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Some peculiarities of synthesis of cysteine-containing peptides

E V Kudryavtseva, M V Sidorova, R P Evstigneeva

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Abstract. Data on protective groups for the thiol function of cysteine and methods of disulfide bonds formation used in modern peptide chemistry are considered and systematised. The advantages and disadvantages of protective groups, of reagents used for cyclisation, and possible side reactions associated with them are described. The bibliography includes 119 references.

I. Introduction

Among other peptides and proteins occurring in nature, those containing cysteine constitute a separate group. Disulfide bridges formed between pairs of cysteine residues play an essential role in the stabilisation of tertiary and quaternary structures of the protein molecules by determining their rigid spatial orientation and giving them a unique shape required for the fulfilment of their biological functions. In low-molecular-mass peptides, all types of noncovalent contacts are weakened; therefore, disulfide bonds are of crucial importance for the structural stabilisation of a molecule and reproduction of its biological activity.

Many peptides are known that contain one, two, or more disulfide bridges and take part in the regulation of physiological processes occurring in the organism.[1,2] Synthetic peptides including those with S—S bonds are widely employed in many areas of medicine and pharmacology.[3,4] Therefore, the methods used for the formation of disulfide bridges are of great practical significance.

Modern peptide chemistry possesses a wide range of methods for synthesis, purification, and analysis, which allow one to obtain virtually any kind of peptides, their analogues, and even miniproteins. However, the synthesis of peptides containing disulfide bonds still presents a challenge because of low yields at the stage of their closure. Very often, all efforts spent to synthesise linear precursors come to an overall negative result. Elucidation of the reason for such failures often goes beyond the scope of synthetic studies. The reviews devoted to the synthesis of peptides having disulfide bridges are very few in number and are often limited to particular aspects of the general problem.

The present review considers modern approaches to the synthesis of cysteine-containing peptides, the groups employed for protection of the thiol function, the methods for their removal, and most popular reagents used for the closure of the disulfide bonds.

E V Kudryavtseva, **M V Sidorova** Russian Cardiological Scientific Centre, Ministry of Health Care and Medical Industry of the Russian Federation, 3-ya Cherepkovskaya ul. 15a, 121552 Moscow, Russian Federation. Fax (7-095) 414 67 86. Tel. (7-095) 414 67 16
R P Evstigneeva M V Lomonosov Moscow Academy of Fine Chemical Technology, prosp. Vernadskogo 86, 117571 Moscow, Russian Federation. Fax (7-095) 430 79 83. Tel. (7-095) 434 86 78

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II. Protective groups for the thiol function

It is necessary that the strongly nucleophilic thiol group of cysteine be protected in the course of peptide synthesis. The protective groups for the SH function of cysteine should meet the same demands as those for the protective groups of the side-chain functions of other amino acids, *viz*., stability in the reactions of peptide chain elongation and deblocking of α-amino groups and retention of configuration of the asymmetric centre in the course of synthesis. The protective groups for the thiol function that have been successfully used in peptide syntheses are listed in Table 1. The choice of a protective group is determined by the procedure of subsequent cyclisation. Depending on conditions of cysteine deblocking, one may obtain free thiols or disulfides. The protective groups should possess different chemical stability, which would enable the selective formation of two or more disulfide bonds if required.

In order to characterise protective groups for the thiol function of cysteine used in modern peptide chemistry, Hiskey [5,6] has proposed to classify them according to the structure of the protected derivatives, *viz*., sulfides, thioacetals, *S*-acylamidomethyl thiazolidine derivatives, thioesters, and disulfides.

1. Protective groups that form sulfides and related compounds

Protective groups that form this type of compounds (RSR′), are the most well-known, numerous, and popular. The benzyl group proposed by Sifferd and du Vigneaud is an example of a classical protective group that forms sulfides (see Ref. 1). Rather drastic conditions necessary for the removal of the protective benzyl group (Na in liquid ammonia or HF at 25 °C) impose certain limitations on its application. It has been observed [1,43] that HF results in desulfurisation of the cysteine residue, which may also occur with Na/NH_3. In addition, deprotection with Na/NH_3 is sometimes accompanied by the cleavage of the peptide bonds; the bonds formed by the amino group of proline appear to be the most labile under these conditions.[1] In this connection, it was necessary to design acid-labile protective groups by introducing electron-donor substituents into the aromatic ring of benzyl alcohols. A great number of such protective groups have been synthesised. In

Table 1. The protective groups for the thiol function of cysteine employed in peptide synthesis.

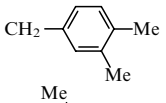
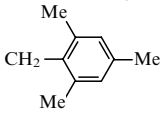
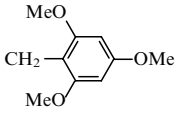
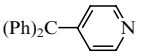
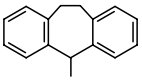
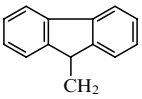
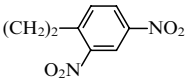
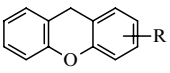
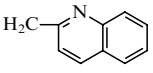
Name [a]	Formula of the protective group	Conditions where the protective group is stable [b]	Conditions for removal of the protective group	Ref.
Benzyl (Bzl)	CH_2Ph	HF, 0 °C; Tl(III); B RSCl; H_2/Pd; HBr/AcOH	Na/NH_3(liq); HF, 25 °C, 30 min	2, 6–8
Methylbenzyl (Meb or Mbzl)	$CH_2C_6H_4Me$-*p*	TFA; Ag(I); RSCl; B	HF/MeOPh, 0 °C, 60 min;[c] Tl(III)	6–9
3,4-Dimethylbenzyl (Dmb)		TFA; Ag(I); B; RSCl	HF/MeOPh, 0 °C, 10 min	8
2,4,6-Trimethylbenzyl (Tmb)		TFA, 23 °C, 2 h	HF/MeOPh, 0 °C, 30 min; TFA, Δ, 1 h	10
4-Methoxybenzyl (Mob)	$CH_2C_6H_4OMe$-*p*	B; RSCl; I_2	HF, 0 °C; Hg(II); Ag(I); Tl(III); $(BrC_6H_4)_3N$; TFA : CH_2Cl_2 (1 : 1), 25 °C [c]	6–8, 10–13
2,4,6-Trimethoxybenzyl (Tmob)		B; Nu	30% TFA/scavengers,[d] I_2; Tl(III), HF	2, 7, 14, 15
tert-Butyl	Bu^t	TFA; HF, 0 °C; B; I_2; Ag(I)	HF, 20 °C; HF/scavengers,[d] 0 °C; Hg(II); NpsCl; Tl(III)	2, 12, 16
Diphenylmethyl (or benzhydryl) (Dpm)	$CHPh_2$	TFA; I_2;[e] Hg(II);[e] Ag(I) [e]	TFA/MeOPh, Δ; HF; Na/NH_3(liq); HBr/AcOH; $(SCN)_2$; NpsCl	5, 17
Di(4-methoxyphenyl)methyl (Ddm)	$CH(C_6H_4OMe\text{-}p)_2$	HCl/AcOH;[e] B; Tl(III)	TFA, 70 °C, 2 h; TFA/MeOPh, 10 min; TFA : PhOH (85 : 15), 25 °C, 2 h	1, 5, 8
Triphenylmethyl (Trt)	CPh_3	B; Nu	TFA/scavengers;[d] Hg(II); Ag(I); I_2; Tl(III); RSCl	1, 2, 5–8, 18
4,4′,4″-Trimethoxytriphenylmethyl (TMTr)	$C(C_6H_4OMe\text{-}p)_3$	B; Nu	TFA (dilute); I_2 (1–2 equiv.), 25 °C, 15–30 min	2, 7
Pyridyldiphenylmethyl		B	Hg(II) pH 4; Zn/AcOH; electrolytic reduction	19
Dibenzosuberyl		HCl(gas) in organic solvents; TFA; B	HBr/AcOH;[c] HF	6
Fluorenylmethyl (Fm)		HF; TFA; Tl(III) or I_2 (stable for 1 h)	NH_3/MeOH; Pip	5, 20
2-(2,4-Dinitrophenyl)ethyl (Dnpe)		HF; I_2; Tl(III); TFA; TFMSA	Pip	21
β,β-Diethoxycarbonylethyl (Dce)	$CH_2CH(COOEt)_2$	HBr/AcOH	1 M KOH, 20 °C, 5–10 min	1
Adamantyl (Ad)		TFA [i]	Tl(III); 1 M TFMSA/PhSMe in TFA, 0 °C	13, 22, 23
Xanthenyl, R = H (Xan), R = 2-MeO (2-Moxan), R = 3-MeO (3-Moxan)		B	dilute acids in the presence of scavengers;[d] I_2; Tl(III)	24
2-Quinolylmethyl		AH	$FeCl_3/DMF/H_2O$; $FeCl_2/DMF/H_2O$; $CuCl_2/DMF/H_2O$	25
Ferrocenylmethyl (Fcm)	$C_5H_5FeC_5H_4CH_2$	NaOH; NaOMe; I_2; N_2H_4; Pip	TFA; $(SCN)_2$; RSCl; Hg(II); Ag(I)	26

Table 1 (continued).

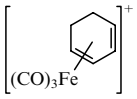
Name [a]	Formula of the protective group	Conditions where the protective group is stable [b]	Conditions for removal of the protective group	Ref.
2-Ferrocenylpropan-2-yl (Fc)	$C_5H_5FeC_5H_4CMe_2$	NaOH; NaOMe; I_2; N_2H_4; Pip	TFA; $(SCN)_2$; RSCl; Hg(II); Ag(I)	26
(η^5-Cyclohexa-3,5-dienyl)tricarbonyl iron		NaOH/Me_2CO	$HBF_4/CHCl_3$	27
Tetrahydropyranyl (Thp)		B	0.5 M $AgNO_3$, 0 °C; TFA; HBr/TFA; $(SCN)_2$	5, 6, 8
Isobutoxymethyl (*i*-Bom)	CH_2OBu^i	B	TFA; HCl/AcOH; Hg(II)	5, 6, 8
p-Chlorophenoxymethyl	$CH_2OC_6H_4Cl$-*p*	B	TFA	5
Benzylthiomethyl (Btm)	CH_2SCH_2Ph	B	HF; $Hg(AcO)_2$/HCOOH (80%), 25 °C, 20 min	5, 6, 8
Phenylthiomethyl	CH_2SPh	B	HF; Hg(II)	5
Acetamidomethyl (Acm)	$CH_2NHCOMe$	HF, 0 °C; B; H_2/Pd	Ag(I); Hg(II); I_2; Tl(III); $AgBF_4$; RSCl	2, 5, 6, 8, 18, 28, 29
Trimethylacetamidomethyl (Tacm)	$CH_2NHCOBu^t$	HF; TFA; 1 M TFMSA; B	$Hg(AcO)_2$, TFA/MeOPh; I_2 (10 equiv.) in AcOH; $AgBF_4$	28–31
Phenylacetamidomethyl (Phacm)	$CH_2NHCOCH_2Ph$	HF; B	Hg(II); I_2; Tl(III); penicilline aminohydrolase	2, 32, 33
Benzamidomethyl (Bam)	$CH_2NHCOPh$	AH, B	Ag(OTf); Hg(II)	6, 18
(2-Oxo-1-pyrrolidino)methyl (Pym)		B	Hg(II); I_2/MeOH	34
Acetyl (Ac)	COMe	TFA (boiling); HBr/AcOH	0.1 M NaOMe/MeOH, 25 °C, 5–10 min; N_2H_4/MeOH	1, 5, 8
Benzoyl (Bz)	COPh	TFA (boiling); HBr/AcOH	0.1 M NaOMe/MeOH, 25 °C; N_2H_4/MeOH; 0.2 M NaOH	1, 5, 8
Benzyloxycarbonyl (Z)	$COOCH_2Ph$		0.1 M NaOMe/MeOH, 25 °C; aqueous alkali; NH_3; Na/NH_3(liq); TFA/PhOH, Δ; HBr/AcOH	1, 5, 8
p-Methoxybenzyloxycarbonyl (pMZ)	$COCH_2C_6H_4OMe$-*p*		TFA, 20 °C; 0.2 N HBr/AcOH; HCl/AcOH; 0.5 N NaOMe in DMF; Na/NH_3(liq)	1, 8
tert-Butoxycarbonyl (Boc)	$COOBu^t$		AH (TFA)	8
p-Nitrophenoxycarbonyl	$COOC_6H_4NO_2$-*p*		AH(TFA); Na/NH_3(liq)	5
Phenylthiocarbonyl	COSPh		AH	5
Butylthiocarbonyl	COSBu		AH	5
Ethylcarbamoyl (Ec)	CONHEt	4.5 M HBr/AcOH; 1 M HCl; TFA, Δ	Na/NH_3; 4 M NH_3; NH_3(liq): MeOH (1 : 1); 1 M NaOH; NaOMe; N_2H_4/MeOH; Hg(II) (pH 7); Ag(I) (pH 7)	5, 6, 8
N-Methoxymethylcarbamoyl	$CONHCH_2OMe$	AH	Na/NH_3(liq); NaOMe/MeOH; aqueous buffers (pH 9.5), 25 °C	8
N-[β-(*N*-Acyl-*N*-methylamino)-ethyl]carbamoyl	$CONHCH_2CH_2N(R)Me$ R = $PhCH_2OCO$, Bu^tOCO		AH	6
Alkylthio (SEt, SPr^i, STrt)	SR R = Et, Pr^i, CPh_3	AH; NH_4OH; NaOH	β-mercaptoethanol; benzenethiol (pH 7.5–9); dithiothreitol; Bu_3P; oxidative sulfitolysis	6, 8, 35
tert-Butylthio (SBu^t)	SBu^t	HCl/dioxane; Pip; TFA; RSCl; B	Na/NH_3; RSH; Bu_3P; 2-pyridinesulfenyl chloride; *o*-nitrobenzenesulfenyl chloride	2, 6, 8, 17, 35–37
Methoxycarbonylthio (Scm)	SCOOMe	HF; AH	RSH; mild alkaline reagents (pH 10.6)	1, 6, 8, 35, 36
(*N*-Methyl-*N*-phenylcarbamoyl)thio (Snm)	SCON(Me)Ph	HF	RSH (especially dithiothreitol and 2-pyridinethiol)	2

Table 1 (continued).

Name [a]	Formula of the protective group	Conditions where the protective group is stable [b]	Conditions for removal of the protective group	Ref.
3-Nitro-2-pyridylthio (Npys)		HF, 0 °C (in the absence of thiols); TFA, 25 °C;[e] HOBT/DCC	RSH or other reducing agents	2, 38–41
tert-Butoxycarbonylthio (SCB)	$SCOOBu^t$		B and AH	36
Benzyloxycarbonylthio (SZ)	$SCOOCH_2Ph$	TFA; weak bases	40% HBr/AcOH; strong bases	36
Sulfonates	SO_3Na	B	Bu_3P; β-mercaptoethanol, dithiothreitol	42

[a] The abbreviations of protective groups cited in Ref. 8 are given; [b] the following notations are adopted: B are bases; TFA is trifluoroacetic acid; Nu are nucleophiles; NpsCl is 2-nitrobenzenesulfenyl chloride; Pip is piperidine; TFMSA is trifluoromethanesulfonic acid; AH are acids; HOBT is *N*-hydroxybenzotriazole; DCC is dicyclohexylcarbodiimide; [c] partial removal; [d] scavengers are compounds that neutralise carbocations formed upon splitting of protective groups; [e] low stability.

Table 1, they are listed in the order of the increase in their acid lability. The most acid-labile group is *S*-2,4,6-trimethoxybenzyl protection,[5,7,14] which is easily removed with 30% trifluoroacetic acid (TFA) in CH_2Cl_2 in the presence of phenol, thioanisole, or water (the so-called scavengers whose purpose is to neutralise the carbocations formed upon cleavage of protective groups) as well as with 5% TFA in CH_2Cl_2 in the presence of 3% triethylsilane or tri(isopropyl)silane. Scavengers are necessary to prevent the reverse addition of the trimethoxybenzyl group to the SH group.[14] The distinctive feature of methoxy- and trimethoxybenzyl protective groups is the possibility of their removal with heavy metal salts, *e.g.*, Ag(I) and Hg(II), which makes it possible to selectively deblock cysteine.[7,11,15] The mechanism of this reaction is analogous to that of splitting of trityl and acetamidomethyl groups and will be considered in more detail in subsequent sections.

S-tert-Butyl protection is extremely acid-resistant. Its removal can be accomplished with HF or $Hg(AcO)_2$ in TFA.[2,12] Quantitative removal of the *tert*-butyl group can be achieved with the use of strongly electrophilic reagents, such as 2-nitrophenylsulfenyl chloride, which binds two cysteine residues through intermediate mixed disulfides [2] (this reaction will be considered below). However, this reagent cannot be used if the peptide chain contains tryptophan because side reactions may occur in the indole ring.[16]

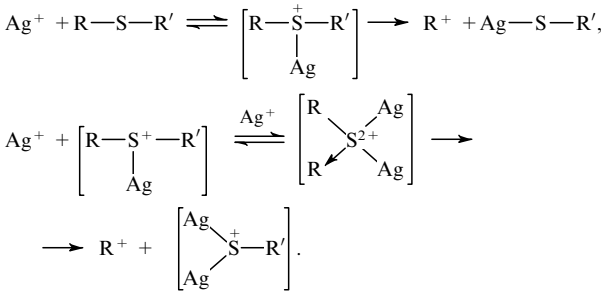
The next type of protective groups includes di- and triarylmethyl groups. In Table 1, they are also presented in the order of the increase in their acid lability:

$$(p\text{-}MeOC_6H_4)_3C > Ph_3C > (p\text{-}MeOC_6H_4)_2CH > Ph_2CH.$$

The ease of removal of these protective groups under mild acidic conditions makes them attractive for solid-phase peptide synthesis, which makes use of N^{α}-9-fluorenylmethoxycarbonyl derivatives of amino acids (the Fmoc-technique).[4,44,45] Cleavage of these sulfides occurs faster than that of their *tert*-butyl and benzyl analogues. This is due to the greater stability of the carbocations formed; however, their higher reactivity can result in the reverse alkylation reaction of cysteine. Thus in the case of the triphenylmethyl protection, an equilibrium between the protected and free cysteine is established in TFA in 30 min and in most cases the degree of *S*-detritylation does not exceed 60%.[1,8] The reaction equilibrium can be shifted to the right by addition of scavengers (especially, sulfur-containing ones) or water to the reaction mixture.[1,5,16]

In terms of its reactivity, the Ddm group occupies an intermediate position between the Trt and Dpm groups [5] and is much more acid-labile than the triphenylmethyl group. This permits selective removal of the Ddm group without affecting the Trt group.[1,5,8] On the other hand, the resistance of Ddm protection to heavy metal salts allows selective splitting of the Trt group in the presence of Ddm deprotection.

Cleavage of the Trt group can be carried out with Hg(II) or Ag(I) ions.[5] The mechanism of cleavage of sulfides with metal ions is similar to the mechanism of the acid-catalysed reaction: the difference between these mechanisms is that in the case of metal ions the last stage is irreversible and leads to a stable carbocation, which can be formed by two routes:



In some cases, silver salts are more efficient than mercuric salts, because mercuric derivatives of peptides (mercaptides) are sometimes difficult to cleave.[5] Metal mercaptides are usually destroyed by thio alcohols. To this end, the reaction product is treated *in situ* with a 20–50-fold excess of a reducing reagent in an appropriate buffer, such as dithiothreitol or β-mercaptoethanol.[2,18] The reduced product is purified by gel filtration, ion-exchange chromatography, or HPLC to remove the excess of the reducing reagents. Hg(II) ions are usually removed with hydrogen sulfide.

Owing to the stabilising effect of three electron-donor methoxy groups, the TMTr protective group produces a more stable carbocation than the Trt group, which determines its specificity. The distinctive feature of this protective group is its extremely high acid lability. Deprotection is performed with dilute solutions of TFA.[7] The tris(methoxyphenyl)methyl carbocation formed, like the trityl carbocation, can enter into the reverse reaction, *viz.*, alkylation; therefore, the use of scavengers is obligatory in this case. Both Trt and TMTr protective groups may be split off together with simultaneous closure of the disulfide bonds with iodine or thallium trifluoroacetate (see Section III).

The *S*-pyridyldiphenylmethyl group is not so popular in peptide synthesis. Only a few examples concerning this protective group are documented.[19] The *S*-dibenzosuberyl protective group is also used rather seldom. In its chemical properties and deblocking conditions, it is similar to the Ddm group.[6] The *S*-9-fluore-

nylmethyl protective group [5, 20] is one of the few groups that is removed under basic conditions, as a rule, this is performed by treatment with piperidine in DMF. The stability of this protective group under conditions of TFA treatment and in anhydrous HF makes possible its application in solid-phase syntheses, whereas its resistance to Tl(III) and I_2 allows its selective removal in the presence of such protective groups as Acm, Trt, Meb, *etc*., and selective formation of disulfide bonds. Yet another example of a base-labile protective group is the 2-(2,4-dinitrophenyl)ethyl group.[21] The electron-acceptor nitro groups in *ortho*- and *para*-positions make this group similar to Fm as regards its stability in the presence of strong acids and splitting by bases by a β-elimination mechanism. The presence of nitro groups allows one to increase the solubility of Dnpe derivatives in polar solvents. Like Fm, this group is completely stable under conditions of classical and solid-phase syntheses, which employ N^{α}-*tert*-butoxycarbonyl derivatives of amino acids (Boc-technique) [4, 45] as well as under conditions of splitting of a peptide from the polymer with HF or trifluoromethanesulfonic acid.

Yet another alkali-labile protective group is *S*-β,β-di(ethoxycarbonyl)ethyl group.[1] It is easily split off by the β-elimination reaction upon treatment with 1 M KOH.[1]

The advantages of the *S*-adamantyl (Ad) protective group,[22] although it is used rather seldom, are best manifested in comparison with Mob protection. The adamantyl protective group is more resistant to TFA: the degree of cleavage is only 10% over 2.5 h at 0 °C. It is quantitatively removed with a mixture of 1 M CF_3SO_3H and PhSMe in TFA (60 min, 0 °C) or $Tl(CF_3COO)_3$ in TFA.[13, 22, 23]

Xanthenyl and its 2(3)-methoxy derivatives are new protective groups for the thiol function of cysteine.[24] These acid-labile groups are eliminated by dilute acids in the presence of appropriate scavengers. An alternative procedure for their removal is the treatment with I_2 or $Tl(F_3CCOO)_3$, which allows direct synthesis of cyclic disulfides.[25]

The use of a novel quinolin-2-ylmethyl protective group has been reported.[25] A new procedure for the direct conversion of its derivatives into symmetrical disulfides includes treatment with $FeCl_3$, $FeCl_2$, and $CuCl_2$.[25]

Two new protective groups (Fc and Fcm) containing a ferrocenylmethyl fragment also deserve special mention.[26] These groups are rather stable under basic conditions and in the presence of reducing reagents, such as $NaBH_4$ and Zn in AcOH, although the Fc group is partly eliminated under these conditions. Both protective groups are rapidly split off by electrophilic reagents, *e.g*., thiocyanogen $(SCN)_2$, methoxycarbonylsulfenyl chloride, and TFA. The Fc group can be also cleaved with formic acid. Iodine only partly destroys the Fc and Fcm protective groups. Removal of both these protective groups by acids is efficient only in the presence of scavengers (*e.g*., benzenethiol), which capture the ferrocenylcarbenium ion formed. Fcm protection, which was used in combination with N^{α}-Boc or N^{α}-Fmoc protections, is of greatest importance for the peptide synthesis. In this case, *p*-toluenesulfonic acid was used for selective removal of Boc protection.[26]

The (η^5-cyclohexadienyl)tricarbonyl iron complex $\{[(\eta^5\text{-}C_6H_7)Fe(CO)_3]^+\}$ was successfully used to protect the thiol function of cysteine in the synthesis of a model peptide Ac-Cys-Gly-Ala-OEt.[27] Owing to the high affinity of this complex for the SH group of cysteine, the formation of the corresponding adduct occurs rapidly and without side reactions. Protected derivatives are bright yellow coloured, which facilitates monitoring of their purification on silica gel.[27]

2. Protective groups that form mono- and di-thioacetals

Protective groups that form mono- (S−C−O) and di-thioacetals (S−C−S) are not numerous and seldom used for cysteine protection.

The protective groups of the thioacetal type, *viz*., tetrahydropyranyl, isobutoxymethyl, *p*-chlorophenoxymethyl, benzylthiomethyl, and phenylthiomethyl groups, are stable under alkaline conditions and can be removed by acids. The ease of their cleavage with acids decreases in the following order: acetals > monothioacetals ≫ dithioacetals, and is determined by the same factors as for sulfides, *viz*., the ease of protonation, the stability of the cation formed, and the possibility of irreversible reactions that shift the equilibrium towards the cleavage products.[5] *S*-Monothioacetal derivatives of cysteine react with acids at the same rates as do trityl sulfides, whereas *S*-dithioacetal derivatives resemble Cys(Bzl) and Cys(Bu^t) under acidolysis conditions. According to the ease of their acidolysis, the protective groups are arranged in the following order:[8]

$$(p\text{-}MeOC_6H_4)_2CH > \text{} \cong Me_2CHCH_2OCH_2 \cong Ph_3C > > Ph_2CH > PhCH_2SCH_2 > PhCH_2.$$

Cleavage of mono- and di-thioacetals is more efficient with Hg(II) and Ag(I) salts.[5] The conditions for selective cleavage have been established for virtually each protective group.[8] The mechanism of cleavage of mono- and di-thioacetals by metal ions seems to be analogous to the mechanism of cleavage of sulfides. The formation of the M−S bond weakens the C−S bond. The formation of a cation is the rate-determining step of this reaction. The rate of cleavage depends of the cation stability.[5]

$$-SCH_2OR \overset{M^+}{\rightleftharpoons} \left[-\overset{M}{\overset{|}{\overset{+}{S}}}CH_2OR \right] \xrightarrow{\text{slowly}} -S-M + ROCH_2^+,$$

$$-SCH_2SR \overset{M^+}{\rightleftharpoons} \left[-\overset{M}{\overset{|}{\overset{+}{S}}}CH_2SR \right] \xrightarrow{\text{slowly}} -S-M + RSCH_2^+.$$

As with sulfides, regeneration of free thiols is achieved by treatment with a great excess of mercaptoalcohols or hydrogen sulfide. It is still uncertain whether selective cleavage of *S*-monothioacetals can be performed in the presence of *S*-dithioacetals, disulfide bonds, and other protective groups that are sensitive to heavy metal ions.[5]

Direct oxidation of monothioacetals into disulfides by halogens is inapplicable to cysteine derivatives. However, there is evidence of removal of the *S*-Thp group from the A-chain of synthetic insulin by its direct conversion into a symmetric disulfide using $(SCN)_2$.[5, 6]

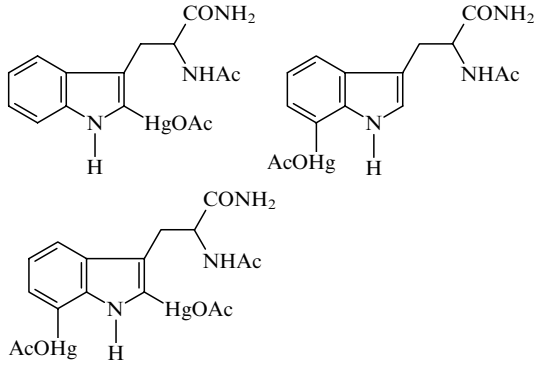
3. *S*-Acylamidomethyl protective groups

The acetamidomethyl protective group the introduction of which can be accompanied by the formation of thiazolidine derivatives is widely employed in the synthesis of cysteine-containing peptides owing to its high stability in acidic and alkaline media and under conditions of catalytic hydrogenolysis. It is used in combination with Boc, Fmoc, and benzyloxycarbonyl protections of the α-amino group in classical and solid-phase syntheses of peptides. However, there are a few reports on the partial loss of the Acm protective group on repeated treatment of the peptide chains with HF needed for their splitting from the polymer.[30] The Acm protection is stable under conditions of acid-catalysed removal of Meb, Mob, Trt, and Tmob groups. Specially selected conditions of deprotection of the thiol function of cysteine with heavy metal salts in appropriate solvents ensure selective removal of the *S*-Acm group in the presence of Bzl and Bu^t groups. Reaction with Hg(II) salts yields an intermediate peptide–metal compound. When Hg(II) salts are used in acidic aqueous solutions, the reaction is completed within 1–2 h.[6] Deblocking can also take place in organic solvents, *e.g*., in DMF.[2]

$$R-\overset{\overset{Acm}{|}}{Cys}-OH \xrightarrow[pH\ 4]{Hg^{2+}} R-\overset{\overset{Hg^+}{|}}{Cys}-OH \xrightarrow{H_2S} R-Cys-OH$$

R is the peptide residue.

As in the case of removal of Trt protection, subsequent treatment with hydrogen sulfide or mercaptoalcohols yields free thiol groups. If Hg(II) salts are used for removal of Acm protection from the SH group of cysteine in tryptophan-containing peptides, side reactions can occur.[46] Thus treatment of a bis-Acm derivative of somatostatin with a large excess of $Hg(AcO)_2$ (5–20 equiv. per one Acm group) in 1%–10% acetic acid results in substitution of hydrogen in positions 2 and 7 by the HgOAc group to form mono- and bis-mercuriacetates of tryptophan.

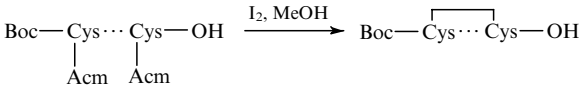


Treatment of these derivatives with β-mercaptoethanol results in the substitution of the $Hg(AcO)^+$ group by $HOCH_2CH_2S$ to yield undesirable side products. Acetoxymercuration can be avoided by decreasing the amount of $Hg(AcO)_2$ to 1–2 equiv. and by using 50% acetic acid.[46]

There is evidence of the possibility of S → N and S → O migrations of the Acm protective group to the side carboxamide groups of asparagine and glutamine[2,47] and to the hydroxy groups of serine and threonine.[2,48,49] The migration of the Acm protective group to the side chain of glutamine (S → N migration) was observed in the deprotection of the thiol function of cysteine with I_2 in MeOH. This side reaction is inhibited by an excess of glutamine as a scavenger.[47] S → O Migration was observed in the deprotection of cysteine with Tl(III) and Hg(II) salts in a peptide containing several serine and threonine residues.[48] This reaction is efficiently inhibited by three-carbon alcohols (*e.g.*, glycerol) as scavengers.[2,48]

The anion plays an important role in the removal of the Acm group with Ag(I) salts. The reaction occurs with silver nitrate,[5] trifluoromethanesulfonate,[18] trifluoroacetate,[18] and tetrafluoroborate.[28] Silver acetate does not remove the Acm protection. Deprotection of cysteine with silver trifluoroacetate requires that the excess of the deblocking reagent to be twice as high as that with silver trifluoromethanesulfonate. The reaction time is also twice as long;[18] therefore the use of AgOTf is preferable. Silver salts are removed by large (up to 50 equiv.) excess of β-mercaptoethanol or dithiothreitol.[18] Then, the reaction mixture is purified by gel filtration. This deblocking procedure does not affect such highly sensitive amino acids as methionine, tryptophan, and tyrosine.

Direct conversion of di-Acm substituted peptides into disulfides upon treatment with iodine is an attractive method for the removal of the Acm protective group.



This method will be considered in more detail in Section III.

Recently, trimethylacetamidomethyl, phenylacetamidomethyl, and benzamidomethyl protective groups, which can be regarded as derivatives of acetamidomethyl protection, have gained wide acceptance. The properties of benzamidomethyl protection are identical with those of the Acm group.[6,18] It is also sensitive to AgOTf. However, the removal of the Bam group takes more time than the removal of the Acm protection.[18]

Yoshida *et al.*[28–31] have proposed to use the trimethylacetamidomethyl group to protect the SH function of cysteine. It was found that under conditions of post-synthetic deblocking of the side functional groups of peptides with HF or NaOH, the Tacm and Acm groups, unlike the Bam group, were sufficiently stable. The authors[30] attribute the ease of introduction of the protective group and high yields of protected cysteine derivatives to salient advantages of Tacm protection. It should be mentioned that the formation of a side product (thiazolidine-4-carboxylic acid) cannot be avoided in the synthesis of *S*-acetamidomethylcysteine.[30]

The phenylacetamidomethyl protective group[32,33] is stable and is split off under the same conditions as the Acm group. Its distinctive feature is the possibility to be cleaved by penicillin aminohydrolase.[2] Therefore, the Phacm group is orthogonal with respect to Acm protection. First, it is possible to remove the Phacm group enzymically and then the Acm group is removed by treatment with heavy metal salts or iodine.[2,33]

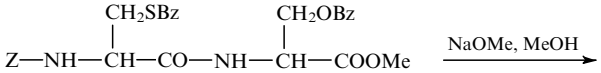
The 2-oxopyrrolidinomethyl group is resistant to reagents of peptide synthesis in a broad range of pH (from 0.5 to 13). In its resistance to alkalis, it surpasses the Bam group.[34] Cleavage of the Pym protection with simultaneous formation of the disulfide bond is achieved by treatment with I_2 in MeOH.[34]

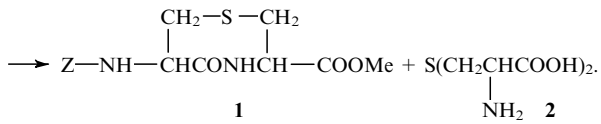
4. Protective groups that form thioesters and related compounds

Protective groups that form thioesters (RCOSR′) include acetyl, benzoyl, benzyloxycarbonyl, *p*-methoxybenzyloxycarbonyl, *tert*-butoxycarbonyl, and *p*-nitrophenoxycarbonyl groups. Dithiocarbonates (RSCOSR′) are represented by derivatives with phenylthiocarbonyl and butylthiocarbonyl protective groups and *N*-(alkyl)thiocarbamates (RNHCOSR′), with *N*-ethylcarbamoyl, *N*-methoxymethylcarbamoyl, and *N*-[β-(*N*-acyl-*N*-methylamino)ethyl]carbamoyl protective groups.

A characteristic feature of thioesters (acetyl and benzoyl protections), thiocarbonates (benzyloxycarbonyl, *p*-methoxybenzyloxycarbonyl, *p*-nitrophenoxycarbonyl protections), and thiocarbamates (*N*-ethylcarbamoyl and *N*-methoxymethylcarbamoyl protections) is their sensitivity to nucleophiles and bases. Similarly to the corresponding esters and urethanes, thiocarbonates containing a benzyloxycarbonyl group and its analogues as well as Boc-protection are also cleaved in acidic media and with even greater ease.[8] Dithiocarbonates (phenylthiocarbonyl and butylthiocarbonyl protections) and thiocarbamate (*N*-[β-(*N*-acyl-*N*-methylamino)ethyl]carbamoyl protection) are cleaved only with acids. Alkaline treatment of thioesters can induce base-catalysed disulfide exchange, which can result in the formation of polymer mixtures and peptide parallel and antiparallel dimers. The side reaction of β-elimination, which is accompanied by elimination of thiobenzoic acid from the corresponding Cys(Bz) derivatives in peptides, can also take place.[5] The occurrence of this reaction depends on the solvent polarity; therefore one must avoid the addition of DMF to the reaction mixtures in the treatment of cysteine-containing peptides with bases, such as NaOMe.[5] Cleavage of *S*-acyl groups can be achieved by treatment with hydrazine in methanol. Under these conditions, Ac protection is split off rapidly as the corresponding acethydrazide without β-elimination.

A peculiar side reaction was observed when the protected peptide Z-Cys(Bz)-Ser(Bz)-OMe was treated with NaOMe in MeOH.[1] The serine residue was converted into an α-aminoacrylic acid residue, and the addition of the deblocked SH group to the double bond yielded products **1** and **2**:



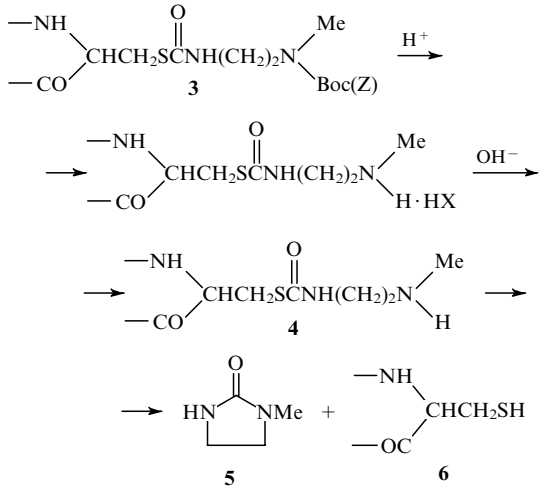


A serious limitation to the use of Ac, Bz, Z, pMZ, Boc, nitrophenylthiocarbonyl, butylthiocarbonyl, *N*-methoxymethylcarbamoyl, Ec-, and [β-(*N*-acyl-*N*-methylamino)ethyl]carbamoyl acyl protective groups is their proneness to S → N acyl migration. Such migration takes place in the course of elongation of the peptide chain as soon as a free amino group appears.[5,8] This side reaction has been observed both in the classical and solid-phase peptide syntheses.[6] When acetyl and benzoyl protective groups were used in solid-phase synthesis for protection of the thiol function of cysteine, a nearly quantitative chain growth termination occurred due to the interaction of the deblocked SH group with the residual chloromethyl groups of the polymeric support after S → N migration.[6] Nevertheless, Ac and Bz groups are used to protect cysteine residues in solid-phase syntheses of protected peptide fragments designed for fragment condensation. The S → N acyl migration can be suppressed if highly reactive derivatives (*e.g.*, mixed anhydrides with pivalic acid) are used for the formation of the amide bond.[5] In this case, the rate of aminolysis significantly exceeds the rate of S → N migration. The benzyloxycarbonyl group also undergoes S → N migration, but this reaction proceeds at a much slower rate. The formation of side products can be avoided in this case if condensation is carried out with pivaloyl chloride or diphenyl phosphorochloridate.[5]

The *p*-methoxybenzyloxycarbonyl protective group is distinguished by higher stability of the cation formed on its splitting and can therefore be removed not only by alkalis, but also by acids. However, its application is limited due to the difficulty of its introduction (the yields are about 30%) and high lability with respect to acids and alkalis.[5]

The *N*-ethylcarbamoyl and *N*-methoxymethylcarbamoyl protective groups are resistant to acids and easily removed by basic reagents (as a rule, without racemisation and desulfurisation), they do not undergo S → N migration. Thus, they present substantial practical interest.[6,8] The possibility of partial splitting of the Ec group with an excess of triethylamine during solid-phase synthesis with the use of the Boc-technique (at the stage of neutralisation of the peptidyl polymer or on repeated condensation) restricts the application of this protective group.

The use of *S*-[*N*-β-(*N*-alkoxycarbonyl-*N*-methylamino)ethyl]-carbamoyl protective group, yet another group of the ethylcarbamoyl type, has been reported.[6] In order to deblock compound **3** in which the thiol function of cysteine is protected by this group the alkoxycarbonyl (Z or Boc) group is removed by acid treatment. The resulting *N*-β-(methylamino)ethylcarbamoyl derivative **4** is cyclised to yield 1-methyl-2-imidazolidone **5** and the target thiol-containing peptide **6**.[6]



5. Protective groups that form disulfides

Disulfide derivatives (R – S – S – R′) obtained after blocking of the SH group include symmetrical and asymmetrical cysteine disulfides, *S*-sulfonates, *S*-alkyl(aryl)thio, *S*-alkoxycarbonylthio derivatives, and sulfamides. Symmetrical disulfides are very seldom used in modern peptide chemistry for synthetic purposes. Ethylthio, isopropylthio, triphenylmethylthio, *tert*-butylthio, methoxycarbonylthio, (*N*-methyl-*N*-phenylcarbamoyl)thio, and 3-nitro-2-pyridinethio protective groups that form asymmetrical disulfides are good alternatives to the protective groups described above owing to their lability with respect to aliphatic and aromatic thiols. A specific feature of some of these groups (Scm, Snm, Npys) is their ability to form intra- and intermolecular disulfide bonds as soon as cysteine is deblocked. Here, the SH groups of peptide thiols play the role of a deblocking reagent (this problem will be considered in detail in Section III).

S-Methoxycarbonylthio derivatives of cysteine are highly reactive compounds.[6,35,36] Their reactivity with respect to thiols is greater than that of *S*-sulfonates and is comparable with that of sulfenyl thiocyanates or sulfenyl iodides, which are intermediates in the closure of disulfide bonds by the corresponding reagents (see Section III). The advantages of the Scm group are the stability of protected derivatives and the irreversibility of their reaction with thiols, unlike the reaction of sulfenyl iodides and sulfenyl thiocyanates.[6] Owing to its stability in acidic media, the Scm group can be used in solid-phase syntheses using the Boc-technique.

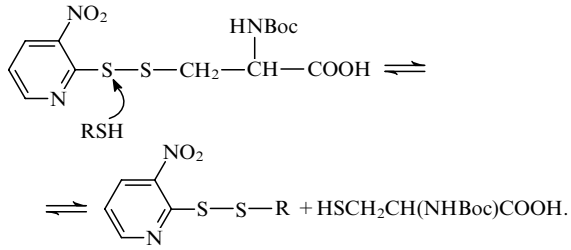
Cleavage of disulfides is performed in aqueous solutions (pH 7.5 – 9.0) with thiols, such as β-mercaptoethanol or benzenethiol, which is a stronger nucleophile.[8] The reaction with thiols is based on the thiol – disulfide exchange. In order to shift the equilibrium in the direction of the target product, thiol must be employed in a large excess. Deblocking can also be achieved with a small excess of dithiothreitol, where the equilibrium is shifted due to the intramolecular cyclisation of the reagent, or with 1 equiv. of tributylphosphine at pH 8.[8] Phosphines are specific reagents for cysteine and reduce only disulfide bonds; this reaction is selective.[17] Disulfides may also be cleaved by oxidative sulfitolysis. The resulting *S*-sulfonates are reduced with thiols or aqueous solutions of thio acids with gentle heating.[8]

It is known that disulfide derivatives are relatively resistant to acids. Their stability decreases in the following order: RSSBu^t > RSSPr^i ~ RSSEt. Thus the SBu^t group is cleaved by treatment with liquid HF for 1 h at 0 °C.[2,6] The SPr^i and SEt groups can be split off with hydrogen chloride in AcOH.[6] Only the SBu^t group is of practical value and has been successfuly used in solid-phase synthesis using the Boc and Fmoc techniques.[36,37] The majority of mixed disulfides (Table 1) are resistant to ammonia and alkalis, which are used to split the peptides from the polymeric supports with phenolic and phenacyl anchor groups.[8]

Alkoxycarbonylthio (*tert*-butoxycarbonylthio and benzyloxycarbonylthio) protective groups were proposed by Nokihara and Berndt.[36] Unfortunately, SCB derivatives proved to be not very stable on storage. Thus the cysteine derivative ZCys(SCB)OMe turned into its cystine analogue within 3 months. In addition, the high lability of SCB protection with respect to alkalis restricts its application in peptide synthesis.[36] The benzyloxycarbonylthio protective group is stable in TFA. It is more resistant to alkalis that the methoxycarbonylthio (Scm) group and can be removed by treatment with strong alkalis, though this is preserved for 2 h at pH 10.6, which results in complete removal of the Scm protection. Therefore, the SZ group can be used both for the protection of the SH group of cysteine and for the selective formation of cystines.[36]

The *N*-methyl-*N*-phenylcarbamoylthio protective group is resistant to acids and can therefore be used in peptide syntheses with the Boc-technique. The possibility of removal of this protective group by alkaline treatment has not been finally clarified.[2]

Like Scm protection, the 3-nitro-2-pyridylthio and *N*-methyl-*N*-phenylcarbamoylthio protective groups may selectively generate disulfide bonds in the presence of free SH groups. These groups are removed by thiolysis, which occurs in accordance with the Scheme:[38, 39]



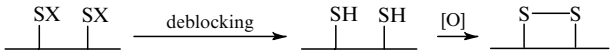
It has been found [39] that Npys protection is the most efficiently split off with aliphatic thiols, such as β-mercaptoethanol or mercaptoacetic acid: when the thiols are taken in a 10-fold excess over 3-nitro-2-pyridylthio derivatives, the reaction is completed within 10 min. The *S*—Npys bond is rather resistant to the action of aromatic thiols, 1-methylimidazole-2-thiol, and pyridine-2-thiol. In this case, selective cleavage of the O—Npys and N—Npys bonds may occur.[39] Albericio *et al.*[40] have shown that the Npys group is more stable under standard conditions of peptide synthesis using the Boc-technique (HF, TFA, and tertiary amines) in comparison with the methoxycarbonylthio group. The Npys protection for the thiol function of cysteine is absolutely stable in solvents and reagents used in solid-phase syntheses using the Boc-technique, *e.g.*, CH_2Cl_2, DMF, *N,N*-dimethylacetamide, *N*-methylpyrrolidone, MeOH, trifluoroethanol, dioxane, in a mixture of 40% TFA and CH_2Cl_2, this also withstands treatment with HF (to which 10% anisole or *p*-cresol is added) for 1 h at 0 °C. Thus, the Npys group is virtually resistant under conditions of HF cleavage by the high technique.[40] The low-high technique is inapplicable in this case, since the use of *p*-thiocresol and dimethyl sulfide results in the loss of lateral Npys groups.[40] The data on the resistance of Npys protection to *N*-hydroxybenzotriazole are contradictory.[40, 41] Partial (12%) cleavage of the Npys group was observed in the treatment with 0.1 M solution of this reagent in DMF.[41] Therefore, the method of activated *p*-nitrophenyl and pentafluorophenyl esters was employed to form the amide bond (condensation).[4, 40, 50] Matsueda *et al.*[41] have successfully used *N,N′*-dicyclohexylcarbodiimide with *N*-hydroxybenzotriazole to attach Boc-Cys(Npys)-OH in the synthesis of a series of peptides. The protective Npys group was found to be very labile under conditions of solid-phase synthesis using the Fmoc-technique, but was sufficiently stable in the photolytic cleavage of peptides from the polymer.[40]

Of interest is the report on the use of base-resistant *S*-sulfonates [Boc-Cys(SO_3Na)-ONa] and [Fmoc-Cys(SO_3Na)-ONa] in peptide synthesis.[42] Deblocking of cysteine may be achieved by treatment with tributylphosphine, β-mercaptoethanol, and dithiothreitol.[42] Splitting of the peptide from the polymer with TFA–*p*-cresol systems leads to partial loss of sulfonates.

III. Methods for the formation of disulfide bonds

Despite the intensive progress in methodology of peptide chemistry, the formation of disulfide bonds still presents a serious problem. Its solution involves: (1) the choice of a protective group for the thiol function of cysteine and (2) the selection of a method for the formation of a disulfide bridge. These two aspects are closely interrelated, for the choice of a protective group is the principal factor that predetermines the selection of the method for the formation of the S—S bonds. This problem becomes more complicated in the case where several disulfide bonds are to be formed. The so-called 'orthogonal' scheme of synthesis allows selective closure of disulfide bonds between definite cysteine residues without involvement of other protected residues or the preformed disulfide bridges. In this case, the choice of protective groups that can be selectively split off and the cyclisation procedure are of crucial importance.[8] In any case, the cyclisation process is very complicated and its successful outcome depends on many factors, such as the correct choice of the solvent, temperature, pH of the reaction medium, concentration of reagents, type of the oxidant, and so on. The methods for cyclisation can be arbitrarily classified into three groups:[2]

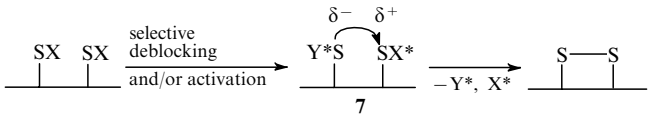
A. Deprotection and formation of a disulfide bond starting from the corresponding SH-derivatives of peptides.



B. Deprotection with simultaneous closure of the ring.



C. Closure of the disulfide bond by thiolysis. This entails two possibilities: (1) The protective group of one of cysteine residues (X*) enhances the electrophilicity of the sulfur atom (activation). This facilitates the cleavage of the bond between the sulfur atom and the protective group in the thiolysis. The free SH group of the other cysteine residue is the necessary thiol. In this case, X* = X = —SCOOMe, —SCON(Me)Ph, 3-nitro-2-pyridylthio, *etc.* (2) The protective groups X = CPh_3, $CH_2NHCOMe$, Bu^t, *etc.*, are split off with $(SCN)_2$, 3-nitro-2-pyridinesulfenyl chloride, 4-nitrobenzenesulfonyl chloride, and other reagents to form activated asymmetrical disulfides (X* = SCN, 3-nitro-2-pyridylthio, 4-nitrophenylsulfonyl) that can enter into the reactions of thiol–disulfide exchange:



7

1. Formation of disulfide bonds in SH-derivatives of peptides

Group A methods envisage the blocking of two cysteine residues by identical protective groups that are removed under identical conditions, purification of linear polythiols, and oxidation of SH groups with any appropriate reagent providing the formation of intra- or intermolecular disulfide bonds. Oxidation of free thiols is usually carried out in highly dilute solutions at the peptide concentration 10^{-4}–10^{-5} M in order to avoid intermolecular coupling and side reactions.[2, 8] Direct formation of the S—S bonds in dilute solutions strongly depends on the peptide structure, particularly on the nature of the amino acids separating two cysteine residues and the number of amino acids between them.[51, 52] If the number of amino acids between two cysteine residues (*n*) is from 4 to 20, the intramolecular S—S bonds are predominantly formed under conditions of high dilution (10^{-3}–10^{-4} M). At $n < 3$, the limited chain flexibility results in dimers and polymers that are formed in addition to the peptides with the intramolecular S—S bonds.[51] The formation of the disulfide bond between the neighbouring cysteine residues demands very strong oxidants, such as thallium(III) trifluoroacetate.[53] The ability to form intrachain disulfide bonds depends also on the structural stability of the disulfide loop. Thus the oxidation of peptides is facilitated by the presence in the amino acid sequence of Pro-Gly residues involved in the formation of β-folds of proteins and peptides. Many peptides (*e.g.*, oxytocin) can be readily oxidised by atmospheric oxygen. Peptide fragments of proteins having the Pro-Pro sequence between two cysteine residues are difficult to oxidise and can form only symmetrical dimers.[51]

a. Oxidation with atmospheric oxygen

Atmospheric oxygen is often used to oxidise thiol groups of cysteine to yield disulfides. This reaction is usually carried out in weakly alkaline solutions at pH 7.5–8.5 with vigorous stirring or by bubbling air through the reaction mixture. The required level of pH is usually maintained by adding ammonia,[54,55] or other reagents, such as ethyldiisopropylamine.[55,56] In some cases, cyclisation occurs much faster when ethyldiisopropylamine is used instead of ammonia.[55] The oxidations may also be carried out in 0.1 M NH_4HCO_3 (pH 8.0)[57] or 0.1 M ammonium acetate buffer (pH 6.8–7.0).[51] Passage of pure oxygen (instead of air) through a solution of a peptide in 0.1 M NH_4HCO_3 did not increase the rate of cyclisation.[57] In the case of peptides that aggregate in aqueous solutions, 0.5–8.0 M urea or 0.1–1.5 M guanidine hydrochloride are added.[2,58,59] As a rule, the oxidation is performed at 5–25 °C. The reaction time varies from several hours to several days.[54–57] Most probably, this reaction occurs by a free-radical mechanism. The cyclisation is usually monitored by HPLC or by a reaction for the presence of a free SH group using Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoic acid).[60] The yields of cyclic disulfides in this oxidation are usually small (9%–15%).[54–56]

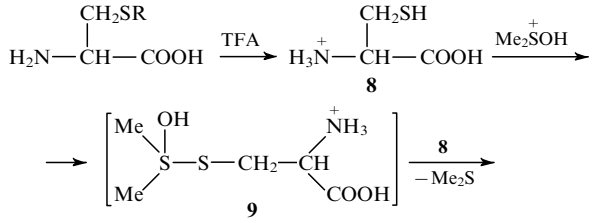
There are some examples of preparation of synthetic toxins, growth factors, and enzyme inhibitors by this method. That this process is lengthy and the product yields are low are serious limitations of this method. However, its simplicity makes it an attractive procedure despite the advent of new efficient reagents.

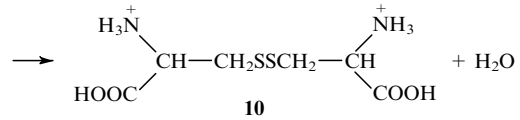
b. Oxidation with potassium ferricyanide

Potassium ferricyanide is a popular cyclisation reagent. Laloo and Mahanti[61] have studied the kinetics of cysteine oxidation with potassium ferricyanide and proposed a mechanism for this reaction. Using ESR spectroscopy they have found that this reaction occurs *via* an intermediate radical, $[-SCH_2C^{\bullet}(NH_2)COO^-]$.[61] Depending on the solubility of a peptide, the oxidation is carried out in aqueous or aqueous-ethanolic solutions with high dilution (pH 8–10).[60,62] Haskell-Lecevano *et al.*[63] performed this oxidation in a saturated solution of NH_4OAc containing methanol and acetonitrile. The termination of the reaction is judged from the appearance of a persistent yellow colouration. Inorganic impurities are removed by ion-exchange chromatography. This method mainly yields homogenous reaction mixtures. Oxidation of sulfhydryl groups occurs virtually quantitatively.[55] This procedure has been used to obtain oxytocin,[2,64] vasopressin analogues,[62] somatostatin,[2,65] and peptides with two S–S bridges (endothelin-1,[58] a bee venom peptide of mast cell degranulation, [66] *etc.*).

c. Oxidation with dimethyl sulfoxide

Dimethyl sulfoxide (DMSO) was used to oxidise peptide thiols for the first time in 1988.[67] This compound has a number of indisputable advantages: it can be used in a wide range of pH (from 3 to 8), ensures higher reaction rates (in comparison with oxidation with atmospheric oxygen), and increases solubility of peptides.[2,59,67–71] This reaction is catalysed by acids or primary and secondary amines. The mechanism of oxidation in an acidic medium was proposed by Otaka *et al.*[68]





R = Trt, Mbzl, Dpm.

The limiting step of this reaction is the formation of the intermediate compound **9**, which rapidly yields the disulfide **10** on reaction with the free thiol **8**. The optimum pH range for the reaction with DMSO is 3–8.[69] In this case, the reaction rate does not depend on pH, because in this pH range both the protonation of DMSO and the formation of a thiolate anion may take place. At pH < 3, methionine can undergo oxidation into the corresponding sulfoxide, whereas at pH > 8 the rate of disulfide exchange increases. However, there are some exceptions. For example, the oxidation of peptides with an N-terminal cysteine should be conducted at pH 6–7. If the amino groups of cysteine are acetylated, the pH is unimportant. Tam *et al.*[69] interpret this phenomenon by the fact that the weakly positive α-amino group of cysteine facilitates the formation of the unstable intermediate adduct **9**. In more acidic media, the α-amino group is protonated and does not exert such an effect.

Oxidation with dimethyl sulfoxide is carried out at its concentration in the reaction mixture from 10% to 50%. An increase in DMSO concentration increases the reaction rate. Thus complete oxidation of a linear precursor of a human growth factor fragment with 10%–30% DMSO requires 2–3 h, whereas with 40%–50% DMSO it takes only 0.5 h. The oxidation-sensitive amino acid residues of tryptophan, tyrosine, and methionine are not modified under these conditions.[69,71]

A 10% DMSO solution in TFA has been used for simultaneous splitting of acid-labile cysteine protective groups (Trt, Mbzl, Dpm) and the closure of disulfide bonds.[68,70] However, in this case the oxidation lasted several hours. Over this time, the unprotected tryptophan was strongly modified and methionine was oxidised into the corresponding sulfoxide. Oxytocin, which has no tryptophan or methionine in its amino acid sequence, was obtained in 73.5% yield from a bis(methoxybenzyl)cysteine derivative by treatment with 10% DMSO in TFA in the presence of anisole (12 h, 25 °C). Human calcitonin (α-hCGRP) was obtained from a bisadamantyl derivative of cysteine under identical conditions (1 h , 17%).[68]

The resistance of the oxytocin derivatives [1,6-Cys(Acm)] and [1-Cys(Acm), 6-Cys(SH)] with respect to 10% DMSO in TFA has been studied.[70] The former proved to be stable for 12 h, whereas the monosubstituted derivative [1-Cys(Acm), 6-Cys(SH)] was converted into oxytocin under identical conditions within 1 h (69%); the product was contaminated with a dimer.

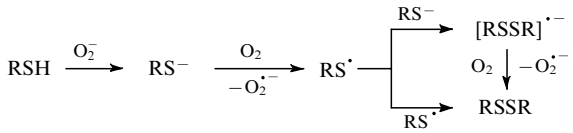
Tamamura *et al.*[59] reported on the closure of the S–S bonds upon action of 10% or 50% DMSO in 1 M HCl. The thiol groups of cysteine protected with Acm of Mbzl groups had been deblocked beforehand with silver trifluoromethanesulfonate in TFA. Two chemical reactions occurred simultaneously in the DMSO–HCl system: conversion of Cys(Ag) derivatives into cysteine and oxidation of cysteine residues with DMSO. This approach was used to obtain oxytocin, urotensin II, tachiplensin I, and endothelin I in 72%, 80%, 79%, and 62% yields, respectively (as calculated per bis-Acm derivatives). No side reactions that could affect methionine, tyrosine, or tryptophan residues were observed under these conditions.[59]

Oxidation by DMSO is especially efficient in cyclisation of basic peptides that are insoluble in weakly alkaline solutions, which are required for oxidation with oxygen or $K_3[Fe(CN)_6]$. This problem appeared in the synthesis of basic hydrophobic growth factors that remained insoluble in weakly alkaline solutions even after addition of 8 M urea.[69,71] The advantages of this method have been also exemplified in the synthesis of a natriuretic peptide (pBNP) and tachiplensin I having two S–S bonds.[67]

However, despite the obvious merits of this method, it has certain disadvantages, which are connected with the difficulty of removal of the excess of DMSO and dimethyl sulfide formed in this reaction.

d. Oxidation with hydrogen peroxide

Hydrogen peroxide is a common component of biological systems and participates in physiological oxidation processes.[72] Therefore, the use of this oxidant in peptide synthesis is fully justified. Oxidation of thiols with H_2O_2 may occur in two ways: *via* generation of a thiolate anion (RS^-) or a radical ($RS^{\bullet}$):[73]



The oxidation performed under alkaline conditions accelerates the generation of the thiolate anion; therefore in this case the formation of the disulfide occurs at higher rate.[74]

Using oxidation of *N*-acetylcysteine and glutathione as examples, it was shown that this reaction proceeds *via* several thiol and disulfide complexes with H_2O_2.[75,76] The important role of H_2O present in the reaction mixture was emphasised, since it stabilises these complexes by creating a solvate shell around them.[75,76]

$$RSH + H_2O_2 \longrightarrow [RSH...H_2O_2],$$

$$RSH + 2H_2O_2 \longrightarrow [RSH...H_2O_2...H_2O_2],$$

$$2[RSH...H_2O_2] \longrightarrow \underset{\mathbf{11}}{[RSSR...H_2O_2]} + 2H_2O,$$

$$[RSSR...H_2O_2] + 2RSH \longrightarrow \underset{\mathbf{12}}{2RSSR} + 2H_2O,$$

$$[RSH...H_2O_2...RSH] \longrightarrow RSSR + 2H_2O.$$

The dependence of the rates of these reactions (in a phosphate buffer, pH 7.0) on the concentration ratio, $K = [RSH]/[H_2O_2]$, has been studied. The formation of the complex **11** takes place at $K < 2.5$, while that of the free disulfide **12** at $K > 2.5$, *i.e.*, where the concentration of the residual thiol is $< 10^{-2}$ mol litre^{-1}.

A systematic study of the applicability of hydrogen peroxide for the closure of disulfide bonds has been carried out by Kudryavtseva *et al.*[77,78] Table 2 lists the yields obtained in the formation of disulfide bonds in peptides of various lengths and structures under the action of both iodine and hydrogen peroxide.[78] Syntheses of some biologically active peptides, such as antigenic determinants of HIV proteins (compounds **13**–**16**, Table 2), atrial (vasodilator) (compounds **18**–**20**, Table 2) and vasoconstrictor peptides, containing oxidation-sensitive tryptophan, tyrosine, and methionine residues demonstrate that the oxidation with H_2O_2 enables the preparation of the target disulfides within only 15–20 min and in high yields (60%–85%) without formation of side products.[78] The oxidation was carried out in aqueous media at pH 6.5–8.0. It was also shown that H_2O_2 can be used for the synthesis of preparative (gram-scale) amounts of antigenic determinants of HIV proteins (**13**–**16**) and oxytocin (**17**).[78]

There are other reports concerning the use of H_2O_2 in the synthesis of oxytocin and vasopressin.[3,79] Hydrogen peroxide was used for cyclisation of methionine-containing peptides (pH 8–11), human natriuretic peptide (α-hNAP), and somatostatin in phosphate buffer (pH 8.5, 10 min). The reaction products were obtained in more than 90% yields and with high purity.

Apparently, the use of hydrogen peroxide in cyclisation has limitations of its own. It is known, for example, that H_2O_2 may induce more profound oxidation of disulfides to sulfones.[72]

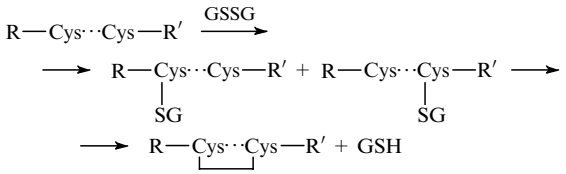
Table 2. Comparative yields of cyclic peptides during the closure of the S–S bridge with iodine (in ACOH or MeOH) and hydrogen peroxide.

No. of compound	Structure	Yields (%)	
		I_2	H_2O_2
13	H-LGLWGCSGKLIC-NHEt	51	75–85[a]
14	H-LGIWGCSGKHIC-NHEt	56	85–90[a]
15	H-NSWGCAFRQVC-NHEt	66	80–87[a]
16	H-WGCSGKLICTTAVPWNA-OH	25	85–90[a]
17	H-CYIQNCPLG-NH_2	50	70–75[b]
18	H-SSCFGGRIDRIGAQSGLGCNSFR-OH	23	83
19	H-SSCFGGRIDRIGAQSGLGCNSFRY-OH	26	50
20	H-SLRRSSCFGGRIDRIGAQSGLGCNSFRY-OH	20	62

[a] The scatter in yields on oxidation of 0.1–1.5 g of the corresponding bis-Acm derivatives of peptides; [b] the scatter in yields on oxidation of 2.0–20.0 g of bis-Acm derivatives of oxytocin.

e. Oxidation with glutathione

Yet another way to close S–S bonds is the use of buffers that simulate physiological conditions. Since *in vivo* disulfide bridges are formed under reductive conditions, and the reducing medium in cells is maintained by the glutathione system (5 mM reduced and 0.1 mM oxidised glutathione),[81] buffer solutions containing reduced and oxidised glutathione at a 10 : 1 ratio are most often used for the formation of S–S bonds.[2] Other ratios of reduced and oxidised forms, *e.g.*, 1 : 1 (Ref. 82) or 2 : 1 (Ref. 83) are also employed. The reaction of peptide polythiols is conducted in dilute solutions at pH 7.3–8.7 and 0.1–0.5 M Tris–HCl buffer is used for dissolution.[83,84] The oxidation of thiols with glutathione occurs in two steps.[85] At first, a 'reduced' peptide having free SH groups reacts with the oxidised glutathione (GSSG) to give mixed disulfides, which undergo intramolecular thiol–disulfide exchange to form disulfide bonds in peptides and the reduced glutathione.[85]



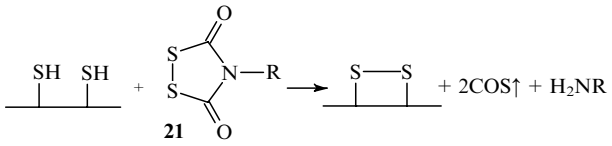
The limiting step of this reaction is the reaction of the peptide with GSSG; therefore, the reaction rate depends on the concentration of the latter. The oxidation is normally carried out for 16–72 h at 25–35 °C.[82,83] The reaction product is purified by affinity chromatography[2] or by HPLC.[83,84] This method was used to obtain a 48-membered neurotoxin of sea anemone (yield 22.8%),[84] the trypsin inhibitor CMTI III (yield 10%),[82] human epidermal growth factor (h-EGF) peptides,[83] and ω-conotoxin.[86] All these peptides contain three disulfide bonds; their closure was performed simultaneously. This method yields predominantly natural, thermodynamically favourable structures.[82–86] However, the procedure that employs a mixture of oxidised and reduced glutathiones is mainly limited by large molecules with a thermodynamically favourable physiologically active conformation and is hardly suitable for the synthesis of short peptides containing disulfide bonds.

f. The use of polymers

Clarc and Pai have proposed an original procedure for cyclisation of linear SH-containing peptides using an Ekathiox™ polymer.[87] The resin (40–80 μ, pore diameter 100 Å) contains 0.2–0.4 mmoles of oxidising functional groups per gram. The oxidising polymer was removed by filtration after 4 h at 20 °C. The efficiency of the polymer was evaluated by the formation of oxytocin (yield 92%). The authors[87] note that the high yield and the absence of dimeric side products were due to pseudo-dilution (see Section III.4), where the reaction was performed on a solid phase.[87] The polymer Ekathiox™ does not modify tryptophan, tyrosine, or methionine residues. Moreover, it allows one to conduct oxidation in both aqueous and organic media. It is difficult to evaluate the advantages of this method, because the nature of the functional groups was not specified by the authors.

An alternative approach to cyclisation using polymeric reagents was proposed by Lin Chen and Barany.[88]

N-Substituted 2,3-dithiasuccinimides **21** were used as oxidants to produce intramolecular disulfide bridges in the synthesis of oxytocin and deaminooxytocin performed under different conditions.



R = CH_3, CH_2CO_2H, CH_2CO_2–Ⓟ,
Ⓟ is the insoluble polymer.

One procedure consisted in the reaction of soluble peptides with a dithiasuccinimidoacetyl polymer, and the other, the reaction of a peptidyl polymer with a soluble derivative of 2,3-dithiasuccinimide. The rate of this reaction depends on the pH of the reaction medium. The reaction kinetics has been studied in aqueous, organic, and aqueous-organic media. The disadvantage of this method is the high reactivity of dithiasuccinimide derivatives, which irreversibly oxidise functional side groups of amino acids.[88]

g. Miscellaneous reagents

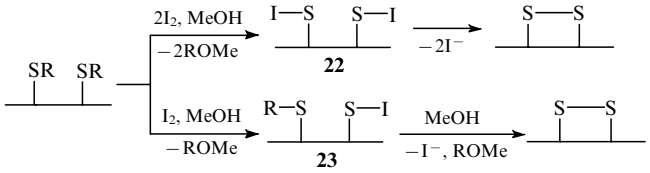
Other reagents, such as 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent[89]) and a CCl_4–NEt_3 mixture,[2,7] have been used to oxidise free thiol groups of cysteine residues. Although these compounds did not gain wide acceptance for oxidation in solution, they have been used for solid-phase cyclisation and therefore will be considered below.

2. Removal of protective groups with simultaneous closure of an S–S bridge

Direct conversion of *S*-protected peptides into the corresponding disulfides is one of the most attractive methods of disulfide bond formation. A number of reagents are now available that can be used for such conversions.

a. Iodine

Iodine is the most popular reagent used in the formation of the disulfide bonds. The reactions of cysteine with Trt, Acm, Tmob, Mob, and other protective groups with iodine have been studied in great detail. Two mechanisms have been proposed to explain the conversion of a sulfide into a disulfide.[5] One of them implies the formation of an intermediate sulfenyl iodide **22**, and the other, of alkyl(aryl)thiosulfenyl iodide **23**.



R = Acm, Trt, Tmob, Mob.

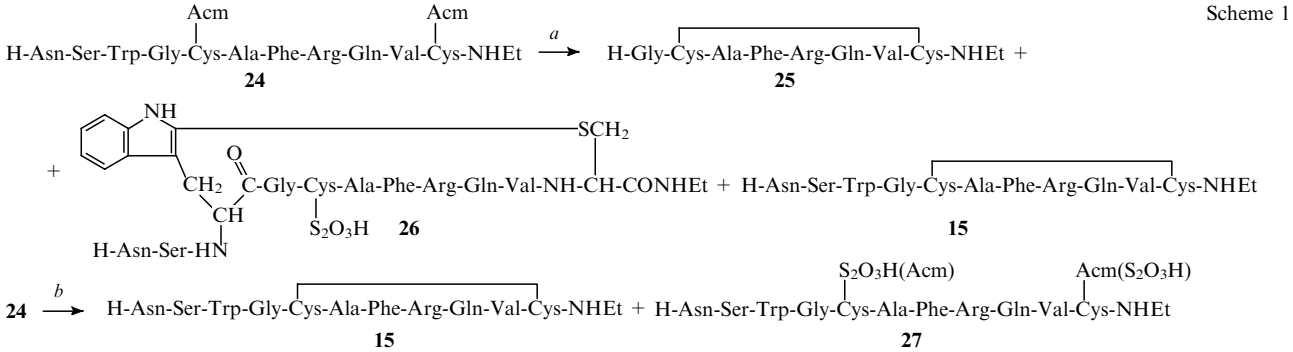
Neither of these mechanisms has been favoured, although both routes have been confirmed by several authors.[5] Since the intermediate sulfenyl iodides are highly reactive, the efficiency of oxidation strongly depends on the amino acid sequence of a peptide.

Several procedures for oxidation of protected peptides with iodine exist, which differ in the ratios of the reaction components, solvents, reagents used to remove the excess of iodine, *etc*. The oxidation is carried out in both neutral solvents (MeOH or aqueous MeOH, trifluoroethanol, dioxane, $CHCl_3$, DMF) and in acids (acetic, propionic, trifluoroacetic) or their mixtures with water. Depending on the type of the thiol function protection and the nature of the solvent, the reaction rates may vary considerably. Thus the rates of oxidation of Cys(Trt) in MeOH, acetic acid, and their mixtures with H_2O are from 4 to 500 times as high as that of oxidation of Cys(Acm) in the same solvents. If oxidation is carried out in chloroform, dichloromethane, and trifluoroethanol, this difference is much higher. This allows selective oxidation of Cys(Trt) in the presence of Cys(Acm). On the contrary, the rate of oxidation of Cys(Acm) in DMF and its mixtures with water is 5–10-fold higher than that of Cys(Trt).[2]

The excess of iodine plays an important role in this process. With equimolar amounts of the oxidant with respect to the [Cys(Acm)]$_2$ derivative of gramicidin S, the reaction in methanol was completed within 3 h and the yield of gramicidin S was 67%.[90] The use of 5–15-fold excess of I_2 in AcOH made it possible to obtain cyclic disulfides of various lengths and structures and in sufficiently good yields.[66,91,92] The use of 40- and 50-fold excess of I_2 in AcOH was reported to result in low yields (10%) of the target products.[93,94]

Using the synthesis of the cyclic disulfude 15 from the bis-*S*-Acm-protected fragment 593–603 of HIV-2 gp41 (**24**) as an example (Scheme 1), the effect of the excess of iodine and of the solvent on cyclisation has been studied.[55,78,95] With 2–13-molar excess of I_2 in AcOH, the yields of the target product **15** did not exceed 39%.[55,78,95] The highest yield (66%) was obtained when

Scheme 1



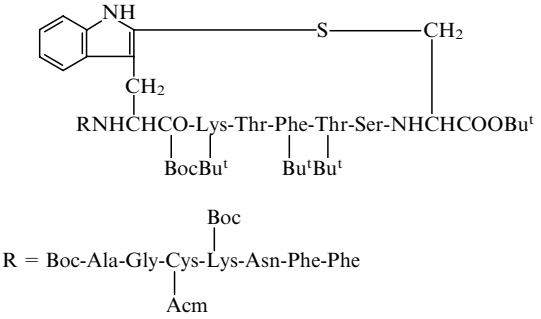
(*a*) 13 moles of I_2 per 1 mole of the peptide in AcOH; (*b*) 5 moles of I_2 per 1 mole of the peptide in AcOH.

oxidation was performed in aqueous methanol using 2 moles of I_2 per 1 mole of the peptide.[78, 95, 96]

The excess of iodine is removed with sodium thiosulfate, ascorbic acid, zinc dust, and by extraction with carbon tetrachloride.[2, 55, 78, 91–95] Removal of the excess of iodine with sodium thiosulfate was accompanied by modification of the cysteine residue with the thiosulfate ion [55, 78, 95, 96] (compounds **26** and **27**, the exact positions of the Acm and S_2O_3H groups in compound **27** were not determined) (Scheme 1).

The limitation of the use of iodine for the disulfide formation is the possibility of side reactions affecting oxidation-sensitive amino acid residues, *e.g.*, iodination of the benzene ring of tyrosine,[8] oxidation of methionine to the corresponding sulfoxide,[8] and scission of the indole ring of tryptophan with subsequent cleavage of the peptide chain.[8, 55, 78, 95–97] Thus the formation of a shortened peptide **25** (Scheme 1) was a result of oxidative degradation of tryptophan by iodine.[78, 95] Since these reactions are catalysed by acids, the cleavage of the peptide chain at the tryptophan residue can sometimes be avoided if oxidation is conducted in neutral organic solvents.[55, 95, 96, 98]

Oxidation of tryptophan-containing peptides with iodine sometimes results in tryptophan-2-yl sulfides as a side product of the type **26**. Apparently, one of the reasons for this side reaction is the formation of highly reactive intermediate sulfenyl iodides. Tryptophan-2-yl sulfides were obtained under various conditions.[42, 95, 98] For example, in the synthesis of somatostatin from a precursor with different protective groups at cysteine residues (Acm in position 3 and Trt in position 14), treatment with iodine in 10% aqueous trifluoroethanol resulted in the formation of a side product in 65% yield.[98]



According to Sieber *et al.*,[98] this reaction occurs due to the different lability of Acm and Trt groups in trifluoroethanol.[2, 98] When oxidation was carried out in a MeOH/DMF mixture, tryptophan-2-yl sulfide was not formed. The distance between the cysteine and tryptophan residues plays a significant role in the formation of tryptophan-2-yl sulfides. Oxidation of model tryptophan-containing peptides with iodine shows that only disulfide dimers are formed if tryptophan and cysteine are separated by one or two glycine residues. If this distance is four glycine residues, the yields of tryptophan-2-yl sulfides are 40% and 70%, respectively.[98]

Alternatively, tryptophan-2-yl sulfides ware formed when sodium thiosulfate was used to remove iodine in the synthesis of a fragment of the HIV-2 protein gp41 (if $Na_2S_2O_3$ is replaced by ascorbic acid, this side reaction does not take place). Blocking of the thiol function of one of cysteine residues with the thiosulfate ion (compound **26**) triggers the reaction of the thiol function of the other cysteine residue on position 2 of the indole ring of tryptophan.[55, 78, 95]

No side reactions involving the indole ring of tryptophan took place during cyclisation with iodine when its N^{im}-formyl derivative was used.[58]

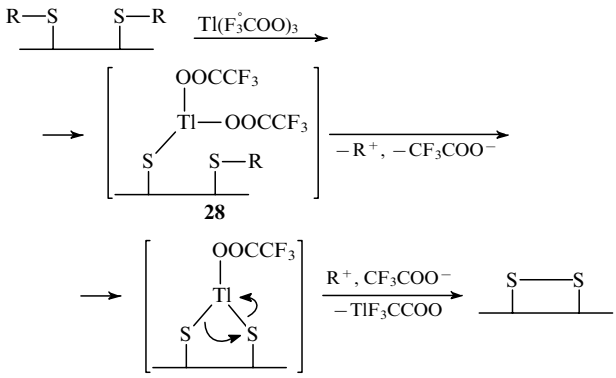
Despite the obvious limitations of this method, there are many examples of its successful application in the synthesis of compounds with several disulfide bonds in particular.[54, 58, 66]

b. Cyanogen iodide and *N*-iodosuccinimide

These compounds also serve as a source of the I^{2+} ion. The mechanism of oxidation with these reagents is identical with that of iodine. Good solubility of ICN in aqueous-organic solvent systems has been reported.[99] The utility of ICN was demonstrated in the cyclisation of several Cys(Acm) peptide derivatives in aqueous methanol. A comparison of oxidation with cyanogen iodide and iodine showed that although large excess of the oxidant (from 25- to 80-fold) is required in the former case and the duration of these reactions was 24–48 h, the yields were about twice as high as with I_2. *N*-Iodosuccinimide was used in the synthesis of oxytocin and vasopressin on a polymer,[100] therefore this reagent will be considered below in more detail.

c. Thallium(III) trifluoroacetate

A new, rather efficient oxidant, $Tl(CF_3COO)_3$, was first used in 1987 for removal of certain cysteine protective groups with simultaneous formation of the disulfide bond.[9, 101] Thallium trifluoroacetate removes many protective groups of the sulfide type (Mob, Tmob, But, Trt, Xan, Ad, Meb) and acylaminomethyl derivatives (Acm, Phacm). The oxidation occurs through the formation of a highly reactive intermediate compound **28**, *viz.*, thallium(III) thiolate. At the final step of this reaction, the protective group is split off from the thiol group of the second cysteine residue with concomitant disulfide bond closure.



The oxidation is carried out in trifluoroacetic acid (1–2 h, 0 °C) in the presence of 2 equiv. of anisole to bind the carbocations formed [9, 101] or in DMF.[2, 7] The use of aqueous solvents is excluded in this case, since the presence of water can bring about the formation of small quantities of cysteic acid.[9] Thallium salts are readily soluble in ether and their removal does not present any problem.[9]

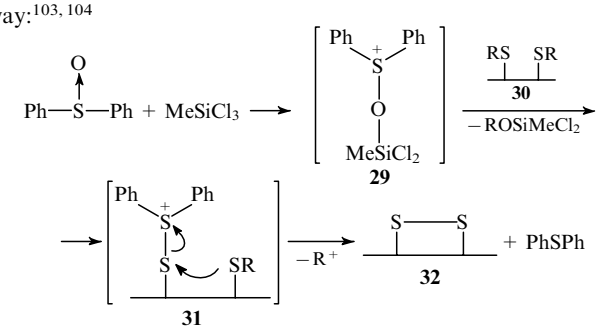
The use of $Tl(CF_3COO)_3$ gave satisfactory results in the synthesis of oxytocin, urotensin II, and human calcitonin. The yields of the products purified by gel filtration were 45%, 35%, and 11%, respectively.[101] In the latter two cases, the decrease in the product yields can be explained by the presence of a tryptophan residue in the amino acid sequences of these peptides, which undergoes considerable modification. It was shown that only 38% of tryptophan remains intact under these conditions. The use of a Trp(Mts) derivative allows one to preserve tryptophan for 60 min on treatment with $Tl(CF_3COO)_3$ at 0 °C. Methionine is partly oxidised (by ~ 38%) to the corresponding sulfoxide with Tl(III) salts.[9]

The use of this reagent is efficient in the case of difficultty oxidisable thiols, where cysteine residues are adjacent as in the peptide fragment of the acetyl choline nicotine receptor (alpha 185–196).[53]

A comparison of efficiencies of atmospheric oxygen, potassium ferricyanide, and thallium(III) trifluoroacetate as oxidants in some syntheses revealed that $Tl(CF_3COO)_3$ gave the highest yields.[17, 53] It has been widely employed for the closure of disulfide bonds on a polymer.[7, 17, 102] The high toxicity of thallium salts imposes certain restrictions on their application, which will hardly go beyond the scope of laboratory experiments.

d. Sulfoxides in the presence of silyl chlorides

This cyclisation method was proposed in 1991 independently by Kiso [103, 104] and Fujii.[105] In the presence of silyl chlorides in trifluoroacetic acid, aryl(alkyl) sulfoxides may split off Acm, Tacm, Bam, Bu^t, Meb, and other cysteine protective groups with simultaneous closure of the S–S bridges in 10–30 min at 4 °C. The mechanism of this reaction can be presented in the following way:[103, 104]



The closure of the disulfide bond occurs through the generation of the sulfonium cation **31** upon treatment of the protected peptide **30** with diphenyl sulfoxide and silyl chloride. Further, electrophilic attack of the second substituted sulfur atom in compound **31** results in the formation of an S–S bridge and the splitting of the protective group, which is similar to the reaction of sulfenyl iodide with *S*-protected cysteine in the oxidation with iodine. The nature of silyl chloride seems to play a key role in the reactivity of the intermediate compound **29**. The ability of the protective group split off to form stable structures similar to the immonium ion derived from the Acm group is also important. These two factors determine the rate of the reaction.[104]

The efficiency of various silyl chlorides ($SiCl_4$, $MeSiCl_3$, Me_3SiCl), and Me_4Si in combination with sulfoxides [PhS(O)Ph, MeS(O)Me, and MeS(O)Ph] has been studied.[104] $SiCl_4$ and $MeSiCl_3$ were found to be the most efficient silylating reagents. There was no difference in reactivities of different sulfoxides.[104] The Ph_2SO–$MeSiCl_3$ system or $SiCl_4$ removes Acm, Mob, Meb, Bzl, and Bu^t protective groups with equal efficiency.[105]

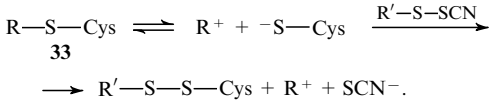
Tryptophan is subject to side reactions under these conditions. Chlorination of the indole ring is the predominant reaction in the treatment of tryptophan-containing peptides with silyl chloride.[104, 106] The addition of scavengers (anisole and 3-methylindole) routinely used to prevent tryptophan modification was inefficient.[104] It is therefore recommended to use tryptophan in the form of a formyl derivative.[103, 104, 106] Nevertheless, successful application of this reagent has been demonstrated in the synthesis of somatostatin (yield 60%),[104, 106] oxytocin (yield 56%–69%),[104] and human natriuretic peptide (hBNP) (yield 50%).[104] This method was also used for regioselective formation of disulfide bonds in the synthesis of conotoxin M1 and human insulin.[107]

3. Closure of disulfide bonds by thiolysis or thiol–disulfide exchange

Implementation of the route C requires that the two protective groups of two cysteine residues be different in stability. This enables selective removal of one of the protective groups (Y), whereas the other group (X*) activates the sulfur atom of cysteine, thus facilitating the formation of a disulfide bond. In this case, the intermediate electrophilic derivative (–S–X* in compound **7**) undergoes a nucleophilic attack by the sulfhydryl group of the second cysteine residue. This approach is used for the closure of both intra- and intermolecular S–S bonds and is particularly widely employed for the preparation of asymmetrical disulfides. Methods A and B are less efficient to this end. The reason is that simultaneous oxidation of two cysteine residues, when the formation of the S–S bonds between two different peptide chains is needed, gives predominantly dimers, whereas asymmetrical disulfides are the minor products. The peptide thiols are activated by introducing electron acceptor substituents, which enhance the ability of sulfur for the nucleophilic substitution. Activation may be accomplished either *in situ*, or as an individual stage with the isolation and characterisation of the intermediate product, or by selection of appropriate protective groups. In the latter case, the protective groups (*e.g.*, of the disulfide type) must be sufficiently stable under conditions of elongation of the peptide chain and simultaneously ensure the formation of a disulfide bond by thiol–sulfide exchange.

a. Thiocyanogen $(SCN)_2$

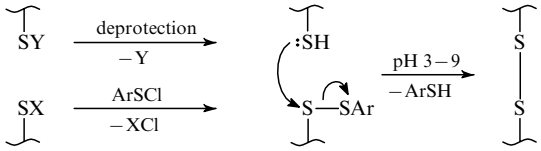
Thiocyanogen [2, 6] reacts both with free and protected thiols. The resulting activated peptide (X* = SCN in compound **7**) reacts with the thiol group of the same or another peptide *in situ* to form a disulfide bond. It has been established that this reagent selectively reacts with Thp and Trt derivatives of peptides in the presence of Dpm and Bzl protections.[5, 6] The reaction of thiocyanogen with sulfides can be presented as a reaction of an electrophile with a dissociated form of a sulfide.[5]



The rate of this reaction is determined by the degree of dissociation of the corresponding protected derivative **33**, which, in turn, depends on the stability of the cation R^+ formed upon splitting. Therefore, the cyclisation of Trt and Mob derivatives of peptides occurs rapidly and specifically even in nonpolar solvents, whereas Dpm sulfides react slowly and are converted into the corresponding disulfides in 60%–75% yields.[5] The degree of dissociation of derivatives **33** may be enhanced by acid catalysis. Cleavage of Dpm and But sulfides requires stronger acids than that of Trt and Mob derivatives. If the reaction with thiocyanogen occurs at a slow rate (as is the case with Bzl derivatives), the formation of homodimeric side products is more probable.[2, 5] High electrophilicity of thiocyanogen favours the occurrence of side reactions.[2] Thiocyanogen is decomposed by amines (including free bases of arginine and histidine), the corresponding reactions may be carried out only in acidic or neutral media.

b. Aromatic sulfenyl halides

2-Nitrobenzenesulfenyl chloride (Nps-Cl) or 2-pyridinesulfenyl chloride (PyS-Cl) activate free or protected cysteine residues. The closure of the disulfide bonds by these reagents may be illustrated by the following general scheme:[2]



In the case of intermolecular closure of the S–S bonds, Y is a protective group of any type, and X is hydrogen or a protective group that can be removed by aromatic sulfenyl halides, *e.g.*, Trt, Dpm, Bu^t, SBu^t, and Acm.

Nps-Cl and PyS-Cl are extremely reactive, they decompose in the presence of moisture. PyS has been proposed for deprotection of cysteine protected with Trt, Dpm, Acm, Bu^t, and SBu^t groups and subsequent preparation of asymmetrical disulfides.[108] Reactions with this reagent are carried out in glacial acetic acid. The reactive mixed disulfide formed reacts with a free SH group to give the target disulfide. The mixed disulfide Cys(PyS) is resistant to acids. Removal of the Boc group in the derivative Boc-Cys(PyS)-OH by treatment with acid is not accompanied by splitting of the PyS group. The activation of thiols and formation of disulfide bonds occur without removal of protective groups, such as Boc, Z, and Bzl. The PyS derivatives of peptides are formed in quantitative yields and can be isolated in a crystalline state. The mixed

disulfides Cys(PyS) and the respective peptides are unstable in alkaline solutions and rapidly decompose to yield symmetrical disulfides and di(2-pyridyl) disulfide, $(PyS)_2$. Free SH groups may be liberated by the action of an excess of thiols (*e.g.*, β-mercaptoethanol).[108]

Nps-Cl was used to remove Trt, Dpm, Ddm, and Acm protections [109] and intermediate mixed disulfides can be isolated. Free SH-derivatives can be obtained under the action of an excess of a thiol (*e.g.*, β-mercaptoethanol or dithiothreitol). Deprotection and formation of disulfide bonds using Nps-Cl occur much more slowly than with PyS-Cl.[108]

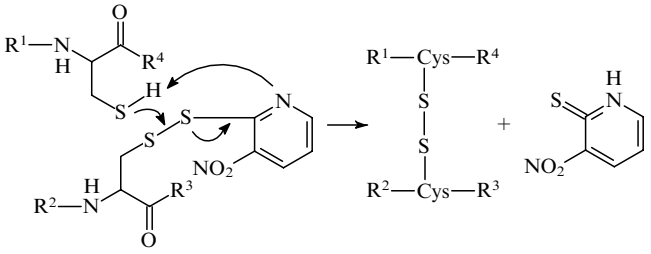
The use of aromatic sulfenyl halides is limited because of difficulties associated with their synthesis and isolation (these compounds are easily hydrolysed even in humid air) and due to the fact that tryptophan can undergo side reactions under the action of these compounds.

Peptides containing cysteine with free SH groups can be activated directly in solution or on a solid phase with di(2-pyridyl) disulfide, $(PyS)_2$. Highly specific conversion of a Cys(Snm) derivative into a Cys(PyS) derivative under the action of 2-mercaptopyridine in chloroform is an alternative procedure for obtaining stable *S*-arylsulfenyl derivatives.[2] And, finally, cysteine-containing peptides may react with Ellman's reagent in buffer solutions at pH 7.4 to give intermediate disulfides. The interaction of the latter with a free SH group of the other cysteine residue also results in the closure of S–S bridges.[2]

c. Disulfide derivatives

The characteristic feature of this method is that disulfides play a dual role. On the one hand, they are used for protection of the thiol functions of cysteine because they are stable under conditions of a multistep peptide synthesis. On the other hand, being strong electron acceptors, they activate the thiol groups in reactions of thiol–disulfide exchange and thiolysis and thus facilitate the closure of the S–S bonds. These groups include alkoxycarbonylthio (Scm, Sce), Snm, and Npys protections.

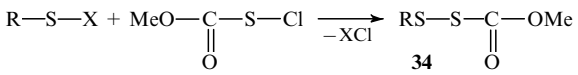
Asymmetrical disulfides have been synthesised from peptides containing Cys(Npys) derivatives.[17, 110]



R^1, R^2, R^3, R^4 are amino acids.

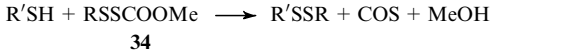
Derivatives containing Npys protective groups are also used to form intramolecular disulfide bonds.[2] This reaction is carried out over a rather broad pH range. The reaction time varies from several hours at pH ~ 4 to 30 min at pH 8–9. However, at pH > 9, disproportionation of disulfides may occur, which shifts the reaction equilibrium towards the formation of symmetrical homodimers. The probability of disulfide exchange between asymmetrical cystines formed and the free SH groups present decreases at low pH. The optimum pH values are 4.45–9.35. The stability of the Npys protective group in acidic media (TFA at 25 °C and HF at 0 °C in the absence of thiols) makes it possible to use these cysteine derivatives in solid-phase syntheses using Boc-technique. This protection was successfully used for the formation of S–S bridges in the synthesis of insulin, its analogues, and relaxin.[17]

Methoxycarbonylsulfenyl chloride (Scm-Cl), like aromatic sulfenyl halides, removes such protective groups as Trt, Acm, Bu^t, Ddm, and Dpm to form Cys(Scm) derivatives:[111]



X is Trt, Acm, Bu^t, Ddm, Dpm.

Compound **34** reacts with free thiols to produce the disulfide bonds.



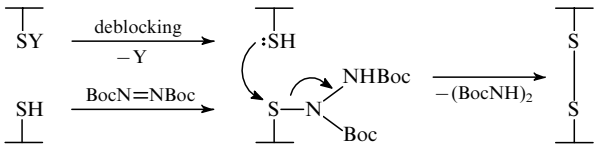
The latter reaction is catalysed by mild bases, such as triethylamine.[111] Sulfenylthiocarbonates are unstable in the presence of alkalis; therefore, experiments with these compounds must be carried out in neutral or acidic media. However, undesirable modification of tryptophan is possible. Conversion of Acm derivatives into Scm derivatives or the reaction of free SH groups with Cys(Scm) may also be carried out on a solid phase[2] with formation of both inter- and intramolecular disulfide bonds.

Ethoxycarbonylsulfenyl chloride (Sce-Cl) was proposed for the formation of intramolecular disulfide bonds. This reagent is identical in structure and mechanism of action with Scm-Cl.[111] The activated cysteine derivatives thus formed react with free thiol groups of cysteine to yield intramolecular disulfide bridges. The optimum results were obtained with ammonium-acetate buffer, pH 7.0. However, this reaction may also be carried out in weakly acidic solutions, but in this case the yields are lower. The rate of this reaction is very high: cyclisation is completed within 10 min, but the reaction conditions could not be found where the yields exceeded 20%–30%.[111] The application of Sce-Cl to tryptophan-containing peptides is problematic.[111] Nevertheless, this reagent was used in the synthesis of somatostatin, urotensin IIA,[111] and a trypsin inhibitor.[56]

d. Bis(*tert*-butyl) azodicarboxylate

The closure of the disulfide bond using bis(*tert*-butyl) azodicarboxylate (Boc–N=N–Boc) occurs *via* thiolysis. Boc–N=N–Boc has not gained wide acceptance despite its successful application in the synthesis of asymmetrical disulfides.[112, 113]

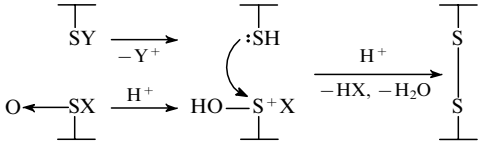
The synthesis of an activated peptide derivative was carried out in DMF with 2–3 equiv. of bis(*tert*-butyl) azodicarboxylate under argon (4–12 h, 25 °C).[112, 113] The resulting compound is rather stable and can be isolated. The activated derivative is dissolved in an appropriate solvent, after which the second peptide containing a free sulfhydryl group is added to form a disulfide heterodimer.



BocN=NBoc is bis(*tert*-butyl) azodicarboxylate.

e. The use of cysteine sulfoxide

This method is used for the closure of both intra- and intermolecular S–S bonds. One of the cysteine residues is used in the form of a sulfoxide.[114] The reaction occurs in a strongly acidic medium (TFA or a trifluoromethanesulfonic acid–TFA mixture). Under these conditions, the sulfoxide is protonated, and the resulting electrophilic derivative readily reacts with the nucleophilic thiol.

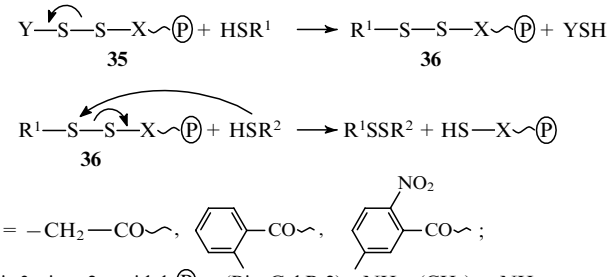


Y is Mbzl, Ad, Bu^t; X is Acm or Mob.

The use of dimethyl sulfide accelerates the reaction and favours regeneration of the SH group. The efficiency of this method was demonstrated by the synthesis of oxytocin from Cys(Acm)(O) and Cys(Mbzl) derivatives (yield 86%).[114]

f. The use of disulfide-containing polymers

An original procedure for the synthesis of asymmetrical disulfides on polymeric supports containing disulfide fragments in the anchor group has been proposed by Ponsati *et al.*[115] This method is based on thiol–disulfide exchange. At the first step, the peptide thiol HSR^1 reacts with the anchor group of the polymer **35** to form a disulfide peptidyl polymer **36**. At the second step, compound **36** reacts with the second peptide HSR^2 as a result of which the target disulfide R^1SSR^2 is cleaved from the polymer.



Y is 3-nitro-2-pyridyl; Ⓟ = (Bio-Gel P-2)–NH–$(CH_2)_2$–NH–;

R^1, R^2 are the polypeptide residues.

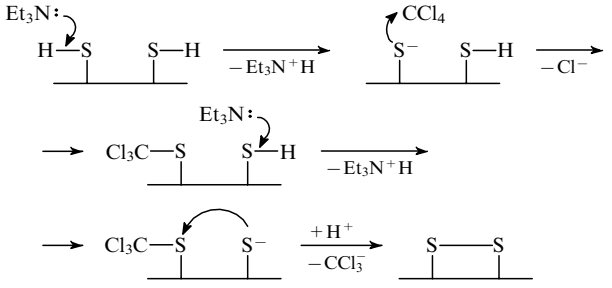
An optimum anchor group for the polymer and conditions for this reaction have been selected. With X = $-CH_2-CO\sim$, the reaction was slow (48 h). If benzoic or nitrobenzoic acid residues were used as X, the reaction proceeded much faster, and the yields of the target heterodimers reached 60%–70%. This method has a number of advantages. The polymer used in this reaction can be easily regenerated; the addition of the peptide to the polymer facilitates the procedure of purification of the reaction mixture from the excess of the reagents, by-products, and other impurities, which are removed by mere filtration.[115]

4. Closure of disulfide bonds on a polymeric support

In a search for the most convenient cyclisation methods, it was suggested to form S–S bonds directly on a polymeric support. This method has a number of advantages. As oxidation requires high dilutions, a solid-phase system may be ideal in this respect. When a peptidyl polymer is swollen in an appropriate solvent, the distance between neighbouring peptide chains increases, which minimises interaction between them. This phenomenon, which is referred to as pseudo-dilution, simulates dilution in solution. The distance between molecules may be increased by decreasing the degree of the polymer substitution by the starting amino acid. Rigid fixation of molecules restricts their conformational mobility and decreases intermolecular contacts, which ensures predominant occurrence of intramolecular reactions.[7] Oxidation on a polymeric support permits one to circumvent the problem of peptide solubility. In many cases, solvent systems the composition of which depends on cyclisation conditions do not provide complete dissolution of peptides to be oxidised, which inevitably results in the loss of the product and decrease in yields. Moreover, solid-phase synthesis significantly facilitates the workup of the reaction mixture. Thus, removal of an excess of the oxidant and side products is achieved merely by filtration. Since the oxidation reaction is often at equilibrium, this may be shifted towards the formation of the target product as a result of repeated treatment of the peptidyl polymer with a fresh portion of the oxidising mixture. The oxidation process can be automated and included into the reaction protocol after elongation of the peptide chain has been completed. However, despite the apparent simplicity of this procedure, the existence of a three-phase (peptide–polymer–solvent) system presents certain problems for cyclisation. At present, there is still no deep understanding of the nature of the interaction between the peptide chains linked with the polymer and between the polymer and the peptide. Nevertheless, examples of successful cyclisation on a solid phase have been reported. The choice of a solvent is of crucial importance in this process. A solvent must afford good swelling of a peptidyl polymer in order to provide the maximum access of the oxidant to the peptide. The most popular solvents are DMF, CH_2Cl_2, CCl_4, AcOH, trifluoroethanol, TFA, and *N*-methylpyrrolidone.[2,7,15,89,100,116–119] The use of aqueous solutions should apparently be excluded with traditional polystyrene supports. As in solution, the closure of the peptide S–S bonds on a polymer may be conducted with both preliminary and simultaneous cleavage of protective groups for the sulfhydryl functions of cysteine. Removal of Hg(II) or Ag(I) ions employed for deblocking of cysteine with heavy metal salts requires the use of reagents that convert them into soluble derivatives. β-Mercaptoethanol, which yields Hg(II) salts soluble in a large amount of DMF, is especially recommended in this case.[2]

Systematic studies of disulfide bonds formation on polymeric supports in the synthesis of model peptides and oxytocin have been carried out.[2,89,116] Cyclisation was conducted on polymeric supports with different anchor groups and using different reagents. Cysteine was used in the form of Cys(Fm), Cys(Trt), Cys(Acm),[89] and Cys(Tmob) derivatives.[14,15] It was shown that the disulfide bond was rather stable under conditions of acid-promoted (TFA and HF) splitting of the cyclic peptide from the supports with *p*-alkoxybenzyl and benzhydrylamino anchor groups.[89,116] However, splitting of peptides from supports with photolabile anchor groups caused modification of the S–S bond; therefore special conditions are required in order to achieve satisfactory results. Closure of disulfide bonds was performed using atmospheric oxygen (DMF, pH 7–8, 1 h), Ellman's reagent (0.1 M *N*-methylmorfolinium acetate in DMF, pH 7.5, 1 h), and diethyl azocarboxylate (0.5 equiv. DMF, pH 7.0, 2 h).[89] It was found that the oxidation of oxytocin on a polymeric support occurred in higher yields than in solution.[89]

Oxidation on a polymer employed a mixture of CCl_4 and NEt_3 (1 : 1) in DMF or *N*-methylpyrrolidone.[2,7,15] The hypothetical mechanism of this reaction is given below.[7]



The target oxytocin was obtained in 70% yield; no side products were detected.

Oxidation of a 26-membered peptide containing free SH groups on a polymer was carried out in 1 M solution of potassium ferricyanide in DMF. The reaction lasted 12 h and gave a cyclic disulfide in good yield.[2]

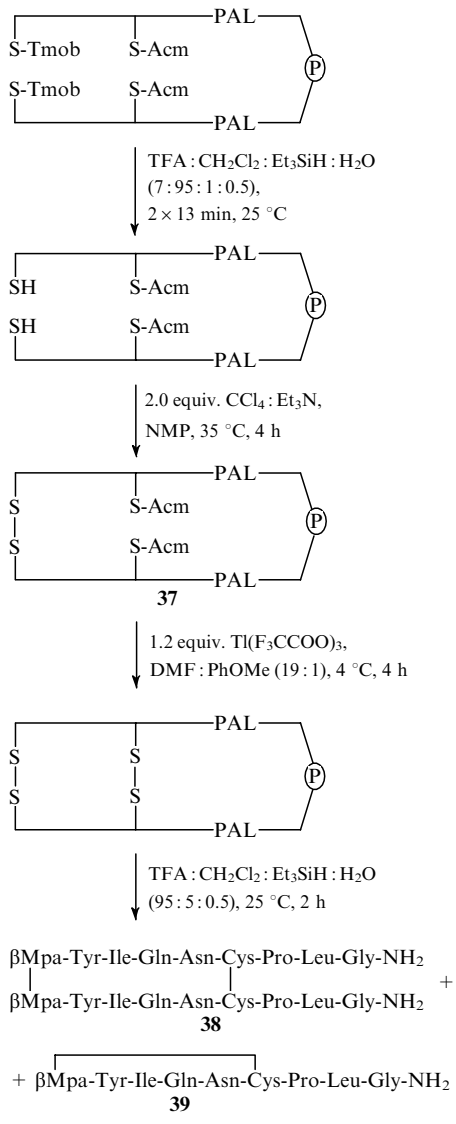
Methods of direct conversion of cysteine-protected peptides into disulfides [(I_2, $Tl(CF_3COO)_3$)] have proved to be very efficient in oxidation on a solid phase. Standard conditions of this oxidation include treatment of cysteine-protected (Acm, Trt, Tmob) peptidyl polymers with 3–10 equiv. of I_2 in an appropriate solvent (TFA, AcOH, DMF, *etc.*) (1–2 h, 25 °C) or with 1.2 equiv. of $Tl(CF_3COO)_3$ (1–2 h, 0 °C). The yields of cyclic products are 60%–90%.[89,117] Treatment of peptidyl polymers with acid-labile anchor groups [*p*-alkoxybenzyl or tris(alkoxy)-benzylamide] with solutions of I_2 or $Tl(F_3CCOO)_3$ in TFA or AcOH results in simultaneous closure of S–S bridges and splitting of the peptide from the polymeric support. This approach was used by Barlos *et al.*[118] for cleavage of the acid-labile

2-chlorotrityl anchor group with an AcOH : trifluoro-ethanol : CH_2Cl_2 mixture (1 : 2 : 7). When this mixture was used in combination with 10 equiv. of I_2 (10–15 min, 20 °C), cyclic disulfides were obtained in ~ 60% yields. Protective groups of the *tert*-butyl type were not removed under these conditions.[118]

The advantage of direct conversion of Cys(Acm) and Cys(Trt) peptide derivatives into the corresponding disulfides by treatment with Tl(III) salts over iodine has been demonstrated by several authors.[89, 102, 116] Treatment of S-Acm or S-Trt derivatives of the model peptide Ac-Cys(Acm)-Pro-D-Val-Cys(Acm)-NH_2 with $Tl(CF_3COO)_3$ in TFA : anisole (21 : 1) with subsequent cleavage from the polymer with HF gave a cyclic product in 91% yield, whereas after treatment with 10 equiv. of I_2 in aqueous AcOH the yield was 52%.[89, 116] In the latter case the decrease in the yield is unrelated to the nature of the oxidant but rather is due to the application of the AcOH–water mixture (1 : 4, v/v), which prevents swelling of the polystyrene-peptidyl polymer.

Cyclisation on a solid phase was highly efficient in the synthesis of parallel dimers of oxytocin and deaminooxytocin.[7, 119] The attempts to obtain these compounds in solution failed, because in dilute solutions it was monomeric products that were predominantly formed, whereas at high concentrations polymers were formed.[119] Rigid fixation of peptides on a polymer has made it possible to obtain target dimers in good yields. The synthesis of a parallel dimer of deaminooxytocin is shown in Scheme 2.

Scheme 2



(P) is the styrene copolymer with 1% divinylbenzene, PAL is the tris-(alkoxy)benzamide anchor group, NMP is *N*-methylpyrrolidone, βMpa is β-mercaptopropionic acid.

The CCl_4–NEt_3 mixture was used for the closure of the S–S bond between Tmob-blocked N-terminal residues of β-mercaptopropionic acid (βMpa) after preliminary treatment of the peptidyl polymer with dilute TFA and scavengers. This procedure gave an intermediate product **37** in more than 90% yield. A mixture of the target parallel dimeric product **38** and deaminooxytocin **39** was obtained in 3 : 1 ratio after closure of the second disulfide bond with $Tl(CF_3COO)_3$ and cleavage of the reaction product from the polymer. Whether deaminooxytocin **39** results from the oxidation of an intermolecular side reaction product with $Tl(F_3CCOO)_3$ or from the dimer–disulfide exchange still remains unclear.[119] A scheme with an alternative combination of protective groups [Cys(Tmob) and βMpa(Acm)] has also been tested. At the first step of this synthesis, the disulfide bond was closed between cysteine residues. It is of note that in this case the yield of the intermediate product having only one disulfide bridge was as low as 20%.

The closure of S–S bonds with *N*-iodosuccinimide in a DMF–CH_2Cl_2 mixture (1 : 1) in the synthesis of the (1–29) fragment of the human growth hormone having one disulfide bond (yield 15.6%) and an 18-membered peptide apamine having two S–S bonds (yield 11%) was reported.[100] In this case, the closure of S–S bridges did not follow the conventional procedure, *viz*., oxidation after the completion of the linear peptide synthesis, but was rather included into the protocol of a solid-phase synthesis on an automatic peptide synthesiser using the Boc-technique. After attachment of the appropriate pair of cysteine residues, the disulfide bond between them was closed and the elongation of the peptide chain was continued. Acm and Mbzl protections were used for the blocking of the thiol functions of cysteine. The use of an analogous procedure for the synthesis of oxytocin resulted in extremely low yields of the cyclic product (~ 1%), although [8 Arg]vasopressin was obtained in 41% yield under identical conditions. The question about the reason for such a low yield of oxytocin is still obscure. In our opinion, the authors' attempts [100] to interpret this phenomenon in terms of intermolecular aggregation of Cys(Acm) derivatives of oxytocin contradicts the results obtained by Albericio *et al*.[89, 116] according to which it is cyclisation leading to oxytocin on a polymer under various conditions that occurs in sufficiently high yields, which often exceed those obtained by cyclisation in solution.

IV. Conclusion

The methods of formation of disulfide bonds considered above usually give compounds having one or two S–S bonds. However, each of the above-described methods has limitations of its own. Most often, they are related to oxidation-sensitive amino acids: tyrosine, tryptophan, and methionine. Despite the explosive development of methods and techniques of modern peptide chemistry, no versatile cyclisation procedure exists, and each time when an S–S bond is to be formed in a new compound, special experiment is required for the selection of the most adequate protection for the thiol function of cysteine and cyclisation reagent. In this context, further development of protective groups for the thiol function, elaboration of selective methods for deblocking of SH groups and specific activation of the thiol groups, and a search for new mild reagents suitable for the formation of S–S bridges are currently central tasks. Their solution assumes special importance in the synthesis of compounds with several intra- and intermolecular S–S bonds. At present, only few syntheses of such compounds have been carried out. Synthesis of complex molecules is a challenge of the nearest future. In this respect, methods of closing S–S bonds on a solid phase hold especially great promise. Further development and improvement in these techniques might facilitate laborious and sometimes unpredictable processes of cyclisation and make them

automatic by including this stage into the program of a peptide synthesiser.

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