96. The Synthesis of Cystine Peptides by Iodine Oxidation of *S*-Trityl-cysteine and *S*-Acetamidomethyl-cysteine Peptides[1])

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Summary

Previously reported studies of the iodine oxidation of *S*-trityl-cysteine peptides and *S*-acetamidomethyl-cysteine peptides, leading directly to cystine peptides, have been extended. Detailed investigations have been made of the reactivities of the *S*-trityl and the *S*-acetamidomethyl group towards iodine in various solvents. In chloroform, methylene chloride, trifluoroethanol, and hexafluoroisopropyl alcohol the differences in the reaction rates of the two groups have been found to be extremely large, allowing the selective conversion of the tritylthio groups to disulfides in the presence of the *S*-acetamidomethyl derivatives. In a second group of solvents, consisting of methanol, acetic acid, dioxane, and mixtures of these solvents with water, simultaneous iodine oxidation of *S*-trityl- and *S*-acetamidomethyl-cysteine peptides leads to a preferential combination of these two residues, resulting in predominantly asymmetrical cystine derivatives. – The suitability of the two sulfur-protecting groups in the synthesis of cyclic cystine peptides has been assessed. – Possible reaction mechanisms are discussed. – The scope and limitations of iodine oxidation in peptide synthesis have been studied.

The applicability of the method has been demonstrated in the preparation of the open-chain asymmetrical cystine peptide **5**, the protected somatostatin derivative **17**, and the A(1–13) segment **19** of human insulin, previously employed in the total synthesis of this hormone.

Some years ago, we reported that *S*-trityl-cysteine peptides and *S*-acetamidomethyl-cysteine peptides [2] could be directly converted into cystine peptides by oxidation with iodine [3] [4]. In the meantime, this method has been applied by us and by others to the synthesis of a variety of structurally different cystine peptides [5] [6].

[1]) Abbreviations are according to the IUPAC-IUB Commission on Biochemical Nomenclature [1]. In addition, the following abbreviations have been adopted in the text: Acm = acetamidomethyl; HFIP = hexafluoroisopropyl alcohol; TFE = trifluoroethanol; DMF = dimethylformamide.

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Since this method can be employed with peptides carrying other permanent and temporary protective groups, it permits the introduction of disulfide bonds to give cystine residues at an early stage of the synthesis. Using the procedure in the synthesis of porcine and human calcitonin, both containing 32 amino acids, we were already able to form the 1–7 disulfide ring in a nonapeptide and a decapeptide, respectively [7] [8]. In the synthesis of peptides containing more than one cystine residue, selective sulfur protection can be avoided in this way by forming the correct disulfide linkages at the stage of monocystine-peptide segments. This approach has its limits, as *e.g.* in the synthesis of insulin where a cyclic cystine peptide sequence is present in which one of the ring members is an additional half-cystine residue. In such cases, the resultant problems of selective temporary protection of amino and carboxyl groups in the segments to be synthesized would be extremely difficult to solve.

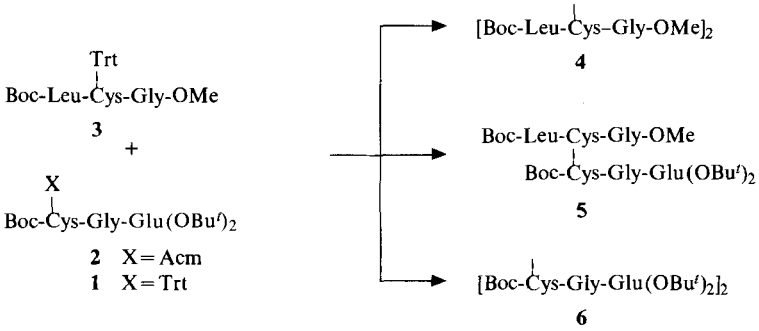
In the present paper, we give a survey of the possible applications of the method in the synthesis of cystine-containing peptides. It will be shown that the *S*-trityl group and the *S*-acetamidomethyl group differ considerably in their reactivity with iodine in some solvents. This behaviour permits the selective conversion of trityl sulfides to the disulfide in the presence of *S*-acetamidomethyl-cysteine residues. This method of selective sulfur protection has been applied by us in the synthesis of human insulin [9] and some of its analogues [10] [11]. For the previously described A(1–13) segment [5d], the key-intermediate in which this selective protection was required, we give an alternative synthetic pathway.

1. The Course of Iodine Oxidation of *S*-Trityl-cysteine and *S*-Acetamidomethyl-cysteine Residues in Various Solvents. – 1.1. *Formation of Open-chain Cystine Peptides.* The time-course of iodine oxidation giving the symmetrical cystine peptide **6** (s. below) was studied with the two model compounds Boc-Cys(Trt)-Gly-Glu-$(OBu^t)_2$ (**1**) [5b] and Boc-Cys(Acm)-Gly-Glu$(OBu^t)_2$ (**2**) (for details see exper. Part). As can be seen in *Table 1*, the two groups *S*-trityl and *S*-acetamidomethyl show marked differences in their reactivities with iodine, depending on the solvent. According to the time t_h, required for the conversion of 50% of the starting material, called 'half-time', the solvents studied may be divided into two groups I and II, with the exception of DMF. In both solvent groups the *S*-trityl reacts faster than the *S*-acetamidomethyl group. In solvents of group I (methanol, glacial acetic acid, and dioxane) the corresponding rates differ by a factor of 10 to 100, and both reactions are accelerated by addition of water. Conversely, in solvents of group II (chloroform or methylene chloride and mixtures of these with HFIP and TFE[3]) the differences in the oxidation rates are much larger, the *S*-acetamidomethyl group reacting only very sluggishly. It is noteworthy that in DMF, in contrast with the other solvents, the *S*-acetamidomethyl reacts faster than the *S*-trityl group, and water doesn't accelerate the reaction but even retards it slightly.

The course of the simultaneous iodine oxidation of an *S*-trityl and an *S*-acetamidomethyl compound was studied with an equimolar mixture of the peptides **3** and **2** *(Scheme 1)*. Because of the extremely large rate differences in the solvents of group II, it was to be expected that selective iodine oxidation should be possible,

[3]) In HFIP and TFE alone iodine is very poorly soluble.

Scheme 1. *Iodine oxidation of* **3** *in the presence of* **2**



i.e. that **3** could be selectively converted to **4** [5e] without formation of **5** and **6** [3]. In experiments similar to those in which the above 'half-time' were determined, the conversion of the *S*-trityl compound **3** was monitored, and upon its complete disappearance the composition of the mixture determined. At the given concentrations, the reaction times varied between 30 s and 2 min[4]). The results of these experiments, summarized in *Table 2*, show that in HFIP/chloroform **3** was converted to the symmetrical cystine peptide **4** with *a selectivity of more than 98%*. Besides unchanged **2**, traces of the asymmetrical derivative **5** were present. A small increase in **5**, and consequently a corresponding decrease in selectivity, was observed when TFE/chloroform or chloroform (methylene chloride) alone was used as solvent; but in no case **6** could be detected.

The results of the iodine oxidation in the solvents of group I are noteworthy. As expected from the data shown in *Table 1*, in these solvents a certain amount of the *S*-acetamidomethyl compound **2** was already converted to **6** before all of the *S*-trityl compound **3** had disappeared. However, the yield of the asymmetrical cystine peptide **5** was surprisingly high, 70–80% of the *S*-trityl component **3** having been converted to this mixed disulfide in methanol[4]). Similarly high yields of **5** were obtained in methanol/chloroform 1:1, although the *S*-acetamidomethyl group should react considerably slower than in methanol (s. *Table 1*). A somewhat lower yield of **5** was obtained in aqueous methanol and in acetic acid; but in all the solvents of group I the yield of **5** was above 50%, *i.e.* even higher than the yield to be expected in a random oxidation (*cf.* footnote 4). This is indicative of *partial selectivity* in these solvents, in the sense that an *S*-trityl component and an *S*-acetamidomethyl component are preferentially combined to the mixed disulfide. It

[4]) If equimolar amounts of the two *S*-trityl compounds **1** and **3** *(Scheme 1)* are completely oxidized with iodine in any solvent, the three cystine peptides **4**–**6** are obtained as a statistical mixture. The yields (in mol-%) of such a random oxidation will be: 50% for the asymmetrical derivative **5** and 25% for each of the two symmetrical cystine peptides **4** and **6**. For the discussion of the selective conversion of the *S*-trityl component **3** to the disulfide **4**, in the presence of the *S*-acetamidomethyl component **2**, it is more convenient to base the yields of **4** and **5**, containing the peptide moiety of the *S*-trityl component **3**, on this starting material. In the above random oxidation, the yields for **4** and **5** are therefore 50% each, meaning that 50% of **3** is converted to **4** and 50% to **5**. In the same way the yields of **6** and **5** are based on the *S*-acetamidomethyl compound **2**.

can be taken advantage of this phenomenon in the synthesis of open-chain asymmetrical and cyclic cystine-peptides (s. below). As can be seen in *Table 2*, there was no preferential formation of **5** in DMF.

Table 1. *'Half-times' (t_h) for the iodine oxidation of Boc-Cys(Trt)-Gly-Glu(OBu^t)$_2$ (**1**) and Boc-Cys(Acm)-Gly-Glu(OBu^t)$_2$ (**2**) in various solvents*[a])

	Solvent	t_h for **1** (*S*-Trt)	t_h for **2** (*S*-Acm)
Group I	MeOH	3–5 s	1 min
t_h(Trt) < t_h(Acm)	MeOH/H_2O 8:2	< 1 s	4–6 s
	AcOH	70–80 s	40–45 min
	AcOH/H_2O 8:2	1–3 s	50–60 s
	dioxane	1 min	1.5–2 h
	dioxane/H_2O 8:2	5–10 s	5–10 min
	MeOH/$CHCl_3$ 1:1	2–4 s	15 min
Group II	$CHCl_3$, CH_2Cl_2	1–2 s	1.5–2 h
t_h(Trt) ≪ t_h(Acm)	HFIP/$CHCl_3$ 1:1	1–2 s	> 2 h[b])
	HFIP/$CHCl_3$ 3:1	< 1 s	> 2 h[b])
	TFE/$CHCl_3$ 1:1	5–6 s	> 2 h[b])
	TFE/$CHCl_3$ 3:1	4–5 s	> 2 h[b])
t_h(Trt) > t_h(Acm)	DMF	25–35 s	2–3 s
	DMF/H_2O 8:2	30–40 s	3–5 s

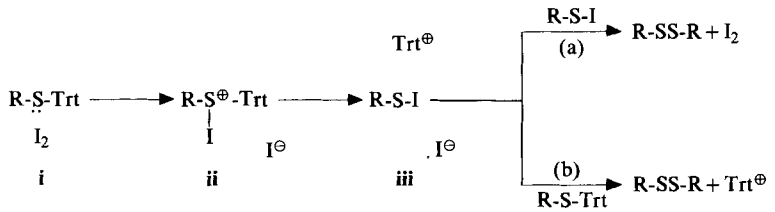
[a]) c(peptide) = $5 \cdot 10^{-3}$ M; c(iodine) = $15 \cdot 10^{-3}$ M; temp. 20–25°.
[b]) Decrease of the reaction rate in the order TFE/$CHCl_3$ 3:1 > TFE/$CHCl_3$ 1:1 > HFIP/$CHCl_3$ 3:1 > HFIP/$CHCl_3$ 1:1.

Table 2. *Composition of the mixture obtained from the iodine oxidation of* **3** *in the presence of* **2** (s. *Scheme 1*)[a])

Solvents	**4** [%]	**5** [%]	**6** [%]	**2** [%]
Group II				
HFIP/$CHCl_3$ 1:1	> 98[b])	< 2[b])	0[c])	> 98
HFIP/$CHCl_3$ 3:1	> 98[b])	< 2[b])	0[c])	> 98
TFE/$CHCl_3$ 1:1	96–98[b])	2–4[b])	0[c])	96–98
TFE/$CHCl_3$ 3:1	95–97[b])	3–5[b])	0[c])	95–97
$CHCl_3$, CH_2Cl_2	94–96[b])	4–6[b])	0[c])	94
Group I				
MeOH	20–30[b])	70–80[b])	10–20[c])	5–15
MeOH/$CHCl_3$ 1:1	20–30[b])	70–80[b])	10–20[c])	5–15
MeOH/H_2O 8:2	25–35[b])	65–75[b])	20–25[c])	5–10
AcOH/H_2O 8:2	40–50[b])	50–60[b])	15–25[c])	20–30
DMF	5–15[b])	15–25[c])	75–85[c])	0[d])

[a]) c(**2**) = c(**3**) = $2.5 \cdot 10^{-3}$ M; c(iodine) = $15 \cdot 10^{-3}$ M; temp. 20–25°.
[b]) Yields based on **3**; *cf.* footnote 4.
[c]) Yields based on **2**; *cf.* footnote 4.
[d]) The reaction was run until **2** had disappeared. The mixture still contained 65–75% of unchanged **3**.

Scheme 2. *Proposed mechanism for the iodine oxidation of a trityl sulfide*



The above results cannot be accounted for by the rate differences of the *S*-trityl and *S*-acetamidomethyl group indicated in *Table 1*. Additional factors that may be involved will be discussed in section 1.2.

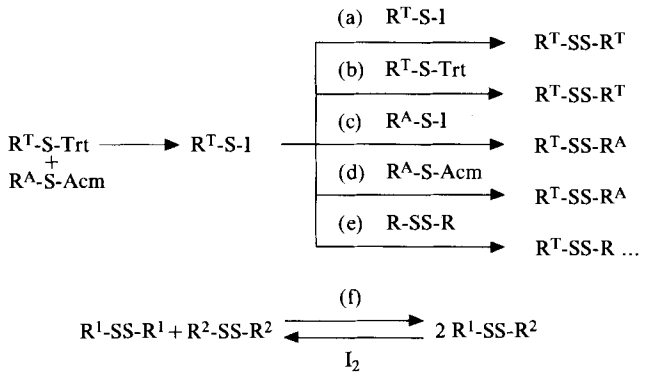
1.2. *Possible Mechanism of the Iodine Oxidation.* A reasonable mechanism for the iodine oxidation of a trityl sulfide is given in *Scheme 2*. The first interaction between iodine and a divalent S-atom should be the formation of the charge-transfer complex **i**, the S-atom being the donor and iodine the acceptor; such molecular iodine complexes are well known (see *e.g.* [12]). Charge separation leads to the iodosulfonium ion **ii** [13] which is cleaved to the sulfenyl iodide **iii** and the trityl carbenium ion. Sulfenyl iodides are assumed to be intermediates in the iodine oxidation of thiols to disulfides [14]. The subsequent formation of the disulfide can occur in two ways: a) two sulfenyl iodides disproportionate to the disulfide and iodine, or b) the electrophilic S-atom of R-S-I attacks the nucleophilic divalent S-atom of an unchanged R-S-Trt. This last step would be analogous to the reaction of a sulfenyl thiocyanate with a trityl sulfide, which has been extensively studied by *Hiskey et al.* [15]. The reaction of *S*-trityl and *S*-acetamidomethyl-cysteine derivatives with sulfenyl chlorides, resulting in the removal of these two sulfur-protecting groups, has also been described [5c] [16] [17].

The mechanism proposed in *Scheme 2* suggests that in this iodine oxidation any sulfur substituent (*i.e.* sulfur-protecting group) capable of forming a stabilized species upon cleavage of the bond between the S-atom and itself may yield a sulfenyl iodide **iii**, and hence a disulfide[5]). In the meantime, it has been shown that the following *S*-protected cysteine derivatives can be directly converted to cystine by iodine: *S*-benzamidomethyl- [18–20], *S*-tetrahydropyranyl- [21], *S*-(5-dibenzo)-suberyl- [22], and *S*-diphenyl(4-pyridyl)methyl-cysteine [23].

As shown in *Table 1*, iodine rapidly oxidized the *S*-trityl compound **1** in all the solvents of group I and II. The *S*-acetamidomethyl compound **2** reacted relatively fast in the solvents of group I, but extremely slowly in the non-polar, or polar but only slightly nucleophilic solvents of group II, such as chloroform, methylene chloride or the fluorinated alcohols HFIP and TFE. Evidently in these solvents one or more reaction steps leading to the generation of a sulfenyl iodide are impeded. It is noteworthy in this connection that the violet colour of iodine in

[5]) In the case of the acetamidomethyl group the stabilized species is obviously the iminium ion $[CH_3-CO-NH=CH_2]^+$. In our iodine oxidations of *S*-trityl compounds, a trityl ether (in alcoholic solvents) or triphenylmethanol was obtained upon work-up. We have not studied the fate of the acetamidomethyl moiety.

Scheme 3. *Possible reaction paths in the simultaneous iodine oxidation of a trityl, and an acetamidomethyl sulfide*



these solvents does not change immediately upon addition of an *S*-acetamidomethyl derivative, but only gradually turns reddish-brown.

Scheme 3 shows the possible course of the reaction when a trityl (R^T-S-Trt) and an acetamidomethyl sulfide (R^A-S-Acm) are simultaneously oxidized. Path (a) and (b) represent the conversion of the trityl component to its symmetrical disulfide as discussed in *Scheme 2*. In the presence of an acetamidomethyl component, the sulfenyl iodide R^T-S-I can moreover react by path (c) and (d): *i.e.* it may combine with the sulfenyl iodide R^A-S-I from the acetamidomethyl compound (if this species has already been formed), or it may react with R^A-S-Acm itself. Both steps lead to the asymmetrical disulfide. The same applies to the sulfenyl iodide generated from R^A-S-Acm. In addition, it is conceivable that a sulfenyl iodide might attack an already formed disulfide bond, giving rise to a disulfide exchange (e), a reaction known to occur with arylsulfenyl chlorides [24]. Finally, it remains to be clarified whether disulfides disproportionate under the influence of iodine (f).

Scheme 4. *Iodine oxidation of* **1** *in the presence of cystine peptide* **4**

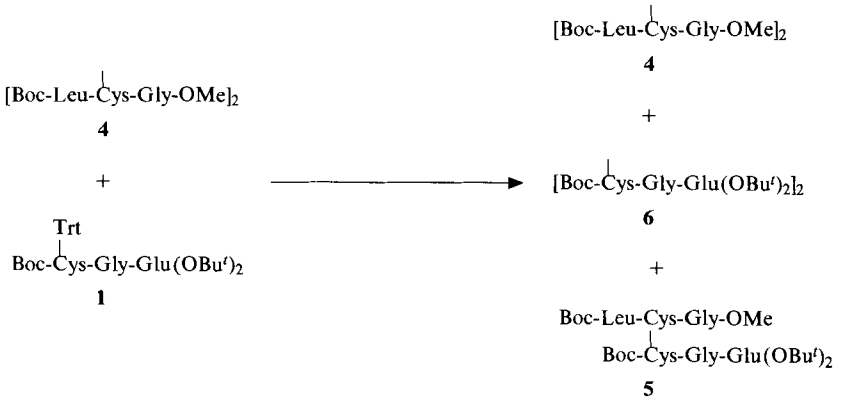


Table 3. *Study of the extent of the side reactions (e) and (f)* (s. *Scheme 3*)

Solvent	Formation of **5** according to *Scheme 4* [%][a])	Formation of **4** and **6** according to *Scheme 5* [%][b])
MeOH	1–3	5–10
MeOH/H_2O 8:2	1–3	5–10
AcOH/H_2O 8:2	3–5	5–10
$CHCl_3$, CH_2Cl_2	1–3	8–12
MeOH/$CHCl_3$ 1:1	1–3	5–10
HFIP/$CHCl_3$ 1:1	< 1	< 1
HFIP/$CHCl_3$ 3:1	< 1	< 1
TFE/$CHCl_3$ 1:1	1–3	2–4
TFE/$CHCl_3$ 3:1	1–3	2–4

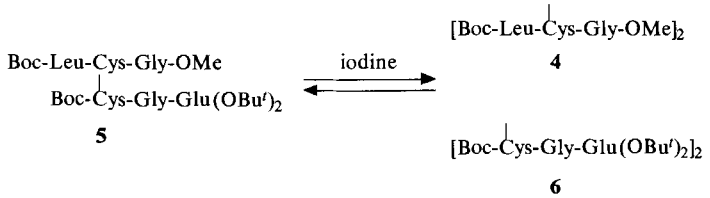
[a]) Yield based on **4**. $c(\mathbf{1}) = 5 \cdot 10^{-3}$M; $c(\mathbf{4}) = 2.5 \cdot 10^{-3}$M; c(iodine) $= 15 \cdot 10^{-3}$M; reaction time 5 min; temp. 20–25°.

[b]) Yield based on **5**. $c(\mathbf{5}) = 5 \cdot 10^{-3}$M; c(iodine) $= 30 \cdot 10^{-3}$M; reaction time 4 h; temp. 20–25°.

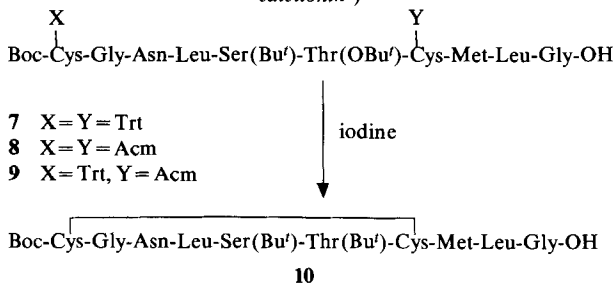
The results in *Table 2* show that in methanol and acetic acid, path (d), *i.e.* attack by the sulfenyl iodide rapidly formed from the *S*-trityl component **3** on the *S*-acetamidomethyl component **2**, is a predominant step. It is also path (d) which is responsible for the fact that **3** was not converted to **4** with absolute selectivity in the solvents of group II (HFIP, *etc.*) but that small amounts of **5** were also formed. Indeed, complete selectivity would have been expected from the enormous differences in the reaction rates of the *S*-trityl and *S*-acetamidomethyl group alone (s. *Table 1*). In DMF, attack by a sulfenyl iodide on the slower-reacting *S*-trityl compound **3** is not a reaction path influencing the composition of the product mixture to any considerable extent.

For the application of the iodine oxidation in preparative work, the extent of the undesired side reactions (e) and (f) of *Scheme 3* was determined in separate experiments: In the model oxidation of **1**–**6** in the presence of **4** (s. *Scheme 4*) the conversion of the symmetrical cystine peptide **4** to the asymmetrical derivative **5** reached at most 5% (*Table 3*, 1st column). The susceptibility of the model cystine peptide **5** to disulfide exchange under the influence of iodine (s. *Scheme 5*) was varying with the solvent used (*Table 3*, 2nd column). However, in these studies the reaction conditions (s. *Table 3*) were much more vigorous than necessary for the iodine oxidation of an *S*-trityl or an *S*-acetamidomethyl cysteine peptide. Nevertheless, these results clearly indicate that the *reaction times have to be kept as short as possible*.

Scheme 5. *Disulfide exchange in* **5** *by the action of iodine*



Scheme 6. *Formation of the disulfide ring in three differently S-protected 1–10 segments of human calcitonin*[a])



[a]) See text and *Table 4* for reaction conditions.

1.3. *Formation of Cyclic Cystine Peptides.* In the preceding experiments, the formation of the disulfide bond has always been an intermolecular reaction. The corresponding intramolecular reaction was studied by the preparation of the cyclic cystine peptide **10** from the precursors **7–9** carrying different protective groups at the cysteine residues *(Scheme 6, Table 4)*. This decapeptide was an intermediate in the synthesis of human calcitonin [5a]. The course of the iodine oxidation of **7–9** was compared under identical conditions.

The peptides were dissolved in methanol/methylene chloride/water 6:2.5:0.4, the only mixture yielding a sufficiently high concentration of the poorly soluble dityrityl derivative **7**. Iodine was dissolved in methylene chloride/methanol 8:1.5[6]). Each of the three segments **7–9** was oxidized at two concentrations (c_1 and c_2), and the reaction was carried out in two different ways: the solution of the peptide (Solution A) was either added to the solution of iodine (Solution B) or *vice versa*. Both solutions were of the same volume. After complete mixing and a further reaction time of 10 min the excess of iodine was reduced and the mixture worked up.

As can be seen in *Table 4*, the derivative **9** carrying both kinds of sulfur-protecting groups was converted in practically quantitative yield to the cyclic mono-

Table 4. *Yields of* **10** *in the oxidations of* **7–9** (s. *Scheme 6*)

	c_1	c_2	
Solution A (peptide)	10^{-3}M	$5 \cdot 10^{-3}$M	
Solution B (iodine)	$4 \cdot 10^{-3}$M	$20 \cdot 10^{-3}$M	
Mode of mixing the solutions	A to B or B to A	A to B	B to A
Precursor			
7 X=Y=Trt	90–100%	90–100%	20% (+80% of polymers)
8 X=Y=Acm	20% (+80% of **8**)	70% (+30% of **8**)	70% (+30% of **8**)
9 X=Trt, Y=Acm	90–100%	90–100%	90–100%

[6]) The 'half-time' in this solvent mixture, determined as above (s. *Table 1*), were <1 s for the *S*-trityl compound **1** and 2–3 min for the *S*-acetamidomethyl compound **2**. Simultaneous iodine oxidation of **2** and **3** (s. *Scheme 1, Table 2*) resulted in a mixture of about the same composition as in methanol, *i.e.* the asymmetrical cystine peptide **5** was formed in a yield considerably higher than 50%.

mer **10** under all conditions used. With **8**, containing two acetamidomethyl groups, the reaction was not yet complete after 10 min; 80 (at c_1) or 30% (at c_2) of **8** was still present. Complete conversion was achieved with the ditrityl derivative **7**. But in more concentrated solution (c_2), the result depended on the mode of mixing: addition of iodine to the peptide produced large amounts of polymers as shown by the poor solubility of the reaction mixture and the formation of 'tails' on thin-layer chromatograms.

The results shown in *Table 4* are in agreement with those described in section 1.1 and 1.2. The cyclization reaction was performed in one of the solvents in which the *S*-atoms of an *S*-trityl and an *S*-acetamidomethyl moiety are preferentially linked (solvents of group I). As discussed above, this partial selectivity results from the fact that a sulfenyl iodide generated from the *S*-trityl group – a fast step – reacts in another fast step with an *S*-acetamidomethyl group, giving the disulfide (path (d) in *Scheme 3*). Therefore derivative **9** is the most suitable precursor for cyclization when oxidation is carried out in a solvent of group I. With the di(acetamidomethyl) derivative **8** the oxidation is more slowly because of the lower reactivity of the *S*-acetamidomethyl group towards iodine. However, once either of the two cysteine residues has been converted to the sulfenyl iodide, the situation is as with **9** above: cyclization to **10** takes place rapidly. The ditrityl derivative **7** yields **10** in a clean reaction only in the more dilute solution (c_1), or at higher concentration (c_2) when iodine is in excess. In the latter case, the intramolecular formation of the disulfide bond presumably occurs essentially by disproportionation of two sulfenyl iodide groups (path (a) in *Scheme 3*). A deficit in iodine will convert **7** mainly to an intermediate with just one cysteine residue transformed to the sulfenyl iodide. As the combination of a sulfenyl iodide with the adjacent *S*-trityl group is a relatively slow step, the intermolecular reaction with a second sulfenyl iodide, and hence the formation of polymers, predominates in more concentrated solution. The results in *Tables 4* and *2* suggest that in solvents like methanol (*cf. Table 1*, solvents of group I) the reactivity of sulfenyl iodides lies in between the one of acetamidomethyl and trityl sulfides, namely RSAcm > RSI > RSTrt.

2. Incorporation of the Iodine Oxidation into Peptide Synthesis. – 2.1. *Behaviour of Amino Acids.* Using the iodine oxidation in a peptide synthesis it has to be taken into account that some amino acids might be iodinated, *i.e.* tyrosine, histidine, and tryptophan. Moreover, the stability of the sulfide group in methionine towards iodine had to be established. For this purpose, either the five model compounds **11**–**15** (s. *Table 5*) were exposed in the indicated solvents to iodine, or the iodine oxidation of Boc-Leu-Cys(Trt)-Gly-OMe (**3**) to the symmetrical cystine peptide **4** was carried out in the presence of **11**–**15**. *Table 5* shows that side chain unprotected tyrosine (compound **11**) was fairly rapidly iodinated to the mono- and diiodo derivatives in aqueous methanol and to a somewhat lesser degree in DMF, but was stable in all other solvents. Protection of the phenolic hydroxyl group of tyrosine as a *t*-butyl ether (compound **12**) prevented iodination. Iodine substitution of histidine occured when the imidazole group was present as a free base, (compound **13**), but not when it was protonated. In the tryptophan derivative **14**, not only iodination did occur in methanol, aqueous acetic acid, and DMF,

Table 5. *Stability of various amino acids towards iodine oxidation*[a])

Model compound	Solvent								
	MeOH	MeOH/H_2O 8:2	AcOH	AcOH/H_2O 8:2	$CHCl_3$, CH_2Cl_2	Dioxane	DMF	TFE/$CHCl_3$ 3:1	HFIP/$CHCl_3$ 3:1
Z-Tyr-OMe (**11**)[b])	–	+++	–	–	–	–	+	–	–
Z-Tyr(Bu^t)-OMe (**12**)	–	–	–	–	–	–	–	–	–
Z-Val-His-OMe (**13**)[b])	+	++	–	–	+	++	+++	+	+
Z-Val-His-OMe · HCl	–	–	–	–	–	–	–	–	–
Z-Trp-NH_2 (**14**)	+++	+++	–	++	–	–	+++	–	–
Boc-Phe-Met-OMe (**15**)	–	–	–	–	–	–	–	–	–

[a]) c(peptide) = $5 \cdot 10^{-3}$ M; c(iodine) = $15 \cdot 10^{-3}$ M; reaction time 30 min; temp. 20–25°. Products formed: – < 5%, + = 5–10%, ++ = 10–20%, +++ > 20%.

[b]) The products are mono-iodo and di-iodo derivatives.

but also oxidation of the indole ring leading in part to unstable products [25]. No side reactions took place in glacial acetic acid, chloroform, dioxane or HFIP and TFE. With tryptophan the possibility had also to be considered that an intermediate sulfenyl iodide might substitute the indole nucleus. Indeed, the formation of a sulfur bridge at C(2) of indole by sulfenyl chloride derivatives of cysteine was demonstrated by *Wieland et al.* (see *e.g.* [26]). In the iodine oxidation of **3** to **4** in the presence of **14**, this reaction was not observed. However, with some other substrates yielding cyclic sulfides, conditions were found under which substitution in the side chain of tryptophan was the main reaction[7]). The observed decomposition of tryptophan in some cases does not mean that iodine oxidation of a peptide containing this amino acid is precluded in these solvents. Indeed, the formation of the disulfide ring in the protected somatostatin derivative **17** (s. below *Scheme 7*), proceeded in high yield in methanol/DMF. The prerequisite is again that the reaction time should be kept short.

The sulfide group of methionine in **15** turned out to be stable in all solvents studied. The known oxidation to the sulfoxide requires much more vigorous conditions [27].

2.2. *Behaviour of Protecting Groups.* We studied the behaviour under the iodine oxidation conditions of the following permanent and temporary acid-sensitive protecting groups: *t*-butyl-oxycarbonyl (Boc), *t*-butyl esters (OBu^t), *t*-butyl ethers (Bu^t), *N*-trityl (Trt), and [1-(*p*-Biphenyllyl)1-methylethoxy]carbonyl (Bpoc). The permanent protective groups derived from *t*-butyl alcohol were not cleaved by the hydrogen iodide produced during the oxidation. The two temporary amino-protecting groups Trt and Bpoc, however, were partially or completely removed by this acid. The iodine oxidation therefore has to be carried out in the presence of

[7]) Results to be published.

a suitable base such as pyridine or sodium acetate [5b]. Recently, *Berndt* described the use of *N*-methylmorpholine for this purpose [28]. Moreover, when the isolation of the cystine peptide requires prior concentration of the reaction mixture instead of simple extraction or precipitation, it is recommended to reduce the excess of iodine in a buffered solution of the reducing agent (see exper. part).

2.3. *Behaviour of Unprotected Amino and Carboxyl Groups.* In an earlier publication we showed that the solvent methanol converted free carboxy groups in part to the methyl esters [3], but the reaction time of 1 h was much longer than that required for the iodine oxidation. With shorter times esterification was not observed.

Iodine oxidation with substrates containing unprotected amino groups could be carried out without substantial formation of by-products; but the resultant products, especially open-chain cystine peptides, are relatively unstable [5b]. Because their isolation and purification may be critical, the *N*-protecting group should better be removed after formation of the disulfide bond.

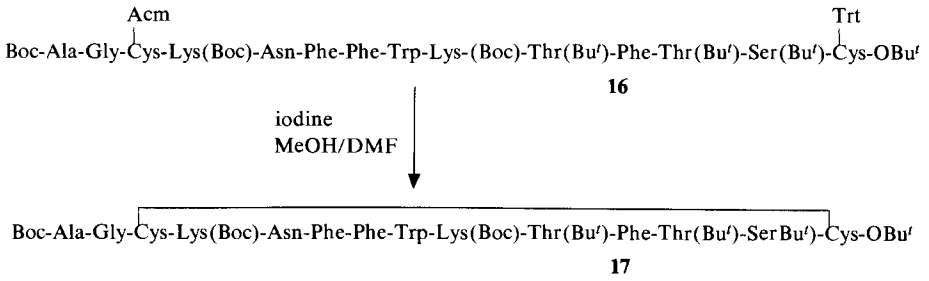
It is also feasible to apply the method to peptides carrying no further protecting groups other than those on the side chains of the cysteine residues. The last step in the synthesis of human insulin is an example [9]. The work-up consisted in a simple desalting of the reaction mixture (after reduction of excess iodine) on a *Sephadex* column. With smaller peptides this method will not be applicable in all cases. As we showed for the iodine oxidation of H-Leu-Cys(Trt)-Gly-OH, reversed-phase chromatography may be the method of choice [5e]; but as a rule it is probably in most cases more convenient to perform the iodine oxidation with small peptides carrying protective groups of the *t*-butyl type, whose removal proceeds without complications in the presence of cystine residues.

Finally, it has to be borne in mind that some of the customary methods of peptide synthesis can hardly be employed once disulfide bonds are present in the substrate. This is particularly true of any reactions requiring basic conditions, such as the hydrolysis of esters with alkali or the conversion of esters to their hydrazides. The use of the two newly developed protecting groups derived from 2-(trimethylsilyl)-ethanol, *i.e.* 2-(trimethylsilyl)-ethoxycarbonyl [29] and 2-(trimethylsilyl)-ethyl-esters [30], is also rendered difficult with cystine peptides. During the removal of these groups with fluoride ions, a rapid disulfide exchange takes place.

3. Preparative Examples. – The observations on the course of iodine oxidation described in section 1 and 2 are the results of experiments performed with very small quantities (micromoles) of the model compounds. The application of the method to the preparation of cystine peptides in gram quantities is exemplified in this final section (for details see exper. part).

3.1. The complete conversion of equimolar amounts of the *S*-acetamidomethyl derivative **2** and the *S*-trityl derivative **3** to **4**–**6**, as depicted in *Scheme 1*, was carried out in methanol. The products were separated by chromatography on silica gel. The asymmetrical cystine peptide **5** was isolated in 76% yield[4]), the symmetrical products **4** and **6** in 21 and 19%[4]), respectively. These results clearly show that simultaneous iodine oxidation of an *S*-trityl and an *S*-acetamidomethyl cysteine peptide in methanol (or another of the group I solvents, s. *Table 1*) is a very satisfactory method for the synthesis of open-chain asymmetrical cystine peptides.

Scheme 7. *Formation of the disulfide ring in the protected 1–14 somatostatin derivative* **16**

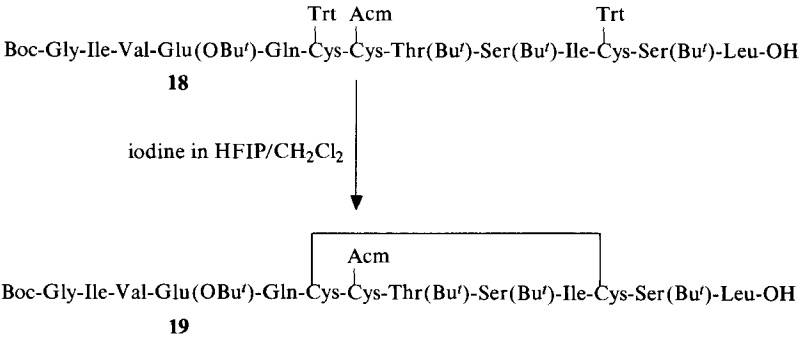


Moreover, it has to be considered that the more easily available of the two components can be used in excess, and the scarcer one consequently converted to the desired asymmetrical compound in a even higher yield.

3.2. The synthesis of cyclic cystine peptides is exemplified by the protected somatostatin **17** *(Scheme 7)*. As previously in the segment **9** of calcitonin (s. *Scheme 6*), one cysteine residue in the precursor **16** was protected by a trityl (position 14) and the second by an acetamidomethyl group (position 3). Cyclization of **16** was carried out in methanol/DMF 9:1 at a peptide concentration of 10^{-3} M with a 12-fold molar excess of iodine. The solution of the peptide was poured in one single portion into the solution of iodine, rather than added dropwise. For complete oxidation a reaction time of 5 min was sufficient. Pure protected somatostatin **17** was obtained after counter-current distribution in a yield of 78%. It is evident that the observed instability of the tryptophan residue *(Table 5)* doesn't seriously reduce the yield.

3.3 The protected A(1–13) segment **19** of human insulin *(Scheme 8)* was a key-intermediate in the total synthesis of this hormone [9]. Its preparation has been described previously [5d]. Compound **19** was obtained by selectively converting the two *S*-trityl cysteine residues in positions 6 and 11 to cystine, leaving the *S*-acetamidomethyl cysteine residue in position 7 unaffected. Previously, this selective iodine oxidation was carried out in a 6–13 segment, using TFE/methylene chloride

Scheme 8. *Selective formation of the disulfide ring A6–A11 in the protected A(1–13) segment* **18** *of human insulin*



as solvent. Now we performed this transformation with the tridecapeptide derivative **18** in HFIP/methylene chloride. Because of the extremely low solubility of **18**, sufficiently high concentrations could only be obtained in the very powerful solvent HFIP. This solution ($25.5 \cdot 10^{-3}$ M) was added to a 7fold molar excess of iodine in methylene chloride ($32.6 \cdot 10^{-3}$ M). After a reaction time of 6 min. excess iodine was reduced with ascorbic acid, and the product isolated. Purification by counter-current distribution, as previously reported, gave **19** in a yield of 68%.

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Experimental Part

1. General. – *HFIP vapours are very toxic; great caution is indicated when using this volatile solvent!* Melting points (m.p.) were determined in open capillaries and are uncorrected. For the preparation of protected amino acids and standard procedures in peptide synthesis, see [31]. Thin-layer chromatography (TLC.) was carried out on *Antec* or *Merck* silica gel plates, using the following solvent systems: 96 (=2-butanol/glacial acetic acid/water 67:10:23); 100 (=ethyl acetate/pyridine/glacial acetic acid/water 62:21:6:11); 157 (=chloroform/methanol/glacial acetic acid/water 70:42:0.5:10); 157A (like 157, but 90:10:0.5:1); 157B (like 157, but 85:13:0.5:1.5). The spots were visualized with *Reindel-Hoppe* reagent modified according to [32].

2. Preparation of New Protected Cysteine Derivatives and Two Other Model Compounds. – 2.1. *Boc-Cys(Acm)-Gly-Glu(OBu^t)$_2$* (**2**) was prepared from Boc-Cys(Acm)-OH (5.84 g, 20 mmol) and H-Gly-Glu(OBu^t)$_2$ [5b] (6.32 g, 20 mmol) in DMF (55 ml), using 1-hydroxybenzotriazole (2.70 g, 20 mmol) and dicyclohexylcarbodiimide (4.95 g, 24 mmol) in the usual manner. The product was chromatographed through a column of silica gel using ethyl acetate/methanol 19:1. After solvent evaporation 9.9 g (84%) of **2** were obtained as an amorphous solid, Rf 0.40 in chloroform/methanol 9:1, $[\alpha]_D^{20} = +18°$ ($c = 1.5$, chloroform).

$C_{26}H_{46}N_4O_9S$	Calc.	C 52.86	H 7.85	N 9.49	S 5.43%
(590.73)	Found	„ 52.57	„ 7.74	„ 9.37	„ 5.11%

2.2. *Preparation of the two protected 1–10 segments* **8** *and* **9** *of human calcitonin* followed the procedure used for the previously described compound **7** [5a]. Analytical data of new intermediates and of **8** and **9**. – 2.2.1. *Boc-Cys(Acm)-Gly-Asn-Leu-NH-NH$_2$.* Crystallization from acetonitrile, m.p. 170–177°, $[\alpha]_D^{20} = -49°$ ($c = 1.7$, methanol).

$C_{23}H_{42}N_8O_8S$	Calc.	C 46.77	H 7.17	N 18.97	S 5.43%
(590.70)	Found	„ 46.44	„ 7.33	„ 18.85	„ 5.17%

H-Ser(Bu^t)-Thr(Bu^t)-Cys(Acm)-Met-Leu-Gly-OH. Precipitation from methanol/water gave a white solid. Rf 0.45 in system 96, $[\alpha]_D^{20} = +22°$ ($c = 0.5$, DMF).

$C_{34}H_{63}N_7O_{10}S_2$ (794.04) Calc. N 12.35 S 8.08% Found N 12.27 S 7.78%

Boc-Cys(Acm)-Gly-Asn-Leu-Ser(Bu^t)-Thr(Bu^t)-Cys(Acm)-Met-Leu-Gly-OH (**8**). Precipitated from methanol/water. Rf 0.48 in system 157, $[\alpha]_D^{20} = -26°$ ($c = 0.5$, DMF).

$C_{57}H_{101}N_{13}O_{18}S_3$ (1352.69) Calc. N 13.46 S 7.11% Found N 13.29 S 7.04%

2.2.2. *Boc-Cys(Trt)-Gly-Asn-Leu-Ser(Bu^t)-Thr(Bu^t)-Cys(Acm)-Met-Leu-Gly-OH* (**9**). Purification by counter-current distribution in methanol/buffer[8])/chloroform/carbon tetrachloride 2.2:0.66:1.1:0.88; *K*-value 0.84. Rf 0.57 in system 157, $[\alpha]_D^{20} = -13°$ ($c = 0.5$, DMF).

$C_{73}H_{110}N_{12}O_{17}S_3$ (1523.93) Calc. N 11.03 S 6.31% Found N 11.02 S 6.03%

2.3. *Z-Trp-NH$_2$* (**14**). Z-Trp-OH (1.7 g, 5 mmol), dissolved in acetonitrile (20 ml), was converted in the usual manner to the mixed anhydride with isopropyl chloroformate (0.67 ml, 5 mmol), using *N*-ethylmorpholine (0.64 ml, 5 mmol) as a base. Bubbling a stream of ammonia through this solution and extraction with chloroform afforded 1.13 g (67%) of **14**. Recrystallization from ethyl acetate, m.p. 185–187°, $[\alpha]_D^{20} = -12°$ ($c = 1.0$, methanol).

$C_{19}H_{19}N_3O_3$ (337.38) Calc. C 67.64 H 5.68 N 12.46% Found C 67.48 H 5.61 N 12.39%

2.4. *Boc-Phe-Met-OMe* (**15**). Boc-Phe-OH (5.3 g, 20 mmol) was condensed according to standard procedures with HCl · H-Met-OMe (4.0 g, 20 mmol) in DMF (100 ml), using dicyclohexylcarbodiimide (4.5 g, 22 mmol), 1-hydroxybenzotriazole (4.0 g, 30 mmol), and *N*-ethylmorpholine (2.55 ml, 20 mmol). Two recrystallizations of the crude material from methanol/water gave 5.9 g (72%) of **15**, m.p. 75–76°, $[\alpha]_D^{20} = -21°$ ($c = 1.9$, methanol).

$C_{20}H_{30}N_2O_5S$	Calc.	C 58.51	H 7.37	N 6.82	S 7.81%
(410.53)	Found	„ 58.50	„ 7.44	„ 6.82	„ 7.95%

For the preparation of the methionine sulfoxide derivative, 1 mg of **15** was dissolved in 0.08 ml of methanol and treated with 0.02 ml of 35% H_2O_2-solution. Rf 0.50 in chloroform/methanol 9:1 (**15**: Rf 0.75).

2.5. *Protected 1–14 Somatostatin Derivative: Boc-Ala-Gly-Cys(Acm)-Lys(Boc)-Asn-Phe-Phe-Trp-Lys(Boc)-Thr(Bu^t)-Phe-Thr(Bu^t)-Ser(Bu^t)-Cys(Trt)-OBu^t* (**16**) was prepared by conventional fragment-condensation procedures. The details of this synthesis will be published elsewhere. Rf of **16** 0.21 in system 157A and 0.45 in 157B. Amino acid analysis (samples were hydrolyzed at 110° for 24 h with 6N hydrochloric acid): Lys 1.93 (2), Asp 1.00 (1), Thr 1.85 (2), Ser 0.62 (1), Gly 1.07 (1), Ala 1.08 (1), Cys 1.14 (2), Phe 3.09 (3).

2.6. *A (1–13) Human Insulin: Boc-Gly-Ile-Val-Glu(OBu^t)-Gln-Cys(Trt)-Cys(Acm)-Thr(Bu^t)-Ser(Bu^t)-Ile-Cys(Trt)-Ser(Bu^t)-Leu-OH* (**18**). To a solution of Boc-Gly-Ile-Val-Glu(OBu^t)-Gln-NH-NH$_2$ [5d] (2.82 g, 4.8 mmol) in 80 ml of DMF at −18° were added 6.0 ml of 2.4N HCl in ethyl acetate and *t*-butyl nitrite (0.63 ml, 5.3 mmol). After 15 min at −15° a solution of HCl · H-Cys(Trt)-Cys(Acm)-Thr(Bu^t)-Ser(Bu^t)-Ile-Cys(Trt)-Ser(Bu^t)-Leu-OH [5d] (7.0 g, 4 mmol) and *N*-ethylmorpholine (3.18 ml, 25.2 mmol) in 40 ml DMF was added. This solution was stirred for 1 h at −10° and for 15 h at 0°, producing a heavy gelatinous precipitate. The mixture was poured into 300 ml of 0.01N HCl at 0°, stirred well, the precipitate collected, washed with water, and dried. The product was triturated twice with warm DMF (30 ml), filtered, washed with ether and dried to yield 7.37 g (82%) of **18**. TLC. analysis showed that impurities had been completely removed by this extraction. The very poorly soluble **18** (clear solutions could only be prepared in HF1P) did not move on TLC. in any of the solvent systems used. Amino acid analysis (samples were hydrolyzed for 24 h with 6N hydrochloric acid): Thr 1.01 (1), Ser 1.71 (2), Glu 1.99 (2), Gly 1.00, Cys 3.16 (3), Val 0.83 (1), Ile 1.55 (2), Leu 1.09 (1).

3. Micro-methods for the Determination of the Course of Iodine Oxidation. – The iodine oxidations leading to the results summarized in *Tables 1–5* were carried out in small test-tubes with 1.0 μmol or 0.5 μmol of the different model compounds. The samples were obtained by evaporating an aliquot part of the peptide solution in chloroform. The composition of the reaction product was determined by comparing the TLC. with those of a series of samples containing the corresponding starting materials and products in known ratios. The procedure is illustrated for the determination of the 'half-times'. All other experiments were carried out in essentially the same fashion.

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[8]) Preparation of the buffer: 19.3 g of ammonium acetate and 28.6 ml of glacial acetic acid were diluted with water to 2 l.

'Half-times' of Iodine Oxidation of **1** *and* **2** (s. *Table 1*). To a series of stirred solutions of 1 μmol of **1** or **2** in 0.14 ml of the indicated solvent were added 0.06 ml of 0.1N iodine in the same solvent. After appropriate times the reaction was stopped by adding 0.1 ml of 0.01N sodium thiosulfate in water. The mixture was extracted with 0.6 ml of chloroform, and the organic layer washed with 0.1 ml of water, dried over anhydrous sodium sulfate and evaporated to dryness. The samples were dissolved in 0.045 ml of chloroform and compared by TLC. with mixtures of **1** or **2** and **6** of known ratios. In this way, the time required for the conversion of 50% of the starting material was determined. Rf (chloroform/methanol 9:1) 0.75 for **1**, 0.40 for **2**, and 0.60 for **6**.

4. Iodine Oxidations in Microsamples. – 4.1. *Iodine Oxidation of* **3** *in the Presence of* **2** (s. *Scheme 1, Table 2*). A solution of 0.5 μmol of **2** and 0.5 μmol of **3** in 0.2 ml of the solvent was treated with 3 μmol of iodine. As above, the time at which **3** had disappeared was determined in a series of samples. It ranged from 30 s to 2 min, depending on the solvent used. The composition of the mixture after this time was determined. Rf (ethyl acetate) 0.12 for **2**, 0.65 for **3**, 0.53 for **4**, 0.42 for **5**, and 0.30 for **6**.

4.2. *Iodine Oxidation of* **1** *in the Presence of Cystine Peptide* **4** (s. *Scheme 4, Table 3*). A solution of 1 μmol of **1** and 0.5 μmol of **4** in 0.2 ml of solvent was treated with 3 μmol of iodine. Reaction time: 5 min TLC. in ethyl acetate as above.

4.3. *Disproportionation of* **5** *by Iodine* (s. *Scheme 5, Table 3*). A solution of 1 μmol of **5** in 0.2 ml of solvent was treated with 6 μmol of iodine for 4 h. TLC. in ethyl acetate.

5. Stability of Various Amino Acids under the Conditions of Iodine Oxidation (s. *Table 5*). – With the model compounds **11**–**15**, the extent of formation of by-products during treatment with iodine alone or during iodine oxidation of **3** to **4** was determined. For this purpose, either 1 μmol of the respective compound in 0.2 ml of solvent was exposed to 3 μmol of iodine, or the oxidation of 1 μmol of **3** was carried out in the presence of the compound. Reaction time: 30 min. Rf (toluene/acetone 1:1) 0.55 for **4**, 0.65 for **11**, 0.85 for **12**, 0.05 for **13**, 0.30 for **14**, 0.70 for **15**, and 0.10 for the methionine sulfoxide derivative of **15**.

6. Cyclic Cystine Peptides. – *Preparation of* **10** (s. *Scheme 6, Table 4*). Iodine oxidation of **7**–**9** was carried out under strictly identical conditions at two concentrations (c_1 and c_2). The reactions were performed in two ways: the solution of the peptide was either added to a vigorously stirred solution of iodine or *vice versa*. The following solutions were used: Solution A (peptide) in methylene chloride/methanol/water 6.0:2.5:0.42: 2 μmol of **7**, **8** or **9** (3.4 mg of **7**, 2.7 mg of **8**, 3.0 mg of **9**) in 2.0 ml for concentration c_1, and in 0.4 ml for concentration c_2. Solution B (iodine) in methylene chloride/methanol 8.0:1.5: 2.03 mg (8 μmol) of iodine in 2 ml for c_1, and in 0.4 ml for c_2. The two solutions were mixed dropwise, with in approximately 50 s at c_1 and 20 s at c_2. After 10 min 2 ml of an aqueous solution of 0.01N sodium thiosulfate was added and the mixture then extracted with 5 ml of chloroform. The organic layer was washed twice with 2 ml of water, dried over sodium sulfate and evaporated. For analysis on TLC. the residue was taken up in 0.24 ml of chloroform/methanol 1:1. For the evaluation of the yield the samples were compared with serial dilutions of **10**. Rf (system 100) 0.70 for **7**, 0.30 for **8**, 0.45 for **9**, and 0.37 for **10**.

7. Preparative Examples. – 7.1. *Iodine Oxidation of an Equimolar Mixture of* **2** *and* **3** (s. *Scheme 1*). To a solution of **2** (3.36 g, 4 mmol) and **3** (2.59 g, 4 mmol) in 10 ml of methanol, iodine (5.1 g, 20 mmol) in 50 ml of methanol was added in one portion. After 10 min the solution was partitioned between chloroform (600 ml) and 0.5N sodium thiosulfate (100 ml), the organic phase washed with water (3 100-ml-portions), dried over sodium sulfate, and the solvent removed. The residue was chromatographed on silica gel (170 g, 3.6×55 cm) with ethyl acetate/petroleum ether 1:1. After 150 ml, which contained trityl methyl ether (995 mg, 3.8 mmol), elution was continued with ethyl acetate/petroleum ether 9:1, 60-ml fractions being taken. Fractions 1–5 contained **4** [5e] (340 mg, 0.42 mmol), fractions 6–11 **5** (2.80 g, 3.04 mmol) and fractions 12–17 **6** [3] (405 mg, 0.39 mmol). **5** was recrystallized from methanol/water, m.p. 133–135°, $[\alpha]_D^{20} = +14°$ ($c = 0.35$, chloroform).

$C_{40}H_{70}N_6O_{14}S_2$	Calc.	C 52.04	H 7.64	N 9.10	S 6.95%
(923.15)	Found	„ 51.92	„ 7.57	„ 8.96	„ 6.76%

In a random oxidation producing the cystine peptides **4–6**, the statistical yield of **5** would have been 2 mmol.

7.2. *Protected Somatostatin Derivative* **17** (s. *Scheme 7*). A solution of **16** (24.8 g, 0.01 mol) in 1 l of DMF was poured, in one portion (requiring about 30 s), into a well-stirred solution of iodine (30.5 g, 0.12 mol) in 9 l of methanol. After 5 min a solution of ascorbic acid (23 g) in 2 l of citrate buffer (pH 5)[9]) was added, producing within about 15 s a colourless and slightly turbid solution. This solution was concentrated to about 1.5 l on a rotary evaporator at a bath temp. of 45°. The resultant suspension was diluted with 2 l of water and stirred at 0° for 1 h yielding a white precipitate. It was collected by filtration and dried. The resultant white solid was triturated 3 times with ether (150 ml) and filtered. The product was then purified by counter-current distribution using methanol/buffer[10])/chloroform/carbon tetrachloride 40:13.3:23.1:10. After 990 steps **17** was in tubes Nos. 160–215, $K = 0.23$. The contents of these tubes were collected, the solvent evaporated *in vacuo*, and the residue lyophilized from *t*-butyl alcohol yielding 16.8 g (77.7%) of **17** as a white solid. Rf 0.30 in system 157A. Amino acid analysis (samples were hydrolysed at 110° for 24 h with 6N hydrochloric acid): Lys 1.88 (2), Asp 1.00 (1), Thr 1.81 (2), Ser 0.89 (1), Gly 1.04 (1), Ala 0.99 (1), Cys 1.55 (2), Phe 2.72 (3).

7.3. *Protected A(1–13) Segment of Human Insulin* **19** (s. *Scheme 8*). A solution of **18** (10.28 g, 4.6 mmol) in HFIP (180 ml) was added dropwise within 5 min to a stirred solution of iodine (8.2 g, 32.2 mmol) in HFIP (85 ml) and methylene chloride (900 ml). After further 6 min the mixture was poured into a cold (0°) solution of ascorbic acid (26.7 g) and ammonium acetate (19.25 g) in 750 ml of water. The organic solvents were removed from the colourless mixture on a rotary evaporator, and the aqueous suspension extracted with 1 l of chloroform/methanol 9:1 in 3 portions. The organic solution was washed twice with 200 ml of water and dried over sodium sulfate, and the solvent evaporated. The residue obtained was purified by counter-current distribution as previously described [5d] to give 5.49 g (68.2%) of pure **19** as a white solid.

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[9]) Preparation of the buffer: 84 g of citric acid hydrate, and 400 ml of 2N sodium hydroxide were diluted with water to 2 l.

[10]) Preparation of the buffer: 19.25 g of ammonium acetate, and 570 ml of glacial acetic acid were diluted with water to 2 l.

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