3,6-bis(trimethylgermyl)-, and 3-*tert*-butyl-6-(trimethylsilyl)cyclohexa-1,4-dienes were prepared by the general method of Eaborn et al., where the corresponding 1,4-disubstituted benzenes were reduced electrolytically in methylamine in the presence of LiCl.[30]

3-Deuterio-6-(trimethylgermyl)cyclohexa-1,4-diene: NMR (60 MHz, CCl_4) δ 0.22 (s, 9), 2.48 (m, 1), 2.72 (m, 1), 5.59 (m, 4); MS *m/e* 199 (M^+). The deuterium content was determined as 86% by MS analysis.

3-*tert*-Butyl-6-(trimethylsilyl)cyclohexa-1,4-diene. The reduction of 1-*tert*-butyl-4-(trimethylsilyl)benzene gave a mixture of two isomeric cyclohexadienes, the title compound and 1-*tert*-butyl-4-(trimethylsilyl)-cyclohexa-1,4-diene (1:0.9 determined by NMR). NMR of the former (60 MHz, CCl_4): δ 0.08 (s, 9), 0.96 (s, 9), 2.32 (m, 1), 2.57 (m, 1), 5.68 (m, 4). NMR of the latter (60 MHz, CCl_4): δ 0.12 (s, 9), 1.10 (s, 9), 2.73 (m, 4), 5.54 (m, 1), 6.06 (m, 1). The mixture was used satisfactorily for ESR experiments.

3,6-Bis(trimethylgermyl)cyclohexa-1,4-diene. The electrolytic reduction of 1,4-bis(trimethylgermyl)benzene afforded 31% of the title compound: bp 65-90 °C (7 mmHg); NMR (60 MHz, CCl_4) δ 0.21 (s, 18), 2.52 (m, 2), 5.42 (br s, 4); MS m/e 314 (M^+). The GLC analysis indicated the presence of two isomers with a ratio of 69/31 which may be assigned to the cis-trans isomers.

3,6-Bis(trimethylsilyl)cyclohexa-1,4-diene was prepared from benzene, Me_3SiCl, and lithium in THF.[31] From the mixture with 1,4-bis(trimethylsilyl)benzene, the desired product was separated by TLC, mp 50-51 °C (lit. mp 50-51 °C). The purified material was used for both ESR and the product studies of the reaction with di-*tert*-butyl peroxide.

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ESR Spectroscopy. ESR spectra were recorded with Varian E-12 equipment (9.5-GHz frequency, 3300-G magnetic field, and field modulation 100 KHz) and are calibrated with the anthracene cation radical in concentrated H_2SO_4.[32] Photolyses were carried out in a cavity of the ESR spectrometer with a 500-W Ushio Super-high-pressure mercury lamp. Temperatures were controlled by a Varian's variable temperature accessory and calibrated with a Cu-constantan thermocouple.

Reaction of 3,6-Bis(trimethylsilyl)cyclohexa-1,4-diene and Di-*tert*-butyl Peroxide. Thermal reaction: A mixture of 3,6-bis(trimethylsilyl)cyclohexa-1,4-diene (0.464 mmol) and DTBP (0.464 mmol) was sealed in a Pyrex glass under argon and was heated in an oil bath at 130 ± 10°C for 6.5 h.

Photoreaction: A mixture of 3,6-bis(trimethylsilyl)cyclohexa-1,4-diene (0.454 mmol) and DTBP (0.473 mmol) in *n*-hexane (1 mL) in a quartz tube was photolyzed by a high-pressure mercury lamp for 3.5 h at 0 °C.

The products were analyzed on a Hitachi K-53 GLC with an Apiezon L (30%, 2 m) or a SE 30 (20%, 2 m) column.

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Registry No. 1a, 79585-24-3; **1b**, 79585-26-5; **1c**, 79585-25-4; **2**, 12169-67-4; **3**, 87088-28-6; **4**, 87088-27-5; **5**, 87088-29-7; **6**, 63470-25-7; **7**, 86472-63-1; **8**, 87012-82-6; **9**, 18090-43-2; 3-deuterio-6-(trimethylgermyl)cyclohexa-1,4-diene, 87012-81-5; 1-*tert*-butyl-4-(trimethylsilyl)-cyclohexa-1,4-diene, 87012-83-7; *cis*-3,6-bis(trimethylgermyl)cyclohexa-1,4-diene, 87012-84-8; *trans*-3,6-bis(trimethylgermyl)cyclohexa-1,4-diene, 87012-85-9.

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S_N2 Deprotection of Synthetic Peptides with a Low Concentration of HF in Dimethyl Sulfide: Evidence and Application in Peptide Synthesis[1]

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Abstract: An S_N2 deprotection reaction for synthetic peptides was observed when the weak base dimethyl sulfide was used as a diluent for HF. Kinetic studies of the deprotection of *O*-benzylserine revealed that there was a sharp changeover in mechanism from $A_{AL}1$ to $A_{AL}2$ when the concentration of HF in dimethyl sulfide was below 55%. The changeover in mechanism was also found in the deprotection of *O*-benzyltyrosine. At higher HF concentrations (>55%), the $A_{AL}1$ cleavage mechanism, which generates carbonium ions, led to significant 3-benzyltyrosine side product. However, at low HF concentrations, the side product was minimal as a result of an $A_{AL}2$ cleavage mechanism in which carbonium ions are not formed. A sharp increase of side product was seen when the HF concentration reached the critical changeover concentration. The HF-dimethyl sulfide reagent was also found to reduce methionine sulfoxide to methionine and, in the presence of a thiol, to deprotect N^{in}-formyltryptophan to tryptophan. Both of these reactions were also dependent on the concentration of HF and were optimal at low concentrations. Furthermore, deprotection of aspartic and glutamic acid side chain benzyl esters at the low HF concentration also minimized the $A_{AC}1$ mechanism and the accompanying acylation side reactions. A practical mixture for the S_N2 deprotection reaction was found to be HF-dimethyl sulfide-*p*-cresol (25:65:10 v/v). For the deprotection of Trp(For)-containing peptides, the reagent was adjusted to HF-dimethyl sulfide-*p*-cresol-*p*-thiocresol (25:65:7.5:2.5 v/v) so that the N^{in}-formyl could be removed concomitantly with other protecting groups. The low-acidity function, S_N2 reaction was also effective for solid-phase peptide synthesis. The same protecting groups were removed as in solution, and in addition the bond holding the peptide to the resin support was cleaved. For more resistant anchoring bonds and protecting groups a combined low-high HF procedure was developed, in which most of the precursors of harmful carbonium ions are removed by a S_N2 mechanism before the final strong-acid, S_N1, step begins. The new deprotection procedure was tested on three synthetic model peptides, methionine-enkephalin, bovine growth hormone fragment (128-131), and C-terminal pentagastrin amide, and was found to provide efficient deprotection and significant reduction in the level of alkylation side reactions, the rearrangement to aspartimide, and the acylation of aromatic scavengers by glutamic acid.

The chemical synthesis of peptides, whether in solution or in solid phase, requires a final step in which all protecting groups and polymeric supports are removed.[2] For this purpose, many methods have been developed, all aiming for a method with

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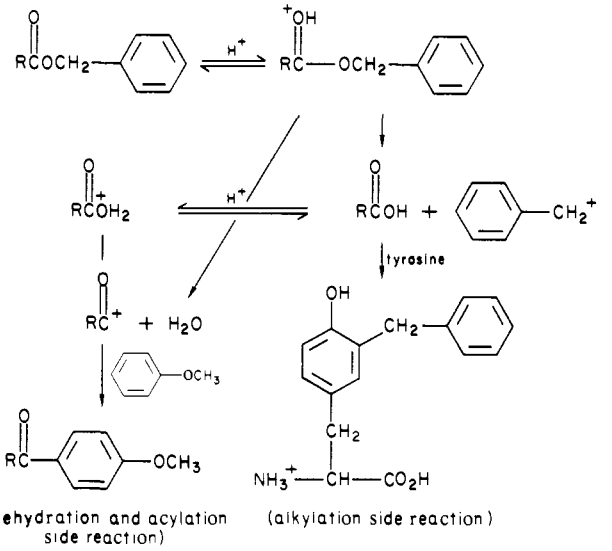
Scheme I. Side Reactions from S_N1 Strong-Acid Cleavage Mechanism



maximal efficiency and minimal side reactions.[3] Among these methods, e.g., sodium in ammonia reduction, hydrogenation, electrolysis, oxidation–reduction, acidolysis, and base-catalyzed elimination, the deprotection by hydrogenation has usually been considered to be the mildest and most suitable for small and relatively simple synthetic peptides. For large and complex synthetic peptides, this method is seldom used, although recent use of liquid ammonia[4] or of transfer hydrogenation in formic acid[5] may improve its generality.

By far, the most popular method of deprotection is by acids, which are often the best solvents for the dissolution of the synthetic protected peptides. The choice of the proper acid, whether a strong acid such as HF or a mild acid such as trifluoroacetic acid, however, remains a controversy. Although trifluoroacetic acid causes fewer side reactions, HF allows the use of a combination of the *tert*-butoxycarbonyl group for N^{α} protection and benzyl alcohol derivatives for side-chain protection, a strategy that would continue to be useful if improvements in the HF removal of protecting groups could be advanced.

Anhydrous HF, a strongly protonating acid with an acidity function (H_o) of −10.8,[6] is nonoxidizing, highly volatile, and an excellent solvent for peptides.[7] In general, anhydrous HF removes all the usual protecting groups efficiently but also can cause serious alkylation and acylation side reactions. The underlying reason for these opposing effects is the strong-acid property of HF, which removes protecting groups by an S_N1 mechanism. This can be illustrated by the HF deprotection of glutamic or aspartic benzyl ester side chains (Scheme I). In an S_N1 process there is a heterolytic alkyl–oxygen cleavage of the protonated benzyl ester ($A_{AL}1$ mechanism)[8] to give the carboxylic acid and the benzyl carbocation. Usually a scavenger such as anisole is present to capture this reactive byproduct. In some cases the nucleophilic side chains of tyrosine, methionine, tryptophan, and cysteine compete with the scavengers for the carbocations, leading to alkylation side reaction.[9-11] These side reactions are well documented and can be minimized by various methods such as modified protecting groups[9] and a combination of scavengers.[1d] Furthermore, anhydrous HF is such a strong protonating acid that the resulting carboxylic acid is again protonated leading to an acyl–oxygen cleavage to give an acylium ion and H_2O ($A_{AC}1$ mechanism). The acylium ion can also be generated directly from the benzyl ester. The resulting acylium ion often forms irreversible side products. In the case of glutamic acid, the acylium ion reacts with the anisole to form a ketone adduct,[12] and in aspartic acid it cyclizes to form aspartimide.[13,14] Thus, there is clearly a need to modify the HF deprotecting procedure so that these two major types of side reactions can be minimized.

In the past few years, our laboratory has undertaken to improve the HF deprotection procedure using a different approach. We recognize that the deprotecting mechanism as well as the resulting side reactions are a consequence of the S_N1 type of mechanism ($A_{AL}1$ and $A_{AC}1$). If the HF reaction could be modified to proceed by an S_N2 mechanism ($A_{AL}2$ and $A_{AC}2$), in which carbocations are not generated, the alkylation and acylation side reactions should be prevented. In this paper, we describe the development of a new HF deprotection method, the evidence supporting the

Table I. Basicities of Protecting Groups of Synthetic Peptides

functional group	representative amino acid	protecting group	pK_a[a]
amine	lysine	amide	−0.36 to 1.80[b]
amine	lysine	carbamate	−2.80[c]
carboxylic acid	glutamic acid	ester	−4.61[d]
hydroxyl	serine	ether	−2.39[e]
	tyrosine	carbonate	−2.50[f]
mercaptan	cysteine	thioether	−6.80[g]
thioether	methionine	sulfoxide	−1.80[h]

[a] pK_a represents the value of the conjugate acid BH^+ corresponding to the base B of the protecting group under consideration. The values referred to standard state in H_2O at 25 °C. Some of the pK_a values given in the table are not those of the protecting groups but are of structurally related compounds that contain the representative functionalities. [b] Taken from ref 17. [c] Taken from ref 19. [d] Taken from ref 17 and 18. [e] Taken from ref 20. [f] Estimated from O-protonation of an analagous carbamate compound. See ref 19. [g] Taken from ref 21. [h] Taken from ref 20.

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view that the reactions proceed by an S_N2 mechanism, and the application of such technique in peptide synthesis.

Results and Discussion

(I) Rationale for the S_N2 Deprotection Mechanism. In a synthetic peptide, the hydroxyl, thiol, carboxyl, and amino functional groups, after being derivatized by the various benzyl-derived protecting groups, become weak organic bases: ethers, thioether, esters, and carbamates. In other words, the normal aqueous titratable functional groups of the peptides have been transformed into weak bases that have pK_a values ranging from 0 to −7 as defined by the Brønsted acid–base equilibrium.[15,16] Table I lists the approximate pK_a values of the protected functionalities in a synthetic peptide.[17-21]

The weak proton affinity of the protecting groups will require a strong acid for their conversion to the conjugate acids. Anhydrous trifluoroacetic acid with an H_0 of −3.3[6] can only partially protonate some of the protecting groups. This is reflected in their relative stability in this acid. Acids with intermediate acid strength such as HBr, whose H_0 is estimated to be −8.0,[22] are strong enough to protonate and remove most of these protecting groups. However, the rate of removal is relatively slow compared with HF, whose acidity function approaches −11. Thus, in anhydrous HF, the protonation of these weak bases is measurably strong, and the Brønsted acid–base equilibrium favors the conjugate acid.

For the acid-catalyzed removal of protecting groups, reversible protonation of the Brønsted weak base by a strong acid to the conjugate acid is the initial step. In an S_N1 process this is followed by a heterolytic alkyl–oxygen cleavage reaction that proceeds via transition states with high carbocation character to ion pair intermediates.[17] Such transition states require the high ionic character of a strongly acidic solution for the stabilization of ion pair intermediates. Thus S_N1 cleavage is usually found in strongly acidic conditions and hence in high concentrations of HF. In an S_N2 process, reactions are usually characterized by reduced carbocation character in the transition state and are aided by the assistance of a nucleophile in the heterolysis of the carbon–oxygen bond. Operationally, the S_N2 reaction will therefore occur at lower acid concentrations and at a lower acidity function than S_N1 reactions. Both of these conditions make S_N2 reactions less prone to side reactions and more desirable as a deprotection mechanism.

In all HF-catalyzed cleavage reactions, only S_N1 deprotection mechanisms have been reported. This is in contrast to HBr-catalyzed cleavage reactions in which the S_N2 deprotection mechanism predominates. The difference between these two acids in their cleavage mechanism is probably due to the fact that the F^- counter ion of HF is very weakly nucleophilic, since it is strongly hydrogen bonded to other HF molecules. Addition of salts such as sodium iodide might confer an S_N2 mechanism but an excessive amount would be required to bring the acidity function of HF to the desirable level. Even so, the byproduct of such reaction, e.g., benzyl iodide, is a strong alkylating agent and is highly undesirable.

We have sought an organic weak base that can act as a solvent for the dilution of HF to lower its acidity function and as a nucleophile in the presence of HF so that the reaction mechanism can be changed to the S_N2 type and at the same time produce a byproduct that is relatively inert and nonalkylating. One important criterion for the selection of such a weak base is that it must be a weaker Brønsted base than the protecting groups, so that it remains partly unprotonated and nucleophilic while the protecting groups are largely protonated and susceptable to cleavage. Sulfides and thiols are both weak organic bases with pK_a's usually 2 units below the common weak bases of the protected functional groups (Table I). It has been demonstrated that thiols and sulfides when complexed with protic acids or Lewis acids[23] are capable of acting as nucleophiles for acid-catalyzed displacement reactions. Furthermore, the expected byproducts will be sulfides or sulfonium salts, which are relatively inert in the highly acidic medium. Dimethyl sulfide, which has a pK_a of −6.8[18] is highly volatile and easily removed after the deprotection reaction, satisfies our criteria for such an organic weak base. Our intention was to use dimethyl sulfide both as a diluent to reduce the acidity function of HF, so the S_N1 reaction is not favored, and as a nucleophile so that the S_N2 mechanism will dominate. A binary mixture of 1:1 molar ratio of HF–DMS solution (HF 25% and DMS 75% by volume) was determined by Hammett indicators to have an H_0 between −4.6 and −5.2, an acidity function strong enough to protonate most protecting group weak bases. Since dimethyl sulfide has a pK_a of −6.8, most of it will be expected to be free, unprotonated, and nucleophilic. This is consistent with the IR studies by Adam and Katz.[24] Thus, this binary mixture should be a suitable S_N2 deprotecting reagent. This is in contrast to the usual high-concentration HF reagent that is maintained at >90% HF and contains 2–10% of anisole, thiols, or sulfides in a fully protonated state. In this paper an HF–DMS–*p*-cresol mixture is recommended as the low HF cleavage reagent.

(II) Evidence for the S_N2 Mechanism. (A) Kinetic Studies of Deprotection of *O*-Benzylserine. Acid-catalyzed reactions, either S_N1 or S_N2, can be distinguished kinetically by following the rates of the reaction over a wide range of acid concentration. A change between two coexisting mechanisms will usually give a sharp upward break in the rate constant as the second mechanism becomes predominant. This was seen in the investigation by Yates and McClelland on the kinetics of the acid hydrolysis of alkyl and aryl acetates in aqueous sulfuric acid media.[25] For the secondary alkyl and benzyl acetates, the rate constant rose slowly with increasing acid concentration and reached a local maximum, characteristic of the $A_{AC}2$ mechanism, but then, before the rate had fallen to zero, the rate rose again very steeply due to the rapidly falling water activity, and the mechanism changed to $A_{AL}1$. The qualitative distinction for the mechanistic acid-rate profile between an S_N1 and S_N2 type of cleavage by strong acid is that the latter occurs at low acid concentrations with a slow increase in rate with increasing acid concentration, and the S_N1 reaction is found at high acid concentrations with the rate rising very steeply.

The changeover from an S_N2 to an S_N1 mechanism over a wide range of acid concentrations is not limited to the sulfuric acid hydrolysis of the acetates. Said and Tillett[26] have recently reported that a changeover in mechanism is observed with the perchloric acid hydrolysis of phenyl acetate. Homer et al.[27] have found that the rate increase of the HBr- and H_2SO_4-catalyzed removal of benzyloxycarbonyl groups from *N*-[benzyloxycarbonyl]glycine ethyl ester in acetic acid is less steep with HBr than with H_2SO_4. This is ascribed to the involvement of the more nucleophilic

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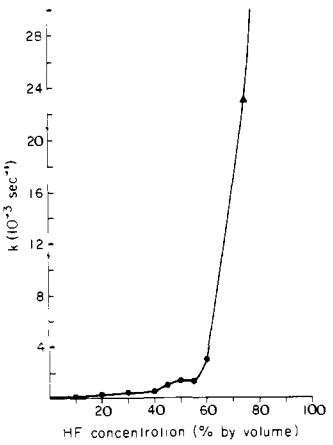
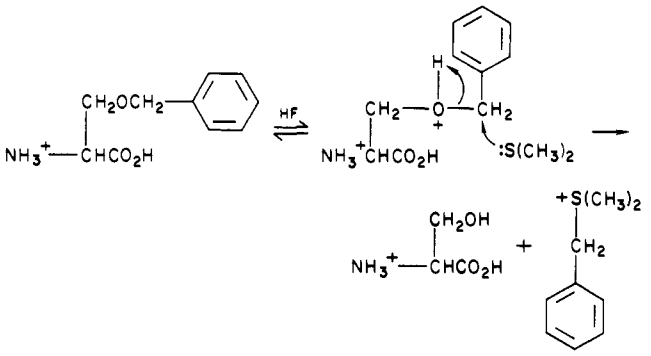


Figure 1. Plot for the apparent first-order rates of deprotection of Ser(Bzl) in different concentrations of HF in dimethyl sulfide at 0 °C: (●) calculated from initial rates; (▲) calculated from best estimates of average rates (see text).

Scheme II. An S_N2 Cleavage Mechanism of Ser(Bzl) by the Low Concentration of HF in Dimethyl Sulfide



bromide ion in the rate-determing step of the S_N2 mechanism. The rate acceleration by a weak base during the acid-catalyzed deprotection has been also observed. Brady et al. have found that dimethyl sulfide in trifluoroacetic acid removed benzyl protecting groups 4-fold faster than trifluoroacetic acid alone.[28] Similar observations with sulfides in trifluoroacetic acid alone or with trifluoromethanesulfonic acid–trifluoroacetic acid have been reported by Kiso et al. and by Yajima et al.[23]

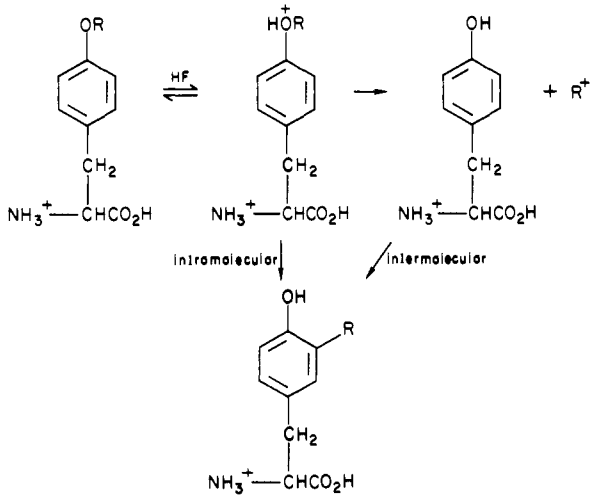
A test of our proposed S_N2 cleavage method using HF–DMS on benzyl protecting groups will presumably be fulfilled if a changeover in mechanism can be observed as the HF concentration is varied. For this purpose, we have chosen the deprotection of Ser(Bzl) as a test model (Scheme II). The cleavage of the benzyl ether protecting group in HF–DMS can only be achieved by an alkyl–oxygen cleavage either through an $A_{AL}1$ or an $A_{AL}2$ mechanism. The kinetic response of the deprotection of Ser(Bzl) to changes in concentration of HF in DMS is shown in Figure 1. It can best be accomodated by two distinct acid–rate profiles similar to the sulfuric acid hydrolysis of benzyl acetate. When HF was below 50%, deprotection was slow and the rate change with concentration was 0.013×10^{-3} s^{-1}/vol %. At high HF concentration (>50%) the initial rate of deprotection of Ser(Bzl) increased much more rapidly with rising HF concentration (Table II). The initial rate of deprotection at 50% HF was found to be 1.35×10^{-3} s^{-1} but increased to 3.01×10^{-3} s^{-1} at 60%. The initial rate was too fast to be measured accurately above 60% but the average rate was calculated to be 23×10^{-3} s^{-1} at 75%, 42×10^{-3} s^{-1} at 80% and 60×10^{-3} s^{-1} at 90% HF. The slope between 60 and 90% was 1.9×10^{-3} s^{-1}/vol %. Such a sudden break in the rate–HF concentration profile is strongly suggestive of a changeover from an S_N2 to an S_N1 mechanism. More precisely, it is a changeover from an $A_{AL}2$ to an $A_{AL}1$ mechanism. There is a mixed mechanism in the range 40–60%, with a crossover at approximately 55% HF–45% DMS.

Table II. Rate Constants[a] for the Deprotection of *O*-Benzylserine at 0 °C in HF–DMS Mixtures

reagents, vol %			
HF	DMS	10^4k, s^{-1}	$t_{1/2}$, min
10	90	1.69	68
20	80	2.70	43
30	70	4.29	27
40	60	5.66	20
45	55	10.1	11
50	50	13.5	8.5
55	45	13.2	8.7
60	40	30.3	3.8
75	25	230[b]	0.50
90	10	600[b]	0.19

[a] Apparent first-order initial rate constants were determined from plots of ln $[a/(a-x)]$ vs. time where a is the starting concentration of Boc-Ser(Bzl) and x is the serine concentration at a given time. The time points were taken from the first 25 min or from the initial 10% of the reactions. [b] Estimated from average rate (see text).

Scheme III. *O*-Benzyltyrosine Side Reaction during HF Deprotection



The changeover in mechanism of HF–DMS is consistent with our strong acid/weak base explanation. Since DMS has a pK_a of −6.8[29] and the effective acidity function of the 1:1 binary mixture (−4.6 to −5.2) was well below this value, DMS was still largely unprotonated and available for the nucleophilic participation required by an $A_{AL}2$ cleavage mechanism. As the HF concentration rises above 50%, DMS becomes strongly protonated, its activity falls, and the $A_{AL}1$ mechanism predominates. Furthermore, as the acidity increases the substrate is more strongly protonated, and the cleavage rate rapidly rises.

(B) Alkylation Side Reaction. Further evidence for the S_N2 deprotection mechanism was obtained from the deprotection of *O*-benzyltyrosine (Scheme III). The normal HF cleavage of *O*-benzyltyrosine produces tyrosine and a benzyl cation. The tendency of the resulting benzyl cation to alkylate the 3-position of the phenolic ring of tyrosine is a direct result of an S_N1 deprotection.[1d,9] The 3-benzyltyrosine side product amounts to approximately 20% during the usual HF procedure (e.g., HF–anisole, 9:1, v/v). Attempts to lower the amount of alkylation by reducing HF concentration to 60–70% failed and, in fact, the side product actually increased to about 40%.[1d,30] These results

(28) Brady, S. F.; Hirschmann, R.; Veber, D. F. *J. Org. Chem.* **1977**, *42*, 143–146.

(29) We have used −5.3 for the H_o of dimethyl sulfide previously (see ref 1); however, after correction by the Bunnett and Olsen treatment, the acidity function will be −6.8.

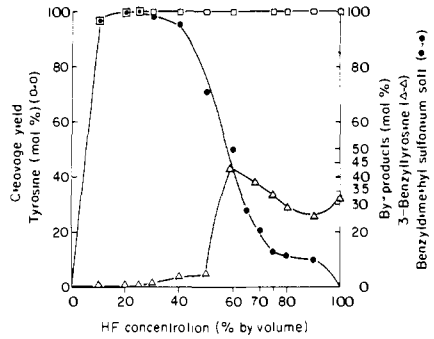


Figure 2. Plot for the products obtained from the deprotection of Tyr(Bzl) in different concentrations of HF in dimethyl sulfide at 0 °C for 1 h: (□) cleavage yield; (●) benzyldimethylsulfonium ion, (△) 3-benzyltyrosine.

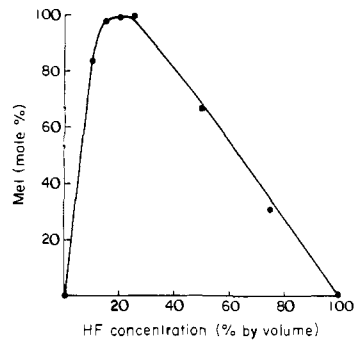


Figure 3. Plot for the reduction of Met(O) in different concentrations of HF in dimethyl sulfide at 0 °C for 1 h.

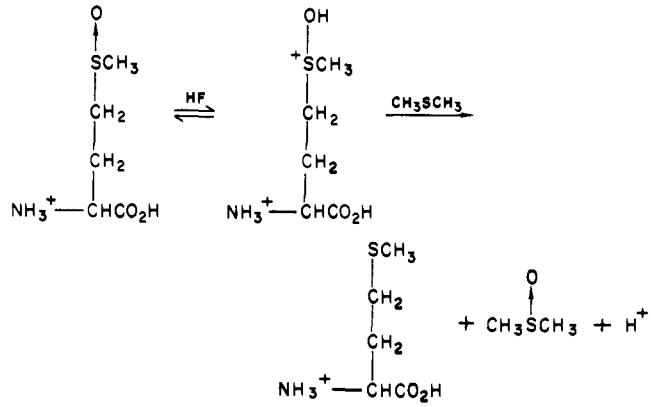
can be explained by our Ser(Bzl) deprotection results, which showed that even at 60% HF, the cleavage mechanism is still S_N1, and by the fact that the cleavage products are ionic intermediates and are sensitive to the polar nature of the acidic medium.[1d] A test for the changeover in mechanism from S_N1 to S_N2 using HF–DMS for the deprotection of Tyr(Bzl) was developed by quantitation of the side product, 3-benzyltyrosine, and the by-product, benzyldimethylsulfonium salt. In an S_N1 mechanism, the side product was expected to be high (20–40%) and the sulfonium salt was expected to be minimal. For the S_N2 deprotection of Tyr(Bzl) we would expect little or no alkylating intermediate since the benzyl moiety is released as an unreactive benzyldimethylsulfonium salt that has little tendency to alkylate the phenolic ring of tyrosine. The overall result is greatly diminished C-benzylation side reaction.[30]

When Boc-Tyr(Bzl)-OH was treated in different concentrations of HF (10–100%) in dimethyl sulfide at 0 °C for 1 h, the products tyrosine, 3-benzyltyrosine, and benzyldimethylsulfonium salt were analyzed and quantitated by reverse-phase HPLC, and the amount of 3-benzyltyrosine was also quantitated by ion-exchange chromatography. The results of the product analysis are shown in Figure 2. When the HF concentration was above 10%, the deprotection of *O*-benzyltyrosine was near quantitative. The amount of 3-benzyltyrosine was observed to be <0.5% when the concentration of HF was 25% or less but increased slowly to approximately 5% at the 50% HF concentration mark. Between 50% and 60% of HF, the amount of 3-benzyltyrosine increased sharply to 38% and slowly decreased to approximately 20% when HF concentration was at 90%, reflecting the ionic and strongly acidic medium. Furthermore, when we examined the amount of benzyldimethylsulfonium salt with the same acid–product profile, we found that the sulfonium salt was nearly quantitatively formed at concentrations below 40% but decreased sharply when HF concentration was above 40%, and the level was maintained at about 10% at concentrations above 70%. Since the formation of 3-benzyltyrosine is a result of an S_N1 mechanism while benzyldimethylsulfonium salt is of an S_N2 mechanism, these data strongly suggest that there is a changeover in mechanism of reaction with HF in dimethyl sulfide in the 50–60% range of HF concentration. Above this concentration, the dimethyl sulfide is largely protonated, and the cleavage mechanism is S_N1. Below this concentration, dimethyl sulfide is largely unprotonated and participates in an S_N2 cleavage mechanism to give the benzyldimethylsulfonium salt, resulting in little alkylation side product.

Since even at the low concentration of HF the alkylation product was still observable, although in much suppressed amount, our results are consistent with the kinetic studies of the deprotection of Ser(Bzl) and further demonstrate that the cleavage in HF dimethyl sulfide mixtures is by a progressive merging of two distinct mechanisms. Thus, in the low concentration range, the S_N2 mechanism dominates and the unprotonated dimethyl sulfide

(30) Tam, J. P.; Heath, W. F.; Merrifield, R. B. *J. Org. Chem.*, submitted for publication.

Scheme IV. Reduction of Methionine Sulfoxide to Methionine by HF in Dimethyl Sulfide



offers nucleophilic assistance, while at the high HF concentration, the strong acid prevails to give a strong carbocation character in the transition state and hence an S_N1 mechanism.

(C) **Reduction of Methionine Sulfoxide.** The reactions of oxygen exchange, racemization and reduction of sulfoxides are known to occur in strong acids.[31] The mechanism, by which S–O bond breakage may occur unimolecularly (S_N1) or by a bimolecular nucleophile displacement (S_N2), depends on both the nucleophilicity of the acid anion and the acidity function. In halo acids, sulfoxides are known to be stable in HF, racemized in concentrated HCl, racemized and partially reduced in concentrated HBr, and completely reduced in HI.[32] Thus, the extent of S–O bond breakage in halo acids reflects the nucleophilicity of the halide ions, with the most nucleophilic anion, iodide ion, giving complete cleavage of the S–O bond and the least nucleophilic anion, fluoride ion, giving no reaction. Furthermore, rates of the acid-catalyzed sulfoxide reactions have been found in a wide range of acid concentration to correlate with their protonated forms and hence with the acid concentration.[33] From these results, one would expect that an S_N2-like oxygen exchange reaction between methionine sulfoxide and dimethyl sulfide in a wide range of concentrations of HF solution is possible provided that the sulfoxide is protonated and the dimethyl sulfide is free. Since methionine sulfoxide has a pK_a from −1.80 to −2.5 and dimethyl sulfide has a pK_a of −6.8 (see Table I), a dilute solution of HF in dimethyl sulfide, which has an H_0 between these two limits, should allow the reduction to occur (Scheme IV). To further facilitate the exchange reaction, a large excess of dimethyl sulfide can be used to bring the reduction of Met(O) to a rapid completion. At acidity function lower than −6.8, both the dimethyl sulfide and sulfoxide will be protonated, and the reduction will be inhibited. Such an assumption is supported by the studies of Landini et al. on the

(31) Scorrano, G. *Acc. Chem. Res.* **1973**, *6*, 132–138.
(32) Landini, D.; Montanari, F.; Hogeveen, H.; Maccagnani, G. *Tetrahedron Lett.* **1964**, 2691–2694.
(33) Landini, D.; Montanari, F.; Modena, G.; Scorrano, G. *J. Am. Chem. Soc.* **1970**, *92*, 7168–7174.

Table III. Reduction of Methionine Sulfoxide in Different Concentrations of HF in Dimethyl Sulfide at 0 °C for 1 h

reagents, vol %[a]		yield, mol %[b]	
HF	DMS	Met	Met(O)
0	100	0	100
10	90	84	16
20	80	98	2
25	75	100	0
30	70	100	0
35	65	100	0
40	60	80	20
50	50	65	35
60	40	56	44
70	30	42	58
80	20	27	73
90	10	15	85
100	0	0	100

[a] Total volume 5 mL. [b] Determined by ion-exchange chromatography (Beckman amino acid analyzer 120B).

Table IV. Reduction of Methionine Sulfoxide with Different Sulfides in HF at 0 °C for 1 h

reagents, vol %[a]		products, mol %[b]		
thiol/sulfide	HF	Met	Met(O)	Met(R)
anisole	90	0	100	0
	25	0	100	0
thioanisole	90	6	94	0
	25	29	1	70[c]
diphenyl sulfide	90	1	99	0
	25	1	99	0
dimethyl sulfide	90	8	92	0
	25	100	0	0
thiacyclopentane[d]	90	10	90	0
	25	100	0	0
1,4-thioxane	90	0	100	0
	25	0	100	0
p-thiocresol	90	1	99	0
	25	3	97	0

[a] Total volume 5 mL. [b] Determined by ion-exchange chromatography (Beckman amino acid analyzer 120B). [c] *S*-Methylmethionine. [d] Tetrahydrothiophene.

protonation of sulfoxides as measured by NMR and UV in aqueous sulfuric and perchloric acids,[34] in which protonation of sulfoxides is linearly related to the acidity function. Therefore, a changeover in mechanism should be observed if the reduction of Met(O) is carried out over a wide range of HF concentrations in DMS. Indeed, when Met(O) was treated with HF containing 0–90% of dimethyl sulfide, at 0 °C for 1 h and the products, Met(O) and Met, were quantitated by ion-exchange chromatography, the results (Figure 3) showed that the rate of reduction reached a maximum at 20–35% of HF. The extent of reduction of Met(O) was nearly quantitative at these concentrations (Table III). At a concentration above 40% HF, the amount of Met(O) reduction declined rapidly, indicating that the dimethyl sulfide was becoming protonated, and the rate of the S_N2 reaction dropped sharply. At 75% of HF, only 30% of reduction was observed, and at 100% HF (no dimethyl sulfide) no reduction of methionine sulfoxide was seen. From these results we can conclude that the reduction of methionine sulfoxide is dependent on the acidity of the mixture and requires the participation of dimethyl sulfide in an S_N2 reaction. Furthermore, the reduction is optimal when the sulfoxide is strongly protonated while the dimethyl sulfide is largely free.

We have also examined other sulfides and thiols in the HF–sulfide reduction of Met(O) in both the low and the high HF concentrations (Table IV). In all cases the sulfide reduction of methionine sulfoxide proved to be effective only at low HF concentrations. Thioanisole was as effective as dimethyl sulfide in the reactions. However, in HF, the protonated thioanisole was an alkylating agent. In the reduction of Met(O) by HF–thioanisole (1:3, v/v) mixture at 0 °C for 1 h, 70% of the product was *S*-methylmethionine. This side reaction was not found in HF–dimethyl sulfide at any concentration. Diphenyl sulfide, with both methyl substituents replaced by the phenyl group, was too sterically hindered and was completely ineffective. The more basic thiacyclopentane was found to be as effective as dimethyl sulfide, but the 6-member oxa ring, 1,4-thioxane, was found to be ineffective. In general, aryl or aliphatic thiols such as thiophenol, *p*-thiocresol, ethanethiol, and 1,2-ethanedithiol were found to be ineffective.[35]

Scheme V. Proposed Mechanism for the Deprotection of N^i-Formyltryptophan by Thiol in HF

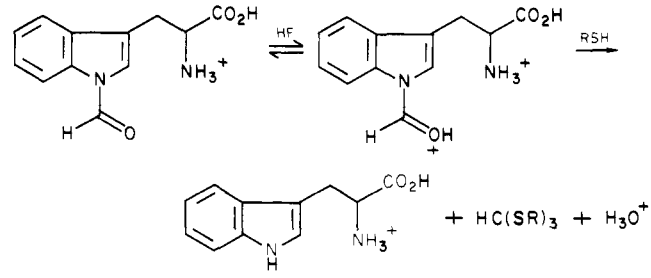


Table V. Deprotection of N^i-Formyltryptophan in HF–Dimethyl Sulfide–*p*-Thiocresol Mixtures at 0 °C for 1 h

reagents, vol %[a]				products, mol %[b]		
HF	DMS	*p*-thiocresol	*p*-cresol	Trp	Trp(For)	Trp(R)[c]
100	0	0	0	0	100	0
95	0	5	0	40	32	28
90	5	0	5	0	100	0
90	0	0	10	0	100	0
90	5	5	0	35	35	30
90	0	5	5	36	32	32
90	0	10	0	18	49	33
75	15	5	5	78	19	3
50	40	5	5	78	22	0
35	55	5	5	85	15	0
25	65	5	5	96	4	0
25[d]	65	5	5	99	1	0
10	80	5	5	62	38	0
0	90	5	5	0	100	0

[a] Total 5 mL of reagent and at 0 °C for 1 h. [b] Determined by ion-exchange chromatography. [c] Side products. [d] For 2 h.

The ability of the low concentration of HF in dimethyl sulfide to reduce Met(O) to Met also helps resolve a difficult problem in the synthesis of methionine-containing peptides. In a synthetic strategy of repetitive weak-acid and final strong-acid treatment, unprotected methionine can be S-alkylated to the sulfonium salt. The use of Met(O) will avoid the side reaction due to the reduced nucleophilicity of the thioether side chain.[36] However, the Met(O) sulfoxide is stable to the usual HF cleavage condition and requires subsequent thiolytic reduction to Met after the removal of all other protecting groups. This reduction is always slow and often accompanied by side reactions.[37] The new HF–DMS deprotection method will reduce methionine sulfoxide and simultaneously deprotect other side chain protecting groups in one single manipulation.

(D) Deprotection of N^i-Formyltryptophan. Another piece of evidence indicating that reactions in low HF concentrations in DMS proceed by an S_N2 mechanism is the observation of an $A_{AC}2$ reaction for the deprotection of N^i-formyltryptophan in the presence of a thiol (Scheme V). Since unprotected tryptophan often leads to serious side reactions when the indole moiety is

(34) Landini, D.; Modena, G.; Scorrano, G.; Taddei, F. *J. Am. Chem. Soc.* **1969**, *91*, 6703–6707.

(35) Reduction of sulfoxide by 2-thiopyridine has been reported recently: Yamashiro, D. *Int. J. Pept. Protein Res.* **1982**, *20*, 63–65.

(36) Iselin, B. *Helv. Chim. Acta* **1961**, *44*, 61–78.

(37) Kessler, W.; Iselin, B. *Helv. Chim. Acta* **1966**, *49*, 1330–1344.

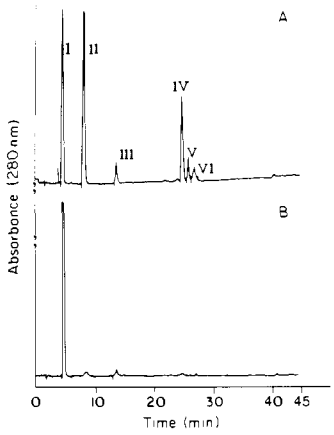


Figure 4. HPLC analysis of HF–thiol deprotection of N^i-formyltryptophan at 0 °C: (A) high HF procedure, HF–*p*-thiocresol–*p*-cresol (90:5:5, v/v), and (B) low HF procedure, HF–DMS–*p*-thiocresol–*p*-cresol (25:65:5:5, v/v, 2 h), and then high HF procedure (same as A). I, Trp; II, Trp(For); III, *p*-cresol; IV-VI, Trp-*p*-thiocresol side products.

exposed to repetitive acid treatments, the N^i-formyl protecting group is often used. It prevents alkylation side reactions by protecting the indole moiety against electrophiles during the synthesis.[38] N^i-Formyltryptophan is stable to strong acids including HF and is usually removed after the strong-acid deprotection of other groups by nucleophiles or aqueous base.[38] In our studies of the HF–DMS deprotection procedure, we found that by the addition of 1–10% of a thiol to this mixture, N^i-formyltryptophan was deprotected to tryptophan (Table V). The deprotection was again HF-concentration dependent and was optimal at low HF concentrations between 20% and 40% of HF in dimethyl sulfide. When a mixture of HF–DMS–thiol was used as the deprotecting agent, the removal of N^i-formyl group of Trp(For) was greatest at 25% HF concentration. The yields of Trp and Trp(For), as quantitated by ion-exchange chromatography and HPLC, were 95% and 5%, respectively. The Trp yield decreased to 86% as HF concentration increased to 50%. At higher concentrations the reaction was inhibited. At 70% of HF, side products besides the starting materials and tryptophan began to appear. At 90% HF the side products became significant and amounted to 35% of the total (Figure 4). Our preliminary results indicate that these are thiol addition products to the Trp(For). They occur only when Trp(For) was present and only at high HF. With the free, unprotected Trp residue, no side reactions were observed. The side reaction was also absent when Trp(For) was completely deprotected in the low HF–DMS–thiol mixture followed by further exposure to high HF concentration in the presence of an aromatic thiol.

The mechanism of the thiolytic removal of formyl from Trp(For) by low concentrations of HF in DMS is a novel reaction; however, a simplified mechanism can be proposed on the basis of our experimental results and limited literature precedent (Scheme V). Since the N^i-formyl–indole bond of Trp(For) is an unusually activated amide bond, a plausible mechanism is that the thiol reacts with the formyl group to form trialkyl or triaryl trithioorthoformate. The formation of triethyl trithioorthoformate from formic acid or formamide with ethanethiol in the presence of catalytic amounts of sulfuric acid has been known since 1907.[39] However, neither acetamide nor *N*-methylacetamide reacts with ethanethiol under similar conditions.[40] Trithioorthoformates are also formed from alkyl or aryl thiols with ethyl formate in HCl.[41] Our experimental evidence supporting the proposed mechanism derives from the isolation and characterization of the products

Table VI. Deprotection of N^i-Formyltryptophan in Different HF–Dimethyl Sulfide–Thiol Mixtures

reagents, vol %[a]		products, mol %[b]		
thiol/sulfide	HF	Trp	Trp(For)	Trp(R)
ethanethiol[c]	90	56	44	0
	25	100	0	0
ethanedithiol[c]	90	79[e]	21[e]	0
	25	100	0	0
3,4-dimercaptotoluene[c]	90	11	89	0
	25	68	32	0
thiophenol[c]	90	16	42	42
	25	100	0	0
thioanisole[d]	90	0	100	0
	25	4	96	0
thiacyclopentane[d]	90	0	100	0
	25	0	100	0
dimethyl sulfide[d]	90	0	100	0
	25	15[f]	85	0

[a] Conditions: 0 °C for 1 h, with 10 mg of Boc-L-Trp(For) in a total volume of 5 mL. [b] Determined by ion-exchange chromatography (see method). [c] 90% HF reaction contained 4.5 mL of HF, 0.25 mL of *p*-cresol, and 0.25 mL of thiol and 25% HF reaction contained 1.25 mL of HF, 0.25 mL of *p*-cresol, 0.25 mL of thiol and 3.25 mL of CH_3SCH_3. [d] 90% HF reaction contained 4.5 mL of HF and 0.5 mL of sulfide and 25% HF reaction contained 1.25 mL of HF and 3.75 mL of sulfide. [e] Values in ref 1c were incorrectly reported. [f] Deprotection due to traces of thiol impurities in dimethyl sulfide.

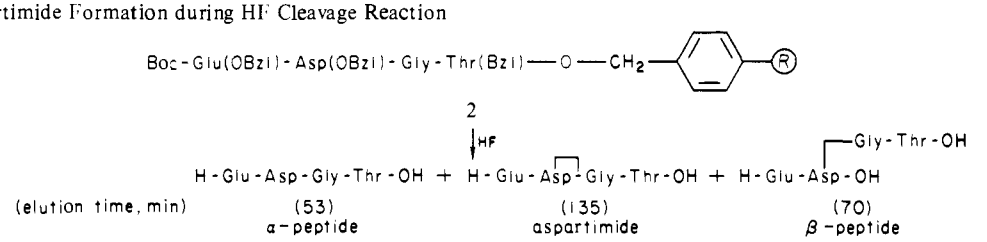
of the reaction. When Trp(For) was treated with HF–DMS–*p*-thiocresol (25:65:10, v/v) at 0 °C for 2 h, Trp and tri(4-methylphenyl) trithioorthoformate were obtained. The identity of the latter was confirmed by comparison with a sample synthesized independently according to a published procedure.[42] Thus, formyl protecting groups, particularly when activated as in the case of Trp(For), are especially labile to an $A_{AC}2$ displacement by acids with nucleophilic anions or acid in nucleophilic solvents such as HF–DMS–thiol. Furthermore, the removal of activated formyl groups of formylimidazole by acids, e.g., HCl in acetic acid, is known to proceed rapidly and efficiently by an $A_{AC}2$ mechanism. This is also true for most amides, which hydrolyze in acid by an $A_{AC}2$ mechanism and in which the slow step is the bimolecular addition of water to the protonated amide. The acidity function relationship is consistent with this mechanism. Our studies also point to the similarity of $A_{AC}2$ acid hydrolysis of amides and our thiolysis of Trp(For).

Trp(For), whose pK_a is estimated to be about −2.0, is strongly protonated at the low HF concentration, while most thiols, whose pK_a is usually −6.0 or lower, are unprotonated. This will again explain the acidity dependence of the reaction because when the HF concentration increases above 70%, both Trp(For) and the thiol will be strongly protonated and the reaction will be inhibited. Under such conditions the $A_{AC}2$ pathway would be expected to be slow and give way to other pathways that lead to side reactions.

We have also examined the effects of different thiols and sulfides on this new reaction using both high and low HF concentrations (Table VI). In all cases examined, thiolytic removal was only effective in the low HF concentration. Arenethiols, such as *p*-thiocresol and thiophenol were observed to be as effective as alkanethiols such as ethanethiol but the danger of thioester formation is considerably less with arenethiols. Alkanedithiols, such as 1,2-ethanedithiol, have been used a scavengers in the synthesis of tryptophan-containing peptides under the normal high HF cleavage conditions, and 1,2-ethanedithiol has recently been used in 90% HF for the deprotection of formyltryptophan.[43] We find that 1,2-ethanedithiol is quite effective for removal of the formyl group in low HF, but we have obtained incomplete deprotection and poor recovery yields of Trp and Trp-containing peptides in high HF containing this dithiol. In addition, this compound

(38) Ohno, M.; Tsukamoto, S.; Makisumi, S.; Izumiya, N. *Bull. Chem. Soc. Jpn.* **1972**, *45*, 2852–2855.
(39) (a) Holmberg, B. *Liebigs Ann. Chem.* **1907**, *353*, 131–138. (b) Holmberg, B. *Chem. Ber.* **1907**, *40*, 1741–1743.
(40) Rinzema, L. C.; Stoffelsma, J.; and Arens, J. F. *Recl. Trav. Chim. Pays-Bas* **1959**, *78*, 354–363.
(41) Kendall, J. D.; Majer, J. R. *J. Chem. Soc.* **1948**, 687–690.
(42) Hull, R. *J. Chem. Soc.* **1957**, 4845–4853.
(43) Matsueda, G. R. *Int. J. Pept. Protein Res.* **1982**, *20*, 26–34.

Scheme VI. Aspartimide Formation during HF Cleavage Reaction



polymerizes extensively in the high HF mixture but does not polymerize significantly at the low concentration. An arenedithiol, such as 3,4-toluenedithiol which was expected to be less prone to polymerization, was very insoluble and was completely ineffective in the deprotection reaction. Sulfides were, in general, found to be ineffective. However, some deprotection of Trp(For) by dimethyl sulfide at the low concentration of HF was observed and was ascribed to the presence of thiol impurities. With tetrahydrothiophene or thioanisole, such side reaction was not observed. Since Trp(For) deprotection is normally desired, this partial removal of the formyl group by DMS is not a problem. However for the purpose of obtaining Trp(For)-containing peptides, using the low concentration of HF procedure, tetrahydrothiophene should be used instead of dimethyl sulfide.

(E) The Dehydration Side Reaction of Aspartyl and Glutamyl Residues. One of the most undesirable effects of the usual high HF deprotection of synthetic peptides bearing aspartyl and glutamyl residues is the $A_{AC}1$ side reaction of the side-chain ester or carboxylic acid to give acylium ion and subsequent side products. This side reaction has been studied extensively and has been found to be dependent on temperature and acid concentration.[12,14] However, these side reactions can be minimized under most circumstances even in high HF concentrations by carrying out the cleavage reaction at temperatures lower than 0 °C or for periods of time shorter than 1 h. Alternatively, HF may be diluted to a lower concentration, usually 70–85%. All these measures are relatively effective in most cases in the reduction of glutamyl and aspartyl side reactions. However, both side reactions are known to be sequence dependent, and with some particular sequences, these side reactions, even with the best precautions, are known to be significant. With glutamic acid or its γ-protected benzyl ester, the acylium adduct with anisole, 4-(*p*-methoxybenzoyl)-2-aminobutyric acid, was about 5% under the normal high HF cleavage condition but increased to 15–17% when the glutamyl residue was at the C terminus. With aspartic acid or its β-protected benzyl ester, the acylation reaction was minimal, but aspartimide formation was as much as 25% under the normal HF cleavage condition when the sequence was Asp-Gly. Recently, our studies on these side reactions indicate that they proceed through both $A_{AC}1$ and $A_{AC}2$ mechanisms.[8] At the low HF concentrations, the side reactions proceeded through an $A_{AC}2$ mechanism, and at the high HF concentrations, the side reactions proceeded through an $A_{AC}1$ mechanism. Thus, these two examples present models for measuring the changeover in mechanism from S_N1 to S_N2 using different concentrations of HF in the cleavage reagent.

Exposure of Glu(OBzl), to different concentrations of HF–DMS–anisole at 0 °C for 1 h resulted in substantially different amounts of side products (Table VII). At the low HF condition (50% or less) no glutamic–anisole addition product was detected. The side product was noticeable at the 0.5% level when the HF was 60% and increased to 15% at 90% of HF.

For the aspartimide side reaction (Scheme VI) a model test tetrapeptide H-Glu(OBzl)-Asp(OBzl)-Gly-Thr(Bzl)-O-CH_2-resin[13c] was used, since we could separate all the byproducts in one single ion-exchange chromatography run (i.e., the desired peptide, the peptide with the β-amide bond at the Asp-Gly sequence, and the peptide with the aspartimide bond (Scheme VI). When the model tetrapeptide was subjected to different HF concentrations in dimethyl sulfide for 1 h at 0 °C we found that at low HF concentration (25% HF) the aspartimide was minimal but detectable at about 0.8% (Table VIII). At 60% of HF the side reaction was 1.8% but rose to 6.7% at 75% and 16.5% at 90% HF. The changeover from $A_{AC}2$ to $A_{AC}1$ was at approximately 75% HF concentration. Thus, the minimal side reactions of aspartyl and glutamyl residues at the low HF concentration clearly indicate that the S_N1 type of mechanism was largely inhibited.

Table VII. Recovery of Glutamic Acid from Glu(OBzl) in Different Concentrations of HF in Dimethyl Sulfide at 0 °C for 1 h

condition			product, mol %
HF	DMS	anisole	Glu
25	70	5	100
30	65	5	100
40	55	5	100
50	45	5	99.5
60	35	5	99.5
70	25	5	99
75	20	5	98
80	15	5	95
85	10	5	88
90	5	5	85
95	0	5	80

Table VIII. Aspartimide Formation from Boc-Glu(OBzl)-Asp(OBzl)-Gly-Thr(Bzl)-OCH_2-resin in Different Concentrations of HF in Dimethyl Sulfide

condition, vol %[a]			temp, °C[b]		time,	product, mol %[c]	
HF	*p*-cresol	DMS	initial	final	min	α peptide	imide
25	10	65	−78	0	45	99.4	0.6
25	10	65	−78	0	60	99.2	0.8
25	10	65	−78	0	120	97.5	2.5
25	10	65	−78	0	240	94.6	5.4
25	10	65	0	0	60	98.8	1.2
40	10	50	−78	0	60	99.3	0.7
50	10	40	−78	0	60	99.2	0.8
60	10	30	−78	0	60	98.2	1.8
70	10	20	−78	0	60	97.5	2.5
75	10	15	−78	0	60	93.3	6.7
85	10	5	−78	0	60	88.8	11.2
90	10	0	−78	0	60	83.6	16.4
90	10	0	0	0	60	75.2	24.8

[a] Total volume of each reaction was 5 mL. [b] HF was distilled into the reaction vessel at the temperature indicated under initial and then placed in a 0 °C ice bath at time 0 and magnetically stirred. [c] Product analyzed by ion-exchange chromatography; β-isomer was usually <0.5%.

(F) Kinetics of the Deprotection Reactions. From the previous supporting data for the S_N2 mechanism for removal of protecting groups in low concentrations of HF in dimethyl sulfide, it is clear that a wide range of HF concentrations can be used. We have chosen 25% HF–75% DMS by volume (~1:1 molar mixture) as the standard reagent, and the apparent first-order rate constants for deprotection of eight amino acid derivatives in this binary mixture were determined (Table IX). The results were in general consistent with the expected lability of these protecting groups as seen in CF_3CO_2H. With a benzyl carbamate (e.g., Z-Gly), a phenyl benzyl ether (e.g., Tyr(Bzl)), and a tosylimidazole (e.g., His(Tos)), the deprotections were rapid and completed quantitatively within 1 h. However, the rate of deprotection for the benzyl ether and benzyl ester derivatives such as Thr(Bzl) or Asp(OBzl) were slow ($t_{1/2}$ ~ 40 min) and would require more than 5 h for completion. To accelerate the deprotection rate we

Table IX. Rate Constants[a] for Deprotection of Different Amino Acid Derivatives in HF–DMS (1:1 Molar) at 0 °C

amino acid derivative	10^4k, s^{-1}	k_{rel}
Z-Gly	54.20 ± 0.59	[100]
Ser(Bzl)	2.87 ± 0.34	5.29
Thr(Bzl)	2.75 ± 0.25	5.07
Asp(OBzl)	2.70 ± 0.22	4.98
Glu(OBzl)	11.10 ± 0.10	20.47
His(Tos)	>1120	213.00
Lys(2-ClZ)	~3.3[b]	6.17

[a] Note: the deprotection rates are accelerated in the ternary mixture of HF–DMS–*p*-cresol (25:65:10, v/v). [b] Average rate constant.

have increased the acidity function of the reagent by adding 10% *p*-cresol to the binary mixture to give HF–DMS–*p*-cresol (25:65:10, v/v). The *p*-cresol, which is a scavenger by itself, also serves as a dissociating agent for the HF–DMS complex. The ternary mixture promotes deprotection by the same mechanism as the HF–DMS (25:75, v/v) but with an increase in rate. Thus, with the new mixture HF–DMS–*p*-cresol (25:65:10, v/v), most benzyl alcohol derived protecting groups were quantitatively removed in 2 h at 0 °C. The differences between the kinetic data shown in Table IX and the deprotection yields given in Table X are due to several factors in the experimental protocol including solvent changes leading to altered acidity function. The kinetic experiments involved removal of samples at given time points and quenching the aliquots to stop the reaction. On the other hand, the deprotection studies in Table X involved performing the reactions and evaporating all of the solvent before isolating the products. Both the binary and ternary mixtures take some time to evaporate due to association of the various components. Therefore, while the reaction times are reported (Table X) as 1 or 2 h, the total time of contact with the reaction mixture includes both reaction time and evaporation time.

The new S_N2 deprotecting method is sensitive to steric and electronic factors. Thus, most benzyl protecting groups of esters, ethers, and carbamates were completely removed at 0 °C for 1–2 h in the solution model. Monohalogen-substituted benzyl protecting groups such as Lys(2ClZ) were also removed smoothly in 1–2 h, but the dihalogen substituted benzyl protecting group *O*-(2,6-dichlorobenzyl)tyrosine[9] was only 59% removed in 1 h. Protecting groups that are hindered or are less carbocationic in nature are also resistant to the cleavage condition. This was evident in the deprotection of Asp(O*c*Hex),[13] Arg(Tos), Arg(NO_2), His(Dnp), Cys(Bzl), and Cys(4-MeBzl); all gave little to no deprotection with the low HF–DMS method. The N^{im}-tosyl protecting group of histidine was removed rather easily by the low HF–DMS method. Other protecting groups that were found to be stable to our new deprotection method are fluorenylmethyloxycarbonyl, trifluoroacetyl, acetyl, methyl ether, and methyl ester (Table X). The N^{i}-formyl of tryptophan was also relatively stable but was removed by the presence of 1–10% thiol.

Similar to the protecting groups, the order of stability of the bond to the functionalized polymeric supports also depends on steric and electronic properties. In general the cleavage rate was found to be about 2-fold slower than for the corresponding solution model. This may be due to the relatively poor swelling property of the HF–DMS mixture alone since the cleavage rate was found to be increased in the HF–DMS–*p*-cresol mixture (25:65:10, v/v), which provides improved swelling of cross-linked polystyrene resins. Simple benzyl esters to copoly(styrene–divinylbenzene) resin supports were satisfactorily cleaved (65–85%) under the low HF conditions in 2 h at 0 °C. However, the cleavage yields of peptides attached through the more acid-stable oxymethylphenylacetamidomethyl resin linkage were lower (40–60%).

(G) Need for a Second-Stage High HF Step or Other Combined Strategies for Complete Removal of All Groups. Conceptually, the new low HF deprotection procedure is ideal for the deprotection of synthetic peptides, but operationally, it is hampered by the lack of available protecting groups for every amino acid that can be removed in one single low HF step. A case in point is that the usual protecting group for arginine, tosyl, is relatively stable to the low HF condition. An alternative to the tosyl protecting group is the benzyloxycarbonyl group for the guanidino group, Arg(Z,Z), which is removable under the S_N2 conditions. A different approach in protecting groups that could fit into our low HF strategy quite well is dinitrophenyl for the protection of the imidazole moiety since it can be removed thiolytically prior to the HF treatment. Similarly, the low HF procedure can be used with other side-chain protecting groups or multidetachable resin supports that require orthogonal deprotection methods to complete the regeneration of the peptide.

Table X. Extent of Deprotection of Amino Acid Derivatives in Low HF Concentrations[a]

protected residue	yield, mol %[b] HF–DMS, 1:3 v/v (1 h)	HF–DMS, 1:3, v/v (2 h)	HF–DMS–*p*-cresol, 25:65:10, v/v (2 h)
Boc-Ala	100	100	100
Z-Ala	100	100	100
Ac-Ala	0	0	0
Tfa-Ala	0	0	0
Fmoc-Ala	0	0	0
Arg(Tos)	0	<5	<10
Arg(NO_2)	0	<5	<5
Asp(OBzl)	80.5	100	100
Asp(OcHex)	0	<5	<5
Cys(4-MeBzl)	0	<5	<5
Glu(OBzl)	93.3	100	100
His(Tos)	100	100	100
His(Dnp)	0	0	0
Lys(2-ClZ)	82.9	100	100
Met(O)	100	100	100
Ser(Bzl)	80.4	100	100
Thr(Bzl)	79.8	100	100
Trp(For)			99.5[c]
Tyr(Bzl)	100	100	100
Tyr(2,6-Cl_2Bzl)	33	65	89
Tyr(BrZ)	92.6	100	100
Tyr(Me)	0	<5	<5
Val-OMe	0	<5	<5

[a] Determined by amino acid analysis. [b] The cleavage yields were in general higher than predicted from the rate constants (Table IX) due to reaction conditions. In these experiments, HF was distilled into the sample and the reaction was brought to 0 °C for 1 or 2 h, HF was then evaporated, and the yields were analyzed. Although the concentration of HF decreased rapidly, the total time of contact with HF was longer than 1 h. However, in accordance with the usual practice, the time is reported as 1 or 2 h. [c] HF–DMS–*p*-cresol–*p*-thiocresol (25:65:5:5, v/v).

(H) General Conditions for the Removal of Protecting Groups and Polymeric Supports. For syntheses using the normal existing protecting groups and polymeric supports, we have derived from our data the following deprotection conditions

(1) For synthetic peptides or peptidyl resins not containing Arg(Tos) or Cys(4-MeBzl), and synthesized on a chloromethyl resin support, a cleavage method with low HF concentration (HF–DMS–*p*-cresol, 25:65:10, v/v) at 0 °C for 2 h is generally sufficient.

(2) For peptides containing Arg(Tos) or other functionality stable in low HF, we favor a two step operation, which we have named in our laboratory as the low–high HF deprotection procedure. The low HF procedure consists of the treatment of the protected peptidyl-resin with the low concentration of HF in dimethyl sulfide for 2 h at 0 °C. Specifically, the following mixture was found to be useful: HF–DMS–*p*-cresol (25:65:10, v/v). When Trp(For) is present, the mixture is modified by including 2.5% of *p*-thiocresol to give HF–DMS–*p*-cresol–*p*-thiocresol (25:65:7.5:2.5, v/v). Under these conditions, Trp(For), Met(O), and most other protecting groups will be removed, but Arg(Tos), Arg(NO_2), Asp(O*c*Hex), His(DNP), and Cys(4-

Table XI. Product Analysis of Methionine-enkephalin from Different HF Deprotection

method[a]	yield,[b] mol %	HPLC analysis[c] of peptides		amino acid analysis[d]			
		Enk	3-Bzl-Tyr-Enk	Tyr	3-Bzl-Tyr	Met(O)	Met
low HF	75	94.0	0.5	0.99	0.01	0	0.98
high HF	90	76	19	0.80	0.18	1.0	0.05
low–high HF	92	94.5	0.5	0.97	0.01	0	0.98

[a] Low HF: HF–DMS–*p*-cresol, 25:65:10, v/v, for 2 h at 0 °C. High HF: HF–*p*-cresol, 90:10, v/v for 1 h at 0 °C. Low–high HF: low HF procedure then recharge vessel to give high HF for 1 h (see text). [b] Yield based on peptide cleaved from resin/peptide cleaved from resin + peptide remaining in resin × 100%. [c] Enk = enkephalin; 3-Bzl-Tyr-Enk = [1-(3-benzyltyrosine)]enkephalin,[1] product analyzed by HPLC (see Experimental Section). [d] Tyr and 3-Bzl-Tyr were determined by ion-exchange chromatography (see ref 9) as well as by HPLC (see Experimental Section) after 6 N HCl hydrolysis; Met(O) and Met were determined by ion-exchange chromatography after aminopeptidase digestion.

MeBzl) will be stable. The high HF procedure is achieved by the evaporation of all the HF and dimethyl sulfide in vacuo at 0 °C and then by recharging the vessel with anhydrous HF so that the final concentration of HF is approximately 90% (note, both *p*-cresol and *p*-thiocresol, which are solids at room temperature and account for 10% of the original volume, will remain in the reaction vessel during the evaporation). If the evaporation of DMS is not complete, the final HF concentration may be too low, and the next stage will be incomplete. The peptide-resin is then treated for 0.5–1 h at −10 to 0 °C depending on the particular peptide sequence and polymeric support, to complete the deprotection and cleavage reactions. The HF is then evaporated, the residue is extracted with EtOAc, ether, or CH_2Cl_2 to remove scavengers and byproducts, and finally the peptide is extracted with dilute HOAc.

(3) For peptides containing acid-resistant polymeric supports such as phenacyl, nitrobenzyl, and some of the benzylhydrylamine resins, the peptide resin is treated first with the low HF reagent for 1–2 h (length depending on the protecting groups) to deprotect the side chains. After evaporation of HF and dimethyl sulfide, the peptide resin is washed with CH_2Cl_2 or EtOAc to remove the byproducts such as sulfonium salts, dimethyl sulfoxide, and thiol derivatives and then pumped dry. The peptide is then cleaved from the dried resin with the high concentration of HF (HF–*p*-cresol, 9:1, v/v) as described before or by other appropriate means.

Since the improved S_N2 deprotection method generates sulfonium salts, dimethyl sulfoxide and trithioorthoformate, it is necessary to remove these byproducts as expediently as possible after the deprotection step, because under neutral or slightly basic conditions, alkylation by sulfonium salts, oxidation by dimethyl sulfoxide and formylation by trithioorthoformate are potential side reactions. We have found that such side reactions do not proceed significantly during the cleavage reaction and proper workup. However, side reactions were actually observed when lyophilized samples containing deprotected peptides together with sulfonium salts and orthothioformate were purposely stored at 0 °C for 2–15 weeks but were completely avoided when the samples were worked up properly and immediately. We have found that dialysis or gel permeation chromatography was suitable for the workup of most peptides. Crude batch separation of the byproducts by C-18 reverse-phase chromatography was also effective since sulfonium salts were elutated by the starting aqueous buffer, and the retained peptides were removed later by organic solvents. Separation of sulfonium salts from the crude peptides by mixed-bed ion-exchange resins such as Amberlite MB-I or Rexyn I-300 was also possible but the recovery yields depended largely on the size and charge of the sample. For peptides that were quite insoluble in aqueous acetic acid, precipitation of the synthetic peptides presented the best procedure since the water-soluble sulfonium salts could be removed easily.

The low–high HF deprotection procedure may seem at first glance not substantially different from the normal HF deprotection method; however, in many test syntheses that we have compared, both the synthetic yield and purity of the product were significantly improved with the new technique. The advantages are (1) most of the carbocation precursors are removed prior to the high HF procedure, and alkylation side reactions are thereby greatly minimized, (2) methionine sulfoxide is reduced during the low HF treatment, (3) N^i-formyltryptophan is deprotected, and (4) side reactions of both aspartic and glutamic acids are significantly reduced since the benzyl protecting groups are removed during the low HF treatment, and the resulting free carboxylic side chains are less prone to side reactions. These advantages are illustrated and discussed in the syntheses of three model peptides.

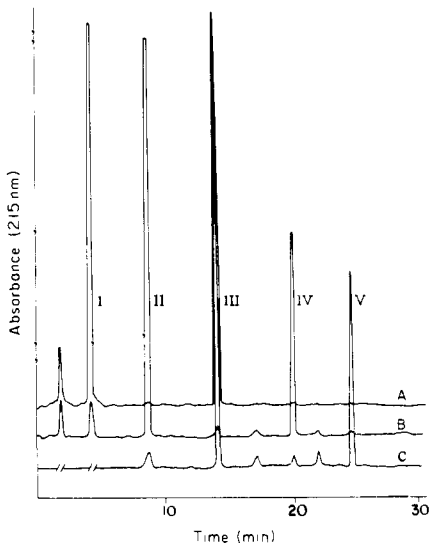


Figure 5. HPLC analysis of crude, unpurified methionine-enkephalin by (A) low–high HF procedure (see text); (B) high HF procedure, HF–*p*-cresol (9:1, v/v, 0 °C, 1 h); (C) sample obtained from (B) and after thiolytic reduction by 2-mercaptoethanol. For clarity, the solvent and the reagent peaks that appeared before the first 5 min of the chromatograph from C were deleted. Peak I, benzyldimethylsulfonium salt; II, H-Tyr-Gly-Phe-Met(O)-OH; III, methionine-enkephalin, H-Tyr-Gly-Gly-Phe-Met-OH; IV tyrosine alkylation of [Met(O)enkephalin, H-(3-Bzl)Tyr-Gly-Gly-Phe-Met(O)-OH; and V, H-(3-Bzl)Tyr-Gly-Gly-Phe-Met-OH.

(III) Applications in Peptide Synthesis. (A) Synthesis of Methionine-enkephalin. Methionine-enkephalin (**1**),[44] a penta-

H-Tyr-Gly-Gly-Phe-Met-OH
1

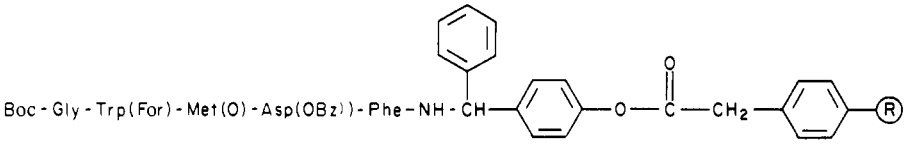
peptide, has been studied in our laboratory as a test peptide, and its synthetic side products have been well characterized by ion-exchange chromatography and HPLC. The two major problems associated with its HF deprotection that we would like to eliminate are (1) the alkylation side reaction of tyrosine and (2) the thiolytic reduction of methionine sulfoxide following HF treatment. Methionine-enkephalin was synthesized on a standard chloromethyl-resin by the stepwise strategy with Boc-Tyr(Bzl) and Boc-Met(O) for the protection of Tyr and Met. The protected peptide-resin was then treated in three ways: (1) with the low HF (HF–DMS–*p*-cresol, 25:65:10, v/v, 2 h, 0 °C), (2) with the

(44) Hughes, J.; Smith, T. W.; Kosterlitz, H. W.; Fothergill, L. A.; Morgan, B. A.; Morris, H. R. *Nature (London)* **1975**, *258*, 577–579.

Table XII. Product Analysis of Pentagastrin Amide from Different HF Deprotection Procedures

peptide	HPLC elution time, min[a] A	B	yield, mol %[b] I	II	III	IV	V
H-Gly-Trp-Met(O)-Asp(Phe-NH_2)-OH	15.7	26.5	<1	<1	<1	<1	<1
H-Gly-Trp-Met(O)-Asp-Phe-NH_2	16.1	27.2	<1	1.2	17	22	24
H-Gly-Trp(For)-Met(O)-Asp-Phe-NH_2	17.8	18.2	<1	85	20	32	29
H-Gly-Trp(For)-Met(O)-Asp-Phe-NH_2	20.6	23.6	<1	1.5	<1	<1	<1
H-Gly-Trp-Met-Asp-Phe-NH_2	21.4	24.7	95	1	19	5	4
H-Gly-Trp(For)-Met-Asp-Phe-NH_2	22.4	27.1	<1	1	14	3	3

[a] Elution condition: C-18 reverse phase (3.9 × 300 mm), linear gradient 2–98% B (condition A) and 10–60% B (condition B) in 45 min, at 280 nm, 0.1 AUFS; buffer A, 0.1% H_3PO_4 95%, CH_3CN 5%; buffer B, 0.1% H_3PO_4 50%, CH_3CN 50%; flow rate at 2 mL/min. [b] I, low–high HF procedure. Low: HF–DMS–*p*-cresol–*p*-thiocresol, 25:65:7.5:2.5, v/v, 2 h, 0 °C. Then, high: HF–*p*-cresol–*p*-thiocresol, 90:7.5:2.5, v/v, 0 °C, 1 h. II, HF–*p*-cresol, 90:10, v/v. III, HF–DMS–*p*-cresol–*p*-thiocresol, 80:10:5:5, v/v. IV, HF–*p*-cresol–*p*-thiocresol, 80:10:10, v/v. V, HF–*p*-cresol–*p*-thiocresol, 90:5:5, v/v. Conditions for II–V are 0 °C for 1 h.



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low–high HF, and (3) with the high HF procedure (HF–*p*-cresol, 9:1, v/v, 0 °C, 1 h). The cleavage yields obtained from the low–high HF and high HF methods were comparable and were 90–92% (Table XI). The cleavage yield from the low HF procedure was somewhat lower (75%). HPLC analysis of the crude, unpurified products obtained from the low HF and low–high HF procedures indicated that methionine-enkephalin accounted for 94% of the crude peptide (Figure 5A). No other products higher than 1.0% were observable. As expected, Met(O) was completely reduced to Met as evidenced from enzymatic digestion. The alkylation product, [1-(3-benzyltyrosine)]methionine-enkephalin, as a result of *O*-benzyltyrosine rearrangement, was barely detectable and estimated to be not higher than 0.5%. In contrast, the usual high HF treatment provided two major product peaks (Figure 5B). Enzymatic digestion of the crude and unpurified peptide product showed that the Met(O) was quantitatively recovered (Table XI). After extensive thiolytic reduction in 2-mercaptoethanol at 38 °C for 3 days, the HPLC analysis of the crude product revealed the presence of three products, with methionine-enkephalin accounting for 76% of the mixture (Figure 5C). The alkylation side product of the terminal tyrosine, [1-(3-benzyltyrosine)]methionine-enkephalin, was found to be 19%. Thus, the low–high procedure achieved our desired goal of minimizing the tyrosine alkylation side reaction and concommitantly reducing Met(O) to Met.

(B) Synthesis of Glu-Asp-Gly-Thr. The tetrapeptide Glu-Asp-Gly-Thr, which corresponds to position 128–131 of bovine growth hormone, is another model test peptide well studied in our laboratory for the glutamic and aspartic acid side reactions. Under the usual high HF condition the glutamic acid acylation of scavenger with this test peptide amounted to about 3% and was reduced about 3-fold when *p*-cresol was used instead of anisole. This side reaction was further reduced to less than 0.5% if the high HF reaction was carried out for short periods of time and at −10 to −5 °C. Thus, the glutamic side reaction is no longer significant under these modified conditions and will not be discussed further. However, aspartimide formation with this peptide provided a critical test for the low–high procedure.

The tetrapeptide was synthesized on a chloromethyl-resin, and the protected peptide-resin (**2**) was treated with the usual high HF condition (0 °C, 1 h). Ion-exchange chromatographic analysis (Table VIII) revealed that aspartimide formation was 16.4%. When the model protected tetrapeptide-resin was treated with the low HF procedure at 0 °C, aspartimide was barely detectable (0.5%) after 1 h but increased to 2.5% in 2 h and 5.4% in 4 h. When the protected peptide-resin was treated with the combined low–high HF procedure (1 h each) aspartimide was 4.5–8%. These results are consistant with a detailed study we have recently undertaken on the rate of aspartimide formation of this particular tetrapeptide, in which it was found that aspartimide formation depends not only on temperature and time but also on the side-chain protecting group.[14] The amount of aspartimide formation was greatest with the benzyl ester and 3–5-fold less with the cyclohexyl ester or with the free carboxylic acid. Since, with the low HF treatment, the unprotected peptide Glu-Asp-Gly-Thr is formed, the subsequent high HF treatment produced far less aspartimide product than the treatment of the benzyl ester protected peptide-resin. Aspartimide formation was further reduced to 1.8% when the high HF treatment of the low–high HF procedure was carried out at −10 °C for 1 h. Thus for the HF cleavage of peptides containing aspartic acid, particularly with the sequences Asp-Gly, Asp-Ser, Asp-Asn, and Asp-His, the low–high HF procedure (with the high HF treatment at −10 °C for 1 h) will effectively minimize aspartimide formation. In the test peptide, Glu-Asp-Gly-Thr, the low–high HF procedure reduced aspartimide formation 10-fold compared to the usual HF deprotection procedure.

(C) Synthesis of Pentagastrin Amide. The C-terminal pentagastrin amide **3** is a potent active fragment of the gastrin hormones.[45] It consists of an unusually rich concentration of difficult amino acids such as Trp, Met, and Asp and was of considerable interest as a test for the efficacy of the low–high HF deprotection procedure. The pentapeptide amide was synthesized on a multidetachable benzhydrylamine-resin[46] with Boc-Trp(For), Boc-Met(O), and Boc-Asp(OBzl) as starting materials. The complete protected peptide-resin **3** was deprotected by the low–high HF procedure with the addition of 2.5% of *p*-thiocresol to remove the N^{in}-formyl group and also by the high HF procedure with HF–*p*-cresol. Both methods gave comparable cleavage yields of about 85%. In order to fully analyze the cleavage products, we have synthesized and established the elution time using HPLC of all five main side products of pentagastrin amide during the HF deprotection (Table XII). These include the products from the incomplete deprotection of Trp(For) or Met(O) and aspartimide rearrangement. In general, side products due to aspartimide rearrangements were small, but products from partial deprotection of Trp(For) or Met(O) were noticeably large. The crude product from the low–high HF procedure as examined by HPLC moni-

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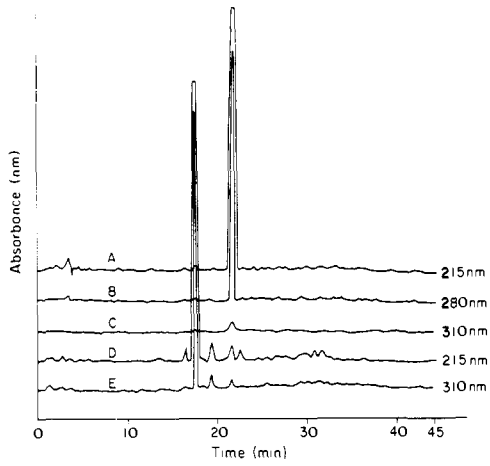

Figure 6. HPLC analysis of crude and unpurified pentagastrin amide by (A–C) low–high HF procedure (see text) and (D + E) high HF procedure alone (see text).

Table XIII. Enzymatic Hydrolysis of Pentagastrin Amide Products by Aminopeptidase M

	amino acid ratio[b]					
products[a]	Trp[c]	Trp(For)[c]	Met	Met(O)	Asp	Gly
I	0.99	<0.01	0.98	<0.01	1.02	1.0
II	0.01	0.99	0.01	0.98	0.98	1.0
III	0.35	0.40	0.33	0.68	0.98	1.0
IV	0.27	0.35	0.08	0.91	1.01	1.0
V	0.28	0.32	0.07	0.91	0.98	1.0

[a] See Table XII footnotes for explanations. [b] Analyzed by ion-exchange chromatography, C-terminal phenylalanine amide not analyzed. [c] Side products of Trp not analyzed.

toring at 215 (Figure 6A) and 280 nm (Figure 6B) was found to contain a single major peak corresponding to the correct pentagastrin amide and accounting for 95% of the crude peptide product. Since Trp(For) absorbs strongly at 310 nm but Trp does not, the HPLC was rerun at 310 nm (Figure 6), which gave a greatly diminished peak indicating that Trp(For) was converted to Trp. This was further confirmed by the enzymatic hydrolysis with aminopeptidase M in which Trp was quantitatively recovered and Trp(For) was not detectable (Table XIII). The enzymatic hydrolysis also confirmed that Met(O) was totally converted to Met (Table XIII).

The cleavage of the pentagastrin peptide-resin (**3**) by the high HF alone gave essentially one major peak accounting for 85% of a protected pentagastrin amide, Gly-Trp(For)-Met(O)-Asp-Phe-NH_2 (Figure 6, D and E). There were two minor peaks of about 5% each. Enzymatic digestion of this crude peptide revealed that Trp(For) and Met(O) were present. The crude, unpurified peptide was treated with 2-mercaptoethanol for 24 h at 50 °C to reduce Met(O) to Met, followed by a brief base treatment to remove Trp(For). Several additional peaks were found in the HPLC analysis. The yield of the desired pentagastrin, as quantitated by HPLC, was reduced to 65%.

Since thiols and dimethyl sulfide are also reported and recommended by others for use in high HF deprotection, we have also experimented with this procedure for the deprotection of the pentagastrin amide peptide-resin. With HF ranging from 80 to 90% and in the presence of dimethyl sulfide, *p*-cresol, and thiocresol, the analysis of the crude products gave very complex patterns of several major peaks. This was not unexpected, since both Met(O) and Trp(For) were partly deprotected to Met and Trp. Although, after thiolytic reduction and base treatment the HPLC analysis of the crude peptide mixture gave a more simplified pattern, multiple peaks remained and the correct pentagastrin amide accounted for only 35–50% of the product mixture. Enzymatic digestion followed by ion-exchange analysis of the amino acids usually revealed low recovery of Trp (Table XIII). This further supported our earlier contention that the cleavage of Trp(For) with thiol at high HF concentration leads to substantial side reactions. Three thiol side products have now been identified, and they are thiol adducts that are relatively hydrophobic and elute as multiplets late in the usual HPLC profile (Figure 4). Presumably they are apt to escape easy detection in the product analysis.

Our results with the low–high HF procedure for the deprotection of the pentagastrin peptide-resin have demonstrated that it is highly efficient and provides a far better product compared to the conventional methods of high HF cleavage.

Experimental Section

Commercial protected amino acids were obtained from Peninsula Laboratories, San Carlos, CA. 3-Benzyltyrosine hydrochloride salt was prepared according to Iselin[36] (mp 239–243 °C (lit. 239–243 °C)). The purity was also assessed by HPLC. Other reagents were: trifluoroacetic acid (Halocarbon Products), HF (Matheson), diisopropylethylamine (Aldrich) was distilled over CaH_2 (Alfa), *N,N'*-dicyclohexylcarbodiimide (Pierce), 1-hydroxybenzotriazole (Aldrich) recrystallized from 80% ethanol, dichloromethane (Eastman) distilled from Na_2CO_3, and acetonitrile HPLC grade (Jackson and Burdick).

Hydrolysis of free peptides was with 6 N HCl in evacuated, sealed tubes at 110 °C, 24 h. Peptide-resins were hydrolyzed in 12 N HCl–phenol–HOAc (2:1:1) at 110 °C, 24 h, and by 4 N methanesulfonic acid, 110 °C, 24 h. For total enzymatic digestion, synthetic peptide (1 mg) was treated in 1 mL of 0.1 M sodium phosphate buffer (pH 7) with 43 μg of leucine aminopeptidase M (Sigma) for 24 h at 37 °C. A 0.2-mL aliquot was removed and diluted with 0.2 N citrate buffer to 2 mL. After filtration, the samples were analyzed on a Beckman 121 amino acid analyzer. HF reactions were carried out in a Diaflon HF apparatus (Toho Co., Osaka, Japan).

Analytical high-pressure liquid chromatography of the peptides was on a thermostated (30 °C), reverse-phase μBondapak C-18 column (4 × 300 mm) in a Waters Associates instrument fitted with a Schoeffel variable-wavelength UV photometer and an automatic Wisp injector. The chromatograms were recorded on a Hewlett-Packard 3380 A integrator (1-mV full scale).

Kinetic Studies of Deprotection of *O*-Benzylserine. The desired amount of dimethyl sulfide and a small magnetic stirring bar were placed in a 50-mL fluorocarbon HF reaction vessel connected to the HF vacuum line. HF was then distilled into the evacuated reaction vessel at −78 °C to bring the total volume to 5 mL. The magnetically stirred solution was equilibrated to 0 °C by the ice bath for 1 h. The closed reaction vessel was then removed from the HF line by loosening the tube connector between the vessel valve and the line, and the entire reaction vessel assembly was mounted on a ring stand. The tube connector was disassembled and replaced with a Teflon-brand disk (od 13 mm, 4 mm thick) as the injector port. A premeasured 0.10-mL aliquot of a CH_2Cl_2 stock solution containing 1 mmol/mL of Boc-L-Ser(Bzl) and 0.3 mmol/mL of Boc-L-Ala was drawn up into a 30-cm steel hypodermic needle (18 gauge) connected to a 3-cm^3 Plastipack Syringe (Luer-Lok tip) with Teflon-brand tape as the seal. The Teflon-brand disk was then pierced with the steel needle and the valve between the reaction vessel and disk was opened. The needle was directed down into the reaction solution and the CH_2Cl_2 solution injected. The reaction mixture was vigorously stirred in the ice bath, and the hypodermic apparatus was used to withdraw aliquots of the reaction solution at the required time, the first aliquot being discarded to clean the needle. Two–three drops (~0.1 mL) of each withdrawn aliquot containing the sample in HF/DMS were quenched in 2.0 mL of 0.2 N sodium citrate, pH 2.2. After dilution, the samples were analyzed on a Beckman Model 120B amino acid analyzer using a 0.9 × 55 cm column packed with Beckman AA-15 sulfonated polystyrene cation-exchange resin operated at 58 °C. The column was eluted using 0.2 N sodium citrate, pH 4.00 buffer, with serine eluting at 40 min and alanine at 48 min. The amount of deprotection of Ser(Bzl) was calculated from the ratio of serine to alanine vs. the known absolute ratio in the stock solution. The results are summarized in Tables II and IX.

Studies of C-Benzylation of *O*-Benzyltyrosine. A typical experiment was as follows: Tyr(Bzl) (10 mg) with a measured volume of dimethyl sulfide in a Teflon-brand vessel was charged with HF to a total volume of 5 mL at −78 °C. While it was stirred vigorously, the reaction mixture was warmed to 0 °C in an ice bath and allowed to react for 1 h. HF and dimethyl sulfide were then removed in vacuo. The residue was taken up in 1–2 mL of trifluoroacetic acid and diluted with buffer A for HPLC analysis. The elution condition was as follows: solution A, 950 mL of H_2O, 50 mL of CH_3CN, 1 mL of H_3PO_4; solution B, 500 mL of H_2O, 500 mL of CH_3CN, 1 mL of H_3PO_4; linear gradient from 2 to 98% B into A in 45 min, 1 mL/min at 225 or 280 nm. The elution times of the

tyrosine derivatives in the standard 45-min gradient were as follows (in min): Tyr (4.6), Tyr(Bzl) (20.3), 3-Bzl-Tyr (17.4), Tyr(2,6-Cl_2Bzl) (29.2), 3-(2,6-Cl_2Bzl)Tyr (24.7), Tyr(*c*Hex) (24.5), 3-*c*Hex-Tyr (21.3), 3-*t*-Bu-Tyr (17.1), 3-(2-ClBzl)Tyr (20.4), anisole (12.1), *m*-cresol (19.2), and *p*-cresol (13.3); 3-(2-BrBzl)Tyr (18.1, in 30-min gradient). Note: these elution times are reported as uncorrected and variations of several minutes were observed depending on buffer and elution conditions. Some of the experiments were repeated under a slightly different protocol: Boc-Tyr(Bzl) (10 mg), Boc-Ala (3 mg), and a known concentration of scavenger in a Teflon-brand vessel was charged with HF to a total volume of 5 mL. The reaction proceeded as above for 1 h at 0 °C. The reaction was stopped by withdrawing 2 separate 0.05-mL portions of HF solution and quenching one in NH_4OH solution (1 mL, 12% NH_3) and the other in sodium citrate buffer (1 mL, pH 2.2). The products in the NH_4OH solution were analyzed by HPLC, and those in sodium citrate buffer solution were analyzed on the Beckman 121 amino acid analyzer with alanine as the standard.

Analyses of tyrosine were performed on a Beckman 121 amino acid analyzer. Analyses of 3-alkyltyrosine were performed on a Beckman 120B amino acid analyzer according to ref 17 and 29 on a sulfonated polystyrene column (0.9 × 11 cm, PA-35) with a thermostated jacket (86 °C) and eluted with 0.35 N citrate buffer pH 7.0, 70 mL/h.

Studies on the Reduction of Methionine Sulfoxide. Methionine sulfoxide (10 mg) was mixed with the appropriate sulfide or other reductant in a Teflon-brand HF reaction vessel into which HF was then distilled at −78 °C. A total volume of 5 mL was used in all reactions which were equilibrated to 0 °C. After the desired length of time elapsed, HF was either removed by evaporation in vacuo and the residual taken up in water or aliquots were taken out and quenched in NH_4OH solution. A portion of this mixture was analyzed by ion-exchange chromatography (Beckman 120B, AA-15 column (0.9 × 55 cm) eluted with 0.2 N sodium citrate, pH 3.49, 58 °C); the elution times were, Met(O), 40 min and, Met, 118 min. For the analysis of *S*-methylmethionine, a PA-35 column (0.9 × 11 cm) eluted with 0.2 N sodium citrate, pH 4.25 at 58 °C, was used. The elution times were the following: Met(O) 15 min, Met 18.5 min, NH_3 105 min, and Met(CH_3^+) 117 min. The results are summarized in Tables III and IV.

Isolation of Tri-*p*-tolyl Trithioorthoformate from the Low HF Reaction between L-Trp(For) and *p*-Thiocresol. L-Trp(For) HCl[38] (0.108 g, 0.402 mmol) was treated with *p*-thiocresol (0.5 g, 4.03 mmol) in 1.25 mL of HF and 3.25 mL of dimethyl sulfide for 120 min at 0 °C. After the HF and DMS were removed, the residue was dissolved in 60 mL of diethyl ether and extracted with 15 mL of 10% acetic acid. The organic layer was washed with water and then dried under reduced pressure to yield a white solid. The solid was recrystallized from CH_3OH and dried over P_2O_5 to yield 0.1 g (65% yield) of tri-*p*-tolyl trithioorthoformate, mp 107.5–109 °C. Analysis of the sample by LC on a 0.4 × 30 cm C-18 reverse-phase column using system II (see below) gave a single peak at 47 min and a single peak at 29.4 min using system III (see below). NMR (300 MHz) in $CDCl_3$ (ppm) shows 7.39, 7.36, 7.14, 7.11 (12 H, CH aromatic, q), 5.27 (1 H, CH, s), 2.34 (9 H, $3CH_3$, s). Anal. Calcd for $C_{22}H_{22}S_3$: C, 69.06; H, 5.80; S, 25.14. Found: C, 69.09; H, 5.84.

Tri-*p*-tolyl tritlioorthoformate synthesized by a literature procedure[42] gave crystals that melted at 107.5–109 °C. The synthetic material cochromatographed with the isolated material on LC using systems II and III. Elemental analysis gave: C, 69.08; H, 5.83.

Chromatographic Analyses of Tryptophan Derivatives and Tri-*p*-tolyl Trithioorthoformate. Ion-exchange chromatography was performed on a Beckman Model 120B amino acid analyzer using a 0.9 × 11 cm column of PA-35 sulfonated polystyrene operated at 58 °C. The column was eluted with 0.35 N sodium citrate, pH 5.26, at a rate of 70 mL/h. Elution times were Trp 36 min, Trp(For) 48 min, NH_3 62 min, and Trp thiocresol side product broad unsymmetrical peak at 135 min.

High-performance liquid chromatography was performed on a μBondapak C-18 column (0.4 × 30 cm) using three different gradient programs of B (H_2O–CH_3OH–CH_3COOH, 10:90:0.25, v/v) into A (H_2O–CH_3OH–CH_3COOH, 90:10:0.25, v/v, pH adjusted to 4.2 with NaOH). The column was kept at 30 °C, eluted at 2.0 mL/min, and was monitored at 280 nm.

The three gradient programs are as follows: *system I*, 45 min 5–85% B linear; *system II*, 45 min 5–98% B linear followed by 15 min 98% B isocratic; and *system III*, 30 min 50 → 98% B linear followed by 15 min 98% B isocratic.

Elution times in minutes are as follows: *system I*, Trp 4.6, Trp(For) 8.2, *p*-cresol 13.7, thiocresol side products of Trp 24.9, 25.9, 27.0, and *p*-thiocresol 28.1; *system II*, Trp 4.0, Trp(For) 7.0, *p*-cresol 9.7, *p*-thiocresol 22.6, and $HC(SC_6H_4CH_3)_3$ 46.8; and *system III*, *p*-cresol 2.8, *p*-thiocresol 6.4, and $HC(SC_6H_4CH_3)_3$ 29.4.

HF Deprotection of Synthetic Peptides. It has become clear to us during the course of our investigations on deprotection and cleavage of peptides in HF that satisfactory and reproducible results can be obtained if the following guidelines and precautions are followed.

(1) Treatment with 50% trifluoroacetic acid prior to the HF treatment. Two treatments with 50% TFA in CH_2Cl_2 (2 × 5 min, then washed 3× with CH_2Cl_2 and followed by an optional treatment of neutralization and removal of the trifluoroacetate salts by diisopropylethylamine) serve to remove *tert*-butyl protecting groups, which would otherwise provide long-lived reactive *tert*-butyl cations during the HF deprotection step. It also serves to remove impurities or precipitates embedded in the resin supports.

(2) Check HF line for leaks: one of the most troublesome problems is a leaky HF-line, which can lead to introduction of water, carbon dioxide, or acetone (from the dry ice–acetone bath) into the reaction vessel. Consequently, the HF reaction mixture will be diluted, with uncertain results. Furthermore, HF or dimethyl sulfide can be lost during the reaction, also leading to unsatisfactory results. To avoid this problem, it is best to check the HF line, with the reaction vessel in place, for leaks before the experiment. This can be carried out by evacuating the HF line and closing the system to the outside. The vacuum should hold for at least 4 h. If vacuum fails to hold, replace O-rings or dismantle the HF line if necessary for thorough cleansing.

(3) Introduction of reagents and precautions to avoid incomplete mixing. The incomplete mixing of the HF reagent and the peptidyl resin or peptide can occur in several ways. One is due to the magnetic stirring bar being frozen by the *p*-cresol or *p*-thiocresol mixture during the introduction of the reagents. This can be alleviated simply by the following order of addition of the reagents: (a) peptide or peptidyl-resin, (b) *p*-cresol, *p*-thiocresol, or both, in a melted form, carefully on top of the resin by a warm pipette, (c) after cooling and the *p*-cresol mixture has solidified, magnetic stirring bar, and (d) dimethyl sulfide.

A typical low-concentration HF experiment was as follows. After the reagents (dimethyl sulfide, *p*-cresol, *p*-thiocresol), total volume 3.75 mL, and peptide-resin were placed in the reaction vessel and connected to the HF line, the vessel was cooled to −78 °C for 0.5 h (longer cooling time for large volume of reagent). The line was evacuated briefly for 0.5 min and HF was quickly distilled into the evacuated reaction vessel to a 5-mL mark (or any desired volume). The reaction was then quickly equilibrated to 0 °C by ice bath and allowed to stir vigorously for 2 h (check stirring constantly). The HF–dimethyl sulfide–*p*-cresol mixture at this point was usually colorless to light yellow. After 2 h, the mixture was then evacuated first with water aspirator (caution: bumping!) with the valve of the reaction to the aspirator only partially opened. After most of the reagent was removed, the mixture was further evacuated by mechanical pump to a light colored liquid (usually at ~0.5-mL mark).

A typical high concentration of HF was prepared as follows. The evaporated reaction vessel was cooled again to −78 °C, evacuated, and charged again with HF to the 5-mL volume mark. [Note: if the removal of HF and dimethyl sulfide after the first stage was incomplete, recharging HF to 5 mL will result in a final HF concentration that is less than 90%. Under such circumstances the more acid-resistant resins, such as pam-resin or benzhydrylamine-resin, or peptides with many acid-resistant protecting groups, such as tosyl or 2,6-dichlorobenzyl, will not be completely deprotected. If there is doubt about the completion of the evaporation step, we recommend charging HF to 7.5- or 10-mL total volume, so that the final HF concentration will be certain to reach at least 90% by volume. A final mixture of 95% HF and 5% cresol plus thiocresol has been found to be entirely satisfactory.] The reaction was then equilibrated to 0 °C and allow to react for 45–60 min. The HF was then removed as described previously. The residue was then extracted 3 times with EtOAc to remove the organic scavengers and organic soluble products, followed by extraction with 1–20% of HOAc or 0.1–1.0 M $(NH_4)_2CO_3$ to take up the peptide. Alternatively, after evaporation of HF, the peptide was taken up in 1–5% HOAc and the aqueous phase was extracted 3 times with equal volumes of ether to remove the hydrophobic organic components.

Syntheses of Test Peptides. General Procedure. The syntheses of test peptides were carried out by stepwise solid-phase methods on chloromethylcopoly(styrene–1% divinylbenzene) similar to those described earlier.[46] The Boc group was moved prior to the final HF deprotection step.

(A) Methionine-enkephalin. The peptide Boc-Tyr(Bzl)-Gly-Gly-Phe-Met(O)-OCH_2-resin was synthesized from Boc-Met(O)-OCH_2-resin (5 g, 0.26 mmol/g). Samples of 250 mg each were treated with (1) the high HF procedure (HF–*p*-cresol, 9:1, v/v, 0 °C, 1 h), (2) the low HF (HF–DMS–*p*-cresol, 25:65:10, v/v, 2 h, 0 °C) and (3) the low–high HF (the low HF procedure followed by the high HF). The results are summarized in Table XI. A portion of the crude product from (3) was purified by ion-exchange chromatography on a Dowex 50X-4 column (0.9 × 55 cm) on a Beckman 120B amino acid analyzer, eluted with 0.5 M pyridine acetate (pH 5.6) at 58 °C at a flow rate of 66 mL/h. Methionine-

enkephalin, eluted at 165 min, was collected and accounted for 92% of the starting material.

(B) Bovine Growth Hormone Fragment (128–131). The tetrapeptide Boc-Glu(OBzl)-Asp(OBzl)-Gly-Thr(Bzl)-OCH_2-resin was synthesized from Boc-Thr(Bzl)-OCH_2-resin (5 g, 0.22 mmol/g). Samples of 100–250 mg each were treated with different HF procedures as required. The results (Table VIII) were quantitated by ion-exchange chromatography on an AA-15 column (Beckman, 0.9 × 54 cm) attached on a Beckman 120B amino acid analyzer, eluted with pH 3.20 citrate buffer at 59 °C. The elution times of the peptides were β-peptide 53 min, α-peptide 70 min, and imide 135 min (Scheme VI).

(C) Pentagastrin Amide. The pentapeptide Boc-Gly-Trp(For)-Met-(O)-Asp(OBzl)-Phe-NH-CH(C_6H_5)-C_6H_4-$OCOCH_2$-resin (**3**), was synthesized from a multidetachable benzhydrylamine-resin, prepared according to ref 46, 5 g, 0.18 mmol/g. Samples of 100–250 mg each were treated with different HF procedures as required. The results are summarized in Tables XII and XIII.

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Registry No. Boc-L-Ser(Bzl), 23680-31-1; Tyr(Bzl), 16652-64-5; Boc-Tyr(Bzl), 2130-96-3; L-Trp(For)·HCl, 38023-86-8; H-Gly-Trp-Met-Asp-Phe-NH_2, 18917-24-3; Boc-L-Trp(For), 47355-10-2; Z-Gly, 1138-80-3; Sor(Bzl), 4726-96-9; Thr(Bzl), 4378-10-3; Asp(OBzl), 2177-63-1; Glu(OBzl), 1676-73-9; His(Tos), 23241-48-7; Lys(2-ClZ), 42390-97-6; Boc-Ala, 15761-38-3; Z-Ala, 1142-20-7; Trp(For), 74257-18-4; Tyr(Bzl), 16652-64-5; Tyr(2,6-Cl_2Bzl), 40298-69-9; Tyr(BrZ), 86902-29-6; H-Gly-Trp-Met(O)-Asp-Phe-NH_2, 86941-81-3; H-Gly-Trp(For)-Met(O)-Asp-Phe-NH_2, 86921-21-3; H-Gly-Trp(For)-Met-(O)-Asp-Phe-NH_2, 86902-30-9; H-Gly-Trp(For)-Met-Asp-Phe-NH_2, 86941-82-4; hydrofluoric acid, 7664-39-3; dimethyl sulfide, 75-18-3; L-methionine sulfoxide, 3226-65-1; tri-*p*-tolyl trithioorthoformate, 17241-10-0; methionine enkephalin, 58569-55-4; bovine growth hormone fragment (125–131), 57680-10-1; anisole, 100-66-3; thioanisole, 100-68-5; diphenyl sulfide, 139-66-2; thiacyclopentane, 110-01-0; 1,4-thioxane, 15980-15-1; *p*-thiocresol, 106-45-6; ethanethiol, 75-08-1; ethanedithiol, 540-63-6; 3,4-dimercaptotoluene, 496-74-2; thiophenol, 108-98-5.

Resonance Raman Spectra of Rubredoxin, Desulforedoxin, and the Synthetic Analogue $Fe(S_2$-*o*-xyl$)_2^-$: Conformational Effects

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Abstract: The resonance Raman (RR) spectrum of the rubredoxin analogue $Fe(S_2$-*o*-xyl$)_2^-$ (S_2-*o*-xyl = *o*-xylylene-α,α′-dithiolate) shows four widely spaced bands (I–IV) in the Fe–S stretching region, 297, 321, 350, and 374 cm^{-1}, instead of the two expected for tetrahedral FeS_4. The RR spectrum of oxidized rubredoxin (Rd_{ox}) is also shown to have four bands in this region, 312, 325, 359, and 371 cm^{-1}, contrary to an initial impression of a tetrahedral spectrum. Normal mode calculations were carried out to explore the possible sources of Fe–S mode splitting. For a FeS_4 model, distortions of the angles split the T_2 mode but only by small amounts for the angles seen in either the protein or the analogue crystal structures; the effect on the A_1 breathing mode was negligible. When coupling between Fe–S stretching and S–C–C bending was included, with $Fe(SCH_2CH_3)_4$ as a model (with point mass methyl and methylene groups), an appreciable effect of the S–C dihedral angles on the Fe–S breathing mode was observed, which accounted satisfactorily for the band I frequency difference between Rd_{ox} and $Fe(S_2$-*o*-xyl$)_2^-$ on the basis of the differing S–C dihedral angles (90°, 180° and 90°, 90°, respectively, for the C_2-related pairs). This coupling also split the T_2 Fe–S components but not by enough to account for the observed spectra. Additional couplings were revealed by perdeuteration of the methylene groups in $Fe(S_2$-*o*-xyl$)_2^-$, which produced 10-cm^{-1} *up*shifts of the middle two Fe–S bands (II and III). These upshifts could be explained by the crossing over of nearby ligand skeletal modes, observed in the IR spectrum, which shifted down strongly on methylene perdeuteration. Differential coupling with these ligand modes might account for the Fe–S mode splitting. Rd_{ox} RR spectra from *Desulfovibrio gigas*, *Desulfovibrio sulfuricans*, and *Megasphera elsdenii* were very similar. The constancy of band I (312 cm^{-1}) implies the same set of S–C dihedral angles. Slight shifts in bands II and III were observed, suggesting subtle conformational differences. The RR spectrum of *D. gigas* desulforedoxin resembled that of rubredoxin but showed an appreciable upshift of band II, to 341 cm^{-1}, possibly reflecting S–Fe–S angle distortions that might be associated with the adjacency of two cysteine side chains in the primary structure.

The iron–sulfur proteins continue to attract much interest from inorganic and biochemists.[1] These ubiquitous electron-transfer proteins encompass several structural classes, with Fe–S centers containing one, two, three, or four iron atoms. They all absorb light strongly in the visible region, reflecting S → Fe charge-transfer electronic transitions. They are therefore good candidates for structural monitoring via resonance Raman (RR) spectroscopy,[2,3] since the Fe–S vibrational modes are expected to be selectively enhanced. Indeed, one of the first biological applications of RR spectroscopy was the study of rubredoxin, a one-Fe protein, by Long and co-workers.[4] In the intervening decade, however,

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