98. Selective Removal of the *o*-Nitrophenylsulfenyl Protecting Group in Peptide Synthesis

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Summary

2-Thiopyridone (2-mercaptopyridine, **1**) was found to be a very suitable reagent for removing the N^{α}-*o*-nitrophenylsulfenyl (NPS-) group in both conventional and solid-support peptide synthesis. A 3- to 5-molar excess of the reagent together with an equivalent amount of glacial acetic acid in methanol, dimethylformamide, or methylene chloride produces the soluble, stable mixed disulfide, 2-nitrophenyl 2-pyridyl disulfide (**2**), and the N^{α}-deprotected amino-acid or peptide. The yields are quantitative in less than 5 min if all educts are dissolved, in about 20 to 30 min in solid-support synthesis. No modifications of either the indole side-chain of tryptophan or of a series of side-chain protecting groups, in particular of the *t*-butyl type, are produced. No adverse side-reactions (insoluble disulfides) were observed. The procedure is illustrated with a series of amino-acid derivatives and with the solid-support synthesis of [5-leucine]-enkephalin.

A particularly successful strategy of peptide synthesis with which rather large, biologically active peptides like corticotropin, α-melanotropin, and β-melanotropin have been prepared [1] utilizes side-chain protecting groups that are easily removed by quite mild acid treatment. Such groups encompass *t*-butoxycarbonyl [2] (for lysine side-chains [3]), *t*-butyl ester [4] (for aspartic and glutamic acid side-chains [5]), and *t*-butyl ether (for tyrosine, serine, and threonine side-chains [6]).

On the other hand, the *o*-nitrophenylsulfenyl group (NPS-), introduced by *Zervas* [7] for protecting the α-amino function, has become very useful in both conventional and solid-support synthesis [8] because of the ease of its introduction and removal under very mild conditions such as acid-catalysed alcoholysis [9] allowing selective fission even in the presence of *t*-butyl-type protecting groups [10]. Unfortunately, in the synthesis of long-chain peptides, it is often impossible to remove NPS repeatedly without affecting other acid-labile groups [11]. Another serious drawback is its reactivity towards the indole ring of tryptophan in the

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presence of hydrochloric acid, 2-(*o*-nitrophenylsulfenyl)-tryptophyl residues being produced from tryptophyl [12].

In view of these restrictions, *Meienhofer* suggested the use of *Raney*-nickel [13], whereas *Scoffone et al.* and *Brandenburg* [14], and *Kessler & Iselin* [15] examined thiol reagents such as thioacetamide, thiourea, and thioglycolic acid which cleave the NPS-group by a direct nucleophilic attack on its sulfur atom, but with formation of many embarrassing by-products. Inorganic thiols, like thiosulfate and thiocyanide [16] have been used with similarly unsatisfactory results. Side-reactions with formation of insoluble or only partly soluble disulfides are particularly interfering in solid-support synthesis [15].

Many of these difficulties have been overcome by *Jüllerat & Bargetzi* [17] with 3-nitro-4-mercapto-benzoic acid and its methyl ester. They demonstrated the selectivity, the prevention of insoluble by-products, and the relative freedom of

Scheme. *Cleavage of the* o*-nitrophenylsulfenyl group from amino-acids and peptides with 2-thiopyridone/2-mercaptopyridine* (**1**) *and an independent synthesis of 2-nitrophenyl 2-pyridyl disulfide* (**2**). R = side-chain (protected or unprotected) of an α-amino-acid, R′ = OH or peptide residue

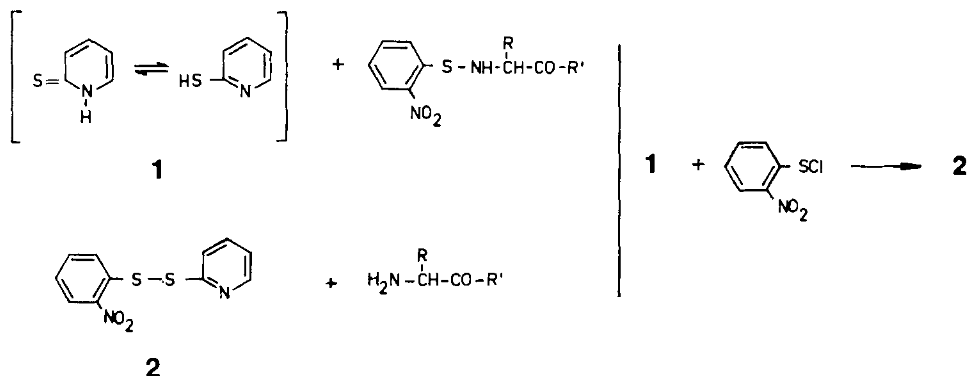

Table. *Selective removal of the* o*-nitrophenylsulfenyl group from protected amino-acids with 2-thiopyridone;* stability of *t*-butoxycarbonyl-groups and *t*-butyl esters under the reaction conditions. Solutions of 0.1 mmol of the amino-acid derivatives in 3 ml of methanol were treated with a solution of 0.5 mmol each of 2-thiopyridone and glacial acetic acid in 1 ml of methanol at RT. for at least 24 h. The course of the reaction was followed by TLC. in the solvent systems A and B (see Experimental part). NPS is completely removed in less than 5 min

Amino-acid derivative	Results
AZOC-Gly · OH [19]	Unchanged: AZOC is stable
BPOC-Gly · OH,DCHA [20]	Unchanged: BPOC is stable
BOC-His(Tos) · OH,DCHA [11]	Unchanged: BOC and Tos are stable
NPS-Lys(BOC) · OH	Lys(BOC) produced quantitatively
NPS-Ser(*t*Bu) · OH	Ser(*t*Bu) produced quantitatively
NPS-Cys(Bzl) · OH	Cys(Bzl) produced quantitatively
H · Asp(O*t*Bu) · OH	Unchanged: *t*-Bu ester is stable
NPS-Asn · OH	Asn produced quantitatively
NPS-Met · OH,DCHA	Met produced quantitatively
NPS-Trp · OH,DCHA	Trp produced quantitatively
H · Lys(MSOC)-Pro-Val · NH_2 [21]	Unchanged: MSOC is stable

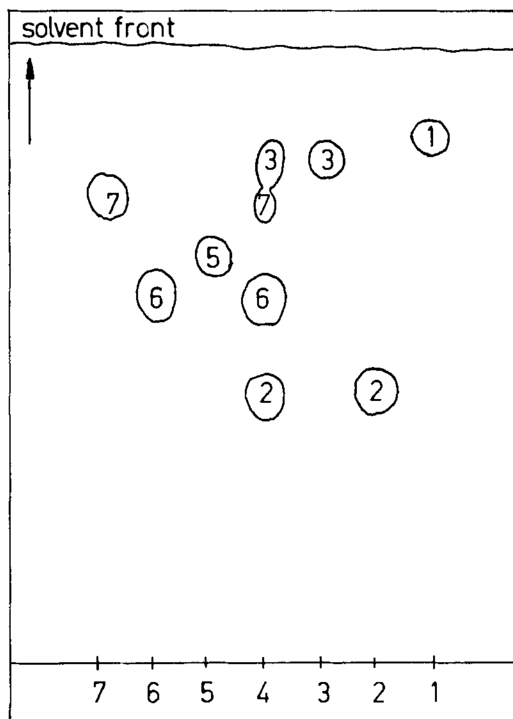

Fig. *Cleavage of NPS-Trp · OH, DCHA with 2-thiopyridone/acetic acid in methanol* (conditions see *Table*). TLC. after 18 h, solvent A. 1) NPS-Trp · OH,DCHA; 2) Tryptophan; 3) **2**; 4) Reaction mixture; 5) Trp(NPS); 6) 2-Thiopyridone; 7) 2,2'-Dipyridyldisulfide

NPS-transfer to the indole ring in both conventional and solid-support synthesis. The short reaction time for NPS-cleavage in solution was somewhat offset by the fact that 4 h are necessary for complete removal of the NPS-group in the solid-support synthesis. Furthermore, the reagents are not yet commercially available.

The great potentialities of the use of the NPS-group in combination with *t*-butyl-type side-chain protection [11] have thus not yet been fully exploited. (The rather energetic conditions necessary for the cleavage of the peptide from most solid-supports constitute another problem not discussed in this context.) One compound that will combine rapid and selective cleavage with lack of undesired side-reactions and solubility of disulfide products is 2,2'-dipyridyldisulfide, which has been used for titrating thiol groups in papain [18]. Therefore 2-thiopyridone was investigated as a cleavage reagent. The reagent is easily soluble in the organic solvents principally used in conventional and solid-support peptide synthesis. A 3- to 5-fold excess of 2-thiopyridone plus an equivalent amount of glacial acetic acid in solvents like methanol or dimethylformamide removes the NPS-group quantitatively and practically without side-reactions in *ca.* 3 min at RT. from NPS-amino-acids and their dicyclohexylammonium salts *(Table)*. The only product besides the deprotected amino-acid or peptide is the mixed disulfide, 2-nitrophenyl 2-pyridyl disulfide *(Scheme)*, stable in the reaction mixture for at least 24 h; no precipitate of bis(2-nitrophenyl)disulfide

is observed, nor can this compound be detected by thin-layer chromatography (TLC.) *(Fig.)*. The indole ring of tryptophan remained unchanged.

In an example of solid-support synthesis, the cleavage is considerably slower but the accelerating effect of glacial acetic acid is very pronounced. Thus, the use of only a 5- to 10-molar excess of 2-thiopyridone and an equivalent amount of glacial acetic acid in methylene chloride removes the NPS-group quantitatively within 30 min. These conditions do not affect the most labile *t*-butoxy-carbonyl, side-chain protecting group even after 24 h *(Table)*.

2-Mercapto-4,6-dimethylpyrimidine, 2-mercapto-1-methylimidazole, and 8-mercaptoquinoline are also capable of removing the NPS-group under similar conditions in less than 5 min. No insoluble disulfides are generated (care must be exercised with 8-mercaptoquinoline, however, because this reagent is easily oxidized in air to the insoluble symmetrical disulfide). One advantage of these compounds over 2-thiopyridone is their better solubility in water which would allow the removal of NPS from substituted proteins or highly water-soluble peptides. These reagents are presently under more careful investigation.

Experimental Part

General procedures and materials. - The *o*-nitrophenylsulfenyl-(NPS-)amino-acid derivatives were synthesized according to *Zervas et al.* [7]. Thin-layer chromatograms (TLC.) were run on fluorescent *Merck* F-254 silica-gel plates with either 1-butanol/acetic acid/water, 5:2:3 (v:v) (A) or 100:15:35 (B), and revealed with I_2 vapour, ninhydrin, or UV. absorption (fluorescence quenching) at 254 nm. Trp(NPS), 2-(*o*-nitrophenylsulfenyl)-L-tryptophan [22] or S-2-amino-3-{3-[2-(*o*-nitrophenylsulfenyl)-indolyl]}-propionic acid, was prepared according to *Juillerat & Bargetzi* [17]. 2-Mercapto-4,6-dimethyl-pyrimidine was obtained from acetylacetone and thiourea with conc. HCl in ethanol after the method of *Hunt et al.* [23]. 2-Mercapto-1-methylimidazole, 8-mercaptoquinoline, 2-thiopyridone/2-mercapto-pyridine, and *o*-nitrophenylsulfenyl chloride were commercially available.

S¹-(2-Nitrophenyl)-S²-(2-pyridyl)-disulfide (**2**). - Equivalent quantities of *o*-nitrophenylsulfenyl chloride (1.9 g) and 2-mercaptopyridine (1.1 g) in $CHCl_3$ solutions were mixed and stirred for 10 min. The product which precipitated as the hydrochloride was gathered by filtration, suspended in ethyl acetate and shaken with sufficient 1M Na_2CO_3 solution for the aqueous phase to remain alkaline. The organic phase was then separated, washed twice with water and finally with saturated NaCl solution, and dried ($MgSO_4$). The solvent was distilled off *in vacuo*, petroleum ether was then added to the residue until yellow crystals started to appear: 2.0 g (80%) **2**. For analysis, a sample was chromatographed on SiO_2 with $CHCl_3$ and recrystallized, m.p. 80-81°.

$C_{11}H_8N_2O_2S_2$	Calc.	C 49.96	H 3.05	N 10.60	S 24.26%
(264)	Found	„ 49.83	„ 3.11	„ 10.47	„ 24.22%

Solid-support synthesis of [5-leucine]-enkephalin, H · Tyr-Gly-Gly-Phe-Leu · OH. - The Cs salt of BOC · Leu · OH (290 mg) reacted with 2.0 g of a commercially available chloromethylated co-polystyrene - 2% divinyl-benzene (*Merrifield*-type) resin (0.7 mmol Cl/g) according to the procedure of *Gisin* [24] and the BOC-groups removed as usual. The following cycle of procedures was used to introduce each N^{α}-*o*-nitrophenylsulfenyl-amino-acid in succession [NPS · Phe · OH; NPS · Gly · OH; NPS · Gly · OH; NPS-Tyr(*t*Bu) · OH]:

1) Wash with CH_2Cl_2 (2× 15 ml, 2 min each).
2) Neutralize with diisopropyl-ethylamine/CH_2Cl_2, 5:95, v:v (15 ml, 7 min).
3) Wash with $CHCl_3$ (3× 15 ml) and CH_2Cl_2 (3× 15 ml) for 2 min each.
4) Add 10 ml of CH_2Cl_2 containing a 3-molar excess of the appropriate NPS-amino-acid; shake for 10 min.

5) Add 3 ml of CH_2Cl_2 containing a 3-molar excess of dicyclohexyl-carbodiimide; react 5 h.
6) Wash with 3 × 15 ml of CH_2Cl_2, abs. ethanol, and again CH_2Cl_2 for 3 min each.
7) Remove the NPS-groups with a 5- to 10-molar excess of 2-mercaptopyridine in 15 ml of CH_2Cl_2 containing an amount of glacial acetic acid equivalent to that of 2-mercaptopyridine. Reaction time: 30 min.
8) Wash with 3 × 15 ml of ethanol, CH_2Cl_2, $CHCl_3$, and CH_2Cl_2 for 3 min each.

At the end of the series of cycles, including the removal of the NPS-group from the NPS-Tyr(*t*Bu)-residue introduced last, the peptide chains were released from the resin and from the *t*-butyl group of the tyrosine side-chain in the usual manner with a mixture of liquid HF and thioanisole. The HF was evaporated, the resin/peptide mixture was washed twice with ethyl acetate to remove excess thioanisole, and the peptide extracted into dilute aqueous acetic acid (10%). The solution was freed of traces of thioanisole by extraction with ether and the crude product isolated by distillation of the solvent *in vacuo*. The product showed at least 3 ninhydrin-positive spots on TLC. (A) and (B), one of which corresponded to that of an authentical sample of [Leu^5]-enkephalin. Purification was effected on a diethylaminoethyl-cellulose column with a gradient of 0.01 M to 0.1 M ammonium bicarbonate. The pure compound emerged at 0.05 M; 150 mg (~20%); pure aspect on TLC. Amino-acid analysis: Leu 1.0 (ref.), Phe 0.9, Gly 2.1, Tyr 1.0.

REFERENCES

[1] *R. Schwyzer*, Naturwissenschaften *53*, 189 (1966).
[2] *L.A. Carpino*, J. Amer. chem. Soc. *79*, 4427 (1957).
[3] *R. Schwyzer & W. Rittel*, Helv. *44*, 159 (1961).
[4] *R.W. Roeske*, Chemistry & Ind. *1959*, 1121.
[5] *R. Schwyzer & H. Dietrich*, Helv. *44*, 2003 (1961); *H. Kappeler & R. Schwyzer*, Helv. *44*, 1136 (1961).
[6] *H.C. Beyermann & J.S. Bontekoe*, Trav. chim. Pays-Bas *81*, 691 (1962).
[7] *L. Zervas, D. Borovas & E. Gazis*, J. Amer. chem. Soc. *85*, 3660 (1963).
[8] *M. Fridkin, A. Patchornik & E. Katchalski*, J. Amer. chem. Soc. *90*, 2953 (1968).
[9] *K. Poduška*, Coll. Czech. chem. Commun. *33*, 3779 (1968).
[10] *L. Zervas & C. Hammalidis*, J. Amer. chem. Soc. *87*, 99 (1965).
[11] *E. Wünsch*, 'Synthese von Peptiden', Bd. 15, *Houben-Weyl*, 'Methoden der organischen Chemie', E. Müller, ed., Georg-Thieme-Verlag, Stuttgart 1975, p. 217.
[12] *J.C. Anderson, A.M. Barton, P.M. Hardy, G.W. Kenner, J.K. MacLeod, J. Preston, R.C. Sheppard & J.S. Morley*, Proc. 7th Europ. Peptide Symposium, Budapest 1964.
[13] *J. Meienhofer*, Nature *205*, 73 (1965).
[14] *A. Fontana, F. Marchiori, L. Moroder & E. Scoffone*, Tetrahedron Letters *1966*, 2985; *D. Brandenburg*, ibid. *1966*, 6201.
[15] *W. Kessler & B. Iselin*, Helv. *49*, 1330 (1966).
[16] *B. Ekström & B. Sjöberg*, Acta chem. Scand. *19*, 1245 (1965).
[17] *M. Juillerat & J.P. Bargetzi*, Helv. *59*, 855 (1976).
[18] *K. Brockelhurst & G. Little*, Biochem. J. *133*, 67 (1973).
[19] *A. Tun-Kyi & R. Schwyzer*, Helv. *59*, 1642 (1976).
[20] *P. Sieber & B. Iselin*, Helv. *51*, 614, 622 (1968).
[21] *A. Eberle*, Dissertation ETH 5735 (1976); *A. Eberle, J.L. Fauchère, G.I. Tesser & R. Schwyzer*, Helv. *58*, 2106 (1975).
[22] IUPAC Commission on the Nomenclature of Organic Chemistry and IUPAC-IUB Commission on Biochemical Nomenclature, Biochemistry *14*, 449 (1975).
[23] *R.R. Hunt, J.F.W. McOmie & E.R. Sayer*, J. chem. Soc. *1959*, 525.
[24] *B.F. Gisin*, Helv. *56*, 1476 (1973).