# Benzylthiols as scavengers in TFA cleavages of peptide resins

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## Introduction

Aliphatic thiols such as EDT[2], DTT[3] and DODT[4] act as efficient scavengers in TFA cleavages of peptide resins prepared by solid-phase peptide synthesis (SPPS).[5] Nevertheless, aliphatic thiols are often malodorous, can form byproducts by reacting with peptides[6] and, due to the lack of chromophores, these scavengers can form impurities that can be difficult to detect by UV during downstream processing.[7] On the other hand, aromatic thiols are easier to detect although they are also less reactive than aliphatic thiols and thereby less effective as scavengers.[8]

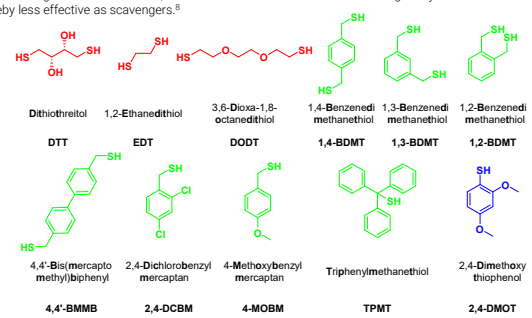



**Fig. 1** Structures, chemical names and abbreviations for thiols evaluated as scavengers in peptide resin TFA cleavages. Aliphatic, benzylic and aromatic thiols in red, green and blue, respectively.

## Results and discussion

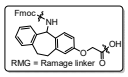
We reasoned that benzylthiols (BTs) could constitute suitable scavengers for cleavages of peptide resins by the virtue of combining the high reactivity of aliphatic thiols with the UV visibility of aromatic compounds. According to SciFinder more than 490,000 BTs are known >8,000 of which are commercially available. Aiming at scavengers useful for large scale manufacturing applications we focused on BTs that would be relatively simple and thus could be made cost efficiently while not containing any sensitive functional groups not compatible with conc. TFA used in peptide resin cleavages. To evaluate the impact of the scavenger structure on the outcome of peptide resin cleavages we selected a series of BTs varying in electronic and steric properties (Fig. 1). The three BDMT isomers and the biphenyl based 4,4'-BMMB were chosen as BT counterparts of the aliphatic dithiols DTT and DODT. Moreover, the electron withdrawing group (EWG) containing 2,4-DCBM, the electron donating group (EDG) based 4-MOBM and the highly sterically congested α-diphenyl substituted TPMT were examined as well. Aliphatic DTT, EDT and DODT as well as the aromatic 2,4-DMOT were used as benchmarks and the 39-mer antidiabetic exenatide (**1**)[9] was selected as the model peptide.

HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPPS-$NH_2$ **1**

The 39-mer **1** was chosen as the substrate as it contains a plethora of sensitive amino acids (AAs) which can undergo various side reactions during the cleavage of the peptide off the resin, for example by forming adducts with the species liberated from side chain protecting groups (PGs). An exenatide resin was synthesized by standard Fmoc SPPS[10] and its cleavages were carried out for 2 h at rt using TFA/TIS/$H_2O$ (95:3:2, v/v/v) in the presence of Fig. 1 thiol scavengers (Scheme 1). A control experiment in the absence of a thiol was carried out as well.

Boc-His(Trt)-Gly-Glu(OtBu)-Gly-Thr(tBu)-Phe-Thr(tBu)-Ser(tBu)-Asp-Leu-Ser(tBu)-Lys(Boc)-Gln(Trt)-Met-Glu(OtBu)-Glu(OtBu)-Glu(OtBu)-Ala-Val-Arg(Pbf)-Leu-Phe-Ile-Glu(OtBu)-Trp(Boc)-Leu-Lys(Boc)-Asn(Trt)-Gly-Gly-Pro-Ser(tBu)-Ser(tBu)-Gly-Ala-Pro-Pro-Pro-Ser(tBu)-RMG — ***exenatide* resin**

i. TFA/TIS/$H_2O$/**thiol**, 2h at rt
ii. $Et_2O$ precipitation




H-His-Gly-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-$NH_2$ **crude *exenatide***

**Scheme 1** TFA cleavages of exenatide resin using thiol scavengers shown in Fig. 1. An aminomethyl functionalized polystyrene resin was used as a solid support, 200 mg of exenatide resin, 1.84 mL TFA, 60 μL TIS and 40 μL $H_2O$ were used in each run. 0.78 mmol of scavengers containing one SH group and 0.39 mmol of those having two SH groups was used.

The yields and HPLC purities of the isolated crude peptides were determined (Fig. 2) revealing that cleavages using DODT, 4,4'-BMMP, EDG containing 4-MOBM, bulky TPMT, aromatic 2,4-DMOT as well as no thiol were all inferior to the cleavages using the standard DTT and EDT reagents. On the other hand, cleavages containing the three BDMT isomers as well as the EWG based 2,4-DCBM compared favorably to those carried out in the presence of the benchmark aliphatic thiols.

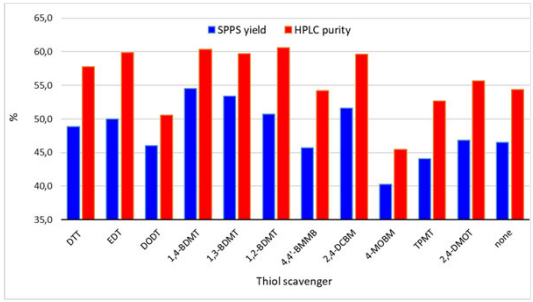



**Fig. 2** Chemical yields and HPLC purities of crude products obtained from TFA cleavages of exenatide resin (Scheme 1).

As the malodorous liquids EDT and 1,3-BDMT and the toxic 2,4-DCBM are undesirable from EHS standpoint DTT, 1,2- and 1,4-BDMT were deemed as best overall among the thiol scavengers tested. To determine contents of specific cleavage related impurities we carried out an extracted ion chromatography mass spectrometry (EIC MS) assessment on the crude exenatide materials (Fig. 3). With regards to minimizing the formation of peptide-*t*-Bu adducts 1,4-BDMT proved to be the most efficient with EDT, 1,2- and 1,3-BDMT being slightly inferior.

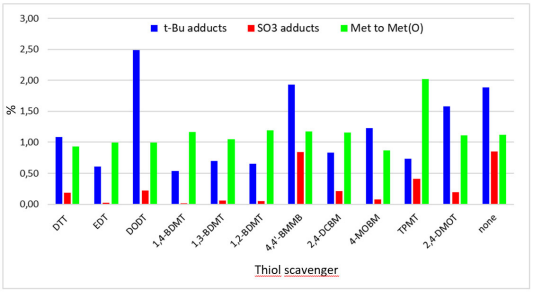



**Fig. 3** EIC MS contents (converted to UV areas at 220 nm) of *t*-Bu (+56 Da) and $SO_3$ (+80 Da) adducts and Met to Met(O) (+ 16 Da) oxidations in exenatide crudes obtained from TFA cleavages of exenatide resin (Scheme 1).

Furthermore, 1,4-BDMT and EDT were the best scavengers in terms of minimizing the sulfonylation of the peptide while 4-MOBM, 1,2- and 1,3-BDMT were only slightly worse. On the other hand, with the exception of TPMT (~2%) the Met(O) content was roughly the same for all other scavengers examined (~1%). On the other words none of the thiols tested affected Met(O) formation appreciably and we propose that suppressing Met(O) is best accomplished by using some of the scavengers developed specifically for this side reaction.[11] Next, we examined properties of the most promising BT scavengers 1,4- and 1,2-BDMT under the conditions encountered in peptide resin TFA cleavages. Previously, it has been shown that alkylthiols such as DTT may decompose during TFA cleavages,[7] decreasing their scavenging ability. We therefore evaluated stability of 1,4 and 1,2-BDMT BTs in a conc. TFA solution, testing the benchmark alkylthiols DTT and DODT as well. The order of stability in TFA/TIS/$H_2O$ (95:3:2) was determined to be 1,4-BDMT>1,2-BDMT>DTT>>>DODT (Fig. 4). Conceivably, the higher stability of 1,4-BDMT compared to the other thiols contributed to its scavenging efficiency in the TFA cleavage of exenatide resin. Regarding solubility we found that adequate solubility (5% w/v) of 1,4-BDMT in TFA could be attained while 1,2-BDMT in TFA remained only partially soluble even after 24 h of vigorous shaking. Again, it is conceivable that the higher solubility in TFA contributed to the better characteristics of 1,4-BDMT as a scavenger in peptide resin cleavages. With respect to solubility it is worth noting that precipitation was observed during TFA cleavages carried out in the presence of some of the BTs tested. We isolated these precipitates from TFA cleavages of exenatide resin using 1,2-, 1,3- and 1,4-BDMT and found that these materials are not BTs but rather adducts of BTs with PGs[12] released from the peptide resin. These poorly soluble BT adducts were easily filtered off together with the spent resin after the cleavage.

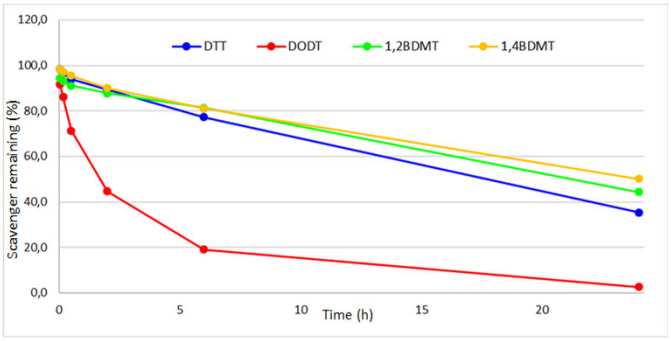
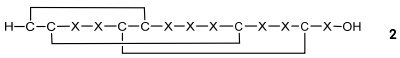



**Fig. 4** Stability of DTT, DODT, 1,2-BDMT and 1,4-BDMT in TFA/TIS/$H_2O$ (95:3:2, v/v/v).

Finally, we set out to examine 1,4- and 1,2-BDMT in a cleavage of a peptide resin containing multiple sensitive Cys residues which are susceptible to undergo a wide range of side reactions.[13] To that end we chose a Cys rich peptide (CRP) **2** (X=AA) as the substrate and carried out TFA cleavages of a resin based on linear CRP **2** using DTT, 1,2- and 1,4-BDMT as scavengers (Scheme 2).




**2**

The crude products thus obtained were analyzed by HPLC, determining the yields and HPLC purities of the linear CRP crudes (Fig. 5). HPLC areas of CRP-t-Bu adducts, the major byproducts in these CRP resin cleavages, were determined as well. These analyses revealed that in the absence of a thiol CRP was formed in an inferior yield and purity and the content of CRP-t-Bu adducts was increased ~2-fold. Regarding scavenging characteristics of the three thiols compared 1,4-BDMT was superior to both DTT and 1,2-BDMT in terms of yield, CRP purity as well as suppressing the formation of CRP-t-Bu adducts.

H-Cys(Trt)-Cys(Trt)-X-X-Cys(Trt)-Cys(Trt)-X-X-X-Cys(Trt)-X-X-Cys(Trt)-X — **CRP resin**

i. TFA/TIS/$H_2O$/**thiol**, 2h at rt
ii. $Et_2O$ precipitation

H-Cys(H)-Cys(H)-X-X-Cys(H)-Cys(H)-X-X-X-Cys(H)-X-X-Cys(H)-X **crude CRP**

**Scheme 2** TFA cleavages of CRP resin using DTT, 1,4-BDMT and 1,2-BDMT as scavengers. A 2-chlorotrityl chloride functionalized polystyrene resin was used as solid support, 100 mg of CRP resin, 1.5 mL TFA, 133 μL TIS, 66 μL $H_2O$ and 0.43 mmol of a thiol scavenger were used.

In summary, we assessed a series of BTs as scavengers for TFA cleavages of peptide resins and found that this class of thiols encompasses useful alternatives to the aliphatic thiols currently used as the state-of-the-art reagents in peptide resin cleavages. Specifically, we found that 1,4-benzendimethanethiol (1,4-BDMT) used as a scavenger in cleavages of exenatide and a Cys-rich peptide resins furnished the crude products in improved yields and purities compared to the cleavages carried out in the presence of the standard aliphatic thiol reagents. Importantly, 1,4-BDMT is easily UV detectable, exhibits suitable solubility and stability in TFA and can be sourced cost efficiently on scales required for GMP production of therapeutic peptides. Studies are underway aiming at implementing 1,4-BDMT to commercial manufacturing as well as examining additional BTs as scavengers and peptide resins as substrates.

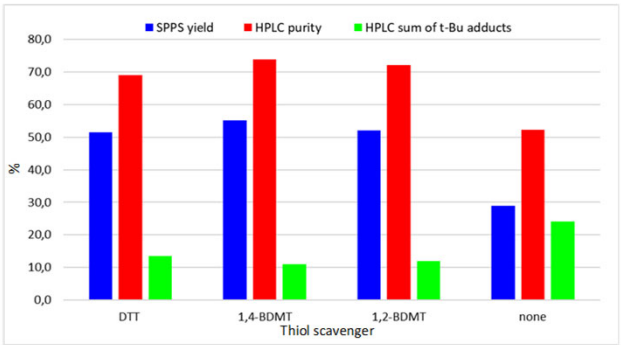



**Fig. 5** Chemical yields and HPLC areas of CRP and CRP-*t*-Bu adducts for TFA cleavages of CRP resin (Scheme 2).

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