New disulfide reducing reagent for solid phase peptide synthesis

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This thesis is submitted to the School of Health Sciences, College of Health Science, University of KwaZulu-Natal, Westville, to satisfy the requirements for the degree of Master of Medical Science in Pharmaceutical Chemistry.

This is to certify that the contents of this thesis are the original research work of Miss Sinenhlanhla Nonhlanzeko Mthembu, carried out under our supervision at Peptide Sciences Laboratory and the Catalysis and Peptide Research Unit, University of KwaZulu-Natal, Westville Campus, Durban, South Africa.

As the candidate’s supervisor, I have approved this thesis for submission.

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ABSTRACT

The special physical and chemical properties of sulfur atom make thiols and disulfide bonds of vital importance in many biological processes. For instance, it is well known, the role of cysteine residues in proteins, acting as key in the catalytic site of enzymes or stabilizing the tertiary structure of proteins through disulfide bonds. In nature, glutathione is responsible for reducing disulfide bonds formed within the cytoplasmic proteins to cysteines. To mimic this function on the bench, many disulfide reducing agents have been developed. β-mercaptoethanol (β-ME) and dithiothreitol (DTT) work in a similar manner as GSH except that DTT forms intramolecular disulfide bond yielding a very stable six-membered ring, making DTT a more effective reducing agent. Recently, a new reducing agent inspired in DTT was developed from aspartic acid, Dithiobutylamine (DTBA), which work faster and because its lower thiol pKₐ value, showed a good reactivity at physiological pH. Due to the high polarity of DTBA, it is only soluble in aqueous solution, thus in this work, a non-polar analogue 2-(dibenzylamino)butane-1,4-dithiol (DABDT) has been developed. The molecule has been successfully obtained in good yield by a five-step process as an almost odorless solid.

In solution, DABDT has good stability and its performance as reducing agent is comparable to DTT. Moreover, its efficacy has been probed as reducing agent of disulfide protected Cys and thiol derivatized peptide on solid phase.
DECLARATION I

Plagiarism declaration

I, Sinenhlanhla Nonhlanzeko Mthembu declare that

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2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other person’s data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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Signed

____________________
Sinenhlanhla Nonhlanzeko Mthembu
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In this competitive world those who are willing to come forward and accept challenges will succeed. The current project is a bridge from theoretical to practical working. With this motivation I joined this research project. Many people have helped me throughout this journey and hence, I take this opportunity to thank them all. I would like to give my sincere thanks to:

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CHAPTER 1

Introduction

1. Thiols and Disulfides

Free thiols and disulfide bonds are principal actors in many biological processes because their unique chemical and physical properties. The major representative of thiol containing molecules in nature is Cysteine (Cys), one of the 20 genetically encoded amino acids that are present in proteins. Although, the amino acid composition of different proteins is variable and proteins vary from different organisms, in general, Cys is one of the less abundant amino acids (Figure 1A). On the other hand, it is a highly conserved residue in the functional sites of proteins. However, not all Cys present in living organisms are in protein sequences, it is also present as one of the constituents of glutathione, the main redox regulator in cells. Glutathione is present in prokaryotic and eukaryotic cells in high concentration; it is a tri-amino acid molecule with the unusual link between the amino group of Cys and the γ-carboxylate of Glu (Figure 1B).

![Figure 1](image)

**Figure 1:** A) Abundance of Cys in different organism. B) Structure of glutathione.

Glutathione is the predominant representative of the so called Low-Molecular-Weight (LMW) thiols, this family of molecules act as redox buffers to protect the cells against reactive oxygen species and nitrogen species, as well as electrophilic species, even some antibiotics, etc. The LMW thiols are structurally diverse, some of them are common in all organism as glutathione, Cys, homocysteine, coenzyme A or lipoic acid and others are organism-specific as for example mycothiol in Actinomycetes, Ergothioneine in fungi and mycobacteria, bacillithiol in Firmicutes, among other (Figure 2).
Regarding the ability of two Cys residues to form disulfide bridges by oxidation, they are essential to stabilize the tertiary structure in proteins and peptides, at the same time that impose rigidity. The number of disulfide bonds in a single molecule can be varied and can be intra- and inter-molecular. As examples to illustrate this we can mention: (i) Human Defensins (HD’s), antimicrobial peptides (AMPs) component of the innate immune system, are small proteins (or big peptides) up to 35 amino acid residues consisting of a triple β-sheet structure stabilized by three disulfide bonds (Figure 3A). (ii) Insulin, a hormone formed by two peptide chains linked by two disulfide bridges: chain A of 21 amino acid residues and chain B of 30 residues. Additionally, insulin has an intra-molecular disulfide bond in its A chain (Figure 3B). (iii) Antibodies, constituted by two heavy chains (Figure 3C, dark purple) and two light chains (Figure 3C, light purple) that contain intra-molecular disulfide bonds and are linked with inter-molecular disulfide bridge. The disulfide bond structure present in antibodies is evolutionarily highly conserved. However, in monoclonal antibodies (mAb), it can be distinguished two types of disulfide bridges, the classical that are the conserved ones and non-classical which varies depending upon the mAb.
Figure 3: Inter and intra molecular disulfide bridges. A) Human defensins, B) Insulin and C) Monoclonal Antibody.

Nowadays, monoclonal antibody (mAb) forms an important part of the pharmaceutical research with further extension to clinical research. Disulfide bridges play a crucial role in the maintenance of their structure and function, however because of the increasing interest in the development of antibody-drug conjugates (ADC) it is very important to develop reducing agents able to liberate some free thiols to allow the conjugation without modification of their functionality.

2. Reducing agents

In the last several decades, disulfide reducing agents have been widely used in many molecular biology protocols to stabilize free sulfhydryl groups and/or to reduce disulfide bonds in peptides and proteins. Broadly, from the chemical and structural point of view they can be classified into two groups: one based in thiols, which include monothiol and dithiols and the second based in phosphine.

2.1. Reducers based in Thiols

The most used reagents to reduce disulfides are thiols, this process is easy and specific. This reaction is essentially a disulfide exchange which involves three steps:

\[
R_1\text{-SH} \overset{pK_a}{\rightleftharpoons} R_1\text{-S}^- + R_2\text{-S-S-R}_3 \rightleftharpoons R_2\text{-S-S-R}_1 + R_3\text{-S}^- \overset{pK_a'}{\rightleftharpoons} R_3\text{-SH}
\]

Scheme 1: Three steps involved in the disulfide exchange.
In the first step the thiolate anion should be formed to act as nucleophile in the second step and finally the new thiolate must be protonated. Thus, the thiolate interchange is a base catalyzed process pH dependant. The pKₐ of the R₁-SH will determine the amount of thiolate anion present in the reaction media, as much acidic the thiol is (lower pKₐ), more thiolate will be released at a given pH (Figure 4)

![Chemical reaction](attachment:image.png)

<table>
<thead>
<tr>
<th>Thiol pKₐ</th>
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<tr>
<td>6</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>7</td>
<td>50</td>
<td>50</td>
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<td>8</td>
<td>90</td>
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**Figure 4:** Effect of pKₐ on formation of thiolate anion at pH 7.

Once the thiolate (R₁-S⁻) is present, the nucleophilic reaction occurs over the sulphur corresponding to the less acidic thiol in the mixed disulfide, and then the most acidic thiol is the one that is released. In our scheme pKₐ(R₂-SH) > pKₐ(R₃-SH).

One of the main drawbacks using thiols as reducing agents is that they should be removed from the media if the sulphydryl released must be quantified or be involved in further reactions.

### 2.1.1. Monothiol

The most common thiol used in the laboratory as reducing agent was β-Mercaptoethanol (β-ME) (Figure 5) and still it is widely used at present. β-ME is the smallest thiol soluble in water and maybe the cheapest one. However, it has a real unpleasant odor and it is considered highly toxic, thus researchers have been always looking for a good reductant to replace β-ME (see the following sections).

![Reduced and oxidized structure of β-ME](attachment:image.png)

**Figure 5:** Reduced and oxidized structure of β-ME.

The presence of the hydroxyl group on the molecule, reduce the thiol pKₐ (9.6) one-unit respect to ethanethiol (10.6) favoring the thiolate formation.
The total reduction of a disulfide bond leading the two sulfhydryl groups is regulated by several equilibrium reactions (Scheme 2). To ensure that the reaction is not stopped in the formation of the mixed disulfide (Scheme 2, blue-red molecule) large excess of the reductive thiol (red) should be used, then equilibria [1] and [2] are pushed to render the two desired free thiols (blue).

Scheme 2: Set of equilibrium reaction during disulfide-thiol interchange.

2.1.2. Dithiol

The first revolutionary introduction of a new reductive thiol was in 1964 by W. W. Cleland. His approach was to design a diol molecule that would adopt a stable cyclic structure in its oxidized form. Having this in mind, Cleland synthesized two epimers of 1,4-dimercaptobutane-2,3-diol, known as dithiothreitol (DTT) and dithioerythritol (DTE) (Figure 6).

Figure 6: Reduced and oxidized structure of DTT and DTE.

Compared with β-ME, reduction using DTT or DTE were faster, and additionally because they are solid, they are less odorous. Among DTT and DTE, DTT is widely accepted as a reducing agent in the field of chemistry and biochemistry mainly due to its ease of synthesis. The stability of the formed cyclic six-membered oxidized product is the driving force to shift the equilibrium in forward direction, avoiding the use of excess of reducing agent unlike as that mentioned above in case of β-ME.
Because of the high stability of cyclic oxidized DTT, the reduction never renders the mixed disulfide species (Scheme 2). However, DTT is mainly associated with two limitations: i) DTT as reducing agent is non-functional at pH below 7 because the pKₐ of two thiol groups are 9.2 and 10.1. ii) DTT has very short half-life.¹⁴ At pH 8.5, the half-life is 1.4 h and 10 h at pH 7.5, which is the limit of it to be working as a reducing agent.

Owing the limitations of DTT, past few decades have witnessed the search of potential reducing agent based on similar strategy. In this regard, Whitesides and co-workers reported potent reducing agents, N, N’-dimethyl-N,N’-bis(mercaptoacetyl)hydrazine (DMH) and meso-2,5-dimercapto-N,N,N’,N’-tetramethyl adipamide (meso-DTA) (Figure 7). However, there usage has not been extended in the field of chemistry and biochemistry.

![Figure 7](image1.png)

**Figure 7**: Reduced and oxidized structure of DMH and meso-DTA.

With further advancement in this area, Raines and co-worker report reducing agent (2S)-2-amino-1,4-dimercaptobutane (DTBA) derived from Aspartic acid.¹⁵ However, they later reported that free amino group obstructs the ability of DTBA as a reducing agent.¹⁶ This led to the synthesis of 2,3-bis(mercaptomethyl)pyrazine (BMMP), another reducing agent by Raines and co-worker (Figure 8) with an idea of eliminating the limitations associated with DTBA.¹⁶ In another report, Inagaki and co-workers also reported the increase of hydrophobicity improves the reducing ability of the reducing agent.¹⁷

![Figure 8](image2.png)

**Figure 8**: Reduced and oxidized structure of DTBA and BMMP.
2.2. Reducers based in Phosphines

It was well known that trialkyl/triaryl phosphines show affinity towards reduction of disulfide bridges. Nevertheless, the application in molecular biology protocols was limited due to poor aqueous solubility. Several attempts were made to improve the solubility of the phosphines until 1991 when Whitesides and co-workers introduced tris(2-carboxyethyl)phosphine (TCEP)\textsuperscript{18} as a water soluble reducing agent. TCEP became good alternative to thiols as reducing agent because of its high stability towards air oxidation, wide range of pH, odourless, and process is irreversible avoiding excess usage of reducing agent (Scheme 3). Additionally, it does not interfere with thiol quantification and/or further reaction of thiol group.

Scheme 3: Mechanism of phosphine derivatives as reducing agent for disulfide bridge: (i) formation of thioalkoxyphosphonium cation, (ii) hydrolysis of cationic intermediate to form the phosphine oxide.

Despite all the advantages of TCEP mentioned above, it cannot be considered as universal reducing agent since its reactivity is limited to aqueous media.
3. References


CHAPTER 2

2-(DIBENZYLAMINO)BUTANE-1,4-DITHIOL (DABDT), A FRIENDLY DISULFIDE REDUCING REAGENT COMPATIBLE WITH A BROAD RANGE OF SOLVENTS

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Abstract

Although Cys is not of an amino acid of general abundance in proteins, it is key for their role in their stabilization. Furthermore, cystine is present in a large number of peptides with interesting biological activities, which has fueled the development of many Cys containing Active Pharmaceutical Ingredients. Finally, the presence of a free thiol in a peptide or other organic molecule is very often used for bioconjugation. The manipulation or synthesis of Cys containing peptides and proteins require the use of disulfide reducing agents. Herein, we describe the synthesis and application of a new reducing agent (DABDT), which is conveniently prepared from the cheap aspartic acid, very friendly to use due to the absence of odor and solubility in a broad range of solvents, and remove the SDMP, which is the thio protecting group of choice in peptide synthesis, as well as other disulfide bridges.
1. Introduction

Cysteine is one of the 20 genetically encoded amino acids that are present in proteins. Although, the amino acid composition of different proteins is variable and proteins vary from different organisms, in general, abundance of Cysteine (Cys) is very low. Nevertheless, Cys play a very important role in nature, its thiol side-chain often is involved in enzymatic reactions and by oxidation of two Cys residues they form disulfide bridges that are essential to stabilize the tertiary structure in proteins and peptides. Disulfide bridges can be intra- and inter-molecular.

Taking into account the importance of the Cys and the disulfide bridge, disulfide reducing agents are widely used in many molecular biology protocols in order to stabilize free sulfhydryl groups and to reduce disulfide bonds in peptides and proteins. The most common dithiol reducers but not the only ones are β-mercaptoethanol (β-ME), dithiothreitol (DTT) and Tris(2-carboxyethyl)phosphine (TCEP). All present some advantages and cons, for example, β-ME, which is very poor user friendly due to its stench and is weakly reducing and kinetically slow compared to DTT, on the other hand its stability is higher. The great reducing ability as well as the low stability of DTT are because of the stability of the cycle in its oxidized form. Nevertheless, the limitation is that at physiological pH its reactivity is lower due to its high pKa and hence the difficulty to reach the thiolate form. In this sense, TCEP represents a big improvement, it is active in a wide pH range (5.0-9.0), it is stable to air oxidation and odorless but unlike the previous ones, its oxidized state is irreversible (Figure 1).

![Figure 1](image_url)

Figure 1: The most used disulfide reducing agent in their reduced and oxidized form.

An ideal reducing agent should be almost odor free, low pKa, and show a good solubility in a broad range of solvents, including organic ones. In this regard, it is important to take into account that the synthesis of cystine/cysteine containing peptides involves very often the concourse of
more than one kind of thiols protecting groups, which can be removed by different chemical mechanism. One of this kind of protecting groups are those where the thiol of the Cys is masked through a disulfide bridge. In the past, the S-tert-butyl (S-tBu) and more recently the S 2,6-dimethoxybenzyl (S-DMP), which is commercially available and was developed by our group and it is more labile than S-tBu and therefore of a broader use. In peptide chemistry, it is very important to have a reducing agent soluble in non-aqueous solvents, because very often the disulfide based protecting group is removed when the peptide if fully protected and even anchored to the solid support where the peptide was elongated using a solid-phase peptide synthesis strategy (SPPS).

With this in mind, we develop a new reducing agent suitable for non-aqueous media. The designed strategy is based in the previous work of Raines and co-workers, which describe a potent disulfide-reducing agent obtained from aspartic acid, the dithiobutylamine (DTBA). This reducing agent is not soluble in organic solvents due to the presence of two thiols and a primary amine. With this background, we propose the synthesis of 2-(dibenzylamino)butane-1,4-dithiol (DABDT) as non-aqueous dithiol reductant (Figure 2).

\[
\text{Figure 2: Chemical structure of 2-(dibenzylamino)butane-1,4-dithiol (DABDT).}
\]

2. Results and Discussion

A priori, DABDT belongs to the family of the reducing agents whose oxidized form is stabilized by a six-membered ring such as DTT and DTBA, the presence of the two benzyl moieties will be balance the solubility properties of the compound, and as it could be synthesized from the aspartic acid, it would be cheaper than DTT.

First of all, we have used the commercially available Z-Asp-OBzl as starting material with idea of hydrolyzing the ester, and then reducing both carboxylic groups to the corresponding alcohols. The main concern about this step was the stability of the Z group in front of the reduction conditions, since this must be carried out with lithium aluminum hydride (LAH). The reduction with LAH of a small sample of the Z-amino protected dicarboxylic acid took place with the
concomitant reduction of the Z group and therefore the free amine was afforded, confirming that
the proposed synthetic scheme was not suitable. A new approach was designed involving three
main transformation, starting by the truly cheap aspartic acid, which first of all was submitted to
perbenzylation, followed by reduction to diol in the second step, and finally the conversion to the
desired dithiol in the third step (Scheme 1).

Scheme 1: Synthetic route to prepare DABDT.

The first step, the perbenzylation of aspartic acid using benzyl bromide (Bzl-Br) in presence of
K₂CO₃ as base, was carried out at 80 °C under microwave (MW) condition using H₂O as solvent.
After 40 min of reaction, TLC revealed total consumption of the aspartic acid (negative ninhydrin)
and the perbenzylated product (2) was obtained in high purity as an immiscible liquid, which was
extracted by ethyl acetate and purified by silica gel chromatography yielding 94 % of pure
dibenzyl N,N-dibenzylaspartate (2). The full analysis and characterization of the new compound
was performed by HPLC, mass spectrometry and different NMR techniques (SI: Figure S1-S8).

In comparison with other benzylation methodologies described previously in the literature⁹-¹⁰,
the present method is advantageous from several points of view. First of all, the use of MW
allows to minimize the reaction time, and the use of H₂O as solvent makes the process eco-
friendly.

The second step consist in the reduction of the two benzyl esters to alcohol using LAH. After
several attempts proceeding as reported in the literature,¹¹ the reaction never reached completion
even after overnight stirring. Nevertheless, by addition of LAH in three equal portions in a period
of 2 h, instead of same amount in one batch, followed by 4 h stirring at room temperature, the
conversion was complete. Then, the excess of LAH was quenched, filtrated and after the work
up, the solution of 2-(dibenzylamino)butane-1,4-diol (3) was passed through a silica gel column
to obtain pure product in high yield (81 %). As previous, the full analysis and characterization of
the new compound was performed by HPLC, mass spectrometry and different NMR techniques (SI: Figure S9-S16).

Finally, the conversion of the diol to thiols was attempted by several different methodologies. First, the reaction was carried out with Lawesson’s reagent. Although the use of this reagent is mainly for the conversion of carbonyl compounds to thiocarboxyls, Nishio reported the direct conversion of alcohols into thiols. In our hands, this protocol did not work under any of the assayed conditions.

Other methodologies reported in literature for conversion of alcohol into thiol involves the previous transformation into other functionality with better leaving group properties like tosylate, mesylate, chloride etc. Our choice was to convert diol into “chlorine” derivative due to several advantages like clean reaction, because its work-up does not involve either extractions or chromatography purification because the by products in the reaction are gaseous. This motivated us to convert the diol (3) into “chlorine” derivative (4) using SOCl₂. In all the attempts, we could successfully convert diol (3) into chlorine at both positions resulting in one product only as monitored by TLC. Hence 4 was used for the next step without purification.

After conversion of alcohol into “chlorine” as a good leaving group, we proceed to the next step by two different approaches i.e., by formation of iso-thiouronium salts or by formation of thioesters. The formation of iso-thiouronium salts is achieved by use of thiourea which upon reaction with NaOH affords sodium salt of thiolate. However, the results obtained were unexpected since upon completion of the reaction the major product formed was the cyclic thioether instead of DABDT.

For better understanding of the results, we hypothesized that the formation of iso-thiouronium salt at both positions is not same due to different reactivity of “chlorine” which could have resulted in the formation of iso-thiouronium salts only in one position. This intermediate upon reaction with NaOH resulted in intramolecular nucleophilic substitution reaction forming cyclic thioether as major product favored because of the high stability of the 5-membered ring (Figure 3).
Figure 3. Formation of DABDT via thiourea approach. Formation of iso-thiouronium salts took place only in one position.

In the second strategy, the thioester formation was attempted using potassium thioacetate. The reaction was fast and clean as the by-products were water soluble which can be easily removed by aqueous workup. The crude obtained after workup was purified by silica gel column chromatography to afford pure 5, which was fully characterized by HPLC, mass spectrometry and different NMR techniques (SI: Figure S17-S24).

Finally, the thioester hydrolysis, which had to render DABDT, was performed in aqueous HCl in MeOH. The conversion was good and fast due to the complete consumption of thioester in 2.5 h. However, upon analysis it was revealed that the oxidized one (DABDT$_{ox}$) was present in significant amount along with DABDT. This led us to attempt the reduction of thioester using reducing agent to avoid formation of DABDT$_{ox}$. First of all, the reduction of the thioester was attempted using LAH. The reaction was completed in 10 min (100% of conversion). After quenching with water and acidification, the extraction with DCM afforded DABDT with > 90% of purity and in high yield. However, to our surprise, after a couple of hours, the DABDT was found to be converted into DABDT$_{ox}$, both in DCM solution and as solid. The reaction was repeated several times with similar fully conversion to the oxidized form. The only plausible explanation is that oxidation was catalyzed by the presence of the aluminium. As the reduction seems to be a good strategy to obtain the dithiol, it was attempted using NaBH$_4$, which is a different and milder. To the best of our knowledge, there is not precedent in the literature about it used to reduce thioesters. Then, NaBH$_4$ (10 equiv.) was added in two portions to the dithioester dissolved in MeOH and the reaction was left to react for 15 mins. After workup DABDT (> 97% pure) was obtained in 96% yield. In this case, it was stable as solid and as well as in solution. DABDT was characterized by HPLC, mass spectrometry and different NMR techniques (SI:}
Figure S25-S32). DABDT upon treating with 6N NaOH affords DABDT$^{\text{ox}}$, which was fully characterized (SI: Figure S33-S40).

**Solubility**

The ideal of any reagent such as a reducing one is to be soluble in a broad range of solvents for a maximum extension of its use. This is still more important in the case of Cys containing moieties, because in some cases the reduction will be carried out when the peptide is still protected and anchored to the resin or when the peptide/protein are totally unprotected and are in solution. While in the first case, organic solvents would be the preferred and, in the second, aqueous based will be required.

In a preliminary solubility screening, we can conclude that DABDT shows a good solubility in organic polar solvents such as $N,N$-dimethylformamide (DMF), dichloromethane (DCM), tetrahydrofuran (THF), ethyl acetate and acetonitrile (ACN).

**Stability**

Ideally, a solid reagent should be kept at room temperature for facilitating its transportation and storage. For practical uses, it is also convenient that the reagent be stable in solution. In this regard, we can conclude that 5% DABDT in ACN is stable for more than 10 days as it shows <30 % oxidized, as quantified by HPLC.

**Screening the reducing availability of DABDT**

For exploring the reducing availability of the new reducing agent, DABDT, we have chosen two different models: (i) Cys containing moieties where the $\beta$-thiol side chain is protected with the dimethoxyphenylthio (SDMP) and (ii) a peptide containing at the N-terminal dithio-dipropionyl moieties.

After being introduced by our group a few years ago, Fmoc-Cys(SDMP)-OH, which is commercially available has become the protecting group of choice among the Cys protecting groups of the disulfide type for the SPPS. This kind of protecting groups are removed by reducing agents and therefore its use is compatible with other protecting groups that are labile in the presence of acids [e.g. trityl (Trt), diphenylmethyl (Dpm)], or oxidants [e.g. acetoamidomethyl (Acm)] or enzymes [e.g. phenylacetamidomethyl (Phacm)]. It is important to highlight that the disulfide protecting group should be removed first in the cascade of Cys protecting groups if more of one is used, because once a disulfide bridge is formed in the peptide/protein, the removal
of the disulfide protecting group such as DMP will provoke scrambling (random formation of intra and inter disulfide bridges, the inter type will render polymerization).

On the other hand, the dithiodipropionic acid is very often used as last residue of the peptide chain. This derivative is of very friendly use, because it is a great acylating moiety, is very cheap, and its reduction renders a thiopropionyl peptide, which is ready for conjugation of the peptide to another biological or chemical entity.

First of all, Fmoc-Cys(SDMP)-OH was subjected to reduction in solution using DABDT (5 equiv.) in 3% of N,N-diisopropylethylamine (DIEA) in ACN at room temperature and the reaction was monitored by HPLC. Under these conditions, the reduction seems to proceed very fast since, at time 0, around of 80% of the protecting group was removed as indicated by the formation of Fmoc-Cys-OH, the protecting group HSDMP in form of free thiol, and the oxidized (cyclic) form of the DABDT (Figure 4, left). However, the removal reaction was reversible if we left the reaction for 80 minutes, the back formation of Fmoc-Cys(SDMP)-OH takes place with the concomitant diminution of the peaks corresponding to the HSDMP and the cyclic form of the DABDT (Figure 4, right).

![Diagram](image)

**Figure 4:** HPLC chromatogram of disulphide bridge reduction in Fmoc-Cys(SDMP)-OH using DABDT, monitored at 0 min, 20 min, 40 min and 80 min.

This problem was overcoming by adding to the reducing cocktail some H₂O. Thus, carrying out the reduction in ACN/DIEA/H₂O (94:3:3), the reaction took place instantly. Thus, at time 0, the
reduction of Fmoc-Cys(SDMP)-OH into Fmoc-Cys-OH was higher than 95 %. Moreover, analyzing the reaction mixture in the same timing as in the previous case, i.e. until 80 min, it was no evidence that the reaction went back. We have hypothesized that the presence of H$_2$O can stabilize the free thiol.

Further, the reaction was studied under two more different conditions. In the first one, the equivalents of DABDT were decreased to 3 using the same solvent mixture, ACN/DIEA/H$_2$O (94:3:3), with no effect in the reaction course, the conversion was instantaneous. However, when DIEA was substituted for a weaker base as N-methylmorpholine (NMM), the reaction became a little slower taking place in 40 min. For comparison purposes, DTT was used as reducing agent, the same behavior was found. This is a confirmation that in terms of reducing capacity DABDT could be a good substituted for DTT.

Next, the removal of the SDMP group was carried out in solid-phase. Thus, the model peptide Ac-Cys(SDMP)-Gly-Phe-Leu-NH-Rink-resin was elongated using typical SPPS conditions. At the end, the peptide resin was treated with 5 equiv. of DABDT in ACN/DIEA/H$_2$O (94:3:3) twice for 5 min. each one. Then, the peptide resin was washed with DMF and DCM, and the peptide was cleaved from the resin with trifluoroacetic acid (TFA)-triisopropylsilane (TIS)-H$_2$O (95:2.5:2.5) for 1 h at room temperature. After lyophilization, the crude peptide was analyzed by HPLC showing only the reduced peptide (Figure 5, upper panel).

Using the second model, the HOOC-CH$_2$-CH$_2$-S-S-CH$_2$-CH$_2$-CO-Gly-Phe-Leu-NH-Rink-resin was prepared and then treated with DABDT (3 eq.) and DIEA-H$_2$O-ACN (3:3:94) twice for 1 min each one. After the cleavage using the same conditions than above and the corresponding work-up, the HPLC showed that the reduction has taken place with 100 % of yield (Figure 5, lower panel).
Figure 5: HPLC chromatogram of disulphide bridge reduction in peptide anchored on solid support using DABDT.

3. Conclusion

Taking as starting product the cheap aspartic acid, we have developed the disulfide reducing agent, DABDT. DABDT belongs to the class of dithiol reducing agents whose oxidized form in stabilized by a six-member ring such as DTT. The preparation of DABDT has been conveniently finned tuned, carrying out all steps with high performance in terms of purity and yield. It is important to highlight that the conversion of the thioester to the thiol is conveniently carried out through a reduction using NaBH₄ as reducing agent. Unexpectedly, the use of the strongest LAH as reducing agent allows the obtention of the target compound, which after purification suffers apparently spontaneous oxidation to the six-member ring compound. We have hypothesized that
this oxidation could be catalyzed by traces of aluminium present in the final compound. The use NaBH₄ allows the formation of the target DABDT, which does not suffer the undesirable oxidation.

DABDT is soluble in a broad range of solvents such as DMF, ACN, THF, DCM and ethyl acetate. DABDT as a solid and dissolved in ACN is stable at least for reasonable period of times. DABDT has demonstrated to remove cleanly the SMDP protecting group of the thiol of the cystine in solution and solid-phase as well. Furthermore, DABDT has reduced N-terminal dithiodipropionyl moieties present in the N-terminal of protected peptides anchored on a resin.

We envisage that due to the cheap and convenient preparation, friendly use due to the absence of odour, and stability, and excellent performance will be rapidly adopted by the scientific community as the as disulfide reducing agent of choice.

4. Experimental

_General:_ All reagents and solvents were purchased from commercial suppliers and were used without further purification, unless otherwise stated. NMR spectra (^1H NMR and ^13C NMR) were recorded on a Bruker AVANCE III 400 MHz spectrometer. Chemical shift values are expressed in parts per million (ppm). Analytical HPLC was performed on Agilent 1100 system using Phenomenex C₁₈ column (3 µm, 4.6 × 50 mm), and Chemstation software was used for data processing over a 5-95 % gradient of CH₃CN (0.1% TFA)/ H₂O (0.1% TFA) over 15 min, flow rate: 1.0 mL/min, detection at 220 nm. High resolution mass spectrometry (HRMS) was performed using a Bruker ESI-QTOF mass spectrometer in positive-ion mode.

_Synthesis of dibenzyl N,N-dibenzylaspartate (2)_

To a solution of K₂CO₃ (4.6 g; 35 mmol) in water, aspartic acid (1 g, 7.5 mmol) was added and the reaction was stirred vigorously. Benzyl bromide (4.1 mL, 35 mmol) was added dropwise to the above stirring solution. The reaction was stirred at 80 °C for 40 mins under MW conditions. The reaction was monitored by TLC until consumption of aspartic acid (as checked by ninhydrin spray). After the complete consumption of aspartic acid, the crude product was extracted with ethyl acetate. Organic layer was washed with water, collected and dried over magnesium sulphate. Organic layer was filtered and concentrated to afford crude product which was purified using silica gel column chromatography using n-Hexane/Ethyl acetate as mobile phase. The pure product was used for next step.
Pale yellow liquid, yield = 3.4 g (94.4 %); HPLC $t_R = 13.5$ min; $^1$H NMR (400 MHz, CD$_3$OD): 2.56 (dd, $J = 7.7$ Hz, 1H), 2.74 (dd, $J = 7.9$ Hz, 1H), 3.36 (d, $J = 13.5$ Hz, 2H), 3.58 (d, $J = 13.5$ Hz, 2H), 3.78 (t, $J = 7.5$ Hz, 1H), 4.78 (d, $J = 12.0$ Hz, 1H), 4.95 (dd, $J = 7.5$ Hz, 2H), 5.10 (d, $J = 11.7$ Hz, 1H), 7.05-7.27 (m, ArH); $^{13}$C NMR (100 MHz, CD$_3$OD): 35.7, 55.8, 59.4, 67.5, 67.6, 128.3, 129.2, 129.3, 129.4, 129.5, 129.7, 129.8, 130.0, 137.3, 137.5, 140.3, 172.5, 172.7. HRMS: m/z: calcd. for C$_{32}$H$_{31}$NO$_4$: 494.2325 [M+H]$^+$; found: 494.2348.

Synthesis of 2-(dibenzylamino)butane-1,4-diol (3)

Cooled a solution of 2 (3.2 g, 6.5 mmol) in diethyl ether and stirred vigorously. To the ice cooled stirring solution added LAH in three portions and then the reaction was stirred for 4 h at room temperature. The reaction was monitored by TLC until complete consumption of 2. After completion, excess LAH was quenched using water maintaining the temperature at 0 °C. The suspension was filtered to remove the salts. The aqueous layer was washed several times with ethyl acetate. Organic layer was collected, dried over magnesium sulphate, filtered and concentrated to afford crude product which was purified using silica gel column chromatography using n-Hexane/Ethyl acetate as mobile phase. The pure product was used for next step.

Pale pink semi solid, yield = 1.5 g (81 %); HPLC $t_R = 5.9$ min; $^1$H NMR (400 MHz, CD$_3$OD): 1.59 (m, 1H), 1.92 (m, 1H), 2.87 (m, 1H), 3.52-3.76 (m, 8H), 7.18-7.33 (m, ArH); $^{13}$C NMR (100 MHz, CD$_3$OD): 31.5, 54.9, 58.6, 62.0, 62.6, 128.2, 129.4, 130.2, 141.3. HRMS: m/z: calcd. for C$_{18}$H$_{23}$NO$_2$: 286.1801 [M+H]$^+$; found: 286.1811.

Synthesis of $S,S'$-(2-(dibenzylamino)butane-1,4-diyl) diethanethioate (5)

3 (1.5 g, 5.3 mmol) was dissolved in DCM and SOCl$_2$ was added in excess. The reaction was stirred at 60 °C and was monitored by TLC until no starting material was observed. After 3 h solvent was removed completely to afford 4 which was used directly for next step. 4 was dissolved in ACN and a solution of potassium thioacetate (5 eq) in ACN with few drops of DMF (for solubility) was added followed by triethyl amine (75 µL, 0.1 mmol). The reaction was further heated to 60 °C and was monitored by TLC for consumption of SM. After 4 h when no starting material was observed (as monitored by TLC), the solvent was removed under vacuum. The crude was washed with water and extracted with ethyl acetate. Organic layer was collected, dried over magnesium sulphate, filtered and concentrated to afford crude product which was purified using silica gel column chromatography using n-Hexane/Ethyl acetate as mobile phase. The pure product was used for next step.
Dark red semi solid, yield = 1.0 g (67 %); HPLC $t_R = 10.1$ min; $^1$H NMR (400 MHz, CDCl$_3$): 1.53 (m, 1H), 1.82 (m, 1H), 2.18 (s, 3H), 2.22 (s, 3H), 2.63 (m, 2H), 2.76 (m, 1H), 2.99 (m, 1H), 3.19 (m, 1H), 3.51 (d, $J = 13.5$ Hz, 2H), 3.59 (d, $J = 13.9$ Hz, 2H), 7.14-7.29 (m, ArH); $^{13}$C NMR (100 MHz, CDCl$_3$): 25.7, 27.9, 29.1, 29.6, 52.2, 56.1, 125.9, 127.2, 128.0, 138.5, 194.4, 194.8. HRMS: $m/z$: calcd. for C$_{22}$H$_{27}$NO$_2$S$_2$: 402.1556 [M+H]$^+$; found: 402.1568.

Synthesis of 2-(dibenzylamino)butane-1,4-dithiol (1)

**Method I:** Using LR

3 (50 mg, 0.2 mmol) was dissolved in toluene and LR (121 mg, 0.3 mmol) was added. The reaction was heated for 12 h (conventional) or 2 h (MW) at 140 °C. Solvent was removed under vacuum. No product formation.

**Method II:** Using thiourea

3 (50 mg, 0.2 mmol) was dissolved in DCM and SOCl$_2$ was added in excess. The reaction was stirred at 60 °C and was monitored by TLC until no starting material was observed. After 3 h solvent was removed completely to afford 4 which was directly used for the next step. Thiourea and 4 was dissolved in EtOH. The reaction was refluxed for 8 h under nitrogen. To this reaction mixture, 6N NaOH (2 mL) was added and further refluxed for 2 h. The reaction was cooled to room temperature and 2N HCl was added until pH 4 was obtained followed by extraction with DCM. The organic layer was collected, dried over magnesium sulphate, filtered and concentrated. Only trace amount of desired product was obtained.

**Method III:** From thioester

Three approaches were made to synthesis DABDT (1) from thioester (5)

**Approach IIIa:** Using HCl

Compound 5 (50 mg, 0.12 mmol) was dissolved in MeOH followed by addition of 5 mL of 3N HCl. The reaction mixture was heated at 70 °C in MW. The reaction was monitored until complete consumption of SM. After 2.5 h, MeOH was removed under vacuum and residue was extracted with DCM. The organic layer was collected, dried over magnesium sulphate, filtered and concentrated. The DABDT$^{ox}$ was also formed along with the desired product.
**Approach IIIb: Using LAH**

LAH was suspended in diethyl ether and compound 5 (50 mg, 0.12 mmol) in diethyl ether was added at 0°C. The reaction mixture vigorously stirred at rt for 10 mins. The reaction was quenched using water followed by addition of conc HCl to make the reaction mixture acidic. The crude was extracted with DCM. The organic layer was collected, dried over magnesium sulphate, filtered and concentrated affording pure DABDT.

**Approach IIIc: Using NaBH₄**

4 (50 mg, 0.12 mmol) was dissolved in methanol and NaBH₄ was added 10 folds in excess. The reaction was stirred vigorously at rt for 30 mins. Conc HCl was added until solution was acidic enough. Crude was washed with water and extracted with DCM. Organic layer was collected, dried over magnesium sulphate, filtered and concentrated to afford pure 1.

Yellow solid, yield = 47 mg (93.0 %); HPLC tᵣ = 8.24 min; ¹H NMR (400 MHz, CDCl₃): 1.20 (s, thiol-2H), 1.79 (m, 2H), 2.32 (m, 1H), 2.50 (m, 2H), 2.70 (m, 1H), 2.86 (m, 1H), 3.46 (d, J = 13.5 Hz, 2H), 3.61 (d, J = 13.5 Hz, 2H), 7.16-7.27 (m, ArH); ¹³C NMR (100 MHz, CDCl₃): 21.0, 22.9, 32.9, 52.4, 58.2, 126.1, 127.4, 128.0, 138.4. HRMS: m/z: calcd. for C₁₈H₂₃NS₂: 318.1601 [M+H]+; found: 318.1601.

**Synthesis of N,N-dibenzyl-1,2-dithian-4-amine (1°)**

6N NaOH solution (1 mL) was added to 1 (15 mg). Reaction was extracted with DCM. Organic layer was collected, dried over magnesium sulphate, filtered and concentrated to afford pure 1°.

Yellow solid, yield = 14.5 mg (97 %); HPLC tᵣ = 7.89 min; ¹H NMR (400 MHz, CDCl₃): 1.82 (m, 2H), 2.26 (m, 1H), 2.53 (m, 1H), 2.82 (m, 2H), 2.95 (m, 1H), 3.54 (d, J = 13.9 Hz, 2H), 3.65 (d, J = 13.9 Hz, 2H), 7.14-7.28 (m, ArH); ¹³C NMR (100 MHz, CDCl₃): 28.7, 34.1, 35.6, 52.6, 56.4, 125.9, 127.3, 127.4, 138.9. HRMS: m/z: calcd. for C₁₈H₂₃NS₂: 316.1188 [M+H]+; found: 316.1182.

**General protocol for synthesis of peptide (Ac-Cys(SDMP)-Gly-Phe-Leu-NH₂ and DTDP-Gly-Phe-Leu-NH₂)**

Fmoc-Rink-Amide AM resin (0.74 mmol/gram, 1 equiv.) was washed with DMF (2 × 1 min), CH₂Cl₂ (2 × 1 min) and DMF (2 × 1 min). Deprotection of the Fmoc group was achieved by treatment of the resin with 20% piperidine/DMF (1 × 1 min and 1 × 7 min) followed by washing with DMF, CH₂Cl₂ and DMF. The protected Fmoc-amino acids (3 eq.) were incorporated using
DIC (3 eq.) and OxymaPure (3 eq.) in DMF, as a coupling system, 30 mins at rt. This was repeated until the final peptide was achieved. Washes between couplings and deprotections were performed with DMF (3 × 1 min), CH₂Cl₂ (3 × 1 min) and DMF (3 × 1 min). After the peptide synthesis, Fmoc was removed from both the peptides. In one case acetylation (acetic anhydride and DIEA in 1:2 ratio using DMF as solvent) was performed for 30 mins. In another case, dithiodipropionic acid (DTDP; 3 eq.) using DIC (3 eq.) and OxymaPure (3 eq.) in DMF for 30 mins. Both the peptides were dried and microcleavage was performed, by treating with TFA/TIS/H₂O (95:2.5:2.5) for 1 h at rt. The cleavage mixture was evaporated with a stream of nitrogen, precipitated with Et₂O, centrifuged and the pellet was re-dissolved in H₂O/CH₃CN (1:1) for analysis by HPLC and LCMS.

**General protocol for study of DABDT as a reducing agent in solution phase for Fmoc-Cys(S-DMP)-OH**

The equimolar solution (10 mmol) of DABDT and Fmoc-Cys(S-DMP)-OH was prepared separately in acetonitrile. Different ratio (1:5, 1:3 and 1:1) of both solutions were mixed for studying the reduction reaction. 5 µL of Fmoc-Cys(S-DMP)-OH and 250 µL of DABDT (150 µL for 1:3 and 5 µL for 1:1) and 10 µM DIEA and 3% water were added and immediately 2 µL of sample was injected in HPLC.

**General protocol for study of DABDT as a reducing agent in solid phase**

10 mg of the resin was washed with DMF. 100 mg/mL solution of DABDT was prepared in DMF. To the washed resin, 50 µL of DABDT solution was added with 5 µL of DIEA and 1 µL water. After 5 mins the supernatant was discarded, and the reaction mixture was again added for 5 mins. The resin was washed with DMF and DCM and dried. Minicleavage was performed using TFA for 30 mins. TFA was evaporated with a stream of nitrogen, precipitated with Et₂O, centrifuged and the pellet was re-dissolved in H₂O/CH₃CN (1:1) for analysis by HPLC and LCMS.

**ACKNOWLEDGMENT**

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5. References


6. Characterization of Synthesized Molecules

**Figure S1**: HPLC for Compound 2

![HPLC graph for Compound 2](image1)

Exact Mass: 493.2253

**Figure S2**: HRMS for Compound 2

![HRMS graph for Compound 2](image2)
Figure S3. $^1$H NMR for 2

Current Data Parameters
NAME May07-2018-FA-51ne
EXPNO 10
PROCNO 1

F2 - Acquisition Parameters
Date_ 20180507
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PROBND 5 mm FABEO BR-
FULPROG zg30
TD 32760
SOLVENT Me0D
NS 64
DS 2
SMP 823.685 Hz
FIDRES 0.250967 Hz
AQ 1.9921944 sec
RG 101
DW 60.800 usec
DE 6.50 usec
TE 290.2 K
E1 1.06000000 sec
TD0 1

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NUCI 1H
F1 10.00 usec
FL1 -3.00 dB
FLW 15.48666575 W
ZFC1 400.224715 MHz

F2 - Processing parameters
SI 16384
SF 400.2200488 MHz
MDW EM
SSB 0
LB 0.30 Hz
GB 0
FC 1.00
Figure S4. $^{13}$C NMR for 2.

Current Data Parameters
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PROCNO 1

F2 - Acquisition Parameters
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Time 21:03
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FURPRO zppg30
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SOLVENT MeOD
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DS 4
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FIDRES 0.366798 Hz
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WG 1293
DW 20.880 usec
DE 6.50 usec
TZ 294.2 K
D1 2.00000000 sec
D11 0.00000000 sec
TDO 1

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PL1 -2.00 dB
PL1W 54.14257431 W
SF01 100.6454626 MHz

-------- CHANNEL f2 --------
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PCOS02 90.00 usec
PL2 -3.00 dB
PL12 15.60 dB
Pl13 15.60 dB
FL2W 0.21377757 W
FL1W 0.120301511 W
SF02 460.22106909 MHz

F2 - Processing parameters
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SF 100.6452561 MHz
MOS EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40
Figure S5. $^{13}$C DEPT 90 for 2
Figure S6. $^{13}$C DEPT 135 for 2
Figure S7. 2D NMR HSQC for 2
Figure S8. 2D NMR HMBC for 2
Figure S9: HPLC for Compound 3

Figure S10: HRMS for compound 3
Figure S11. $^1$H NMR for 3
Figure S12. $^{13}$C NMR for 3
Figure S13. $^{13}$C DEPT 90 for 3
Figure S14. $^{13}$C DEPT 135 for 3
Figure S15. 2D NMR HSQC for 3
Figure S16. 2D NMR HMBC for 3
Figure S17: HPLC for Compound 5

![HPLC Graph]

Exact Mass: 401.1483

Figure S18: HRMS for compound 5

![HRMS Graph]
Figure S19. $^1$H NMR for 5
Figure S20. $^{13}$C NMR for 5

Current Data Parameters
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EXPN0    10
PROCNO   1

F2 - Acquisition Parameters
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SOLVENT  CDCl3
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FIDRES   0.366798 Hz
AQ       1.3631488 sec
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F1       11.00 usec
PLW1     54.14300156 W

-------- CHANNEL f2 --------
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NUC2     1H
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PCED2    90.00 usec
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PLW12    0.21483000 W
PLW13    0.10806000 W

F2 - Processing parameters
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WDM      EM
SSB      0
LB       1.00 Hz
GB       0
PC       1.40
Figure S21. $^{13}$C DEPT 90 for 5
Figure S22. $^{13}$C DEPT 135 for 5
Figure S23. 2D NMR HSQC for 5
Figure S24. 2D NMR HMBC for 5
Figure S25: HPLC for Compound 1

Figure S26. HRMS for compound 1

Exact Mass: 317.1272
Figure S27. $^1$H NMR for 1

Current Data Parameters
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PROCNO 1

F2 - Acquisition Parameters
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SOLVENT CDC13
NS  64
DS  2
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FDRES 0.122266 Hz
AQ  4.0894465 sec
RG  203
DW  62.400 usec
DE  6.50 usec
TE  29.6 K
D1  1.00000000 sec
TD0  1

--------- CHANNEL f1 ---------
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NCHI  1H
P1  12.00 usec
PLW1  16.29999924 W

F2 - Processing parameters
SI  65536
SP  400.2200408 MHz
WDW  BM
SSB  0
LB  0.30 Hz
GB  0
PC  1.00
Figure S28. $^{13}$C NMR for 1
Figure S29. $^{13}$C DEPT 90 for 1
Figure S30. $^{13}$C DEPT 135 for 1
Figure S31. 2D NMR HSQC for 1
Figure S32. 2D NMR HMBC for 1
Figure S33. HPLC for $1^{\text{ox}}$

![HPLC graph]

Figure S34. HRMS for $1^{\text{ox}}$

![HRMS graph]
Figure S35. $^1$H NMR for 1$^{ox}$
Figure S36. $^{13}$C NMR for 1

Current Data Parameters
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EXPNO: 11
PROCNO: 1

F2 - Acquisition Parameters
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NS: 1024
DS: 4
SWH: 24038.461 Hz
FIDRES: 6.366798 Hz
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RG: 2050
DW: 20.800 usec
DE: 6.50 usec
TE: 299.9 K
D1: 2.00000000 sec
D11: 0.03000000 sec
TD0: 1

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PLW1: 54.14300156 W

--------- CHANNEL f2 ---------
SF02: 400.2216009 MHz
NUC2: 1
CPD2: 90.00 usec
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PLM12: 0.21483000 W
PLM13: 0.10860000 W

F2 - Processing parameters
SF: 32768
SF: 100.6355027 MHz
WDW: EM
LSB: 0
LB: 1.00 Hz
CR: 1.40
Figure S37. $^{13}$C DEPT 90 for 1\textsuperscript{ox}
Figure S38. $^{13}$C DEPT 135 for $^{1}$Cox
Figure S39. 2D NMR HSQC for 1\textsuperscript{ox}
Figure S40. 2D NMR HMBC for $\text{I}^{\text{ox}}$
CHAPTER 3
CONCLUSION

In this work, a new disulfide reducing agent, DABDT, has been developed. As DTT, the dithiol DABDT belongs to the family of reducing agents which form a stable six-member ring in its oxidized form.

After a careful optimization, the synthetic route proposed is a five-step process with an overall yield of 52%. Furthermore, this process is based on the use of the very affordable and cheap starting material, aspartic acid, and other convenient reagents such as LAH as well. Some green aspects such as water as a solvent and the use of LAH have been introduced in some of the steps of the process. Overall, it is envisaged that DABDT will be very competitive from a commercial point of view.

Starting from aspartic acid, the first step involves a perbenzylation in water (four benzyl groups were incorporated), which always is a convenient reaction, because selectivity is not required.

The reduction of the two benzyl esters to the corresponding alcohols is performed with LAH. The conversion of the diol to the dichloro derivative is easily carried out using thionyl chloride. This reaction does not imply any purification step, because the side products are gas and easily removed by evaporation.

The formation of the acetyl thioesters is done with potassium thioacetate, which requires the only chromatography purification of the whole process.

Finally, and after several attempts, DABDT is nicely obtained by reduction of the dithio acetyl derivatives with NaBH₄, which to the best of our knowledge it is not commonly used for this kind of reactions. Use of LAH as reducing agent render first the target compound that is spontaneously converted to its oxidized six-membered ring form. It has been speculated that aluminium traces can catalyze this undesirable oxidation. Hydrolysis with HCl solution require long reaction times or use of microwave but rendering in all cases the desired product with several impurities.

DABDT is a solid, which facilitates its use, and does not show a deep hurtful smell. As a solid, it is stable and even in ACN solution at room temperature, it is stable for many days.

DABDT is soluble in a broad range of organic solvents such as MeOH, ACN, DMF or DMSO. As expected, it is not soluble in water.
DABDT has shown to reduce the SDMP, which is the disulfide protecting group of choice for cysteine, in both solution and solid-phase. Furthermore, it also reduces in solid-phase and solution other disulfide bridges between cysteine and/or cysteine analogues.

In conclusion, DABDT is a promising disulfide reducing agent able to be used in organic solvents in both solution and solid-phase.