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NEW STRATEGY FOR THE CHEMICAL SYNTHESIS OF PROTEINS

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Summary: For the chemical synthesis of proteins, an efficient deprotecting procedure by combination of a hard acid, trimethylsilyl trifluoromethanesulfonate, and a soft nucleophile, such as thioanisole, is described. For disulfide bond formation, two new procedures are presented; the one by oxidation with thallium(III) trifluoroacetate and the other by the acid-catalyzed reaction, involving S-substituted cysteine sulfoxides.

In 1983, we reviewed actidolytic deprotecting procedures in peptide synthesis[1] and presented several advantageous features of a new deprotecting procedure with TFMSA/TFA for chemical syntheses of larger peptides and possibly proteins. As an example, the usefulness of the thioanisole-mediated deprotection[2,3] with TFMSA/TFA was demonstrated by the chemical synthesis of a crystalline protein with full enzymatic activity of ribonuclease A.[4] However, in order to improve the yield of the final product, we fully realized it necessary to explore more efficient synthetic procedures, mainly in two respects; i.e., a more efficient deprotecting procedure than TFMSA/TFA, with less side reactions and a more efficient disulfide bond-forming reaction than the usual time-consuming air-oxidation reaction. At present, biosynthesis of proteins by recombinant DNA technique still suffers several restrictions. Present situations for the disulfide bond-forming reaction have to be improved, even if proteins are synthesized by either chemical methods or biological methods. We wish to present a new strategy for the chemical synthesis of proteins containing several disulfide bonds.

1. Hard Acid Deprotecting Procedure:

(A) Deprotection with TMSOTf. Chemical synthesis of proteins requires a large number of protecting groups for functional amino acids. Various protecting groups employed have to be removed quantitatively, with less side reactions, in the final step of the synthesis. For this purpose, the practical problem which we have to take into consideration is that more rigorous cleavage conditions are needed than those employed for removal of protecting groups from small peptides. The thioanisole-mediated deprotecting procedure with 1 M TFMSA-thioanisole/TFA described above is based on the hard-soft acid-base concept.[5] We have considered to replace TFMSA, a strong Bronsted acid, by a silyl compound, as a source of hard acids.

The use of sily compounds, such as Me_3SiI[6] or Me_3SiCl,[7] as peptide deblocking reagents, has been examined. However, somewhat elevated temperature or prolonged

Table 1. Removal of various protecting groups by 1 M TMSOTf/TFA in the presence of a soft base

Treated amino acid derivatives (0°C, 30 min)	Parent amino acid regenerated (soft base added)		
	thioanisole	MeSMe	PhSPh
Z(OMe)-Lys(Z)-OH	98.7	87.0	73.1
Z(OMe)-Ser(Bzl)-OH	91.7	90.2	97.8
Z(OMe)-Glu(OBzl)-OH	99.3	97.3	100.0
Z(OMe)-Asp(OBzl)-OH	99.2	97.7	100.0
Boc-Asp(OChp)-OH	100.0		100.0
Boc-Tyr(Bzl)-OH	100.0		87.5
Boc-Tyr(Cl_2-Bzl)-OH	100.0	88.3	86.6
Boc-His(Bom)-OH	88.9	50.5	90.2
Boc-Trp(Mts)-OH	100.0	25.6	100.0
Boc-Trp(For)-OH*	100.0		
Z(OMe)-Arg(Mts)-OH	97.6	67.2	100.0
Z(OMe)-Arg(MBS)-OH	93.5		84.3
Z-Arg(Tos)-OH	62.1	0	8.3
Z-Arg(NO_2)-OH	11.0		10.8
H-Cys(MBzl)-OH	100.0		100.0
Boc-Cys(tBu)-OH	87.3	32.9	97.1
Z(OMe)-Cys(Ad)-OH	100.0	11.0	100.0
Boc-Cys(Acm)-OH	0		
H-Cys(Bzl)-OH	0		
Boc-Ile-PAM-resin**	81.9	12.4	23.8

* In the presence of EDT.
** 60 min treatment

treatment was required for complete removal of the Bzl group and even the Z group. We found that TMSOTf in TFA has an ability to cleave, not only the Boc group,[8] but also readily various other protecting groups currently employed in peptide synthesis. The resulting trimethylsilylated compounds can be hydrolyzed easily with water or ammonium fluoride[9] to regenerated parent amino acids (Table 1). Of amino acid derivatives so far tested, only few derivaitves, Cys(Bzl), Cys(Acm) and Arg-(NO_2), resisted the action of this reagent. The rate of this cleaving reaction with 1 M TMSOTf-thioanisole/TFA was judged to be much faster than that of 1 M TFMSA-thioanisole/TFA. TMSOTf, a silyl ester, seems to play a role in this cleavage reaction as a super hard acid[10] and thioanisole seems to take part acting as a soft base (Fig.1)

Thioanisole is a good scavenger to suppress side reactions in TFMSA/TFA deprotection[2] and a good accelerator of the cleavage reaction.[3] This thioether was judged to be still a better soft base than PhSPh and MeSMe in the present deprotecting reaction. When PhSPh (at a concentration of 0.5 M, due to its lower solubility in TFA) was employed, the recoveries of Lys and Tyr were slightly low. MeSMe (at a concentration of 1 M) was not effective enough to cleave the Mts[11] and MBS[12] groups. The results suggested the existance of a subtle relationship between soft acids (protecting groups) and soft bases.

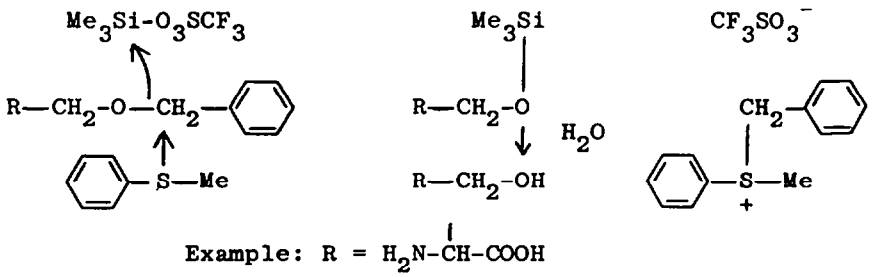

Fig. 1. Deprotecting reaction by TMSOTf-thioanisole/TFA

Table 2. Removal of protecting groups by trimethylsilyl compounds in the presence of thioanisole

Hard acid (22°C, 60 min)	^{29}Si ppm*	Ser(Bzl)	Lys(Z)	Arg(Mts)	Asp(OBzl)
1 M TMSOTf/TFA#	46.1**	91.7	98.7	97.6	99.2
2 M TMSOMs/TFA	37.9	93.5	92.4	80.0	99.5
3 M TMSOTa/TFA	35.6	7.1	16.9	0	1.6

* Me_4Si was used as an internal standard.

** Lit.(14): 44.6 ppm in benzene.

\# Reaction at 0°C for 30 min (Table 1).

It seems noteworthy that Ile, possessing a bulky side chain, could be cleaved from Boc-Ile-OCH_2-PAM-resin[13] by treatment with 1 M TMSOTf-thioanisole/TFA in fairly good yield. The result seems to demonstrate the usefulness of this deprotecting procedure for solid-phase peptide synthesis.

When the ^{29}Si-NMR signal of three trimethylsilyl compounds in TFA were measured, using Me_4Si as an internal standard,[14] the signal of TMSOTf apeared at the lowest magnetic field, compared with those of TMSOMs and TMSOTa. These values seem to correlate well with cleaving efficiencies of these trimethylsilyl compounds tested as sources of a hard acid. As seen in Table 2, the protecting groups of four compounds, Ser(Bzl), Asp(OBzl), Lys(Z) and Arg(Mts), were cleaved by TMSOMs by increasing the concentration and elevating the temperature (22°C), but complete removal of these protecting groups by treatment with 3 M TMSOTa-thioanisole/TFA was unsuccessful. Thus, of the hard acids tested, TMSOTf was judged to be the most attractive reagent for deprotection.

We have synthesized several biologically active peptides by applying this new deprotecting procedure based on the hard acid principle. As an example, we wish to present the synthesis of human GIP (glucose-dependent insulinotropic peptide).[15] Protected GIP,[16] prepared by the solution method, was treated with 1M TMSOTf-thioanisole/TFA in the presence of additional scavengers, m-cresol and EDT (for protection of Trp) in an ice-bath for 120 min (Fig. 2). After purification by gel-

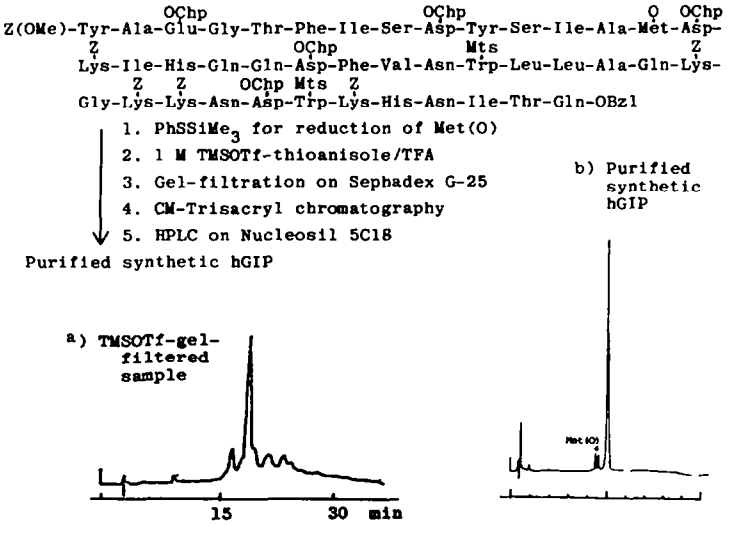

Fig. 2. HPLC of synthetic hGIP prepared by TMSOTf/TFA deprotection

filtration on Sephadex G-25, followed by HPLC on a Nucleosil 5C18 column, the desired 42-residue peptide was obtained in a better yield (28%) than that obtained by deprotection with 1 M TFMSA-thioanisole/TFA (10%).

From this result and others, including the synthesis of neuromedine U-25, a porcine spinal cord peptide,[17] it can be judged that this procedure may serve to improve the final deprotecting step for syntheses of larger and more complex peptides. In solid phase peptide synthesis, the anchoring linkage on a support is the Bzl ester, which is easily cleavable by TMSOTf/TFA treatment. We are accumulating useful informations along this line.

(B) Deprotection with TMSBr. Removal of Bzl-type protecting groups by HBr/AcOH was first demonstrated by Ben-Ishai and Berger[18] in 1952. However, this procedure did not gain wide acceptance in practical peptide synthesis, since preparation and handling of HBr/AcOH or TFA reagent are rather laborious. We wish to report that Bzl-type protecting groups can be cleaved, as by HBr/AcOH, when the trimethylsilyl group is supplied as a source of hard acid in TFA, in lieu of the proton of HBr, and thioanisole is used as a soft nucleophile. When amino acid derivatives were treated with 1 M TMSBr/TFA in the presence of various cation scavengers in an ice-bath for 60 min, thioanisole was again found to be most effective to accelerate the cleavage reaction, compared with other scavengers examined (Table 3). The Bzl and Z groups were cleaved in nearly quantitative yields by 1 M TMSBr-thioanisole/TFA. In addition, the Cl_2-Bzl group[19] and the Mts group were cleaved in nearly 90% yield under these conditions. However, removal of the Chp group from Asp[20] was unsuccessful. Of various S-protecting groups of cysteine, the MBzl group was cleaved completely, but other were not cleaved under the conditions stated above. Thus, the rate of cleavage by 1 M TMSBr-thioanisole/TFA was judged to be much slower than that by 1 M TMSOTf-thioanisole/TFA. However, it seems worthwhile to note that Met(O)[21] was reduced back to Met in 91% yield by this TMSBr/TFA system, while the TMSOTf/TFA system reduced Met(O) in only 27% yield under identical conditions.

Deprotection with HBr/TFA is reported to suppress well acid catalized succinimide-formation of the Asp residue[22] linked to particular amino acids, such as Gly, Ser, Ala and Asn. A model peptide, Z(OMe)-Ala-Asp(OBzl)-Gly-OBzl, was treated with 1 M TMSBr/TFA or 1 M TMSOTf/TFA in the presence of thioanisole in an ice-bath for 120 min and the succinimide formed was quantitated with a dual-wavelength TLC scanner. The amount of the side product formed by TMSBr/TFA treatment was judged to be negligible, while the TMSOTf/TFA system gave the side product in 4.6% yield. In this respect, the TMSBr/TFA system seems to be superior to the TMSOTf/TFA sys-

Table 3. Removal of various protecting groups by 1 M TMSBr/TFA in the presence of a soft base

Amino acid deriv. (0°C, 60 min)	Parent amino acid regenerated (%) anisole	thioanisole	MeSMe	PhSPh
Z(OMe)-Lys(Z)-OH		87.8		
Z(OMe)-Ser(Bzl)-OH	64.1	85.7	82.3	82.3
Z(OMe)-Glu(OBzl)-OH		93.9		
Boc-Asp(OBzl)-OH	27.6	100.5	45.2	46.0
Boc-Asp(OChp)-OH		4.2	3.9	5.0
Boc-Tyr(Cl_2-Bzl)-OH	32.9	96.9	74.7	76.2
Boc-Trp(Mts)-OH	3.3	86.1	13.6	11.4
Z(OMe)-Arg(Mts)-OH	23.4	87.0	19.0	40.8
H-Cys(MBzl)-OH		98.7		
Z(OMe)-Cys(Ad)-OH		0		
Boc-Cys(tBu)-OH		7.6		
Z(OMe)-Met(O)-OH		91.3		

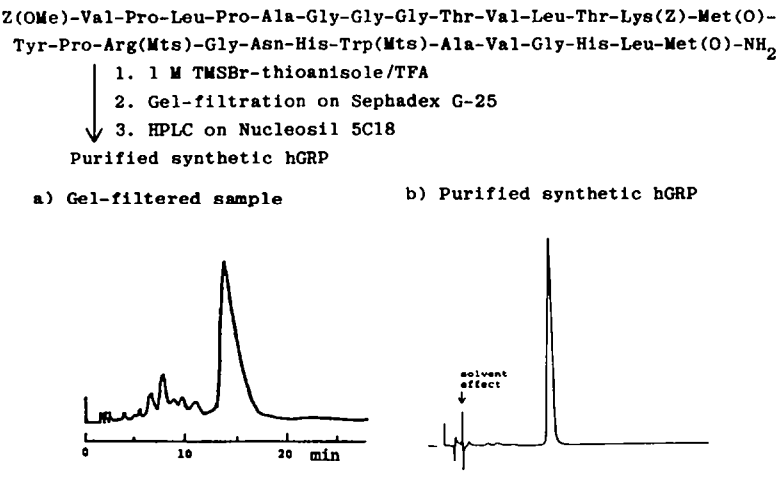

Fig. 3. HPLC of synthetic hGRP prepared by TMSBr/TFA deprotection

tem. The deprotecting procedure based on the hard acid principle was found not to be entirely free from the ring closure reaction of the Asp residue, depending on the nature of hard acid employed.

The above results suggested that this 1 M TMSBr-thioanisole/TFA deprotecting procedure can be applied to syntheses of relatively small peptides containing Asp or Met. As a model peptide, hGRP (human gastrin-releasing polypeptide)[23] containing two Met residues was selected. In the previous synthesis,[24] the two Met(O) residues employed were reduced by treatment with phenylthiotrimethylsilane, prior to TFMSA/TFA deprotection. However, in the present experiment, protected hGRP was directly treated with 1 M TMSBr-thioanisole/TFA in an ice-bath for 3 h to ensure the complete derotection of three protecting groups, Z and two Mts, and at the same time the complete reduction of the two Met(O) residues. When the crude product, obtained after gel-filtration, was examined by HPLC, the product possessing an identical retention time with that of an authentic sample of hGRP emerged from the column as a main component (Fig. 3). After preparative HPLC purification, the desired peptide was obtained in a much better yield (52%), than before (30%), without particular treatment for the reduction of Met(O), before or after deprotection.

Next, this reagent was applied to the solid phase synthesis of dynorphin,[25] a 17-residue peptide containing the Asp-Asn sequence which is known to be sensitive to cyclization by both base and acid. Protected dynorphin attached on the resin was synthesized by the Fmoc strategy[26] using an automated peptide synthesizer (LKB model 4170). The protected peptide-resin was treated with 1 M TMSBr-thioanisole/TFA in the presence of the additional scavengers, m-cresol and EDT, in an ice-bath for 60 min, to cleave the peptide from the resin, together with other protecting groups attached; Mtr[27] from Arg, Boc from Lys, and tBu from Asp and Tyr. The deprotected peptide was purified to homogeneity by gel-filtration, followed by HPLC. As shown in Fig 4-a, the desired compound was obtained in a fairly good yield (33%, from Gln loaded on the resin). Succinimide formation was negligible. The Mtr group was judged to be cleanly cleaved from Arg by this TMSBr/TFA treatment, while the sample obtained by TFA-thioanisole treatment (25°C, 60 min) gave a complicate elution patten on HPLC (Fig. 4-b), presumably due to incomplete cleavage of the Mts group.

Thus, the 1 M TMSBr/TFA-thioanisole/TFA deportecting procedure seems to have

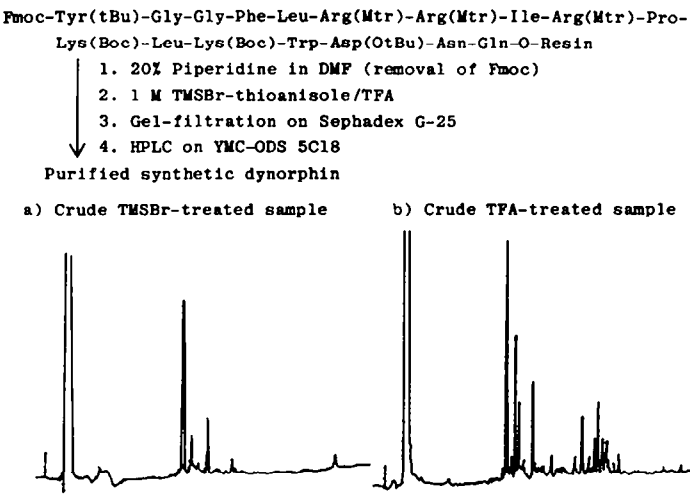

Fig. 4. HPLC of synthetic dynorphin prepared by TMSBr/TFA deprotection

some attractive features for both solution and solid phase peptide syntheses. We intend to evaluate further whether this hard acid deprotecting procedure with TMSBr can be applied to the synthesis of more complex peptides by increasing its concentration in TFA.

2. New Disulfide Bond-Forming Reaction.

(A) By oxidation with thallium(III) trifluoroacetate: Iodine has been used as an oxidant to convert directly Cys(R)-peptides (R=Tri and Acm) into cystine-peptides.[28] In this reaction, solvent effects have to be taken into consideration to minimize iodination at several amino acid residues, such as Tyr, His, Met, and Trp. We have found that $(CF_3COO)_3Tl$ in TFA can cleave various S-protecting groups of cysteine, including the above two groups, to form spontaneously cystine. This metal seems to act first as a soft acid to cleave S-protecting groups, then as a mild oxidant[29] to form the disulfide bond as shown in Fig. 5.

Each Cys-derivative in TFA was treated with $(CF_3COO)_3Tl$ (1 equiv.) in an ice-bath for 60 min. Except for Cys(Bzl), other S-protecting groups so far examined here (MBzl, tBu,[30] Ad,[31] Acm,[32] Tri,[33] and Dbs[34]) were cleaved to form cystine as

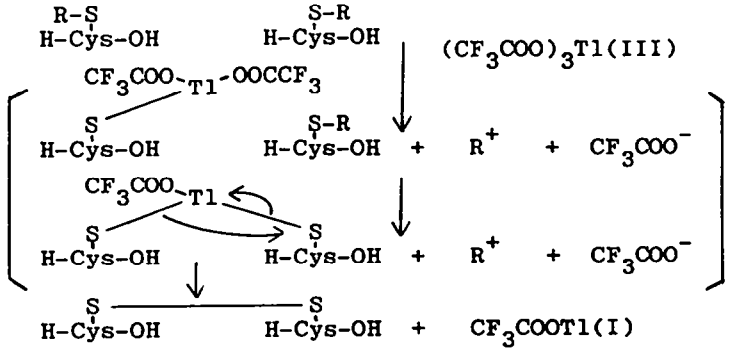

Fig. 5. Disulfide bond-formation by $(CF_3COO)_3Tl$ oxidation

Table 4. Oxidative cleavage of various S-protecting groups of cysteine by $(CF_3COO)_3Tl$

Cys(R) deriv.	Cystine formed (%)	Cysteine regenerated after reduction (%)
Cys(MBzl)	86.7	98.5
Cys(tBu)	80.5	96.9
Cys(Ad)	83.0	89.3
Cys(Acm)	81.0	95.3
Cys(Tri)	80.2	93.6
Cys(Dbs)	81.1	87.0
Cys(4-Me-Bzl)	74.5*	89.7
Cys(Bzl)	0	0

* A by-product, presumably the sulfoxide, was detected.

the sole product (Table 4). Cys(4-MeBzl)[19] generated cystine, but a small amount of a by-product (presumably the sulfoxide) was detected. After incubation of each cleaved sample with EDT(40°C, 5 h), cysteine was regenarated quantitatively. These results suggested that the $(CF_3COO)_3Tl$ oxidation procedure can be applied to convert S-protected cysteine-peptides to cystine-peptides, as well as into cysteine-peptides, if necessary.

Prior to applying this newly found disulfide bond-forming reaction to practical peptide synthesis, the side effects of this oxidant on other functional amino acids were examined. Unmasked Trp suffered modification to produce several unidentified products (recovery of Trp, 38%), but Trp(Mts) remained intact, after a 60 min treatment at 0°C. Met was partially oxidized to the corresponding sulfoxide (34%), but not to the sulfone. Other amino acids, Tyr and His, survived unchanged after this $(CF_3COO)_3Tl$ treatment. From these model experiments, we reached the conclusion that two amino acids, Trp and Met, must be protected during the $(CF_3CO-O)_3Tl$ treatment.

Next, the stability of the disulfide bond of cystine under conditons required for removal of the Mts group from Trp and reduction of Met(O) was examined. As a model experiment, cystine was treated with 1 M TMSOTf/TFA or 1 M TFMSA/TFA in an ice-bath for 60 min and the effect of an added soft base was examined. When the recovery of cystine was measured, PhSPh gave a much better result (recovery, 80-85%) than thioanisole (recovery, 64%). In this case, MeSMe[35] is also a good soft base also, but not effective enough to assist the cleaving reaction of Trp(Mts) and Arg(Mts), as described above. PhSPh is not freely soluble in TFA. Thus, a concentration of 0.5 M PhSPh in TFA was judged to be suitable for practical use.

Next, selective deoxygenation of Met(O) in the presence of cystine was examined. Thioanisole or MeSMe is known to reduce Met(O) partially during TFMSA/TFA or TMSOTf/TFA treatment, but PhSPh which we selected above was found not to exert any noticeable reducing effect on Met(O) during TFMSA/TFA or TMSOTf/TFA treatment. Apart from sulfur compound, we were interested in examining the reducing properties of ammonium iodide.[36] When a mixture of Z(OMe)-Met(O)-OH and cystine in TFA was treated with ammonium iodide in an ice-bath for 60 min, tolerably high recoveries of Met (89.7%) and Cys (90.2%) were obtained. Thus, the combination of ammonium iodide and MeSMe in TFA was found to be effective to reduce Met(O) to Met, without affecting the cystine disulfide bond.

The usefulness of $(CF_3COO)_3Tl$ for the intramolecular disulfide bond-forming reaction was examined by preparing three model peptides. Synthesis of oxytocin[37] is the most simple example of this application. When protected oxytocin, Z(OMe)-

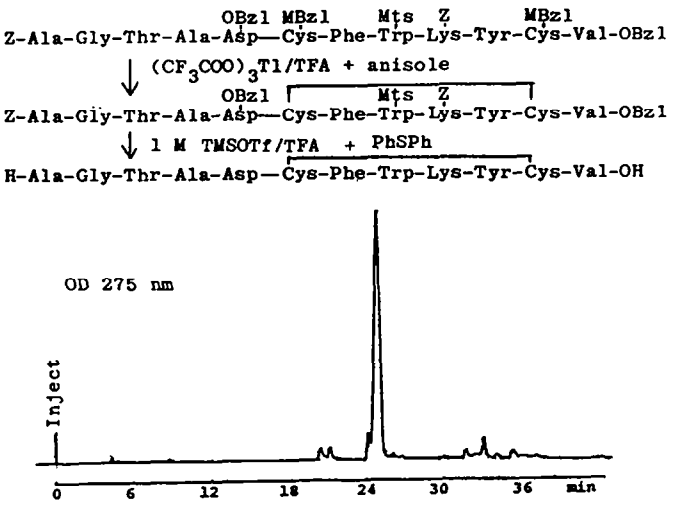

Fig. 6. HPLC of crude synthetic urotensin II prepared by $(CF_3COO)_3Tl$ oxidation, followed by TMSOTf/TFA deprotection

Cys(R)-Tyr-Ile-Gln-Asn-Cys(R)-Pro-Leu-Gly-NH_2 (R=MBzl or Acm), in TFA was treated with $(CF_3COO)_3Tl$ (1.2 equiiv.) in an ice-bath for 60 min, oxytocin was directly obtained as a main product in both cases (isolation yield, 27% and 22% respectively), without the laborious air-oxidation reaction.

Next, as an example of a Trp-containing peptide, urotensin II, a caudal neurosecretory 12-residue peptide hormone of the toleost fish,[38] was prepared. Protected urotensin II, prepared by the solution method, was treated with $(CF_3COO)_3Tl$ in TFA as described above to establish the disulfide bond between Cys(MBzl) residues, then with 1 M TMSOTf/TFA in the presence of PhSPh and m-cresol in an ice-bath for 120 min to remove the rest of the protecting groups attached. When the crude product obtained after gel-filtration, was examined by HPLC, a product possessing a retention time identical with that of an authentic sample of synthetic urotensin II[39] emerged from the column as a min component, as shown in Fig. 6. The desired product was isolated by preparative HPLC in 34% yield, while the yield of the authentic sample prepared by the usual air-oxidation procedure was 16%.

Next, as an example of a Met-containing peptide, human β-calcitonin gene-related peptide (β-hCGRP)[40] was synthesized. Protected 37-residue peptide corresponding to the entire amino acid sequence of β-hCGRP was synthesized by assembling seven peptide fragments. This protected peptide, dissolved in TFA, was treated with $(CF_3COO)_3Tl$ in the presence of anisole in an ice-bath for 60 min to establish the disulfide bond between two Cys(Ad) residues. The product was next treated with 1 M TMSOTf/TFA in the presence of PhSPh for 180 min to remove all protecting groups attached and after addition of NH_4I and MeSMe, for additional 30 min to reduce Met(O) to Met without affecting the disulfide bond. The HPLC pattern of the crude sample obtained at this stage is shown in Fig. 7. After three steps of purification, the product was obtained in a better yield (12%) than that obtained by the usual air-oxidation procedure (7%).

We confirmed that no thallium contamination occured in the synthetic peptides by X-ray energy spectroscopy. An attractive feature of this mild oxidant is that disulfide bond-formation can be carried out in TFA without any solubility problem, since TFA dissolves most of the peptides freely. A new rout has thus been opened to synthesize peptides containing one disulfide bond without laborious and time-consuming air-oxidation.

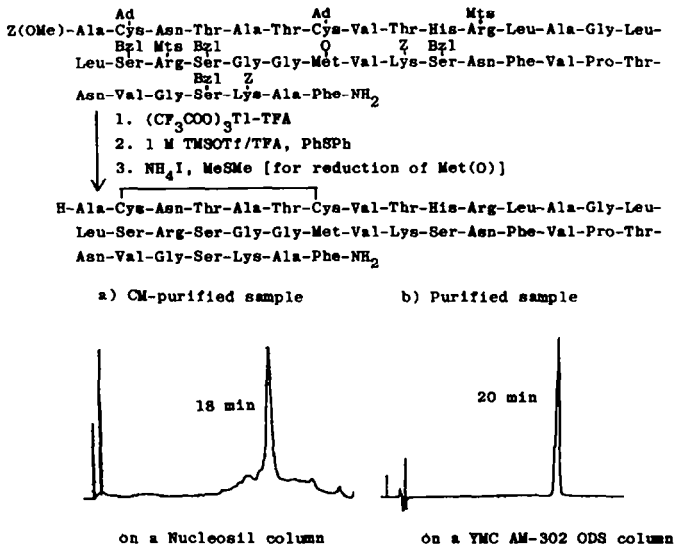

Fig. 7. HPLC of synthetic β-hCGRP

(B) Sulfoxide-directed disulfide bond-forming reaction. Considering the synthesis of peptides containing more than one disulfide bond, we have explored a new acid catalized disulfide bond-forming reaction, involving S-substituted cysteine sulfoxides, Cys(R)(O).

In 1979, we reported that treatment of Z(OMe)-Cys(MBzl)(O)-OH with MSA in the presence of a cation scavenger, anisole, afforded p-methoxyphenylcystine as a major product.[41] We found that when anisol was replaced by cysteine in the above MSA treatment, cystine formed in 86% yield (Table 5). The yield was improved, when MSA was replaced by 1 M TFMSA/TFA or by 1 M TMSOTf/TFA and MeSMe was used as an alternative scavenger. However, on treatment with TFA alone, the yield was less than 10%. Cystine was obtained in fairly good yields, when an equimolar mixture of Boc-Cys(Acm)(O)-OH and cysteine was treated with the foregoing acids, including TFA.

It was found further that this sulfoxide-directed disulfide bond-forming reaction proceeded, even if cysteine was replaced by Cys(R')(R'=acid cleavable S-protecting groups, such as MBzl, Ad, and tBu). A sulfur compound added, such as MeSMe, is known to act as a soft base in acid media, accelerating the cleavage reaction, and then regenerating the SH group (Fig. 8). Thus, it seems worthwhile to note that TFA-treatment of a mixture of Boc-Cys(Acm)(O)-OH and Z(OMe)-Cys(MBzl)-OH afforded cystine in a nearly quantitative yield. Other combination of the sulfox-

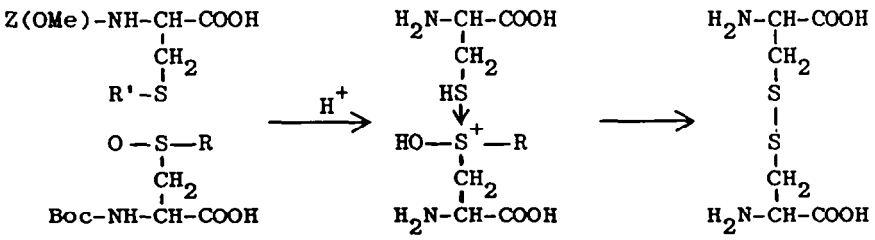

Fig. 8. Sulfoxide-directed disulfide bond-forming reaction

Table 5. Acid-catalized disulfide bond-forming reaction between Cys(R)(O) and cysteine or Cys(R')

Sulfoxide	Acid treated (0°C, 60 min)	Cystine formed (%) Cysteine	Cys(MBzl)	Cys(Ad)	Cys(tBu)
Cys(MBzl)(O)	TFA	9.1	7.3	2.8	2.7
	MSA	86.4	83.3	85.8	
	1 M TFMSA/TFA	100.0	92.2	92.4	92.1
	1 M TMSOTf/TFA	100.0	84.7	84.8	89.8
Cys(Acm)(O)	TFA	97.9	85.3*	1.7	7.6
	1 M TFMSA/TFA	84.9	70.3	75.9	59.5
	1 M TMSOTf/TFA	80.0	84.3	86.3	87.8
Cys(Ad)(O)	1 M TFMSA/TFA	20.8	15.5	10.5	

* Reaction at 25°C.

ide and Cys(R') required more stronger acid (or hard acid) treatment in order to obtain cystine in satisfactory yields.

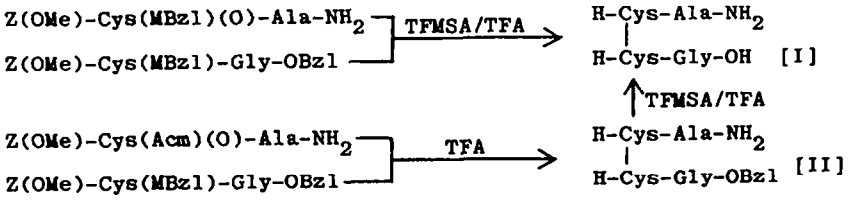

Fig. 9. Synthesis of an open-chain unsymmetrical cystine peptide

As a model experiment, we wish to demonstrate that a open-chain unsymmetrical cystine peptide can be obtained in two ways (Fig. 9). Treatment of an equimolar mixture of Z(OMe)-Cys(MBzl)(O)-Ala-NH_2 and Z(OMe)-Cys(MBzl)-Gly-OBzl with 1 M TFMSA/TFA in the presence of MeSMe (0°C, 60 min) afforded the free form of the unsymmetrical cystine-peptide amide (I) (yield 86%), while TFA-treatment of an equimolar mixture of Z(OMe)-Cys(Acm)(O)-Ala-NH_2 and Z(OMe)-Cys(MBzl)-Gly-OBzl (25°C, 60 min) afforded the unsymmetrical cystine-peptide ester (II) [oil, FAB-MS m/z:

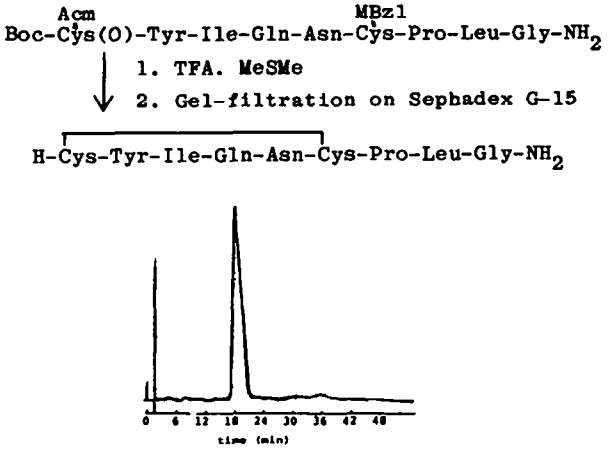

Fig. 10. HPLC of synthetic oxytocin prepared by application of the sulfoxide-directed disulfide bond-forming reaction

458 $(M + H)^+$]. For confirmation of its structure, (II) was converted into the free amide (I) by treatment with 1 M TFMSA/TFA (overall yield, 71%). Samll amount of two symmetrical cystine-peptide by-products were removed from the desired peptides by HPLC on a YMC-R ODS column using aqueous 0.1% TFA.

This disulfide bond-forming reaction was next applied to the synthesis of oxytocin. We were able to obtain oxytocin by simple TFA-treatment (25°C, 60 min) of Boc-Cys(Acm)(O)-Tyr-Ile-Gln-Asn-Cys(MBzl)-Pro-Leu-Gly-NH_2 in 86% yield, as seen in the HPLC elution pattern (Fig. 10).

These model experiments seem to open a new way to establish the disulfide bond at the sulfoxide position intramolecularly, as well as intermolecularly. In addition to the methods hitherto employed for synthesis of unsymmetrical cystine peptides,[42] the present two alternative disulfide bond forming reactions presented here may be useful for synthesis of peptides containing several disulfide bonds.

Experimental Section

Amino acid analysis and HPLC were conducted with a Hitachi 835-02 analyzer and a Waters 204 compact model, respectively. Rotation and ultraviolet absorption (UV) were determined with a Union PM-101 polarimeter and a Hitachi 100-20 model, respectively. FAB-MS was obtained on a ZAB SE instrument. Thin layer chromatography (TLC) was determined on silica gel (Kieselgel G, Merck) and Rf values refer to the following solvent systems: Rf_1 n-BuOH-AcOH-pyridine-H_2O (4:1:1:2), Rf_2 n-BuOH-AcOH-pyridine-H_2O (30:20:6:24). Leucine-aminopeptidase (LAP, Lot. No. L-6007) was purchased from Sigma.

Synthesis of hGIP by TMSOTf/TFA deprotection: Protected hGIP (50 mg) was treated with 1 M TMSOTf-thioanisole/TFA (5 ml, the amount of TMSOTf was 45 equiv./protecting group) in an ice-bath for 120 min. m-Cresol and EDT (20 equiv. each) were added to protect Tyr and Trp residues. Dry ether was added and the resulting powder, after being washed with ether, was dissolved in H_2O. The solution was adjusted to pH 8.0 with Et_3N and mercaptoethanol (200 µl) and 1 M NH_4F (400 µl) were added. The solution was stirred in an ice-bath for 30 min, then the pH was adjusted to 5 with 1 N AcOH. The solution was applied to a column of Sephadex G-25, which was eluted with 1 N AcOH. Lyophilization of the desired fraction (monitored by UV absorption measurement at 280 nm) gave a fluffy powder; yield 36 mg (96%). The gel-filtered sample was next purified by ion-exchange chromatography on CM-Trisacryl using gradient elution with 0.2 M NaCl in 0.01 M $AcONH_4$. Desalting of the desired fraction by gel-filtration on Sephadex G-25 and subsequent lyophilization gave a fluffy powder; yield 13.6 mg (37%). The product was next purified by HPLC using a Nucleosil 5C18 column with a gradient elution of MeCN (30-50%) in 0.1% TFA aq.; yield 10.2 mg (28% from protected GIP). Rf_1 0.26. $[\alpha]_D^{18}$ - 43.2° (c=0.3, 1 N AcOH). Amino acid ratios in a LAP digest: Asp 3.68(4), Ser 1.75(2), Glu 1.09(1), Gly 2.19(2), Ala 3.21(3), Val 1.17(1), Met 0.98(1), Ile 3.98(4), Leu 2.00(2), Tyr 1.95(2), Phe 2.19(2), Lys 4.76(5), His 1.98(2), Trp 2.11(2), Thr Gln and Asn were not determined (recovery of Leu, 78%).

Synthesis of human GRP by TMSBr/TFA deprotection.
Protected hGRP (50 mg, 15.7 µmol) was treated with 1 M TMSBr-thioanisole/TFA (5 ml) in the presence of m-cresol (10 equiv. per Tyr) and EDT (10 equiv. per Trp) in an ice-bath for 3 h, then dry ether was added. the resulting powder was treated with base as stated above and purified by gel-filtration on Sephadex G-25, fol-

lowed by HPLC using a Nucleosil 5C18 column with isocratic elution of MeCN (27%) in 0.1% TFA aq.; yield 23.6 mg (52% from protected hGRP). Rf_2 0.45, $[\alpha]_D^{21}$ - 99.7° (c=0.1, 1 N AcOH). Amino acid ratios in a LAP digest: Thr 2.16(2), Pro 2.93(3), Gly 4.88(5), Ala 2.08(2), Val 2.92(3), Met 1.95(2), Leu 3.00(3), Tyr 1.02(1), Lys 1.12(1), His 1.78(2), Trp 0.88(1), Arg 1.00(1), Asn was not detemined (recovery of Leu, 77%).

Solid phase synthesis of dynorphin by TMSBr/TFA deprotection.
Starting from the Gln-bounded resin (content of Gln, determined as Glu after 6 N HCl hydrolysis, 0.08 mmol/g of the resin), protected dynorphin was synthesized by the Fmoc strategy using an automated LKB peptide synthesizer. The peptide-resin (200 mg) was treated with 1 M TMSBr-thioanisole/TFA (3.5 ml) in an ice-bath for 60 min, the solution was filtered and resin was washed with TFA. Dry ether was added to the combined filtrate and washing. The resulting powder was treated with base as described above and purified by gel-filtration on Sephadex G-25, followed by HPLC on a YMC-ODS column with a gradient of MeCN (20-40%) in 0.1% TFA aq.; yield 7.8 mg (33% from Gln loaded on the resin). Rf_1 0.34, $[\alpha]_D^{28}$ - 59.1° (c=0.2, 1% AcOH). Amino acid ratios in a 6 N HCl hydrolysate: Tyr 0.93(1), Gly 1.99(2), Phe 1.00(1), Leu 2.04(2), Arg 2.94(3), Ile 0.81(1), Pro 1.04(1), Lys 2.00(2), Trp N.D., Asp 2.08(2), Glu 1.04(1) (recovery of Lys, 81%).

Synthesis of urotensin II by application of $(CF_3COO)_3Tl$ oxidation. Protected urotensin II (50 mg) in TFA (5.0 ml) was treated with $(CF_3COO)_3Tl$ (14.6 mg, 1.2 equiv.) in the presence of anisole (50 µl) in an ice-bath for 60 min, then TFA was removed by evaporation in vacuo and dry ether was added. The resulting powder was treated with 1 M TMSOTf/TFA (4 ml) in the presence of PhSPh (0.79 ml) and m-cresol (0.25 ml) in an ice-bath for 120 min, then isopropyl ether was added. The resulting powder was treated with 5% NH_4OH as stated above and purified by gel-filtration on Sephadex G-25, followed by HPLC on a cosmosil 5C18 column with a gradient of MeCN (23 to 35%) in 0.1% TFA; yield 10.4 mg (34%), $[\alpha]_D^{20}$ - 59.0° (c=0.2, 1% AcOH). FAB-MS m/z: 1361 $(M+H)^+$. Amino acid ratios in a 6 N HCl hydrolysate: Asp 1.02(1), Thr 0.92(1), Gly 1.01(1), Ala 1.96(2), Cys 0.86(1), Val 1.00(1), Tyr 1.00(1), Phe 1.02(1), Lys 1.05(1) (recovery of Val, 78%).

Synthesis of β-hCGRP by application of $(CF_3COO)_3Tl$ oxidation.
Protected β-hCGRP (50 mg) in TFA was treated with $(CF_3COO)_3Tl$ (6.23 mg, 1.2 equiv.) in the presence of anisole (50 µl) in an ice-bath for 60 min, then TFA was removed by evaporation and dry ether was added. The resulting powder was treated with 1 M TMSOTf/TFA (4.6 ml) in the presence of PhSPh (at a concentration of 0.5 M) and m-cresol (234 µl) in an ice-bath for 180 min. NH_4I (27 mg, 20 equiv.) and MeSMe (14 µl, 20 equiv.) were added and treatment was continued for additional 30 min. After addition of dry ether, the resulting powder was treated with 5% NH_4OH at pH 8.0 as stated above and purified by gel-filtration on Sephadex G-15; yield 31.1 mg (86%). Next, the product was purified by ion-exchange chromatography on CM-Trisacryl column using a liner gradient of 0.2 M NaCl in 0.01 M $AcONH_4$. After desalting of the desired fraction on Sephadex G-15, a fluffy powder was obtained; yield 7.5 mg (24%). Subsequent purification was performed by HPLC on a Nucleosil 5C18 column, which was eluted with a gradient of MeCN (25-40%) in 0.1% TFA; yield 4.4 mg (12% from protected hCGRP), Rf_2 0.82, $[\alpha]_D^{23}$ - 78.8° (c=0.1, in 0.5N AcOH). FAB-MS m/z: 3794 $(M+H)^+$. Amino acid ratios in a LAP digest: Thr 3.48(4), Pro 1.16(1), 4.38(4), Ala 3.94(4), Cys 0.78(1), Val 3.46(4), Met 0.93(1), Leu 3.04(3), Phe 2.00(2), Lys 2.12(2), His 0.86(1), Arg 1.98(2) (recovery of Phe, 82%).

Synthesis of oxytocin by application of the sulfoxide-directed disulfide bond-forming reaction. Boc-Cys(Acm)(O)-Tyr-Ile-Gln-Asn-Cys(MBzl)-Pro-Leu-Gly-NH_2 (50 mg) was treated with TFA (5 ml) in the presence of MeSMe (0.5 ml) in an ice-bath for 60 min and at 25°C for additional 60 min, then TFA was removed by evaporation and dry ether was added. The resulting powder was purified by gel-filtration on Sephadex G-15 using 1 N AcOH as an eluant. Lyophilization of the desired fractions (monitored by UV absorption measurement at 275 nm) gave a fluffy powder; 24 mg (86%), FAB-MS m/z: 1007.3 $(M+H)^+$. Amino acid ratios in a 6 N HCl hydrolysate: Cys 0.65, Tyr 0.86, Ile 0.96, Glu 0.98, Asp 1.00, Pro 0.95, Leu 0.99, Gly 1.00 (reovery of Gly, 70%).

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References and Notes

Amino acids used in this investigation are of the L-configuration. The following abbreviations are used: Z=benzyloxycarbonyl, Boc=tert.-butyloxycarbonyl, Z(OMe)=p-methoxybenzyloxycarbonyl, Bzl=benzyl, Cl_2-Bzl=2,6-dichlorobenzyl, Tos=p-toluenesulfonyl, Mts=mesitylenesulfonyl, MBS=p-methoxybenzenesulfonyl, Bom=benzyloxymethyl, MBzl=p-methoxybenzyl, tBu=tert.-butyl, Ad=1-adamantyl, Acm=acetamidomethyl, Tri=triphenylmethyl, Mtr=4-methoxy-2,3,6-trimethylbenzenesulfonyl, Dbs=dibenzosuberyl, Chp=cycloheptyl, MSA=methanesulfonic acid, TFMSA=trifluoromethanesulfonic acid, TMSOTf=trimethylsilyl trifluoromethanesulfonate; TMSOMs=trimethylsilyl methanesulfonate; TMSOTa=trimethylsilyl trifluoroacetate, TMSBr=trimethylsilyl bromide, PAM=4-(oxymethyl)phenylacetamidomethyl, TFA=trifluoroacetic acid, EDT=ethanedithiol, Fmoc=9-fluorenylmethyloxycarbonyl.

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