiol moiety (Table 3). While the analysis of Cys in DMF sample (entry 1) revealed insignificant Fmoc loss at rt, at elevated temperature (ET) a large ratio of de'Fmocing occurred (~ 27%), conceivably due to heat induced breakdown of DMF forming dimethylamine. In addition, substantial amounts of impurities were formed as well, indicating that stability of Cys side chain in DMF is limited. On the other hand, while in the presence of Oxyma (pKa 4.6) the loss of Fmoc was fully suppressed the formation of other impurities was still quite significant (entry 2). In fact, several impurities which were not appreciable in the entry 1 DMF/Cys experiments were quite large in the DMF/Cys/Oxyma runs. MS analysis revealed that these byproducts were all related to oxidative breakdown of the Cys thiol side chain (Figure 1) including the thiosulfonate dimer Fmoc-$CySO_2$-S-Cy-Fmoc.[24-25] In a stark contrast to the extensive breakdown of Cys observed with DMF/Cys and DMF/Cys/Oxyma respectively, no appreciable degradation of the AA was observed upon addition of 10 mol% DTT or DITU, not even at ET (entries 3 – 4). We propose that the remarkable stability of Cys in the presence of DTT or DITU is due to a scavenging effect these sulfur species have on the ROS.[26] In the absence of a scavenger (entry 2) the Oxyma based ROS triggers a reaction cascade resulting in the formation of Cys byproducts depicted in Figure 1. With respect to the stability of Cys in 10%DMSO/EtOAc no appreciable AA breakdown was observed irrespective of temperature, presence of Oxyma and/or sulfur scavengers (entries 5 – 8). A possible explanation of the increased Cys stability in DMSO/EtOAc even in the presence of Oxyma and the absence of sulfur scavengers (entry 6) is the high dependency of occurrence of ROS on the reaction medium.[27-28] With the data on AA stability in 10%DMSO/EtOAc in hand we carried out a scaled-up, green SPPS of Aib-ACP decapeptide (Table 4). Starting with 10 mmol of Ramage linker tethered AMS resin this synthesis was assisted by mild heat (45 – 50 °C) throughout[29] and utilized 4-MP in 10% DMSO/EtOAc (2 x 20 min) for all de'Fmocings. To keep 4-MP consumption low, all first Fmoc removals were done using 1% v/v 4-MP (ca 1.3 equiv vs amount of starting resin), followed by 5% v/v 4-MP treatments. The residual Fmoc content on the resin was determined[9b] after completion of each second deprotection and from the de'Fmocing of Fmoc-5-10 resin onwards a third, 5% 4-MP de'Fmocing had to be used to attain a satisfactory conversion (>99.7%). All couplings were carried out using 1.3 equiv AA/Oxyma/DIC in 10% DMSO/EtOAc for 30 min except for the highly congested Aib[3] for which 2 x 1.0 equiv AA was used. In keeping with the preceding small scale experiments capping was carried out until Asp[6] and all washes were done using 2% DMSO/EtOAc: four washes after each de'Fmocing and one wash after each coupling. The total solvent consumption for the whole synthesis was ~20 L, >95% of which was recyclable EtOAc. The synthesis furnished 51.24 g of Aib-ACP peptide resin (91% of theory) and per test TFA cleavages carried out on 0.5 g scale[31] the content of crude Aib-ACP on the resin was 9.82 g (85% of theory). An LC-HRMS analysis on the crude **1** (Figure 2)[32] confirmed the identity of the product. The LC-MS purity of the target peptide was determined to be 75.9% which constitutes a threefold improvement over a previous green SPPS of 10-mer **1** on a PS resin.[9b] Per the MS data the largest impurities were des Asn (2.7 %), des Ile (2.3% and 1.1%) and des Gln (1.3%) and suppressing these byproducts would further improve the green Aib-ACP SPPS process herein.[33]

Table 4. 10 mmol green SPPS of Aib-ACP 10-mer[1]



AA cycle	de'Fmocings (1)	Couplings (3)	Cappings (4)
Gly[10]	1% 4-MP (20 min) + 5% 4-MP (20 min)	1.3 equiv 0.1 M Fmoc-Gly-OH	yes
Asn[9]	1% 4-MP (20 min) + 5% 4-MP (20 min)	1.3 equiv 0.1 M Fmoc-Asn(Trt)-OH	yes
Ile[8]	1% 4-MP (20 min) + 5% 4-MP (20 min)	1.3 equiv 0.1 M Fmoc-Ile-OH	yes
Tyr[7]	1% 4-MP (20 min) + 5% 4-MP (20 min)	1.3 equiv 0.1 M Fmoc-Tyr(*t*Bu)-OH	yes
Asp[6]	1% 4-MP (20 min) + 5% 4-MP (20 min)	1.3 equiv 0.1 M Fmoc-Asp(O_tBu)-OH	yes
Ile[5]	1% 4-MP (20 min) + 5% 4-MP (20 min)	1.3 equiv 0.1 M Fmoc-Ile-OH	no
Aib[4]	1% 4-MP (20 min) + 5% 4-MP (20 min) + 5% 4-MP (20 min)	1.3 equiv 0.1 M Fmoc-Aib-OH	no
Aib[3]	1% 4-MP (20 min) + 5% 4-MP (20 min) + 5% 4-MP (20 min)	2 x 1.0 equiv 0.07 M Fmoc-Aib-OH[2]	no
Gln[2]	1% 4-MP (20 min) + 5% 4-MP (20 min) + 5% 4-MP (20 min)	1.3 equiv 0.1 M Fmoc-Gln(Trt)-OH	no
Val[1]	1% 4-MP (20 min) + 5% 4-MP (20 min) + 5% 4-MP (20 min)	1.3 equiv 0.1 M Boc-Val-OH	no

[1] RMG, Ramage (tricyclic amide) linker; AMS aminomethyl PS/DVB(1%) resin; before each de'Fmocing and coupling the resin was rinsed with 100 mL 10% EtOAc/DMSO (45 - 50 °C); in all couplings, 40% of DIC was added at the outset and the remaining 60% at t= 15 min; the final Boc-1-10 resin was washed with 2 x 200 mL 10% EtOAc/DMSO (45 - 50 °C), 3 x 200 mL *i*- PrOH (25 °C) and dried *en vacuo* to constant weight. [2]The resin was washed 2 x with 150 mL 0.1M Oxyma in 10% DMSO/EtOAc between the 1st and 2nd Aib[3] coupling.

Summary

In summary, it is reported that it is indeed possible to generate an efficient, scalable Fmoc SPPS method that is compatible with i) less expensive PS resins, ii) use of environmentally more acceptable solvents, iii) replacement of piperidine for 4-MP and iv) use of 4-MP in lower concentrations compared to standard piperidine de'Fmocings. With the exception of highly cumbersome couplings such as Aib[3] in Aib-ACP the method allows for the use of only 1.3 equiv AA derivatives in couplings.[34]

The fact that i) the vast majority of the waste stream consists of the easier-to-recycle EtOAc ii) the chemistry can be tuned by altering temperature and/or solvent composition iii) AA raw materials exhibit excellent stability in the reaction media render the methodology suitable for a further advancement of sustainable synthesis of peptides.

Finally, based on our investigation of the breakdown of Fmoc-Cys(Trt)-OH we propose that small amounts of a suitable scavenger (sulfur-based or other) is added to reaction mixtures in SPPS whenever ROS induced side reactions (and not restricted to Cys) are a concern. The concept of addressing unwanted side reactions in solid phase chemistry by addition of auxiliary components is not restricted to the above example and will be further developed.

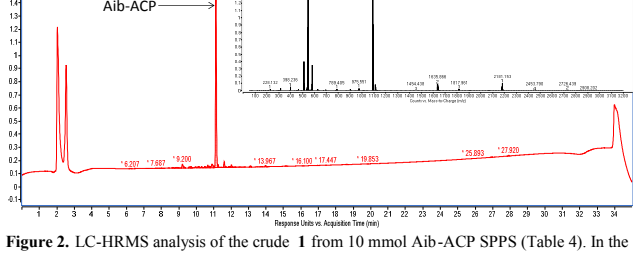


Figure 2. LC-HRMS analysis of the crude **1** from 10 mmol Aib-ACP SPPS (Table 4). In the insert, MS spectrum of the target peptide (m/z 1090.5897) is shown.

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[18] This Fmoc-5-10 resin was prepared from a 0.29M Fmoc-RMG AMS resin at 45 – 50 °C using 2 x 15 min 5% 4-MP in DMPU/EtOAc (1:2) for de'Fmocings, 1.3 equiv AA/Oxyma/DIC (1:1:3) 30 min in DMPU/EtOAc (1:2) for couplings and 2 equiv AcOH/DIC (1:2) 5 min for one-pot cappings (until Asp[6]). DMF was used for all solvent washes and a test TFA cleavage of the final resin revealed the purity of the crude Fmoc-5-10-NH_2 was ~90%.
[19] El-Faham, A.; Funosas, R. S.; Prohens, R.; Albericio, F. *Chem. Eur. J.* **2009**, *15*, 9404.
[20] See for example Loffredo, C.; Assunção, N. A. Gerhardt, J.; Miranda, M. T. M. *J. Pept. Sci.* **2009**, *15*, 808.
[21] Jeong, J.; Jung, Y.; Na, S.; Jeong, J.; Lee, E.; Kim, M.-S.; Choi, S.; Shin, D.-H.; Paek, E.; Lee, H.-Y.; Lee, K.-J. *Mol. Cell. Proteomics* **2011**, *10*, 1.
[22] Cleland, W. W. *Biochemistry* **1964**, *3*, 480.
[23] DITU is a precursor to the coupling agent DIC, see for example Zhu, C.; Xu, D.; Wei, Y. *Synthesis* **2011**, *5*, 711. We found that peptide syntheses mediated by DIC contaminated with DITU gave lower contents of oxidation related impurities than corresponding syntheses using DITU-free DIC did. A full account on the role of DITU in preventing side reactions in peptide synthesis will be disclosed elsewhere.
[24] For a kinetic study on thiol oxidation involving the formation of a thiosulfonate dimer see for example Nagy, P.; Ashby, M. T. *J. Am. Chem. Soc.* **2007**, *129*, 14082.
[25] A formation of a symmetrical, cysteine disulphoxide dimer is conceivable as well, for the mechanism of formation of this species see for example Subbarao, T. L. M. V.; Syamasundar, B.; Radhakrishnamurti, P. S. *Oxid. Commun.* **2007**, *30*, 376.
[26] It is conceivable that this putative Oxyma based N-oxyl radical is formed in an analogous manner to which HOBt is converted to the BTNO radical. HOBt is known to form N-oxyl species (BTNO) which can in turn abstract protons from ArNMe$_2$ methyl groups, see: Baciocchi, E.; Bietti, M.; Lanzalunga, O.; Lapi, A.; Raponi, D. *J. Org. Chem.* **2010**, *75*, 1378.
[27] For a study on solvent effects in the formation of oxyl radicals see: Bietti, M.; Martella, R.; Salamone, M. *Org. Lett.* **2011**, *13*, 6110.
[28] It is worth noting that we exposed Fmoc-Gly-OH and Fmoc-Met-OH respectively to similar stress stability experiments as the one shown for Cys in Table 3. In keeping with the results obtained for Cys no appreciable breakdown of Gly and Met in 10%DMSO/EtOAc was observed.
[29] For a recent review on the use of heat in improving of SPPS of difficult peptides see: Paradis-Bas, M.; Tulla-Puche, J.; Albericio, F. *Chem. Soc. Rev.* **2016**, *45*, 631.
[30] An Fmoc quantification assay based on a literature method was used: Gude, M.; Ryf, J.;White, P.D. *Lett. Pep. Sci.* **2003**, *9*, 203.
[31] These 0.5 g test TFA cleavages were done in quadruplicate in TFA/TIS/H_2O (92.5:5.0:2.5) at rt using 1 and 2 h cleavage times and Et$_2$O and CPME as precipitation solvents. 95±1 mg of crude **1** of identical purity was obtained in all four runs.
[32] An Agilent Q-TOF MS system operated in a positive mode (ESI) was used. Analytical separation was achieved using a Waters Acquity UPLC Peptide CSH C18 column (30 °C, 150x2.1mm, 1.7um), TFA/H_2O (0.1:100, **A**), and TFA/MeCN (0.1:100, **B**) mobile phases, gradient of 94% B over 30 min and flow rate of 0.25 ml min^{-1}.
[33] As it is conceivable for EtOAc to acetylate a growing peptide chain during SPPS it is worth noting that we exposed i) an Fmoc-5-10 resin to 5% 4-MP in 10%DMSO/EtOAc ii) an H-5-10 resin to Fmoc-Aib-OH/Oxyma/DIC in 10%DMSO/EtOAc for 16 h at 50 °C followed by TFA cleavages of the stressed peptide resins which resulted in a negligible content (< 0.5%) of the acetylation product (Ac-5-10-NH_2) in the crude peptide material.
[34] For a SPPS method using DMF as solvent and 1.5 equiv AAs in couplings see Mándity, I.; M.; Olasz, B.; Orvös, S. B.; Fülöp, F. *ChemSusChem.* **2014**, *7*, 3172.