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Use of 2,2'-dithiobis(5-nitropyridine) for the Heterodimerization of Cysteine Containing Peptides. Introduction of the 5-nitro-2-pyridinesulfenyl Group

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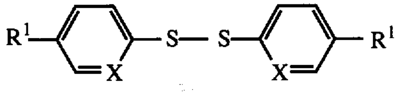
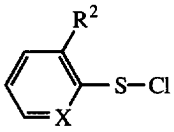
Abstract. 2,2'-Dithiobis(5-nitropyridine) has proven to be a suitable reagent for the activation of the thiol function of cysteine in peptides and subsequent asymmetric disulfide formation. The utility of this reagent is tested in the preparation of *de novo* designed cytochrome model heterodimeric 62-mer peptides.

The formation of an asymmetric disulfide bond between two different peptide chains generally requires activation of the thiol function of one of the peptides, followed by addition of the second peptide in the free thiol form. This approach is necessary to promote the formation of the desired heterodimer, otherwise unwanted homodimeric molecules are obtained in appreciable yields. Several procedures have been described for the formation of mixed disulfides. One of the most successful and therefore widely used method employs aromatic sulfenyl protecting/activating groups, such as 2-nitrophenyl (Nps), 2-pyridinesulfenyl (PyrS) and 3-nitro-2-pyridinesulfenyl (Npys).[1,2] The formation of the disulfide bond is driven by the low pKa of the aromatic thiol, and hence the reaction can be performed under the mildly acidic conditions required to minimize disulfide exchange reactions once the desired Cys-Cys disulfide is formed. The Npys group can be introduced during the synthesis of a peptide via the commercially available cysteine derivative, Boc-Cys(Npys)-OH, which is readily incorporated into a Boc/Bzl peptide synthesis strategy.[3] However, the Npys and other activated Cys derivatives are not compatible with the Fmoc/But synthesis strategy as they are not stable to the strongly basic conditions required for the removal of the Fmoc group. Consequently, the activation of cysteine for the synthesis of heterodimers in peptides assembled by Fmoc/But strategy is often performed with Npys-Cl.[3] However, sulfenyl chlorides have limited stability, are moisture-sensitive and can react with other functionality such as the indole ring of tryptophan.[4] Cysteines can also be activated by reacting thiol-containing peptides with 2,2'-dithiobispyridine, although it results in a rather modest level of activation.[2,5]

We describe in this article the use of 2,2'-dithiobis(5-nitropyridine) or DTNP[6] as an activating reagent for the thiol function of cysteine in order to synthesize heterodimeric peptides. Furthermore, the protecting/activating group of the thiol function generated, 5-nitro-2-pyridinesulfenyl or pNpys, is an isomer of





$R^1 = NO_2$, X = N } 2,2'-dithiobis(5-nitropyridine)

$R^2 = NO_2$, X = N } Npys-Cl

$R^1 = H$, X = N } 2,2'-dithiopyridine

$R^2 = H$, X = CH } Nps-Cl

the known Npys, retaining essentially all of its valuable properties but adding the simplicity, availability and chemical advantages of being introduced by a disulfide reagent instead of a sulfenyl chloride. In addition, the *para* nitro group increases the acidity of the corresponding thiol, making it a better leaving group than the 2-pyridinesulfenyl, and therefore able to react at lower pH.[7]

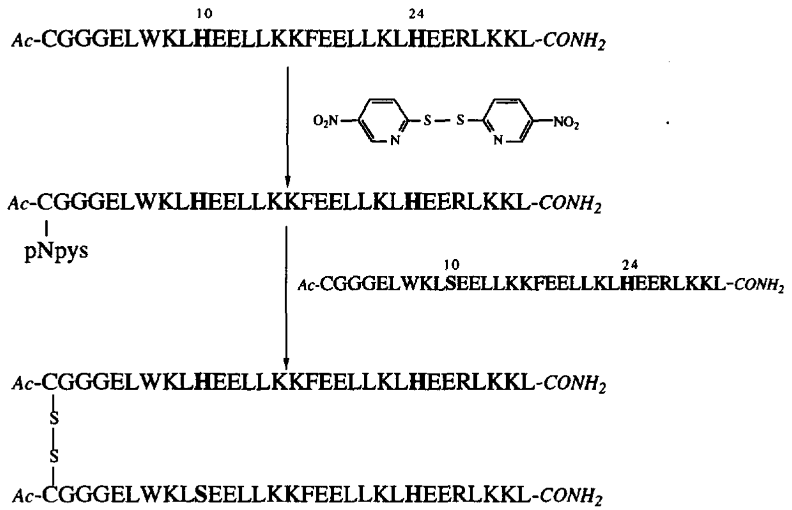
Our aim was the synthesis of *de novo* heterodimeric amphiphilic di-α-helical peptide molecules that coordinate heme groups as models for the different cytochromes found in nature. We designed and synthesized the following peptide chains:[8]

H10H24 [1-31]: *Ac*-CGGGELWKL**H**EELLKKFEELLKL**H**EERLKKL-$CONH_2$

S10H24 [1-31]: *Ac*-CGGGELWKLSEELLKKFEELLKL**H**EERLKKL-$CONH_2$

The peptides were assembled following a Fmoc/tBu scheme of protection[9] using 5-(4-aminomethyl-3, 5-dimetoxyphenoxy)valeric acid (the PAL™ handle) as the handle in order to obtain the corresponding terminal carboxamides.[10, 11] H10H24 (in the free thiol form) was then reacted with DTNP to activate its Cys residue. It is important to note that a sulfenyl halide would not be a suitable reagent for that purpose due to the presence of a tryptophan in the sequence.[4, 12]

Generally speaking, the group 5-nitro-2-pyridinesulfenyl is easily introduced in cysteine containing peptides by treating them with a 3-5 times excess of 2,2'-dithiobis(5-nitropyridine) in acetic acid:water (3:1) for 4-6 hours with excellent yields. A simple workup yields a crude product which is pure enough (>95%) to be used in the next step.[13]



The heterodisulfide formation takes place in a wide range of pH conditions under argon atmosphere. We found that the reaction is extremely fast (less than a minute) between pH=3.5 and pH=6.5 (1M ammonium acetate buffer) rendering high yields of the target molecule (90-95 % judged from integrated areas of HPLC traces)[14]. The reaction proceeds much slower in 0.1 M acetic acid (pH *ca* 2.7) needing about three hours to be completed but again with excellent yields. This last point demonstrates the low pKa of the conjugated acid thiol of the leaving group as can be expected from the structure, isomeric to Npys. The ability to form disulfides at low pH should be advantageous for the synthesis of heterodimers that are less stable than the corresponding homodimers, in which case the symmetrical products would predominate at higher pH values due to disulfide exchange reactions.

In conclusion, 2,2'-dithiobis(5-nitropyridine) is a very convenient and efficacious reagent for the formation of unsymmetrical disulfide bonds at acidic pH. Moreover, it provides a new protecting group 5-nitro-2-pyridinesulfenyl (pNpys), isomeric to the well known 3-nitro-2-pyridinesulfenyl (Npys). It is also a valuable alternative reagent to sulfenyl chlorides since it is stable, convenient, and unreactive towards tryptophan. The preparation of a pNpys protected cysteine and its evaluation as a suitable derivative for peptide synthesis by Boc/Bzl strategy is also underway.

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References and Notes

1. Abbreviations used for amino acids follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature in J. Biol. Chem. **1972**, *247*, 977-983. Single letter code for amino acids used in this article are: C, cysteine; E glutamic acid; F, phenylalanine; G, glycine; H, histidine; K, lysine; L, leucine; R, arginine; S, serine; W, tryptophan. Other abbreviations are: Ac, acetyl; Boc, *tert*-butyloxycarbonyl; But, *tert*-butyl; Bzl, benzyl; DIPCDI, *N,N'*-diisopropylcarbodiimide; DMF, *N,N*-dimethylformamide; DTNP, 2,2'-dithiobis(5-nitropyridine); Fmoc, 9-fluorenylmethyloxycarbonyl; HOBt, 1-hydroxy-

benzotriazole; Nps, 2-nitrophenyl; Npys, 3-nitro-2-pyridinesulfenyl; Pmc, pentamethylchromansulfonyl; pNpys, 5-nitro-2-pyridinesulfenyl; PyrS, 2-pyridinesulfenyl; Trt, trityl or triphenylmethyl;

2. For a recent review in formation of disulfide bonds in synthetic peptides and proteins see: Andreu, D.; Albericio, F.; Sole, N. A.; Munson, M. C.; Ferrer, M.; Barany, G. *Methods in Molecular Biology: Peptide Synthesis Protocols, Vol. 35*; Pennington, M. W.; Dunn, B. M., Eds; Humana Press Inc.; Totowa, NJ, 1994; pp 91-169.
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5. Castell J. V.; Tun-Kyi, A. *Helv. Chim. Acta* **1979**, *62*, 2507-2510; Akaji, K.; Fujino,K.; Tatsumi, T. ; Kiso, Y. *J. Am. Chem. Soc.* **1993**, *115*, 11384-11392.
6. 2,2'-dithiobis(5-nitropyridine) is a reagent generally used for the selective detection of thiols. See for instance: Grassetti, D. R.; Murray, D. R. *J. Chromatogr.* **1969**, *41*, 121-123. However, during the preparation of this manuscript, a search on the uses of this reagent revealed than it was actually used in one case as a reagent for unsymmetrical formation in the preparation of polymer supports for the synthesis of sulfhydryl containing oligonucleotides: Gupta, K. C.; Sharma, P.; Kumar, P.; Sathyanarayana, S. *Nucleic. Acids. Res.* **1991**, *19*, 3019-25.
7. Ponsati, B.; Ruiz-Gayo, M.; Giralt, E.; Albericio, F.; Andreu, D. *J. Am. Chem. Soc.* **1990**, *112*, 5345-5347.
8. The synthesis of this heterodimer is given as a example among the several successfully performed in our laboratory using this procedure. The *de novo* design of these peptides is discussed extensively in the following references of our groups: Robertson, D. E.; Farid, R. S.; Moser, C.C.; Urbauer, J. F.; Mulholland, S. E.; Pidikiti, R. ; Lear, J. D.; Wand, A. J.; DeGrado, W. F.; Dutton, P. L. *Nature* **1994**, *368*, 425-432; Choma, C. T.; Lear, J. D.; Nelson, M. J.; Dutton, P. L.; Robertson, D. E.; DeGrado, W. F. *J. Am. Chem. Soc.* **1994**, *116*, 856-865; Rabanal, F.; DeGrado, W. F.; Dutton, P. L. *J. Am. Chem. Soc.* **1995**, in press.
9. The amino acids were coupled as their pentafluorophenyl esters in the presence of HOBt. Protection was as follows: Trt for Cys, Pmc for Arg, Boc for Lys and His, *t*-But ether for Ser and *t*-But ester for Glu.
10. Albericio, F.; Kneib-Cordonier, N.; Biancalana, S.; Gera, L.; Masada, R. I.; Hudson, D.; Barany, G. *J.Org. Chem.* **1990**, *55*, 3730-3743.
11. Both peptides were cleaved with TFA:thioanisole:anisole:ethanedithiol (90:5:3:2) for 2 hours yielding crudes with the corresponding right product as a major component (about 85-90% according to the integrated area of the HPLC traces). Both peptides were purified to homogeneity by preparative reverse phase HPLC in acetonitrile-water-0.1% TFA eluent gradients and characterized by analytical HPLC, UV-spectroscopy (indole absorption) and laser-desorption mass spectrometry.
12. We attempted to prepare des-acetyl H10H24 with an Npys group on its Cys residue by coupling Boc-Cys(Npys)-OH at the last coupling step (DIPCDI mediated coupling, 5 eq., 1 h). However, the use of the PAL handle and the presence of both a tryptophan residue and a Pmc protected arginine in the sequence require good scavenging such as that provide by thiols at the final acidolytic treatment [see for instance: Choi H.; Adrich, J. V. *Int. J. Peptide Protein Res.* **1994**, *42*, 58-63; Sieber, P. *Tetrahedron Lett.* **1987**, *28*, 6147-6150]. The lability of Npys to thiols prevents their use in the cleavage mixture. Therefore, the thiol-based cleavage mixture was substituted for a silane-based one. The corresponding Boc-Cys(Npys)-H10H24[2-31] was then cleaved with TFA:triisopropylsilane:phenol:water (88:2:5:5) [Sole N. A.; Barany, G. *J. Org. Chem.* **1992**, *57*, 5399-5403]. This procedure provided the target peptide in approximately 37% yield in a mixture difficult to purify. The byproducts were not analyzed.
13. DTNP (Aldrich or Sigma, 3-5 equiv.) was dissolved in the minimum amount of acetic acid:water (3:1, v/v) and the thiol containing peptide (1 equiv.) was added in one portion with vigorous stirring. After the reaction was complete (4-6 h), acetic acid was added to reach a proportion of 9 to 1 and the solvent was eliminated by lyophilization. The solid was extracted with aqueous 0.1% TFA, sonicated, centrifuged, and the supernatant freeze-dried. The pNpys-peptide still contained traces of DTNP and 5-nitro-2-pyridinethiol that were finally fully removed by washing (5x) the lyophilized powder with diethyl ether:methylene chloride (7:3, v/v).
14. To an ammonium acetate buffered solution (1M, pH 3.5-6.5, bubbled with argon) of the Cys(pNpys) containing peptide (1 equiv.), the corresponding free thiol peptide (1 equiv.) was added in one portion either solid or predissolved in aqueous TFA (0.1%, bubbled with argon). The reaction was easily monitored by the intense yellow coloration due to the release of 5-nitro-2-pyridinethiol. HPLC monitoring of the reaction helps in adjusting the actual amounts of peptide added and therefore optimizing yields.

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