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SOLID PHASE SYNTHESIS OF TRYPANOTHIONE DISULFIDE

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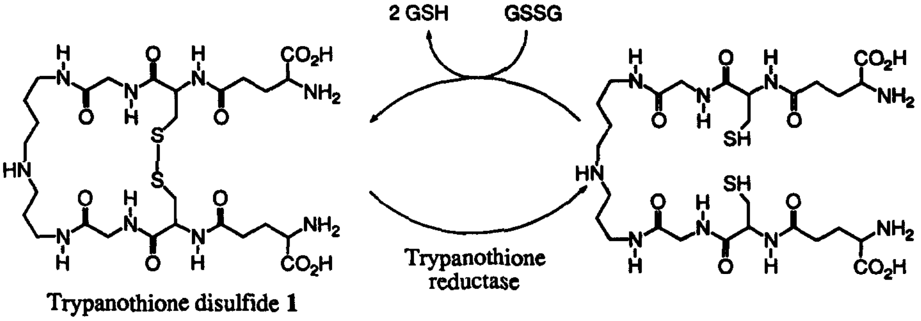
Abstract : The synthesis of **trypanothione disulfide 1** was performed according to an original solid phase strategy. The key step involves the selective protection of the primary amines of spermidine with tertiobutyldiphenylsilyl chloride followed by coupling the disilyl derivative on a methylchloroformylated polystyrene resin. After deprotection and assembly of the peptidic chain, treatment of the resin with HF, followed by oxidation and purification afforded 1 in 38% yield.

In mammalian cells, the tripeptide L-γ-glutamyl-L-cysteinylglycine (glutathione, GSH) plays a pivotal role against many types of potential cellular damage[1], these reactions result in the oxidation of GSH to the disulfide form (GSSG). In order to complete the cycle, GSSG is in turn reduced by a specific ***glutathione disulfide reductase***. Trypanosomidae, including the pathogenic species responsible for South American Chagas' desease and African sleeping sickness do not possess a classical GSH-based redox system. They use instead a glutathione-spermidine conjugate N^1,N^8-bis(glutathionyldisulfide)spermidine named trypanothione disulfide 1[2] reducible by a *trypanothione disulfide reductase* (figure 1). Thus trypanothione itself and the enzymes that control its metabolism represent an important target for the development of new non-toxic anti-parasitic drugs.

Figure 1



Trypanothione disulfide 1

Up to now, only one synthesis of 1 in homogenous phase has been described, involving eight steps and an approximately 10% overall yield[2,3]. Here we report the preparation of 1 by a convenient solid phase peptide synthesis (SPPS) method. A key step of this synthesis is the selective protection of the N^1 and N^8 primary amino groups of spermidine 2 (figure 2) to allow its attachment to a resin through the N^4 secondary amino group. After deprotection of the primary amino groups, the symmetrical peptide chain elongation can be continued according to a classical SPPS procedure.

Few reports are available in which the amino group of an amino acid is linked to a resin for SPPS. As a linkage between polymer and secondary amine, a benzyl carbamate structure analogous to the benzyloxycarbonyl amino-protecting group appeared promising because this protection is compatible with both Boc and Fmoc[4] strategies and can be cleaved by treatment with anhydrous hydrogen fluoride. Although an early paper on the use of a methylchloroformylated styrene divinylbenzene[5] resin has been published in 1970 by Merrifield[6] in an attempt to develop a $NH_2 \rightarrow COOH$ stepwise elongation strategy for SPPS, the only other report which we found in the literature was made in 1993 and concerned the preparation of para-nitroanilides peptides[7]. To obtain this functionalized polymer, a chloromethylated resin (0.7 meq/g) is first treated with potassium acetate; the product is converted to the hydroxymethylated form by saponification and a final treatment with phosgene in toluene affords the methylchloroformylated resin in quantitative yield[8].

A convenient and selective protection of the N^1 and N^8 primary amines of spermidine **2** has been reported using N-ethoxycarbonylphtalimide[9]. Using this reagent introduced by Nefkens[10], we obtained in a good yield N^1,N^8-diphtaloylspermidine **3** which was easily coupled on the chloromethylated resin as judged by determination of chloride ions liberated from the resin and IR spectroscopy of the resulting polymer. However, the spermidine-resin linkage proved to be unstable to the different hydrazinolysis conditions used to deprotect the phtaloyl groups (figure 2).

Therefore, an other specific protective group of primary amines, the bulky tert-butyldiphenylsilyl (TBDPS)[11] was used. Spermidine **2** was treated with TBDPS-Cl (3 equivalents) and triethylamine (4.5 equivalents) in acetonitrile (figure 2). N^1,N^8-disilylated spermidine **4** was characterized by time of flight mass spectroscopy but was not stable enough to be purified and was thus directly coupled after addition of dichloromethane (CH_2Cl_2/CH_3CN 4/1 v/v) to increase the swelling of the resin. The efficiency of the coupling was determined by a quantitative Kaiser test[12] (the TBDPS protecting group is labile in these conditions); a quantitative yield (with respect to the initial load of the methylchloroformyl resin) was evaluated by measurement of the absorbance (ε=30000 $M^{-1}cm^{-1}$ for the spermidine-ninhydrine complex at 570 nm).

Regeneration of the primary amino groups from their TBDPS derivatives under various acidic conditions[11] [0.5N HCl/THF (1/2 v/v); 2N HCl (dioxane); CH_3COOH/toluene (4/1 v/v); pure CF_3COOH or CF_3COOH/ CH_2Cl_2 (1/1 or 1/9 v/v)], as well as with 1M tetrabutylammonium fluoride (THF)[13] was studied using increasing reaction times. The stability of the spermidine-resin linkage in these conditions was evaluated by determination of amino groups available on the resin. Due to the lability of the silyl protective group in the Kaiser test conditions, these values correspond both to protected and deprotected primary amino groups (table 1).

table 1 : Stability of the spermidine-resin linkage[a] .

reagents time (mn)	HCl 0,5N[b]	HCl 2N[c]	CH_3COOH 80%[d]	TFA	TFA 50%[e]	TFA 10%[e]	$Bu_4N^+F^-$ [f]
5	78	67	62	31	31	35	-[g]
10	72	67	58	28	29	35	-[g]
30	60	65	56	15	12	36	-[g]
60	59	59	56	11	11	35	-[g]
120	58	54	56	9	10	28	28
240	52	39	55	7	9	24	28

Notes: a: percentage of amino groups remaining on the resin after deprotection compared to the load evaluated before the deprotection step; b: in THF 1/2 v/v; c: in dioxane; d: in toluene; e: in dichloromethane v/v; f: 1M in THF; g: the deprotection of amino groups was not complete. Each value was the average value for four deprotection reactions, the coefficient of variation was less than 5%.

The efficiency of the deprotection was then checked using a qualitative Kaiser test after coupling of the first protected amino acid (Fmoc-glycine). Except for tetrabutylammonium fluoride, in all other cases the deprotection was complete after a reaction time of 5 minutes. These results showed that, whatever the conditions used, the number of amino groups available on the resin decreases with time, confirming the previously observed lability of the spermidine-resin linkage. However, reasonable yields could be obtained using mild acidic conditions (HCl 0.5N/THF 1/2 v/v) and a short deprotection time, followed, as soon as possible by the coupling step.

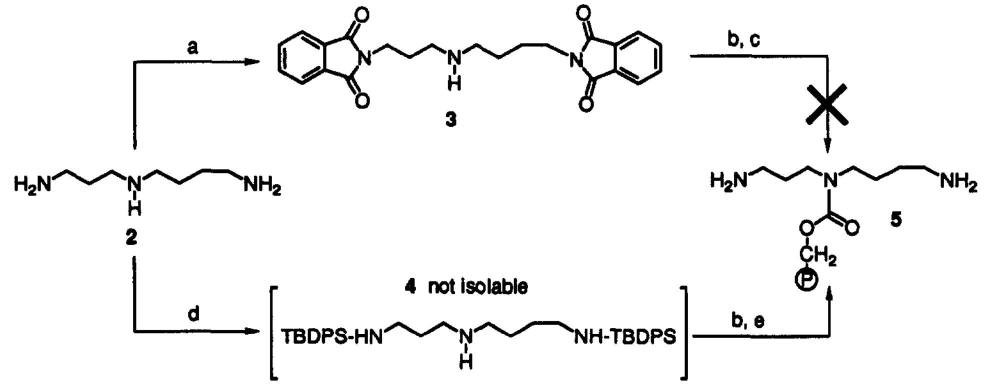


Figure 2: Reagents and conditions: a)N-ethoxycarbonylphtalimide, $CHCl_3$, 45 mn, rt; b)polymer-OCOCl, DIEA, CH_2Cl_2, 72h, rt; c)N_2H_4, CH_2Cl_2, 10 mn, rt; d)TBDPS-Cl, TEA, MeCN, 4h, rt; e)THF/HCl 0,5N 2/1 v/v, 15 mn.

The peptide chain was then elongated by successive coupling of Fmoc-Gly-OH, Fmoc-Cys(Trt)-OH and Z-Glu-OBn(γ-OH). Coupling reactions were performed in DMF using HOBt and BOP as coupling agents (HOBt/BOP/protected amino acid in a 1/1/1 ratio) and DIEA; Fmoc cleavages were achieved with piperidine in DMF (figure 3). Coupling yields were determined after deprotection by the quantitative Kaiser test. At the end of the synthesis, aminoacid analysis, performed after acidic hydrolysis of the peptidyl-resin yielded the expected ratio. Quantification of glutamic acid, the last amino acid incorporated on the resin indicated an overall 67% yield calculated on the basis of the initial load of the methylchloroformylated resin.

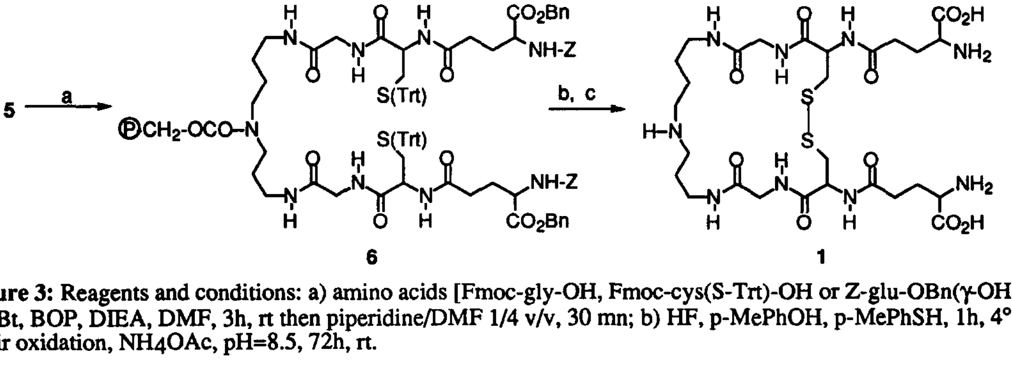


Figure 3: Reagents and conditions: a) amino acids [Fmoc-gly-OH, Fmoc-cys(S-Trt)-OH or Z-glu-OBn(γ-OH)], HOBt, BOP, DIEA, DMF, 3h, rt then piperidine/DMF 1/4 v/v, 30 mn; b) HF, p-MePhOH, p-MePhSH, 1h, 4°C, c) air oxidation, NH_4OAc, pH=8.5, 72h, rt.

The cleavage of the peptidyl-resin 6 was achieved according to the standard anhydrous hydrogen fluoride procedure (10 ml HF/g of resin bound peptide) with *para*-cresol (75 mg/ml HF) and *para*-thiocresol (25 mg/ml HF) as scavengers. After filtration and washing of the cleaved mixture (diethyl ether), the acidic extract (5%

aqueous acetic acid) was lyophilized. The resulting crude dihydrotrypanothione was directly oxidized. The oxydation was carried out at 0.1 mg/ml concentration in ammonium acetate 0.04M (adjusted to pH=8.5 with aqueous ammonia) under atmospheric oxygen at room temperature and monitored by reversed phase-HPLC[14] (RP-HPLC). Preparative RP-HPLC[14] affords trypanothione disulfide **1** with a 38% overall yield.

The purity was checked by analytical RP-HPLC and capillary electrophoresis[15]. Co-injection of the purified product with a sample synthesized according to Fairlamb [2,3] affords a single peak. The time-of-flight plasma desorption mass spectroscopy[16] of the oxidized and reduced trypanothione gave expected peaks at respectively 723 and 725 Da (MH^+). The synthetic material has also shown to be fully active with recombinant *trypanothione disulfide reductase* from *T. cruzi*; the K_m for **1** was determined to be 50 μM in good agreement with the value reported previously[17].

In conclusion, we have shown that solid phase synthesis was suitable to prepare trypanothione disulfide **1** which offers an easy access to the preparation of various analogues.

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References and notes

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4. Abbreviations: Boc, tert.butyloxycarbonyl; BOP, benzotriazol-1-yloxytrisdimethylaminophosphonium hexafluorophosphate; DIEA, diisopropylethylamine; DMF, N,N-dimethylformamide; Fmoc, fluorenyl--methoxycarbonyl; HOBt, 1-hydroxybenzotriazole; Trt, trityl; Z, benzyloxycarbonyl; Bn, benzyl.
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8. The yield was determined after coupling of β-alanine on the resin followed by total acidic hydrolysis and aminoacid analysis.The I.R. spectra of the hydroxymethylated and methylchloroformylated resins were identical with those reported by Letsinger [5]. The use of the less harmful triphosgene in the last step of the synthesis did not yield the desired product.
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14. Chromatographic conditions: a) preparative Nucleosil C18 column (25 x 200 mm). Detection at 250 nm by UV absorbance. The solvent system used was as follows: solvent A was 0.05% TFA in water and solvent B was 50% acetonitrile in solvent A. The product **1** was eluted at 4.9 ml/min with a run of 35 min of solvent A then a linear gradient of 0.11%/min solvent B in solvent A during 90 mn (rt=63.5 min). b) Analytical Nucleosil C18 column (4.6 x 250 mm) with a flow of 0.5 ml/min and a run of 5 min solvent A, then a linear gradient of 3.33%/min solvent B in A.UV detection at 215 nm: dihydrotrypanothione, rt=24.2 min; trypanothione disulfide, rt=23.4 min.
15. Capillary electrophoresis was performed on an Applied Biosystem 270 A-HT apparatus. Separation was carried at 30°C in a 20 mM sodium citrate buffer solution (pH=2.5) with a potential difference of 30 kV. Detection by UV absorbance at 200 nm.
16. PDMS analysis were performed with a Bio-Ion/Applied Biosystems 20K time-of-flight spectrometer using an accelerating voltage of 15 kV and a ^{252}Cf ionization source. Sample were adsorbed on the nitrocellulose target for analysis.
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